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*J Clin Invest.* 1969;48(4):674-684. <https://doi.org/10.1172/JCI106025>.

### Research Article

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Active sodium outflux rate constants were depressed in animals with malaria ( $0.202 \pm 0.012$ ), as compared to controls ( $0.325 \pm 0.027$ ). Passive sodium influx rate constants were higher in infected monkeys ( $0.028 \pm 0.002$ ) than in control animals ( $0.019 \pm 0.002$ ). The cross incubation of malarial plasma with normal red blood cells induced a 22% diminution in active sodium outflux but no changes were observed in sodium influx.

It is concluded that malaria alters erythrocytic sodium transport in all erythrocytes. The elevated intracellular sodium concentration is the net result of decreased sodium outflux and increased sodium influx. The plasmodium organism or the affected host may produce a circulating substance that is deleterious to erythrocytic membrane cation transport.

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# Alterations of Red Blood Cell Sodium Transport during Malarial Infection

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**ABSTRACT** Previous studies have suggested that malaria induces changes in erythrocytic membrane permeability and susceptibility to osmotic lysis. The present study investigated erythrocytic transport of sodium with cells from Rhesus monkeys infected with *Plasmodium knowlesi*. Red blood cell sodium concentration was significantly elevated in 37 parasitized animals ( $21.8 \pm 1.2$  mM; mean  $\pm$  SEM), as compared to 23 control animals ( $10.0 \pm 0.38$  mM). The cellular sodium increased with the density of parasitemia and the cellular potassium decreased in proportion to the elevation of sodium. Nonparasitized as well as parasitized erythrocytes possessed this abnormality of cation metabolism. Effective chloroquine therapy reversed the changes over a period of 4 days.

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This work was presented in part before a joint meeting of the American Federation of Clinical Research and The American Society of Clinical Investigation, Inc., 5 May 1968. An abstract of this work appeared in *Clin. Res.* 1968. 16: 382.

This paper is contribution No. 479 from the Army Research Program in Malaria.

Received for publication 20 September 1968 and in revised form 11 December 1968.

## INTRODUCTION

The normal erythrocyte of most animal species, including man, is known to transport sodium and potassium against their electrochemical gradients, thereby maintaining an intracellular environment low in sodium and high in potassium. The transport of these cations has been studied in a variety of human illnesses, such as hereditary spherocytosis (1, 2) and sickle-cell anemia (3), as well as nonhematologic diseases, such as severe uremia (4), disseminated neoplastic disorders (5), and extensive burns (5). Overman (6) studied the effects of *Plasmodium knowlesi* malaria upon the erythrocytic cation concentration in Rhesus monkeys. He found that red blood cell sodium increased and red blood cell potassium decreased as the parasitemia increased, and postulated the existence of a circulating toxin that increased membrane permeability. Other investigators have postulated that a circulating factor may be present in malarial plasma, since the rate of hemolysis exceeds that which would be expected from the number of parasitized erythrocytes (7) and the osmotic fragility of nonparasitized cells is abnormal (8). Normal erythrocytes show a markedly shortened life span after transfusion into a malarious recipient (9, 10). On the other hand antibodies cannot be demonstrated on the surfaces of red blood cells from malarious animals (11).

The present studies were performed to make detailed observations of erythrocytic cation transport in malarious blood and to ascertain if circulating factors played a role in the etiology of the observed red blood cell cation transport defects.

## METHODS

The experimental malarial infections were induced with the following species of parasites and hosts: *Plasmodium knowlesi* and *P. coatneyi* (Rhesus monkey), *P. falciparum* (splenectomized chimpanzee), and *P. berghei* (hamster). The principles of laboratory animal care as promulgated by the

National Society for Medical Research were observed. Before venipuncture, the primates were tranquilized with either Sernylan<sup>1</sup> (phencyclidine hydrochloride) or Innovar<sup>2</sup> (droperidol). Quantitative parasite counts were done on thin smears of whole blood stained with Leishman-Giesma stain; 1000 erythrocytes were counted and the density of parasitemia was expressed as the per cent of erythrocytes parasitized.

**Solutions: red blood cell sodium and potassium.** All solutions were made up to an osmolality of 295 mOsm. The buffer used was glycylglycine (270 mM)-MgCO<sub>3</sub> (44 mM) adjusted to pH 7.4 at 37°C. Unless otherwise stated in the text, the extracellular medium for the outflux and influx experiments contained the following solute concentrations: 142 mM sodium; 5.0 mM potassium; buffer 10% v/v; 1.2 mM phosphate; glucose, from 10 to 11 mM; and albumin 0.1 g/100 ml. Red blood cell sodium (Na<sub>c</sub>) and potassium (K<sub>c</sub>) concentrations were determined on heparinized cells after a triple washing with buffered isosmotic MgCl<sub>2</sub>. The cells were then lysed with deionized, distilled water and analyzed on a lithium internal standard flame photometer. Intracellular cation concentration was calculated either by using the method of Sachs and Welt (12) or by hemolyzing a known volume of pipetted cells as determined by microhematocrit. These two methods showed close agreement.

**Separation of parasitized cells and parasites.** Infected whole blood (*P. knowlesi*, mature forms) was centrifuged at 2000 g for 30 min. The less dense parasitized erythrocytes form the upper layers of the column of blood. The upper- and lowermost 10% (by volume) of cells were removed for analysis of cations; comparison of the density of parasitemia in these layers provided an estimate of the degree of separation.

Parasites were separated from their host-cells with an Aminco-French pressure cell (13). The red blood cells were ruptured at 1500-2000 psi and the parasites were separated, washed with MgCl<sub>2</sub>, and finally fragmented at 18,000-20,000 psi. Total Na content of the parasites could then be determined.

