*Biochimica et Biophysica Acta* 938 (1988) 334–344
Elsevier

BBA 73893

# Hepatocellular uptake of aflatoxin $B_1$ by non-ionic diffusion. Inhibition of bile acid transport by interference with membrane lipids

N. Müller and E. Petzinger

*Institut für Pharmakologie und Toxikologie der Justus Liebig Universität Giessen, Giessen (F.R.G.)*

(Received 26 October 1987)

Key words: Aflatoxin $B_1$; Membrane transport; Cholestasis; Phallotoxin; Bile acid; (Rat liver)

**Aflatoxin $B_1$ permeates isolated rat hepatocytes by non-ionic diffusion. Its uptake is neither saturable nor influenced by metabolic energy and not inhibited by treatment of cells with proteases. The initial rate of aflatoxin $B_1$ uptake measured at 7°C is between 40 and 50% compared to that at 37°C. However, after an incubation period of 7 minutes identical equilibrium uptake is reached at both temperatures. The apparent activation energies, calculated for aflatoxin $B_1$ uptake by Arrhenius diagrams ranged between 1.69 and 4.5 kcal/mol. A $Q_{10}$ value of 1.34 was calculated for a temperature interval of 7–17°C but decreased to 1.05 for the interval of 27–37°C. Liposomes or lipoproteins added to the cell suspension inhibited the aflatoxin $B_1$ uptake into hepatocytes. Liposomes mainly composed of unsaturated fatty acids bind twice as much aflatoxin $B_1$ as those composed of saturated ones, indicating that the lipophilicity of the mycotoxin is crucial in the determination of its uptake into liver cells. At concentrations above 5 μg/ml, aflatoxin $B_1$ inhibited the carrier-mediated uptake of cholic acid and of phalloidin into hepatocytes. This effect was reversible and abolished by washing the cells after preincubation with aflatoxin. In concentrations below 5 μg/ml the uptake of phallotoxin and cholic acid was however stimulated by 15–25%. These results indicate, that a carrier-mediated uptake into hepatocytes via the multispecific bile salt transporter is not responsible for the organoselective clearance of aflatoxins by the liver. On the other hand, the cholestatic effect of aflatoxin $B_1$ results at least partially from the inhibition of the multispecific bile acid transport system. This inhibition may arise from affinity of aflatoxins to lipid domains of the cell membrane.**

## Introduction

Among the naturally occurring aflatoxins, aflatoxin $B_1$ is the most abundant and toxic one [1,2]. Aflatoxin $B_1$ is carcinogenic for animals [3–5] as well as for man [6]. During chronic exposure hepatocellular carcinomas are frequent, whereas in acute aflatoxicosis bile duct hyperplasia and periportal liver cell necrosis are observed [7]. Aflatoxin $B_1$ is predominantly excreted into bile [8] thereby inducing cholestasis [9]. The hepatic clearance of aflatoxin $B_1$ is very rapid and oral ingestion is followed by first pass elimination. In chicken blood a half-life of radiolabelled aflatoxin $B_1$ of 1.5 min was reported [10]. The rapid clearance by the liver as well as the organ specific disposition of the mycotoxin might be due to a liver cell specific transport mechanism for the uptake of aflatoxins. In favour of this assumption was the finding that in liver slices a correlation between aflatoxin $B_1$ uptake and toxicity was observed. Livers from mice with a higher resistance against aflatoxins took up aflatoxin $B_1$ far more slowly than rat livers [11]. Comparably, primary

Correspondence (present address): E. Petzinger, Max-Planck-Institut für Systemphysiologie, Rheinlanddamm 201, D-4600, Dortmund 1, F.R.G.

liver cell cultures derived from resistant mice showed slower uptake rates when compared to cultured rat hepatocytes [2]. Recently a saturable accumulation of aflatoxin $B_1$ into isolated rat hepatocytes was observed [12]. Although not explicitly proven in the literature, we assumed a carrier-mediated transport of aflatoxins into hepatocytes and speculated that aflatoxins might be taken up into liver cells by the multispecific bile acid transport system, which in the past was found to be responsible for the uptake of several cholephilic xenobiotics [13] and which was responsible for the liver specific toxicity of phalloidin [14,15]. In the present investigation aflatoxin $B_1$ uptake into isolated rat hepatocytes was characterized. For the kinetic measurement of the early phase of aflatoxin $B_1$ uptake the silicon oil centrifugation method was used, which enabled us to measure uptake in 30-s intervals in contrast to a time interval of 5 min in two earlier reports [16,17].

## Methods

*(a) Preparation of hepatocytes*

Male Wistar rats of 260–300 g body weight were kept on a standard diet and water ad libitum. Hepatocytes were prepared using a modification of the method of Berry and Friend [18]. The liver was perfused for 20 min with a calcium-free Krebs-Henseleit-buffer containing 0.05% collagenase (clostridiopeptidase A, EC 3.4.21.4; Boehringer Mannheim, purity grade II). The liver tissue was then disintegrated mechanically for 2 min by introduction of gas bubbles. The yield was $(1–2) \cdot 10^8$ cells per liver. Only 10–15% of isolated hepatocytes took up 0.2% Trypan blue. The cell suspensions were incubated in slowly shaking Erlenmeyer flasks at 37 °C in the presence of 95% $O_2$ and 5% $CO_2$. One ml of the cell suspension contained $2 \cdot 10^6$ hepatocytes, which corresponds to a protein value of $3.91 \pm 0.65$ mg cell protein/ml. The mean cell diameter was $20 \pm 2$ $\mu$m.

*(b) Protein analysis*

Protein concentrations were determined according to the 'biuret method' published by Weichselbaum [19] (standard: bovine serum albumin).

