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DETECTION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF POLYAMINES FORMED BY CLOSTRIDIAL PUTREFACTION OF CASEINS

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SUMMARY

Casein incorporated in building materials is degraded by species of alkali-tolerant *Clostridia*. A whole range of compounds have previously been detected in degraded building materials containing casein as an additive by gas chromatography (GC), including volatile and non-volatile organic acids, alcohols, ketones, aldehydes and monoamines. Using high-performance liquid chromatography (HPLC) it was possible, however, also to detect polyamines formed in degraded caseins. Histamine, agmatine, serotonine, tyramine, tryptamine, putrescine and cadaverine were detected in solutions containing casein in which the alkali-tolerant *Clostriadia* had been grown. Uninoculated, sterile incubated caseins contained no detectable amounts of polyamines. This gives clear evidence of the role of the biotic environment in the degradation of caseins. A combination of GC and HPLC therefore, provides a convenient set of techniques for studying the degradation products of casein.

INTRODUCTION

Polymers are subject to degradation by various agents. Both synthetic and native polymers are degraded by, *e.g.*, radiation, oxidation, heat and hydrolysis. Native polymers, *i.e.*, those polymers which are found in nature and which have been synthesized by living organisms, are susceptible also to attack by biological organisms. Some synthetic polymers can also be degraded by biological agents, especially those polymers having a carbon sequence resembling that of native polymers. Polyesters, for example, have a repetitive part, poly(β-hydroxybutyrate), that is used by microorganisms as a nutrient storage substance, and polyesters are therefore readily degraded in a biotic environment.

The use of native polymers in industrial systems can cause problems if no precautions are taken against their attack by microorganisms. Reports of discomfort and temporary health complaints during sojourns in newly built apartments, office buildings and hospitals constructed in the late 1970s in Scandinavia led to thorough investigations. Degraded malodorous self-levelling floor concrete mixtures containing a biopolymer, casein, were analysed by various gas chromatographic (GC) techniques

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and the presence of isobutylamine, triethylamine, *n*-pentylamine, diisobutylamine, di-*n*-butylamine, putrescine and β-phenylethylamine in the concrete mixtures was confirmed[1,2]. In addition, short-chain organic acids such as propionic, isobutyric and butyric acid were also found[1].

Both groups of substances represent characteristic putrefactive fermentation products of *Clostridia,* which we also isolated from various caseins and concrete mixtures during the early 1980s[1–3]. The *Clostridia* are a well known group of bacteria and numerous papers describing their characteristics and their putrefaction products have been published. The term "putrefaction" denotes their capability to degrade proteins anaerobically with the resulting production of odorous substances.

Our work has been concentrated on the detection of various monoamines present both in cultures of *Clostridia* (isolated from the degraded concrete mixtures), in degraded concrete mixtures and in indoor air samples from malodorous buildings[1–4]. We also found that the isolated *Clostridia* were alkali-tolerant[3], which explains why *Clostridia* could survive and degrade the casein in the concrete mixture despite its high pH (*ca.* 12).

We used the method developed by Dalene *et al.*[5], which permits the direct detection by GC of aliphatic monoamines such as triethylamine, 2-ethylhexylamine and pyrrolidine and of aromatic amines such as β-phenylethylamine and α-phenylethylamine. The method was adjusted to our purposes, which meant not only the analysis of a highly complex material such as concrete mixtures but also the analysis of bacterial media and degraded caseins. These monoamines, however, are only some of those produced by *Clostridia.* Others of interest include putrescine, cadaverine, spermidine, histamine, agmatine, tyramine, tryptamine and serotonine. The major drawback of the method cited above in relation to the problem described here is, however, that it does not allow the detection of these polyamines.

The aim of this work was, therefore, to develop a method that permits the detection of non-volatile polyamines in degraded caseins. Using high-performance liquid chromatography (HPLC) in parallel with GC, we developed a convenient method for studying the degradation products of caseins. Mechanisms for the degradation of the casein molecule, which is a polypeptide, are also presented.