**Sodium outflux.** The sodium outflux studies were conducted using the method of Sachs and Welt (12). This technique measures the rate of appearance of intracellular <sup>24</sup>sodium or <sup>22</sup>sodium from previously labeled cells into a buffered extracellular solution at 20 min intervals over a 60 min sampling period. A correction for hemolysis was applied if hemolysis in the in vitro system exceeded 1%; the experiment was discarded if hemolysis exceeded 3-5%. The components of the transport system for sodium were examined through the use of ouabain (strophanthin-G), 1 × 10<sup>-4</sup> M, and ethacrynic acid [2-3 dichloro-4-(2-methylene butyl)phenoxyacetic acid],<sup>3</sup> 1 × 10<sup>-3</sup> M. All studies utilized paired normal control red blood cells which were handled identically to the cells from the malarious monkeys and were studied simultaneously. The outflux rate constant for sodium (<sup>0</sup>k<sub>Na</sub>) expresses the portion of intracellular sodium extruded per hr. This rate constant is calculated according to the method of Hoffman as described by Sachs and Welt (12). The basic assumption underlying this method is that Na outflux is a first order process so that,

$$\frac{dNa_c^*}{dt} = {}^0k_{Na}Na_c^*,$$

where Na<sub>c</sub>\* is the radioactivity of the cells expressed in cpm/ml of cells. Determinations of radioactivity were made with a well-type gamma counting system. Total sodium outflux (<sup>0</sup>M<sub>Na</sub>) is calculated from the relationship

$${}^0M_{Na} = Na_c^*k_{Na}$$

and is expressed in mmoles of sodium/liter of cells per hr. In accordance with the suggestion of Hoffman and Kregenow (14), pump I was defined as that component of active sodium outflux inhibited by ouabain, and pump II as a smaller fraction of active Na outflux which was further inhibited by the addition of ethacrynic acid to ouabain. The Na<sub>c</sub> was experimentally increased and the K<sub>c</sub> decreased in normal simian erythrocytes through a 24 hr incubation in a shaker bath at 37.5°C. The removal of extracellular potassium and manipulation of extracellular sodium from 100 to 140 mM allowed the cells to accumulate varying amounts of intracellular sodium. MgCl<sub>2</sub> was used as a counter ion for Na to maintain the osmolality of the incubation solution at 295 mOsm. The incubation solutions contained glycyl glycine buffer, phosphate, glucose, and albumin as described earlier. Inosine (10 mM) and adenine (3 mM) were added to help maintain ATP stores; streptomycin was used to inhibit bacterial growth. This extracellular medium was changed after 8 hr. After the incubation, the cells were loaded with radiosodium and outflux studies were done in identical fashion to those studies just described.

**Sodium influx.** Sodium influx experiments also utilized heparinized blood, washed triply with 295 mOsm sodium chloride solution. These cells were then added to flux solutions identical to those described for outflux, so that the final hematocrit was 3-5%. Approximately 10 μc of <sup>22</sup>sodium were added to each influx flask. These cells were sampled at 30, 60, and 90 min and 8 ml of the suspension was immediately iced, centrifuged at 0-5°C, and washed triply with iced MgCl<sub>2</sub> solution. The washed cells and the original extracellular solution were then analyzed for radioactivity.

All influx experiments on erythrocytes from malarious monkeys were accompanied by concomitant study of normal control erythrocytes, and by sodium outflux measurements on the same cells. All values of measured sodium influx were then corrected for sodium outflux in order to compensate for the loss of <sup>22</sup>sodium from the cells.

The corrected sodium influx was calculated according to the method of Sachs and Conrad (15) from the following relationship:

$$\frac{dNa_c^*}{dt} = {}^1k_{Na}Na_c^* - {}^0k_{Na}Na_c^*$$

where Na<sub>c</sub>\* is the concentration of radioactive sodium in the cells, <sup>1</sup>k<sub>Na</sub> is the influx rate constant for sodium, Na<sub>o</sub>\* is the concentration of radioactive sodium in the supernatant or extracellular fluid and <sup>0</sup>k<sub>Na</sub> is the outflux rate constant for sodium. Total sodium influx (<sup>1</sup>M<sub>Na</sub>) is expressed in mmoles of sodium transported/liter of cells per hr and is calculated from the relationship

$${}^1M_{Na} = Na_o^*k_{Na}$$

where Na<sub>o</sub> is the concentration of nonisotopic sodium in the extracellular fluid. All outflux and influx measurements were made in duplicate.

**Cross-incubation technique.** The cross-incubation experiments were designed to study the effect of cell-free heparinized malarial plasma, obtained from monkeys with parasitemias of approximately 50% (*P. knowlesi*), upon normal red blood cells. The control plasma was obtained from a

<sup>1</sup> Parke, Davis & Co., Detroit, Mich.

<sup>2</sup> McNeil Pharmaceuticals, Ft. Washington, Pa.

<sup>3</sup> Merck, Sharpe & Dohme Research Laboratories, West Point, Pa.

TABLE I  
Red Blood Cell Sodium ( $Na_e$ ) and Potassium ( $K_e$ ) in the Rhesus Monkey Infected with *Plasmodium knowlesi*

	$Na_e^*$	$K_e^*$
Control monkeys (23)	10.0 $\pm$ 0.38	102.1 $\pm$ 1.2
Infected monkeys (37)†	21.8 $\pm$ 1.2	92.4 $\pm$ 1.5
	$P < 0.001$	$P < 0.001$

\* Expressed in mmoles/liter of cells  $\pm$ SEM.

† Mean parasitemia, 25%.

monkey other than the donor source of normal erythrocytes. These normal erythrocytes were collected, separated, and washed as described for the flux studies. These cells were then separated into two fractions and 8 ml of cells was incubated for 22 hr with the control and experimental plasmas. The incubation mixture contained: erythrocytes, 5 ml; plasma, 50 ml; glycyl glycine buffer, 10 ml; glucose, 20 mM; phosphate 2.0 mM; inosine 10 mM; adenine, 3 mM; penicillin, 100,000 U, and streptomycin, 100 mg. After the 22-hr incubation, the cells were separated, washed with 295 mOsm NaCl solution and subjected to outflux and influx experiments as already described.

