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Studies on the Ripening of Stilton Cheese: Proteolysis

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ABSTRACT

The pH of fresh Stilton curd, which 2 h after coagulation was ~6·7, decreased to ~4·8 in 4 day-old cheeses but increased gradually thereafter to ~6 at the end of ripening (70 days). At the end of the normal ripening period, ~60% of the total N (TN) was water soluble (WSN) and ~58% of the WSN was soluble in 70% ethanol, while 40% was diffusible on dialysis against water; free amino acid N (phosphotungstic acid-soluble, PTA-N) reached ~4·8% of TN. The levels of TNBS-reactive amino groups were highly correlated with the values of WSN and 12% TCA-N but showed a greater change in the PTA extract than the value of PTA-N. Dialysable WSN was resolved into five peptide fractions by chromatography on Sephadex G-10; these fractions were very heterogeneous and contained up to eight ninhydrin-positive peptides when examined by TLC. Chromatography on DEAE cellulose revealed a high level of heterogeneity in the 70% ethanol-soluble and -insoluble fractions and this was confirmed by the wide range of N-terminal groups. The free amino acids (FAA, as measured by ion exchange chromatography) represented 6·8% and 9·9% of TN in the 70 day-old experimental cheeses and in commercial samples, respectively. Valine, leucine, lysine and glutamic acid accounted for ~50% of total FAA throughout the ripening period.

INTRODUCTION

Blue cheese varieties undergo extensive proteolysis during ripening; perusal of the literature indicates that the protein in Blue cheese varieties is more

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extensively hydrolysed than that in most other common varieties (Ismail & Hansen, 1972; Marcos *et al.*, 1979). Hewedi & Fox (1984) noted that about 57% of the total N in commercial mature Danish-type Blue cheese was water soluble, ~65% of which was soluble in 70% ethanol, 72% was soluble in 0·1M $CaCl_2$ and 12% was soluble in 5% phosphotungstic acid. The quantitative estimation of free amino acids and related compounds in 30 commercial cheese varieties (Kosikowski & Dahlberg, 1954) revealed that Blue cheese varieties, i.e. Roquefort and Gorgonzola, were distinguished by the greatest concentration of free amino acids (FAA). Sato *et al.* (1966) reported that different strains of *P. roqueforti* have different proteolytic activities which may affect the patterns of FAA in the final cheese. Thus, Danablu was characterized by a high content of aspartic acid and low concentrations of arginine, glutamic acid and leucine (Ismail & Hansen, 1972). In Gorgonzola there was a greater increase in methionine than in α-amino butyric, glycine and glutamic acid (Salvadori *et al.*, 1976).

Stilton, which is one of the main subclasses of Blue cheese, has been studied much less extensively than other Blue varieties, e.g. Roquefort and Danablu. Hiscox *et al.* (1951) showed that during the period of active mould growth in Stilton cheese there was extensive breakdown of casein with the formation of high levels of peptones and amino acid nitrogen. They also noted a decrease in pH during the white stage (pre-mould growth) although no starter was used and the cheese milk was renneted while still sweet. Scott (1968) noted a decrease in the pH of Stilton from 5·5 to 5·3 during the first 2 weeks of ripening followed by a slight increase when blue veining began and the pH reached 6·5 after 6 months of ripening. The increase in the pH of Blue cheeses during ripening has been attributed mainly to the proteolysis (Godinho & Fox, 1982).

The present work was undertaken to study the extent and nature of proteolysis in Stilton cheese.

MATERIALS AND METHODS

Cheese samples

Samples of two lots of Stilton cheese, manufactured (under commercial conditions) from fresh milk (cheese A) and 24 h old milk (cheese B) by St. Ivel Creameries, Melton Mowbray, Leicestershire, Great Britain, were obtained 4, 20, 28, 45, 55 and 70 days after manufacture. Samples of curd were also obtained 2, 8 and 24 h and 3 days after manufacture from the same source. For comparative purposes, samples of Stilton and Danish Blue cheese were purchased at a local market. The samples used were the same as those used in the study on lipolysis described in the previous paper.

pH measurement

Curd or cheese (10 g) was macerated in a mortar with 10 ml of distilled water and the pH of the slurry measured using a pH meter-26 (Radiometer, Copenhagen, Denmark).

Fractionation of cheese nitrogen

The cheese nitrogen was fractionated by the procedures described by Kuchroo & Fox (1982*a*,*b*, 1983) for the fractionation of water-soluble nitrogen in Cheddar cheese. Phosphotungstic acid-soluble N was determined as described by Reiter *et al.* (1969).

Analytical techniques

The method of Fields (1971), as modified by Kuchroo *et al.* (1983), was used to quantify the amino groups in water-soluble extracts and fractions thereof. The method of Woods & Wang (1967), using dansyl chloride, was used to characterize the N-terminal amino acids of peptides separated by the preceding fractionation scheme. Electrophoresis in polyacrylamide disc gels containing sodium dodecylsulfate (SDS) was performed as described by Laemmli (1970).

Thin-layer chromatography (TLC) was performed on glass plates precoated with a silica gel layer, as described by Kuchroo & Fox (1982*b*). The free amino acids were separated and quantified by the procedure of Weaver *et al.* (1978) using a Locarte Amino Acid Analyzer (Locarte Company, London).

