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

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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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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.

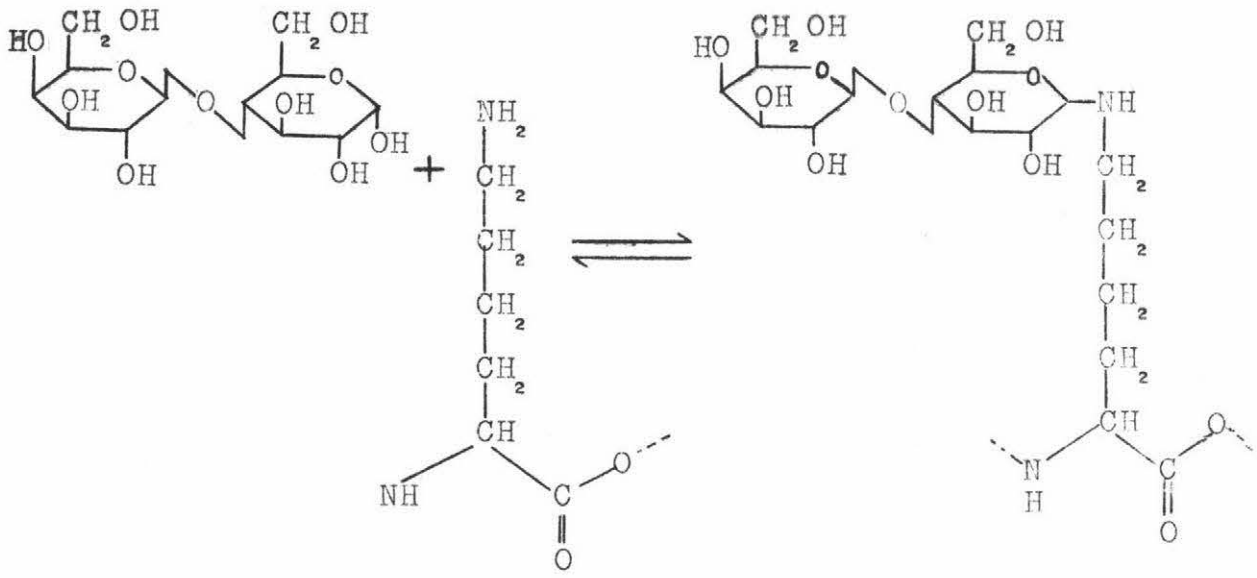
While free amino acids can become involved with reducing sugars in the Maillard reaction, in foods the free amino groups are largely the ϵ-amino groups of lysine, as

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

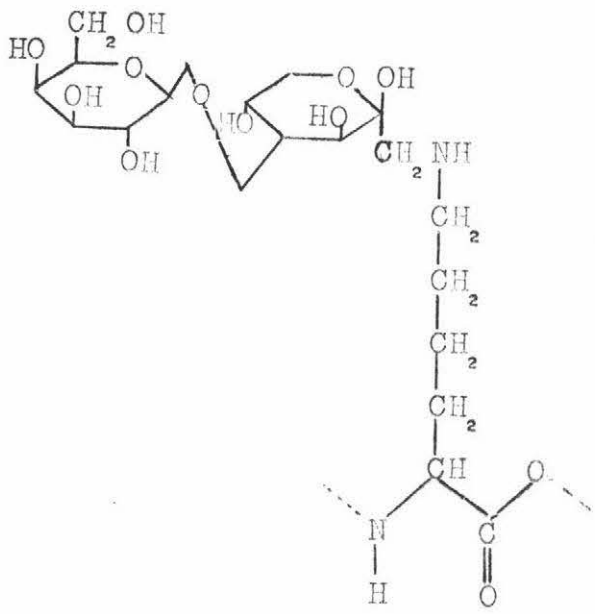


Lactose lysine in peptide chain ϵ - N - lactosyl - lysine

(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 $\longrightarrow$ 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 $\xrightarrow[-3H_2O]{\text{Acid conditions}}$ Furosine + Pyridosine + 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

$$ALV = 2.70\ TLV - 167 \qquad (1.1)$$
$$(r = 0.99)$$

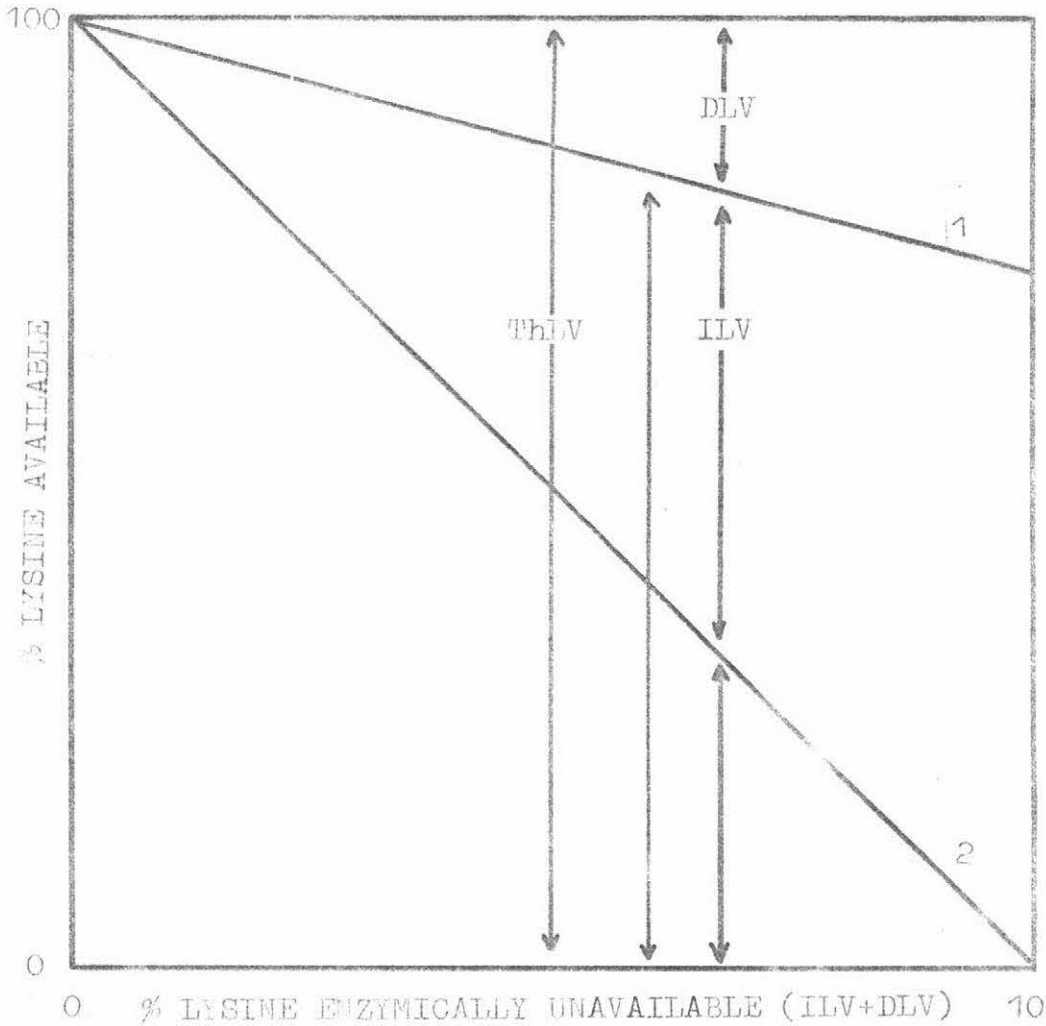
where ALV = enzymically available lysine, and
TLV = total lysine, by conventional acid hydrolysis

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,

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

and in relation to figure 1.1,

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

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

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

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)

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

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

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

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.

CHAPTER 2

EXPERIMENTAL GAS CHROMATOGRAPHIC ANALYSIS OF LYSINE

2.1 INTRODUCTION

The successful development of a method of analysing amino acids by gas-liquid chromatography (GLC) depended on the finding of a method of quantitatively converting the amino acids in a protein hydrolysate to volatile derivatives that could be separated on a GLC column. Several methods using a variety of derivatives have been developed including

(a) N-trifluoroacetyl n-butyl esters (Gehrke, et al., 1968)

(b) Trimethylsilyl derivatives (Gehrke, et al, 1969)

(c) N,O-diisopropyl derivatives (Pettitt and Stouffer, 1970)

(d) N-isobutyloxycarbonyl methyl esters (Makita, et al., 1975)

(e) Bisneopentylidene ether ester of lysine (Zscheile and Brannaman, 1972)

(f) n-propyl, N-acetyl derivatives (Adams, 1974)

This list is not intended to be exhaustive but rather to indicate the variety of methods developed, some of which have only a restricted specific application. Of the methods developed the most commonly used appears to be the direct esterification method developed from method (a). This was reported by Gehrke and Roach, 1969, and its use by them has been reported many times. (Gehrke, et al., 1971a, 1971b, 1971c, Zumwalt, et al, 1971). The method has also been used by many other workers on food and biological materials (Bognar, 1970, Lachovitzki and Bjorklund, 1970 Conkerton, 1974). Very good precision and accuracy has been claimed by comparison with conventional ion exchange column chromatography (Kaiser, et al 1974 Conkerton, 1974).

While the GLC method offers a worthwhile reduction in time over the time required for analysis by the traditional ion exchange analyzer, both procedures are still lengthy when allowance is made for the necessary protein hydrolysis step. The conventional hydrolysis technique using HCl (6N, under reflux, or in a sealed tube in an atmosphere of

nitrogen, at a temperature of 110°) takes 24h.

To complement the speed of GLC analysis a study was made by Roach and Gehrke, 1970, into possible ways of speeding up the hydrolysis step. Using 6N HCl a variety of times and temperatures, for protein hydrolysis were investigated and compared with the results given by the conventional method. It was found that hydrolysis at 145° for 4h compared favourably with the 110° for 24h process, especially for proline, threonine, serine, methionine, hydroxyproline, and arginine which are particularly prone to hydrolytic losses. With regard to the present study it was found that the hydrolysis of bovine serum albumin at 145° for 4h gave a lysine recovery that was less than 3% higher than the recovery given by the conventional process. Additionally for a standard mixture of 18 AA's after treatment with 6N HCl at 145° for 4h the average recovery was 99.7% with an average relative standard deviation of 0.92%. The recovery of lysine was only 97.4%, but for this planned study provided all hydrolyses are carried out under identical conditions and the recovery is consistent, the actual response is of little consequence in terms of studying the kinetics of lysine loss in heat treated milk.

Therefore because of the time reduction in the hydrolysis process, and because of the claimed accuracy, reproducibility and acceptance of the Gehrke GLC method it was decided to use the Gherke and coworkers methods of protein analysis.

SECTION I

AN EXAMINATION OF THE GEHRKE METHOD OF AMINO ACID ANALYSIS BY GAS LIQUID CHROMATOGRAPHY

2.2 EQUIPMENT REQUIRED

(a) Gas liquid chromatograph: a twin column temperature programmable instrument fitted with two flame ionisation detectors is needed.

(b) Integrator: required to give accuracy. The most suitable is an electronic digital type.

(c) Recorder: a twin pen type capable of matching the GLC/integrator output, typically a minimum sensitivity of 1mV is necessary.

(d) Ultrasonic bath: to assist mixing during derivatization.

(e) Oil baths: for esterification and acylation steps.

(f) Rotary evaporator: is essential for vacuum evaporation of solvents.

(g) Vacuum pump: for dearating protein/acid mixtures prior to hydrolysis.

(h) Hydrolysis tubes: for protein hydrolysis.

(i) Reaction vessels (glass): for esterification and acylation steps. Must be capable of withstanding and maintaining pressure.

2.3 REAGENTS REQUIRED

(a) Chromatographically pure amino acids for use as standards, and for quantification.

(b) 6N HCl, AR grade for protein hydrolysis.

(c) Internal standard: Butyl stearate, ornithine, and trans-4-(amino methyl) - cyclohexanecarboxylic acid (tranexamic acid) are all claimed to be suitable internal standards.

(d) 7N NH_4OH, AR grade for elution of amino acids from the cation exchange column.

(e) Cation exchange resin for hydrolysate clean up.

(f) n- butanol, AR grade, for the production of 3N HCl in butanol reagent.

(g) 3N HCl in butanol is made by dissolving dry HCl (produced by slowing adding conc.HCl into conc.H_2SO_4, followed by bubbling to the gas through conc.H_2SO_4) in butanol until 3N in HCl (checked by titration).

(h) Anhydrous methylene chloride.

(i) Trifluoroacetic anhydride, AR grade, for acylation.

2.4 SELECTION OF COLUMN PACKINGS

Roach and Gehrke, 1969 used three different stationary phases for the separation of the AA N-TFA-n-butyl esters viz, ethylene glycol adipate (EGA) on acid washed Chromosorb W, OV-17 and OV-22 siloxane phases both on high performance Chromosorb G. It was claimed that the OV-17 and OV-22 columns could be used interchangeably as they gave essentially the same elution pattern. Later, Zumwalt, et al., 1971 reported the use of OV-101 as well as EGA and OV17. In a following paper Gehrke, et al., 1971b, reported that a mixed phase packing of 2% OV-17 and 1% OV-210 on 100/120 mesh Supelcoport was superior to OV-17 alone. It was found to give quantitative elution and highly efficient and complete separation of histidine, internal standard (trans-4-aminomethylcyclohexanecarboxylic acid, commonly referred to as "tranexamic" acid), lysine, arginine, tryptophan, and cystine. Using the two different column packings enabled the separation and quantification of the 20 protein amino acids.

The mixed OV-17/OV-210 packing appears to have been accepted, together with the EGA packing as the most suitable for the separation of the N-TFA n-butyl esters of AA. It can be purchased ready made as either a packing, or in pre-packed columns from some of the major GLC supplies companies (Regis Chemical Company of Chicago; Supelco, Inc. of Belefonte, Pennsylvania).

Two column packings are required to separate the 20 common amino acids released by acid hydrolysis from biological proteins.

(a) 0.65%W/W ethylene glycol adipate on Chromosorb W (80/100 mesh, acid washed)

(b) 2.0% W/W OV-17 and 1.0% W/W OV-210 on Gas Chrom Q (100/120 mesh)

2.5 PREPARATION OF PROTEIN HYDROLYSATES

(a) Hydrolysis Procedure

This procedure was developed and examined by Roach and Gehrke, 1970, and has also been examined by Kaiser, et al., 1974.

(i) Samples containing 20-25 mg of protein are weighed into 25 x 200mm pyrex glass screw top culture tubes.

(ii) 6N HCl (25ml) is then added.

(iii) Using a glass "T" piece and appropriate valves the hydrolysis tube is evacuated and then nitrogen flushed alternately for three cycles. The evacuation is done with the tube in an ultrasomic bath.

(iv) The hydrolysis is then carried out at 145° for 4 hours.

(v) The hydrolysate is filtered through glass fibre paper and made up to 50ml.

(In the initial application of Gehrke's methods to milk the following changes were made:

(i) An aliquot (1ml) of 1:2 diluted milk was transferred to a hydrolysis tube fitted with a gas connection and teflon valve. These tubes were made locally.

(ii) The pressure inside the hydrolysis tube was reduced to about 0.2mm Hg by a vacuum pump. After the initial bubbling off of the dissolved air had ceased, the tube with the vacuum still applied was placed in an ultrasonic bath causing the release of further dissolved air which was pumped out. The headspace was then flushed with nitrogen.)

(b) Cation Exchange Clean-Up of Hydrolysates

(i) To aliquot containing about 10mg of amino acids an internal standard (0.5 mg of tranexamic acid is added and the whole is dried under vacuum using a rotary evaporator (when butyl stearate was used as an internal standard it was added after the ion exchange cleanup).

(ii) After dissolving the dried mixture in HCl (10ml of 0.1N), it is placed onto a cation resin bed (7ml of Amberlite IR 120) in an ion exchange column and left in contact for approximately 10 min.

(iii) After the solution has slowly passed through the bed (1ml/min) the resin is washed with deionized water (50 ml).

(iv) The amino acids and internal standard are then eluted from the column with ammonia (20ml of 7N), followed by a deionized water rinse.

(Preparation of Cation-Exchange Resin

(i) Amberlite IR-120 (ea 10g) was covered with glass distilled water and periodically stirred over a period of 2-3h. The water was then decanted.

(ii) The resin was then covered with 7N NH_4OH and stirred periodically for ca 2h, after which the resin was allowed to settle and the NH_4OH decanted. This NH_4OH treatment was repeated twice, and then the resin washed with glass distilled water until the effluent was neutral.

(iii) The resin was regenerated into the H^+ form by the addition of enough 3 N HCl to form a slurry. This was stirred for about 1h, the resin settled and the 3N HCl decanted off. This was repeated twice, and washed with glass distilled water until the effluent was neutral. The resin was kept wet with glass distilled water until ready for use.)

2.6 PREPARATION AND SEPARATION OF N-TRIFLUOROACETYL n-BUTYL ESTERS OF AMINO ACIDS

The initial method of obtaining the N-trifluoroacetyl n-butyl esters (hereafter N-TFA n-butyl esters) of amino acids involved the formation of methyl esters followed by an interesterification step (Gehrke, et al., 1968) which made the process time consuming, requiring 3.5h for the complete derivatization. However the same workers developed the following method for direct esterification of the protein amino acids (Roach and Gehrke, 1969). The procedure below is represented in the block diagram, figure 2.1a, and the reaction scheme in figure 2.1b.

(a) To a protein hydrolysate, or pure amino acid mixture, containing 1-20mg of amino acids (AA), add an internal standard (0.2-4mg n-butyl stearate or 0.5mg tranexamic acid)

(b) Dry the mixture under a partial vacuum in a rotary evaporator in a 60° water bath.

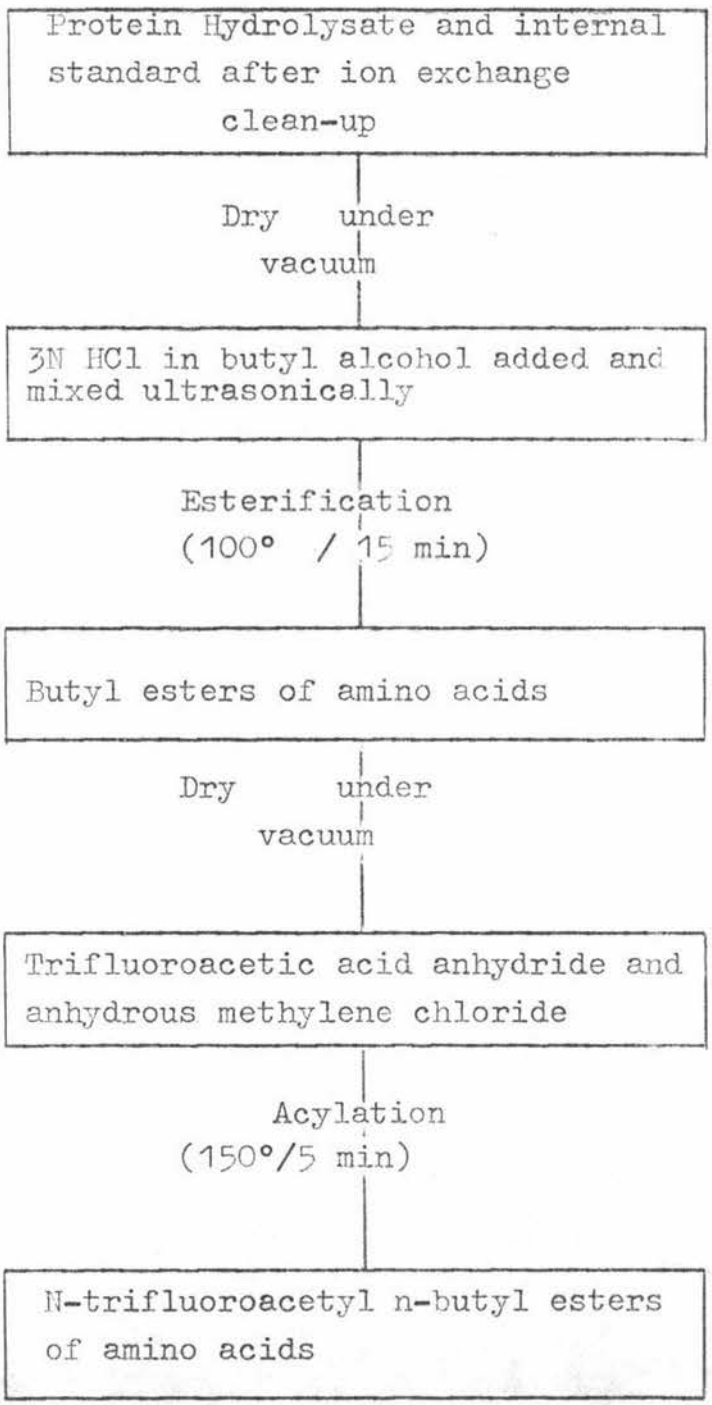
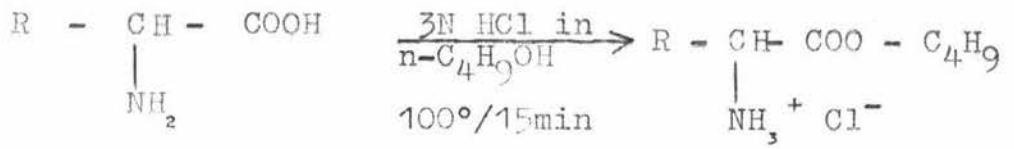


FIGURE 2.1a BLOCK DIAGRAM FOR THE PRODUCTION OF N-TFA n-BUTYL ESTERS OF AMINO ACIDS (GEHRKE METHOD)

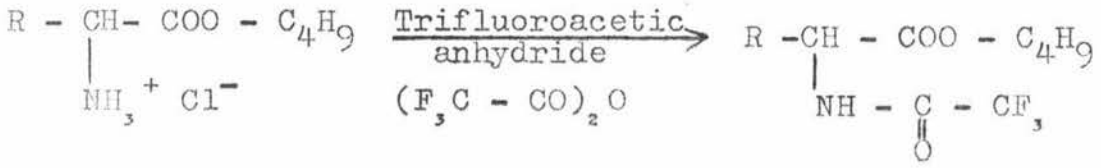
ESTERIFICATION



Amino acid

Amino acid butyl ester

ACYLATION



Amino acid butyl ester

N-trifluoroacetyl
amino acid butyl ester

FIGURE 2.1b REACTION SCHEME FOR THE PRODUCTION OF N-TFA n-BUTYL ESTERS OF AMINO ACIDS

(c) To the dried mixture add 3N HCl in dry n-BuOH (1.5ml/1.0mg AA) and then mix ultrasonically for 15 sec before esterification (100°/15 min).

(d) After esterification evaporate the butyl ester mixture to dryness under partial vacuum in a rotary evaporator in a 60° water bath.

(e) Add dry methylene chloride (ca 10ml) to form an azeotrope with any remaining water, and then dry again under vacuum in a rotary evaporator in a 60° water bath.

(f) Add dry methylene chloride (ca 2ml), and trifluoroacetic anhydride (1ml) to the butyl esters in the flask, ultrasonically mix then transfer to two culture tubes, and cap with teflon lined screwcaps.

(g) Immerse the tubes in an oil bath (150°C) for 5 min, cool the derivatives, and store at 0° until chromatography is performed.

Separate the derivatives using both the EGA and mixed OV columns with the chromatograph temperature programmed from 70° to 235°, at 6°/min.

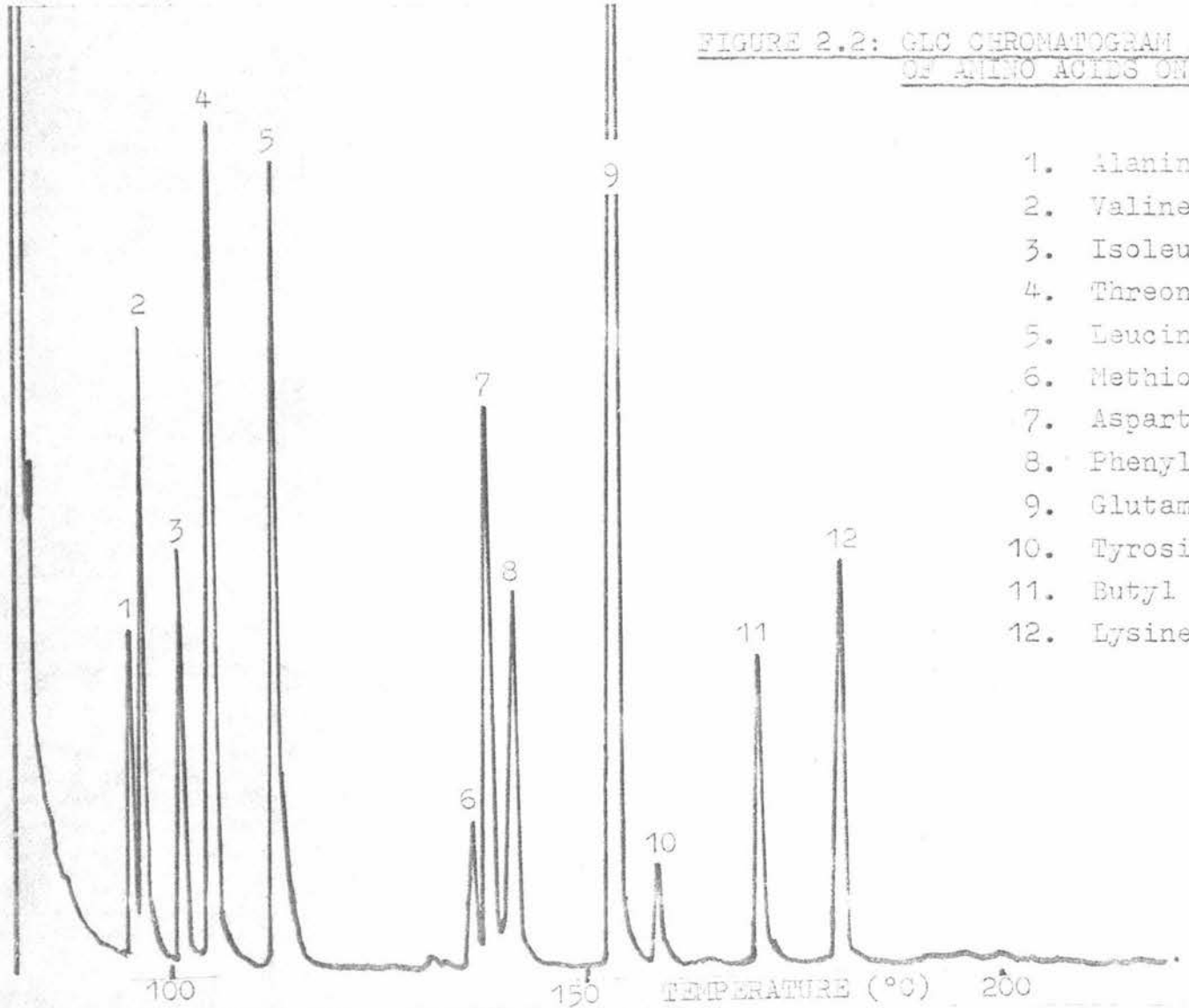
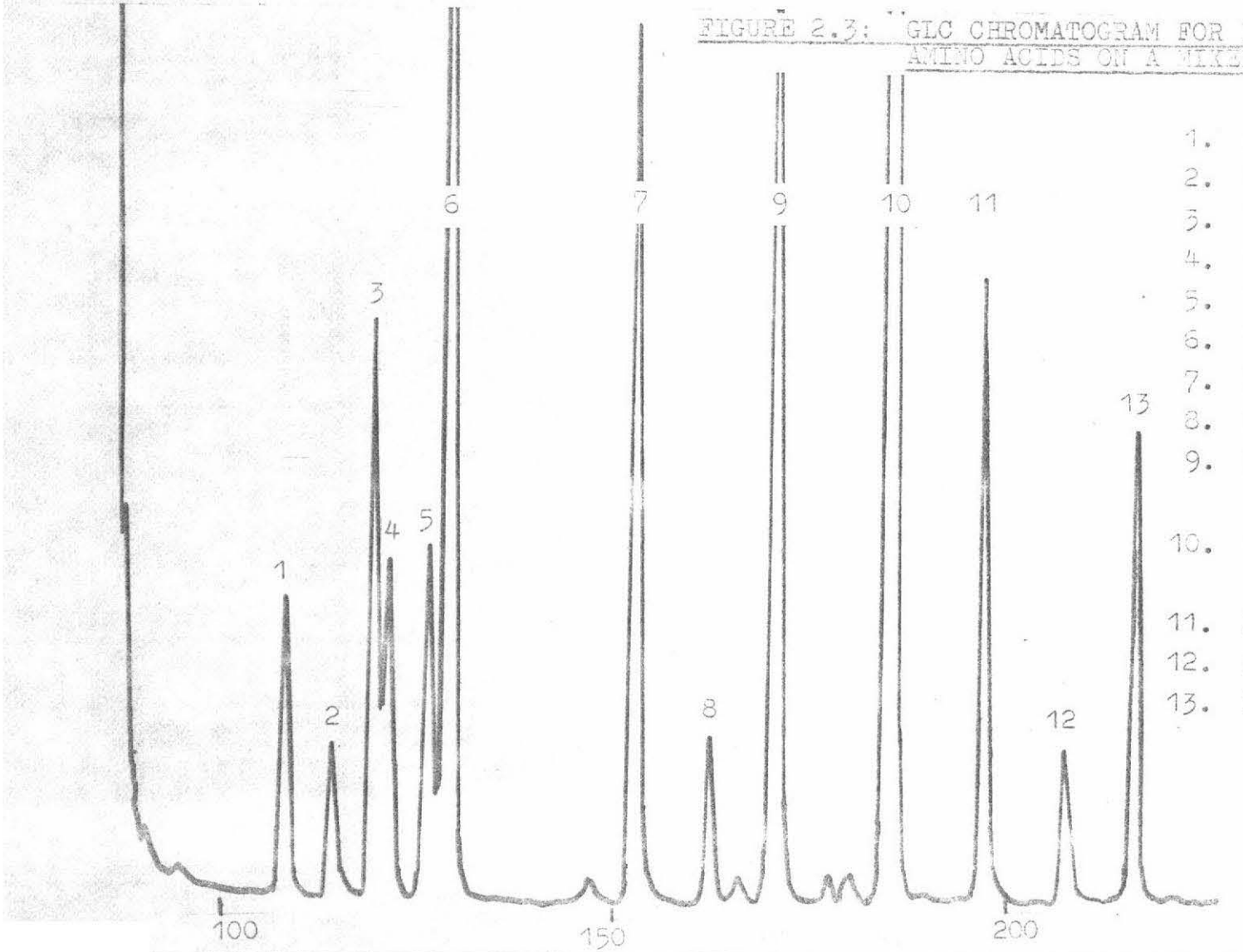


FIGURE 2.2: GLC CHROMATOGRAM FOR N-TFA-n-BUTYL ESTERS OF AMINO ACIDS ON AT EGA COLUMN

1. Alanine
2. Valine
3. Isoleucine
4. Threonine, Proline and Glycine
5. Leucine and Serine
6. Methionine
7. Aspartic Acid
8. Phenylalanine
9. Glutamic Acid
10. Tyrosine
11. Butyl Stearate (IS)
12. Lysine

FIGURE 2.3: GLC CHROMATOGRAM FOR N-TFA-n-BUTYL ESTERS OF AMINO ACIDS ON A MIXED SP-1710+OV-210 COLUMN



1. Alanine
2. Glycine
3. Valine
4. Threonine
5. Serine
6. Isoleucine and Leucine
7. Proline
8. Methionine
9. Phenylalanine and Aspartic Acid
10. Glutamic Acid and Tyrosine
11. Lysine
12. Arginine
13. Butyl Stearate (IS)

2.7 RESULTS

Figures 2.2 and 2.3 are typical chromatograms for milk produced by the Gehrke method. Figure 2.2 is the separation achieved on the EGA column whereas Figure 2.3 is that for the same sample on the mixed OV column.

2.8 DISCUSSION

Comparing the two figures shows a considerable difference between them in elution order of the amino acids. It is this difference which enables certain of the amino acids (AA's) to be identified and quantified, since where there is simultaneous elution of two AA's on one column, their pairing does not occur on the other column.

In the case of the EGA column, there are also some significant changes in the elution order when compared with that achieved by Gehrke and coworkers, as shown in Table 2.1.

TABLE 2.1 ELUTION ORDER OF DERIVATIVES FROM EGA COLUMN

Experimental	Gehrke
Alanine	Alanine
Valine	Valine
Isoleucine	Glycine
Threonine, Proline and Glycine	Isolencine
Leucine and Serine	Leucine
Methionine	Proline
Aspartic Acid	Threonine
Phenylalanine	Serine
Glutamic Acid	Methionine
Tyrosine	Phenylalanine
(Butyl Stearate)*	Aspartic Acid
Lysine	Glutamic Acid
	Tyrosine
	(Butyl Stearate)*
	Lysine

* Internal Standard

The reason for the marked differences could have been that the experimental column was locally packed and was made with Varaport 30 instead of the acid washed Chromosorb W used by Gehrke and coworkers (Kaiser, et al., 1974), although Chromosorb G has been used (Zumwalt, et al., 1971) with no change to the elution order.

For the OV-17/OV-210 column there is better correspondence in the elution order, and in fact better separation was achieved (although as the column aged the separation of valine and threonine, and serine, isoleucine and leucine decreased). Table 2.2 gives the elution order.

TABLE 2.2 ELUTION ORDER OF DERIVATIVES FROM OV-17/OV-210 COLUMN

Experimental	Gehrke
Alanine	Alanine
Glycine	Glycine
Valine	Valine and Threonine
Threonine	
Serine	Serine
Isoleucine and leucine	Isoleucine and Leucine
Proline	Proline
Methionine	Methionine
Phenylalanine and Aspartic Acid	Phenylalanine and Aspartic Acid
Glutamic Acid and Tyrosine	Glutamic Acid and Tyrosine
Lysine	Lysine
Arginine	Arginine
(Butyl Stearate)*	(Butyl Stearate)*

* Internal Standard

In this case the separation was achieved using a commercially prepared column (Varian Aerograph, California, U.S.A.). In the initial investigation a laboratory prepared column was used and this also gave poor separation. Of the first five peaks glycine and valine almost co-eluted, and serine, isoleucine, and leucine did co-elute. However tyrosine was separated from glutamic acid, eluting about 30 sec. or 2° earlier. When the commercial column became

available it was used in preference. Further, as only one integrator was available this was used almost exclusively with the commercial OV-17/OV-210 column. Separations were performed using the EGA column which was occasionally connected to the integrator but all the major studies were performed with the OV-17/OV-210 column.

SECTION II

INVESTIGATION OF GEHRKE'S METHOD, FOR APPLICATION TO MILK PROTEINS

2.9 ION EXCHANGE CLEAN-UP

As outlined earlier the Gehrke method included an ion exchange clean-up step. This was to selectively adsorb the AA from the hydrolysed protein and allow removal of other hydrolysis products some of which may have reacted with the derivatizing agents giving spurious interfering peaks on the chromatogram.

Because skim milk has a limited range of non protein constituents it was thought that the interference from non-amino acid peaks may be limited. Therefore a comparison trial was made in which the ion exchange clean up step was omitted.

The resulting chromatograms appeared to show no significant difference and particularly there was no increase in any spurious peaks in the region of lysine, or the IS which at this stage was butyl stearate.

Because the ion exchange step involved further sample handling, and possible losses which would not be measured since the IS (butyl stearate) was added after the clean-up, and because there was no improvement in the separation by the inclusion of the clean-up process, this step was omitted with a consequent saving of time.

There was one major difference between the chromatograms before and after clean-up. On the OV-17/OV-210 column the threonine peak increased in size by a factor of

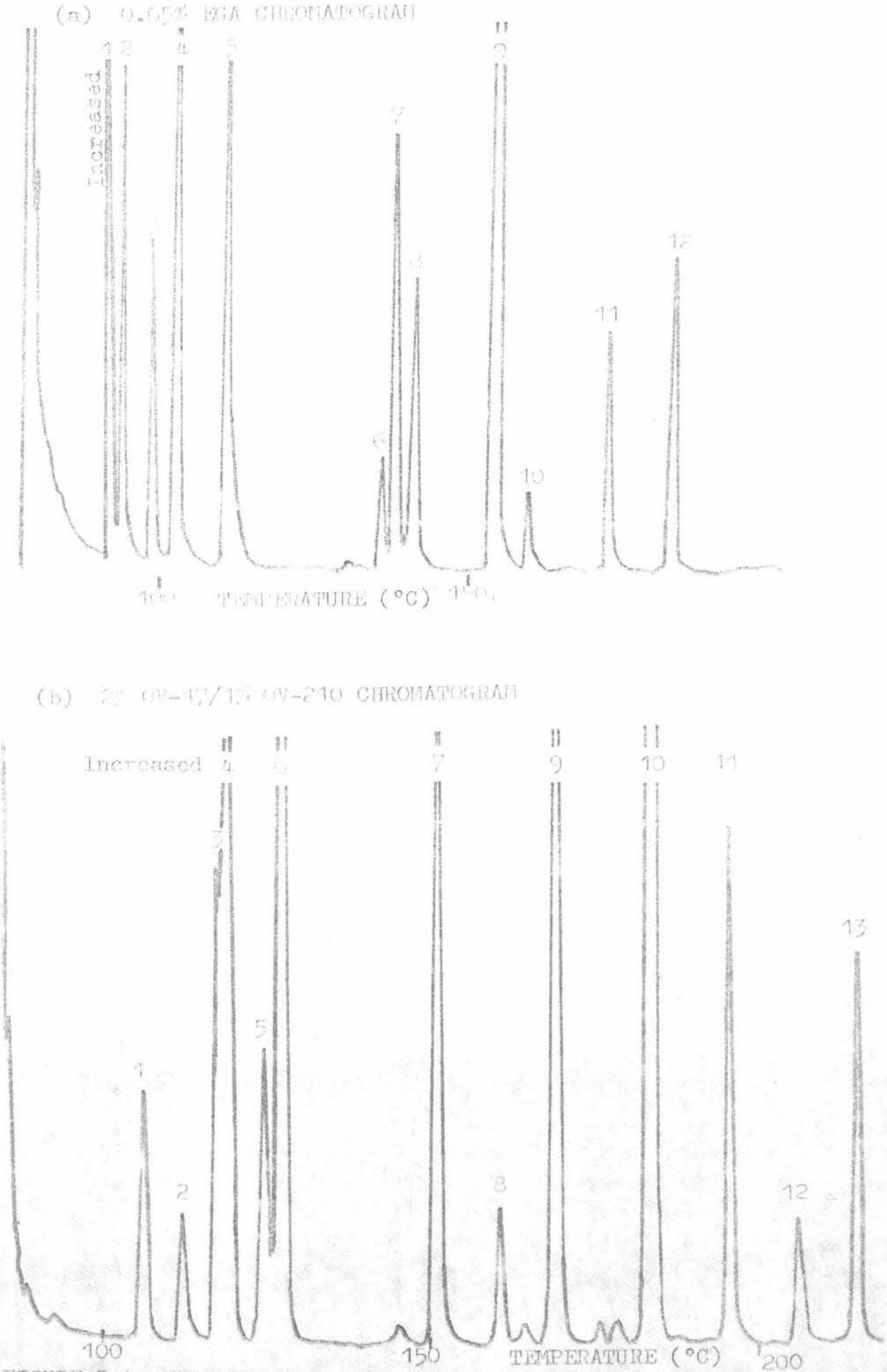


FIGURE 2.4: THE EFFECT ON THE GLC CHROMATOGRAM OF THE AMINO ACIDS OF MILK OF OMITTING THE ION

about 3, and a similar increase occurred in the alanine peak on the EGA column with the omission of the clean-up step. (See figures 2.4a, 2.4b)

2.10 INTERNAL STANDARD

(a) Butyl Stearate

When butyl stearate was used as internal standard and the ion exchange clean-up step was included, the IS was added to the sample after the clean-up step. However when this clean-up step was omitted the IS was added immediately after the hydrolysate had been filtered and dried. It was important to dry the filtrate first as the butyl stearate was dissolved in $MeCl_2$ and this tended to cause over-vigorous evaporation with occasional loss of IS and/or sample.

Butyl stearate proved to be an unsatisfactory internal standard. There was greater consistency between the ratios of the areas of the various amino acid peaks in duplicate derivatizations, than there was between the amino acid: internal standard peak area ratios.

This inconsistency was probably caused by the insolubility of butyl stearate in the hydrolysate. It appeared to form a greasy coating on the internal surfaces of the esterification tube, and it was not easy to transfer quantitatively. (It was not completely soluble in the 3N HCl in n-BuOH).

Butyl stearate was dropped as an internal standard when tranexamic acid became available.

(b) Tranexamic Acid

This latter internal standard was soluble in 0.1 N HCl and therefore it was possible to add this directly to the hydrolysate once it was cooled and prior to filtration. Any transfer losses occurring subsequent to this stage were then of much less consequence. The tranexamic acid proved to be a much more satisfactory internal standard than the butyl stearate originally used.

2.11 REAGENT PREPARATION

Careful reagent preparation and storage is necessary. As the esters are hydrolysed by water all reagents must be dry, and to prevent artefacts and spurious peaks they should

be of high purity.

While the preparation method ultimately used will be given later, together with other details, one aspect of the preparation can be discussed appropriately here.

In the preparation of the 3N HCl in butanol it was found essential to use an all glass system. When some polythene tubing was incorporated in the system some of the plasticizers from the tubing dissolved in the 3N HCl BuOH reagent. These carried through into the final derivatives and gave numerous peaks, especially at the higher elution temperatures in the region of the lysine derivative and butyl stearate peaks.

2.12 HYDROLYSIS PROCEDURE

A simplification was made to the hydrolysis technique. As this investigation was primarily concerned with lysine, and not cystine or methionine the hydrolysis procedure could be speeded up by omitting the deaeration /N_2 flushing step that Roach and Gehrke, 1970, employed. Lysine is stable to oxidation under hydrolysis and therefore the presence of dissolved air is of little consequence. In a comparative study no significant difference was detected for the recovery of lysine from hydrolysates that had not undergone deaeration compared with hydrolysates that had been deaerated prior to hydrolysis.

This also had the advantage that it was now easy to use glass culture tubes, for the hydrolysis as a side arm and valve was no longer needed for deaeration (N_2 flushing).

The filtration of the hydrolysates was also modified. Gherke and coworkers used glass fibre filter paper to filter the hydrolysate, before it was made up to volume. This was replaced with hydrophobic glass fibre filter paper (also used in the Foss Electric Pro-Milk instrument, see Chapter 4). The hydrolysate was filtered through a small (ca 1 cm^2) piece of this filter paper held in a two piece stainless steel mini-Buchner funnel. The filter paper was washed with distilled water (ca 2ml) after the hydrolysate/ internal standard mixture had been put through it. The

hydrolysate mixture and washings were collected in a glass culture tube within the Buchner flask, so that the transfer losses were kept to a minimum while still retaining the speed of vacuum filtration. (The hydrolysate would not filter through the hydrophobic paper without vacuum being applied.) After the water wash only 'dry' humin particles remained on the filter paper with no moisture remaining in the paper.