## RESULTS

*Red blood cell sodium and potassium during malarial infection.* Red blood cells from 23 normal Rhesus monkeys contained a sodium concentration ( $Na_e$ ) of 10.0  $\pm$  0.38 mmoles/liter of cells  $\pm$ SEM and a potassium concentration ( $K_e$ ) of 102.1  $\pm$  1.2 mmoles/liter of cells. Erythrocytes from 37 malarious monkeys, with a mean parasitemia of 25%, contained significantly more sodium, 21.8  $\pm$  1.2 mmoles/liter of cells, and less potassium, 92.4  $\pm$  1.5 mmoles/liter of cells ( $P < 0.001$ , Table I). It was

generally observed that the cellular potassium decreased as the cellular sodium increased; however, the earliest changes in erythrocytic cation composition could be detected through measurement of  $Na_e$  since small sodium increments caused a greater percentage change of the measured sodium concentrations than did equimolar potassium decrements on the potassium concentration. Table II shows the values for  $Na_e$  and  $K_e$  in different animal species infected with different malarial strains. The study of splenectomized chimpanzees, infected with human *P. falciparum* malaria showed similar changes of  $Na_e$ , which rose to levels as high as 42 mM, and  $K_e$ , which fell to levels as low as 67 mM, as the parasitemia increased to 35%. Additional experiments utilized *P. coatneyi* infections of Rhesus monkeys; the results were qualitatively similar to those described for the other malarial strains (Table II). The density of parasitemia rose most slowly with *P. coatneyi*, requiring approximately 14 days for peak parasitemias (always less than 10%). The  $Na_e$  was highest for this strain at any given level of parasitemia with  $Na_e$  as high as 26 mM when only 2% of circulating erythrocytes were parasitized. *P. berghei* infections in hamsters provided the smallest quantitative changes of  $Na_e$ ; although  $Na_e$  doubled, the absolute increase was only 3–6 mM since the normal values are low as compared to primates. Fig. 1 depicts a plot of  $Na_e$  on the ordinate and per cent parasitemia with *P. knowlesi* on the abscissa. It can be seen that  $Na_e$  increased as the density of parasitemia increased and the highest value of 44 mM was found with 90% parasitization of circulating erythrocytes. Detectable elevations of  $Na_e$  were found with parasitemias of less than 5%. Red blood cell potassium ( $K_e$ ) diminished in equimolar proportion to the increase in  $Na_e$  and was found to be

TABLE II  
Effect of Different Malarial Species on Different Hosts: Red Blood Cell Sodium and Potassium

Type of infection	Host animal	Per cent parasitemia (range)	$Na_e^*$		$K_e^\ddagger$	
			Normal	Malarious	Normal	Malarious
			mmoles/liter		mmoles/liter	
			(8)§	(10)	(7)	(9)
<i>P. falciparum</i>	Chimpanzee	1–40%	18.2 $\pm$ 1.1	30.4 $\pm$ 2.1	88.7 $\pm$ 3.9	74.7 $\pm$ 2.1¶
			(23)	(22)	(23)	(22)
<i>P. coatneyi</i>	Rhesus monkey	0.1–8%	10.0 $\pm$ 0.38	21.4 $\pm$ 0.38	102.1 $\pm$ 1.2	97.9 $\pm$ 0.79¶
			(8)	(4)	(8)	(4)
<i>P. berghei</i>	Hamster	26–32%	3.2 $\pm$ 0.06	7.8 $\pm$ 0.54	109.4 $\pm$ 1.63	110.3 $\pm$ 3.36

\*  $Na_e$  = red blood cell sodium; mean  $\pm$ SEM.

†  $K_e$  = red blood cell K; mean  $\pm$ SEM.

§ No. of observations.

|| Difference significant with  $P < 0.001$ .

¶ = Difference significant with  $P < 0.01$ .

as low as 65 mM (normal = 102 mM) in terminally ill animals.

Although the magnitude of the increase in  $\text{Na}_e$  exceeded that which could be predicted if only the parasitized cells were abnormal, the erythrocyte population was separated by centrifugation into nonparasitized and parasitized cells, and the cation concentration of these cell groups was measured. Parasite counts on these populations of cells indicated the efficiency or degree of separation of the parasitized from the nonparasitized cells. Table III shows results which are representative of three experiments. Although a 12-fold change in concentration of parasitized cells was achieved, no differences in  $\text{Na}_e$  were found. It was therefore concluded that all circulating erythrocytes, parasitized and nonparasitized alike, shared the abnormality of sodium transport.

Measurements of sodium in the parasites, after they were separated from the erythrocytes in an Aminco-French pressure cell, showed that the parasites contributed no more than 1% of the total sodium content of a liter of cells which were 50% parasitized.

*Effects of chloroquine therapy.* The effects of antimalarial therapy on erythrocytic sodium and potassium in a representative (three experiments) monkey infected with *Plasmodium knowlesi* are shown in Fig. 2. The density of parasitemia increased rapidly to 45% on day 5 after inoculation and decreased immediately after the

TABLE III  
Red Blood Cell Sodium ( $\text{Na}_e$ ) after Concentration of Parasitized Erythrocytes

	$\text{Na}_e$ *	% Parasites†
Whole blood	27.6	18
Top layer	27.0	20
Bottom layer	28.2	1.6

\* Expressed in mmoles/liter of cells.

† Per cent of red blood cells parasitized.

onset of chloroquine treatment. As the density of parasitemia rises, the cellular sodium is tripled and the cellular potassium falls in equimolar proportion. Although the parasites disappear quickly after chloroquine therapy, the cellular cation changes return to normal more slowly and the red blood cell sodium is not normal until 2-4 days after the circulating parasites have disappeared. The cellular potassium concentration seems to rise to normal levels 1-2 days before the cellular sodium returns to its normal concentration. These data suggest that the parasite induces a membrane alteration that is slowly reversible and this membrane-defect can temporarily persist despite the absence of the inciting agent, the parasite.

