Effects of Digoxin-Specific Antibodies on Accumulation and Binding of Digoxin by Human Erythrocytes

JERRY D. GARDNER, DIANE R. KIINO, TIMOTHY J. SWARTZ, and
VINCENT P. BUTLER, JR.

From the Digestive and Hereditary Diseases Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014, and from the Department of Medicine, Columbia University College of Physicians and Surgeons, New York 10032

ABSTRACT The present studies indicate that accumulation of digoxin by intact human erythrocytes is the result of two processes: binding of digoxin to the erythrocyte membrane and uptake of digoxin across the membrane into the cell. In contrast, accumulation of ouabain by human erythrocytes is entirely attributable to binding of this glycoside to the plasma membrane. Digoxin binding to the erythrocyte membrane involves a single class of binding sites, is a saturable function of the extracellular digoxin concentration, reversible, temperature-sensitive, dependent on the cation composition of the incubation medium, inhibited by other cardioactive steroids, and correlates with the inhibition of erythrocyte potassium influx. Digoxin uptake across the membrane into the cell is also temperature-sensitive and reversible but is a linear function of the extracellular digoxin concentration, not altered by changes in the cation composition of the incubation medium, not inhibited by other cardioactive steroids, and does not correlate with inhibition of erythrocyte potassium influx. Digoxin-specific antibodies can both prevent and reverse effects of digoxin on potassium influx in human erythrocytes by virtue of the capacity of the antibodies to decrease the amount of digoxin that is bound to the erythrocyte membrane. These antibodies also reduce uptake of digoxin across the plasma membrane into the erythrocyte; however, this portion of cellular digoxin is not responsible for the observed inhibition of potassium influx. In the presence of digoxin-specific antibodies, the changes in digoxin binding to the erythrocyte membrane and in digoxin uptake across the membrane into the cell reflect the ability of the antibodies to form complexes with

"free" digoxin molecules in the incubation medium and thereby decrease the effective concentration of digoxin.

INTRODUCTION

Previous studies have documented the ability of digoxin-specific antibodies to prevent and to reverse established cellular effects of digoxin (1-6). Watson and Butler (5) reported that digoxin-specific antibodies would prevent completely both the accumulation of [³H]digoxin by human erythrocytes and the associated inhibition of potassium influx. These authors also reported that addition of digoxin-specific antisera to erythrocytes, which had been preincubated with [³H]digoxin, resulted in complete removal of all detectable glycoside during the subsequent 10 min but reversal of the digoxin-induced inhibition of potassium influx was still incomplete as long as 4 h after addition of the antibody. The basis for the delay between the removal of digoxin and restoration of potassium influx was not established but these authors suggested, among other possibilities, that a small fraction of the [³H]digoxin, which was not detectable with their technique, might have been bound with a high affinity to, and released more slowly from, a specific cellular cation transport system than was the majority of the cellular digoxin that was promptly removed by antibody.

The cardiac glycoside, ouabain, is also accumulated by intact human erythrocytes (7-10) as well as by erythrocyte ghosts (7, 8). Uptake of ouabain by intact human erythrocytes is attributable to binding of the glycoside to the plasma membrane (9-11). This binding is reversible, exhibits a high degree of chemical specificity, can be detected at ouabain concentrations as low as 10⁻¹⁰ M, is dependent on the cation composition of the incubation solution, and correlates with inhibition of potassium influx (10). These observations raise the possibility that

Dr. Butler is the recipient of a Research Career Development Award from the U. S. Public Health Service (HL-11315).

Received for publication 6 October 1972 and in revised form 29 January 1973.

the discrepancy observed by Watson and Butler (5), between the time course of [^3H]digoxin loss from erythrocytes and that of restoration of potassium influx after addition of digoxin-specific antisera, might be attributable to these authors having measured primarily the release of digoxin molecules that were not bound to the erythrocyte plasma membrane and that did not produce inhibition of erythrocyte potassium influx.

In the present study, we explored the accumulation of digoxin by intact human erythrocytes and, when appropriate, compared this accumulation with that of ouabain. We also explored the relation between cellular accumulation of these two glycosides and the inhibition of potassium influx. Finally, we investigated the effect of digoxin-specific antibodies on accumulation and binding of digoxin by human erythrocytes as well as the effect of these antibodies on digoxin-induced alterations in erythrocyte potassium transport.

METHODS

Erythrocytes obtained from normal male and female volunteers (20–31 yr of age) were washed three times in isotonic choline chloride (pH 7.4). All incubations were performed at 37°C unless otherwise specified and the hematocrit of the incubation mixture was 5–10%. The standard incubation solution had the following composition (millimolars): NaCl, 150; Tris buffer (pH 7.4), 10; glucose, 11.1.

Total glycoside accumulation was measured using the technique described previously (10). Erythrocytes were added to incubation solutions containing the appropriate [^3H]glycoside. After mixing thoroughly, triplicate 100- μl samples were taken at appropriate times, placed in polyethylene micro test tubes (Beckman Instruments, Inc., Fullerton, Calif.) and washed four times with 300 μl of isotonic choline chloride by alternate centrifugation and resuspension. Centrifugation was performed using a Microfuge (Beckman Instruments, Inc.) at 10,000 g for 15 s. After the final wash, each sample was treated with 100 μl of 10% perchloric acid, agitated, and centrifuged for 30 s. The tube and its contents were inverted and placed in a vial containing 20 ml of liquid scintillation solution. When the vial was capped and shaken the supernate passed from the sample into the scintillator and the precipitate remained in the tip of the sample tube. At some time during the incubation, triplicate 100- μl samples of the incubation mixture were added to 100 μl of 10% perchloric acid, agitated, centrifuged, inverted, and placed in a vial containing liquid scintillation solution.

To measure the release of glycoside molecules, which had been accumulated by the cell, erythrocytes were preincubated at 37°C with the appropriate [^3H]glycoside. At the end of the preincubation period the cells were washed three times as rapidly as possible with at least 50 vol of iced incubation medium that contained no glycoside. Washing was performed by alternate centrifugation at 1,500 g for 1 min and resuspension. The cells were then resuspended in fresh incubation medium and, at appropriate times, the total amount of radioactivity present in the cells was determined using the wash technique described in the preceding paragraph.

To measure glycoside binding to the plasma membrane, erythrocytes were added to incubation solutions containing the appropriate [^3H]glycoside and mixed thoroughly. At appropriate times, triplicate 100- μl samples were added to tubes containing 10 ml of iced, distilled water and mixed thoroughly. The resulting hemolysate was then filtered using a Millipore filter (RAWP-02500, Millipore Corp., Bedford, Mass.) and washed three times with 10 ml of iced, distilled water. The filter plus 100 μl of 10% perchloric acid was then added to 20 ml of liquid scintillation solution and radioactivity determined.

