9-(*S*)-(2,3-DIHYDROXYPROPYL)ADENINE INHIBITS THE TRANSFORMATION OF CHICK EMBRYO FIBROBLASTS INFECTED WITH ROUS SARCOMA VIRUS

Evidence for inhibition of enzymatic activity of isolated cellular protein kinases by the drug

J. KÁRA, P. VÁCHA and A. HOLÝ+

Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Flemingovo nám. 2, 16610 Prague 6
and +*Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Flemingovo nám. 2, 16610 Prague 6, Czechoslovakia*

Received 12 September 1979

1. Introduction

The antiviral activity of 9-(*S*)-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA] against some types of both RNA and DNA viruses including vaccinia, herpes simplex (types 1 and 2), measles, and vesicular stomatitis has been demonstrated recently [1]. This novel nucleoside analog, the sugar moiety of which is replaced by an aliphatic chain [2], also inhibits the activity of adenosine deaminase (EC 3.5.4.4) [3] thus enhancing the antiviral effect of adenine arabinoside [1]. The experiments carried out on different cells in tissue culture did not show any significant effect of (*S*)-DHPA either on DNA, RNA or protein synthesis [1], and no signs of toxicity were noted in mice that had been injected intraperitoneally or intravenously with (*S*)-DHPA at a dose as high as 1 g/kg. In vivo, (*S*)-DHPA is metabolically very stable [4], and the unchanged drug is rapidly excreted by urine. The selective antiviral effect of (*S*)-DHPA substantiates further efforts to elucidate its biological role and inhibitory mechanism.

Chick embryo fibroblasts infected in culture with Rous sarcoma virus (RSV) have been shown to acquire certain new morphologic, growth and metabolic properties [5–7]. An increased glycolytic capacity, one of the most commonly reported alterations in malignant cells [8], has also been found in tissue from Rous sarcomas [9], in slices from chorioallantoic membranes infected with RSV [10], and in cells from cultures of chicken embryo fibroblasts infected in vitro with RSV [6]. Enzymatic activity of certain glycolytic enzymes, lactate dehydrogenase (EC 1.1.1.27), phosphoglucomutase (EC 2.7.5.1), and glucosephosphate isomerase (EC 5.3.1.9), is markedly higher in the transformed cells than in the normal tissue [10].

In this study we have used two transformation parameters of the RSV-transformed tumor cells:

(1) The morphological transformation of chick embryo fibroblasts to the round-shaped tumor cells;
(2) The level of enzymatic activity of lactate dehydrogenase induced in the transformed cells with concomitant increase of aerobic glycolysis.

Transformation of chick fibroblasts by avian sarcoma viruses requires the function of a single viral gene (*src*) which is responsible for both the induction and maintenance of neoplastic transformation [11]. A protein of 60 000 daltons that is apparently encoded in *src* has been isolated by immunoprecipitation from cytosol of avian and mammalian cells transformed by RSV and identified [12–14]. This protein, which is not a structural component of the virion, is thought to be responsible for RSV-induced cell transformation and has been named $p60^{src}$ [15]. A protein kinase activity in $p60^{src}$ has been demonstrated [15–18]. This significant observation suggests that virus-specific protein kinase $p60^{src}$ is critical in the transformation of cells by RSV.

Here we describe the inhibitory effect of (*S*)-DHPA on the transformation of chick embryo fibroblasts infected in culture with Schmidt-Ruppin strain of

