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Mechanisms of heat damage in proteins. 8. The role of sucrose in the susceptibility of protein foods to heat damage*

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1. A high-protein yeast cake, based on ovalbumin and lactalbumin, and containing 200 g sucrose/kg was baked and toasted as previously described by Block, Cannon, Wissler, Steffe, Straube, Frazier & Woolridge (1946). This caused a severe reduction in its protein quality for rats, and in its fluorodinitrobenzene (FDNB)-reactive lysine content. The damage appeared to be caused by inversion of sucrose to glucose and fructose during fermentation by the yeast followed by Maillard reactions. Processing an albumin–sucrose mixture in a similar way but without fermentation caused no loss in nutritional value for rats and a small reduction in FDNB-reactive lysine.

2. Sucrose–lysine solutions were prepared and heated as previously described by El-Nockrashy & Frampton (1967). Contrary to their findings, we detected no loss of lysine after storage for 16 h at 35° or after heating for 2 h at 100°, although after heating for 4 h at 121° about 0·7 of the original lysine remained. At an alkaline pH, sucrose hydrolyses only slowly even at 121°, and this is catalysed to some extent by the presence of lysine.

3. A 'dry' albumin-sucrose mixture (10–200 mg moisture/g) was heated in sealed glass ampoules under a range of conditions. The loss of FDNB-reactive lysine was stongly dependent on the processing conditions; in particular it was decreased by an increase in pH. After 2 h at 121°, lysine damage caused by sucrose was similar to that caused by glucose. Lysine damage due to sucrose was believed to follow on from its break-down to glucose and fructose.

4. Although the presence of sucrose does not make proteins highly sensitive to Maillard reactions and loss of nutritive value, it must not be considered entirely inert. Our results confirm earlier work indicating that its presence at relatively high levels in oil seeds may be largely responsible for the damage to protein quality observed when they are severely processed.

Protein foods heated in contact with reducing sugars are easily damaged as a result of Maillard reactions involving primarily the ϵ-amino groups of lysine. Milk, however, is the only naturally occurring protein food that has a high content of reducing sugars. The non-reducing sugar, sucrose, occurs more widely in protein foods and its role during heat damage is less well understood. Lea (1948) reported that sucrose did not react with free amino groups in casein held at 37° for 6 months, whereas reducing sugars had reacted almost to completion within 1 month. An early study whose results have been taken to indicate that sucrose might make a protein food sensitive to heat damage was that of Block, Cannon, Wissler, Steffe, Straube, Frazier & Woolridge (1946). These workers prepared a high-protein cake, based on ovalbumin and lactalbumin, which was designed to provide for all the nutritional needs of convalescents. The ingredients of the cake mix contributed virtually no reducing sugars; the carbohydrate consisted mostly of sucrose and of starch from wheat flour. However, after baking, the protein efficiency ratio (PER) of the mixture decreased significantly and after further toasting the PER was reduced to a very low value. The reduction in nutritional value was largely counteracted by addition of lysine.

Heat damage that occurs during excessive 'toasting' of soya-bean meal or the roasting of groundnuts has been attributed to the sucrose present in these materials (Evans & Butts,

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1949; Varela, Moreiras-Varela, Vidal, Murillo & Luque, 1967; Anantharaman & Carpenter, 1971). It has been generally accepted that the rate-limiting step in the reactions responsible for the damage was the inversion of sucrose to glucose and fructose, and that the Maillard reactions then proceeded rapidly with these reducing sugars.

El-Nockrashy & Frampton (1967) have suggested that the initial reaction between lysine and sucrose is the 'aminolysis' (cf. Walker, 1951) of the 1,2 glycosidic linkage between the glucose and fructose residues by the ϵ-amino group of lysine. Presumably El-Nockrashy & Frampton (1967) envisaged the reaction of one monosaccharide unit with lysine and the release of the other. Their main evidence was that 0·28 of the lysine in an aqueous lysine–sucrose solution was lost when the solution was stored overnight at room temperature and that reducing sugars were detected in the stored solution.

The present paper describes experiments that were designed to investigate further the nature of protein–sucrose heat damage and to study the conditions under which it occurs. As preliminary steps, the 'cake-mix' experiment of Block *et al.* (1946) and the experiments of El-Nockrashy & Frampton (1967) with heated sucrose–lysine solutions were repeated. Then, using model mixes of albumin and sucrose, the effects of time and temperature of heat processing, moisture level and pH on FDNB-reactive lysine content were investigated.

EXPERIMENTAL

Preparation of test materials

High-protein cake. The cake-mix contained the following ingredients (g/kg): wheat flour (McDougall's McD Plain flour; RHM Foods Ltd, London) 510, sucrose (castor sugar; British Sugar Corporation, London) 197, ovalbumin (Burtons Henalder; Holland and Sons Ltd, Cambridge) 115, lactalbumin (Nutritional Biochemicals Corporation, Cleveland, Ohio, USA) 76, hydrogenated vegetable oil (Trex, J. J. Bibby and Sons Ltd, Liverpool) 52, dried yeast (Eastern Counties Farmers Ltd, Cambridge) 30, molasses (Fowlers West Indian Treacle; Fowler Ltd, London) 11, sodium chloride 9. Except for the source of supply, these ingredients corresponded to those used by Block *et al.* (1946). The further processing also corresponded to their description.

