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REVERSIBLE INHIBITION OF THE REPRODUCTON OF DNA VIRUSES BY GLYCOSIDES OF 2-PHENETHYL ALCOHOL AND RELATED COMPOUNDS

R. WEIL,* G. MAASS,† K. WALLENFELS* and R. HAAS†

Universität Freiburg, Germany

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Abstract—Phenylethyl alcohol and related compounds are able to inhibit the reproduction of DNA viruses but not the reproduction of RNA viruses in tissue culture. Since phenylethyl alcohol and particularly the analogous compounds *p*-nitrophenylethyl alcohol and phenylpropyl alcohol have a high toxicity for tissue cultures, an attempt was made to attain a similar inhibition of virus synthesis but a lower level of toxicity through administration of the glycosides of these aromatic alcohols. For this purpose the following glycosides were synthesized: Phenylethyl galactoside; *p*-Nitrophenylethyl galactoside; Phenylpropyl galactoside; Phenylethyl glucoside; Phenylethyl glucuronide. The corresponding glycosidases, β-galactosidase, β-glucosidase and β-glucuronidase could be detected and their activity measured in monkey kidney and HeLa cell tissue cultures. In addition, it was possible to localize the enzyme activities histochemically by using glycosides of 4-methylumbelliferone. The effect of the synthesized glycosides on the reproduction of DNA viruses (Vaccinia virus, Herpes simplex virus, SV-40) and RNA viruses (Poliovirus type 1) was investigated. The efficacy of the glycosides is dependent upon the type of glycone. The galactosides have the greatest inhibitory effect while the glucuronide of phenylethyl alcohol shows no effect at all on the replication of Vaccinia virus. The glycosides investigated possess a lower toxicity than the free alcohol; HeLa cells are more sensitive towards the compounds than monkey kidney cells. The use of virus inhibiting compounds in the form of glycosides therefore makes it possible for the active substances to be brought into the cell in a non-toxic form and to be transformed into the active compound by the cell enzymes.

IN NUMEROUS investigations in the last years it has been shown that almost all acid hydrolases which have been found to occur in mammalian cells are localized in cytoplasmic particles, the lysosomes. All agents which alter the permeability of the lysosome membrane effect an activation of the enzymes.[1, 2] Infection of the cells *in vitro* with various DNA and RNA viruses also leads to changes in the permeability of the lysosomes.[3–7] The first stage of this process of infection is characterized by an increased permeability of the membrane for certain substrates of the hydrolases. In the second stage the lysosomal enzymes are released into the cytoplasm, where a distinct increase in the activity of acid hydrolases can be observed.[5]

From these findings the question arose as to whether the activation of the lysosomal glycosidases could be used to liberate an inhibitor of virus synthesis administered in form of its glycoside. In this way the virus inhibitor would be formed intracellularly

* Chemisches Institut.
† Hygiene-Institut.

at the site of virus synthesis, and it would be expected that a particular efficacy of the inhibitor and a smaller degree of toxicity for the entire cell population would be attained. In order to test this hypothesis, several glycosides of phenylethyl alcohol were synthesized and the inhibitory effect of these compounds on the synthesis of some DNA viruses in tissue culture was investigated. Phenylethyl alcohol, as well as other aromatic alcohols, inhibits the reproduction of DNA viruses such as Herpes simplex-, Vaccinia- and SV-40-Virus but does not affect the synthesis of Poliovirus type 1.[8, 9] Little is known about the mechanism of this inhibition. In *E. coli* it could be shown that phenylethyl alcohol selectively inhibits DNA synthesis.[10, 11] A more exact investigation of this effect, however, led to the conclusion that not the course of the DNA synthesis itself but a step at the beginning of chromosome replication is blocked.[12, 13] In contrast to these findings it has been shown by other authors that the compound preferentially inhibits the synthesis of messenger RNA[14, 15] or of total RNA.[16] An effect of phenylethyl alcohol on the physico-chemical properties of calf thymus and bacterial DNA cannot be demonstrated.[15, 17] Experiments in which an attempt was made to reverse the effect of phenylethyl alcohol by simultaneous administration of *p*-aminobenzoic acid or phenylalanine, which have a certain structural similarity to the aromatic alcohol, were unsuccesful. From these findings it was concluded that phenylethyl alcohol interfere neither with the synthesis nor with the utilization of these metabolites.[15] All investigations, however, are in agreement that the inhibitory effects are completely reversible when certain concentrations of phenylethyl alcohol are used, so that no irreversible damage occurs in the microorganisms and animals cells treated with this compound.

MATERIALS AND METHODS

Virus. Vaccinia virus, which was isolated from the commercial smallpox vaccine from the Bayerische Landesimpfanstalt, Herpes simplex virus (strain Hofmeister), SV-40 (strain Vac/111/L) and Poliovirus type 1 (strain Mahoney).

Tissue cultures. All investigations were carried out in cultures of HeLa cells and in primary kidney tissue cultures of *Cercopithecus aethiops*, which were prepared by treatment with trypsin and set up in prescription bottles or in tissue culture tubes. A 0·5% lactalbumin hydrolysate (Difco Co., Detroit) in Hanks solution, supplemented with 5% calf serum served as a growth medium. For the preservation of the tissue cultures after inoculation with virus the same medium, but without calf serum, was used.

