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Energy Linked Uptake of Demethylphalloin by Isolated Rat Liver Cells*

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Summary. Isolated hepatocytes accumulate demethylphalloin (DMP) under aerobic conditions. In the absence of oxygen the initial rate of the DMP uptake is reduced to less than 20%, while reoxygenation restores the transport. Liver cells release previously accumulated phallotoxin when the oxygen supply is interrupted. DMP uptake is blocked by oligomycin, antimycin A, carbonylcyano-chlorophenylhydrazon (CCCP) or dinitrophenol and is partially inhibited by carboxyatractyloside. Depletion of ATP in hepatocytes by replacement of glucose by fructose reduces the accumulation of toxin too. Below 22° C no uptake was measurable. Between 22° and 37° C an apparent activation energy of 76.6 kJ/mol toxin and a Q_{10} of 2.6 was calculated for the carrier mediated uptake of DMP. The results suggest that the uptake of demethylphalloin is an energy dependent substrate transfer very similar to that of cholate.

Key words: Isolated hepatocytes – Metabolic inhibitors – ATP dependence – Temperature dependence – Phallotoxin-uptake

Introduction

Earlier studies on the sensitivity of hepatocytes to phalloidin suggested that integral membrane proteins are responsible for the recognition and uptake of phalloidin by hepatocytes (Frimmer and Petzinger 1977). Some serine proteases (Frimmer et al. 1977a), some protein reagents e.g. DIDS and glutardialdehyde (Petzinger et al. 1978a) and phospholipase (Petzinger et al. 1978b) protect isolated rat liver cells against phalloidin. Experiments at various temperatures (Frimmer and Rufeger 1977) gave evidence for the importance of lipid areas in the membrane which are involved in the recognition of phalloidin too. Recently uptake studies with the labelled toxin (^{3}H-DMP) were performed supporting the concept of a carrier mediated uptake of phallotoxins into liver cells (Petzinger et al. 1979; Petzinger 1980). The insensitivity of hepatoma cells is due to a lack of transport of (^{3}H)-DMP (Grundmann et al. 1978; Petzinger et al. 1979). Thus the membrane of nonliver cells is supposed to be a barrier for the entry of phallotoxins while hepatocytes contain membrane components which carry phalloidin to the intracellular space. Intracellular phalloidin, either artifically injected into nonliver cells (Wehland et al. 1977; Stockem et al. 1978) or transported by a carrier into liver cells binds to microfilaments (Govindan et al. 1973; Wulf et al. 1979; Jahn et al. 1980). However there is no correlation between the amount of microfilaments and the amount of toxin taken up by hepatocytes (Faulstich et al. 1977). Thus the rate limiting step of phalloidin transport is more likely the translocation across the cell membrane than intracellular binding by F-actin or by other proteins e.g. ligandin (Ziegler et al. 1982). To get more information on the transport mechanism in liver cells for phallotoxins, efforts to characterize the energy and temperature dependence were made in this study. The following experiments demonstrate that the phallotoxin transport is an active one linked to the oxidative energy supply.

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Abbreviations: DMP = demethylphalloin, CCCP = carbonylcyano-chlorophenylhydrazon, DNP = 2,4-dinitrophenol

Methods and Materials

Preparation of Hepatocytes. Rat liver cells were isolated according to Berry and Friend (1969). By perfusing the liver for 15 min with 0.05% collagenase in Krebs-Henseleit buffer in the absence of Ca^{2+}, $1-2 \times 10^8$ hepatocytes per preparation were isolated. After an equilibration period of 30 min in Tyrode buffer, pH 7.4 at 37° C in O_2/CO_2 (95%/5%) atmosphere about 85–90% of the cells were intact refering to the exclusion of 0.2% trypan blue. 1 ml of suspension contained 2×10^6 hepatocytes corresponding to 3.8 mg of cell protein.