To measure the release of glycoside molecules from the erythrocyte membrane, cells were preincubated with the appropriate [^3H]glycoside and then washed three times with iced incubation medium. After resuspension in fresh incubation medium, at appropriate times, the amount of radioactivity bound to the cell membrane was determined by the filtration technique described in the preceding paragraph.

When digoxin binding to the erythrocyte membrane was determined on cells incubated with antiserum, the cells were washed three times with at least 50 vol of iced incubation medium before the hemolysis step. This was necessary because it was found that the digoxin-specific antibodies are retained on the filter and thereby, will give falsely high values for [^3H]digoxin binding to the erythrocyte membrane if not removed before the filtration step. Appropriate control experiments using the standard incubation solution indicated that addition of these extra washing steps did not alter significantly the values for [^3H]digoxin binding. Unless otherwise specified, digoxin-specific antiserum and control antiserum were present in the incubation solutions at a concentration of 0.03 ml antiserum/ml incubation solution. The digoxin-specific antiserum used for the present studies was obtained from a sheep immunized with a digoxin-bovine serum albumin conjugate prepared by the periodate oxidation method (12–14); this serum was denoted S-62 in a previous report by Watson and Butler (5). Control antiserum was obtained from a sheep (S-301) immunized in a similar manner with a 1-methyladenosine-bovine serum albumin conjugate also prepared by the periodate oxidation method (12). All sera were heated at 56°C for 30 min to inactivate complement, and absorbed three times with washed human erythrocytes to remove heterophile antierythrocyte antibodies.

The volume of counted cells was calculated from the hemoglobin concentration of the incubation mixture and the hematocrit determined on a separate tube containing the incubation solution and erythrocytes at a hematocrit of approximately 25%. The hematocrit was measured using a Drummond microhematocrit centrifuge (Drummond Scientific Co., Broomall, Pa.) and hemoglobin concentration was measured using the cyanmethemoglobin method (15).

The accumulation of glycoside was calculated from the counts per milliliter cells and the specific activity of glycoside in the incubation medium. The glycoside concentration in the tritiated material supplied by the manufacturer was determined by measuring the binding of radioactivity to the cell membrane in the presence of constant radioactivity and varying concentrations of the appropriate nonradioactive glycoside (10). The concentrations thus determined for [^3H]digoxin and for [^3H]ouabain were within 9% of values calculated from the concentrations given by the commercial supplier.

Liquid scintillation counting was performed using 20 ml of a solution composed of 15 parts toluene (J. T. Baker Chemical Co., Phillipsburg, N. J.), 5 parts Triton X-100

FIGURE 1 Time course of total accumulation and membrane binding of digoxin by human erythrocytes. The digoxin concentration in the incubation medium was 1.2×10^{-7} M. Open circles represent total cellular accumulation of digoxin. Closed circles represent digoxin bound to the erythrocyte membrane. Binding and cellular accumulations were determined simultaneously from the same incubation mixture. Each point represents the mean and the vertical line ± 1 SD of four experiments.

(New England Nuclear Corp., Boston, Mass.), and 1 part Liquifluor (New England Nuclear Corp.). The observed counts were usually such that their standard deviation was less than 2%. Variation in quenching was determined from the ratio of counts in two channels produced by an automatic external standard. Standard curves were constructed for [^{3} H]digoxin and for [^{3} H]ouabain using acetone as a quenching agent. Similar curves were obtained using hemoglobin as the quenching agent.

For potassium influx determination, ^{42} K (final concentration approximately 20 μ Ci/ml) was added to cells that had been incubated with or without [^{3} H]glycoside (hematocrit, 5–10%). After mixing thoroughly, duplicate 100- μ l samples were placed in polyethylene micro test tubes and washed four times with 300 μ l of iced (4°C) isosmotic choline chloride. After the final wash, each sample was treated with 100 μ l of 10% perchloric acid, agitated, centrifuged, inverted, and placed in 20 ml of liquid scintillation fluid for counting. At some time during the incubation, triplicate 100- μ l samples of the incubation mixture (i.e., cells plus medium) were added to a micro test tube containing 100 μ l of 10% perchloric acid, agitated, centrifuged, inverted, and placed in a liquid scintillation solution. The volume of cells counted was calculated from the hemoglobin concentration in the incubation mixture and the previously measured hemoglobin content per volume of cells. Erythrocyte sodium and potassium concentrations were determined as described previously (16), and the incubation solution concentrations were determined at the end of the incubation period. Sodium and potassium concentrations were measured

with an Instrumentation Laboratory model 143 flame photometer (Instrumentation Laboratory, Inc., Lexington, Mass.). Initially, the uptake of ^{42} K was determined at 0, 15, 30, and 45 min; however, since the uptake was observed to be constant over this period, potassium influx was calculated from samples taken at 0 and 40 min unless otherwise specified. Potassium influx was calculated using the method described by Sachs and Welt (17) and the average of the potassium concentrations in the incubation solutions at the beginning and end of the incubation period. All counts were corrected for decay.

Digoxin was kindly supplied in crystalline form by Dr. Stanley T. Bloomfield, Burroughs Wellcome Co., Research Triangle Park, N. C. All other reagents were of the highest grade of purity obtainable. [^{3} H]Digoxin (lot no. 184-165, sp act 12 Ci/mmol) and [^{3} H]ouabain (lot no 184-194, sp act 13 Ci/mmol) were obtained from the New England Nuclear Corp. ^{42} K was obtained as the chloride from ICN Corp., Chemical & Radioisotopes Div., Irvine, Calif.

RESULTS

Fig. 1 illustrates the time course for total digoxin accumulation by human erythrocytes and for digoxin binding to the erythrocyte membrane. Both processes reached a steady state by approximately 80 min; however, values for total cellular digoxin accumulation were significantly greater than those for digoxin binding to the cell membrane. The possibility that this difference was only apparent and instead, attributable to entrapment of extracellular radioactivity was excluded by the observation that when [^{3} H]mannitol or [^{14} C]polyethylene glycol was used as a marker for extracellular fluid, less than 5% of the [^{3} H]digoxin accumulated by the cells could be attributed to trapped extracellular radioactivity. When the same experiment was performed using [^{3} H]ouabain instead of [^{3} H]digoxin, values for total cellular ouabain accumulation were not significantly different from those for ouabain binding to the cell membrane. This observation is further evidence against the difference between digoxin accumulation and digoxin binding being attributable to trapped extracellular digoxin.

As the temperature of the incubation medium was reduced, there was a progressive decrease in binding of digoxin to the cell membrane and at 4°C, binding of digoxin was barely detectable. Reducing the incubation temperature produced a similar change in total cellular accumulation of digoxin. However, at each incubation temperature studied, values for cellular accumulation were significantly greater than those for digoxin binding, and at 4°C, there was appreciable accumulation of digoxin by the cells even though binding of digoxin to the cell membrane was abolished.

