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DIFFERENTIAL EFFECTS OF METABOLIC INHIBITORS ON CELLULAR AND MITOCHONDRIAL UPTAKE OF ORGANIC CATIONS IN RAT LIVER

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Abstract—The effects of several metabolic inhibitors on the uptake of tri-*n*-butylmethylammonium (TBuMA) were studied in isolated rat liver mitochondria, isolated rat hepatocytes and isolated perfused rat livers, in order to characterize further the mechanisms for carrier-mediated uptake and cellular accumulation of organic cations in the liver. Treatment of isolated hepatocytes with valinomycin, carbonylcyanide-*m*-chlorophenyl-hydrazone (CCCP), dinitrophenol, oligomycin or antimycin resulted in a rapid decrease in cellular ATP within 3 min of addition. The initial uptake rate of TBuMA was generally largely affected by these treatments. However, fructose at 10 mM had no effect at all on the uptake rate of the cation whereas cellular ATP was decreased to an extent comparable to that after treatment with the metabolic inhibitors. Consequently it was hypothesized that the metabolic inhibitors affected the initial cellular uptake rate of organic cations due to either altered intracellular sequestration (e.g. mitochondria) or alternatively to direct effects on the plasma membrane rather than by decreasing cellular ATP. Isolated rat mitochondria were shown to take up organic cations very efficiently. Accumulation in this organelle is probably driven by the negative membrane potential as measured by the uptake of the lipophilic cation [^{3}H]tetraphenylphosphonium. Treatment of the isolated mitochondria with various metabolic inhibitors decreased the membrane potential in parallel to the effects on the uptake of TBuMA. Since mitochondria constitute a considerable intracellular volume, they may contribute largely to the storage of the organic cation in the hepatocyte. In isolated perfused livers, preloaded with either TBuMA or tetraphenylphosphonium (TPP^+), the addition of valinomycin or CCCP leads to a marked backflux of the cations from the liver into the perfusion medium. This suggests strongly that a large part of the intracellular storage capacity is lost after metabolic inhibitor treatment, probably as the consequence of dissipation of the mitochondrial membrane potential. Since the metabolic inhibitors in contrast to TBuMA uptake did not decrease the initial uptake rate of TPP^+ into isolated hepatocytes, it was concluded that mitochondrial uptake (mitochondria are the major storage sites for TPP^+) is not an essential determinant of the initial uptake rate in intact hepatocytes. It is concluded that: (i) carrier-mediated uptake of TBuMA in the rat hepatocyte is not directly dependent on cellular ATP; (ii) unlike uptake into the cells, uptake of this cation into rat mitochondria is electrogenic; (iii) since the metabolic inhibitors largely affect mitochondrial uptake of TPP^+ without influencing its initial uptake rate in hepatocytes, these processes (including those for TBuMA) should be seen as unrelated phenomena; (iv) metabolic inhibitors as used in the present study and many other studies, apart from ATP depletion, may directly influence hepatocyte uptake of organic compounds, e.g. by aspecific interactions with the carriers involved in translocation across the plasma membrane.

For organic cations, carrier-mediated uptake mechanisms have been described in liver plasma membrane vesicles, isolated hepatocytes, isolated perfused livers and in various animals *in vivo* [1, 2]. For amphipathic exogenous cationic compounds at least three uptake systems have been described in isolated rat hepatocytes [3]. With regard to the driving forces for uptake into hepatocytes, it was reported that manipulations of the trans-membrane ion gradients such as Na^+, K^+ or Cl^- did not basically alter the initial uptake rate of vecuronium [4] nor that of tri-*n*-butylmethylammonium (TBuMA†) [5]. Uptake of these cations was therefore suggested to occur via an electroneutral process. Uptake of organic cations into hepatocytes was shown to be inhibited by metabolic inhibitors such as KCN and the protonophores dinitrophenol (DNP) and carbonylcyanide-*m*-chlorophenyl-hydrazone (CCCP) [4], as well as the K^+-ionophore valinomycin [5] but the actual mechanisms underlying these effects were not further elucidated. Since steep bile to medium concentration gradients were observed for vecuronium [6], TBuMA [7, 8] and several other cationic compounds [2] at least one active transport step should be operative in the overall hepatobiliary excretion process. Recently it was found that protein phosphorylation via protein kinase C may be essential in the canalicular transport of TBuMA whereas on the sinusoidal domain of the hepatocyte no such effects could be detected [9].

In the present paper, we further characterized the energy dependency of hepatic uptake of the organic

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† Abbreviations: TBuMA, tri-*n*-butylmethylammonium; TPP^+, tetraphenylphosphonium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCCP, carbonylcyanide-*m*-chlorophenyl-hydrazone; DNP, dinitrophenol.

cation TBuMA with special reference to the question as to whether mitochondria play a significant role in the overall cellular uptake rate and sequestration.

MATERIALS AND METHODS

Chemicals. [*methyl*-^{3}H]TBuMA (21 Ci/mmol) and TBuMA were synthesized in our laboratory, according to procedures described by Neef *et al.* [7]. Radiochemical purity was checked by TLC and exceeded 99%. [^{3}H]Tetraphenylphosphonium ([^{3}H]-TPP^+) (45 Ci/mmol) was provided by New England Nuclear (Boston, MA, U.S.A.). Collagenase-H (type I), oligomycin, antimycin, DNP, valinomycin and TPP^+ were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). CCCP was purchased from Roth (Karlsruhe, F.R.G.). Taurocholate was from Fluka Chemie AG (Buchs, Switzerland); Dextran T 40 was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were from Merck (Darmstadt, F.R.G.).

Animals. Male Wistar rats, weighing 240–270 g and maintained on a standard diet and tap water, were used in all experiments. Animals used for the isolation of mitochondria and perfused livers were fasted for 16 hr before the experiment.

Hepatocyte preparation. Hepatocytes were obtained by using a modification of the procedure of Ref. 10 as described previously by Steen et al. [5]. Cells were finally collected in standard incubation medium containing (millimolar): NaCl, 118; KCl, 5; $MgSO_4$, 1.1; $CaCl_2$, 2.5; KH_2PO_4, 1.2; glucose, 10; HEPES, 10; $NaHCO_3$, 25; Dextran T 40, 1% (w/v); adjusted to pH 7.4.