Experiments in the Absence of O_2. Following an equilibration in O_2/CO_2 (95%/5%) hepatocytes were incubated in the presence of N_2/CO_2 (95%/5%) for 5, 30 and 60 min prior to mixing with 30 ng (^{3}H)-demethylphalloin/5 µg phalloidin (refered as "phallotoxin" in the text). In these experiments phalloidin which is chemically very similar was used because demethylphalloin was not available in sufficient amounts to achieve micromolar toxin concentrations. The procedure is tolerable as was shown in earlier experiments (Frimmer et al. 1980). Aliquots of 100 µl cellsuspension were withdrawn by a syringe through the diaphragma of the incubation vial. The cells were pelleted by rapid centrifugation through silicon oil. Hepatocytes were dissolved in 3 M KOH and the radioactivity was measured in Lipoluma/Lumasolve/water mixture (100:10:2 v/v).

Experiments with Metabolic Inhibitors. Metabolic inhibitors, dissolved in ethanol were added 10 min prior to (^{3}H)-DMP/phalloidin. The final concentration of ethanol was

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below 2% and had no measurable influence on the uptake kinetics. In the experiments derived from Fig. 4 the cell concentration was 2×10^6/ml. For the measurement of the effect of metabolic inhibitors on ATP liver cells were 10 fold concentrated. In order to compare the effect to phallotoxin uptake hepatocytes were exposed to equimolar concentrations as well as to 10-fold higher concentrations of inhibitors. ATP was measured by an enzymatic method (Lamprecht and Trautschold 1970).

Materials. Oligomycin, antimycin A, carboxyatractyloside and CCCP were from Sigma, Munich, FRG. (^{3}H)-DMP, specific activity 3.5 Ci/mmol was a gift from Prof. Dr. Th. Wieland and Dr. H. Faulstich, MPI Heidelberg, FRG.

Results

Effect of Anaerobic Conditions on (^{3}H)-DMP Uptake

By changing the atmosphere from oxygen to nitrogen the amount of (^{3}H)-DMP taken up into liver cells was reduced depending on the duration of the nitrogen exposure (Fig. 1 a). Thirty minutes incubation in 95% N_2 + 5% CO_2 reduced the uptake rate to about 20% of the control without influencing the zero time value of the uptake curve. This value was significantly raised when the exposure to nitrogen was prolonged to 60 min and indicates that an increasing fraction of the cells had become freely permeable for the toxin (Petzinger 1981). When the period of anaerobiosis was limited to 30 min the blocked transport was restored to 75% after subsequent reoxygenation (Fig. 1 b). In the presence of oxygen, liver cells accumulate phallotoxin within 30 min 8–10-fold. They loose part of the internalized toxin when subsequently incubated in the absence of O_2 (Fig. 2 a).

The same effect was observed when CCCP was added to liver cells preloaded with (^{3}H)-DMP (Fig. 2 b).

Because cholate hinders the uptake of phallotoxins into isolated hepatocytes by competive inhibition (Petzinger 1981) it was of interest whether the small amount of O_2 insensitive uptake of DMP still reflects carrier mediated uptake and should therefore be inhibited by bile acid. However cholate did not influence the phallotoxin uptake under anaerobic conditions (Fig. 1 b). The O_2 insensitive DMP uptake is probably different from the O_2 dependent uptake and might be due to damaged cells with passive permeability to DMP.

Effect of ATP Depletion by Fructose on the Uptake of (^{3}H)-DMP

The ATP dependence of the transmembrane transport of (^{3}H)-DMP was tested by replacement of glucose by fructose in the Tyrode buffer. Fructose is reported to cause a rapid depletion of ATP in liver parenchymal cells (Mäenpää et al. 1968; Woods et al. 1970; van den Berghe et al. 1977). In the presence of fructose, liver cells took up smaller amounts of (^{3}H)-DMP compared to those in the presence of glucose (Fig. 3). The ATP content of isolated hepatocytes was diminished to 65% by fructose (Table 1).

