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Inverse changes in erythroid cell volume and number regulate the hematocrit in newborn genetically hypertensive rats

(microcytosis/erythrocytosis/spontaneously hypertensive rat)

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ABSTRACT **Erythrocytosis and microcytosis have been described in strains of genetically hypertensive rats and in essentially hypertensive humans. Published discussion of these phenomena has centered around their relationship to observed alterations in ionic transport and the pathogenesis of hypertension. In presenting data for another strain of spontaneously hypertensive rats in which these findings are exhibited, we note that erythroid cell size decreases concurrently with the increase in cell numbers so that the hematocrit and the mean corpuscular hemoglobin concentration remain constant. Data from the literature support the hypothesis that erythroid cell size is inversely proportional to cell count in a large number of species. Erythrocytosis, as it develops in the neonatal rat, is a consequence of the marked immaturity of this species at birth. Erythrocytosis in the spontaneously hypertensive rat is not due to a difference in the affinity of its hemoglobin for oxygen or to significant tissue anoxia. Microcytosis in the spontaneously hypertensive rat is the consequence of a continuation of the linear volume decrease with age of its erythroid cells seen in the normotensive animals and may be accounted for by the production of smaller cells with concomitant regulation of individual cell volume.**

Sen *et al.* (1) have described erythrocytosis in a strain of spontaneously hypertensive rats bred at Cleveland Clinic Foundation and designated SH. The erythroid cell (RBC) count of the young rats increased "concomitantly and in direct proportion to blood pressure." The authors noted that the RBCs of these SH rats became progressively smaller as hypertension developed so that the mean cell volume (MCV) was significantly less than that of normotensive rats (Sprague–Dawley or Wistar) or renal (clipped) hypertensive rats at comparable body weights.

Microcytosis has been documented also by Bianchi *et al.* (2) in their Milan hypertensive strain compared to its normotensive control. Cell numbers were not reported in that study. A strain of the spontaneously hypertensive rat (SHR) derived from the National Institutes of Health colony was examined by Bruschi *et al.* (3) and found to exhibit erythrocytosis, microcytosis, and larger platelets compared to its normotensive Wistar–Kyoto control (WKY). Bruschi *et al.* (3) extended their investigation to include patients with essential hypertension (170 males and 120 females) versus normotensives (103 males and 93 females). The hematologic indices of these two groups showed the same differences found in the rats.

In the present study, we follow the time course of erythrocytosis and microcytosis in another strain of SHR (Laboratory Supply, Indianapolis) from the 6th to the 28th week of life. These values are compared to those of its normotensive control (WKY) and to those of the normotensive domestic Wistar rat, a strain not specifically bred for low arterial pressure. With respect to the erythrocytosis, we present evidence that the (presumed) increase in erythropoietin levels in SHR is not due to differences in oxygen affinity for hemoglobin or to plasma levels of 2,3-bisphosphoglycerate. Recent advances in our understanding of the mechanisms of cell volume regulation now permit an informed discussion of the microcytosis of SHRs in terms of observed alterations in ionic flux across the erythrocyte membrane of this strain and possible alterations in the membrane itself. Published data on hematologic indices for a number of animal species are used (16, 17) to support the hypothesis that RBC size and cell number are inversely regulated resulting in constancy of the hematocrit.

METHODS

The three strains of rat were obtained from Laboratory Supply. Data shown are from seven rats in each strain for weeks 6–21 and six rats in each strain thereafter. They were kept isolated from other rats, in a separate room, where they remained free of infection and appeared to thrive. Systolic blood pressure was recorded by the tail-cuff method (Narco Bio-systems, Houston), the animals being warmed, unanesthetized, and quiescent.

RBC counts (Coulter counter, model ZBI) and duplicate or triplicate hematocrits (10-min centrifugation, IEC microcapillary centrifuge) were done on freely flowing tail vein blood from previously warmed animals. Samples (10 μl) of blood were rinsed immediately into Isoton II dilutent (Coulter) with a second dilution into 20 ml of Isoton for counting. We discovered that there are time constraints on the counting procedure. Cells should remain in the first dilution for <30 min and in the second for <10 min. Longer periods lead to clumping and consequent decrease in the number of cells counted, a behavior not observed in human RBCs so treated.