Uptake measurements in hepatocytes. The initial uptake velocity of the various radiolabeled compounds was determined by preincubating 3 mL cell suspension, in standard incubation medium (2.5–3.0 × 10^6 cells/mL), for 30 min at 37° under continuous shaking and gassing with 95% O_2, 5% CO_2. The experiment started after addition of the radiolabeled compound. Subsequently, samples were removed from the cell suspension at 15 sec intervals for a 1 min period (for the TPP^+ experiments) and at 30 sec intervals for a 3 min period (for the TBuMA experiments). The uptake of the compounds by the cells was rapidly stopped by dilution with 3 mL of an ice-cold solution, containing (millimolar): NaCl, 143; KCl, 5; $MgSO_4$, 1.1; $CaCl_2$, 2.5; KH_2PO_4, 1.2; HEPES, 10; adjusted to pH 7.4. Separation of the cells from the medium was performed by rapid filtration over Whatman GF/C filters under a constant vacuum of 600 mbar. Then the filters were washed twice with 3 mL of the same ice-cold solution. To determine uptake of the radiolabeled compound by the cells, radioactivity remaining on the filters was counted. Therefore, filters were transferred into scintillation vials and 3 mL Safe Fluor (Packard, Groningen, The Netherlands) was added. The vials were counted for 5 min in a Beckman LS 1800 liquid scintillation counter.

The effect of various metabolic inhibitors on the uptake of the radiolabeled compounds was determined by addition of the compounds 3 min prior to the start of the experiment. The compounds were dissolved in either incubation medium, ethanol or dimethyl sulfoxide. Final concentrations of ethanol or dimethyl sulfoxide never exceeded 0.1%, and were shown to have no effect on the uptake of the radiolabeled compounds.

Viability tests. The viability of the cell suspension was determined by the exclusion of Trypan blue as described before [5]. The viability and the integrity of the cells were not affected by the several metabolic inhibitors used within the time period when uptake of the radiolabeled compounds was measured.

Isolation of rat mitochondria. Mitochondria were isolated from rat livers by a modified method of Rickwood *et al.* [11]. In brief, starved rats were killed by decapitation. The liver was homogenized in isolation medium containing (millimolar): sucrose, 75; mannitol, 225; HEPES, 5; EGTA, 1; at pH 7.4. The homogenate was centrifuged for 10 min at 1000 *g* at 4°. Mitochondria from the supernatant were collected by centrifugation for 10 min at 10,000 *g*. The pellet was washed twice. Protein concentration was determined according to procedures of Lowry *et al.* [12] and ranged between 20 and 40 mg/mL. The isolated mitochondria were checked for morphological appearance by electron microscopy (see legend to Fig. 2), for purity by enrichment of mitochondrial enzyme markers [11] and for viability by respiration measurements and membrane potential estimations.

Uptake measurements in isolated rat mitochondria. To determine uptake of cationic compounds into mitochondria, 20 μL mitochondria suspension (0.4–0.8 mg protein) and 80 μL incubation medium were incubated for 5 min at 28°. Final concentrations were (millimolar): mannitol, 75; sucrose, 25; EGTA, 1; KCl, 95; KH_2PO_4, 5; Tris, 20; succinate, 10; malate, 5; ATP, 1; at pH 7.4. The uptake started after the addition of 20 μL incubation medium containing the radioactive compound, at a final concentration of 1 μM. The uptake was rapidly stopped at indicated time points by dilution with 3 mL ice-cold solution containing (millimolar): mannitol, 75; sucrose, 25; EGTA, 1: KCl, 95; KH_2PO_4, 5; Tris, 20; at pH 7.4. Mitochondria were separated from the medium by rapid filtration over Whatman GF/C filters. Determination of radioactivity was performed as described above for uptake in isolated hepatocytes.

Estimation of the membrane potential. To estimate the effects of the several metabolic inhibitors on the membrane potential of cells and mitochondria, the initial uptake velocity of the lipophilic cation [^{3}H]-TTP^+ bromide [13, 14] was determined over a 1 min period [15], in both isolated hepatocytes and isolated mitochondria.

Determination of cellular ATP. To measure the amount of cytosolic ATP in isolated cells after treatments with metabolic inhibitors, cells were separated from the medium by rapid centrifugation and resuspended in 10% trichloroacetic acid. After extractions with diethyl ether and neutralization by KOH, samples were determined for ATP by HPLC on an anion exchange column, with 0.01 M NH_4Cl as eluent.

Kinetics in the isolated perfused liver. A perfusion technique was used as described by Meijer *et al.* [16], with some slight modifications. Briefly, rats were anaesthetized with pentobarbital (60 mg/kg,

i.p.). After cannulation of the portal vein and the superior vena cava the liver was excised and placed in the perfusion apparatus. The recirculating medium consisted of Krebs bicarbonate buffer supplemented with 0.5% bovine serum albumin and was constantly gassed with 95% oxygen and 5% carbon dioxide. The perfused flow was maintained at 35 mL/min at a hydrostatic pressure of 12 cm of water to assure sufficient oxygen supply. The pH was monitored on line and ranged between 7.35 and 7.45. The temperature was kept at 38°. After a 30 min recovery period following the surgical procedure, the radiolabeled compound was added to the perfusion medium. Metabolic inhibitors were administered either 3 min prior or 30 min after the addition of the radiolabeled compound (see also legends to the figures). In one experiment the bile duct was ligated (in both the control and the treatment group) to exclude effects due to bile flow differences. The volume of the perfusion medium used in all experiments was 100 mL. Perfusion was performed over a 1 hr period. During the experiment the viability of the liver was checked by measuring pH and flow of the recirculating medium. Perfusate and bile samples were collected at indicated time points in scintillation vials and 3 mL Safe Fluor was added. The vials were counted for 5 min in a Beckman LS 1800 liquid scintillation counter.

Statistical analysis. In the experiments with isolated hepatocytes, values are expressed as mean percentage uptake ± SEM. Each treatment was compared to the control in the same experiment using the paired Student's *t*-test. Differences were considered to be significant if $P < 0.05$.

RESULTS

TBuMA uptake studies in isolated hepatocytes

To investigate the dependency on metabolic energy of the hepatic uptake of organic cations, the initial uptake rate of the model organic cation TBuMA in isolated hepatocytes was studied in the presence of a range of metabolic inhibitors. For hepatic uptake of TBuMA, the involvement of two different carrier systems has been reported. At low concentrations (<1 μM) the cation is mainly taken up by the type I cation uptake system, whereas at 25 μM of the cation a multispecific uptake system also contributes to the total uptake rate [3, 5]. In the present study 1 μM of the cation was used to specifically investigate the type I organic cation system.

