Mechanisms of heat damage in proteins

4. The reactive lysine content of heat-damaged material as measured in different ways*

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1. Analyses have been made of materials in which proteins were caused to react with sugars so as to cause a severe fall in their nutritionally available lysine content as assayed with both rats and chicks.

2. With materials in which the reactions had proceeded under mild conditions (37°), the direct use of either fluorodinitrobenzene (FDNB), *o*-methylisourea or sodium borohydride to measure selectively those lysine units which had not engaged in Maillard reactions gave similar values which, in turn, appeared to reflect the full extent of the nutritional damage.

3. Analysis by a procedure using trinitrobenzenesulphonic acid (TNBS), or the use of FDNB indirectly to measure 'bound lysine' failed to indicate the full extent of the Maillard reaction in mildly heated materials, although the same procedures did appear to do so when applied to materials in which sugar and proteins had been allowed to react at a higher temperature.

4. With a pure protein and with fat-extracted, dried muscle that had been severely heated, all the procedures for measuring reactive lysine gave similar results, with the exception of the procedure using sodium borohydride which proved ineffective in measuring the type of damage that had occurred in these materials.

5. The findings are discussed in relation to the nature of the reactions believed to take place in different types of heat damage.

6. Suggestions are made of the types of materials with which different procedures can be satisfactorily used. The direct FDNB procedure and that using *o*-methylisourea appeared to be applicable to all our samples.

A number of different methods have been in use for the determination of those lysine units in processed protein foods which have escaped reactions that make them nutritionally unavailable. It had been implicitly assumed that the methods should give similar results, but it has recently been demonstrated with a model Maillard compound that this is not necessarily so (Finot & Mauron, 1972): the work was done using α-*N*-formyl-(ϵ-*N*-deoxyfructosyl)-lysine (FFL). The intention of the work to be described here has been to investigate whether similar differences are obtained when the various procedures are applied to proteins that have been heat-damaged both in the presence and absence of sugars.

In earlier papers, beginning with Carpenter & Ellinger (1955), lysine units that reacted with fluorodinitrobenzene (FDNB) and then yielded ϵ-*N*-dinitrophenyl-lysine (DNP-lysine) on acid-hydrolysis have been termed 'available lysine'. This implied that the value was equivalent to the level of biologically available lysine, but it is clear now that this is not always the situation (cf. Carpenter, 1973). To avoid misunderstanding we will refer to those lysine units measured in chemical procedures that have reacted to form derivatives prior to hydrolysis of the protein as 'reactive

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lysine', and prefix the measure with a term identifying the reagent used, e.g. 'FDNB-reactive' lysine, 'trinitrobenzenesulphonic acid-reactive' lysine ('TNBS-reactive' lysine), etc.

EXPERIMENTAL

Test materials

Albumin mix. Ovalbumin, a commercial product, 'Burton's Henalder' (Holland & Sons Ltd, Cambridge) and lactalbumin, purified grade (Nutritional Biochemicals Corporation, Cleveland, Ohio) were mixed 3:2 by weight. This was the protein mix that had been used in an early study of heat-damage by Block, Cannon, Wissler, Steffee, Straube, Frazier & Woolridge (1946).

The ovalbumin–lactalbumin mix was itself blended with an equal weight of glucose or sucrose for further experiments. This was achieved by adding water to make a slurry, mixing vigorously and freeze-drying. These, and all other test materials, were then adjusted to approximately 15% moisture content, by gradually dropping water from a pipette onto a constantly and rapidly tumbled material and then leaving it in a sealed container overnight to equilibrate. At the slurry stage the mixes had a pH of 5·4.

The glucose–albumin mix was packed into glass jars which were sealed and stored in an incubator for 10 and 30 d at 37°. It was also sealed into glass ampoules as previously described by Carpenter, Morgan, Lea & Parr (1962) and autoclaved for 15 min at 121°. The sucrose–albumin mix was sealed into ampoules and autoclaved for 1 or 2 h at 121°.

Chicken muscle. This material was obtained from Batchelor's Foods Ltd, Ashford, Kent and consisted of freeze-dried white muscle. It was previously used by Varnish & Carpenter (1970, 1971). Fat was extracted by refluxing in a Soxhlet apparatus for 24 h with chloroform–hexane (3:1, v/v) then for 3 h with diethyl ether, which was removed by exposing the material overnight in a room at 27°. The extracted material was adjusted to 15% moisture, packed into glass ampoules, which were flushed with nitrogen before sealing, and then autoclaved for 27 h at 121°.

Bovine plasma albumin (BPA). This material, BPA obtained from Koch-Light Laboratories, Colnbrook, Bucks., was the same as that used by Bjarnason & Carpenter (1969, 1970). It was adjusted to 15% moisture and packed into ampoules which were evacuated before sealing and then heated for 27 h at 145° in a hot-air oven.

FFL. FFL, synthesized by the method described by Finot & Mauron (1969), was kindly supplied by these authors.

