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DRUG TRANSPORT, VIABILITY AND MORPHOLOGY OF ISOLATED RAT HEPATOCYTES PRESERVED FOR 24 HOURS IN UNIVERSITY OF WISCONSIN SOLUTION

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Abstract—Isolated hepatocytes are a valuable tool to study liver functions. Suitable methods to preserve the isolated cells with good maintenance of viability and functions are crucial to extend experiments with hepatocytes from a single isolation over 2 or more consecutive days. We investigated whether University of Wisconsin (UW) solution, which was designed to preserve organs for transplantation, is also suitable for preservation of isolated rat hepatocytes. Viability, as determined by Trypan blue exclusion and reduction of the tetrazolium 3(4,5-dimethyl-thiazoyl-2-yl)2,5 diphenyltetrazolium bromide to a purple formazan, morphological appearance using electron microscopy, ATP levels and uptake and storage of three model drugs ([^{3}H]vecuronium, [^{3}H]taurocholic acid and [^{3}H]ouabain) were determined directly after isolation and after 22 hr of storage in UW solution at 0–4°. The present study shows that cold storage of rat hepatocytes for 22 hr in UW can be performed without significant loss of viability and with maintenance of proper morphology, cellular ATP and transport functions. In contrast, after storage in Krebs–Henseleit buffer the normal morphology, ATP content and transport functions were strongly affected. These results imply that hepatocytes from a single isolation and stored in UW solution can be used for experiments on 2 consecutive days.

Suspensions of isolated hepatocytes are a valuable tool for studying liver functions. So far a limitation in the use of hepatocyte suspensions is that experiments cannot be extended for more than a few hours after isolation due to deterioration of cell functions. Often, the yield of viable cells from one rat liver is so abundant that it cannot be fully used: the actual experiments can be so time-consuming that some of the available hepatocytes have to be discarded. Deterioration of cell functions usually occurs within a few hours after isolation of hepatocytes, that is when storing cells at 4° in a physiological salt solution even when including foetal calf serum [1, 2] or when cells are incubated at 37° [3]. Moreover, at 37° undesired clumping of hepatocytes occurs with time, which makes it almost impossible to obtain homogeneous cell samples [3]. Storing cells for a period of weeks to months is possible by cryopreservation, but by this method a considerable number of cells is lost and the metabolic capacity of the viable hepatocytes is decreased after the storage period [4, 5]. During monolayer culture hepatocytes lose some of their specific hepatocytic functions, for instance, cytochrome P450 activity [6, 7], albumin synthesis [8] and transport functions [9, 10].

Table 1. Composition of UW solution

Compound	Amount
K-lactobionate (mmol/L)	100
Raffinose (mmol/L)	30
KH_2PO_4 (mmol/L)	25
$MgSO_4$ (mmol/L)	5
Hydroxyethyl starch (g/L)	50
Allopurinol (mmol/L)	1
Adenosine (mmol/L)	5
Glutathione (mmol/L)	3
Insulin (U/L)	40
Penicillin-G (U/L)	200,000
Dexamethasone (mg/L)	16

Recently, methods have become available for isolating hepatocytes from human liver tissue [2, 5, 7, 11]. Because human liver tissue available for cell isolation is scarce, suitable methods to preserve and make optimal use of the isolated cells are indispensable. Therefore, we have been looking for a method to preserve hepatocytes for at least 24 hr.

A new preservation solution [University of Wisconsin (UW‡) cold storage solution] has become available for the cooling and storage of organs to be used for transplantation. This cold storage solution has been used very successfully in the preservation of the liver, pancreas and kidney of animals and humans [12–16]. The composition of the UW solution is presented in Table 1. We investigated whether UW solution is also a good storage solution for isolated rat hepatocytes. Viability of the hepatocytes

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‡ Abbreviations: MTT, 3(4,5-dimethyl-thiazoyl-2-yl)2,5 diphenyltetrazolium bromide; BSA, bovine serum albumin; KHB, Krebs–Henseleit buffer with 1% BSA; TB, Trypan blue; UW, University of Wisconsin.

was tested by electron microscopy, the exclusion of Trypan blue (TB) and the capacity to reduce the tetrazolium dye 3(4,5-dimethyl-thiazoyl-2-yl)2,5 diphenyltetrazolium bromide (MTT) to a blue formazan [17]. In addition, cellular ATP levels were determined and the capacity for uptake and storage of the cationic drug [^{3}H]vecuronium, the anionic bile acid [^{3}H]taurocholic acid and the uncharged drug [^{3}H]ouabain was studied to test plasma membrane transport functions. In comparison, we stored hepatocytes in Krebs–Henseleit buffer (KHB) and determined the exclusion of TB, the transport capacity for taurocholic acid, the morphology and the ATP content.