EXPERIMENTAL

Commercial casein (Kebo) was used as a model substance. To simulate the conditions in the concrete mixtures, casein was incorporated as the sole carbon source (250 mg per litre of water) in a salt solution consisting of (per litre) KH_2PO_4 (1.0 g), K_2HPO_4 (1.0 g), $MgSO_4$ (0.5 g), NaCl (0.5 g), $FeSO_4$ (0.1 g) and $MnSO_4$ (0.1 g). To this solution were added pure cultures of *Clostridia* originally isolated from degraded concrete mixtures[1]. The microorganisms were *Clostridium bifermentans* (GA 06, NZ 06) and *Clostridium sporogenes* (VP 04, DB 01). The characteristics of the microorganisms used are presented in Table I, in which the macroscopic appearance is given and also those carbohydrates which each organism ferments. The volatile organic acid products are also listed. A more thorough description was given by Karlsson *et al.*[3].

To test-tubes, 10 ml of the sterile solutions described above were added. Two different pH levels (7.5 and 10.0) and two temperatures (20 and 30°C) were used for

TABLE I

SUMMARY OF CHARACTERISTICS OF *C. SPOROGENES* AND *C. BIFERMENTANS*

The + denotes capability to ferment the saccharides. Ind^- does not produce indole; Ure^- = does not produce urease; Cat^- = does not produce catalase.

Organism	*Colonial morphology; Gram stain*	*Biochemical tests*	*Volatile acids*
C. sporogenes (VP 04, DB 01)	Small, grey, dull, rhizoid; Gram positive	$Glucose^+$, $maltose^+$, liquefies gelatine, hydrolyses esculine, H_2S^+, Ind^-, Cat^-	Acetic, propionic, isobutyric, *n*-butyric, isovaleric
C. bifermentans (GA 06, NZ 06)	Yellow-white, dull, rhizoid; Gram positive	$Glucose^+$, $maltose^+$, $salicine^+$, $mannose^+$, liquefies gelatine, H_2S^+, hydrolyses esculine, Ind^-, Ure^-, Cat^-	Acetic, propionic, isobutyric, *n*-butyric, isovaleric, isocaproic

incubation. For each pH, strain and temperature four tubes were prepared. The incubation took place in an anaerobic atmosphere (Gas Generating Box; BioMerieux) for a period of 2–12 weeks. After 2 weeks, two 1-ml samples were withdrawn from the supernatant of one test-tube of each pH, strain and temperature. The remaining three tubes of each pH, strain and temperature were incubated for another 10 weeks. One sample was analysed by GC after extraction with diethyl ether containing 500 μg/l of ammonia as described previously[2,3]. The other sample was derivatized as described below and analysed by HPLC.

Derivatization of samples for HPLC was described by Buteau *et al.*[6]. *o*-Phthaldialdehyde (OPT) is used as a derivatizing agent. To 0.5 ml of an amine standard solution, 10 ml of OPT reagent [20 ml of borate buffer (pH 10.4 ± 0.02), 180 mg of OPT, 2 ml of methanol and 0.4 ml of mercaptoethanol] was added. The mixture was allowed to react in the dark for 5 min, then 2 g of sodium chloride were added, followed by 4 ml of ethyl acetate. The contents were mixed for 1 min and the two phases were allowed to separate. The supernatant was used for injection into the HPLC system. Depending on the concentration of amines, the size of the sample was varied from 0.5 to 1 ml. The amount of ethyl acetate was also varied, between 2 and 4 ml. The standard OPT derivative was prepared about once a week and was kept in the dark at −20°C before use. A standard mixture was run each day before the samples in the HPLC.

