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Transport of Dehydroepiandrosterone and Dehydroepiandrosterone Sulphate into Rat Hepatocytes

Stephan Reuter and Doris Mayer*

Abteilung für Cytopathologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

The purpose of the present study was to characterize the transport of dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS) into hepatocytes at physiological and pharmacological concentrations. Hepatocytes were isolated from female Sprague–Dawley rats by collagenase perfusion. Uptake of [^{3}H]DHEA and [^{3}H]DHEAS at increasing concentrations (3.5 nM–100 μM) was measured by the rapid filtration technique at 30 s intervals up to 120 s. The uptake of DHEAS by hepatocytes was saturable ($K_m = 17.0\ \mu M$; $V_{max} = 3.7$ nmol/min/mg cell protein). In contrast, a specific saturable transport system for DHEA could not be detected in rat hepatocytes. It is suggested that DHEA enters the cell by diffusion. The uptake of DHEAS could be inhibited by antimycin A, carbonylcyanide-*m*-chlorophenylhydrazone, and dinitrophenol (inhibitors of the mitochondrial respiratory chain), by dinitrofluorobenzene and *p*-hydroxymercuribenzoate (NH_2- and SH-blockers, respectively), and by monensin (Na^+-specific ionophore). No inhibition was seen in the presence of ouabain (inhibitor of Na^+–K^+-ATPase) and phalloidin (inhibitor of cholate transport and actin-blocker). Interestingly, DHEAS uptake was inhibited by bile acids (cholate, taurocholate and glycocholate). Conversely, [^{3}H]cholate uptake was strongly inhibited by DHEAS, which indicates a competition for the same carrier. Replacement of sodium ion with choline markedly decreased uptake velocity at pharmacological DHEAS concentrations. The results suggest that DHEAS uptake is a saturable, energy-dependent, carrier-mediated, partially Na^+-dependent process, and that DHEAS may be taken up via the multispecific bile acid transport system.

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INTRODUCTION

Dehydroepiandrosterone (5-androsten-3β-ol-17-one; DHEA) and its sulphate (DHEAS) represent the main secretory steroidal products of the adrenal cortex and major circulating steroids in the blood. In human plasma, DHEA concentrations between 5 and 24 nM have been measured, while DHEAS concentration can be up to 9 μM [1–3]. In rats basic plasma DHEA concentrations between 4–23 ng/ml were found [4]. While DHEAS forms a circulating reservoir, DHEA probably is more active at the tissue level [5]. Despite the abundance of DHEA and DHEAS their physiological role is far from being clear. *In vitro* and *in vivo* data suggest oestrogen or androgen like effects, depending on sex hormone homoeostasis [6]. DHEA has several effects of pharmacological and clinical interest, including cancer-preventive actions, [1, 7–12]. On the other hand recent studies demonstrated that in rat liver DHEA induces a strong peroxisome proliferation, and after long-term treatment with high doses, the development of hepatocellular carcinomas [13–17a].

The mechanism of action of DHEA has not yet been elucidated. Several groups have been working on cellular binding of DHEA. A specific receptor has not been observed so far. In contrast to other steroids, DHEA does not bind to nuclei from isolated rat hepatocytes [17b]. Kalimi and Regelson [18] published data indicating the existence of a DHEA-binding macromolecule with unproven specificity in rat liver cytosol. Nicollier *et al.* [19] characterized a low molecular weight protein with high affinity for DHEAS and other sulphated steroids but not for free steroids including DHEA in guinea pig liver cytosol. Recent findings by Yamada

*Correspondence to D. Mayer.
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et al. [20] indicate the presence of a highly specific DHEAS-binding protein with an apparent molecular mass of 160–230 kDa in rat liver cytosol. In competitive binding studies they demonstrated that DHEA and other free steroids had no effect on [^{3}H]DHEAS binding.

The mechanisms by which DHEA and DHEAS enter hepatocytes have not been studied so far. The purpose of our work was to elucidate the uptake mechanism of DHEAS and DHEA into isolated rat hepatocytes at physiological and pharmacological concentrations and to characterize potential specific transport systems for these steroids.

MATERIALS AND METHODS

Reagents

[^{3}H]DHEA (54.9 Ci/mmol), [^{3}H]DHEAS (23.5 Ci/mmol) and [^{3}H]cholate (12.9 Ci/mmol) were obtained from NEN (Dreieich, Germany). Soluene 350 tissue solubilizer, Hionic-Fluor scintillant and plastic scintillation minivials were purchased from Canberra-Packard (Frankfurt, Germany). Silicone oils AR 20 and AR 200 were from Wacker Chemie GmbH (München, Germany). DHEA, DHEAS, antimycin A, oligomycin, carbonylcyanide-*m*-chlorophenylhydrazone, dinitrofluorobenzene, *p*-hydroxymercuribenzoate, cholic acid, taurocholic acid, glycocholic acid and choline chloride were obtained from Sigma Chemical Co. (Deisenhofen, Germany). Monensin, dinitrophenol, HEPES and EGTA were from Serva (Heidelberg, Germany); ouabain, bovine serum albumin (BSA) and ATP-bioluminescence-CLS kit from Boehringer (Mannheim, Germany). Phalloidin was a gift from Professor Faulstich (Heidelberg, Germany). DMEM/HAM's F12-medium (1:1) and collagenase were purchased from Biochrom K.G. (Berlin, Germany).