The effects of the metabolic inhibitors on the initial uptake rate of TBuMA are depicted in Fig. 1, upper panel. It can be seen that all of the agents decreased the uptake rate of the cation significantly. However, oligomycin which has been reported to block mitochondrial ATP-synthetase and antimycin which decreases mitochondrial respiration [11, 17] showed only a moderate inhibition of TBuMA uptake rate, yet the combination of both compounds showed an additive effect. The protonophores, DNP and CCCP, and the K^+-ionophore valinomycin (all known as uncouplers of oxidative phosphorylation) inhibited TBuMA uptake rate by about 55%.

Metabolic inhibitors are commonly used to characterize cellular transport processes, in particular to establish the energization by compounds such as ATP. For fructose, a completely different ATP-depleting mechanism was described [18]. The addition of 5 mM fructose to the cell suspension did not decrease TBuMA uptake rate whereas at 10 mM of the sugar, even a slight stimulation was observed (see Fig. 1, upper panel).

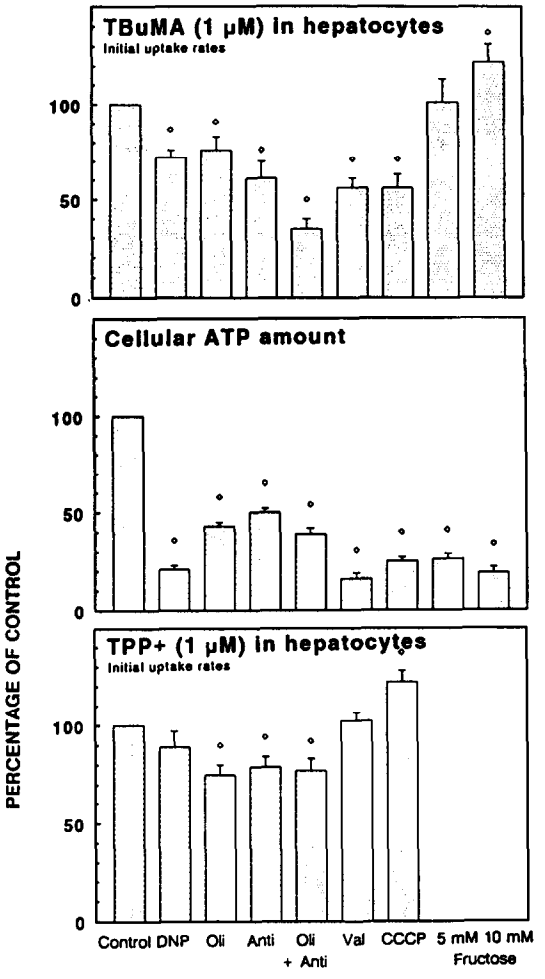


Fig. 1. Effects of a range of metabolic inhibitors (all at 10 μM) as well as fructose on the initial uptake of TBuMA at 1 μM (upper panel), the initial uptake rate of TPP^+ at 1 μM (lower panel) and the amounts of cellular ATP after treatments of cell suspensions with the metabolic inhibitors and fructose for 5 min (middle panel). The effects of fructose on the initial uptake rate of TPP^+ are not determined (Oli, oligomycin; Anti, antimycin; Val, valinomycin). Effects are expressed as mean percentages + SEM, compared to controls. Each value is the mean of at least three independent experiments. Values were significant if $P < 0.05$ (◇).