o-Phthaldialdehyde (OPT), mercaptoethanol and amines of analytical-reagent grade were obtained from Sigma and Merck (and ethyl acetate of analytical-reagent grade from Merck). Stock solutions of amines in water were prepared and stored at 2°C. These stock solutions had a concentration of 100 μg/ml and were further diluted as required. Three stock solutions containing mono-, di- and polyamines were prepared: solution A consisted of polyamines (histamine, agmatine, serotonine, tyramine and tryptamine) solution B of diamines (putrescine, cadaverine and spermidine) and solution C of monoamines (isobutylamine, *n*-pentylamine, 2-ethylhexylamine, α-phenylethylamine, β-phenylethylamine and *n*-octylamine). The standard mixture

for GC analysis consisted of isobutylamine, triethylamine, pyrrolidine, *n*-pentylamine, diisobutylamine, 2-ethylhexylamine, *n*-octylamine, α-phenylethylamine and β-phenylethylamine. The concentrations were 1, 5, 10 and 20 μg/ml. The standard and samples were extracted at high pH (13) with diethyl ether and the ether layer was injected into the GC column.

The HPLC instrument consisted of two Waters Model 45 pumps, a Rheodyne manual injector and a Waters Model 720 System Controller. The detector was a Waters Model 420 fluorimeter. The gain was set at 64 or 128, depending on the amount of amines present. The column (25 cm × 4.6 mm I.D.) contained Nucleosil C_{18} with a particle size of 5 μm. The water used for the mobile phase was glass-distilled and then filtered through a Schleicher & Schüll ME 25 0.45-μm membrane filter. Solutions of acetic acid (0.08 *M*) were prepared by adding the required amount of analytical-reagent grade acetic acid to water prepared as above. Methanol and acetonitrile were of HPLC grade (Merck and Rathburn Chemicals). The methanol was filtered through the membrane filter; the acetonitrile was already filtered when purchased. The solvents were degassed prior to use.

For mobile phases, two solvents (A and B) were prepared. Solvent A was methanol–0.08 M acetic acid–acetonitrile (45:40:15) and solvent B was 100% methanol. The mobile phase was kept stable (isocratic) for 15 min using solvent A and was thereafter changed from relatively polar to non-polar (A to B) during 10 min and then kept stable (isocratic) with solution B for 20 min.

The gas chromatograph was an Antek 464 LP, equipped with a nitrogen–phosphorus-specific ceramic bead (TSD), which allows the detection of underivatized amines. The column (2.5 m × 2 mm I.D.) was packed with 28% Pennwalt 223 + 4% KOH on Gas-Chrom R (80–150 mesh). The column was maintained at 140°C.

RESULTS AND DISCUSSION

Typical monoamines detected in degraded caseins are isobutylamine, triethylamine, diisobutylamine, di-*n*-butylamine and β-phenylethylamine. An example of a gas chromatogram of the detected monoamines is shown in Fig. 1. The actual monoamine pattern obtained depends on the type of microorganism responsible for the degradation.

Fig. 2–5 show examples of HPLC traces obtained from samples of degraded caseins. The mobile phase contains up to 15% acetonitrile, which results in sharper peaks and shorter retention times. The more polar amines are eluted by gradually increasing the methanol content.

Fig. 2 shows casein degraded by *C. bifermentans* at pH 7.5 and an incubation temperature of 20°C. The following polyamines were detected: histamine, agmatine, serotonine, tyramine, tryptamine and putrescine. Fig. 3 shows degradation by the same organism but at a higher pH of the incubation medium. The same amines were detected, the main differences being in the relative amounts produced At pH 10 the amounts of serotonine and tryptamine are much higher than at pH 7.5. At pH 7.5, the putrescine peak is larger. Some peaks are unidentified, but some of them no doubt come from derivatized ammonia, which is always present in media in which *Clostridia* have been grown.

