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(Na,K)-ATPase and Ouabain Binding in Reticulocytes from Dogs with High K and Low K Erythrocytes and Their Changes during Maturation*

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The present study demonstrated that dog reticulocytes had considerable amounts of (Na,K)-ATPase, but lost it rapidly during maturation into erythrocytes. Furthermore, reticulocytes from dogs possessing erythrocytes characterized with high (Na,K)-ATPase activity and high K, low Na concentrations (HK dogs; Maede, Y., Inaba, M., and Taniguchi, N. (1983) *Blood* 61, 493–499) had more ouabain binding sites than cells from normal dogs (LK dogs). Our results were as follows: i) The maximal binding capacities (B_{max}) for ouabain binding at equilibrium were approximately 0 and 1,500 binding sites/cell in LK and HK dog erythrocytes, respectively. ii) Reticulocytes from LK dogs possess approximately 5,700 ouabain binding sites/cell. iii) The B_{max} value for ouabain in HK reticulocytes was about 10,000 sites/cell, being 2-fold that in LK reticulocytes. iv) Ouabain-sensitive fluxes of ^{24}Na and ^{42}K in each type of reticulocyte were compatible with the number of ouabain binding sites on the cells. v) Ouabain binding capacity, as well as (Na,K)-ATPase activity, in the reticulocytes from LK dogs fell rapidly to nearly zero during the maturation into erythrocytes. vi) Although reticulocytes from HK dogs also showed a similar regression of (Na,K)-ATPase during maturation, they retained a certain number of ouabain binding sites even after maturation, resulting in the high activity of (Na,K)-ATPase in HK erythrocyte membrane.

Although most mammals, including humans, have high potassium (K) and low sodium (Na) concentrations in their erythrocytes, some species, dog as well as cat, possess erythrocytes having low K, high Na concentrations (1). This is due to lack of a Na/K exchange pump, the (Na,K)-ATPase which is a membrane-bound enzyme playing an active role in transporting Na out of and K into cells to maintain intracellular high K, low Na concentrations (2). Recently, however, we have demonstrated that some dogs have erythrocytes characterized by high activity of the (Na,K)-ATPase and high K, low Na concentrations (3, 4). Such characteristics are genetically determined and inherited by an autosomal recessive mode. These phenomena seen in the dogs resemble those in ruminants, especially in sheep. Sheep are well-known to be divided, even in the same species, into two distinct types with regard to the intracellular K concentration of their red cells, the high K (HK) and low K (LK) types (5). It has also been shown that HK cells in sheep had higher (Na,K)-ATPase activity and different kinetic properties from LK cells with differing internal cation affinities (6). Furthermore, it is very interesting that the (Na,K)-ATPase activity in reticulocytes from HK and LK type sheep was much higher than in mature cells and the kinetics of the enzyme were similar to one another in the two types of reticulocytes (7). Similarly, it has been reported that reticulocytes in dogs had a high K concentration, close to that of human red cells (8).

The maturation of reticulocytes into erythrocytes, the final stage of the differentiation process of erythroid cells, is associated with major alterations in membrane structure and function, involving changes in the composition of proteins and lipids (9–12), and transport of some materials, including Na and K (13). These findings strongly suggest that reticulocytes of dogs may possess the (Na,K)-ATPase activity in their membranes, but may lose it rapidly during maturation into erythrocytes, and that the (Na,K)-ATPase activity in those dog erythrocytes with hereditary high activity of the enzyme may still remain even in their mature erythrocyte stage. The present study was done to examine these mechanisms.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Ouabain (18.0 Ci/mmol) was obtained from New England Nuclear. The purity of [^{3}H]ouabain used in this study was checked by the procedure of Joiner and Lauf (14), and taken into account in calculating the specific binding to red cells. $^{24}NaCl$ (260 mCi/g of Na) and ^{42}KCl (36 mCi/g of K) were purchased from the Japan Atomic Energy Research Institute (Tokai-mura, Ibaraki, Japan). The sources of other chemicals were as follows: ouabain octahydrate, Na_2ATP, NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase from Boehringer Mannheim; PMSF[1] from Calbiochem; Percoll and dextran density marker beads from Pharmacia Fine Chemicals; RPMI 1640 medium and fetal calf serum from GIBCO. All other chemicals used were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or from Dojindo Laboratories (Kumamoto, Japan).

Experimental Reticulocytosis in Dogs—Two types of dogs with differing (Na,K)-ATPase activity and concentrations of Na and K in erythrocytes were used in the present study. One possessed erythrocytes with hereditary high activity of the (Na,K)-ATPase and high K, low Na concentrations (HK dogs), and the other type had red cells with low K, high Na concentrations and lacked the enzyme activity (LK dogs). Two dogs of each type, weighing 10–15 kg, were bled about 200 ml once daily from the jugular vein for 7 days. The hematocrit value in each dog was reduced from 46 to 22%, on the average, in 7 days. For separation of reticulocytes, about 50 ml of whole blood was

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[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin (fraction V).

taken into a heparinized syringe from each dog on the first and third day after the last bleed. At these times, the reticulocyte count in the peripheral blood from each dog was 7–15%, averaging 13%. All dogs recovered from anemia 7 days after the last bleed.