List of abbreviations used:

Phenylethyl alcohol	2-phenylethyl alcohol
phenylpropyl alcohol	3-phenylpropyl alcohol
p-nitrophenylethyl alcohol	2-(p-nitrophenyl)-ethyl alcohol
phenylethyl galactoside	2-phenylethyl-β-D-galactoside
phenylpropyl galactoside	3-phenylpropyl-β-D-galactoside
p-nitrophenylethyl galactoside	2-(*p*-nitrophenyl)-ethyl-β-D-galactoside
phenylethyl glucoside	2-phenylethyl-β-D-glucoside
phenylethyl glucuronide	2-phenylethyl-β-D-glucuronide
phenylethyl thiogalactoside	2-phenylethyl-β-D-thiogalactoside
p-nitrophenylethyl thiogalactoside	2-(p-nitrophenyl)-ethyl-β-D-thiogalactoside
methylumbelliferone	4-methylumbelliferone
methylumbelliferyl galactoside	4-methylumbelliferyl-β-D-galactoside
methylumbelliferyl glucoside	4-methylumbelliferyl-β-D-glucoside
methylumbelliferyl glucuronide	4-methylumbelliferyl-β-D-glucuronide
o-nitrophenyl galactoside	*o*-nitrophenyl-β-D-galactoside
o-nitrophenyl glucoside	*o*-nitrophenyl-β-D-glucoside

Inoculation. After a confluent layer has been formed in the prescription bottles, the cells were washed once with Hanks solution and each flask were inoculated with 2 ml of the virus suspension. The multiplicity of infection was between 0·1 and 1 $TCID_{50}$ per cell in case of SV-40 and about 10 $TCID_{50}$ per cell in case of Vaccinia-, Herpes simplex-, and Poliovirus type 1 virus. With SV-40 the adsorption time was 2 hrs., with the other viruses mentioned above 1 hr at 37°. After removal of the rest of the inoculum the tissues were washed several times and 20 ml of maintenance medium was added. This medium contained the various concentrations of the glycosides under investigation.

Determination of virus concentrations. 96 Hr after infection the tissue cultures were repeatedly frozen (−60°) and thawed (37°), the cell debris was removed by centrifugation and the supernatent liquid was used for the determination of infectivity. Dilution series in $\log_{10}$-steps were set up with Hanks solution and were used to inoculate tissue culture tubes containing monkey kidney tissue cultures. The inoculation dose was 1 ml per tissue culture tube and 5 tubes per dilution step were used. Calculation of the virus titer was carried out according to the method.[18]

Immunfluorescence. For detection of virus-specific antigen in the infected cells with fluorescein-labelled antibodies the indirect method[19] was applied. We used an anti-vaccinia serum which was obtained by immunization (four biweekly intramuscular injections followed by two i.v. injections 2 weeks apart of a partially purified vaccinia-virus suspension) of rabbits and an anti-rabbit serum from duck (three weekly injections of 5 mg rabbit γ-globulin up to a total of 150 mg antigen). The coupling of fluorescein isothiocyanate with the anti-rabbit γ-globulin was carried out according to the procedure;[20] the unreacted fluorescein was removed by filtration on sephadex G-25. Infected cover slips cultures were washed in phosphate buffered saline, fixed in cold acetone, washed again and covered with antivaccinia serum for 20 min at room temperature. After additional repeated washings the labeled anti-rabbit γ-globulin was added and the material was bedded in buffered glycerol.

Complement fixation test (CFT). HeLa cell cultures were infected with vaccinia virus. After 72 hr the nutrient medium was withdrawn from the cells, the cultures were broken up by repeated freezing and thawing and were taken up in medium, and the cell debris was removed by centrifugation. The supernate served as the antigen for the CFT. The reaction was carried out according to the Kolmer technique with an overnight incubation at 4°. The highest antigen dilution which showed more than a 50 per cent visible inhibition of hemolysis is designated as the antigen titer.

Histochemical detection of glycosidases. Cover slip cultures of monkey kidney cells were incubated for 10 min at 37° in a nutrient medium containing methylumbelliferyl galactoside, methylumbelliferyl glucoside or methylumbelliferyl glucuronide in a concentration of 6×10^{-3} M. Subsequently the cells were washed repeatedly with phosphate buffered glycerol. For the fluorescence microscopic investigation a Zeiss fluorescence microscope and an Osram HBO-200 mercury high pressure lamp were used. In control experiments the cover slip cultures were fixed in buffered osmium tetroxyde for 15 min before adding the substrate.

Determination of enzyme activities. The cell material from 10 prescription bottles was used for these determinations. The cell layer was washed with distilled water, peeled off with a rubber scraper, and placed in a Potter–Elvejhem homogenizer with 15 ml of distilled water. The cells were homogenized for 5 min with cooling in ice. The homogenate was used for the enzyme tests without prior centrifugation. The

activity of β-galactosidase and β-glucosidase was determined according to the procedure.[21] The hydrolysis of phenylethyl galactoside was measured under the same conditions with 10^{-2} M of the galactoside as substrate. After a 2-hr incubation at 37° the test tube was heated for 5 min in a boiling water bath. After centrifugation the supernatant liquid was brought to pH 7·0 by addition of 6 N sodium hydroxide. The liberated galactose was determined with the aid of galactose dehydrogenase from Pseudomonas saccharophila according to the procedure.[22] The activity of β-glucuronidase was measured according to the method.[23] The enzyme activities are given in terms of moles of hydrolysed substrate/mg of protein/hr. Protein was determined according to the procedure.[24]

Synthesis of glycosides. Phenylethyl galactoside was prepared from α-acetobromogalactose and phenylethyl alcohol in the presence of Ag_2O according to the method[25] and subsequent deacetylation with sodium methylate. The same procedure was used for the synthesis of the following analogous *O*-glycosides.