Analytical methods

Nitrogen. Micro-Kjeldahl digestion was followed by an automated N determination using a Technicon AutoAnalyzer (Technicon Ltd, Basingstoke, Hants). The method used was essentially that of Davidson, Mathieson & Boyne (1970) and involved reacting the diluted digest with solutions of sodium phenate and sodium hypochlorite and then measuring the intensity of the indophenol blue colour which developed.

Total lysine. This was determined on acid-hydrolysates of test materials as described

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by Roach, Sanderson & Williams (1967), using short (60 mm) column chromatography and reaction with ninhydrin in a Technicon AutoAnalyzer system. Under this system ornithine appeared as a separate peak ahead of lysine.

FDNB-reactive lysine (*direct method*). The procedure was that of Carpenter (1960) as revised by Booth (1971) in which the test materials are treated with FDNB, hydrolysed with acid and the resulting DNP-lysine is measured colorimetrically. Methoxycarbonyl chloride was used for the 'blank' determination and a factor of 1·09 was used to correct for loss of DNP-lysine during hydrolysis.

For analysis of FFL the working procedure was scaled down tenfold and acid-hydrolysis was carried out by autoclaving for 1 h at 121° in covered test-tubes instead of refluxing for 16 h as in the normal method. A further factor of 1·16 was used to correct for the lower values obtained by microanalyses, in comparisons run with the albumin mix.

FDNB-reactive lysine (*difference method*). Using the method of Roach *et al.* (1967) free lysine (rather than DNP-lysine) was estimated in the acid-hydrolysates that had been prepared for the 'direct' FDNB method. This value is taken as a measure of inaccessible or 'bound' lysine which has not reacted with FDNB, but is released by acid-hydrolysis. 'Reactive lysine by difference' is then calculated as 'total minus bound' lysine. No correction for possible losses is applied.

TNBS-reactive lysine. The procedure as applied to FFL was that of Kakade & Liener (1969) in which the test materials are treated with trinitrobenzenesulphonic acid (TNBS), autoclaved with acid and the resulting ε-trinitrophenyl lysine (TNP-lysine) is measured colorimetrically. For the remaining samples it was scaled up tenfold, as suggested by Ousterhout & Wood (1970). We found values for the molar extinction of TNP-lysine that had been autoclaved for 1 h at 115° which were close to that of $1{\cdot}46 \times 10^4$ reported by Kakade & Liener (1969) and we used their value throughout our work.

o-Methylisourea (*MIU*)*-reactive lysine*. This approach to the measurement of reactive lysine groups in foods was suggested by Mauron & Bujard (1963). It involves treatment of test materials with MIU which transforms lysine units with reactive ε-amino groups into homoarginine units (Chervenka & Wilcox, 1956; Shields, Hill & Smith, 1959). On acid-hydrolysis, homoarginine is released.

Our working procedure was based on that used by J. Mauron & E. Bujard (personal communication). A solution of MIU (free base) was prepared by taking 4·3 g *o*-methylisourea hydrogen sulphate (Ralph N. Emanuel Ltd, Wembley, Middx) and 8·5 g $Ba(OH)_2.8H_2O$ and making up to 100 ml with water. The suspension was centrifuged to remove the precipitated barium sulphate and the resulting clear solution adjusted to pH 10·8 with 1 M-NaOH. Test material, containing about 200 mg protein, was weighed into a 100 ml graduated conical flask; 20 ml water was added and the mixture was adjusted to pH 10·8 with 1 M-NaOH. Twenty ml of the freshly-prepared MIU solution was then added, the pH readjusted to 10·8 and the total volume made up to 50 ml with water. A crystal of thymol was added as a preservative and the reaction was allowed to proceed at room temperature for 4 d, re-adjusting to pH 10·8 each day with 1 M-NaOH.

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The contents of the flask were then transferred to a dialysis bag, together with two washings each of 10 ml water and dialysed against running tap-water for 2 d. After this they were transferred to a 1 l flask, washing twice with 50 ml water, and concentrated HCl and water were added to give a final volume of approximately 420 ml 6 M-HCl. This was refluxed for 16 h, cooled, and made up to 500 ml with water. The resulting hydrolysate was analysed for homoarginine by the method of Spackman, Stein & Moore (1958), using an EEL amino acid analyser (model no. 193, Evans Electroselenium Ltd, Halstead, Essex) with a 150 mm column and the conditions for basic amino acids recommended by the manufacturer. Homoarginine is eluted after arginine and furosine.

Furosine. This basic amino acid, ϵ-*N*-(2-furosyl methyl)-L-lysine, which was first observed by Erbersdobler & Zucker (1966) occurs as one of the breakdown products of the acid-hydrolysis of FFL and other Maillard-derivatives of lysine (Finot, Bricout, Viani & Mauron, 1968; Heyns, Heukeshoven & Brose, 1968). The amount of furosine in acid-hydrolysates was determined using the same ion-exchange column as that used for homoarginine. It is eluted between arginine and homoarginine.

Total lysine after borohydride treatment. This procedure has been used by Thomas (1972) for the determination of the reactive lysine content of cottonseed meal – a material in which lysine units may combine with the aldehydic compound gossypol unless processing is carefully controlled.

