acidified by addition of 0.5 ml of 0.2 N H_2SO_4, and a carborundum chip to minimize bumping is added. The tubes are immersed to half their length in a boiling water bath for 10 to 12 minutes until the odor of ethanol has disappeared. The tubes are cooled and the glycerol is converted to formaldehyde by oxidation with 0.05 ml of 0.025 M $NaIO_4$ at room temperature for 10 minutes. Excess $NaIO_4$ is reduced by treatment with 0.05 ml of 0.5 M Na AsO_2 for 10 minutes.

Five ml of 0.2% chromotropic acid in sulfuric acid are added to each tube and stirred mechanically.‡ The tubes are placed in an aluminum block heater§ adjusted to 100-105°C and heated for 30 minutes. The tubes are cooled and the intensity of color is measured spectrophotometrically‖ at 570 mμ. The reading of the blank against water, using 19 mm cuvets, ordinarily does not exceed 0.060.

The precision of the micromethod for determination of serum triglycerides was compared with that of the macromethod by analyzing the same serum sample on 24 separate occasions in duplicate by each method. The results are shown in Table I. The mean value obtained by the macromethod was 121.5 mg per 100 ml, with a standard deviation of 3.5 mg/100 ml while corresponding values for the microprocedure were 122.4 ± 2.6 mg/100 ml. The coefficients of variation for the macro- and micromethods were 2.8 and 2.1 respectively. Thus, the microprocedure yields essentially the same values for serum triglyceride determination as does the macromethod and has a slightly lower standard deviation and coefficient of variation.

TABLE I. Summary of 24 Duplicate Determinations of Serum Triglycerides.

Method	Triglycerides Mean, mg/100 ml	S.D., mg/100 ml	C.V.
Macro	121.5	3.5	2.88
Micro	122.4	2.6	2.12

S.D. = Standard deviation.

C.V. = Coefficient of variation = $\frac{\text{S.D.}}{\text{mean}} \times 100$.

Summary. A micromethod for direct determination of serum triglyceride concentration is described by means of which duplicate measurements may be carried out on 0.1 ml of serum.

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‖ Coleman Jr. Model 6 Spectrophotometer, Coleman Instruments, Maywood, Ill.

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Potassium Loss from Human Erythrocytes Exposed to Amphotericin B. (29825)

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Amphotericin B, a heptaene antibiotic derived from *Streptomyces nodosus,* is effective in the treatment of a number of systemic fungal infections. Because of recent reports that the drug causes hemolysis of canine erythrocytes *in vivo*(1), and hemolysis of human erythrocytes *in vitro*(2,3), the effect of the drug on human erythrocytes was studied further using an *in vitro* system.

Materials and methods. Freshly drawn human erythrocytes were freed of plasma and buffy coat by centrifugation, washed 3 times in buffered saline solution (pH 7.4, containing 135 mM NaCl, 10 mM Na_2HPO_4, and 5 mM KCl) and then suspended in 1½ volumes of buffer. Amphotericin B (Fungizone for Infusion, Lot #3A80431, E. R. Squibb & Sons) was dissolved in distilled water and

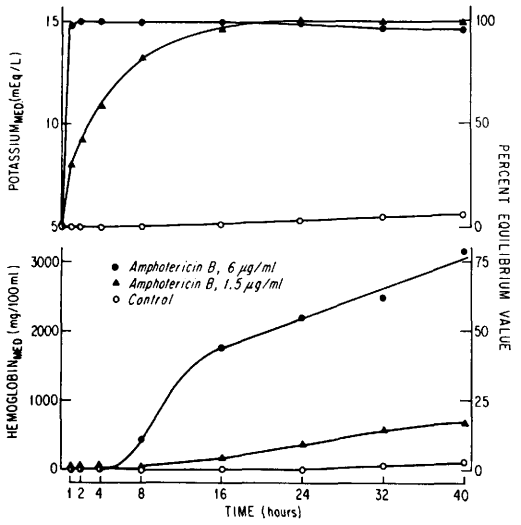

Fig. 1. Potassium ion concentration (upper panel) and hemoglobin concentration (lower panel) of buffered medium at 20°C containing human erythrocytes, expressed as functions of time after addition of amphotericin B. MED = medium. Potassium ion concentration, $[K^+]$ varies with time according to $y(t) = b-(b-a)\exp(-ct)$, where $y(t)$ is $[K^+]$ at time t, a is $[K^+]$ initially, b is $[K^+]$ at equilibrium, and c is a constant expressing the hourly rate of attainment of equilibrium. Values of the constants for system containing (1) 6 μg/ml amphotericin B: a = 5.9, b = 15.0, c = 4.1; (2) 1.5 μg/ml amphotericin B: a = 5.4, b = 15.0, c = 0.23.

then diluted with an equal volume of twice concentrated buffer (pH 7.4). Immediately after dilution 3 volumes of the mixture were added rapidly to one volume of the erythrocyte suspension and the resulting 10% suspension of erythrocytes was incubated at 20°C. Since "Fungizone for Infusion" contains sodium desoxycholate, a compound known to be surface active, 2 control incubation flasks were included in each experiment, one with equivalent amounts of sodium desoxycholate in buffer and the other with buffer alone.

At timed intervals portions of well-mixed cell suspension were removed for measurement of hematocrit, and determination of potassium, sodium, and hemoglobin concentrations of the cell suspension and supernatant medium after centrifugation. Potassium and sodium were measured by flame photometry using an Autoanalyzer (Technicon Co., Chauncey, N. Y.). Hemoglobin was determined by the cyanmethemoglobin method. Hematocrits were determined by centrifugation of cell suspension in Wintrobe tubes at 1800 *g* for 30 minutes.