p-Nitrophenylethyl galactoside

m.p. 155–156°	$(\alpha)_{546}^{22}$: −7·1 (c = 2·37;H_2O)	
Anal.: $C_{14}H_{19}O_8N$	Calcd.: 51·06% C	5·86% H
(329·3)	Found: 51·00% C	5·94% H

Phenylpropyl galactoside

m.p. 100–102°	$(\alpha)_{546}^{22}$: −6·4 (c = 2·5;H_2O)	
Anal.: $C_{15}H_{22}O_6$	Calcd.: 60·39% C	7·43% H
(298·3)	Found: 60·26% C	7·54% H

Phenylethyl glucoside

m.p. 120–122°	$(\alpha)_{546}^{22}$: −36·0 (c = 2·5;H_2O)	
Anal.: $C_{14}H_{20}O_6$	Calcd.: 59·15% C	7·03% H
(284·3)	Found: 59·05% C	7·18% H

Phenylethyl glucuronide

This compound was synthesized from 2,3,4-triacetyl-l-bromoglucuronic acid methyl ester[26] and phenylethyl alcohol. The free glucuronide as prepared according to the method described.[27]

m.p. 154–155°	$(\alpha)_{546}^{22}$: −60·0 (c = 2·4:H_2O)	
Anal.: $C_{14}H_{18}O_7$	Calcd.: 56·37% C	6·08% H
(298·3)	Found: 56·39% C	6·25% H

Methylumbelliferyl galactoside and methylumbelliferyl glucoside were synthesized according to the method described,[28] methylumbelliferyl glucuronide was prepared according to the procedure given.[29] Phenylethyl thiogalactoside was prepared from 2,3,4,6-tetraacetyl-l-thiogalactose and 2-phenylethyl iodide following the procedure described.[30] *p*-Nitrophenylethyl thiogalactoside was synthesized according to[31], *o*-nitrophenyl galactoside and *o*-nitrophenyl glucoside were prepared according to the method given.[32] Phenolphthalein glucuronide was a product of SERVA, Heidelberg.

RESULTS

Determination of enzyme activities

The use of glycosides of phenylethyl alcohol and related alcohols presupposes the presence of the corresponding hydrolases in the cell cultures used. The measurements of the lysosomal enzymes β-galactosidase, β-glucosidase and β-glucuronidase in homogenates of monkey kidney tissue cultures and HeLa cells are given in Table 1. It can be seen that activities of the three enzymes investigated can be detected in monkey kidney cells. The values obtained agree in magnitude with corresponding data for mammalian tissues in the literature.[33, 21] The galactoside of phenylethyl alcohol is a good substrate for β-galactosidase. γ-Galactonolactone, a specific inhibitor,[34] effects a 50 per cent inhibition of the enzyme with *o*-nitrophenyl galactoside as a substrate and a 65 per cent inhibition with phenylethyl galactoside as a substrate.

TABLE 1. ACTIVITY OF β-GALACTOSIDASE, β-GLUCOSIDASE AND β-GLUCURONIDASE IN MONKEY KIDNEY CELLS AND HELA CELLS

Enzyme	Substrate	Activity·10⁷* Kidney cells	HeLa cells
β-Galactosidase	oNPGal	0·81	1·97
	oNPGal + γ-lactone 10^{-2} M	0·44	n.d.
	PEGal	0·46	n.d.
	PEGal + γ-lactone 10^{-2} M	0·15	n.d.
β-Glucosidase	oNPGlu	0·16	0·13
β-Glucuronidase	PPGluc	0·32	0·80

* given in moles of hydrolyzed substrate/mg of protein/hr. The activities were measured according to the methods described in.[21, 22, 23]

Abbreviations: oNPGal : *o*-nitrophenyl-β-D-galactoside
PEGal : 2-phenylethyl-β-D-galactoside
oNPGlu : *o*-nitrophenyl-β-D-glucoside
PPGluc : phenolphthalein-β-D-glucuronide
γ-lactone : γ-galactonolactone

A β-thioglycosidase which has been reported to occur in various animal tissues[35] could not be detected. The enzyme, which hydrolyzes thioglycosides to form an aglyconic thiol, should produce galactose and phenylethyl mercaptan from phenylethyl thiogalactoside. In experiments with this compound as a substrate under conditions of the test given,[35] no galactose could be detected either by the enzymatic method described[22] or by the gas chromatographic procedure described.[36] In order to determine whether the substrate might have been hydrolyzed to form thiogalactose and phenylethyl alcohol, the enzymatic test mixture was extracted with diisopropyl ether and the ether was examined by gas chromatography for the presence of phenylethyl alcohol. This procedure also gave no indication of an enzymatic hydrolysis of the thiogalactoside.

Histochemical detection of the glycosidases

Since for the hydrolases under investigation there are substrates available which contain a fluorescing aglycone, an attempt was made to observe the enzyme activities *in vivo*. Therefore the galactoside, glucoside and glucuronide of 4-methylumbelliferon, which can be detected by its fluorescence after the enzymatic hydrolysis of the glycosides, were synthesized. When cover slip cultures of monkey kidney cells were

incubated with solutions of these glycosides and examined under a fluorescence microscope, the pictures shown in Figs. 1, 2 and 3 were obtained. As the pictures show, the glycosides used are taken up and hydrolyzed *in vivo*. The location of the enzymatic reaction is marked by the fluorescence of the liberated 4-methylumbelliferone.

In control experiments the cover slip cultures were fixed with osmium tetroxide before incubation with the substrates. This treatment completely inactivates the hydrolases, so that in these experiments no hydrolysis of substrates could be detected. No fluorescence could be detected in cultures incubated without substrate.