RESULTS AND DISCUSSION

The cheeses were unwrapped during ripening and hence the composition changed (Table 1); presumably such changes influence ripening rate and pattern. Sporulation of mould was first detected at ~28 days in a zone corresponding roughly to the outer third of the cheese. Salt concentration influences mould germination and growth in surface-salted Blue cheeses (Godinho & Fox, 1981*a*,*b*) but since salt distribution was uniform throughout the experimental Stilton cheese from the start, such variation in growth was probably due to the availability of O_2 (Washam *et al.*, 1979). After 55 days, mould growth had extended to the rind of the cheeses and after 70 days almost uniform mould growth had occurred throughout the cheese wheels.

TABLE 1
Gross Composition of Stilton Curd and Cheese

Age (days)		*Moisture* (%)	*Fat* (%)	*Salt* (%)	*Nitrogen* (%)	*pH*
Curd						
Rennet gel, 2 h	A					6·7
after renneting	B					6·7
Curd, 8 h	A	69·8	16			6·3
after cutting	B	67·1	17			6·1
Curd, 24 h	A	54·1	26			4·8
	B	53·0	26			4·6
Curd, 3 days	A	49·8	27			4·5
	B	49·4	28			4·3
Cheese						
4	A	49·0	28	2·3	3·9	4·7
	B	48·4	28	2·5	3·9	4·4
20	A	47·6	28	2·3	4·0	4·8
	B	46·9	29	2·5	4·0	4·8
28	A	46·2	29	2·4	4·1	4·9
	B	46·3	30	2·7	4·2	4·8
45	A	44·5	30	2·4	4·2	5·2
	B	45·2	31	2·6	4·4	5·2
55	A	42·6	31	2·6	4·4	5·8
	B	42·2	31	2·7	4·6	5·9
70	A	41·6	33	2·6	4·5	6·0
	B	41·0	32	2·7	4·6	6·1
Commercial samples						
Stilton		39·6	34	3·7	3·9	6·6
Danablu		43·6	31	3·4	3·6	6·5

The pH of the fresh curds was 6·7 and 6·3 at 2 and 8 h after manufacture, respectively, and had decreased to ~4·7 after 24 h and to ~4·5 after 3 days. The pH changed little during the first 28 days of ripening, after which it increased significantly (Table 1), as already observed by Hiscox *et al.* (1951). The initial decrease in pH is attributed to the production of lactic acid by the starter bacteria during the first week after manufacture; since the curd was very moist (~69%) when placed in hoops and as no pressure was applied, drainage was slow and the curd was in close contact with the acid whey for a long time (~3 days) which leads to the development of acidity and falling pH. With the progress of ripening, the pH increased gradually to 6·1 at 70 days and was 6·6 in the Stilton sample purchased at the local market. Similar changes in pH were observed by Scott (1968) and by Godinho & Fox (1982) who suggested that extensive proteolysis with subsequent deamination of amino acids and the metabolism of organic acids leads to an increase in pH.

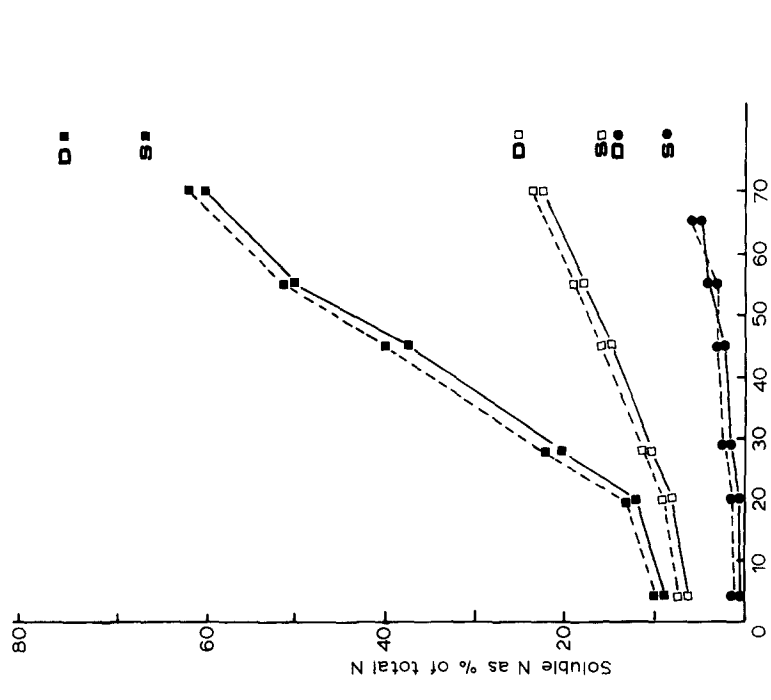


Fig. 1. Formation of water-soluble N (■), 12% ICA-soluble N (□) and 5% phosphotungstic acid-soluble N (●) in Stilton cheese during ripening. Cheese A ——; Cheese B – – –; commercial Stilton: S, commercial Danablu: D.