MATERIALS AND METHODS

Materials. The following compounds were obtained from the indicated soruces: collagenase (c 2139) and MTT from the Sigma Chemical Co. (St Louis, MO, U.S.A.); bovine serum albumin (BSA), vecuronium bromide and [16*β*-*N*-*methyl*-^{3}H]vecuronium (sp. act. 9.9 Ci/mmol) from Organon (Oss, The Netherlands); taurocholic acid [^{3}H(G)] (sp. act. 8.1 Ci/mmol) and ouabain [^{3}H(G)] (sp. act. 18 Ci/mmol) from Du Pont NEN Research Products (Boston, U.S.A.); sodium taurocholic acid from Fluka Chemie AG (Buchs, Switzerland); ouabain from Merck (Darmstadt, Germany), UW solution from Du Pont Critical Care (Waukegan, IL, U.S.A.); Percoll from Pharmacia AB (Uppsala, Sweden); and Safe Fluor S from Lumac (Landgraaf, The Netherlands). All other chemicals were of analytical grade and were obtained from commercial sources.

Isolation of hepatocytes. Non-fasted male Wistar rats (270–315 g) were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). Hepatocytes were isolated by collagenase perfusion. The procedure was a modification of that of Berry and Friend [18] which we have described previously in more detail [19]. The viability of the cells was tested using TB, MTT and electron microscopy, and the uptake of [^{3}H]taurocholic acid, [^{3}H]vecuronium and [^{3}H]-ouabain and cellular ATP were measured.

Preservation of hepatocytes. Hepatocytes were stored in UW solution (without Penicillin-G) (Table 1) or KHB (118 mM NaCl, 50 mM KCl, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 10 mM *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid, 1% (g/v) BSA, saturated with O_2/CO_2 [95:5], pH 7.42) by suspending the cells in 30 mL storage solution at 0–4°. After 22 hr of storage, the UW solution or KHB was discarded and cells were washed twice with KHB. Non-viable cells were separated from viable cells by centrifugation in 40 mL of 45% Percoll solution (10 min at 100 *g*). The Percoll solution with non-viable cells on top was discarded, the pellet consisting of viable cells was resuspended in KHB and the recovery, viability and transport functions of the hepatocytes were determined.

Viability tests. Viability was tested by TB exclusion (0.2% final concentration) and reduction of the tetrazolium dye MTT by mitochondrial dehydrogenases. After incubation with MTT, the percentage of cells that had reduced the yellow tetrazolium dye MTT to a purple formazan was determined light microscopically. These purple cells were considered viable [17].

ATP determination. Immediately after isolation and also after 30 min of incubation under carbogen gassing in a shaking waterbath at 37° the ATP level of the cells was determined. Briefly, 300 μL of 10% trichloric acid were added to 2×10^6 cells. The protein precipitate was pelleted and 250 μL of the supernatant fluid were extracted four times with diethylether saturated with water. KOH was added to a final pH of 7 and ATP was measured by HPLC. Separation was performed on an anion exchange column (Supelco LC-SAX) with 0.04 M ammonium-phosphate pH 3.5 as the elution solvent and was followed by UV detection at 254 nm.

Electron microscopy. Directly after isolation, as well as after preservation and centrifugation in Percoll, cells were fixed by suspending them in 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 and prepared for electron microscopy as described earlier [20].

Uptake experiments. After 3.2 mL of cell suspension in KHB (1.5×10^6 cells/mL) were pre-incubated under carbogen for 30 min, substrate was added and samples of 200 μL were taken. Cells were immediately separated from the medium by filtration under vacuum (600 mbar) on Whatman GF/C filters (25 mm) and washed twice with 3 mL of KHB without BSA. The radioactivity on the filters was counted after addition of 4 mL of Safe Fluor S with a Beckman LS 1701 Liquid Scintillation System. The uptake of two concentrations of [^{3}H]vecuronium (7.5 and 15 μM), [^{3}H]taurocholic acid (10.5 and 21 μM) and [^{3}H]ouabain (100 and 200 μM) was determined. The concentrations of the substrates were chosen at the K_m and $0.5 \times K_m$ for uptake. After preservation in KHB only the uptake of 21 μM [^{3}H]taurocholic acid was determined. Non-specific binding of label to the filters was determined and appeared negligible. Results were compared statistically using the Student's *t*-test.

RESULTS

The viability of the hepatocytes directly after isolation was 97% ± 1 as determined with TB and 93% ± 1 as controlled by the MTT test.