Effect of phenylethyl galactoside on the reproduction of Vaccinia virus, Herpes simplex virus, SV-40 and polio virus Type I

In a first experiment the effect of increasing concentrations of phenylethyl galactoside on the production of infectious Vaccinia virus in tissue cultures of monkey kidney and HeLa cells was examined. The results are shown in Fig. 4. It can be seen from these results that phenylethyl galactoside leads to an inhibition of the virus synthesis, that the inhibition is dependent on the concentration of the inhibitor in the medium, and that the concentration necessary for a maximum effect is different in the two types of cells. Concentrations above $2{\cdot}25 \times 10^{-2}$ M in the case of monkey kidney cells and above $7{\cdot}5 \times 10^{-3}$ M in the case of HeLa cells caused toxic damages and led to vacuolization, rounding and detachment of cells. Since γ-galactonolactone is a very effective inhibitor of animal β-galactosidase and strongly inhibits the hydrolysis of phenylethylgalactoside, an attempt was made to reverse the inhibition of the virus synthesis by addition of the lactone. A 10^{-2} M concentration of the lactone in the medium did not, however, affect the action of 10^{-2} M phenylethyl galactoside on the reproduction of the virus. This result is probably due to the transformation of the lactone into the free acid, which occurs at pH > 7.[37]

Addition of phenylethyl galactoside to cultures of monkey kidney cells which had been infected with poliovirus type 1 had no effect on the yield of infectious particles. On the other hand the reproduction of Herpes simplex virus and SV-40 was inhibited

TABLE 2. INFLUENCE OF DIFFERENT CONCENTRATIONS OF PHENYLETHYL GALACTOSIDE ON THE REPRODUCTION OF VACCINIA VIRUS, HERPES SIMPLEX VIRUS, SV-40 AND POLIO VIRUS TYPE I.

Concentration Phenylethyl galactoside	Vaccinia	Herpes simplex	SV-40	Polio virus Type I
0	5·7	4·2	4·8	8·2
$0{\cdot}3 \times 10^{-2}$ M	5·5	3·7	4·8	8·3
$0{\cdot}7 \times 10^{-2}$ M	5·5	3·2	4·5	8·3
$1{\cdot}3 \times 10^{-2}$ M	4·6	2·5	3·5	8·2
$2{\cdot}0 \times 10^{-2}$ M	2·7	1·5	2·6	7·7

(Virus concentrations 72 hr p.i. given in terms of neg. log. TCD_{50}/1 ml).

in the same manner as Vaccinia virus (Table 2). Phenylethyl galactoside thus has the same action spectrum as the free alcohol,[8, 9] but higher concentrations of the glycoside are necessary to achieve the same degree of efficacy. The cytopathic effect in the infected tissue cultures is inhibited along with the virus synthesis. The production of

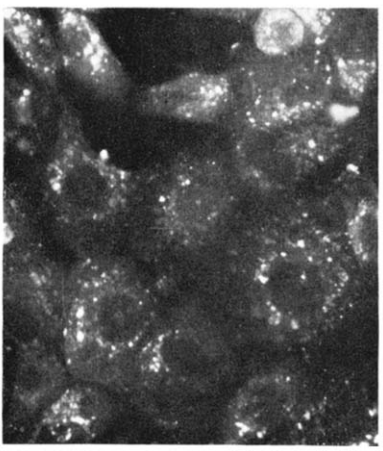

FIG. 1. Histochemical demonstration of β-galactosidase in monkey kidney cells.

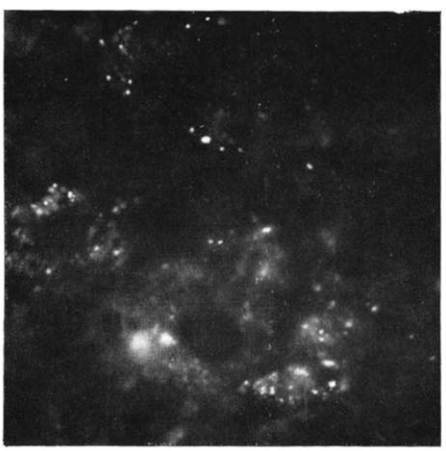

FIG. 2. Histochemical demonstration of β-glucosidase in monkey kidney cells.

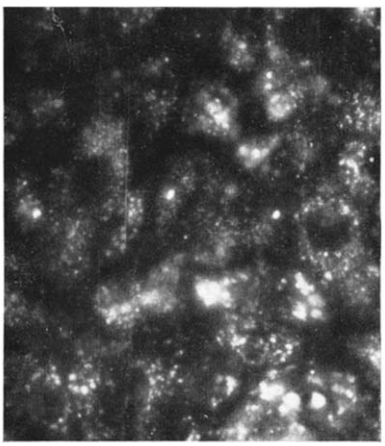

FIG. 3. Histochemical demonstration of β-glucuronidase in monkey kidney cells.