20 g compressed baker's yeast was added to 1 kg cake-mix, together with one level teaspoonful (about 4 ml) of both grated lemon rind and nutmeg, and sufficient water (about 320 ml) to make a dough. The dough was left to rise at 35° overnight (16 h) and then portions were baked for 30 min at 200° on aluminium-foil sheets. The resulting cakes were sliced into 12 mm thick sections and 'toasted' in a hot-air oven for 50 min at 130°. Any slightly burnt material was discarded, and the remainder was ground in a hammer mill with a 1 mm screen.

Albumin–sucrose mix. Sucrose, ovalbumin and lactalbumin were mixed in the proportions 5:3:2 (by wt) (i.e. in approximately the same proportions as these ingredients were present in the cake-mix). A dough was prepared and baked as before but with the omission of lemon rind, nutmeg and yeast. Although the dough was held at 35° overnight, it did not rise. The baked material was toasted and ground as described previously.

Albumin mix. Ovalbumin-lactalbumin (3:2, w/w) were baked and toasted in the same way as for the albumin–sucrose mix.

Sucrose syrups. Sucrose was adjusted to 14 mg moisture/g and pH 5 (the pH of the albumin–sucrose–water mixture) by gradually adding pH 5 buffer (0·05 M-potassium hydrogen phthalate) (BDH Chemicals Ltd, Poole, Dorset) dropwise to a constantly and rapidly tumbled material. It was then packed into cans (80 mm deep, 100 m diameter) and autoclaved for 1 h and 4 h at 121°, producing thick, slightly golden syrups.

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Controlled heating of model mixes

Lysine in aqueous sugar solutions. Portions (10 ml) of sucrose-lysine and glucose-lysine solutions containing (/ml) 260 mg sugar component and 12·5 mg lysine hydrochloride (both reagents from BDH Chemicals Ltd, Poole, Dorset) each of which had a pH of 5·4, were heated in screw-capped bottles for 16 h at 35°, for 2 h at 100° in a boiling water-bath and for 4 h at 121° in an autoclave. Identical sugar–lysine solutions, with sodium bicarbonate added in a quantity calculated to be equivalent to the lysine HCl, had a pH of 7·7 and were heated in the same way. The exception was the composition of the sucrose-lysine-$NaHCO_3$ solution heated for 4 h at 121°; this contained (/ml) 150 mg sucrose, 6·25 mg lysine hydrochloride and the equivalent of $NaHCO_3$. It had a pH of 7·9. All this was done to follow the experimental conditions described by El-Nockrashy & Frampton (1967). The solutions were subsequently analysed for lysine by ion-exchange chromatography and for the presence of reducing sugars. Where reducing sugars were present they were determined quantitatively. In addition, sucrose solutions, without the addition of lysine, and at either pH 5·4 or pH 7·9 (with additions of $NaHCO_3$) were autoclaved and analysed for their reducing sugar content.

Heating at different times and temperatures. Finely ground sucrose, 'processed sucrose' (sucrose syrup autoclaved for 4 h at 121°), or glucose, were mixed with ovalbumin and lactalbumin in the proportions 5:3:2 (by wt). The moisture content of each mixture was adjusted to 140 mg/g by gradually adding water dropwise from a pipette onto a constantly and rapidly tumbled material and then leaving it overnight in a sealed container to equilibrate. Each mixture was sealed into small glass ampoules as previously described by Carpenter, Morgan, Lea & Parr (1962). The test material was compressed into the closed end of a 150 mm × 12 mm Pyrex test-tube; a small section of glass rod and a plug of glass-wool were then placed on top of the test material in order to fill the head-space and avoid loss of moisture during processing, and the tubes were sealed by 'drawing-off' in a hot flame.

Three ampoules of each mixture and three ampoules containing the protein component alone (adjusted to 140 mg moisture/g were autoclaved) for each of the following periods: 1 h at 100°, 1 h at 121°, 2 h at 121°. After processing, the glass ampoules were carefully broken open, taking care to remove all the glass from the test material, and the three replicates of each mixture were ground and mixed together in a mortar and pestle.

The albumin–sugar mixtures heated for 2 h at 121° became solid after processing and were passed through a 16 mesh BSS sieve, by gently rubbing the sample across the wires, before mixing. All materials were stored at −20° in sealed containers until analysed.

Heating at different moisture levels. Finely ground sucrose, ovalbumin and lactalbumin were mixed in the proportions 5:3:2 (by wt). The moisture levels of different batches were adjusted to 50, 100, 150 and 200 mg/g as described previously. To obtain a moisture level of about 10 mg/g, the protein component was dried in a vacuum oven for 2 d at 30° before mixing with sucrose. The protein component alone was adjusted to the same moisture levels. All model systems were sealed into glass ampoules and autoclaved for 1 h at 121°. The ampoules were opened and the test materials were ground, as before, and stored at −20°.