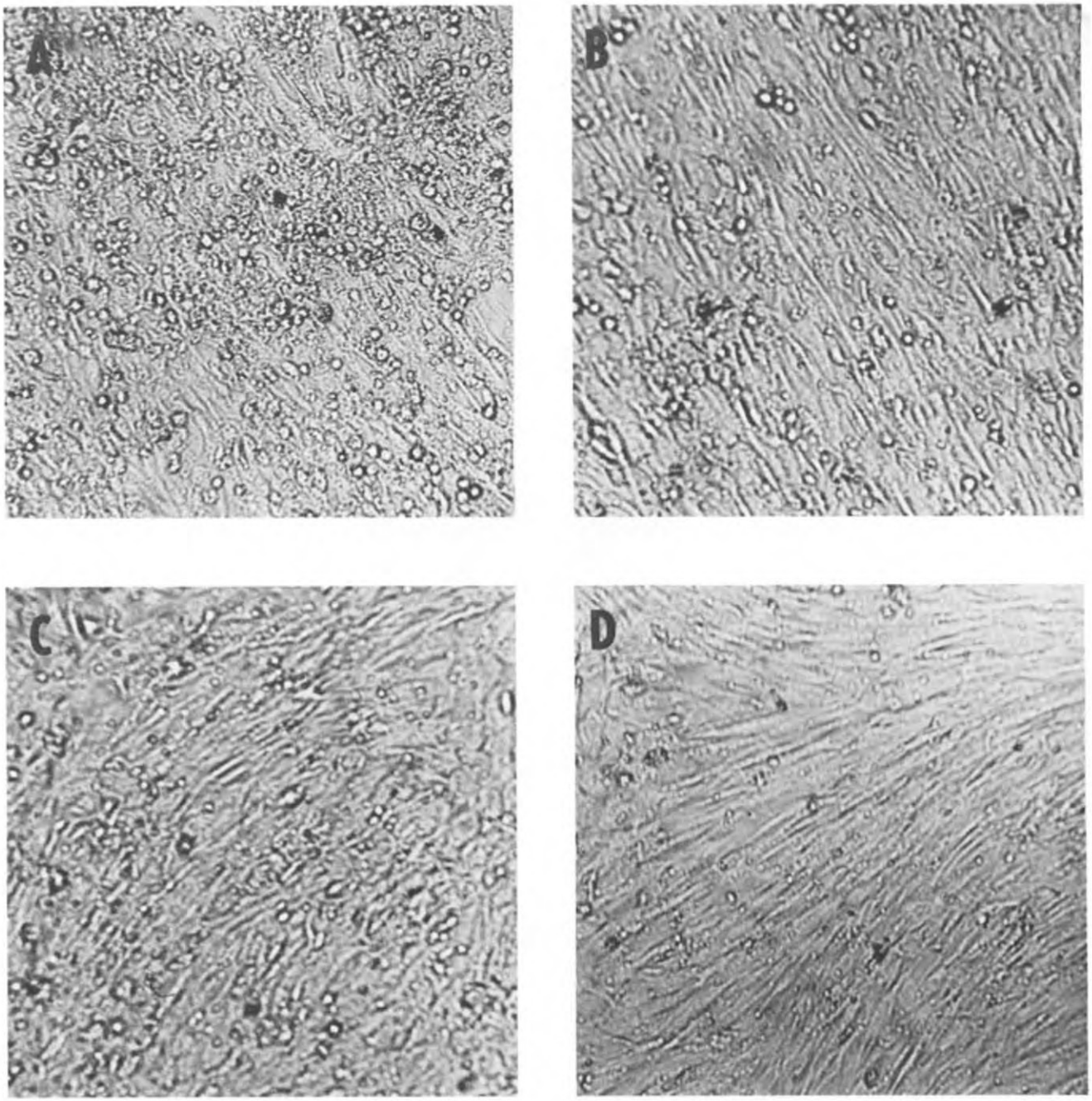

Fig.1. Inhibition of transformation by (*S*)-DHPA of chick embryo fibroblasts infected in culture with Schmidt Ruppin strain of Rous sarcoma virus. Microphotographs of cultures 72 h after infection with SR-RSV. (A) Chick embryo fibroblasts (CEF) infected with SR-RSV and grown after infection in medium without inhibitor. Many transformed, round-shaped cells are present in this culture. (B) CEF infected with SR-RSV and grown after infection in medium containing 25 μg (*S*)-DHPA/ml. (C) CEF infected with SR-RSV and grown in medium containing 50 μg (*S*)-DHPA/ml. (D) CEF infected with SR-RSV and grown after infection in medium containing 100 μg (*S*)-DHPA/ml. Transformed cells are not present in this culture and no increase of aerobic glycolysis was observed.