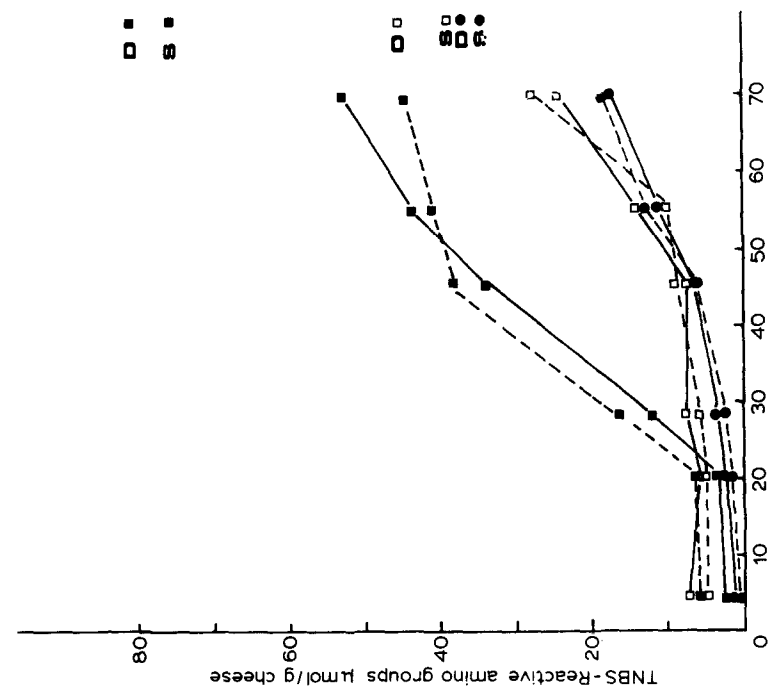
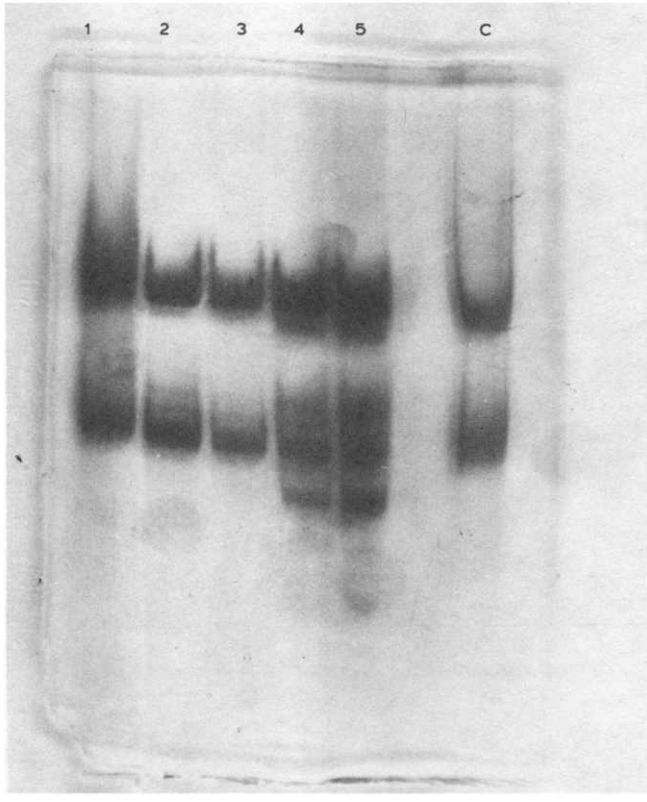


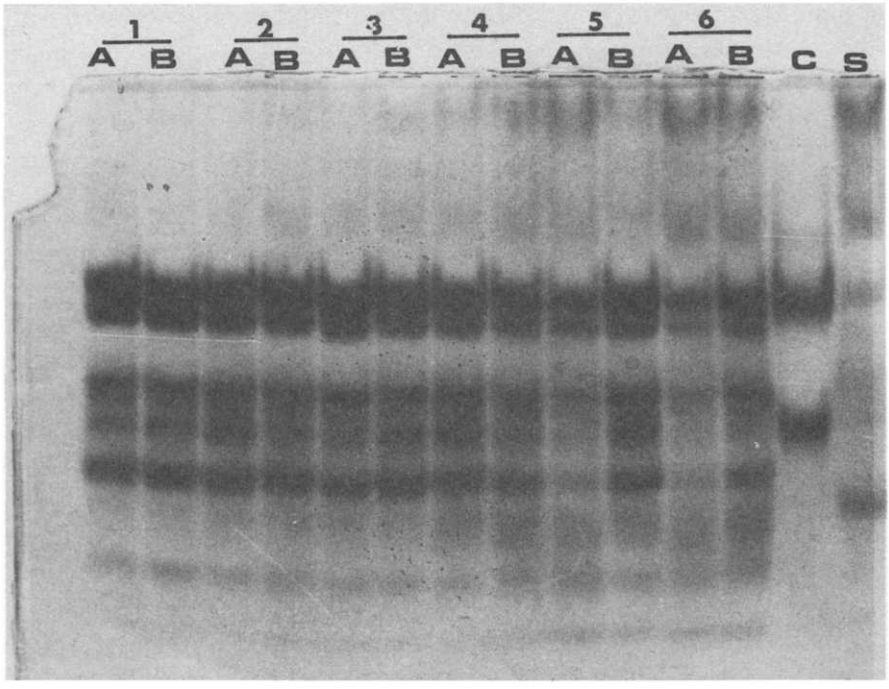
Fig. 2. Formation of TNBS-reactive amino groups in the water extract (■), 12% TCA- (□) and 5% phosphotungstic acid- (●) supernatant of Stilton cheese during ripening. Cheese A ——; Cheese B – – –; commercial Stilton: S, commercial Danablu: D.