Ground sucrose was similarly adjusted to 0, 50, 100 and 200 mg moisture/g with buffer (pH 5). About 25 g of each mix was then accurately weighed into 200 mm × 24 mm glass test-tubes. These were sealed into ampoules and three ampoules of each moisture level were autoclaved for 1 h at 121°. Each ampoule was separately broken open in a mortar and the sugar dissolved from the glass in hot distilled water. When cool, the solutions were made up to 100 ml in a volumetric flask and analysed for invert sugar.

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Heating at different levels of pH. Albumin–sucrose mix (100 g) were made into a paste with 25 ml distilled water. The pH of the paste was 5. Subsequent pastes were adjusted to pH 7 and pH 9 by addition of 0·1 M-sodium hydroxide. All samples were freeze-dried, ground in a hammer mill, adjusted to 150 mg moisture/g and autoclaved for 1 h at 121° in sealed glass ampoules. The resulting materials were ground, mixed and then stored at −20°.

Analytical methods

'Kjeldahl' nitrogen, total lysine and FDNB-reactive lysine by the 'direct' method were determined as described previously (Hurrell & Carpenter, 1974). With the 'direct FDNB method' (Booth, 1971) all values, except those for the cake-mix, were multiplied by a factor of 1·09 as an approximate correction for losses of dinitrophenyl (DNP)-lysine during hydrolysis. Values obtained for the cake-mix, which contained a large proportion of wheat flour, were multiplied by a factor of 1·2 as recommended by Booth (1971). For moisture determination samples were dried in an oven at 100° until constant weight.

Qualitative test for reducing sugars. Approximately 4 ml portions of heated lysine–sugar solutions and 5 ml Benedict's reagent (Association of Official Agricultural Chemists, 1975) were boiled vigorously in a 50 ml beaker for 2 min. Reducing sugars, if present, form a voluminous yellow, red or orange precipitate on cooling. Under our conditions 2·5 mg glucose gave a definite positive test: this amount is equivalent to detecting inversion of less than 0·005 of sucrose.

Paper chromatography of sugars. Portions of unfermented cake-mix dough (5 g) and the fermented cake-mix dough (5 g) were accurately weighed, in duplicate, into 100 ml conical flasks and extracted four times with 25 ml boiling aqueous methanol (850 ml/l) (Southgate, 1969). The extractions were pooled, filtered hot into 100 ml volumetric flasks and, when cool, made up to volume.

They were examined by descending paper chromatography using butanol–acetic acid–water (4:1:1, by vol.) as the solvent. Each extract was applied to Whatman No. 4 paper, with solutions (10 mg/ml) of glucose, fructose, sucrose, lactose and maltose. The chromatograph was developed overnight, after which the paper was dried, sprayed with aniline phthalate (0·93 g aniline + 1·6 g phthalic acid dissolved in 100 ml water-saturated *n*-butanol) and heated for 5 min at 105°. Aniline phthalate stains reducing sugars green–brown (Partridge, 1949). Fructose does not stain well but gives a fluorescent 'spot'. Lactose was used as a standard to check whether lactalbumin contained this sugar; maltose was used to check whether this sugar was present in the final product as a result of diastatic enzyme activity in wheat flour during fermentation. The solvent was allowed to run off the paper and the distance each spot travelled from the origin relative to the distance travelled by glucose (R_g value), taking glucose to have an R_g value of 100 (Smith, 1958) was calculated to aid in identification of the compounds.

Invert-sugar analysis. Methanol was removed from methanol extracts, prepared as described previously, by rotary evaporation and the volume re-adjusted to 100 ml with water. Each extract was analysed in duplicate for invert sugar by the method of Lane & Eynon (Association of Official Agricultural Chemists, 1965).

Animal experiments

A series of test materials, as set out in Table 1, were assayed for their value as the only protein source for young rats, so as to measure their 'protein efficiency ratio' (PER) and 'net protein ratio' (NPR) (Bender & Doell, 1957). Forty-eight, 21-d-old white female rats of CFY strain (Anglia Laboratory Animals, Huntingdon, Cambs.) were randomly allocated into twenty-four cages so that each cage contained two rats, and three cages of rats were allotted to the N-free diet and three cages to each of the seven experimental diets (Table 1).

The N-free diet contained the following ingredients (g/kg): sucrose 835, cellulose powder 60, maize oil 48, mineral mix 47, vitamin mix 10. The mineral mix contained (g/kg): KH_2PO_4 292·5, $MgCO_3$ 30·2, $MgSO_4.H_2O$ 37·9, $MnSO_4.H_2O$ 5·2, $ZnCO_3.2ZnO.3H_2O$ 0·9, $CaCO_3$ 197·5, $CaHPO_4$ 230, calcium citrate 10·2, NaCl 153·5, ferrous ammonium citrate 8·9, $CuSO_4.5H_2O$ 0·8, NaF 0·1, KIO_3 0·05, maize starch to 1000. The vitamin mix contained (g/kg): thiamine HCl 0·4, riboflavin 0·5, pyridoxine 0·6, nicotinic acid 1·0, calcium pantothenate 1·2, biotin 0·1, choline chloride 100, menaphthone 0·2, Rovimix A (Roche Products Ltd, Welwyn, Herts; containing 15 μg retinol/mg) 1·6, Rovimix D_3 (2·5 μg cholicalciferol/mg) 0·3, Rovimix E (100 μg DL α-tocopheryl acetate/mg) 25, cyanocobalamin 0·002, maize starch to 1000.

