Volume 220, number 1, 236-242 FEB 05010 August 1987

Protein-bound polyamines in the plasma of mice grafted with the Lewis lung carcinoma

J.G. Delcros, A.M. Roch, V. Thomas, S. El Alaoui, J.P. Moulinoux* and G. Quash

Unité de Virologie fondamentale et appliquée, INSERM U51, UA.613 CNRS, 1 Place du Professeur Joseph Renaut, 69371 Lyon Cédex 08 and **Centre hospitalier Universitaire de Rennes, Groupe de recherche en Thérapeutique Anticancéreuse, 2, avenue du Professeur Léon Bernard, 35043 Rennes Cédex, France*

Received 21 May 1987; revised version received 26 June 1987

Protein-bound polyamines were isolated from the plasma of mice using antipolyamine antibodies covalently linked to magnetic latex spheres. Their subsequent separation by polyacrylamide gel electrophoresis (PAGE) showed that in plasma from normal mice, 3 proteins (27, 55 and 82 kDa) carrying polyamines could be visualized, whereas in mice bearing the Lewis lung carcinoma at least 8 other proteins of higher molecular mass (5 of 94, 110, 130, 145 and 160 kDa, and 3 of > 170 kDa) had bound polyamines. These protein-bound polyamines could be detected from the first week after tumour graft; they increased during the second and third week but decreased thereafter. These proteins were not bound by immunolatex spheres preincubated with spermine bound to a protein-carrier insulin. Moreover, the appearance of these protein-bound polyamines was not a consequence of the inflammatory process since in mice infected with heat-inactivated *Brucella abortus*, with the exception of a 65 kDa protein, polyamines were bound to the same proteins found in normal mice. In mice grafted with the Lewis lung carcinoma the concomitant decrease in transglutaminase-mediated polyamine (e.g. putrescine) binding capacity of plasma proteins provides additional evidence for the presence in vivo of polyamines already bound to plasma proteins.

Protein-bound polyamine; Cancer; Transglutaminase

1. INTRODUCTION

Since the first report in 1967 of antibodies to polyamines prepared by injecting laboratory animals with the hapten spermine chemically coupled to lysozyme or bovine serum albumin [1], the production of antispermine antibodies has been reported by many others [2,3]. These antisera formed precipitates with certain human sera and this led to the identification of the antigen responsible for the precipitation as an α_2-lipoprotein with bound spermine. This lipoprotein-spermine complex was found only in the sera of patients with cancer or chronic infection [4]. Confirmation of these results was provided by the finding that naturally occurring antibodies to polyamines exist in human sera [5] and rabbit sera [6,7].

To determine the level of bound polyamines in sera, Quash et al. [8] developed methods for linking antipolyamine antibodies linked via their carbohydrate residues to substituted latex spheres. This new approach permitted us to present evidence for bound polyamines in human plasma and furthermore demonstrated that their amount was greater in patients with gastrointestinal cancers than that in patients with non-malignant gastrointestinal disorders [9].

Now it is well established that polyamines can be bound enzymically to proteins by the action of tissue transglutaminases and factor XIII [10,11].

Correspondence address: J.G. Delcros, Unité de Virologie, INSERM U 51, 1 place J. Renaut, 69371 Lyon Cédex 08, France

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

When the factor XIII-mediated incorporation of putrescine into plasma proteins derived from 44 patients with bronchopulmonary cancer was compared to that from 18 patients with sarcoidosis or tuberculosis 2 types of kinetic data were obtained. Whereas the plasmas from all the patients of the control group showed linear kinetics, those from only 24 cancer patients showed linear kinetics, the others showing kinetics typical of substrate inhibition. PAGE analysis of [^{14}C]putrescine-bound plasma proteins showed around 15 different bands. Among them we identified, with the help of the corresponding specific antisera, 4 protease inhibitors: α_1-antitrypsin, α_2-macroglobulin, α_1-antichymotrypsin and antithrombin III [12].

This finding is not restricted to plasma since other authors have also identified numerous cellular proteins able to bind polyamines under the action of transglutaminase (review [13]). However, experimental data which would provide direct evidence for plasma proteins already containing bound polyamines in vivo are not available.

We therefore undertook a study of plasma from mice bearing the Lewis lung carcinoma (3LL), in order to determine whether protein-bound polyamines can be found in plasma after grafting tumour cells to the animal, if they are specific for tumour growth or inflammation and whether they are formed early or later after tumour graft.