*(c) Uptake experiments*

2 ml of isolated hepatocytes were preincubated in tight glass vessels under permanent gassing with $O_2/CO_2$ (95%/5%) at 37 °C. Thereafter the incubation conditions were modified in temperature, gassing conditions and in the amount of the unlabelled toxin concentration. Hepatocytes were incubated with a constant concentration of [$^3$H]aflatoxin $B_1$ and varied concentrations of the unlabelled compound (1.6–32 $\mu$M). At the indicated times aliquots of 100 $\mu$l of the cell suspensions were withdrawn and the cells were pelleted by rapid centrifugation through silicon oil, according to the method described [20,21]. Silicon oil layer (density 1.0325 g/l) was obtained by mixing silicon oil AR 20 with AR 200 at a ratio of 1 : 3. This technique was used to terminate uptake at the indicated time intervals. The initial velocity of the aflatoxin $B_1$ uptake was calculated from four measured uptake data, obtained during the initial 1–105 s (after 15 s, 45 s, 75 s and 105 s) by the application of logarithmic regression analysis. The resulting curve of the regression analysis was linear for the mentioned time periods and allowed the precise determination of $v_i$ as well as determination of the point of intersection with the ordinate. This intersection point indicates the amount of aflatoxin $B_1$ which was bound at time point 0 s = '$t_0$' value. The logarithmic regression analysis was performed according to the following equations:

$$y = A + B \cdot \ln x$$

with

$$A = \frac{\Sigma y - B \cdot \Sigma \ln x}{n}$$

and

$$B = \frac{n \cdot \Sigma \ln xy \cdot \Sigma \ln x \cdot \Sigma y}{n \cdot \Sigma \ln x^2 - (\Sigma \ln x)^2}$$

Prior to radioactivity count the pelleted hepatocytes were dissolved overnight in 3 M KOH. Radioactivity was measured in a Lipoluma/Lumasolve/$H_2O$ mixture (100 : 10 : 2, v/v, Baker Chemicals, Phillipsburg, NJ, U.S.A.).

Liposomes (dissolved in $H_2O$) as well as lipoproteins (dissolved in $NaN_3$ 0.01%) were added 5

min prior to the incubation of [$^3$H]aflatoxin $B_1$ (3 nM). Since liposomes were dissolved in $H_2O$, controls received the addition of 250 $\mu$l $H_2O$/ml cell suspension. Although decreasing the osmolality of the Tyrode buffer this didn't measurably influence the Trypan blue index of the hepatocyte suspension.

*(d) Experiments in the absence of oxygen*

After a period of 15 min equilibration, hepatocytes were incubated in the presence of $N_2/CO_2$ (95%/5%) for 30 min prior to the addition of [$^3$H] aflatoxin $B_1$ (1.125 nM)/aflatoxin $B_1$ (16 $\mu$M). Aliquots of 100 $\mu$l cell suspension were withdrawn by a syringe through a tight rubber-diaphragma at the incubation vial. For these experiments we used Warbug-type incubation vessels. The complete tightness of the vessels, as well as pregassing the incubation system with nitrogen for at least 30 min was crucial.

*(e) Permeabilization of hepatocytes*

Permeabilization was achieved by freezing the isolated hepatocytes immediately after preparation in liquid nitrogen and subsequent thawing at room temperature. The thawing procedure took at minimum 10 min. Thereafter, at least 99% of the liver cell plasma membranes were permeabilized, as was indicated by staining the hepatocytes with Trypan blue (0.2%). The cells were incubated at 37°C in an $O_2/CO_2$ (95%/5%) atmosphere and were used for uptake experiments.

*(f) Temperature dependence of aflatoxin $B_1$ uptake*

Isolated rat liver cells, preincubated at 37°C, in an $O_2/CO_2$ atmosphere, were incubated subsequently at 7, 17, 27 and 37°C in the presence of [$^3$H]aflatoxin $B_1$ (1.125 nM)/aflatoxin $B_1$ (1.6–32 $\mu$M). The initial velocity of the aflatoxin $B_1$ uptake was calculated from the logarithmic slope of the uptake curves by the application of the logarithmic regression analysis for a $\Delta$ uptake within 1–105 s.

*(g) Measurement of [$^{14}$C]cholate and [$^3$H]phallotoxin uptake*

2 ml of hepatocytes in suspension were incubated with increasing concentrations of aflatoxin $B_1$ (1.6 $\mu$M, 16 $\mu$M and 36 $\mu$M, dissolved in DMSO) for 30 s prior to the addition of a constant concentration of [$^3$H]demethylphalloin (85 nM)/phalloidin (6 $\mu$M) or [$^{14}$C]cholate (1 $\mu$M), respectively. Aliquots of 100 $\mu$l of the cell suspension were withdrawn at the indicated times and the cells collected and separated from the supernatant as described. The final concentration of DMSO was below 2% and had no measurable effect on the uptake kinetics.

In the case of [$^3$H]demethylphalloin-uptake measurements, mixtures of [$^3$H]demethylphalloin (85 nM) with phalloidin (6 $\mu$M) were added to the cell suspension, since unlabelled demethylphalloin was not available. Since demethylphalloin and phalloidin are chemically very similar, phalloidin gives identical uptake results compared to demethylphalloin, if the described mixing ratio is used [22].

*(h) Calculation of the permeability coefficient*

The permeability coefficient $P$ (cm/s) was calculated according to the equation: $-P = I/c$, where '$I$' means the substrate flux indicated in mol · (mg cell protein)$^{-1}$ · min$^{-1}$ for a given concentration difference $\Delta c$. For the calculation of flux '$I$', uptake measurements with increasing aflatoxin concentrations were performed similar to those shown in Fig. 4 and the initial rate of uptake $v_i$ was calculated from the uptake within the first minute by logarithmic regression. The initial rate of uptake was taken as it represents the unidirectional flux of aflatoxin $B_1$ across the membrane. In order to obtain the correct dimension for $P$ which is cm/s the flux dimensions mol · (mg cell protein)$^{-1}$ · min$^{-1}$ had to be transformed in mol · cm$^{-1}$ · s$^{-1}$. For this calculation the following data were taken: mean diameter of hepatocytes is 19.3 $\mu$m [23]; 1 mg cell protein represents $0.5 \cdot 10^6$ hepatocytes (own results) for which a total surface area of 5.85 cm$^2$ was calculated.