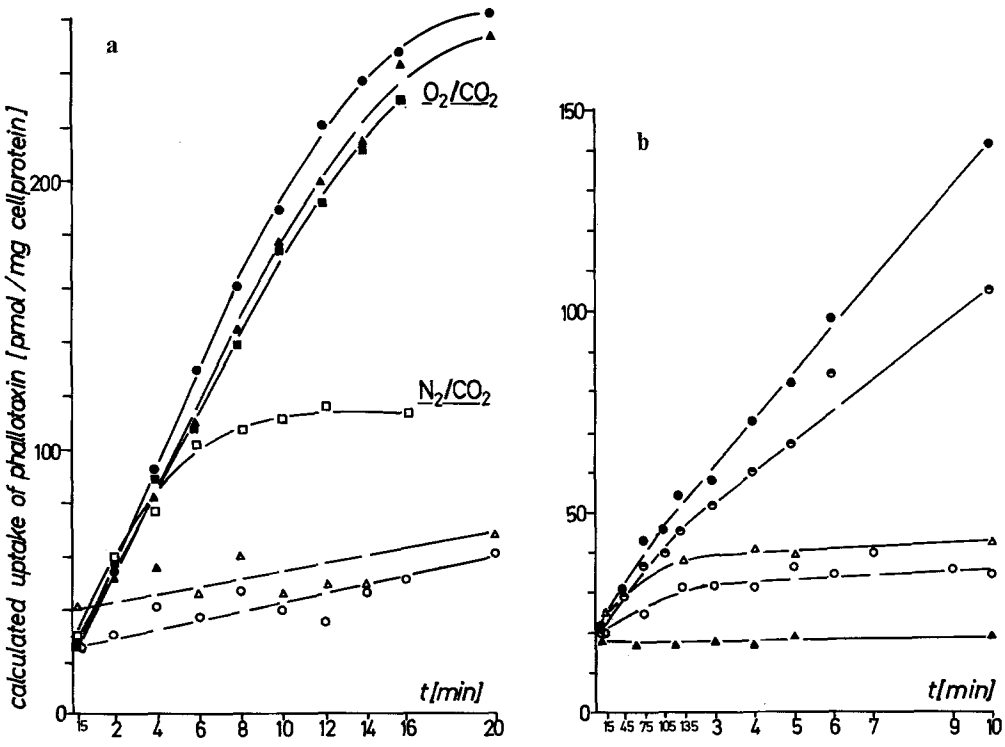

Fig. 1. (a) Effect of anaerobiosis on phallotoxin uptake by isolated rat hepatocytes. 2×10^6 hepatocytes/ml were incubated in the presence (● ■ ▲) and in the absence (○ □ △) of oxygen 5 min (■ □), 30 min (● ○) and 60 min (▲ △) prior to (^{3}H-)DMP (30 ng) plus phalloidin (5 µg). Cell aliquots were withdrawn at the time indicated and phallotoxin uptake was calculated as described. (b) Effect of cholate on the uptake of phallotoxin in the presence and absence of oxygen. Isolated hepatocytes were equilibrated 30 min either with 95% O_2/5% CO_2 (● ▲) or with 95% N_2/5% CO_2 (○ △). 100 µM cholate (▲ △) was added 30 s prior to (^{3}H-)DMP (30 ng/ml) plus phalloidin (5 µg/ml). (●) uptake phallotoxin under aerobic conditions (control); (▲) uptake phallotoxin under aerobic conditions in the presence of 100 µM cholate; (○) uptake phallotoxin under anaerobic conditions (control); (△) uptake phallotoxin under anaerobic conditions in the presence of 100 µM cholate. (◓) uptake phallotoxin by liver cells, exposed 30 min to N_2/CO_2 followed by 30 min O_2/CO_2 in the absence of cholate

Effect of Metabolic Inhibitors on the Uptake of (^3H)-DMP

Ten minutes preincubation of intact liver cells with oligomycin, (10 µg/ml) antimycin A (10 µg/ml), carbonylcyanochlorophenylhydrazon (20 µM) or dinitrophenol (100 µM) inhibited the uptake of (^{3}H)-DMP by intact liver cells completely. In contrast, in the presence of carboxyatractyloside (100 µM) an inhibitor-insensitive part of transport was measurable (Fig. 4). If the cells were washed after CCCP treatment the inhibition was partially reversed (Fig. 5). Extrapolation of the uptake to zero time gives positive intercepts on the ordinate which are identical to the zero time values of the uptake curves from controls. This means that only the translocation step is inhibited but not the binding to the carrier. The transport inhibition was not caused by cell damage as judged by microscopy but more likely by ATP reduction. Oligomycin, CCCP and antimycin A at appropriate concentrations reduced the amount of ATP within 10 min to at least one tenth of that in control cells (Table 1).