2.13 EQUIPMENT CHANGES AND MODIFICATIONS

With the hydrolysis, esterification, and acylation steps all capable of being performed in the same type of culture tube there was an advantage in performing the drying operations also in the same tube, thereby keeping transfers to a minimum. This reduced time, contamination and helped ensure good quantification. An adaptor was made up for the rotary evaporator so that the culture tube could be fitted directly to it and operated without loss of vacuum. A quickfit flass reduction adaptor was modified by removal of the male end and replaced by a short length (ca 2.5cm) of 2 cm diameter glass tubing which was slightly flared. It was then possible to fit a short piece (ca 2.5 cm) of polythene tubing of suitable diameter past the screw thread of the culture tube, and splayed by the shoulders of the culture tube. This polythene tubing then formed a seal between the culture tube and the splayed end of the glass adaptor on the rotary evaporator. This was the most satisfactory solution out of numerous systems tried. The tube was easy to centre so that it rotated on the axis of the rotary evaporator, the seal did not leak, and the tube was easy to fit and remove. Virtually a glass to glass seal was achieved by this arrangement. (See figure 2.5)

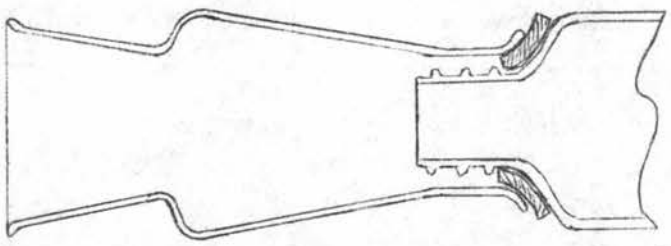


FIGURE 2.5: ADAPTOR FOR FITTING CULTURE TUBES TO A ROTARY EVAPORATOR

The polythene seal was kept soft and flexible by a stream of hot air (ca 90°) from an electric tangential fan heater. Initially this was required as it was found that during the removal of the BuOH after esterification, the BuOH tended to condense on and reflux from the cooler internal upper parts of the rotary evaporator. (This effect occurred with both flasks and culture tubes, and was not a consequence of evaporating from a culture tube.) Not only did this refluxing increase the time for solvent removal but also it increased the risk of contamination from within the evaporator. When the heater was placed under the adaptor joint any refluxing BuOH was evaporated again before it could return to the culture tube (or flask) containing the butyl esters.

An additional advantage of the fitting of culture tubes to the rotary evaporator was that it was now possible to evaporate the milk sample under vacuum prior to hydrolysis. This meant that there was no change in acid normality (which can affect the yield of furosine, pyridosine, and lysine from lactulose lysine during hydrolysis, see Section 1.4 and Table 1.1)

(It is worthy of note that the culture tubes proved to be more satisfactory a vessel for the acylation reaction than the special vessels designed by the Regis Chemical Company for the purpose.)

2.14 MODIFICATION OF THE HYDROLYSIS PROCEDURE: DIALYSIS STUDY

During the course of a computer analysis of some early chromatograms of derivatives made without the inclusion of the ion exchange clean-up of the protein hydrolysate it was realised that the OV-17/OV-210 results indicated the presence of excessive amounts of threonine in milk protein. Data from compositional tables suggested that the valine and threonine peaks should be of similar size, whereas the valine peak was about 1/5 to 1/3 the size of the threonine peak. Examination of the EGA column chromatogram revealed an apparent increase also in alanine, but a multiple peak of about the expected normal size for the glycine and threonine which eluted together. Reference

to the earlier chromatograms of the derivatives prepared from hydrolysates that had been cleaned-up by ion exchange showed no such large peaks for alanine and threonine on the EGA, and OV-17/OV-210 columns respectively. Apparently the contaminant peak was not an amino acid. Subsequent investigation showed that this peak was due to a lactose degradation product produced during the hydrolysis stage (See Appendix 9 for details of this investigation.)

To prevent this contaminant peak from appearing the removal of the lactose before hydrolysis was considered necessary and this was achieved by dialysis (See Section III). It was also thought that the lysine released from the protein during hydrolysis might react with the lactose during subsequent processing thus altering the apparent 'total lysine value' (TLV), but investigation showed no significant difference in TLV figures obtained from non heat treated and heat treated milks that were hydrolysed in the presence and absence of the naturally present lactose (see Appendix 10 for details and results of the dialysis study).

As noted in Appendix 10, that as a result of the dialysis procedure the ratios of internal standard peak area to the areas of proline, phenylalanine, and tyrosine plus glutamic acid peaks can be used as indicators of the change in concentration of the milk protein. It was therefore unnecessary to perform Kjeldahl protein determinations to follow any dilution changes.

As a result of this dialysis study all subsequent hydrolysates were made from dialysed milk.

2.15 REMOVAL OF TRIFLUOROACETIC ANHYDRIDE

The TFAA remaining in the acylation reaction mixture after the acylation step attacked the rubber backing of the teflon liner of the culture tube screw cap, even when stored at 0°. The rubber backing caused some contamination, and atmospheric moisture could now penetrate the tube causing hydrolysis of the esters.

To overcome this problem of derivative storage the TFAA-$MeCl_2$ solvent was removed under vacuum at room

temperature on a rotary evaporator. The derivatives were then redissolved in dry $MeCl_2$ (1ml). The derivatives were stable, when stored at 4° for up to a year, and no deterioration was noticed in the condition of teflon liner. This process also had the advantage of decreasing the sample volume thereby reducing the volume injected onto the column of the GLC. The solvent peak was smaller and the return to base line was rapid.

SECTION III

THE METHOD ADOPTED FOR THE ANALYSIS OF LYSINE IN MILK PROTEIN BY GLC PROCEDURE

2.16 EQUIPMENT

(a) GLC - Varian Aerograph Model 2100 gas chromatograph fitted with two glass columns, two flame ionization detectors (FID), and a dual differential electrometer. This GLC has an oven capable of taking up to four 2m x 0.4mm I.D. U-shaped glass columns, and has direct on column injection. It is fitted with separate, controllable injector and detector blocks. An essential feature is the linear temperature programmer.

(b) Integrator. For accurate work with amino acid analysis it is essential to use an electronic integrator. A Varian Model 485 Digital Integrator was used in this study to determine peak areas.

(c) Recorder. Rikadenki Model B261, two pen, (Rikadenki Kogyo Co., Tokyo).

(d) Ultrasonic cleaner (Varian Aerograph, Walnut Creek, Calif.) was used as a mixer agitator for the contents of tubes at various stages of sample preparation and mixing.

(e) Constant temperature oil bath set at 150° for acylation of the butyl esters.

(f) A hot air oven controllable to 100° and 145° for sample esterification, and hydrolysis respectively. It was convenient to use the GLC oven for these purposes.

(g) Rotary evaporator: a variable speed Buchi Rotovapor-R fitted with an adaptor for the culture tubes.

(h) Culture tubes: glass culture tubes fitted with teflon lined rubber back screw caps were used for hydrolysis, esterification, acylation, and evaporation steps (Kimax, 16 x 150mm Catalogue No. 45066A)

(i) Ultrafiltration cell: an Amicon 402 ultrafiltration cell (Amicon Corp., Lexington, Mass.U.S.A.) fitted with a PM10 membrane (M.Wt. cut off 10,000) was used for the dialysis of the milk samples. The cell (400ml, max.capacity) was fitted to a 2L 316 stainless steel pressure vessel used as a reservoir for the dialysis wash water. The pressure was maintained by a cylinder of compressed nitrogen.

(j) Filter paper: for the filtration of the protein hydrolysates hydrophobic glass fibre filter paper (Foss Electric, Denmark).

2.17 REAGENTS AND REAGENT PREPARATION

(a) Methylene Chloride, AR grade was refluxed over anhydrous calcium sulphate ('Drierite') for 3h before being triple distilled, with the fraction distilling over between 40° and 41° being collected. This was stored over anhydrous calcium sulphate in a glass bottle fitted with a ground glass top.

(b) n-Butanol (BuOH), AR grade was also refluxed over anhydrous calcium sulphate, distilled, and then stored over further anhydrous calcium sulphate, in a glass bottle fitted with a ground glass top.

(c) The 3N HCl in BuOH was made as discussed in Section 2.11, by bubbling dry HCl gas into the purified n-BuOH. To make 3N HCl in n-BuOH, 14.6g of HCl/100g of BuOH is required.

(d) 6N HCl for protein hydrolysis was made up from concentrated HCl, AR grade.

(e) Trifluoroacetic anhydride, AR grade, was obtained from various sources including BDH, and Eastman Chemicals. When not in use it was stored at -20° in a freezer to reduce hydrolysis by atmospheric moisture.

(f) Internal standard: The 4-(amino methyl)- cyclohexanecarboxylic acid (tranexamic acid) was obtained from Aldrich Chemical Co. Inc., New York.

2.18 PREPARATION OF CHROMATOGRAPHIC COLUMN PACKINGS

The packings were made according to the method of Gehrke, et al., 1971, and Kaiser et al., 1974, but used Varaport 30 as the support.

(a) Column packing number 1. 2.0% W/W OV-17, 1% W/W OV-210

Materials OV-17 (Varian Aerograph, Walnut Creek, Calif.)
OV-210 (")
Varaport 30 ")

Varaport 30, 100-120 mesh (29.1g) was weighed into an 0.5l round bottom quickfit flask, and then covered by distilled anhydrous AR grade acetone until the liquid level was ca 3-4mm above the packing. Then 30ml of solution (i) containing OV-17 (0.6g) and 30ml of solution (ii) containing OV-210 (0.3g) in the same grade of AR acetone were added to the flask containing the Varaport 30 in acetone. The solvent was slowly removed with the rotary evaporator under partial vacuum over a period of about 0.75h. Care was taken as dryness was approached not to rotate the flask on the evaporator too quickly thus minimising the fracturing of the Varaport 30 support.

The prepared packing was then transferred into clean, dry glass chromatograph columns (ca 2m x 4 mm I.D.). This was most easily accomplished by plugging one end of the column with silanized glass wool, and this end then attached to a water operated vacuum pump. To the other end of the column a small filter funnel was connected by a short piece of rubber tubing. With the vacuum pump drawing a small quantity of air through the column the dry, free flowing column packing was poured into the funnel. The air flow progressively packed the packing material from the glass wool blocked end back to the funnel end. Gentle tapping

assisted the settling. The funnel was then removed and that end plugged with silanized glass wool as well.

Prior to use the columns were conditioned in the GLC oven at 250° for 12h with a gas flow of about 25ml/min of dry, oxygen free nitrogen carrier gas.

It was found that the commercially prepared OV17/OV210 column (Supelco. Inc., Bellefonte, Pennsylvania) gave superior separation (See 2.8).

(b) Column packing number 2. 0.65% EGA

Materials: EGA (ethylene glycol adipate, from Varian Aerograph, Varaport 30 80/100 mesh

The method used was very similar to that used for packing 1. Varaport 30, 80/100 mesh (29.805g) was weighed into an 0.5l round bottom quickfit flask and then covered to about 3-4mm by anhydrous AR grade acetonitrite. Then 25ml of a solution of EGA (0.195g) in anhydrous AR grade acetonitrite was added to the contents of the flask which was then attached to a rotary evaporator. The packing was then dried slowly over about 0.75h. When the packing was still damp but not adhering to the side of the flask, the flask was removed from the evaporator and the inside walls of the flask were washed with a few ml of anhydrous acetonitrite. The flask was returned to the evaporator for further evaporation of solvent. When the packing was only slightly damp the vacuum was increased and the flask immersed in a 60° water bath until solvent removal was complete.

The glass chromatography columns (ca 2m x 4mm I.D.) were packed by the method described for packing 1.

Prior to use the columns were conditioned in the GLC oven at 215° for 12h with a carrier gas (oxygen free dry nitrogen) flow of about 25ml/min.

2.19 PREPARATION OF THE PROTEIN HYDROLYSATE

(a) Dialysis

Skim milk (30ml) was pipetted into the ultrafiltration cell and diluted with distilled water (ca 120ml) from the pressurized reservoir. The distilled water in the reservoir

maintained the total volume in the cell at 150ml. The reservoir and cell were run at a pressure of 2 bars (ca 30 p.s.i) for about 10h during which time a total of 1.2 l of wash water passed through the cell. After 10h the wash water flow was stopped while the pressure on the cell was maintained until the total volume in the cell was maintained until the total volume in the cell had dropped to just under 3 times that of the original aliquot of milk, i.e. a final volume of about 80ml.

A biuret test on the permeate gave a negative result, and a Molisch test on the final few millilitres of permeate was also negative, indicating complete, or near complete removal of lactose had been achieved without detectable loss of protein.

The dialysed milk was then made up to 90ml, and an aliquot (1ml) transferred to a culture tube for hydrolysis, and another aliquot (15ml) to a Kjeldahl digestion flask. (As was mentioned in 2.14 it was actually unnecessary to make the protein determination, but it was used as a backup check)

(b) Hydrolysis

The sample of milk was dried under vacuum in a 60° water bath, and then 6N HCl (12ml) was added. The tubes were then put in a hot air oven at 145° for 4h, removed, cooled, and then internal standard (5ml of 0.1mg/ml tranexamic acid in 0.1N HCl) was added. The tube was recapped and the contents thoroughly mixed before being filtered through hydrophobic filter paper (see 2.12) and the filter washed with distilled water (ca 2ml). After thorough mixing the solution was evaporated to dryness in a rotary evaporator in a water bath (60°).

Anhydrous $MeCl_2$ (ca 5ml) was added to form an azeotrope with any remaining water, and this was then removed under vacuum.

2.20 PREPARATION OF THE DERIVATIVES

(a) Esterification

To the dry hydrolysate/I.S. mixture, 3N HCl in n-BuOH (10ml) was added, and mixed in the ultrasonic bath

for 1min, before being put into a hot air oven at 100° for 15min. After cooling the excess 3N HCl in n-BuOH reagent was removed under vacuum in a rotary evaporator in a 60° water bath.

A further aliquot (ca 10ml) of $MeCl_2$ was added and then removed under vacuum as before, to assist with complete BuOH removal.

(b) Acylation

Trifluoroacetic anhydride (ca 1ml) and methylene chloride (ca 2ml) were added to the butyl esters in the culture tube. The screw cap was tightened firmly and then the tube was put in an oil bath at 150° for 5 min. (The bath was behind a safety screen in case of explosion.) The tubes were only 25% immersed and the reaction mixture refluxed inside the tube on the cooler, exposed tube walls.

After cooling the mixed solvent was removed under vacuum, using a water bath at room temperature (ca 20°), and anhydrous $MeCl_2$ (1ml) was added to redissolve the N-TFA-n-butyl esters. The derivatives were then stored at 0° until chromatographed.

The overall process is outlined in block diagram from in figure 2.6.

2.21 REPRODUCIBILITY

Although difficulty was experienced in the initial development of the method once the tranexamic acid was adopted as the internal standard the method became much more reproducible.

In one study four samples of the same milk were independently hydrolysed and derivatized. (The preparation of the samples did not include the dialysis step as that development had not been investigated at that time.) The derivatives of each sample were chromatographed once and the ratio of peak areas for lysine:internal standard calculated. The coefficient of variation between the four ratios was 0.63%. This is exceptionally low and is not considered to be typical, even though the investigation was made in order to determine reproducibility. It should be noted that these samples were all hydrolysed and

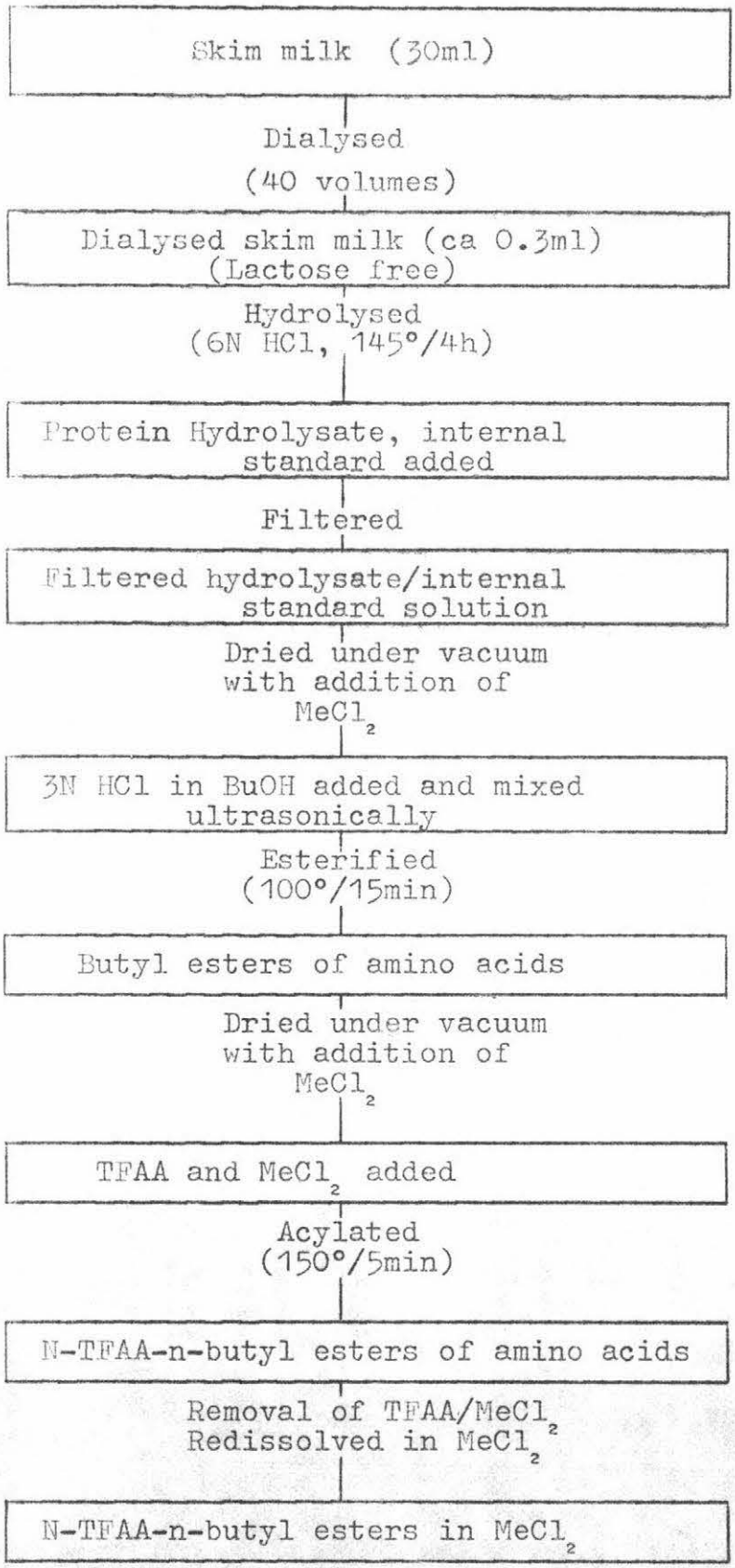


Figure 2.6 BLOCK DIAGRAM FOR THE PRODUCTION OF N-TFA-n-BUTYL ESTERS OF AMINO ACIDS (METHOD DEVELOPED FOR SKIM MILK)

derivatized simultaneously and this would reduce the variability of the results.

Analysis of a total of 10 duplicates gave a coefficient of variation between duplicates of 2.4%, i.e. a standard error of the mean of duplicate analyses of 1.7%, or a 95% confidence range for the mean ratio of lysine area: I.S. area of $\pm$ 3.4% (based on the results of single injections).

A study was made of the reproducibility of the chromatograph/integrator combination by injecting the same derivatized sample 6 times over 3 days. Standard deviations of the amino acid peak areas:I.S. peak areas ranged from 1% to 12%. The ratios with the largest standard deviation were for small peaks (such as methionine), or else peaks which were not readily separated from another peak eluting at about the same time (such as serine which elutes just prior to the isoleucine plus leucine peak).

In the same study it was found that the ratios of the lysine peak area to the areas of proline, phenylalanine, tyrosine, isoleucine, and alanineall had coefficients of variation of about 1.9%. This figure is not significantly different from the figure of 1.6% obtained for the ratio of lysine to internal standard. Therefore a significant proportion of the error between duplicate samples is due to variability between injections of the same sample derivatives. To give mean values for a given sample accurate to 1% with 95% confidence would require 16 repeat injections to be made.

Because of the number of variables it is difficult to effectively assess the reproducibility of the method, but on the basis of the analysis of simultaneously derivatized duplicates, the confidence range for the mean of one injection of each duplicate is likely to be approximately 3% to 3.5%.

2.22 QUANTIFICATION OF THE LYSINE RESPONSE

(a) Preparation

A standard solution of lysine monohydrochloride (0.25mg/ml in 0.1N HCl) was prepared, various aliquots (from 0.0ml to 10.0ml) were derivatized in the presence of I.S. (5ml of 0.1mg/ml tranexamic acid), and the ratios of lysine

I.S. peak areas determined.

The moisture content of the lysine monohydrochloride was determined by drying at 100° ± 1° for 16h.

(b) Results and Calculations:

(i) Moisture content of the lysine monohydrochloride = 3.9%

Therefore the effective dry lysine content of the standard solution was

$$0.25\frac{mg}{ml} \times \frac{146.19}{182.69} \times \frac{96.1}{100}$$

= 0.1922 mg/ml

(ii) Peak area ratios. The values of the ratios found for the corresponding number of millilitres of standard solution derivatized are given in table 2.3.

TABLE 2.3 LYSINE RESPONSE STANDARDISATION

Lysine (ml of std)	0	0	2	2	
mg of pure lysine	0	0	0.3844	0.3844	
Lysine area / I.S. area	0	0	0.723	0.706	

Lysine (ml of std)	5	5	5	10	10
mg of pure lysine	0.9610	0.9610	0.9610	1.922	1.922
Lysine area / I.S. area	1.793	1.696	1.733	3.424	3.452

Regression analysis of this data gives equation 2.1 for the determination of pure lysine (L), for a given ratio (R) for lysine: I.S. peak areas.

$$L = 0.5606R - 0.0092 \qquad (2.1)$$

$$(\ r = 0.9998\ )$$

Using equation 2.1 it is possible to calculate the number of mg of lysine in an aliquot of milk tested.

2.23 LYSINE CONTENT OF MILK PROTEIN

An aliquot (1ml) of diluted (1ml milk + 2ml water)

raw milk (3.7% protein content) was tested by four separate hydrolyses and subsequent derivatizations. The average ratio of the lysine peak area to the internal standard peak area (5ml of 0.1mg/ml tranexamic acid was added to each hydrolysate) was 1.854. Substituting this value in equation 2.1 the lysine content of the aliquot as 1.030mg.

Allowing for dilution, and converting to the basis of protein content the lysine content of the milk protein is 83.5mg/g, or in terms of nitrogen content, 8.53g/16g N_2.

This figure is higher than the average given in compositional tables (FAO, 1970) viz. 76.3 mg/g or 7.79g/16g N_2 but close to the upper value viz. 8.50g/16g N_2 quoted in the same tables.

2.24 CHROMATOGRAPHIC CONDITIONS USED

Gehrke and coworkers have reported using slightly different conditions in different papers but generally they are similar to the following. For EGA columns an initial temperature of 60°, rising at a programme rate of 6°/min to a final temperature of 210°. For the OV-17/OV-210 columns the initial and final temperatures were 90° and 235°, with the same programme rate. (Temperature programming is essential.) The injector and detector temperatures were typically 250°.

In this study an initial temperature of 70° was used when the EGA column, or both columns were being used, otherwise for OV-17/OV-210 columns a temperature of 90° or 100° was found to be satisfactory. A programme rate of 4°/min was found to give good separation and reproducibility. The final temperature was generally 235°. The injector and detector temperatures were both 250°.

The carrier gas (N_2) flow rate was 30ml/min. Flame ionization detectors were used exclusively. The glass U-tube columns were about 2m x 4 mm I.D.

The amplifier was set at 1×10^{-10} a.f.s. attenuation. The integrator was set to give optimum results according to the manufacturers instructions. The recorder was set, as required at between 5 to 20mV.f.s. About 3 to 4 µl of sample was injected in a typical separation.

CHAPTER 3

THE KINETICS OF LYSINE LOSS DURING THE HEAT TREATMENT OF SKIM MILK: EXPERIMENTAL

3.1 INTRODUCTION

Having adapted a method for determining lysine so that it could be used to determine lysine in milk, a trial was made in which some skim milk was canned, heat treated under known conditions, and then the acid available lysine level determined.

3.2 HEAT TREATMENT OF THE RAW SKIM MILK

Raw skim milk (3.7% protein) was filled into 301 x 407 cans (16oz) and sealed under vacuum. The cans had previously had thermocouples fitted to them so that the temperature of the milk could be measured.

The cans were then heated in a horizontal retort for various times and temperatures as indicated in the results section (3.4). The thermocouples were connected to a Honeywell multipoint chart recorder calibrated in degrees Celsius, so that the overall heat treatment could be evaluated.

3.3 HYDROLYSIS, DERIVATIZATION AND SEPARATION

All heat-treated samples and the unheat-treated control sample were handled in an identical way. All were dialysed, hydrolysed, and derivatized according to the method given in 2. The separation of the derivatives was made using the OV-17/OV-210 column, fitted with an F.I.D. detector connected to an integrator and recorder.

As it was not possible to process all the milk samples concurrently, repeat samples of the control milk, and occasionally the most severely heat-treated milk were included with each group that were hydrolysed and derivatized. This provided a check and reference on the uniformity of the hydrolysis and derivatization procedures. The standard deviation between duplicates was less than 2%.

3.4 RESULTS

(a) Colour

The heat treatments used caused definite browning of

the colour, as is shown in the photograph, figure 3.1. No attempt was made to measure the colour because no suitable instruments were available. However, it was observed that the 100°/2.67h treated sample was of a similar colour to the 110°/0.905h, treated sample, while the 110°/2.72h sample was almost identical to the 120°/0.980h sample. Within a given temperature series all samples were noticeably different in colour.

1. Control
2. 100°/2.67h
3. 120°/0.86h
4. 110°/2.72h
5. 115°/2.11h
6. 120°/2.14h

FIGURE 3.1: A PHOTOGRAPH OF A SELECTION OF RETORTED MILKS

(b) Evaluation of Heat Treatment

The temperature/time history of each set of processing conditions for the canned milk was evaluated from the Honeywell recorder chart. Three different temperatures were used in the heat treatment, and a number of different times were used at each temperature.

Although the retort was fitted with an automatic temperature control, the temperature fluctuated about the set temperature by up to $\pm$ 0.5°. For this reason, and because of the need to allow for the come-up-time for the temperature of the milk in the can, in order to evaluate the heat treatment it was necessary to assume a value for

the energy of activation of the reaction causing the loss of TLV. From the literature (reported in 1.8)a typical energy of activation appeared to be about 28Kcal/mole. Using the Arrhenius equation, 1.9, in a modified form

$$\frac{k_2}{k_1} = e^{\left[\frac{E.(T_2 - T_1)}{RT_1T_2}\right]} \qquad (3.1)$$

and using the fact that for a given change in concentration of a component, occurring at two different temperatures, T_1 and T_2, the ratio of the times required for this change to occur is related to the rate constants at the two temperatures by equation 3.2

$$\frac{t_1}{t_2} = \frac{k_2}{k_1} \qquad (3.2)$$

where t_1 is the time required at temperature T_1,
t_2 is the time required at temperature T_2,
k_1 is the rate constant at temperature T_1,
k_2 is the rate constant at temperature T_2

and by combining equations 3.1 and 3.2 to give equation 3.3

$$\frac{t_1}{t_2} = e^{\left[\frac{E\,(T_2 - T_1)}{RT_1T_2}\right]} \qquad (3.3)$$

it is possible to calculate the effective time at the nominal processing temperature. The effect of the come-up-time, and any deviations from the nominal process temperature can in this way be allowed for in calculating the overall effective heating time.

All times reported in Table 3.1 have been calculated using equation 3.3, and are therefore equivalent times.

(c) Chromatographic Separations

The chromatograms produced are typically represented by figures A10.3 and A10.4, and to that of the ion exchange hydrolysate shown in figures 2.2 and 2.3.

The area ratios were analysed using as 'internal' internal standards the five peaks, alanine, isoleucine plus

leucine, proline, phenylalanine plus aspartic acid, and tyrosine plus glutamic acid as discussed in 2.14, 2.21 and A10.3. The ratio of the lysine peak area to the area of each of these five peaks was calculated for each sample, the results being averaged for replicate injections and duplicate samples appropriately. These average ratios were then calculated as a proportion of the corresponding ratio in the unheated control milk sample. As a result five estimates of the proportion of acid available lysine (TLV) left in the milk after heat treatment were obtained for each set of processing conditions. These five values were averaged to give an overall estimate of the TLV in the processed milk relative to the TLV of the control. Table 3.1 gives the relative TLV (RTLV) values and the corresponding heat treatment conditions.

In addition to the series of times at the three temperatures, 100°, 110°, and 120°, one run was done at 115°, as is shown in Table 3.1.

TABLE 3.1 RELATIVE LYSINE CONTENT OF HEAT TREATED MILK

Temperature (°C)	Time (h)	RTLV
Control		1.000
100	0.707	0.986
	1.690	0.975
	2.670	0.957
110	0.548	0.988
	0.905	0.974
	1.920	0.888
	2.720	0.876
120	0.417	0.923
	0.657	0.905
	0.863	0.862
	0.980	0.831
	2.135	0.767
115	2.110	0.828

3.5 CALCULATIONS

Accuracy

It is difficult to statistically analyse the results for reproduceability and accuracy because of the complex way in which the RTLV values were estimated, and also because some milk samples were hydrolysed and derivatized more often than others (such as the control, and the 120°/2.135h samples), and also the number of separations made of each sample was variable for many reasons. As shown in Appendix 10, Tables A10.2a and A10.2b, the average coefficient of variation in the ratios was found to be 1.7% and 2.4% respectively. The average of these is 2.1% and this is probably a reasonable estimate for the RTLV values in Table 3.1. For the case where duplicate analyses were performed and multiple injections were made the standard error of estimates of RTLV in Table 3.1 would be approximately 0.5 to 1%, so that the 95% confidence limits would be about 1% to 2% either side of the values given.

3.6 DISCUSSION

In examining the data more weight should be put on the lowest RTLV values as the relative accuracy of these results will be greater than for the higher values where the change in RTLV is smallest. This means that generally the results of the 120° trial should be considered to be more important than those of the 110° trial which in turn should be weighted more than those of the 100° trial.

(a) Zero Order Reaction

Plotting the RTLV values against heat treatment time on natural scale graph paper gives three lines corresponding to the processing conditions (figure 3.2), that fit the data poorly, except for the 100° data, which fits reasonably closely. No clear trends are visible and it is not possible to state that the reaction has been found to be of zero order, nor is it possible to state unequivocally that it is not zero order.

(b) First Order Reaction

The rate equation for a first order rate reaction is equation 1.10, i.e.

FIGURE 3.2: RELATIVE ACID AVAILABLE LYSINE VALUES VERSUS HEAT TREATMENT TIME

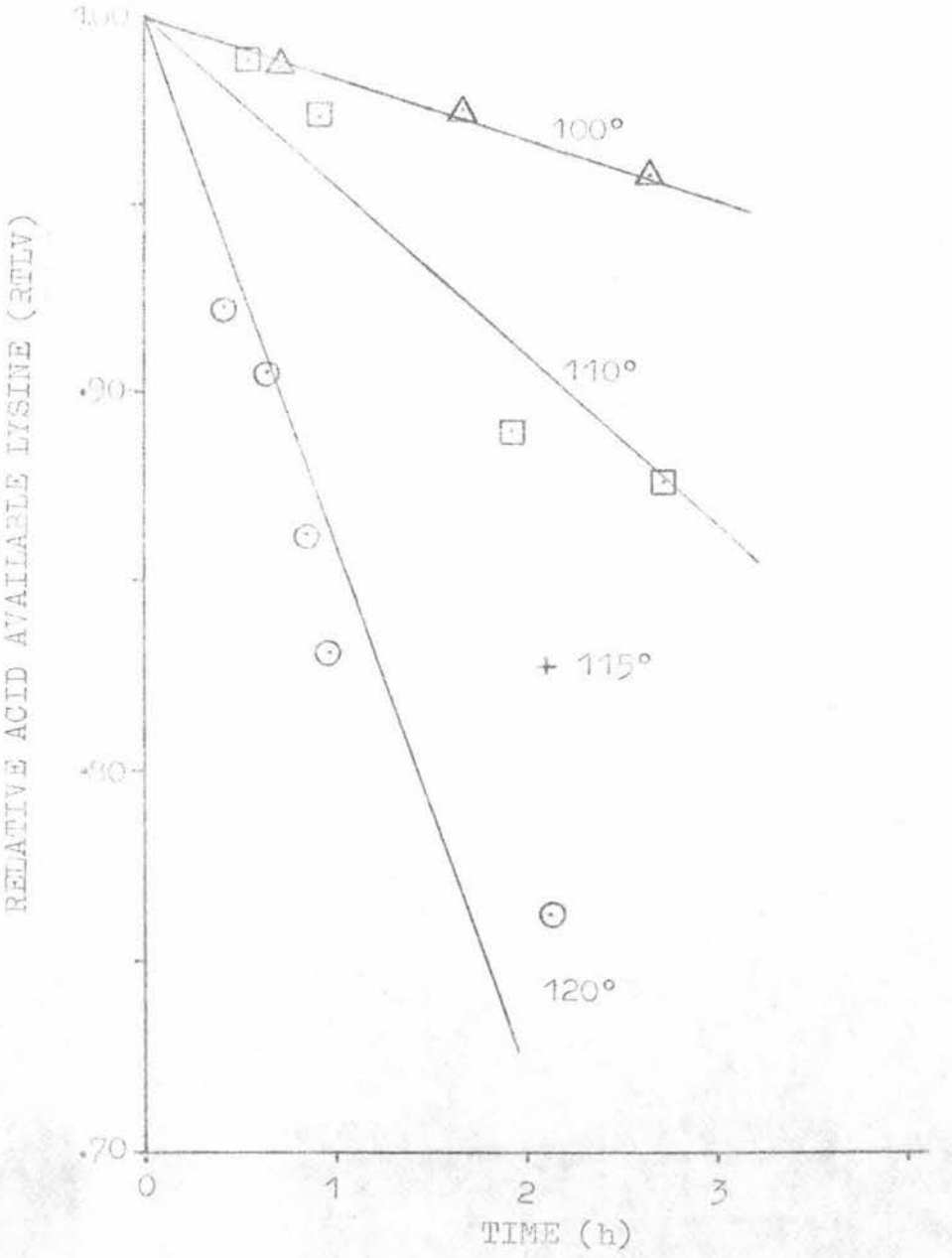
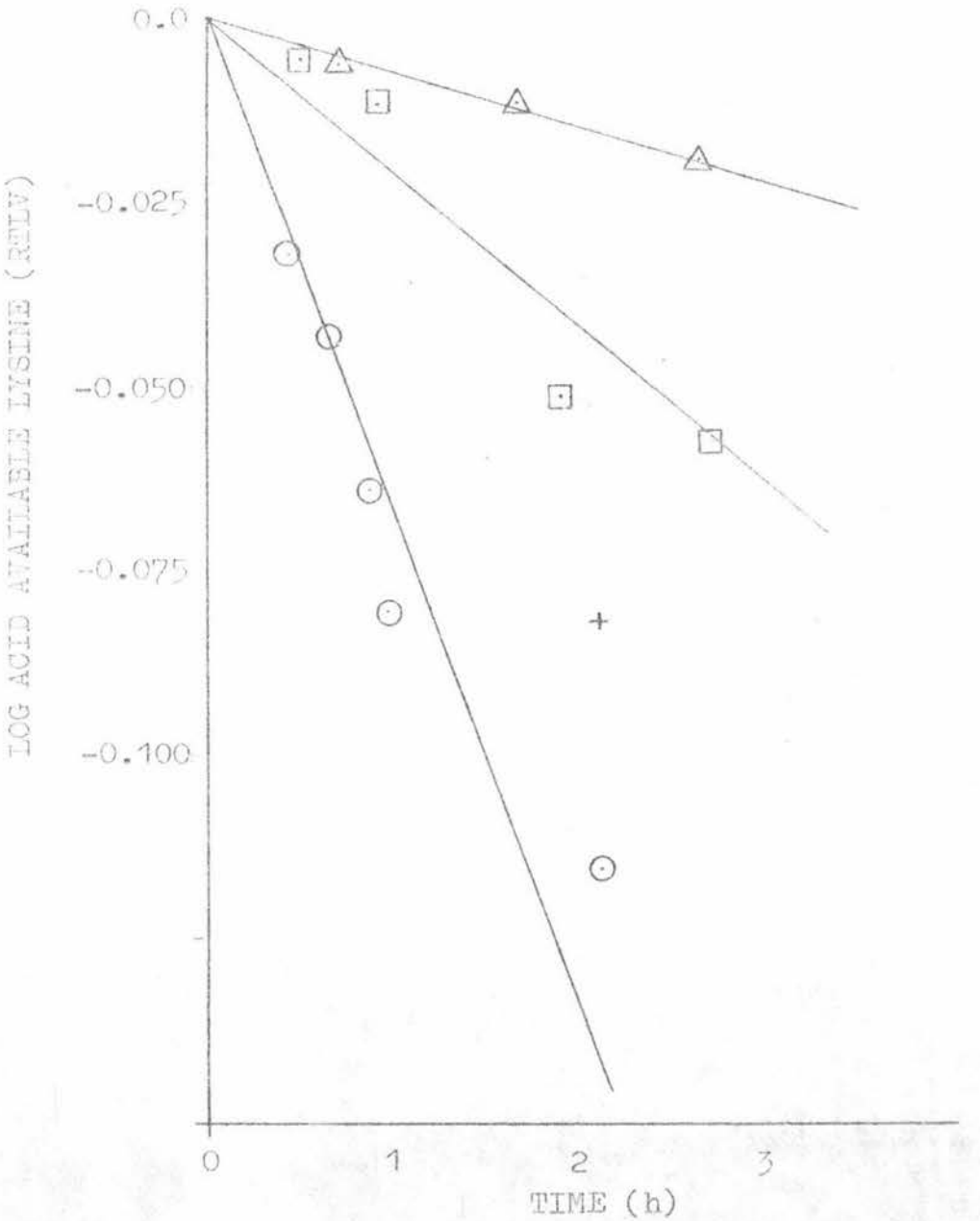


FIGURE 3.3: LOG. RELATIVE ACID AVAILABLE LYSINE VALUES VERSUS HEAT TREATMENT TIME



$$\frac{-dc}{dt} = kc \qquad (1.10)$$

or in its logarithmic form

$$\ln \frac{C}{C_O} = kt \qquad (3.4)$$

(for reactant loss, k is negative)

which implies that if the logarithm of the RTLV values is plotted against time, and if the reaction is of the first order then, at a given temperature a straight line will result.

When this is done for the RTLV values (figure 3.3) while the fit is better than for the zero order plot, it is not good enough to conclude that the reaction is definitely a first order one.

(c) Second Order Reaction

Consideration of the reaction believed to be involved in the loss of lysine availability suggests that being a bimolecular reaction it may well be a second order reaction.

For a reaction of the type

$$A + B \longrightarrow \text{products}$$

if C_{A_o} is the concentration of A at time t_o

C_{B_o} is the concentration of B at time t_o

X_A is the fraction of A converted to product and

X_B is the fraction of B converted to product then

$$\frac{-dC_A}{dt} = k\, C_A\, C_B = \frac{-dC_B}{dt} \qquad (3.5)$$

and

$$C_{A_o}\, X_A = C_{B_o}\, X_B \qquad (3.6)$$

from which it is possible to derive

$$\ln \left(\frac{1 - X_B}{1 - X_A} \right) = -(C_{B_o} - C_{A_o})kt \qquad (3.7)$$

where k is the rate constant, and

t is time.

But as C_{B_o} and C_{A_o} are constant, equation 3.7 can be rewritten as

$$\ln\left(\frac{1 - X_B}{1 - X_A}\right) = -Kt \qquad (3.8)$$

substituting the relationship 3.6 in 3.8 gives

$$\ln\left(\frac{C_{B_o} - C_{A_o} X_A}{C_{B_o} - C_{B_o} X_A}\right) = -Kt \qquad (3.9)$$

While for the milk used it is possible to calculate C_{A_o}, and C_{B_o} where A, and B are lysine and lactose respectively because of the lactose is present in a 7 times greater concentration than the lysine (on a molar basis), the change in the numerator of the log.term is small, i.e. the numerator is effectively constant and equation 3.9 effectively becomes

$$\ln\left(\frac{C_{B_o}}{C_{B_o} - C_{B_o} X_A}\right) = -Kt$$

or equation 3.8 becomes

$$\ln\left(\frac{1}{1 - X_A}\right) = -Kt \qquad (3.10)$$

or in concentration terms

$$\ln\left(\frac{C_A}{C_{A_o}}\right) = Kt \qquad (3.11)$$

Equation 3.11 is in the form of the first order rate relationship 3.4. Therefore although a second order reaction may be possible it is unlikely to give changes in lysine concentration that are significantly different to those calculated by equation 3.4. In light of the general inaccuracy of the data as shown by the previous two figures (3.2 and 3.3), there is no point in considering the second order possibility any further.

It is therefore not readily apparent what reaction order is followed by the loss of lysine availability in heat treated milk.

3.7 DEVELOPMENT OF A MODEL

Since neither the first order (and the pseudo first order), nor the zero order rate expressions give a good fit to the data and while the measurement errors are large and hence a close fit cannot be expected it may still be possible to derive a model that can be used to predict values for TLV more closely than either the zero order or first order rate equations tried.

(a) Theoretical Considerations

According to earlier discussion (Section 1.5 et seq) Mauron and coworkers, and Erbersdobbler demonstrated that acid hydrolysis of heated milk proteins gave a lysine recovery that was considerably higher than that given by nutritional, and certain other chemical tests. This is due to the partial regeneration of lysine from lactulose lysine formed during the Maillard reaction.

If the initial stage of the Maillard reaction, in which lactulose lysine and its intermediates are formed, is first order (or pseudo first order with lactose present in excess) then the loss of nutritionally available lysine, ALV can be calculated from the rate expression, 3.12

$$\ln\left(\frac{ALV}{ThLV}\right) = kt \qquad (3.12)$$

where ThLV is the theoretical, initial lysine content.