Results. The findings are shown in Fig. 1. At a concentration of 1.5 μg of amphotericin B/ml of cell suspension, the potassium concentration of the medium increased slowly and after 16 hours approached equilibrium. At a drug level of 6 μg/ml the potassium concentration in the medium increased rapidly, reaching equilibrium in one hour. In another experiment at the higher drug level increases of 1.0 mEq/l in potassium concentration were detected within the first 3 minutes of incubation.

Measured equilibrium values for potassium concentration in the medium approximated the calculated value, 14.6 mEq/l, which is based on the normal potassium and water content of the cells (97 mEq potassium and 710 g water/liter of cells) and the assumption that potassium ion is evenly distributed throughout the liquid phase of the system. In other experiments using concentrations of amphotericin B of 1 μg/ml, the potassium level in the medium increased slightly but did not reach equilibrium within 40 hours of observation. At a concentration of 0.1 μg/ml, potassium increased in the medium at a rate identical to that observed in the control tubes.

The concentration of sodium in the medium decreased by approximately the same amount as the potassium increased (Table I); thus, the sum of the sodium and potassium ion concentrations in the medium remained nearly constant. These findings show that intracellular loss of potassium tended to be balanced by a corresponding gain of sodium.

Water began to enter erythrocytes during the first hour of incubation with amphotericin B, but substantial water shifts occurred only after potassium equilibrium had been attained. At the 6 μg/ml dose, the hematocrit increased from a value of 10.8% at the start of incubation to 11.2% at one hour. Thereafter, the hematocrit rose to 12.5, 14.4, and 20% at 2, 4 and 8 hours, respectively. With this expansion of cell volume, some hemolysis appeared at 8 hours. Subsequently, as hemol-

TABLE I. Potassium and Sodium Concentrations in Medium During Incubation of Human Erythrocytes with Amphotericin B at 20°C.

Amphotericin B (μg/ml)	Time* (hr)	Potassium in medium	Sodium in medium	Potassium + sodium in medium
		(mEq/l)		
0†	0	5.1	154.5	159.6
	40	5.7	154	159.7
1.5	0	5.4	153.5	158.9
	1	7.9	151	"
	2	9.2	150	159.2
	4	10.8	147.5	158.3
	8	13.2	146	159.2
	16	14.6	144.5	159.1
	24	15.0	143.5	158.5
	32	"	142.5	157.5
	40	14.8	143	157.8
6.0	0	5.9	153.5	159.4
	1	14.9	145	159.9
	2	15.0	"	160.0
	4	15.1	144.5	159.6
	8	15.0	"	159.5

* Time at which sample was removed from incubation flask. A maximum of 2 minutes passed before suspensions were subjected to centrifugation, including the zero-time sample.

† Control tube contained 5.0 μg/ml of sodium desoxycholate.

ysis continued to increase, the hematocrit decreased. At the lower dose of 1.5 μg of amphotericin B/ml, hematocrit changes were delayed in appearance and of lesser magnitude, but followed the same pattern. Values of 11.2, 11.8, 12.2, 13.0 and 17.0% were observed at zero, 2, 4, 8 and 16 hours respectively.

Hemoglobin was first detected in the medium in concentrations greater than 0.004 g/100 ml only after potassium values approached equilibrium. This amount of hemoglobin reflects hemolysis of less than one-thousandth of the cells. In fact, with the 6 μg/ml dose of amphotericin B the electrolyte shift was complete 2 hours before detectable amounts of hemoglobin appeared in the medium.

Control incubation of erythrocytes with sodium desoxycholate in buffer showed a very slow potassium loss which did not differ from the incubation in buffer alone. Sodium uptake was correspondingly slow. The hematocrit remained constant, and hemoglobin was not detected in the medium until the 32-hour sample.

Discussion. Amphotericin B not infrequently causes severe toxic reactions in man (1,4,5). Although certain aspects of these reactions have been described, the mechanism of the toxicity at the cellular level remains to be elucidated. The present report describes a model *in vitro* system using human erythrocytes to study effects of amphotericin B on cell permeability and electrolyte content. In this system, the drug exhibits an effect on the cell membrane, markedly increasing its permeability to potassium and sodium ions. The enhanced rate of cation leak greatly exceeds the opposing active transport. From the results shown in Fig. 1 for a drug level of 6 μg/ml, the average rate of leak (passive loss) of potassium during the first hour is approximately 80 mM/liter of cells/hour, compared with a normal active transport uptake of approximately 0.5 mM/liter of cells/hour at 20°C(6,7). The loss of ionic regulation by the cell impairs its volume regulation after a lag period. Then, increasing water uptake due to colloid osmotic swelling leads to cell disruption and hemolysis.

The sustained blood level of amphotericin B in treated patients is usually between 0.5 and 1.5 μg/ml, as measured by bioassay of serum(8). Serum, which was not present in our *in vitro* system, has about a 10-fold protective effect against hemolysis caused by amphotericin B(3). Therefore, a serum level of 1.0 μg/ml corresponds to an *in vitro* level of approximately 0.1 μg/ml, a concentration which did not cause increased potassium loss from erythrocytes. On the basis of these results, it is unlikely that the hemolysis we observed *in vitro* occurs to the same extent in patients under treatment with amphotericin B for systemic fungal disease. The anemia occurring during amphotericin B therapy has been studied recently by Brandriss *et al,* who concluded that the condition was a result of bone marrow suppression rather than hemolysis(5).

Summary. When fresh human erythrocytes are suspended in buffered solution containing amphotericin B, potassium loss begins within 3 minutes and continues for some time before appreciable hemolysis occurs. The kinetics of the potassium loss indicate that ampho-

tericin B markedly increases cell membrane permeability. The concentration difference of potassium ion across the cell membrane decays at a rate which depends on the dose of amphotericin B.

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