Animals

Female Sprague–Dawley rats (200–300 g) were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany). Animals were maintained on a standard diet with free access to food and water.

Hepatocyte preparation

Hepatocytes were isolated by recirculating perfusion with 0.05% collagenase essentially as described previously [21]. Preperfusion buffer (oxygen-saturated) was 20 mM HEPES, pH 7.4, 6.7 mM KCl, 145 mM NaCl and 0.5 mM EGTA. Perfusion buffer (oxygen-saturated) consisted of 100 mM HEPES, pH 7.4, 6.7 mM KCl, 77 mM NaCl and 3 mM $CaCl_2$. After 25–30 min perfusion the liver capsule was removed and hepatocytes were suspended in perfusion buffer, filtered through a nylon tissue with 50 μm mesh, centrifuged at 50 *g* (Beckman GP 50) and washed in DMEM/HAM's F12 (1:1) medium. Finally they were suspended in DMEM/HAM's F12 buffered with 10 mM HEPES, pH 7.4, at 4×10^6 cells/ml. When sodium dependence was studied cells were washed twice and resuspended in perfusion buffer where NaCl (77 mM) was replaced by choline chloride. Cell number was determined with a Neubauer haemocytometer, and viability was assayed by trypan blue exclusion. Hepatocyte preparations used in this study contained 85–97% viable cells. Protein concentration was determined by an amidoschwarz assay [22].

Measurement of uptake of DHEA, DHEAS and cholate into isolated hepatocytes

Uptake into hepatocytes was determined by incubating hepatocytes (final cell number 2×10^6 cells/ml medium) with 60 nCi/ml of the [^{3}H]-labeled substrates and various amounts of the unlabeled substrates in a total volume of 4 ml. Cells were preincubated at 37°C for 10 min, then substrates dissolved in medium were added. When the influence of metabolic inhibitors, sulfhydryl inactivators and bile acids was assayed, these were also preincubated with the cells for 10 min before addition of the substrate. When ethanol or methanol were used as solvents, control incubations with the same amount of solvent were run. Incubations were carried out in duplicate at 37 or 4°C with gentle shaking. Duplicate samples of the cell suspension (100 μl each) were removed at 30, 60, 90 and 120 s and placed in 400 μl polyethylene tubes containing 150 μl of a 1:1 mixture of silicone oils AR 20/AR 200, layered on top of 20 μl of 0.7 M perchloric acid [23]. The samples were immediately centrifuged for 5 s in a table-top microfuge (Beckman Instruments). The microfuge tube was cut at the silicone oil–perchloric acid interface after freezing in liquid nitrogen. The tip containing the cellular pellet was placed into a scintillation minivial and 500 μl of Soluene 350 tissue solubilizer were added. Samples were shaken overnight at 37°C. Radioactivity was determined after addition of 4 ml of Hionic-Fluor scintillant in a liquid scintillation counter (Tricarb 2200 CA, Canberra-Packard, Frankfurt, Germany) equipped with a chemiluminescence correction option.

The ATP content of hepatocyte preparations was determined using an ATP-bioluminescence kit.

Statistics

The initial uptake rates of the substrates examined were determined from the differences of the values obtained at 60 and 30 s. Specific transport was calculated from the difference of uptake values at 37 and 4°C. Significant differences between groups were estimated by the means of the non-parametric Wilcoxon-test for non-correlated samples. Kinetic parameters were calculated by the transformation-weighting model [24].