Fig. 2 illustrates digoxin accumulation and binding as a function of the digoxin concentration in the incubation medium. Digoxin binding by the erythrocyte membrane appeared to be saturable because there was a digoxin concentration above which no further increase in

digoxin binding was observed (also see Fig. 4). A double-reciprocal plot of these data gave a straight line with intercepts on the x - and y -axis that were significantly different from zero (also see Fig. 3). As demonstrated previously for ouabain (10), digoxin binding to the erythrocyte membrane can be related to digoxin concentration by the equation:

$$A_B = \frac{B_{\max}[A]}{K_B + [A]}$$

where A_B is the amount of digoxin bound, $B_{\max}$ is maximum digoxin binding, $[A]$ is the digoxin concentration in the incubation medium, and K_B is the digoxin concentration at which binding is half-maximal. As observed previously for ouabain (10), values for digoxin binding at digoxin concentrations ranging from 10^{-9} to 10^{-8} M indicated that only one class of binding sites is involved in this reaction.

Addition of 10^{-8} M ouabain to the incubation medium abolished $[^3\text{H}]$ digoxin binding to the erythrocyte membrane (Fig. 2). A similar abolition was observed when $[^3\text{H}]$ digoxin binding was measured in the presence of

FIGURE 2 Total accumulation and membrane binding of digoxin by human erythrocytes as a function of digoxin concentration. Cells were incubated in the presence of various concentrations $[^3\text{H}]$ digoxin for 3 h. Closed boxes and closed circles represent total accumulation and membrane binding of digoxin, respectively, in the standard incubation medium. Open boxes and open circles represent total accumulation and membrane binding of digoxin, respectively, in the presence of 10^{-8} M ouabain. Each point represents the mean of four experiments.

FIGURE 3 Double-reciprocal plots of the binding of digoxin (open circles) and of ouabain (closed circles) as a function of their respective concentrations. Erythrocytes were incubated in the presence of various concentrations of $[^3\text{H}]$ glycoside for 3 h. Each point represents the mean of three experiments.

10^{-4} M nonradioactive digoxin. Fig. 3 illustrates a double-reciprocal plot of the binding of ouabain and of digoxin to the erythrocyte membrane as a function of their respective concentrations. The slope of the line for digoxin was significantly greater than that for ouabain; however, both plots had the same intercept on the y -axis. The concentration at which binding was half-maximal (K_B) was $7.8 (\pm 0.9) \times 10^{-9}$ M (mean $\pm$ SD) for ouabain and $15.0 (\pm 1.3) \times 10^{-9}$ M for digoxin. For both glycosides, the value for maximal binding ($B_{\max}$) was 18.3 ± 2.4 pmol/ml cells (i.e., approximately 1,000 molecules/cell). These data agree with previous findings that ouabain and digoxin bind to the same site on the erythrocyte membrane, and that the affinity of the membrane for ouabain is greater than that for digoxin (10). Our value for maximal binding is higher than that reported by others (7-9) and as we have discussed previously (10), the major source of this discrepancy is probably attributable to differences in the method for extracting bound radioactivity from the cells, in the composition of the liquid scintillation solution and in the procedure used to correct for variable counting efficiency.

At all concentrations of digoxin studied, values for total cellular accumulation were significantly greater than those for binding to the erythrocyte membrane (Fig. 2). Furthermore, the shape of the curve describing digoxin accumulation as a function of digoxin concentration was different from that obtained for digoxin binding. The values for digoxin accumulation did not plateau at higher digoxin concentrations but instead, increased in a linear fashion (also see Fig. 4). Addition

of 10^{-3} M ouabain to the incubation medium reduced, but did not abolish cellular accumulation of digoxin (Fig. 2), and under these conditions there was a linear relation between digoxin accumulation and digoxin concentration. Similar values were obtained when [^{3} H]-digoxin accumulation was measured in the presence of 10^{-4} M nonradioactive digoxin. In the presence of 10^{-3} M ouabain and using a 10-fold greater digoxin concentration range, there remained a linear relation between digoxin accumulation and digoxin concentration whereas under the same conditions, there was negligible binding of digoxin to the erythrocyte membrane.

The experiments thus far presented, demonstrate that total cellular accumulation of digoxin is significantly greater than binding of digoxin to the erythrocyte membrane. Because the radioactivity which was accumulated in excess of that bound to the erythrocyte membrane was a linear function of the [^{3} H]digoxin concentration in the incubation medium, it may represent binding of digoxin to a site so abundant or with so low an affinity for the glycoside that it cannot be saturated over the range of digoxin concentrations used. However, because this linear component of [^{3} H]-digoxin accumulation was not detected using the filter technique, we have assumed that this component represents uptake of digoxin across the erythrocyte mem-

TABLE I
Specificity of Digoxin Binding and Uptake
by Human Erythrocytes

	[^{3} H]Digoxin	
	Bound	Uptake
	% of control	
Control	100 $\pm$ 11	100 $\pm$ 17
Digitoxin	3 $\pm$ 0.6*	97 $\pm$ 16
Digitoxose	106 $\pm$ 7	114 $\pm$ 24
Digitoxigenin	4 $\pm$ 2.5*	107 $\pm$ 18
Ouabain	3 $\pm$ 1.0*	—
Digoxin	2 $\pm$ 1.8*	93 $\pm$ 8
Progesterone	103 $\pm$ 6	98 $\pm$ 8
Cortisone	102 $\pm$ 3	97 $\pm$ 12
Hydrocortisone	97 $\pm$ 11	92 $\pm$ 26
17- β -Estradiol	102 $\pm$ 8	100 $\pm$ 25

Human erythrocytes were incubated for 3 h (37°C) with [^{3} H]digoxin (8×10^{-8} M). Each incubation tube also contained ethanol 0.5% (vol/vol). The uptake of [^{3} H]digoxin across the plasma membrane into the cells was estimated by measuring total cellular accumulation of [^{3} H]digoxin in incubation medium containing ouabain (10^{-3} M). All test substances were present at 5×10^{-5} M. Each value represents the mean of 3 experiments $\pm$ 1 SD.

* $P < 0.01$ using Student's t test (18).

