Volume 245, number 1,2, 150–154 FEB 06910 March 1989

Apoptotic hepatocytes become insoluble in detergents and chaotropic agents as a result of transglutaminase action

Laszlo Fesus, Vilmos Thomazy*, Francesco Autuori+, Maria P. Ceru+, Edit Tarcsa and Mauro Piacentini+

*Departments of Biochemistry and *Pathology, University School of Medicine, Debrecen H-4012, Hungary* and *+Department of Biology, 2nd University of Rome ('Tor Vergata'), 00173 Rome, Italy*

Received 19 January 1989

Physiological deletion of cells ensues programmed death which involves formation of apoptotic bodies with fragmented DNA. Here we report that apoptotic hepatocytes are insoluble in detergents, urea, guanidine hydrochloride, reducing agents and thereby can be isolated from rat liver following collagenase treatment. They are wrinkled, spherical structures similar to cornified envelopes of epidermis by phase-contrast microscopy and show irregular, globular morphology by scanning-electron microscopy. Part of their DNA content is cleaved into nucleosomal and oligonucleosomal fragments. Their insolubility, like that of the cornified envelope, is evoked by ε-(γ-glutamyl)lysine and N^1,N^8-bis(γ-glutamyl)spermidine protein cross-linking bonds formed by transglutaminase.

Apoptosis; Transglutaminase; Protein crosslinkage; Terminal differentiation

1. INTRODUCTION

Apoptosis is a basic cellular phenomenon whereby cells are deleted from living tissues in a physiological manner [1]. It occurs as programmed death of cells during embryogenic development, normal tissue turnover, hormone-induced atrophy, involution of hyperplasia, removal of preneoplastic cells and regression of tumors [1–3]. Cardinal elements of the process involve rapid volume reduction, chromatin condensation, change of the cell surface (which permits recognition by phagocytic cells) and dependence on active protein synthesis [1,4,5]. The program is associated with increased intracellular Ca^{2+} concentration [6] and activation of a Ca^{2+}-dependent endonuclease which cleaves chromatin at internucleosomal sites [5,7]. We have recently reported that coincidence of the induction as well as activation of tissue transglutaminase (EC 2.3.2.13; Ca^{2+}-dependent protein-glutamine γ-glutamyltransferase) and a high number of apoptotic hepatocytes was observed during the involution of rat liver hyperplasia and in glucocorticoid-treated rat thymocytes [8,9]. In the present study structures which are insoluble in urea, chaotropic and reducing agents as a result of transglutaminase-catalyzed cross-linkage of proteins were isolated from rat liver and identified as apoptotic hepatocytes.

Correspondence address: L. Fesus, Department of Biochemistry, University School of Medicine, Debrecen H-4012, Hungary

2. EXPERIMENTAL

To obtain isolated hepatocytes livers of male Wistar rats (200–250 g) were perfused with collagenase [10] after removal of Ca^{2+} by an in situ preperfusion with a chelator. As an alternative procedure the liver was perfused in situ with an isotonic buffer containing Ca^{2+}-chelator as described [10], then it was removed, cut into small pieces and the hepatocytes were dissociated by rubbing through a steel mesh at 4°C. After both procedures the dispersed cells were filtered through cotton gauze to remove remaining connective tissue and clumps of cells. 2–3 × 10^8 cells, corresponding to about 3 g of liver, were sedimented by centrifugation (600 × *g*, 10 min), then suspended at 0°C in 25 ml lysis buffer (10 mM KCl, 2 mM $MgCl_2$, 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.5) containing 0.2 mM phenylmethylsulfonyl fluoride to inhibit proteases and 0.4 mM

Published by Elsevier Science Publishers B.V. (Biomedical Division)

iodoacetamide to inhibit transglutaminase. After centrifugation (600 × g, 5 min), the pellet was washed in cold lysis buffer three times, then suspended in 6 M guanidine hydrochloride to dissolve nuclei. A subsequent centrifugation (600 × g, 10 min) resulted in a pellet consisting of structures which we consider to be apoptotic hepatocytes.

