Storage of milk powders under adverse conditions

1. Losses of lysine and of other essential amino acids as determined by chemical and microbiological methods

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1. Whole-milk powders containing 25 g water/kg were stored for up to 9 weeks in sealed aluminium containers at elevated temperatures. Lysine and other essential amino acids were measured by chemical and microbiological methods.

2. Storage at 60° resulted in the progressive formation of lactulosyl-lysine. After 9 weeks, 30% of the lysine groups were present in this form. The powders still retained their natural colour and the levels of tryptophan, methionine, cyst(e)ine and leucine remained unchanged.

3. Storage at 70° resulted in the formation of lactulosyl-lysine followed by its complete degradation with the development of browning. Available tryptophan, methione, leucine and isoleucine decreased progressively during storage.

4. The different methods for lysine determination gave widely dissimilar results. The direct fluorodinitrobenzene (FDNB) technique and reactive lysine from furosine were considered to be the most reliable methods. The FDNB-difference, dye-binding lysine, *Tetrahymena* and *Pediococcus* methods all seriously underestimated reactive or available lysine in heat-damaged milk powders. *Tetrahymena* and *Pediococcus* appeared to utilize lactulosyl-lysine as a source of lysine.

5. The results are discussed in relation to storage and distribution of milk powders in hot climates.

During prolonged storage of milk powders, Maillard reactions between milk proteins and lactose may reduce the level of lysine and other essential amino acids. The loss of lysine, the most sensitive amino acid to Maillard damage, increases with increase in temperature and moisture content (Erbersdobler, 1970) and hermetic packaging and cool storage conditions are necessary to reduce the reaction to a minimum. Lysine has been reported to be reasonably stable for at least 1 year in low-moisture milk powders stored in moisture-resistant containers at temperatures up to 37° (Henry *et al.* 1948; Rolls & Porter, 1973; Womack & Holsinger, 1979). In temperate climates, distribution and storage would therefore appear to present little possibility of large losses of amino acids. In hot countries, however, where distribution networks are often less developed, milk powders may be subjected to high temperatures (above 40°) at some stage during their transport and storage. Little information is available on the losses of nutrients under these more extreme conditions.

In the present paper, we report the losses of lysine and of other amino acids in low-moisture milk powder (25 g water/kg) during storage at high temperatures, and high-moisture powder (100 g water/kg) stored at 37°. At the same time, we have extended earlier studies (Finot & Mauron, 1972; Finot, 1973; Hurrell & Carpenter, 1974, 1981) on the ability of different lysine assay methods to accurately predict the extent of lysine damage after Maillard reactions. The dye-binding lysine and furosine techniques, and microbiological methods with *Tetrahymena* and *Pediococcus*, have been evaluated.

EXPERIMENTAL

Test materials

Spray-dried whole-milk powder containing 25 g water/kg was packed under air into aluminium cans (40 mm high, 75 mm diameter) which were then sealed and stored in incubators at 60° and 70° for up to 9 weeks. Whole-milk powder, adjusted to 100 g moisture/kg, was also stored at 37° in sealed glass jars. Samples were taken at intervals and stored at −20° until used for analysis.

Analytical methods

Nitrogen. This was determined by an automatic Kjeldahl procedure using the Kjelfoss apparatus (Foss Electric, Hillerod, Denmark).

Total amino acids. Acid-hydrolysates of test materials were analysed by ion-exchange chromatography using a Beckman Multichrom amino acid analyser (Beckman Instruments, Munich). Methionine and cyst(e)ine were determined as methionine sulphone and cysteic acid respectively after preliminary oxidation with performic acid (Moore, 1963).

Fluorodinitrobenzene (FDNB)-reactive lysine (direct method). The procedure was that of Carpenter (1960) as modified by Booth (1971) in which the test materials are treated with FDNB, hydrolysed with acid and the resulting dinitrophenyl (DNP)-lysine is measured colorimetrically. Methoxycarbonyl chloride was used for the 'blank' determination. Analyses were made in duplicate and a factor of 1·09 was used to correct for loss of DNP-lysine during acid-hydrolysis. To minimize the loss of DNP-lysine due to the presence of lactose, a 400 mg sample was hydrolysed in 100 ml 6 M-hydrochloric acid instead of 40 ml as recommended.

FDNB-reactive lysine (difference method). 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 the inaccessible or 'bound' lysine which has not reacted with FDNB, but is released as lysine on acid-hydrolysis. 'Reactive lysine by difference' is then calculated as 'total minus bound' lysine (Rao *et al.* 1963; Roach *et al.* 1967).

