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# Uptake of Duck Hepatitis B Virus into Hepatocytes Occurs by Endocytosis but Does Not Require Passage of the Virus through an Acidic Intracellular Compartment

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**The infectious entry pathway of duck hepatitis B virus (DHBV) was investigated with primary duck hepatocytes. Virus uptake was measured by a selective PCR technique which allows for the detection of a successful infection without the need for viral replication or gene expression. To test whether DHBV uptake occurs by endocytosis, the effects of energy depletion were analyzed. The requirement for an acidic intracellular pH was tested with the lysosomotropic agent ammonium chloride. The data show that energy depletion prevents the uptake of DHBV into primary hepatocytes whereas ammonium chloride has no effect. From these data, we conclude that DHBV is taken up by its host cells by endocytosis. However, in contrast to that of most other enveloped viruses, escape of DHBV from the endocytotic route does not depend on an acidic intracellular compartment.**

Despite considerable efforts, information about the early events of the hepadnaviral life cycle, especially the interaction with the cellular receptor, the penetration step, and the uncoating process, is still scarce. This dearth is mostly due to the fact that the primary hepatocytes of the natural host are the only cells which can be infected with a hepadnavirus in tissue culture. Of the four hepadnaviruses which have been well characterized to date—the human, woodchuck, ground squirrel, and duck hepatitis B viruses (DHBV)—the duck virus is the only virus for which such cells can be obtained with the efficiency which is required for systematic studies (1, 9). Therefore, most of the previous work and also the experiments discussed here were carried out with this model virus.

Since hepadnaviruses belong to the group of enveloped viruses, one obvious question is whether they enter the host cell by direct fusion with the outer cell membrane or whether the penetration step is preceded by endocytosis. To date, two reports which tried to answer this question indirectly by analyzing the requirement for a low intracellular pH for DHBV infection (6, 7) have been published. In both studies, primary duck hepatocytes were infected with DHBV in tissue culture in the presence of several widely used lysosomotropic agents. However, conflicting results were obtained. Offensperger et al. (6) found a strong reduction of DHBV infection if the hepatocytes were treated with chloroquine or ammonium chloride. This result would suggest that DHBV is taken up by endocytosis and fuses with the membrane of the endocytotic vesicle after acidification. On the other hand, Rigg and Schaller (7) observed a normal infection in the presence of ammonium chloride or monensin. This finding would be compatible with both direct fusion with the outer cell membrane or pH-independent fusion after endocytosis. The reasons for these contradictory findings are unclear.

Here, we have examined this issue by a PCR technique which allows the successful detection of DHBV uptake without the need for replication or gene expression (1). In particular, we directly addressed the question of endocytosis by testing the effects of energy depletion. The data show that DHBV enters primary duck hepatocytes by endocytosis but that the virus does not require an acidic intracellular compartment to escape from the endocytotic pathway.

## MATERIALS AND METHODS

**Primary hepatocyte cultures.** The method used for the preparation of primary hepatocytes was described previously (1). Liver tissue was obtained from duck embryos and digested with 0.5% collagenase (type CLS II; Biochrom KG, Berlin, Germany) in 5 ml of serum-free Williams' E medium (Gibco, Eggenstein, Germany) supplemented with 2 mM L-glutamine, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.2]), 100 U of penicillin per ml, 100 μg of streptomycin per ml, $10^{-5}$ M hydrocortisone, 1 μg of insulin per ml, and 1.5% dimethyl sulfoxide (hepatocyte medium; all reagents were from Sigma, Deisenhofen, Germany) for 20 min at 37°C. After two washes with 10 ml of medium, the cells were resuspended in medium, seeded onto 35-mm-diameter culture dishes (six-well plates), and cultivated at 37°C with 5% $CO_2$. The medium was first changed 30 min after seeding, and further medium changes were done daily. The amount of cells seeded per well was estimated so that after about 2 days a confluent cell layer was obtained.