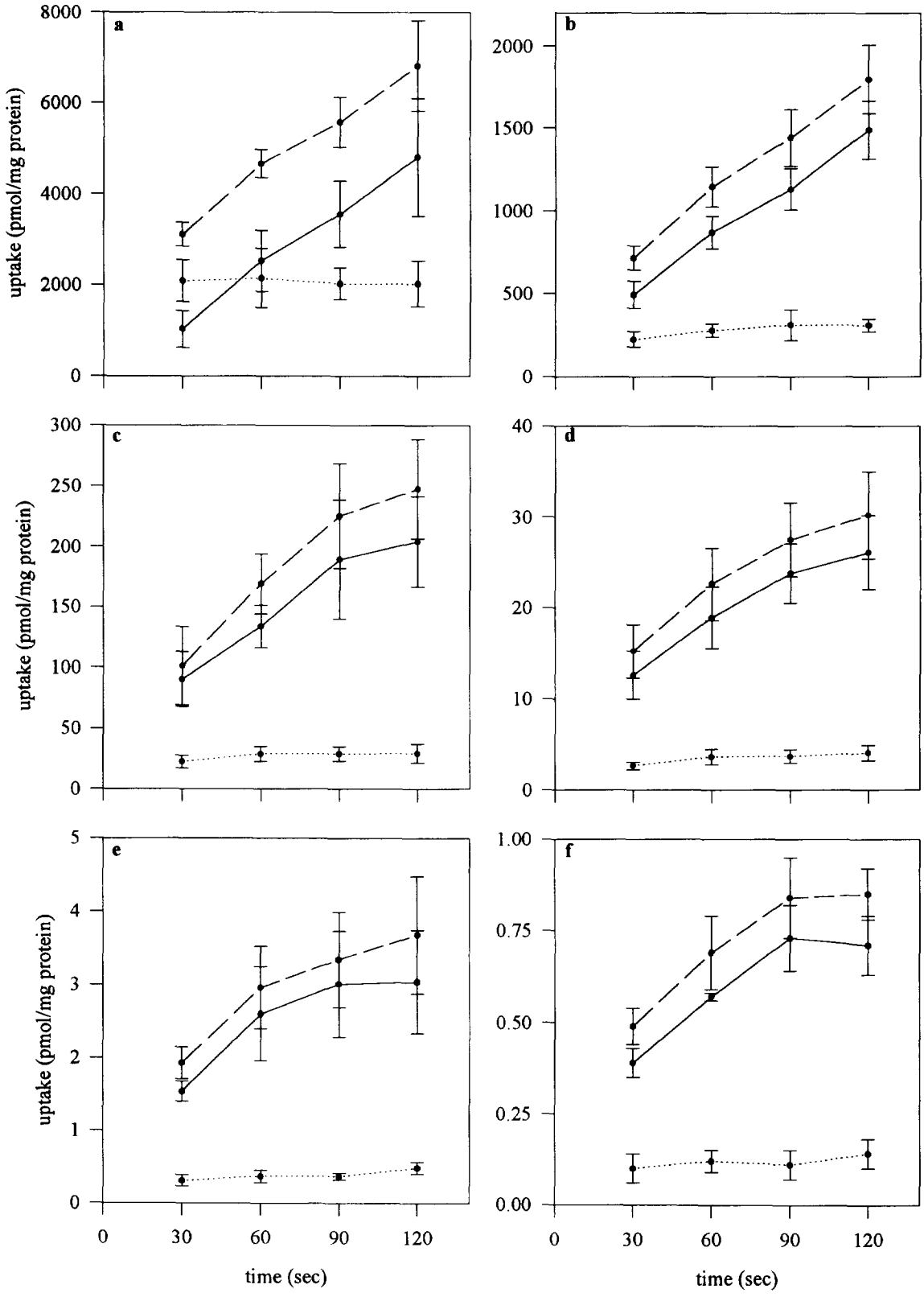

Fig. 1. Uptake of DHEAS into isolated hepatocytes. Hepatocytes were incubated with 60 nCi [^{3}H]DHEAS and unlabeled DHEAS to yield the indicated concentrations. (a) 100 μM, (b) 10 μM, (c) 1 μM, (d) 100 nM, (e) 12.5 nM and (f) 3.5 nM. Samples were removed at the time points indicated and uptake determined as described in Materials and Methods. Each point is the mean ± SD of three to seven separate preparations with duplicate determinations. ●···● binding at 4°C, ●---● uptake at 37°C, ●——● specific uptake (difference between values at 37 and 4°C).

RESULTS

Time-course of DHEAS uptake and uptake velocities at different substrate concentrations

The uptake rates of DHEAS into hepatocytes at concentrations from 3.5 nM to 100 μM are shown in Fig. 1. The uptake process was very rapid and was linear for at least 90 s, with the exception of 12.5 nM DHEAS. At pharmacological concentrations >1 μM, uptake was linear over 120 s. Unspecific binding observed at 4°C was nearly constant with time at all concentrations studied.

Saturation kinetics of the transport system of DHEAS was determined from uptake rates for 1–200 μM DHEAS (Fig. 2). Kinetic data calculated with the transformation-weighting model [24] showed that DHEAS-transport followed Michaelis–Menten kinetics. The apparent K_m is 17.0 ± 1.9 μM and V_{max} is 3.7 ± 0.3 nmol/min/mg protein.

Effect of metabolic inhibitors on DHEAS uptake

In order to determine whether the transport of DHEAS into isolated hepatocytes requires metabolic energy, aliquots of cells were preincubated for 10 min with several compounds known to decrease metabolic energy supplies. DHEAS concentration was 12.5 nM in these experiments (about physiological concentration). Initial uptake rate in the absence of inhibitors was 2.88 ± 0.74 pmol/min/mg protein. The inhibitory effects are summarized in Fig. 3. At a concentration of 0.5 mM the cardiac glycoside ouabain, which inhibits Na^+–K^+-ATPase and thus transport systems that are coupled to canalicular sodium export, had no effect on initial uptake rate (V_0 = 2.72 ± 1.60 pmol/min/mg protein). Antimycin A (10 μM), which blocks mitochondrial electron transport, caused a decrease in DHEAS-uptake to 1.16 ± 0.26 pmol/min/mg protein. Oligomycin (20 μM), an inhibitor of oxidative phosphorylation, reduced V_0 to 0.44 ± 0.22 pmol/min/mg protein. The two uncouplers 2,4-dinitrophenol (0.5 mM) and carbonylcyanide-*m*-chlorophenylhydrazone (10 μM) also had a significant effect on the initial velocity of DHEAS uptake, reducing V_0 to 1.76 ± 0.60 and 1.36 ± 0.40 pmol/min/mg protein, respectively.

