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Hepatocellular uptake of cyclosporin A by simple diffusion

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Cyclosporin A is known to be eliminated mainly via the biliar pathway after biotransformation. Whether liver cells take up the drug by simple diffusion across the lipid barrier or by carrier-mediated transport, as shown for some other peptides, was unknown up to the present. Experiments with [^{3}H]cyclosporin A on isolated rat hepatocytes indicate that the uptake of cyclosporin A is neither saturable nor is driven by metabolic energy. Cholestasis caused by cyclosporin A treatment is therefore not the result of mutual competition for a carrier protein. Nevertheless, cyclosporin A interacts with the bile acid transport system by non-competitive inhibition of bile salt uptake.

Introduction

Cyclosporin A, an immunosuppressive cyclopeptide [1], leads to moderate cholestasis and bilirubinemia in the course of biliary excretion of the drug [2,3]. Recently, we observed that cyclosporin A non-competitively inhibits the uptake of cholate and of demethylphalloin by liver cells [4], whereas some cyclic somatostatin analogs inhibit the same transport system in a competitive manner [5]. That means that cyclosporin A cannot be taken up by carrier systems responsible for the uptake of phalloidin, cyclosomatostatins and bile acids [5–7]. The question remained: is the hepatocellular uptake of cyclosporin A mediated by an individual carrier different from the bile salt transporter (e.g., bilirubin carrier [8]) or by simple diffusion across the lipid barrier? The hydrophobic properties of cyclosporin A could indicate the latter mechanism. In the following, kinetics of hepatocellular uptake of cyclosporin A in vitro are presented.

Materials

[^{3}H]Cyclosporin A (specific activity 310.8 GBq/mmol) and nine different cyclosporin A analogs were generous gifts from the Sandoz AG, Basel. Lipoluma, Lumasolve was purchased from Baker Chemicals, Gross-Gerau, F.R.G.; silicon oil from Wacker-Chemie, München, F.R.G. Lipoproteins were provided by Professor Stoffel Köln, and liposomes from Nattermann and Cie. GmbH, Köln, F.R.G. All other chemicals were of at least analytical grade purity.

Methods

Isolation of rat liver parenchymal cells

Rat liver cells were isolated according to Berry and Friend [9]. $(1–2) \cdot 10^8$ hepatocytes were iso-

Abbreviations: DMSO, dimethylsulfoxide; Cremophor EL, poly(oxyethylene)-40 ricinoleic acid.

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lated by perfusion of rat liver for 15 min with 0.05% collagenase in a Ca^{2+}-free Krebs-Henseleit buffer. After an equilibration period of 30 min in a shaking water bath in O_2/CO_2 atmosphere, the viability of the liver cells was tested by Trypan blue exclusion. 80–90% of the cells were found intact and were used within 2 h of cell isolation.

Isolation of AS-30D ascites hepatoma cells and Ehrlich ascites cells

AS-30D ascites cells were prepared as described by Frimmer et al. [10]. Ehrlich ascites cells from white mice were harvested 10 days after inoculation. The washing procedure was the same as for AS-30D ascites cells.

[^{3}H]Cyclosporin A uptake studies

The uptake of [^{3}H]cyclosporin A was measured by a rapid centrifugation technique according to Klingenberg and Pfaff [11]. Isolated hepatocytes ($2 \cdot 10^6$/ml) were incubated with varying concentrations of [^{3}H]cyclosporin A with or without unlabelled cyclosporin A (dissolved in dimethylsulfoxide (DMSO); DMSO never exceeded 10 μl per ml of cell suspension).

Aliquots of 100 μl cell suspension were withdrawn after 15, 45, 75, 105, 135 s, 3, 4, 5 and 10 min and centrifuged through a silicon oil layer. Other conditions are indicated in the individual experiments. The radioactivity associated with the cell pellet was measured in a liquid scintillation counter (Packard Tricarb 2660) after addition of Lipoluma/ Lumasolve/ H_2O, vigorous shaking and heating at 40 °C as prescribed by the manufacturers (Baker Chemicals).