After 22 hr of storage in KHB a major proportion of the cells appeared on top of the Percoll, indicating the presence of largely damaged cells. The mean recovery of viable cells was only 38% of which 94% excluded TB (Table 2). After 22 hr of storage in UW solution much fewer cells remained on top of the Percoll layer after centrifugation in Percoll. The recovery of the hepatocytes in the pellet fraction after centrifugation in Percoll was 85% ± 5, of which 97% ± 1 excluded TB and 91% ± 2 appeared viable in the MTT test (N = 7, Table 2).

Electron microscopy studies showed that after preservation in UW solution the morphology of the hepatocytes in the pellet fraction after Percoll centrifugation was not discernable from that before the preservation period. Figure 1a shows a representative example of a hepatocyte directly after

Table 2. Viability of rat hepatocytes after isolation and after 22 hr of preservation in UW solution or KHB

	Recovery (%)	Viability TB (%)	Viability MTT (%)
Direct		97 ± 1	93 ± 1
After preservation			
in UW	85 ± 5	97 ± 1	91 ± 2
in KHB	38 ± 12	94 ± 4	—

Viability was determined directly after isolation and after preservation in cold UW solution or in KHB and removal of non-viable cells by centrifugation in Percoll. Viability was measured by TB exclusion and reduction of MTT.

Results are expressed as means ± SEM (N = 7).

isolation and Fig. 1b after 22 hr in UW solution. The cells have numerous microvilli on the surface and normal rough and smooth endoplasmic reticulum and intact mitochondria. This is in contrast with the altered morphology of cells after preservation in KHB and centrifugation in Percoll (Fig. 1c). In some of these cells there are not even any organelles left.

Table 3 shows uptake characteristics of the three substrates tested, immediately after isolation and after preservation in UW solution and centrifugation in Percoll. The initial uptake rate and the "plateau value" are shown. The initial uptake was determined during the first 2.5 min for taurocholic acid and the first 5 min for vecuronium and ouabain. During these time intervals the uptake was linear with time. Plateau values were taken from 15–21 min for taurocholic acid, 30–60 min for vecuronium and 45–60 min for ouabain. Neither the initial uptake rates nor the plateau values were changed during preservation in UW solution. In contrast, after storage in KHB both transport characteristics for taurocholic acid were decreased significantly (Table 4, $P < 0.01$). Because of the very low recovery of cells after preservation in KHB and centrifugation in Percoll, only the uptake of taurocholic acid was measured in KHB preserved cells. Preservation for 22 hr in UW did not alter the ATP level of the cells, but preservation in KHB lowered the ATP level of the cells significantly from 12.8 ± 1.2 to 8.5 ± 1.3 $\mu g/10^6$ cells (Table 5). The ATP level of the cells after incubation for 30 min at 37° was the same in freshly isolated cells as in cells preserved in UW solution, and after removal of non-viable cells by centrifugation in Percoll (16.2 $\mu g/10^6$ cells). The ATP level after 30 min of incubation was lower in cells preserved in KHB and centrifuged in Percoll than in cells on the day of isolation (10.8 ± 2.9 and 16.2 ± 1.1, respectively, Table 5). However, this difference was not significant.

DISCUSSION

The results presented show that UW solution is not only a good preservation solution for intact organs, but also for isolated rat hepatocytes. After 22 hr of storage of rat hepatocytes in UW solution and removal of a small amount of non-viable cells, viable hepatocytes with normal morphological appearance and intact transport functions were obtained.

We have chosen to study the transport of three compounds that are generally considered as model compounds for the carrier-mediated transport of uncharged compounds [21, 22], organic anions [23–25] and organic cations [26]. The classical view on the specificity of plasma membrane carriers is that there is a subdivision in transport carriers based on the charge of the substrate [27, 28]. However, many interactions in uptake have been observed between substrates with different charges [29–31] and overlapping substrate specificity is likely.

The initial uptake rate was determined from that part of the time curves where uptake was linear. The plateau value in the cellular content is determined by the balance between uptake in and (biliary plus sinusoidal) excretion from the cells. Transport characteristics of the isolated hepatocytes (both uptake and excretion) of these three compounds were not altered at all after 22 hr of storage in UW solution at the concentrations chosen. In contrast, after storage in KHB the transport capacity of hepatocytes was decreased, as evidenced by the lower uptake of 21 μM [^{3}H]taurocholic acid after storage in KHB. Because of the very low recoveries and the deteriorated morphology, as seen by electron microscopy, the uptake of the two other substrates was not measured after preservation of cells in KHB. The unaltered transport functions after storage in UW solution show that this preservation method is superior to storage of hepatocytes in culture where it was shown that the uptake rate of bile acids declines considerably with time [9, 10].