(a)

Fig. 3. (a) Electrophoretograms of two sets (A, B) of Stilton cheese milk and curds during manufacture and the early stage of ripening. 1. Cheese milk; 2. 2 h curds; 3. 8 h curds; 4. 24 h curds; 5. 3 day curds; C. Na-caseinate.

The levels of water-soluble-, 12% TCA- and 5% PTA-soluble N as indices of proteolysis revealed extensive proteolysis during the ripening of Stilton (Fig. 1). A relatively high content of water-soluble N (WSN) was observed in the cheese on day 4, which is presumed to be due mainly to the proteolytic activity of the coagulant which was enhanced at the low pH and the high moisture content of the cheese of that time. WSN increased rapidly after 20 days of ripening, reaching 60·6% and 62·4% of TN for cheeses A and B, respectively, at the end of the experimental period (70 days). The rate of formation of 12% TCA- and 5% PTA-soluble N increased after 20 days of ripening and they increased almost linearly thereafter to reach 23·0% and 4·8%, respectively, at the end of the ripening period. While the values for WSN and 5% PTA-N were considerably higher in the commercial Stilton sample, the value for 12% TCA-N was lower than those for the experimental cheeses after 70 days of ripening. This may be a reflection of conversion of peptides to free amino acids which attained a high level, as indicated by the



(b)

Fig. 3—*contd.* (b) Electrophoretograms of two sets (A, B) of Stilton cheese during ripening. Age of cheese: 1. 4 days, 2. 20 days; 3. 28 days; 4. 45 days; 5. 55 days; 6. 70 days; S. commercial Stilton; C. Na-caseinate.

level of 5% PTA-N. The soluble nitrogen fractions were slightly higher in cheese B (from aged milk) than in cheese A at most times during ripening, particularly during the early stages of ripening (4 days). Commercial Danablu had higher amounts of soluble N by all three indices used. Danablu undergoes the most extensive proteolysis among several types of cheese, including sub-types of blue cheese (Marcos *et al.*, 1979). Obviously, the proteolytic agents in Stilton are capable of bringing about a large increase in the amount of soluble nitrogen, in agreement with the results for other blue cheese varieties (Kinsella & Hwang, 1976).

The values for TNBS-reactive amino groups were highly correlated (Fig. 2) with those for WSN and 12% TCA-N, but the concentration of NH_2-groups in the PTA extract showed a greater change than PTA-N. This could be expected since hydrolysis of several peptide bonds may be required to release very small peptides and amino acids soluble in 5% PTA while the cleavage of each peptide bond results in the formation of a TNBS-reactive group (Kuchroo *et al.*, 1983).

Characterization of proteolysis in Stilton cheese and curd by gel electrophoresis (Fig. 3*a* and *b*) revealed that considerable breakdown of both

α_{s1}- and β-caseins to α_{s1}-I and β-I, respectively, had already occurred within 24 h after manufacture and extensive degradation of α_{s1}- and β-caseins had occurred after 4 days. Relatively little further change occurred up to 45 days but α_{s1}-casein was completely hydrolyzed at 55 days in cheese A and at 70 days in cheese B. Proteolysis of β-casein was slower than that of α_{s1}-casein and a considerable amount of the former remained unhydrolyzed at the end of the experimental period (70 days). In the commercial Stilton sample, both α_{s1}- and β-caseins were extensively degraded and only low concentrations of peptides were detectable on the electrophoretograms.

Stacking gel electrophoresis confirmed that α_{s1}-casein in the water-soluble fractions of cheese was extensively hydrolyzed in the 4 day-old cheeses but β-casein had undergone very little degradation up to 45 days, after which both α_{s1}- and β-caseins underwent further hydrolysis. In the water-soluble fraction, traces of a degradation product with an electrophoretic mobility corresponding approximately to α_{s1}-I and which may or may not be a product of α_{s1}-casein, appeared as a faint band in the young cheeses but its intensity decreased with the progress of ripening and it had disappeared at the end of the ripening period. While β-I was not apparent in the electrophoretograms of the water-insoluble fractions, a band with an electrophoretic mobility corresponding to β-I appeared as the principal peptide in the water-soluble fractions. In the case of the commercial Stilton and Danablu samples, both α_{s1}- and β-caseins were extensively hydrolyzed and only faint bands were detectable on the gel. In fact, the electrophoretic patterns of the water-soluble fraction of Danablu revealed a very complex pattern of protein degradation; the degradation products of α_{s1}-casein were present at much higher concentrations in the WS-fraction of Danablu than in that from the experimental or commercial Stilton.