Maillard-type compounds of aldehyde and lysine units break down on acid-hydrolysis to give a variable, but often high, recovery of lysine. However, when they are treated with sodium borohydride they are reduced to acid-stable compounds: thus when proteins are treated with formaldehyde followed by borohydride treatment, ϵ-*N*,*N*-dimethyllysine units are formed (Means & Feeney, 1968, 1971). The 'total' lysine released on acid-hydrolysis following such treatment may, therefore, be a measure of those lysine units in a test material that had not reacted with aldehydes. Dixon (1972) presented results indicating a similar effect of borohydride in preventing the liberation on acid digestion of an amino acid that had reacted at its ϵ-NH_2 group with glucose.

The following procedure was a slight modification of that used by Thomas (1972). The test material (50–200 mg) was weighed into a boiling-tube and 5 ml of water added. The mixture was first cooled by placing the tube in crushed ice for 5 min and then it was mixed for 90 s using a whirlimixer (Fisons Scientific Apparatus, Loughborough, Leics.). It was then recooled for a further 15 min in the ice before adding 25 mg sodium borohydride at the rate of 5 mg every 5 min. The tube was equilibrated at room temperature for 1 h. The contents were then washed into a 500 ml flask with 210 ml 6 M-HCl and refluxed for 16 h. The hydrolysate was cooled, filtered through glass wool and made up to 250 ml with water. The total lysine in the hydrolysate was determined by the method of Roach *et al.* (1967).

Animal experiments

The glucose–albumin mix unheated and the same material stored for 30 d at 37° were assayed for available lysine with rats and with chicks.

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The procedure for the rat assay was similar to that of Waibel & Carpenter (1972). The test materials were assayed at three levels and the response of the rats over 7 d was measured both as 'g weight gain' and as 'g weight gain/g food eaten' relative to the level of supplementary lysine in the diet. The potency of the test materials was compared to that of lysine using the slope-ratio method of Finney (1964).

The chick assay was a development of that used by Carpenter, March, Milner & Campbell (1963). One hundred and fifty 1-d-old male Apollo chicks were purchased from a commercial hatchery. They were reared to 7-d-old on a commercial ration and then transferred to a pre-experimental ration for a further 5 d. At 12 d the chicks were weighed and ninety-six of average weight were randomized into thirty-two cages with three chicks in each cage. Four cages were allocated to each of the eight diets.

Sesame-seed meal was unobtainable and as the lysine-deficient protein source, heat-damaged groundnut meal (Varnish, 1971) was used instead. The meal was enclosed in aluminium-foil packets (about 450 mm × 300 mm × 25 mm) which were autoclaved in clearly separated layers for 4 h at 121°. After unwrapping, the groundnut meal was spread out to cool overnight and finally the whole batch was remixed using a mechanical mixer. The basal diet consisted of (g/kg): autoclaved groundnut meal 455, ground wheat 200, arachis oil 100, zein 46·7, dried-grass meal 20, L-threonine 2·4, DL-methionine 3·2, vitamin mix 5, choline chloride 1·5, *myo*-inositol 1, mineral mix 31·3, calcium carbonate 20, $CaHPO_4.2H_2O$ 28·8, and maize starch to 1000. The mineral mix contained (g/kg): 515 K_2HPO_4, 163 $MgSO_4.7H_2O$, 268 NaCl, 44 ferric citrate.$5H_2O$, 1·3 KI, 8·0 $MnSO_4.4H_2O$, 0·48 $ZnCl_2$, 0·48 $CuSO_4.5H_2O$ and the vitamin mix (g/kg): Rovimix A (15 mg retinol/g) 35·3, Rovimix D_3 (2·5 mg cholecalciferol/g) 4·4, Rovimix E (100 mg DL-α-tocopheryl acetate/g) 100, menaphthone 0·4, biotin 0·04, pteroylmonoglutamic acid 0·6, thiamin 0·6, pyridoxine 2·0, riboflavin 2·0, nicotinic acid 10·0, calcium D-pantothenate 6·0, cyanocobalamin 0·004 and glucose to 1000. The pre-experimental diet was the same as the basal diet except that no zein was added and 400 g groundnut meal/kg were added instead of the autoclaved groundnut meal.

For the experimental period the basal diet was supplemented with L-lysine hydrochloride: 0, 1·4, 2·8 and 4·2 g lysine/kg and the test materials were added at levels that contributed 20 and 40 g crude protein/kg respectively at the expense of maize starch and zein to keep the diets isonitrogenous. The birds were fed for 8 d *ad lib.* and the response was measured by the method used in the rat assay.

RESULTS

The growth assay results for the biologically available lysine content of the albumin mix + glucose (unheated) and for the same mix stored 30 d at 37° are shown in Table 1. Although the rat and the chick assays differed for individual values they gave similar estimates for the amount of lysine made unavailable by the heat process.