Temperature Dependence of the Uptake of (^3H)-DMP

Earlier findings on perfused rat livers (Frimmer 1972) and on isolated hepatocytes (Frimmer and Rufeger 1977) demonstrated that the response to phalloidin is markedly delayed at temperatures lower than 21° C. In studies of Gries et al. (1968)

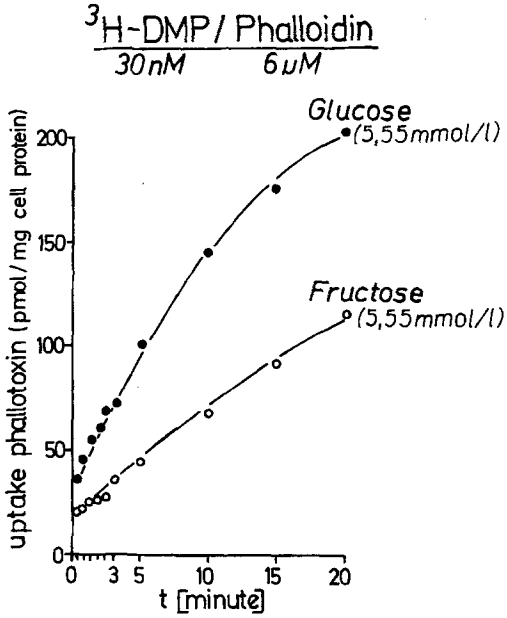

Fig. 3. Effect of fructose on the uptake of phallotoxin by isolated rat hepatocytes. 1 ml cell suspension was incubated either in Tyrode buffer supplemented by 5.55 mmol/l glucose (●) or by 5.55 mmol/l fructose (○) for 60 min prior to the addition of (^{3}H-)DMP/phalloidin

