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Biological Activity of 2-Phenylethanol and Its Derivatives

VIII. Influence on Herpesvirus DNA-Synthesis in Rabbit Kidney Cells

By

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With 3 Figures

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Summary

The biosynthesis of herpesvirus DNA in rabbit kidney cells is inhibited to 50% by PEA (2-Phenylethanol) at 0.65 mg PEA/ml. The inhibition of cellular DNA synthesis in uninfected cells by PEA is about twice as sensitive as that of viral DNA synthesis.

The cellular DNA-dependent DNA polymerase is inhibited in a non-competitive way. The 50% inhibitory concentration amounts to 0.8 mg PEA/ml.

In contrast the herpesvirus induced DNA-dependent DNA polymerase is 10-fold more resistant towards PEA.

It is assumed that, contrary to the synthesis of cellular DNA, the PEA induced inhibition of viral DNA synthesis is not caused by an inhibition of the virus-induced DNA-dependent DNA polymerase.

1. Introduction

Lilly *et al.* (18) reported that 2-phenylethanol (PEA) exerts an inhibitory effect on the growth of gram-negative microorganisms. PEA was shown subsequently to be able to inhibit also proliferation in bacteria (1), moulds (17), yeast (4), plants (22) and mammalian cells (survey: 24).

Results from previous studies have demonstrated the inhibition of DNA synthesis as well as production of RNA virus in bacteria (15, 23) and animal cells (27, 3) by PEA.

In two publications Roizman (27) and Maass *et al.* (19) previously studied PEA inhibition of multiplication in herpesvirus. Roizman (27) postulates on the basis of his results, a block in the virus DNA synthesis by this drug. Direct evidence for his hypothesis, however, is not yet available.

We approached the problem by determination of the differential influence of PEA on cellular and on viral DNA synthesis, by comparing the inhibitory concentrations blocking DNA synthesis in the intact biological model *i.e.* mammalian cells with the isolated enzyme system.

2. Materials and Methods

2.1. Source of Materials

The following materials were purchased: Unlabeled deoxyribonucleoside triphosphate (dATP, dGTP, dCTP and dTTP) from Boehringer Mannheim, Tutzing; ^{3}H-dATP (spec. activity 13.5 Ci/mmole) and Thymidine-^{3}H (spec. activity 24 Ci/mmole) from The Radiochemical Centre, Amersham; ribonuclease (pancreas; 3000 units/mg, EC number: 2.7.7.16) from Worthington Biochemical Corp., Freehold; cesium chloride from E. Merck, Darmstadt; insta-gel from Packard Instruments, Zürich; penicillin and streptomycin from Bayer-Farbenfabriken, Leverkusen and moronal from v. Heyden, München.

Herring-DNA, isolated according to Zahn *et al.* (28) was a gift from H. Mack, Illertissen (Bayern).

2.2. Rabbit Kidney Cells

Rabbit kidney cells were obtained from the kidneys of 6—12 days old rabbits by trypsinization. These primary cells were cultivated in Hanks's solution containing 10% calf serum, 0.5% lactalbumine-hydrolysate and bicarbonate as well as antibiotics (penicillin: 100 units/ml, streptomycin: 0.1 mg/ml and moronal: 25 units/ml).

After 4 or 5 days the cells were used in the experiments.

2.3. Infection of Cells with Herpesvirus

The cells were infected with the IES-strain of herpesvirus hominis (6) after changing the culture fluid. Eagle's basal medium (BME) with nonessential amino acids was used throughout the experiments. 2 hours after infection the nonadsorbed virus was washed away. Further incubation in BME was for different times.

2.4. Labelling of Cellular and Viral DNA

The labelling was effected with 10 μCi of thymidine per Petri dish, holding 10 ml of BME. At the end of the experiment, the cells were scraped off, washed two times and frozen at −20° C and kept until extraction of the DNA. For each experiment 3 Petri dishes containing about 6×10^6 cells were used.

2.5. Extraction of DNA

DNA from uninfected and virus-infected cells was extracted by the method of Kirby (12). The DNA fraction was further freed of RNA by treatment with 20 μg/ml ribonuclease (pancreas) for 120 minutes at 37° C. The DNA finally was precipitated by the addition of ethanol (50% final concentration). The precipitate was dissolved in 0.015 M Tris-HCl (pH 8.0).