Reactive lysine and lactulosyl-lysine from furosine. Furosine (ε-*N*-2-furoyl methyl)-L-lysine) was determined by ion-exchange chromatography (Finot *et al.* 1968). It is formed as one of the breakdown products on acid-hydrolysis of lactulosyl-lysine and other deoxyketosyl-lysine derivatives (Finot & Mauron, 1972; Finot, 1973). Bujard & Finot (1978) reported that lysine units in milk products, which are in the form of lactulosyl-lysine, generate on acid-hydrolysis under closely-specified conditions, 40% lysine and 32% furosine. From these values, lysine as lactulosyl-lysine was calculated as 3·1 × furosine and reactive lysine was calculated as total lysine minus 1·24 × furosine (Finot *et al.* 1981).

Dye-binding lysine (DBL). The method was that of Hurrell *et al.* (1979) in which the dye-binding capacity (DBC) of the basic groups in the protein for Acid Orange 12 is measured before (DBC_A) and after (DBC_B) reaction with propionic anhydride which blocks the lysine groups. DBC measurements were made using the Pro-Meter MK11 (Foss Electric). DBL was calculated as DBC_A minus DBC_B.

Tryptophan using p-dimethylaminobenzaldehyde. The method was that of Spies & Chambers (1949) after a papain predigestion (Mauron *et al.* 1960).

Assays with Streptococcus zymogenes. Available methionine, tryptophan, leucine and isoleucine, and relative nutritional value (RNV), were assayed with *Strep. zymogenes* by the general procedure described by Boyne *et al.* (1975), but a different method of enzyme predigestion was employed, as follows. The test sample (100 mg N) was suspended in 20 ml sodium β-glycerophosphate solution (20 g/l) in a screw-stoppered 50 ml bottle. The pH value was adjusted to 8·2 and 1 ml solution of pronase (B grade; Calbiochem Ltd, Hereford: 100 mg/ml in the sodium β-glycerophosphate solution) was added. The mixture was incubated at 48° for 30 min in an 'end-over-end' shaker (Ford, 1964) and then readjusted

to pH 8·2 with 1 M-NaOH. A further 1 ml pronase solution was added and incubation continued for 1·5 h. The digest was then adjusted to pH 7·2 and further diluted as appropriate for the different amino acid assays.

For the assay of total tryptophan the test samples were hydrolysed with alkali, as follows. Each sample (50 mg N) was mixed with 15·4 g barium hydroxide octahydrate and 9 ml boiled water in a 150 ml polypropylene flask and heated for 7 h at 121° in a steam autoclave. After cooling the mixture was adjusted to pH 6·0 by slow addition of 5 M-sulphuric acid, diluted to 200 ml and centrifuged. The supernatant fluid was decanted and the residue stirred with 100 ml water and centrifuged. The supernatant fluids were combined, made to 300 ml at pH 7·2, and assayed with *Strep. zymogenes* using a solution of DL-tryptophan as the reference standard.

The RNV values give an over-all measure of the relative efficiency with which amino acids in the control (100) and test samples were utilized for bacterial growth in the assay cultures. *Strep. zymogenes* has no requirement for exogenous lysine and so these values tend to discount any depression of nutritional quality caused specifically by the loss of lysine.

Assays with Tetrahymena. The assay procedure for lysine and methionine was that of Shorrock (1976), except that the test samples were predigested with pronase as described previously. The same digests were used both for *Tetrahymena* and *Strep. zymogenes.*

Assays with Pediococcus cerevisiae P60. This micro-organism requires exogenous lysine but, unlike *Strep. zymogenes* and *Tetrahymena*, it is non-proteolytic. Therefore, the assay values are in effect a measure of the release of free and small-peptide-bound lysine during digestion with pronase under the conditions specified previously.

The design of all these microbiological assays was such that no simple statistical procedure was applicable. In the experience of this laboratory with *Strep. zymogenes* assays, the between-assays variance is not more than 10%. With *Tetrahymena* the coefficient of variation ranged between 9·2 and 14·6%, depending on the type of sample (Shorrock, 1976). The present *Tetrahymena* values for available lysine in the 60°-stored milk powders (Table 1) are means of three independent assays. An analysis of variance was done, from which the standard error of the difference between means (16 df) was found to be 4·3 and the least significant difference at $P > 0{\cdot}05$ was 9·2. Despite what appears to be a small fall with time of storage, the over-all regression was not significant.

Results with *P. cerevisiae* are means representing four independent assays. The standard error of the difference between means (11 df) was 1·7 and the least significant difference at $P > 0{\cdot}05$ was 3·8. There was a significant linear regression ($P < 0{\cdot}05$).