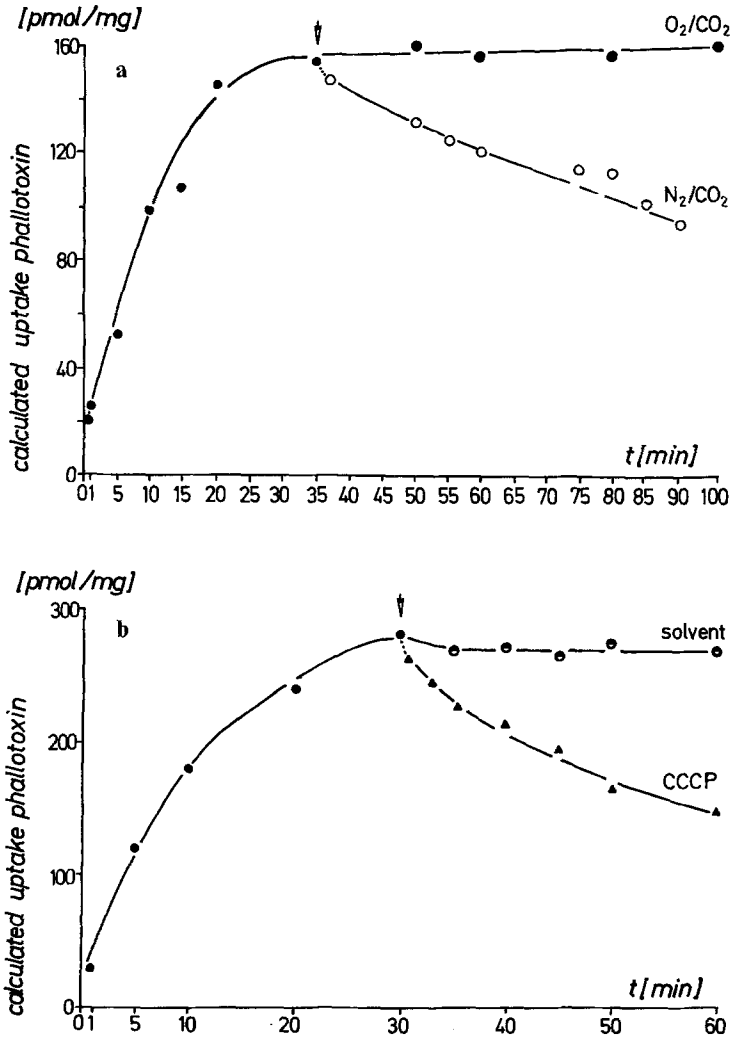

Fig. 2
(a) Release of phallotoxin by intact hepatocytes in the absence of oxygen. 2×10^6 hepatocytes/ml were incubated with (^{3}H-)DMP (30 ng) plus phalloidin (5 µg) 35 min in the presence of oxygen to accumulate phallotoxin (●). The cell suspension was devided into equal parts (*arrow*) and incubated for 100 min in the presence of N_2/CO_2 (○) as well as in the presence of O_2/CO_2 (●). (b) Release of phallotoxin by ATP depletion. Experimental conditions as above except 4 µg CCCP/ml were added after 30 min incubation (*arrow*) in the presence of O_2/CO_2. (●) uptake of phallotoxin in the presence of oxygen; (◓) release of phallotoxin; *arrow:* plus buffer volume (control). (▲) release of phallotoxin; *arrow:* plus 20 µM CCCP

Table 1. Loss of ATP by metabolic inhibitors and fructose on isolated hepatocytes. 20×10^6 hepatocytes/ml were incubated in the presence either of 10 or of 100 µg oligomycin and antimycin A as well as in the presence of 4 µg CCCP for 10 min and for 10 min and 60 min in fructose supplemented Tyrode. Controls were incubated in glucose supplemented Tyrode 10 min and 60 min. Prior to ATP measurement cells were washed twice with the appropriate buffer, boiled 2 min in 10^{-2} n $HClO_4$ and stored in liquid nitrogen; controls: $n = 16$, all others: $n = 3$

Compound	[ATP] µMol/g cell prot.	Residual [ATP] %
None (control)	3.35 ± 1.15	100
Oligomycin 10 µg/ml	0.18 ± 0.20	5
Oligomycin 100 µg/ml	0.13 ± 0.19	4
Antimycin A 10 µg/ml	0.03 ± 0.06	1
Antimycin A 100 µg/ml	0.06 ± 0.09	2
CCCP 4 µg/ml	0.03 ± 0.06	1
Glucose 5.55 mmol/l 60 min (control)	3.68 ± 1.26	100
Fructose 5.55 mmol/l 10 min	1.83 ± 1.18	49
Fructose 5.55 mmol/l 60 min	2.40 ± 1.31	65

merely moderate binding of (^{3}H)-DMP in the perfused rat liver was measured at 10°C. In order to evaluate whether altered transport functions are responsible for the insensitivity of liver cells at low temperature, uptake studies with (^{3}H)-DMP at 2° – 37°C were performed. No net transfer was measured at 2° – 17°C within 20 min (Fig. 6a). Below 22°C only a slight increase of toxin uptake occured. However at 22° – 37°C the initial rate of uptake increased steadily (Fig. 6b) by the factor $Q_{10} = 2.6$ for each 10°C increment. The apparent activation energy was calculated $A_{app} = 76.6$ kJ/mol by means of the Arrhenius plot (Fig. 6c). The results agree with the assumption of carrier-involved uptake of phallotoxins across the plasma membrane of liver cells.