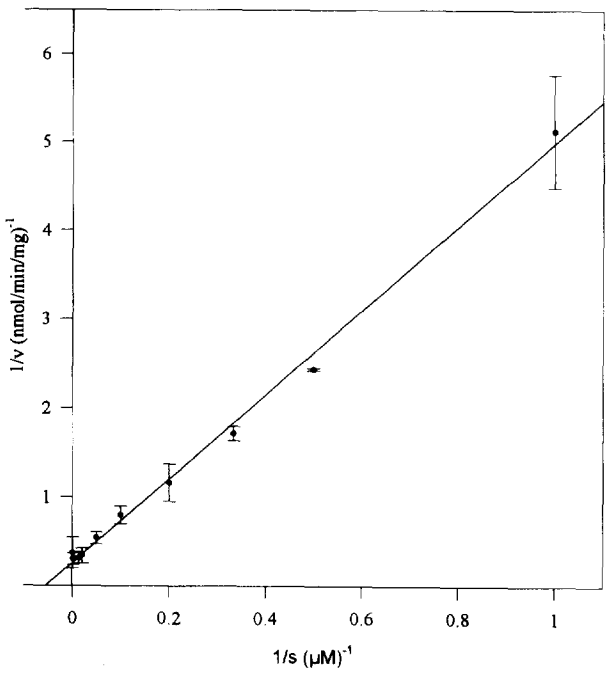

Fig. 2. Lineweaver–Burk plot of initial velocities of DHEAS uptake. DHEAS concentrations were 1, 2, 3, 5, 10, 20, 50, 75, 100 and 200 μM. Each point represents the mean ± SD of three separate preparations with duplicate determinations. Values of V_{max} and K_m were determined from the initial uptake rates (30–60 s) using the transformation-weighting model [24].

The influence of metabolic inhibitors on the cellular ATP levels was determined by incubating cells with 10 μM antimycin A or with 10 μM carbonylcyanide-*m*-chlorophenylhydrazone. Control cells had an ATP content of 11.98 ± 2.64 pmol/mg protein (three cell preparations with three separate determinations). ATP level was decreased to 4.94 ± 3.17 pmol/mg protein (41%; $P < 0.05$) by antimycin A, and to 0.63 ± 0.14 pmol/mg protein (5%; $P < 0.05$) by carbonylcyanide-*m*-chlorophenylhydrazone.

Two compounds which may inactivate carrier proteins by binding to functional amino acids, dinitrofluorobenzene and *p*-hydroxymercuribenzoate, were examined in this system. Dinitrofluorobenzene (50 μM), which binds to free amino groups, reduced V_0 to 1.12 ± 0.56 pmol/min/mg protein, *p*-hydroxymercuribenzoate (50 μM), which binds to free sulfhydryl groups, also had a significant inhibitory effect (V_0 = 1.34 ± 0.43 pmol/min/mg protein).

Phalloidin (0.5 μM), an inhibitor of bile acid transport [25] and actin blocker [26], showed no effect on V_0 of DHEAS uptake (2.78 ± 0.40 pmol/min/mg protein). The Na^+-specific ionophore [27], monensin (5 μM), reduced V_0 to 1.16 ± 0.10 pmol/min/mg protein.

Effect of bile acids on DHEAS uptake

Three bile acids, which are substrates of both the bile acid specific transporter and the multispecific bile acid transporting system [28] were examined for their inhibiting capacity on DHEAS uptake (Fig. 4). Initial uptake rate in the absence of inhibitors was 2.88 ± 0.74 pmol/min/mg protein. The inhibitor/substrate (I/S) ratio was 8000 (100 μM of bile acid/12.5 nM DHEAS) and 4000 (50 μM of bile acid/12.5 nM DHEAS). At these high inhibitor concentrations DHEAS uptake was strongly reduced by cholate (to 0.58 ± 0.20 and 0.90 ± 0.42 pmol/min/mg protein, respectively), taurocholate (to 0.98 ± 0.46 and 1.24 ± 0.66 pmol/min/mg protein, respectively) and glycocholate (to 0.86 ± 0.36 and 1.40 ± 0.56 pmol/min/mg protein, respectively).

Effect of DHEAS on cholate uptake

The results described above that cholate inhibits DHEAS uptake prompted us to investigate if conversely DHEAS is capable to inhibit the uptake of [^{3}H]cholate. As shown in Fig. 5 DHEAS is a strong inhibitor of [^{3}H]cholate uptake at different inhibitor/substrate ratios. A significant inhibitory effect of DHEAS already occurs at a I/S-ratio of 1.