*(i) Measurement of the affinity properties of aflatoxin B to lipoproteins and liposomes*

For the determination of the affinity of [$^3$H]aflatoxin $B_1$ to liposomes and lipoproteins a dialysis chamber with a dialysis membrane of pore diameter of $(1.5–2) \cdot 10^{-9}$ m, (penetration limit: 10–15 kDa) was used. The dialysis chamber consists of two compartments with a maximum volume

of 450 µl each. 0,7 µCi $^3$H-AFB$_1$ diluted in double-distilled water (400 µl) was added to one chamber and dialysed against a 5% liposome (10% lipoprotein) solution (400 µl) for 36 h in the dark. The composition of liposomes and lipoproteins was as follows:

(1) Liposomes mainly composed of unsaturated fatty acids: palmitic acid 10–15%, stearic acid 1.5–4%, oleic acid 6–13%, linolic acid 61–71%, linoleic acid 4–7%.

(2) Liposomes mainly composed of saturated fatty acids: palmitic acid 10–15%, stearic acid 85–90%.

(3) High-densitiy lipoproteins (particle size $(90–120) \cdot 10^{-10}$ m): (a) apolipoproteins: 70% $A_I$, 20% $A_{II}$, 1–3% $C_I$, 1–3% $C_{II}$, 5–10% $C_{III}$ (all together 50%).

(b) lipids: 60% phospholipids, 36% cholesterol, 4–10% triacylglycerols (all together 50%).

(4) Low-density lipoproteins: (particle size: 250 $\cdot 10^{-10}$ m): (a) apolipoproteins: 95% B, 5% E (all together 20%).

(b) lipids: 29.5% phospholipids, 58% cholesterol, 12.5% triacylglycerols (all together 80%).

## Materials

[$^3$H]Demethylphalloin (spec. act. 3.5 Ci/mmol = 129 GBq/mmol) was a gift from Professor Dr. H. Faulstich, MPI Heidelberg, F.R.G.; lipoproteins were obtained from Professor Dr. Stoffel, Köln, F.R.G.; liposomes: PPC-Phospholipon 100®, PPC-H-Phospholipon 100 H® from Nattermann, Düsseldorf, F.R.G., [$^{14}$C]cholic acid sodium salt (spec. act. 52 mCi/mmol = 1.92 GBq/mmol) and [$^3$H]aflatoxin $B_1$ (spec. act. 39 Ci/mmol = 1.44 TBq/mmol) were purchased from Amersham Buchler, Braunschweig, F.R.G.; aflatoxin $B_1$, cholic acid sodium salt and valinomycin were purchased from Sigma, Munich, F.R.G. or Sigma, F.O.B., St. Louis, MO, U.S.A.; collagenase and trypsin were from Boehringer, Mannheim, F.R.G., ouabain was from SERVA, Heidelberg, F.R.G. Silicon oil was obtained from Wacker-Chemie, München, F.R.G. and lipoluma/lumasolve from Baker-Chemie, Gross-Gerau, F.R.G.

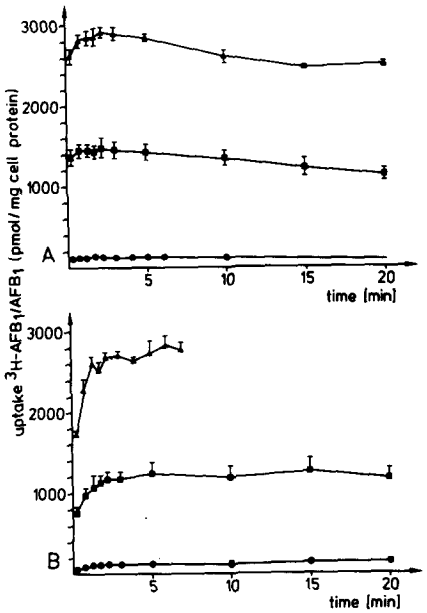



Fig. 1. (A) Kinetics of [$^3$H]aflatoxin $B_1$/aflatoxin $B_1$ at 37 °C, pH 7.4. $2 \cdot 10^6$ hepatocytes/ml cell suspension were kept at 37 °C in 95% $O_2$/5% $CO_2$ atmosphere, suspended in Tyrode buffer. After addition of 1.125 nM [$^3$H]aflatoxin $B_1$ mixed with 1.6 µM (●), 16 µM (■) and 32 µM (▲) aflatoxin $B_1$, aliquotes of 100 µl were withdrawn after the indicated periods. Hepatocytes were immediately separated from the buffer supernatant by rapid centrifugation through silicon oil. The uptake curves represent mean values ($x \pm$S.E.) derived from four experiments. (B) Delayed uptake velocity of aflatoxin $B_1$ by reduction of the incubation temperature to 7 °C. All further experimental conditions are identical to A.

## Results

### *Kinetics of initial transport of aflatoxin $B_1$ uptake into isolated rat hepatocytes*

At 37 °C, $AFB_1$ uptake into isolated rat hepatocytes was very rapid (Fig. 1A). With concentrations of 1.6, 16 and 32 µM aflatoxin $B_1$, uptake is accomplished within 15, 45 and 180 s, respectively. At aflatoxin $B_1$ concentrations below 1.5 µM the kinetic curve reached a steady plateau during an incubation period of 20 min. In the presence of 16 µM and, more pronounced, with 32 µM aflatoxin $B_1$, cell associated radioactivity reached the maximum within 3 min thereafter it declined slowly (Fig. 1A). Thus aflatoxin $B_1$ uptake was biphasic for these concentrations with a subsequent efflux after 3 min. The decline in cell

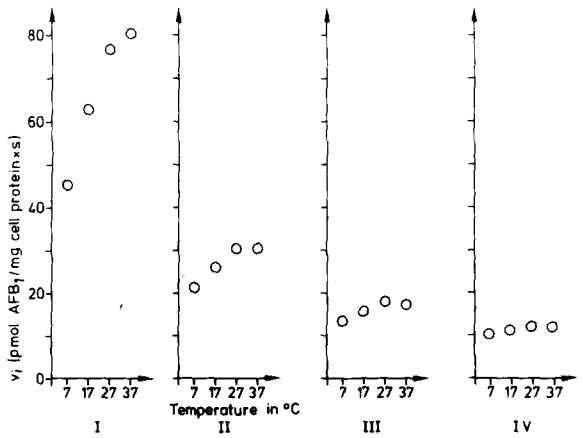



Fig. 2. Effect of temperature on the initial uptake velocity $v_i$, calculated for the time intervals: I (0–15 s), II (0–45 s), III (0–75 s), IV (0–105 s). Note that the time unit is 'second'. The incubation conditions are equal to those described in the legend to Fig. 1. Initial uptake rates were calculated by logarithmic regression analysis from the results shown in Fig. 1.

trapped radioactivity is the result of the release of metabolites of aflatoxin $B_1$. Loss of cell-associated radioactivity was, however, not observed when the incubation temperature was 7 °C (Fig. 1B). Under this condition the kinetic curve was monophasic and the amount of radioactivity taken up within the first minute (0–1 min uptake) was reduced for 17–18% when compared with the uptake at 37 °C. However, at all incubation temperatures it reached identical values within the first 7 minutes.