Discussion

Various properties of the uptake of phallotoxins into isolated liver cells resemble those of cholate uptake (Petzinger and Frimmer 1980). Because cholate inhibited the response of liver cells to phalloidin (Frimmer et al. 1977b) as well as the uptake of (^{3}H)-demethylphalloin competitively (Petzinger 1981) it is probable that phallotoxins are translocated by a carrier system of the liver cell which normally handles bile acids. It is well known that the uptake of bile acids is carrier mediated (Glasinovic et al. 1974; Reichen and Paumgartner 1975) and linked to the metabolic energy supply (Schwarz et

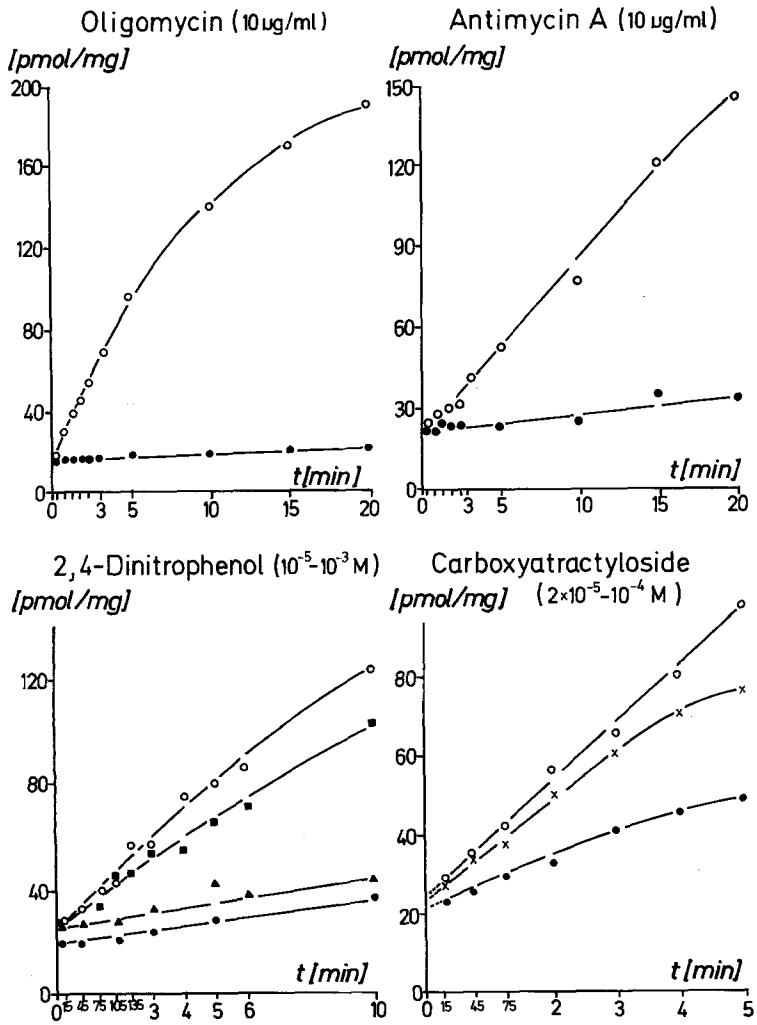

Fig. 4
Effect of metabolic inhibitors on the uptake of phallotoxin by isolated rat hepatocytes. 2×10^6 hepatocytes/ml were incubated in the presence of oligomycin (10 µg/ml, ●), antimycin A (10 µg/ml, ●), 2,4-dinitrophenol (10^{-3} M ●, 10^{-4} M ▲, 10^{-5} M ■) and carboxyatractyloside (10^{-4} M ●, 2×10^{-5} M ×) 10 min prior to 30 ng (^{3}H-)DMP/5 µg phalloidin. Controls (○) received the appropriate solvent volume