The test materials were added to the N-free diet at the expense of sucrose to give a crude protein content (N × 6·25) of 100 g/kg. All diets were fed to rats *ad lib.* for 14 d, with the exception of the N-free diet which was fed for 12 d. PER values were calculated after 14 d as g body-weight gain/g protein eaten, and net protein ratio (NPR) values were calculated after 12 d as:

$$\frac{\text{weight loss of animals on N-free diet (g)} + \text{weight gain of test animals (g)}}{\text{crude protein (N} \times 6{\cdot}25\text{) consumed by the test animals (g)}}.$$

RESULTS

Baking and toasting experiments

Animal test. The results of the rat feeding trial are shown in Table 1, together with the results of Block *et al.* (1946) for comparison. Baking and toasting the cake-mix greatly reduced the PER and NPR values and the addition of 6·3 g lysine/kg to the diet containing the heated material resored about half the loss. However, there was no significant effect of baking and toasting of either the albumin mix or the albumin–sucrose mix on their respective PER or NPR values.

Reactive lysine measurements. FDNB-reactive lysine determinations were made in duplicate on all materials and the results are set out in Table 1, together with a pooled estimate of the standard error of each estimate. The results indicated that baking and toasting had caused a much greater reduction in the reactive lysine content of the cake-mix than of the albumin–sucrose mix. Further, only 0·54 of the original total lysine content of the unheated material was recovered from acid-hydrolysates of the heated cake-mix.

The heated albumin–sucrose mix shows little loss of total lysine, although the direct FDNB-reactive lysine value was reduced to 0·76 of the unheated value, as compared to 0·93 when the albumin mix was heated alone.

Paper chromatography of sugars. Methanol extracts from cake-mix dough, before and after fermentation, were examined, and the results are shown in Table 2. Only one sugar was detected in the unfermented-dough extract; this had the R_g value of sucrose and, like sucrose, gave only a faint colour reaction with aniline phthalate. Two compounds were detected with the fermented-dough extract and these had the R_g values obtained for glucose and fructose respectively. 'Glucose' stained a dark green–brown with the colour reagent used, whereas 'fructose' gave a much fainter colour and was also fluorescent under ultra-violet light. No sucrose was detected after fermentation. Neither lactose nor maltose was detected in extracts of the cake-mix dough.

Invert-sugar analyses. The results are shown in Table 3. They confirm that the sucrose present in the cake-mix was almost completely inverted as a result of yeast fermentation. The invert-sugar content of the cake-mix dough was increased from 13 mg/g before fermentation to 150 mg/g after fermentation. The sucrose content of the unfermented dough was also about 150 mg/g.

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Table 1. *The effect of baking and toasting on the nutritional value and on the lysine content (mg/g crude protein [nitrogen × 6·25]) of the test materials*

(Values in parentheses represent the values for heat-treated samples as proportions of the corresponding values for the unheated controls)

Test material*	Treatment	NPR	PER	PER values of Block *et. al.* (1946)	Total lysine	FDNB-reactive lysine†
Cake-mix	Unheated	4·9[a]‡	3·9[a]‡	[3·7]	71·8	59·2
	Baked and toasted	1·9[b] (0·39)	0·8[b] (0·21)	[0·7 (0·19)]	38·6 (0·54)	21·6 (0·36)
	Baked and toasted +6·3 g lysine/kg diet	3·5[c] (0·71)	2·6[c] (0·67)	[3·2 (0·86)]	nd	nd
Albumin mix	Unheated	5·4[a]§	4·1[a]§	nd	88·5	83·4
	Baked and toasted	4·6[a] (0·85)	3·7[a] (0·90)	nd	89·7 (1·02)	77·6 (0·93)
Albumin–sucrose mix	Unheated	5·4[a]	4·1[a]	nd	85·3	80·1
	Baked and toasted	5·0[a] (0·93)	4·1[a] (1·00)	nd	78·9 (0·92)	61·0 (0·76)
	Baked and toasted +6·3 g lysine/kg diet	5·3[a] (0·98)	4·2[a] (1·02)	nd	nd	nd
Pooled standard error of treatment means		0·27	0·27	nd	1·7	1·5

NPR, net protein ratio (for details see p. 289); PER, protein efficiency ratio (for details see p. 289); FDNB, fluorodinitrobenzene; nd, not determined.

* For details, see p. 286.

† All values, except those for the cake-mix, were multiplied by a factor of 1·09. Values obtained for the cake-mix were multiplied by a factor of 1·2. See p. 288 for details.