In Figs. 4 and 5 the corresponding curves for casein degraded by *C. sporogenes*

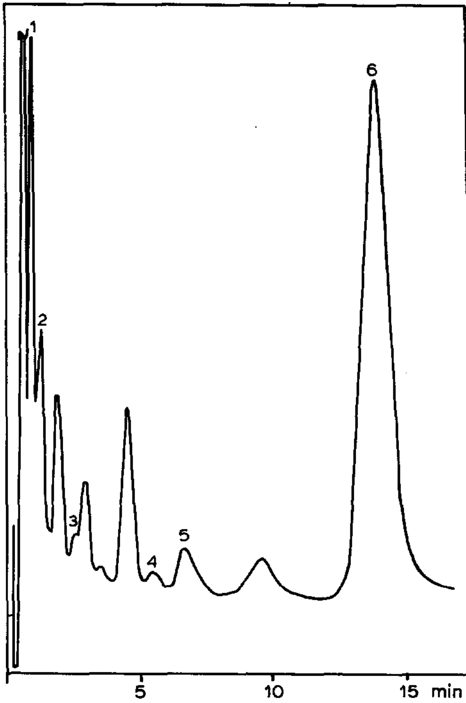

Fig. 1. Monoamines detected in gas chromatograms of casein degraded by *Clostridia*. 1, Isobutylamine; 2, triethylamine; 3, diisobutylamine; 4, 2-ethylhexylamine; 5, *n*-octylamine; 6, *β*-phenylethylamine.

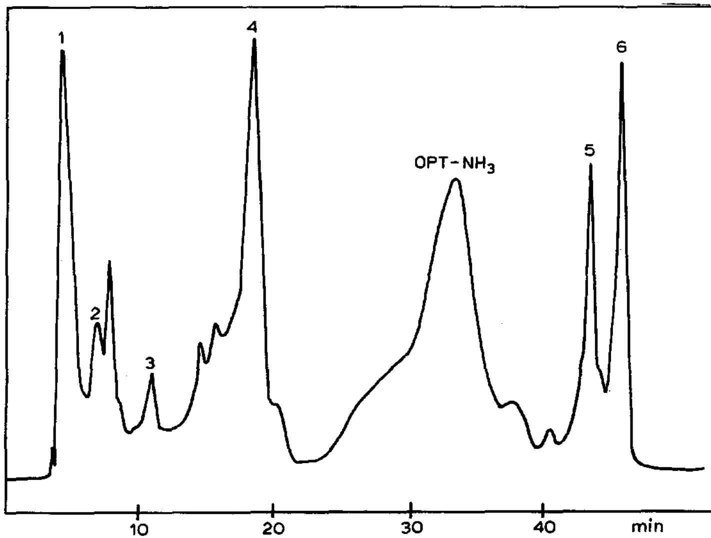

Fig. 2. HPLC trace of casein degraded by *Clostridium bifermentans* at 20°C, pH 7.5. 1, Histamine; 2, agmatine; 3, serotonine; 4, tyramine; 5, tryptamine; 6, putrescine.

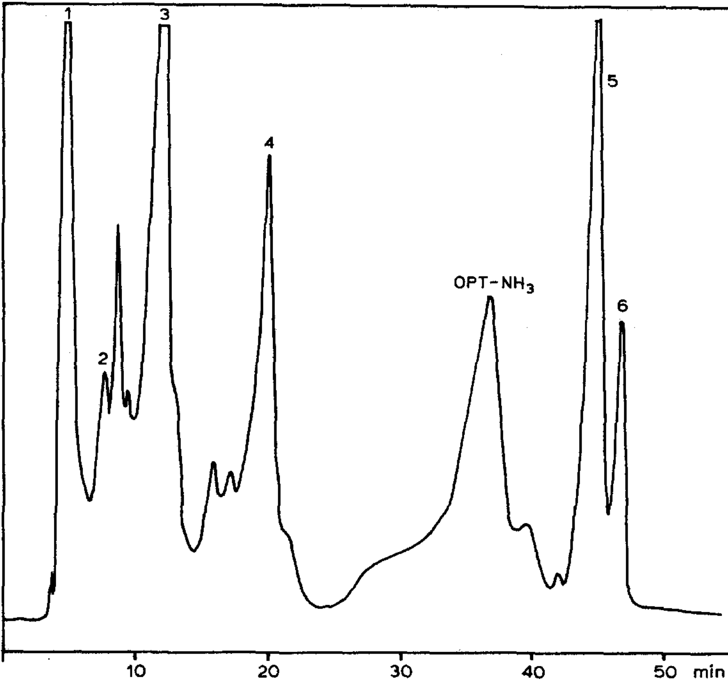

Fig. 3. HPLC trace of casein degraded by *Clostridium bifermentans* at 20°C, pH 10. 1, Histamine; 2, agmatine; 3, serotonine; 4, tyramine; 5, tryptamine; 6, putrescine.