ATP measurements in isolated hepatocytes

To investigate whether the metabolic inhibitors and

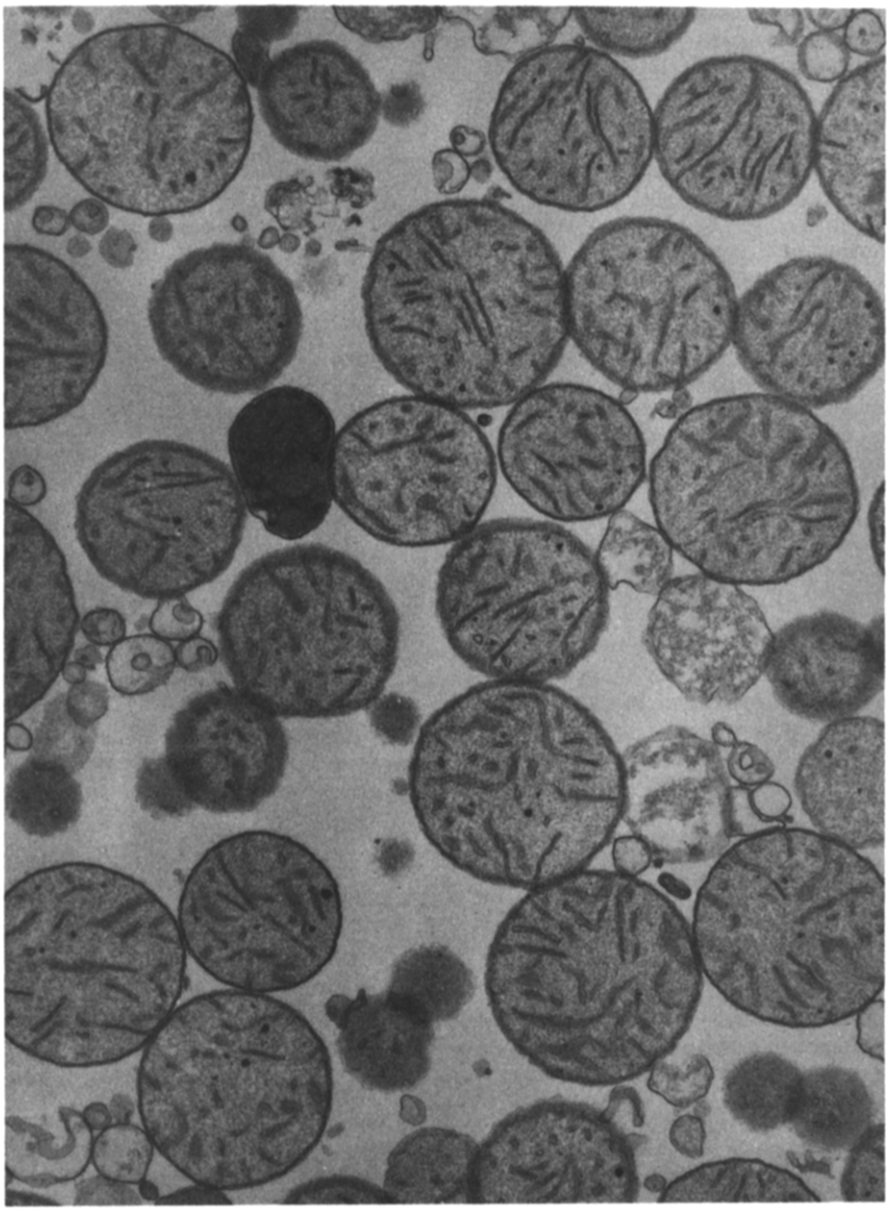


Fig. 2. Ultrathin section of mitochondria, isolated as described in Materials and Methods. Mitochondria were incubated for 5 min at 28°, spun down by rapid centrifugation and subsequently fixed by the addition of 5% glutaraldehyde.

fructose exerted their effect on the uptake rate of TBuMA by decreasing the amount of cellular ATP, these levels were measured in hepatocytes after the indicated treatments. The method described in Materials and Methods appeared to be a simple, sensitive and reproducible one. From Fig. 1, middle panel, it can be seen that ATP levels were decreased by all treatments but not to the same extent. Oligomycin, antimycin and their combination decreased the amount of ATP to about 60% of controls. DNP, CCCP and valinomycin even decreased the ATP content to about 20%, an effect comparable to that of fructose at both 5 and 10 mM.

Fructose was reported to deplete the amount of ATP in cytosol via rapid phosphorylation through fructose-kinase. Fructose-phosphates however are substrates for glycolysis and therefore the amount of ATP secondarily increases after 10 min of incubation, a phenomenon that was confirmed in the present study.

Isolation of rat mitochondria

The above-mentioned data rendered it unlikely that metabolic inhibitors exerted their effect on uptake of TBuMA only by decreasing the amount of cellular ATP. Consequently, alternative sites of

Table 1. Purity of the mitochondrial preparation as calculated by enzymatic characterization of the homogenate and the final mitochondrial pellet

	Relative enrichment of marker enzymes in the mitochondrial fraction	Percentage of total homogenate present in the mitochondrial fraction
Succinate cytochrome *c* reductase	4.09 ± 0.78 (N = 13)	81.8
Acid phosphatase	2.71 ± 0.89 (N = 22)	2.7

Relative enrichment is defined as the ratio of specific activity in the rat liver homogenate and the mitochondrial fraction. The mitochondrial marker enzyme succinate cytochrome *c* reductase and the lysosomal marker enzyme acid phosphatase were used.

Relative enrichment is given as the mean ± SD.

interaction should be present. Since all the metabolic inhibitors (apart from fructose) used in the present study do interfere in mitochondrial function, we investigated whether sequestration in this organelle is an important determinant in the initial cellular uptake rate of organic cations. Therefore, we isolated mitochondria from rat liver and performed uptake studies with TBuMA. Mitochondria were isolated and purified by the method of Rickwood *et al.* [11]. Morphological appearance, enrichment of mitochondrial marker enzymes and respiration measurements revealed that a highly purified and viable mitochondria fraction was obtained. Figure 2 shows that our preparation contained properly shaped mitochondria with small cristae while contamination by other membrane structures was low. From Table 1 it can be seen that the marker enzyme succinate cytochrome *c* reductase is enriched 4-fold in the mitochondrial fraction compared with total homogenate, whereas the lysosomal marker enzyme acid phosphatase was only slightly enriched. It was calculated that 80% of protein in the suspension was of mitochondrial origin [19]. Measurement of the respiration rate of the mitochondrial suspension indicated that the mitochondria steadily consumed O_2 (data not shown). The capability of building a membrane potential was checked by measuring the uptake of the lipophilic organic cation TPP^+, a compound that passively distributes across membranes according to the membrane potential [13].

Uptake studies in isolated mitochondria

To investigate whether alterations in the membrane potential of isolated mitochondria influence the uptake of organic cations, the membrane potential (as indicated by the distribution of TPP^+ [13–15]) was measured in the presence of the several metabolic inhibitors. As can be seen from Fig. 3, upper panel, the protonophore CCCP largely inhibited the accumulation of TPP^+ into energized mitochondria which is in line with the observation that the membrane potential in mitochondria is determined mainly by the proton gradient (ΔpH) [11]. The K^+-ionophore valinomycin also clearly decreased the uptake of TPP^+ which can be explained by dissipation of the K^+ gradient. Uptake of TPP^+ in the presence of CCCP was almost completely blocked and

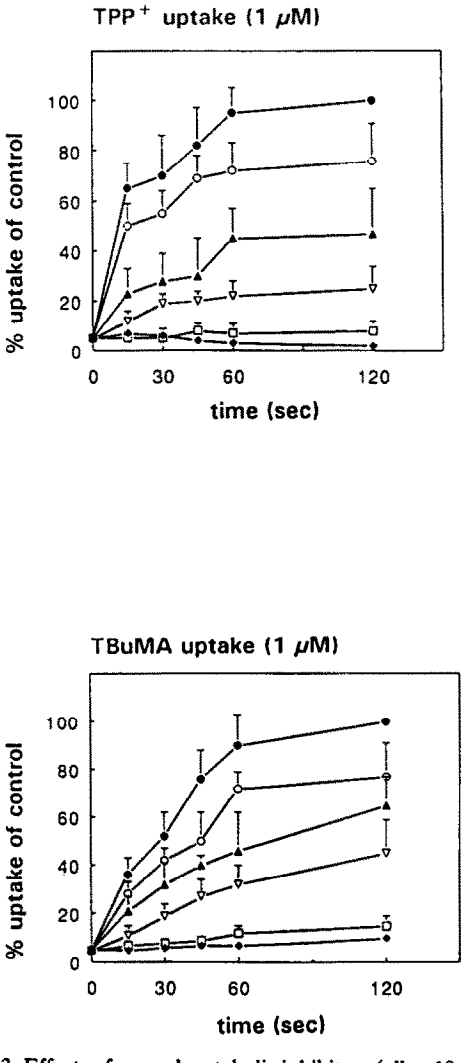


Fig. 3. Effects of several metabolic inhibitors (all at 10 μM) on the uptake of 1 μM TPP^+ (upper panel) and 1 μM TBuMA (lower panel) into isolated mitochondria. Each value represents the mean of at least three independent experiments. Values are expressed as mean percentages + SEM compared to controls. (Mitochondrial content at t = 120 sec of the control curve is set to 100%.) (●) Control; (○) + oligomycin; (▲) + antimycin; (▽) + oligomycin and antimycin; (□) + valinomycin; (◆) + CCCP.