‡ Values, within a column, which do not contain a common superscript were significantly different ($P < 0·95$).

§ These values were not determined separately, they are those determined for the unheated albumin–sucrose mix.

Table 2. *Paper chromatography* of sugars from fermented and unfermented cake-mix doughs†*

Sample	R_g†
Glucose	100
Fructose	134
Sucrose	64
Lactose	30
Maltose	38
Methanol extract from unfermented dough (one detectable compound)	62
Methanol extract from fermented dough (two detectable compounds)	
compound *a*	102
compound *b*	136

R_g, distance travelled from origin by the compound, relative to the distance travelled by glucose, taking glucose to have an R_g value of 100. The glucose spot travelled 53 mm under the conditions of the test.

* For details of procedures, see p. 288.

† For details, see p. 286.

The inversion also took place when sucrose was heated in cans at 140 mg moisture/g and pH 5. The invert-sugar concentration increased from 9 mg/g after 1 h at 121°, to 228 mg/g after 4 h at 121°. The latter corresponded to 0·25 of the sucrose originally present.

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Table 3. *Inverted sugar analysis* of fermented and unfermented cake-mix doughs† and of various heated sucrose samples*

Test material		Invert sugar (mg/g sample)
Cake-mix dough‡		
Unfermented		13
Fermented		150
Sucrose heated in cans at pH 5, 140 mg moisture/g:		
1 h at 121°		9
4 h at 121°		228
Sucrose heated in glass ampoules, pH 5, 1 h at 121°		
Moisture content (mg/g):	0	0
	50	4
	100	5
	200	8
SE of analytical value		1

* For experimental details, see p. 288.
† For details see p. 286.
‡ Initial sucrose content of unfermented dough was about 150 mg/g.

Table 4. *The proportion of original lysine remaining in sugar-lysine solutions after heat treatment**

	Sucrose		Glucose	
Solution ...	Lysine HCl	Lysine HCl + $NaHCO_3$†	Lysine HCl	Lysine HCl + $NaHCO_3$†
Heat treatment				
16 h at 35°	1·01	0·98 (0·73)‡	1·01	0·93
2 h at 100°	1·01	0·97	0·92	0·24
4 h at 121°	0·69	0·71§ (0·67)‡§	0·50	0·07

* For details, see p. 287.
† An equivalent amount of $NaHCO_3$ was added to lysine HCl.
‡ Values in parentheses are from El-Nockrashy & Frampton (1967). They did not report any value for the solution kept for 2 h at 100°.
§ This solution contained (/ml) 150 mg sucrose and 6·25 mg lysine HCl (plus bicarbonate), all other solutions contained (/ml) 260 mg respective sugar and 12·5 mg lysine HCl.

Controlled heating of model mixes

Lysine in aqueous sugar solutions. Lysine added to sucrose solutions, either with Na HCO_3 or as the hydrochloride salt, was almost completely recovered by ion-exchange chromatography after storage for 16 h at 35° or after heating for 2 h at 100° (Table 4). After autoclaving sucrose–lysine solutions for 4 h at 121°, we recovered approximately 0·7 of the lysine whether the pH of the solution was 7·9 or 5·4. Much more destruction of lysine occurred when it was heated in glucose solutions, especially when the pH was increased by the addition of bicarbonate.

In agreement with El-Nockrashy & Frampton (1967), we found that lysine was the only ninhydrin-positive constituent eluted from the column when the stored and heat-treated sucrose–lysine–HCO_3-solutions kept at 35° and at 100° were added. But, whenever there was a significant loss of lysine under our conditions, an unidentified peak was eluted from the column after about 60 min. Lysine was eluted at 100 min.

Of the heated sucrose–lysine solutions, only those autoclaved for 4 h at 121° gave positive tests for reducing sugars with Benedict's reagent. The solution autoclaved at pH 5·4 gave a value for invert sugar equivalent to 0·97 of the original sucrose; the corresponding

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Table 5. *FDNB-reactive lysine values* (mg/g crude protein [nitrogen × 6·25]) of unheated albumin–sugar mixes and the proportion of lysine remaining after different heat treatments†*

Test material ...	Albumin	Albumin–sucrose	Albumin-processed sucrose‡	Albumin–glucose
Lysine in unheated mix	83·4	80·1	(80·9)§	81·6
Proportion of lysine remaining after heating for:				
1 h at 100°	0·97	0·85	0·40	0·13
1 h at 121°	0·97	0·49	0·18	0·12
2 h at 121°	0·97	0·12	0·12	0·06

FDNB, fluorodinitrobenzene.
* SE of reactive lysine values was 1·7 mg/g crude protein (N × 6·25).
† For details, see p. 286. All the samples were at pH·5 and 140 mg moisture/g.
‡ Sucrose autoclaved with 140 mg moisture/g for 4 h at 121°.
§ Not determined; value given is the mean of the values obtained for the unheated albumin–sucrose and for the albumin–glucose mixes which did not differ significantly.