For animals from 21 to 29 weeks of age, MCVs, in addition to being calculated from the above cell counts and hematocrits, were measured with a Channelizer (Coulter, model C1000) using latex microspheres (5-μm diameter; 65.45 μm^3; Coulter 2426552A) as standards. Also for these age groups, hemoglobin determinations were done using the Drabkin (Sigma) method and a Gilford model 250 spectrophotometer.

Plasma volume and RBC mass were measured in six rats from each of the three strains during the 27th week of life, after RBC counts and MCVs had become stable. These animals were anesthetized with Inactin [10 mg/100 g (body weight)]. For plasma volume determinations, 0.25 μCi (10 μCi/ml; 1 Ci = 37 GBq) of ^{125}I-labeled albumin was injected

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Abbreviations: SHR, spontaneously hypertensive rat; P_{50}, total pressure of oxygen at half saturation of hemoglobin; RBC, erythroid cell; MCV, mean cell volume; WKY, Wistar–Kyoto.

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through an intrajugular venous catheter. Three blood samples, 5, 10, and 15 min after injection, were taken from a contralateral carotid artery catheter and centrifuged, and the activity in plasma measured (Chicago Nuclear γ counter) in a duplicate sample with the zero-time intercept was used to calculate the ^{125}I-labeled albumin distribution space. In a similar fashion, RBC mass was estimated in the same rats 20–30 min after the injection of ^{51}Cr-labeled RBCs (prepared by a 20-min incubation with $NaCrO_4$). Samples of cells used for injection and those recovered were washed with 150 mM NaCl solution before determination of activity.

Oxygen equilibrium curves for the determination of total pressure of oxygen at half saturation of hemoglobin (P_{50}) values were carried out by the thin-film method at constant partial pressure of CO_2 (4, 5). 2,3-Bisphosphoglycerate concentrations were measured by an enzymatic method that couples 2,3-bisphosphoglycerate phosphatase, phosphoglycerokinase, and glyceraldehyde-3-phosphate dehydrogenase to NADH oxidation (Sigma; Technical Bulletin 35-UV).

Data were analyzed by unpaired two-tailed Student's *t* test and are given as mean ± SEM. Differences were deemed significant when $P < 0.05$.

RESULTS

Blood Pressure. Hypertension is clearly established in the SHR by the 11th week of life and reaches maximum levels usually by the 20th week (Fig. 1). The figure demonstrates a slightly but consistently higher pressure in domestic Wistar rats than in WKY rats at each age. Blood pressure values for SHR and WKY rats have been reported from this laboratory (6).

RBC Counts. The time course of erythrocytosis in the maturing rats of each strain is graphically displayed in Fig. 2. It is apparent that all three strains experience a progressive increase in the number of RBCs per mm^3 of blood from the 7th to the 21st week of life. Data from weeks 7 to 21 are from the Buffalo laboratory (J.B.V.L.). The break in the abscissa at week 21 in Figs. 2–4 indicates continuation of the study at the Newington laboratory (J.W.B. and P.U.F.), using the same strains and identical methodologies. Data from the 21st week were repeated at Newington and demonstrate a satisfactory concordance between results of the two laboratories. At week 21 and thereafter the RBC counts of the SHR are significantly increased over those of the normotensive strains (Fig. 2).

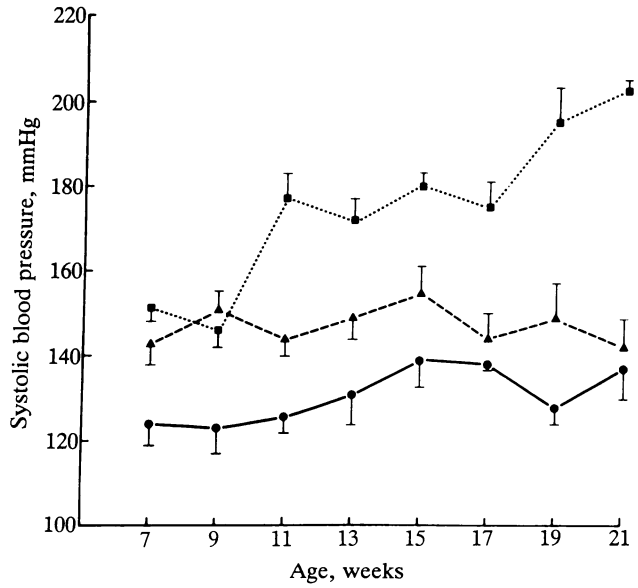

FIG. 1. Systolic blood pressure versus age in rat strains SHR (■), WKY (●), and Wistar (▲). Pressure was by the tail-cuff method, the animals being warmed, unanesthetized, and quiescent ($n = 7$ for each strain).