**DHBV infection. (i) General protocol.** Cells were infected the day after seeding by adding 20 μl of DHBV-positive duck serum per dish in 2 ml of hepatocyte medium. The serum contained about 100 pg of DHBV DNA per μl as determined by dot blot analysis.

**(ii) Infection in the presence of sodium azide–2-deoxy-D-glucose.** The cells were preincubated with 0.1% sodium azide and 50 mM 2-deoxy-D-glucose (Sigma, Deisenhofen, Germany) for 1 h at 37°C. Virus was added directly to the medium containing the inhibitors, and the cells were incubated for various times at 37°C to allow for virus uptake.

**(iii) Inactivation of external virus.** To inactivate membrane-bound virus, the cells were incubated for 2 min with glycine buffer (50 mM glycine, 150 mM NaCl [pH 2.2]). The cells were washed, normal medium was added, and the cultures were further incubated at 37°C.

**(iv) Removal of external virus by trypsin digestion.** The inoculum was removed, the cells were washed with medium, and 2 ml of 0.5% trypsin solution per 35-mm-diameter dish was added. The cells were incubated for 1 min at room temperature, the trypsin solution was removed, and the cells were incubated for an additional 5 min at 37°C without medium. The cells were then resuspended in medium, transferred to Eppendorf tubes, pelleted by centrifugation, and analyzed by PCR as described below. Trypan blue staining revealed that the viability of the cells after trypsin treatment was better than 95%.

**(v) Infection in the presence of ammonium chloride.** Stock solutions for each experiment were freshly prepared in water. The infection protocols are given in the figure legends. In some experiments, 2′,3′-dideoxyguanosine (ddG; Pharmacia, Freiburg, Germany) was added to the cultures at a final concentration of 20 μg/ml.

**FPV and HSV-1 infection.** Confluent primary duck hepatocyte cultures were infected with fowl plague virus (FPV; generously provided by G. Herrler, Uni-

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versity of Marburg) and herpes simplex virus type 1 (HSV-1) with or without the addition of ammonium chloride (0 to 30 mM). The cells were preincubated with the drug at 37°C for 1 h, after which the virus inocula were added. Infection was allowed to proceed for 16 h in the continuous presence of the drug. Total RNA was prepared with the Qiagen RNeasy kit according to the instructions of the manufacturer. Aliquots of each sample corresponding to 5 μg of total RNA were separated on a 1% agarose–0.7% formaldehyde gel, blotted onto nylon membranes, and hybridized with an FPV- or HSV-1-specific $^{32}$P-labelled probe.

**Preparation of tissue culture samples for PCR.** Primary duck hepatocytes grown in a 35-mm-diameter dish were digested in 0.25 ml of lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 0.45% Tween 20, 0.45% Nonidet P-40) with 0.1 mg of proteinase K per ml for 2 h at 56°C. The proteinase K was inactivated by heating the samples for 10 min at 95°C, and a 5-μl aliquot was used for PCR.

**PCR amplification.** Viral DNA sequences were amplified with the following oligonucleotide primers: GCG CTT TCC AAG ATA CTG GAG CCC AA (P1; nucleotide positions 1426 to 1451), CTG GAT GGG CCG TCA GCA GGA TTA TA (P2; nucleotide positions 2445 to 2420), and CCC TGT GTA GTC TGC CAG AAG TCT TC (P3; nucleotide positions 2843 to 2818). Primer pair P1-P2 amplifies both the open circular DNA and the repaired viral DNA with the same efficiencies, whereas primer pair P1-P3 selectively amplifies the repaired DNA (1). A single PCR sample (total volume, 50 μl) contained 5 μl of cell lysate, 50 pmol of each primer, 2.5 U of *Taq* polymerase (Perkin-Elmer, Langen, Germany), 0.2 mM (each) deoxynucleoside triphosphates, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.01% gelatin. The samples were subjected to 25 amplification cycles (of 1 min at 94°C and 3 min at 72°C). The annealing step was left out to reduce self-annealing of the amplification products. After amplification, 10 μl of each sample was separated on a 1% agarose gel and analyzed by ethidium bromide staining and Southern blotting.