Dialysis of the water-soluble fraction of Stilton cheese showed that ~40% of the water-soluble N of the 70-day cheeses was dialysable against water. Chromatographic fractionation (Fig. 4) on Sephadex G-10 showed that the diffusate from most samples was resolved into three main peaks (1, 2, 3) and with the progress of ripening, two further major peaks (4, 5), representing very small peptides and probably amino acids, developed. Thin-layer chromatography showed that all fractions from Sephadex G-10 were very heterogeneous and contained as many as eight ninhydrin-positive peptides (Fig. 5). Peaks 4 and 5, which represented ~40% of total diffusible N in mature cheese, appeared to contain only free amino acids, as shown by chromatography before and after hydrolysis in 6M-HCl at 105°C for 24 h.

Approximately 35% and 48% of the retentate fractions of the WSN were soluble in 70% ethanol at the end of the ripening period (70 days) for cheeses A and B, respectively.

The SDS-PAGE patterns of 70% ethanol-soluble and -insoluble fractions

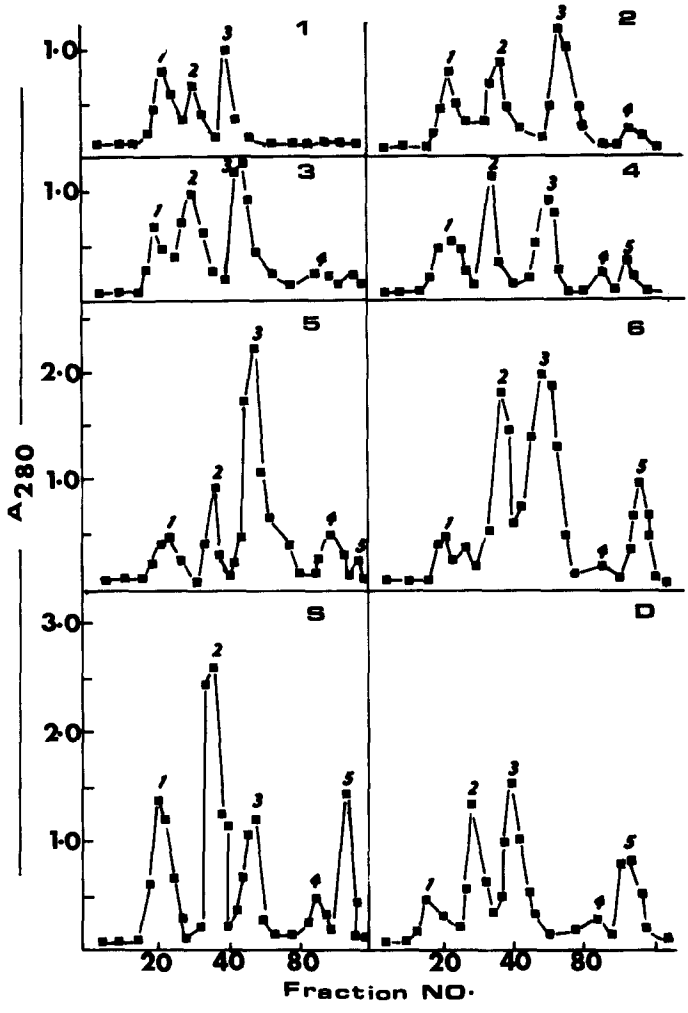


Fig. 4. Elution profile of the water-soluble nitrogen diffusate of Stilton cheese (B). Age of cheese: 1. 4 days; 2. 20 days; 3. 28 days; 4. 45 days; 5. 55 days; 6. 70 days. Commercial Stilton: S; commercial Danablu: D.

of the retentate from WSN demonstrated (Fig. 6) the predominance of fast-moving peptides as ripening progressed.

Chromatography on DEAE-cellulose indicated that the ethanol-soluble and -insoluble fractions were heterogeneous. A considerable number of peptide fractions (up to 8) were resolved on DEAE-cellulose (Fig. 7). The heterogeneity of these fractions, as well as the elution profiles, differed significantly for the two fractions with stage of ripening but only slightly between the two sets of cheeses, A and B. Thus, amino terminal labelling