Hematocrits. Hematocrits for the three strains studied are presented in Fig. 3. The values for the three strains are closely grouped and virtually identical from weeks 21 to 28.

MCV. Increasing RBC counts in the presence of constant hematocrits defines RBCs of diminishing size and this is clearly demonstrated in the calculated MCVs set out in Fig. 4. The MCVs of all strains fall steadily to week 21, when the values of normotensive strains appear to stabilize. By and after the 13th week, the MCVs of SHRs are consistently below those of the normotensive strains and the gap widens after week 19 as the cells of the hypertensive strain continue to decrease in size. Fig. 4 *Inset* gives values by electronic sizing for weeks 21–28. Although these absolute values for the SHR are larger than those calculated, the significantly smaller size of the cells of the hypertensive strain is confirmed by this technique.

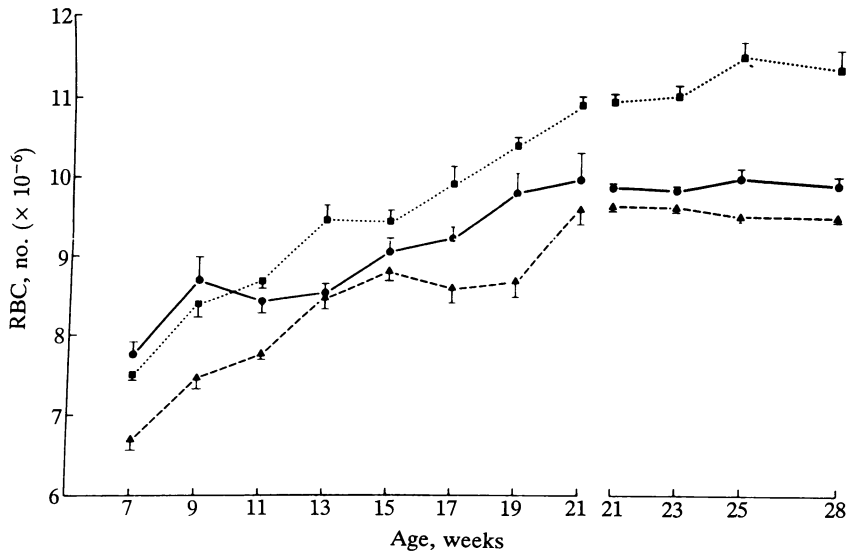

FIG. 2. RBC counts versus age in rat strains SHR (■), WKY (●), and Wistar (▲). Data for weeks 7–21 are from the Buffalo laboratory (J.B.V.L.); data for weeks 21–28 are from the Newington laboratory (J.W.B. and P.U.F.).

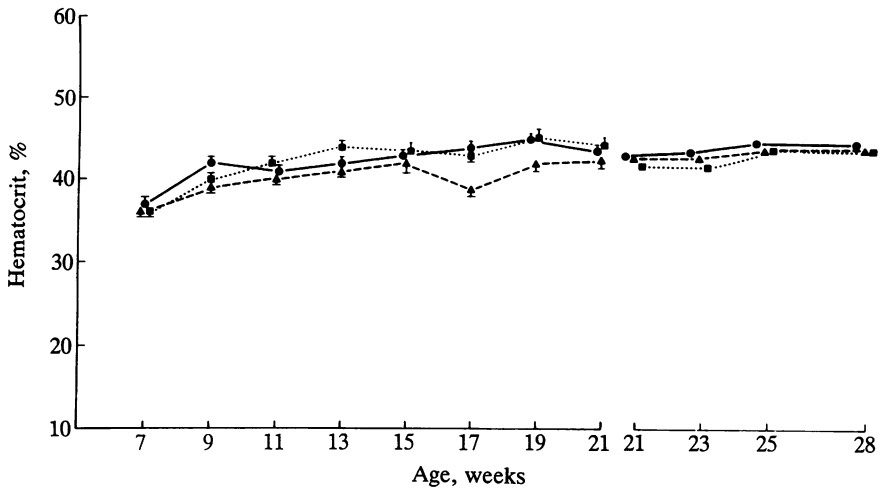

FIG. 3. Hematocrit versus age in rat strains SHR (■), WKY (●), and Wistar (▲).