The results of this investigation will be reported along with evidence showing that the increase in protein-bound polyamines is accompanied by a concomitant decrease in the putrescine-binding capacity of plasma proteins in mice grafted with the Lewis carcinoma.

2. MATERIALS AND METHODS

2.1. *Mice*

26 female C57B16 × DBA_2 F_1 hybrid mice were used for the experiment: 20 were grafted with the Lewis lung carcinoma, 3 non-grafted mice were injected with heat-inactivated *Brucella abortus* and 3 untreated mice served as controls [14]. Plasma samples were collected using sodium citrate (0.129 M), dialyzed with 2 changes against 10 mM Tris (pH 7.5), 0.14 M NaCl (in order to eliminate free polyamines), distributed into aliquots of 50 μl and stored at −70°C.

2.2. *Preparation of antipolyamine antibodies covalently bound to magnetic latex*

Aminopolystyrene magnetic latex spheres of 0.5–1.5 μm diameter were obtained from Advanced Magnetics (USA). Antipolyamine antibodies were partially purified by ammonium sulphate precipitation from a human serum which was chosen on the basis of its response in an automated nephelometric assay to latex-spermidine and latex-spermine [15]. This reaction can be specifically inhibited by 35, 43 or 48% with free putrescine, spermidine or spermine, respectively. This human serum behaves as a heterogeneous population of antibodies with different specificities for tetra-, tri- and diamines as demonstrated in [16] for an experimental antipolyamine antiserum.

Coupling of human oxidized antipolyamine immunoglobulins to hydrazine-substituted magnetic latex particles was accomplished as described [8]. Determination of the protein content [17] before and after coupling permitted us to calculate that there were 121 μg immunoglobulins linked per mg latex.

The reactivity of antipolyamine-bound latex was checked by a visual agglutination test on glass slides with spermine linked to poly(L-glutamic acid) (PLG) as described in [8].

2.3. *Immunopurification of protein-bound polyamines*

(i) 3 mg latex-antipolyamine were incubated with 10 μl mouse plasma diluted in 300 μl GBL buffer [0.1 M glycine, 50 mM borate, pH 8.5, 0.14 M NaCl, 0.1% Synperonic PE/L62 (ICIS.A., Clamart, France)]. After gentle shaking for 2 h at 37°C, the latex-antipolyamine was washed 7 times in GBL buffer. The absence of protein in washings was verified using the Coomassie blue technique [18].

(ii) To control specificity, latex-antipolyamine was preincubated for 1 h at 37°C with 0.7 mM spermine as insulin-spermine prepared as described for PLG-spermine [19], then added to other aliquots of the same plasma examined in (i) above. After washings, the magnetic latex spheres were heated in a boiling water bath for 5 min with 50 mM Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol (βME) to release any proteins with bound polyamines. After magnetization,

supernatants were put onto slots in the slab gel, prepared according to conditions described in [20]. Gels were stained with silver nitrate [21].

2.4. *Putrescine-binding capacity of plasma proteins by action of exogenous transglutaminase*

Transglutaminase was purified from guinea pig liver [22]. 1 unit corresponds to the amount of enzyme capable of inserting 2.1 nmol putrescine into 114 μg methylcasein in the presence of 67 μM putrescine over 1 h at 37°C.

To experimental tubes containing 30 μl dialysed plasma diluted to ¼ in Tris buffer (10 mM Tris-HCl, pH 7.5, 0.14 M NaCl) were added [^{14}C]-putrescine (125 nCi) in amounts ranging from 90 to 540 μM. 20 μl transglutaminase (0.2 U) was added to the mixture. The reaction was initiated by the addition of calcium (8 mM) in the presence of DTT (8 mM). The volume was adjusted to 95 μl with the Tris buffer. The control contained all reagents except plasma proteins which were replaced by buffer. After incubation for 2 h at 37°C with gentle shaking, 30 μl incubation mixture were spotted onto a Whatmann 3MM filter paper disk presoaked with 100 μl of 100 mM EDTA and treated as in [12]. Protein determination on plasma was according to Lowry et al. [17].

3. RESULTS

3.1. *Control of the immunolatex*

3.1.1. PAGE analysis of proteins dissociated from immunolatex not incubated with plasma

Densitometer tracings of the protein bands separated after treatment of immunolatex by βME show 3 proteins of 70, 53 and 22 kDa (fig.2A). These protein bands correspond to heavy chains H (μ and γ) and light chains L of immunoglobulins immobilized on latex.