For the measurement of ε-(γ-glutamyl)lysine content the isolated structures were subjected to exhaustive proteolytic digestion [11], the protein-free fraction of the digest was passed through an AG-50W-X8 column to remove lysine, then the sample was desalted on a silica HPLC column using water as eluent. The fraction collected at the position of ε-(γ-glutamyl)lysine was derivatized by phenylisothiocyanate [12], then analyzed by HPLC (ISCO) on a 3.9 mm × 30 cm μBondapak C_{18} column using the following elution system: eluent A was 0.14 M sodium acetate with 0.5 ml/l triethanolamine (pH 6.35), eluent B was acetonitrile; isocratic separation at 10% B was carried out for 10 min followed by a linear gradient up to 25% within 2 min and a subsequent isocratic separation for 8 min. As an alternative method prepurification of the isodipeptide on an ion-exchange chromatography system was followed by a precolumn derivatization with *o*-phthalaldehyde and reversed-phase separation [13]. In both cases the isodipeptide peak as well as the lysine peak, obtained after acid hydrolysis in a parallel sample, were used for the calculations and radiolabelled ε-(γ-glutamyl)lysine was present throughout the procedure to follow recovery. The amount of N^1,N^8-bis(γ-glutamyl)spermidine in the digest was determined by the method of Beninati et al. [13]. Amino acid and lysine content was assayed following fluorescamine derivatization [14].

DNA content of the isolated structures was assayed [15] following digestion by proteinase K [16]. For DNA electrophoresis [16] the digested samples were subjected to centrifugation (13000 × g, 10 min). The pellets and supernatants were precipitated separately with 50% isopropanol and 0.5 M NaCl overnight at −20°C. The precipitates were centrifuged, then air dried at room temperature and redissolved in 10 mM EDTA, pH 7.4, at 37°C overnight before elctrophoresis [16] in 1.5% agarose gels.

3. RESULTS AND DISCUSSION

Hepatocytes isolated from liver following collagenase perfusion were essentially free of nonparenchymal cells [10]. When they were disrupted by non-ionic detergent and, after low speed centrifugation, the resulting fraction (mostly nuclei) was suspended in 6 M guanidine hydrochloride, a subfraction could still be sedimented. It consisted of spherical or wrinkled structures as viewed by phase-contrast microscopy (fig.1a) very similar to cornified envelopes (fig.1b), which are formed by keratinocyte transglutaminase in epidermis [17]. 80–90% of the structures mimicked the keratinocyte envelopes containing diffusely distributed DNA (fig.1c,d). The structures from liver appeared as irregular, globular entities of relatively smooth surface as viewed by scanning-electron microscopy (fig.1e,f). Extensive proteolytic digestion [11] resulted in their dissolution. Studying their size distribution 70–75% was 12–13 μm in diameter, 20–25% was 15–16 μm and the size of 5–10% scattered in the range between 8 and 30 μm outside of the two main peaks as established by flow cytometry. The number of the isolated structures was 48 ± 11 per 10^5 hepatocytes (n:12) and 3.8–5.2 × 10^4 could be isolated from 1 g of liver. Somewhat higher numbers (15–25% increase) were obtained when hepatocytes were isolated by mechanical dispersion rather than by collagenase treatment; 20–25% of the structures prepared this way were large ones showing sizes between 20 and 35 μm in diameter (fig.1g).