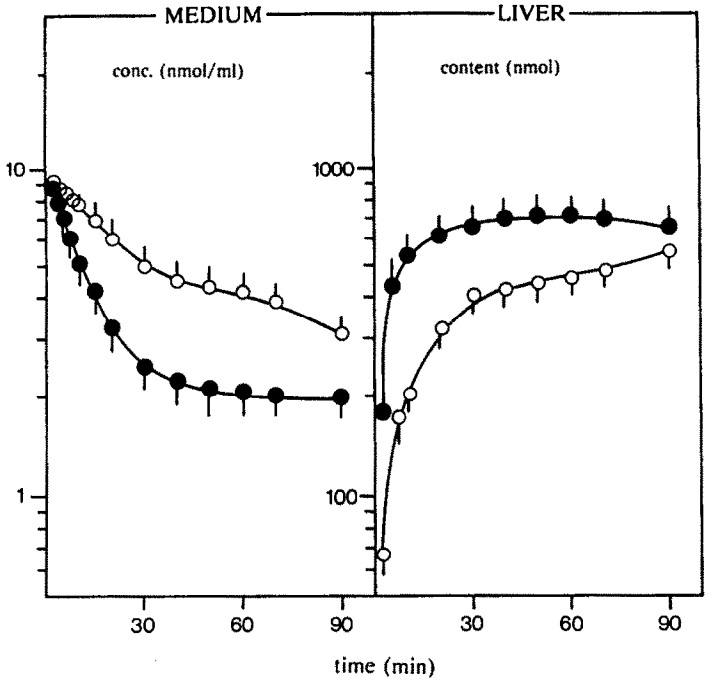


Fig. 4. Effect of CCCP (10 μM) on the hepatic disposition of TBuMA (dose is 1 μmol) studied in the isolated perfused liver. The bile duct was ligated in both control and CCCP experiments to prevent effects on uptake due to bile flow differences. (● Control; (○) CCCP treatment.

comparable with the uptake of the cation at 0°. Residual uptake under these conditions is likely to be due to simple passive diffusion and binding.

The influence of the various inhibitors on the uptake of TBuMA in isolated mitochondria was very similar to that on the uptake of TPP^+ (Fig. 3, lower panel). The uptake of this cation in mitochondria was completely inhibited by CCCP and valinomycin, and to a lesser extent by antimycin and oligomycin. From experiments in which an artificial diffusion potential was generated by using different K^+ concentrations in the incubation medium in the presence of valinomycin, we were able to demonstrate that the uptake of TBuMA (as compared to the uptake of TPP^+) is electrogenic and very likely driven by the membrane potential (data not shown).

Uptake of TPP^+ in isolated rat hepatocytes

To investigate whether mitochondrial uptake and initial cellular uptake are separate processes, the initial uptake rate of TPP^+ was studied in isolated hepatocytes and compared to that observed in isolated mitochondria. From Fig. 1, lower panel, it can be seen that the effects of the various metabolic inhibitors on initial uptake rate of TPP^+ in isolated cells are quite different compared to those in isolated mitochondria. The K^+-ionophore valinomycin and the protonophore CCCP slightly stimulated TPP^+ uptake rate whereas the protonophore DNP only moderately decreased the uptake rate. Oligomycin, antimycin and their combination only reduced TTP^+ uptake by about 20%.

Isolated perfused rat liver studies

Figure 4 shows that in the isolated perfused liver, clearance of TBuMA (dose 1 μmol) from the medium in the presence of CCCP is reduced compared to controls. In this preparation, bile duct was ligated in control as well as in treated livers to exclude effects of bile flow *per se*. It can be seen that TBuMA accumulated to a high degree in the liver, since 70% of the cation is cleared from the medium within 90 min.

To investigate whether the accumulated amount of TBuMA in the liver is stored in a compartment sensitive to metabolic inhibitors, isolated livers were preloaded with TBuMA or TPP^+, and subsequently CCCP or valinomycin was added to the perfusate. After addition of the inhibitors, the concentration of both TBuMA and TPP^+ in the perfusate clearly increased (Fig. 5). The observed effects were more pronounced in the case of valinomycin than CCCP. From the figures it can be seen that in the presence of CCCP bile flow was reduced only to about 50%. Since bile flow is an energy-dependent process [2], this might indicate that mitochondrial function was only partly affected at the CCCP concentration used. On the other hand, valinomycin treatment led to a rapid and complete blockade of bile formation and therefore excretion of the cation to bile.

DISCUSSION

Many efforts have been made to elaborate the driving forces for hepatic uptake of organic compounds, including the dependency on metabolic energy [1, 2]. In the present study we provide new insight into the mechanisms for hepatic uptake of the organic cation TBuMA in several preparations

by testing a range of metabolic inhibitors. Previously, we reported that the metabolic inhibitors KCN (1 mM), DNP (1 mM) and CCCP (10 μM) decreased the initial uptake velocity of the cationic drug vecuronium [4] as well as that of TBuMA [5]. Here we show that in isolated hepatocytes an extended range of metabolic inhibitors interferes with the initial uptake rate of TBuMA (1 μM) specifically via the type I organic cation uptake system [3]. Treatment with these compounds depleted the amount of cellular ATP, although to a variable extent. These effects raised the question as to whether ATP itself is necessary for the particular uptake process. Interestingly, when we depleted the cellular amount of ATP with fructose [18], the uptake rate of TBuMA was not inhibited at all. This low level of ATP was continued for at least 10 min. Since in hepatocytes the major part of cellular ATP is present in the cytosol [18] we concluded that cellular ATP *per se* is not an essential factor in the initial uptake rate of TBuMA.