Table 6. *Proportion of FDNB-reactive lysine* remaining in albumin and albumin-sucrose mixes heated at different moisture levels for 1 h at 121°†*

Test material ... Moisture content (mg/g)	Albumin	Albumin–sucrose
10	0·97	0·84
50	0·96	0·71
100	1·01	0·60
150	0·96	0·49
200	1·01	0·38

FDNB, fluorodinitrobenzene.
* SE of reactive lysine values was 1·1 mg/g crude protein (nitrogen × 6·25); the FDNB-reactive lysine content of unheated albumin was 83·4 mg/g crude protein and the FDNB-reactive lysine content of unheated albumin–sucrose was 80·1 mg/g crude protein.
† For details, see p. 287; pH of each sample was 5.

solution of sucrose autoclaved without lysine present at pH 5·4 gave a value of 0·94. For the solutions autoclaved at pH 7·9, that with sucrose and lysine gave an invert-sugar value equivalent to 0·012 of the original sucrose; whilst that autoclaved without lysine gave a value of 0·007.

Variation in duration and temperature of heat treatment. Heating the albumin–sucrose mix for 1 h at 100° only slightly reduced its reactive lysine content (Table 5). However, as the heat process was increased, so progressively more lysine became unreactive to FDNB until, after 2 h at 121°, only 0·12 of the original reactive lysine remained. The reactive lysine in the albumin–glucose mix decreased to this low level after only 1 h at 100°. Except for the most severe heat treatment (2 h at 121°), when losses of reactive lysine in all albumin–sugar mixes were similar, the losses of reactive lysine in the 'albumin–processed sucrose' mix were between those which had occurred in the sucrose and in the glucose mixes. The reactive lysine in the albumins heated alone remained virtually unchanged.

Variation in moisture content. Increasing the moisture level had no effect on the reactive lysine content of the albumin mix heated alone (Table 6). However, as the moisture level in the albumin–sucrose mix was increased from 10 to 200 mg/g, so the heat treatment rendered progressively more lysine units inaccessible to FDNB. At 200 mg moisture/g, the reactive lysine remaining after heating was 0·38 of its original value.

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Invert-sugar analyses of sucrose heated for 1 h at 121° in sealed ampoules, at pH 5 and different moisture levels are reported in Table 3. Inversion was negligible when water was absent and increased only slightly as the moisture level was increased until at the highest moisture level (200 mg/g) 0·008 of the sucrose had been inverted.

Variation in pH level. Lysine damage to the albumin–sucrose mix was considerably decreased as the pH was increased. After heating for 1 h at a moisture level of 150 mg/g, 0·27 of the original FDNB-reactive lysine was recovered when the pH was 5. At pH 7, the amount recovered was increased to 0·64 and at pH 9, 0·79. The albumin mix alone was not investigated under the same conditions.

The value of 0·27 for material heated for 1 h at 121° and 150 mg moisture/g at pH 5 in this experiment is lower than the value of 0·48 obtained with some heat treatment in the previous 'moisture' experiment. But in this instance the proteins and sugar had been brought into intimate contact by mixing as a paste with added water, so as to be able to adjust the pH, and then freeze-drying, whereas in the previous experiment the ingredients were dry-mixed in powder form.

DISCUSSION

The 'cake-mix' experiment

Rat feeding results. Our PER values for the cake-mix, and for the same material after baking and toasting, were very similar to those obtained by Block *et al.* (1946) and confirmed its susceptibility to nutritional damage from heat treatment. The NPR test is a more critical one than PER since it allows for maintenance as well as growth and is less liable to distortion by any change in palatability of the diet and the NPR values likewise indicated a severe decrease in the nutritional value of the cake-mix after baking and toasting. The main reason for this decrease appeared to be a loss of nutritionally available lysine. This amino acid was present in the protein of the raw cake-mix diet at well above the level required by growing rats (Warner & Breuer, 1972), but in the rat diet containing the baked and toasted material it was the first limiting factor. Although addition of 6·3 g lysine/kg to the diet containing the heated material (an amount that would by itself meet the rats' requirements) gave a large response, NPR and PER values did not increase to the corresponding value for the unheated cake-mix. It appears therefore that there was also some other form of damage to the protein.

These results would appear to be characteristic of Maillard damage; although lysine is known to be the most susceptible amino acid, especially under mild conditions, other amino acids are also involved as the heat treatment is increased (Evans & Butts, 1949; Lea & Hannan, 1950; Rao, Sreenivas, Swaminathan, Carpenter & Morgan, 1963; Ananthanaraman & Carpenter, 1971). It has been hypothesized that profuse cross-link formation takes place within the protein involving advanced Maillard reaction products, and that such changes decrease protein digestibility and so reduce the biological availability of all amino acids (Valle-Riestra & Barnes, 1970). The occurrence of Maillard reactions during baking and toasting of the cake can be explained by inversion of sucrose to glucose and fructose by the yeast during fermentation, followed by reactions between these sugars and the cake-mix proteins on heating.

