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AGE-DEPENDENT DECREASE IN THE HEPATIC UPTAKE AND BILIARY EXCRETION OF OUABAIN IN RATS

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Abstract—The biliary excretion of i.v. injected ouabain was examined in male and female Wistar-derived rats in relation to age. The hepatic uptake velocity for ouabain was also determined in isolated hepatocyte preparations obtained from male rats of various ages. Biliary recovery values of ouabain (percent of the dose) were fairly comparable for young male and female rats (3–4-month old). Recovery progressively decreased with age, the first 10-min recoveries at 24 months being about one-third those of respective young values in both sexes. A significant linear relation was demonstrated between the first 10-min recovery (Y, percent of the dose) and rat age (X, month), yielding the relations of $Y = 17.75 - 0.43X$ for males and $Y = 18.99 - 0.43X$ for females respectively. Similarly, the initial uptake velocity (Y, nmol/mg/min) for ouabain decreased in a linear fashion with age (X, month), yielding a significant negative correlation ($Y = 0.704 - 0.0021X$, $r = -0.839$, $P < 0.005$, $N = 21$) at an ouabain concentration of 8 μM. Kinetic studies using non-linear regression analysis revealed a significantly lower V_{max} value (0.533 ± 0.041 nmol/mg/min) in old (24–29 months) rats compared to the young (4–4.5 months) value (1.193 ± 0.105 nmol per mg/min, $P < 0.05$), while the affinity constant (K_m, μM) did not differ significantly between young and old animals (203.12 ± 25.42 μM in young rats vs 283.68 ± 28.90 μM in old rats, mean ± SE, $0.05 < P < 0.1$). The results of the present study suggest that the age-dependent decrease in the biliary recovery of i.v. injected ouabain in rats can be largely explained by the decrease with age in the hepatic uptake of ouabain. Furthermore, the results provide further support for our previous thesis that the decrease in the lateral mobility of hepatocyte plasma membrane proteins, as revealed by the fluorescence recovery after photobleaching technique, may play a signfiicant role in the age-dependent decrease in the physiological function(s) of the hepatocyte plasma membrane, such as the hepatobiliary transport of ouabain.

Drug metabolism by the hepatic microsomal cytochrome monooxygenase system has been extensively studied in relation to age in experimental animals [1–3]. In contrast, little has been done in the past on the effect of age on the hepatobiliary transport mechanisms of drugs, despite its importance in pharmacokinetic alterations during aging. We previously showed that the initial maximal uptake velocity of indocyanine green *in vivo* changed little with rat age from 6 months to 24 months, in contrast to the progressive decrease in the biliary transport maximum of sulfobromophthalein [4]. Kroker *et al.*, using isolated rat liver perfusion, also found a significant decrease in the biliary transport and a relatively stable hepatic uptake process of taurocholate during aging [5]. Although there were other sporadic studies suggesting that the hepatic uptake process for drugs may be affected by aging [6, 7], these studies used relatively young rats (6–12 months) as the so-called old age groups and compared them to immature rats. Thus, these studies do not provide any significant evidence for the senescent related decrease in the hepatic uptake of drugs.

On the other hand, we found that the biliary recovery of i.v. injected ouabain progressively decreases with age in male Wistar derived rats [8, 9]. Furthermore, we recently observed a decline in ouabain excretion with age in male and female Fischer-344 rats [10]. Since the biliary recovery of ouabain for the first 10-min period after injection declined markedly with age, we suggested that the hepatic uptake process for ouabain may also be affected by aging in addition to the biliary excretory system [9, 10]. However, since these studies examined sequential biliary recoveries of ouabain after a bolus i.v. injection, it was not clear whether the decrease was due to a change in the true hepatocyte uptake mechanism or to a diminution of hepatic blood flow with age. More recently, we demonstrated that the lateral diffusion constant of hepatocyte plasma membrane proteins, as examined by fluorescence recovery after photobleaching (FRAP), steadily decreases with age to the extent of an almost straight line negative correlation between age and the diffusion constant value [9, 11–13]. These studies indicated that lateral protein mobility in the membrane may become more restricted as age advances. We suggested therefore that the decrease in the membrane proteins diffusion constant could be a partial cause of the decrease in biliary ouabain excretion with age by means of presumed decline in ouabain uptake by hepatocytes [9, 10]. In order to more directly prove this hypothesis, we obviously need to examine whether the velocity of the hepatic uptake of ouabain really decreases with age. For this purpose, the present study used isolated hepatocyte preparations from rats of different ages. This eliminates the influence of any hepatic blood flow change

with age on *in vivo* ouabain uptake. For comparison with the uptake data in the present study as well as with our previous excretion studies in rats of a different strain (F-344) [10], we also examined the biliary excretion of i.v. injected ouabain in both male and female Wistar derived rats of various ages.