The analytical results are summarized in Table 2. For all the procedures, except that for furosine, the results are the means of at least duplicate analyses and for each

Table 1. *The lysine potency (mg/g crude protein (nitrogen × 6·25)) of test materials determined by rat assay and by chick assay**

(Mean values with their standard errors)

		Albumin mix† + glucose				
		Unheated		Stored 30 d at 37°		
Rat assay	Response measured	Mean	SE	Mean	SE	Potency of stored sample: potency of unheated control
	g Wt gain	64·5	5·7	18·3	5·6	0·28
	g Wt gain/g food eaten	75·2	4·7	25·9	5·0	0·34
Chick assay						
	g Wt gain	107·4	3·2	23·6	3·1	0·22
	g Wt gain/g food eaten	103·3	3·7	25·4	3·7	0·25

* For details of experimental procedure see p. 593.
† Ovalbumin–lactalbumin (3:2, by wt).

procedure a pooled estimate of the standard error of these means was obtained with at least nine degrees of freedom. Looking first at the absolute results for the unheated samples, it is seen that both methods using FDNB and that using borohydride gave values a little lower but similar to the corresponding values for total lysine. The values obtained with TNBS and MIU were considerably lower; they were particularly low in the instance of TNBS for materials containing sugars.

DISCUSSION

Nutritional value of the samples. In the present experiments we have only tested the effect of storage with glucose at 37° on the nutritional availability of the lysine. It seems clear from the results that 30 d storage has reduced the value to about 25–30% of the original. As is often the situation, biological assays have shown imperfections that introduce reservations about the individual estimates. Thus, for undamaged proteins we have previously reported chick-assay values that were in excess of the results obtained from total lysine estimation of acid-hydrolysates (Carpenter *et al.* 1963; Miller, Carpenter & Milner, 1965; Varnish & Carpenter, 1970), as have Smith & Scott (1965). It has not yet been possible to advance a satisfactory explanation for this phenomenon.

However, in the present experiments there is no doubt about the great reduction in the nutritional value of the product during storage. This finding is comparable with the results of sole-protein assays on similarly stored materials (Henry & Kon, 1950; Rao, Sreenivas, Swaminathan, Carpenter & Morgan, 1963).

In an earlier study (Bjarnason & Carpenter, 1969) the potency of the BPA samples heated at 145° for 27 h was found, using a rat-growth assay for lysine, to be approximately 15% that of unheated BPA. More than half the total lysine in the heated sample was recovered in the rat's faeces.

Total-lysine values. For mildly heated albumin–glucose mix and for BPA heated for 27 h at 145°, the materials for which we have the results of animal assays, the

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Table 2. *The total- and reactive-lysine content (mg/g crude protein (nitrogen × 6·25)) of test materials determined by different procedures*

(Values in parentheses for the heated samples are expressed as percentages of the values obtained by the same procedure for the corresponding unheated samples)

	Total lysine	Total lysine after borohydride	FDNB-reactive lysine (difference method)	FDNB-reactive lysine (direct method)§	TNBS-reactive lysine	MIU-reactive lysine	Furosine (as lysine equivalents)
Albumin mix*	88·5	82·4	87·2	83·4	61·1	69·7	nd
Albumin mix + glucose	86·3	81·1	84·7	81·6	53·6	71·9	0
Stored 10 d at 37°	66·5 (77)	26·0 (32)‡	61·8 (73)	32·6 (40) (37)‡	41·8 (78)	29·5 (41)	10·1
Stored 30 d at 37°	50·9 (59)	12·2 (15)‡	45·7 (54)	19·6 (24) (20)‡	35·9 (67)	15·8 (22)	16·2
Heated 15 min at 121°	29·3 (34)	18·7 (23)‡	19·5 (23)	12·2 (15) (12)‡	11·3 (21)	6·5 (9)	8·7
Albumin mix + sucrose	82·0	82·2	80·0	80·1	49·3	nd	nd
Heated 1 h at 121°	53·5 (65)	46·9 (57)	40·0 (50)	40·9 (51)	30·0 (61)	nd	3·5
Heated 2 h at 121°	32·0 (39)	23·8 (29)	nd	17·6 (22)	nd	nd	2·7
Bovine plasma albumin	130	128	127	122	113	115	nd
Heated 27 h at 145°	101 (78)	104 (81)	24·1 (19)	17·1 (14)	15·8 (14)	6·9 (6)	nd
Extracted chicken muscle	96·5	87·5	93·3	89·4	78·2	81·0	0
Heated 27 h at 121°	88·8 (92)	83·1 (95)	62·6 (67)	62·3 (70)	48·5 (62)	48·6 (60)	0
α-Formyl-fructosyl-lysine (FFL)†	(53)†	(0)†	(53)†	(3)†	(80)†	(0‖)†	(20‖)†
SE of analytical values	1·7	1·7	1·7	0·7	0·7	0·9	nd

FDNB, fluorodinitrobenzene; TNBS, trinitrobenzenesulphonic acid; MIU, *o*-methylisourea; nd, not determined.

* Ovalbumin–lactalbumin (3:2, w/w).

† Values for FFL are calculated as a percentage of the theoretical lysine content of this molecule, which is itself 43·5%.

‡ Values obtained after a further period of storage under refrigeration.

§ All values multiplied by a constant correction factor (1·09).

‖ Further values for FFL taken from Finot & Mauron (1972) for comparison.