Aflatoxin $B_1$ uptake was not linear at any time interval within the first uptake minute. This is shown in Fig. 2 for uptake experiments which were performed at four different incubation temperatures. When the measured uptake data during time intervals between 0 and 15 s (Fig. 2 I), 0 and 45 s (Fig. 2 II), 0 and 75 s (Fig. 2 III) and 0 and 105 s (Fig. 2 IV) were calculated as uptake (in pmol aflatoxin/mg cell protein) per second this value increased, as expected, for increasing incubation temperature. However, for a given temperature this uptake per second decreased depending on the time interval which was used for the calculation. The uptake rate was highest when calculated from the uptake during 0–15 s and lowest when calculated from the uptake between 0 and 105 s. Thus any calculation of $v_i$, the uptake rate during the first minute, was obtained by logarithmic regression using the measured data for

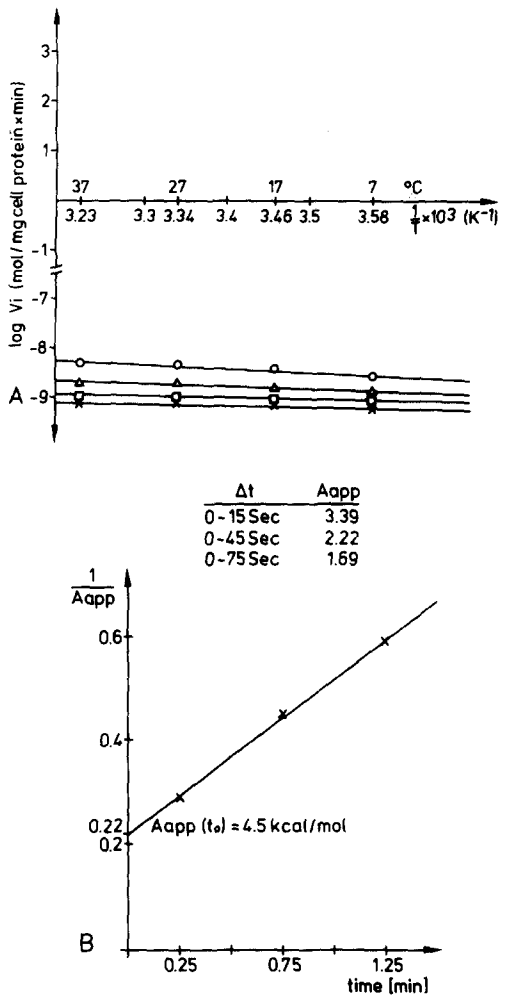



Fig. 3. (A) Arrhenius diagram of the temperature dependence of aflatoxin $B_1$ uptake into isolated rat hepatocytes. Apparent activation energy was determined according to the equation: $2.303 \cdot R \cdot \log v_i \cdot (1/T)^{-1}$; $R$ = gas constant. Aflatoxin $B_1$ initial uptake velocity ($v_i$) was measured as described in the legend to Fig. 2. Note that the time unit is 'min'. The data for log $v_i$ were calculated for the different time intervals: 0–15 s (○), 0–45 s (△), 0–75 s (□) and 0–105 s (×). (B) The reciprocal values of $A_{app}$, calculated according to the equation mentioned above, are plotted versus time. The intersection between the linear curve and the ordinate represents the theoretical $A_{app}$ value for time $t = 0$.

the uptake intervals 0–15 s, 0–45 s, 0–75 s and 0–105 s. The uptake rate $v_i$ increased linearly between 7 and 27 °C but declined between 27 and 37 °C. This increase of $v_i$ between 7 and 27 °C indicates a temperature-sensitive reduction of membrane properties which reduces its character

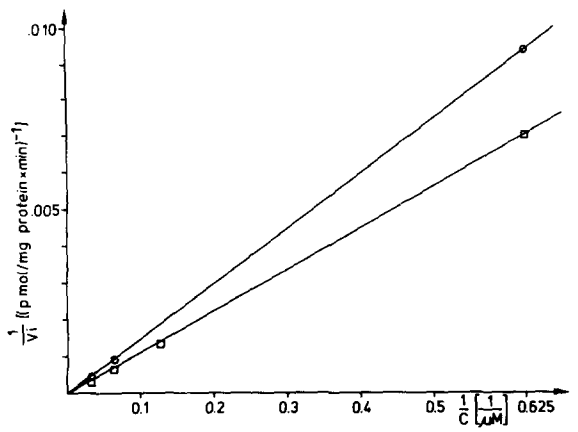



Fig. 4. Lineweaver-Burk plot (double reciprocal) for aflatoxin $B_1$ uptake into isolated hepatocytes at 7 °C and 37 °C. Uptake of aflatoxin $B_1$ was measured in 2 ml hepatocyte suspension with 1.125 nM [$^3$H]aflatoxin $B_1$ plus 1.6, 8, 16 and 32 $\mu$M aflatoxin $B_1$. The reciprocal value of the initial aflatoxin $B_1$ uptake rate, calculated for the first minute after toxin incubation, was plotted versus the reciprocal value of the applied toxin concentration. The results are mean values obtained from at least three experiments performed either at 7 °C (○) or at 37 °C (□). The standard deviation values of $v_i$ range below 10% of the mean values. The intersection of both plots with the zero mark shows nonsaturation for aflatoxin $B_1$ uptake.

of a permeation barrier and thus enhances the permeation rate of aflatoxins. For aflatoxin uptake during a time interval of 0–15 s (the earliest possible measurement), a $Q_{10}$ value of 1.34 was found for 7–17 °C (1.34 gives the increase in the uptake rate for a given time interval (here 0–15 s) if the temperature difference is 10 °C). This value, however, was significantly reduced to $Q_{10}$ of 1.05 for the temperature range between 27 and 37 °C. For the uptake at 37 °C the apparent activation energies for aflatoxin $B_1$ uptake were calculated from Arrhenius diagrams (Fig. 3A). Using different time periods for the calculation of $v_i$ (0–15, 0–45 and 0–75 s), the apparent activation energies increased the shorter the time interval was. For the uptake between 0–15 s $A_{app}$ was 3.39 kcal/mol, for the uptake between 0–45 s $A_{app}$ was 2.22 kcal/mol and 1.69 kcal/mol for the uptake between 0–75 s. When these values were plotted versus time (Fig. 3B) a linear correlation was obtained. By extrapolation at $t = 0$ the apparent activation energy for the initial contact of aflatoxin $B_1$ with the cell membrane was found to be 4.5 kcal/mol.