MATERIALS AND METHODS

Tritiated water (0.25 mCi/g), [carboxyl-^{14}C]dextran (1.24 mCi/g, molecular weight 50,000–70,000) and [^{3}H]ouabain (1 mCi/0.028 mg, generally labeled) were purchased from New England Nuclear (Boston, MA). The possible contamination of lower molecular weight materials to the [^{14}C]dextran preparation which may penetrate hepatocytes was examined by means of ultrafiltration using Centricon membrane filters (Amicon, Danvers, MA). Forty-eight percent of the radioactivity of the dextran solution passed the filter of Centricon-30 which prevents the filtration of materials with molecular weights higher than 30,000 daltons, while only 5.22% passed the filter of Centricon-10 which prevents the filtration of materials higher than 10,000 daltons in their molecular weights. The preparation was used without further purification. No correction for this factor was made either. Ouabain octahydrate was from Merk (Rahway, NJ), collagenase (Type I) from Sigma (St. Louis, MO). As an incubation medium, Waymouth MB 752/1 medium (GIBCO, Ohio) was added to 25 mM HEPES (Dojindo Laboratories, Kumamoto, Japan). 0.5 mM alanine and 0.21 mM serine were used. The pH (7.4) and osmolality (300 mOsm/kg) of the medium were carefully adjusted. All chemical agents were of analytical grade.

Male and female Wistar derived rats were originally purchased from Shizuoka Jikken Dobutsu (Hamamatsu, Japan). They were bred and maintained on commercial rat pellets (CRF1 Oriental, protein content 23%, Tokyo) in the SPF animal aging colony of the institute. The husbandry conditions, survivals and pathologies of the later period of their lives have been described elsewhere [14]. We used animals of ages ranging from 3 to 29 months. For the *in vitro* kinetic study, we used two groups (4–4.5 month and 25–29 month) of the male sex as representative young and old groups.

In vivo *ouabain excretion study*. The biliary excretion of i.v. injected ouabain was studied in male and female rats of various ages essentially by the same experimental procedure reported previously by the authors [8–10]. In brief, animals were anesthetized with pentobarbital sodium (Nembutal, Abbott, North Chicago, IL) and the bile duct was cannulated with PE-50 or PE-10 tubings (Cray Adams, Parsippany, NJ) in male and female rats respectively. After basal bile collection for 10 min, the [^{3}H]ouabain saline solution mixed with ouabain octahydrate was injected i.v. through a femoral vein cannula in the course of 10–15 sec. The ouabain dose was 0.1 mg/100 g b.wt. The venous cannula was immediately flushed with a small amount of saline solution, and then the bile was collected at 10-min intervals for 60 min. The biliary ouabain excretion was calculated as the product of the bile flow rate and the ouabain concentrations of bile samples as reported previously [8, 10].

In vitro *uptake study*. The isolation of hepatocytes was performed as described previously by the authors [15, 16]. After washing, hepatocytes were stored in suspension (about 1.25×10^6 cells/ml) at 4° in Waymouth medium for about 2 hr before uptake study. Ouabain uptake velocity determination was performed by the method of Eaton and Klaassen [17, 18].

Suspensions of hepatocytes (4 ml) were preincubated for 3 min at 37° under an atmosphere of 95% O_2 and 5% CO_2 with constant shaking (100 oscillations/min). After preincubation, the preparation was incubated with the addition of 1 ml Waymouth medium containing about 4–5 μCi of [^{3}H]ouabain with unlabeled ouabain of different concentrations for different time periods (0, 1, 2, 3, 4, 5 min) in order to obtain the initial uptake velocities.