Several pieces of evidence suggest that the structures which we isolated are apoptotic bodies formed during regular cell turnover in liver. Their incidence in liver (40–60 per 10^5 hepatocytes) closely correlates with the number of apoptotic bodies (60–70 per 10^5 hepatocytes) scored by histological techniques [2,18]. As expected on the basis of morphologic observations [3,9] significantly higher number of bodies was isolated from livers undergoing involution than from normal ones (table 1). When DNA, obtained from the structures following digestion by proteinase K, was analyzed by electrophoresis the typical sign of the effect of a Ca^{2+}-dependent endonuclease [6,7] became apparent: part of the DNA was converted to a 'ladder' of fragments of integer multiples of 180 base pairs (fig.2a, lane 4). This is the only specific biochemical feature disclosed so far which is characteristic for apoptosis [6,7].

Beside nonionic detergents and guanidine hydrochloride, the apoptotic bodies were resistant to 8 M urea with or without 5% 2-mercaptoethanol and boiling in 2% SDS suggesting that covalent bonds are involved in their maintenance. In two biological systems covalent bonds formed by transglutaminase among polypeptide chains render structures insoluble in detergents and chaotropic agents. Fibrin is cross-linked by a plasma transglutaminase, i.e. blood coagulation factor XIII in clots [19,20]. The cornified envelopes are formed by keratinocyte transglutaminase in the outermost epidermal layers marking the final stage of keratinocyte terminal differentiation