Koch, Smith & Gedes (1954) reported that the inversion of sucrose is very rapid during fermentation of a dough. Although yeast uses invert sugar for further metabolism to ethanol and carbon dioxide, there was such a large amount of sucrose originally present in the cake mix that it is not surprising that most of the invert sugar remained after fermentation. The diastatic enzymes present in wheat flour also act, with the disaccharidases of yeast, to produce glucose from starch. According to Cathcart (1944), the ideal situation during fermentation of a sweet, yeast-raised product is to have glucose being produced from starch at the

same rate as yeast is using it; in this way, the concentration of sugar present at the beginning of fermentation will remain and give sweetness to the final product.

When an albumin–sucrose mixture (1:1, w/w) was baked and toasted without prior fermentation with yeast, its nutritional value for rats was not reduced; and the reactive lysine although reduced, was still at a level to meet the rats' requirement (Table 1). It would appear that, without prior fermentation, sucrose caused relatively little nutritional damage under our conditions of baking and toasting. This is in contrast to the findings of Clarke, Howe, Mertz & Reizt (1959) who prepared baking-powder biscuits from mixtures containing approximately 100, 190 and 310 mg sucrose/g. After mixing with water and baking for 20 min at 232°, the proportions of the original total lysine they recovered by microbiological assay were 0·93, 0·66 and 0·40 respectively. A possible explanation for the difference is that the biscuits, being thinner, had reached a temperature high enough to cause considerable hydrolysis of sucrose whereas the interior of the albumin–sucrose 'cake' did not. Bender (1970) has pointed out that, although bread is baked in an oven held at approximately 230°, the interior of the loaf rarely reaches a temperature greater than 95°.

It should be mentioned that the levels of pyridoxine, nicotinic acid, retinol, cholecalciferol and α-tocopherol used in our diets were lower than the published recommendations for the growing rat (Warner & Breuer, 1972). However, these values include a safety margin and the lower levels that we used did not appear to have an adverse effect on the growth of our rats over the 14 d period of the assay. Even the simple albumin mix supported rapid growth although its vitamin contribution would have been less than that of the cake-mix.

Total lysine analyses. There appears to be some contradiction between our findings and those of Block *et al.* (1946) as to the loss of total lysine from the baked and toasted cake. They reported that total lysine, measured by microbiological assay, decreased to 0·86 of its original value after baking and toasting. Using ion-exchange chromatography however, we recovered only 0·54 of the original total lysine (Table 1). Obviously it is impossible to reproduce processing conditions exactly but it does appear from the PER values that the damage in the original experiment was as great as in our repetition of it, and we do expect the reduction in total to be at least 0·35 of the reduction in reactive lysine after protein–sugar reactions (Mottu & Mauron, 1967; Hurrell & Carpenter, 1974). Block *et al.* (1946) do not give details of their microbiological assay procedure, but it seemed a possibility that if they had measured bacterial growth turbidometrically the darker colour of the toasted material might have led to an artificially high result. However, in a check on this point using raw and severely-processed bread dough, the proportional decrease in total lysine value was similar whether it was determined by ion-exchange chromatography (as used in our laboratory) or by microbiological assay using either pH or turbidity as the response measured (R. F. Hurrell, unpublished result).

Reactive lysine values. We have seen that the proportional decrease in direct FDNB-reactive lysine values was greater for the baked and toasted cake mix than for the albumin–sucrose mix and that this difference was in line with the extent of nutritional damage indicated by the rat feeding experiments. The direct FDNB reactive lysine test has again proved to be a sensitive indicator of heat damage in protein foods.

However, the validity of absolute values obtained from this method of analysis (Carpenter, 1960; Booth, 1971) for materials high in carbohydrates is still uncertain. The problem comes from the effect of carbohydrates increasing the loss of DNP-lysine during the acid-hydrolysis stage. Under the conditions used sugars have relatively little effect: thus the albumin–sucrose mix gave a value 0·96 of that obtained for albumin mix alone. But polysaccharides give greater losses and recovery factors obtained by the addition of free DNP-lysine to replicate digests over-estimate losses from protein-bound DNP-lysine (cf. review by Carpenter & Booth, 1973). Booth (1971) suggested a correction factor of 1·2 for materials rich

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in polysaccharides. Applying this factor to the results obtained with the cake-mix gives a value for the unheated sample which is still only 0·82 of the corresponding value for total lysine. How far this reflects the need for a higher correction factor and how far the existence of 'bound' ϵ-lysine groups in the unheated ingredients is a complex question. Similar results are obtained with unprocessed cereal grains and grain legumes and we hope to pursue the matter in a further paper.

It had been hoped that the introduction of the 'by difference' methods for the estimation of FDNB-reactive lysine would obviate this difficulty, but it is now clear that they are not sensitive indicators of Maillard damage (Finot & Mauron, 1972; Hurrell & Carpenter, 1974). Unfortunately the 'borohydride procedure' (Hurrell & Carpenter, 1974) had not been developed when our 'cake-mix' experiment was carried out.