## RESULTS

**Infection of hepatocytes with DHBV is independent of intracellular pH.** Viruses can penetrate the membrane barrier of a cell either directly or after endocytosis (3). To examine which route is used by the hepadnaviruses, we first tested whether the infection of duck hepatocytes with DHBV is sensitive to a lysosomotropic agent. If infection can be blocked with such a drug, the virus must be endocytosed, since acid-dependent fusion always takes place in an intracellular compartment, usually an endosome. To clarify this point, we performed infection experiments with an experimental system which is based on the detection of repaired viral DNA. Since this repair reaction is complete within 24 to 48 h, all potential problems which can arise from prolonged incubation of the cells with the drug or the amplification of small amounts of viral DNA because of virus which was taken up in spite of the inhibitors can be avoided.

The most widely applied lysosomotropic agent is ammonium chloride, because it has a relatively low short-term toxicity. In order to determine the effective dose of this drug for primary duck hepatocytes, we performed infection experiments with FPV. FPV is one of the viruses for which it has been unambiguously demonstrated that membrane fusion occurs only after endocytosis and requires an acidic intracellular pH (4). To test for possible unspecific toxic effects, we performed control infections with HSV-1. For HSV-1, it has been shown that this virus infects cells by direct fusion with the outer cell membrane in a pH-independent manner (10).

As can be seen from Fig. 1A, ammonium chloride very efficiently prevented the infection of primary hepatocytes with FPV. At ammonium chloride concentrations of 10 mM or more, no FPV RNA could be detected. The sensitivity of the Northern (RNA) blot is too low to detect the RNA in the virus inoculum. There was also no visible cytopathic effect. That this finding was not due to an unspecific reduction of cell metabolism can be demonstrated with the HSV-1-infected cells (Fig. 1B). Here, the ammonium chloride had no effect, as was demonstrated by the amount of HSV-1-specific mRNA in the different samples. In summary, the data show that ammonium chloride at concentrations between 10 and 30 mM blocks the infection of primary duck hepatocytes with a virus which requires an acidic intracellular compartment for penetration and does not significantly affect cellular metabolism. We therefore decided to use ammonium chloride to test whether infectious entry of DHBV is pH dependent.

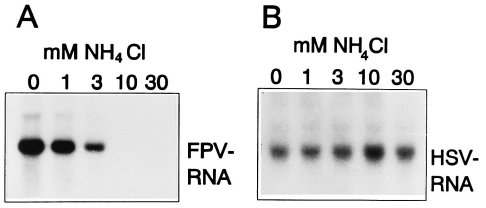


FIG. 1. Infection of primary duck hepatocytes with FPV and HSV-1 in the presence of ammonium chloride. Cells were preincubated for 1 h with ammonium chloride (0 to 30 mM) at 37°C. The virus inocula were added, and the cells were infected for 16 h at 37°C in the continuous presence of the drug. The cells were harvested, total RNA was prepared, and 5 μg of each RNA sample was analyzed by Northern blotting with an FPV- or HSV-1-specific $^{32}$P-labelled probe. (A) FPV-infected cells. (B) HSV-1-infected cells.