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total-lysine values have failed to indicate the true extent of the nutritional damage. This is in line with all former experience (see review Carpenter, 1973).

Frangne & Adrian (1972) have made a large study of total-lysine values in different proteins heated with sugars for varying times, and have suggested that there is a proportion of lysine units which does not undergo these reactions. On the other hand, Finot & Mauron (1972) have shown that even FFL, a pure Maillard compound which has no nutritional value as a source of lysine (Finot, 1973), can give approximately a 50% yield of lysine on acid-hydrolysis. We feel that the relatively high total-lysine values obtained with our heated materials are also accounted for by 'bound' lysine units breaking down to give a proportion of free lysine.

As pointed out by Mottu & Mauron (1967), total-lysine values can be used as indicators of heat damage of the Maillard type in materials such as dried-milk powders where the corresponding value for undamaged material can be accurately estimated; but even this is not a sensitive estimate, because the reactive lysine falls to a much greater extent.

FDNB-reactive lysine (*direct method*). This determination appears to have measured the full extent of the nutritional damage in both the protein stored with glucose for 30 d and the BPA heated by itself at a high temperature. For protein–sugar reactions of the type occurring in overheated milk powders this is only a confirmation of the results of Mottu & Mauron (1967). Earlier, Ross & Krampitz (1960) and Pion (1961) had both drawn the opposite conclusion, i.e. that FDNB-reactive lysine was not a significantly more sensitive indicator of protein–sugar damage, but Bujard, Handwerck & Mauron (1967) showed that this was due to their use of the Schober & Prinz (1956) procedure for estimating FDNB-reactive lysine content; this procedure is less specific and more subject to interference from 'caramel' colours in damaged materials.

Our value for FFL, which has been checked and repeated, corresponds to only 3% of its theoretical lysine content. We have no explanation for low levels compared with those of *c.*12 and 15% reported by Booth (1971) and Mauron (1972) respectively. The analyses were, however, carried out on different batches of material.

The particular procedure used is reasonably straightforward when applied to model materials of the type studied here. The addition of high levels of either glucose or sucrose to unheated samples has led to only a 3–4% fall in the values obtained. Significantly lower recoveries of DNP-lysine may be obtained with materials containing polysaccharides. The method is also liable to interference from hydroxylysine and from 'humin' colour, though these problems have been overcome by modifications introduced in other laboratories, as reviewed elsewhere (e.g. Matheson, 1968; Booth, 1971; Carpenter, 1973).

FDNB-reactive lysine (*difference method*). This type of procedure, first used by Rao, Carter & Frampton (1963), Blom, Hendricks & Caris (1967) and Roach *et al.* (1967) was developed with the thought that it was, in principle, equivalent to the direct FDNB procedures but avoided the problem of recovering DNP-lysine quantitatively from acid digestion in the presence of starch and structural carbohydrates. The inherent assumption was that the difference between total lysine (as measured

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after acid-hydrolysis of a test material) and 'reactive lysine' measured as DNP-lysine (after treatment with FDNB and acid digestion) consisted of 'bound lysine' which failed to react with FDNB and would therefore, on subsequent acid digestion, yield free lysine in the same proportion as in the 'total-lysine' estimation.

The workers mentioned above and Sasano, Yoshiwara & Oki (1968) all found reasonably good agreement between results obtained by the 'direct' and 'difference' procedures. The 'difference' procedure has been considered the method of choice, apart from the factor of convenience, and a reference against which to judge the 'direct' procedure (Ostrowski, Jones & Cadenhead, 1970; Booth, 1971; Milner & Westgarth, 1973). Looking back, we can see that all these workers who found general agreement had used either animal-protein samples (essentially carbohydrate-free) or vegetable materials that were either unprocessed or heated at relatively high temperatures (as in oilseed processing). In the present series of tests, materials of these types have also all given similar results for the two procedures, as did heated lactalbumin and bovine plasma albumin in earlier trials (Bjarnason & Carpenter, 1969).

The type of damage which the 'difference' procedure fails to measure adequately is that occurring in materials where lysine and reducing sugars have been in prolonged contact at relatively low temperature (37° in our experiments), and in which 'early' Maillard compounds, of which FFL is a model (Finot & Mauron, 1972), would be expected. The same type of damage would be expected in mildly damaged milk powders but, by chance, no such samples have been included in the previous comparisons.

Finot & Mauron (1972) have demonstrated that, for FFL, FDNB can react with the material in several ways with the result that very little free lysine is recovered on subsequent acid digestion, whereas over 50% of the lysine present is recovered as such when there is no pretreatment with FDNB.

It must be concluded that, despite the advantages of the procedure in some respects, it cannot be used for quality control where there is a possibility of early Maillard compounds being present in significant amounts.

TNBS-reactive lysine. Kakade & Liener (1969) adopted TNBS, which reacts with free amino groups to form trinitrophenyl (TNP) derivatives, as an alternative reagent to FDNB for the measurement of reactive lysine in foods, because of its greater convenience as a chemical reagent. It was implicitly assumed that it would give no reaction with Maillard compounds and this seemed to be confirmed by the low value reported by Kakade & Liener (1969) for a 'protein–carbohydrate mixture heated at 100°'.