RESULTS

The results of the various lysine assays on the milk powders stored at 60° and 70° are set out in Table 1 and further illustrated in Figs. 1 and 2, which show the values obtained for the test samples as percentages of those for the unheated control.

Storage at 60° led to the formation of the Amadori compound lactulosyl-lysine (early Maillard damage), which increased progressively with the period of incubation, even though the milk powder retained its natural colour. After 9 weeks, approximately 36% of the lysine groups were judged to be present as lactulosyl-lysine, which is biologically unavailable to the rat (Finot *et al.* 1977).

From Fig. 1 it appears that the results obtained by the different methods fell into two distinct groups. One group comprised direct-FDNB and reactive-lysine from furosine. The direct-FDNB values were compatible with the levels of lactulosyl-lysine present, and they fell progressively over the storage period to about the same extent as did the reactive-lysine values derived from the furosine estimations. The other group, comprising *Tetrahymena*, *P. cerevisiae*, total lysine, FDNB by difference, and dye-binding lysine, all predicted a much smaller fall in biologically-available lysine.

Table 1. *Lysine values (mg/g crude protein (nitrogen × 6·25)) of milk powders stored at 60° and 70° and 25 g moisture/kg*

(Chemical analyses are means of duplicate values, microbiological analyses are means of three replicate values)

Storage temperature and time (weeks)	Total lysine	Bound* lysine	FDNB-reactive lysine		Furosine method‡		Dye-binding lysine	*Tetrahymena*-available lysine	*Pediococcus cerevisiae*-available lysine
			By difference†	Direct	Lysine as reactive lysine	Lysine as lactulosyl-lysine			
Control (no treatment)	85·4	1·9	83·5	86·0	85·4	0	79·4	77·4§	63·0
60°									
1	82·5	2·6	79·9	72·6	76·1	11·1	77·8	75·9§	nd
2	80·4	2·6	77·8	70·1	72·6	19·5	74·6	75·3	57·3
3	75·1	2·5	72·6	64·0	68·0	17·9	71·5	75·3	nd
4	73·1	2·9	70·2	60·9	64·2	22·3	69·6	68·7	54·5
5	67·5	nd	nd	nd	57·4	25·4	nd	70·7	nd
6	69·6	3·9	65·7	59·9	57·8	29·7	66·5	59·7	50·3
7	67·7	nd	nd	nd	55·3	31·0	nd	67·0	nd
8	67·8	nd	nd	nd	55·3	31·3	nd	70·0	48·5
9	71·0	3·8	67·2	50·8	58·6	31·0	62·8	66·0	nd
70°									
1	64·4	5·7	58·7	47·3	49·8	36·5	58·0	64·0	41·5
2	56·3	9·1	47·2	37·8	37·9	46·1	53·6	66·0	37·5
3	26·9	16·6	13·0	15·1	15·9	27·5	22·8	36·0	12·8
4	18·9	18·7	0·2	12·3	14·2	11·8	14·5	9·0	5·3
5	16·0	nd	nd	nd	12·7	8·3	14·3	16·0	nd
6	15·3	18·3	0	12·6	13·3	5·0	10·3	11·0	1·5
7	16·0	nd	nd	nd	14·0	5·0	nd	6·0	nd
8	14·6	nd	nd	nd	14·6	0	nd	11·0	0·3
9	14·1	15·0	0	7·3	14·1	0	10·9	12·0	nd
SE of analytical value	‖	1·1	‖	1·0	‖	‖	0·9	‖	‖

nd, not determined.

FDNB, fluorodinitrobenzene

* Bound lysine refers to those lysine units which are released as lysine on acid-hydrolysis after dinitrophenylation; for details, see p. 344.

† Total lysine minus bound lysine.

‡ Reactive lysine = total lysine $-(1{\cdot}24 \times \text{furosine})$; lysine as lactulosyl-lysine $= 3{\cdot}1 \times$ furosine.

§ Mean of two rather than three replicate values.

‖ Total lysine and furosine were single values. For SE of microbiological values, see p. 345.