We first performed infection experiments in which primary duck hepatocytes were incubated for 30 h with DHBV in the continuous presence of ammonium chloride. Successful virus uptake was measured by PCR detection of covalently closed circular (CCC) DNA, which is formed only after penetration and uncoating of the incoming DHBV particles. This method has the advantage of being able to detect successful virus uptake at a very early time point. To exclude the possibility that CCC DNA could result from amplified DHBV genomes generated from a small amount of virus which entered the cells because the ammonium chloride block was not 100% tight, we also added 20 μg of ddG per ml to the cultures. In a previous study, we have shown that at this concentration, ddG completely prevents the amplification of viral DNA but has no effect on the repair reaction (1). Thus, if infection is performed in the presence of ddG, any CCC DNA detected in the selective PCR must be due to virus which was successfully taken up by the cells and cannot be an amplification product. As can be seen from Fig. 2, ammonium chloride had no inhibitory effect on CCC DNA formation. Thus, in spite of the continuous presence of the lysosomotropic drug, DHBV particles were taken up and the genome was uncoated, transported into the nucleus, and repaired.

**Ammonium chloride can interfere with late steps of DHBV replication.** According to the paper in which an inhibitory

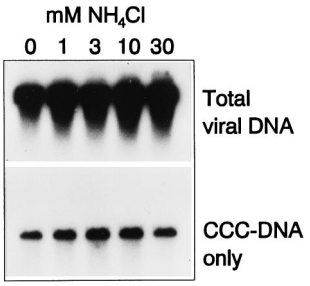


FIG. 2. Uptake of DHBV into primary duck hepatocytes is not affected by ammonium chloride. Hepatocytes were preincubated for 1 h at 37°C with various concentrations of ammonium chloride (0 to 30 mM) and 20 μg of ddG per ml. Twenty microliters of DHBV-positive duck serum was added, and the cells were infected for 6 h at 37°C. The inoculum was removed, and the cells were washed and incubated for another 24 h at 37°C in fresh medium containing the inhibitors. The cells were then harvested and analyzed by PCR as described elsewhere, and the amplification products were detected by Southern blotting. The upper panel shows total viral DNA, and the lower panel shows CCC DNA only.

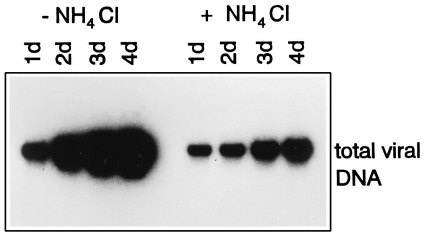


FIG. 3. Ammonium chloride inhibits late steps of DHBV replication. Duck hepatocytes were seeded in 35-mm-diameter dishes and infected with 20 μl of DHBV-positive duck serum per dish for 6 h at 37°C. The inoculum was removed, the cells were washed, and fresh medium was added. After an incubation period of 24 h at 37°C, ammonium chloride was added to a final concentration of 30 mM to half of the dishes; this time point was defined as day 1 (1d). The cells were then further incubated at 37°C, harvested on days 2, 3, and 4, and analyzed by PCR and Southern blotting. The left side of the panel shows total viral DNA of the control infection experiment without ammonium chloride, whereas the right side of the panel shows total viral DNA of hepatocytes maintained in the presence of the drug.

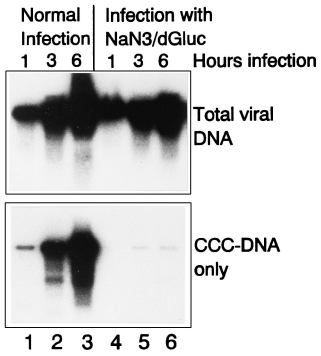


FIG. 4. Uptake of DHBV into primary hepatocytes requires cellular energy. Primary duck hepatocytes were infected for 1, 3, and 6 h with DHBV with or without the addition of sodium azide ($NaN_3$) and 2-deoxy-D-glucose (dGluc). After the infection period, the cells were washed, external virus was inactivated by low-pH treatment, new medium without inhibitors was added, and the cells were harvested 24 h later and analyzed for total (upper panel) and repaired (lower panel) viral DNA by selective PCR. The amplification products were detected by Southern blotting.