3.1.2. Reactivity

As shown in fig.1, 500 μg immunolatex were agglutinated in the presence of 230 nmol spermine bound to PLG (PLG-spermine) but no aggregation was observed in the presence of PLG only.

3.2. *PAGE analysis of proteins dissociated from immunolatex incubated with normal mouse plasma*

As shown in fig.2B, antipolyamine antibody-bound latex spheres isolated 3 proteins from normal mouse plasma. In addition to the 3 protein bands corresponding to the μ, γ and light chains of covalently bound antipolyamine antibodies, there exist 2 proteins of 82 and 27 kDa which are well separated and a third which comigrates with the Hγ chains at approx. 55 kDa.

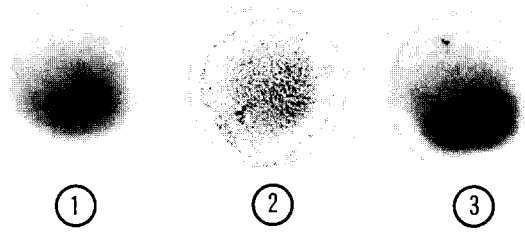

Fig.1. Agglutination on a glass slide of latex antipolyamine suspended at 3.3 mg latex/ml in 50 mM Tris-citrate, pH 8.1, 0.14 M NaCl, 0.1% Tween 20 with buffer alone (1), with spermine (0.2 μmol) bound to PLG (300 μg) (2) or with non-substituted PLG (3).

3.3. *PAGE analysis of proteins dissociated from immunolatex incubated with plasma from tumour-bearing mice*

In mice bearing the Lewis tumour, at the third week after graft, in addition to the three proteins isolated from normal mouse plasma, at least 8 other proteins of higher molecular mass (5 of 94, 110, 130, 145 and 160 kDa, and 3 of >170 kDa) had also bound polyamines (fig.2D).

Before continuing, the specificity of antipolyamine latex spheres and the contribution of the inflammatory process to the protein profile on PAGE had to be established.

3.3.1. Specificity

Specificity was verified by preincubating antipolyamine latex spheres with 0.7 mM spermine as insulin-spermine before adding plasma. As shown in fig.2D, preincubation of latex with insulin-spermine inhibits the binding of all these proteins to the antipolyamine-latex, including those found in normal mice plasma, thereby indicating that spermine was one of the haptens recognized by bound antibodies.