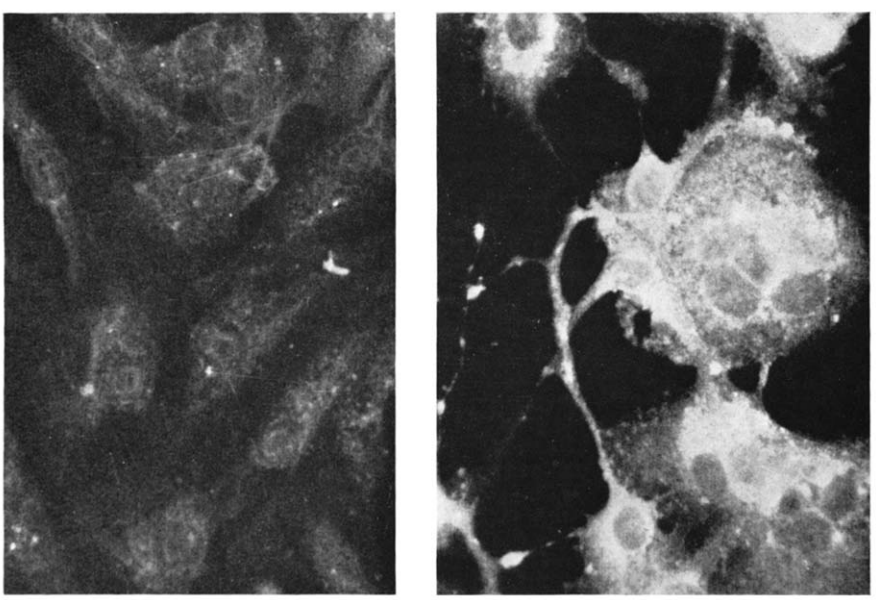

FIG. 5. Detection of viral antigen in HeLa cells with fluorescein-labeled antibodies.
(a) 48 hr p.i. in the presence of $7{\cdot}5 \times 10^{-3}$ M phenylethyl galactoside.
(b) 48 hr p.i. without phenylethyl galactoside.

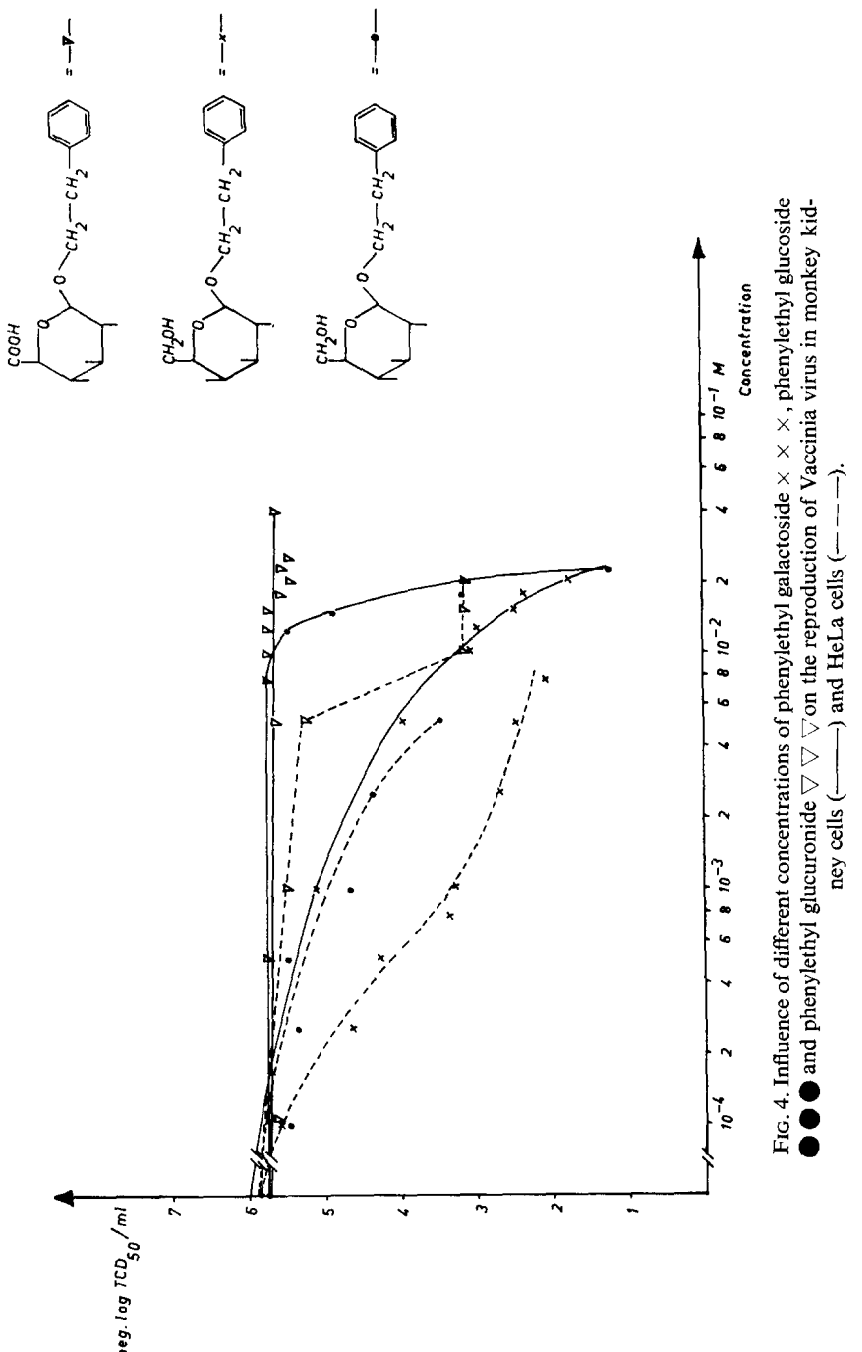

FIG. 4. Influence of different concentrations of phenylethyl galactoside × × ×, phenylethyl glucoside ●●● and phenylethyl glucuronide ▽ ▽ ▽ on the reproduction of Vaccinia virus in monkey kidney cells (——) and HeLa cells (— — —).

the virus-specific coat protein is greatly reduced too. In infected HeLa cells no viral antigen can be detected in the presence of $7{\cdot}5 \times 10^{-3}$ M phenylethyl galactoside by the indirect method applying an anti-vacciniavirus serum from the rabbit and a fluorescein-conjugated anti-rabbit serum from the duck (Fig. 5). A corresponding result is obtained from the detection of the Vaccinia viral antigen in infected HeLa cells with the aid of the complement fixation test. As shown in Table 3, the synthesis of this antigen, demonstrable by CF-test, decreases with increasing concentrations of the inhibitor.