Rous sarcoma virus (SR-RSV) and present experimental evidence that (*S*)-DHPA inhibits enzymatic activity of cellular protein kinases (EC 2.7.1.37) isolated from the cytoplasmic proteins of Rous sarcoma tissue. These facts suggest the possible inhibitory effect of (*S*)-DHPA on viral protein kinase $p60^{src}$ in vivo.

2. Materials and methods

The cultures of chick embryo fibroblasts were prepared and cultivated in plastic Petri dishes in a CO_2 incubator as in [19]. The cultures were infected 24 h after plating with SR-RSV, 1×10^6 p.f.u./dish, and the infected cells were cultivated at 37°C in Eagle's minimal essential medium containing 10% inactivated calf serum and antibiotics [19] or in the medium containing various concentrations of (*S*)-DHPA. The number of transformed cells per microscopic field in the infected cultures was determined 72 h after infection, as in [20]. The extracts of soluble proteins were prepared 3 days after infection from SR-RSV-infected and uninfected chick embryo fibroblasts and from the cells infected with SR-RSV and cultivated after infection in the presence of (*S*)-DHPA (100 μg/ml). The enzymatic activity of lactate dehydrogenase (EC 1.1.1.27) (expressed in units/100 μg protein) in the extracts was determined by the colorimetric method in [21], using a kit of chemicals for lactate dehydrogenase determination (Bio-LA-test, Lachema). Protein concentration in the cell extracts was determined by the method in [22], and in the protein kinase preparations by the method in [23].

Rous sarcoma tissue was isolated from Brown Leghorn chickens infected with SR-RSV [20]. The tumor tissue was homogenized, and high-speed supernatant was prepared as in [24]. Chromatography of protein kinases isolated from Rous sarcoma on DEAE–Sephadex A-25 column and protein kinase assays were performed essentially as in [25]. Casein prepared according to [26] was used as substrate in protein kinase assays. [γ-^{32}P]ATP (spec. act. 25 Ci/mmol), prepared by the method in [27], was kindly provided by Dr V. Pačes.

3. Results

3.1. *Inhibition by (S)-DHPA of malignant conversion of chick embryo fibroblasts infected with SR-RSV*

When the chick embryo fibroblasts infected in culture with a high titre SR-RSV were cultivated after infection in medium containing increasing concentrations of (*S*)-DHPA, a markedly decreased number of transformed cells was observed 72 h after infection in these cultures, depending on the concentration of (*S*)-DHPA present in the medium (fig.1, table 1). In comparison with the infected cells grown in normal medium without inhibitor (fig.1A), there was a com-

Table 1
Inhibition by 9-(*S*)-(2,3-dihydroxypropyl)adenine of transformation of chick embryo fibroblasts infected with SR-RSV and inhibition of induction of lactate dehydrogenase in the infected cells growing in the presence of (*S*)-DHPA

Culture	(*S*)-DHPA in medium (μg/ml)	No. transformed cells in one microscopic field	Inhibition of transformation (%)	Lactate dehydrogenase activity in cell-extract (units/100 μg protein)
CEF inf. SR-RSV	100	0	100	13.6
CEF inf. SR-RSV	50	75	82.5	–
CEF inf. SR-RSV	25	140	67.3	–
CEF inf. SR-RSV	0	428	0	25.5
CEF (uninfected)	–	–	–	13.3

Cultures of chick embryo fibroblasts (24 h after plating) were infected with SR-RSV as in section 2 and cultivated 3 days in the presence or absence of (*S*)-DHPA in the medium as indicated in table 1. The transformed cells were counted 72 h after infection under the microscope [20], and lactate dehydrogenase activities in cell extracts were determined [21]

plete inhibition of cell transformation in the cultures growing in the presence of 100 μg (*S*)-DHPA/ml (fig.1D). A significant decrease in number of transformed cells was observed at the concentration of (*S*)-DHPA as low as 25 μg/ml (fig.1C, table 1). These results were reproducible in several experiments. Under extreme conditions (100 μg/ml), (*S*)-DHPA did not exhibit any marked cytotoxic effect upon uninfected cells or untransformed cells after viral infection (cf. [1]).