Plasma Volume and RBC Mass. Plasma volumes and RBC masses were determined in six rats of each strain during the 27th week of life; these data are shown in Table 1. There is no significant difference between these values in the SHR and either normotensive strain.

P_{50} and 2,3-Bisphosphoglycerate Concentrations. Values for P_{50} of erythrocytes from SHR and WKY rats (n = 7 of each strain), determined at 6–8 and 12–15 weeks of age, were 37.9 ± 1.2 and 37.1 ± 1.1 torr, respectively (1 torr = 133.3 Pa). 2,3-Bisphosphoglycerate concentrations, determined in erythrocytes of the same animals at 11 weeks of age, were 22.9 ± 0.5 (SHR) and 22.3 ± 1.0 μmol/g of hemoglobin (WKY).

Hemoglobin Content. Values per volume and number of cells for the three strains are given in Table 2.

DISCUSSION

Erythrocytosis. It is apparent from Fig. 2 that a brisk erythropoiesis is a prominent feature of the neonatal maturing rat, a phenomenon that has been recognized with little comment for many years (7). From weeks 7 to 21 there is little difference in the rate of erythrocyte production among the three strains studied; from weeks 21 to 28 there is a leveling off of the erythrocyte count (production equals destruction) in the two normotensive strains whereas the SHR continues at a rate of erythropoiesis that results in significantly higher RBC counts than either normotensive strain (Fig. 2). In agreement with Sen *et al.* (1), we find the increase in the number of RBCs to be absolute as shown by the data for plasma volumes and RBC masses (Table 1).

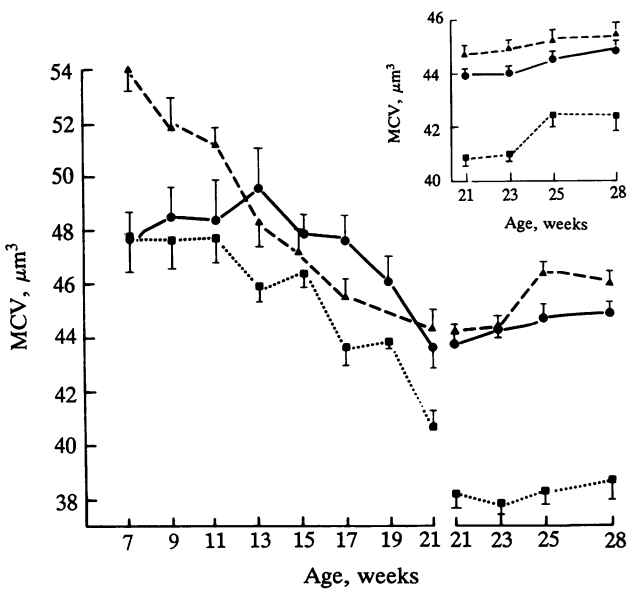

FIG. 4. MCV versus age in rat strains SHR (■), WKY (●), and Wistar (▲), calculated from RBC counts and hematocrits. (*Inset*) Repetition of MCVs for weeks 21–28 done by electronic sizing.

It is reasonable to assign this postnatal erythropoiesis to the marked prematurity of the newborn rat. The human fetus doubles its RBC count during the final 16 weeks of intrauterine life (8), and there is a progressive fall in RBC count in the first 12 weeks after birth (9). Postnatal erythropoiesis during maturation of the newborn rat is analogous to the burst of erythropoietic activity in the final weeks of human fetal life and is probably in response to the same stimuli. Why does the process persist in the SHR, leading to the erythrocytosis noted by Sen *et al.* (1), others cited above, and the present study?

Erythropoietin was thought by Sen *et al.* (1) to be the effective stimulus for erythrocytosis in their hypertensive strain. Using a bioassay method that employed exhypoxic polycythemic mice, they reported 0.27–0.17 international reference preparation unit/ml of plasma in younger and older SH Rats, respectively, whereas titers in the normotensive strain were undetectable. No similar study incorporating erythropoietin analyses has been reported in SHR. In the newborn human infant, erythropoietin levels become undetectable during the first several weeks of life (10) and the decline in erythropoietic activity is accepted as a physiological adaptation to the greater availability of oxygen after birth. The premature human infant, to which the newborn rat may be more properly compared, continues to exhibit erythropoietin levels appropriate to its anemic state (11).