Results

Kinetics of uptake of [^{3}H]cyclosporin A

[^{3}H]Cyclosporin A enters liver cells in a concentration- and time-dependent manner. Time-dependent saturation is reached after 3 min of incubation. In contrast, initial uptake is not saturable as shown by the Lineweaver-Burk diagram (Fig. 1). Higher concentrations of cyclosporin A (above 800 nM) could not be tested because of the insolubility of the compound. The distribution of the cell-associated radioactivity was distinguished by cell fractionation studies. After 5 min preincubation with [^{3}H]cyclosporin A and removing free radioactivity by washing, isolated hepatocytes were broken by ultrasonication. Soluble proteins were separated from cell membranes by 100 000 × *g* centrifugation. 50% of the radioactivity was associated with the fraction of soluble proteins and 50% with cell membranes.

Inhibition of [^{3}H]cyclosporin A uptake by cyclosporin analogs

A prerequesite for carrier-mediated uptake is a mutual inhibition by substrate analogs. The uptake of [^{3}H]cyclosporin A is neither inhibited by nonradioactive cyclosporin A nor by different cyclosporin A analogs in a 100–8000-fold molar excess. We tested nine different cyclosporin A analogs, e.g., valin 2-dihydrocyclosporin, valin 2-cyclosporin, 3′-acetylcyclosporin, 3′-desoxycyclosporin, compound 05 in which the amino acid in position 8 is changed and compound 06 in which the amino acid in position 2 is changed. The [^{3}H]cyclosporin uptake inhibition of two analogs is shown (Fig. 2). From these results we conclude that a specific transport system does not exist for cyclosporin A.

Recently, we reported that cyclosporin diazirine, a photolabile cyclosporin analog binds to several proteins in isolated rat liver plasma membranes [12]. As was shown for other cyclosporin A analogs, this photoreactive derivative did not inhibit the uptake of [^{3}H] cyclosporin A in isolated rat hepatocytes. Even after covalent binding of cyclosporin diazirine to the plasma membrane the permeation of [^{3}H]cyclosporin A remained unaltered (data not shown).

Inhibition of [^{3}H]cyclosporin A uptake by Cremophor EL (poly(oxyethylene)- 40-ricinoleic acid)

With respect of the hydrophobic properties of cyclosporin A, different solvents were tested. DMSO in a concentration of 1% was used throughout the studies, because in this concentration it had no effect on cell viability or uptake of cyclosporin A. In contrast, Cremophor EL (poly(oxyethylene)-40-ricinoleic acid), a solvent contained in Sandimmun from Sandoz AG inhibits the uptake of [^{3}H]cyclosporin A. The same is true for a mixture of Cremophore/ethanol 66 : 33% (which is the mixture for cyclosporin A in Sandimmun) (Fig. 3).

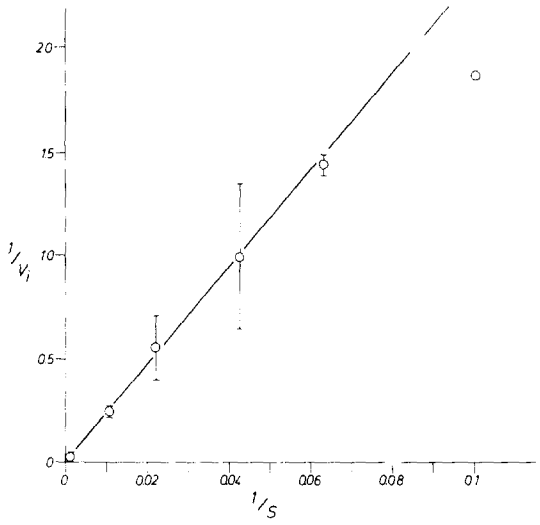

Fig. 1. Lineweaver-Burk diagram of the uptake of [^{3}H]cyclosporin A. Isolated hepatocytes ($2 \cdot 10^6$/ml Tyrode buffer (NaCl 137 mmol/l; KCl 2.7 mmol/l; $MgCl_2$ 1.05 mmol/l; $CaCl_2$ 1.8 mmol/l; $NaHCO_3$ 12 mmol/l; glucose 5.55 mmol/l; NaH_2PO_4 0.42 mmol/l) were incubated with 740, 80, 42, 23, 16 and 10 pmol of [^{3}H]cyclosporin A per ml of cell suspension. The initial rate of uptake (pmol/mg protein per min) was determined during the period of linear [^{3}H]cyclosporin A uptake ($n = 6$).