Concomitantly with the inhibition of cell transformation, (*S*)-DHPA also inhibits the virus-induced metabolic alterations in the infected cells, e.g., induction of lactate dehydrogenase (table 1) and aerobic glycolysis. In comparison with the untreated infected cells (fig.1A) where the medium was quickly acidified by lactic acid produced by very active aerobic glycolysis, the infected cells growing in the presence of 100 μg (*S*)-DHPA/ml do not acidify the medium and exhibit a markedly lower level of lactate dehydrogenase activity in the cytosol (table 1).

However, this inhibitory effect of (*S*)-DHPA on the oncornavirus-specific function, i.e., morphological and metabolic transformation of the infected cells, was found to be reversible. If the infected cells growing for 3 days after infection in medium containing 100 μg (*S*)-DHPA/ml were washed with normal medium and overlayered with normal medium containing 1.5% agar and cultivated further at 37°C, many transformed, round-shaped cells appeared in this culture within 48 h (data not shown). Inhibition of cell transformation in this system thus requires a constant presence of the inhibitor in the medium (50–100 μg (*S*)-DHPS/ml). This suggests that (*S*)-DHPA does not inhibit the integration of proviral DNA into the cellular genome [28], nor the synthesis of viral RNA, but probably inhibits a virus-specific function responsible for cell transformation, e.g., the phosphorylation of some cellular proteins catalyzed by the virus-specific protein kinase $p60^{src}$ [15–17].

3.2. *Inhibition by (S)-DHPA of the enzymatic activity of protein kinases present in high-speed supernatant from Rous sarcoma tissue homogenate*

In a model experiment we have first studied the effect of (*S*)-DHPA upon the enzymatic activity of cellular protein kinases present in the cytosol proteins of Rous sarcoma. As shown in table 2, the activity of protein kinases in the extract was very significantly inhibited by (*S*)-DHPA, the inhibition being dependent on the concentration of the (*S*)-DHPA present in the enzymatic assay.

By chromatography on a DEAE Sephadex A-25 column of the protein kinases present in the extract from Rous sarcoma tissue, 3 distinct protein kinase fractions have been isolated (fig.2) during the elution by linear gradient of 0.04–0.5 M ammonium sulfate in TGMED buffer. The protein kinases were

Table 2
Inhibition by (*S*)-DHPA of enzymatic activity of protein kinases in cytosol proteins extracted from Rous sarcoma tissue

(*S*)-DHPA (μg/assay)	[^{32}P]phosphate incorporated into casein (cpm)	Inhibition of protein kinase activity (%)
0	12 794	0
100	9946	22.3
200	7270	43.2
400	4832	62.5

The assay mixture contained in 225 μl total vol.: 50 mM Tris–HCl buffer (pH 7.4), 0.15 M NaCl, 10 mM $MgCl_2$, 2 × 10^6 cpm [γ-^{32}P]ATP (spec. act. 25 Ci/mmol), 150 μg casein, 240 μg cytosol proteins extracted from Rous sarcoma tissue, and (*S*)-DHPA as indicated in the table. After 10 min incubation at 30°C the enzymatic reaction was stopped by addition of 3 ml ice-cold 10% trichloroacetic acid solution containing 40 mM sodium pyrophosphate. Acid-precipitable radioactivity was collected on fiberglass filters GF/C (Whatman) and washed 6-times with 5 ml 10% trichloroacetic acid. The radioactivity on the dried filters was quantitated by liquid scintillation counting