Rats, like man, modify the affinity of their hemoglobin for oxygen by using 2,3-bisphosphoglycerate. A difference in oxygen affinity of the blood of the SHR could be responsible for tissue anoxia at a site capable of stimulating erythropoietin production. Also, significant tissue anoxia should effect an increase in 2,3-bisphosphoglycerate concentration in

Table 1. Plasma and RBC volumes of rat strains (n = 6 for each strain) at 27 weeks of age

	Volume, ml/100 g (body weight)		
Type	Wistar	WKY	SHR
Plasma	3.40 ± 0.19	3.14 ± 0.23	3.19 ± 0.29
RBC	2.27 ± 0.30	2.06 ± 0.16	2.05 ± 0.26

Data are mean ± SEM.

Table 2. Hemoglobin content in erythrocytes of three strains of rats

Age, weeks	Strain	Hemoglobin	
		g/dl of cells	g per 10^{12} cells
21	Wistar (4)	34.2 ± 0.5	15.1 ± 0.2
	WKY (4)	33.3 ± 0.5	14.6 ± 0.2
	SHR (4)	35.3 ± 0.5	13.3 ± 0.1
23	Wistar (6)	34.2 ± 0.3	15.2 ± 0.1
	WKY (6)	33.2 ± 0.4	14.7 ± 0.2
	SHR (6)	35.3 ± 0.4	13.4 ± 0.2
25	Wistar (6)	33.9 ± 0.4	15.3 ± 0.1
	WKY (6)	33.9 ± 0.3	14.8 ± 0.1
	SHR (6)	35.5 ± 0.2	13.5 ± 0.2
27	Wistar (6)	33.1 ± 0.1	15.2 ± 0.1
	WKY (6)	32.8 ± 0.3	14.8 ± 0.2
	SHR (6)	33.9 ± 0.3	13.2 ± 0.3

Numbers in parentheses are *n* (number of rats used).

whole blood. In the two strains examined, we found no age-related change and no significant difference in P_{50} values measured at 6–8 and 12–15 weeks of age, or for 2,3-bisphosphoglycerate measured at 11 weeks (see *Results*).

Familial erythrocytosis in man is associated with various hemoglobinopathies (12). However, the same P_{50} and 2,3-bisphosphoglycerate values already cited exclude a structural difference in the hemoglobin of the SHR, a difference that might be responsible for a greater oxygen affinity in that strain. Of other possibilities, primary production of excessive erythropoietin by the SHR, although not excluded, is rendered less likely for the rat by Brecher and Stohlman (13) who showed that macrocytosis was the response of this species to excessive erythropoietin stimulation or injection. A remaining possibility is an abnormal sensitivity of the erythropoietic stem cell of SHR to normal levels of erythropoietin.

Microcytosis. The increase in RBC counts (Fig. 2) and the virtually constant hematocrit (Fig. 3) define a decreasing MCV for all strains as they mature and a leveling off as the adult cell size is reached after the 21st week of life (Fig. 4). At this point the MCV is significantly smaller in the SHR than in the normotensive strains. Direct measurements of the cells by electronic sizing are confirmatory of the calculated values (Fig. 4 *Inset*).

Human RBC indices during gestation (8) and after birth (9) document a steady decline in MCV from the 10th week of gestation (when mean values are ≈200 μm^3) to the 12th week of neonatal life when the MCV of 88 ± 7.9 μm^3 (mean ± SD) approximates that of the normal adult. Standard texts of hematology suggest that smaller cells are produced during succeeding stages of gestation and postnatal maturation. Valet *et al.* (14, 15) describe the successive appearance of five populations of erythrocytes in the newborn rat. The first three of these are present at birth and disappear by the third week postpartum, coincident with the appearance of populations IV and V. Population IV disappears by the 12th week and number V constitutes the final adult population. Erythrocytes of populations I through IV show a linear decrease in MCV until their disappearance from the circulation. In population V this decrease begins to level off at weeks 4–5 and there is no further reduction after the 12th week. Our study, which begins at the 7th week of life, must deal almost exclusively with population V and, for the normotensive strains, documents no further decrease in MCV after the 20th week.