Uptake of [^{3}H]cyclosporin A in intact and permeabilized rat hepatocytes

[^{3}H]Cyclosporin A is concentrated by intact hepatocytes due to intracellular binding proteins, e.g., cyclophilin, which has been detected in all tissues (Hiestand, personal communication). This is evident by studies using cells with permeable membranes. In such cells cyclosporin A was not concentrated (Fig. 4). Cyclosporin A binding to plasma membranes remains constant over 200 s. Intracellular soluble binding proteins are lost across the permeabilized plasma membrane. This can be shown by electrophoretic separation of cells before and after freezing and thawing.

Cell specificity of [^{3}H]cyclosporin A uptake

Comparison of [^{3}H]cyclosporin A uptake by rat liver cells, AS-30D ascites hepatoma cells and Ehrlich ascites cells. AS-30D ascites hepatoma and Ehrlich ascites cells are deficient in bile acid transport [13]. In contrast, [^{3}H]cyclosporin A enters both cell types by nonsaturable diffusion (Fig. 5A, B). The initial uptake in Ehrlich ascites cells is 2-fold higher at 700 nM [^{3}H]cyclosporin A than in

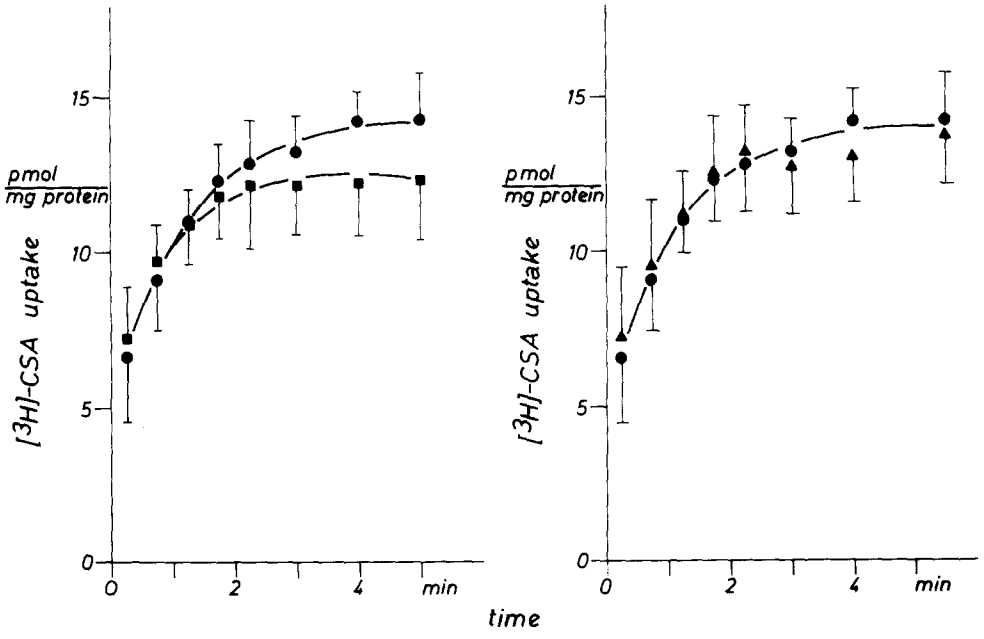

Fig. 2. Cyclosporin analogs do not inhibit [^{3}H]cyclosporin A uptake by isolated hepatocytes. Isolated rat liver parenchymal cells were incubated for 30 s with an 100-fold molar excess of cyclosporin A (CSA) analogs 05 and 06 before addition of 80 pmol of [^{3}H]cyclosporin A. At timed intervals 100-μl aliquots were withdrawn and centrifuged through silicon oil. Cell associated radioactivity was counted in a liquid scintillation counter ($n = 5$). Control, ●; 05, ■; 06, ▲.

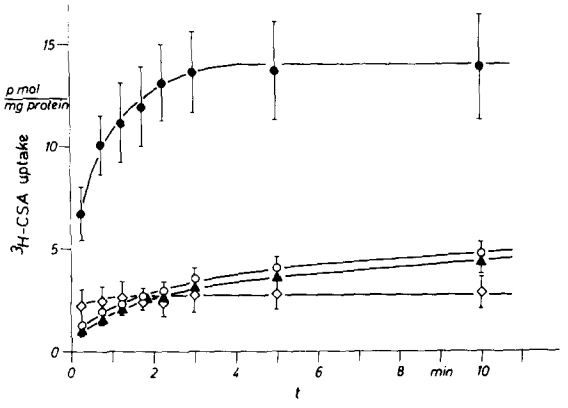

Fig. 3. Inhibition of [^{3}H]cyclosporin A uptake by isolated hepatocytes by Cremophor EL, placebo from Sandoz and Sandimmun. Isolated hepatocytes were incubated with DMSO (control) or Cremophor (poly(oxyethanol)-40-ricinoleic acid)/ethanol (67 vol%/33 vol%), 10 μl placebo (of unknown composition from Sandoz) or 10 μl Sandimmun (containing cyclosporin A (CSA) plus 67 vol% Cremophor EL/33 vol% ethanol). Uptake measurements were started by addition of 80 pmol/ml [^{3}H]CSA. (n = 4.) control, ●; Cremophor/ethanol, ○; placebo, ▲; Sandimmun, ◇.