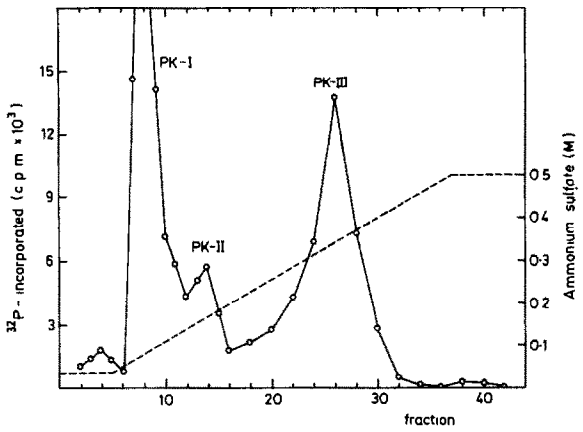

Fig.2. Chromatography of protein kinases present in high-speed supernatant from Rous sarcoma tissue on a DEAE-Sephadex A-25 column. DEAE–Sephadex A-25 column (2.5 × 20 cm) was equilibrated with TGMED buffer (50 mM Tris–HCl (pH 8.0) containing 25% glycerol (v/v), 5 mM $MgCl_2$, 0.1 mM EDTA and 5 mM dithiothreitol) containing 0.04 M ammonium sulfate. Proteins extracted from Rous sarcoma tissue (85 mg protein in 11 ml TGMED buffer) were applied on the column and fractions were eluted at +3°C in a linear gradient of 0.04–0.5 M ammonium sulfate in TGMED buffer (total vol. 140 ml). Fractions (4 ml) were collected (flow rate 1 ml/min.) and aliquots 50 μl from each fraction were used for protein kinase assay, as in the legend to table 2.

designated PK-I (eluted by 0.1 M ammonium sulfate), PK-II (eluted by 0.17 M ammonium sulfate) and PK-III (eluted by 0.35 M ammonium sulfate). When assayed in the presence of 1.9×10^{-3} M (*S*)-DHPA, the enzymatic activities of the protein kinases were inhibited by 53%, 63% and 27%, respectively, exhibiting different sensitivity to (*S*)-DHPA. Protein kinase PK-II was the most inhibited enzyme. These results indicate that the cellular protein kinases may be inhibited by (*S*)-DHPA to a different extent.

4. Discussion

Our results clearly show that 9-(*S*)-(2,3-dihydroxypropyl)adenine inhibits the morphological and metabolic transformation of chick embryo fibroblasts infected in culture with a high titre of SR-RSV. This inhibition of the biological function of avian sarcoma virus is apparently reversible as shown by the fact that permanent presence of (*S*)-DHPA in the medium is necessary in order to inhibit the transformation of RSV-infected cells.

The fact that (*S*)-DHPA inhibits the enzymatic activity of cellular protein kinases (table 2) suggests that virus-specific protein kinase $p60^{src}$, which is responsible for the cell transformation by avian sarcoma virus [15–18] might be inhibited by (*S*)-DHPA inside the infected cells at relatively lower (*S*)-DHPA concentrations. This is supported by the finding that morphological transformation of chick embryo fibroblasts infected with SR-RSV is inhibited already in the presence of (*S*)-DHPA at 25 μg/ml (1.25×10^{-4} M) (table 1). Direct experimental evidence of the inhibition of the virus-specific protein kinase $p60^{src}$ by (*S*)-DHPA is necessary in order to prove this hypothesis. Cellular protein kinases are probably not inhibited significantly by (*S*)-DHPA in vivo at the low concentration (1.25×10^{-4} M) of the inhibitor in medium.

Furthermore we have found that the *R*-enantiomer, (*R*)-DHPA, is also active as inhibitor of CEF transformation by SR-RSV, the inhibition being somewhat less as compared with the same molar concentration of (*S*)-DHPA, unlike the marked difference observed with other viruses [1,29]. There is no difference in the transformation inhibiting potency between the two enantiomers and the racemic mixture (*R,S*)-DHPA.

In a preliminary experiment we have found that (*S*)-DHPA at 100 μg/ml inhibits completely the production of virions labeled with [^{3}H]uridine in cultures of chick embryo fibroblasts infected with SR-RSV (data not shown). The mechanism of this inhibition of virus replication may involve the inhibition of protein kinase present inside the oncornavirus particles [30–32], which exhibit different characteristics in comparison with $p60^{src}$. The possible effect of (*S*)-DHPA on these virion-associated protein kinases or its higher affinity to these enzymes may be one of the principles of the selective antiviral effect of this drug and deserves further experimental study.