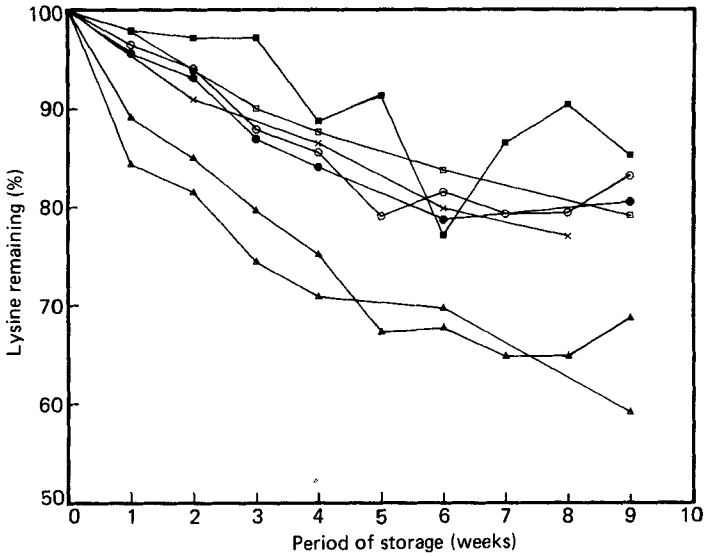

Fig. 1. Change in lysine values during storage at 60°. (○) Total lysine, (●) fluorodinitrobenzene (FDNB) by difference, (△) direct FDNB, (▲) reactive lysine from furosine, (□) dye-binding lysine, (■) *Tetrahymena*, (×) *Pediococcus*.

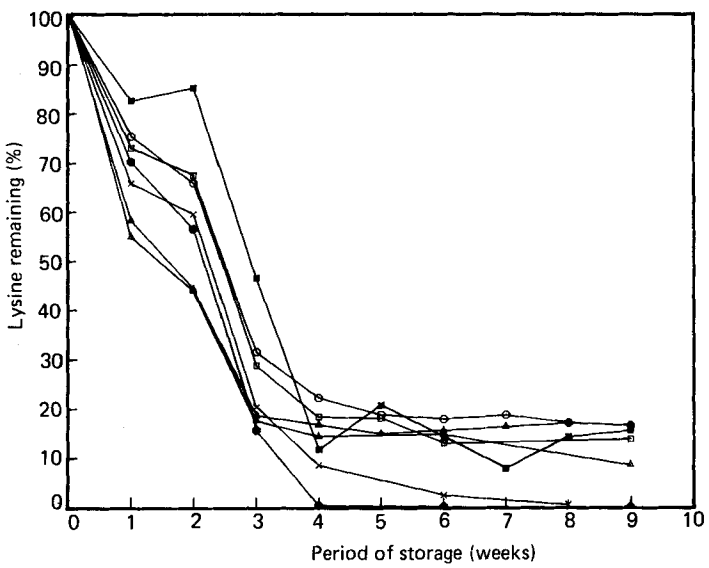

Fig. 2. Change in lysine values during storage at 70°. (○) Total lysine, (●) fluorodinitrobenzene (FDNB) by difference, (△) direct FDNB, (▲) reactive lysine from furosine, (□) dye-binding lysine, (■) *Tetrahymena*, (×) *Pediococcus*.

Fig. 2 illustrates the results obtained for storage at 70°. By 3 weeks the product had darkened to a deep red-brown colour, indicative of advanced Maillard reactions. At 2 weeks 50% of the lysine units were in the form of lactulosyl-lysine (Table 1). This amount had fallen to 10% by 4 weeks and, at 8 weeks, no lactulosyl-lysine was detected. Meanwhile, direct-FDNB and reactive-lysine values from furosine had fallen sharply and to the same extent, to 44% at 2 weeks and 18% at 3 weeks. During these early weeks of storage the

product was rich in lactulosyl-lysine, and *Tetrahymena*, *P. cerevisiae*, dye-binding lysine, total lysine and FDNB by difference all indicated a lesser extent of lysine damage. The discrepancy was most marked with *Tetrahymena*, which indicated only 15% loss at 2 weeks, as against 56% loss with direct-FDNB and reactive-lysine from furosine. From 4–9 weeks storage, when little or no lactulosyl-lysine remained, all the methods tended to give similar low values.

It is of interest to compare the 'bound lysine' values with the corresponding values for lactulosyl-lysine. Bound lysine is here defined as those lysine units that fail to react with FDNB but yet are measured as lysine after reaction with FDNB followed by acid-hydrolysis. At 60° and for the first 2 weeks at 70°, bound lysine was only 15–20% of the lysine units present as lactulosyl-lysine. In the coloured products however (4–9 weeks at 70°), bound lysine increased progressively even when there was little or no lactulosyl-lysine left.

The dye-binding results are given in greater detail in Table 2. Over the whole storage period at 60° and for the first 2 weeks at 70°, while the products still retained their natural colour, the DBC_A reading was not reduced. In the coloured products (3–9 weeks at 70°), there was a slight reduction in DBC_A. DBC_B (after propionylation) on the other hand was gradually increased both at 60° and 70°. At 60° and for the first 2 weeks at 70°, the increase in DBC_B was approximately 50% of the lysine units bound as lactulosyl-lysine. In the coloured products, even though lactulosyl-lysine was degraded, DBC_B continued to increase.