The ATP content and synthesis of cells is considered a sensitive viability marker and a good estimate of the functional metabolic capacity [3, 32, 33]. Preservation of hepatocytes for 22 hr in UW solution did not influence the ATP level in the cells (see Table 5). Preservation in KHB however resulted in a lower cellular ATP content. Cellular ATP in the KHB-stored cells could be only partly restored by 30 min of normothermic incubation under carbogen gassing. Our results are in agreement with those of Michell *et al.* [34], who found that UW solution is superior to physiological salt solutions for the storage of hepatocytes based on TB exclusion, lactate dehydrogenase release and cellular ATP [34]. Sorrentino *et al.* [35] were also in agreement that cellular ATP is better preserved in cells stored in UW solution than in cells stored in a physiolological salt solution. However, in contrast to our results, they saw no extensive deterioration in the viability and transport functions after 24 hr preservation of hepatocytes in a physiological salt solution. Fox *et al.* [36] described recently the storage of isolated rat hepatocytes at 4° in culture medium Liebovitz-15 supplemented with 5% (w/v) polyethylene glycol. After 24 hr of storage at a pH between 7 and 8, lactate dehydrogenase leakage during normothermic incubation was in the normal range. However, these

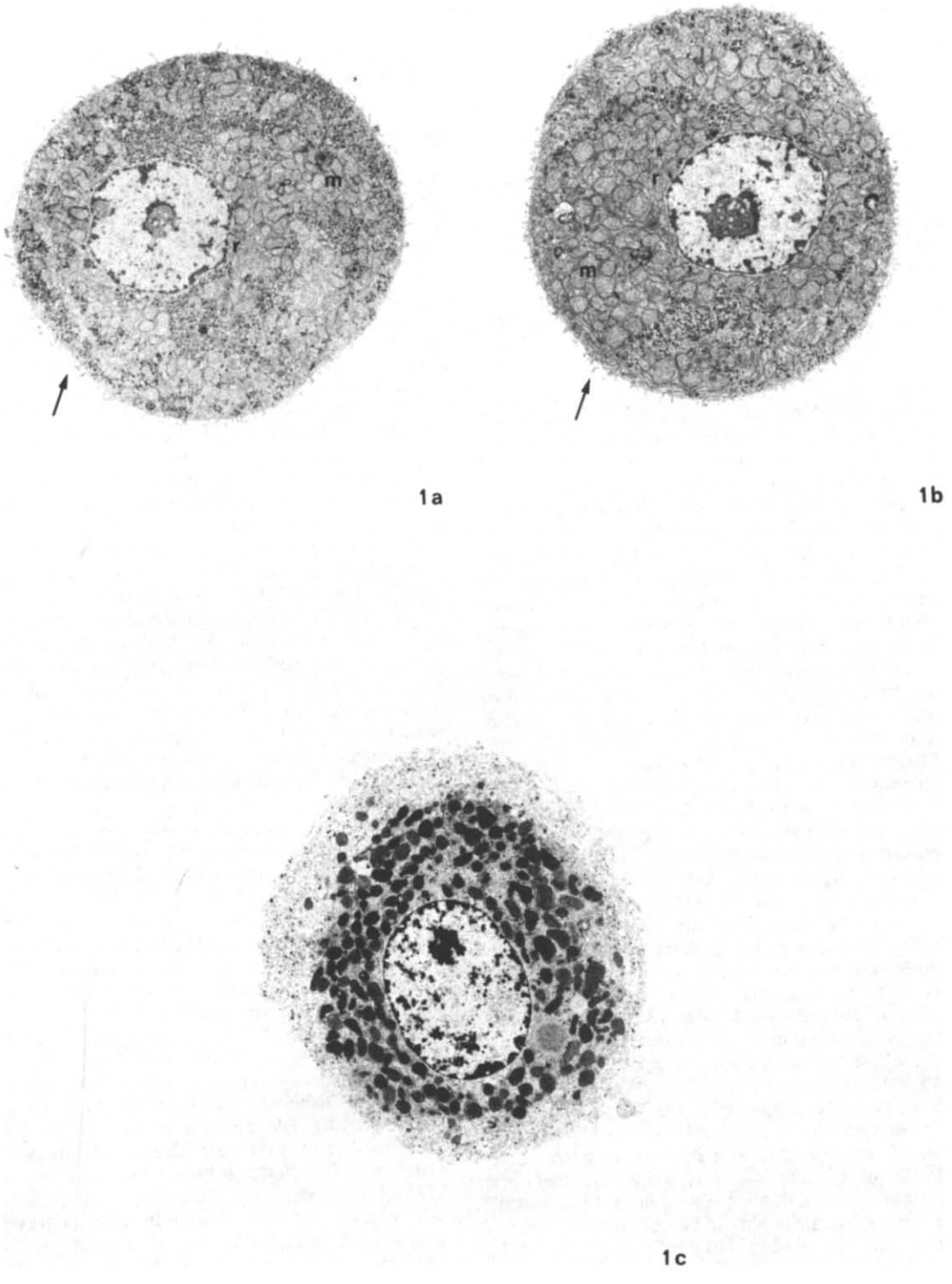

Fig. 1. (a) Isolated hepatocyte immediately after isolation showing numerous microvilli (arrow) and normal morphology; m = mitochondria, r = rough endoplasmic reticulum (3600×). (b) Isolated hepatocyte after 22 hr of preservation in UW solution from the pellet fraction after Percoll centrifugation. The morphology is similar to that before preservation. Arrow indicates microvilli; m = mitochondria, r = rough endoplasmic reticulum (3600×). (c) Isolated hepatocyte after 22 hr of preservation in KHB from the pellet fraction after Percoll centrifugation. In part of the cell the organelles have disappeared (3600×).

Table 3. Uptake characteristics of taurocholic acid, vecuronium and ouabain after isolation and after 22 hr of preservation in UW solution

Substrate (μM)	Initial uptake rate* (pmol/10^6 cells/min) Directly after isolation	After 22 hr‡	Plateau† (pmol/10^6 cells) Directly after isolation	After 22 hr
Taurocholic acid				
10.5	416 ± 36	520 ± 88	1590 ± 119	1939 ± 309
21.0	765 ± 52	736 ± 41	3497 ± 188	3347 ± 123
Vecuronium				
7.5	39 ± 5	35 ± 14	939 ± 92	889 ± 25
15.0	96 ± 14	69 ± 6	2055 ± 194	1974 ± 135
Ouabain				