But if $RALV = \frac{ALV}{ThLV}$

then $\ln(RALV) = kt \qquad (3.13)$

$\therefore \quad \ln(1 - \Delta RALV) = kt \qquad (3.14)$

But from equation 1.3

$$\Delta ALV = 2.70\ \Delta TLV \qquad (1.3)$$

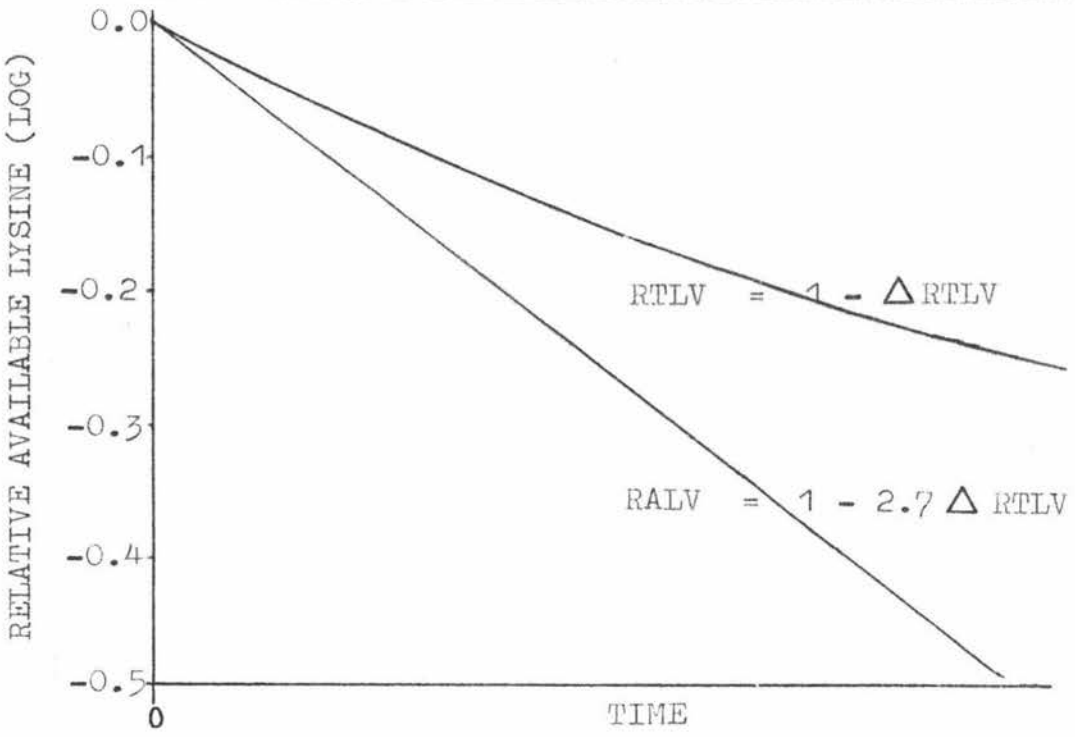
$$\therefore \quad \Delta RALV = 2.70\ \Delta RTLV \qquad (3.15)$$

Substituting equation 3.15 in equation 3.14 gives 3.16

$$\ln (1 - 2.70 \Delta RTLV) = kt \qquad (3.16)$$

i.e. a semilog plot of $(1-2.70 \Delta RTLV)$ against time is linear with slope k. If this is linear then a semilog plot of $(1 - \Delta RTLV)$ i.e. RTLV against time would not be linear but rather concave upwards as is shown in figure 3.4

FIGURE 3.4: THE RELATIONSHIP BETWEEN RALV AND RTLV



If the mechanism for the loss of lysine in heated fluid milk is the same as in the powders used by Mauron and coworkers (they also had one sample of heat treated fluid milk), and the same regeneration of lysine occurred during acid hydrolysis then this relationship (3.16) would be applicable to this study.

3.8 THE MODEL DEVELOPED

In view of equation 3.16 a study was made to find if the experimental data would give an improved fit to a first order model if the observed $\Delta RTLV$ were first multiplied by a constant greater than unity. A computer programme was developed that would increase all the $\Delta RTLV$ values by a constant multiplying factor and show at which value for all data the optimum fit to a first order model occurred. (See Appendix 8 for the computer programme and the basis of its operation.)

The optimum fit occurred when the observed values for ΔRTLV were increased by a factor of 3.43, i.e. the data showed an optimum fit to the model described by equation 3.17

$$\ln (1-3.43\Delta RTLV) = kt \qquad (3.17)$$

For this model the three rate constants for the three trial treatment temperatures are listed in Table 3.2.

TABLE 3.2 MODEL RATE CONSTANTS

Temperature (°C)	100	110	120
Rate Const.(h^{-1})	-0.058	-0.208	-0.760

For the single trial made at 115°, applying the same multiplying factor of 3.43, the k value obtained is - 0.423.

These values can be used to predict RTLV values if the time of heating at one of these temperatures is known, using a different form of equation 3.17. Changing the subject of the formula gives equations 3.18a and 3.18b from which the predicted RTLV values in Table 3.3 have been calculated.

$$\Delta RTLV = \frac{1 - e^{kt}}{3.43} \qquad (3.18a)$$

and as $\Delta RTLV = 1 - RTLV$

$$RTLV = \frac{2.43 + e^{kt}}{3.43} \qquad (3.18b)$$

TABLE 3.3 MEASURED AND PREDICTED RTLV VALUES

Treatment		RTLV Values	
Temperature (°C)	Time (h)	Observed	Predicted
100	0.707	0.986	0.988
	1.69	0.975	0.972
	2.67	0.957	0.958
110	0.548	0.988	0.969
	0.905	0.974	0.950
	0.920	0.888	0.949
	2.72	0.876	0.874

FIGURE 3.5: GRAPH OF OBSERVED AND MODEL PREDICTED VALUES FOR RTLV

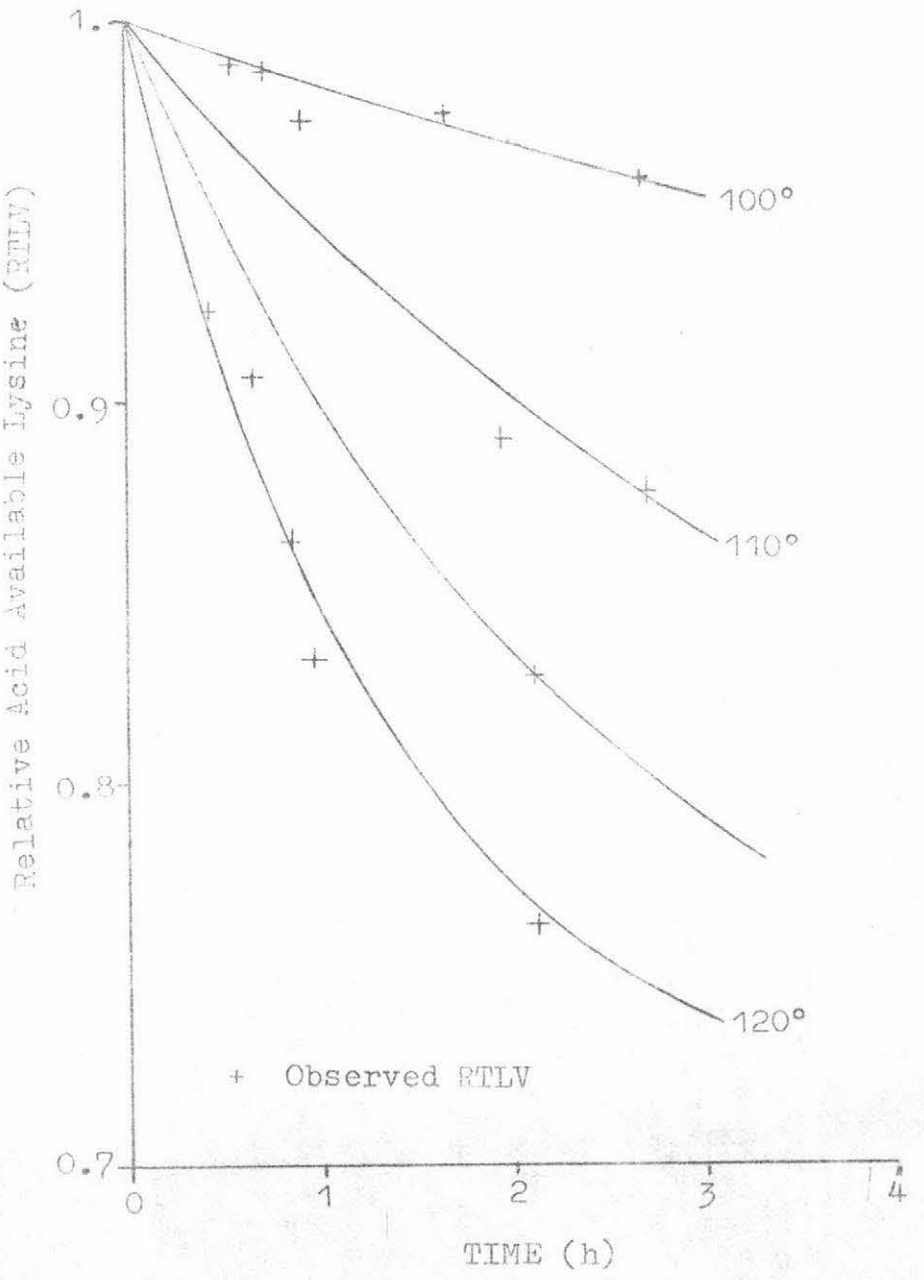


Table 3.3 (cont)

Treatment		RTLV Values	
Temperature (°C)	Time (h)	Observed	Predicted
120	0.417	0.923	0.918
	0.657	0.905	0.882
	0.863	0.862	0.856
	0.980	0.831	0.843
	2.135	0.767	0.762

These figures have been plotted in figure 3.5

3.9 EXAMINATION OF THE MODEL DEVELOPED

(a) Multiplying Factor (F-Value)

Table 3.3 and figure 3.5 show that the model predicts the observed values reasonably well, and that the data overall is a better fit, than is given by the zero order, and first order rate equations as plotted in figures 3.2 and 3.3. The improvement in fit is largely in the results for the 120° trial. The computer analysis showed that for the 100° and 110° trials the best fit of the data occurred for a multiplying factor of 1.0, i.e. the best fit occurred when the log. RTLV values were plotted against time without any modification. (i.e. time or pseudo first order reaction was involved). However the improvement in fit for the 120° data when a multiplying factor was applied to the RTLV values more than compensated for the loss of goodness of fit for the other two sets of data. This is shown in Table 3.4 which lists the rate constants, and their standard error expressed as percentage of the rate constants listed for the multiplying factors (F) of 1.0, and 3.43

TABLE 3.4 IMPROVEMENT IN FIT PROVIDED BY THE MODEL

Temp. (°C)	F = 1.0		F = 3.43	
	Rate Const. (h^{-1})	% Std error	Rate Const (h^{-1})	% Std error
100	-0.0162	15.2	-0.0584	15.5
110	-0.0507	27.3	-0.2085	29.2
120	-0.1419	21.0	-0.7595	9.3

(b) Accuracy

The weighted mean value for the % standard error for the rate constants at F = 3.43 is 19.1%. Therefore the % standard error associated with the geometric mean rate constant (-0.210) found for the model is given by

$$\text{Std. error of GMRC} = \frac{19.1}{\sqrt{n}}$$

where n is the number of determined rate constants on which GMRC is based

$$\therefore \text{ Std.error of GMRC} = 11.0\%$$

Applying a range of $\pm$ 2 standard errors for 95% confidence limits, the GMRC could have a value in the range - 0.210 $\pm$ 0.046, i.e. the GMRC lies in the range -0.164 to -0.256. These values correspond to the GMRC values for F = 2.87 and F = 3.86 respectively. Therefore the 95% confidence limits for the optimum F are 2.87 to 3.86. This wide range is a reflection of the high standard error associated with the calculated value for the GMRC.

This range almost includes the 2.70 value determined by Mauron and coworkers, and if the 99.7% confidence range is examined, the lower F value is 2.54 which then includes the Mauron value. An additional factor to be considered is the possible error in the literature value. No indication is given of the confidence interval for the 2.70 factor found. However Finot, 1973 also derived the relationship (equation 3.19)

$$\text{ILV} = 1.93 \quad \text{DLV} - 6.2 \qquad (3.19)$$

where ILV is the % lysine available to acid hydrolysis but not nutritionally available, and
DLV is the % lysine lost to acid hydrolysis.

Equation 3.19 was derived by Finot from rather more data than was used in calculating equation 1.1 and yet for some reason equation 1.1, from which the 2.70 factor was extracted, was used in the remainder of his examination.

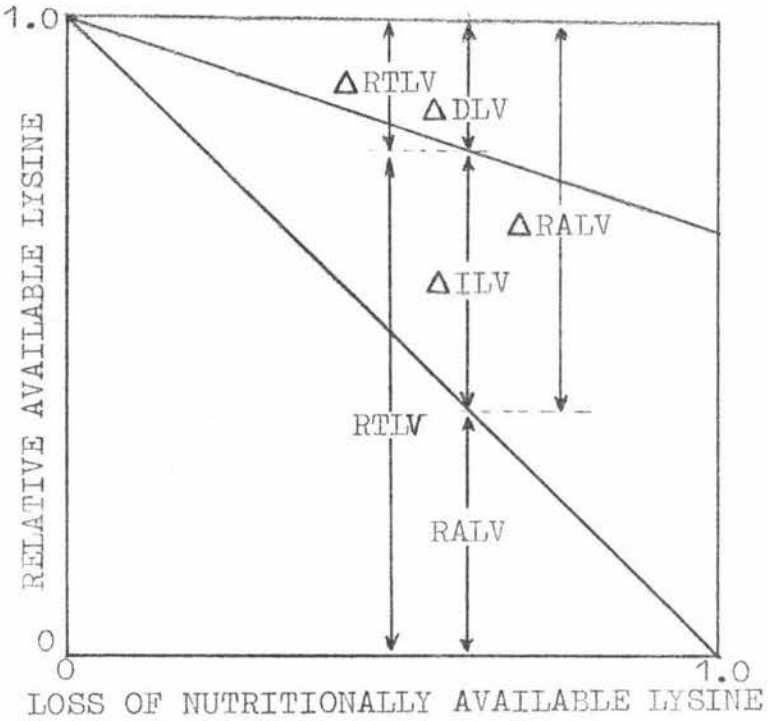
Equation 3.19 implies that a change of 1 unit in DLV gives a change of 1.93 in the ILV value, i.e.

$$\Delta ILV = 1.93 \Delta DLV \qquad (3.20)$$

Reference to figure 3.6 shows that

$$\Delta ILV = \Delta RALV - \Delta RTLV, \text{ and}$$
$$\Delta DLV = \Delta RTLV$$

FIGURE 3.6 THE RELATIONSHIP BETWEEN DIFFERENT LYSINE VALUES



Therefore equation 3.20 becomes

$$\Delta RALV = 2.93 \Delta RTLV \qquad (3.21)$$

The factor of 2.93 is within the 95% confidence range of the F value found in this study. It also shows that some variation in the coefficient has been found.

(c) Energy of Activation for the Model

Using the Arrhenius relationship, in the form of equation 1.9

$$E = \left(\frac{RT_1 T_2}{T_2 - T_1}\right) \ln \left(\frac{k_2}{k_1}\right) \qquad (1.9)$$

and substituting the relevant k, and T values from Table 3.4 gives two estimates for the energy of activation for the reaction represented by the model, viz. 38.5 Kcal/mole, and 36.0 Kcal/mole, based on the ratio of rate constants

at 120° and 110°, and 110° and 100° respectively, the average being 37.2 Kcal/mole.

The standard error of the GMRC is 11%, and using this as an indicator of the standard error for the energy of activation then the 95% confidence range for E is 29.0 to 45.4 Kcal/mole. This is higher than many of the values reported in Table 1.2 but the lower limit still includes some of the literature values. For the temperature range 100° to 120°, for the model, Q_{10} is given by

$$Q_{10} = \sqrt{\frac{k_{120}}{k_{110}}}$$

$$\sqrt{\frac{-0.7595}{-0.0584}}$$

$$\therefore \quad Q_{10} = 3.6$$

However the energy of activation for the loss in RTLV, calculated using equation 1.19, and the corresponding Q10, calculated from the unmodified data (i.e. F = 1.0) are found to be 31.5Kcal/mole, and 3.0 respectively.

The effect on the treatment time of the energy of activation being higher than the assumed 28 Kcal/mole is not great. On average it will reduce the process times by about 1%. As the reaction being measured (the change in RTLV) has an energy of activation of 31.5 Kcal/mole, no alteration to the treatment times is required, as other errors are greater.

3.10 Comparison with Literature Findings

Only a limited amount of data has been reported for changes in either ALVand/or TLV for heated fluid milk, especially for prolonged periods of heat treatment. Most work has concentrated on examining the effect of normal commercial heat treatments, such as pasteurization, evaporation and sterilization. The majority of these studies have been non quantitative in terms of the heat treatments

given, and for most treatments including pasteurization and evaporation no significant change has been reported in either TLV or ALV (Carpenter and Booth, 1974). Adrian, 1974, states that "pasteurization and spray drying are looked upon as being inoffensive. Evaporation however along with sterilization, condensation, ultra high treatment and roller drying provoke 5 - 15% lysine destruction."

Other reports have examined the nutritional effects by following NPU, PER, and BV values for heated milk and these are not readily converted into reliable estimates of lysine content (Porter, 1964; Mauron and Mottu, 1962). In one study (Bujard, et al., 1967) in which a sample of sterilised evaporated milk was used, although the final sterilization conditions were given, the effect if any of the initial evaporation on lysine availability is not stated. Further as a comparison with this study the values for ALV, and TLV quoted are of limited value as the protein and lactose concentration were much higher due to the evaporation prior to sterilization. (Increasing the concentration increases the reaction rate.)

Using an FDNB method that has since been criticised on the grounds that it gives high readings for heated milk proteins, Schober and Prinz (1956) found that after 20 min. heating at 100°, 110°, 120° the FDNB available lysine reduction was, for the average of three samples 1.1%, 3.6%, and 9.6% respectively. This gives approximately a three fold increase in rate for a 10° rise in temperature. These losses are slightly greater than the losses found in this study, but this is to be expected as the FDNB method is sensitive to the free ϵ-amino groups of lysine. (The criticism of the technique used was that it gives higher values due to the FDNB reacting with some Maillard reaction products. Therefore the results could be expected to be higher than those of the more recent FDNB methods as covered in Chapter 1, but lower than those given by acid hydrolysis.) Therefore Schober and Prinz's work is in line with the results of this experiment.

De Vuyst et al., 1972 made a study of the effects of boiling fluid milk on the ALV and TLV levels. Although the precise temperature is not stated, the results are given in Table 3.5 (de Vuyst et al., 1972).

TABLE 3.5 EFFECT OF HEAT TREATMENT ON LYSINE

(after de Vuyst et al., 1972)

Sample	Lysine	Boiling Time			
		0.5	1.0	2.0	4.0
Fresh milk	RTLV	0.98	0.93	0.84	0.81
	RALV	0.90	0.82	0.74	0.67
Fresh milk plus 5% sucrose	RTLV	0.96	0.89	0.76	0.74
	RALV	0.94	0.86	0.80	0.69

The loss of RTLV as is shown by the results in Table 3.5 is much higher than the present study (as is shown by Table 3.1). Even allowing for some temperature increase in the boiling point of milk above 100° due to the effect of the dissolved milk solids this appears insufficient to cause the far greater loss of RTLV observed by de Vuyst et al. De Vuyst, et al., also concluded that the presence of sucrose did not increase the rate of loss of lysine. (They did find that 5% lactose and 5% glucose increased the rate of loss by a factor of about 2.)

Plotting the data of de Vuyst, et al., on semilog paper shows that neither the change in RTLV or RALV follows first order kinetics.

For the fresh milk de Vuyst et al., found

$$\Delta RALV = 1.98 \Delta RTLV,$$

and for milk plus 5% sucrose

$$\Delta RALV = 1.58 \Delta RTLV.$$

They did not examine this aspect and consequently no comment is made about this relationship. These values are below the 2.70 value used by Finot, 1973, and considerably less than the optimum F of 3.43 found in this study. Comparison with the latter value is not entirely valid as de Vuyst, et al., did not obtain RALV values that reduced in a first order fashion whereas in this study such a model

has been used, and it is just supposition that the model actually may represent de Vuyst's RALV values. The fact thatde Vuyst's data is still noticeably curved on semilog paper indicates that if the data were fitted to a first order model, then the multiplying factor required would be larger than the 1.98 and 1.58 values found.

3.11 CONCLUSIONS

(a) In determining the acid available lysine in heated milk there is a large error associated with a single analysis.

(b) Heating of liquid milk causes progressively larger losses of acid available lysine.

(c) The rate of loss of acid available lysine increases threefold for a 10° increase in temperature in the range 100° to 120°. The energy of activation for the reaction giving this loss is about 30 Kcal/mole.

(d) The reaction is of indeterminate order.

(e) A model has been devised which enables the relative acid available lysine values to be calculated after a given heat treatment.

CHAPTER FOUR

DYE BINDING BY PROTEINS

4.1 INTRODUCTION

The ability of proteins to bind dyestuffs has long been recognised and used. Dyeing of textiles including proteinaceous woollen goods has been practised for thousands of years. However, the use of dye binding for the determination of protein content is a much more recent development. In 1927 (Chapman et al., 1927) the first major study on the nature of the combination between acid dyes and protein was reported. Orange G was later found (Fraenkel-Conrat and Cooper, 1944) to combine nearly quantitatively with basic amino groups of proteins, and nearly all the dye binding procedures that have since been developed are based on their method.

4.2 BASIC TECHNIQUE

Although there have been many modifications and adaptions to the original procedure the fundamentals have remained the same: viz.

a) The material to be tested is intimately mixed with a buffered (ca pH2) solution of dye.
b) The reaction mixture is allowed time for the binding to reach completion. This may take a matter of less than a minute or some hours depending on the dye and the proteinaceous material being tested.
c) A clear supernatant is obtained by filtration or centrifugation.
d) The absorbance of the supernatant containing the free unreacted dye is measured by spectrometric means. The amount of dye bound by the protein is calculated by difference between the dye concentration in the initial dye solution and in the supernatant.

4.3 DYES USED

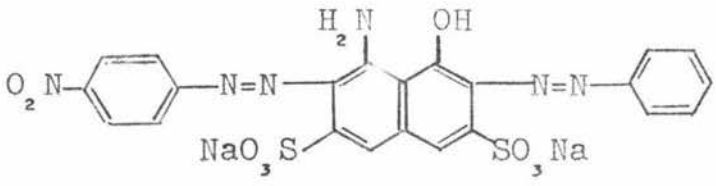
A number of dyes have been investigated but the three most widely used are the following acid azo dyes:

(i) Amido black 10B, also known as C.I. Acid Black 1, Naphthalene Black 10B, Acid Blue Black 58685, Napthol Blue Black, Naphthylamine Black, which willhereafter be referred to as AB.

(ii) Orange G, also known as C.I. Acid Orange 10, which will hereafter be referred to as OG.

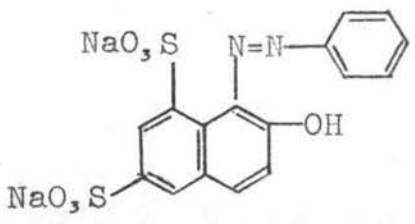
(iii) Acid Orange 12, also known as C.I. Acid Orange 12, Acilane Orange G, which will hereafter be referred to as AO12.

These dyes have the following structures:



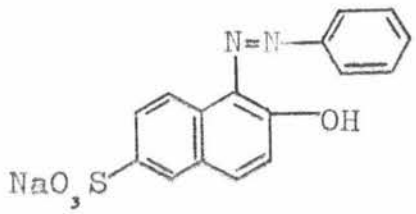
(i) Amido Black 10B

M.W. = 616



(ii) Orange G

M.W. = 452



(iii) Acid Orange 12
M.W. = 350

All these dyes have been extensively studied. (Udy 1956a, 1956b, 1964, 1971; Dolby 1961; Tarassuk et al., 1967; Ashworth et al., 1960; Ashworth and Chandry 1962; Lakin 1970, 1973 a,b,c, 1974, 1975; Sherbon 1967,1974,1975), and applied to a wide range of animal and vegetable proteins including milk, whey, casein, fish meal, wheat, barley, soybeans and rice.

4.4 REACTION THEORY

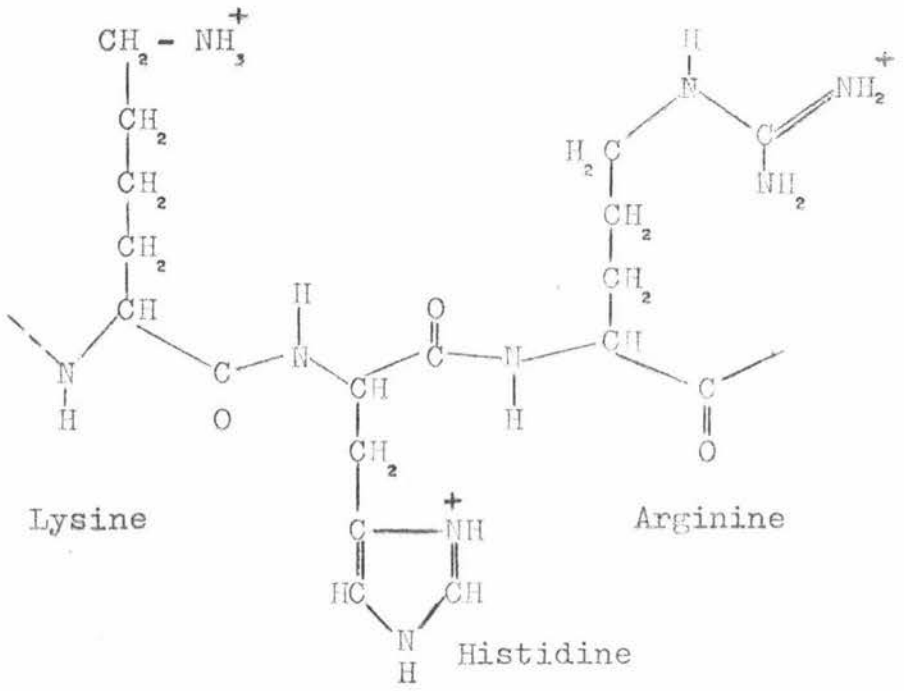
(a) Fundamental Principle

In principle the following reaction occurs (Udy 1971)

$$\text{Protein} + \text{dye}^{-}\ (\text{XS}) \rightleftharpoons \text{Protein: dye}^{+} + \text{dye}^{-}$$

In particular in milk at the normally used pH's (2.0 - 2.2) the dye sulphonic groups are negatively charged, and the ϵ-amino group of lysine, guanidine group of arginine, and the imidazole group of histidine are positively charged. The α-amino terminal groups on the protein chains are also positively charged, but the number of such groups is insignificant in relation to the total number of positively charged basic amino acid groups (hereafter referred to as BAA). In fact it has been shown that α-amino groups have no significant effect on dye binding by protein, almost all α-amino-groups being bound within the peptides (Cohn and Edsall, 1943).

The cationic BAA binding sites are shown in the simulated protein chain



Further at the typically used pH's the protein-dye complex is insoluble and can be readily separated to allow accurate spectrometric determination of the free dye concentration. It is then possible to correlate the dye bound with protein content as determined by the Kjeldahl procedure, and thereafter use the dye binding technique to measure protein levels.

b) Practical Considerations and Findings

(i) Ratio of Dye:Protein

It is important that there is both an excess of dye present, and that this dye is above a limiting concentration, for complete protein precipitation. For AB 6mg of dye would precipitate satisfactorily up to 12 mg of milk protein, provided the final free dye concentration was about 130mg/ℓ (Dolby, 1961). Ratios in excess of the above ratio and free dye concentrations below this figure resulted in significant changes in the linearity of the relationship between the amount of protein and the change in absorbance. Figures are also available for OG (Dolby 1961, Udy, 1956).

(ii) Dye Preferences

The literature indicates that European workers favour AB while American workers first favoured OG and then

AO12 (Sherbon, 1967). Both AB and AO12 are used in commercial protein testing systems. Opinion is divided as to which dye represents the optimum. The relative absorbance indexes for the three dyes are, for AB:AO12: OG, 1.91:1.26:1.00 (Sherbon, 1967), while the relative binding capacities are 1.8; 1.7:1.0 (from Lakin, 1974), showing that overall not only does AB have a greater extinction coefficient, but more dye is bound/g of protein, so that it has a far greater sensitivity than either AO12 or OG. Other workers substantiate these findings. AB is difficult to purify, although a technique has been devised to remove salt, the major contaminant other than water (Lakin, 1970). AO12 on the other hand is readily available with greater purity, is easier to purify and is not as hygroscopic (Udy, 1971). Where relative changes in the absorbance are required however the greater extinction coefficient of AB gives greater changes and it therefore appears to be the most suitable dye.

(iii) Stoichiometry

Almost all workers have found that the extent a given protein binds dye varies with the concentration of free dye remaining in the supernatant liquid. While various theories have been proposed to explain this phenomenon none have been entirely satisfactory. The mechanism of dye binding by milk protein was investigated (Alais et al., 1961) by adding increasing amounts of dye to a known amount of milk protein. The reaction was found to be irreversible, and to progress with the formation of a protein/dye complex involving increasing amounts of dye as further dye was added. At some point during the dye addition the protein/dye complex became insoluble in the reaction medium and precipitated.

The nature of the protein: dye bond has also been examined in the form of a multiple equilibrium situation and various mathematical treatments were developed to explain the equilibrium state (Rosenberg and Klotz, 1960), but none are entirely satisfactory when applied to dye binding by milk because of the wide variety of proteins present in milk. It is stated that "the experimental quantity which is most useful in describing dye-protein

interaction is r, the average number of moles of dye bound per mole of protein". Most other workers have preferred to use the dye binding capacity (hereafter DBC) which is usually quoted as mg. of dye bound/g. of protein. The DBC has also been reported as milliequivalents of dye bound/100g of protein (Lakin, 1973 a,b,c). This latter unit while making comparisons between dyes easier, is not so satisfactory as it mixes the units of measurement, but is useful when discussing bonding stoichiometry.

While some workers have referred to Rosenberg and Klotz, none have successfully applied their theories to the practical situation of dye binding by milk protein. For example it was shown that the linear relationship between the inverse of the free dye concentration in the supernatant and the inverse of the DBC of pure serum albumen found by Rosenberg and Klotz does not hold for milk (Tarassuk et al., 1967). In explanation of this it may be that a certain proportion of binding sites have a lower affinity for dye than others, or alternatively they may be more inaccessible leading to preferential binding at some sites, or it may be that interaction between sites, such as steric blocking or charge reduction occurs as more sites are occupied. This latter view is supported by Vickerstaff, 1954, and also by Rosenberg and Klotz, 1960 who state "it is doubtful that there is a fixed number of binding sites per protein molecule". Deviations from the linear relationship have also been attributed to increasing electrostatic interaction between the bound dye and free dye. It is doubtful whether this is significant in the case of milk protein because the primary binding is believed to be electrovalent involving the ionized sulphonic group and this would not then be 'available' for repulsion of further dye molecules.

In contrast to the possible repulsion it has been stated that non stoichiometry at higher dye concentrations could be due to increased association between dye anions from solution and the dye already bound to the protein by the primary binding mechanism (Lakin, 1973b). The primary

binding is believed to be an electrostatic association, as discussed earlier, and an additional amount of dye is also removed from solutions by hydrophobic association. It has been observed, with pure proteins, that dibasic dyes show greater non linearity than monobasic dyes. However this generalisation is not borne out in an examination of some work on milk in which AB, a dibasic dye, shows great variation in DBC with variation in free dye concentration, while OG, also dibasic shows less variation, but AO12, a monobasic dye, still shows some variation in DBC.

While non-stoichiometry may not be explained by the monobasic, dibasic differences, it has been found that secondary binding will be affected by the structure of the dye, in a manner similar to the way structure is believed to affect substantivity.

Substantivity is the property of a dye being able to bond 'fast' to cellulose fibre. High substantivity dyes have a structure which apparently allows the dye molecule to align itself along the cellulose chain, and any residual attractive forces can then be effective. Many substantive dyes conform to the general type:

$$R_1 - N{:}N - X - N{:}N - R_2$$

where R_1 and R_2 are benzene or naphthalene derivatives,
and X can be based on naphthalene, or some other residues (Vickerstaff, 1954)

Nearly all substantive dyes can be formulated as extended molecules, and deviations from linearity are generally accompanied by a reduction in substantivity.

Clearly AB could be expected to be substantive and AO12 and OG less so. In fact OG has poor substantivity (Lakin, 1973a). Care must be exercised in transposing this effect with cellulose to the binding of dye by milk protein, as it is believed that the primary form of dye: cellulose binding is due to the formation of hydrogen bonds rather than through ionic binding (Vickerstaff, 1954), whereas in milk the primary binding is believed to be an electrovalent

association between the sulphonic acid groups and the BAA sites as outlined earlier.

Substantive dyes give solutions containing aggregates and this shows that such dyes have a tendency to intermolecular association which could be due to hydrogen bonds between the $-NH_2$ group of one molecule and the -OH group of another. Such forces of association are effective only at very short distances so that for aggregation to occur the two molecules must be capable of approaching each other closely, especially in the region of the $-NH_2$ and -OH groups. This would be possible with AB as the $-NH_2$ and the -OH groups which could be involved are in the trans position relative to the primary binding $-SO_3^-$ groups.

Azo dyes by nature are hydrophobic and are made soluble by the addition of sulphonic acid groups. As well as leading to aggregation, this could give dye to protein hydrophobic bonding since substantial parts of the casein molecules are hydrophobic.

It is therefore not surprising that the binding of AB by milk protein has been found to be non-stoichiometric, especially at high free dye concentrations (Dolby 1961, Tarassuk et al., 1967, Sherbon 1967, Lakin, 1973a,b,c, 1974). It is interesting to note that milk protein binds the three dyes, in terms of milliequivalents/g of protein, in the order of their substantivities, viz. AB> AO12> OG (Lakin 1973a).

It was found that while the dye-protein complex, when suspended in the same buffer solution as used in the buffer-dye solution, did not lose any dye on dialysis, but when the complex was suspended in buffer to which urea (2M) had been added, dye in fact did dialyze out. This indicates that hydrogen bonding is involved (Tarassuk et al., 1967). It was not reported whether the separation by dialysis in the presence of urea was complete. If it were it would be contrary to expectations if the primary binding is electrovalent. Lakin, however, does include the possibility of hydrogen bonding occuring as a secondary mode of bonding between already absorbed dye and free dye.

c) Summary

Work reported suggests that the acid azo dyes bind largely to the BAA groups of the proteins. However, the binding of acid azo dyes is not constant for all dyes in terms of milliequivalents of dye bound/g. of protein. Further, for a given dye the DBC of a protein is not constant with varying supernatant dye concentrations. Of the three dyes examined, AB is the most sensitive to a given change in protein concentration.

It seems that there may be up to three types of binding in the protein-dye complex:

(i) Primary electrovalent, between the BAA and dye sulphonic acid groups.

(ii) Hydrogen bonds between the free dye and the bound dye and/or the protein.

(iii) Hydrophobic bonds between the free dye and the bound dye and/or the protein.

4.5 DYE BINDING CAPACITY OF MILK PROTEIN

a) Non-Heat Treated Milk

There is considerable variation reported in the literature for the DBC of unheated milk. Variations due to district, breed, and season have been demonstrated(Tarassuk et al., 1967). Late lactation milk has a different DBC to early and mid-season milk (Dolby, 1961). This may be due to variations in non-protein nitrogen which is determined by Kjeldahl digestion but not by dye binding methods. Also different protein fractions have different DBC values (Tarassuk, et al., 1967), and the relative proportions of the protein fractions change throughout the lactation period.

Some of the variation reported may be due to variations in the purity of the dyes used, and the purity quoted must be closely examined. This is especially important for AB which is more difficult to purify and is hygroscopic. This topic has been dealt with separately in Appendix 5.

The concentration of dye, the protein:dye ratios, and pH used each can, to some extent affect the DBC value, but most workers have used very similar general conditions.

The dye concentrations used lie within the range 0.44g/l to 0.62g/l, and about 10ml of dye is used in the testing of 0.5 ml of milk. The pH values of the buffers used were in the range 2.0 to 2.35 (various workers, reported by Tarassuk et al., 1967). Using a short path length cell the commercial Pro-Milk Mk.II uses AB at a concentration of 0.9384g/l and 20ml of this dye is used in testing 1ml of milk. This gives a working range of 2.0% to 5.5% protein (A/S N.Foss Electric, 1976). Under these general conditions the DBC changes non-uniformly by up to about 3% for protein values in the range 2.8% to 4.5%, i.e. the DBC changes by an amount equivalent to between 0.1% to 0.14% protein (Tarassuk et al., 1967).

The values reported in the literature for the AB, DBC of milk protein, in mg dye/g. protein include

Fresh skim milk	342 to 352	(Ashworth and Chaudry, 1962)
Whole milk	344 to 354	" "
Non-fat milk powder	345 to 367	" "
Milk protein	314	(Lakin, 1973b)
Fresh whole milk	325	(Hadland and Johnson, reported by Tarassuk et al., 1967)
Various whole milks	290 to 342.7	(Tarassuk et al ., 1967)

Examination of the reported results indicates that unless experimental conditions are specified the pseudo-scientific term DBC has little meaning, and may even prove misleading (Lakin, 1973a).

b) Heat Treated Milk

(i) Theory.

The major chemical reaction that the protein in milk undergoes on heating is the Maillard or 'Non Enzymic Browning' Reaction which has been examined elsewhere in this thesis. This reaction in milk principally involves lactose and the ϵ-amino groups of the lysine moieties in the various milk proteins. As dye binding is also believed to involve the ϵ-amino groups of lysine it is reasonable to assume that a milk which has undergone sufficient heat treatment for the Maillard Reaction to occur would show

a decreased DBC and that the decrease in DBC should bear some relationship to the extent of browning, and therefore the heat treatment. Further since such heat treatment is known to affect the nutritional availability of lysine, the DBC should also give some indication of this. This expectation is complicated by the dye binding to both arginine and histidine which also become nutritionally less available after protein heat treatment. Regardless of this complication many workers (Lakin, 1973a; Tarassuk, et al., 1967; Carpenter, 1974; Udy, 1971; Hurrell and Carpenter, 1975; Holsinger and Posati, 1975) have expressed an expectation of a change in nutritional availability of lysine correlating with a change in DBC.

(ii) Reported Findings.

The correlation of dye binding with the BAA's as well as with whole proteins has been extensively reported and a good summary has been compiled (Gullord, 1974). A typical BAA,DBC correlation coefficient found is that for barley, wheat, oats, and triticale of 0.940 (Mossberg, 1966). This correlation has been used in the selection of barley varieties rich in protein, and with a high lysine content. Heat processed cereals and protein foods have shown reduced DBC, although the reductions have not been very large. Autoclaving of soybean at 120° for 45 min., and 120 min. resulted in DBC changes of 3% and 13% respectively (Moran and McGinnis, 1963), while amongst many values reported (Hurrell and Carpenter, 1974) was that for a mixture of albumen and glucose which after heating at 121° for 15 min. showed a 21% drop in DBC.

Unfortunately little real quantitative data is available for heat treated milk. Much of the work using dye binding techniques has been directed towards developing methods for determining the whey protein nitrogen index of skim milk powders (Sanderson, 1970; A/S N.Foss Electric, 1973; T.C.A. McGann et al., 1972 a,b). Some work has been done on liquid milk (Tarassuk, et al., 1967) and it was found that skim milk heated at 120° for 8 min, and 10 min., showed a decrease in DBC (for AB) of 1.8% and 2.8% respectively.

It was found that pasteurization of milk has no effect on DBC, (Vanderzant and Tennison, 1960; Alais, et al., 1961; Tarassuk, et al., 1967). In view of the small differences in DBC detected in milk heated to 120° for 8 and 10min normal kinetic considerations would suggest that under these milder processing conditions the Maillard reaction will not have progressed measurably and no significant reduction in DBC could be expected. In fact no change was detected for milk heated at 90° for 15 min. It was suggested that the denaturation of casein during the initial heating could counter a fall in DBC (Tarassuk, et al., 1967) but this is not likely in view of the fact that casein is considered to be unfolded in its natural state and is little affected by mild heat treatment. It is of interest to note that commercially sterilized milk does not show a significantly reduced DBC for O.G. (Ashworth, 1966) while in contrast it was found that such milk has suffered a 20% drop in biologically available lysine (Mauron and Mottu, 1962). A 10% drop in available lysine in heat sterilized evaporated milk has also been reported (NIRD, 1966). These findings are also supported by the observed 26% reduction in available lysine (as determined by the trinitrobenzene sulphonic acid), in sterilized evaporated milk (Erbersdobler, 1970 reported by Holsinger and Posati, 1975).

Table 4.1 summarises the reported findings;

TABLE 4.1 RELATIVE DYE BINDING CAPACITY OF HEAT TREATED MILK

Sample	Treatment	Dye	RDBC	Reference
Mixed Herd Milk	62.8°/60 min 71.7°/15 min 87.8°/ 5 min	Buffalo Black	N.S.C. " "	Vanderzant and Tennison, 1960
Mixed Milk	63°/60 min 100°/10 min 115°/20 min	AB	N.S.C. 0.989 0.976	Alais et al 1961
Mixed Herd Milk	63°/60 min 74°/15 min 85°/15 sec	AB	N.S.C.	Tarassuk et al, 1967

Table 4.1 (cont)

Sample	Treatment	Dye	RDBC	Reference
Mixed Milk	88°/15 min	AB	N.S.C.	Tarassuk et al., 1967
	93°/1 sec			
	120°/8 min		0.982	
	120°/10 min		0.972	
Skim Milk	3:1 Evaporation	AB	N.S.C.	Tarassuk et al, 1967
Milk	Commercial Evaporated	OG	N.S.C.	Ashworth, 1966
Condensed Skim Milk (Diluted 2.33:1)	120°/2 min	AB	0.987	Extracted from a graph in Tarassuk et al, 1967
	120°/4 min		0.979	
	120°/6 min		0.977	
	120°/8 min		0.971	
	120°/10 min		0.961	

N.S.C. - no significant change

Although the reported changes in DBC for heat treated liquid milk are not large, significant changes have been found in heated skim milk powders. Changes of 13%, and 31% in OG DBC was reported for skim milk powder heated at 100° for 1 hour and 5 hours respectively (NIRD, 1961), while a treatment of 150°C/1hr resulted in 95% destruction of lysine (Adrian, 1972). Changes have also been reported for whole and skim milk powder stored at room temperature. Unfortunately these studies are of little benefit as the moisture content and/or equilibrium relative humidity values were not reported. (As shown by Ebersdobler 1970 and reported by Adrian, 1972, the moisture content of milk powder can greatly influence the rate of loss of available lysine.) It has been found that at 68% ERH, (ca 7.6% moisture) skim milk powder shows a drop in AB DBC of 23% after heating at 70° for 6 hours (Chalmers and Him 1976).