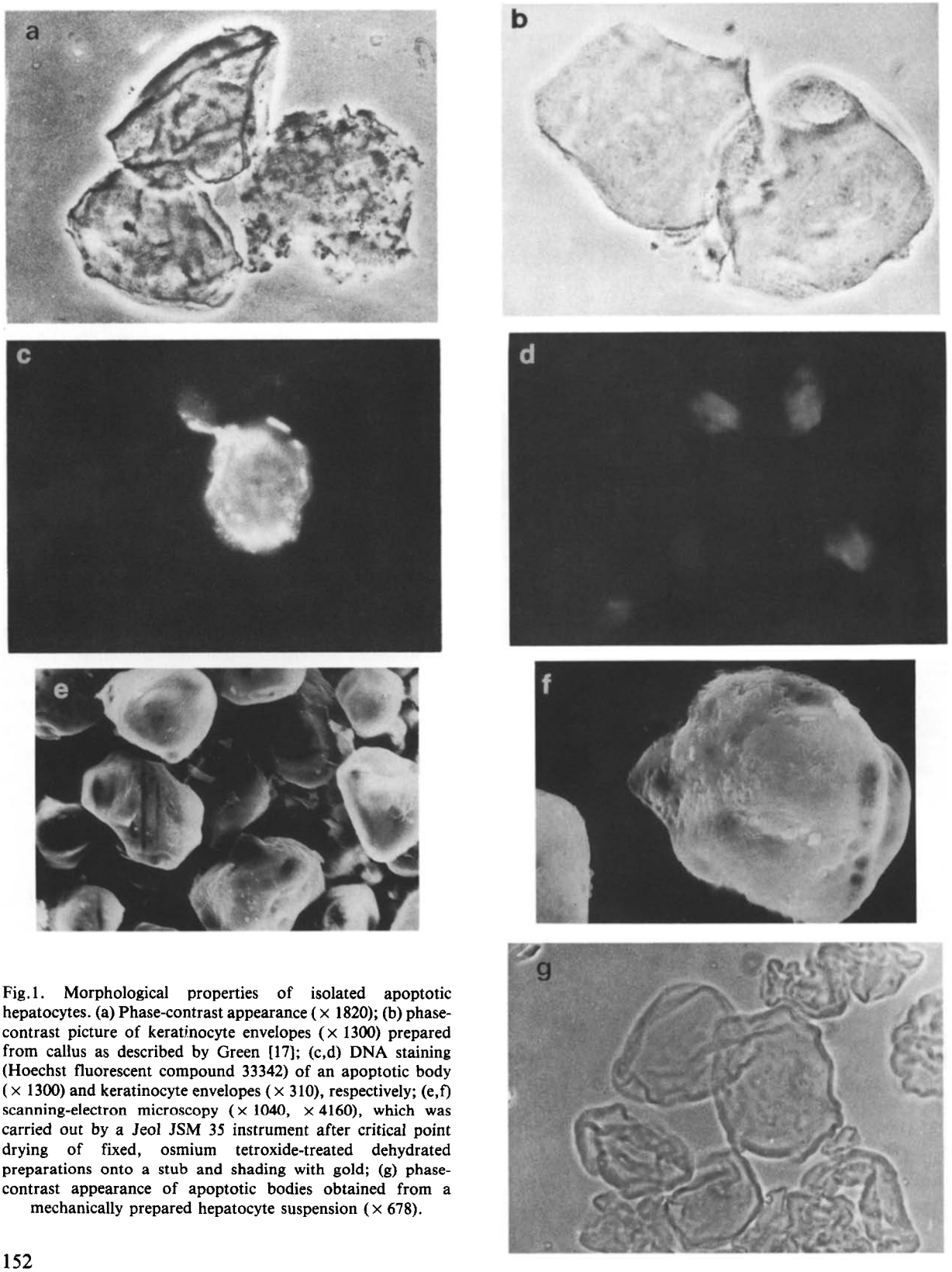


Fig.1. Morphological properties of isolated apoptotic hepatocytes. (a) Phase-contrast appearance (× 1820); (b) phase-contrast picture of keratinocyte envelopes (× 1300) prepared from callus as described by Green [17]; (c,d) DNA staining (Hoechst fluorescent compound 33342) of an apoptotic body (× 1300) and keratinocyte envelopes (× 310), respectively; (e,f) scanning-electron microscopy (× 1040, × 4160), which was carried out by a Jeol JSM 35 instrument after critical point drying of fixed, osmium tetroxide-treated dehydrated preparations onto a stub and shading with gold; (g) phase-contrast appearance of apoptotic bodies obtained from a mechanically prepared hepatocyte suspension (× 678).

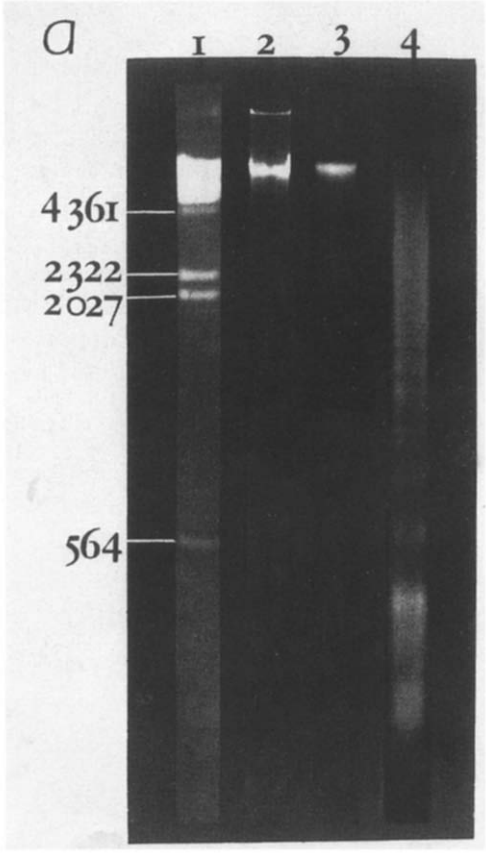
Table 1

Quantitative analysis of isolated apoptotic hepatocytes

Size	12–16 μm
Number[a] per 1 g liver ($\times 10^4$)	4.8 ± 0.7
Number[a] per 1 g involuting liver ($\times 10^4$)	38.5 ± 8.0
nmol ϵ-(γ-glutamyl)lysine	
per 10^5 apoptotic bodies	7.5 ± 1.0
per μmol amino acids	5.0 ± 1.0
nmol N^1,N^8-bis(γ-glutamyl)spermidine	
per 10^5 apoptotic bodies	1.0 ± 0.3
per μmol amino acids	0.6 ± 0.2
μg DNA in 10^5 apoptotic bodies	0.5 ± 0.3

