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The present study was undertaken to standardize the effect of propranolol on normal human red cells and thus establish certain parameters enabling us to evaluate propranolol's effect on pathological cells. Normal human erythrocytes lost 40 mEq. of potassium, decreased the intracellular pH by 0.06 units, and shifted the oxyhemoglobin dissociation curve 6.0 mm. Hg to the right in the presence of propranolol. The series of events and magnitude of the response induced by propranolol was time dependent and sensitive to temperature, pH, drug concentration, and erythrocyte concentration. Calcium was an absolute requirement for maximal propranolol action with simultaneous incorporation of trace amounts of radioactive calcium into the cell. Chelation of calcium with EDTA or EGTA inhibited the response to propranolol.

Several separate groups of investigators have studied the effect of propranolol on normal human erythrocytes. One group used propranolol to protect erythrocytes from hypotonic hemolysis.[1–3] A second group studied its effect on cation permeability.[4–6] A third group worked on the oxyhemoglobin dissociation curve alterations induced by propranolol.[7–10] Although it is also recognized that calcium is required for propranolol to alter cation permeability, its relationship to the over-all events has never been studied.

The simultaneous measurement of osmotic fragility, cation permeability, and the oxyhemoglobin dissociation curve or the sequence of these events has never been reported. In order to study the effect of propranolol on various pathologic red blood cells, it is necessary to standardize its response to normal human red blood cells. The concentration of propranolol for maximum response has been studied by many investigators.[1, 3, 5] The effect of different variables, such as hematocrit, temperature, and pH, on the action of propranolol toward normal human red blood cells has never been documented and is reported in this paper.

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Table I. Effect of propranolol on heparinized whole blood*

Time (min.)	*Na (mEq./LPC)*	*K (mEq./LPC)*	*Mean hemolysis (% NaCl)*	P_{50}† *(mm. Hg)*	ΔpH‡ *(pHp − pHc)*
0	9	103	0.45	25	0.20
5	15	85	0.38	27	0.28
10	20	76	0.33	29	0.36
20	23	70	0.30	30	—
30	25	66	0.26	—	—
60	30	58	0.25	31	—
60§	9	100	0.45	25	0.20

The results are of a single experiment which are representative of at least 10 normal donors obtained from laboratory personnel.
*Whole blood incubated at 37° C., pH 7.4, in propranolol (0.5 mM.) with hematocrit 45 per cent.
‡ΔpH represents the difference between plasma pH (pHp and the intracellular pH (pHc).
†Partial pressure of oxygen at which hemoglobin is 50 per cent saturated.
§EGTA (2.0 mM.) added at beginning of incubation.

Materials and methods

Blood was collected in heparinized vacutainers. Whole blood or washed red blood cells were incubated with the drug at 37° C. in a shaking water bath, maintaining a constant pH of 7.4, with adequate gas equilibration of Po_2 and Pco_2.

Red blood cells were washed three times with a Krebs-Ringer bicarbonate buffer modified by removal of calcium chloride from the formula. In the experiment with stored blood, whole blood was stored at 4° C. for 3 weeks in acid-citrate-dextrose (ACD) solution (7.3 Gm. of citrate, 22 mg. of trisodium citrate dihydrate, and 14.7 Gm. of dextrose per liter) and then washed with the above Krebs-Ringer buffer.

Osmotic fragility was performed on the incubated samples periodically by the standard multiple tube method of Dacie and Lewis.[11] The degree of hemolysis in each tube was expressed as a percentage of total hemolysis. The mean hemolysis per cent was the salt concentration in which 50 per cent of the cells hemolyze.

Intracellular cation content was measured with a flame photometer on the cells washed three times with 0.11M magnesium chloride and expressed as milliequivalents of cation per liter of packed red cells (LPC). Extracellular cation content was measured with a flame photometer directly on the clear suspension after separation by centrifugation. D,L-Propranolol (1[isopropylamino]-3-1-naphthyloxyl-2-propranolol hydrochloride), ouabain, dibutryl-cyclic adenosine monophosphate (AMP) and epinephrine were obtained from the Sigma Chemical Co. (St. Louis, Mo.). These compounds were dissolved in either isotonic sodium chloride or modified Krebs-Ringer buffer before addition to the incubation suspension. EGTA [ethylene-bis-(oxyethylenenitrilo)-tetra-acetic acid] and other chemicals were purchased from Fisher Scientific Co. (Pittsburgh, Pa.).