Table 3 shows, for the same milk powders, results obtained for methionine, cyst(e)ine, tryptophan, leucine and isoleucine, and RNV. *Strep. zymogenes* and *Tetrahymena* assay values for available methionine were closely similar. Methionine in the control milk powder was highly available, as it was in the 60°-stored preparations. On storage at 70° beyond 2 weeks there was a progressive fall, to 55% at 9 weeks. Total methionine values followed the same general pattern.

Cyst(e)ine was notable in that the content showed little or no change during storage at 60° or 70°. Similarly, with total tryptophan, the *Strep. zymogenes* assays indicated little or no change at 60° or 70°. The chemical test and available tryptophan by *Strep. zymogenes*, however, indicated a progressive fall at 70°, to 60% of the original value after 9 weeks.

Available leucine and isoleucine, like tryptophan and methionine, were highly available in the control milk powders. The levels declined only marginally at 60°, but at 70° they fell to approximately 67% at 9 weeks.

The protein nutritional value of *Strep. zymogenes* (RNV) was not impaired at 60° and, indeed, tended to improve. However, after 2 weeks at 70° the RNV declined to 66% after 8 weeks.

The lysine analyses of milk powders containing 100 g moisture/kg stored at 37° are shown in Table 4. This series of powders was set up to investigate further the contrasting results obtained with the different lysine procedures in the powders stored at 60°. The higher moisture content was used to accelerate the reaction. The FDNB-reactive lysine and reactive lysine from furosine values fell progressively throughout the storage period. They fell to almost the same extent as in the milk powders containing 25 g moisture/kg stored at 60°. After 67 d, approximately 60% of the original reactive lysine remained. *Tetrahymena*, *Pediococcus* and total lysine values were again much less reduced, confirming the results obtained with the powders stored at 60°. The powders became slightly coloured, with the advent of advanced Maillard reactions, from 46 d onwards.

DISCUSSION

Losses of lysine as measured by different methods. The Maillard reaction had proceeded to a much greater extent in the milk powders stored at 70°. At 60° there was a progressive increase in lactulosyl-lysine over the storage period, but at 70° lactulosyl-lysine was formed

Table 2. *Dye-binding measurements (mmol/kg crude protein (nitrogen × 6·25)) on stored milk powders containing 25 g moisture/kg*
(Mean values for duplicate determinations)

Storage temperature and time (weeks)	Dye-binding capacity (DBC)		Dye-binding lysine (A − B)	Increase in DBC of propionylated sample (C)*	Lysine as lactulosyl-lysine (from furosine) (D)	Increase in DBC after propionylation as proportion of lysine as lactulosyl-lysine (C/D)
	Untreated (A)	Propionylated (B)				
Control (no treatment)	865	322	543	—	—	—
60°						
1	868	336	532	14	76	0·18
2	873	363	510	41	133	0·31
3	885	395	490	73	122	0·60
4	874	398	476	76	153	0·50
6	871	415	456	93	203	0·46
9	864	435	429	113	212	0·53
70°						
1	853	456	397	134	250	0·54
2	876	510	366	188	315	0·60
3	677	521	156	199	188	1·06†
4	639	540	99	218	81	2·69†
6	624	554	70	232	34	6·82†
9	663	588	75	266	0	—
SE of analytical value	4·0	5·1	6·5	—	‡	—

* DBC of propionylated stored milk powder minus DBC of propionylated control sample.

† These products were highly coloured and contained advanced Maillard reaction products which, like lactulosyl-lysine, also seem to bind the dye Acid Orange 12 before and after propionylation.

‡ Single values.

Table 3. *Storage of milk powders at 60° and 70° and 25 g moisture/kg. Change in content of methionine, cyst(e)ine, tryptophan, leucine and isoleucine (mg/g crude protein (nitrogen × 6·25)), and relative nutritional value (RNV)*

(Values in parentheses represent the values of the stored samples as percentages of the control)