FIGURE 4 Total accumulation and membrane binding of digoxin and simultaneously determined potassium influx on cells incubated in various concentrations of digoxin. Erythrocytes were incubated for 3 h at 37°C with various concentrations of [^{3} H]digoxin after which time total cellular accumulation (open circles) and membrane binding (closed circles) of digoxin were determined. ^{42} K was then added to each incubation tube and potassium influx (boxes) was determined during the subsequent 40 min. The mean potassium concentration in the incubation solution at the end of the influx period was 11.3 mM. Each point represents the mean of three experiments.

brane into the cell. Since 10^{-3} M ouabain abolishes digoxin binding to the cell membrane, the accumulation of digoxin by the cell in the presence of 10^{-3} M ouabain can be used as a measure of digoxin that has been taken up into the cell. The data in Table I summarize the results of experiments designed to test the specificity of the two processes by which erythrocytes accumulate digoxin. [^{3} H]Digoxin binding was inhibited by the cardioactive steroids digoxin, ouabain, digitoxin, and digitoxigenin, but not by other structurally similar steroids or digitoxose. In contrast, none of the tested steroids or digitoxose altered the uptake of [^{3} H]digoxin into the cell.

Fig. 4 illustrates cellular accumulation and binding of digoxin as a function of digoxin concentration and values for potassium influx measured on the same cells. There was a good correlation between the decrease in potassium influx and values for digoxin binding to the erythrocyte membrane, but not between the decrease in potassium influx and values for cellular digoxin

accumulation. The digoxin concentration where digoxin binding was half-maximal ($2.2 [\pm 0.3] \times 10^{-8}$ M) was not significantly different from the digoxin concentration where inhibition of potassium influx was half-maximal ($2.3 [\pm 0.2] \times 10^{-8}$ M). In Fig. 5, glycoside-sensitive potassium influx is plotted as a function of glycoside-binding to the erythrocyte membrane. The line describing this relation for digoxin was not significantly different from that for ouabain indicating that although these two glycosides are bound with different affinities, once they have been bound to the cell membrane they are equipotent in their inhibition of potassium influx.

We previously demonstrated that the affinity with which the erythrocyte membrane binds ouabain is increased by addition of sodium ions to the incubation solution and is decreased by addition of potassium ions (10). We observed similar effects of sodium and potassium on digoxin binding, but observed no effects of these cations on digoxin uptake across the plasma membrane into the cell (Table II).

Fig. 6 illustrates the time course of the loss of digoxin that had been accumulated by the cell and the

FIGURE 5 Glycoside-sensitive potassium influx as a function of glycoside binding to the erythrocyte membrane. Erythrocytes were incubated for 3 h with various concentrations of [^{3} H]ouabain or [^{3} H]digoxin after which time binding of radioactivity to the erythrocyte membrane was determined. ^{42} K was then added to each incubation tube and potassium influx determined. The mean potassium concentration at the end of the influx period was 13.6 mM. Values for glycoside-sensitive potassium influx are expressed as the fraction of potassium influx which was inhibitable by 10^{-8} M ouabain. Values for glycoside binding are expressed as the fraction of the total amount of glycoside which can be bound by the cell. Closed and open circles represent values for ouabain and digoxin, respectively. This experiment is representative of three others.

TABLE II
Effect of Extracellular Cations on Uptake and Binding of Digoxin by Human Erythrocytes

Incubation medium	Digoxin	
	Bound	Uptake
	pmol/ml cells	
Na, 150 mM	19.2 $\pm$ 2.1	31.6 $\pm$ 4.4
Choline, 150 mM	7.0 $\pm$ 0.8	30.9 $\pm$ 3.2
K, 20 mM; choline, 130 mM	4.0 $\pm$ 0.6	32.5 $\pm$ 4.0

Erythrocytes were incubated for 2 h (37°C) with [^{3} H]digoxin (1×10^{-7} M). The uptake of [^{3} H]digoxin across the plasma membrane into the cell was estimated by measuring total cellular accumulation of [^{3} H]digoxin in incubation solutions containing ouabain (10^{-8} M). All values represent the mean of six experiments ± 1 SD.

loss of digoxin that was bound to the cell membrane. Results are also given for potassium influx determined on the same cells. Bound digoxin was released from the erythrocytes at a constant, relatively slow rate (2.7%/h). In contrast, the loss of total cellular digoxin was composed of two components: an initial rapid component that was complete in approximately 10 min followed by a slower component that was identical with the line describing the values for bound digoxin. The values for potassium influx increased steadily during the incubation period and their time course appeared to correlate with the decrease in membrane-bound digoxin. In particular, there was no detectable, initial rapid increase in potassium influx to correspond to the initial rapid loss of digoxin that had been accumulated by the cells. When the same experiment was performed using [^{3} H]ouabain, the loss of cellular ouabain was attributable entirely to the decrease in ouabain bound to the cell membrane, and the rate at which bound ouabain was lost (7.1%/h) was significantly greater ($P < 0.01$) than that at which bound digoxin was lost.

The initial loss of total cellular digoxin was so rapid that a substantial amount of digoxin was probably lost from the cells during the sequential washing and re-suspension steps used to remove extracellular digoxin before determining the amount of radioactivity accumulated by the cells. To explore this possibility, experiments were done in which the loss of digoxin accumulated by the cells was determined by measuring the appearance of [^{3} H]digoxin in the supernate after centrifuging a portion of incubation mixture at 10,000 *g* for 10 s. At 37°C there was a rapid appearance of digoxin in the supernate and this process was complete in approximately 6 min. Using this technique the values obtained for the amount of digoxin lost from the cells were significantly greater than those obtained

FIGURE 6 Loss of digoxin from erythrocytes and simultaneously determined potassium influx. Erythrocytes were preincubated with [^3H]digoxin (1.7×10^{-7} M) for 3 h (37°C), washed rapidly three times with iced incubation medium and, resuspended in fresh incubation solution which initially contained no radioactivity. At appropriate times total radioactivity present in the cells (open circles), and the radioactivity bound to the cell membrane (closed circles) was determined. Samples of the incubation mixture were also taken and added to tubes containing ^{42}K for determination of potassium influx over a 15 min period (boxes.) Potassium influx was also determined on a sample of cells taken from the preincubation mixture just before washing the cells. Potassium influx was 2.51 mmol/liter cells per h in cells preincubated without digoxin and this value did not change significantly during the incubation period. The mean potassium concentration in the medium for potassium influx was 13.7 mM. Each point represents the mean of four experiments.

by measuring the disappearance of radioactivity from the cells using the wash technique. Reducing the temperature of the incubation medium produced a progressive decrease in the rate of appearance of radioactivity in the supernate; however, at 4°C there was still a substantial loss of radioactivity from the cells. In contrast, when the same experiment was performed using [^3H]ouabain, no appreciable radioactivity could be detected in the supernate during the 40 min incubation period. When the temperature of the incubation medium was reduced from 37° to 22°C or to 4°C , there was no significant loss of digoxin bound to the cell membrane.