RBC counts (RBC) were performed on the Model S Coulter counter. The mean corpuscular erythrocyte volume (MCV) was calculated by dividing the hand hematocrit by the RBC count. Oxygen-hemoglobin affinity was quantitated by the P_{50}, the partial pressure of O_2 in millimeters of mercury corresponding to 50 per cent oxyhemoglobin saturation at pH 7.40 and 37° C.

The Po_2 was corrected for the Bohr effect to a pH of 7.40 by employing the Sevringhaus nomogram as previously described[12]

The P_{50} (7.40 pH) was then determined graphically from a log-log cycle plot of the three corrected Po_2-oxyhemoglobin saturation points employing 11, 21, and 36 mm. Hg, respectively for oxygen tension (Po_2) with a constant 40 mm. Hg for CO_2 (Pco_2). The intracellular pH was measured on red cell lysate after freezing and thawing, in an Instrumentation Laboratory Model 113 pH meter at 37° C.

Influx of radiocalcium. ^{45}Ca from New England Nuclear Co. (Boston, Mass.), $CaCl_2$ in 0.5N HCl, specific activity ranging from 6.1 to 14.5 mCi. per milligram was added to 20 ± 1 per cent red cell suspensions in plasma to adjust specific activity of plasma calcium to 0.02 to 0.03 mCi. per milligram. The suspensions were equilibrated under sterile conditions with a prewarmed humidified gas mixture

Table II. Effect of propranolol on washed RBC's*

Time (min.)	*$CaCl_2$ ($mM \cdot$)^2*	*EGTA ($mM \cdot$)*	*Na (mEq./LPC)*	*K (mEq./LPC)*	*Mean hemolysis (% NaCl)*	*P_{50} (mm.Hg)*
0	—	—	10	100	0.45	25
10	2.0	—	15	72	0.35	29
20	2.0	—	22	64	0.26	—
30	2.0	—	27	59	0.25	30
30	2.0	2.0	10	100	0.45	25
60	2.0	—	15	59	0.25	30
60†	2.0	—	10	74	—	—
60	2.0	2.0	10	100	0.45	25
60	—	—	10	100	0.45	25

*Washed red blood cells suspended in modified Krebs-Ringer bicarbonate at 37° C., pH 7.4, in DL-propranolol 0.5 mM. with a hematocrit of 45 per cent.

†Washed red blood cells suspended in modified Krebs-Ringer bicarbonate at 37° C. but equilibrated in a higher CO_2 gas equilibration to achieve a pH of 7.0 in propranolol concentration of 0.5 mM. and final hematocrit of 45 per cent.

Table III. Effect of propranolol on washed RBC's at 25° C.*

Time (min.)	*K (mEq./LPC)*	*Na (mEq./LPC)*	*K susp. (mEq./L.)*	*Mean hemolysis (% NaCl)*
1	105	8	5	0.47
10	88	12	15	0.35
20	80	18	18	0.32
30	71	19	21	0.30
60	60	24	24	0.27

*Washed red blood cells suspended in modified Krebs-Ringer bicarbonate at 25° C., pH 7.4, in DL-propranolol 0.5 mM. with a hematocrit of 40 per cent. The calcium chloride concentration was 2.0 mM.

containing 95 per cent air–5 per cent CO_2 for 5 minutes at 37° C. and incubated in a water bath under constant shaking at 120 c.p.s.

At times indicated, samples were taken and cooled to 0° C. and red cells isolated by centrifugation at 40,000 × g for 2 minutes. The cells were then washed four times with 10 volumes of ice-cold isotonic saline solution and centrifuged (40,000 × g, 10 minutes, 0° C.) on a Sorvall BC-5 high-speed refrigerated centrifuge. The remaining layer of leukocytes and platelets was carefully removed after each washing.