Storage temperature and time (weeks)	Methionine			Cyst(e)ine*	Tryptophan			Leucine	Isoleucine	
					Total					
	Total*	Available *Strep. zymogenes*	Available *Tetrahymena*		Colorimetric†	*Strep. zymogenes*	Available *Strep. zymogenes*	Available *Strep. zymogenes*	Available *Strep. zymogenes*	RNV‡ *Strep. zymogenes*
Control (no treatment)	25·0	31	30	8·6	14·8	13	14	103	49	100
60°										
1	25·4 (102)	31 (100)	30 (100)	8·9 (103)	14·8 (100)	nd	14 (100)	99 (96)	49 (100)	104 (104)
2	26·5 (106)	31 (100)	31 (103)	9·1 (106)	14·1 (95)	nd	14 (100)	96 (93)	46 (93)	109 (109)
3	25·8 (103)	29 (94)	31 (103)	9·2 (107)	13·7 (93)	nd	13 (93)	96 (93)	45 (91)	101 (101)
4	25·5 (102)	31 (100)	30 (100)	9·0 (105)	14·5 (98)	nd	14 (100)	95 (92)	45 (91)	111 (111)
5	nd	31 (100)	31 (103)	nd	nd	nd	14 (100)	98 (95)	47 (95)	112 (112)
6	25·0 (100)	31 (100)	31 (103)	8·8 (102)	14·5 (98)	nd	14 (100)	92 (90)	45 (91)	116 (116)
7	nd	31 (100)	34 (113)	nd	nd	nd	14 (100)	94 (91)	45 (91)	113 (113)
8	nd	31 (100)	31 (103)	nd	nd	nd	14 (100)	98 (95)	44 (89)	113 (113)
9	25·9 (104)	31 (100)	31 (103)	8·8 (102)	14·3 (97)	12 (92)	14 (100)	98 (95)	42 (85)	115 (115)
70°										
1	22·2 (89)	29 (94)	29 (97)	8·3 (97)	14·6 (99)	11 (84)	12 (86)	96 (93)	46 (93)	96 (96)
2	19·9 (80)	32 (103)	32 (107)	7·9 (92)	13·2 (89)	12 (92)	14 (100)	94 (91)	46 (93)	112 (112)
3	17·9 (72)	28 (90)	29 (97)	8·1 (94)	12·9 (87)	12 (92)	12 (86)	91 (88)	42 (85)	80 (80)
4	16·5 (66)	24 (77)	22 (73)	8·1 (94)	10·4 (70)	12 (92)	12 (86)	88 (85)	39 (79)	69 (69)
5	nd	19 (61)	24 (80)	nd	nd	11 (84)	11 (79)	86 (83)	35 (71)	70 (70)
6	16·9 (68)	21 (68)	21 (70)	8·1 (94)	9·7 (67)	12 (92)	11 (79)	82 (79)	34 (69)	67 (67)
7	nd	20 (65)	18 (60)	nd	nd	11 (84)	10 (71)	77 (74)	32 (65)	68 (68)
8	nd	19 (61)	20 (67)	nd	nd	11 (84)	9 (64)	77 (74)	31 (63)	66 (66)
9	17·0 (68)	18 (58)	16 (53)	8·2 (95)	8·9 (60)	11 (84)	8 (57)	74 (71)	32 (63)	nd
SE of analytical value	0·8	§	§	0·2	0·6	§	§	§	§	§

nd, not determined.

* By ion-exchange chromatography after performic acid oxidation and acid-hydrolysis.

† By reaction with *p*-dimethylaminobenzaldehyde after papain digestion.

‡ Expressed relative to control milk = 100.

§ For SE of microbiological values, see p. 345.

Table 4. *Lysine values (mg/g crude protein (nitrogen × 6·5)) of milk powder stored at 37° and 100 g moisture/kg*
(Values in parentheses represent the values of the stored samples as percentages of the untreated control)

Storage time (d)	Total lysine	Direct FDNB reactive lysine	Furosine method*		*Tetrahymena*-available lysine	*Pediococcus cerivisiae*-available lysine
			Lysine as reactive lysine	Lysine as lactulosyl-lysine		
Control, 0	82·2	79·8	79·7	2·5	96	63
5	79·3 (96)	74·9 (94)	77·7 (97)	4·0	93 (96)	71 (113)
11	76·9 (94)	73·7 (92)	74·5 (94)	5·6	94 (98)	66 (105)
17	77·6 (94)	66·7 (84)	74·6 (94)	7·4	96 (99)	66 (105)
23	71·7 (87)	65·5 (82)	68·2 (86)	8·7	92 (95)	63 (100)
29	69·3 (84)	64·7 (81)	64·9 (81)	11·1	89 (93)	61 (97)
32	71·3 (87)	62·8 (79)	66·8 (84)	11·4	93 (96)	59 (94)
46	63·6 (77)	56·2 (70)	58·6 (74)	12·4	79 (82)	57 (90)
53	62·0 (75)	53·9 (68)	56·5 (71)	13·7	80 (83)	58 (87)
67	55·5 (68)	50·1 (63)	49·2 (62)	16·7	84 (87)	51 (81)
SE of analytical value	0·7	1·1	0·7	0·2	†	†

FDNB, fluorodinitrobenzene.
* Reactive lysine = total lysine − (1·24 × furosine); lysine as lactulosyl lysine = 3·1 × furosine.
† For SE of microbiological values, see p. 345.

more rapidly and subsequently degraded. After peaking at 2 weeks the content of lactulosyl-lysine declined to zero by 9 weeks, with the production of advanced Maillard reaction products including coloured pigments.