Effect of sodium replacement on DHEAS uptake

In order to determine whether the V_0 of DHEAS uptake is a sodium-dependent process, the sodium ion concentration in the incubation medium was strongly reduced by equimolar substitution of choline chloride for sodium chloride in the buffer (Fig. 6). Sodium replacement had no significant effect on DHEAS uptake at 12.5 nM and 1 μM, while it reduced V_0 at 100 μM DHEAS from 6.10 $\pm$ 1.38 to 3.34 $\pm$ 1.14 nmol/min/mg protein (55% of control).

Time-course of DHEA uptake

In contrast to DHEAS uptake a specific saturable transport system for DHEA could not be detected in rat hepatocytes at DHEA concentrations varying between 11 nM and 100 μM [Fig. 7(a–c)]. DHEA was taken up by the cells at 37°C with a high velocity. A linear increase during the 120 s incubation period could never be observed. The binding observed at 4°C incubation temperature was very high at all DHEA concentrations investigated.

Reduction of DHEA concentrations by further reducing the ratio of unlabeled DHEA and [^{3}H]DHEA resulted in an unacceptable rise of background. Therefore BSA (0.5 and 2.5%) was added to the assay in order to bind DHEA and to further reduce the concentration of free DHEA. Figure 7(d) shows a linear time-course of DHEA uptake (11 nM) for about 90 s in the presence of 2.5% BSA, but not in the presence of 0.5% BSA.

DISCUSSION

The adrenal steroid, DHEA, and its sulphate-conjugate, DHEAS, both induce enzymes of peroxisomal β-oxidation and peroxisomal proliferation in rat hepatocytes [13, 14, 16], which indicates that both substances can enter hepatocytes. The two compounds