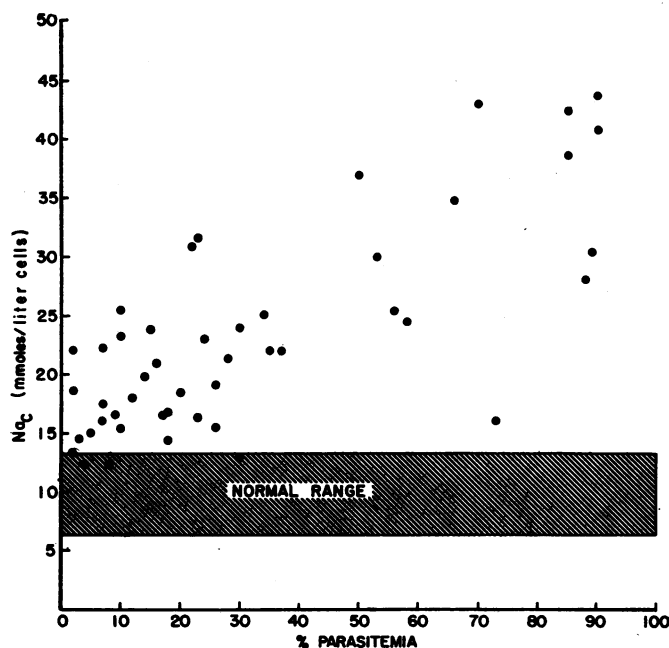


FIGURE 1 Relation of red blood cell sodium concentration ( $\text{Na}_e$ ) to the per cent of cells parasitized with *P. knowlesi*. The normal range of  $\text{Na}_e$  in 23 control monkeys is indicated within the cross-hatched area. No increase of  $\text{Na}_e$  was found before patent parasitemia.

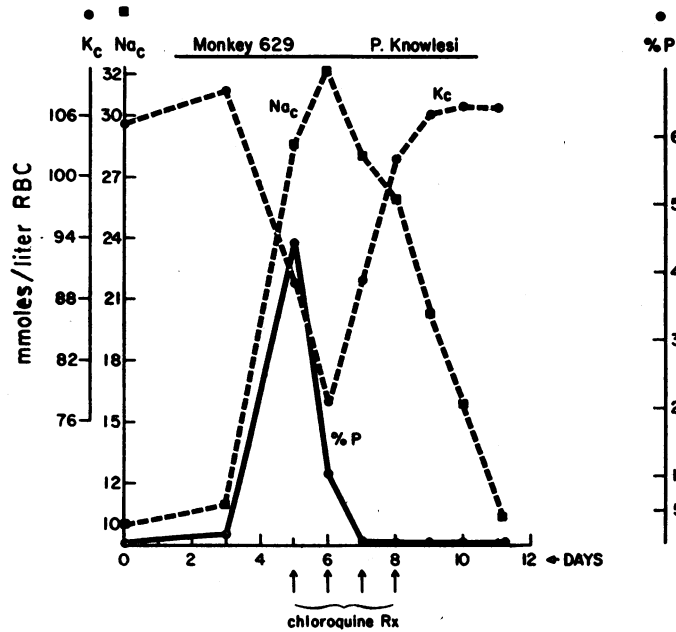


FIGURE 2 Effect of chloroquine therapy on the density of parasitemia (% *P*), and on the red blood cell potassium ( $K_c$ ) and sodium ( $Na_c$ ) concentration. Day zero is the day of infection. The hematocrit fell precipitously to 15% on day 6 after inoculation, was 11.5% on day 8 as the  $Na_c$  was decreasing and  $K_c$  increasing, and had reached 20% on day 11 when erythrocytic cation concentrations were normal.

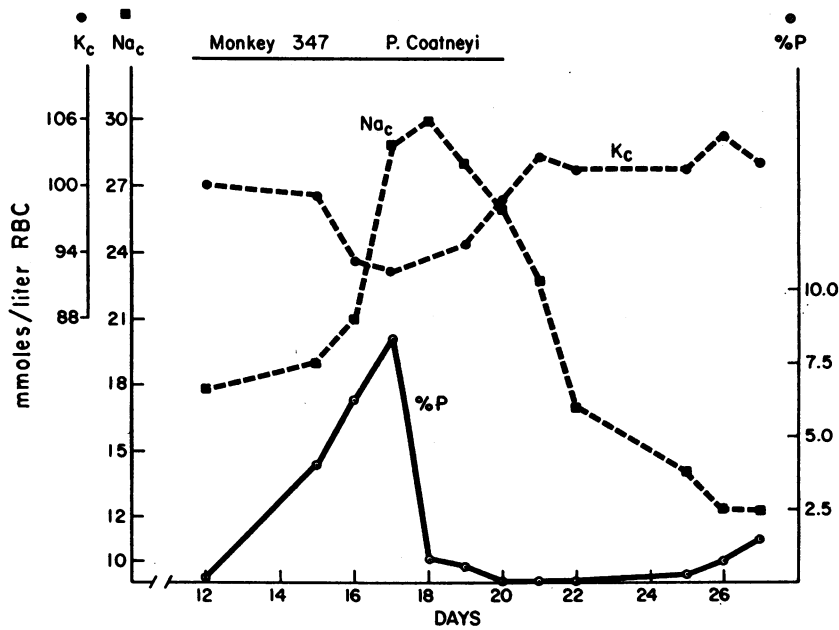


FIGURE 3 Effect of a spontaneous remission of *P. coatneyi* malaria (legend as in Fig. 2). In the absence of chloroquine therapy, the spontaneous disappearance of parasites causes cellular cation changes similar to those depicted in Fig. 2.