Separation of Dog Reticulocytes from Blood—Heparinized whole blood was filtered through an α-cellulose/microcrystalline cellulose column to remove leukocytes and platelets (15). The filtered cells were washed twice with 150 mM NaCl, 20 mM Hepes/Tris (pH 7.5) containing 0.1% (w/v) BSA, then resuspended in the same buffer to yield a hematocrit value of 15–20%. In the preliminary experiment using a Percoll continuous density gradient as described by Pertoft and Laurent (16), most of the reticulocytes (80–97%; average, 90%) were concentrated at the fraction with a density of 1.070–1.087 g/ml. Thus, two kinds of Percoll solution, 43.5 and 58.5% (v/v) Percoll in 150 mM NaCl, 0.1% (w/v) BSA, and 20 mM Hepes/Tris (pH 7.5), were prepared for isolation of reticulocytes. The solutions had specific gravities of 1.070 and 1.090 g/ml, respectively. The red cell suspension was carefully layered over the Percoll gradient, and centrifuged at 800 × *g* for 15 min at room temperature. The cells distributed in the interface of the two Percoll solutions were collected and washed 3 times with the medium for each experiment.

The reticulocytes were counted after supravital staining with new methylene blue.

[^{3}H]Ouabain Binding Studies—Venous blood was heparinized and filtered as mentioned above. The red cells were washed 3 times with a medium consisting of 150 mM NaCl, 3 mM $MgCl_2$, 2 mM Na_2HPO_4, 10 mM D-glucose, 0.1% (w/v) BSA, and 20 mM Hepes/Tris (pH 7.5). The cells were resuspended in the same medium to yield a hematocrit value of 9–10%. [^{3}H]Ouabain binding to the dog red cells and reticulocytes was carried out by the method of Erdmann (17) with the following modifications. The cells (final count, 0.9–1.2 × 10^9 cells/ml) in the medium containing [^{3}H]ouabain and unlabeled ouabain with various concentrations as indicated, were incubated with shaking for the appropriate time at 37 °C. Aliquots, 0.4 ml in most experiments, were transfered into Eppendorf microcentrifuge tubes on ice and immediately centrifuged at 15,000 × *g* for 10 s using a microcentrifuge MC-15A (Tomy Seiko Co., Ltd., Japan). The cells were washed with 1.25 ml of the same medium 3 times by rapid centrifugation at 15,000 × *g* for 10 s. After the last wash, packed cells were lysed with 1.25 ml of ice-cold 5 mM Tris/Cl (pH 7.6) containing 0.5 mM PMSF and centrifuged at 15,000 × *g* for 8 min, and the resultant cell membranes were washed 3 times using the same procedure. The membranes obtained were solubilized by the addition of 0.5 ml of 1% (v/v) Triton X-100 in water, and then added to 6 ml of Scintizol EX-H (Dojindo Laboratories) and counted for radioactivity using a liquid scintillation spectrometer LSC-651 (Aloka Ltd., Japan). The counting efficiency averaged 34%.

Nonspecific [^{3}H]ouabain binding to membranes was determined by counting radioactivity bound to cells in the presence of 0.1 mM unlabeled ouabain, and these values were less than 1% of total cell bound radioactivity in all experiments.

(Na,K)-ATPase Assay—The (Na,K)-ATPase activity of red cell membranes was measured by a pyruvate kinase, lactate dehydrogenase coupled assay as described by Cantley and co-workers (18). The assay mixture consisted of 100 mM NaCl, 20 mM KCl, 3 mM ATP, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM Hepes/Tris (pH 7.5), 1.4 mM phosphoenolpyruvate, 0.3 mM NADH, 10 μg/ml pyruvate kinase, 10 μg/ml lactate dehydrogenase, and 0.1 mM ouabain when present. The enzyme activity was measured using a spectrophotometer Hitachi 220A (Hitachi, Ltd., Japan) at 37 °C, and defined as the difference between activities measured in the presence and absence of ouabain.

Red cell membranes were prepared as described previously (3) using 5 mM Tris/Cl (pH 7.6) containing 0.5 mM PMSF instead of Veronal buffer. The membranes prepared were stored at −80 °C until use. The protein concentration of the membrane was determined by the method of Lowry *et al.* (19) using BSA as a standard. In most experiments, the membrane protein was present at concentrations of 25 or 50 μg/ml in the (Na,K)-ATPase assay system.

Measurement of ^{24}Na and ^{42}K Flux in the Dog Red Cells—The influx and efflux of ^{24}Na and the influx of ^{42}K in reticulocytes and mature erythrocytes from both HK and LK dogs were examined. The suspensions of each type of cell were prepared in the same manner as mentioned for the [^{3}H]ouabain binding experiment with the same medium, which in the final stage contained 165 mM NaCl, 5 mM KCl in the ^{24}Na flux assay and 150 mM NaCl, 20 mM KCl in the ^{42}K influx experiments, respectively. Prior to the flux experiments, the cells were incubated for 30 min at 37 °C with or without 0.1 mM ouabain.