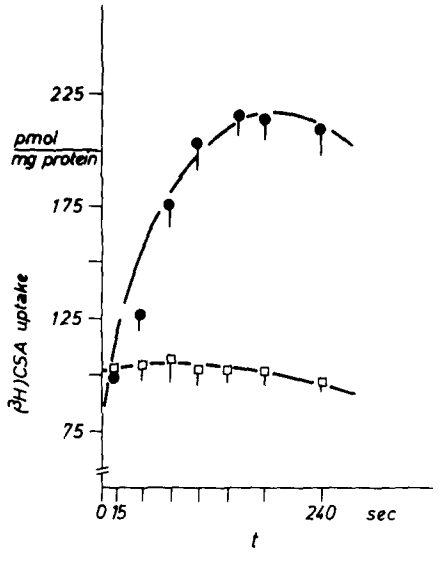

Fig. 4. Uptake of [^{3}H]cyclosporin A [^{3}H]CSA into intact hepatocytes and binding of [^{3}H]cyclosporin A to hepatocytes with a freely permeable membrane. Isolated hepatocytes were incubated with a mixture of 1 μM cyclosporin A plus 10 pmol [^{3}H]cyclosporin A. At the times indicated 100 μl aliquots were withdrawn and uptake was measured as described. Binding of [^{3}H]cyclosporin A was estimated by incubation of 1 μM cyclosporin A plus 10 pmol [^{3}H]cyclosporin A with hepatocytes which had been subjected to a freezing procedure (freezing in liquid nitrogen and subsequent thawing). Those cells have a free permeable cell membrane, indicated by Trypan blue staining (n = 4). Control, ●; freely permeable, □.

hepatocytes. Cyclosporin A association with AS-30D ascites hepatoma cells (700 nM = 25 pmol/mg protein per min) is lower as observed in liver cells (700 nM = 32.2 pmol/mg protein per min).

Energy dependence of [^{3}H]cyclosporin A uptake by isolated hepatocytes. The uptake of [^{3}H]cyclosporin A does not depend on the metabolic energy supply of liver cells. Incubation of cells in a mixture of N_2/CO_2 did not reduce the uptake. Time-dependent saturation was reached after 3 min either in N_2/CO_2 or O_2/CO_2 atmosphere. Preincubation of isolated hepatocytes with metabolic inhibitors (e.g., oligomycin, antimycin A) or uncouplers (e.g., carbonylcyanide *m*-chlorophenylhydrazone) had no effect on [^{3}H]cyclosporin A transport (data not shown). Under our experimental conditions, the ATP levels were reduced to 1–5% of the controls. Cell viability, determined by Trypan blue exclusion, was not significantly reduced.

Temperature dependence of [^{3}H]cyclosporin A uptake. The initial uptake of [^{3}H]cyclosporin A is temperature dependent (Fig. 6A). Below 10 °C no uptake was measurable. Above 10 °C an increase of transport could be seen with a maximum at 37 °C. The activation energy was calculated to be 21 kcal/mol from the linear Arrhenius diagram (Fig. 6B). This is uncommon for a physical diffusion. The reason for this behaviour is unknown at present.

Na^+ or Cl^- dependence of [^{3}H]cyclosporin A uptake. In contrast to the uptake of bile acids [14] and of phalloidin [15] the uptake of cyclosporin A does not depend on the Na^+ or the Cl^- gradient (data not shown).

Bile acids (taurocholate or cholate), iopodate and phalloidin in a 100–800-fold molar excess had no effect on cyclosporin A transport (data not shown). The same is true for bilirubin and bromosulfophthalein.