Red cells were solubilized and decolorized as follows: 0.1 ml. of washed red cells was transferred by SMI micropettors into 20 ml. liquid scintillation glass vials containing 0.5 ml. of 70 per cent $HCLO_4$ and 0.3 ml. of 30 per cent H_2O_2 and incubated at 37° C. for 2 hours. Liquid scintillation cocktail (Aquasol; New England Nuclear) was added to a final volume of 10 ml. and samples were counted in a Packard Tricarb liquid scintillation counter (Model 3375) with an external standard of ^{45}Ca. The counting efficiency was in the range of 72 to 95 per cent. A quench curve was prepared by adding variable volumes of solubilized red cells to the above liquid scintillation cocktail, containing standard amounts of ^{45}Ca. Within the above range of efficiencies, this curve approached a straight line.

Determination of red cell calcium content. Red cell and plasma calcium was determined by atomic absorption spectrophotometry (AAS) of ethanol-HCl extracts of dry-ashed red cells with a single-beam atomic absorption spectrophotometer (Varian Techtron Model AA-5), nitrous oxide–acetyl flame, and a hollow cathode lamp for calcium, as described by Harrison and Long.[13]

Determination of plasma trapping by washed red cells. Since red cells have a very low calcium content and take up only small amounts of ^{45}Ca, we have investigated as to whether differences in ^{45}Ca uptake were not related to differences in trapping of washed red cells by residual plasma. An 0.1 ml. quantity of 5 per cent albumin labeled by ^{125}I (specific activity, 25 μCi per milligram of albumin)

Table IV. Effect of hematocrit on potassium loss*

Preincubation Hct. (%)	*Postincubation*		
	K (mEq./LPC)	*K (mEq./L. suspension†)*	*Cell: suspension ratio‡*
60	79	39	1.8
40	66	28	1.0
30	60	26	0.6
20	44	20	0.4

*Normal washed red cells were incubated for 1 hour with 0.2 mM. propranolol and 2.0 mM. Ca^{++}, hematocrits were adjusted to clinical level with Krebs-Ringer bicarbonate buffer, pH 7.4.

†Results are expressed in milliequivalents per liter of suspension medium after the cells were removed by centrifugation.

‡The ratio is the amount in milliequivalents intracellularly divided by the amount of potassium extracellularly in 1 ml. of cell suspension, calculated using the hematocrit at the end of the incubation and the results from columns 1 and 2. The original cell: suspension ratio of potassium prior to incubation with propranolol was 30 when the hematocrit was 60 per cent.

was added to 10 ml. of red cell suspension in plasma, the hematocrit of which had been adjusted to 20 ± 1. An appropriate volume of plasma was taken to count the radioactivity in a Beckman gamma scintillation counter. Cells were washed as outlined above and their radioactivity was counted.

$$\text{Plasma trapped by washed red cells (\%)} = \frac{\text{d.p.m./ml. washed red cells}}{\text{d.p.m./ml. plasma}} \times 100$$

Results

Whole blood. After the addition of propranolol (0.5 mM.) to heparinized whole blood, the hematocrit and mean corpuscular volume decreased approximately 20 per cent and the cells became resistant to osmotic lysis with a shift of the mean hemolytic salt concentration from 0.45 to 0.25 per cent. The shape of the osmotic fragility curve was not altered but just displaced. The cells lost over 40 mEq. of potassium per liter of packed cells (LPC) with a smaller gain in sodium ions. P_{50} shifted to the right with an increase of 6 mm. Hg over the normal controls.

The initial pH of the plasma was 7.40 and increased to 7.50 and the intracellular pH decreased from 7.20 to 7.14 with a net change of 0.36 units (Table I). These events occurred simultaneously and rapidly with exponential kinetics and remained constant for several hours thereafter. Blood collected in EDTA vacutainers did not respond to propranolol. The addition of EGTA to the heparinized whole blood also inhibited all three events, suggesting a role of calcium in the series of events (Table I).

Washed red blood cells. Red blood cells washed in modified Krebs-Ringer bicarbonate buffer did not respond to propranolol without $CaCl_2$ present (Table II). EGTA added prior to incubation inhibited all three events similar to that observed with whole blood incubation. The kinetics of potassium loss and the final equilibrium achieved were similar in both washed red cells or whole blood. Osmotic fragility and P_{50} changes occurred simultaneously and were similar in both washed red cells or whole blood. Inhibition of the net potassium loss and sodium gain was observed at pH 7.0 (Table II) as others have observed.[5]

Temperature. A decrease in the temperature decreased the rate of potassium loss, but not the final equilibrium (Table III). The initial rate of net potassium loss was 120 mEq. liter of packed cells per hour at 37° C. compared to 60 mEq. liter of packed cells per hour at 25° C. Potassium loss and osmotic fragility changes were parallel at the lower temperature.