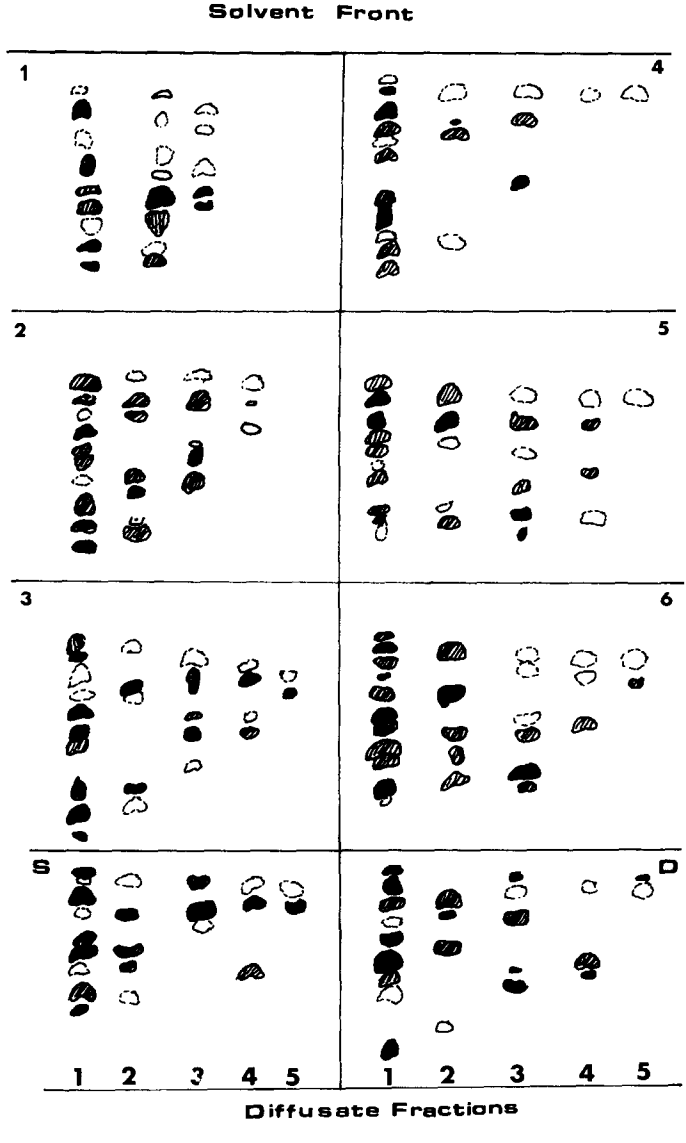


Fig. 5. Thin-layer chromatograms of fractions obtained from chromatograms on Sephadex G-10 of diffusate (WSN-D) from Stilton cheese at different stages during ripening. Age of cheese: 1. 4 days; 2. 20 days; 3. 28 days; 4. 45 days; 5. 55 days; 6. 70 days; S. commercial Stilton; D. commercial Danablu.

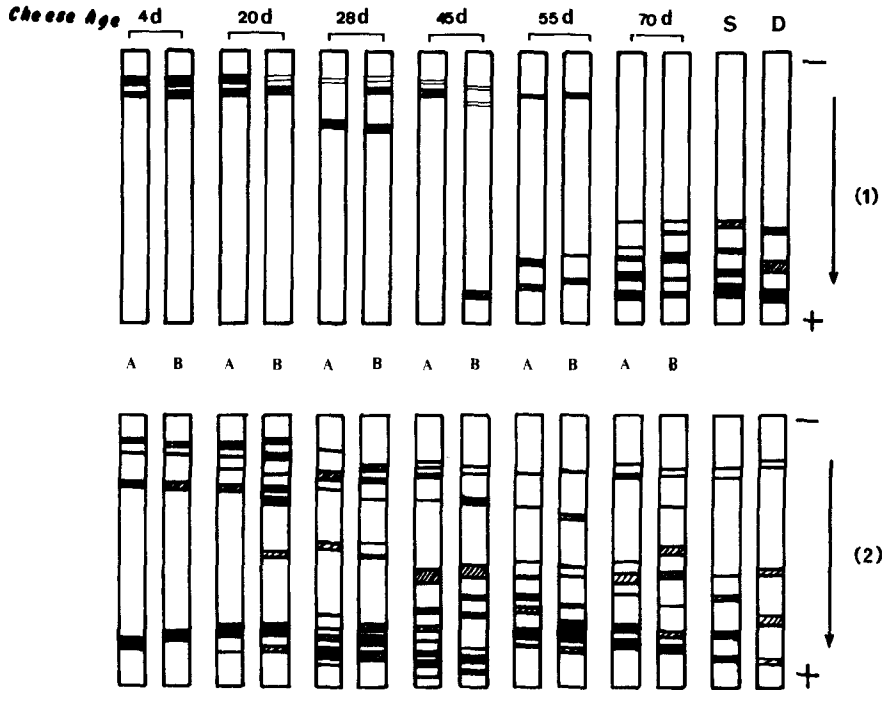


Fig. 6. SDS-gel electrophoretograms of the retentate fractions of Stilton cheese at different stages during ripening. (1) 70% ethanol-soluble fraction. (2) 70% ethanol-insoluble fraction.

showed (Table 2) that the fractions of the ethanol-soluble N from DEAE-cellulose contained many peptides which differed according to the stage of ripening and batch of cheese. Leucine, glutamic acid, isoleucine, valine and phenylalanine were the dominant end-terminal residues in the DEAE-ethanol-soluble fractions. The heterogeneity, evident from the wide range of terminal amino groups, probably reflects the non-specific proteolytic system in Stilton cheese since it seems that there is a highly active endopeptidase which results in the release of many small peptides.