For the ^{24}Na flux experiments, an equal volume of medium containing ^{24}NaCl (10 μCi/ml) was added to the suspension, and incubated at 37 °C. At the appropriate time, aliquots of the cell suspension (0.2 ml) were pipetted into Eppendorf microcentrifuge tubes and washed with the same medium 3 times by rapid centrifugation at 15,000 × *g* for 10 s. The radioactivity of the packed cells was counted in a γ-counter (TDC-6, Aloka, Ltd., Japan). After 100 min of incubation, the cells were washed 4 times and resuspended in the medium with or without 0.1 mM ouabain to yield the initial volume and further incubation was carried out at 37 °C. At the indicated times, 0.2 ml of suspension was removed and centrifugated at 15,000 × *g* for 10 s. The efflux of ^{24}Na from the cells was examined for γ-emission in 150 μl of each supernatant.

The ^{42}K influx experiment was carried out in the same manner as described for the measurement of ^{24}Na influx. In this experiment, the medium contained ^{42}KCl (10 μCi/ml) at a concentration of 20 mM.

Reticulocyte Maturation in Vitro and [^{3}H]Ouabain Binding—The changes of [^{3}H]ouabain binding capacity during the reticulocyte maturation *in vitro* were studied. Reticulocyte culture was carried out by the method of Tucker and Young (20) with some modification.

Reticulocytes were purified from HK and LK dogs with reticulocytosis as described above under sterilized conditions. The cells were suspended in RPMI 1640 medium (pH 7.4) supplemented with 20% (v/v) fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2.5 μg/ml of Fungizone, 20 mM Hepes, and 137 mM NaCl. The cells in the culture medium (1 × 10^8 cells/ml) were dispensed in 15-ml portions into plastic culture vessels (50-ml total capacity; Nunc) and incubated at 37 °C in humidified air containing 5% CO_2. Aliquots were removed at the indicated times and [^{3}H]ouabain binding, total cell counts, and the percentages of the reticulocyte count were measured. To determine [^{3}H]ouabain binding, [^{3}H]ouabain was added to the cells in the culture medium to give a final concentration of 100 nM and incubated for 90 min at 37 °C with gentle shaking. Then, [^{3}H]ouabain bound to the cell membrane was measured by the same procedure as described above, without replacing the washing solution (5 mM Tris/Cl (pH 7.5), 170 mM NaCl).

RESULTS

Demonstration of the (Na,K)-ATPase Activity in Dog Reticulocyte

Ouabain Binding to HK and LK Dog Erythrocytes—We first examined the capacity of ouabain binding to HK and LK dog erythrocytes. Fig. 1 shows that the HK dog erythrocytes bound [^{3}H]ouabain in a time-dependent manner and that equilibrium was reached after incubation for 4 h, while no ouabain binding was observed in LK erythrocytes in spite of prolonged incubation.

We then determined the effect of the concentration of [^{3}H]ouabain on the binding to HK erythrocytes. It was estimated that [^{3}H]ouabain binding to HK cells was dominated by a saturable component obeying Michaelis-Menten kinetics. The half-maximal binding seemed to be reached with approximately 5 nM of [^{3}H]ouabain (data not shown).

Thus, long time incubation and a low level of [^{3}H]ouabain (4.5 h and 3.7 nM, respectively) were used in the following studies to estimate the capacity of ouabain binding at true equilibrium.

Fig. 2 shows an example of ouabain binding to HK dog erythrocytes at equilibrium. In this Scatchard plot, the total number of binding sites (B_{max}) was ascertained to be 32.7 pmol/ml of cells, with 1660 binding sites/cell, and the dissociation constant (K_D) was 1.51 nM (see also Table I). The value for B_{max} is 3.6-fold that in human erythrocytes as shown in Table I, and this is in good agreement with the results which showed that the (Na,K)-ATPase activity of HK dog erythrocytes was about 3-fold greater than that of human erythrocytes described previously (3).

Capacity and Ouabain Binding and (Na,K)-ATPase Activity in HK and LK Dog Reticulocytes—Fig. 3 shows the Scatchard plot of ouabain binding to reticulocytes from HK and LK

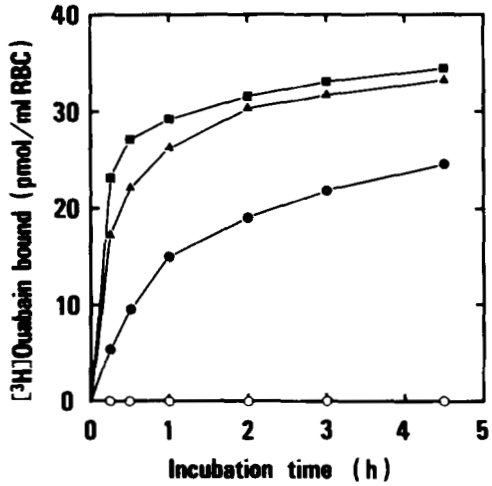

FIG. 1. **Time course of [^{3}H]ouabain binding to erythrocytes from HK and LK dogs.** Erythrocytes from HK (●, ▲, and ■) and LK (○) dogs were incubated in the medium containing [^{3}H]ouabain at 37 °C. At various intervals, 0.4 ml of suspension was removed and the bound [^{3}H]ouabain was measured as described under "Experimental Procedures." [^{3}H]Ouabain was used at three different concentrations of 10 nM (●), 50 nM (▲), and 100 nM (■). Data are expressed as the specific binding of [^{3}H]ouabain and values are given as the mean of two experiments. Since no specific binding was observed in the erythrocytes from LK dogs in all experiments, the values are indicated as one symbol (○). *RBC*, red blood cells.