activity of ammonium chloride on DHBV infection was described, the primary hepatocytes had been incubated with the drug for up to 7 days (6). To test whether this period of incubation could have resulted in a toxic effect, primary duck hepatocytes were infected with DHBV for 6 h. The inoculum was removed, and the cells were washed and incubated in fresh medium for another 24 h. Ammonium chloride was added (final concentration, 30 mM), and the cultures were further incubated for up to 4 days. Control cultures were treated in the same way, with the only exception that no ammonium chloride was added. The cells were harvested on days 1, 2, 3, and 4 and analyzed by PCR for the presence of total DHBV DNA. As can be seen from Fig. 3, the amount of viral DNA in the ammonium chloride-treated cultures was strongly reduced. This result shows that ammonium chloride can exert a negative effect on DHBV replication if it is present for a very long time. This effect is probably due to negative interference with a late step of DHBV replication, e.g., protein biosynthesis or particle assembly, or to an unspecific toxic effect on cellular metabolism.

**DHBV uptake requires cellular energy.** Since infection of hepatocytes with DHBV was not pH dependent, the question remained whether the virus particles have to be endocytosed before membrane penetration can occur. Endocytosis is an energy-driven, ATP-dependent process and therefore can be blocked with inhibitors of ATP synthesis. Therefore, we examined the effects of energy depletion on the uptake process.

Cells in tissue culture generate energy not only by oxidative phosphorylation but also by glycolysis and also exhibit various susceptibilities to drugs which block these pathways. To establish conditions which inhibit ATP synthesis as completely as possible, primary duck hepatocyte cultures were treated with sodium azide and 2-deoxy-D-glucose, which block oxidative phosphorylation and glycolysis, respectively. Control experiments in which a plaque reduction assay with FPV, whose infectivity relies on endocytosis, was used showed that with a combination of both drugs, a more than 90% plaque reduction can be achieved (data not shown).

To test the effect of these drugs on DHBV infection, primary duck hepatocytes were preincubated with sodium azide–2-deoxy-D-glucose for 1 h. Afterwards, DHBV-positive duck serum was added, and the cells were incubated for 1, 3, or 6 h at 37°C with the inhibitors still present. Virus attached to the outside of the cells was inactivated by a brief incubation with a low-pH buffer. New medium without inhibitors was added, and 24 h later, the cells were harvested and analyzed for total and CCC DNA. As can be seen from the top panel of Fig. 4, treatment with sodium azide–2-deoxy-D-glucose did not reduce virus binding (compare lanes 1 to 3 and lanes 4 to 6). However, only in the case of the untreated cells was CCC DNA synthesized, indicating successful uptake of virus particles (Fig. 4 [lower panel]).

Although it appeared most likely that the lack of genome repair was due to a blockade of virus uptake, the possibility remained that the sodium azide–2-deoxy-D-glucose treatment irreversibly damaged the cells. If so, even if virus was taken up during the incubation period, there would be no genome repair after the inhibitors were removed, leading to the false conclusion that virus uptake is energy dependent. This possibility can easily be tested by omitting the low-pH treatment. In this control experiment, the cells were again preincubated with sodium azide–2-deoxy-D-glucose for 1 h and then incubated with DHBV for 1, 3, or 6 h in the presence of the inhibitors. Afterwards, the cells were either treated with the low-pH buffer before normal medium was added or were only washed. Washing the cells removes virus still present in the medium but does not affect virus that has already bound to the cells and that therefore can be taken up during another incubation step at 37°C, which will result in a normal infection. Two days later, the samples were harvested and analyzed for CCC DNA. As can be seen from Fig. 5, there was again only very little detectable CCC DNA if virus bound to the outside of the cells was inactivated before the inhibitors were removed and the medium was changed (upper panel; compare lanes 4 to 6 and lanes 7 to 9; please note that only the results of the CCC DNA analysis are shown). However, if this inactivation step was left out, CCC DNA formation for treated and untreated cells was comparable (Fig. 5 [lower panel]). This result proves that the sodium azide–2-deoxy-D-glucose treatment was fully reversible.