The changes in nutritional properties of skim milk powder as a consequence of storage and heating have been reported elsewhere (Mauron and Mottu, 1962) but as the

reaction medium is in quite a different state compared with liquid milk, and as the Maillard reaction is affected by the moisture content of the powder, any further discussion would be of only minor relevance.

Therefore in the absence of adequate information a study of the effect of heat on liquid milk and on its DBC was undertaken.

CHAPTER FIVE

EXPERIMENTAL: DYE BINDING BY HEAT PROCESSED MILK

5.1 INTENTION

To determine the effect of heat treatment on the DBC of milk protein by processing skim milk for a variety of temperature/time combinations, and then testing for AB DBC.

5.2 HEAT TREATMENT

Culture tubes (Kimax 16 x 150mm, cat no. 45066-A) fitted with teflon lined screw caps were filled with skim milk and preheated to 85° in a boiling water bath. They were then transferred to a hot high velocity air oven, set at 20° above the ultimate treatment temperature. This temperature was maintained for 5 minutes, to bring the temperature of the milk rapidly to the desired treatment temperature, after which the oven was cooled to the desired treatment temperature. The nett effective treatment given during the temperature come up time was calculated as being equivalent to 3 minutes at the treatment temperature.

The oven temperature was stable to $\pm$ 0.3°.

5.3 ANALYTICAL METHOD

(a) Reagent: Amido Black solution. This was made by

(i) dissolving AB (9.87g) in distilled water (ca 3l) heating to 70°, and allowing to cool, and

(ii) dissolving citric acid monohydrate (158.40g) dibasic sodium phosphate dihydrate (19.80g), thymol (3g), and Triton X (1g) in distilled water (ca 2l).

The two solutions were mixed and made up to volume (10l) with distilled water.

This is essentially the formula of Sherbon, 1974.

(b) Spectrophotometer: A Bausch and Lomb Spectronic 20 fitted with a flow through cell adjusted to have an 0.2mm light path.

(c) Centrifuge: A BTL Bench Centrifuge.

(d) Technique Used:

(i) Dye blank. Distilled water (1.0ml) was pipetted into a stoppered cylinder (25ml) and AB solution (20.0ml) was added, the mixture shaken and an aliquot (10.0ml) was centrifuged.

(ii) Milk. After thorough mixing to resuspend any solid that had separated out during the heat processing, a portion (1.0ml) was pipetted into a stoppered cylinder and AB solution (20.0ml) was added and then the mixture was vigorously shaken. An aliquot (10ml) was transferred to a polypropylene conical centrifuge tube, centrifuged (1000g) for 5 minutes to settle the protein-dye precipitate. All tests were done in duplicate.

The supernatant from the milk-dye reaction mixtures were sampled directly into the short path length cell. An initial sample was drawn through the cell, followed by a plug of air and then another sample of supernatant, and the absorbance noted. The duplicate reaction mixture was then sampled similarly. All the samples from a given processing temperature were read in order of increasing time of heating, and then all the reaction mixtures were tested again in the reverse order. After every six readings the cell was washed out thoroughly and the zero absorbance reading checked using distilled water.

The four readings per temperature/time treatment from the replicate readings on the duplicate samples were averaged.

5.4 RESULTS

Absorbance Readings:

Dye blank = 1.3029

TABLE 5.1 ABSORBANCE READINGS FOR HEAT TREATED MILK

Treatment Temp (°C)	Treatment Time (h)	Sample	Absorbance Readings		Mean Absorbance (A)
Untreated		i	0.447	0.448	0.4473
		ii	0.448	0.446	
100	1	i	0.464	0.467	0.4623

Table 5.1 (cont)

Treatment Temp.(°C)	Treatment Time (h)	Sample	Absorbance Readings		Mean Absorbance (A)
		ii	0.459	0.459	
	2	i	0.476	0.476	0.4748
		ii	0.474	0.473	
	3	i	0.492	0.492	0.4888
		ii	0.484	0.487	
	15	i	0.570	0.570	0.5703
		ii	0.571	0.570	
110	1	i	0.480	0.480	0.4810
		ii	0.481	0.483	
	2	i	0.503	0.505	0.5063
		ii	0.509	0.508	
	3	i	0.528	0.527	0.5300
		ii	0.532	0.533	
	6	i	0.578	0.577	0.5783
		ii	0.578	0.580	
120	1	i	0.532	0.534	0.5308
		ii	0.529	0.528	
	2	i	0.574	0.578	0.5763
		ii	0.577	0.576	
	3	i	0.613	0.611	0.6140
		ii	0.615	0.617	

5.5 CALCULATIONS

(a) Reproducibility:

(i) Between Replicate Readings:

The mean difference between replicate readings = 0.0013

The standard deviation of the difference between replicate readings = 0.0011

(ii) Between Duplicate Means:

The mean difference between duplicate means = 0.0026

The standard deviation of the difference between duplicate means = 0.0016

(b) Relative Dye Binding Capacity (RDBC)

The RDBC is the DBC of milk relative to the DBC of non-heat treated milk, i.e.

$$RDBC = \frac{A_{dye} - A_{treated}}{A_{dye} - A_{untreated}} \qquad (5.1)$$

$$= \frac{1.3029 - A_{treated}}{0.8556}$$

TABLE 5.2 RELATIVE DYE BINDING CAPACITY OF HEAT TREATED MILK

Treatment Sample	100/1	100/2	100/3	100/15	110/1
RDBC	0.982	0.968	0.951	0.856	0.961

Treatment Sample	110/2	110/3	110/6	120/1	120/2	120/3
RDBC	0.931	0.903	0.847	0.902	0.849	0.805

5.6 DISCUSSION

Theoretical Considerations:

Plotting the RDBC values against the time of heat treatment (figure 5.1) gives three curves corresponding to the processing temperatures indicating that the loss of DBC does not follow zero order kinetics. The equivalent log plot (figure 5.2) also results in three curves, indicating that the reaction is not first order either. This is not surprising as there are at least 3 BAA's binding the dye, and possibly 3 mechanisms involved in the actual binding of the dye to the milk protein.

The extent to which arginine and histidine react with lactose when liquid milk is heated has not been reported in the literature. Lysine is the major amino acid that reacts with lactose, and it is this reaction that has been widely reported. The lack of literature data would suggest that histidine and arginine in milk are more stable than lysine to heat treatment, and therefore even if all the ϵ-amino lysine moieties have reacted with lactose, there would still be available some

FIGURE 5.1: EFFECT OF HEAT TREATMENT CONDITIONS ON RDBC VALUES

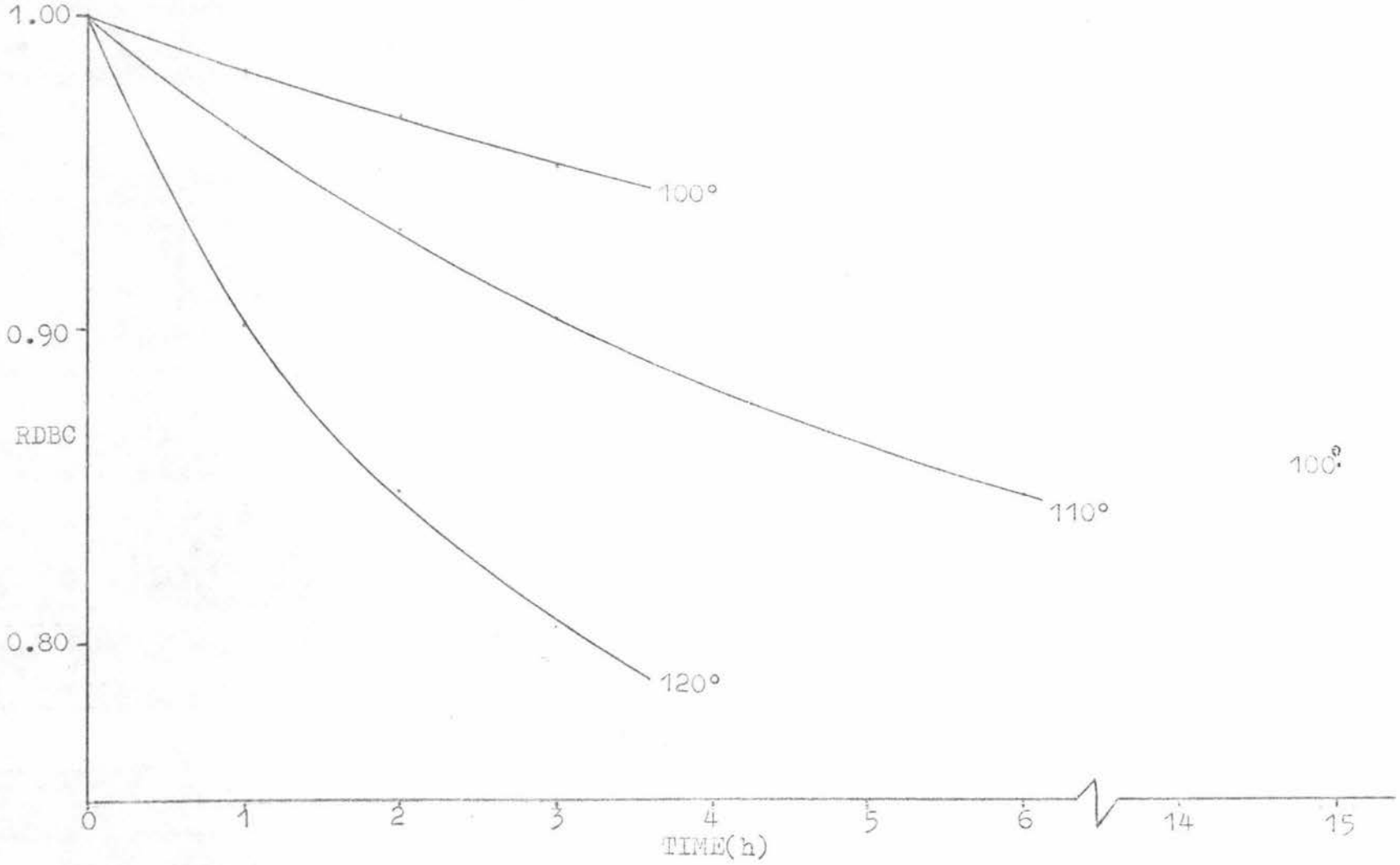
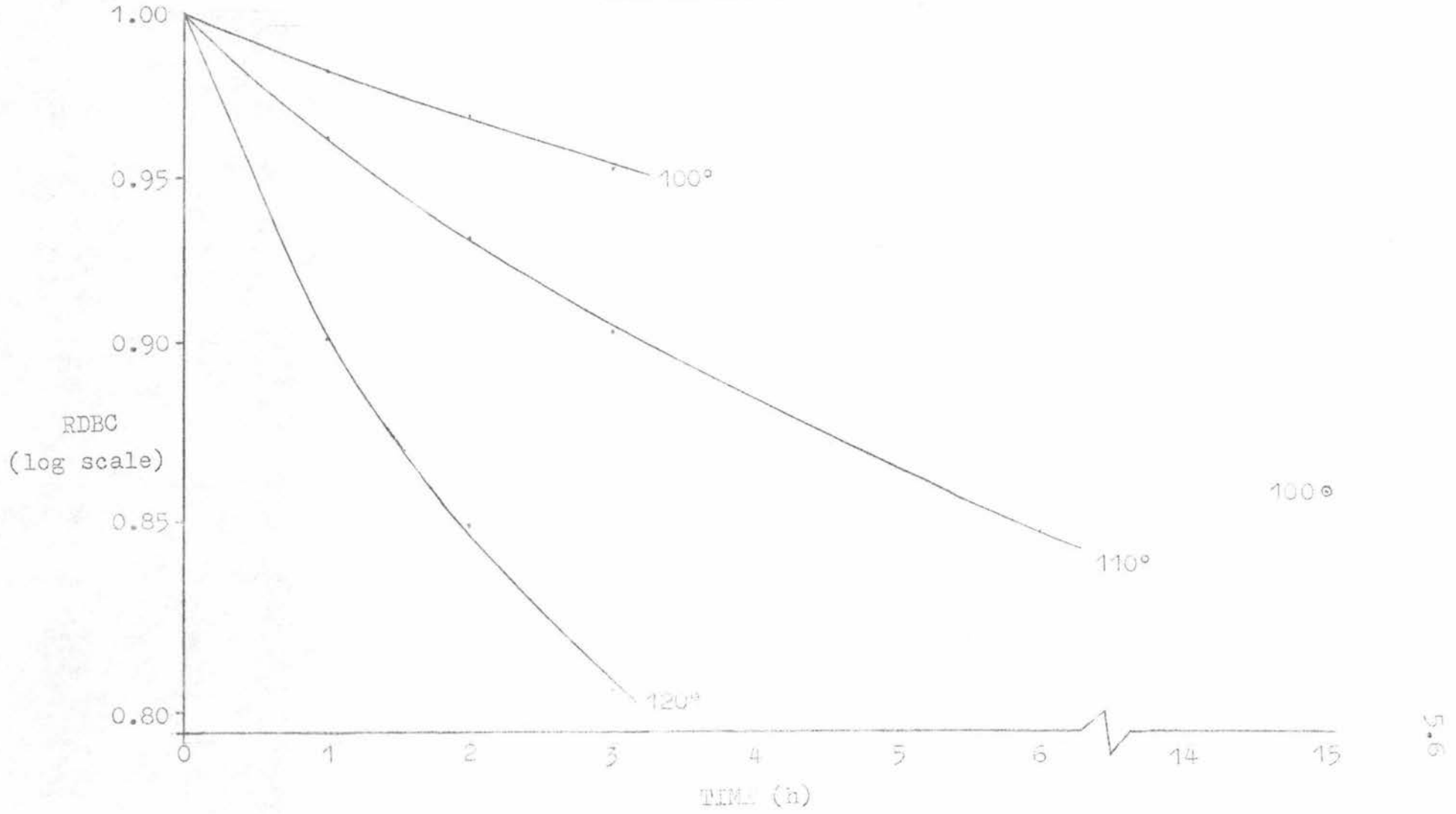


FIGURE 5.2: EFFECT OF HEAT TREATMENT CONDITIONS ON LOG RDBC VALUES



BAA sites for dye binding and so even when the majority of the lysine binding sites are blocked by lactose, the milk protein would be expected to show a considerable DBC. Assuming that the arginine and histidine binding properties are not significantly affected by the processing then the residual DBC after the loss of the lysine sites would be in proportion to the relative number of moles of arginine and histidine to the total moles of BAA's. Typically the composition of cow's milk, with regard to the BAA's, is as follows, (FAO, 1970):

TABLE 5.3 BASIC AMINO ACID COMPOSITION OF MILK

BAA	mg/gN	mM/gN	% of BAA
Arginine	205	1.18	21.1
Histidine	167	1.08	19.2
Lysine	487	3.33	59.7

Assuming that (i) the three BAA's have equal dye affinities,
(ii) the arginine and histidine binding sites are not significantly affected by the heat treatment, then the limiting value for the RDBC will be 40.3%. Therefore if the change in DBC represents the change in lysine binding sites, the relative loss of lysine available for binding (ΔRDL) will be related as below

$$\Delta RDL = \left(\frac{100}{100 - 40.3}\right) (\Delta RDBC)$$

$$= 1.675\ \Delta RDBC \qquad (5.2)$$

where ΔRDL is the change in relative dye available lysine (RDL)

This is represented in diagramatic form in figure 5.3.

Two other forms of this relationship are

$$\Delta RDL = 1.675\ (1.0 - RDBC) \qquad (5.3)$$

$$\text{and } RDL = 1.675\ RDBC - 0.675 \qquad (5.4)$$

The values calculated from the experimental data using equations 5.3 and 5.4 are listed in Table 5.4.

FIGURE 5.5: THE RELATIONSHIP BETWEEN RELATIVE DYE BINDING CAPACITY (RDBC) AND RELATIVE DYE AVAILABLE LYSINE (RDL)

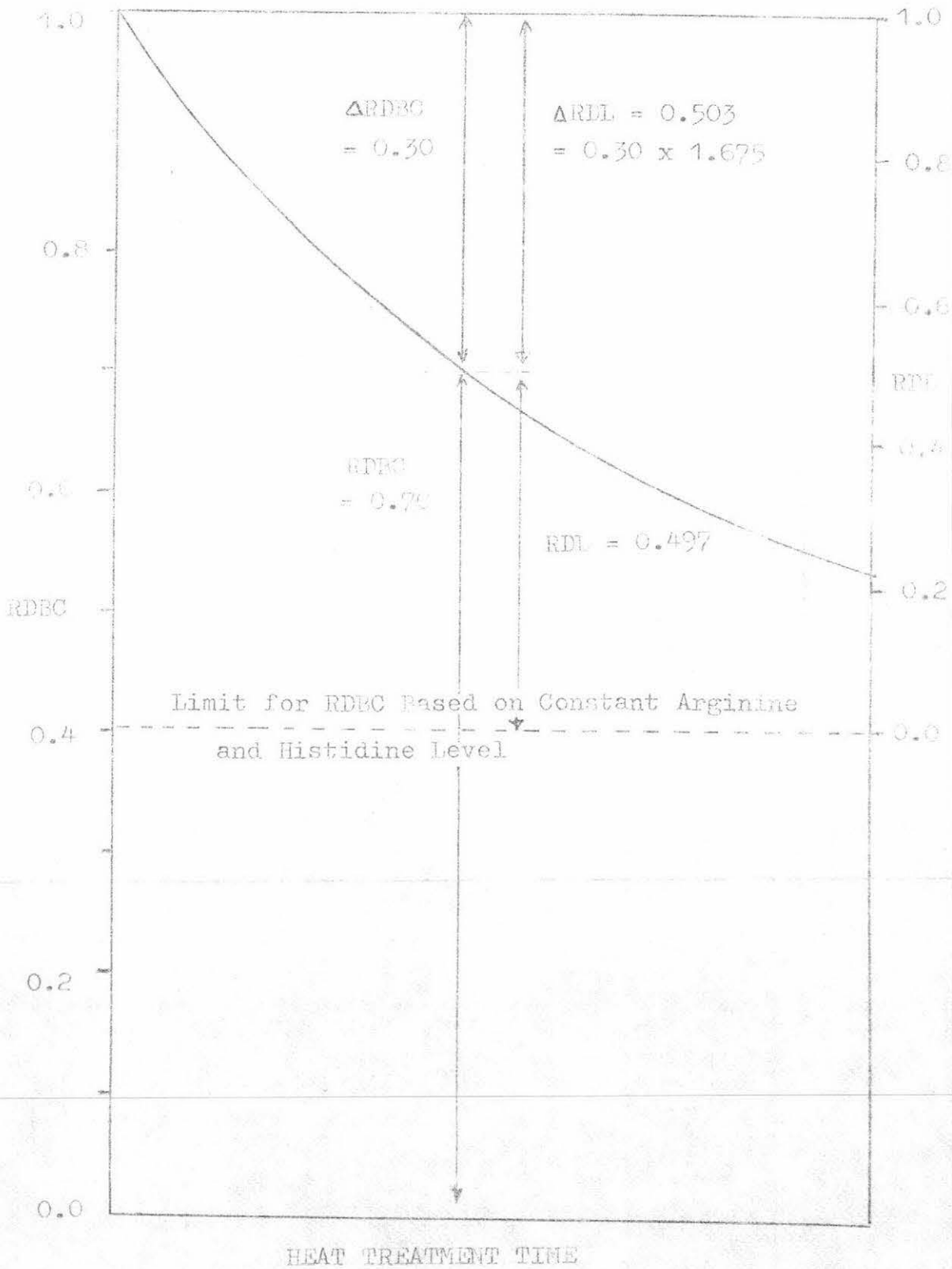


FIGURE 5.4: EFFECT OF HEAT TREATMENT CONDITIONS ON RDL VALUES

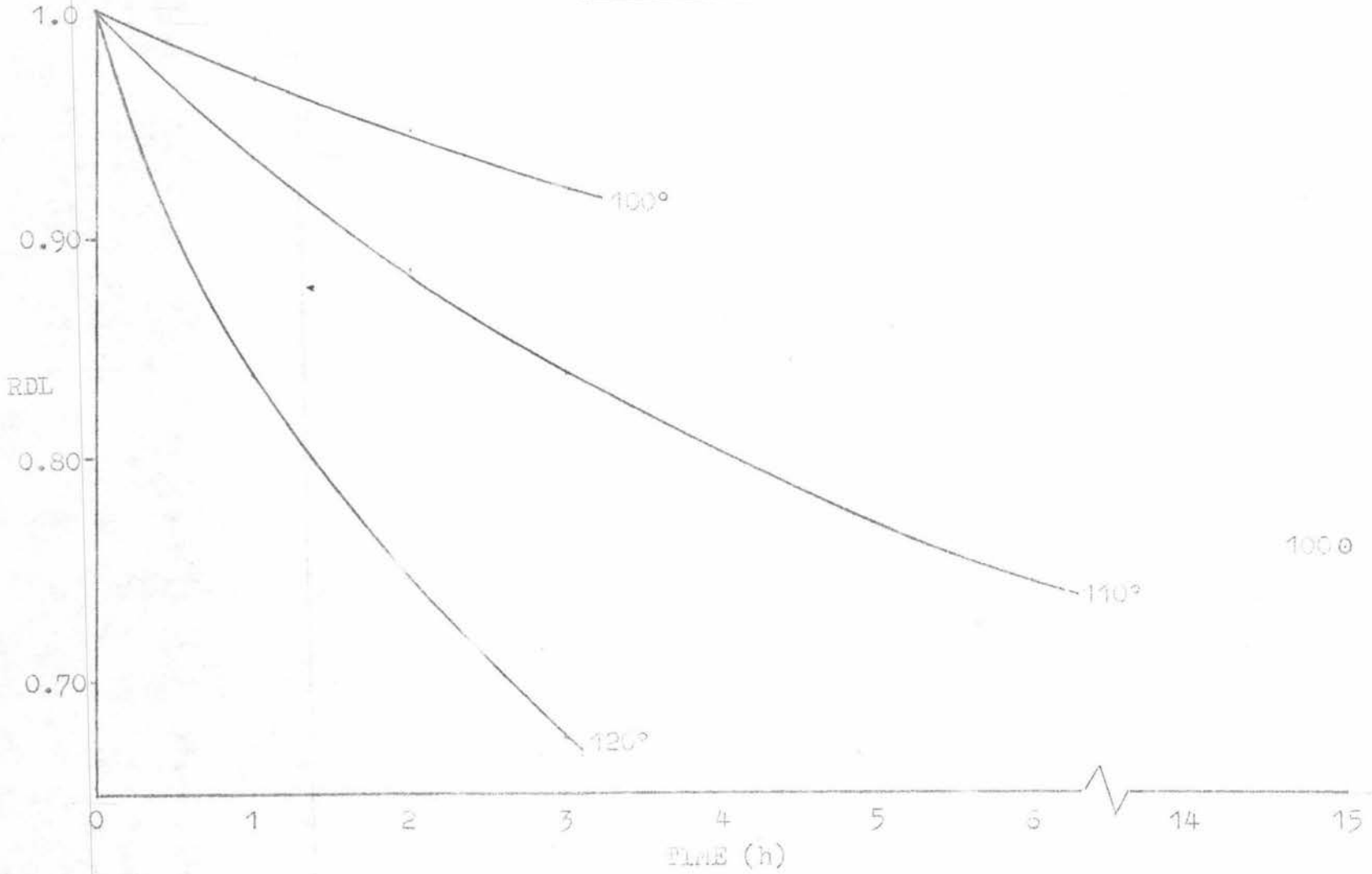


FIGURE 5.5: EFFECT OF HEAT TREATMENT CONDITIONS ON LOG RDL VALUES

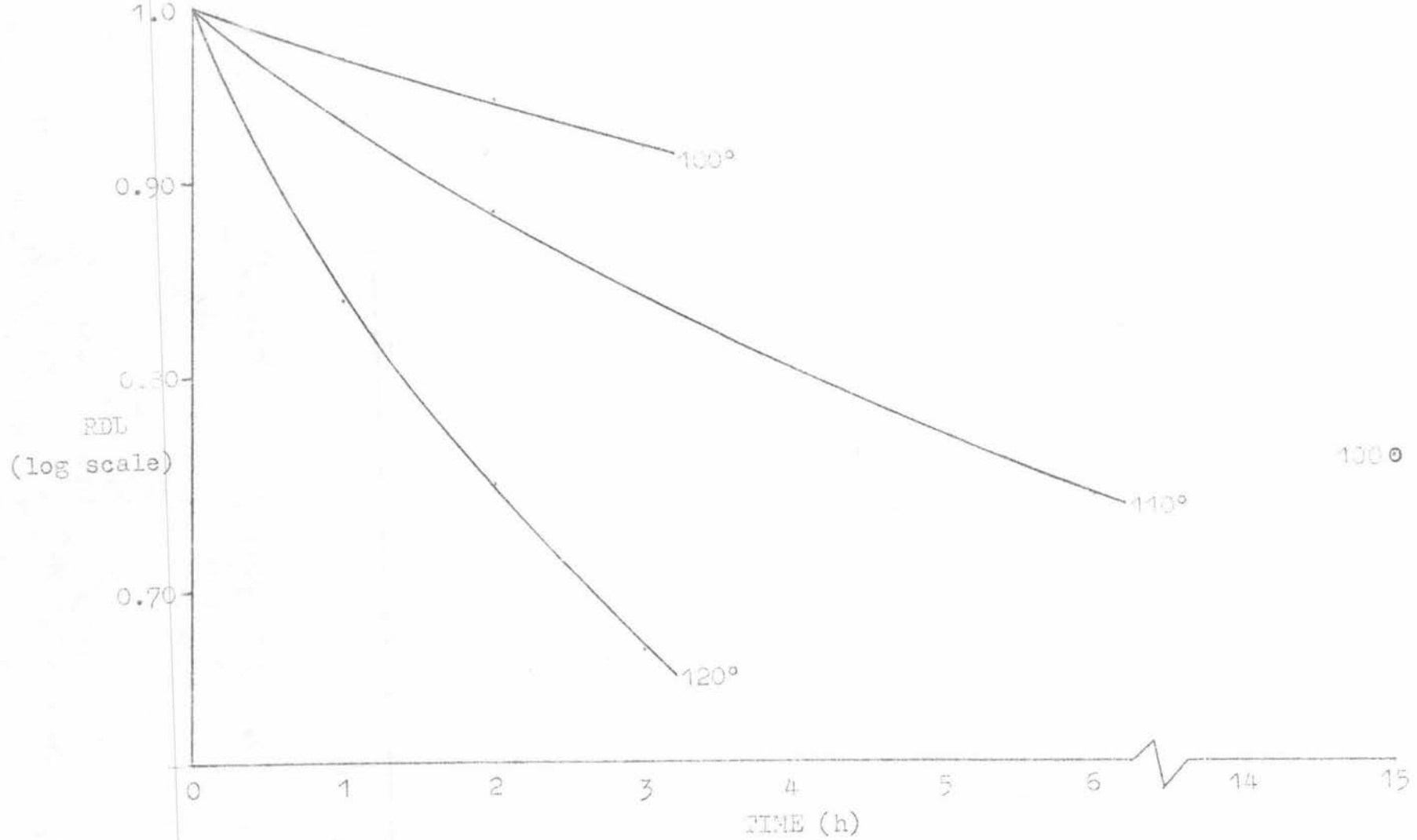


TABLE 5.4 RELATIVE AVAILABLE AND CHANGE IN RELATIVE AVAILABLE LYSINE BY DYE BINDING

Treatment	100/1	100/2	100/3	100/15	110/1
ΔRDL	0.029	0.054	0.081	0.241	0.066
RDL	0.971	0.946	0.919	0.759	0.934

Treatment	110/2	110/3	110/6	120/1	120/2	120/3
Δ RDL	0.116	0.162	0.256	0.163	0.253	0.326
RDL	0.884	0.838	0.744	0.837	0.747	0.674

Plotting these results for RDL (figure 5.5) on semilog paper gives a series of curves, instead of straight lines indicating that the order of the loss of lysine dye binding capacity through lysine/lactose interaction is not first order.

The kinetics of the Maillard reaction has been discussed earlier in this thesis and a variety of reaction orders and activation energies from the literature were noted. (Chapter 1, Section 8).

While there is no compelling reason for presuming that the lysine/lactose reaction, insofar as the formation of ϵ-lactulosyl lysine is concerned should be of second order it would not be surprising if in fact that were the case, as it involves two reacting species in a single step (the aldosylamine step is stated to be an unstable intermediate which rapidly forms the deoxyketoseamine) in unimolecular amounts (see section 1.2.a)

As was shown in section 3.6 for a second order reaction, the rate equation is

$$\ln\left(\frac{C_{B_o} - C_{A_o} X_A}{C_{B_o} - C_{B_o} X_A}\right) = -Kt \qquad (3.9)$$

where C_{A_o} is the concentration of A at time to
C_{B_o} is the concentration of B at time to
X_A is the fraction of A converted to product, and
X_B is the fraction of B converted to product.

Applying this to milk, if A is equivalent to lysine, and B is equivalent to lactose, for the approximate lysine to lactose molar ratio of 1 to 7 that exists in milk, the relationship becomes

$$\ln\left(\frac{7 - X_A}{7 - 7X_A}\right) = Kt \qquad (5.5)$$

Substituting the calculated ΔRDL values for X_A in this relationship estimates of the rate constant, K, can be found. If the reaction is second order then the estimates of K at any one temperature should be constant. When this substitution is done the following K values are found.

TABLE 5.5 RATE CONSTANTS FOR THE CHANGE IN RDL BASED ON SECOND ORDER KINETICS

Temperature	100°			
t(h)	1	2	3	15
ΔRDL	0.029	0.054	0.081	0.241
K	0.025	0.024	0.024	0.016

Temperature	110°			
t(h)	1	2	3	6
ΔRDL	0.066	0.116	0.162	0.256
K	0.059	0.053	0.051	0.043

Temperature	120°		
t(h)	1	2	3
ΔRDL	0.163	0.253	0.316
K	0.154	0.127	0.116

There is a definite reduction in K as the reation progresses. It is to be expected that for small, similar X_A (i.e. ΔRDL) values the computed K values will be similar even if the reaction is not second order. The similarity for the first three K100 values is not unexpected, and they should be viewed in relation to K100 for the 15h heat

treated sample.

To summarise the reduction in RDBC does not follow zero or first order kinetics, and the reduction in RDL as determined by dye binding does not follow zero, first, or second order kinetics.

CHAPTER SIX

DEVELOPMENT OF A PREDICTIVE MODEL

6.1 KINETIC CONSIDERATIONS

The intention of this experiment was as much to formulate a predictive model to represent the protein/lactose reaction. While the most obvious starting point for a model is the second order supposition just examined, the simplest starting point is the first order model. Further justification for using the first order reaction as a base for a model is that in a bimolecular second order reaction where there is a relatively large excess of one reactant, the reaction becomes a one of pseudo-first order due to the nearly constant concentration of one of the reactants.

i.e. in the second order rate equation

$$\ln \left(\frac{C_{B_o} - C_{A_o} X_A}{C_{B_o} - C_{B_o} X_A} \right) = Kt \qquad (3.9)$$

where $C_{B_o} \gg C_{A_o}$, the equation becomes

$$\ln \left(\frac{1}{1 - X_A} \right) = Kt \qquad (6.1)$$

i.e. $$- \ln (1 - X_A) = Kt$$

which is the conventional form of the rate equation for a first order reaction in fractional conversion (X_A) terms.

Returning therefore to the first order study, in which a semilog plot of RDL was made against time, comparison of figures 5.2 and 5.5 shows that multiplying the term RDBC by 1.675 to obtain ΔRDL resulted in a more linear plot. Therefore it appeared possible that by increasing the multiplying factor for ΔRDBC a series of values which when plotted on a semilog basis against time may result in three straight lines.

6.2 DYE BINDING CONSIDERATIONS

There could be a number of reasons why the multiplying factor should be larger than 1.675, including

(i) the lysine content of the milk being tested is lower than the FAO average, which would mean that overall the constant binding of dye by arginine and histidine would reduce the proportional change due to changes in lysine availability.

(ii) The lactulosyl lysine complex or its subsequent derivatives exhibit an affinity for dye giving higher than expected RDBC and RDL values.

(iii) another component in the milk, other than the BAA's binds dye

(iv) the characteristics of the dye binding mechanism change with the change brought about by the Maillard Reaction on the milk protein.

The first point, that the lysine content of the milk is considerably lower than that given in the FAO table is not supported by the value found by the GLC analysis of the milk. The FAO values are used in this section as no attempt was made to measure the arginine or histidine in the GLC study. As shown later in order to give a straight line the change in RDBC needs to be increased by about 3.7 times indicating that the lysine is binding only 27% of the total dye bound by non heat treated milk protein. While this might be possible there seems to be no reason why the ϵ-amino group of lysine is less available than the equivalent sites on arginine and histidine in which case the lysine should be binding about 60% of the total dye.

The second possibility, that the lactulosyl lysine complex or its subsequent products exhibit an affinity for dye has been suggested. It was reported (Hurrell and Carpenter, 1974) a glucose-albumin mixture in which Maillard damage had occurred under mild conditions, i.e. 37° for 10d and 30d, the DBC value for A012 showed virtually no change although the reactive lysine, determined by the fluorodinitrobenzene method, had fallen by 63% and 80% respectively. It has also been reported that casein stored at 37° for 5d showed no change in DBC for OG, whereas the reactive lysine had fallen by 70%, while after 30 days

the DBC for OG had dropped by only 10%, but the reactive lysine content had fallen by 91% (Lea and Hannan,1950). With this type of damage the 'early' Maillard products such as fructosyl-lysine still predominate, and it is probable that they are basic in nature and so react with the dye (Hurrell and Carpenter, 1975). Further, it was stated that "when protein-glucose or protein-sucrose mixtures were more severely heated, some reduction in DBC resulted, but this was much less than the fall in the reactive lysine content. These materials still contained small amounts of fructosyl-lysine units which could combine with the dye and in addition, some of the 'advanced' Maillard products that they contained may also have been basic." They further reported that a sample of 29 year old milk powder had the same DBC value as that which would be expected for undamaged milk powder, and yet its reactive lysine was 70% lower than that of an undamaged powder. From this it was concluded that "the dye binding is unable to detect the serious deterioration in such materials as a source of lysine."

Further evidence that Maillard products may not be entirely unreactive to acid azo dyes is provided in the behaviour of fructose lysine and lactulose lysine in which the α-amino group was protected to simulate a protein chain) towards FDNB. It was found (Finot and Mauron, 1972) that these deoxyketoseamines gave dinitrophenylated compounds i.e. were reactive . Likewise TNBS also is bound by α protected fructose lysine. Both FDNB and TNBS were originally believed to be specific for free ϵ-amino groups. Possibly an acid azo dye, such as AB, could also be partially bound by the deoxyketoseamines. Such behaviour could account for some or all of the observed deviation from first order linearity.

The third possibility that there are other materials present (other than Maillard Reaction products discussed above) which bind dye can be ruled out by the many literature reports to the contrary. (Dolby, 1961; Alais, 1961; Tarassuk, et al., 1967; Ashworth, 1971; Hurrell and Carpenter, 1975). These workers have established that the

minerals, lactose, and non-protein nitrogen which make up the majority of non-protein solids in skim milk do not bind dye.

The only remaining possibility would be that there is a concurrent reaction not involving the lysine, arginine or histidine which results in the formation of products which have the ability to bind dye. This seems unlikely.

The fourth point that the dye binding mechanism changes requires a close examination of the current theories on the dye binding mechanism and the reported values.

6.3 POSSIBLE CHANGES IN THE DYE BINDING MECHANISM OF HEATED MILK PROTEIN

It is proposed that the principal mode in which the dye binding mechanism could alter would be in the stoichiometry of the dye to BAA reaction. As stated earlier the DBC of milk protein is not constant and is dependent on many factors. However, there is substantial agreement amongst workers that the DBC of milk protein is in the range 300 to 340 mg of dye/g of protein. Although there are figures outside of this range, the basis of these figures in terms of dye purity (see Appendix 5) has either not been clearly stated or not allowed for. For example, the DBC for casein is given as 360 mg/g, but this was for undried impure dye, which when converted to a dry, pure dye basis changes to 306 mg/g. (Alais, 1961). The same worker quotes milks having DBC values in the range 359 to 390 mg/g, which on allowing for dye moisture and purity convert to 305 to 331.5 mg/g.

In milk of the FAO composition quoted there is a total of 0.8755 millimoles of BAA per g of protein. M.W. of AB is 616.5 and as it has two binding groups per molecule, for protein dye binding its E.W. is 308.25. Therefore the DBC range of 300 to 340 is equivalent to 0.97 to 1.10 meq/g protein or in stoichiometric terms for milk of average composition the equivalent dye bound by one equivalent of BAA is in the range 1.11 to 1.26. This value is similar to those reported in the literature, viz 1.02 meq/g milk protein (Lakin, 1973b), which converts to 1.17 meq of AB to 1 meq of BAA. For casein a near perfect

equivalence was reported with the experimentally found DBC of 306 being very close to the calculated value of 307 (Alais, 1961). This range of values reported for milk DBC is a reflection of the different milks, different experimental conditions (amounts of dye, milk, reaction mixture volume, and pH), brands and purity of dyes used. In this study the emphasis is on the changes to RDBC values, and the actual DBC values found is of lesser importance and has been presented in Appendix 5. However, as shown in Appendix 5 the DBC value found for non-heat treated milk of 268 is approximately equal to a 1:1 binding ratio on the basis of molecular equivalents.

It has been shown that for a given protein the ratio of the milliequivalents bound per milliequivalent of BAA varies with the dye used, and that the relative difference between the dyes is not constant for all proteins, i.e. while with milk protein the AB DBC : OG DBC is 1:1.42, for wheat protein this ratio is 1:1.74 (Lakin, 1973b). Therefore while the nature of the dye affects the DBC there is also a change in the relative DBC between dyes with different proteins. Change the nature of the protein and the basis of the dye binding mechanism is altered.

It is possible then that when milk protein is heated the protein is changed in such a way as to affect the dye binding mechanism and the stoichiometry in relation to the remaining free BAA binding sites. That the physical nature of the protein is changed is shown by the gelation of milk on heating for prolonged periods, but the extent of casein denaturation under milk processing conditions is considered to be small (Webb and Johnson 1965) so this is unlikely to be important. However, the loss of free ϵ-amino groups of lysine would increase the distance between dye binding sites on a protein chain making it more difficult for the dibasic AB molecule to utilise both binding sites while binding to just one protein chain. Further the binding of the lactose molecules to the protein chain could sterically interfere with the binding of the AB molecule to the remaining free BAA sites. It is thus likely that not all AB molecules would be able to use both of the sulphonic

groups for binding, and that some may only be able to use 1 such group. The effect of this would be to increase the ratio of dye bound to sites available.

The DBC is influenced by the protein/dye ratio, increasing as the quantity of protein is decreased for a constant quantity of dye and in this experiment the protein of the unheated milk is 3.7% and by dye binding the 'apparent protein' of the most severely heated milk is 3.04%. This represents a change in the ratio of protein to dye of 17.8% (i.e. the ratio changes from 2.42 to 1.99 mg/mg) which from the literature (Tarassuk, et al., 1967) will increase the DBC by about 3%. This means that the observed ΔRDBC of 0.185 may in fact be larger by 3%, or 0.006 which is not significant. Further this increase in DBC should not apply in this experiment as the true protein level did not in fact change from 3.7% during processing.

6.4 THE KINETIC MODEL DEVELOPED

All of the above arguments support the observed deviation from non linearity in the semilog plot of both RDBC, and 'available lysine' against heat treatment time but none actually offer an obviously correct solution. In practical terms the exact solution is not necessary but it can be useful to derive a model that satisfactorily predicts the observed results. In view of this, and the obvious linearizing effect that multiplying the loss in RDBC had on the semilog plot, a computer programme was developed to find the optimum multiplying factor required togive the best fit of the data to a first order model (See Appendix 8 for a full discussion of the basis and solution of the computer programme.)

The computer analysis shows that the optimum multiplying factor for ΔRDBC was 3.68, i.e. the optimum fit of the data to straight lines occurs when (1-3.68ΔRDBC) is plotted against heat treatment time, as indicated in figure 6.1.