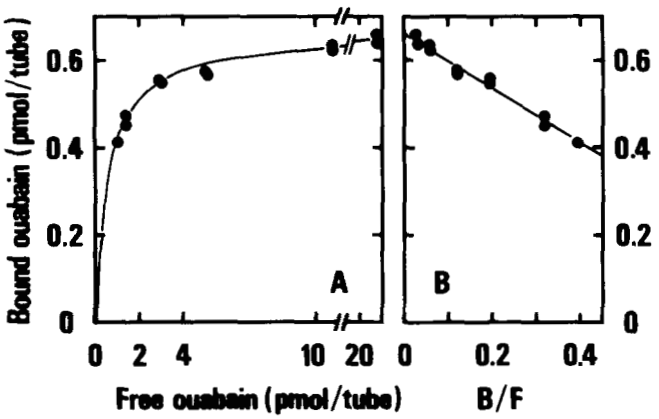

FIG. 2. **Ouabain binding to HK dog erythrocytes at equilibrium.** *A*, erythrocytes from a HK dog were incubated at 37 °C for 4.5 h in the media containing 3.7 nM [^{3}H]ouabain and increasing amounts (0–50 nM) of nonlabeled ouabain. Data are plotted for the specific binding obtained from each tube (0.4 ml of suspension in each tube). *B*, experimental data in *A* are plotted according to Scatchard (21). *B/F*, bound/free.

dogs at equilibrium. The B_{max} and K_D values were shown in Table I. The mean reticulocyte purity of the cell suspensions was 93% in HK dogs and 91% in LK dogs. It was clear that LK dog reticulocyte had a larger number of ouabain binding sites, being 3.6-fold that of HK dog mature erythrocyte. The K_D value for the binding in LK reticulocytes showed a value about half that of HK erythrocytes. Furthermore, the B_{max} value for the HK reticulocytes was increased to about 2-fold that of the LK reticulocytes.

Similar results were obtained in (Na,K)-ATPase activity of the membranes from those cells (Table II). The activity of LK reticulocytes, however, was not as high as expected from the results of the ouabain binding experiments. It was almost identical to that of HK erythrocytes.

^{24}Na and ^{42}K Fluxes in Reticulocytes and Mature Erythrocytes from HK and LK Dogs—The flux of Na and K was examined in practice using ^{24}Na and ^{42}K as tracers of these ions. Fig. 4, *A* and *B* show the fluxes (influx and efflux) of ^{24}Na and in the reticulocytes (*A*) and mature erythrocytes (*B*)

TABLE I

Kinetic constants for ouabain binding to dog red cells

Subjects	B_{max}		K_D
	pmol/ml cells	*sites/cell*	*nM*
Erythrocytes from			
HK (n = 4)[a]	30.5 ± 1.9	1,584 ± 60	1.75 ± 0.33
LK (n = 3)	≃0	≃0	
Reticulocytes from			
HK (n = 2)[b]	167.9	10,165	2.07
LK (n = 2)[b]	86.8	5,698	0.84
Erythrocytes from human (n = 2)[c]	6.6	435	2.66

[a] Data are expressed as mean ± S.D.

[b] Mean values of the two experiments. The purities of each reticulocyte preparation were 90 and 95% for HK cells and 86 and 96% for LK cells, respectively.

[c] [^{3}H]Ouabain binding to erythrocytes from healthy adult men were also determined. The results are expressed as mean values of the two experiments.

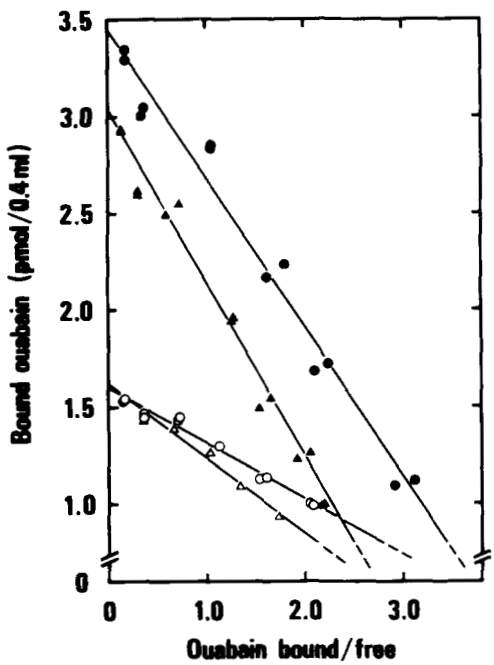

FIG. 3. **Ouabain binding to reticulocytes from HK and LK dogs.** Reticulocytes from two HK (● and ▲) and two LK (○ and △) dogs were incubated as described under "Experimental Procedures." Data are plotted as Fig. 2*B*. The purities of each reticulocyte preparation were as follows: 95% (●), 90% (▲), 96% (○), and 86% (△).