The yield of the extracted DNA amounted to about 20% of the total.

2.6. Isolation of Cellular DNA-Dependent DNA Polymerase

For preparation of crude extracts from rabbit kidney cells a modified method of Keir *et al.* (10) was used. All steps were carried out at 0—4° C. Five g of the sediment of isolated rabbit kidney cells are washed three times with physiological saline and subsequently suspended in 5 ml buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol).

The cells are lysed by rapid freezing and thawing three times. The lysate is centrifuged at 100,000 × *g* for 60 minutes and the supernatant which contains the DNA-dependent DNA polymerase (EC number: 2.7.7.7), is decanted. In some assays this supernatant is dialysed against buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol, 4 mM $MgSO_4$ and 150 mM KCl). The enzyme preparation (1 mg protein/ml) was free from DNA.

2.7. Isolation of Virus-Induced DNA Dependent DNA Polymerase

As described for control cells, cell batches 11 hours after infection were lysed and centrifuged. The supernatant contains cellular DNA-dependent DNA polymerase as well as the virus induced enzyme (EC number: 2.7.7.7). The cellular DNA-dependent DNA polymerase can be inactivated completely by heating the enzyme preparation at 50° C for 10 minutes (10).

In this preparation (0.8 mg protein/ml) no DNA contamination was detectable.

2.8. Polymerase Assay

The assays for the determination of DNA-dependent DNA polymerase activities were based on the measurement of the conversion of nucleoside triphosphate into acid-insoluble form. The reaction mixture of 0.08 ml contained: 0.1 mM ^{3}H-dATP (4×10^6 counts/minute per μmole), 0.1 mM dGTP, dCTP and dTTP, 20 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol and different amounts of native and heat denatured DNA, respectively. The reaction was started by addition of 0.02 ml enzyme preparation. After incubation times of 0 and 60 minutes in most cases at 37° C, 20 μl were withdrawn, applied to a filter disc (Whatman No. 1) and processed as described by Bollum (2).

2.9. Analytical Methods

DNA was determined by the method of Kissane *et al.* (13), protein by the biuret reagent of Gornall *et al.* (7).

Preparative centrifugation was performed at 20° C in a 40 rotor of the Spinco model L for 90 hours at 30,000 r.p.m. The initial density of the CsCl was adjusted to 1.700 g/cm^3; CsCl was dissolved in 15 mM Tris-HCl (pH 8.0). Gradients were analysed by collecting 37 fractions by displacement with saturated CsCl solution. The optical density was measured with a Zeiss PMQ II spectrophotometer. For determination of radioactivity samples of 50 μl were dissolved in 10 ml instagel and counted in a scintillation counter (TriCarb Packard Inst., La Grange).

2.10. Evaluation of Differential Inhibition of Cellular DNA and Herpesvirus DNA Synthesis

DNA was extracted from virus infected rabbit kidney cells. The two DNA species (cellular and viral DNA) were separated by CsCl density gradient (see there). The total radioactivity of the different DNA peaks obtained was calculated using the areas of the Gaussian curves (14).

The ratio specific activity of cellular DNA to specific activity of viral DNA can indicate the differential inhibition of DNA-synthesis in case a particular substance had been added.

3. Results

3.1. Influence of PEA on DNA-Synthesis in Intact Rabbit Kidney Cells

3.1.1. Inhibition of Synthesis in Uninfected Rabbit Kidney Cells

Table 1 shows the inhibition of DNA-synthesis by PEA in uninfected cells expressed by a decrease in specific activity (^{3}H-thymidine incorporation per mg DNA extracted from cells). It is evident that the DNA synthesis of uninfected cells is already reduced to 41% by 0.55 mg PEA/ml.