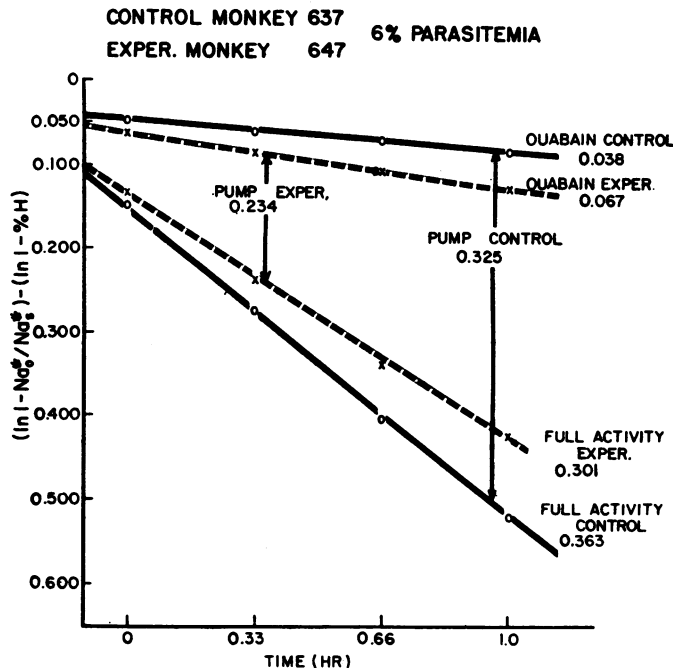


FIGURE 4 Plot of a representative red blood cell sodium outflux study showing the cumulative appearance of the tracer in the extracellular solution after correction for hemolysis.  $Na_o^*$  = isotopic Na in the extracellular solution;  $Na_t^*$  = isotopic Na in the total suspension of cells and extracellular solution; and %H = per cent hemolysis. The figures given for each slope are the outflux rate constants, before and after ouabain inhibition, as determined by the sum of least squares method. The red blood cell sodium concentration was 10.6 mM in the control and 16.1 mM in the malarious monkey.

Fig. 3 depicts one of three experiments with *P. coatneyi*, the results of which show that the temporary persistence of a high  $Na_e$ , after circulating parasites have disappeared, is not an effect of the chloroquine. Since *Plasmodium coatneyi* causes only a mild infection in our Rhesus monkeys, spontaneous remissions can be studied. Fig. 3 shows a spontaneous elimination of parasites 18–20 days after inoculation; the  $Na_e$  diminishes slowly and the  $K_e$  increases to normal concentration more rapidly.

**Sodium outflux experiments.** In order to investigate the causes of the elevated  $Na_e$ , sodium outflux (efflux) studies were done. Red blood cells from normal monkeys and from monkeys with from 5 to 10% levels of parasitemia were studied in simultaneous experiments. The use of such cells with a low density of parasitization served to minimize in vitro hemolysis as well as to emphasize the abnormalities of sodium flux of nonparasitized cells. Screening of the serum urea nitrogen in the infected animals showed only an occasional value in excess of 60 mg/100 ml; no correlation existed between the presence of a transport defect and an elevation of the serum urea nitrogen. Table IV shows the results

of eight outflux experiments. The active sodium outflux rate constants ( $^*k_{Na}^{pump}$ ) were  $0.325 \pm 0.027$  (mean  $\pm$  SEM) in eight control animals and  $0.202 \pm 0.012$  in eight infected animals. Fig. 4 shows a representative experiment. If the amount of isotope appearing in the extracellular solution is plotted against time, the slope of the plot is the outflux rate constant. The degree of ouabain-inhibition, depicted as pump, is clearly less in the cells

TABLE IV  
Sodium Outflux Rate Constants ( $^*k_{Na}$ ) and Total Active Sodium Outflux ( $^0M_{Na}^{pump}$ ) of Erythrocytes from Monkeys Infected with *P. knowlesi*\*

	$^*k_{Na}^{pump} \dagger$	$^*k_{Na}^{residual} \ddagger$	$^0M_{Na}^{pump} \parallel$
Control monkeys (8) $Na_e = 10.4$ mM	$0.325 \pm 0.027$	$0.052 \pm 0.007$	$3.30 \pm 0.22$
Infected monkeys (8) $Na_e = 18.5$ mM	$0.202 \pm 0.012$	$0.125 \pm 0.021$	$3.70 \pm 0.28$
	$P < 0.01$	$P < 0.01$	$P > 0.20$

\* All values expressed as mean  $\pm$  SEM.

† Ouabain-inhibited outflux rate constant.

‡ Ouabain-resistant or uninhibited outflux rate constant.

|| mmoles/liter of cells per hr.

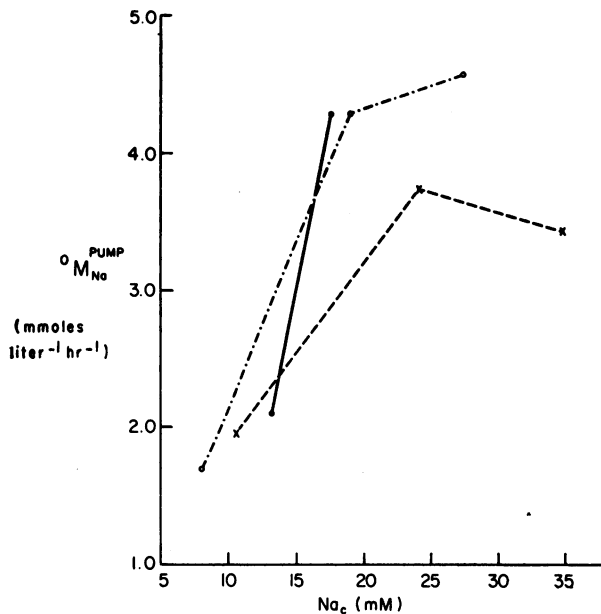


FIGURE 5 Active sodium outflux ( $^{\circ}M_{Na}^{pump}$ ) as a function of erythrocytic sodium concentration ( $Na_e$ ).  $Na_e$  was elevated in normal simian erythrocytes through an overnight incubation in a solution containing no potassium, 142 mM sodium, 10% v/v buffer, 11 mM glucose, 1.2 mM phosphate, 0.125 g/100 ml albumin, 0.2 g/100 ml Streptomycin, 10 mM inosine, and 3 mM adenine.