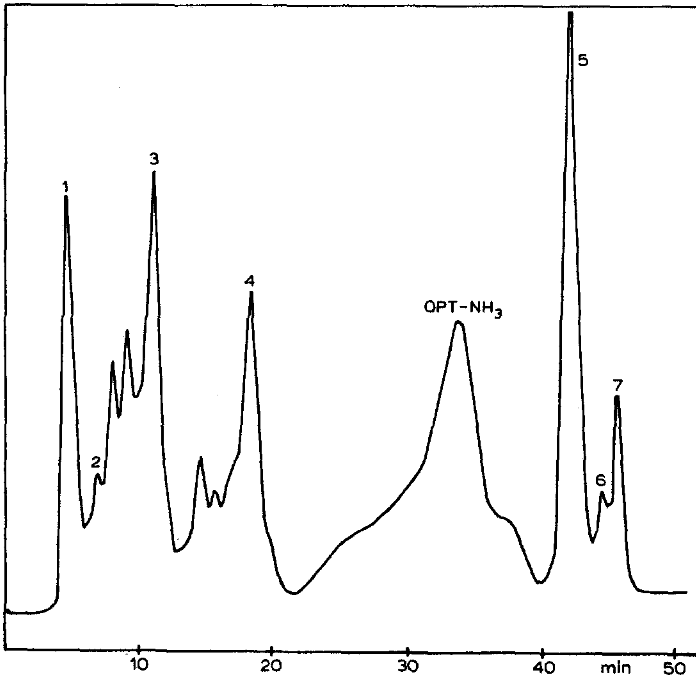

Fig. 4. HPLC trace of casein degraded by *Clostridium sporogenes* at 20°C, pH 7.5. 1, Histamine; 2, agmatine; 3, serotonine; 4, tyramine; 5, tryptamine; 6, putrescine; 7, cadaverine.

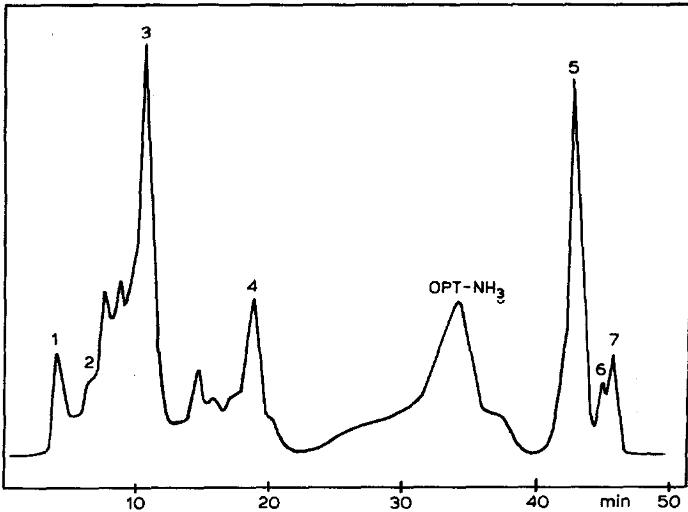

Fig. 5. HPLC trace of casein degraded by *Clostridium sporogenes* at 20°C, pH 10. 1, Histamine; 2, agmatine; 3, serotonine; 4, tyramine; 5, tryptamine; 6, putrescine; 7, cadaverine.

are presented. Here histamine, agmatine, serotonine, tyramine, tryptamine, putrescine and cadaverine are detected. The peaks are larger at pH 7.5 than at pH 10.