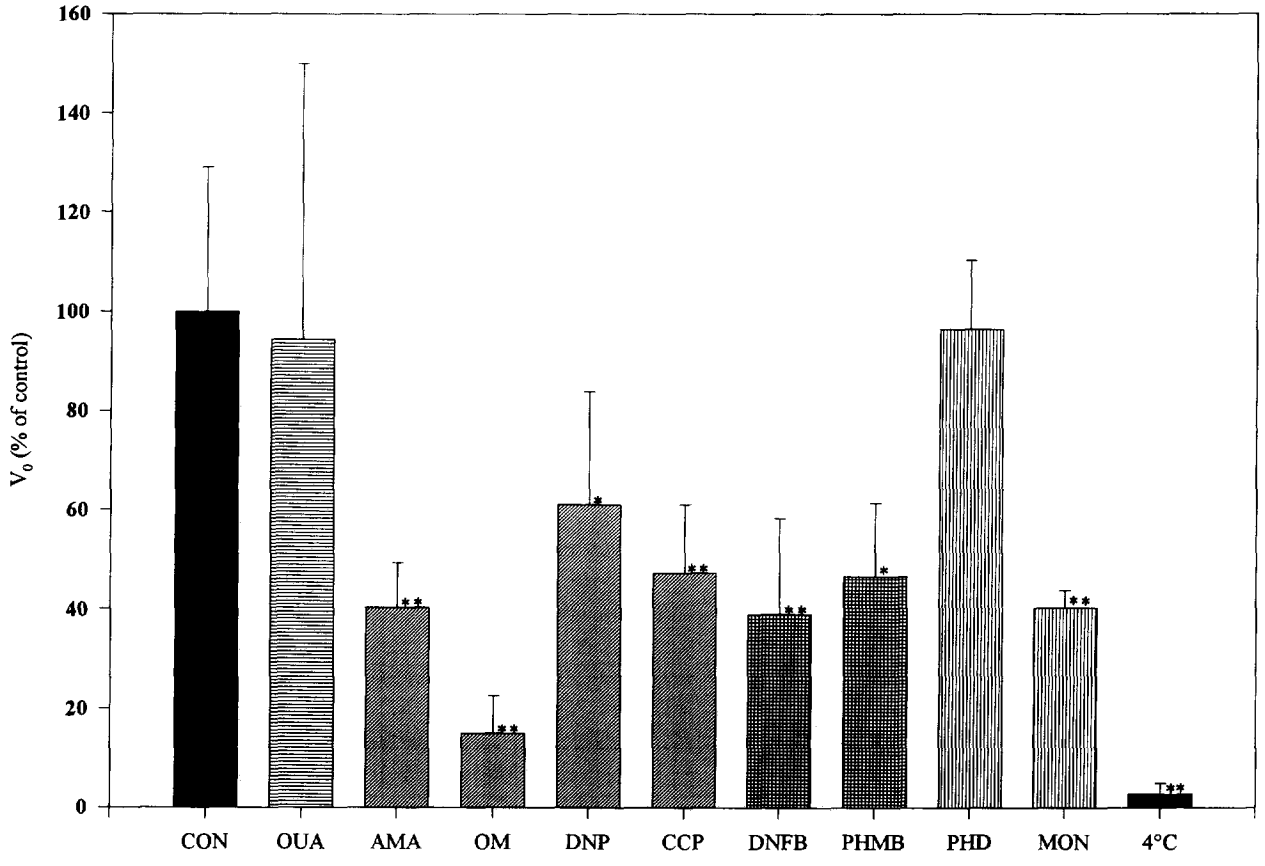

Fig. 3. Effect of metabolic inhibitors and inhibitors of amino and sulfhydryl groups on DHEAS uptake. DHEAS concentration was 12.5 nM. CON, control; OUA, ouabain (0.5 mM); AMA, antimycin A (10 μM); OM, oligomycin (20 μM); DNP, 2,4-dinitrophenol (0.5 mM); CCP, carbonylcyanide-*m*-chlorophenylhydrazone (10 μM); DNFB, dinitrofluorobenzene (50 μM); PHMB, *p*-hydroxymercuribenzoate (50 μM); PHD, phalloidin (0.5 μM); MON, monensin (5 μM). Each bar represents the mean $\pm$ SD of 3–5 separate preparations with duplicate determinations. $^*P < 0.02$, $^{**}P < 0.002$.

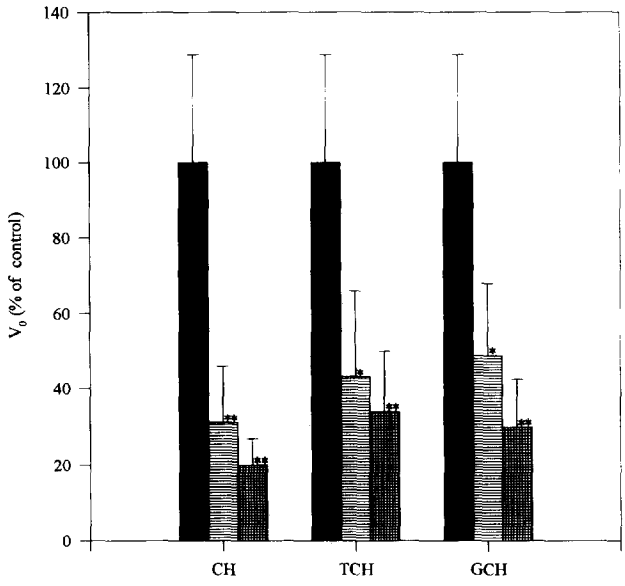

Fig. 4. Inhibition of [^{3}H]DHEAS uptake by bile acids. DHEAS concentration was 12.5 nM. Cholate (CH), taurocholate (TCH), and glycocholate (GCH) concentrations were 0 μM (control, ■), 50 μM (▤) and 100 μM (▦). Each bar represents the mean ± SD of 3–5 separate preparations with duplicate determinations. *$P < 0.01$, **$P < 0.005$.

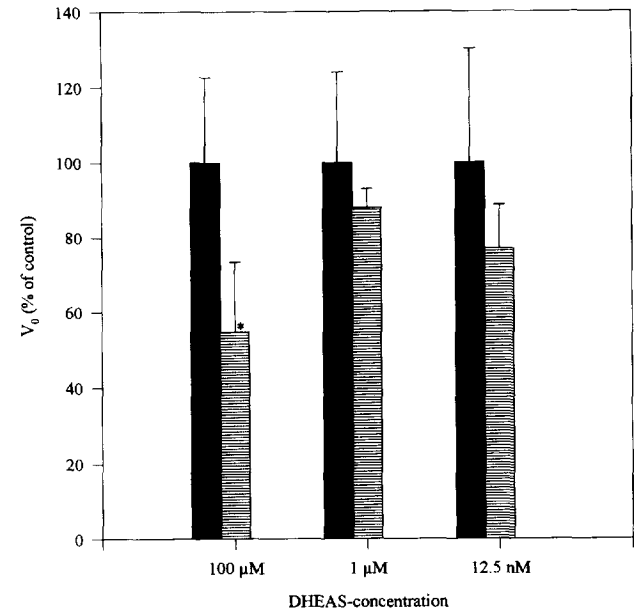

Fig. 6. Effect of sodium replacement on DHEAS uptake. Hepatocytes were incubated in perfusion buffer (■) or in buffer where Na^+ was replaced by choline (▤). DHEAS concentration was 100 and 1 μM and 12.5 nM. Each bar represents the mean ± SD of 3–4 separate preparations with duplicate determinations. *$P < 0.05$.

have different physicochemical properties. While the free unconjugated steroid is a highly lipophilic uncharged compound, DHEAS is an organic anion. It is therefore unlikely that the two substances enter the cell by the same mechanism. Organic anions are usually taken up into hepatocytes by a carrier-mediated mechanism [29–32]. As to the free steroids they can enter the cell by lipophilic diffusion. There are, however, some reports on energy-dependent [33] and carrier-mediated uptake. Since free steroids can inhibit bile acid transport to some extent, it was concluded that steroids may be taken up by the same carrier system [for reference see 31].