The initial rapid loss of radioactivity from erythrocytes, which had been preincubated with [^3H]digoxin (even when the cells were cooled to 4°C), indicates that the values for total cellular accumulation of digoxin are underestimates of the actual amount of digoxin in the cell at the time the sample was taken. Efforts were made to make constant the total time elapsing between the time a sample was taken from the incubation mixture and the time when the supernate was aspirated after the fourth wash (approximately 3 min); however, relatively small differences in the time required to process the samples could result in relatively large differences in the values for radioactivity present in the cell. These qualifications do not apply to our values for glycoside binding to the erythrocyte membrane since the amount of radioactivity lost during the 3 min period required to process the samples was negligible.

It seemed possible that the initial rapid loss of radioactivity, which had been taken up by the cells, might represent the release from the [^3H]digoxin of tritium (or a tritiated portion of the molecule) that was able to cross the erythrocyte membrane rapidly. To evaluate this possibility, we tested the ability of the radioactivity, which was lost from the cells during the initial portion of the incubation period, to bind to the membrane of fresh erythrocytes. The results in Table III indicate that the radioactivity lost to the supernate from cells preincubated with [^3H]digoxin could be bound to the erythrocyte membrane to the same extent as the stock

TABLE III
*Binding of Radioactivity Released
from Human Erythrocytes*

Source of radioactivity	Binding
	cpm/ml cells
Supernate	$17,228 \pm 1,803$
Supernate + ouabain	239 ± 176
Stock [^3H]digoxin	$16,574 \pm 1,503$
Stock [^3H]digoxin + ouabain	293 ± 136

Cells were incubated for 2 h in the presence of [^3H]digoxin (3.6×10^{-7} M) and washed three times with iced incubation medium. The cells were reincubated for 20 min at 37°C in fresh incubation medium that was originally free of radioactivity. The cells and supernate were then separated by centrifugation and fresh, washed erythrocytes were added to a portion of the supernate. After incubation for 60 min at 37°C in the presence and absence of ouabain (10^{-3} M), binding of radioactivity to the erythrocyte membrane was determined. For comparison fresh, washed cells were also incubated with an equivalent amount of radioactivity from the stock [^3H] digoxin both with and without ouabain (10^{-3} M). The concentration of radioactivity in each tube was 32,500 ($\pm 1,083$) cpm/ml (i.e., 9×10^{-9} M). The values given represent the mean of four experiments ± 1 SD.

FIGURE 7 Effect of digoxin-specific antiserum on the time course of total cellular accumulation of digoxin by human erythrocytes. The digoxin concentration in the incubation medium was 1.9×10^{-7} M. Antiserum was added to the incubation solution containing [^{3} H]glycoside just before the addition of erythrocytes. Closed circles represent values obtained in the standard incubation medium. Open circles represent values obtained in the presence of control antiserum. Boxes represent values obtained in the presence of digoxin-specific antiserum. Each point represents the mean of three experiments.

[^{3} H]digoxin (i.e., radioactivity that had not been previously exposed to erythrocytes). Furthermore, this binding was specific in the sense that it was blocked by the addition of 10^{-3} M ouabain to the incubation medium.

Fig. 7 illustrates the effect of digoxin-specific antiserum on the time course of total cellular accumulation of [^{3} H]digoxin by human erythrocytes. Addition of control antiserum to the incubation medium produced no detectable effect on the time course of digoxin accumulation. Digoxin-specific antiserum abolished cellular accumulation of digoxin. Similarly, digoxin-specific antiserum completely blocked binding of [^{3} H]digoxin to the erythrocyte membrane, while control antiserum had no effect. Potassium influx measured from the same incubation tubes showed a progressive decrease during the incubation period and this decrease was abolished by digoxin-specific antiserum, but not by control antiserum.

Fig. 8 illustrates the effect of digoxin-specific antiserum on the time course of the loss of total cellular digoxin that had been accumulated. The initial rapid component was complete by approximately 10 min and was not altered by digoxin-specific antiserum; the slower component, which represents the release of bound digoxin, was significantly more rapid in the presence of digoxin-specific antiserum. In the presence

of control antiserum, bound digoxin was lost at a rate of $1.7 (\pm 0.1)\%/h$ (mean ± 1 SD) and in the presence of digoxin-specific antiserum, the rate was $4.1 (\pm 0.4)\%/h$. There was no initial rapid increase in potassium influx to correspond to the rapid initial loss of digoxin accumulated by the cells and the accelerated increase in potassium influx that occurred in the presence of digoxin-specific antiserum correlated with the effect of the antiserum on the loss of digoxin from the cell membrane.

FIGURE 8 Effect of digoxin-specific antiserum on the loss of digoxin that had been accumulated by erythrocytes and simultaneously determined potassium influx. Erythrocytes were preincubated with [^{3} H]digoxin (3.1×10^{-7} M) for 3 h (37°C), washed rapidly with iced incubation medium, and resuspended in fresh incubation solution that initially contained no radioactivity. At appropriate times the cellular radioactivity was determined on cells incubated with control antiserum (closed boxes) and on cells incubated with digoxin-specific antiserum (open boxes). Samples of incubation mixture were also taken and added to tubes containing ^{42}K for determination of potassium influx over a 15 min period. Potassium influx was also determined on a sample of cells taken from the preincubation mixture just before washing the cells. Potassium influx was 2.86 mmol/liter cells per h in erythrocytes preincubated without digoxin and this value did not change significantly during the incubation period. The mean potassium concentration in the influx tubes was 13.9 mM. Closed and open circles represent potassium influx with control antiserum and digoxin-specific antiserum, respectively. Each point represents the mean of two experiments.

TABLE IV
Effect of Digoxin-Specific Antiserum on Digoxin Release and
Potassium Influx in Human Erythrocytes

Incubation	Digoxin		Potassium influx
	Total accumulation	Bound	
	<i>pmol/ml cells</i>		<i>mmol/liter cells per h</i>
Standard incubation solution			
15 min	38.3±4.1	19.4±1.4	0.72±0.05
5 h	37.6±3.8	19.2±1.0	0.75±0.06
Control antiserum			
15 min	37.5±4.0	20.1±1.6	0.76±0.03
5 h	38.8±2.9	18.8±2.0	0.72±0.08
Digoxin-specific antiserum			
15 min	19.3±2.1*	19.8±2.0	0.80±0.07
5 h	14.1±1.3*†	13.9±0.8*†	1.36±0.14*†
Ouabain			
15 min	39.1±4.6	20.4±1.7	0.74±0.10
5 h	33.5±2.8*†	13.6±1.2*†	0.79±0.08

Erythrocytes were incubated for 3 h in the standard incubation solution at 37°C with [³H]digoxin (1.28×10^{-7} M). Control antiserum, digoxin-specific antiserum, or ouabain (10^{-3} M) was added to three of the four tubes. 15 min and 5 h after these additions total cellular digoxin accumulation, the amount of digoxin bound to the cell membrane and potassium influx were determined. Potassium influx was determined over a 20 min period and the mean potassium concentration in the incubation medium used to measure influx was 9.8 mM.