$v_i$ was plotted according to Lineweaver-Burk (Fig. 4). Saturation kinetics was neither observed at 7 °C nor at 37 °C in contrast to earlier reports [16,17]. Furthermore, uptake of $^3$H-labelled aflatoxin $B_1$ into hepatocytes was not inhibited in the presence of a 3555- and 6400-fold excess of nonlabelled aflatoxin $B_1$, final concentration 16 $\mu$M at an incubation temperature of 37 °C (Table I). These results indicate that aflatoxin $B_1$ permeation across the membrane of isolated hepatocytes is due to physical diffusion without the involvement of saturable carrier proteins.

*Lack of inhibition of aflatoxin $B_1$ uptake by proteases*

Whether membrane proteins are involved in

TABLE I

EFFECT ON [$^3$H]AFLATOXIN $B_1$ UPTAKE OF PREINCUBATION WITH EXCESS NONLABELLED TOXIN

Effect on the [$^3$H]aflatoxin $B_1$ uptake into intact isolated rat hepatocytes by preincubation of the cells with an 3555-fold and an 6400-fold excess of nonlabelled toxin for 5 min. The concentration of nonlabelled aflatoxin $B_1$ was 16 $\mu$M, that of [$^3$H]aflatoxin $B_1$ 2.5 nM (Expt. No. 1) or 4.5 nM (Expt. No. 2). The results are mean values obtained from at least three experiments and are expressed as % inhibition of [$^3$H]aflatoxin $B_1$ uptake in the presence of the nonlabelled toxin concentrations.

| Conditions | fmol/mg cell protein | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 min | | | 5 min | | | 10 min | | |
| | uptake | Δ | % | uptake | Δ | % | uptake | Δ | % |
| 1 control (2.5 nM [$^3$H]$AFB_1$) | 154 ± 37.5 | 8 | 5.1 | 189 ± 40.6 | 26 | 13 | 189 ± 46.8 | 15 | 7.9 |
| plus $AFB_1$ (16 $\mu$M) | 146 ± 21.8 | | | 163 ± 24.3 | | | 174 ± 30.0 | | |
| 2 control (4.5 nM [$^3$H]$AFB_1$) | 430 ± 43.5 | 10 | 2.3 | 501 ± 27.07 | 6 | 1.1 | 495 ± 63.7 | 19 | 3.8 |
| plus $AFB_1$ (16 $\mu$M) | 420 ± 18.4 | | | 495 ± 54.5 | | | 476 ± 34.3 | | |

$AFB_1$ uptake was further tested by an incubation of isolated rat hepatocytes in the presence of 5 $\mu$M trypsin. Trypsin only slightly reduced aflatoxin $B_1$ uptake. $v_i$ was reduced by 3%, the uptake maximum after 105 s was reduced for 12%. Other proteases, e.g. 250 $\mu$g collagenase/ml cell suspension, 3000 IE pepsin/ml or papain (100 $\mu$g/ml) did not reduce $AFB_1$ uptake significantly (not shown).

*Lack of effect of anoxia on aflatoxin $B_1$ uptake into isolated rat hepatocytes*

Incubation of hepatocytes for 30 min under $N_2/O_2$ (95%/5%) atmosphere, did not reduce the uptake of 1.6 $\mu$M aflatoxin $B_1$/[$^3$H]aflatoxin $B_1$ (not shown). Under the same experimental conditions the uptake of cholic acid, which is an active, carrier-mediated transport and which was measured as an internal test for anoxia effects on active transports, was inhibited by 70–80%.

*Uptake of aflatoxin $B_1$ into permeabilized hepatocytes*

Permeabilization of isolated hepatocytes was achieved by immediate freezing the cells in $N_2$ liquid and subsequent thawing at 37°C.

By this procedure approximately 100% of the cells became permeable to Trypan blue due to membrane leaks. Aflatoxin $B_1$ uptake into permeabilized hepatocytes was compared with its uptake into intact hepatocytes (Fig. 5). Permeabilized hepatocytes should be unable to concentrate free aflatoxin in its cytosol. However, they are able to bind the toxin to cell macromolecules which remain in their cytosol. Also the kinetics of the uptake of a compound into an intact cell, with a permeation barrier by an intact membrane, should differ from the uptake kinetics into cell 'ghosts' if an ion gradient drives the uptake of aflatoxins by a carrier. Permeabilization reduced the absolute amount of cell-associated aflatoxin $B_1$ radioactivity by 44%. However, the kinetic curve for the uptake into permeabilized hepatocytes resembled the uptake into intact hepatocytes; e.g. the initial rate of aflatoxin $B_1$ uptake was identical for both cell preparations. It was worth mentioning that the efflux rate was significantly lower on permeabilized hepatocytes. While intact hepatocytes lost during 20 min 30.7% of the maximum (reached within the first 3 minutes) of cell-associated aflatoxin $B_1$ radioactivity, permeabilized hepatocytes lost only 20% during 20 min. Thus permeabilization of hepatocytes does not reduce the initial uptake rate for aflatoxin $B_1$ but slows down the release rate of its metabolites.