3.3.2. Inflammation

The 3LL tumour is known to induce an inflam-

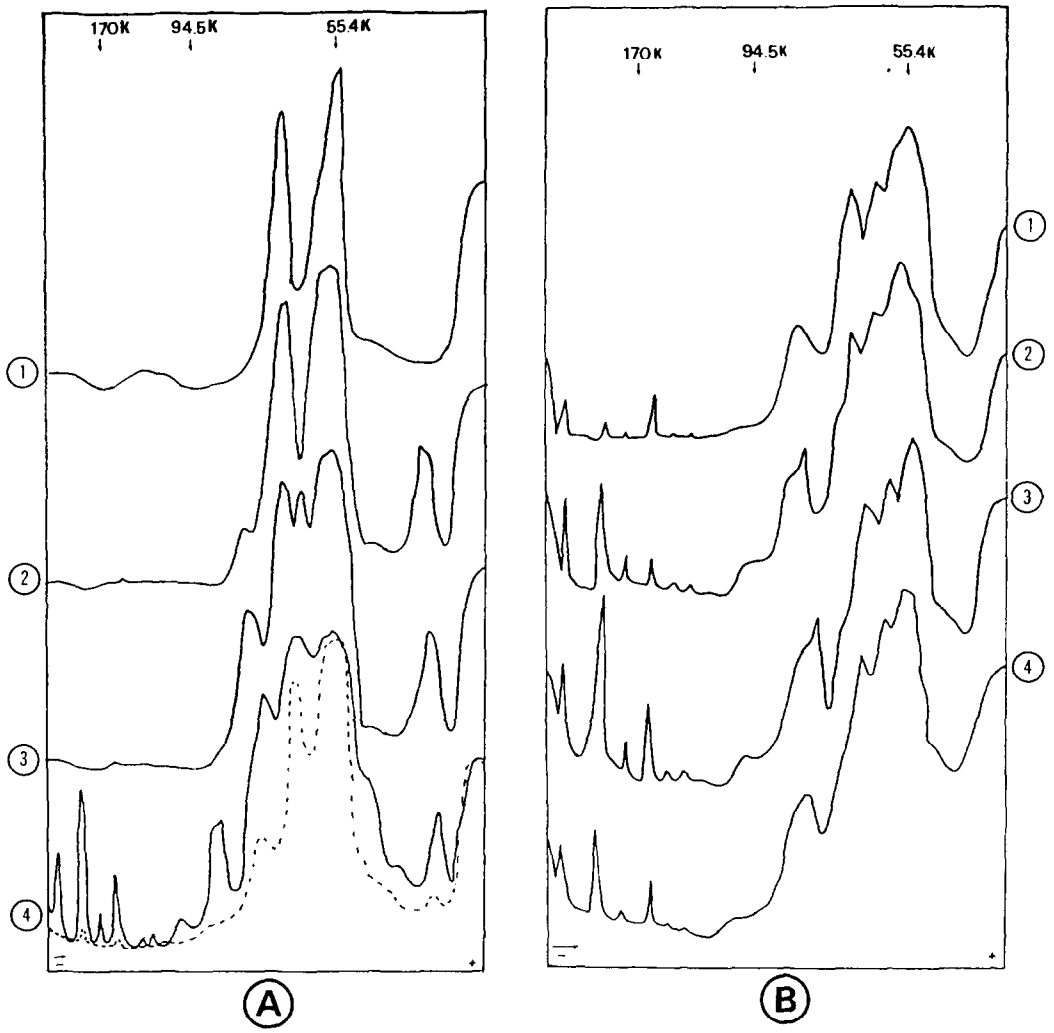

Fig.2. Densitometer tracings of proteins isolated by antipolyamine antibodies after incubation with: buffer alone (A1), plasmas of normal mice (A2) or mice infected with *B. abortus* (A3), or mice bearing the Lewis carcinoma 3 weeks after the graft (A4, ---: immunolatex preincubated with spermine bound to insulin) or 1 (B1), 2 (B2), 3 (B3) or 4 (B4) weeks after the graft. Separation on 9% (A) or 7.5% (B) polyacrylamide gel.

matory hepatomegaly and a non-metastatic splenomegaly. It was therefore possible that protein-bound polyamines were a reflection of the inflammatory rather than of the malignant state. To investigate this, protein-bound polyamines were studied in mice in which liver and spleen hypertrophy was induced by intravenous injection of *B. abortus* as in [23]. Fig.2C shows that polyamines are carried by four proteins: 3 which correspond to those found in normal mice, but with amplification of the band at 82 kDa; a fourth of 65 kDa, absent in normal mice.

Thus, with the exception of polyamines bound on a 65 kDa protein which could be specific for the inflammatory process, protein-bound polyamines are apparently nog influenced by the inflammatory condition accompanying hepato-splenomegaly.

3.4. *Protein-bound polyamines during the growth of the Lewis tumour*

With specificity of interaction assured and the contribution of the inflammatory process evaluated, the question as to whether the binding of polyamines to plasma proteins is an early or late

Table 1

Transglutaminase-mediated putrescine-binding capacity of plasma proteins in mice

Mice		K_m (mM)	V_{max} (nmol PUT bound/ μg protein)
	Controls	0.27 ± 0.01[b]	1.04 ± 0.22
Lewis tumour	1[a]	0.33 ± 0.09	0.72 ± 0.15
	2	0.28 ± 0.13	0.46 ± 0.10[c]
	3	0.40 ± 0.17	0.42 ± 0.07[c]
	4	0.26 ± 0.11	0.45 ± 0.05[c]
B. abortus	10 days	0.72 ± 0.28	3.73 ± 1.52[c]

[a] No. of week(s) after Lewis tumour grafting
[b] Values are means ± SD
[c] Significant difference from control group

The kinetics of the transglutaminase-mediated insertion of putrescine (PUT) into plasma proteins was linear in the Lineweaver-Burk plots and so V_{max} and K_m could be determined

event after tumour graft could be approached.

A study of protein-bound polyamines in the plasma of mice at weeks 1, 2, 3 and 4 after tumour graft was undertaken. From the results in fig.3, it is apparent that polyamines bound to high-M_r proteins can be detected from as early as the first week after tumour graft. An increase of these protein-bound polyamines takes place up to the third week and by the fourth week decreases.