Each value represents the mean of five experiments.

* Significantly different from the corresponding value determined in standard incubation solution ($P < 0.01$ using Student's *t* test [18]).

† Significantly different from the corresponding value determined in the same incubation medium at 15 min ($P < 0.01$).

To explore further the mechanism by which digoxin-specific antiserum accelerated the loss of digoxin bound to the erythrocyte membrane, the effects of adding ouabain (10^{-3} M) were compared with those produced by adding digoxin-specific antiserum. The initial rapid loss of total cellular digoxin accumulation was not altered detectably by addition of control antiserum, digoxin-specific antiserum, or ouabain (10^{-3} M) to the incubation medium. However, both ouabain and digoxin-specific antiserum, but not control antiserum, produced an equivalent increase in the rate of loss of membrane-bound digoxin. In similar experiments using [³H]ouabain, there was no detectable effect of ouabain (10^{-3} M) on the rate at which radioactivity was lost from the cell membrane. The experimental conditions were then modified as follows: erythrocytes were preincubated with [³H]digoxin for 3 h (37°C) and washed rapidly three times with iced, standard incubation medium (containing no glycoside); the cells were incubated with digoxin-specific antiserum for 20 min (37°C) during which time membrane-bound digoxin remains

unchanged, but intracellular digoxin passes out of the cells into the incubation solution where it can combine with antibodies (see Figs. 6 and 8 and Table IV); the erythrocytes were again washed rapidly three times to remove the antiserum and antibody-bound glycoside and resuspended in the appropriate incubation solutions; total cellular digoxin and membrane-bound digoxin were determined at hourly intervals for the subsequent 4 h. Under these conditions, in the standard incubation medium, the rate at which bound digoxin was lost from the erythrocyte membrane ($4.2 \pm 0.8\%/h$) was not significantly different from that in media containing control antiserum (3.7 ± 0.5), digoxin-specific antiserum (4.3 ± 0.6), or ouabain, 10^{-3} M (3.9 ± 0.7). Appropriate control experiments indicated that the 20 min incubation with digoxin-specific antiserum significantly increased ($P < 0.01$) the observed rate at which digoxin was lost when the cells were subsequently incubated in the standard incubation medium or with control antiserum but did not significantly alter values obtained with ouabain or with digoxin-specific antiserum.

Fig. 9 illustrates values for cellular digoxin accumulation when digoxin-specific antiserum was added at different times after the incubation began. Addition of digoxin-specific antiserum to the incubation medium produced an initial rapid decrease in total cellular digoxin after which it decreased at a more gradual rate. Furthermore, the values for cellular digoxin accumulation obtained initially after addition of digoxin-specific antiserum were progressively greater as the addition of the antibody was made at later times during the incubation. Determination of digoxin bound to the erythrocyte membrane from these same incubation tubes showed that the initial rapid decrease in cellular radioactivity represented loss of [^3H]digoxin, which had been taken up across the membrane into the cell, and that after addition of digoxin-specific antiserum, values for total cellular accumulation of digoxin were not significantly different from those for digoxin bound to the erythrocyte membrane. Addition of control antiserum did not detectably alter total cellular digoxin accumulation or membrane-bound digoxin.

The experiments summarized in Table IV further explore the effects of adding digoxin-specific antiserum to incubation medium containing cells incubated with [^3H]digoxin for 3 h. Adding control antiserum to the incubation medium produced no significant change in the values for digoxin accumulation, digoxin binding, or potassium influx determined 15 min and 5 h after the addition was made. 15 min after adding digoxin-specific antiserum to the incubation medium, values for total cellular accumulation of digoxin were significantly lower than corresponding values obtained in the presence of buffer or control antiserum. However, at this time, no significant effect of digoxin-specific antiserum was observed on digoxin binding or on potassium influx. Furthermore, 15 min after adding digoxin-specific antiserum, the value for total cellular accumulation was not significantly different from that for digoxin binding determined on the same cells. 5 h after adding digoxin-specific antiserum, values for digoxin accumulation and digoxin binding were significantly lower than corresponding values obtained after 15 min from the same incubation tube. Values for potassium influx at 5 h were significantly greater than values from the same incubation tube obtained after 15 min. After 5 h, values for total cellular accumulation of digoxin were not significantly different from those for digoxin binding.

In contrast to the effect of digoxin-specific antiserum, 15 min after adding ouabain (10^{-8} M) to the incubation medium, values for cellular accumulation and binding of digoxin as well as for potassium influx were not significantly different from corresponding control values. 5 h after adding ouabain, values for total cellular accumulation of digoxin were significantly lower than

FIGURE 9 Effect on total cellular digoxin accumulation of adding digoxin-specific antiserum at various times during the incubation period. Human erythrocytes were added to eight tubes containing the standard incubation medium (37°C) and [^3H]digoxin (1.1×10^{-7} M). At the times indicated by the vertical arrows at the top of the figure control antiserum or digoxin-specific antiserum was added to each of two incubation tubes. Open circles represent values for total cellular accumulation of digoxin by erythrocytes from the tubes to which digoxin-specific antiserum was added. Closed circles represent values from tubes to which control antiserum was added. Addition of control antiserum did not detectably alter values for total digoxin accumulation. This experiment is representative of two others.

values from the same incubation tube at 15 min, but were significantly greater ($P < 0.01$) than values for total accumulation obtained 5 h after adding digoxin-specific antiserum. Values for digoxin binding obtained 15 min after adding ouabain were not significantly different from control values; however, values obtained 5 h after adding ouabain were significantly lower than corresponding control values obtained after 15 min. These values were also significantly lower ($P < 0.01$) than values for total digoxin accumulation measured on the same incubation tube. Adding ouabain also produced no detectable change in potassium influx at 15 min or at 5 h. After adding digoxin-specific antiserum the magnitude of the decrease in total cellular digoxin accumulation between 15 min and 5 h was similar to that for digoxin binding during the same time period. Similarly, after adding ouabain, the magnitude of the decrease in total cellular digoxin accumulation between 15 min and 5 h was similar to that for

digoxin binding during the same time period. Furthermore, the magnitude of the decreases in total cellular digoxin accumulation and in digoxin binding produced by digoxin-specific antiserum was similar to that produced by ouabain.