[a] Number of apoptotic bodies isolated from normal and involuting (5th day following i.v. injection of liver mitogen lead nitrate; [3,9]) liver was counted

[17,21]. Transglutaminases form ϵ-(γ-glutamyl)-lysine bonds between proteins [22,23]. This bond is resistant to proteases [23]. When the proteolytic digest of the isolated bodies was analysed by high-pressure liquid chromatography the presence of ϵ-(γ-glutamyl)lysine was detected (fig.2b). The isodipeptide was identified by its comigration with synthetic ϵ-(γ-glutamyl)lysine in two reverse-phase chromatography systems, namely separations after precolumn derivatization by phenylisothiocyanate as well as by *o*-phthalaldehyde, and by releasing lysine with a specific enzyme [24], γ-glutamyl-amine cyclotransferase (fig.2b: lower profile). Quantitative determination of ϵ-(γ-glutamyl)lysine resulted in values (table 1) similar to those found in cross-linked fibrin and cornified envelope [23]. 6–9% of the total lysine residues of apoptotic bodies was found in ϵ-(γ-glutamyl)lysine bond. The N^1,N^8-bis(γ-glutamyl)spermidine bond was also found in the apoptotic bodies (table 1). It has



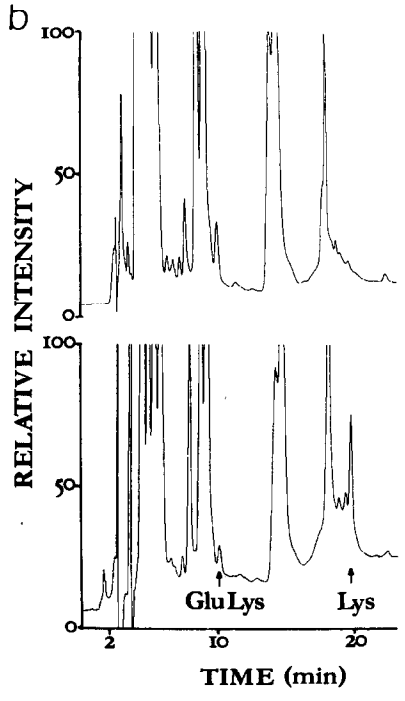


Fig.2. Characteristic biochemical features of isolated apoptotic bodies. (a) Demonstration of the presence of fragmented DNA in the bodies by DNA electrophoresis. Lanes: (1) DNA *Hin*dIII digest, numbers show sizes of fragments in base pairs; (2) DNA, corresponding to 10^5 nuclei, from the fraction obtained after lysis of hepatocytes; (3) pellet and (4) supernatant fractions of DNA from apoptotic bodies. DNA was visualized by staining with ethidium bromide. (b) Detection of ϵ-(γ-glutamyl)lysine isodipeptide (GluLys) in apoptotic hepatocytes by HPLC analysis. Protein-free digest of 10^4 apoptotic bodies were incubated with (lower profile) or without (upper profile) γ-glutamylamine cyclotransferase before derivatization and HPLC separation. Lys shows the elution position of lysine. Detection is at 254 nm with sensitivity range 0.05.

been demonstrated that di- and polyamines are natural substrates for transglutaminases and they participate in the formation of highly cross-linked protein polymers [22,25,26]. A high level of N^1,N^8-bis(γ-glutamyl)spermidine has recently been detected in the insoluble cornified envelope fraction isolated from cultured mouse keratinocytes [26] and from human callus [27].

The major form of transglutaminase in liver is the ubiquitous tissue transglutaminase [22]. Induction, i.e. increased level of tissue transglutaminase mRNA as well as protein, and activation, i.e. elevated concentration of ϵ-(γ-glutamyl)lysine in liver proteins, of the enzyme have been demonstrated during the involution of liver hyperplasia [9]. The results presented here suggest that tissue transglutaminase forms a cross-linked protein shell in apoptotic cells of normal rat liver rendering them resistant to detergents, reducing agents and chaotropic solvents. One may speculate that the cross-linked protein scaffold is needed to keep harmful intracellular enzymes and DNA tightly packed until phagocytosis and final degradation of apoptotic cells occur.