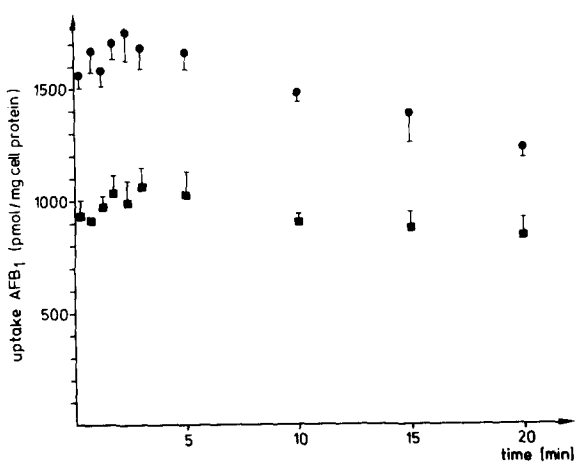



Fig. 5. Aflatoxin $B_1$ uptake into intact parenchymal liver cells and into hepatocytes with permeabilized cell plasma membranes. Permeabilization of liver cell plasma membranes was achieved by freezing hepatocytes into liquid nitrogen and thawing at room temperature. Aflatoxin $B_1$ uptake was measured into intact liver cells (●) as well as into permeabilized hepatocytes (■) at 37°C under $O_2/CO_2$ atmosphere. The concentration was 1.125 nM [$^3$H]aflatoxin $B_1$/1.6 $\mu$M $AFB_1$. The values represent mean values ± S.E. from three experiments with different cell preparations, the bars indicate the standard errors.

*Inhibition of aflatoxin $B_1$ uptake into hepatocytes by addition of liposomes and lipoproteins*

Our results so far indicated passive permeation of aflatoxin $B_1$ into hepatocytes by non-ionic physical diffusion. Because physical diffusion of compounds is linked to the permeation of an undissociated molecule through lipid domains in the membrane, the effect of a co-incubation of liposomes together with isolated hepatocytes was investigated. Only liposomes containing predominantly unsaturated fatty acids inhibited significantly ($P < 0.05$ Student's $t$-test) aflatoxin $B_1$ uptake into hepatocytes (Fig. 6B). With liposomes containing saturated fatty acids significant inhibition was not observed (Fig. 6A).

Significant uptake inhibition was also observed in the presence of low density– but not in the

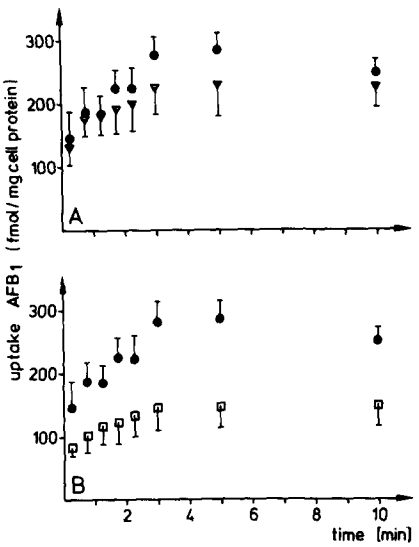


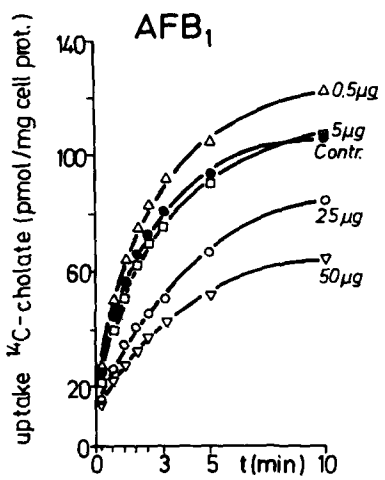

Fig. 6. Aflatoxin $B_1$ uptake into isolated rat hepatocytes: effect of liposomes, containing predominantly saturated fatty acids (sfa) (▽) (A) or unsaturated fatty acids (ufa) (□) (B), respectively. Isolated hepatocytes were incubated at 7 °C and were preloaded with liposomes (100 µl contained 5 µg phospho*enol*phosphatidylcholine) 5 min prior to the addition of 3 nM [$^3$H]aflatoxin $B_1$. The controls (●) (A, B) were treated similar by preincubating with 250 µl $H_2O$ (the liposome solvent)/ml cell suspension. The values represent mean values ± S.E. from four different cell preparations.

presence of high density lipoproteins (not shown).

In order to measure the affinity of aflatoxin $B_1$ to liposomes and lipoproteins equilibrium dialysis was performed. The $K_d$ for liposomes containing unsaturated fatty acids (ufa-liposomes) was $(19 \pm 0.7) \cdot 10^{-3}$ M, the $K_d$ for liposomes with saturated fatty acids (sfa-liposomes) $(31.7 \pm 1.28) \cdot 10^{-3}$ M. The specific binding capacity, which gives the amount of aflatoxin molecules bound by one molecule of phospho*enol*phosphatidylcholine was $(4.63 \pm 0.7) \cdot 10^{-7}$ M for ufa-liposomes but $(2.03 \pm 0.28) \cdot 10^{-7}$ M for sfa-liposomes. The affinity of aflatoxin $B_1$ to lipoproteins decreased with a decrease in the lipid moiety of the lipoproteins and both lipoproteins tested had a lower affinity for aflatoxin $B_1$ than liposomes. The results point out, that for the permeation of aflatoxin $B_1$ through the membrane of hepatocytes lipid domains are essential. Thus it is probably the composition of the lipid domains which influence its permeation rate.

*Effect of aflatoxin $B_1$ on the uptake of cholate and phalloidin*

Under acute aflatoxicosis bile secretion is inhibited [9]. One possible explanation of a chole-



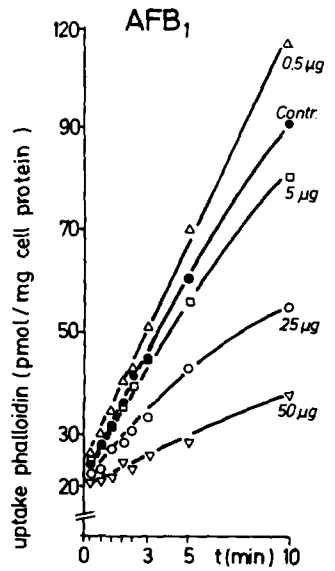




Fig. 7. Uptake of cholate (A) and phallotoxin ([$^3$H]demethylphalloin/phalloidin) (B) into isolated hepatocytes in the presence of aflatoxin $B_1$. Liver parenchymal cells were preincubated with aflatoxin $B_1$ at a concentration of 1.6 (△); 16 (□); 80 (○) and 160 µM (▽) 30 s prior to the addition of [$^{14}$C]cholate (1 µM) or to the addition of the phallotoxin mixture (85 nM [$^3$H]demethylphalloin/6 µM phalloidin). Controls (●) were performed by preincubating 1 ml cell suspension with 10 µl DMSO, which was the solvent of aflatoxin $B_1$. The values represent mean values $x$ derived from four experiments. The S.E. values are not shown for reasons of clarity and were ≤ 15% of each $x$.