DISCUSSION

The present experiments indicate that digoxin accumulation by intact human erythrocytes is the result of two processes. A portion of the accumulated digoxin appears to be clearly attributable to binding of digoxin molecules to the plasma membrane since this accumulation was detected by incubation of erythrocytes with [^3H] digoxin, hemolysis, and collection of the erythrocyte membranes by filtration. Values for accumulation of [^3H] digoxin by human erythrocytes, obtained by repeated washing of the cells, were significantly greater than those obtained with the filter technique. The difference between values obtained with these two procedures cannot be attributed to trapping of extracellular radioactivity with the wash technique. Furthermore, this "extra" radioactivity could be bound specifically to the erythrocyte membrane to the same extent as the original stock [^3H]digoxin (Table III) and its accumulation was abolished by addition of digoxin-specific antiserum to the incubation medium. These findings indicate that the radioactivity accumulated in excess of that bound to the erythrocyte membrane does not represent the accumulation of tritiated contaminants or of tritiated metabolites of [^3H]digoxin and instead, represents uptake of digoxin across the membrane into the cell. One unlikely possibility, which the present studies do not exclude, is that the two processes by which erythrocytes accumulate digoxin reflect the existence of two populations of erythrocytes: one binds digoxin molecules to the erythrocyte membrane and the other takes up digoxin across the plasma membrane into the cell.

Digoxin binding by the erythrocyte membrane reaches a steady state by approximately 80 min and digoxin once bound is lost from the membrane at a relatively slow rate. Digoxin binding is increased by sodium and decreased by potassium in the incubation medium, it is abolished by reducing the temperature to 4°C or by high concentrations of cardioactive steroids, involves a single class of glycoside-binding sites, and correlates with inhibition of erythrocyte potassium influx. Digoxin uptake across the membrane into the cell reaches a steady state in approximately 80 min and the digoxin so accumulated leaves the cell at a relatively rapid rate. Digoxin uptake is not altered by varying the sodium or potassium concentrations in the incubation medium, is reduced, but not abolished by lowering the incubation temperature to 4°C, is not affected by either cardioactive or other steroids, is a linear function of the extracellular digoxin

concentration, and does not correlate with inhibition of erythrocyte potassium influx.

In contrast to digoxin, ouabain accumulation by intact human erythrocytes can be accounted for entirely by binding of this glycoside to the plasma membrane. As observed previously, ouabain is a more potent inhibitor of erythrocyte potassium influx than is digoxin in the sense that the ouabain concentration at which inhibition of potassium influx is half-maximal is lower than the digoxin concentration required to produce half-maximal inhibition (19). The present studies demonstrate that the difference in potency observed for these two agents on erythrocyte potassium influx reflects a difference in the affinity with which they are bound to the cell membrane and that once they are bound to the cell membrane, they are equipotent in terms of inhibition of potassium influx (see Figs. 3 and 5).¹

Baker and Willis (9) reported that accumulation of [^3H]ouabain by a variety of cell types was composed of a component that saturated at relatively low ouabain concentrations and a component that increased linearly up to the highest ouabain concentrations examined. These authors postulated that the saturable component represented binding of the glycoside to the cell membrane and that the linear component represented uptake of ouabain into the cell interior. Our findings with [^3H] digoxin agree with the major conclusions of Baker and Willis. However, in contrast to these authors' findings in both human and guinea pig erythrocytes, we have been unable to demonstrate a component of [^3H]ouabain uptake by human erythrocyte that is a linear function of the extracellular ouabain concentration. It is possible that the linear uptake found in both human and guinea pig erythrocytes by Baker and Willis actually represented uptake of a tritiated contaminant or entrapment of extracellular [^3H]ouabain molecules. Using the techniques described in the present paper, there is an apparent component of [^3H]ouabain uptake that is a linear function of the extracellular ouabain concentration (up to 10^{-4} M); however, we found this radioactivity to be attributable entirely to trapped extracellular fluid determined using [^3H]mannitol or [^{14}C]polyethylene glycol as a marker for extracellular fluid. On the other hand, our techniques may not be sufficiently sensitive to allow us to detect a

¹ Although the digoxin concentration, which produced half-maximal digoxin binding (15.0×10^{-9} M), was approximately twice that for ouabain (7.8×10^{-9} M), ouabain dissociated from the erythrocyte membrane two times more rapidly than did digoxin (7%/h vs. 4%/h). Preliminary experiments show that the rate of association of ouabain with the erythrocyte membrane is greater than that for digoxin; however, our data are not sufficient to allow us to determine whether the association rate for ouabain is four times greater than that for digoxin as predicted by the present results.

linear component of [^3H]ouabain uptake that is of relatively small magnitude since, at a given ouabain concentration, the magnitude of this component would be less than 5% of that observed for [^3H]digoxin uptake into the cell at a corresponding digoxin concentration.

In addition to the present results, further evidence that the glycoside receptor of human erythrocytes is located on the plasma membrane is provided by the studies of Dunham and Hoffman (11) who found that erythrocyte membranes "labeled" with [^3H]ouabain and Na, K-dependent ATPase activity (ATP phosphohydrolase EC 3.6.1.3) could be solubilized with sodium dodecyl sulfate and copurified on sucrose density gradient centrifugation. Smith, Wagner, Markis, and Young (20) found that both ouabain and digoxin when coupled to various large protein molecules exerted only negligible effects on ^{86}Rb uptake by human erythrocytes but were fully active in their inhibition of Na-, K-ATPase activity of solubilized erythrocyte plasma membranes. These observations suggested that the glycoside receptor might lie deep within the erythrocyte plasma membrane and that the glycoside-protein complex was too large to allow the glycoside portion of the molecule to reach the receptor.

The data showing that digoxin-specific antiserum will prevent completely accumulation and binding of [^3H]digoxin by human erythrocytes and will prevent the effect of digoxin on erythrocyte potassium influx extend and confirm previous observations of Watson and Butler (5). The present studies also demonstrate that during the initial 15–20 min after addition of digoxin-specific antiserum to cells, which have been preincubated with [^3H]digoxin, there is a rapid loss of radioactivity from the cells, but no detectable change in the amount of digoxin bound to the cell membrane or in potassium influx. If the incubation period is allowed to continue, there is a further, relatively slow decrease in cellular radioactivity that represents the release of digoxin bound to the cell membrane and a corresponding increase in potassium influx. Watson and Butler (5) reported a discrepancy between the time course of digoxin release from erythrocytes and that of recovery of digoxin-inhibited potassium influx. As these authors suggested, this apparent discrepancy can be attributed to their experimental conditions being such that they were unable to detect the fraction of cellular digoxin that is responsible for inhibiting potassium influx, i.e., digoxin which is bound to the erythrocyte membrane. Watson and Butler preincubated erythrocytes with a much higher concentration of [^3H]digoxin (10^{-5} M) than we used in the present studies (10^{-7} M) and at 10^{-5} M digoxin less than 1% of the total digoxin accumulated by the cells would be bound to the cell membrane. Detection of [^3H]digoxin bound to erythrocyte membrane was further minimized by these authors using [^3H]digoxin at a specific activity

that was 1000-fold less (0.01 Ci/mmol) than that used in the present studies (12 Ci/mmol).