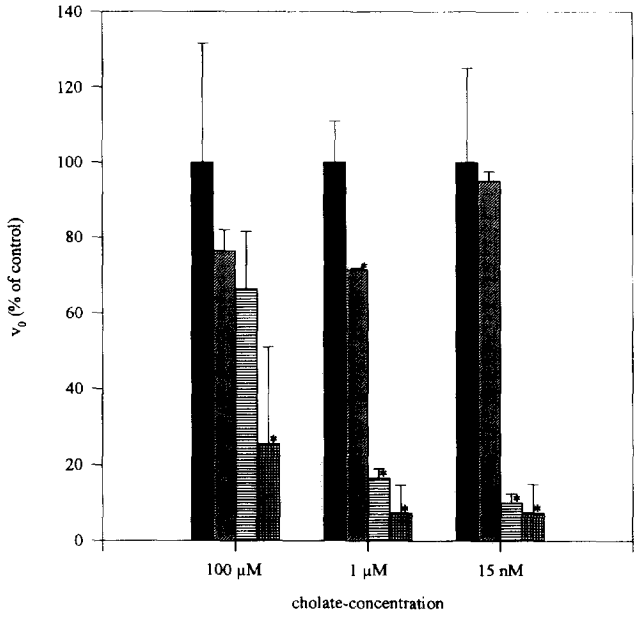

Fig. 5. Inhibition of [^{3}H]cholate uptake by DHEAS. Cholate concentrations were 100, 1 and 15 nM. DHEAS concentration was 0 μM (control, ■), 1 μM (▨), 50 μM (▤) and 100 μM (▦). Each bar represents the mean ± SD of three separate preparations with duplicate determinations. *$P < 0.05$.

DHEA which occurs in the blood at nanomolar concentrations shows a strong binding to hepatocytes at 4°C at these concentrations [Fig. 7(c)] but only little uptake (difference between 37 and 4°C). The same was found for pharmacological concentrations [Fig. 7(a,b)]. The lacking time dependence of DHEA uptake could mean that the transport is so fast that it cannot be measured with the technique chosen. Another explanation would be that DHEA cannot be accumulated in the cells because of a lack of, or a low amount of, cytosolic binding protein. When hepatocytes were incubated with [^{3}H]DHEA at 37°C for up to 6 min (not shown) the curves declined, suggesting a rapid metabolization and excretion of DHEA by hepatocytes. This agrees with observations *in vivo* [4, 34] that after injection of a single dose of [^{3}H]DHEA, DHEA metabolites rapidly appear in the bile and urine. The addition of 2.5% BSA to the uptake assay at 11 nM DHEA [Fig. 7(d)], which mimics physiological conditions and reduces the concentration of free DHEA, resulted in an uptake increasing with time. Although this points to a specific transport of DHEA at low concentrations, it does not prove the presence of a specific carrier for DHEA. It is assumed that DHEA enters hepatocytes by diffusion rather than by carrier-mediated uptake