Hepatocytes were separated from the incubation medium by centrifugal filtration. Each 200 μl aliquot was placed in a 0.5 ml centrifuge tube containing a 50 μl 3 M KOH-solution, covered by a 100 μl silicone layer with a density of 1.015 which was prepared by mixing silicone oil (d = 1.05, Aldrich Chemical Company, WI), and decahydronaphthalene (d = 0.876, Wako Pure Chemical Ind., Osaka, Japan). Centrifugation by a Beckman microfuge B resulted in the precipitation of hepatocytes through the silicone layer into the KOH layer.

Rates of initial ouabain uptake by hepatocytes were calculated from the uptake values obtained from incubations of different periods after the addition of ouabain. The incubation medium adherent to the sedimented cells and the aqueous volume of the cells were separately measured by the use of [^{3}H]H_2O and [carboxyl-^{14}C] dextran respectively, as described by Bauer *et al.* [19]. All values were corrected for the amount of ouabain in the adherent fluid. The radioactivity of the cell precipitates was determined in Aquasol-2 (NEN, Boston, MA) by an Aloca liquid scintillation counter. The viability of hepatocytes was estimated by the Trypan blue exclusion test. Preparations with viabilities below 90% were not used for the experiments. Cellular protein was determined by the method of Lowry *et al.* [20]. One millilitre hepatocyte suspension was washed with a 0.9% NaCl solution 3 times. After centrifugation, cell pellets were lysed by an addition of 8 ml H_2O and 2 ml of a 5 M NaOH solution. Bovine serum albumin was used as a standard. Kinetic studies of ouabain uptake velocities were performed in two age groups using different ouabain concentrations. For the calculation of kinetic parameters (V_{max} and K_m), the non-linear least-squares method described by Wilkinson [21] was used with minor modifications for a Vax-11 computer. We then selected the best fit to the Michaelis–Menten equation.

Statistical analysis of kinetic parameters was performed by the method of Cleland [22]. One way analysis of variance (ANOVA) was used for the comparison of different age groups. If a difference was found significant with respect to animal age, Sheffe's test was applied for the comparison of the two age groups. In some studies the least-squares

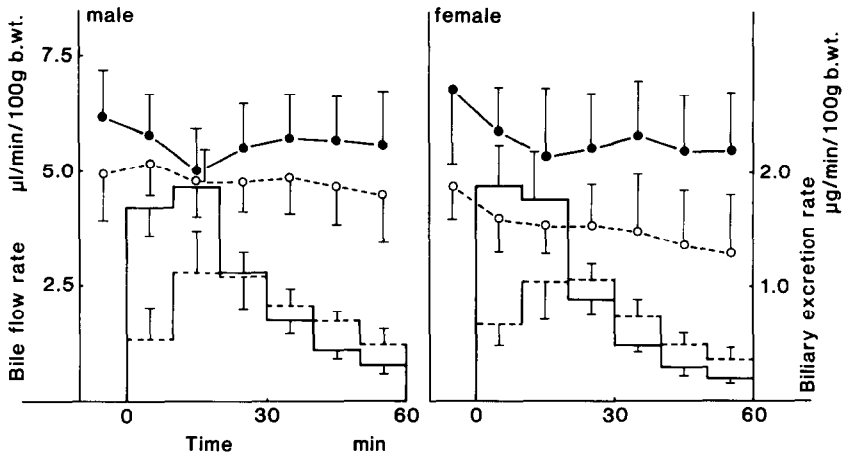

Fig. 1. Sequential biliary excretion of ouabain in young (3.5–4-month-old) and old (24-month-old) male rats (left panel) and in young (3-month-old) and old (28-month-old) female rats (right panel). Number of rats of each group is indicated in Table 1. Vertical bar indicates 1 SD. Circles indicate bile flow rate and columns excretion rate. Solid lines indicate young values and broken lines old values.

linear regression analysis was also applied. For all studies, P values lower than 0.05 were judged to be significant.