Summation of the free amino acids (FAA) following separation on the autoanalyzer showed that they represented 6·8 and 7·7 g of 16 g TN for cheeses A and B, respectively, at the end of ripening (Table 3), i.e. 6·8–7·7% of total N; these values were considerably higher than those for PTA-soluble N ($\sim$ 5% of total N) suggesting that some free amino acids are insoluble in 5% PTA which is in agreement with the results of Jarrett *et al.* (1982). The commercial Stilton sample had a somewhat higher concentration of FAA than the 70-day experimental cheeses but the Danablu had the greatest quantities of free amino acids ($\sim$ twice the concentration in the 70-day experimental Stilton). Similar results by Ismail & Hansen (1972) showed that Danablu has a very high content of free amino acids compared with most

TABLE 2

N-Terminal Group of Alcohol-Soluble Peptides Fractions Separated on DEAE Cellulose

		Fraction 1	*Fraction 2*	*Fraction 3*	*Fraction 4*	*Fraction 5*	*Fraction 6*	*Fraction 7*
4 days	A	Ile Leu Phe Pro	Glu + Asp Ile Pro Leu Phe Ala Val	Pro Ile Glu DNS – NH_2				
	B	Ile Leu Phe Val Glu	Ile Leu Pro Phe Glu + Asp Lys Val	Pro Val Ile Glu + Asp Leu DNS – NH_2				
45 days	A	Ile Leu Phe DNS – NH_2 Pro Val His	Glu Met	DNS – NH_2 Ile DNS – OH Leu Pro Phe Glu	Val Alu	Pro DNS – NH_2 Ile Glu + Asp Leu	Ile Leu Val Tyr DNS – OH	Asp + Glu DNS – OH Pro Leu	Ile Leu Asp + Glu Gly DNS – OH DNS – NH_2 Prop	Val Met	Ile Val Ser DNS – NH_2 DNS – OH Glu
	B	Ile Leu Phe Val DNS – NH_2 Tyr Glu	Met Lys Met Ala Glu Met	Ile Leu Phe Pro DNS – NH_2 DNS – OH Ala	Met Val Glu	Ile Leu DNS – NH_2	Ile Leu Val Tyr Pro Lys DNS – OH	Ile Leu His DNS – OH	Ile Leu Asp + Glu Gly DNS – OH DNS – NH_2 Tyr Lys	Val Met His Ala Pro Thr Ser Arg	Ile Leu Glu Ser Tyr DNS – OH

Commercial Sample 70 days	A	Arg + His DNS – NH_2 Ile Leu Phe Val Tyr Glu	Glu Met Lys Met Ala Pro Asp		Arg + His DNS – NH_2 Ile Leu Phe Val Tyr Glu	Met Lys Ala Glu Pro	DNS – NH_2 Val Leu	DNS – OH Ile Leu Val Pro Lys Tyr	His Alu Glu	Ile Leu DNS – OH Glu		Leu Asp + Glu DNS – DH DNS – NH_2 Gly Tyr Lys Val	Met His Ala Pro Thr Ser Arg	Ile Leu Glu + Asp DNS – OH DNS – NH_2		
	B	Ile Leu Arg + His Ser + Thr DNS – NH_2 DNS – OH	Phe Val Tyr Glu Met Ala Pro	Asp Met	DNS – OH DNS – NH_2 Ile Leu Phe Val Pro	Glu Met Lys Ala Gly Tyr His	Val Leu Glu + Asp DNS – NH_2	Ile Leu Val Lys Tyr DNS – OH Glu + Asp	His Met	Leu Ile His DNS – OH Lys Glu		Leu Val Tyr DNS – OH Arg + His DNS – NH_2 Ser	Glu Ala	Ile Leu Glu Val DNS + OH His + Arg Ala	Tyr Lys Met Pro Ser	
	S	Pro Ile Leu Phe Tyr Arg	Met Glu		Ile Leu Pro Phe Glu His		Glu + Asp Ile Leu Val Tyr His	Tyr Pro Leu Ile DNS – OH		Leu Val DNS – OH Lys Glu		Leu Val Glu DNS – OH		Ile Leu Val Glu + Asp DNS – OH		
	D	Pro Ile Leu Val Ala Arg	Lys Glu		Ile Leu Val Lys DNS – OH DNS – NH_2	Glu	Glu + Asp Ile Leu Lys DNS – NH_2 Val	Leu Ile Lys Glu + Asp DNS – OH DNS – NH_2		Glu + Asp DNS – OH Leu Lys Tyr Met Pro	Val Ala Ile	Leu Ile Val DNS – NH_2 Pro				

Residues underlined referred to the principal N-terminal group.