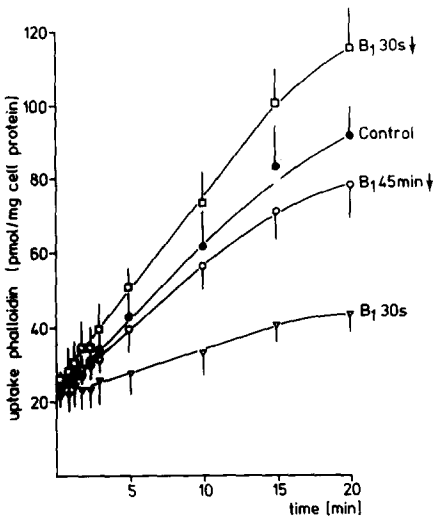



Fig. 8. Reversibility of the inhibition of phallotoxin (85 nM [$^3$H]demethylphalloin/6 $\mu$M phalloidin) uptake into rat hepatocytes by aflatoxin $B_1$. $2 \cdot 10^6$ hepatocytes/ml cell suspension were incubated in the presence of 160 $\mu$M aflatoxin $B_1$ under carbogen atmosphere, at 37 °C and pH 7.4 for 30 s (□) as well as 45 min (○). As indicated by the arrow symbol hepatocytes were washed twice with Tyrode buffer (by centrifugation at $50 \times g$) in order to remove the mycotoxin before phallotoxin uptake was measured. Phallotoxin uptake, measured either in the absence of aflatoxin $B_1$ (control (●)) or in the presence of 160 $\mu$M aflatoxin $B_1$, which was added 30 s prior to the phallotoxins (▽), served as controls. The values represent mean values ± S.E. derived from three experiments, the bars indicate S.E.

static effect by aflatoxin $B_1$ might be an inhibition of the uptake of bile acids into hepatocytes. In the presence of concentrations above 5 $\mu$g/ml, aflatoxin $B_1$ inhibited cholic acid uptake markedly. However, in the presence of 0.5 $\mu$g the $\Delta$ 10 min uptake of cholate is stimulated for 15% (Fig. 7A). As phalloidin is taken up by this bile acid transport system too, the same experiments were performed with phalloidin (Fig. 7B). Again 0.5 $\mu$g aflatoxin $B_1$/ml stimulated uptake (by 28%) while higher concentrations inhibited phalloidin uptake. In these experiments aflatoxin $B_1$ was given 30 s prior to the addition of [$^{14}$C]cholate or [$^3$H]phallotoxin but remained in the incubation buffer during the whole experiment. In order to test the reversibility of the inhibition of the bile acid uptake system by the aflatoxin, hepatocytes were exposed for 30 s and 45 min to 50 $\mu$g aflatoxin $B_1$/ml but were washed thereafter in order to remove free mycotoxin. Independent on the incubation period the inhibition of phalloidin uptake was reversible in both cases (Fig. 8). Only during long time exposure a slight inhibition remained after three cell washes, whereas after short time exposure even a stimulation of phalloidin uptake was observed. This reversibility of the inhibition on this uptake system points out, that irreversible damage of the cells was not responsible for the marked inhibition of bile acid uptake by aflatoxin $B_1$.

## Discussion

The results presented clearly indicate that aflatoxin $B_1$ permeates the membrane of hepatocytes by non ionic physical diffusion. As depicted in Fig. 4 neither saturability of radiolabelled aflatoxin uptake nor competition with nonlabelled aflatoxin $B_1$ was found (Table I). Neither protein structures nor the cellular energy supply were involved in mediating the passage of aflatoxin $B_1$ across the membrane of hepatocytes. Our results exclude that the permeation process itself determines the organospecificity of the mycotoxin's clearance and toxicity.

The time course of the uptake curves presented in Fig. 1 differs from the measurements with isolated hepatocytes by Ch'ih et al. [16]. These authors reported the maximum of aflatoxin $B_1$ incorporation after 3 h in the presence of 1.4 $\mu$M aflatoxin $B_1$, while in our hands the maximum of cell associated radioactivity was reached within 15 s by 1.6 $\mu$M aflatoxin $B_1$. In both cases the measured maxima were, however, comparable, namely 135 pmol/$10^6$ cells [16] versus 180 pmol/mg cell protein in this paper ($10^6$ cells correspond to 0.9 mg cell protein). The reason for the discrepancy in the uptake kinetics is not understood. However, because even in situ in the perfused rat liver as well as in vivo the hepatic clearance of aflatoxin $B_1$ was extremely rapid [24,25,10,9] we assume that the prolonged transport kinetics resulted from the method used for its determination by Ref. 16. While we used the rapid silicon oil centrifugation method, which circumvents washing of the cells (the extracellular volume is totally separated from the cell pellet by an oil layer), Ch'ih et al. used a filtration technique with subsequent washing steps

in order to remove the extracellular fluid. If a rapid physical diffusion is assumed, each washing step will alter the equilibrium of aflatoxin $B_1$ uptake into the cells markedly and any kinetic analysis becomes falsified. After a washing step only that radioactivity remains, which is trapped by binding to macromolecules or enclosed into vesicles.