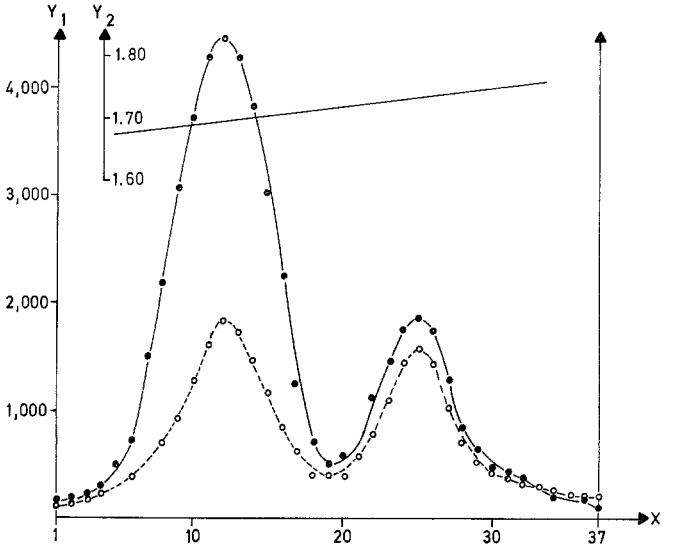

Fig. 1. Buoyant densities of rabbit kidney cell DNA and herpesvirus DNA
DNA was isolated 7 hours after infection. 0.1 mg DNA per tube were centrifuged (see under methods). 37 fractions (0.4 ml) were collected. The buoyant density is given (———). For radioactivity 50 µl aliquots were counted and their activities in dpm are plotted for DNA isolated from controls (●——— ●) and for an assay incubated with 0.8 mg/ml PEA (○ – – – ○) added 2 hours after virus infection. x-axis: number of fractions. y_1-axis: radioactivity in dpm/50 µl. y_2-axis: buoyant density in g/cm³. The left peak corresponds to host DNA

Table 1. *Dependence of Specific Activity of DNA Isolated from Rabbit Kidney Cells on Different PEA Concentrations*

Noninfected cells and cells infected with herpesvirus were analysed. After removal of nonadsorbed virus particles, 10 µCi thymidine methyl-^{3}H and different concentrations of PEA were added to the assays. 5 hours later the cells were harvested and DNA was extracted. The specific activity of DNA was determined (in dpm/mg DNA and in per cent, respectively)

Material	Concentration of PEA (dpm/mg)	Specific activity of DNA (dpm/mg)	(%)
Noninfected rabbit cells	0	$5.9\times10^6\pm0.2$	100 ± 3
	0.55	$2.4\times10^6\pm0.1$	41 ± 4
	0.8	$1.7\times10^6\pm0.1$	29 ± 6
	1.0	$1.5\times10^6\pm0.1$	25 ± 7
	1.3	$1.4\times10^6\times0.1$	24 ± 7
Herpesvirus infected rabbit kidney cells	0	$4.6\times10^6\pm0.2$	100 ± 4
	0.55	$2.5\times10^6\pm0.1$	54 ± 4
	0.8	$1.9\times10^6\pm0.1$	41 ± 5
	1.0	$1.6\times10^6\pm0.1$	35 ± 6
	1.3	$1.2\times10^6\pm0.1$	26 ± 8

3.1.2. Inhibition of DNA Synthesis in Rabbit Kidney Cells, Infected by Herpesvirus

The specific activity of DNA extracted from virus infected cells is higher at comparable concentrations of PEA than in uninfected cells (Table 1). This indicates that the amount of DNA produced by herpesvirus shows a smaller decrease in incorporation rate than cellular DNA. This assumption could be checked after separation of the two DNA fractions in a CsCl gradient (Fig. 1). The average buoyant density of cellular DNA is determined to 1.695 g/cm^3 in comparison to herpesvirus DNA, which is 1.727 g/cm^3. Figure 1 shows the distribution patterns of DNA extracted from kidney cells without and with 0.8 mg PEA/ml added. The radioactivity in the host DNA peak from untreated cells is 5.9×10^4 dpm, that from PEA treated cells 1.5×10^4 dpm. The respective radioactivity of virus DNA in the assays without PEA is 1.5×10^4 dpm and in the assay treated with PEA 1.2×10^4 dpm. The resulting ratio of radioactivity of DNA from treated to untreated assays for the cellular DNA is 0.39 and for the virus DNA 0.80. From this it can be concluded that by 0.8 mg PEA/ml cellular DNA-synthesis is inhibited stronger (61%) than the viral DNA-synthesis (20%). This result is supported by a dose-response experiment (Table 2). In these experiments it is evident that primarily the cellular DNA-synthesis is blocked by low PEA concentrations. In the concentration range of 0.55—1.30 mg/ml the cellular DNA-synthesis is inhibited stronger (33—48%) than viral DNA-synthesis.