TABLE 3. CONCENTRATION OF VACCINIA VIRUS ANTIGEN IN HELA CELLS DETERMINED BY CF-TEST.

Concentration Phenylethyl galactoside	Antigen titer (CF-test)
0	1:16
$1{\cdot}0 \times 10^{-4}$ M	1:16
$2{\cdot}5 \times 10^{-4}$ M	1:8
$5{\cdot}0 \times 10^{-4}$ M	1:4
$1{\cdot}0 \times 10^{-3}$ M	1:4
$2{\cdot}5 \times 10^{-3}$ M	1:2
$7{\cdot}5 \times 10^{-3}$ M	negative

Different concentrations of phenylethyl galactoside were added to infected cell cultures after the adsorption time of the virus. 96 Hr after infection the cells were destroyed by repeated freezing and thawing and the antigen titer in the liquid supernate was determined by CF-test applying an anti-vaccinia serum from rabbits.

From these results it can be concluded that the virus induced protein synthesis in the cell is also blocked by phenylethyl galactoside.

After it has been ascertained that the galactoside inhibits the reproduction of DNA viruses, experiments were carried out to find whether this inhibition is reversible. Prescription bottles with monkey kidney tissue cultures were inoculated in the usual way with Vaccinia virus, and at the end of the adsorption time maintenance medium containing $2{\cdot}25 \times 10^{-2}$ M phenylethyl galactoside was added. After 48 hr the medium was removed and fresh nutrient medium without phenylethyl galactoside was added. In every case the cell cultures began to synthesize infectious viruses. After certain time intervals prescription bottles were taken out and frozen, whereupon the virus concentration was determined. The results of this experiment are shown in Fig. 6. The virus synthesis set in again with full activity when the phenylethyl galactoside was removed from the cell culture. When the cells were incubated with phenylethyl galactoside for 24 hr before infection and were then infected and suspended in medium without inhibitor, no difference in the virus synthesis compared to that in control cultures could be observed.

Activity of the galactosides of 3-*phenylpropyl alcohol and* p-*nitrophenylethyl alcohol.*

Earlier investigators had examined various compounds which have a structural similiarity to phenylethyl alcohol for a comparable inhibitory action.[15, 38, 39] The

most effective analogous compounds proved to be 3-phenylpropyl alcohol, methoxyphenylethyl alcohol and cinnamic alcohol. When used in cell cultures, however, 3-phenylpropyl alcohol showed such a high degree of toxicity that virus-inhibiting concentrations could not be attained.[9] Therefore this alcohol seemed suitable to find out whether a compound administered in the form of its glycoside has a smaller

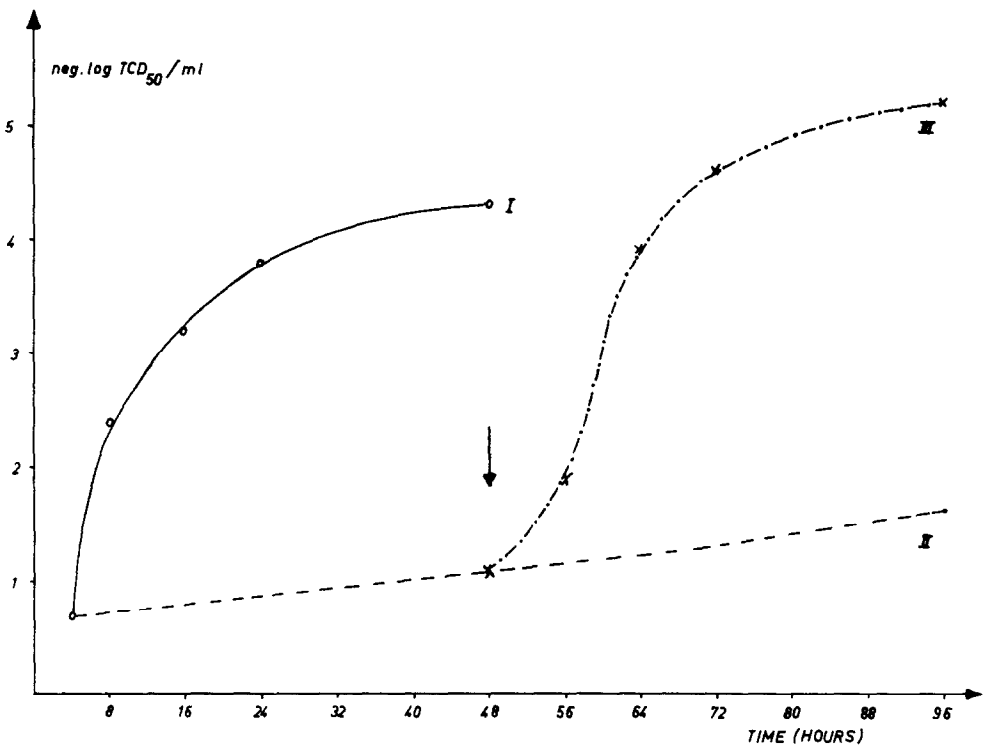

FIG. 6. Reversibility of the inhibition of Vaccinia virus reproduction caused by phenylethyl galactoside in monkey kidney cells.