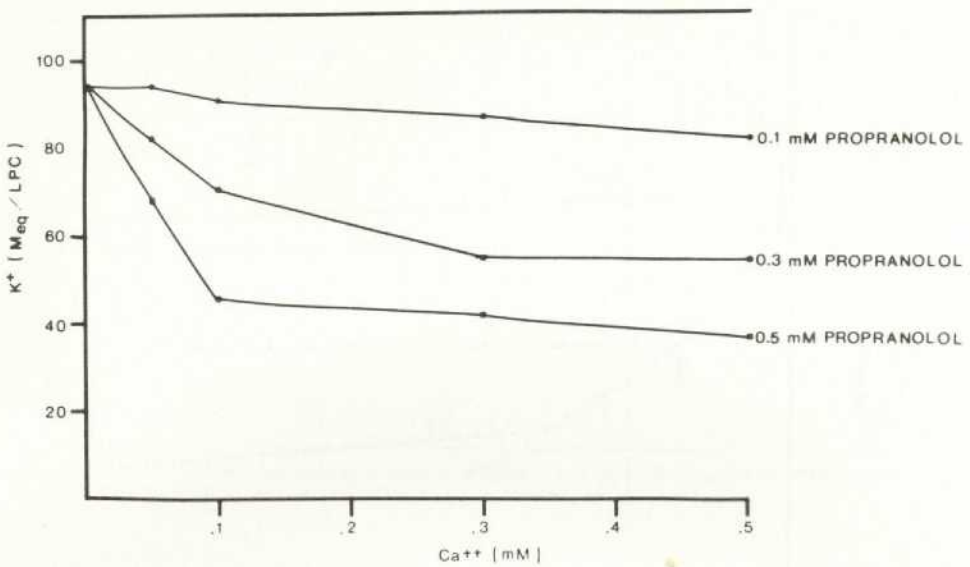

Fig. 1. Normal washed RBC's adjusted to a hematocrit of 20 per cent with Krebs-Ringer bicarbonate, pH 7.4, incubated at 37° C. for 1 hour with fixed DL-propranolol concentrations and varying Ca^{++} concentrations.

Table V. Compounds that inhibit the effect of propranolol*

	Addition (mM ·)	*% saline for 50% hemolysis*
Normal control	—	0.45
Propranolol control†	—	0.25
EGTA	2.0	0.40
KCl	10.0	0.30
KCl	24.0	0.38
$MgCl_2$	10.0	0.32
M Blue	2.0	0.41
DNFB	3.0	0.35

*Normal washed RBC's adjusted to hematocrit of 50 per cent with modified Krebs-Ringer bicarbonate. Incubated at 37° C., pH 7.4, for 60 minutes containing DL-propranolol 0.5 mM. and $CaCl_2$ 2.0 mM. with various additives.

†Propranolol control contained DL-propranolol 0.5 mM. and $CaCl_2$ 2.0 mM. only.

Hematocrit. A change in the packed red cell volume of the incubation suspension altered the response of potassium loss from propranolol. The equilibrium of potassium between cell and suspension medium was effected by the hematocrit level. The lower the hematocrit, the greater was the shift of net potassium from cell to suspension medium (Table IV). The high hematocrit with more total potassium available in the system achieves the highest concentration of potassium in the medium. The loss of potassium from the cell in low hematocrit suspension was responsive to both calcium and propranolol concentrations (Fig. 1).

Inhibition. Several compounds prevented the osmotic fragility changes from occurring in the presence of propranolol, including extracellular potassium itself (Table V). Divalent cation chelators, EGTA and EDTA, inhibited net potassium loss and were com-