Assuming that this first order model represents the actual loss of free ϵ-amino groups of lysine caused by the Maillard reaction (ΔRAL) then from the computer

FIGURE 6.1: THE OPTIMUM FIRST ORDER MODEL FOR THE MODIFIED EXPERIMENTAL RDBC VALUES (1 - 3.68 ΔRDBC) (Semilog plot)

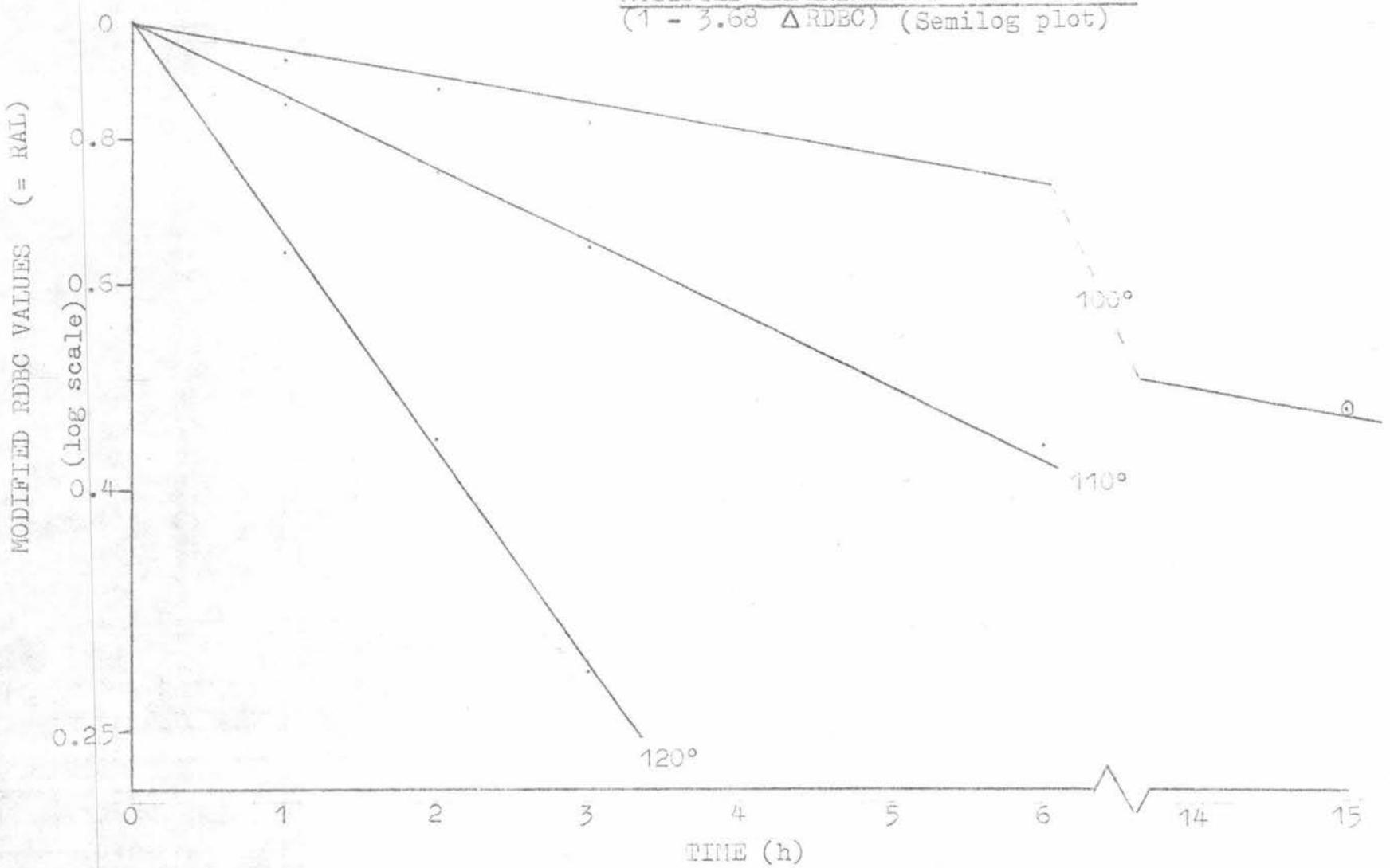
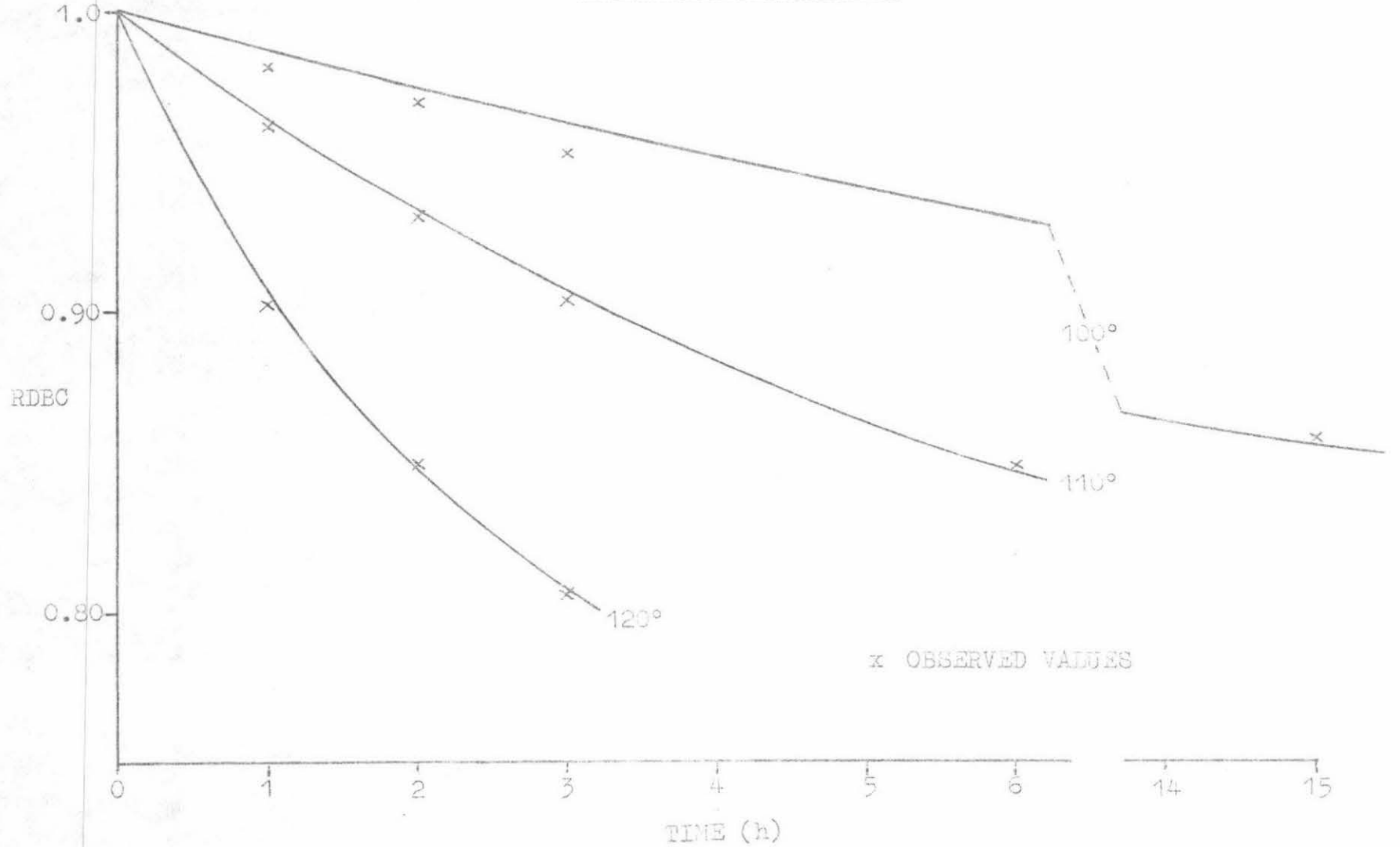


FIGURE 6.2 THE PREDICTIVE MODEL FOR RDBC VALUES FOR HEAT TREATED MILK



solution the rate constants listed in Table 6.1 are obtained for the reaction,

TABLE 6.1 MODEL RATE CONSTANTS

Temperature (°C)	100	110	120
Rate Constant (h^{-1})	-0.051	-0.141	-0.418

$$\Delta RAL = 3.68 \Delta RDBC \qquad (6.2)$$

$$\text{or} \quad RAL = 1.0 - 3.68 \Delta RDBC \qquad (6.3)$$

But for a first order reaction the following rate equation is applicable

$$\frac{C}{C_O} = e^{kt}$$

where C_O is the initial concentration of a reactant
C is the concentration at time t and
k is the rate constant

$$\therefore \quad RAL = \frac{C}{C_O} \qquad (6.4)$$

Eliminating RAL from equations 6.3 and 6.4 gives

$$RDBC = \frac{2.68 + e^{kt}}{3.68} \qquad (6.5)$$

From equations 6.3 and 6.5 it is possible to predict, for the experimental conditions used, the RAL and RDBC values listed in Table 6.2 . (See figures 6.1 and 6.2)

TABLE 6.2 ACTUAL AND PREDICTED RDBC AND RAL VALUES

Treatment		RDBC VALUES		RAL	
Temp (°C)	Time(h)	Observed	Predicted	Observed*	Predicted
100	1	0.982	0.986	0.934	0.950
	2	0.968	0.974	0.882	0.903
	3	0.951	0.961	0.819	0.858
	15	0.856	0.854	0.469	0.464
110	1	0.961	0.964	0.856	0.868

Table 6.2 (cont)

Treatment		RDBC VALUES		RAL	
Temp (°C)	Time(h)	Observed	Predicted	Observed*	Predicted
110	2	0.931	0.933	0.745	0.754
	3	0.903	0.906	0.642	0.655
	6	0.847	0.845	0.435	0.429
120	1	0.902	0.907	0.638	0.657
	2	0.849	0.846	0.443	0.432
	3	0.805	0.806	0.280	0.284

*(Calculated from 1 - 3.68 Δ RDBC)

6.5 EXAMINATION OF THE MODEL DEVELOPED

(a) On a Stoichiometric Basis

The fact that this model fits the observed data so well, justifies a closer examination. If the model is correct the multiplying factor of 3.68 would suggest that the change in DBC is not very sensitive as an indicator of the changing availability of the lysine ϵ-amino groups. However, this situation is at least partly remedied when allowance is made for the assumed constant levels of histidine and arginine. As lysine provides only 59.7% of the BAA in milk of average composition, the 3.68 factor is at least partly due to the theoretical multiplication factor of 1.675 for converting theroetical changes in DBC to changes in dye available lysine (RDL). This still leaves a multiplication factor of 2.20 to be accounted for.

Although the model fits the observed data it does not necessarily reflect accurately the practical situation. The model incorporates two assumptions which may not be entirely valid, namely that it is first order and that the change in ϵ-lysine groups are a constant multiple of the ΔRDBC values. Any measurement errors are increased by the 3.68 multiplication factor.

The model reliably predicts the RDBC values, but further experiments are needed to confirm that it also represents the change in lysine ϵ-amino groups availability.

For the model to be correct the stoichimetry of the dye binding mechanism must change. The theoretical RDBC

(TRDBC) of the milk protein is given by

$$TRDBC = \frac{\text{available (arginine + lysine + histidine)}}{\text{total BAA}}$$

$$= \frac{0.211 + 0.192 + 0.597}{1.0} \qquad (6.6)$$

Initially for non-heat treated milk the TRDBC = 1.0 and in heated milk, assuming only the available lysine is affected, then

$$TRDBC = 0.211 + 0.192 + 0.597 \times RAL$$
$$= 0.403 + 0.597 \times RAL \qquad (6.7)$$

As an example the model predicts for milk heated at 120° for 3h will have an RDBC = 0.806, and an RAL = 0.284, from which the TRDBC = 0.573. In terms of the model the ratio RDBC:TRDBC is a measure of the required change in stoichiometry in the dye binding mechanism. This indicates that after this heat treatment the available binding sites are now binding 1.41 times the number of dye molecules that they bound in the unheated milk.

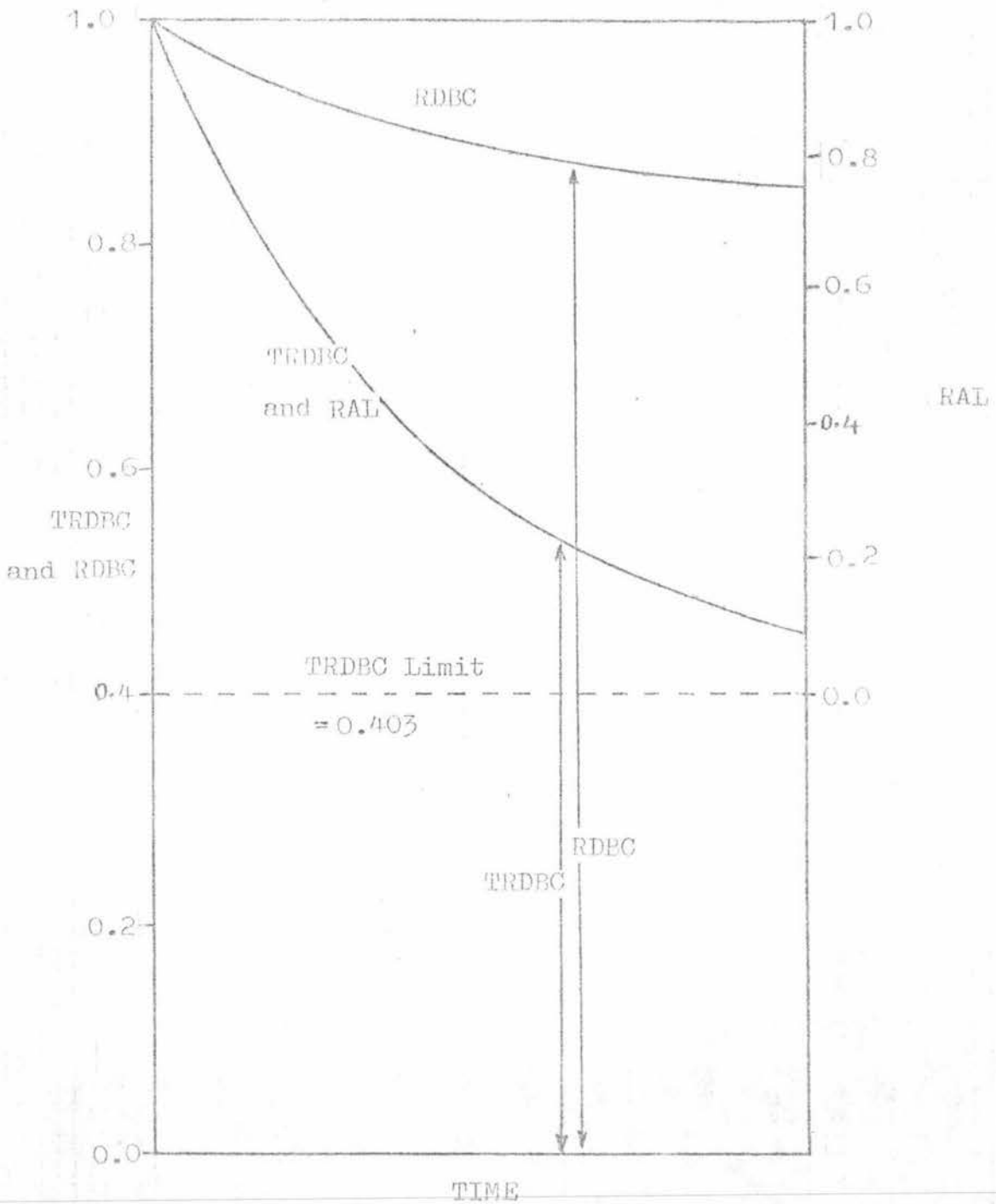
When there is complete loss of available lysine ϵ-amino groups, the RDL = 0, the TRDBC = 0.403. However, the model predicts an RDBC = 0.729, indicating that the binding stoichiometry will change by a factor of 1.81, after sufficient heating to reduce the available lysine ϵ-amino groups to zero. (See figure 6.3)

This increase in binding stoichiometry could be explained as outlined earlier, i.e. that lactosyl lysine or its subsequent products exhibit an affinity for dye; and that heating brings about a change in the protein causing a change in dye binding mechanism. It is possible that it is a combination of both these effects.

(b) On the Basis of Dye Binding by a Maillard Reaction Product

If the reaction between lysine and lactose were first order and was represented by the model, then to explain the greatly reduced change in dye binding on other than

FIGURE 6.3 GRAPHICAL REPRESENTATION OF STOICHIOMETRIC CHANGES REQUIRED BY THE PREDICTIVE MODEL



The ratio of RDBC : TRDBC is a measure of stoichiometry change required.

stoichimetric/steric interference grounds, would require that a product or products of the Maillard reaction has the capacity to bind AB. The observed change in dye binding is only 1/3.68 (i.e. 27.2%) of that predicted by the model. This indicates that 72.8% of the change is obscured by the production of a new species able to bind AB. The possibility of lactulosyl lysine binding dye was discussed earlier (section 6.2) and considered to be possible. Further, it has been calculated that of the nutritionally unavailable lysine, in heated milk 71.5% is present as the deoxyketoseamine (i.e. lactulosyl lysine)(section 1.7). If this reaction product does bind AB on approximately a 1:1 stoichiometric basis, then the small observed drop in RDBC is explained. This does not allow for any further reaction the lactulosyl lysine may undergo itself. The fact that colour develops, and that deoxyketoseamines are important in this pathway (Reynolds, 1969) indicates that dye binding by lactulosyl lysine is only a partial explanation.

6.6 ACCURACY OF THE MODEL

While a multiplication factor (F) of 3.68 for RDBC gives the best fit of the data to a straight line, the accuracy of the model must be kept in perspective. As outlined in the discussion of the computer programme (Appendix 8), the average coefficient of variation for the rate constants was a minimum at F = 3.68, and this can be used to calculate confidence limits for the geometric mean rate also calculated by the programme.

Table 6.3 is the computer printout for the calculated values for F = 3.68

TABLE 6.3 COMPUTER PRINTOUT AT OPTIMUM F

F = 3.68

TEMP	RATE CON	MEAN SQUARE	COV
100	-0.4178852	0.0007530	-6.57
110	-0.1406754	0.0003075	-4.08
120	-0.0510577	0.0009543	-6.53

GEO MEAN RATE COEF = -0.144249
TOTAL SUM SQUARES = 0.005292
GEO MEAN RATE COEF = 0.000667
AVERAGE COEF OF VARN = 5.69548

The 95% confidence limits for the geometric mean rate coefficient are $\pm$ 2 standard errors ,i.e.$\pm$2(Ave. coeff. of variation/ $\sqrt{3}$

i.e. $- 0.144 \pm 2\ (5.7\%)/\sqrt{3}$

$= - 0.144 \pm 0.010$

$= - 0.154$ to $- 0.134$

The lower limit corresponds to the geometric mean rate for F = 3.51, and the upper limit corresponds to the geometric mean rate for F = 3.83. Therefore the 95% confidence limits for F are 3.51 and 3.83. This indicates that the fit of the data to first order kinetics at F = 1, (for RDBC), and F = 1.68 (for apparent lysine) are significantly worse than the fit at F = 3.68 (the model for RDL).

6.7 ENERGY OF ACTIVATION

The energy of activation for a reaction can be calculated as developed in Chapter 1 by applying equation 1.9

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

Applying this equation to the three 'reactions, viz

(a) loss of DBC for which F = 1,

(b) apparent loss of lysine -amino groups, for which F = 1.68.

(c) loss of free lysine ϵ-amino groups using a first order model at which F = 3.68 the following values for E are found

(a) $E = \frac{1.98 \times 373 \times 393}{20} \cdot \ln\left(\frac{-0.07712}{-0.01072}\right)$

$= 28.7$ Kcal/mole

(b) $E = \frac{1.98 \times 373 \times 393}{20} \cdot \ln\left(\frac{-0.13950}{-0.01893}\right)$

$= 29.0$ Kcal/mole

(c) $E = \frac{1.98 \times 373 \times 393}{20} \cdot \ln\left(\frac{-0.41789}{-0.05126}\right)$

$= 30.5$ Kcal/mole

While there may be doubt regarding the validity of the model (i.e. case (c)), as far as the reaction is

concerned it does not make a great deal of difference to the E value calculated for the reaction that causes a change in DBC through heat treatment.

Another way of interpreting these values is to show the relative increase in the rate of reaction for a 10° increase in reaction temperature. From equation 1.9, equation 6.8 can be derived.

$$\frac{k_1}{k_2} = e^{\frac{E}{R}\left(\frac{T_1 - T_2}{T_1 T_2}\right)} \qquad (6.8)$$

While the value $T_1 - T_2$ may be constant (such as here where a 10° change is being examined), the actual value of the ratio of the rate constants will vary according to the value of T_1, or T_2, i.e. at 0°C (273°K), and at 100°C (373°K), and so the value of the ratio will vary. It is therefore necessary to specify at what temperature the ratio between the rate constants is being examined.

Within a narrow temperature range, however, the ratio is relatively constant, especially when the temperatures under consideration are above about 370°K. Therefore the ratio of the rate constants for 100°C (373°K), and 110°C (383°K), and the ratio for the rate constants for 110°C (383°K) and 120°C (393°K) will be nearly identical. For this experiment, using the two temperatures of 105°C (378°K) and 115°C (388°K) as being typical for the three 'reaction' situations for which E values were calculated it is found that for a 10° rise, the ratio of the reaction rate constants are

(i) $\frac{k_1}{k_2} = e^{(28,700 \times 10)/(1.98 \times 378 \times 388)}$

$= 2.69$

(ii) $\frac{k_1}{k_2} = e^{(29,000 \times 10)/(1.98 \times 378 \times 388)}$

$= 2.71$

(iii) $\frac{k_1}{k_2} = e^{(30,500 \times 10)/(1.98 \times 378 \times 388)}$

$= 2.86$

Therefore regardless of which reaction is considered, a change that takes 1 hour to occur at one temperature will take about 2.75 hours to occur at a temperature that is 10° lower than the original. This is illustrated in figures 6.1 and 6.2.

6.8 COMPARISON WITH LITERATURE FINDINGS

Because of the limited data reported in the literature, and because the reported changes in DBC are small, it is not possible to completely corroborate the data found in this experiment.

Alais, et al., 1961, reported RDBC's of 0.989, and 0.976 for milk after 100°/10 min, and 115°/20 min.

The experimental model gives $k_{100°} = 0.051$ and

$$RDBC = \frac{2.68 + e^{kt}}{3.68}$$

which predicts that for a treatment of 100° for 10 min,

$$RDBC = 0.998$$

Applying the Arrhenius equation (6.8) to the experimentally determined reaction data to find a rate constant for 115°, it is found that

$$k_{115} = k_{120}\, e^{\frac{30500}{1.98}\left(\frac{-5}{388 \times 393}\right)}$$

$$= -0.252$$

Using this value for k_{115}, after heating at 115° for 20 min., the predicted RDBC is 97.8. This compares more favourably than does the first calculated figure with its corresponding literature figure.

It is apparent from the rate constants, and the first RDBC calculation above that at temperatures below 100° long holding periods will be required before any significant change could be expected in the RDBC (assuming the reaction mechanism is the same in all cases). The model predicts that heating at 70°C for 100h would give an RDBC of 0.972.

Tarassuk, et al., 1967 reported that heating at

120° for 8 min and 10 min resulted in RDBC values of 0.982, and 0.972 respectively. Applying the model to these conditions gives corresponding RDBC values of 0.985, and 0.981. Considering the magnitude of the changes and possible experimental errors in the reported data, the model fits the observations of Alais, et al., and Tarassuk, et al., satisfactorily, as well as predicting no significant change in milk that has undergone only mild heat treatment such as pasteurization.

6.9 SUMMARY

(a) Experimental evidence published has established that the BAA's of proteins bind acid azo dyes and under conditions of excess dye a protein/ dye precipitate is formed. The amount of dye bound under excess dye conditions is nearly stoichiometric.

(b) Of the acid azo dyes used, opinion is divided as to which is the optimum. For milk protein determination both AO12 and AB are used in commercial equipment.

(c) The dye binding mechanism is not just a simple BAA/dye bond but is believed to involve up to 3 different types of bonds; (a) primary electrovalent, between the BAA and dye sulphonic acid groups, (b) hydrogen bonds between the free dye in the supernatant and the bound dye and/or the protein, (c) hydrophobic bonds between the free dye and the bound dye and/or the protein.

(d) The protein/dye stoichiometry is affected by the protein, the dye, and the experimental reaction conditions.

(e) A wide range of values has been reported in or can be extracted from the literature for the AB DBC of milk protein. However, comparisons are difficult because these figures are often based on dyes of unknown or unstated purity.

(f) Heating liquid milk decreases the DBC of the protein and this is most likely to be due to the Maillard reaction occuring between the lactose present, and the ϵ-amino groups of the lysine in the protein, although there may be a contribution from changes in the arginine and histidine moieties.

(g) The change in DBC depends on the severity of the heat treatment, with a process such as pasteurization giving no noticeable change in DBC.

(h) The loss of DBC does not follow first order kinetics.

(i) If the loss of DBC is assumed to be due solely to changes in ϵ-amino groups in lysine, and if allowance is made for this, then the loss of ϵ-amino group availability as determined by dye binding also does not follow first order kinetics.

(j) A model, in the form of a first order reaction was established and this could be used to satisfactorily predict changes in RDBC caused by heat treatment.

(k) Application of the model to various heat treatments in the literature predicted RDBC values that were in good agreement with the literature reported values.

(l) The energies of activation for the three possible 'reactions' were found to be similar (see Table 6.4) and all in the region of the reported values for the energy of activation of the Maillard reaction.

TABLE 6.4 CALCULATED VALUES FOR E AND Q10

Reaction	Ea (Kcal-mole)	Q10 for range 100-120°C
Change in DBC	28.6	2.68
Change in DBCx1.68 (equivalent to change in lysine only)	29.0	2.71
Change in DBCx3.68 (first order model)	30.8	2.88

6.10 CONCLUSIONS

(a) Heat treatment reduces the AB DBC of protein in liquid milk in non first order manner.

(b) Allowing for other dye binding amino acids and assuming only the lysine is affected by heating of the protein in the presence of lactose, the loss of lysine for AB dye binding is not first order.

(c) A first order model can be fitted to changes of up to 0.2 in RDBC, if it is assumed that

(i) the dye binding reaction stoichiometry is affected by the change in nature of the protein brought about by the reaction between the lactose and ϵ-amino groups of lysine. The stoichiometry of dye binding using this model, is such that when all the ϵ-amino groups are unavailable to AB the amount of dye bound per BAA is increased by a factor of 1.81. There is a progressive increase to this level as the reaction proceeds and under the most severe heat treatment process used in this experiment required the dye binding per BAA to increase by a factor of 1.41;

or (ii) the change in free ϵ-amino groups is represented by the model but the Maillard reaction results in 71.5% of the unavailable lysine being present as lactulosyl lysine and that this compound can bind AB on a 1:1 basis.

APPENDIX 1

THE CORRELATION BETWEEN DYE CONCENTRATION AND ABSORBANCE

A1.1 Theory

Beer's Law states that the absorbance of light passing through a solution is proportional to the concentration of solute in the solution.

$$A = Ebc$$

where A is absorbance

E is molar absorptivity, a const. for a given substance

b is light path length in cm.

c is concentration in g/l

It is however not uncommon to find at high concentrations that there is some deviation from this law, with absorbance being lower than the law would predict.

It was therefore important to determine the behaviour of the AB solution at the concentrations used in the dye binding tests. Steinsholt (1957) claimed that AB follows Beer's Law only at concentrations below 6.2mg/l. It was also reported that Beer's Law is followed by AB solutions which have absorbances of up to 1.0, which under the conditions used corresponded to 13mg/l (Dolby, 1961; Tarassuk, et al., 1967). In these tests a 1cm light path length cells was used and with this path length the accuracy of the absorbance reading would tend to become the limiting factor rather than the deviation from Beer's Law. This is a consequence of the relationship between % transmission of light and the absorbance, viz:

$$A = 2 - \log T$$

where T = % transmission

and the fact that the instrument scale is normally linearly calibrated for % transmission. Thus the relative accuracy of a reading is constant over the transmission scale but not the absorbance scale. For example, it might be possible to make a reading with an accuracy of $\pm$ 0.2 units on the transmission scale. A typical T reading may be 75%, with error limits of 74.8% to 75.2% which convert to absorbance

values of 0.1261 and 0.1238, a range of 0.0023 absorbance units, but at T = 10% then the corresponding limits of error for the absorbance readings are 1.0088 and 0.9914, giving a range of 0.0174 absorbance units. Further the absorbance scale of most spectrophotometers are not finely calibrated for absorbance readings in excess of 1.0 which makes the determination of accurate concentrations difficult where the absorbance of the solution is in excess of this figure.

In more recent studies this problem has in part been reduced by the used of cells with light paths as short as 0.2mm, which enables reasonably accurate measurements to be made of the absorbance of solutions that are 50 times more concentrated than those that could be handled by the older 1cm light path cells. Such short light path cells, usually of a flow through type are now readily available, and in fact have been the basis of the successful development of such instruments as the Foss Pro-Milk and Udy Analyzer protein determining instruments.

A1.2 Practical Study

A solution of AB made up, as previously described (Section 5.3) for use with the Foss Pro-Milk instrument was diluted to give 10 lower concentrations as listed in the Table below. The absorbance of the standard dye, the 10 diluted dye samples, and water was determined using a Bausch and Lomb Spectronic 20 spectrophotometer fitted with an 0.2mm flow through cell. The average of two readings was taken, the first set of readings being made starting with the lowest concentration, and the second set with the highest concentration. The cell was cleaned after every three measurements.

A1.3 Results

The absorbance readings are listed in Table A1.1

FIGURE A1.3: ABSORBANCE VERSUS CONCENTRATION OF AMINO NITROGEN IN SOLUTION

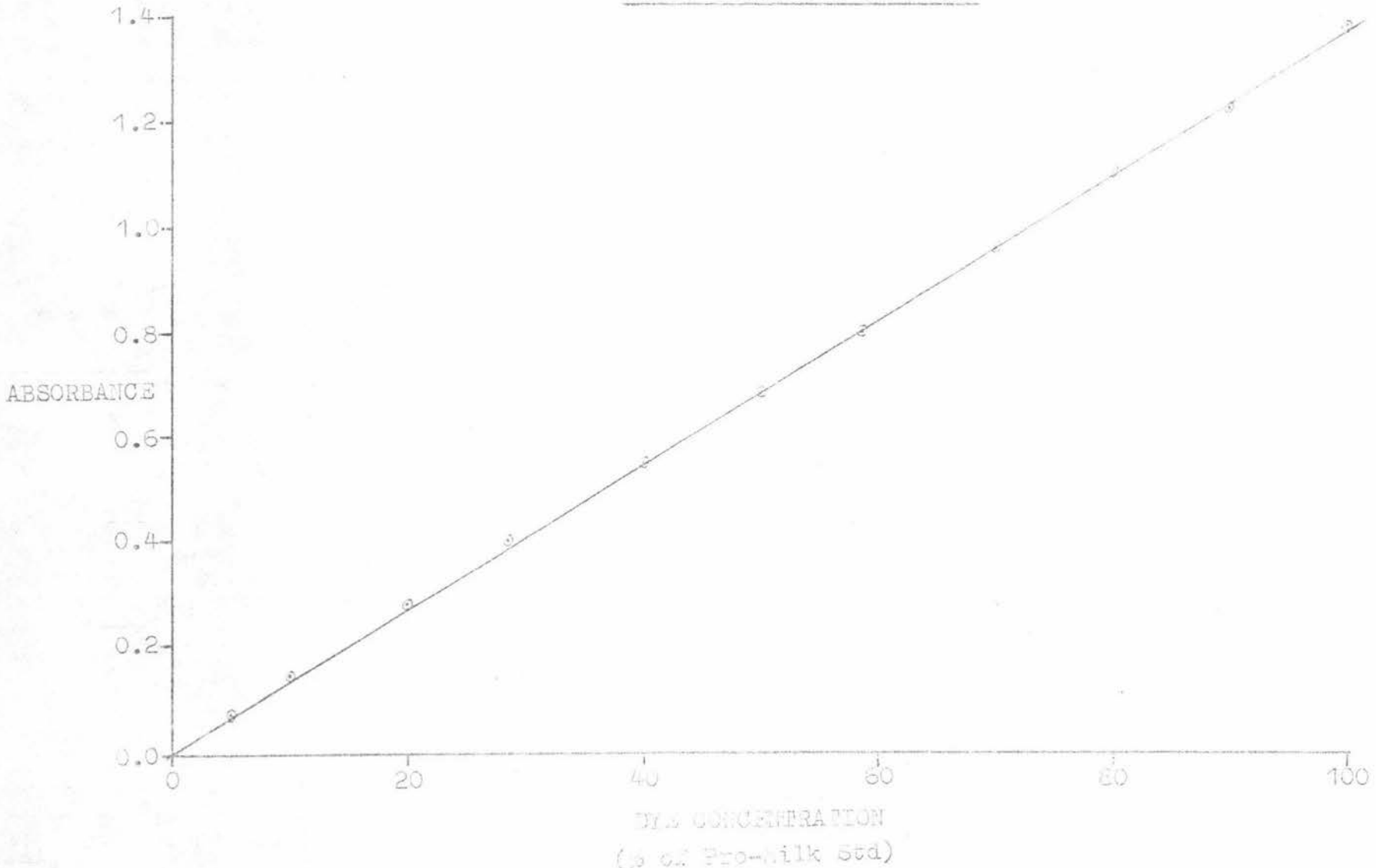


TABLE A.1.1 ABSORBANCE OF DILUTE PRO-MILK DYE SOLUTIONS

Dye Conc (D) % of Std	Transmission Readings (%) Ascending	Descending	Mean T %	Absorbance (A)	Ax Dil. Factor
0.0	100.0	100.0	100.0	0.0	-
5.0	84.6	84.5	84.55	0.0729	1.458
10.0	72.0	72.1	72.05	0.1424	1.424
20.0	52.2	52.6	52.40	0.2807	1.404
28.6	39.8	39.8	39.80	0.4001	1.399
40.0	28.3	28.0	28.15	0.5505	1.376
50.0	21.1	21.0	21.05	0.6767	1.353
60.0	15.8	15.8	15.80	0.8013	1.336
70.0	11.2	11.0	11.10	0.9547	1.364
80.0	8.1	8.0	8.05	1.0942	1.368
90.0	6.0	6.0	6.00	1.2218	1.358
100.0	4.2	4.2	4.20	1.3768	1.377

This concentration corresponds to the concentration used in setting the '45' calibration reading for the Pro-Milk protein tester.
Figure A1.1 is a plot of these results.

A1.4 Analysis

As a result of the method of calibration the correlation line must pass through the point, 0.0 concentration, 100% transmission. Therefore the regression equation was forced through this origin. The equation obtained, is

$$A = 0.01368\ D \qquad (A1.1)$$

where A is the absorbance of the dye, and
D is the % of the original dye concentration.

Equation A1.1 implies that the absorbance of the undiluted dye is 1.368, and the predicted absorbance values are listed in Table A1.2.

TABLE A1.2 PREDICTED AND MEASURED ABSORBANCE VALUES

Dye %	0.0	5.0	10.0	20.0	28.6	40.0
A measured	0.0	0.0729	0.1424	0.2807	0.4001	0.5505
A predicted	0.0	0.0684	0.1368	0.2736	0.3908	0.5472

Table A1.2 (cont)

Dye %	50.0	60.0	70.0	80.0	90.0	100.0
A measured	0.6767	0.8013	0.9547	1.094	1.222	1.377
A predicted	0.6840	0.8210	0.9576	1.094	1.231	1.368

For % transmittance and % dye concentration the relationship is

$$T = 100\ (0.9690)^{D} \qquad (A1.2)$$

A1.5 Discussion

Beer's Law implies a linear relationship between absorbance and concentration. The form of the regression equation indicates that over the range of concentrations studied such a straight line relationship exists and this is confirmed when the values predicted by the correlation equation are compared with the experimental values.

As the standard dye used in the experimental work on DBC of the milk protein is equal to the maximum used in this Beer's Law study it can be concluded that the dye concentration encountered elsewhere in the DBC study will be in the range complying with Beer's Law.

APPENDIX 2

THE RELATIONSHIP BETWEEN THE PRO-MILK PROTEIN AND TRANSMITTANCE SCALES

A2.1 THEORY

Beer's Law as discussed in Appendix 1 is expressed as the following equation,

$$A = Ebc$$

and as $$A = 2 - \log T$$

then $$C = \frac{2 - \log T}{Eb}$$

If the initial concentration of the AB dye is C_i, and the protein binds dye to the extent that the concentration changes by C_p then the resultant final concentration, C_f, is given by

$$C_f = C_i - C_p$$

Further if the reaction between the milk protein and the AB dye does not change its stoichiometric behaviour with a change in protein or dye concentration, the in excess dye

$$C_p = B.[P]$$

where B is a proportionality constant

P is % protein in the milk

$$\therefore \quad C_i - B.[P] = \frac{2 - \log T}{Eb} \qquad (A2.1)$$

From which can be derived

$$[P] = \text{const} \quad K^1 \log T$$

where K^1 is a constant.

There is a slight complication with the Pro-Milk instrument in that the instrument's transmission scale is zeroed with the dye that is used for the protein tests. This allows about 4.2% transmittance of the incident light through an 0.2mm path length cell (the length used in the Pro-Milk). Therefore the correlation between the Pro-Milk transmission scale (hereafter Pro-Milk transmission values will be designated PT values, as distinct from T representing transmission values on the Bausch and Lomb Spectronic 20), and the protein (P) scale will not be of the exact form formulated above.

Additionally as many workers have shown, (Dolby, 1961; Tarassuk, et al, 1967; Lakin, 1973a) milk protein has a DBC value that is a function of the excess free dye concentration (i.e. C_f), and this will give further deviations from the theoretical equation.

A2.2 PRO-MILK MARK II INSTRUMENT SCALE

The Pro-Milk MkII instrument has two graduated scales, one of which is linear, the transmission (PT) scale reading from 0 to 100, and the other, the protein (P) scale, which is graduated from 2.0% to 5.5%

From the dial of the Pro-Milk MkII the corresponding readings of PT and protein were taken, as listed in Table A2.1.

TABLE A2.1 TRANSMISSION AND PROTEIN READINGS FROM PRO-MILK INSTRUMENT

Transmission (PT)	14.4	20.5	27.4	35.7	45.6	57.3	71.2	87.7
Protein (P)	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5

It was later confirmed that two equivalent points on the scale are 20.5%, 2.5%; and 87.65%, 5.5% (A/S N.Foss Electric, 1976).

A2.3 ANALYSIS

Plotting the log PT scale values against the P values, (figure A2.1)shows that the relationship deviates significantly from the linear form developed in the theory, but as stated then this is not entirely to be unexpected. Although not linear the deviation from linearity is not large and it seemed possible that the relationship between PT and P could be approximated by a function of the following type:

$$Y = a + b \cdot c^{X}$$

which applied to PT and P becomes

$$PT = a + b \cdot c^{P}$$

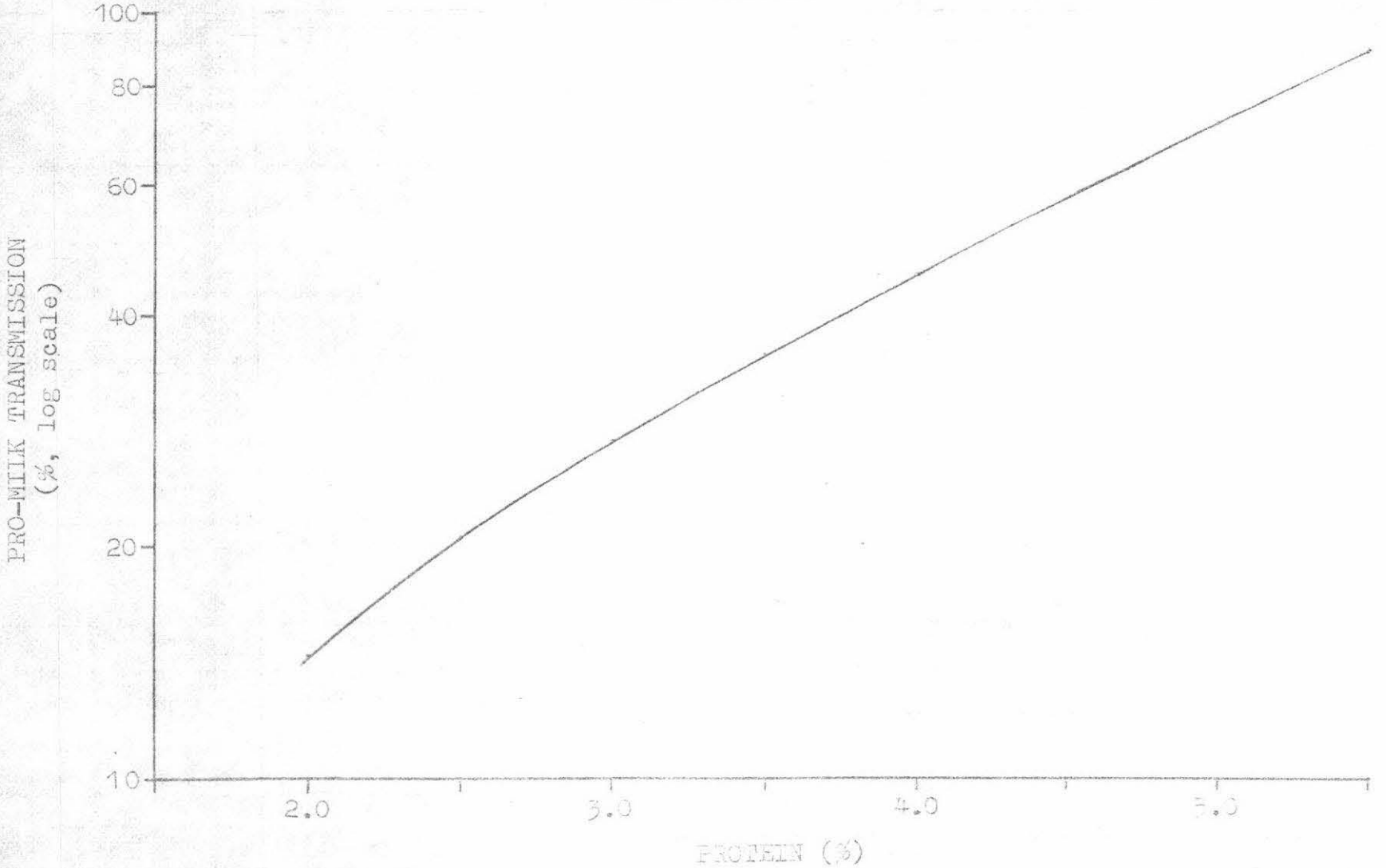
or

$$P = b' + c' \ln (PT + a)$$

where a,b,b',c and c' are constants

Using asymptotic regression analysis (Snedecor and Cochran, 1965), it is found that

FIGURE A2.1: PRO-MILK TRANSMISSION VALUES VERSUS PROTEIN VALUE



$$PT = -17.76 + 16.293\,(1.40424)^{P} \qquad (A2.2)$$

or $$P = -8.221 + 2.9458 \ln (PT + 17.76) \qquad (A2.3)$$

Substituting PT values into equation A2.3, gives the calculated values for P listed in Table A2.2.

TABLE A2.2 MEASURED AND CALCULATED PROTEIN VALUES FOR PRO-MILK TRANSMISSION VALUES

PT	14.4	20.5	27.4	35.7
P measured	2.00	2.50	3.00	3.50
P calculated	2.00	2.51	3.00	3.50

PT	45.6	57.3	71.2	87.7
P measured	4.00	4.50	5.00	5.50
P calculated	4.00	4.50	5.00	5.50

For the two scale points stated by A/S N.Foss to be equivalent (20.5%, 2.5%; and 87.65%, 5.5%), the function predicts P values of 2.51% and 5.50%. These predicted values as well as the others in the Table are in very close agreement with the observed scale values, therefore the function satisfactorily represents the PT and P relationship.

The fact that the logarithmic function contains a constant is a result of the unusual method of zeroing the transmission scale, and the changing stoichiometry of the protein/dye reaction. The equation also implies that the dye is at a concentration equivalent to 5.826% protein.

The standard dilution of the working fluid to provide the PT = 45 calibration solution is equivalent to P = 3.972, by the equation. This compares with the figure quoted by McGann, et al., 1973 of 3.97.

APPENDIX 3

THE RELATIONSHIP BETWEEN THE PRO-MILK TRANSMISSION SCALE AND

(A) AMIDO BLACK DYE CONCENTRATION

(B) THE BAUSCH AND LOMB SPECTRONIC 20 TRANSMISSION SCALE

A3.1 PRACTICAL COMPARISON

Using the same diluted samples of the Pro-Milk AB solution as were used for the Beer's Law study (Appendix 1), the readings in Table A3.1 were found on the Pro-Milk MkII (average of two readings).