Table II lists the polyamines produced by the different strains at different pHs and temperatures. Uninoculated, incubated casein medium gave no detectable amounts of polyamines or monoamines. Fig. 6 shows a standard chromatogram of the amines investigated. Mono-, di- and polyamine standards are incorporated in the same figure.

Brooks and Moore[7] used GC to study the production of acids, alcohols, phenols, ketones, aldehydes, mercaptans and amines in several species of *Clostridia*. Larsson *et al.*[8] used headspace liquid chromatography to study amines and other bacterial products. Pons *et al.*[9] described a method for identifing volatile amines in *Clostridia* cultures by the use of GC–mass spectrometric (GC–MS) techniques. The combination of a flame ionization detector in GC and the electron-impact and chemical ionization modes in MS gave positive results, although much consideration must normally be given to the selection of the detectors, columns and derivatization techniques when identifying amines by GC–MS.

Dalene *et al.*[5], as mentioned above, developed a rapid and simple method for detecting underivatized monoamines at low concentrations (μg/l–ng/l) by the use of GC with a nitrogen-sensitive ceramic detector. This was also the technique we used to study degraded concrete mixtures containing casein. HPLC has, however, often been used to replace GC as it provides improved detection and separation and also increased sensitivity.

Buteau *et al.*[6] studied the presence of polyamines in wine and must by HPLC with fluorescence detection based on three derivatization techniques using *o*-phthaldialdehyde, dansyl chloride and fluorescamine. Allison *et al.*[10] used *o*-phthaldialdehyde derivatization of amines in HPLC with electrochemical detection and Michin and Hanau[11] used a modification of the above techniques to detect putrescine, ca-

TABLE II

POLYAMINES PRODUCED BY CULTURES OF *Clostridia* DURING INCUBATION IN CASEIN MEDIA FOR TWO WEEKS

+ = Amine detected; (+) = small amount amine detected; 0 = no amine detected.

Amine	*VP 04**				*DB 01**				*GA 06**				*NZ 06**			
	pH 7.5		*pH 10*		*pH 7.5*		*pH 10*		*pH 7.5*		*pH 10*		*pH 7.5*		*pH 10*	
	20°C	*30°C*	*20°C*	*30°C*	*20°C*	*30°C*	*20°C*	*30°C*	*20°C*	*30°C*	*20°C*	*30°C*	*20°C*	*30°C*	*20°C*	*30°C*
Histamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Agmatine	+	0	+	0	+	0	+	0	+	+	+	+	+	+	+	+
Serotonine	+	+	+	+	+	+	+	+	0	0	0	0	+	+	+	+
Tyramine	+	+	(+)	+	(+)	(+)	+	+	+	+	+	+	+	+	+	+
Tryptamine	+	+	+	+	+	+	+	+	+	+	0	0	+	0	0	0
Putrescine	+	+	+	+	+	+	+	+	0	+	0	+	0	0	+	0
Cadaverine	+	+	+	+	+	0	+	0	+	0	+	0	+	+	+	+

* VP 04, DB 01 = *C. sporogenes*; GA 06, NZ 06 = *C. bifermentans*.