In a second control experiment, we tested the influence of energy depletion on the infection of primary duck hepatocytes with HSV, which penetrates cells by direct membrane fusion (10). The cultures were incubated with HSV ($10^5$ 50% infectious doses as tested with Vero cells) for 1 h at 4°C (adsorption period) and 3 h at 37°C (infection period) with or without the addition of sodium azide–2-deoxy-D-glucose. External virus was inactivated by incubating the cells with low-pH buffer;

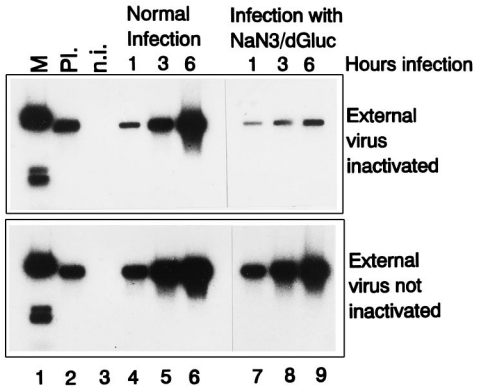


FIG. 5. Energy depletion of primary duck hepatocytes with sodium azide–2-deoxy-D-glucose is fully reversible. Primary hepatocytes were infected for 1, 3, and 6 h with DHBV with or without prior addition of sodium azide ($NaN_3$) and 2-deoxy-D-glucose (dGluc). The cells were washed, and external virus was either inactivated by low-pH treatment (upper panel) or left on the cells (lower panel). New medium without inhibitors was added, and 2 days later, the cells were harvested and analyzed for repaired viral DNA by selective PCR. The amplification products were detected by Southern blotting. M, molecular size marker; Pl., plasmid control; n.i., noninfected control culture.

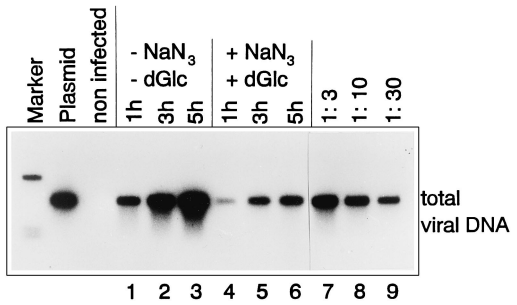


FIG. 7. Energy depletion blocks DHBV uptake. Duck hepatocytes were seeded in six-well plates, and half of the wells were preincubated for 1 h at 37°C with 50 mM 2-deoxy-D-glucose (dGlc) and 0.1% sodium azide ($NaN_3$). Twenty microliters of DHBV-positive duck serum was added to each of the wells, and infection was allowed to proceed at 37°C for 1, 3, and 5 h. The cells were washed, and external virus was removed by trypsin digestion. The cells were then resuspended in medium, transferred to Eppendorf tubes, and lysed. Trypsin-resistant viral DNA was detected by PCR and subsequent Southern blotting. Total viral DNA of duck hepatocytes infected in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of the inhibitors is shown. Lanes 7 to 9 show dilutions of the sample in lane 3.

fresh normal medium was added, and the cells were further incubated at 37°C for 16 h. Total RNA was prepared, and HSV RNA was detected by Northern blotting as described in the legend to Fig. 1. No effect of energy depletion on HSV infection could be observed (Fig. 6). This finding also demonstrates that treatment of the cells with sodium azide–2-deoxy-D-glucose does not exert an unspecific toxic effect on the primary hepatocytes.

In the control experiments described above, we could exclude the possibility that treatment of the primary hepatocytes with sodium azide–2-deoxy-D-glucose for up to 6 h irreversibly damaged the cells. However, the possibility remained that immediately after virus entry, an energy-dependent event has to occur. For instance, it could be imagined that the nucleocapsid protein has to be modified to induce the uncoating process. If this event does not occur shortly after virus entry, the infection might be abortive rather than successful.