Mitochondrial uptake

The rapid depletion of cellular ATP by the metabolic inhibitors is mainly the consequence of effects on mitochondrial function. Since accumulation in mitochondria has been demonstrated for various mono- and bivalent organic cations [17, 20, 21], the observed effects of the metabolic inhibitors in whole cells may be due in principle to effects on these organelles. Accumulation of organic cations in mitochondria can be anticipated since a large membrane potential of about 220 mV, inside negative [11], is present. Therefore, the organelles may even provide sink conditions for the initial uptake process. The lipophilic organic cation TPP^+, its derivatives and other lipophilic cationic drugs such as methyldeptropine were previously used as probes for measuring the membrane potential in isolated cells and membrane vesicles [13–15, 22]. In this study we observed that both TPP^+ and TBuMA are efficiently taken up into isolated energized mitochondria whereas virtually no uptake was observed at 0° or in the absence of metabolic substrates. In the presence of a diffusion potential ($[K^+_{in}] > [K^+_{out}]$ + valinomycin) the uptake of both TBuMA and TPP^+ was stimulated, whereas in the presence of a reversed diffusion potential ($[K^+_{in}] < [K^+_{out}]$ + valinomycin) no uptake was observed (data not shown). It can thus be concluded that the negative membrane potential is driving the cation uptake. Similar observations were reported for the uptake of the neurotoxic organic cation *N*-methyl-4-phenylpyridinium (MPP^+) in isolated rat liver mitochondria [17].

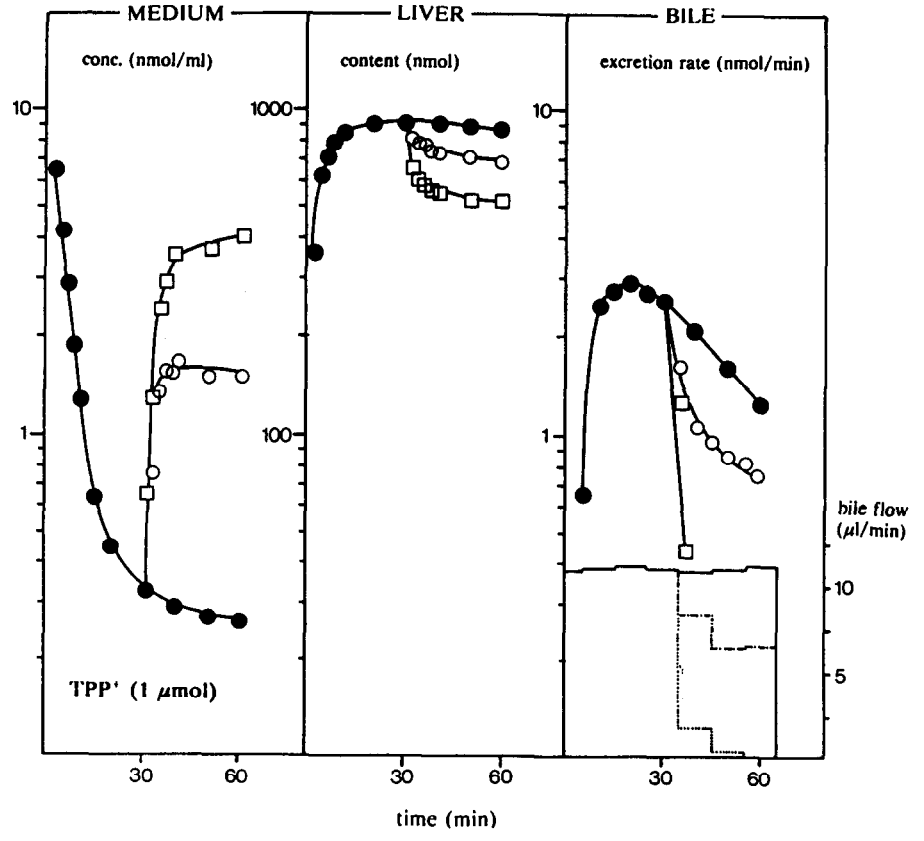
TBuMA and TPP^+ essentially showed the same uptake and inhibitory patterns after treatment with the metabolic inhibitors. Under ion gradient conditions as described in Materials and Methods, the effects of both valinomycin and CCCP can be explained by the breakdown of the mitochondrial membrane potential due to rapid K^+ and H^+ fluxes, respectively. It is known that antimycin blocks mitochondrial respiration at site 3 [11], preventing the establishment of the proton gradient, whereas oligomycin blocks mitochondrial ATP-synthetase [11]. It is likely therefore that the metabolic inhibitors affect uptake of TBuMA in mitochondria only by dissipating the organelle's membrane potential.

Whole cell uptake

If mitochondria are (at least partly) involved in the cellular accumulation of TBuMA, it is of interest to investigate whether mitochondrial uptake is an essential determinant of the initial uptake rate in intact hepatocytes. In other words, can any compound that would decrease the uptake in mitochondria also alter the initial uptake rate of cations in the whole cell? TPP^+ is an excellent probe to test this hypothesis since it may pass through the plasma membrane passively due to its high lipophilicity, and also distributes passively into mitochondria according to the membrane potential [13–15]. However, our data make clear that the sensitivity to metabolic inhibitors with regard to the cellular uptake of TPP^+ is completely different compared to that of mitochondrial uptake. For the protonophores CCCP and DNP these results may be explained by the fact that cytoplasm is more acidic (pH = 7.2) than blood (pH = 7.4) [5]. Protons will initially leak out of the cell and will produce a more negative diffusion potential. The K^+-ionophore valinomycin will, similar to CCCP, produce a more negative diffusion potential due to backflux of K^+ ions ($[K^+_{in}] > [K^+_{out}]$). In any case, these results indicate that dissipation of the mitochondrial membrane potential, as produced by these inhibitors (also confirmed by the rapid loss of cellular ATP), is not reflected in the initial uptake rate of TPP^+ in intact hepatocytes. If CCCP indeed initially elevated the cellular membrane potential as indicated by the increased cellular accumulation of TPP^+, one may ask why uptake rate of a cation like TBuMA was not increased by the inhibitor but in contrast clearly decreased. From a previous study we showed that (in contrast to TPP^+) TBuMA uptake into hepatocytes was not electrogenic and that differences in membrane potential did not alter the TBuMA uptake rate [5]. Major effects on TBuMA uptake rate were only observed in the presence of lipophilic agents such as verapamil, rhodamine B and bulky organic cations, and were explained by competition for the supposed carrier site [5]. Lipophilic compounds such as CCCP, valinomycin and the other metabolic inhibitors used in this study may also produce such (non) competitive inhibiting effects on the level of carrier-mediated uptake of TBuMA. The lack of inhibition of the metabolic inhibitors on initial cellular uptake of TPP^+ was anticipated, since the cation can cross membranes passively due to its lipophilic character.