Permeation of aflatoxin $B_1$ by physical diffusion means that the intracellular concentration of free, nonbound aflatoxin is identical to the concentration of free, nonbound aflatoxin $B_1$ in the extracellular space. An accumulation of aflatoxins within the cytosol of intact cells must be due to trapping by binding to cytosolic binding structures. If these binding structures are washed out of permeabilized cells these 'ghosts' should take up less radioactivity than do intact cells. As shown in Fig. 5 permeabilization of hepatocytes reduced the uptake of cell associated radioactivity by about 44% when compared with intact liver cells. Indeed when subcellular fractions were prepared from intact hepatocytes after an uptake experiment, 49% of the total cell associated radioactivity was found in the cytosolic compartment (not shown). It is reasonable to assume that the 44% reduction of aflatoxin $B_1$ uptake in permeabilized hepatocytes is predominantly due to a loss of a soluble cytosolic binding fraction for aflatoxins during the permeabilization procedure. Other authors [12] also found the highest amount of cell-associated aflatoxin radioactivity, namely 69% of the total cell lysate radioactivity, in the cytosol fraction. As in those studies two-thirds of the cytosol associated radioactivity were bound noncovalently to proteins the existence of aflatoxin binding proteins in the liver cytosol was proposed [16], an assumption which was supported by similar results of Ref. 26.

The determinants for the uptake of aflatoxin $B_1$ into isolated hepatocytes are most likely lipid domains within the membrane. The experiments with liposomes containing either mainly saturated or unsaturated fatty acids (Fig. 6) indicated that partitioning into domains with unsaturated fatty acids was prefered by aflatoxin $B_1$. Affinity to lipids might explain the effects of the mycotoxin on the membrane transport of bile acids and phalloidin whereby low aflatoxin $B_1$ concentrations stimulated the transport rate by the carrier proteins, while high concentrations inhibited. Other transport systems were not investigated presently but it is tempting to speculate that the reported inhibition of nucleoside and leucin uptake into hepatocytes [17] may at least partially be due to an alteration of lipid domains by the aflatoxins. Although in this case a toxic effect (with decreased cell viability and increased lactate dehydrogenase release) [17] was measured concomitantly, the observed inhibition of the uptake of diverse substrates might at least partially be due to such an alteration of lipid domains by aflatoxins. The inhibition of the multispecific bile acid transport system was not due to nonspecific toxic effects since after cell washing bile acid and phalloidin uptake returned to control levels (Fig. 8). A small amount of cell associated aflatoxin $B_1$ even stimulated this transport system. This was observed either in the presence of 1.6 $\mu$M aflatoxin or on washing hepatocytes after preincubation with 160 $\mu$M aflatoxin $B_1$, the latter probably being the result of a small remaining portion of the toxin in the cell suspension. The mechanism for this uptake facilitation of a protein carrier by small amounts of aflatoxin is unknown. As is known from reconstitution experiments lipids are in some cases regulators of transport properties of carriers [27]. One might therefore assume that low amounts of the hydrophobic aflatoxin alter lipid–protein interactions in such a way that the mobility of the carrier protein is facilitated whereas high amounts have an opposite effect.

## Acknowledgements

We are gratefully indebted to Professor Dr. Stoffel, Cologne, for kindly supplying the lipoproteins. We acknowledge the encouragement by Professor Dr. M. Frimmer, Giessen during the experimental work. This work was partially supported by the Deutsche Forschungsgemeinschaft, grant PE 250/5-1.

## References

1 Nesbitt, B.F., O'Kelly, J., Sargeant, K. and Sheridan, A. (1962) Nature 195, 1068–1069.
2 Hanigan, H.M. and Laishes, B.A. (1984) Toxicology 30, 185–193.

3 Wogan, G.N. and Newberne, P.M. (1967) Cancer Res. 27, 2370–2376.
4 Butler, W.H. and Clifford, J.I. (1965) Nature 206, 1045–1046.
5 Burguerra, J.A., edds, G.T. and Osuna, O. (1983) Am. J. Vet. Res. 44, 1714–1717.
6 Stoloff, L. (1983) Nutr. Cancer 5, 165–186.
7 Clifford, I. and Rees, K.R. (1967) Biochem. J. 102, 65–75.
8 Bassir, O. and Osiyemi, F. (1967) Nature 215, 882.
9 Unger, P.D. and Mehendale, H.M. (1977) Toxicol. Appl. Pharmacol. 41, 523–534.
10 Harland, E.C. and Cardeilhac, P.T. (1975) J. Vet. Res. 36, 909–912.
11 Portman, S.R., Plowman, K.M. and Campell, C.T. (1970) Biochim. Biophys. Acta 208, 487–495.
12 Ch'ih, J.J. and Devlin, T.M. (1984) Biochem. Biophys. Res. Commun. 122, 1–8.
13 Petzinger, E., Ziegler, K. and Frimmer, M. (1987) in Bile Acids and the Liver (Paumgartner, G., Stiehl, A. and Gerok, W., eds.), pp. 111–124, MTP Press Ltd., Lancaster U.K.
14 Petzinger, E. and Frimmer, M. (1982) Naunyn-Schmiedebergs Arch. Pharmacol. 319, 87–92.
15 Frimmer, M. (1987) Toxicol. Lett. 35, 169–182.
16 Ch'ih, J.J., Lin, T. and Devlin, T.M. (1983) Biochem. Biophys. Res. Commun. 110, 668–674.
17 Ch'ih, J.J., Lin, T. and Devlin, T.M. (1983) Biochem. Biophys. Res. Commun. 115, 15–21.
18 Berry, M.N. and Friend, D.S. (1969) J. Cell Biol. 43, 506–529.
19 Weichselbaum, T.E. (1946) Am. J. Clin. Pathol. Tec. Sec. 10, 40–90.
20 Klingenberg, M. and Pfaff, E. (1967) Methods Enzymol. 10, 680–684.
21 Schwarz, L.R., Burr, R., Schwenk, M., Pfaff, E. and Greim, H. (1975) Eur. J. Biochem. 55, 617–623.
22 Frimmer, M., Petzinger, E., Ziegler, K. and Veil, J.B. (1980) Naunyn-Schmiedebergs Arch. Pharmacol. 311, 91–94.
23 Drabkin, D.L. (1975) Ann. N.Y. Acad. Sci. 244, 603–623.
24 Falk, H.L., Thomson, S.J. and Kotin, P. (1965) Proc. Am. Assoc. Cancer Res. 6, 18.
25 Wogan, G.N., Edwars, G.S. and Schank, R.C. (1967) Cancer Res. 27, 1729–1736.
26 Mainigi, K.D. and Sorof, S. (1977) Cancer Res. 37, 4304–4312.
27 Koepsell, H. (1986) Rev. Physiol. Biochem. Pharmacol. 104, 65–137.