To investigate this possibility, we developed an assay which allowed us to quantitate the amount of virus which crossed the membranes of the hepatocytes in the presence or the absence of the inhibitors. If the sodium azide–2-deoxy-D-glucose effect is in fact due to a blockade of virus endocytosis, no or only a minimal amount of virus should be taken up by the cells when

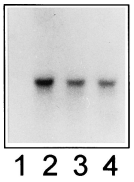


FIG. 6. Energy depletion does not interfere with HSV infection. Primary hepatocytes were seeded in six-well plates and infected with HSV as described in the text. HSV RNA was detected by Northern blotting after 16 h. Lane 1, noninfected control. Lane 2, normal infection. Lane 3 shows cells that were incubated with HSV for 3 h. External virus was inactivated by low-pH treatment, and the cells were further incubated for 16 h at 37°C. Lane 4 shows cells that were preincubated with sodium azide and 2-deoxy-D-glucose for 1 h. Virus was added, and the cells were infected for 3 h at 37°C. The cells were washed, external virus was inactivated by low-pH treatment, and the cells were further incubated for 16 h at 37°C.

ATP synthesis has ceased. Moreover, if no virus is taken up, there can also be no later step which could be affected by the inhibitors.

In this test, primary hepatocytes are incubated with DHBV in the presence and absence of sodium azide–2-deoxy-D-glucose for up to 5 h. External virus is removed by trypsin treatment, and the remaining viral DNA, which must be due to endocytosed virus, is quantitated. However, there are two problems. First, the trypsin digestion must be very efficient. Since DHBV uptake is very slow, only a small fraction of adsorbed virus is taken up during the infection period. To make this fraction visible, nonendocytosed virus must be removed as completely as possible. Second, because of the low amount of trypsin-resistant virus, the viral DNA can only be detected by PCR. This PCR has to be quantitated, which is complicated by the well-known fact that the relationship between the amount of template and the amplification product is not linear.

In pilot experiments, we could show that by the method described here, more than 95% of adsorbed virus can be removed (data not shown). By titration of the DNA samples and quantitative evaluation of the Southern blots with a phosphorimager, we were also able to achieve a very good correlation between the amount of template DNA and the amplification product. We therefore used this assay to test whether in cells treated with sodium azide–2-deoxy-D-glucose, trypsin-resistant viral DNA can be detected. Primary duck hepatocytes were preincubated for 1 h at 37°C either in normal medium or in medium containing the inhibitors. DHBV was added to all of the cultures, and infection was allowed to proceed for 1, 3, and 5 h. Cells were then subjected to protease treatment and analyzed for total viral DNA by PCR. The results are given in Fig. 7 and 8. As is clear from Fig. 7, the amount of trypsin-resistant viral DNA was much reduced after infection was carried out in the presence of the inhibitors (compare lanes 1 to 3 and lanes 4 to 6). To quantitate this effect, the radioactivity in the bands was determined with a phosphorimager. A calibration curve was generated with the sample shown in Fig. 7, lane 3 (no inhibitors and 5-h infection) and the serial dilutions of this sample shown in lanes 7 to 9. This calibration curve then was used to calculate the amount of trypsin-resistant viral DNA after 1, 3, and 5 h of infection. As can be seen from Fig. 8, energy depletion resulted in a reduction in DHBV uptake of

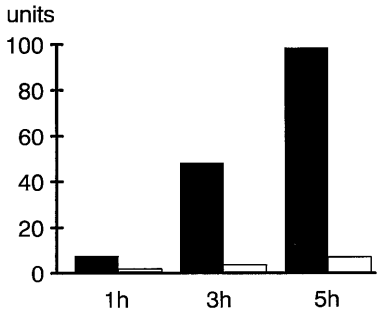


FIG. 8. DHBV uptake in the absence or presence of sodium azide–2-deoxy-D-glucose. After hybridization with a DHBV-specific probe, the Southern blot shown in Fig. 7 was exposed to a phosphorimager, and the intensity of the bands was quantitated by densitometry. The values obtained with samples 3, 7, 8, and 9 were used to generate a calibration curve which was then used to determine the amounts of trypsin-resistant DNA in arbitrary units. Solid bars, normal infection; open bars, infection in the presence of sodium azide–2-deoxy-D-glucose.