I Normal reproduction curve of Vaccinia virus.
II Reproduction of Vaccinia virus in the presence of $2{\cdot}25 \times 10^{-2}$ M phenylethyl galactoside.
III Reproduction of Vaccinia virus after removal of phenylethyl galactoside (48 hr p.i.)

toxicity for the cell culture and a greater therapeutic effect than the free compound. A further glycoside investigated was the galactoside of p-nitrophenylethyl alcohol, which had also been observed in preliminary experiments to be relatively toxic. This substance was of interest for the reason that the previously investigated group of phenylethyl alcohol analogues modified in the nucleus and the side chain did not include a nitro compound. The effect of phenylpropyl galactoside and *p*-nitrophenylethyl galactoside on the reproduction of Vaccinia virus is shown in Fig. 7.

It can be seen that both galactosides can be used in cell cultures in spite of the high toxicity of the free alcohols. The inhibitory effect is comparable to that of phenylethyl galactoside and the toxicity is only slightly higher.

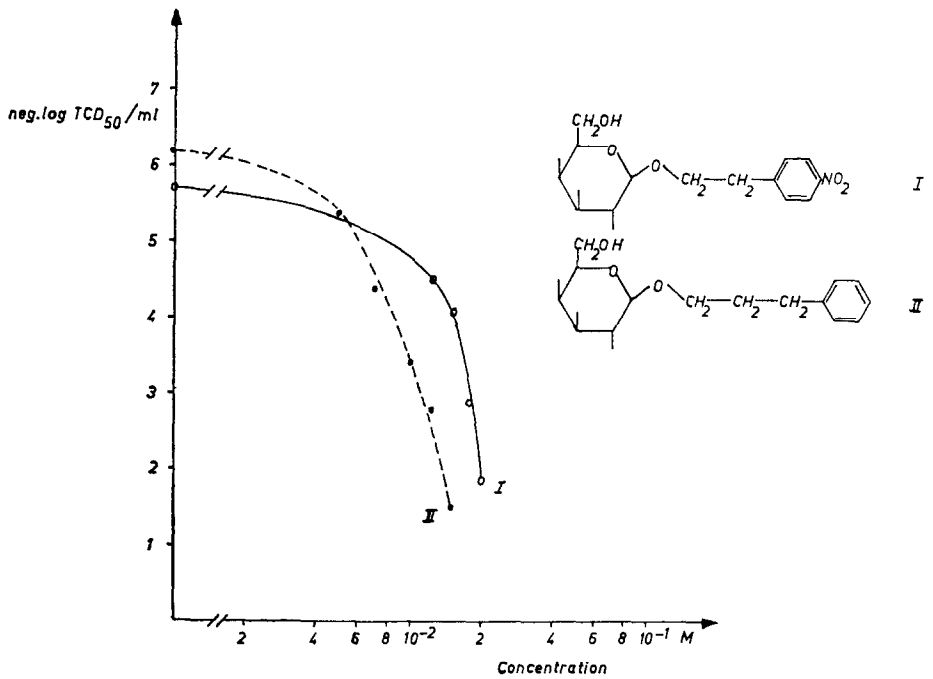

FIG. 7. Inhibition of Vaccinia virus reproduction in monkey kidney cells by various concentrations of *p*-nitrophenylethyl galactoside (I) and phenylpropyl galactoside (II).

Activity of phenylethyl glucoside and phenylethyl glucuronide

Since β-glucosidase and β-glucuronidase activity have been detected in monkey kidney and HeLa cells, it would be expected that phenylethyl glucoside and phenylethyl glucuronide have virus-inhibitory activity.

In Fig. 4 the effect of these compounds on the reproduction of Vaccinia virus in monkey kidney and HeLa tissue cultures is shown. In the case of the glucoside the maximum concentration which can be used in both cell types is comparable to the maximum concentration of the galactoside; at lower concentrations, however, the inhibitory effect of the glucoside is significantly smaller than the inhibition caused by the galactoside. This difference is particularly marked in the case of monkey kidney cells, in which concentrations of the glucoside under 10^{-2} M practically no longer inhibit virus synthesis. In contrast to phenylethyl glucoside the corresponding glucuronide showed no inhibitory activity and no toxic effect in monkey kidney cells in concentrations up to 4×10^{-2} M, whereas in HeLa cells the glucuronide inhibited virus reproduction by 3 logs in concentrations upwards of 10^{-2} M. However, at these concentrations slight degenerative changes in the cells can already be observed, so that the decrease in virus synthesis is possibly due to a damage of cell metabolism.

Activity of thiogalactosides

Phenylethyl thiogalactoside is not hydrolyzed in homogenates of monkey kidney cells. It can therefore be assumed that this compound as well as the analogous *p*-nitrophenylethyl thiogalactoside is not hydrolized *in vivo* either. In fact no inhibition

of virus reproduction could be observed upon administration of these thiogalactosides. Phenylethyl thiogalactoside does, however, cause toxic damage of the cell cultures in concentrations above $2{\cdot}5 \times 10^{-2}$ M. This effect is not observed with the less soluble analogous nitro compound.

In contrast to phenylethyl thiogalactoside, ethyl thiogalactoside has no toxic effect in concentrations up to $1{\cdot}5 \times 10^{-1}$ M. It can therefore be concluded that the toxicity of phenylethyl thiogalactoside is not due to the thioglycoside moiety but is dependent on the presence of the phenylethyl residue in the molecule.