TABLE II

(Na,K)-ATPase activity of HK and LK dog red cells

Subjects	Reticulocytes	(Na,K)-ATPase activity
	%	*nmol ATP hydrolyzed/ mg protein/min*
Erythrocytes from		
HK (n = 6)[a]	<0.2	45.1 ± 3.03
LK (n = 4)	<0.2	≃0
Reticulocytes from		
HK (n = 2)[b]	90	134.1
LK (n = 2)[b]	95	37.9

[a] Data are expressed as mean ± S.D.

[b] Mean values of the two experiments.

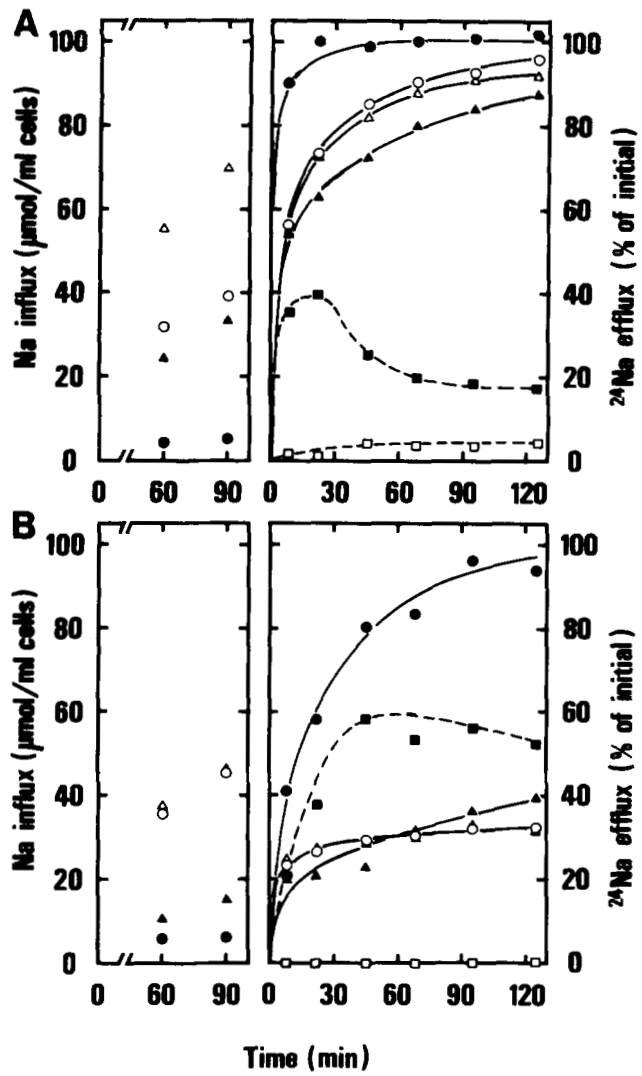

FIG. 4. **^{24}Na influx and efflux in reticulocytes (*A*) and erythrocytes (*B*) from HK (●, ▲, and ■) and LK (○, △, and □) dogs.** The influx and efflux of ^{24}Na in the cells were examined in the presence (▲ and △) and in the absence (● and ○) of 0.1 mM ouabain as described under "Experimental Procedures." The efflux of ^{24}Na from the cells (*right panels*) are expressed as the released radioactive percentages of ^{24}Na initially (at time 0) accumulated in the cells, and the ouabain-sensitive component of the efflux at each time are also plotted (■ and □). The purities of reticulocyte in this experiment were 65, 70% and 92, 98% for HK and LK dogs, respectively. Data are expressed as mean values of two experiments.

from HK and LK dogs with or without ouabain treatment. The amount of Na influx observed in reticulocytes and mature erythrocytes from HK dogs in the absence of ouabain was very small compared to that in cells from LK dogs. An increase of Na influx was observed when HK cells were treated with 0.1 mM ouabain. The Na influx in LK reticulocytes was similar to that in HK dog cells, but the values of Na influx in LK reticulocytes were apparently much higher than in HK cells. Similarly, the influx of Na into LK erythrocytes was distinctly accumulative as compared to HK mature cells, and it was not affected by ouabain treatment.

For the efflux of Na, both reticulocytes and mature erythrocytes from HK dogs showed very potent efflux; that is, total ^{24}Na accumulated in immature and mature cells was pumped out in 30 and 125 min, respectively. In addition, at least 40% of the reticulocytes and 60% of the mature cells in the ^{24}Na efflux were shown to be ouabain-sensitive, that is, (Na,K)-ATPase-dependent (shown in Fig. 4, *A* and *B*). On the other hand, it was also demonstrated that the mature erythrocytes from LK dogs had no ouabain-sensitive ^{24}Na efflux, which was in agreement with a previous report (22). Additionally, LK reticulocytes showed a very low level of ouabain-sensitive ^{24}Na efflux.

Fig. 5 shows the active, ouabain-sensitive ^{42}K influx in the reticulocytes and mature cells from HK and LK dogs. It was demonstrated that the reticulocytes from both types of dog and mature cells from HK dogs transported ^{42}K into their cells rapidly, but complete saturation was not observed even after 60 min of incubation at 37 °C. In contrast, mature erythrocytes from LK dogs only showed passive, ouabain-insensitive uptake for ^{42}K. The apparent ratio of this active ^{42}K influx in immature and mature cells in both types of dog corresponded well to the number of ouabain binding sites. From the values described in Fig. 5 and the number of maximal ouabain binding sites per cell (Table I), K ion turnover numbers for each type of cell were calculated as follows: 4000, 2900, and 2400 K ions/site/min for HK mature cells, HK reticulocytes, and LK reticulocytes respectively, which was consistent with the values in human erythrocytes (14).