Although these results provide strong evidence for the binding of polyamines to high-M_r plasma proteins during growth of the Lewis tumour in mice, they do not give information on the type of bond linking the PA to proteins. In this context it has been well established that polyamines can be bound by a transglutaminase-mediated type of reaction [24] to glutamine residues of proteins in γ-glutamylamine links. Were this the case here, these glutamine residues in plasma proteins should be less reactive towards exogenous transglutaminase.

3.5. *Polyamine-binding capacity of plasma proteins in tumour-grafted mice*

Since the human antibodies used here showed extensive cross-reaction with spermine, spermidine and putrescine [1,16], we can define them as anti-polyamine antibodies. Furthermore, as spermine but not putrescine precipitates proteins such as fibronectin [25], the accessibility of glutamine residues in plasma protein to transglutaminase was investigated with putrescine.

A Lineweaver-Burk plot was used to express the results ($1/V = f(1/S)$). In all cases, the kinetics of polyamine binding was linear and permitted the V_{max} and apparent K_m to be calculated.

As shown in table 1, when compared to control values V_{max} decreased progressively as tumour growth took place during the first and second weeks. In contrast, the V_{max} of plasma proteins from mice injected with *B. abortus* was significantly higher (about 2.5-fold) than those in controls. These results suggest that the decrease in V_{max} observed in tumour-grafted mice is independent of the hypertrophic process accompanying tumour growth. It should also be noted that no differences in K_m values could be detected between control and 3LL-grafted mice, whereas a significant increase in K_m appeared in mice infected with *B. abortus*.

The decrease of V_{max} observed in mice bearing Lewis lung carcinoma could be related to a decrease in the number of transglutaminase-sensitive residues in proteins, resulting from increased insertion of primary amines (polyamines, ϵNH_2 protein-bound lysine) on glutamine residues in plasma proteins.

4. DISCUSSION

Our immunochemical approach associated with molecular mass determination on PAGE permitted the separation of protein-bound polyamine from

the plasma of mice and the unambiguous demonstration of an increase in the number of these proteins bearing polyamines during growth of the 3LL tumour. These new proteins of high molecular mass were: (i) not present when immunolatex was preincubated with 0.7 mM spermine bound to insulin and (ii) independent of any concomitant inflammatory and hypertrophic process as experimentally induced inflammation with heat-inactivated *B. abortus* showed only one band at 65 kDa in addition to the 3 found in normal plasma.

These polyamines could be bound to cellular proteins within the tumour and then released into the circulation as demonstrated for fibronectin in cellular culture media [26], or could be excreted in the free state in plasma and bound to existing plasma proteins by the action of factor XIII as we have shown previously [12] or that of intrahepatic transglutaminase. There is evidence in the literature for this latter hypothesis. Indeed, Rosenblum et Russell [27] have shown that in rats injected with [^{14}C]polyamine, hepatic transglutaminase is responsible for the binding of polyamines to proteins within 5 min following injection. Our results do not permit us to choose between these two alternatives.

As regards the nature of the polyamine-protein bond, γ-glutamyl-amine bonds were suspected. Their direct measurement would have required quantities of mouse plasma substantially greater than the 1 ml which was obtained per mouse. We therefore tested this hypothesis indirectly by measuring the accessibility of any remaining free amide groups of glutamine residues in plasma proteins to purified exogenous tissue transglutaminase. The results (table 1) show clearly that the capacity of plasma proteins to bind polyamines decreases with tumour growth, independently of the inflammatory process.

These observations lend support to the existence of naturally occurring γ-glutamyl-polyamine bonds. However, they do not allow us to differentiate between a decrease in individual glutamine residues of the same or different proteins. These plasma proteins with bound polyamines can be detected as early as 1 week after tumour graft and increase until the third week but fall off at the fourth week. One reason for this decrease may be that, following the binding of polyamines, the protein carriers may be altered in secondary structure and hence their antigenicity and thus their capabilities of eliciting an antibody response. These antibodies could then react with the very proteins responsible for their induction, forming immune complexes and as such their bound polyamines would no longer be available for interaction with the antipolyamine antibodies bound to magnetic latex spheres. This is a distinct possibility, since we have previously presented evidence for the existence of immune complexes containing polyamines in human plasma [9].

At this stage of our investigations, it seems clear that bound polyamines merit further study in the light of the 'polyamine status' [28] of individuals under conditions of physiological and pathological growth. The identification of their protein component is a sine qua non for understanding circulating polyamine homeostasis.

ACKNOWLEDGEMENTS.