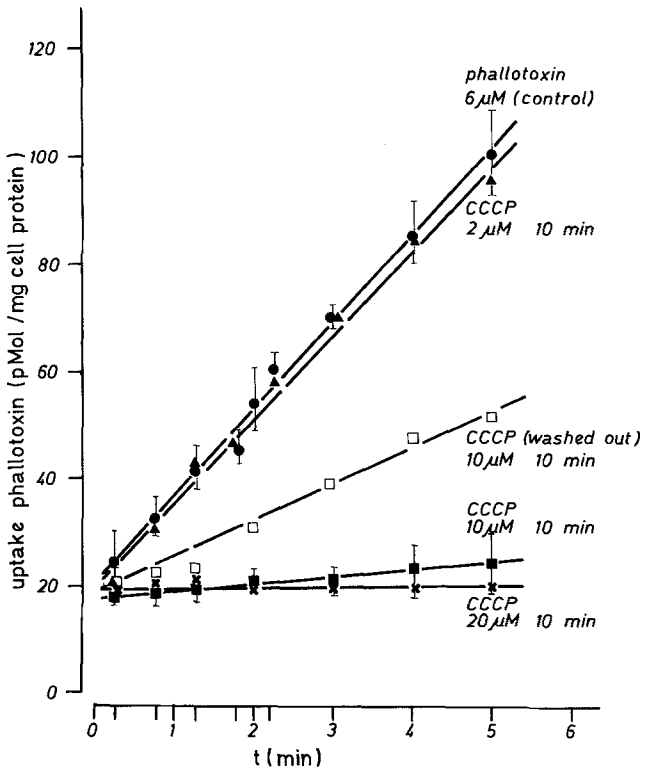

Fig. 5. Influence by carbonylcyano-m-chlorophenylhydrazon (CCCP) on the uptake of phallotoxin (phalloidin/3H-DMP) in isolated hepatocytes. Dose response of the inhibition of phallotoxin uptake by carbonylcyano-chlorophenylhydrazone (CCCP). Isolated liver cells were exposed to 2 µM CCCP (▲), 10 µM CCCP (■) and 20 µM CCCP (×) 10 min prior to the addition of (^{3}H-)DMP/phalloidin. The inhibitory effect of 10 µM CCCP was partially reversed if the inhibitor was removed after 10 min and the liver cells were allowed to regenerate for a period of 15 min in glucose supplemented Tyrode buffer prior to phallotoxin uptake (□)

al. 1975; Anwer et al. 1976). However corresponding data for the uptake of demethylphalloin have not been available until now.

The data in this paper give some convincing and some supporting arguments for the thesis that phallotoxins are translocated in liver cells in an energy linked manner too.

1. The most convincing results in favor of an energy linked uptake of demethylphalloin comes from experiments under anaerobic conditions (Fig. 1); liver cells were unable to accumulate phallotoxin in N_2/CO_2 atmosphere but restored at least 2/3 of the original transport rate after reoxygenation. The reversibility implies that most of the hepatocytes are still intact after the period of anaerobiosis. Thus the lack of transport is not the result of an impaired transport system, but more probable due to an impaired energy supply.

2. The phallotoxin uptake into liver cells is a concentrative one. Under aerobic conditions isolated hepatocytes accumulate phallotoxins 8–10-fold intracellularly. Part of the already internalized toxin was lost when the oxygen atmosphere was switched off or CCCP was added (Fig. 2). Considering that 40–60% of the toxin is tightly bound to intracellular F-actin (Faulstich et al. 1977; Petzinger 1981) the released demethylphalloin obviously stems from a pool of free unbound toxin available for the cytoplasmic side of the carrier. Because the intracellular concentration of that pool of toxin is at least 4 times that of the extracellular one, the toxin flux is reversed downhill and demethylphalloin traverses the membrane from the inside outward. In the presence of oxygen, however, the high intracellular toxin concentration can only be maintained as long as the ATP concentration is sufficiently high.