Liver perfusions

In the isolated perfused liver we further studied the effects of valinomycin and CCCP on the uptake and accumulation of TBuMA. In the presence of CCCP the uptake of TBuMA from the medium was decreased (Fig. 4). However, hepatic accumulation was not completely diminished under these conditions. From livers preloaded with TBuMA a backflux to the medium was observed after addition


MEDIUM
LIVER
BILE
conc. (nmol/ml)
content (nmol)
excretion rate (nmol/min)
10
1
1000
100
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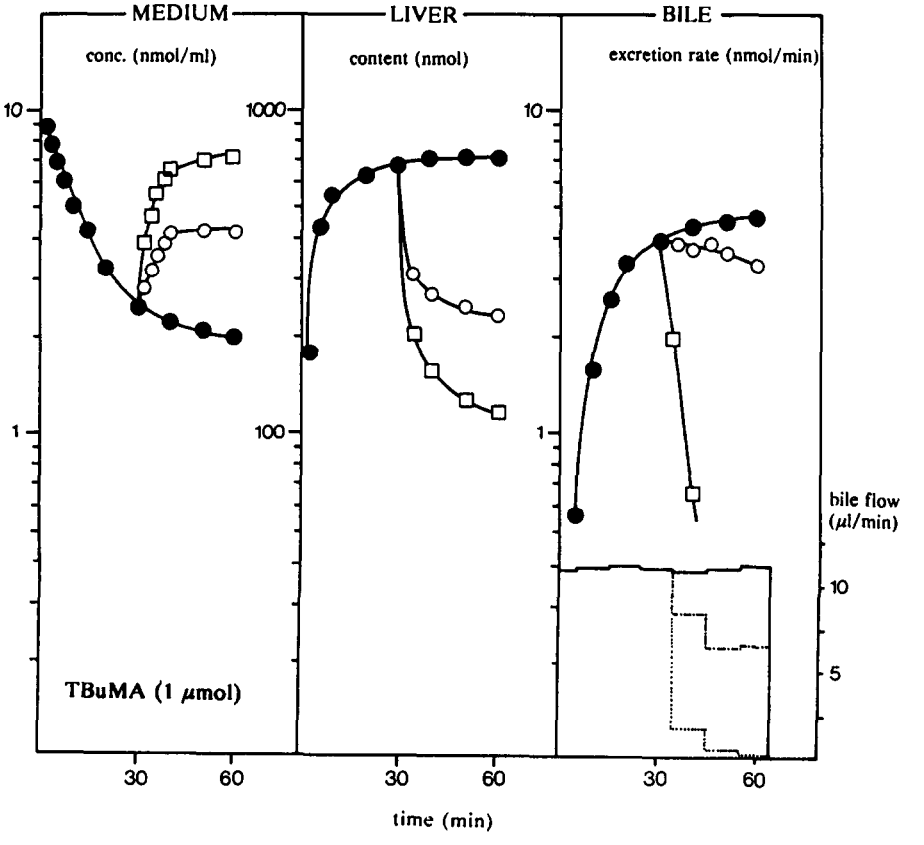
time (min)


MEDIUM
LIVER
BILE
conc. (nmol/ml)
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10
1
1000
100
10
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bile flow (μl/min)
10
5
TBuMA (1 μmol)
30
60
30
60
30
60
time (min)

of one of the ionophores (Fig. 5, lower panel). Although accumulation is the result of both influx and backflux, the observed effects cannot be explained solely by inhibition of influx (=re-uptake) of TBuMA: competing organic cations that have been shown to block initial uptake of TBuMA in perfused livers and hepatocytes almost completely did not show a backflux pattern as for CCCP and valinomycin (unpublished data). This idea is confirmed by identical experiments in which a similar backflux of TPP^+ is observed (see Fig. 5, upper panel). An inhibition of re-uptake of TPP^+ by the metabolic inhibitors is unlikely since TPP^+ can pass through membranes passively. In these experiments, the bile ducts of the livers were not ligated since backflux is anticipated as being directed to both the perfusate and the bile. However, backflux to the bile was not observed, probably due to inhibition of ATP-dependent processes such as bile flow (which was indeed decreased) and the actual excretion process.

From these latter experiments it can be concluded that mitochondria are indeed the main source of hepatic accumulation of these cations. Since previous studies from our laboratory have shown that organic cations can also be concentrated in acidified organelles such as endosomes and lysosomes [23–26], secondary release from these organelles may also contribute to the hepatic backflux of TBuMA and TPP^+ induced by CCCP.

Finally, it has to be noted that, parallel to the plasma membrane, the involvement of a carrier-mediated uptake system for organic cations in mitochondria, which was proposed earlier by Ramsay and Singer [17], cannot be excluded. Since the metabolic inhibitors used in this study also markedly lowered the final mitochondrial content of TPP^+ the dissipation of the membrane potential probably plays the most significant role in the decreased mitochondrial accumulation of TBuMA. Future studies should be designed such that the breakdown of the membrane potential as well as (non)competitive inhibition at the supposed carrier level can be distinguished.

With regard to the hepatic uptake mechanisms for organic cations, we conclude that: (i) the initial cellular uptake of the organic cation TBuMA via the type I uptake system is not (directly) dependent on cellular ATP; (ii) TBuMA is actively taken up by mitochondria. In contrast to plasma membrane transport, mitochondrial uptake is electrogenic and driven by the negative membrane potential; (iii) sequestration of TBuMA into mitochondria is important for cellular storage but shows features that are different in principle from the initial cellular uptake process; (iv) the metabolic inhibitors as used in the present study and many other studies, apart from ATP depletion, may influence directly hepatocyte uptake of organic compounds, e.g. by aspecific interactions with the carriers involved in translocation across the plasma membrane.

Fig. 5. Effects of valinomycin (10 μM) and CCCP (10 μM) on the plasma concentration, hepatic content and biliary excretion rate of TPP^+ (dose 1 μmol, upper panel) and TBuMA (dose 1 μmol, lower panel) after a preloading period of the isolated perfused livers with the particular organic cation for 30 min. Each value of the curve is the mean of two independent experiments and is plotted on a semilogarithmic scale, (●) Control; (○) + CCCP; (□) + valinomycin. Effects of the metabolic inhibitors on bile flow are depicted in the figures on a linear scale; (——) control; (– · – · –) + CCCP; (· · · · · · · ·) + valinomycin.