RESULTS

Figure 1 shows sequential biliary excretion of ouabain in young (3.5–4-month-old) and old (24-month-old) male (left panel) and female (3 months vs 28 months) rats (right panel). In all studies, the biliary excretion of ouabain was highest in the first or second 10-min collection period. In both sexes, old rats exhibited a markedly lower excretion rate compared to the respective young groups in the first 10-min period. In Table 1, the biliary excretion for the first and second 10-min periods as well as the total (60-min) excretion values are summarized for all age groups. Values in young males and females were fairly comparable. In both sexes, the first and second 10-min excretion values are significantly lower in the old than corresponding young, the old groups' first 10-min values being almost one-third of young (3–3.5 month-old) values. It is, however, noted that in both male and female rats, the oldest groups (28-month in males and 31-month in female) showed slightly higher first 10-min values. This tendency became more marked in subsequent collection periods. Thus, the second 10 min values and total 60-min collection values in oldest groups were not significantly different from respective youngest values in either sex.

Figure 2 shows sequential changes in plasma ouabain concentration in male rats of different ages. The plasma ouabain concentration at corresponding times tended to increase with age except for the oldest rats which showed slightly lower values compared to the value in the group next to the oldest in both sexes.

In Fig. 3. the relationship between the first 10-min excretion value (Y) and rat age (X month) is shown for male rats. A significant negative correlation is demonstrated. A least squares linear regression analysis yielded relations of $Y = (17.75 \pm 2.93) - (0.430 \pm 0.049)X$ ($t = 8.86$, $P < 0.005$, $r = -0.855$, $N = 31$) and $Y = (18.99 \pm 3.24) - (0.426 \pm 0.06)X$ ($t = 7.534$, $P < 0.005$, $r = -0.854$, $N = 23$) for males and females respectively, a decline in biliary excretion of ouabain for the first 10-min period of 2.4% in males and 2.2% in females per month.

Figure 4 is an example of ouabain uptake by isolated hepatocyte preparations. The increase in the ouabain uptake with time was virtually linear for all ouabain concentrations used.

In Figure 5 the ouabain uptake velocities of isolated hepatocyte preparations at the concentration of 8.0 μM are shown as a function of animal age (month). A least squares regression yielded the relation, $Y = (0.0645 \pm 0.0134) - (0.001798 \pm 0.000296)X$, $r = -0.812$, $t = 6.06$, $N = 21$, ($P < 0.05$ from analysis of covariance) demonstrating, a significant decline of 2.8% per month in the ouabain uptake velocity with age.

In Fig. 6 kinetic studies for young and old rats are summarized. In both studies, the uptake velocity followed the Michaelis–Menten saturation kinetics. The V_{max} values thus calculated were 1.193 ± 0.105 nmol/mg protein/min (mean ± SE, N = 8) for young rats and 0.533 ± 0.0401 nmol/mg protein min for old rats (N = 5), the difference between the two groups being statistically significant ($P < 0.05$, t-test, Cleland). The Michaelis constant, K_m, on the other hand was not significantly different between the young (203.12 ± 25.42 μM) and old (283.68 ± 28.90 μM) rats ($0.05 < P < 0.1$).

DISCUSSION

The present study demonstrated that the biliary excretion of i.v. injected ouabain was about 20% of the dose for the first 10 min and 60% for the first 60 min in both male and female Wistar derived rats. These values agree with a previous study of males of the same Wistar derived strain using a 4-fold higher ouabain dose (0.4 mg/100 g b.wt) [8]. Interestingly, the 10-min recovery value and the 60-min total recov-

Table 1. Bile flow rate and biliary excretion of ouabain in rats of various ages

Age months (N)	Body weight (g)	Liver weight (g)	Basal bile flow rate		Biliary ouabain recovery (% of the dose)		
			(μl/min/100 g b.wt.)	(μl/min/g liver wt.)	0–10 min	10–20 min	0–60 min
Male							
3.5–4 (12)	319.2 ± 22.95†	10.1 ± 0.90†	6.21 ± 1.02†	2.03 ± 0.34†	16.89 ± 2.47†	18.72 ± 2.49	61.57 ± 5.57
13 (6)	463.3 ± 8.76*	13.59 ± 0.91*	4.77 ± 0.33	1.56 ± 0.18*	11.35 ± 1.49*	15.88 ± 1.63	59.74 ± 3.75
24 (7)	435.1 ± 32.20*	14.66 ± 2.07*	4.96 ± 1.05	1.49 ± 0.36*	5.43 ± 2.70*†	11.17 ± 3.73*	47.14 ± 11.06*†
28 (6)	382.5 ± 63.62*	14.84 ± 1.24*	6.58 ± 2.59	1.64 ± 0.43*	7.98 ± 2.83*	16.36 ± 4.79	63.04 ± 8.04
Female							
3 (7)	172.4 ± 6.93	5.80 ± 1.36	6.80 ± 1.55	2.13 ± 0.61	18.89 ± 3.67	17.66 ± 4.31	55.46 ± 6.60
14 (6)	269.2 ± 13.57*	6.94 ± 0.55*	4.82 ± 0.73	2.00 ± 0.41	10.83 ± 2.58	16.19 ± 2.85	52.58 ± 7.55
28 (7)	297.7 ± 23.56*	8.40 ± 0.53*	4.70 ± 0.71	1.65 ± 0.15*	6.76 ± 1.80*	12.01 ± 3.38*	45.44 ± 6.23
31 (3)	264.0 ± 61.88*	8.88 ± 0.55*	5.80 ± 0.79	1.70 ± 0.31*	8.11 ± 1.24*	15.59 ± 0.50	54.95 ± 2.63