The changes depicted in the three indices, RBC count, hematocrit, and MCV (Figs. 2–4), are concurrent, masking the underlying mechanisms. It appears that the hematocrit is closely regulated (Fig. 3), and this constancy requires either a volume decrease of circulating erythrocytes or the production of smaller cells *pari passu* with the rise in their number. Reduction of MCV by the known mechanism of KCl and water loss would in turn require an increase in hemoglobin concentration per cell; but since the mean cell hemoglobin concentration remains constant and comparable in the three strains (Table 2) this is not observed (14, 15).

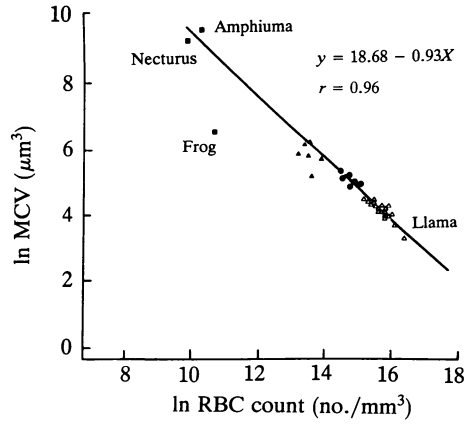

FIG. 5. ln–ln plot of MCV versus RBC counts in 32 species of vertebrates. △, Mammals; ●, fowl; ▲, reptiles; ■, amphibia. The line of regression was calculated for warm-blooded species only.

Regulation of Hematocrit. An inverse relationship between RBC size and numbers is apparent across a large number of animal species. Wintrobe *et al.* (ref. 16; see table B-2) catalog hematologic indices for 33 subprimate vertebrates. In 31 of these, data for RBC count and MCV are given. Data for a number of primates are supplied by Huser (17). By using these data, a plot of RBC against MCV generates a curve in the form $y = 1/x$, containing the inherent corollary that the product of the coordinates (in this case equal to the hematocrit) is a constant. A log–log transformation of the data is displayed in Fig. 5. At the extremes of the calculated line of regression are amphiuma (RBCs, 0.03 × 10^6 cells per μl; MCV, 13,860 μm^3; hematocrit, 40%) and the llama (RBCs, 15.0 × 10^6 cells per μl; MCV, 25 μm^3; hematocrit, 36.9%). For the 22 warm-blooded species the hematocrit values are closely grouped [41.51 ± 6.06% (mean ± SD)] and the mean cell hemoglobin concentration in all species is even more so [30.46 ± 3.25% (mean ± SD)]. The number of RBCs under normal conditions is governed by the oxygen needs of the organism; cell size in the relationship described in Fig. 5 appears to be adjusted to yield a constant hematocrit.

Size of the RBC. The past decade has been notable for a lively interest in the ionic basis of cell volume regulation (18–20). Ionic flux rates across RBC membranes of genetically hypertensive man and rat show alterations from those of normotensive subjects that could affect steady-state cell size (13). One of the most consistent findings is an increased rate constant for both sodium and potassium efflux. The greater part of this increase is in the ouabain-insensitive component of total efflux in both its furosemide-sensitive and furosemide-resistant portions (21, 22). From cross-incubation experiments, Van de Van and Bohr (22) and Feig *et al.* (21) concluded that the elevated flux of Na and K from erythrocytes of SHR was due to an intrinsic difference in the cell membrane rather than to a humoral factor present in plasma. Although Orlov *et al.* (23) found regulatory volume decrease of erythrocytes in response to hypoosmotic challenge to be absent in the rat, ionic fluxes responsible for cell volume maintenance may serve to perpetuate the microcytosis caused by the production of smaller cells in the SHR.

In summary, the early postpartum weeks of the rat's life are marked by active erythropoiesis and a concurrent volume decrease of the maturing erythrocytes. Na^+–K^+ cotransport is known to be increased in RBCs of the SHR and both this

and the leak component of potassium loss may be magnified by a genetic membrane defect in this strain (2, 21, 22, 24, 25) with consequent persistence of microcytosis. All animal species for which data are available appear to regulate RBC size in inverse proportionality to numbers so that packed cell volume is kept within narrow limits (Fig. 5). The exaggerated erythropoiesis of the SHR (Fig. 2) may reflect an increased sensitivity of the stem cell to erythropoietin in this strain. The appearance of progressively smaller RBCs, resulting in MCVs significantly smaller than are found in normal maturation (Fig. 4), seems to be linked to this increase in their number. The sensor and effector mechanisms that accomplish this interrelationship are subjects for future investigation.

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