From our results in Table 1, it is clear that the different methods for lysine determination give widely dissimilar results. The difficulties in measuring reactive lysine after early Maillard reactions have recently been discussed in detail by Hurrell & Carpenter (1981). Deoxyketosyl-lysine compounds are biologically unavailable for the rat (Finot *et al.* 1977) but are measured as 'available' by some reactive-lysine methods. This was first demonstrated by Finot & Mauron (1972) and Finot (1973) with the model compound α-formyl-ϵ-deoxyfructosyl-lysine. Hurrell & Carpenter (1974) subsequently showed, with model systems of casein heated with glucose which had undergone early Maillard reactions, that the direct-FDNB, total lysine after borohydride and the guanidination procedures gave similar lysine values to animal assays but that the trinitrobenzene sulphonic acid, total lysine and FDNB-reactive lysine by difference procedures failed to predict the full extent of lysine damage. Problems existed only when the slightly basic deoxyketosyl lysine was present. After advanced Maillard reactions and the degradation of deoxyketosyl lysine all the methods gave similar values. Bujard & Finot (1978) confirmed these results with heated milk. Mottu & Mauron (1967) had already shown that the direct-FDNB procedure and rat assays gave similar values for heat-damaged milk powders.

We have now compared the furosine, *Tetrahymena*, *Pediococcus*, DBL and FDNB-reactive lysine by difference procedures with the direct-FDNB method, in the analysis of lysine in milk powders stored at high temperatures. The direct-FDNB and furosine procedures gave similar results. For milk powders and for milk-based infant formula, the furosine procedure is the method of choice as it gives a measure of lysine bound as lactulosyl-lysine as well as the level of reactive lysine. It is, therefore, possible to measure lysine damage without knowing the lysine content of the untreated product.

The *Tetrahymena*, *Pediococcus*, DBL and FDNB-reactive lysine by difference procedures all failed to predict the full extent of lysine damage in milk powders containing lactulosyl-lysine. It would appear that, unlike the rat, *Tetrahymena* and *Pediococcus* can utilize lactulosyl-lysine as a source of lysine. The utilization of deoxyketosyl-lysines by man remains to be studied in detail. According to Niederwieser *et al.* (1975), 16% of the protein-bound ϵ-deoxy-fructosyl-lysine given to babies in an infant formula was excreted in the urine and 55% in the faeces. In the rat, 10–15% is also absorbed and excreted in the urine (Finot & Magnenat, 1981); the rest is destroyed in the caecum (Finot *et al.* 1977).

The DBL procedure is in general a useful screening procedure for the reactive lysine content of foodstuffs (Hurrell *et al.* 1979), but it is now evident that it should not be used for foods containing early Maillard reaction products. Lactulosyl-lysine is still sufficiently basic to bind with Acid Orange 12 in the DBC_A reading. This might not be a problem if the dye was still bound with lactulosyl-lysine after propionylation. Although some of our earlier results indicated that this may be so (Hurrell *et al.* 1979), it would now appear that lactulosyl-lysine is partially propionylated and consequently only approximately half the lactulosyl-lysine units present react with the dye. In a further study using the model compounds α-formyl-lactulosyl-lysine and α-formyl-fructosyl-lysine, we have found that 29 and 42% respectively of these compounds were propionylated by our procedure. The net result is that the DBC_B reading increases less than the level of lactulosyl-lysine and consequently DBL over-estimates the reactive lysine. After advanced Maillard reactions in which lactulosyl-lysine has been degraded, the DBL procedure has given similar results to the direct-FDNB, furosine and other procedures. It would appear that advanced Maillard reaction products react with the dye in the same way before and after propionylation.