However, the work of Finot & Mauron (1972) indicated that TNBS can still react at the ε-N position in a Maillard derivative of lysine. This is confirmed in the present study both with FFL and with protein stored in contact with glucose at 37° for which it actually appears to be a procedure less sensitive to damage than is the determination of total lysine. Thus, although the present results confirm that TNBS is as sensitive as FDNB to changes brought about by processing at relatively high temperatures, it cannot be used for quality control of materials where early Maillard compounds may be present.

TNP-lysine also appears to be less stable than DNP-lysine (Okuyama & Satake,

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1960; Moodie, Marshall & Kieswetter, 1970) and we have found it to be particularly sensitive to destruction in the presence of carbohydrates. This, presumably, explains the low absolute values obtained for our unheated materials. Although we used the original procedure on a tenfold scale, we did not change the sample:acid ratio for digestion.

On the other hand the TNBS procedure seemed as sensitive as any method for the assessment of damage to lysine in the models which did not contain added sugar. Hall, Trinder & Givens (1973) have reported a further study of the use of TNBS with animal-protein materials.

MIU-reactive lysine. The recovery of homoarginine from the unheated test materials was equivalent to 80–90% of their total-lysine values. This compares with about 90% recovery of lysine groups as homoarginine when a pure sample of ribonuclease was treated under similar conditions (Klee & Richards, 1957). J. Mauron & E. Bujard (personal communication) have pointed out that the optimal pH for the reaction can differ slightly with different proteins.

It appears to be a highly sensitive indicator of all types of lysine binding, including the formation of early Maillard compounds. Although it cannot be recommended in its present form as a routine quality-control measure because of the time taken for an individual determination, and also because different pH values are needed for different materials, it is of special interest as a reference method. It might also be possible to modify reaction conditions to shorten the procedure.

Lysine after borohydride treatment. This test appears to be sensitive both for FFL and for the mildly heated samples in which early Maillard compounds were expected. There is evidence that these are stabilized by reduction to derivatives which do not hydrolyse to lysine on digestion in acid (Means & Feeney, 1968, 1971).

The results with heated BPA and heated chicken muscle are in complete contrast; borohydride has had no influence on the recovery of total lysine. This is further evidence that the linkage preventing reaction of FDNB, TNBS and MIU with lysine units in such heated, pure protein is of quite a different nature from that of the simple Maillard compounds.

The procedure can be carried out with little more trouble and equipment than is needed for the determination of total lysine by short-column chromatography and it may prove useful for quality control where early Maillard compounds are the only form of lysine-binding to be expected. It may also be possible to simplify the procedure further.

Furosine. This material can be measured by ion-exchange chromatography as part of the normal amino acid analysis of a feedstuff (Erbersdobler & Zucker, 1966; Finot *et al.* 1968) and Erbersdobler (1970) has shown that it may prove a convenient indicator of the degree of heat damage in milk powders. Our own results show an increase in the furosine produced from 30 d as opposed to 10 d storage of a protein–glucose mix at 37°. However, there was not the same relationship with materials heated with sugars at higher temperatures. Thus 2 h autoclaving with sucrose resulted in less furosine production than did 1 h. Presumably this is because the more complex

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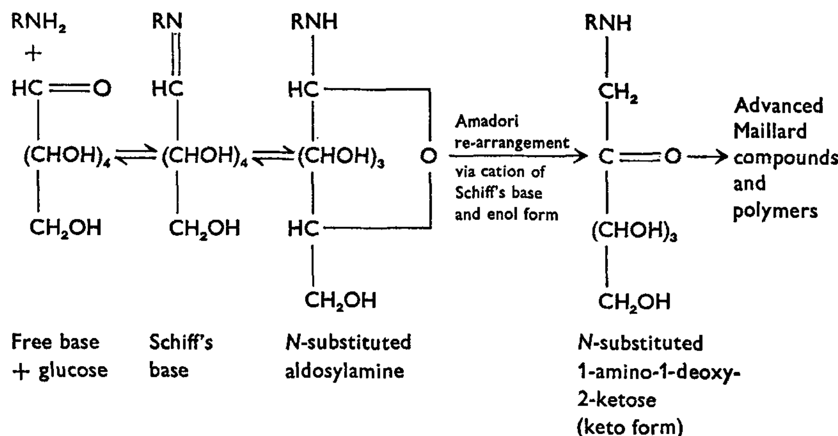

Fig. 1. A simplified representation of the early stages of the Maillard reaction (after Hodge, 1953).

polymers formed in the advanced stages of the Maillard reaction do not break down to furosine on acid-hydrolysis. Sulser (1973) has reported similar results.

It is concluded therefore that, while the presence of furosine in the acid-hydrolysate of a test material indicates that some of its lysine has been involved in Maillard reactions we cannot use it as a quantitative indicator in all instances of protein–sugar damage.

The nature of the reactions involving lysine in the heated samples

It has been a common feature of heat damage to proteins that it results in a fall in their reactive-lysine content. The discussion so far has been concerned with the relative usefulness of different chemical procedures for detecting the extent to which such reactions have occurred. We will now consider what the results, already discussed, can tell us about the nature of the reactions occurring under different conditions in our model materials.