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REFERENCES

1. Meijer DKF, Transport and metabolism in the hepato-biliary system. In: *Handbook of Physiology. The Gastrointestinal System, Vol. III* (Eds. Schulz J, Forte G and Rauner BB), pp. 717–758. Oxford University Press, New York, 1989.
2. Steen H and Meijer DKF, Biliary excretion of endogenous and exogenous compounds: organic cations. In: *Biliary Excretion of Drugs and Other Chemicals* (Eds. Siegers CP and Watkins III JB), pp. 239–272. G. Fischer, Stuttgart, 1991.
3. Steen H, Merema M and Meijer DKF, A multispecific uptake system for taurocholate, cardiac glycosides and cationic drugs in the liver. *Biochem Pharmacol*, **44**: 2323–2331, 1992.
4. Mol WEM, Fokkema GN, Weert B and Meijer DKF, Mechanisms for the hepatic uptake of organic cations. Studies with the muscle relaxant vecuronium in isolated rat hepatocytes. *J Pharmacol Exp Ther* **244**: 268–275, 1988.
5. Steen H, Oosting R and Meijer DKF, Mechanisms for the uptake of cationic drugs by the liver: a study with tributylmethylammonium (TBuMA). *J Pharmacol Exp Ther* **258**: 537–543, 1991.
6. Mol WEM and Meijer DKF, Hepatic transport mechanisms for bivalent organic cations; subcellular distribution and hepato-biliary concentration gradients of some steroidal muscle relaxants. *Biochem Pharmacol* **39**: 383–390, 1990.
7. Neef C, Keulemans KTP and Meijer DKF, Hepatic uptake and biliary excretion of organic cations. I. Characterization of three new model compounds. *Biochem Pharmacol* **33**: 3977–3990, 1984.
8. Neef C, Oosting R and Meijer DKF, Structure–pharmacokinetics relationship of quaternary ammonium compounds. Elimination and distribution characteristics. *Naunyn Schmiedebergs Arch Pharmacol* **328**: 103–110, 1984.
9. Steen H, Nijholt A, Merema M and Meijer DKF, Modulators of protein kinase C as well as substrates for the P-glycoprotein (MDR) system have a major influence on the biliary excretion of the cationic drug tri-*n*-butylmethylammonium (TBuMA). *Hepatology* **16**: 147A, 1992.
10. Berry MN and Friend DS, High yield preparation of isolated rat liver parenchymal cells; a biochemical and fine structural study. *J Cell Biol* **43**: 506–520, 1969.
11. Rickwood D, Darley-Usmar VM and Wilson MT, *Mitochondria, A Practical Approach*. IRL Press, Oxford, 1987.
12. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
13. Hoek JB, Nicholls DG and Williamson JR, Determination of the mitochondrial protomotive force in isolated hepatocytes. *J Biol Chem* **255**: 1458–1464, 1980.
14. Yamazaki M, Sugiyama Y, Suzuki H, Iga T and Hanano M, Utilization of ATP-depleted cells in the

analysis of taurocholate uptake by isolated rat hepatocytes. *J Hepatol* **14**: 54–63, 1992.
15. Restrepo D and Kimmich GA, The mechanistic nature of the membrane potential dependency of sodium–sugar cotransport in small intestine. *J Membr Biol* **87**: 159–172, 1985.
16. Meijer DKF, Keulemans K and Mulder GJ, Isolated perfused rat liver technique. In: *Methods in Enzymology* (Ed. Jakoby WB), pp. 81–94. Academic Press, New York, 1981.
17. Ramsay RR and Singer TP, Energy-dependent uptake of *N*-methyl-4-phenyl pyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetra hydropyridine, by mitochondria. *J Biol Chem* **261**: 7585–7587, 1986.
18. Van den Berghe G, Bronfman M, Vanneste R and Hers HG, The mechanism of adenosine triphosphate depletion in the liver after a load of fructose. *Biochem J* **162**: 601–609, 1977.
19. Weibel ER, Staubli W, Gnagi HR and Hess FA, Correlated morphometric and biochemical studies on the liver cell. I. Morphometric model, stereologic methods and normal morphometric data for rat liver. *J Cell Biol* **42**: 68–91, 1969.
20. Gallagher CH, The effects of neuromuscular blocking agents on mitochondria IV. Effects of *d*-tubocurarine, pyrrolizidine alkaloids and magnesium on oxidative phosphorylation. *Biochem Pharmacol* **17**: 533–538, 1968.
21. Bakeeva LE, Grinius LL, Jasaitis AA, Kuliene VV, Levitski DO, Liberman EA, Severina II and Skulachev VP, Conversion of biomembrane-produced energy into electric form II. Intact mitochondria. *Biochim Biophys Acta* **216**: 13–21, 1970.
22. Ruifrok PG, Uptake of quaternary ammonium compounds into rat liver plasma membrane vesicles. *Biochem Pharmacol* **31**: 1431–1435, 1982.
23. Weitering JG, Mulder GJ, Meijer DKF, Lammers W, Veenhuis M and Wendelaar Bonga SE, On the localization of *d*-tubocurarine in rat liver lysosomes *in vivo* by electron microscopy and subcellular fractionation. *Naunyn Schmiedebergs Arch Pharmacol* **289**: 251–256, 1975.
24. Weitering JG, Lammers W, Meijer DKF and Mulder GJ, Localization of *d*-tubocurarine in rat liver lysosomes. Lysosomal uptake, biliary excretion and displacement by quinacrine *in vivo*. *Naunyn Schmiedebergs Arch Pharmacol* **299**: 277–281, 1977.
25. Meijer DKF, Weitering JG and Vonk RJ, Hepatic uptake and biliary excretion of *d*-tubocurarine and trimethylcurarine in the rat *in vivo* and in isolated perfused rat livers. *J Pharmacol Exp Ther* **198**: 229–239, 1976.
26. Van Dyke RW, Faber ED and Meijer DKF, Sequestration of organic cations by acidified hepatic endocytic vesicles and implications for biliary excretion. *J Pharmacol Exp Ther* **261**: 1–11, 1992.