The FDNB-difference method, with some modifications in experimental detail, is the

'available' lysine method recommended by the Association of Official Analytical Chemists (1980). However, as shown in Table 1, and as suggested by Finot & Mauron (1972) and Hurrell & Carpenter (1974), it is clearly unsuitable for lysine determinations in heat-damaged milk powders. Protein-bound lactulosyl-lysine releases 40% of its lysine content as lysine on simple acid-hydrolysis (Finot *et al.* 1981) but, if treated first with FDNB, it appears to react to give compounds that liberate little or no lysine or DNP-lysine on acid-hydrolysis (Finot, 1973). Using the FDNB-difference procedure, 40% of the biologically-unavailable lactulosyl-lysine is therefore determined as available lysine. The increase in 'bound' lysine (Table 1) in the advanced Maillard milk powders is difficult to explain, especially as lactulosyl-lysine itself is progressively reduced to zero after 9 weeks storage at 70°. It would seem that other forms of lysine are produced during advanced Maillard reactions and that these regenerate lysine with or without prior FDNB-treatment.

Losses of other essential amino acids. It is of interest to compare these lysine results with those for the other amino acids measured (Table 3). Storage of the milk powder at 60° for 9 weeks caused no change in the methionine content. But at 70°, both total and available methionine fell progressively, possibly because of interaction of methionine with advanced Maillard reaction products.

Unlike methionine, cyst(e)ine was stable throughout storage at 60° and 70°. This finding was unexpected, as it is known that the interaction of protein with glucose (Miller *et al.* 1965) and with sucrose (Evans & Butts, 1949) may involve loss of cyst(e)ine.

The microbiological tests showed that total tryptophan, like cyst(e)ine, was little changed by storage at 70°. However, available tryptophan and the colorimetric assay indicated a marked fall. A likely explanation for this inconsistency is that both tests involved an enzymic predigestion, and the decline in apparent tryptophan content with increase in storage time reflects a progressive impairment of in vitro digestibility rather than any real loss of tryptophan. A similar explanation must account for the fall, after pronase digestion, of leucine and isoleucine, both of which present unreactive paraffinic side chains, and for the decline in RNV. The low levels of tryptophan as measured by the colorimetric method could, alternatively, be due to the brown Maillard pigments interfering with the colorimetric test. However, a reaction between tryptophan and lactose is also possible. Using aqueous model systems of N-acetyl-[^{14}C]tryptophan and glucose, Finot *et al.* (1982) have recently shown that, although the indole ring of tryptophan does not react with glucose itself, it does react with advanced Maillard reaction products. It is therefore possible that the alkaline-hydrolysis before the measurement of total tryptophan releases tryptophan from unavailable tryptophan complexes so that it may be utilized by *Strep. zymogenes*. This is analogous to the release of lysine from lactulosyl-lysine on acid-hydrolysis.

Practical considerations. With regard to the distribution and storage of milk powders in hot climates, our results indicate that even at very low moisture contents there can be considerable losses of amino acids. The extensive browning which occurred at 70° would clearly make the product inedible so that the large losses of lysine, methionine and tryptophan are of little practical importance. In whole-milk powders stored at 60° under our conditions, methionine and tryptophan were stable. The loss of lysine was less than 10% after 3 weeks but rose to 40% after 9 weeks. These products still retained their natural colour, indicating that browning alone is not a good indicator of the evolution of the Maillard reaction. A temperature of 60° is already extremely high and would not be reached during storage in properly-equipped warehouses. However, if during their transport the powders are left in direct sunlight on quaysides or in railway sidings, or held up in customs sheds or left in the sun when being sold on market stalls, etc., then it is possible that such temperatures might be reached.

The extent of the Maillard reaction depends not only on the time and temperature of

storage but also on the moisture content and the composition of the product. Moisture content is reduced to a low level during manufacture of milk powders (20–40 g/kg) and will remain low provided that the product is stored in moisture-resistant containers which can be resealed efficiently after opening. Breast-milk-substitute formulas contain a much higher proportion of lactose than whole-milk powders and they are more sensitive to Maillard reactions.

Our experiments were made at 60° and 70° for up to 9 weeks storage. At 40° and 50° Maillard reactions will be less rapid; above 70° they will be more rapid. For a more critical interpretation of our findings, we need more details concerning the temperature range and storage time to which products might be subjected. This information was not available to us. We have shown, however, that lysine, but neither methionine nor tryptophan, can be damaged in low-moisture powders which have retained their natural colour and that when measuring reactive or available lysine in heat-damaged powders, care should be taken when choosing the analytical method. The most reliable methods are direct-FDNB and the furosine technique.The FDNB-difference method (as recommended by the Association of Official Analytical Chemists, 1980), dye-binding lysine, the *Tetrahymena* and *Pediococcus* techniques are all unsuitable for measuring reactive or available lysine in heat-damaged milk powders.

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