3.2. Influence of PEA on DNA-Dependent DNA Polymerases

3.2.1. Inhibition of the Cellular Enzyme

The optimal temperature of the cellular enzyme in the *in vitro* assay with denatured as well as with native DNA is near 37° C (Fig. 2). The PEA inhibition experiments were conducted at this temperature. The inhibition of the cellular enzyme in the assay using heat-denatured DNA is of the non-competitive type, with an inhibition constant of $K_I = 0.83$ mg PEA/ml (Fig. 3).

Table 2. *Differential Inhibition of DNA Isolated from Herpesvirus Infected Rabbit Kidney Cells by PEA*

PEA and thymidine were added as described in legend of Table 1. DNA was extracted from noninfected and herpesvirus infected cells. For details of DNA extraction and CsCl gradient centrifugation see under methods. In each centrifugation tube about 0.1 mg DNA were run

Concentration of PEA (mg/ml)	^{3}H-thymidine incorporation into cellular DNA (dpm/peak)	^{3}H-thymidine incorporation into viral DNA (dpm/peak)	^{3}H-thymidine incorporation ratio: cellular DNA / viral DNA (absolute)	(%)
0	32.0×10^4	12.2×10^4	2.62	100
0.55	14.0×10^4	11.1×10^4	1.26	48
1.0	7.6×10^4	8.7×10^4	0.87	33
1.3	5.1×10^4	4.7×10^4	1.09	42

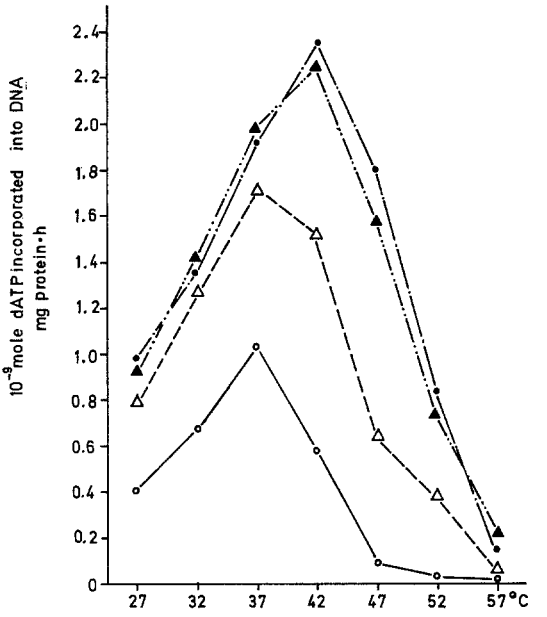

Fig. 2. Effect of temperature on cellular and virus-induced DNA dependent DNA polymerase
The cellular enzyme was tested *in vitro* with heat-denatured (○) as well as with native (△) DNA as template. Activity of virus-induced enzyme was also tested with heat-denatured (●) and with native (▲) DNA. The assays were performed under template saturating conditions (200 µg/ml)