Changes of the (Na,K)-ATPase Activity during Maturation of Dog Reticulocytes—From the results described above, it was supposed that reticulocytes from LK dogs might rapidly lose the Na pump, that is, the (Na,K)-ATPase, during maturation for unknown reasons, while the cells from HK dogs might substantially retain it even after the maturation into erythrocytes. Thus, we examined the decrease in the number of ouabain binding sites accompanying cellular maturation *in vivo* and *in vitro*.

Ouabain Binding to Dog Red Cells Fractionated with Percoll Continuous Gradient—Red cells from HK and LK dogs taken at the second day after the successive bleeding for 7 days were separated by centrifugation with Percoll continuous gradient and fractionated, and [^{3}H]ouabain binding was measured for each fraction. It is known that the age of erythrocytes is directly related to density, young cells having lighter gravity

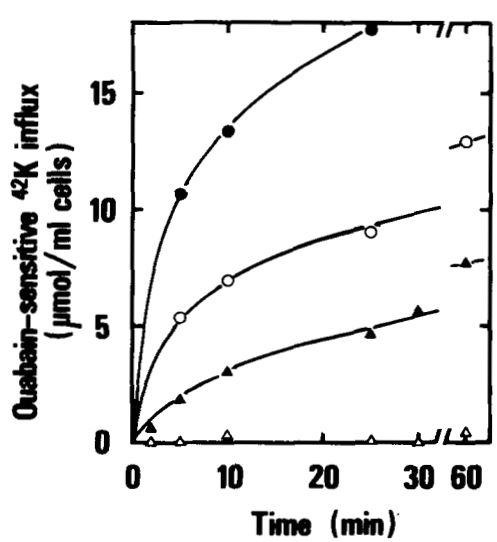

FIG. 5. **Ouabain-sensitive ^{42}K influx into reticulocytes (● and ○) and erythrocytes (▲ and △) from HK (● and ▲) and LK (○ and △) dogs.** The influxes of ^{42}K into each type of cells were measured as described under "Experimental Procedures." Data were obtained by subtracting the influx in the presence of 0.1 mM ouabain from the influx in the absence of ouabain, and expressed as mean values of two experiments. The purities of reticulocytes were the same as described in the legend for Fig. 4.

than old cells. Fig. 6 shows the distribution of cells which bound [^{3}H]ouabain. It was found that ouabain binding sites in LK dog red cells decreased nearly to zero as the density of cells increased. In contrast, HK cells maintained the number of binding sites at a high level throughout aging, although they gradually lost it during maturation.

Changes of Ouabain Binding to Reticulocyte during Maturation in Culture—As shown in Fig. 7, both the reticulocyte counts from HK and LK dogs fell rapidly with linear or curvilinear components within 40 h after the onset of incubation, and then gradually decreased to less than 5% at 140 h in all experiments, while no decrease was observed in total cell count. The profiles of the regression of [^{3}H]ouabain binding capacity, reflecting the inactivation of (Na,K)-ATPase, were in good agreement with those changes in reticulocyte counts; that is, the number of ouabain binding sites in LK reticulocytes decreased rapidly within 40 h and fell to about 5% of the initial number after 140 h in culture. The HK cells also showed a similar loss of the capacity of [^{3}H] ouabain binding by 80–100 h, but after that they retained a certain number of ouabain binding sites, approximately 1500 sites/cell, which was almost equal to the number of sites in HK mature erythrocytes as shown in Fig. 2 and Table I. These results are consistent with the above observations concerning the decrease of ouabain binding in proportion to red cell aging *in vivo*.

Moreover, HK reticulocytes treated with [^{3}H]ouabain prior to culture developed in the same way as the cells not treated with [^{3}H]ouabain, and no differences in the regression of retained [^{3}H]ouabain were observed during incubation (Fig. 8). Similar results were obtained for LK cells (data not shown). Thus, it is suggested that the inactivation of the (Na,K)-ATPase by such a functional inhibition by ouabain might have no direct effect on the maturation of reticulocytes into erythrocytes.

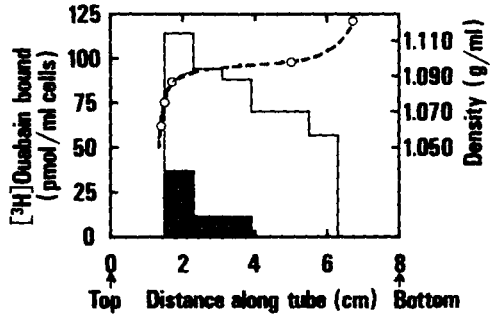

FIG. 6. **Distribution of [^{3}H]ouabain binding in density separated HK and LK dog red cells.** Red blood cells from HK (□) and LK (□) dogs with reticulocytosis induced by bleeding were separated by Percoll continuous gradient centrifugation technique (16) and fractionated. [^{3}H]Ouabain binding was measured for each fraction by incubating the cells with [^{3}H]ouabain at a concentration of 100 nM for 1 h at 37 °C. Other methods for [^{3}H]ouabain binding assay were the same as described under "Experimental Procedures." The densities of the gradient (○) were determined using dextran density marker beads.