Interaction of cyclosporin A with lipoproteins and liposomes. Cyclosporin A binds to lipoproteins: in

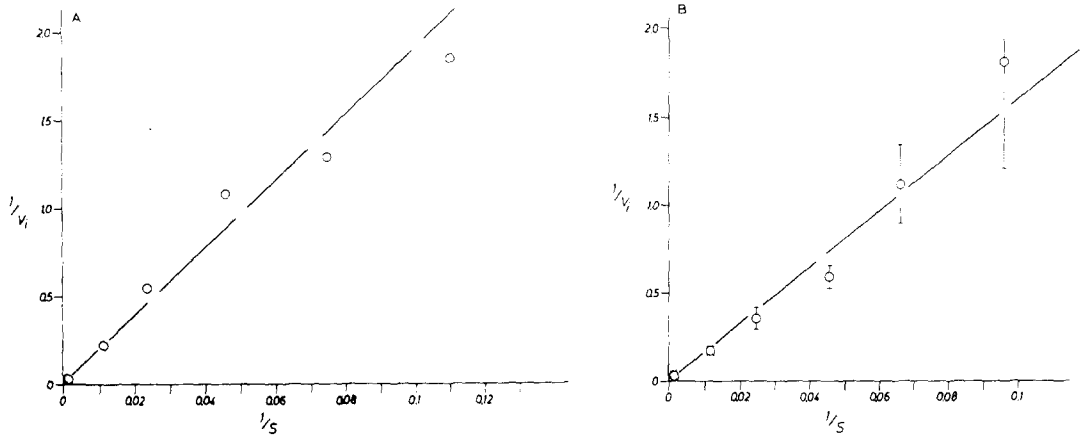

Fig. 5. Lineweaver-Burk diagram of the uptake of [^{3}H]cyclosporin A by AS-30D ascites hepatoma (A) and Ehrlich ascites cells (B). The uptake of 700, 80, 40, 20, 15 and 19 pmol of [^{3}H]cyclosporin A was meausred in isolated rat AS-30D ascites hepatoma cells ($10 \cdot 10^6$ cells/ml Tyrode buffer corresponding to 4 mg of cell protein) or mouse Ehrlich ascites cells ($10 \cdot 10^6$ cells/ml Tyrode buffer = 4 mg of cell protein). Uptake was measured at 37 °C, pH 7.4 in O_2/CO_2 atmosphere. Initial uptake rates were determined and plotted according to Lineweaver and Burk ($n = 4$).

vivo 57% were detected on the high-density lipoprotein, 25% on the low-density lipoprotein and 2% on the very-low-density lipoprotein [16]. In our studies addition of lipoproteins to rat liver cells reduced the uptake of cyclosporin A. Uptake of cyclosporin A was also blocked by addition of liposomes consisting of phosphatidylcholine with unsaturated or saturated fatty acids (data not shown). This was due to binding of cyclosporin A to liposomes or lipoproteins.

Discussion

Apparently cyclosporin A penetrates hepatocytes by simple diffusion as recently shown for T-lymphocytes [17]. Several lines of evidence sug-