* Significantly different from respective youngest rat values ($P < 0.05$). Values are expressed as mean ± SD.
† Significantly different from respective middle aged (13–14 month) rat values ($P < 0.05$).

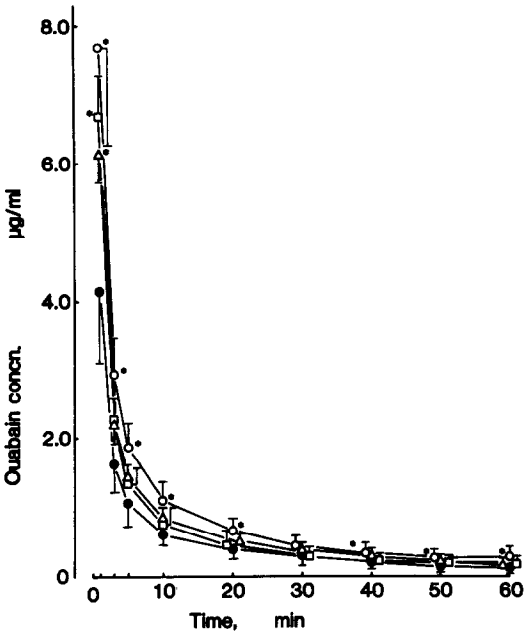

Fig. 2. Sequential changes in plasma ouabain concentration in male rats of different ages. Number of rats of each group is indicated in Table 1. Vertical bar indicates 1 SD. *Significantly different from respective youngest (3.5–4 month) values. ●, 3.5–4 month-old; △, 14-month-old; ○, 24-month-old; ■, 28-month-old.

eries in both studies are not very different when expressed as the percent of the dose, suggesting that the hepatobiliary excretory process is not saturated in the dose range of 0.1–0.4 mg/100 g. b.wt. Young female rat values for the first 10 min were slightly higher than young male values. This agrees with another recent study of ours on Fischer-344 rats of both sexes which found a significantly higher excretion value in young females [10], although in the present study the difference between male and female was not statistically significant. Although the biliary transport maximum (T_m) of sulfobromophthalein (BSP) was generally higher in male than in female rats when unconjugated BSP was infused [23, 24], we have recently found that the T_m of conjugated BSP was higher in females [25]. Thus, it appears that the more efficient transport system in females is not specific to ouabain.

In addition, the present study has shown that in rats of both sexes biliary excretion for ouabain progressively decreases with age, which agrees with our recent study of F-344 rats [10]. The only exceptions are the values in the oldest groups which were higher than respective values in the groups next to the oldest. This problem will be discussed later. The present observation indicates that in the rat liver the ouabain transport function generally decreases with age. This finding is in marked contrast with our previous observations on microsomal P-450 functions [26–29] or cytosolic enzyme activities (e.g. glutathione *S*-transferase) [24, 30] which decrease with age in male rats but remain quite stable in females. Since the first 10-min recovery values showed the most