DISCUSSION

Some investigators have suggested that intracellular K concentration is an essential requirement for RNA and protein synthesis (23, 24), and that the (Na,K)-ATPase, through its control of the intracellular concentration of Na and K, may play an important role in the differentiation of erythroid cells (25). It is known that reticulocytes are still capable of hemoglobin synthesis, whereas mature erythrocytes are unable to do this (26). It is, therefore, not surprising that, as has been observed in other species (7), dog reticulocytes also had increased (Na,K)-ATPase activity in order to maintain a high K environment in their cells. To our knowledge, however, no report on this phenomenon has appeared in literature, although the possibility has been suggested (8). In the present study, we clearly demonstrated that dog reticulocytes have a large amount of (Na,K)-ATPase, approximately 12-fold that of human erythrocytes (Table I), while it was rapidly and completely lost during maturation into erythrocytes (Fig. 7). It is unclear at present what the controlling mechanism for such dramatic change of the (Na,K)-ATPase activity during maturation of reticulocytes in dog is. In this respect, Bernstein *et al.* (25) observed that the binding of ouabain to the (Na,K)-ATPase in Friend erythroleukemic cells induced hemoglobin synthesis and thus suggested that changes in the intracellular concentration of K might be involved in the control of erythroid differentiation. Although maturation of reticulocytes into erythrocytes is the final step of erythroid development, we

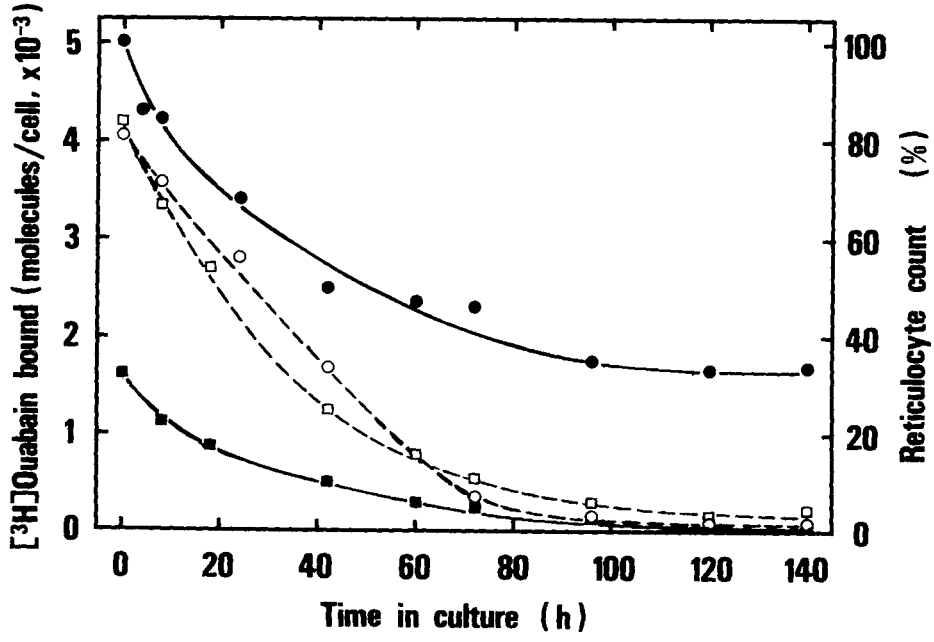

FIG. 7. **Changes of [^{3}H]ouabain binding during reticulocyte maturation into erythrocyte.** Reticulocytes from HK (● and ○) and LK (■ and □) dogs were separated and cultured. At the appropriate time, aliquots of cells were removed and [^{3}H]ouabain binding (● and ■), reticulocyte counts (○ and □), and total cell counts (data not shown) were estimated. For detailed procedures, see "Experimental Procedures." No considerable change in the total cell counts (within ±4%) were observed during the culture.

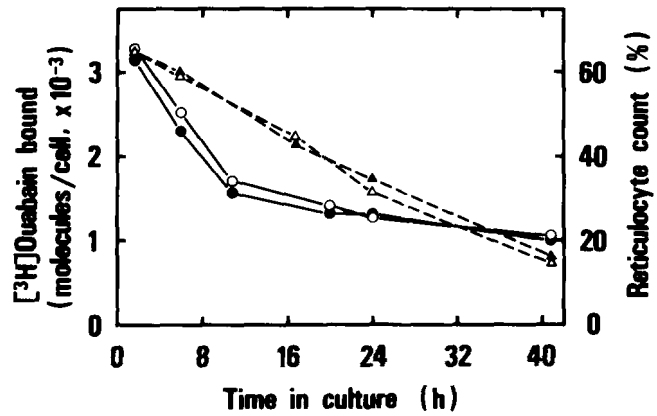

FIG. 8. **Effect of ouabain on reticulocyte maturation into erythrocyte *in vitro*.** HK dog reticulocytes were collected and cultured as described under "Experimental Procedures." [³H]Ouabain binding was assayed by incubating the cells for 90 min at 37 °C with 100 nM [³H]ouabain at the indicated times (●). Simultaneously, aliquots of the cells were treated with 100 nM [³H]ouabain in the same way prior to the cultivation, then washed with culture medium and incubated, and the retained [³H]ouabain was estimated at the indicated time (○). Data are expressed as mean values of two experiments. Reticulocyte counts (treated with (△) or without (▲) [³H] ouabain prior to the culture) were measured for both experiments. No significant change in total cell counts were observed throughout the culture (within ±2.5% of the total cell counts).