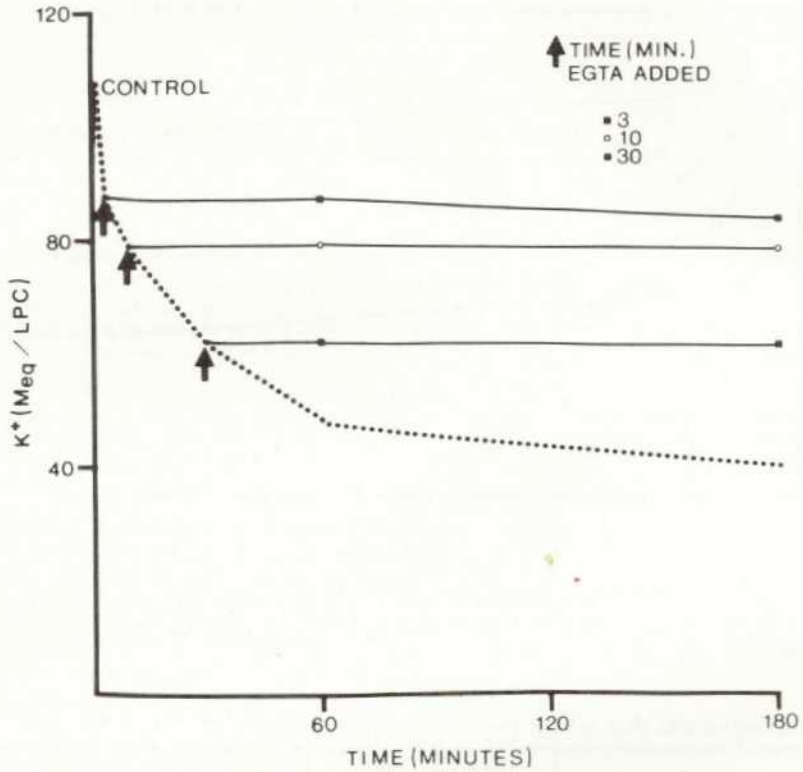

Fig. 2. Normal washed RBC's adjusted to a hematocrit of 20 per cent with Krebs bicarbonate, pH 7.4, incubated at 37° C. with 0.3 mM. DL-propranolol and 0.8 mM. Ca^{++}. EGTA (1.0 mM.) was added at various times as indicated.

petitive with the $CaCl_2$ concentration (Tables I and II). The addition of EGTA at any time during the reaction prevented any further loss of potassium (Fig. 2), indicating a role of calcium throughout the incubation period. Similar results were observed when the cells were washed free of external calcium chloride and allowed to continue incubation in the presence of propranolol alone.

Methylene blue (2.0 mM.) inhibited net potassium loss and was competitive with $CaCl_2$ concentration (Table VI).

Ouabain, furosemide, epinephrine, phentolamine, and dibutryl-cyclic AMP did not inhibit the reaction over a wide range of concentrations (10^{-3} to 10^{-6}M).

Stored blood. Whole blood stored in ACD for 3 weeks lost a major portion of its organic phosphate content and contained less than 1 per cent of its original adenosine triphosphate (ATP) level. Whole blood in contrast to fresh blood contained an increased level of sodium and a decreased content of potassium inside the cell. In spite of this alteration in cation ratio and energy potential, stored blood responded to propranolol. Stored blood gained a small amount of sodium but lost significant amounts of potassium with simultaneous alteration in the osmotic fragility curve. Stored blood was also sensitive to 0.1 mM. propranolol concentration (Table VII).

Influx of radiocalcium. Fig. 3 depicts the influx of ^{45}Ca into red cells during incubation of 20 per cent red cell suspensions in plasma. During the first 4 hours of incubation, there was a small reproducible rise in the red cell Ca^{45}:external Ca^{45} ratio (RBC

Table VI. Effect of Ca^{++} on methylene blue inhibition of K loss*

Hematocrit %	*Propranolol (mM ·)*	*$CaCl_2$*	*Methylene blue (mM ·)*	*% NaCl for 50% hemolysis*	*K (mEq./LPC)*	*Na (mEq./LPC)*
45	0.5	2.0	—	0.25	62.9	17.1
	0.5	2.0	2.0	0.40	82.1	12.8
	—	—	2.0	0.45	97.1	8.8
	0.5	2.0	3.0	0.43	87.2	12.8
17	—	—	2.0	0.45	97.8	8.8
	0.5	2.0	—	—	37.0	50.0
	0.5	2.0	2.0	—	67.0	28.0
	0.5	4.0	2.0	—	53.0	14.0
	0.5	8.0	2.0	—	37.0	16.0

*Normal washed RBC's adjusted to hematocrit of 20 per cent with Krebs-Ringer bicarbonate buffer, pH 7.4, incubated with 0.5 mM. propranolol, 2.0 mM. methylene blue, and varying CA^{++}.