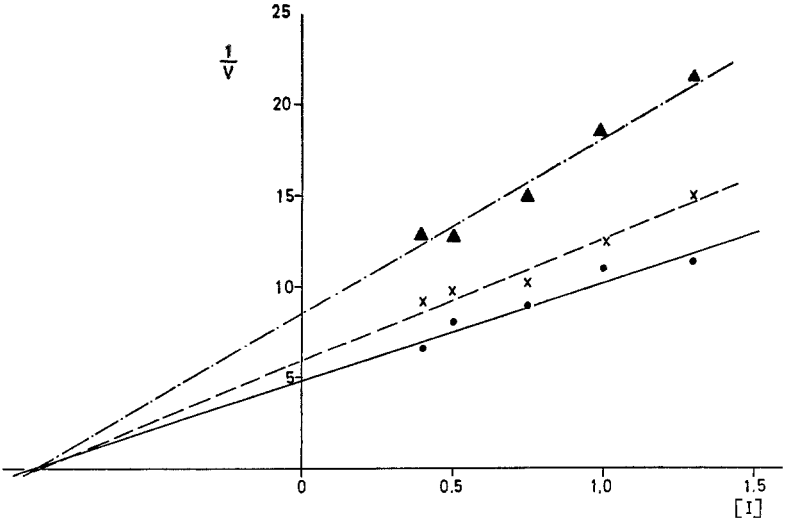

Fig. 3. Inhibition of rabbit kidney DNA-dependent DNA polymerase by PEA

Plot according to Dixon *et al.* (5). x-axis: [I], inhibitor concentrations in mg PEA per ml; y-axis: $\frac{1}{v}$, reciprocal values of the initial reaction velocity in $10^9 \cdot h \cdot ml \cdot moles^{-1}$ dATP. The assay was performed under non-saturating template concentrations with heat-denatured DNA. Linear regressions: ▲— · —▲, template concentration: 150 µg DNA per ml; × – – ×, template concentration: 100 µg DNA per ml; ●———●, template concentration: 50 µg DNA per ml; $K_i = 0.83 \pm 0.47$ mg PEA per ml; $K_m = 92 \pm 12$ µg DNA per ml

3.2.2. Inhibition of Virus Induced Enzyme

Contrary to results of KEIR *et al.* (10), the DNA-dependent DNA polymerase induced by *herpesvirus hominis* in our experiments is inhibited by $MgSO_4$ and KCl (Table 3). Therefore in the inhibition experiments buffers free of KCl and $MgSO_4$

Table 3. *Influence of $MgSO_4$ and KCl on Cellular (Non-Infected) and Virus-Induced DNA-Dependent DNA Polymerase*

The crude preparations were dialysed against 4 mM $MgSO_4$ and 150 mM KCl for 10 hours at 2° C. The enzyme activities of the treated and untreated fraction are compared. The assays were performed under template saturating conditions (200 µg/ml)

DNA-dependent DNA polymerase		10^{-9} mole incorporation into DNA / mg · h
Cellular enzyme	Without dialysis	11.4
	Dialysed against $MgSO_4$ and KCl	1.4
Virus induced enzyme	Without dialysis	15.3
	Dialysed against $MgSO_4$ and KCl	1.4

were used. The virus-induced polymerase shows no significant preference for neither native nor heat-denatured DNA (Fig. 2). In contrast to cellular DNA-dependent DNA polymerase the optimal temperature in the incubation assay for native and for heat-denatured DNA is about 42° C. In order to make the results compatible with the cellular enzyme, the inhibition experiments of virus-induced enzyme are performed at both their own temperature optimum (42° C) and at 37° C (Table 4). The inhibitory concentrations determined for the virus-induced

Table 4. *Quantitative Comparison of PEA Action on Virus-Induced DNA-Dependent DNA Polymerase*

The PEA concentration that causes a 50% reduction in incorporation rate has been determined under the conditions of template saturation (200 µg/ml). As template native as well as heat-denatured DNA has been used. For heat-denatured DNA the 50% inhibition has been determined at 37° and 42° C. The values are derived from 8 independent experimental series

DNA template	Incubation temperature (°C)	PEA concentration (±S.D.) Causing 50% inhibition (mg/ml)
Heat-denatured	42	8.15±2.93
Native	42	8.50±3.12
Heat-denatured	37	7.15±2.71

enzyme are 10 times higher than those found for the cellular enzymes. Apparently the inhibition in the range of the temperature optimum of the enzyme at 42° C (50% inhibition: 8.15 mg PEA/ml) is less sensitive as at 37° C (50% inhibition: 7.15 mg/ml).

4. Discussion

The results presented demonstrate the inhibition of the DNA biosynthesis from *herpesvirus hominis* in rabbit kidney cells by PEA. However, the inhibition of viral DNA-synthesis is less than that of cellular DNA-synthesis. The cellular synthesis is inhibited by 50% at 0.65 mg/ml while the same reduction for viral synthesis is observed at 1.20 mg/ml.