also consider it possible that the inactivation of the (Na,K)-ATPase could be a potent inducer of such maturation as they suggested (25). In dog reticulocytes, however, ouabain which was bound to the cells prior to the culture caused no distinguishable stimulation for reticulocyte maturation and had no effect on the decrease in [³H]ouabain binding capacity (Fig. 8) in either HK or LK dogs. This result shows that the functional inhibition of the (Na,K)-ATPase had no effect on reticulocyte maturation. Thus, it is possible to conclude that the decrease of the (Na,K)-ATPase activity during maturation in dogs is one of the various phenomena observed during red cell aging, such as the fall in activity of other enzyme (27, 28) and of membrane transport (29, 30).

The present study also demonstrated that the reticulocytes from HK dogs have an intensity over 2-fold that of LK reticulocytes in regard to active ^{24}Na and ^{42}K transport, the number of ouabain binding sites, and (Na,K)-ATPase activity. This is quite different from the findings seen in HK and LK sheep reticulocytes (6, 7, 13). Reticulocytes from LK sheep, as mentioned elsewhere, have activity and (Na,K)-ATPase kinetics similar to those of HK sheep, while the enzyme activity of LK sheep reticulocytes is more reduced than that of HK sheep during maturation. The maturation-associated loss of this enzyme in HK dog reticulocytes seems to occur in the same way as in LK reticulocytes, but to cease in the early stage of aging (80–100 h in culture, see Fig. 7), and the activity may be at a constant level which maintains high K, low Na concentrations. As reported previously (3), in HK dog erythrocytes (mature cells) mean corpuscular volume was higher and mean corpusular hemoglobin concentration was lower than in LK dogs, indicating a significant increase in cell volume such as has been observed in young red cells (31). Furthermore, the concentration of reduced glutathione, which is higher in young red cells (32), was about 5–7 times greater in HK cells than in LK cells (3, 33). These findings indicate that the "mature" erythrocytes in HK dogs may have remained at an immature stage of their development. From these results, we can attribute the presence of high (Na,K)-ATPase activity in HK mature erythrocytes to two causes: i) the precursor of erythroid cells in HK dogs possess much more (Na,K)-ATPase than the LK immature cells because of a genetical control, and ii) the inactivation of this enzyme during maturation of the cells may be suppressed for some reason.

Another explanation may be possible in regard to the high activity of the enzyme both in reticulocytes and erythrocytes of HK dogs. Previously, some investigators have demonstrated that reticulocytes contain a soluble, ATP-dependent proteolytic system (34–36). This degradation of proteins in reticulocytes appears to be responsible for the selective elimination of many normal proteins during reticulocyte maturation into erythrocyte (35, 36), as well as for the selective degradation of abnormal protein (34). In addition, Weigensberg and Blostein (30) recently reported that the loss of Na^+-dependent glycine transport activity and [³H]ouabain binding sites during reticulocyte maturation in sheep was retarded by ATP depletion, suggesting that an ATP-dependent proteolytic system may modulate the number of ouabain binding sites, the (Na,K)-ATPase. In our preliminary study, total D-[^{14}C] glucose uptake and intracellular concentration of ATP in HK erythrocytes showed no significant differences from LK cells.[2] These findings suggest that HK dog red cells, as compared to LK cells, might be in an energy(ATP)-starved condition due to increased consumption of ATP resulting from high activity of the (Na,K)-ATPase in the HK cells. In addition, it is known that the intracellular ATP concentration in dog red cells is maintained at a low level, which is about one-half to one-fourth that of the level in human red cells (8). If dog reticulocytes also have an ATP-dependent proteolytic system as described in sheep reticulocytes (30) and others (34–36), the loss of the (Na,K)-ATPase activity as well as other enzyme activities during maturation in HK dog reticulocytes may be retarded by the depletion of ATP for the stimulation of the proteolytic system in the cells.

Kinetic analysis for ouabain binding to dog red cells showed a slight difference in K_D values between HK and LK reticulocytes (Table I). It is unclear at present whether this discrepancy derived from an alteration of the (Na,K)-ATPase itself in the HK cells, or from other causes, *e.g.* the difference in the intracellular concentration of cations, especially K, between the two types of cells. Many investigators have reported changes in the number of ouabain binding sites and no altered K_D values in erythrocytes of normal (17, 37) or abnormal subjects (38, 39). However, there is no denying the presence of multiple receptor sites on HK reticulocytes as reported for human erythrocytes (40), and further studies may be necessary.

It would be very interesting to know what the mechanism of the dramatic change in the (Na,K)-ATPase activity in dog reticulocytes during their final maturation step is. This important problem remains unclear at present, but it seems possible to conclude that dog reticulocyte is one of the most useful subjects not only for making a study of the enzyme itself, but for unraveling some of the more complex problems concerning the current investigation of cellular aging.

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