from the infected monkey. If the value for  $^{\circ}k_{Na}^{pump}$  is multiplied by the  $Na_e$ , the resultant value represents the total active sodium outflux ( $^{\circ}M_{Na}^{pump}$ ). It can be seen from Table IV that the control cells ( $3.30 \pm 0.22$  mmoles/liter of cells per hr) and the experimental or infected cells ( $3.70 \pm 0.028$ ) were not significantly different in regards to  $^{\circ}M_{Na}^{pump}$ . This is clearly an abnormal response in the experimental cells since the increased intracellular sodium would be expected to increase  $^{\circ}M_{Na}^{pump}$  proportionally. (See below.) Notice should also be taken of the considerable difference in the  $^{\circ}k_{Na}^{residual}$  between these groups of cells. The residual rate constant ( $^{\circ}k_{Na}^{residual}$ ) is a measure of the outflux of sodium after pump-inhibitors are added to the system. The  $^{\circ}k_{Na}^{residual}$

TABLE V  
Effects of Ouabain Concentration and Extracellular Sodium ( $Na_o$ ) on the Outflux Rate Constants

	$^{\circ}k_{Na}^{pump*}$		$^{\circ}k_{Na}^{residual\dagger}$	
	Ouabain		$Na_o$	
Control $Na_e = 9.4$ mM	$1 \times 10^{-4}$ M 0.428	$1 \times 10^{-3}$ M 0.427	142 mM 0.049	1.0 mM 0.027
Malaria $Na_e = 24.5$ mM	0.228	0.228	0.145	0.123

\* Ouabain-inhibited outflux rate constant.

† Ouabain-resistant or uninhibited outflux rate constant.

TABLE VI  
Effects of Elevating Red Blood Cell Sodium ( $Na_e$ ) in Normal Simian Erythrocytes

	$Na_e$		
	8.0 mM	18.8 mM	27.4 mM
$^{\circ}k_{Na}^{pump}$	0.212	0.228	0.167
$^{\circ}k_{Na}^{residual}$	0.049	0.064	0.030
$^{\circ}M_{Na}^{pump*}$	1.70	4.29	4.58

\* mmoles/liter of cells per hr.

was 2.5 times greater in the cells from the infected animals and accounted for more than one-third of all tracer sodium outflux. Human erythrocytes have been shown to possess a small fraction of sodium outflux that is not inhibited by ouabain but is inhibited by ethacrynic acid. Hoffman and Kregenow (14) have termed this pump II. Since it was conceivable that the large  $^{\circ}k_{Na}^{residual}$  in the red blood cells from malarious monkeys reflected increased activity of pump II, the effects of ethacrynic acid inhibition were studied.

Four studies of normal monkey red blood cells showed the inhibitory effect of ethacrynic acid,  $1 \times 10^{-8}$  M, on the outflux rate constant when added to ouabain  $1 \times 10^{-4}$  M, to be so small ( $\Delta = 0.012 \pm 0.006$ ) as to be indistinguishable from zero. This is not attributable to a complete lack of effect of ethacrynic acid on monkey erythrocytes since this inhibitor, when used alone, inhibits only slightly less than ouabain. When the presence of a pump II was investigated in four malarial monkeys, it could not be identified using ethacrynic acid ( $\Delta = 0.033 \pm 0.017$ ). Hence it was not possible to significantly reduce the  $^{\circ}k_{Na}^{residual}$  by the addition of another pump-inhibitor.

In order to test the possibility that the malaria parasite interfered with or inactivated the ouabain and therefore raised the inhibitory  $k_m$  for ouabain, a 10-fold increase in ouabain concentration ( $1 \times 10^{-3}$  M) was used in two studies. This produced no further inhibition of sodium outflux in either control or experimental cells. Table V shows one such experiment. Table V also shows that exchange diffusion (16) of an intracellular sodium ion for an extracellular sodium ion without any net transport of sodium does not explain the large  $^{\circ}k_{Na}^{residual}$  since the removal of extracellular sodium ( $Na_o = 1.0$  mM) did not materially reduce this value. It is also unlikely that the Na exchange diffusion recently described in human cells by Garrahan and Glynn (17) explains these results since ouabain and extracellular K would inhibit this mechanism of Na transport.

The  $Na_e$  was experimentally elevated in normal simian erythrocytes and the outflux measured in order to answer two questions: (a) Is the high  $^{\circ}k_{Na}^{residual}$  simply a func-



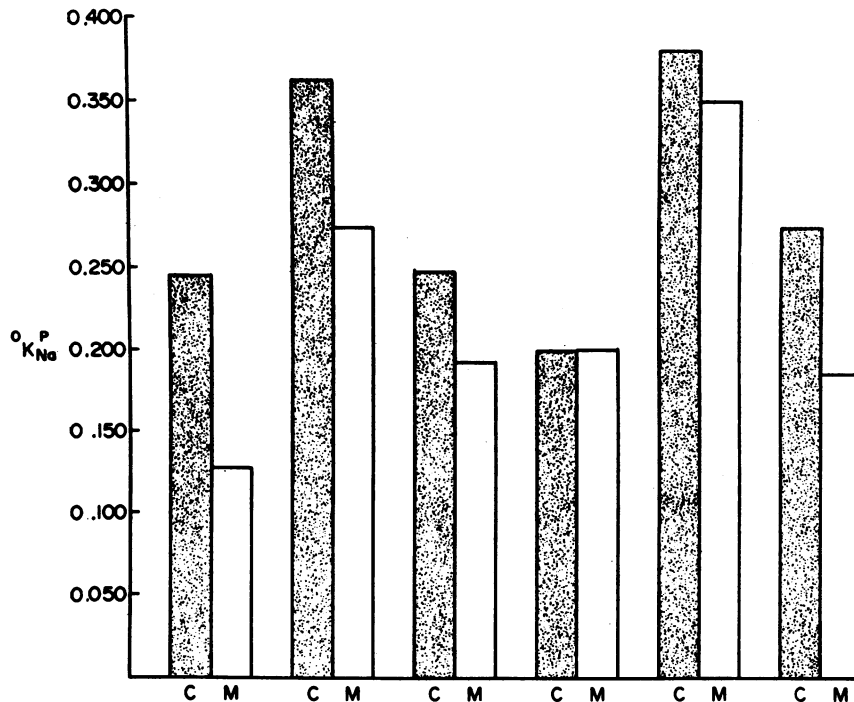


FIGURE 6 Active sodium outflux rate constants ( $^{\circ}k_{Na}^P$ ) of normal simian erythrocytes after incubation with control (C) or malarial plasma (M). The average depression of outflux, induced by malarial plasma, was 22% ( $P < 0.02$ ). Sodium influx was unchanged.