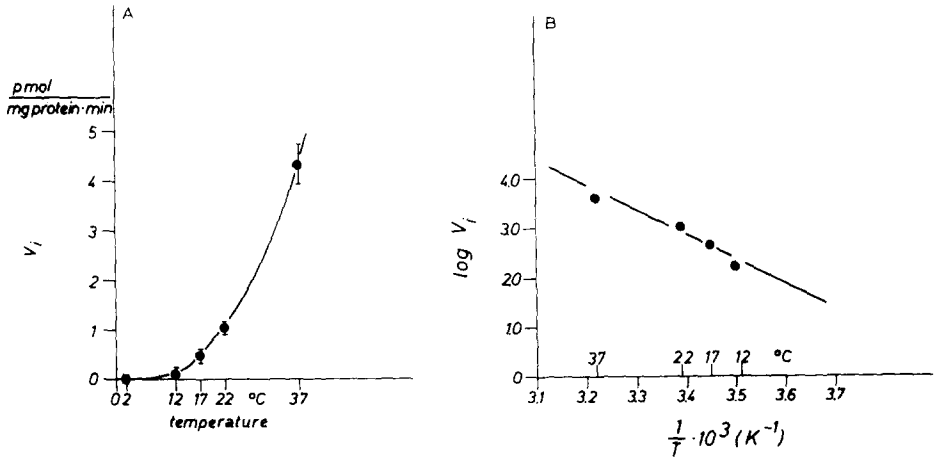

Fig. 6. Temperature dependence of [^{3}H]cyclosporin A uptake by isolated rat liver cells. After a preincubation period of 10 min at the indicated temperatures $2 \cdot 10^6$ hepatocytes/ml Tyrode buffer were incubated with 80 pmol/ml [^{3}H]cyclosporin A at 2, 12, 17, 22 and 37 °C. At timed intervals 100-µl aliquots were withdrawn. The initial rate of uptake (V_i = pmol/mg protein per min) was determined at different temperatures ($n = 4$). (A) Plot: initial rate of uptake versus temperature. (B) Arrhenius diagram. The experimental values from A were plotted according to Arrhenius: log V_i versus 1/T. The apparent activation energy was calculated $E_{a,app} = 2.303 \times R \times (\log V_i)/(1/T)$.

gest this mechanism: transport is not saturable in the concentration range tested. Uptake increases linearly with the extracellular concentration. Non-radioactive cyclosporin A or cyclosporin A analogs did not inhibit the uptake of [^{3}H]cyclosporin A. Furthermore, uptake does not depend on metabolic energy supply. Anaerobic incubation did not inhibit [^{3}H]cyclosporin A permeation. Pre-incubation of isolated hepatocytes with metabolic inhibitors had no effect on [^{3}H]cyclosporin A penetration in contrast to the uptake of phalloidin [18] or cyclosomatostatin (unpublished results). On the other hand, cyclosporin A is concentrated within hepatocytes 30-fold. This is due to intracellular binding proteins (e.g., cyclophilin) and to the solubility of cyclosporin A in the lipid phase of membranes. At present no inhibitors of [^{3}H]cyclosporin A uptake are known. The inhibition of cyclosporin A permeation in the presence of lipoproteins or liposomes is due to binding of cyclosporin A to lipids, as already reported [17]. Two uncommon properties of cyclosporin A uptake are its temperature dependence and the activation energy calculated for its permeation. The calculated activation energy of 21 kcal/mol seems to be very high and resembles that of carrier-mediated processes. The insolubility of cyclosporin A at temperatures below 15 °C might be a possible explanation [19] for the high activation energy. Binding to lipoproteins is also markedly dependent on temperature: 70% of the drug is bound at 4 °C, 98% at 37 °C [20].

In the course of the above studies we observed that the Cremophore EL used for commercial preparations of cyclosporin A inhibits the hepatocellular uptake of the drug. If such preparations were used for kinetic studies, curves with 'saturation kinetics' were obtained (Fig. 7).

Cyclosporin diazirine, a photoaffinity label, used in our laboratory binds to several membrane proteins of liver cells (200, 85, 54, 50, 37 kDa; see Ref. 12) without inhibition of [^{3}H]cyclosporin A uptake. Apparently, none of the identified [^{3}H]cyclosporin diazirine binding proteins in rat liver plasma membranes are responsible for the uptake of cyclosporin A. That means that not every high affinity binding of a photolabile derivative is evident for the existence of a transport system for the compound. For proof of the existence of a transport system it is always necessary to do both kinetic and photoaffinity labeling studies.

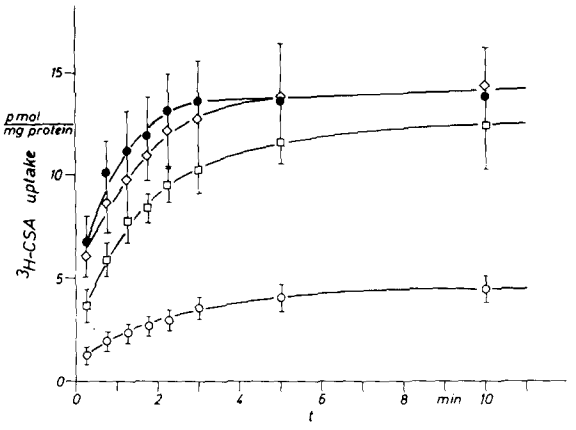

Fig. 7. Inhibition of [^{3}H]cyclosporin A uptake by isolated hepatocytes by Cremophor EL/ethanol (67 vol%/33 vol%). Isolated hepatocytes were incubated for 30 s without (control) or with 0.1, 1 or 10 μl Cremophore EL/ethanol. 80 pmol/ml [^{3}H]cyclosporin A ([^{3}H]CSA) were added and uptake was measured as described in Methods ($n = 4$). Control, ●; 0.1 μM ◇; 1.0 μM, □; 10.0 μM ○.

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