In the present experiments a yield of only 20% has been achieved for extraction of DNA from uninfected and infected cells. Nevertheless, cellular and viral DNA respectively are most likely represented correctly because similar dose-responses were obtained by simultaneous incorporation measurements using the Schmidt-Thannhauser-method. In addition, higher extraction yield of DNA from Herpesvirus infected cells by different modifications (e.g. diethylpyrocarbonate 2%) which resulted in a higher overall yield led to the same viral/cellular DNA ratio.

From investigations of Higgins *et al.* (8) it is known, that at high PEA concentrations the lysosomal membranes become modified. They could demonstrate that at 5 mg PEA/ml enzymes are released from lysosomes. Up to now no data are available on the dose-response relationship of PEA on lysosome stability.

Nevertheless also at concentrations below 5 mg/ml a release of lysosomal enzymes may be supposed to occur, followed by a solubilization of the DNA (16). On the other hand, the results of Leach *et al.* (16) indicate that this effect may not be important at conditions inhibitory for cellular DNA synthesis (0.65 mg PEA/ml) in our system, possibly in contrast to conditions at the higher PEA concentrations inducing a decrease for viral DNA-synthesis.

The experiments discussed below using isolated virus-induced DNA-dependent DNA polymerase support this suggestion. They point to a decrease of cellular DNA-synthesis by inhibition of cellular DNA-dependent DNA polymerase by PEA.

The DNA-dependent DNA polymerase from uninfected rabbit kidney cells is inhibited at the same low level as the same enzyme extracted from mouse lymphoma cells and thymus glands (21). The inhibition kinetics (non-competitive type) of the 3 enzyme preparations isolated from different mammalian tissues are very similar. The K_1 value, for non-competitive inhibition corresponds to an inhibitory concentration that reduces the enzyme activity to 50% (5). For the rabbit kidney enzyme a K_i value of 0.83 mg/ml has been obtained. At about the same concentration (0.45 mg/ml) DNA-synthesis of intact cells is reduced by 50% (Table 2). This suggests the inhibition of DNA-synthesis in intact cells by PEA to be caused by DNA-dependent DNA polymerase inhibition.

In contrast to this correspondence of active PEA levels in uninfected cells, there are large differences in the system of viral DNA-biosynthesis: Isolated DNA polymerase is inhibited by 50% only at about 8 mg PEA/ml while the synthesis of viral DNA in intact cells is already inhibited by 50% at 1.20 mg/ml. Thiolgroup inhibitors showing differential inhibitions of the same pattern as PEA in the virus and cell DNA polymerase system have been described by Keir (9). At 1—2 mg/ml PEA concentration Roizman (27) too found the formation of infectious progeny in cells infected with herpesvirus to be inhibited.

It may be speculated that under the influence of PEA less DNA-dependent DNA polymerase is induced in herpesvirus infected rabbit kidney cells. Yet there is no conclusive experimental evidence available. PLAGEMANN (25) has shown that PEA can block the induction of a RNA polymerase by mengovirus. This might be caused by a PEA-induced inhibition of protein synthesis. A similar mechanism could be present in the herpesvirus system, since at concentrations of 1.20 mg/ml, reducing viral DNA-synthesis by 50%, protein synthesis of mammalian cells is markedly reduced (25, 26).

On the other hand, ROIZMAN (27) could not demonstrate any decrease in viral antigen production under conditions inhibitory for viral DNA formation. In view of these inconclusive facts on protein synthesis involvement in differential PEA inhibition, results on template preference of DNA-dependent DNA polymerase may gain some importance. In contrast to the results of MORRISON (20) the viral induced polymerase accepts native DNA and heat-denatured DNA equally well. Since only a crude extract has been used, no conclusive interpretation is possible so far. The finding, however, that the temperature optimum of 42° C is 5° C higher for viral than the one for the cellular enzyme is rather remarkable. This fact together with the results on the differential inhibition of the two DNA polymerases clearly point to the fact that in infected cells a distinct DNA polymerase is induced (11).

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