TABLE A3.1 TRANSMISSION READINGS OF THE SPECTRONIC 20 AND THE PRO-MILK INSTRUMENTS

D	0	5.0	10.0	20.0	28.6	40.0
T	100.0	84.55	72.05	52.40	39.80	28.15
PT	100.0	86.50	75.80	57.40	45.00	32.90

D	50.0	60.0	70.0	80.0	90.0	100
T	21.05	15.80	11.10	8.05	6.0	4.2
PT	24.00	17.40	11.90	7.20	4.00	0.0

A3.2 ANALYSIS

(a) For Pro-Milk and Amido Black

It is evident from the above Table that if a plot of logT vs D is linear then the equivalent plot logPT vs D will not be linear, figure A3.1. The PT curve is near linear for D values between 0 and 40%, and thereafter becomes progressively curvilinear. This is due in part at least, to the peculiar method of calibrating the Pro-Milk instrument. To find the relationship between PT and D, use was made of the same asymptotic regression method (Snedecor and Cochran, 1967) as was used to find the relationship between PT and P. (Appendix 2).

The relationship found was

$$PT = 107.72\,(0.9762)^{D} - 8.548 \qquad (A3.1)$$

which gives the predicted PT values in Table A3.2

FIGURE A3.1: PRO-MILK TRANSMISSION VALUES VERSUS CONCENTRATION OF AMIDO BLACK DYE SOLUTION

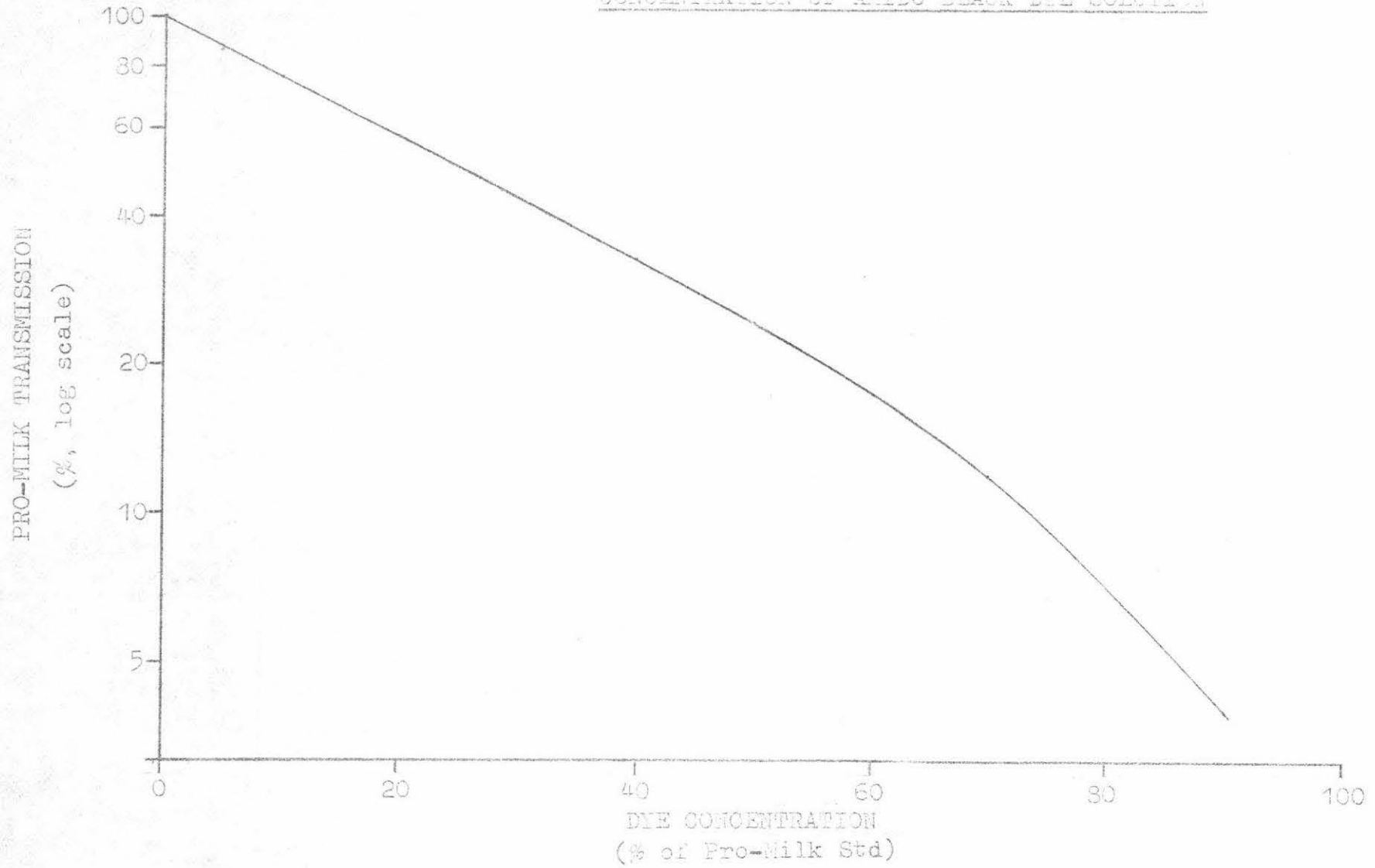


TABLE A3.2 PREDICTED AND MEASURED PRO-MILK TRANSMISSION VALUES

D	0	5.0	10.0	20.0	28.6	40.0	50.0
PT(predicted)	99.2	86.9	76.1	58.0	45.5	32.5	23.7
PT(measured)	100	86.5	75.8	57.4	45.0	32.9	24.0

D	60.0	70.0	80.0	90.0	100.0
PT(predicted)	16.8	11.4	7.10	3.8	1.1
PT(measured)	17.4	11.9	7.2	4.0	0.0

The fit of equation A3.1 is not as good as for either the T vs D, or PT vs P relationships derived earlier (equations A1.1, and A2.2).

While equation A3.1 gives the line of best fit it is apparent that there is quite a high measure of error in the predicted values. As most of the protein testing work results in the remaining supernatant solutions having PT values of less than 60%, and since below this value the relationship is more linear, the regression relationship between PT and D was calculated for D values between 0 and 60. The relationship found was

$$PT = 103.68 \ (0.9742)^{D} - 4.034 \qquad (A3.2)$$

from which the values in Table A3.3 can be calculated

TABLE A3.3 PRO-MILK TRANSMISSION VALUES CALCULATED FROM EQUATION A3.2

D	0	5.0	10.0	20.0	28.6	40.0
PT(pred.)	99.65	86.9	75.8	57.4	45.1	32.4
PT(meas.)	100	86.5	75.8	57.4	45.0	32.9

D	50.0	60.0	70.0	80.0	90.0	100
PT(pred.)	24.0	17.6	12.6	8.8	5.8	3.56
PT(meas.)	24.0	17.4	11.9	7.2	4.0	0.0

There has been a significant improvement in the fit of predicted values in the range of values used for the repression. This indicates that although an equation of the form

$$Y = a + b(c)^X$$

does not adequately represent the relationship between PT and D for all D values, it would be possible to derive two equations to cover different ranges of D, or the addition of another parameter, or an extra term involving X would give an improved fit.

(b) For Pro-Milk and Spectronic 20 Transmission Values

Relationships between T and D, and PT and D have been derived, and by elimination of the term D, a relationship between T and PT can be found. Although the initial relationships have been individually optimised, it is quite likely that a third relationship derived from them will not necessarily be the optimum for the third pairing of the variables.

The relationship between PT and T obtained by eliminating D, using equations A1.2 and A3.2 is

$$PT = 2.2693\ T^{0.82973} - 4.03 \qquad (A3.3)$$

This gives the values in Table A3.4 which only covers the relationship of PT and T corresponding to D values of 0 to 60% as equation A3.2 is applicable only over that range.

TABLE A3.4 PRO-MILK TRANSMISSION VALUES CALCULATED FROM EQUATION A3.3

T	100.0	84.55	72.05	52.40
PT predicted	99.6	86.1	74.9	56.6
PT measured	100.0	86.5	75.8	57.4

T	39.80	28.15	21.05	15.80
PT predicted	44.2	32.2	24.4	18.4
PT measured	45.0	32.9	24.0	17.4

Even when the range of application is restricted the fit of the predicted values is not very good, and on

average the predicted PT values are low by 0.33. In fact the two sets of values give lines with different slopes.

An examination of the logarithmic form of equations A1.2 and A3.2, viz:

$$\ln T = -D \ln 1.0320 + \ln 100$$

$$\ln (PT - 4.034) = -D \ln 1.02648 + \ln 103.68$$

suggests that the form of the relationship between PT and T would be

$$\ln (PT + c) = \ln a + b \ln T$$

where a, b, and c are constants

and that the log T, log (PT + C) relationship would be linear. Therefore application of normal linear regression techniques should give the relationship, and by trial and error a value of c found that would result in the minimum error in the predicted values. The relationship found, for all pairs of PT, T values was

$$\ln (PT - 8.2) = \ln 3.070 + 0.7736 \ln T$$

or

$$T = 0.2346 (PT + 8.2)^{1.29266} \qquad (A3.4)$$

or

$$PT = 3.070\, T^{0.7736} - 8.20 \qquad (A3.5)$$

from which the values in Table A3.5 can be calculated. Unlike the earlier Table equation A3.5 is accurate for all T (or all D) values.

TABLE A3.5 PRO-MILK TRANSMISSION VALUES CALCULATED FROM EQUATION A3.5

T	100.0	84.55	72.05	52.4	39.8	28.15
PT(predicted)	100.02	86.84	75.78	57.44	44.86	32.39
PT(measured)	100.0	86.50	75.80	57.40	45.00	32.90

T	21.05	15.8	11.10	8.05	6.0	4.2
PT(predicted)	24.22	17.76	11.56	7.21	4.08	1.12
PT(measured)	24.00	17.40	11.90	7.20	4.0	0.0

The values in this Table are in better agreement, with the difference in mean PT values for the predicted and observed values being less than 0.1. In statistical terms the standard devn. is 0.41, which while being large, is mostly caused by the deviations of two readings from their corresponding predicted values, viz. 32.9 and 32.39, and 0.0 and 1.12. These two readings contribute 75% of the standard error. It is likely that these readings are in error. The general close agreement suggests that where the deviations are large this would be of experimental origin rather than being actual differences. The relationship found can be used to satisfactorily predict PT values from given T values.

APPENDIX 4

THE RELATIONSHIP BETWEEN THE BAUSCH AND LOMB SPECTRONIC 20 TRANSMISSION SCALE, AND THE PRO-MILK PROTEIN SCALE

A direct comparison of readings covering the whole range of the scale was not made, but from the derived relationships between P and PT, and PT and T, viz.

$$P = -8.22 + 2.9458 \ln (PT + 17.76) \qquad (A2.2)$$

and $$PT = -8.20 + 3.070\ T^{0.7736} \qquad (A3.5)$$

elimination of PT gives the following equation

$$P = -4.917 + 2.945 \ln (T^{0.7736} + 3.114) \qquad (A4.1)$$

Some direct comparisons were made over a limited range for protein values between 3.1% to 3.8%. In all 17 pairs of P and T values were available. Substitution of the T values in A4.1 gave P values which were on average 0.06% lower than the observed P values. Typical examples are given in Table 4.1

TABLE 4.1 MEASURED AND CALCULATED VALUES OF P FOR GIVEN T VALUES

T	36.2	35.1	35.1	34.1	33.6
P(predicted)	3.78	3.72	3.72	3.67	3.64
P(measured)	3.79	3.74	3.72	3.69	3.64

T	32.1	31.5	29.4	27.1	25.8
P(predicted)	3.55	3.52	3.39	3.24	3.15
P(measured)	3.57	3.48	3.36	3.24	3.11

This Table indicates that equation A4.1 can be used to predict P values from Bauch and Lomb T values.

A further comparison can be made utilising equation A3.4. For a PT value of 87.65%, the dial calibration of P is 5.5% (Foss Electric, 1976). Using A3.4, the predicted T value corresponding to a PT of 87.65%, is 85.48%, which when substituted into A4.1 gives a P value of 5.50%. The equivalent calculations for PT of 20.5%, P of 2.50% (Foss

FIGURE A4.1: BEE RELATIONSHIP BETWEEN TRANSMISSION VALUES AND PROTEIN VALUES

Electric, 1976), gives a T of 17.98% and from A4.1 a P of 2.51%.

Therefore equation A4.1 can be used to determine the protein content of milk when the experimental conditions used (volume of milk, volume and composition of dye) are the same as for the Foss Electric Pro-Milk. The relationship would need to be adjusted for any change in conditions.

APPENDIX 5

DYE PURITY AND ABSORBANCE, AND THE DYE BINDING CAPACITY OF MILK PROTEIN

A5.1 INTRODUCTION

Because of the BAA's in the protein chain, milk protein will bind acid azo dyes, as previously discussed (Chapter 4). Many workers in this field have investigated and reported this reaction in terms of the DBC of the milk protein. Some of the causes for variation in the DBC of milk protein have been discussed earlier, but one of the major sources of variation in the quoted figures appears to be the purity of the dyes used.

A5.2 THEORY

In Appendix 1, it was shown that Beer's Law applies to solutions of AB at concentrations used in milk protein studies. However in dye binding work rather than use the Beer's Law constants and symbols it has been found useful to use the system suggested by Ashworth and Chaudry, 1962, which was to define an absorbancy index K, for a dye, where

$$K = \frac{A}{C} \qquad (A5.1)$$

where A is the absorbance

C is the dye concentration in mg/ml (or g/l)

hence from Beer's Law $Eb = K$

Hereafter the Ashworth and Chaudry system will be adhered to.

It is therefore possible by knowing K for pure dye to determine the purity of dye of unknown purity by measuring the absorbance of a solution of the dye, provided the weight of dye and the cell path length are known. This technique has been used (Dolby, 1961; Lakin, 1973; Alais, 1960) to examine dye purity. Where published papers give suitable information it is possible to use this relationship to estimate the purity of the dyes used, and so make valid comparisons of the DBC figures obtained.

A5.3 LITERATURE

(a) Dye Purity

As has been noted (Lakin, 1970), the difficulties in making comparisons are almost entirely associated with

variations in the purity of the dyestuffs used. Not only do dye samples vary according to their source (Dolby, 1961; Alais, 1961; Lakin, 1970) but separate batches from the same manufacturer have been shown to differ (Alais, 1961; Radcliffe, 1966). Of the dyes commonly used, AB, is the most difficult to purify and the most impure (Lakin, 1970). The main impurities in AB which is hygroscopic, are water and sodium chloride. However, a method for purifying AB has been developed (Lakin, 1970), and this has enabled an absorbancy constant (K) to be determined.

The values used in the following discussion will all be in terms of K, but may have been derived from values quoted in terms of other units.

Lakin, 1970, found that 'Merck's AB 10B for electrophoresis' contained, on a dry basis, 6.7 % NaCl, and that after purification the dye which still contained 0.1% NaCl, had a K value of 92.1. Different brands, after purification but still containing up to 0.3% NaCl, had K values varying from 85.6 to 91.1. This indicates that water, and sodium chloride are not the only impurities in some brands of AB, and that these may be considerable amounts of other dyestuff which is not entirely removed by purification. These dyestuff impurities could possibly be a problem in dye binding studies as they may compete for BAA sites on the milk protein (Dolby, 1961). Lakin therefore decided that the dye which was initially the purest, and which after purification was the purest, would be used to determine the K value for AB. This was found to be 92.1.

Sherbon, 1967, reported a K value of 89.6 for AB, and referred to other publications in which he, and another worker (Herrington) compared the properties of AB, AO12, and OG. It was also stated the purity of AB is 90%, but it was not stated if this was on a moisture free basis.

Tarassuk, et al., 1967 found that commercially prepared dyes were not 100% pure. The best grade of AB 10B available was only 86% pure. In addition it was hygroscopic and changed in moisture content on storage. A solution of 0.4400 g/l of Merck AB 10B in a buffer solution (pH2.35), when diluted 100 to 1 had an absorbance of 0.320 in a 1 cm

cell. This gave a K value of 72.7, corresponding to 79.7% purity based on Lakin's K value, or 81.2% based on Sherbon's K value. No statement was made regarding moisture, but it is probable that undried dye was used as no reference is made to any drying process.

It is clearly difficult to extract consistent data on the purity and absorbance constants for the dyes used. Hadland and Johnson appear to have been optimistic in quoting figures of 95% to 97% purity for their dye, while the analysis of Alais, et al, (1961) gives a guide to the composition: 85% purity on a wet basis, 92% on a dry basis. This allows up to 8% impurity on a dry basis which is comparable with Lakin's 6.7% NaCl content in the best sample of dye used (Merck AB 10B for electrophoresis).

Ashworth and Chaudry, 1962, using Merck AB 10B, standardized their dye solutions by using an experimentally determined K value of 81.5. Using a vacuum oven the dye was dried at 75° for 4.5h, and solutions made up from the dry dye at concentrations of 0.005 to 0.015 mg/ml. The highest value found for K was 81.6 and this was assumed to be the value for pure dye. However, it is stated that "since this work was completed, a letter from E.Merck, Darmstadt, states that the pure dye has an absorbancy index of 89.6 at a pH of 2.0." Merck further state that "on the average the assay of our product is 80-86%". Ashworth and Chaudry calculated their data from a K value of 81.5. Based on Merck's K value, which agrees with Sherbon's figure quoted above, Ashworth and Chaudry's dye was 91% pure, on a moisture free basis.

Dolby, 1961, quotes Hadland and Johnson (1959) as finding 'Merck's AB for electrophoresis' as being of 95-97% purity. Dolby found that a 10mg/l (0.01 mg/ml) solution of AB to have an absorbance of 0.75, which gives a K value of 75. Unfortunately it is not stated if the weight of dye is on a moisture free basis. The purity of Dolby's dye appears to be 81.4% relative to Lakin's purified dye, or 83.7% relative to Sherbon's dye. Dolby also found some samples that were only 58% of the purity of the one having a K of 75. Elsewhere in his paper a graph shows

that the absorbance of a solution of AB at 1 g/l was 50 (determined by dilution), which after allowing for other procedural dilutions, gives a K of 75 as well.

Alais, et al., 1961, reported for Merck AB 10B for electrophoresis (No. 1167) that a solution of dye, diluted to 0.7% of its original concentration of 0.6165 mg/ml, had an absorbance of 0.440, in a 1.25 cm cell. This gives a K value of 81.6. Elsewhere in their paper it is stated that AB typically contained 6% to 8% water, and that all dye weights reported in paper were on an undried basis. It appears therefore that the dye used was 89.5% pure relative to Lakin's, or 91.1% relative to Sherbon's dye. Figures are also given for various other brands of dye and these contained up to 48% more impurities. A chemical analysis was also performed on the dye which indicated that the Merck dye, No. 1167, was about 85% pure in the undried state, and 92% after drying.

(b) Dye Binding Capacity of Milk Protein

Not all workers have calculated a DBC value from their work and it is only possible in these cases to make an estimate from a graph or regression equation provided sufficient other data is available.

Lakin, 1973b, quotes milk powder as having an AB DBC of 102 meq/100g which is equivalent to 314.4 mg/g. Because this same worker reported earlier (Lakin, 1970) a method for purifying AB it is likely that this is reported on a pure, dry weight basis. No experimental conditions were stated. This assumption is supported by a statement in Lakin 1973a, that "AB was purified by recrystallisation", and a graph in that paper appears to show a DBC value of about 310 for skim milk powder protein.

Sherbon, 1967, quotes without qualification an AB DBC value of 315, and that the purity of AB is about 90%. It is not clear, but is possible that an allowance has been made for the 10% impurity.

Tarassuk, et al., 1967, while stating that the purity of the best grade of AB is only 86%, does not make

it clear whether subsequent dye weights and/or results are reported in terms of impure, wet dye, impure dry dye, or pure dry dye substance. Throughout his paper, the DBC values vary from 240 to 380 depending on the conditions used. Under the conditions adopted for experimental investigation reported values for skim milk range from 289.9 to 342.7, without any comments being made as to the cause of the variation. For one Table the regression equation linking % protein and absorbance given by Tarassuk, et al., 1967, is

$$P = 5.111 - 4.4893\ A$$

From this for a P of 1%, the absorbance would change by 0.2228, which for an absorbancy value, K, of 89.6 (Merck's figure), means that the AB concentration will alter by 0.002486 mg/ml. Allowing for the dilution (26X) before the readings were taken, and for the total volume of the reaction mixture (20.5ml) the nett change is 1.325 mg of AB. As only 0.5 ml of milk was tested the change of 1% protein is equivalent to a change of 5 mg of milk protein. Therefore the apparent DBC is 265. Applying the same procedure to another regression equation ($P = 5.905 - 4.684\ A$) given elsewhere in the same paper, a calculated DBC of 254 is obtained. Yet another similar equation ($P = 5.155 - 4.289A$) gives a DBC of 277. In one part of the paper where differences between milk producing areas was being investigated, one area produced milk which resulted in the equation $P = 5.6629 - 5.3445\ A$, giving a DBC of 223! No reason for this difference could be found. However it is also stated that a solution of 0.4400 g/l, diluted 100:1 had an absorbance of 0.320 ± 0.005 in a 1 cm cell which corresponds to a K value of 72.7 which is 81.2% of the K given by Merck for their AB. This would change a DBC value of 265 calculated above to a value of 326.5, which is in the region of those quoted by Tarassuk et al. This highlights the importance of the need to state the dye purity.

Dolby, 1961, derives from his work the regression equation

$$P = 6.90\ A + 0.09$$

Therefore a change of 1% in protein would give a change of 0.1449 in the absorbance reading, which for a K value of 89.6, and allowing for dilution (33.33X), reaction volume (15ml) and volume of milk used (equivalent to 0.25ml, diluted to 5 ml) implies that milk protein has a DBC of 323.5 Another equation is also given (P = 7.274 A - 0.05) from which the DBC is apparently 306.9. Dolby does not give any figures for DBC, but he investigated the binding of AB by milk protein using the following procedure: "Buffered solutions of AB at three different concentrations (o.62 g/l, 0.75 g/l and 1 g/l) were prepared. To a number of 10 ml portions of each were added 5 ml volumes of diluted milk containing various quantities of the same whole milk. After centrifuging (the 15 ml of reaction mixture), 3 ml of the supernatant liquid were diluted to 100 ml and the absorbance measured. The absorbance of the undiluted supernatant liquid was calculated." The results were plotted as the change in absorbance against the number of mg of protein added to 10 ml of dye solution. The graph shows that at lower levels of protein to dye ratio the change in absorbance is constant. The regression line passes through the origin, and the point A = 0.48, and 10 mg of protein.

Therefore if the addition of 10 mg of protein changes the absorbance in the dilute supernatant by 0.48, then the true change in the supernatant, applying equation A5.1 is

$$\triangle A = \frac{0.48 \times 100}{3}$$

Using Merck's absorbancy index of 89.6 this corresponds to a dye concentration change, C given by

$$\triangle C = \frac{0.48 \times 100}{3 \times 89.6} \quad \text{mg/ml}$$

Given that the volume of the reaction mixture was 15 ml, the dye bound ($\triangle D$) by the 10 mg of protein, is given by

$$\triangle D = \triangle C \times V$$
$$= \frac{0.48 \times 100 \times 15}{3 \times 89.6} \quad \text{mg}$$
$$= 2.678 \text{ mg}$$

$\therefore$ For milk protein, the DBC = 268

Ashworth and Chaudry, 1962, quote DBC values of 344, 349 and 354 but this is based on dye which had a K value of 81.5, The values become 312.9, 317.4, and 322.0 when converted to pure dye using a K of 89.6. In the same way as has been calculated for other workers, using from their paper the equation

$$P = 4.70 - 1.72\ A$$

the DBC is found to be 308 (based on K = 89.6), or 338 (based on K = 81.5) the latter being identical to a value quoted in their paper.

Alais, et al., 1961 report that their standard dye solution, containing 0.6165 g/l of AB, when diluted to 0.7% of this concentration (i.e. 0.0043155 g/l) had an absorbance of 0.440 in a 1.25 cm path length cell. This gives a calculated K value of 81.6 for a 1 cm cell. The dye used was Merck AB for electrophoresis, number 1167. This dye was not dried, or purified, and was the same batch as had been chemically analysed at 85% purity on an undried basis, and 92% on a dry weight basis. This would indicate that the K value for pure, dry dye should be 94.9. Their equation for liquid milk was

$$P = 4.632 - 5.5d$$

where P is total protein precipitated from 0.75 g of liquid sample (diluted to 10 ml), expressed as a % of 0.75 g, and d is the absorbance of the excess dye in the supernatant solution.

This implies that when 4.632% of 0.75 g of protein is precipitated the supernatant absorbance is zero, i.e. 34.74 mg of protein is precipitated by the quantity of AB used in the test, viz. 20 ml x 0.6165 mg/ml = 12.33 mg, from which the DBC of the precipitated proteins is 355. This, however, is not the same as the DBC of milk protein fraction given by other workers since non protein nitrogen, and some of the soluble proteins which do not bind dye (Alais et al., 1961; Tarassuk, et al., 1967) are included when the protein content of milk is determined by the conventional Kjeldahl procedure. This non dye binding nitrogenous fraction of milk is equivalent to about 6% of the total nitrogen content.

Worker	Crude Dye Purity	K	Purity Basis for K	Milk Protein DBC	Purity Basis for DBC	On basis of K = 89.6 for pure dye
Lakin, 1970	92.3 % after drying	92.1	99.9	314	Presumably 99.9%	323
Sherbon, 1967	90%, basis unknown	89.6	Presumably dye assumed to be 90% pure	315	Presumably dye assumed to be 90% pure	315
Tarassuk, et al., 1967	86%	72.7	Presumably undried, impure	290-343 av. approx. 330	Presumably undried, impure	269
Merck, 1962	80- 86%	89.6	100%	-	-	-
Ashworth & Chaudry, 1962	7.3% H_2O on average	81.5	Dry basis	342-352	Dry basis	315
Dolby, 1961	-	75	Presumably undried, impure dye	268 307-324 av. 315	Absorbance difference with K=89.6, i.e. 100% pure	268 307-324
Alais, et al. 1961	85%, 6-8% H_2O	81.6	Undried, impure (85% purity approx)	334	Undried impure	306 (284 for their pure dye)
Hadland & Johnson, 1959	95 to 97%	-	-	325	-	-

Therefore on a conventional protein content basis, the DBC of milk protein as determined by Alais, et al., 1961, is 333.7 and allowing for dye moisture and impurities, the DBC value is reduced to 283.6 (using their K of 96 for pure dye based on K of 81.6 for 85% purity dye).

The literature data for dye absorbance constants, dye purity and DBC is summarised in Table A5.1.

A5.4 PRACTICAL INVESTIGATION

(a) Dye Purity

(i) Moisture: Four samples (ca. 1g) of AB (Merck, No. 1166 and No. 1167) were accurately weighed into aluminium drying dishes, and dried in a vacuum oven at 80° for 4 h. After cooling in a desicator the samples were weighed and the moistures determined by difference.

(ii) AB Content: The purity of the AB supplied by A/S N.Foss Electric, Hillerod, Denmark for use with the Pro-Milk MkII was determined by establishing the absorbance characteristics of dilute samples of the standard Pro-Milk solution used for the Beer's Law Study (Appendix 1). The weight of dye supplied by Foss Electric was 9.81 g, to be used to make 10 l of working dye .

(b) Dye Binding Capacity

The dye binding capacity of the milk protein in the milk used for the DBC kinetic studies was established from the results of that study.

A5.5 RESULTS AND DISCUSSION

TABLE A5.2 MOISTURE CONTENT OF MERCK AMIDO BLACK 10B

Dye	'Age'	Moisture(%)
Merck No.1166	Freshly opened	4.9
Merck No.1166	Previously opened	15.3
Merck No.1167	Previously opened	10.6
Merck No.1167	Previously opened,old	16.0

As reported extensively in the literature, and noted elsewhere in this thesis, AB is hygroscopic, as clearly indicated by these results. There does not appear to be

any difference in the hygroscopic nature of the two AB dye types, but rather the moisture content appears to be a function of age. No data as to how long the previously opened samples had actually been opened was available. These moisture levels are in line with literature values.

AB Content: From Appendix 1, equation A1.1 states

$$A = 0.01368\ D$$

where A is absorbance in a 0.2mm path length cell,
and D is % of the concentration of the working dye.

Therefore the absorbance of the working dye is 1.368. This is equivalent to an absorbance of 68.40 in a 1 cm path length cell. This is for a solution containing 9.81 g of crude dye in 10 l of solution, i.e. 0.981 mg/ml.

But using the manufacturers absorbancy index of 89.6 (quoted by Ashworth and Chaudry, 1962), the absorbance of a solution containing 0.981 mg/ml of pure dye is

$$A = KC = 87.90 \qquad (A5.1)$$

Therefore the purity of the dye supplied and used was

$$= \frac{68.4}{87.90} \times \frac{100}{1}$$

Based on the K of 92.1 found by Lakin, 1970, for purified dye, the purity of the dye supplied becomes 74.3%.

These figures, while being low, are not impossible given that AB was found to have up to 16% moisture, and that Lakin found about 7% NaCl.

A5.5 EXPERIMENTALLY DETERMINED MILK PROTEIN DYE BINDING CAPACITY

(a) Based on the Pro-Milk Scale

From the derived relationship between P and T (equation A4.1) it is possible to find the DBC of milk protein. As A4.1 was based on two other relationships, equations A2.2 and A3.5, the first of which is for P and PT, the second for PT and T, the value of DBC found will be that on which the Pro-Milk instrument is based. From the regression equation A4.1

$$P = -4.917 + 2.945 \ln (T^{.7736} + 3.114) \qquad (A4.1)$$

it is seen that the relationship is curvilinear as there is a constant included in the logarithmic term. If this constant were 0 the term would become an expression of the absorbance of the supernatant of the form

$$A = 2 - \log T$$

The appearance of the constant indicates that the protein scale on the Pro-Milk is adjusted to allow for the increase in DBC which occurs when the concentration of dye in the supernatant solution increases. (The effect of the constant is greater at low T values, i.e. higher dye concentrations).

It is therefore necessary to express the DBC with reference to the protein level at which the DBC is being measured.

When the formula A4.1 is changed to make T the dependent variable, and this then converted to an expression for A, it is found that

$$A = 2 - 1.2927 \log \left[e^{\left(\frac{(P + 4.317)}{2.945}\right)} - 3.113 \right] \quad (A5.2)$$

When the protein level in the milk is 3.7%, the absorbance of the excess dye in the supernatant is 0.45987. The drop from the initial dye absorbance, corrected for dilution by the addition of 1 ml of milk to 20 ml of dye, of 1.3029 to 0.45987 is due to the 37 mg of milk protein. As the Pro-Milk instrument uses an 0.2 mm light path cell, the appropriate correction factor (50) must be used in calculating the change in dye concentration.

$$\therefore \quad C = \frac{(1.3029 - 0.4599) \times 50}{89.6}$$

$$= 0.47044 \text{ mg/ml}$$

The total amount of dye bound by 37 mg of milk protein is given by

$$D = \Delta C \times V$$

$$= 9.879 \text{ mg}$$

which gives a DBC value of 267.

(b) From the Spectronic 20 Measurements

While the above calculation necessarily involves the use of the Spectronic 20, as this was used to determine the P, T relationship, a single point estimation can also be made from the absorbance value determined from the Spectronic 20 for the non heat treated milk sample of 3.70% protein. This value was 0.4473, which gives by the above procedure a DBC of 271. The difference is not a real difference but one due to the Pro-Milk derived value being based on a series of observations and regression equations, and the latter value being based on one sample. The values of 267, and 271 are amongst the lowest reported in the literature, but given the number of factors that influence the DBC, and the highly variable nature of the dye used, the values are not unusual or contradictory in relation to the literature values.

The DBC value of 267, expressed in milliequivalents of AB is 0.866. As given earlier 1g of milk protein contains 0.8785 milliequivalents of BAA groups. The DBC value of 271 corresponds to 0.879 milliequivalents of dye. On this basis in unheated milk there is effectively 1:1 binding between the binding groups available on the dye, and the sites available on the protein. This does not necessarily mean that all potential bonds are actually formed because there are other mechanisms by which dye can be bound to the protein. If these are operating then not all direct, primary bonds are being formed. This has been discussed earlier.

A5.6 CONCLUSIONS

The dye, amido black, is highly variable in purity on both a dry and undried basis. The purity of the dye can alter to such an extent that the apparent DBC values can be greatly affected. When DBC values are being compared the purity of the dye used, as well as the other experimental conditions must be taken into account.

In the study of the effect of heating on DBC of milk this problem was avoided by converting values to one relative to the DBC of the unheated milk. It was not necessary to actually have an absolute value for that study.

APPENDIX 6

THE EFFECT OF HEAT ON THE COLOUR OF SKIM MILK, AND ITS RELATIONSHIP WITH THE DYE BINDING CAPACITY

A6.1 LITERATURE REPORTS

The progression of the Maillard reaction is characterised by the formation of brown pigments, related to the poorly defined class of high molecular weight pigments often termed melanoidins (Webb and Johnson, 1965; Reynolds, 1969) (See also 1.2) The weight of experimental evidence supports the view that this browning in heated milk is a result of the Maillard condensation of lactose with the milk proteins, principally with the ϵ-amino groups of lysine, rather than as a result of lactose caramelisation.

Brown pigments would be expected to show an absorbance peak in the blue region of the visible spectrum, i.e. about 400 to 480 nm, and that this absorbance would increase with the increase in pigment formation. This in fact has been found (Burton, 1954; Dura, et al., 1958).

With increasing concentration, temperature, and pH the rate of browning is greatly increased (Burton, 1954), but in normal fluid milk browning was not significant below 80° under normal pH conditions, although sugar-protein complexing has been detected (Tumerman and Webb, 1965). For normal skim milk in the temperature range of 95° to 120°, the rate of browning increases 3.1 times for an increase of 10° in temperature, (Burton, 1954) indicating that the reaction has an energy of activation of 30Kcal/mole. It was found that reflectance of heat treated milk relative to the reflectance of non heat treated milk is approximately linear with heat treatment time. The maximum change in this relative reflectance covered in the experiment reported was about 50%.

Similar behaviour was noted for goats milk (Burton, 1963) but in that case the Q10 was 2.6 for homogenised milk, and 2.7 for non homogenised milk. This means that the energy of activation for the development of colour was 27 Kcal/mole.

Other literature reports tend to gloss over the quantitative measurement of the colour of browned food products and tend to report the colour in subjective visual terms such as "straw coloured", "slightly browned," "scorched", and so on (Finot, et al., 1971; Tarassuk, et al., 1967). For pure mixtures kinetic relationships have been devised such as, for glucose and glycine (Mollah, 1968)

$$Cp = K C_{gl}^{2} C_{g} t^{2} \qquad (1.12)$$

and for fructose and glycine

$$Cp = K C_{gl} C_{f} \qquad (1.13)$$

where C_p is pigment concentration
C_{gl} is glycine concentration
C_g is glucose concentration
C_f is fructose concentration, and
t is time.

The method of pigment measurement, or the identity of the pigments was not given. The possible range of pigments would probably be more limited than in heated milk due to the limited and pure nature of the reaction substrates.

As discussed earlier in this thesis (Chapter 1) lactose reacts with the available ϵ-amino groups of lysine in a 1:1 ratio, forming heat labile, colourless complexes, which breakdown on further heating to form a wide range of compounds many of which are brown or may serve as browning intermediates. While the reaction pathways are many, complex, and still not fully understood, the ϵ-amino groups of lysine are significantly involved (see figure A6.1). It can therefore be expected that as the formation of brown pigments progresses the DBC of the milk protein will be affected. Consequently it may be expected that some type of relationship may be found to exist between the RDBC and the extent of browning. For evaporated skim milk which was subsequently diluted to about 20% solids, and then heated at 120° for a range of times, up to 10 minutes it was found that browning, as measured by a reflectance colorimeter using a blue filter was linearly related to DBC (Tarassuk, et al., 1967)

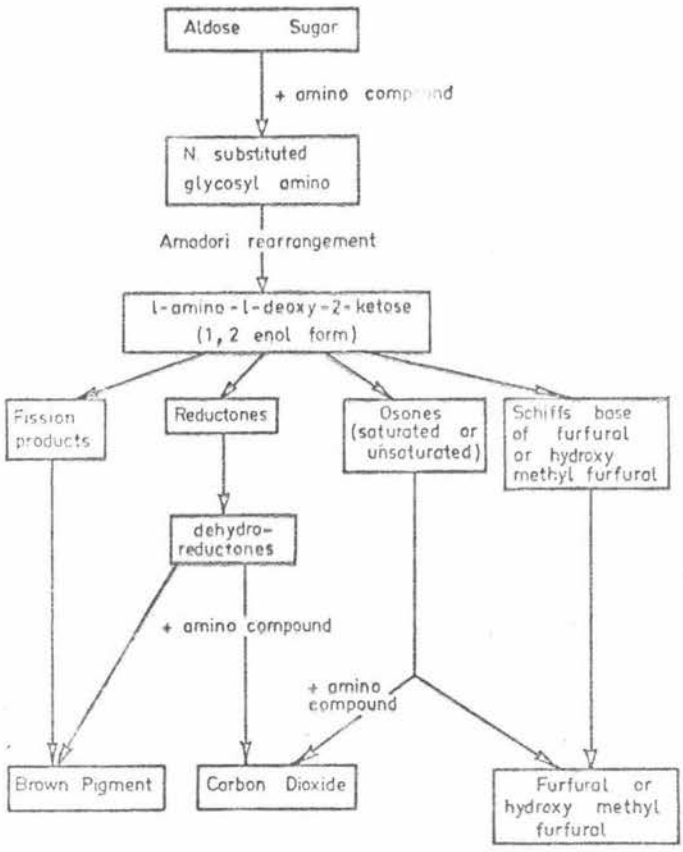


Figure A6.1 RELATIONSHIPS BETWEEN PIGMENT, CARBON DIOXIDE, AND FURFURAL PRODUCTION (COLE, 1967)

A6.2 PRACTICAL INVESTIGATION

The same samples that were used for the dye binding tests were also tested for their C.I.E. tristimulus values (X, Y, Z), using a Neotec Du-Color reflectance colorimeter. The samples were put in a sample holder which had uniform optical density over the visible spectrum, and the X,Y,Z values were determined through the base of the sample holder. The depth of fluid in the sample holder was at least 1.5 cm. This depth was found to be critical, as at lesser depths the colour coordinates were found to be a function of the depth of sample. The sample holder was shielded to prevent daylight penetrating the sample and thereby affecting the reading.

The samples were also usually ranked by several observers in order of increasing browning. This was done without the experimenter being aware of either the heat treatments used or the X,Y,Z values for any of the samples.

A6.3 RESULTS

TABLE A6.1 C.I.E. REFLECTANCE VALUES FOR HEAT TREATED MILK

Heat Treatment		C.I.E. Reflectance			Visual Ranking
Temp (°C)	Time(h)	X	Y	Z	(1 = lightest)
No heat treatment		62.9	66.1	74.0	Not ranked
100	1	61.5	65.2	72.2	1
	2	59.4	62.7	61.7	2
	3	54.7	57.1	51.1	4
	15	27.3	26.1	15.8	8
110	1	61.7	65.0	60.6	3
	2	47.4	47.6	35.0	5
	3	37.2	36.0	22.9	7
	6	27.7	26.0	14.7	10
120	1	40.2	39.6	27.8	6
	2	29.0	27.3	15.3	9
	3	24.9	23.1	12.2	11

A6.4 DISCUSSION

The visual ranking agreed exactly with the order of the Z values even though the differences between some samples is very small, e.g. between samples 100/15 and 120/2. Although not shown in the table, nine of the samples were duplicated in the visual grading, and all pairs were correctly identified. Figure A6.2 shows the samples arranged in order of increasing browning and they can be identified by reference to the Results table. The visual similarity of samples of similar Z values is readily appreciated even allowing for some colour distortion during the photographic processing.

Figure A6.2 The heat processed milk samples in order of visual browning.

No.	1	2	3	4	5	6
Process	100/1	100/2	110/1	100/3	110/2	120/1

No.	7	8	9	10	11
Process	110/3	100/15	120/2	110/6	120/3

All the tristimulus values show a decrease with an increase in heat treatment time, and the rate of decrease increases with an increase in temperature. This is demonstrated in figure A6.3, a plot of Z values relative to that of unheated milk, against time of heating. The greatest change in reflectance occurs in the Z value which in the C.I.E. system corresponds to the blue region of the visible spectrum. Therefore the following analysis is based on the Z values.