Table VII. Effect of propranolol on stored blood*

Time (min.)	*Propranolol (mM ·)*	*$CaCl_2$ (mM ·)*	*Na (mEq./LPC)*	*K (mEq./LPC)*	*Mean hemolysis (% NaCl)*
Exp. I:					
20	—	—	38	61	0.58
20	—	—	38	66	0.57
20	0.5	2.0	45	42	0.44
Exp. II:					
20	—	—	45	62	0.56
20	0.1	2.0	48	53	0.51
20	0.2	2.0	47	47	0.48

*Three week stored ACD blood washed in Krebs-Ringer bicarbonate and incubated at 37° C., pH 7.4, with a hematocrit of 40 per cent. The results were obtained from two different experiments from separate stored whole blood samples.

^{45}Ca:ext ^{45}Ca) which subsequently remained unchanged until red cell ATP decreased below 15 to 20 per cent of the initial level. The net red cell calcium content as determined by atomic absorption spectrophotometry (AAS) was 18 ± 4 μmoles per liter of cells and remained unchanged after 4 hours of incubation (17 ± 5 μmoles per liter). The specific activity of the red cell calcium at 4 hours of incubation (17 ± 5 μmoles per liter). The specific activity of the red cell calcium at 4 hours of incubation was 17 per cent of the specific activity of plasma calcium. These data indicate that the slow initial rise in radiocalcium resulted from an influx of calcium into cells associated with an exchange of radiocalcium with an exchangeable red cell calcium pool which represented approximately 17 per cent of the total red cell calcium.

Red cell suspensions incubated with propranolol (0.5 mM.) differed from control red cells in that a rapid rise of the RBC ^{45}Ca:ext ^{45}Ca ratio occurred within the first 10 minutes of incubation, indicating an increased influx of calcium into cells. No further increase in radioactivity occurred during the subsequent 4 hours of incubation. The net red cell calcium content of propranolol-treated cells was unchanged after 4 hours of incubation (18 ± 6 μmoles per liter). The specific activity of calcium in propranolol-treated cells at 4 hours was higher than the specific activity of control red cells (25 per cent of the specific

The loss of potassium would result in a loss of water and a decrease in the mean corpuscular volume. This results in a simultaneous decrease in osmotic fragility.[15]

The oxyhemoglobin affinity changes that occur simultaneously may be explained by the increase of hydrogen ion concentration inside the cell.[9]

The effect of propranolol on cation permeability does not appear to be mediated via an active cation transport system. Its action was not altered by ouabain, an inhibitor of active cation transport, nor was ATP required for its action, since stored blood still responded to propranolol.

Most compounds that can selectively increase potassium permeability in the red blood cell require calcium for their action.[14]

Chelation of divalent cations by EGTA or EDTA caused marked inhibition of the propranolol effect. The inhibition of potassium loss by EGTA was immediate in its action and could be produced at any time during the incubation.

Radiocalcium studies indicate an increase in the exchangeable fraction of cellular calcium upon addition of propranolol as concluded from an increased uptake of radiocalcium and unchanged net cellular calcium content. However, because of the limited sensitivity in the determination of net cellular calcium content, we are unable to determine if propranolol increases or exchanges a small calcium pool.

The role of membrane-bound calcium vs. ionized intracellular calcium in potassium permeability, however, is still unknown. Calcium alone can induce potassium permeability changes in energy-depleted red blood cells or ghosts.[17, 18]

Chlorpromazine, a lipid-soluble anesthetic that protects erythrocytes from hypotonic hemolysis, has been shown to displace membrane-bound Ca^{++}.[19] Propranolol may also displace or exchange calcium in the membrane in a similar manner.

Our studies also indicate that active calcium is required throughout the incubation since chelation of calcium at any time will interrupt the process.

The equilibrium achieved between potassium inside the cell and outside was effected by the hematocrit of the suspension and the total available potassium in the system. The equilibrium of potassium between cell and suspension was close to 1.0 in high-hematocrit suspensions with 0.5 mM. propranolol, as others have observed.[5] In the low-hematocrit systems, however, more potassium came out of the cells and achieved a lower equilibrium with the medium. The hematocrit and total available potassium are important variables in assessing the response of propranolol.

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