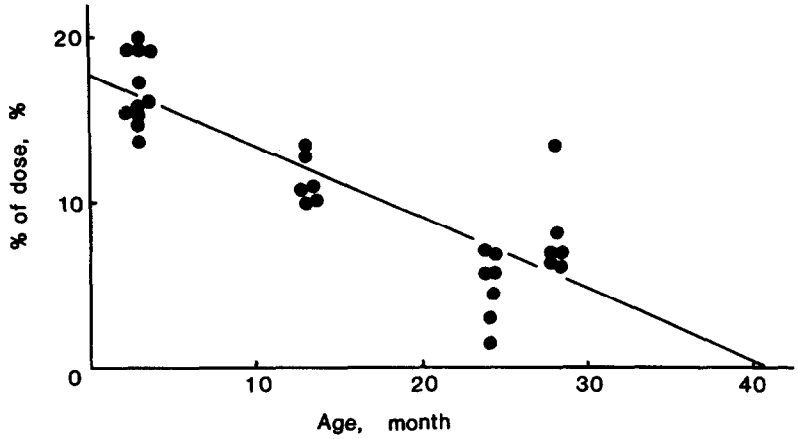

Fig. 3. The relation between the individual rat age (X, month) and the first 10-min biliary recovery of ouabain in male rats. The least squares linear regression analysis yielded the relation; $Y = 17.92 - 0.437X$, $r = -0.8404$, $P < 0.01$.

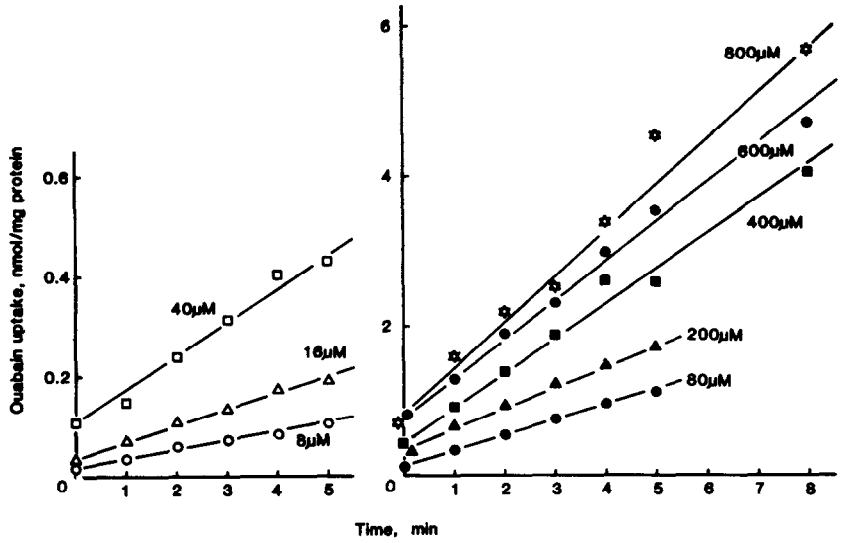

Fig. 4. An example of ouabain uptake study using different ouabain concentrations.

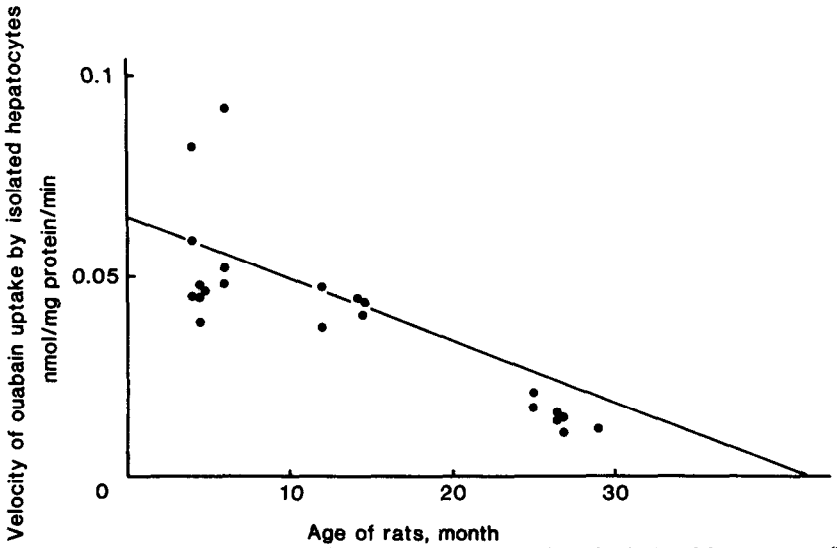

Fig. 5. The relation between the initial uptake velocity of ouabain by isolated hepatocytes (Y) and animal age (X) examined in male rats of various ages. A significant negative correlation is shown. ($Y = 0.065 - 0.002X$, $r = -0.812$, $P < 0.005$).