Insufficient data is available to show clearly whether the relative reflectance heat treatment time relationship is linear in the initial stages for milk

FIGURE A6.3: RELATIVE REFLECTANCE OF HEATED MILK VERSUS HEAT TREATMENT

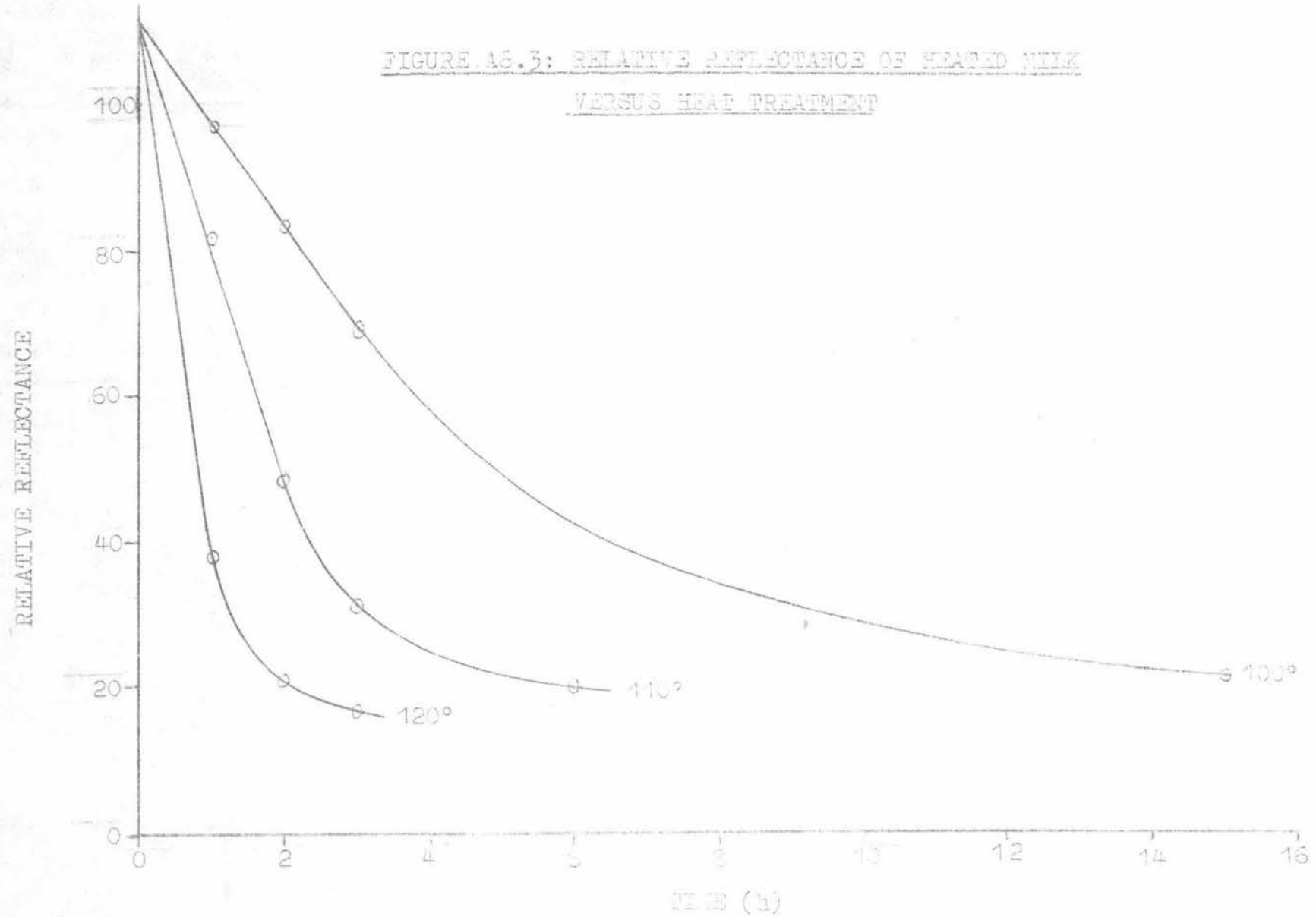
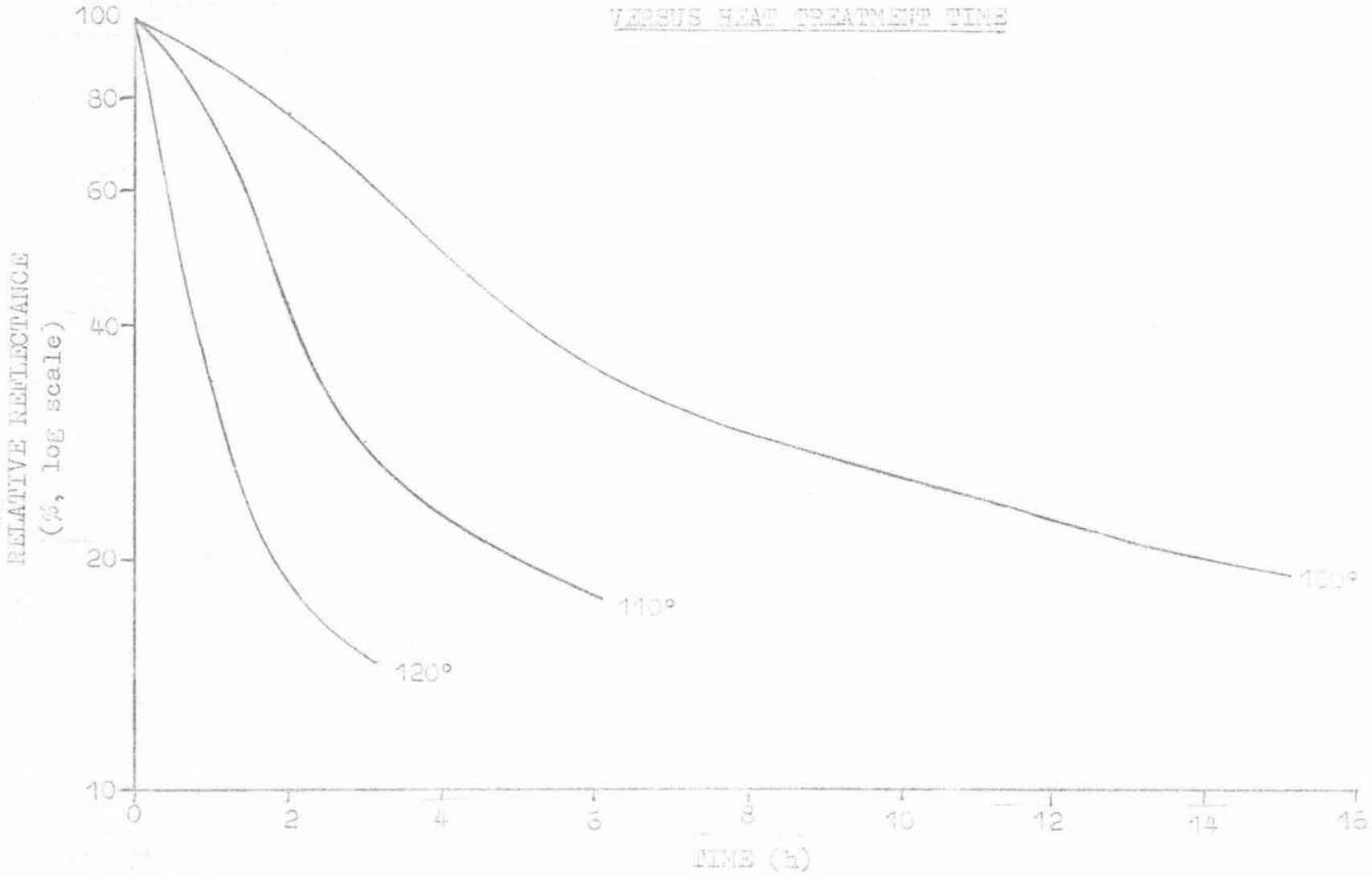


FIGURE A6.4: RELATIVE REFLECTANCE OF HEATED MILK VERSUS HEAT TREATMENT TIME



heated at 120°. This is the case for milk heated at 110° and 100°, and has been found (Burton, 1954) to be the case for temperatures between 95° and 120° inclusive, for changes in relative reflectance of up to 50%. Therefore a corresponding linear portion for the 120° relationship between 111% and 54% relative reflectance has been plotted in figure A6.3. The development of browning appears to be zero order during the initial stages.

Extrapolation of the linear section of the 100° and 110° relationships gives a pseudo relative reflectance of non heat treated milk of 111% of that actually found. This was also reported by Burton, 1954. In fact during the initial stages of heating fluid milk there is a noticeable increase in the X,Y,Z values. (Burton, 1954; Reeves,1976). This rise probably masks the lag phase usually noted with colour development in model systems and foods undergoing browning. (Burton, 1963; Cole, 1967; Spark, 1969; Schnickels, 1976). From figure A6.3, the reflectance values show a 'lag phase' insofar as the reflectance readings are less than the initial readings only after 40 min. for the 100° treatment, 20 min. for the 110° treatment, and 7 min. for the 120° treatment. At 100° the maximum reflectance values occur after about 7 minutes and are about 8% to 10% higher than for the non heat treated milk. After this time all values decreased. Therefore a pseudo reflectance value for zero time of heat treatment obtained by extrapolation will be used where appropriate in this analysis.

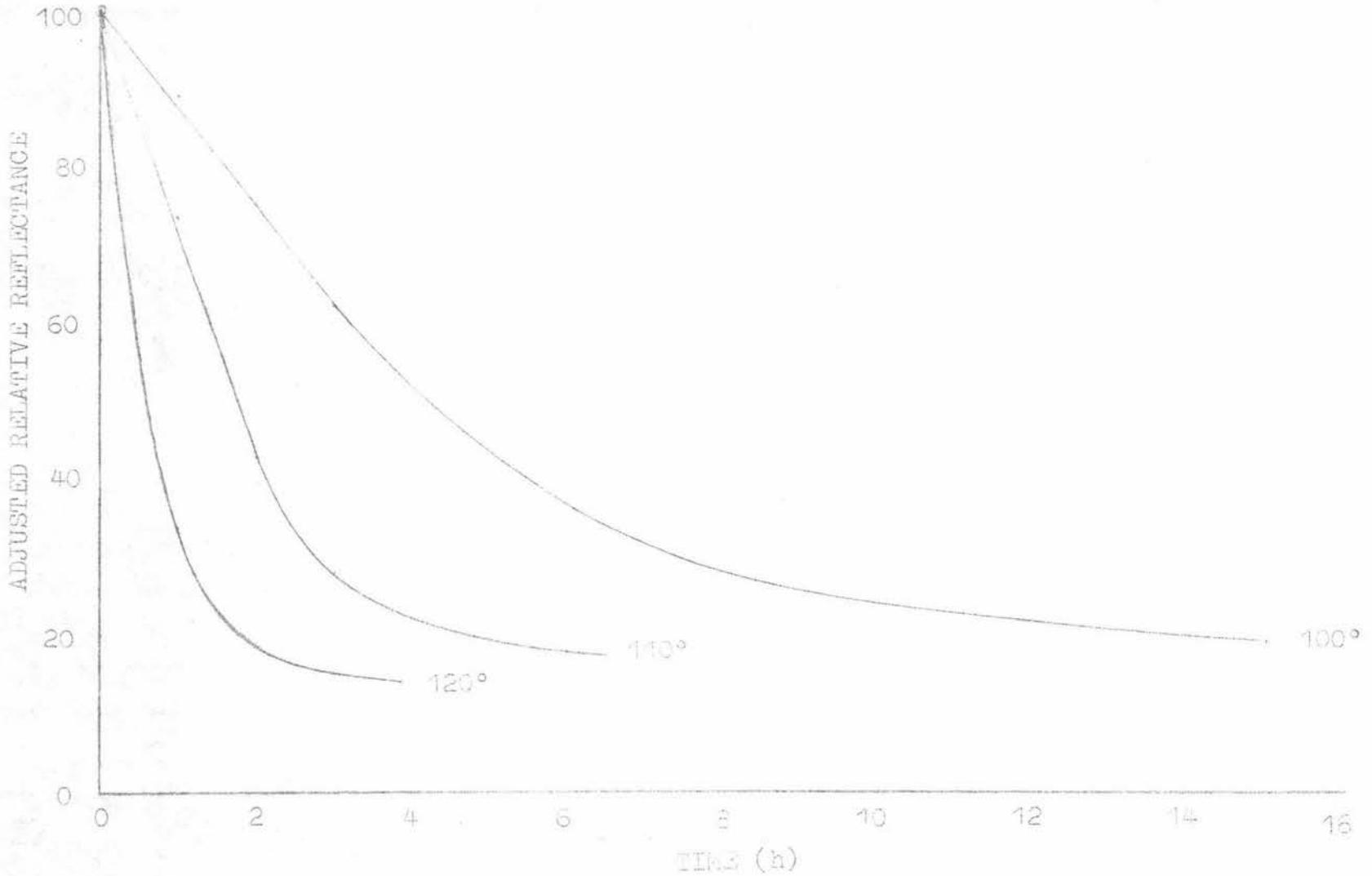
Recalculating the Z values relative to the pseudo initial Z value of 111% x 74, i.e. 82.14 gives the adjusted relative reflectance values listed in Table A6.2.

TABLE A6.2 ADJUSTED RELATIVE REFLECTANCE VALUES

Temperature (°C)	-	100°				110°	
Time (h)	-	1	2	3	15	1	2
Relative Reflectance (%)	100	87.9	75.1	62.2	19.2	73.8	42.6

Temperature (°C)	110°		120°		
Time (h)	3	6	1	2	3
Relative Reflectance(%)	27.9	17.9	33.8	18.6	14.9

FIGURE A6.5: ADJUSTED RELATIVE REFLECTANCE OF HEAT TREATED MILK



A semilog plot of these values gives a series of sigmoid shaped curves, figure A6.4, indicating that browning as measured by Z values does not follow first order kinetics. When this plot is made on natural graph paper, figure A6.5, as would be expected from figure A6.3, the initial stages during which there is approximately a 50% change in relative reflectance is linear. From this it is possible to derive the rate equation A6.1

$$R_Z = 100 + kt \qquad (A6.1)$$

where R_Z is % relative reflectance for Z
k is the zero order rate constant, and
t is time.

The three rate constants for the linear (hence zero order) sections are

$$k_{100} = -12.5\ h^{-1}$$
$$k_{110} = -27.9\ h^{-1}$$
$$k_{120} = -81.0\ h^{-1}$$

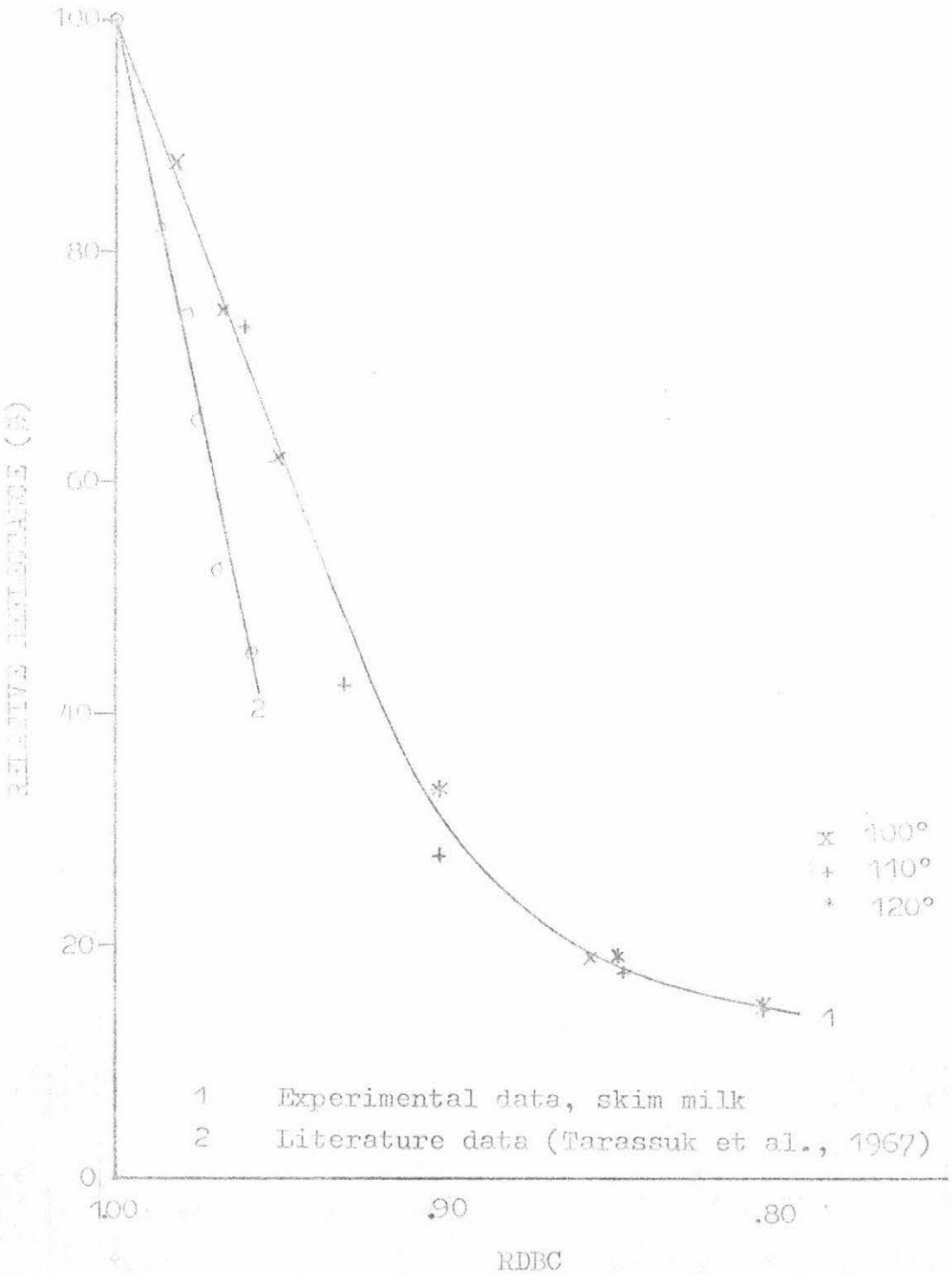
Burton, 1954 found that the rate constants of browning, as determined by reflectance colorimetry using a filter with a peak at 426 nm, to be

$$k_{100} = -17.4\ h^{-1}$$
$$k_{110} = -56\ h^{-1}$$
$$k_{120} = -185\ h^{-1}$$

(These figures were calculated from Burton's published graph and were not included in the text of the paper.) These figures are different from those calculated in this experiment. The % reflectance loss depends on the nature of the filter used (see the X,Y,Z values given earlier in this Appendix), and as the rate of browning is greatly influenced by the solids content and pH of the milk, the differences are not unreasonable.

From the three experimentally derived rate constants for brown colour development two estimates of Q10 (the increase in reaction rate when the reaction temperature is increased by 10°) are 2.2, calculated from the ratio of

FIGURE A6. 6 : RELATIVE REFLECTANCE VERSUS RDBC FOR HEAT TREATED MILK



k_{110} to k_{100}, and 2.9 calculated from k_{120} and k_{110}. The latter figure agrees quite well with the literature figure of 3.1 (Burton, 1954). The corresponding two estimates from this experiment for the energy of activation for the reaction giving colour development are 22.3 Kcal/mole, and 30.1 Kcal/mole.

Theoretically it was predicted that because the free ϵ-amino lysine groups are involved in both the dye binding mechanism, and the browning reaction there could be some relationship between RDBC and the colour as determined by reflectance values. Plotting the figures from Table A6.3 gives figure A6.6

TABLE A6.3 RELATIVE REFLECTANCE AND RDBC FOR HEATED MILK

Temperature	100			
Time	1	2	3	1[illegible]
R Z	87.9	75.1	62.2	19.2
R.D.B.C.(%)	98.2	96.8	95.1	85.6

Temperature	110			
Time	1	2	3	6
R Z	73.8	42.6	27.9	17.9
R.D.B.C.(%)	96.1	93.1	90.3	84.7

Temperature	120		
Time	1	2	3
R Z	33.8	18.6	14.9
R.D.B.C.(%)	90.2	84.9	80.5

This shows that there is a very definite relationship between the two variables and that it does not matter at what temperature the milk is treated, the results follow a consistent pattern.

While Tarassuk, et al., 1967, do not quote their results in the same terms, and used a different colorimeter, and therefore a filter that is likely to have different

characteristics, it is possible to extract from their graph the results in Table A6.4 for a condensed skim milk that had been partially diluted, to give 22.5% total solids, and then heated at 120°.

TABLE A6.4 LITERATURE VALUES FOR RELATIVE REFLECTANCE AND RDBC

Time (min)	0	2	4	6	8	10
Relative Reflectance	100	81.8	74.5	65.5	52.7	45.5
RDBC	100	98.7	97.9	97.6	97.0	95.9

These values have also been plotted on Figure A6.6. The figures are not expected to be directly comparable with the results of this present study as at increased concentration of solids browning is more rapid, and as shown the filter used can affect the rate of decrease in relative reflectance. The main point in the comparison is the fact that the relationship found was also essentially linear over the range of heat treatments used.

The shape of the line found in this study does not readily suggest a possible kinetic relationship of any mechanistic basis. A log plot does not result in any recognisable relationship. This should not be unexpected as the loss of free amino groups is not proportional to colour intensity nor does it indicate the extent of the reaction since the loss occurs mainly in the initial stages of the Maillard reaction (Spark, 1969; Hurrell and Carpenter, 1975) and pigment formation begins after a lag phase. Further the absorbance (or reflectance) of a brown mixture cannot be taken as a measure of the amount of pigments present, nor as a quantitative indicator of the extent of a particular reaction due to the many pathways leading to brown colour formation, and furthermore it is likely that the absorbance of the end products varies from pigment to pigment. The proportions of pigment formed can be changed simply by altering conditions such as the temperature, sugars present, solids, water content and protein type (Mollah, 1968). However for a given product under a given

set of experimental circumstances it should be possible to experimentally derive a relationship, such as figure A6.6, which would be applicable to a similar product that has undergone similar treatment.

APPENDIX 7

OBSERVATIONS ON THE TIME/TEMPERATURE RELATIONSHIP IN THE COAGULATION OF MILK

In the experimental dye binding section of this investigation it was observed that some of the heated milks had coagulated. Although no attempt was made to quantify the coagulation it was noticeable that there was very slight coagulation in milk heated at 100° for 15h, and in the 110°/6h, while in the 120°/3h sample there was slight coagulation. A sample heated at 120° for 4h was so coagulated that it was impossible to satisfactorily break the gel for dye binding tests.

These observations fit the reported figures of coagulation occuring at 100° after 12 h, and at 130° in 1h (Tumerman and Webb,1965). From this it appears that for coagulation the Q10 is about 2.5, i.e. approximately the same as for the colour change (Appendix 6), and for the rate of change of TRDBC. Thus these very diverse properties are all affected in approximately the same manner as the temperature is increased. While this does not mean that they are necessarily related, it is interesting to record that these Q10 values are all significantly lower than typical Q10 values for protein denaturation. If is therefore unlikely that protein denaturation is important in the way heat affects the three properties studied.

APPENDIX EIGHT

COMPUTER PROGRAMMES FOR THE DEVELOPMENT OF THE KINETIC MODELS

A8.1: PURPOSE

The following computer programmes were devised to find the optimum factor required to modify the observed changes in

(a) acid available lysine (TLV), and

(b) dye binding capacity (DBC),

to give first order models. There is little reason in either case to believe that the first order model represents an actual change in the heat treated milk, and its main use is to provide a basis for the derivation of predictive relationships for TLV and DBC.

A8.2: PROGRAMME STRUCTURE

(a) For Acid Available Lysine (TLV)

The RTLV values are read in and the changes in RTLV found (CTLV). These changes are then multiplied by a factor (F) greater or equal to 1, from which the model RTLV are calculated (1 - F x CTLV). To develop first order models the modified data must be converted to natural logarithm values (ALFTLV). The data for the three treatment temperatures must be fitted to three first order models. This is done by the regression subroutine sum (T,L,N) which provides the data for the calculation of the three rate constants (RC(I)).

To determine which F value gives the optimum fit of the modified data to a first order rate expression the sums of squares (SSQ) for each of the three temperatures is determined, which when added together gives the total sums of the squares at a given F value. While this provides an indication of the overall goodness of fit for the three models at that F value it places equal weighting on all results. As the slopes of the three model rate equations are the rate constants for the three temperatures the covariance of the slopes (COV) provides the

best indicator for the model for each temperature. These can then be averaged with weighting dependent on the degrees of freedom associated with each estimate of COV.

Other parameters calculated to provide additional statistical information are the total sum of the squares (TSSQ), and the weighted mean square (WMSQ). This latter quantity is based on the sum of the sum of the squares (WSSQ) at a given temperature multiplied by the corresponding rate constant. This weighting puts more emphasis on the results of the higher temperature run which because the measured differences are greater overall, could be expected to be relatively the most accurate. The WMSQ is then given by WSSQ divided by the sum of the three rate constants (SRC).

The geometric mean rate constant (GMRC) is calculated in order to follow the average change in model rate constant as the F value is increased. If the Arrhenius relationship is followed, the GMRC value should be close in value to the RC for the model at 110°.

(b) For Dye Binding Capacity (DBC)

This programme is essentially the same as for RTLV with the addition of a subroutine (TRANS(D)), to convert the Spectronic 20 absorbance data to RDBC values which are then analysed in the same way as the RTLV data in the first programme.

ACID RELEASED LYSINE PROGRAMME

```
      DIMENSION TIME(12),TLV(12),CTLV(12),ALFTLV(12),RC(3),N(3)
      DIMENSION SQ(3),SS(3),COV(3)
      COMMON/C1/SUMT(3),SUML(3),SSL(3),STL(3),SST(3)
      DATA N/3,4,5/
      READ(5,/) TIME
      READ(5,/) TLV
C* PRINT TEMPERATURES, TIMES, TOTAL LYSINE VALUES
C*
      DO 1 I=1,12
1     WRITE(6,101) TIME(I),TLV(I)

C* CHANGE TLV TO CTLV
      DO 2 I=1,12
2     CTLV(I)=1-TLV(I)
      DO 99 F=3.3,3.7,0.01
      WRITE(6,102)F
C* INITIALIZATION
      DO 3  I=1,3
      RC(I)=0;SST(I)=0;STL(I)=0;SSL(I)=0;SUMT(I)=0;SUML(I)=0
3     CONTINUE
      TSSQ=0;WSSQ=0;SRC=0;GMRC=0
C* MULTIPLY CTLV(I) BY F FACTOR AND CONVERT TO LOGS
      DO 4 I= 1,12
4     ALFTLV(I)=ALOG(1-F*CTLV(I))
      DO 7 I =1,3
7     CALL SUM(TIME(I),ALFTLV(I),1)
      DO 8 I=4,7
8     CALL SUM(TIME(I),ALFTLV(I),2)
      DO 9 I=8,12
9     CALL SUM (TIME(I),ALFTLV(I),3)
      DO 10 I=1,3
      RC(I) =STL(I)/SST(I)
      SSQ(I) =(SSL(I)-STL(I)**2/SST(I))/(N(I)-1)
      SS(I) =(SSL(I)-STL(I)**2/SST(I))/(N(I)-2)
      SSLOPE=SQRT(SS(I)/(SST(I)-SUMT(I)**2/N(I)))
      COV(I)=(SSLOPE/RC(I))*100
      L=90+I*10
10    WRITE(6,103)L,RC(I),SSQ(I),COV(I)
C*
C* CALCULATE GEOMETRIC MEAN RATE CONSTANT
      GMRC=(ABS(RC(1)*RC(2)*RC(3)))**(1.0/3.0)
      WRITE(6,105)GMRC
C* CALCULATE SUM OF SQUARES FOR ALL TEMPERATURES, WEIGHTED MEAN
C- SQUARE AND AVERAGE COEFFICIENT OF VARIATION FOR ALL TEMP
C*
      DO 11 I=1,3
      TSSQ= TSSQ+SSQ(I)*(N(I)-1)
      WSSQ=WSSQ+SSQ(I)*RC(I)
11    SRC=SRC+RC(I)
      WMSQ =WSSQ/SRC
      ACOV =SQRT(((COV(1)**2)*1+(COV(2)**2)*2+(COV(3)**2)*
      WRITE(6,104)TSSQ
      WRITE(6,106)WMSQ
      WRITE(6,107)ACOV
99    CONTINUE
101   FORMAT(2F10.5)
102   FORMAT(///'   TEMP.    RATE CON      MEAN SQUARE    COV
103   FORMAT(I5,F14.7,F13.7,F9.2)
104   FORMAT(/'  TOTAL SUM OF SQUARES = ',F8.6)
106   FORMAT(/'  WEIGHTED MEAN SQUARE = ',F8.6)
105   FORMAT(/'  GEO MEAN RATE CONST  = ',F8.6)
107   FORMAT(/'   AVERAGE COEF OF VARN =',F9.5)
      END
      SUBROUTINE SUM(T,L,N)
      REAL L
      COMMON/C1/SUMT(3),SUML(3),SSL(3),STL(3),SST(3)
      SUMT(N) =SUMT(N)+T
      SST(N)  =SST(N)+T*T
      SUML(N)  =SUML(N)+L
      SSL(N)  =SSL(N)+L*L
      STL(N)=  STL(N)+T*L
      END
```

SAMPLE PRINTOUT

TEMP.	RATE CON	MEAN SQUARE	COV	F= 3.42
100	=0.0582215	0.0000784	=15.49	
110	=0.2077467	0.0071414	=29.14	
120	=0.7548503	0.0065745	=9.34	

GEO MEAN RATE CONST = 0.209006

TOTAL SUM OF SQUARES = 0.047079

WEIGHTED MEAN SQUARE = 0.006319

AVERAGE COEF OF VARN = 19.14834

TEMP.	RATE CON	MEAN SQUARE	COV	F= 3.43
100	=0.0584039	0.0000789	=15.50	
110	=0.2085397	0.0072002	=29.15	
120	=0.7595354	0.0066267	=9.32	

GEO MEAN RATE CONST = 0.209923

TOTAL SUM OF SQUARES = 0.048265

WEIGHTED MEAN SQUARE = 0.006371

AVERAGE COEF OF VARN = 19.14796

TEMP.	RATE CON	MEAN SQUARE	COV	F= 3.44
100	=0.0585863	0.0000794	=15.50	
110	=0.2093343	0.0072594	=29.16	
120	=0.7642658	0.0066830	=9.30	

GEO MEAN RATE CONST = 0.210644

TOTAL SUM OF SQUARES = 0.048669

WEIGHTED MEAN SQUARE = 0.006425

AVERAGE COEF OF VARN = 19.14017

DYE BINDING PROGRAMME

```
      DIMENSION DATA(11),TEMP(11),RC(3),SQ(3), N(3),SS(3),COV(3)
      COMMON/C1/ST(3),SAB(3),SAB2(3),SABT(3),ST2(3)
      DATA N/3,4,4/
      READ(5,7) DATA
C*    PRINT ABSORBANCE DATA
      DO 1 I=1,11
1     WRITE(6,101)I,DATA(I)
C*    TRANSFORM DATA TO % ORIGINAL DOC*VALUE (RDOC)
      CALL TRANSF(DATA)
C*
C*    INCREASE MULTIPLICATION FACTOR (F) STEPWISE
      DO 9 F=3.60,3.80,.01
      WRITE(6,102)F
C*
C*    INITIALIZATION
      TSSQ=0;WMSQ=0;SRC=0;GMRC=0
      DO 2 I=1,3
      ST(I)=0;SAB2(I)=0;SABT(I)=0;ST2(I)=0
2     CONTINUE
C*
C*    MULTIPLY CHANGE IN  RDOC BY F ;CONVERT TO LOGS
      DO 3 I=1,11
      IF(1-F*DATA(I) .LE. 0) GOTO 9
3     TEMP(I)=ALOG(1-F*DATA(I))
      DO 4 I=1,3
      T=1
4     CALL SUM(T,TEMP(I),1)
      DO 5      I=4,7
      T=I-3
      IF(T.EQ.4)T=6
5     CALL SUM(T,TEMP(I),2)
      DO 6 I=8,11
      T=I-7
      IF(T.EQ.4)T=15
6     CALL SUM(T,TEMP(I),3)
C*
C*    CALCULATE AND WRITE:RATE CONST,SUM OF SQUARES AND COEFF
C*    OF VARIATION FOR EACH TEMPERATURE ;CONVERT I TO TEMP
      DO  7     I=1,3
      RC(I)=SABT(I)/ST2(I)
      SSQ(I)=(SAB2(I)-SABT(I)**2/ST2(I))/(N(I)-1)
      SS(I)=(SAB2(I)-SABT(I)**2/ST2(I))/(N(I)-2)
      SSLOPE =SQRT(SS(I)/(ST2(I)-ST(I)**2/N(I)))
      COV(I) =(SSLOPE/RC(I))*100
      L=130-I*10
7     WRITE(6,103)L,RC(I),SSQ(I),COV(I)
C*
C*    CALCULATE GEOMETRIC MEAN RATE CONSTANT
      GMRC =(ABS(RC(1)*RC(2)*RC(3)))**(1.0/3.0)
      WRITE(6,106)GMRC
C*
C*    CALCULATE SUM OF SQUARES FOR ALL TEMPERATURES, WEIGHTED
C*    MEAN SQUARE AND AVERAGE COEFFICIENT OF VARIATION FOR
      ALL TEMPERATURES
      DO 8 I = 1,3
      TSSQ=TSSQ + SSQ(I)*(N(I)-1)
      WSSQ=WSSQ +SSQ(I)*RC(I)
8     SRC =SRC + RC(I)
      WMSQ =WSSQ/SRC
      ACOV =SQRT(((COV(1)**2)*2+(COV(2)**2)*2+(COV(3)**2)*1)/5)
      WRITE(6,104)TSSQ
      WRITE(6,105)WMSQ
      WRITE(6,107)ACOV
9     CONTINUE
C*
101   FORMAT (I5,F10.5)
102   FORMAT(///'  TEMP.    RATE CON       MEAN SQUARE    COV
103   FORMAT(I5,F14.7,F13.7,F9.2)                          F = ',F4.2)
104   FORMAT(/'   TOTAL SUM SQUARES      = ',F8.6)
105   FORMAT(/'   WEIGHTED MEAN SQUARE = ',F8.6)
106   FORMAT(/'   GEO MEAN RATE COEF    =-',F8.6)
107   FORMAT(/'   AVERAGE COEF OF VARN = ',F9.5)
      END
```

```
      SUBROUTINE SUM(T,AB,N)
      COMMON /C1/ST(3),SAB(3),SAB2(3),SABT(3),ST2(3)
      ST(N)=ST(N)+T
      ST2(N)=ST2(N)+T*T
      SAB(N)=SAB(N)+AB
      SABT(N)=SABT(N)+AB*T
      SAB2(N)=SAB2(N)+AB*AB
      END
      SUBROUTINE TRANSF(D)
      DIMENSION D(11)
      DO 1 I=1,11
    1 D(I)=1+(D(I)-1.3029)/.8556
      END
```

SAMPLE PRINTOUT

```
TEMP.    RATE CON       MEAN SQUARE    COV     F = 3.67
120     -0.4158233      0.0007429     -6.55
110     -0.1401192      0.0003136     -4.14
100     -0.0508552      0.0009557     -6.56

GEO MEAN RATE COEF    =-0.143631

TOTAL SUM SQUARES     = 0.005294

WEIGHTED MEAN SQUARE  = 0.005131

AVERAGE COEF OF VARN  =   5.71327
```

```
TEMP.    RATE CON       MEAN SQUARE    COV     F = 3.68
120     -0.4178852      0.0007530     -6.57
110     -0.1406754      0.0003075     -4.08
100     -0.0510577      0.0009543     -6.53

GEO MEAN RATE COEF    =-0.144249

TOTAL SUM SQUARES     = 0.005292

WEIGHTED MEAN SQUARE  = 0.005174

AVERAGE COEF OF VARN  =   5.69548
```

```
TEMP.    RATE CON       MEAN SQUARE    COV     F = 3.69
120     -0.4199590      0.0007647     -6.58
110     -0.1412353      0.0003014     -4.02
100     -0.0512608      0.0009529     -6.50

GEO MEAN RATE COEF    =-0.144969

TOTAL SUM SQUARES     = 0.005292

WEIGHTED MEAN SQUARE  = 0.006421

AVERAGE COEF OF VARN  =   5.68054
```

```
      SUBROUTINE SUM(T,AB,N)
      COMMON /C1/ST(3),SAB(3),SAB2(3),SABT(3),ST2(3)
      ST(N)=ST(N)+T
      ST2(N)=ST2(N)+T*T
      SAB(N)=SAB(N)+AB
      SABT(N)=SABT(N)+AB*T
      SAB2(N)=SAB2(N)+AB*AB
      END
      SUBROUTINE TRANSF(D)
      DIMENSION D(11)
      DO 1 I=1,11
    1 D(I) =14(D(I)-1.3029)/.8556
      END
```

SAMPLE PRINTOUT

TEMP.	RATE CON	MEAN SQUARE	COV	F = 3.67
120	-0.4158233	0.0007429	-6.55	
110	-0.1401192	0.0003136	-4.14	
100	-0.0508552	0.0009557	-6.56	

GEO MEAN RATE COEF = -0.143631

TOTAL SUM SQUARES = 0.005294

WEIGHTED MEAN SQUARE = 0.005131

AVERAGE COEF OF VARN = 5.71327

TEMP.	RATE CON	MEAN SQUARE	COV	F = 3.68
120	-0.4178852	0.0007530	-6.57	
110	-0.1406754	0.0003075	-4.08	
100	-0.0510577	0.0009543	-6.53	

GEO MEAN RATE COEF = -0.144249

TOTAL SUM SQUARES = 0.005292

WEIGHTED MEAN SQUARE = 0.005174

AVERAGE COEF OF VARN = 5.69548

TEMP.	RATE CON	MEAN SQUARE	COV	F = 3.69
120	-0.4199590	0.0007647	-6.58	
110	-0.1412353	0.0003014	-4.02	
100	-0.0512608	0.0009529	-6.50	

GEO MEAN RATE COEF = -0.144969

TOTAL SUM SQUARES = 0.005292

WEIGHTED MEAN SQUARE = 0.006421

AVERAGE COEF OF VARN = 5.69054

APPENDIX 9

INVESTIGATION OF CHROMATOGRAM CONTAMINATION

A9.1 INTRODUCTION

Because of the apparent contamination of the chromatographs by a non amino acid derivative (see 2.9) a brief, non quantitative investigation was made into the source of the observed contaminant, to establish if there were any other contaminant peaks, other than the major one that coincided with alanine (on the EGA column), and threonine (on the OV-17/OV210 column), and particularly if any were coincident with or in the region of the lysine derivative. A further aspect was to determine if lysine was affected by the production of the contaminant peak since it was thought that the contaminant may have been a product of browning in the milk, although this was thought unlikely as the contamination was detected in chromatograms of non heat treated milk.

A9.2 EXPERIMENTAL

Four trials were set up.

(a) Lactose (15mg), and lysine monohydrochloride (0.75mg) in distilled water (3ml) were heated at 130° for 16h. After heating the mixture was dried under vacuum on a rotary evaporator and then derivatized.

The resulting OV-17/OV210 and EGA chromatograms are shown in figure A9.1.

(b) The same mixture as (a) was first dried and then heated in the presence of 6N HCl (12ml) at 130° for 1h, after which time the solution was dried and derivatized. The corresponding chromatograms are shown in figures A9.2 and A9.3.

(c) To each of two culture tubes, each containing lactose (15mg) and lysine monohydrochloride (0.75mg), 6N HCl (12ml) was added. One was deaerated and flushed with CO_2, and the other was left with an air headspace. Both were then heated at 145° for 4h (as per the hydrolysis procedure used for milk). The hydrolysates were then derivatized. The chromatograms are reproduced as figures A9.4, A9.5, A9.6 and A9.7.