Acknowledgement

The authors wish to thank Dr V. Pačes, Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Prague, for the kind gift of [γ-^{32}P]ATP preparation.

References

[1] De Clercq, E., Descamps, J., De Somer, P. and Holý, A. (1978) Science 200, 563–565.
[2] Holý, A. (1975) Coll. Czech. Chem. Commun. 40, 187–214.
[3] Schaeffer, H. J., Vogel, D. and Vince, R. (1965) J. Med. Chem. 8, 502–506.
[4] Čihák, A. and Holý, A. (1978) Coll. Czech. Chem. Commun. 43, 2082–2088.
[5] Rubin, H. (1960) Virology 10, 29–49.
[6] Morgan, R. H. and Ganapathy, S. (1963) Proc. Soc. Exp. Biol. Med. 113, 312–315.
[7] Temin, H. M. (1968) Int. J. Cancer 3, 273–282.
[8] Aisenberg, A. G. (1961) The glycolysis and respiration of tumors, Academic Press, New York.
[9] Burk, D., Sprince, H., Spangeler, J. M., Kabat, E. H., Furth, J. and Claude, A. (1941) J. Natl. Cancer Inst. 2, 201–240.
[10] Weber, G., Banerjee, G., Levine, S. and Ashmore, J. (1961) J. Natl. Cancer Inst. 27, 869–873.
[11] Vogt, P. K. (1977) in: Comprehensive Virology (Fraenkel-Conrat, H. and Wagner, R. eds) pp. 341–455, Plenum Press, New York.
[12] Brugge, J. S. and Erikson, E. (1977) Nature 269, 346–348.
[13] Purchio, A. F., Erikson, E., Brugge, J. S. and Erikson, R. (1978) Proc. Natl. Acad. Sci. USA 75, 1567–1571.
[14] Beemon, K. and Hunter, T. (1978) J. Virol. 28, 551–566.
[15] Collett, M. S. and Erikson, R. L. (1978) Proc. Natl. Acad. Sci. USA 75, 2021–2024.
[16] Levinson, A. D., Oppermann, H., Levintow, L., Varmus, H. E. and Bishop, J. M. (1978) Cell 15, 561–572.
[17] Rübsamen, H., Friis, R. R. and Bauer, H. (1979) Proc. Natl. Acad. Sci. USA 76, 967–971.
[18] Sefton, B. M., Hunter, T. and Beemon, K. (1979) J. Virol. 30, 311–318.
[19] Kára, J. (1968) Folia Biol. (Praha) 14, 249–265.
[20] Mach, O. and Kára, J. (1971) Folia Biol. (Praha) 17, 65–72.
[21] Babson, A. L. and Phillips, G. E. (1965) Clin. Chim. Acta 12, 210–215.
[22] Bradford, M. M. (1976) Analyt. Biochem. 72, 248–254.
[23] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[24] Keller, R. K., Chandra, T., Schrader, W. T. and O'Malley, B. W. (1976) Biochemistry 15, 1958–1967.
[25] Desjardins, P. R., Lue, P. F., Liew, C. C. and Gornal, A. G. (1972) Canad. J. Biochem. 50, 1249–1259.
[26] Reiman, E. M., Walsh, D. A. and Krebs, E. G. (1971) J. Biol. Chem. 246, 1986–1995.
[27] Glynn, I. M. and Chappell, J. B. (1964) Biochem. J. 90, 147–149.
[28] Varmus, H. E., Vogt, P. K. and Bishop, J. M. (1973) Proc. Natl. Acad. Sci. USA 70, 3067–3071.
[29] De Clercq, E. and Holý, A. (1979) J. Med. Chem. 22, 510–513.
[30] Hizi, A., Wunderli, W. and Joklik, W. K. (1979) Virology 93, 146–158.
[31] Houts, G. E., Miyagi, M., Ellis, C., Beard, D., Watson, K. F. and Beard, J. W. (1978) J. Virol. 25, 546–552.
[32] Rubin, C. S. and Rosen, O. M. (1975) Annu. Rev. Biochem. 44, 831–887.