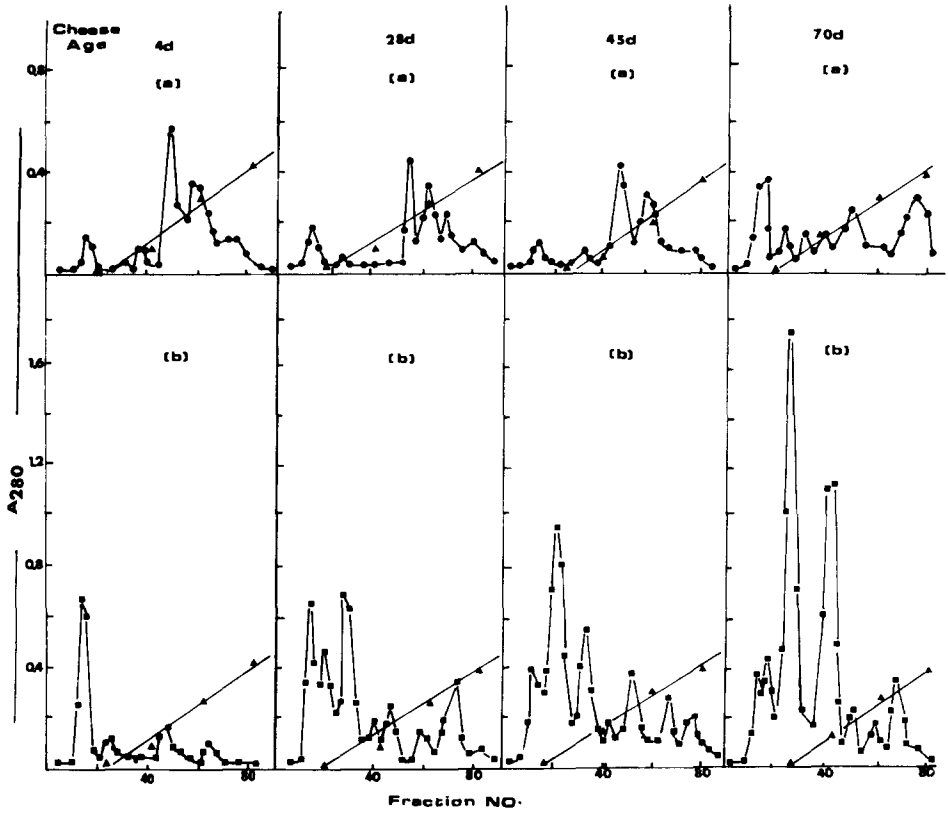


Fig. 7. Elution profiles of the retentate fractions of water-soluble nitrogen-free Stilton cheese at different stages during ripening on DEAE cellulose chromatography (80 × 2·5 cm column) eluent 0·02 Na-phosphate buffer, pH 6·5, with a linear NaCl gradient 0·0–0·5M; fraction size, 15 ml. (a) 70% ethanol-insoluble fractions: (b) 70% ethanol-soluble fractions.

other types of cheese. Although the concentration of individual amino acids showed increases ranging from 5- to 36-fold, some of the amino acids (i.e. Ser, Leu, Arg, His) decreased as a percentage of the total free amino acids during the later stages of ripening. This probably indicates that amino acids are in a dynamic state in cheese during ripening due to alteration to other constituents (acids, amines and NH_2) or as substrates for mould growth (Schormuller, 1968). Valine, lysine, leucine and glutamic acid represented 50% of total free amino acids in most samples. The high percentages of leucine, phenylalanine and valine probably indicate the preferential cleavage of peptide bonds involving hydrophobic residues by the proteolytic agents in the cheese (Pelissier *et al.*, 1974). Although the total quantity of FAA was higher in cheese B than in cheese A at all stages of ripening, particularly in the later stages, the ratio of individual amino acids in both cheeses showed only relatively small differences.

TABLE 3

Concentration of the Individual Free Amino Acids in Stilton Cheese at Different Stages of Ripening

Age (days)	*Cheese*	*Free amino acid (mg/16 g N)*																
		Lys	*His*	*Arg*	*Asp*	*Thr*	*Ser*	*Glu*	*Pro*	*Gly*	*Ala*	*Val*	*Ile*	*Leu*	*Tyr*	*Phe*	*Met*	*Total*
	A	36	7	13	14	16	11	120	52	5	22	52	32	130	56	88	16	670
								18[a]				7·8[a]		19·4[a]	8·3[a]	13·1[a]		
4	B	21	12	19	—	11	6	98	39	—	43	87	27	123	62	129	12	689
								14[a]				12·6[a]		18[a]	9[a]	18·7[a]		
20	A	45	5	5	46	16	14	175	67	9	38	88	43	246	36	131	27	991
	B	52	4	24	29	28	25	102	38	—	53	111	58	320	58	228	18	1 148
28	A	131	21	20	31	67	56	511	126	306	63	238	101	637	58	344	92	2 802
	B	109	29	35	63	50	73	390	88	62	84	379	142	706	105	461	66	2 842
45	A	296	81	72	79	88	95	381	317	423	127	392	299	772	338	432	187	4 388
	B	315	67	118	96	110	120	425	287	95	167	523	187	812	497	591	117	4 527
55	A	432	121	194	83	132	108	1 130	227	88	115	403	198	970	148	522	153	5 004
	B	402	146	261	91	139	126	813	289	71	172	601	172	1 105	457	578	107	5 530
70	A	713	189	124	195	238	209	691	475	92	294	525	428	1 139	579	592	361	6 844
								10[a]				7·6[a]		16·6[a]	8·4[a]	8·6[a]		
	B	811	262	253	221	308	312	718	594	89	360	683	348	1 237	614	729	248	7 787
								9[a]				8·8[a]		15·8[a]	7·8[a]	9·4[a]		
Commercial samples	S	1 008	190	115	216	344	255	1 739	520	267	326	1 066	704	1 198	711	979	381	9 959
	D	1 227	443	409	488	679	398	814	147	216	587	1 491	1 082	1 648	1 040	1 195	747	12 611

[a] As % of the total free amino acids.

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