tion of a high  $Na_e$ ? and (b) Does the simian erythrocyte respond to an elevated  $Na_e$  in a similar fashion to the human erythrocyte in which elevation of cellular sodium stimulates active sodium outflux? Table VI shows the results of one of three sodium outflux studies with graded increases of  $Na_e$ . The  $^{\circ}k_{Na}^{residual}$  shows no significant change despite levels of  $Na_e$  which are two- and three-fold greater than the control value of 8.0 mM. Hence the high  $^{\circ}k_{Na}^{residual}$  observed in the high sodium malarial erythrocytes could not be directly attributed to the elevation of sodium in the cell per se. These findings are in agreement with recently published work with lactose-treated human cells in which Maizels (18) found no change in the rate constant for "passive efflux" when cellular sodium is increased. Fig. 5 depicts the response of the sodium outflux pump to changes of  $Na_e$  and provides an affirmative answer to the second question. Active sodium outflux is increased twofold as the  $Na_e$  is doubled; this means, therefore, that the sodium outflux rate constant did not change when  $Na_e$  was increased to approximately 20 mM. When  $Na_e$  approached 30 mM, the outflux rate constant began to decrease.

**Sodium influx experiments.** Since  $Na_e$  is a function of the net balance between active sodium outflux and passive sodium influx, it is apparent that the observed elevation of  $Na_e$  may be attributable to increased influx

of sodium into the cell, as well as to the measured decrease of active sodium outflux. Sodium influx was measured in five experiments and the results are shown in Table VII. The control influx rate constants ( $^i k_{Na}$ ) had a mean value of  $0.019 \pm 0.002$  as compared to  $0.028 \pm 0.002$  in the cells from malarial animals. When these influx rate constants are multiplied by the extra-cellular sodium concentration (142 mM), it can be seen that the total sodium influx ( $^i M_{Na}$ ) is 1.28 mmoles/liter of cells per hr greater in the experimental erythrocytes, 3.98, than in the control cells, 2.70.

**Cross-incubation of malarial plasma and normal cells.** Since the changes of cellular sodium, sodium outflux, and

TABLE VII  
Sodium Influx Rate Constants ( $^i k_{Na}$ ) and Total Sodium Influx ( $^i M_{Na}$ ) in Monkeys Infected with *P. knowlesi*\*

	$^i k_{Na}$	$^i M_{Na} \ddagger$
Control monkeys (5)	$0.019 \pm 0.002$	$2.70 \pm 0.47$
Infected monkeys (5)	$0.028 \pm 0.002$ $P < 0.02$	$3.98 \pm 0.48$ $P < 0.02$

\* All values expressed as mean  $\pm$  SEM.

‡ mmoles/liter of cells per hr.

sodium influx suggested the presence of a circulating toxin which adversely affected most, if not all, red blood cells, it was decided to cross-incubate normal monkey erythrocytes with cell-free, malarial plasma, harvested from terminally ill animals with dense parasitemias (> 50%). Survey of the serum urea nitrogen and creatinine showed no significant azotemia. The cells were incubated in control and malarial plasmas for 20–22 hr and thereafter sodium flux measurements were made as previously described.

Figure 6 depicts the results of six cross-incubation studies. The ouabain-inhibited sodium outflux rate constants are compared after incubation with control or with malarial plasma; the average inhibition induced by the malarial plasma was 22% (paired  $P < 0.02$ ). The mean  $\text{Na}_e$  was 10.6 mM after incubation in control plasma, whereas the  $\text{Na}_e$  was elevated to 13.2 mM after incubation in the experimental plasma (paired  $P < 0.05$ ).<sup>4</sup> The measured hemolysis that occurred during these outflux studies was generally greater in those cells previously exposed to the malarial plasma. The influx rate constants for sodium were similar in both groups after cross-incubation with the control value of  $0.016 \pm 0.001$  and the experimental value of  $0.013 \pm 0.000$ . Hence these in vitro incubations only partially induced the alterations of sodium transport which were observed in erythrocytes from an infected animal.

## DISCUSSION

The present study confirms the original observations of Overman and Overman, Bass, Davis, and Golden (6, 19) in regard to an increase of Na and a decrease of K in the red blood cells of Rhesus monkeys infected with *P. knowlesi*. These observations were extended to show that the accumulation of sodium and loss of potassium from the erythrocyte, during malarial infection, occurred with *P. falciparum* infections in the chimpanzee and with *P. coatneyi* infections in the Rhesus monkey. Although the present report does not include any human experiments, Overman's investigations suggested that similar although less dramatic changes occurred particularly in severe human infections (20). It was not likely that the observed changes of red blood cell cation concentration and transport were restricted to only the parasitized cells. The increase of  $\text{Na}_e$  seen at levels of parasitemia less than 10% (Fig. 1) was greater than could be accounted for by assuming disproportionate changes in only the parasitized cells. This possibility was ruled out by the experiments depicted in Table III which show that fractionation of parasitized blood into

<sup>4</sup> These values of  $\text{Na}_e$  were obtained immediately before the outflux studies after the cells were further incubated for 3 hr in a solution of <sup>22</sup>Na, sodium chloride, sodium phosphate, glucose, and no potassium.