The damaging conditions for which there has been most previous chemical study are those of storage of a protein in contact with a reducing sugar at a moderately low temperature. The preparation of our own samples stored with glucose at 37° for 10 and 30 d followed the conditions used by Lea & Hannan (1950) in their study of casein–glucose reactions. It has been the general finding with such stored materials that the dominant effect is a fall in the level of available lysine with little change in the over-all digestibility of N (Henry, Kon, Lea & White, 1948; Henry & Kon, 1950). It has been thought that in these materials Maillard reactions have occurred between the free carbonyl group of a reducing sugar and the free NH_2 groups of the protein (most of which would consist of ϵ-NH_2 groups of lysine units) and that these reactions would not go beyond the deoxyketosyl derivative (cf. Hodge, 1953; Mauron, 1972). Materials of this type show little actual browning. Fig. 1 shows the initial steps of the Maillard reaction.

Certainly, the pattern of our results, obtained with the different procedures for the sample stored with glucose for 30 d at 37°, is entirely consistent with the corresponding

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pattern obtained with FFL. Thus, approximately 50% of the lysine failing to be measured by the direct FDNB or MIU procedures is recovered by acid digestion. But, equally, this regeneration is prevented by treatment prior to digestion with the reducing agent sodium borohydride. Also, values for 'FDNB-reactive lysine (difference method)' and for TNBS-reactive lysine are as high as, or even higher than, total lysine values both for the stored protein and for FFL. The furosine value is also at least as high as would be expected if all the lysine failing to be measured with MIU (i.e. approximately 80%) were in the deoxyketosyl form.

In the sample stored with glucose for 10 d at 37°, the furosine level is again consistent with virtually all the 'bound' lysine being in the deoxyketosyl form. However, if we apply the formula of Finot (1973), derived using milk powders, that deoxyketosyl forms give 50% recovery of lysine on acid-hydrolysis, and that the even earlier Maillard compounds (aldosylamines and Schiff's bases) give 100% recovery, it can be estimated that 41% of the lysine units are unbound, 12% are in the earliest Maillard forms and the remaining 47% are in the deoxyketosyl combination. Nevertheless these first two models may be considered to exemplify early Maillard damage.

We will now consider the model prepared to illustrate protein–protein damage, i.e. a pure protein (bovine plasma albumin) heated in the absence of sugar but at a high temperature for a long time. There has again been a severe fall in the level of ϵ-N-lysine derivatives formed by reaction with either FDNB or MIU, followed by acid digestion. But, in this instance, there is a much higher recovery of the 'unreactive' lysine fraction on 'total-lysine' analysis; approximately 75% of the lysine unreactive to FDNB or MIU is recovered on acid digestion. The second difference from the results with the first models is that pretreatment with borohydride does not reduce the recovery of total lysine. These findings are, at any rate, consistent with lysine having reacted under these conditions mainly to form amide, or 'iso-peptide' linkages. Bjarnason & Carpenter (1970) suggested that these might result from condensation (eliminating ammonia) with asparagine and glutamine residues although there is no direct confirmation of this. With such linkages one would expect virtually no loss of total lysine, but the second reaction suggested was with the carbonylic breakdown-products of cystine units and the loss of total lysine might come from this reaction.

A further point of difference between the properties of the models considered so far relates to their dye-binding capacity (Hurrell & Carpenter, 1974). The protein stored with glucose at 37° showed virtually unchanged absorption of the acid azo dye Orange 12. This would be consistent with the ϵ-N position in the lysine units that have been involved in early Maillard reactions being still basic in character, as one would expect of a secondary amine. On the other hand it seems clear that the dye is not absorbed by the 'bound' lysine units in BPA; and one would not expect them to be at a neutral amide grouping.

This same point may explain the contrasting results obtained with TNBS for material damaged in different ways. Presumably it reacts with the basic secondary-amine groups of the early Maillard compound and then, on acid digestion the presence of the TNP group weakens the sugar linkage which splits off to give a high yield of

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TNP-lysine. On the other hand, with an amide group one would expect no reaction with TNBS and the heated BPA does give a low value with TNBS.

The 'FDNB-reactive lysine (difference method)' results are similar to those for TNBS-reactive lysine. Again it seems that FDNB must react with the early Maillard compounds. This has been demonstrated by Finot & Mauron (1972) for FFL. The FDNB-lysine–sugar complex breaks down on acid-hydrolysis in ways that yield neither lysine nor DNP-lysine to any significant extent. With heated pure protein, FDNB does not apparently interfere in the release of 'bound' lysine on acid-hydrolysis.