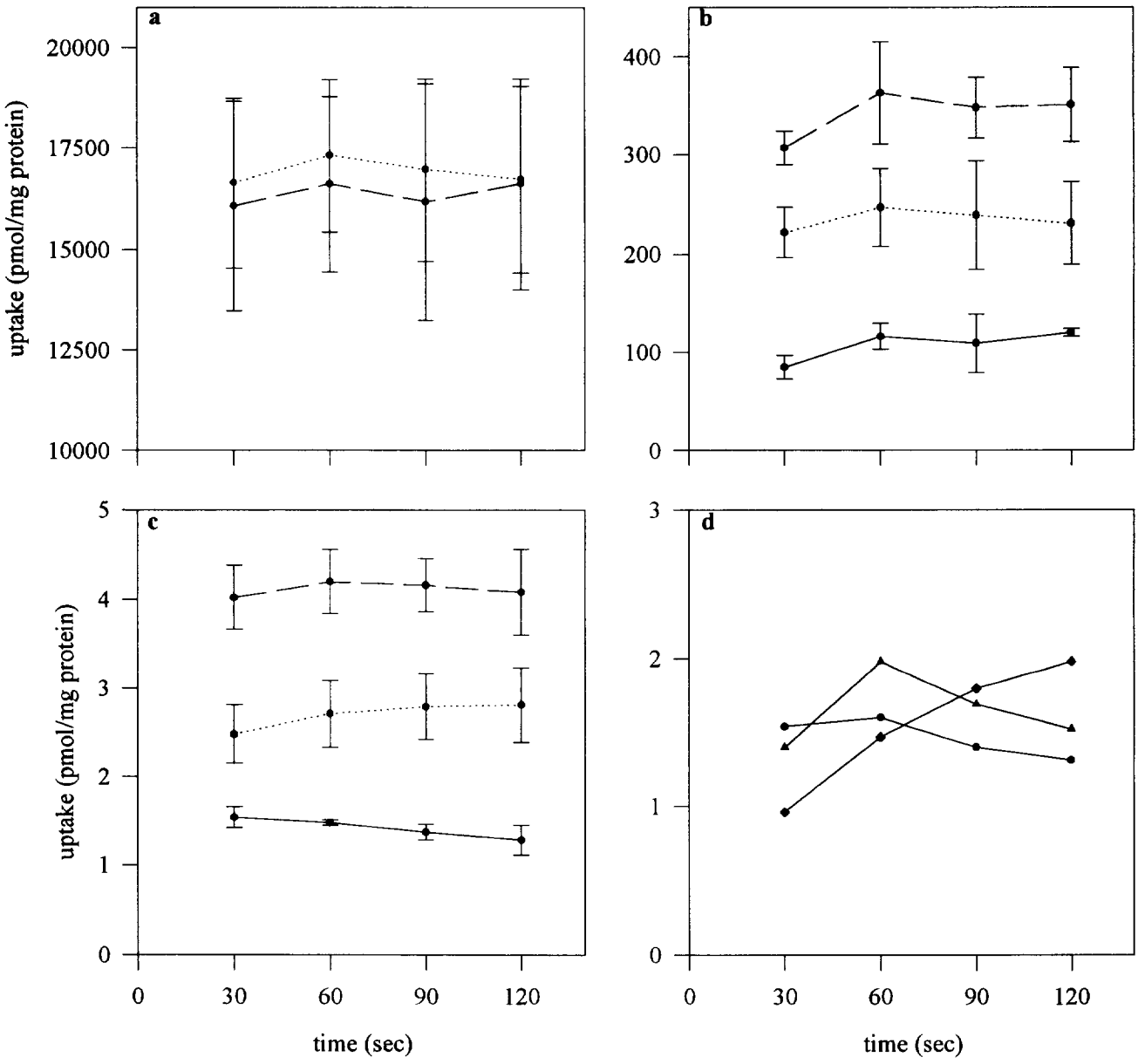

Fig. 7. Uptake of DHEA into isolated hepatocytes. Hepatocytes were incubated with 60 nCi [^{3}H]DHEA and unlabeled DHEA to yield the indicated concentrations. (a) 100 μM, (b) 1 μM, (c) and (d) 11 nM. Samples were removed at the time points indicated and uptake determined as described in Materials and Methods. Each point is the mean $\pm$ SD of 3–7 separate preparations with duplicate determinations. ●···● binding at 4°C, ●---● uptake at 37°C, ●——● specific uptake (difference between values at 37 and 4°C). (d) Specific uptake measured in the presence of 0% (●), 0.5% (▲) and 2.5% BSA (◆).

and that at very low DHEA concentrations [Fig. 7(d)] diffusion or binding to a cytosolic binding protein may become rate limiting. The final clarification of this question, however, will require more detailed investigation.

The inhibition of DHEAS uptake by inhibitors of mitochondrial electron transport and ATP-formation, and by blockers of free amino and sulfhydryl groups indicates that DHEAS is taken up into hepatocytes by an energy-dependent carrier-mediated process. The kinetic data (K_m = 17 μM; V_{max} = 3.7 nmol/min/mg protein) obtained for DHEAS uptake are very similar to that observed for cholic acid and glycocholic acid uptake (K_m = 13.1 and 14.7 μM, respectively; V_{max} = 0.83 and 1.3 nmol/min/mg protein, respectively [35]). The structural similarity between DHEAS and bile acids suggests that these compounds may be transported by the same carrier. Bile acids are taken up at the sinusoidal membrane of the hepatocytes by different transporters. One of them is saturable and Na^+-dependent and seems to be specific for bile acids [28, for further reference see 31]. A second bile acid transporter which is (partly) Na^+-independent, saturable and energy-dependent, is shared by other organic

anions such as glucuronides, glutathione conjugates, dyes, ouabain, phallotoxins etc. [25, 28–31, 36, 37]. Our finding that DHEAS uptake is inhibited by the Na^+-ionophore monensin shows that DHEAS transport requires an intact sodium gradient. The Na^+-dependence of DHEAS uptake is further supported by the finding that sodium replacement by choline leads to inhibition of DHEAS uptake at pharmacological concentrations. The lacking inhibition at low DHEAS concentrations might be due to Na^+-traces left in the incubation medium. Another explanation may be that at low DHEAS concentrations the Na^+-independent component of the multispecific bile acid carrier is active whereas at higher concentrations the Na^+-dependent species of the transporter is involved. The lacking effect of ouabain indicates that the biliary Na^+–K^+-ATPase is not involved in DHEAS uptake. Na^+-dependent excretion of DHEAS at the biliary pole of the cells obviously does not occur during the incubation period.

If DHEAS and bile acids share a common carrier, it would be expected that bile acids (cholate, taurocholate and glycocholate) inhibit DHEAS uptake. This is actually the case, but high bile acid/DHEAS ratios are required (Fig. 4). Conversely, DHEAS inhibited cholate uptake at lower ratios. This suggests that more than one carrier component with different affinities to bile acids and DHEAS are involved in DHEAS uptake, as it has already been concluded from the partial sodium dependence of the process.

In conclusion our data indicate that DHEA is taken up into hepatocytes mainly by diffusion, while DHEAS uptake requires a saturable, energy-dependent, partially Na^+-dependent carrier which is probably identical with the multispecific bile acid transporter system.

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