Financial support for this work was provided in 1986 and 1987 by subventions from the Association pour la Recherche sur le Cancer (France) and from the Fédération Nationale des Centres de Lutte Contre le Cancer (France).

REFERENCES

[1] Quash, G. and Jonard, J. (1967) CR Acad. Sci. Paris 265, 934–936.
[2] Bartos, D., Campbell, R.A., Bartos, F. and Grettie, D.P. (1975) Cancer Res. 35, 2056–2060.
[3] Fujiwara, K., Asada, H., Kitagawa, T., Yamamoto, K., Ito, T., Tsuchiya, R., Sohda, M., Nakamura, N., Hara, K., Tomonaga, Y., Ichimaru, M. and Takahashi, S. (1983) J. Immunol. Methods 61, 217–226.
[4] Quash, G. and Wilson, M.B. (1967) West Indian Med. J. 16, 81–91.
[5] Roch, A.M., Quash, G. and Huppert, J. (1978) CR Acad. Sci. Paris 287D, 1071–1074.
[6] Bartos, D., Bartos, F., Campbell, R.A. and Grettie, D.P. (1980) Science 208, 1178–1180.
[7] Furuichi, K., Ezoe, H., Obara, T. and Oka, T. (1980) Proc. Natl. Acad. Sci. USA 77, 2904–2908.
[8] Quash, G., Roch, A.M., Niveleau, A., Grange, J., Keolouangkhot, T. and Huppert, J. (1978) J. Immunol. Methods 22, 165–174.

[9] Roch, A.M., Quash, G., Ripoll, H., Vigreux, B. and Niveleau, A. (1981) in: Advances in Polyamine Research (Caldarera, C.M. et al. eds) vol. 3, pp. 225–235, Raven, Yew York.
[10] Clarke, D.D., Mycek, M.J., Neidle, A. and Waelsh, H. (1959) Arch. Biochem. Biophys. 79, 338–351.
[11] Folk, J.E. (1980) Annu. Rev. Biochem. 49, 517–531.
[12] Roch, A.M., Thomas, V., Quash, G., Moulinoux, J.P. and Delcros, J.G. (1984) Int. J. Cancer 33, 787–793.
[13] Conrad, S.M. (1985) in: The Enzymology of Post-translational Modifications of Proteins (Freedman, R.B. and Hawkins, H.G. eds) vol. 2, pp. 339–368, Academic Press, London.
[14] Moulinoux, J.P., Quemener, V., Larzul, J.J., Le Calve, M., Roch, A.M., Toujas, L. and Quash, G. (1984) Int. J. Cancer 34, 277–281.
[15] Ripoll, J.P., Roch, A.M., Quash, G.A. and Grange, J. (1980) J. Immunol. Methods 33, 159–173.
[16] Quash, G., Gresland, L., Delain, E. and Huppert, J. (1973) in: Polyamines in Normal and Neoplastic Growth (Russell, D.H. ed) pp. 157–166, Raven, New York.
[17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[18] Rylatt, D.B. and Parish, C.R. (1982) Anal. Biochem. 121, 213–214.
[19] Bonnefoy-Roch, A.M. and Quash, G. (1978) in: Advances in Polyamine Research (Campbell, R.A. et al. eds) vol. 2, pp. 55–63, Raven, New York.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Ansorge, W. (1985) J. Biochem. Biophys. Methods 11, 13–20.
[22] Brookhart, P.P., McMahon, P.L. and Takahashi, M. (1983) Anal. Biochem. 128, 202–205.
[23] Martin, A., Toujas, L., Le Garrec, Y., Dazord, L. and Amice, J. (1979) J. Natl. Cancer Inst. 62, 123–127.
[24] Folk, J.E., Park, M.P., Chung, S.I., Schrode, J., Lester, E.P. and Cooper, H.L. (1980) J. Biol. Chem. 255, 3695–3700.
[25] Vuento, M., Vartio, T., Saraste, M. Von Bonsdorff, C.H. and Vaheri, A. (1980) Eur. J. Biochem. 105, 33–42.
[26] Roch, A.M., Quash, G., Ripoll, H. and Mardon, M. (1983) Biochem. J. 210, 137–144.
[27] Rosenblum, M.G. and Russell, D.H. (1977) Cancer Res. 37, 47–51.
[28] Campbell, R.A., Grettie, D.P., Bartos, D. and Bartos, F. (1981) in: Advances in Polyamine Research (Caldarera, C.M. et al. eds) vol. 3, pp. 409–423, Raven, New York.