3. Actually DMP uptake is ATP dependent as was shown by ATP depletion with fructose and by various metabolic inhibitors (Table 1). The observed inhibition of DMP transport was not the result of any nonspecific membrane injury

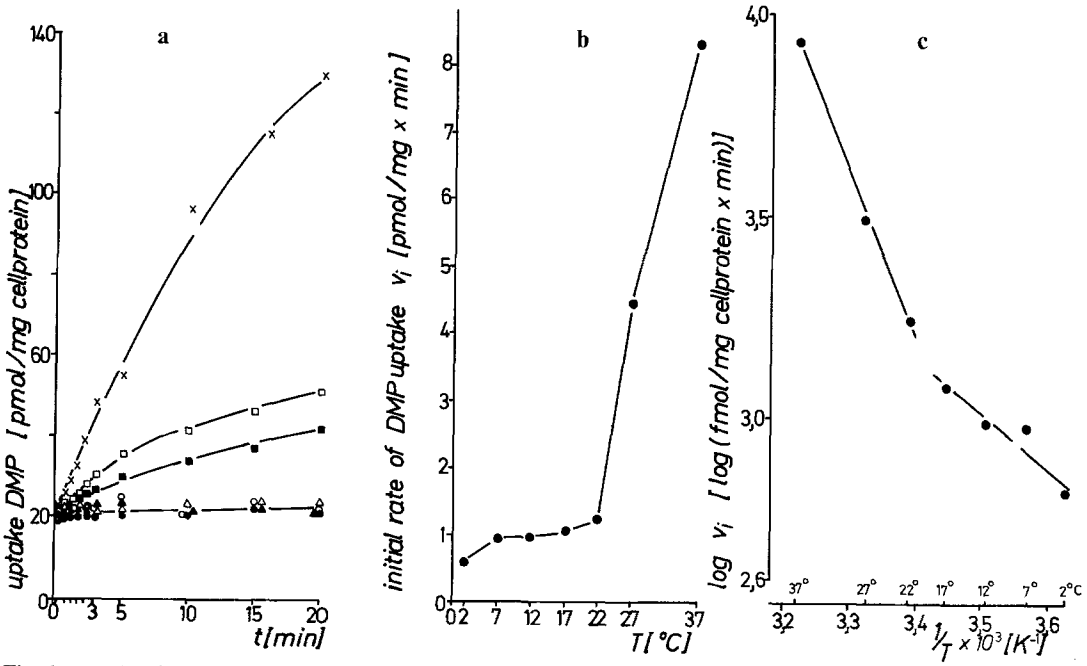

Fig. 6a–c. The effect of temperature on the uptake of phallotoxin by isolated rat hepatocytes. (**a**) Plot: phallotoxin uptake versus time of incubation Isolated hepatocytes were incubated in the presence of 30 ng (^{3}H-)DMP/ml plus 5 µg DMP/ml for 20 min at 2° C (●), 7° C (○), 12° C (▲), 17° C (△), 22° C (■), 27° C (□) and 37° C (×). (**b**) *Plot:* initial rate of uptake (V_i) versus temperature. The rate of DMP uptake during the initial 2 min of incubation (V_i) was used to calculate $Q_{10} = V_{i(T+10)} \times V_i^{-1}$. Experimental values from (**a**). (**c**) *Plot:* log V_i versus $1/T$. Experimental values from (**b**). The apparent activation energy was calculated according to $A_{app} = 2.303\ R \times \text{tlog}\ V_i \times (11/T)^{-1}$; R = gas constant

because in all experiments (Fig. 4) the zero time value of the uptake diagrams was identical with controls. This starting point usually increases 3-fold if the cell membrane is destroyed and permeable for trypanblue (Petzinger 1981). Finally the inhibition by CCCP was partially reversed by washing the cells. (About the effect of metabolic inhibitors on the viability of isolated hepatocytes see Eckel et al. 1979.)

4. Our experiments at various temperatures agree with an active transport mechanism but do not prove it (Le Fevre 1975). However the experiments are incompatible with the assumption of a passive diffusion. No increase of uptake between 2° and 17°C, a remarkable onset of transport at temperatures higher than 22°C, the $Q_{10} = 2.6$ and the apparent activation energy of 76.6 kJ/mol are consistent with an active transport of phallotoxins. The nonlinear Arrhenius plot shows a deflection at 21° – 22°C. This is indicative for the participation of lipids involved in the activity of a carrier protein (Boheim et al. 1980). The lipid domain surrounding the carrier allows only transport activity in a fluid state. It is therefore unclear whether the calculated high activation energy can be attributed to the lipid or to the protein component of the carrier system.

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