Acknowledgements: This work was partially supported by a grant from the Hungarian Academy of Sciences (project OTKA 537/1988) and by the Italian CNR (Bilateral Projects, no.87.0007304).

REFERENCES

[1] Wyllie, A.H., Kerr, J.F.R. and Currie, A.R. (1980) Int. Rev. Cytol. 68, 251–306.
[2] Bursch, W., Laner, B., Timmerman-Trosiener, I., Barthel, G., Schuppler, G. and Schulte-Herman, R. (1984) Carcinogenesis 5, 453–458.
[3] Columbano, A., Ledda-Columbano, G.M., Coni, P.P., Faa, G., Liquori, C., Santa Cruz, G. and Pani, P. (1985) Lab. Invest. 52, 670.
[4] Morris, R.G., Hargreaves, A.D., Duvall, E. and Wyllie, A.H. (1984) Am. J. Pathol. 115, 426–436.
[5] Wyllie, H., Morris, R.G., Smith, A.L. and Dunlop, D. (1984) J. Pathol. 142, 67–77.
[6] Cohen, J.J. and Duke, R.C. (1984) J. Immunol. 132, 38–42.
[7] Wyllie, A.H. (1980) Nature 284, 555–557.
[8] Fesus, L. and Thomazy, V. (1988) Adv. Exp. Med. Biol. 231, 119–134.
[9] Fesus, L., Thomazy, V. and Falus, A. (1987) FEBS Lett. 224, 104–108.
[10] Moldeus, P., Hodberg, L. and Orrenius, S. (1978) Methods Enzymol. 52, 60–65.
[11] Fesus, L., Szucs, E.F., Barrett, K.E., Metcalfe, D.D. and Folk, J.E. (1985) J. Biol. Chem. 260, 13770–13778.
[12] Bidlingmeyer, B.A., Cohen, S.A. and Tarvin, T.L. (1984) J. Chromatogr. 336, 93–104.
[13] Beninati, S., Martinet, N. and Folk, J. (1988) J. Chromatogr. 443, 329–335.
[14] Tiller, J.M. and Bloxam, D.L. (1983) Anal. Biochem. 131, 426–429.
[15] Burton, K. (1956) Biochem. J. 62, 315–323.
[16] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[17] Green, H. (1980) Harvey Lect. 74, 101–139.
[18] Bursch, W., Taper, H.S., Lauer, B. and Schulte-Hermann, R. (1985) Virchows Arch. (Cell. Pathol.) 50, 153–166.
[19] Lorand, L., Losowsky, M.S. and Miloszewski, K.J.M. (1980) Progr. Haemost. Thrombos. 5, 245–290.
[20] Tanaka, Y. and Aoki, N.J. (1982) J. Clin. Invest. 69, 536–542.
[21] Rice, R.H. and Green, H.J. (1977) Cell 11, 417–422.
[22] Folk, J.E. (1980) Annu. Rev. Biochem. 49, 517–531.
[23] Loewy, A.G. (1984) Methods Enzymol. 107, 241–257.
[24] Fink, M.L., Chung, S.I. and Folk, J.E. (1980) Proc. Natl. Acad. Sci. USA 77, 4564–4568.
[25] Piacentini, M. and Beninati, S. (1988) Biochem. J. 249, 813–817.
[26] Piacentini, M., Martinet, N., Beninati, S. and Folk, J.E. (1988) J. Biol. Chem. 263, 3790–3794.
[27] Beninati, S. and Folk, J.E. (1988) in: Polyamines in Biochemical and Clinical Research (Zappia, V. ed.) Plenum, New York, in press.