densely and sparsely parasitized layers produces no differences in the measured  $\text{Na}_e$ . These results might have been anticipated from the studies of Danon and Gunders (21) and of Fogel, Shields, and vonDoenhoff (8) who showed that nonparasitized erythrocytes, obtained from an infected animal, have an increased osmotic fragility and are therefore abnormal. Zuckerman (7, 22, 23) has stressed the occurrence of in vivo hemolysis of erythrocytes that are not directly invaded with a malarial parasite; it is not unusual for red cell destruction to be three-fold greater than the number of cells parasitized. These findings have been explained as a result of antibody production secondary to the immunological changes induced by the malarial parasite; however, no specific red blood cell antibody has been isolated or demonstrated on the erythrocytic membrane. In addition, it is quite unlikely that the presence of antibodies on the red blood cells can explain the findings of the present study, since the changes of cation concentration and transport appear very early in the acute infection (day 2 to 3 of parasitemia) before significant levels of antibodies would be expected to appear and disappear quite rapidly after chloroquine therapy. Furthermore, hemolysis due to complement-fixing hemolysin antibodies is associated with large tears or pores in the erythrocyte membrane with sudden rather than gradual disruption of the erythrocyte cation gradient (24–26). Hence, although immunological processes of red blood cell destruction may occur in malaria and may play a significant role in producing the anemia, it is not probable that such antibodies cause the gradual and progressive deterioration of erythrocytic membrane function which results in sodium accumulation and potassium loss from the cell.

The alterations of sodium transport that are described in this study may be compared to those changes described in other illnesses. Whereas many hemolytic disorders (1–3, 26) display increased passive permeability of the red blood cell membrane to cations with subsequent increased movement of the cation down its electrochemical gradient, these disorders are accompanied by a compensatory increase of the sodium outflux in an attempt to extrude sodium from the cell and maintain a normal intracellular environment. The intracellular sodium and potassium generally remain normal in these illnesses until just before the onset of hemolysis. On the other hand, diseases such as severe uremia and extensive neoplastic invasion (4, 5) may be accompanied, in approximately 25% of cases, by an impairment of the sodium outflux and subsequent elevation of the intracellular sodium concentration. Sodium influx has not been directly measured in these illnesses. It seems likely, therefore, that the elevation of  $\text{Na}_e$  (and depression of  $\text{K}_e$ ) during malarial infections is primarily a function of the impaired active transport mechanisms and only secondarily

TABLE VIII

A Comparison of the Similarities in Erythrocytic Sodium Transport in Malaria and Uremia

	Malaria	Uremia
Cellular sodium	Increased	Increased
Active sodium outflux rate constant	Decreased	Decreased
Cellular ATP	Normal or increased*	Normal or increased
Evidence for circulating "toxin"	Inhibition of outflux after cross-incubation	Inhibition of ATPase after cross-incubation
Effect of therapy	Chloroquine reverses the defects	Dialysis reverses the defects

\* Results to be published.

a consequence of the 50% increase in sodium influx. Studies with human hereditary spherocytic cells (2) have shown that sodium influx may increase 35% with no increase of  $\text{Na}_e$  because of the compensatory increase of sodium outflux. The experiments summarized in Fig. 5 and Table VI indicate that monkey erythrocytes behave similarly to human cells when  $\text{Na}_e$  is experimentally elevated; if  $\text{Na}_e$  is increased to approximately 20 mM, the outflux rate constant is unchanged and, hence, the active sodium outflux increases with the  $\text{Na}_e$ . These findings are in essential agreement with related experiments using human erythrocytes (5, 17, 18, 27-29). Since the red blood cells from the malarious monkeys used in the outflux studies had a mean  $\text{Na}_e$  of 18.5 mM, and an active sodium outflux similar to the controls (3.70 vs. 3.30 mmoles/liter of cells per hr in the controls), the response of the sodium transport system is clearly abnormal. In this regard the impairment appears quite similar to the pump-defect described in uremia (4, 5, 30). Table VIII lists the major similarities between the transport alterations observed in malaria and in uremia. In addition, there is a quantitative defect of membrane ATPase in the high cell-sodium uremics; similar studies have not been done with erythrocytes from a malarious host. The major dissimilarity is that changes described in malaria are predictable since they appear early in the disease and eventually occur in all infected monkeys, whereas these same changes occur in only 25% of all severe, chronic uremics.

The cross-incubation experiments seem to provide direct evidence that a circulating toxic factor is present during malarial infection; this substance inhibits the sodium outflux in normal erythrocytes. It is unknown whether this circulating material is directly produced by the parasite or whether it is produced by the host in response to parasitization. However, these cross-incuba-

tion experiments produced transport changes that differed in two ways from the pattern of altered sodium transport found in erythrocytes taken from an infected monkey: a) The sodium influx was not increased by exposure to malarial plasma; b) the residual outflux rate constant ( $^{\circ}k_{\text{Na}}^{\text{residual}}$ ) was not increased after incubation of normal cells and malarial plasma. There is no entirely acceptable explanation for these discrepancies but it is certainly possible that the in vivo influence of the spleen and reticuloendothelial system, upon cells with early changes of membrane function or structure, is necessary to produce further changes in the membrane and subsequent increases in the leak parameters (31).

It is possible that the changes of sodium outflux, after incubation in malarial plasma, are due to the absence of a critical substance or nutrient rather than to the presence of a toxic factor. In order to minimize this possibility, the following compounds were added to the plasma before the 22 hr incubation: adenine, inosine, glucose, phosphate, and buffer. These compounds, under normal circumstances, are adequate to maintain intracellular ATP stores and thereby to sustain normal rates of sodium transport.

#### ACKNOWLEDGMENTS

The author gratefully acknowledges the constructive advice and criticisms of Doctors John Sachs, Marcel Conrad, and Paul Teschan, as well as the encouragement and support provided by Dr. Teschan. Valuable technical assistance was given by Robert Bevins and Susie P. Willet. The ethacrynic acid was kindly supplied by Merck, Sharpe & Dohme.

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