Controlled heating of simple model mixtures

Lysine in aqueous sugar solutions. Our results (Table 4) did not agree with those of El-Nockrashy & Frampton (1967). We found no loss of lysine in an aqueous sucrose–lysine–HCO_3^- solution kept at either 35° for 16 h or at 100° for 2 h; nor could we detect reducing sugars in these solutions after heating. Our results do not therefore support the conclusion of El-Nockrashy & Frampton (1967) that lysine in aqueous solution at room temperature catalyses the breakdown of sucrose by 'aminolysis' and is itself destroyed. We can offer no explanation for these conflicting results, except that just possibly their solutions had undergone some kind of microbial fermentation. In our work only 0·07 of the lysine in an aqueous glucose–lysine–HCO_3^- solution was lost after storing at 35° for 16 h.

The 0·3 loss of lysine after autoclaving the sucrose–lysine solution at acid pH can be explained by Maillard reactions between lysine and reducing sugars, since sucrose is almost completely inverted under these conditions even in the absence of lysine. The slower rate of Maillard reactions at an acid pH (Lea & Hannan, 1949; Adrian, 1963) is presumably the factor limiting the loss of lysine: this is indicated by the results with glucose–lysine solutions. The sucrose–lysine solution autoclaved at an alkaline pH also showed 0·3 loss of lysine. At this pH the extent of inversion found with sucrose autoclaved alone is not sufficient to explain this amount of damage to lysine. When allowance is made for a 1:1 reaction between monosaccharide and lysine that is lost, the total sucrose estimated to have inverted is 0·021 of that originally present compared with 0·007 for the sucrose autoclaved alone.

'Dry' albumin–sugar mixes. Our results confirm earlier findings that adding sucrose to proteins which are then severely heated will further reduce the level of FDNB-reactive lysine in the heated samples (Anantharaman & Carpenter, 1971), and are in line with work showing a reduction in enzymically-available lysine (Evans & Butts, 1949). It also confirms the findings that more severe heat is needed with sucrose to produce the amount of damage that would be obtained under relatively mild conditions with glucose.

It has been generally assumed that the damage which occurs with sucrose follows on from its inversion to glucose and fructose and is limited by the rate at which this occurs. Certainly our finding that the damage is greater at acid pH is in line with this, since this favours inversion but greatly retards the early Maillard reactions. The same applies to our finding that increasing the moisture content of mixtures above 150 mg/g results in increased damage to lysine.

But as with free lysine autoclaved in solution with sucrose at alkaline pH this concept does not explain everything. When equal weights of sucrose and albumin mix at pH 5 with 200 mg moisture/g were autoclaved for 1 h at 121°, the effect of the sucrose was to cause a decrease in FDNB-reactive lysine (in excess of that produced by autoclaving the proteins alone) of 37 mg/g protein. There is reason to believe that one molecule of reducing sugar will bind only one amino group under these conditions (cf. Carpenter *et al.* 1962). On that

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assumption, and the molecular weights of lysine and glucose (or fructose) being very similar, this would require a production of at least 37 mg reducing sugar/g sucrose originally present. But when sucrose alone was autoclaved under similar conditions of moisture and pH, analysis of the end-material showed only 8 mg invert sugar/g moist mix, i.e. 10 mg/g sucrose originally present, or 0·27 of the quantity calculated to be required for the damage found to the lysine in the first experiment. It does seem that there is an increased breakdown of sucrose in the presence of proteins, which might result either from 'aminolysis' as suggested by El-Nockrashy & Frampton (1967) or from conditions on the surface of the protein in some way catalysing hydrolysis.

It was surprising that Anantharaman & Carpenter (1971) found that added sucrose had a significant effect on the lysine in a groundnut-protein isolate even during storage at 55°. But Karel & Labuza (1968) had already shown that sucrose will invert during storage at this temperature in combination with cellulose and citric acid at 75% relative humidity.

In conclusion, it appears that lysine is stable for long periods in protein–sucrose mixtures (with about 100–150 mg moisture/g) held at $\leqslant$ 37°. At $\geqslant$ 55° sucrose can be inverted under suitable conditions of moisture and pH, and Maillard reactions with lysine and other amino acids can then take place. Although sucrose is less reactive than the reducing sugars it clearly can be responsible for significant damage during high-temperature processing of natural products, for example in the toasting of soya-bean meal and roasting of groundnuts, both materials of approximately 0·1 sucrose content (Pickett & Holley, 1952; Kawamura, 1967).

It is interesting finally to look back and compare our conclusions with those from the classical work of Maillard (1916) on the 'browning' of sugar–amino acid solutions at 100°, which may be summarized as follows: 'After 25 minutes the tube with sucrose is still colourless and even after 4 hours it is no more coloured than was the tube with glucose after 10 minutes: this demonstrates that it is only as the sucrose begins to split into reducing sugars that it becomes reactive. The present experiments demonstrate that a free aldehyde (or ketone) group is essential to the reaction of sugars with amino acids.' Maillard did not, of course, know the nutritional effects of the changes he had observed, but he ended by urging that their biological implications should be examined.

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