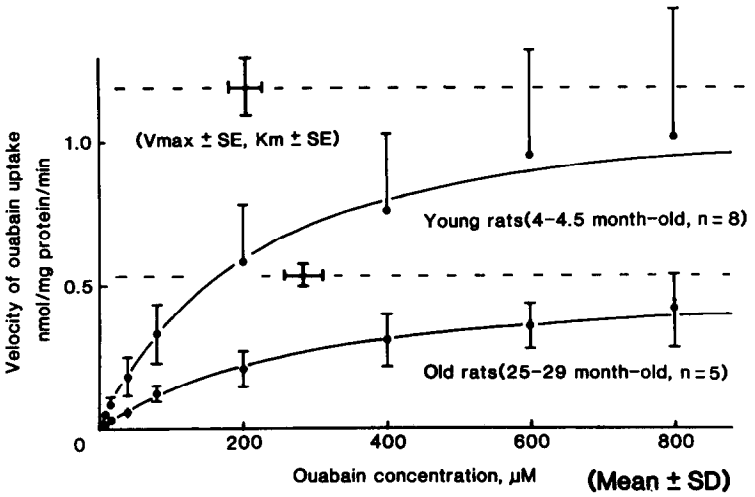

Fig. 6. Kinetic studies of ouabain hepatic uptake velocities in young (4–4.5 months) and old (24–29 months) male groups. In both groups, a dose-dependent saturation curve following Michaelis–Menten kinetics is shown. Kinetic parameters are described in the text.

striking age-dependency, it is highly likely that aging was the paramount factor in the decrement of ouabain uptake. However, with an *in vivo* study we were unable to determine whether the cause was a decline in the hepatic blood flow or an alteration of the hepatic uptake mechanism *per se*. Although hepatic blood flow decrease with age is well established in man [31], there is no definite data showing that this occurs in rodents. Varga *et al.* [32] found little hepatic blood flow change after maturity in rats and the problem is sufficiently important to warrant a restudy.

The present *in-vitro* study of isolated hepatocyte preparation, however, clearly demonstrates that the ouabain uptake process by hepatocytes steadily decreases with age. The decrease in the biliary recovery for the first 10 min was 2.4% per month, while that in the ouabain uptake velocity by isolated hepatocytes was 2.8% per month in male rats. There is no necessity for the agreement of a decrease rate of uptake velocity with age and that of biliary excretion values for an arbitrarily determined period (10 min). However, the above values are sufficient to suggest that the decrease with age in the hepatic uptake velocity can account for most of the age differences in excretion, although additional factors are not excluded.

Klaassen [33, 34] showed that the hepatobiliary excretory function for ouabain was very low in immature rats though it rapidly increased during development. Furthermore, Iga and Klaassen [35] recently reported a large difference in ouabain distribution volume between 2- and 6-month-old male rats, which largely explained the differences in the plasma ouabain profile. They further reported that there is no significant difference in either the initial biliary recovery or in the total 60-min recovery of ouabain between the two age groups. However, examination of their data, shows that there is a difference in the initial 10 and 20-min recovery values between the two age groups similar to the one we found in the present study, though the difference was not statistically significant in their study. The lack of significance in their study may very well be due to the small age difference between the two groups (2- and 6-month-old) and the small number of rats they used (4 rats in each group). Although they discussed their data in terms of senescent change in ouabain pharmacokinetics, it is clear from the age groups they used that this is not a factor at all. The steadily increasing plasma ouabain concentration with age shown in Fig. 2 suggests that the ouabain distribution volume also declined with age as pointed out previously [35]. However, at the first time point (1 min), the ouabain remaining in plasma is already 10% of the dose if we assume that the plasma volume is 3 ml/100 g b.wt. This hampers the correct estimation of the initial volume of distribution from the available data since the area under the curve may not be correctly estimated. Therefore, no attempt was made for calculating this parameter. If we take plasma samples at earlier time intervals, the complete mixing of the ouabain is not assured. Thus, the precise estimation of the volume of distribution is technically difficult. Since a significant age-dependent decrease in the hepatic uptake as well as the biliary excretion of ouabain was demonstrated in the present study, there is no doubt that the higher ouabain concentration in plasma in older rat groups is at least partly due to the decreased hepatic clearance of ouabain, although the lower distribution may well be an additional factor. Except for the above study by Iga and Klaassen [35], no information is available about changes in ouabain's hepatobiliary transport process during aging.