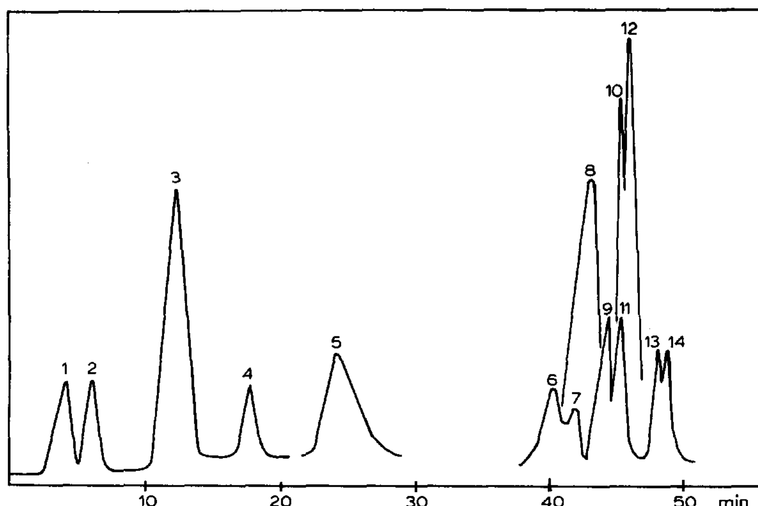

Fig. 6. Summary of monoamines, diamines and polyamines detected by HPLC, showing all three groups of amines. 1, Histamine; 2, agmatine; 3, serotonine; 4, tyramine; 5, spermidine; 6, isobutylamine; 7, *n*-pentylamine; 8, tryptamine; 9, 2-ethylhexylamine; 10, putrescine; 11, α-phenylethylamine; 12, cadaverine; 13, β-phenylethylamine; 14, *n*-octylamine.

daverine, spermidine and spermine in biological tissues. Carteni-Farina *et al.*[12] studied polyamines in halophilic Archaebacteria. Most of the HPLC papers published so far, however, have been related to studies of biological materials in medical applications[13–15] or foods[16].

GC and more recently HPLC have also come into use in polymer technology. Traditional methods used here to investigate degradation involve the detection of the changes in mechanical properties, a decrease in molecular weight or the evolution of $^{14}CO_2$. These methods are well developed and also reliable. Recently, however, papers have been published on the detection of, *e.g.*, monomers by GC, and also on the detection of degradation products[17,18]. The use of alternative techniques in the studying degradation will lead to a better understanding of degradation mechanisms and also give better lifetime predictions.

The aim of our work was to develop a method that permits the detection of a wide range of amines present in degraded caseins. The use of HPLC enables a broader range of amines to be detected; both volatile and non-volatile amines can be identified. Histamine, agmatine, serotonine, putrescine and cadaverine were among the products we were able to find in samples of degraded caseins. As a control, sterile uninoculated samples of caseins yielded no detectable amounts of amines. This provides clear evidence of the role of the biotic environment in the degradation of caseins.

Fig. 7 presents a mechanism for the degradation of polypeptides. This mechanism is general for polypeptides and therefore also applies to casein. All the steps are catalysed by different enzymes, evolved by microorganisms. Amino acids are released from the ends of the polypeptide chains, and these amino acids are then in turn easily decarboxylated to amines. These steps are also catalysed by microbial enzymes. Table III lists the amines formed during the putrefactive degradation of different amino acids. The amines listed in Table III were also among those we

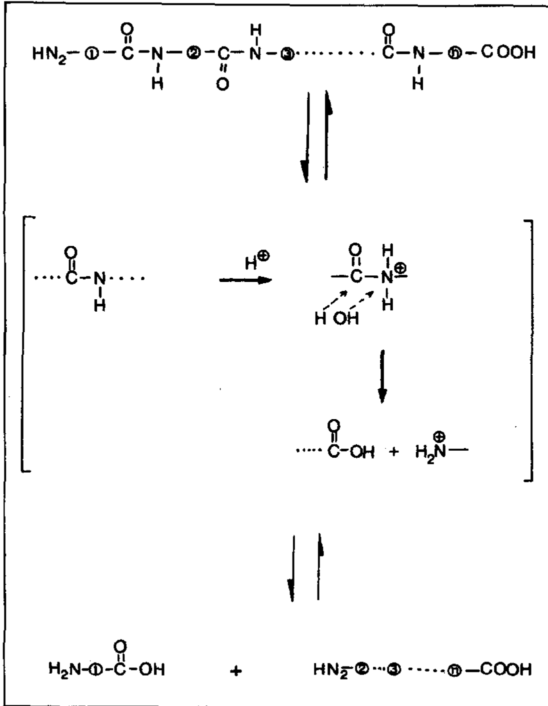

Fig. 7. Mechanism of the degradation of caseins. Casein is shown as a polypeptide where 1, 2, 3, ..., *n* denote different R groups corresponding to amino acid parts.

detected both in degraded cement mixtures containing casein and in cultures of alkali-tolerant *Clostridium bifermentans* and *Clostridium sporogenes* and in a simulated test where casein was used as sole carbon source.