FIGURE A9.1a: OV-17/OV-210 CHROMATOGRAM FOR DERIVATIZED, HEATED LYSINE/LACTOSE SOLUTION

1 Coincides with threonine
3 Variable, complex
4 Lysine

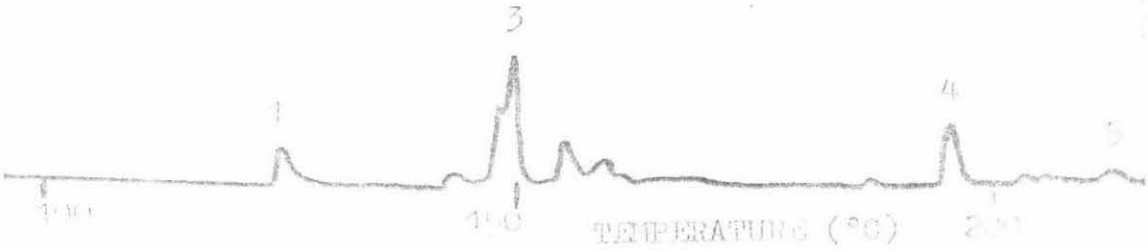


FIGURE A9.1b: EGA CHROMATOGRAM FOR DERIVATIZED, HEATED LYSINE/LACTOSE SOLUTION

1 Coincides with alanine
3 Variable, complex
4 Lysine

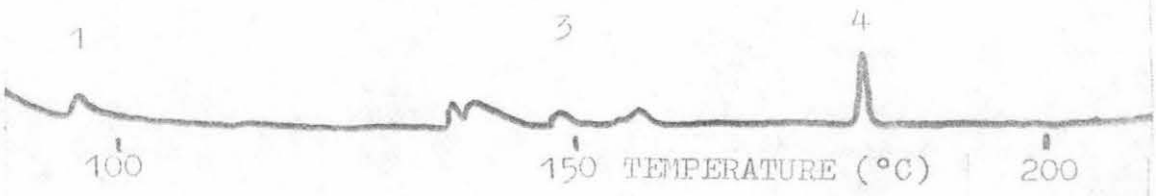


FIGURE A9.2: DT-17/DY-210 CHROMATOGRAM FOR DERIVATIZED, HEATED LYSINE/LACTOSE IN NOT SOLUTION

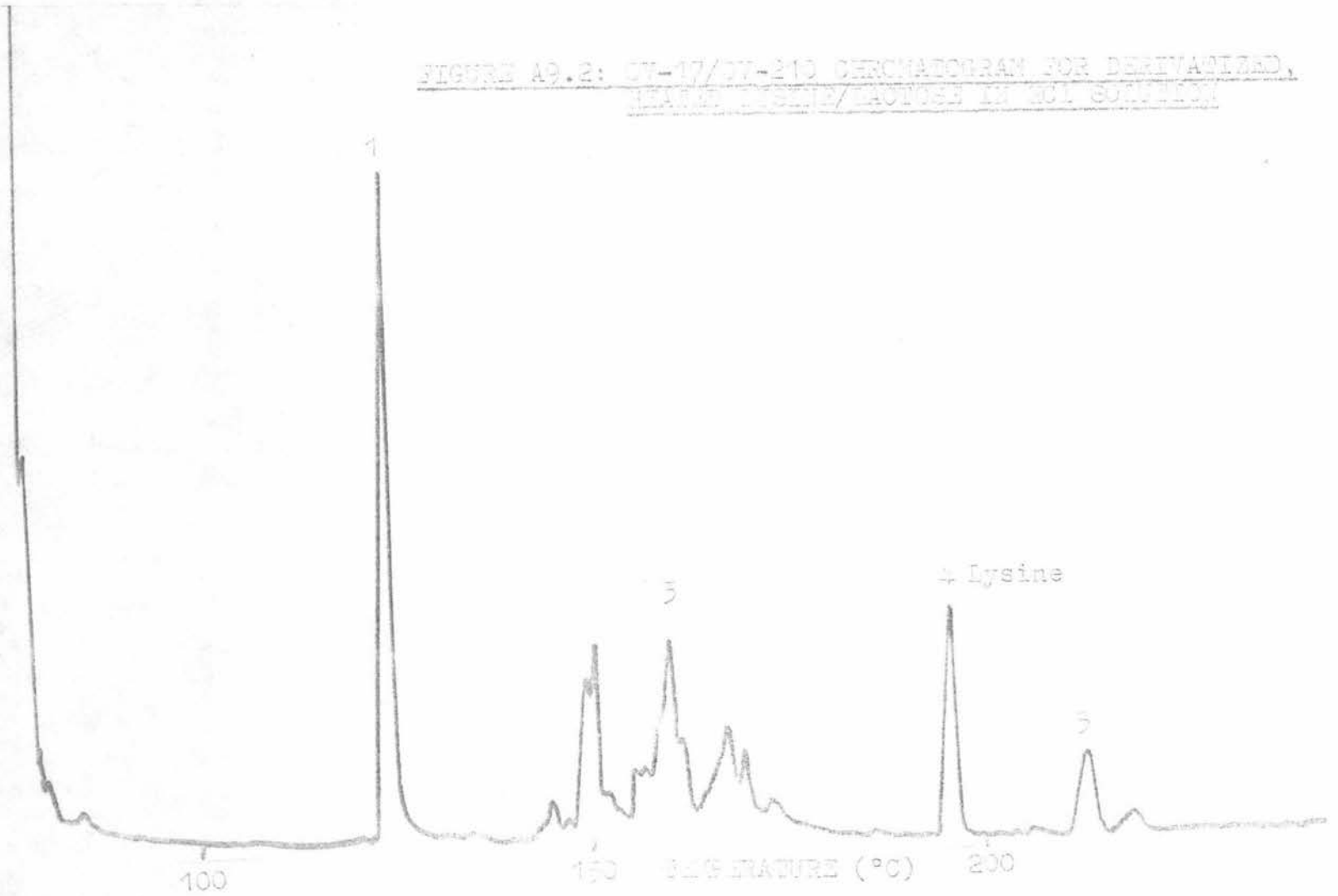


FIGURE A9.5: EGA CHROMATOGRAM FOR DERIVATIZED, HEATED LYSINE/LACTOSE IN HCl SOLUTION

FIGURE A9.4: OV-17/OV-210 CHROMATOGRAM FOR DERIVATISED, ACID HYDROLYSED (AIR HEADSPACE) DRIED LYSATE/LACTOSE MIXTURE

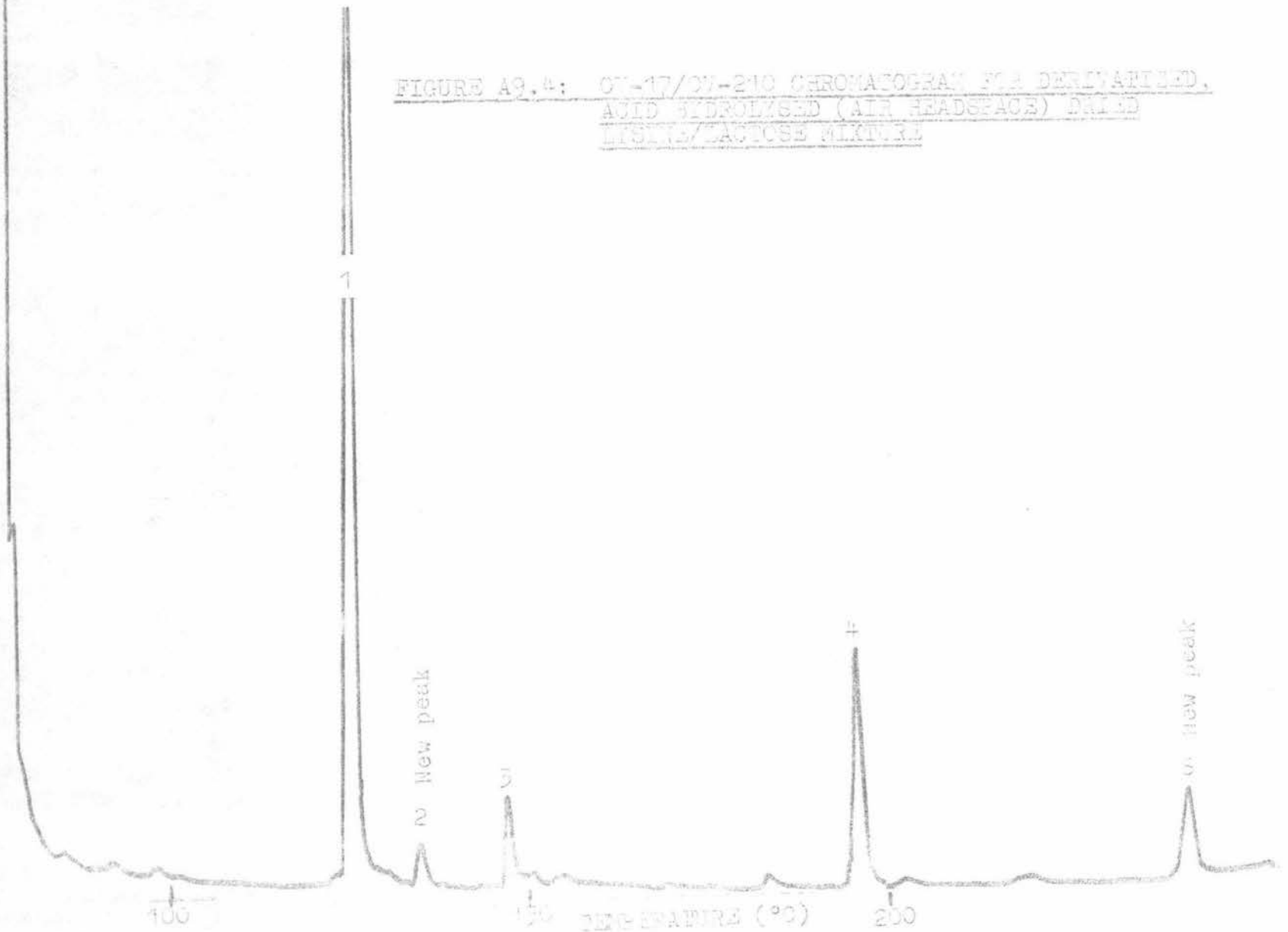


FIGURE A9.5: GAS CHROMATOGRAM FOR DERIVATIZED, ACID HYDROLYZED (ALKALI HEADSPACE), DAIRY CHEESE/FACTICE MIXTURE

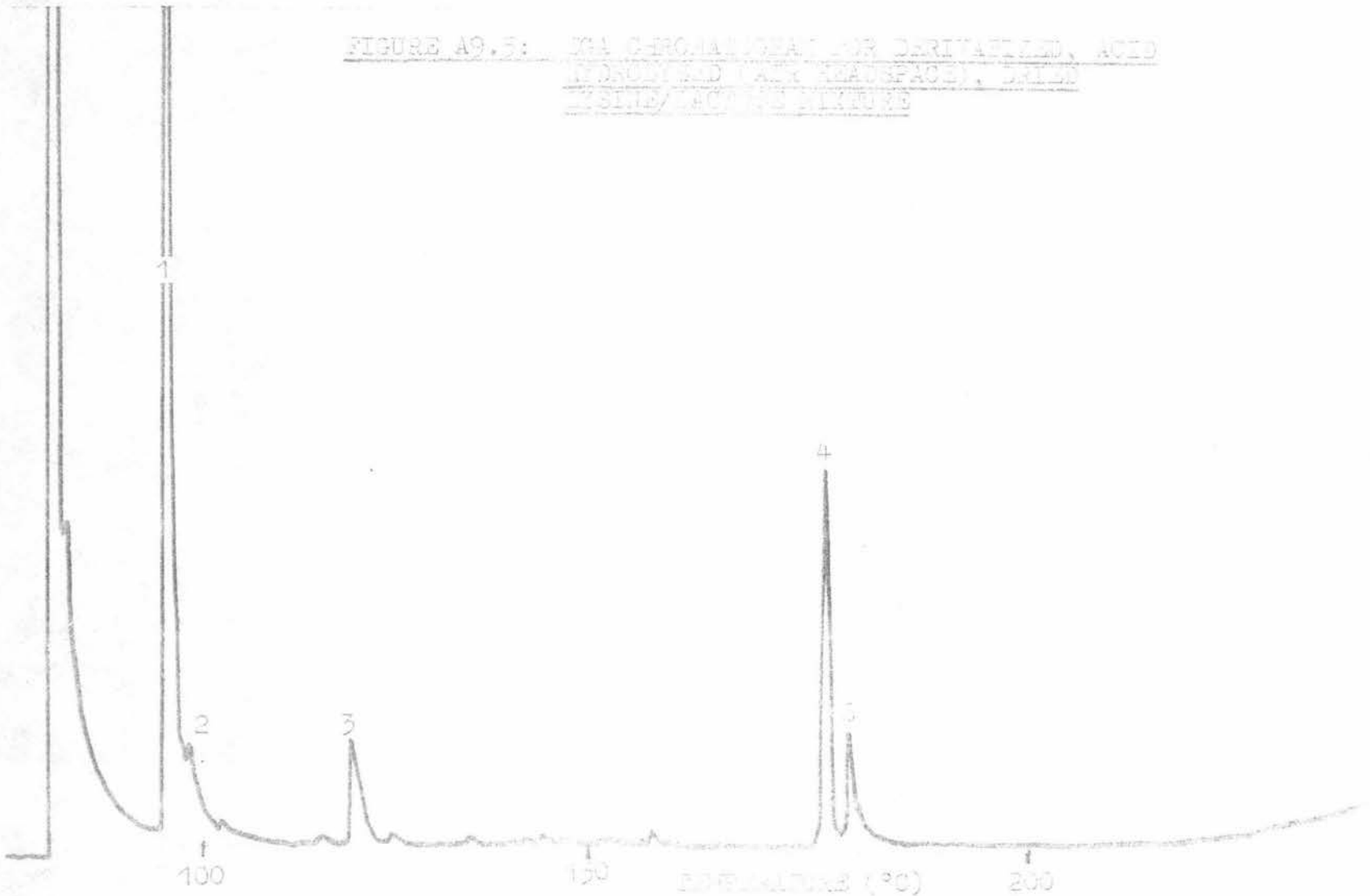


FIGURE A9.6: OV-17/OV-210 CHROMATOGRAM FOR DERIVATIZED, ACID HYDROLYSED (CO_2 HEADSPACE) DRIED LYSINE/LACTOSE MIXTURE

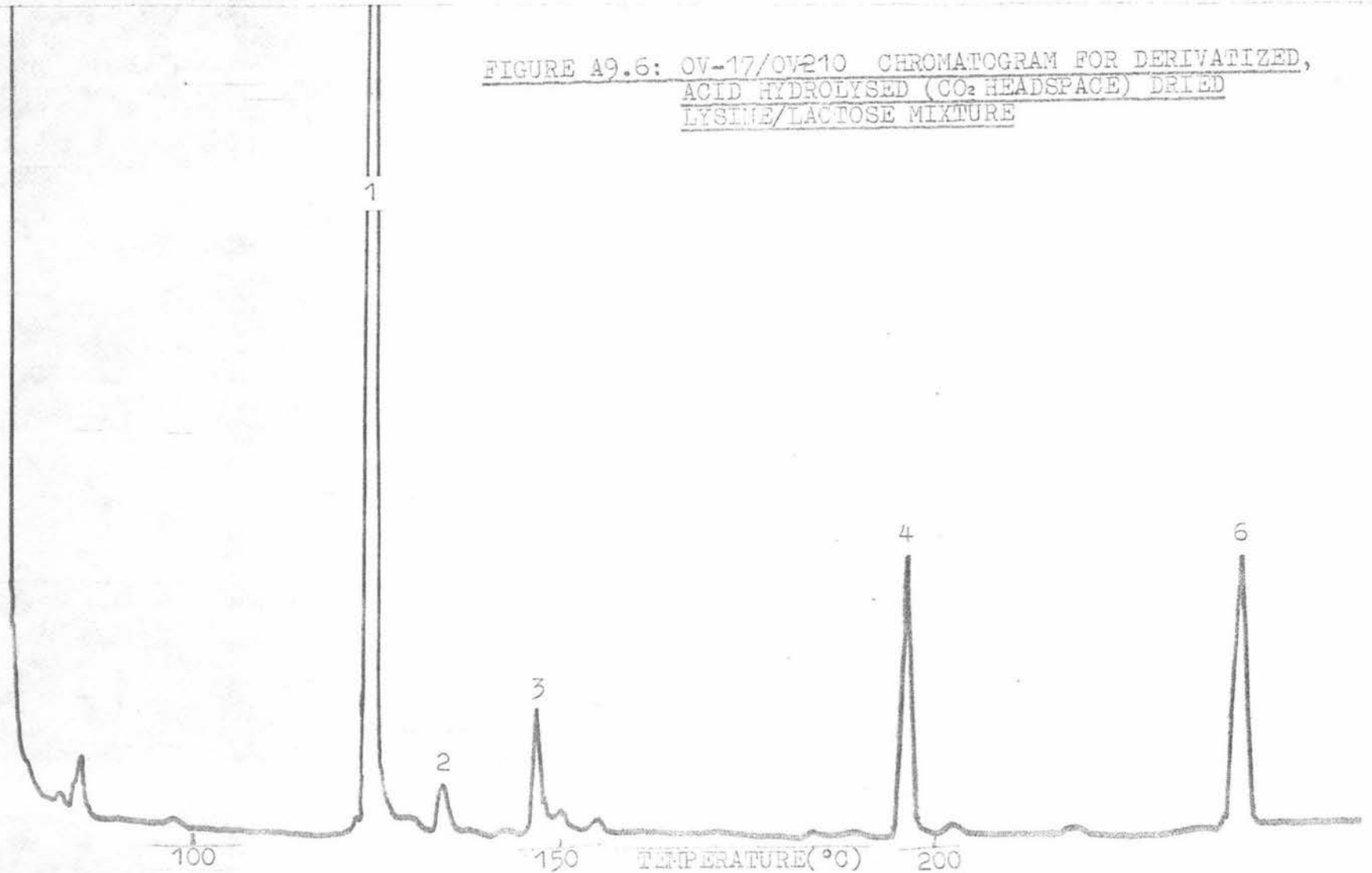
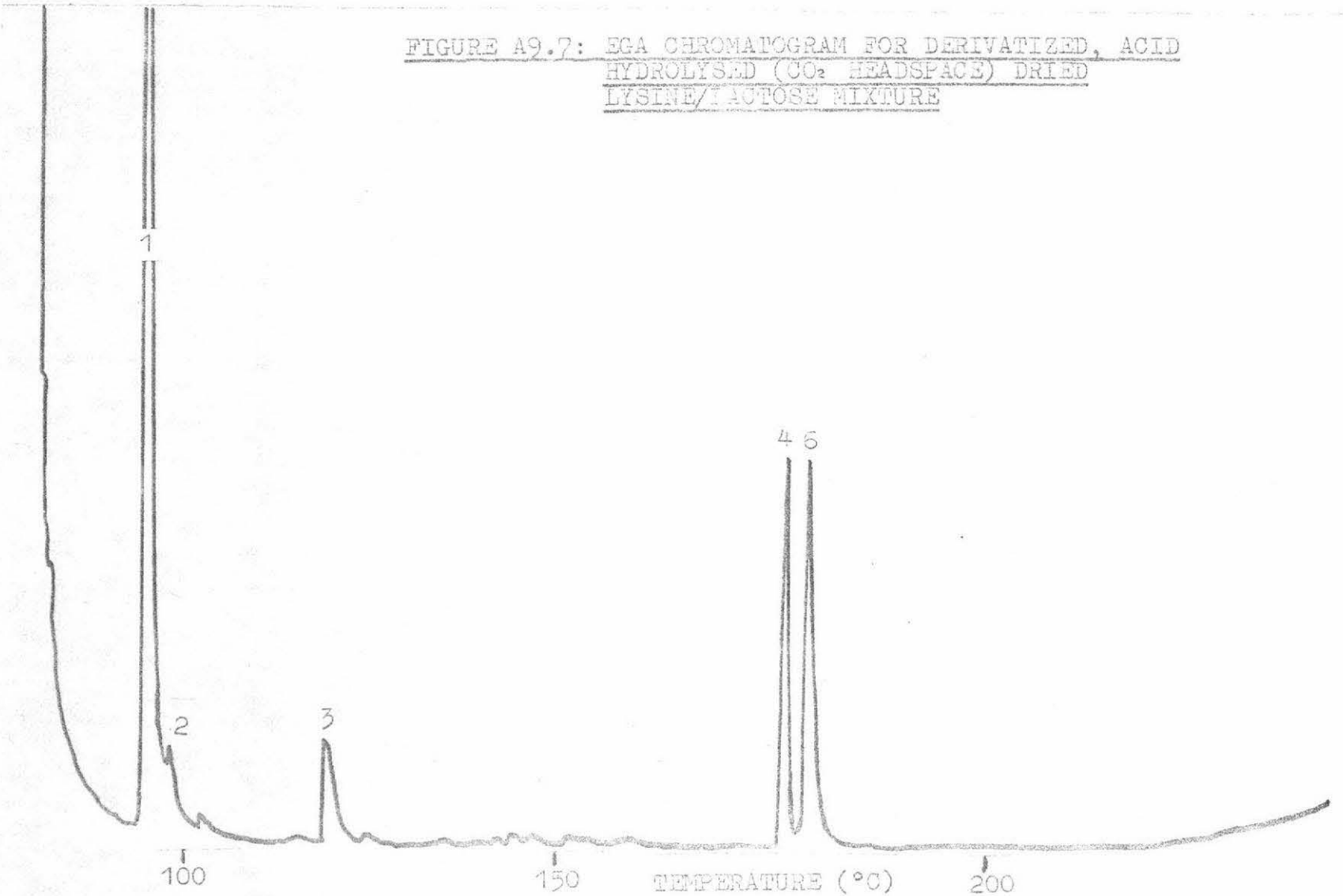


FIGURE A9.7: EGA CHROMATOGRAM FOR DERIVATIZED, ACID HYDROLYSED (CO_2 HEADSPACE) DRIED LYSINE/LACTOSE MIXTURE



A9.3 RESULTS AND DISCUSSION

The major peaks, or peak groups have been labelled from 1 to 6 for reference.

It is clear that the major contaminant peak has been produced during this investigation.

The results of the chromatograms are summarised in Table A9.1.

TABLE A9.1 SEMI-QUANTITATIVE EVALUATION OF THE CHROMATOGRAMS

Trial	Reaction colour	Peak Number		
		1	2	3
a(130°/16h)	None	v.small	absent	small
*b(130°/16h)	Brown	medium	absent	med.
*c(145°/4h) Air	V.brown	large	small	small
*c(145°/4h) CO_2	V.brown	large	small	small

Trial	Reaction colour	Peak Number			
		4	5	1	2
a(130°/16h)	none	small	vv.small		Absent
*b(130°/16h)	Brown	med.	small		Absent
*c(145°/4h) Air	V.brown	med.	vv.small		Small
*c(145°/4h) CO_2	V.brown	med.	vv.small		medium

*6N HCl present

Peak 1 Coincides with alanine on EGA, threonine on OV-17/OV-210

Peak 3 Often a complex group on OV-17/OV210 and coincides with methionine, but ill defined on EGA.

Peak 4 Lysine, almost absent in trial (a)

Peaks 2, and 5 Irregular and small

Peak 6 Occurs close to lysine on EGA, formation promoted by CO_2 atmosphere.

Trial (a) developed little colour, and most of the lysine had been lost from the system. The lysine and lactose have probably reacted but had not produced coloured pigments at the time the heating was stopped. Possibly colourless lactulose lysine had been formed and as there was no hydrolysis step before the derivatization the lysine would not have been regenerated. If this were the case then the major contaminant (peak 1) is apparently not lactulose lysine.

Trial (b) shows that mild heat treatment in the presence of acid is sufficient to cause visible browning and the production of the major contaminant. Also produced are a variety of other contaminants (peak 3 and peak 5 on OV-17/OV210).

Trial (c) showed that under normal hydrolysis conditions the development of brown pigments is extensive, and also the production of the major contaminant is promoted significantly. Peak 3 is noticeably reduced in area and complexity while a new peak (number 6) appears, particularly in the case of the deaerated/carbon dioxide headspace trial.

In trials (b) and (c) the recovery of lysine does not appear to have been affected, hence it is most unlikely that a significant amount of lysine has been incorporated into the contaminants produced, and the contaminants are thus likely to be lactose derivatives.

A follow up trial in which lactose was hydrolysed alone at 145° for 4h, resulted in peaks 1 and 6 being produced, as well as traces of the other peaks confirming this conclusion.

Earlier lysine had been hydrolysed alone, and the resulting chromatogram had no spurious peaks.

A9.4 CONCLUSION

During the hydrolysis of skim milk the lactose present undergoes a series of reactions which cause colour and humin development. Some or all of the products of this reaction are separated during the chromatographic analysis, causing interference particularly to alanine in the case of EGA chromato-

gram, and threonine in the case of the OV-17/OV-210 chromatogram.

Lysine does not appear to be affected by this reaction.

It appears desirable to remove the lactose prior to or to use an ion-exchange clean-up step after hydrolysis. The former alternative has the advantage of reducing colour and humin development during hydrolysis, and also ensuring that there are no possible complicating reactions occurring between lactose and the lysine liberated from the protein during hydrolysis.

APPENDIX 10

AN INVESTIGATION INTO THE DIALYSIS OF MILK, AND ITS EFFECT ON AMINO ACID CHROMATOGRAMS

A10.1 INTRODUCTION

With the realization that lactose undergoes a reaction during hydrolysis and that this causes spurious peaks on the chromatograms of the amino acid derivatives, a study into the possibility of lactose removal by dialysis was necessary.

A10.2 EXPERIMENTAL

(a) Equipment: An ultrafiltration cell (Amicon, 402, Amicon Corp., Lexington, Mass. U.S.A.) fitted with a membrane with a molecular weight cut off value of 10,000 (Amicon PM10) was used.

(b) Dialysis Procedure

An aliquot (30ml) of milk was placed in the assembled UF cell and diluted with distilled water (ca 80ml). This volume was maintained by distilled water being forced into the cell from a nitrogen pressurized (30 p.s.i.) reservoir. After about 10h the water flow was cut off, but the pressure maintained until the volume in the cell had fallen to less than 30ml. (The dialysis was carried out on diluted milk to reduce membrane clogging, and to increase the flow rate.) In 12 hours of dialysis the volume of filtrate, containing lactose and minerals, collected was about 1.25l. The milk sample was transferred as quantitatively as possible to a flask and the UF cell was washed, the washings being collected and added to the sample until about 90ml have been collected.

An aliquot (15ml) was used for protein determination using the conventional Kjeldahl method, and a further aliquot (1ml) transferred to a culture tube for hydrolysis and subsequent derivatization. This the quantity of milk protein transferred for hydrolysis was established.

SECTION I

A.10.3 RESULTS AND DISCUSSION

(a) Browning and Chromatogram Changes

In the initial trial comparison, colour was the obvious difference between the samples throughout the hydrolysis/derivatisation procedure. The colour of the hydrolysate and the derivative of the dialysed milk sample was much paler than that of the non dialysed, lactose containing normal milk sample. Associated with this reduction in lactose and colour, the amount of humin formed during the hydrolysis was greatly reduced. The difference made by the dialysis step is seen in figure A10.1, which shows the filtered hydrolysate, and their corresponding filter papers. So little humin was formed in the hydrolysis of the dialysed samples that filtering could have been omitted.

The difference in colour also carried through to the final derivatives as is shown in figure A10.2.

The chromatograms of the derivatives of the dialysed and non-dialysed milk samples showed the differences expected from the results of the investigation reported in Appendix 9, i.e. the spurious peaks, especially the coeluate of alanine (on the EGA column), and threonine (on the mixed OV column) had been significantly reduced, if not removed completely, as is shown in figures A10.3 and A10.4.

(b) Lysine Recovery

The removal of lactose did not affect the recovery of lysine by acid hydrolysis. The ratios

$$\frac{\text{Lysine Peak area/internal standard peak area}}{\% \text{ Protein}}$$

for the dialysed and non-dialysed unheated milks are not significantly different from each other, nor are the ratios for the dialysed and non-dialysed heated milks, as shown in Table A10.1

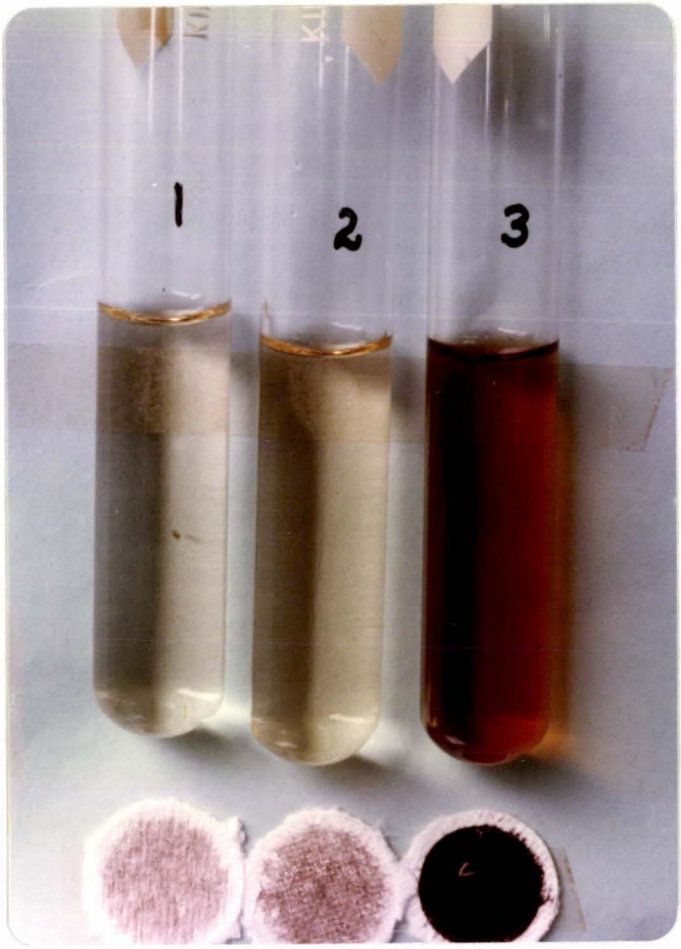


FIGURE A10.1: THE APPEARANCE OF THE HYDROLYSATES OF DIALYSED AND NON-DIALYSED SKIM MILK

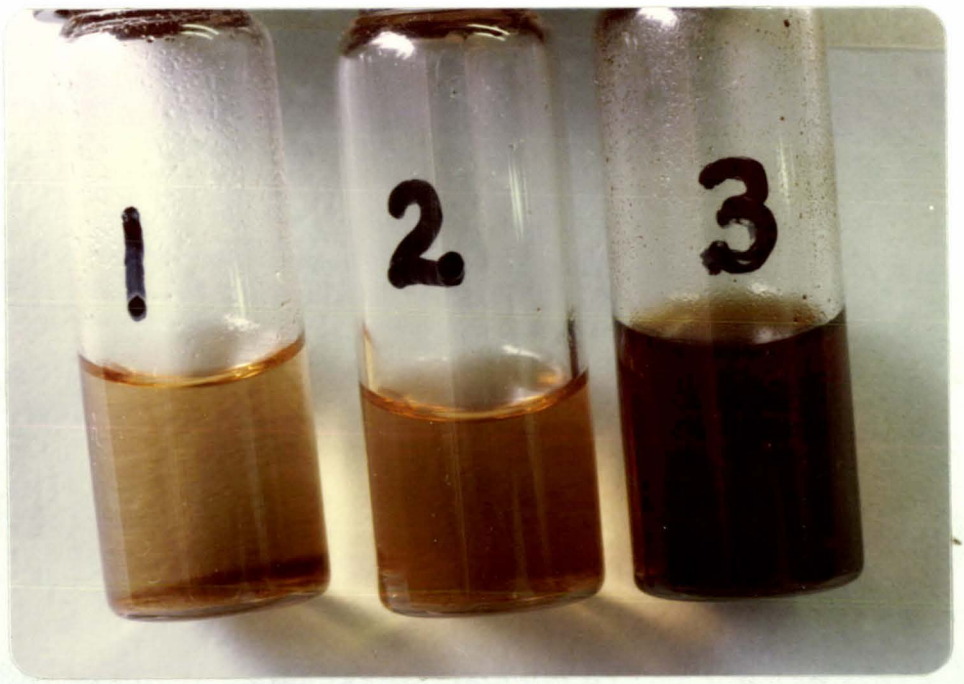
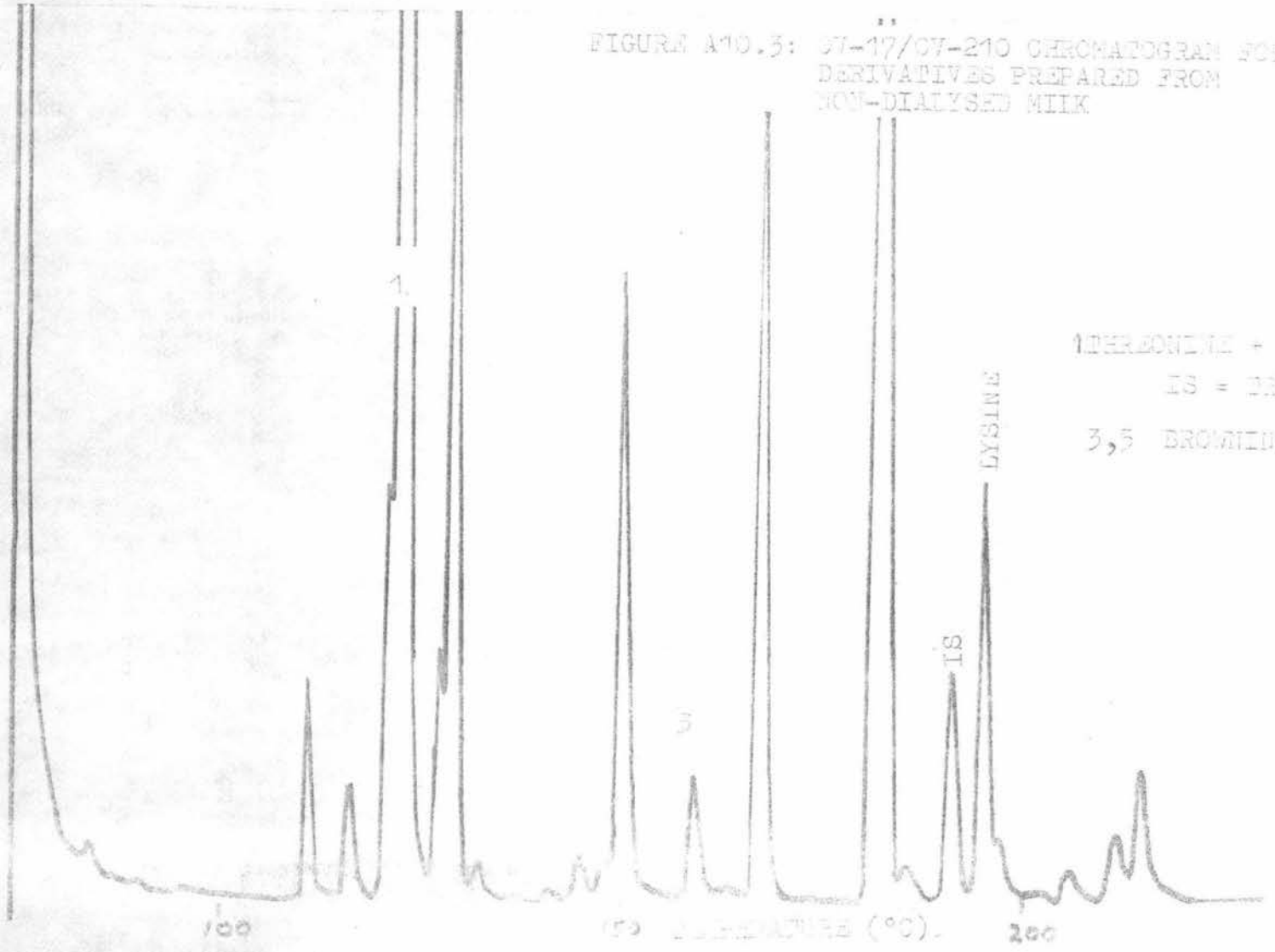


FIGURE A10.2: THE APPEARANCE OF THE DERIVATIVES PREPARED FROM DIALYSED AND NON DIALYSED SKIM MILK

1,2 Prepared from dialysed milk

3 Prepared from dialysed milk

FIGURE A10.3: OV-17/OV-210 CHROMATOGRAM FOR DERIVATIVES PREPARED FROM NON-DIALYSED MILK



1 = THREONINE + BRANCHED CHAIN

IS = BRANDEXAMIC ACID

3,5 BROMINED PEAKS

FIGURE A10.4: OV-17/OV-210 CHROMATOGRAM FOR DERIVATIVES PREPARED FROM DIALYSED MILK

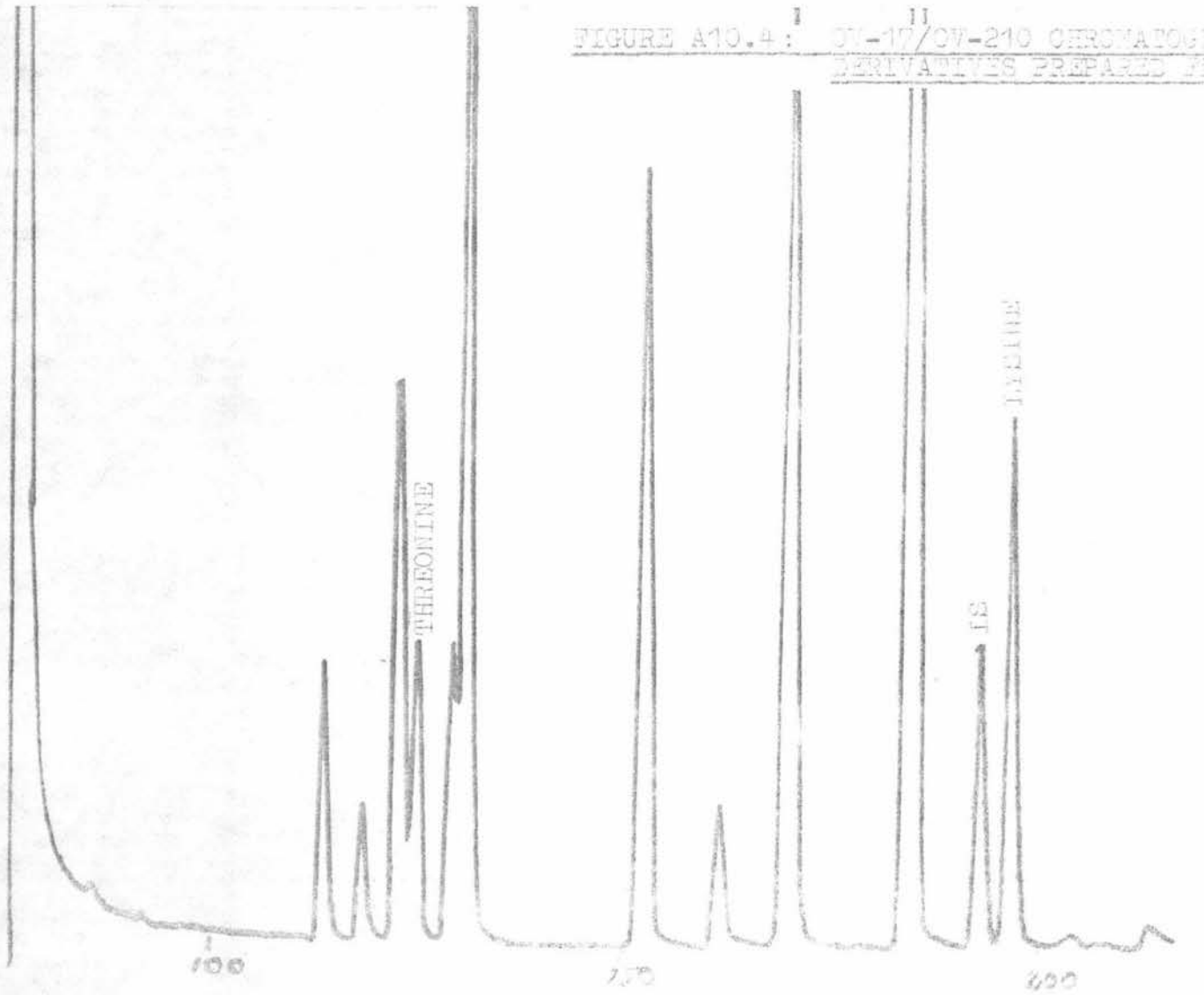


TABLE A10.1 LYSINE RECOVERY IN DIALYSED AND NON-DIALYSED MILK

Treatment	Protein %	Lysine: Internal Standard*	Lysine ratio: Protein
Non heat treated, not dialysed	3.64	1.856	0.5093
Non heat treated, dialysed	3.42	1.722	0.5035
Heat treated, not dialysed	3.64	1.420	0.3901
Heat treated, dialysed	3.16	1.223	0.3869

* Results of duplicate hydrolysis.

SECTION II

(a) The Effect of Heating and Dialysis on other Amino Acids relative to Lysine

If some of the amino acids in milk proteins are not affected by heat treatment, or by dialysis then it should be possible by following the changes in the ratio of lysine peak area: other amino acid peak areas to follow the relative changes in lysine constant, i.e. to use the knowledge of this ratio in unheated milk as a reference value, and the value of the same ratio in heated milk to indicate processing losses. In effect the other amino acids would be used as 'internal' internal standards.

Before this can be done it is necessary to establish which amino acids are unaffected in their relative proportions by heating and by dialysis. Also these amino acids must show consistency during chromatography, and must be free from interference from nearby peaks. This limits the suitable amino acids (on the mixed OV column chromatogram) to alanine, isoleucine plus leucine, proline, phenylalanine plus aspartic acid, tyrosine plus glutamic acid.

The ratio of peak areas for lysine to these selected amino acids is shown in Tables A10.2a and A10.2b. These have been taken from chromatograms of both unheated and heated milks which have been either dialysed or not dialysed,

as is indicated in the table. Each column represents the results of a single injection of the derivatives of a separate hydrolysis.

TABLE A10.2a: AREA RATIO FOR LYSINE: OTHER AMINO ACIDS IN UNHEATED MILK

Amino Acid	Trial					Mean	Coef. of Var.
	Non Dialysed			Dialysed			
	1	2	3	1	2		
Alanine	2.464	2.387	2.421	2.322	2.348	2.388	2.7
Isol + leucine	0.479	0.478	0.482	0.474	0.477	0.478	0.6
Proline	0.695	0.706	0.713	0.725	0.731	0.714	2.1
Phen + Asp.	0.541	0.539	0.544	0.527	0.535	0.537	1.1
Tyro.+ Glut.	0.264	0.269	0.271	0.261	0.266	0.266	1.4

Average coefficient of variation = 1.7%

TABLE A10.2b: AREA RATIO FOR LYSINE: OTHER AMINO ACIDS IN HEATED MILK

Amino Acid	Trial				Mean	Coef. of Var.	% of Unheated
	Non Dialysed		Dialysed				
	1	2	1	2			
Alanine	1.757	1.833	1.854	1.892	1.834	3.1	76.8
Isol.+ Leucine	0.368	0.377	0.380	0.377	0.375	1.4	78.4
Proline	0.577	0.589	0.556	0.563	0.571	2.6	79.9
Phen.+ Asp.	0.418	0.430	0.422	0.428	0.425	1.3	79.0
Tyro.+ Glut	0.201	0.208	0.208	0.215	0.208	2.9	78.2

Average coefficient of variation = 2.4

These tables show no significant differences across the rows within a given table, hence dialysis does not affect the relative proportions of the amino acids.

Between the tables there is a difference between the means of corresponding rows, but as is shown in Table A10.2b this difference is nearly constant, at 78.5% for heated

against the unheated. The variation in this difference is not significant as the standard deviation for the difference in row means between the tables is 1.1%. Therefore the five peaks selected are either not affected by heat treatment, or are all affected equally.

But, as reference to Table A10.1 shows, the heat treatment used in this study resulted in the lysine content in the heated milk being reduced to 76.7% of that in unheated milk. The standard deviation for this figure is about 2%, from which the standard error of the difference between the two means is 1.6%, i.e. the difference of 1.8% between the means calculated in tables A10.1, and A10.2b for % lysine retained after heat treatment are not significantly different. It can be concluded therefore that in milk the acid available amino acids of the five peaks selected are not significantly reduced by the same heat treatment that results in a reduction in acid available lysine to 77.6% of the original value.

(b) Comparison of the Selected Amino Acid Peaks between themselves

By dividing the row means of Table A10.2a and Table A10.2b by the other rowmeans in the same table, the consistency between the peaks can be examined. This gives Tables A10.3a and A10.3b respectively.

For example from Table A10.2a: (unheated milks)

$$\frac{\text{Isoleucine + leucine mean}}{\text{Alanine mean}} = \frac{0.478}{2.388}$$

$$= 0.2002$$

(as is given in Table A10.3a)
and from Table A10.2b (heated milks)

$$\frac{\text{Isoleucine + leucine mean}}{\text{Alanine mean}} = \frac{0.375}{1.834}$$

$$= 0.2045$$

The effect of heating, if any is indicated by the ratio of the two calculated values, i.e.

$$\frac{0.2045}{0.2002} = 1.02$$

i.e. there is an apparent increase in this ratio due to

heat treatment. The values for this latter ratio for all the values in Tables A10.3a and b are given in Table A10.4.

TABLE A10.3a: RATIO OF SELECTED AMINO ACID PEAK AREA RATIOS (UNHEATED MILK)

	Alanine	Isol + Leucine	Proline	Phen.+ Asp.	Tyro.+ Glut.
Alanine	1.00				
Isol + Leucine	0.2002	1.00			
Proline	0.2975	1.489	1.00		
Phen.+ Asp.	0.2249	1.123	0.7558	1.00	
Tyro. + Glut.	0.1114	0.5567	0.3745	0.4954	1.00

TABLE A10.3b: RATIO OF SELECTED AMINO ACID PEAK AREA RATIOS (HEATED MILK)

	Alanine	Isol. + Leucine	Proline	Phen.+ Asp.	Tyro.+ Glut.
Alanine	1.00				
Isol.+ Leucine	0.2047	1.00			
Proline	0.3117	1.522	1.00		
Phen.+ Asp	0.2315	1.131	0.7428	1.00	
Tyro. + Glut.	0.1133	0.5537	0.3637	0.4896	1.00

TABLE A10.4: RELATIVE CONSISTENCY IN SELECTED AMINO ACID PEAK AREAS AFTER HEAT TREATMENT OF MILK

	Alanine	Isol. + Leucine	Proline	Phen.+ Asp.	Tyro.+ Glut.
Alanine	1.00				
Isol.+ Leucine	1.022	1.00			
Proline	1.048	1.022	1.00		
Phen.+ Asp.	1.029	1.007	0.983	1.00	
Tyro.+ Glut.	1.017	0.995	0.971	0.988	1.00

From Table A10.4, the average recovery of the selected amino acids in heated milk relative to the level in unheated milk is 1.008 or 100.8%. But as the standard deviation of this average is 2%, 95% confidence limits for this recovery figure (based on the 10 estimates in Table A10.4) are 98.7% to 101.3%. Thus the recovery of 100.8% is not significantly different from 100%. There is thus no change in availability of these amino acids to acid hydrolysis after skim milk has been severely heated.

A10.4 CONCLUSIONS

(a) The browning of the hydrolysates and derivatives is largely caused by the presence of lactose.

(b) The presence of lactose during hydrolysis does not affect the recovery of lysine, but does give rise to several peaks, including a major one, in the final chromatogram.

(c) The amino acids, alanine, isoleucine, leucine, proline, phenylalanine, tyrosine, and glutamine are not affected in their availability to acid hydrolysis by the heating of milk. It is therefore possible to use these peaks as 'internal' internal standards while following changes to lysine. This would remove the need to take into account any dilution that occurs during the dialysis of the milk, thus saving a further analytical step in the investigation.

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