more than 90%. From this result, it is obvious that energy depletion almost completely blocks the generation of trypsin-resistant viral DNA. This result is strong independent evidence that DHBV is in fact taken up by the hepatocytes via the endocytotic route. It also excludes the possibility that energy depletion irreversibly blocks an event which has to occur after uptake. It rather blocks uptake itself.

## DISCUSSION

The mechanisms used by viruses to cross cellular membranes are the subject of intensive research (3). Because of the lack of an efficient in vitro infection system, such studies could not be performed with hepadnaviruses for a long time. Only recently, two reports in which the pH dependence of DHBV infection was investigated were published (6, 7). However, contradictory results were obtained.

A potential problem of such studies is that the lysosomotropic drugs which are used to raise the intracellular pH can have severe side effects and also often do not completely block virus penetration. This problem can be of particular importance if the test system requires virus replication to prove an infection. For instance, prolonged treatment of cells with lysosomotropic agents can interfere with late stages of virus assembly (3). Moreover, a small amount of virus which was taken up by the cells in spite of the drug treatment could be extensively amplified. These complications can be avoided if a marker which indicates successful virus uptake even in the absence of genome expression and amplification is used. In the case of the hepadnaviruses, formation of CCC DNA is such a marker.

Using a selective PCR technique, we could show that the efficiency of DHBV CCC DNA formation is not affected by ammonium chloride at concentrations which reliably block the infectious entry of a virus which requires an acidic intracellular compartment for membrane fusion. We could also show that this drug can interfere with the late steps of the DHBV replication cycle. The latter finding can at least in part explain the contradictory results published previously. Whereas Rigg and Schaller (7), who observed no pH dependence of DHBV infection, treated the hepatocytes only for a few hours with ammonium chloride, Offensperger et al. (6) used incubation times of up to several days. Taken together, the available data strongly favor the conclusion that DHBV penetrates the cellular membrane in a pH-independent fashion.

Since we found no evidence that an acidic intracellular compartment is required for DHBV infection, we had to directly test whether DHBV is endocytosed by the hepatocytes. To this end, we analyzed the effects of energy depletion. Our data clearly show that no virus is taken up if ATP synthesis is blocked. In several control experiments, we could exclude the possibility that the cells were irreversibly damaged by the drug treatment or that energy depletion acts on a step which occurs after membrane penetration. We therefore conclude that DHBV, and probably also the other hepadnaviruses, is taken up by host cells by receptor-mediated endocytosis but penetrates the membrane by a pH-independent mechanism.

A pH-independent membrane fusion which requires endocytosis is rather uncommon for enveloped viruses. Usually, fusion either is pH independent and takes place at the outer cell membrane (e.g., with HSV or Sendai virus) or is pH dependent and must be preceded by endocytosis (e.g., with influenza virus or vesicular stomatitis virus). Some viruses, such as human immunodeficiency virus, can alternatively fuse with the cell surface or with internal membranes (2, 8). However, internal fusion is optional and is not essential for the infectivity of human immunodeficiency virus. The only example of which we are aware and for which endocytosis was found to be essential for the infectivity of an enveloped virus which fuses at neutral pH is Epstein-Barr virus. Whereas this virus can infect certain tissue culture cells by direct membrane fusion, infection of normal B cells appears to depend on endocytosis (5). The reason for this differential behavior is unclear. Currently, we can only speculate why DHBV must be endocytosed before penetration of the cellular membrane can occur.

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