The use of HPLC to detect degradation products in degraded native polymers exemplified by casein will also be adopted for the study of synthetic polymers. The method must permit the detection of monomers and also shorter fragments of the

TABLE III

CONVERSION OF SOME AMINO ACIDS TO THE CORRESPONDING AMINES BY THE ACTION OF MICROORGANISMS

Amino acid	*Amine*
Alanine	Ethylamine
Valine	Isobutylamine
Phenylalanine	Phenylethylamine
Arginine	Agmatine
Ornithine	Putrescine
Lysine	Cadaverine
Histidine	Histamine
Tryptophan	Tryptamine

polymer chain. Special demands must therefore be fulfilled. Initial tests on degraded polyethylenes showed the presence of a wide range of degradation products in media where polyethylene was the sole carbon source.

CONCLUSION

The use of HPLC made it possible to study diamines and polyamines present in degraded caseins. HPLC proved to be very useful, owing both to its sensitivity and to its potential to detect a wide range of different amines.

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REFERENCES

1 A.-C. Albertsson and Z. Banhidi, *Kemiska och Bakteriologiska Undersökningar i Samband med Golvproblem och Flytspackel*, Swedish Council for Building Research, Stockholm, Sweden, 1983.
2 S. Karlsson, E. Banhidi, Z. Banhidi and A.-C. Albertsson, in B. Berglund, T. Lindvall and J. Sundell (Editors), *Proceedings of the 3rd International Conference on Indoor Air Quality and Climate*, Vol. 3, Swedish Council for Building Research, Stockholm, Sweden, 1984, p. 287.
3 S. Karlsson, Z. Banhidi and A.-C. Albertsson, *Appl. Microbiol. Biotechnol.*, 27 (1988) in press.
4 S. Karlsson, Z. Banhidi and A.-C. Albertsson, *RILEM Materials and Structures*, in press.
5 M. Dalene, L. Mathiasson and J.-Å. Jönsson, *J. Chromatogr.*, 207 (1981) 37.
6 C. Buteau, C. L. Duitschaever and G. C. Ashton, *J. Chromatogr.*, 284 (1984) 201.
7 J. B. Brooks and W. E. C. Moore, *Can. J. Microbiol.*, 15 (1969) 1433.
8 L. Larsson, P.-Å. Mårdh and G. Odham, *Acta Pathol. Microbiol. Scand., Sect. B*, 86 (1978) 207.
9 J.-L. Pons, A. Rimbault, J. C. Darbord and G. Leluan, *J. Chromatogr.*, 337 (1985) 213.
10 L. A. Allison, G. S. Mayer and R. E. Shoup, *Anal. Chem.*, 56 (1984) 201.
11 R. F. Michin and G. R. Hanau, *J. Liq. Chromatogr.*, 7 (1984) 2605.
12 M. Carteni-Farina, M. Porcelli, G. Cacciapuoti, M. De Rosa, A. Gambacorta, W. D. Grant and H. N. M. Ross, *FEMS Microbiol. Lett.*, 28 (1985) 323.
13 H. G. Hadfield, P. Crane, M. E. King, E. A. Nugent, C. Milio and N. Narasimhachari, *J. Liq. Chromatogr.*, 8 (1985) 2689.
14 R. J. Martin, B. A. Bailey and R. G. H. Downer, *J. Chromatogr.*, 278 (1983) 265.
15 K. Oka, K. Kojima, A. Togari, T. Nagatsu and B. Kiss, *J. Chromatogr.*, 308 (1984) 43.
16 H. M. L. J. Joosten and C. Olieman, *J. Chromatogr.*, 356 (1986) 311.
17 A. Hagman and S. Jacobsson, *J. Chromatogr.*, 395 (1987) 271.
18 D. Munteanu, A. Isfan and D. Bratu, *Chromatographia*, 23 (1987) 412.