In contrast to the results of the present study, our previous study of indocyanine green [4] and the report of Kroker and coworkers on taurocholic acid [5] suggested that for these organic anions the hepatic uptake mechanism(s) changes very little with age after maturity. Since it is well established that the transport pathways (or carriers) are different for

organic anions and neutral compounds [36, 37], it would not be surprising if these functions responded differently to aging. On the other hand, it is also possible that the age-dependent decline in ouabain uptake by hepatocytes is a phenomenon resulting from common (age-dependent) alterations in the mobility of proteins in the hepatocyte plasma membranes.

It has been shown recently that the lateral diffusion constant of hepatocyte membrane proteins, as determined by fluorescence recovery after photobleaching (FRAP) [38], declines with age in an almost linear fashion in rats [9, 11–13] and mice (I. Zs.-Nagy *et al.* unpublished observation). We used a peroxide-induced autofluorescence (PIAF) in these experiments [9, 11, 12] that is thought to come from surface membrane proteins of different kinds. Since the maximal velocity of a carrier mediated transport system is a function of the number of carriers and the diffusion constant of the carrier–ligand complex [39], the presently observed age-dependent decrease of ouabain uptake velocity by hepatocytes can be explained as a result of either (a) the decreased diffusion constant of the presumed ouabain uptake carrier in the hepatocyte membrane, or (b) the loss of specific receptor protein(s) for ouabain uptake. These two possibilities are not mutually exclusive and it is possible that both mechanisms underlie the decrease of ouabain uptake with age. Since the relationship between ouabain uptake and both excretion and diffusion shows a striking linearity, the present data on ouabain uptake change with age is an indication that our previous proposal that ouabain transport via the hepatobiliary route is at least partially regulated by the lateral mobility of hepatocyte surface membrane proteins [9]. The progressive restriction of mobility of cell surface membrane proteins with age has been predicted from previous experimental works as well as on the basis of membrane hypothesis for aging [40, 41].

If the above hypothesis is valid, the uptake processes of many (if not all) substances which are mediated by specific carriers in the plasma membrane would have to be affected by the same mechanism of decreased protein mobility, regardless of their chemical characteristics. This can be tested by examining the age related differences in hepatic uptake velocities for many other substances (organic anions in particular) by the same procedure used in the present study. Studies of this type are in progress in this laboratory.

Finally, unusually high excretion values found in the oldest groups of both sexes need some comments. Interestingly, our recent study also showed rather high values in the oldest rat groups of both sexes of F-344 strain [10]. Although, the exact nature of this phenomenon is not known, we suggested in the previous study [10] several possibilities as its explanation, which included (1) a natural selection by survival which has made the oldest group a special super elite, (2) the increase in the liver/body weight ratio, (3) up-regulation of liver function of unknown mechanism(s) in senescence. It is clear that ouabain excretion itself does not determine the survival of animals, so ouabain excretion and survival do not have a direct causal relationship. However, as we suggested above, it is possible that ouabain excretion is regulated by membrane protein mobility and the latter also may play a significant role in the aging and eventually the survival of animals. Therefore, it is possible, though not proven, that exhibited higher ouabain excretion was the result of the survival of an elite minority. The second possibility of relative liver size increase in the oldest groups can be a partial explanation of the phenomenon but does not appear to explain the whole situation, since if it is the cause, it should most significantly affect the first 10-min excretion value, while actually the subsequent excretion values showed a more striking difference from respective values in age groups next to the oldest.

Furthermore, though we have no direct proof, we suggested the third possibility in addition to the other two [10]. Whatever the real mechanism(s) may be, a curious rise in very old rats has been reported for various hepatic functional parameters such as albumin synthesis rate [42], biliary T_m for sulfobromophthalein [23], bile flow and bile salt excretion rate [23] and even membrane proteins' lateral mobility [12]. Future studies are needed to solve this question.

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