DISCUSSION

The above results show that glycosides of phenylethyl alcohol and related compounds inhibit the production of infectious DNA viruses in cultures of HeLa and monkey kidney cells. This effect is due with great probability to the intracellular liberation of the aromatic alcohol, since the presence of the corresponding glycosidases β-galactosidase, β-glucosidase and β-glucuronidase in the investigated cell types can be demonstrated. The activities of β-galactosidase and β-glucuronidase are higher in HeLa cells than in monkey kidney cells. This finding is in agreement with the observation that the activity of the lysosomal glycosidases is often particularly high in tumor tissue. In contrast the activity of β-glucosidase is the same in both cell types and is considerably lower than that of β-galactosidase in both cases.

The enzymatic hydrolysis of the phenylethyl glycosides was investigated only in the case of the galactoside. Phenylethyl galactoside is easily hydrolyzed in homogenates of monkey kidney cells; the reaction is considerably inhibited by γ-galactonolactone, a specific inhibitor of β-galactosidase. The activity of the investigated glycosidases can also be detected in tissue cultures with the aid of glycosides of 4-methylumbelliferone. When cover slip cultures of monkey kidney cells are treated with methylumbelliferyl galactoside, methylumbelliferyl glucoside or methylumbelliferyl glucuronide, the activity of the corresponding glycosidases appears localized in fine granula. These granula are similar to lysosomes vitally stained with euchrysin.[40, 45] Thus the fluorescence technique confirms the intracellular distribution of these enzymes which has been demonstrated by various authors with fractionation methods[41, 42] or histochemical investigations.[43] A localisation of β-glucuronidase in the cytoplasm which has been demonstrated in fixed cells,[44] cannot be observed with methylumbelliferyl glucuronide as a substrate. Since the fluorogenic substrates are obviously hydrolyzed by the lysosomal hydrolases *in vivo*, an activation of the enzymes apparently is not necessary. This observation is not consistent with the well known latency of the lysosomal enzymes, which can be detected histochemically only after fixation of the cells. The question therefore arises as to whether the glyocosides of methylumbelliferone overcome the permeability barrier of the lysosome membrane especially easily. It is known that certain dyes and fluorescing substances are concentrated in the lysosomes *in vivo*,[40, 45] and it may be possible that derivatives of methylumbelliferone are taken up by the lysosomes in a similar manner.

Thioisologs of the *O*-galactosides, which are not hydrolyzed enzymatically, cannot inhibit the reproduction of Vaccinia virus. This fact is a further indication that the activity of the phenylethyl glycosides is dependent on the presence of the corresponding glycosidases. Apparently the free OH group of the aromatic alcohol is important for the inhibitory effect; the structure of the phenylethyl residue alone is not sufficient.

Since the glycosides have an inhibitory effect only after enzymatic hydrolysis, it is to be expected that the efficacy of the glycoside is dependant on the activity of the corresponding glycosidase. This expectation seems to be justified in the case of the glycosides investigated. In HeLa cells which have a greater β-galactosidase activity than monkey kidney cells, the maximum inhibitory effect of phenylethyl galactoside is attained at concentrations which are considerably lower than those required to produce the maximum effect in monkey kidney cells. A $7{\cdot}5 \times 10^{-3}$ M concentration of the galactoside has the same effect in HeLa cells as a $2{\cdot}25 \times 10^{-2}$ M concentration in kidney cells; the toxic concentrations are lower for HeLa cells than for kidney cells.

The inhibition of virus synthesis caused by phenylethyl glucoside is comparable to that caused by the galactoside, but the effect of the glucoside is smaller, and at low concentrations there is almost no inhibition. This result is consistent with the small activity of β-glucosidase in the tissue cultures investigated. Whereas the efficacy of the different galactosides and glucosides has a certain relationship to the activity of the corresponding glycosidases, phenylethyl glucuronide surprisingly has no effect at all on virus reproduction. The toxicity of the compound is very low. In monkey kidney cells no toxic damage can be detected even in the presence of a 5×10^{-2} M concentration of the glucuronide, whereas the same concentration of the analogous galactoside and glucoside very quickly leads to vacuolization, rounding and detachment of the cells. HeLa cells seem to be slightly more sensitive, but here too concentrations can be used which in the case of the galactoside or glucoside would lead to the destruction of the cell culture within hours.

These findings are not consistent with the high activity of β-glucuronidase found in homogenates of monkey kidney and HeLa cells. Moreover, the histochemical demonstration of the enzyme with methylumbelliferyl glucuronide proves that glucuronides are taken up by the cells *in vivo* and are hydrolysed in the lysosomes. Therefore it can be assumed that phenylethyl glucuronide also gets into the cell, but it is not possible to determine the extent of permeation and enzymatic hydrolysis solely on the basis of these experiments. The possibility may exist that only small amounts of the phenylethyl glucuronide penetrate into the lysosomes and that effective intracellular concentrations of phenylethyl alcohol are not attained. Since numerous toxic substances are transformed into glucuronides, it seems reasonable that the uptake of these detoxicated products should be inhibited. Another explanation for the weak inhibitory effect of phenylethyl glucuronide is that this compound may not be a good substrate for β-glucuronidase. The activity of the enzyme was determined with phenolphthaleine glucuronide which is an excellent substrate, but phenylethyl glucuronide may be hydrolyzed to a much smaller extent.

No conclusions about the mechanism of action of the aromatic alcohols can be drawn from the observations reported. The fact that these compounds effect a characteristic reversible inhibition of the synthesis of DNA viruses but do not affect the reproduction of RNA viruses is in agreement with the original suggestion[10] that these substances preferentially affect DNA synthesis.

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