We now turn to model materials heated in the presence of added sugars but at relatively high temperatures. Even the short period of heating, 15 min at 121°, in the presence of added glucose has produced severe changes. The very low values for MIU- and FDNB-reactive lysine indicate that little lysine has escaped reaction of some kind. On the other hand, the furosine value is approximately half that obtained with the material stored 30 d at 37°. If this is taken as an indication that there is also only half as much of the deoxyketosyl derivative (i.e. approximately 40% rather than 80% of the original lysine) the remainder of the reacted lysine has presumably gone to later stages of the Maillard reactions with Strecker degradations (cf. Mauron, 1972). The advanced Maillard reactions do, of course, involve actual destruction of the lysine units, so that every measure falls – which is what we find. The over-all results for this sample are generally consistent with some 40% of the lysine units being in the deoxyketosyl form and some 50% having been destroyed in later stages of the Maillard reactions. Only the TNBS value is considerably below that expected from such an interpretation.

The samples with added sucrose were heated for 1 or 2 h. Even this period would be expected to result in almost negligible change in a pure protein (cf. Carpenter, 1973). At both times there has been a relatively high loss of total lysine in relation to the fall in FDNB-reactive lysine and the formation of furosine. This is consistent with late Maillard products predominating and would be expected if the sucrose is gradually inverting under these conditions to glucose and fructose and then reacting first to deoxyketosyl derivatives and then to late Maillard products. It is interesting in this respect that the furosine levels after 1 and 2 h are similar. The rate of inversion of sucrose must presumably be the factor limiting the rate of damage otherwise its magnitude after 1 h would be much greater. We take these materials as exemplifying advanced Maillard damage. Further results on the role of sucrose in heat damage to proteins will be reported and discussed in a later paper.

Lastly there is the sample of solvent-extracted chicken muscle heated at 121° for 27 h. The rough equivalence between the reactive-lysine values as measured by FDNB, TNBS and MIU would be consistent with either protein–protein damage (as seen in heated BPA) or with advanced Maillard damage. However, the very slight change in total-lysine value makes it comparable only with the former. This conclusion is consistent with previous calculations that the rate of damage to meat and fish-muscle preparations is explicable by protein–protein reactions (cf. Carpenter, 1973). However, some animal tissues may contain higher levels of carbohydrate that

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Table 3. *The lysine contents (determined by different procedures) of test materials selected to illustrate three types of heat damage, together with assessments of the usefulness* of each procedure*

(Each value, expressed as a percentage of the value obtained by the same procedure for a corresponding unheated sample, is taken from Table 1 or 2)

Type of damage ...	Early Maillard		Advanced Maillard		Protein–protein	
Test material ...	Albumin†–glucose, 30 d at 37°		Albumin†–glucose, 15 min at 121°		Bovine plasma albumin, 27 h at 121°	
Analytical test						
Biological	22–34		nd‡		15§	
Total lysine	59	C	34	C	78	D
Total lysine after borohydride	15	A	23	B	81	D
FDNB (difference method)	54	C	23	B	19	B
FDNB (direct method)	24	A	15	B	14	B
TNBS	67	C	21	B	14	B
MIU	22	A	9	B	6	B

nd, not determined; FDNB, fluorodinitrobenzene; TNBS, trinitrobenzenesulphonic acid; MIU, *o*-methylisourea.

* Grading of usefulness: A, a sensitive method, believed to measure the full extent of changes in biologically available lysine; B, a sensitive method, but may not measure the full nutritional change; C, a less sensitive method, but still capable of serving as an indicator of damage; D, a method too insensitive for practical use.

† Ovalbumin–lactalbumin (3:2, w/w).

‡ No protein–sugar mixture heated at a high temperature was assayed biologically in this study. Our assessments have been made with reference to other studies (for explanation see p. 602).

§ The biological assay has been carried out on a different sample but prepared in exactly the same way (Bjarnason & Carpenter, 1969). The assessments are based on these and other results as discussed on p. 602.

account for significant damage occurring under less severe conditions (Skurray & Cumming 1974).

Choice of analytical procedures. The present authors appreciate that heat damage to protein foods, particularly at high temperatures, involves more amino acids than lysine. Cystine is always easily destroyed and, under severe conditions, the availability of all amino acids is reduced and even some of the lysine units that still react with FDNB become indigestible and unavailable (Miller *et al.* 1965; Boctor & Harper, 1968; Valle-Riestra & Barnes, 1970). This is largely the result of a general fall in the digestibility of the proteins. There are also results indicating that under some conditions the value of animal proteins processed or stored in different ways may change to some extent without any fall in reactive lysine (e.g. Moodie & Wessels, 1972).

Nevertheless, there does not, at the moment, appear to be any more sensitive alternative to measurement of reactive lysine that can be used in a general analytical laboratory to detect processing damage in materials valued as a source of dietary protein.

The usefulness of the different procedures for determining reactive lysine has also already been discussed in some detail and in Table 3 we attempt to summarize the conclusions. The direct FDNB and MIU procedures are judged to be the methods of choice for the full range of possible damage. The other procedures are useful for detecting particular types of damage but are of less general application.

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The direct FDNB and MIU procedures are thought to give a good estimate of the available lysine in 'early Maillard' damage, as seen in roller-dried milk powders (Mottu & Mauron, 1967). With 'advanced Maillard' and protein–protein damage, where there is a decrease in over-all protein digestibility, these procedures may still over-estimate available lysine, but they are, of course, more sensitive indicators of damage than is total-lysine determination.

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