100	275 ± 70	209 ± 79	7314 ± 592	8280 ± 201
200	459 ± 50	511 ± 90	10189 ± 183	12253 ± 234

Results are expressed as means ± SEM (N = 3).

* Initial uptake rate is determined during the first 2.5 min of incubation for taurocholic acid, and during the first 5 min for vecuronium and ouabain.

† Cellular content when uptake and efflux are in equilibrium at an incubation time for taurocholic acid of 15–21 min, for vecuronium of 30–60 min and for ouabain of 45–60 min.

‡ After 22 hr of preservation and removal of non-viable cells by Percoll centrifugation.

Table 4. Uptake characteristics of 21 μM taurocholic acid after isolation and after 22 hr of preservation in KHB

	Directly after isolation	After preservation*
Initial uptake‡ (pmol/10^6 cells/min)	765 ± 52	211 ± 38†
Plateau§ (pmol/10^6 cells)	3497 ± 188	1881 ± 39†

* After 22 hr of preservation and removal of non-viable cells by Percoll centrifugation.

† $P < 0.01$ vs directly after isolation.

‡ Determined during the first 2.5 min of incubation.

§ Determined after 15–21 min incubation.

Results are expressed as means ± SEM (N = 3).

Table 5. ATP content of isolated hepatocytes

	Directly after isolation	After preservation* UW (μg/10^6 cells)	KHB
Immediately‡	12.8 ± 1.2	11.2 ± 1.4	8.5 ± 1.3†
After incubation	16.2 ± 1.1	16.2 ± 0.9	10.8 ± 2.9

* After 22 hr of preservation and removal of non-viable cells by Percoll centrifugation.

† $P < 0.01$ vs directly after isolation.

‡ ATP content was determined in the cells at 0° (immediately) and after 30 min of incubation in a shaking waterbath at 37° (after incubation).

Results are expressed as means ± SEM (N = 3).

authors did not measure other parameters of cell quality nor did they measure drug transport or metabolic functions. In any case, the combined results of Fox *et al.* [36] and of Jamieson *et al.* [37], who used the isolated perfused rabbit liver as a model suggest that the presence of impermeants such as lactobionate, raffinose, hydroxyethyl starch and polyethylene glycol and of free radical scavengers such as glutathione is important for successful preservation of isolated hepatocytes in cold storage solutions.

In conclusion, UW solution makes it possible to use isolated rat hepatocytes for transport studies up to at least 22 hr after isolation, reducing the number of experimental animals needed. Furthermore, the preservation method described in the present study is easy and time saving. Moreover, it is also a promising method for making optimal use of the scarce human hepatocytes.

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