The results in Fig. 8 show that addition of digoxin-specific antiserum to cells that had been preincubated with [^3H]digoxin and washed, produced an apparent increase in the release of digoxin that was bound to the erythrocyte membrane. This finding raised the possibility that the antiserum is facilitating the rate at which digoxin molecules move from the plasma membrane to the incubation medium. That is, the antibodies in addition to binding digoxin molecules that are "free" in the incubation medium, might also interact with digoxin molecules that are bound to the membrane and in so doing, facilitate their release.

Erythrocytes which were preincubated with [^3H]digoxin and then washed to remove extracellular radioactivity, contained a substantial amount of [^3H]digoxin that was not bound to the cell membrane. This digoxin, which accumulated in excess of that bound to the cell membrane, left the cells at a rapid rate and accumulated in the supernate from which it could then be bound to the cell membrane. Thus, in Fig. 8, the observed decrease in the amount of [^3H]digoxin bound to the cell membrane underestimated the rate at which digoxin molecules were passing from the cell membrane to the supernate by an amount dependent on the rate at which [^3H]digoxin was moving from the supernate to the cell membrane. In the case of ouabain, there was no significant cellular accumulation of ouabain molecules in excess of those bound to the cell membrane. Therefore, fewer ouabain molecules accumulated in the incubation medium from which they could then bind to the erythrocyte membrane and the observed decrease in the amount of radioactivity bound to the cell membrane more nearly reflected the rate at which ouabain molecules pass from the cell membrane to the incubation medium.

That adding ouabain (10^{-3} M) also produced an apparent increase in the rate at which digoxin was released from the cell membrane and that values obtained with ouabain were the same as those obtained with digoxin-specific antiserum is evidence that digoxin-specific antibodies do not "pry" digoxin molecules off the membrane. Furthermore, if erythrocytes were allowed to accumulate and bind [^3H]digoxin and then preincubated with digoxin-specific antiserum for 20 min to remove [^3H]digoxin which had been taken up across the membrane into the cell, then the rates at which bound digoxin was lost from erythrocytes after they were washed and incubated with buffer or control antiserum were significantly increased (compared with values obtained on cells that were not preincubated with digoxin-specific antiserum) and were the same as the rates for digoxin loss observed in the presence of ouabain or digoxin-specific antiserum. These

findings indicate that the apparent acceleration of the loss of digoxin from the erythrocyte membrane and of the recovery of potassium influx produced by digoxin-specific antiserum (Fig. 8) is attributable to the antibodies forming a complex with the [^{3} H]digoxin which was released from the cells during the initial portion of the incubation period and thereby, preventing this [^{3} H]digoxin from binding to the erythrocyte membrane. Additional evidence against the possibility that digoxin-specific antibodies can directly facilitate the release of bound digoxin molecules from the erythrocyte membrane can be obtained from the studies of Smith et al. mentioned previously (20). Since digoxin-specific antibodies are presumably larger than the glycoside-protein molecules used by Smith et al., they should likewise be unable to reach the glycoside receptor.

Although the present studies indicate that digoxin molecules, which have been taken up across the plasma membrane into the cell can then leave the cell, enter the extracellular solution, bind to the erythrocyte membrane, and inhibit potassium influx, we do not know whether these intracellular digoxin molecules can gain access to the glycoside-binding site directly without first entering the extracellular solution. In fact, we cannot exclude the possibility that digoxin molecules can bind to the erythrocyte membrane only after they have first entered the cell, i.e., that digoxin molecules do not bind directly from the extracellular fluid. Our inability to detect uptake of ouabain across the erythrocyte membrane into the cell argues against, but does not exclude this possibility. Furthermore, Hoffman (21) has reported that strophanthidin, which was incorporated into erythrocyte ghosts at the time of hemolysis, did not inhibit potassium influx, but upon release from the ghosts, it inhibited cation transport in intact erythrocytes when added to the extracellular solutions.

The observed ability of digoxin-specific antibodies to reduce the net amount of [^{3} H]digoxin, which was bound to the cell membrane, and to accelerate the recovery of potassium influx argues against the possibility that the digoxin, which is accumulated in excess of that bound to the cell membrane, actually produces the inhibition of potassium influx and that the recovery of potassium influx, which appears to correlate with the release of bound digoxin from the cell, is actually due to the slow loss of some inhibitory substance whose accumulation is promoted by the digoxin taken up by the cell (5) (see Fig. 8). This correlation of the ability of digoxin-specific antiserum to reduce the amount of bound digoxin and to accelerate the recovery of potassium influx also argues against the recovery of potassium influx being due to the reaccumulation of some necessary substance whose depletion was produced by the digoxin molecules that were

taken up across the erythrocyte membrane into the cell (5).

The mechanism by which digoxin-specific antibodies reverse the effects of digoxin on human erythrocytes can be conceptualized by comparing digoxin-specific antiserum and ouabain in terms of their effects on total cellular digoxin accumulation, digoxin binding, and potassium influx.

After a certain period of time in erythrocytes incubated with [^{3} H]digoxin (of sufficient concentration to occupy all of the glycoside-binding sites with [^{3} H]digoxin), the amount of [^{3} H]digoxin bound to the erythrocyte membrane and the amount taken up across the cell membrane into the cell will become constant. That is, the system will reach a steady state and the rates at which [^{3} H]digoxin molecules move from the intracellular fluid and from the erythrocyte membrane into the incubation solution will be equal to the rates at which [^{3} H]digoxin molecules move from the incubation solution into the cell and onto the erythrocyte membrane, respectively. Addition of excess (at least 1000-fold) ouabain to the incubation solutions alters the system so that it is much more likely that a [^{3} H]digoxin molecule, which dissociates from a membrane-binding site, will be replaced by a ouabain molecule than by another [^{3} H]digoxin molecule. The rate at which [^{3} H]digoxin molecules are replaced by ouabain molecules is determined primarily by the rate at which [^{3} H]digoxin molecules dissociate from their binding sites. Furthermore, occupation of a glycoside-binding site by ouabain will not change potassium influx since once they are bound to the cell membrane, ouabain and digoxin are equipotent in terms of their inhibition of potassium influx (Fig. 5). However, addition of excess ouabain does not alter the probability that a [^{3} H]digoxin molecule, which leaves the intracellular fluid, will be replaced by another [^{3} H]digoxin molecule since uptake of digoxin across the cell membrane into the cell is not altered by excess ouabain.

Under similar conditions, addition of excess digoxin-specific antibodies to the incubation medium will also result in a decrease in the number of [^{3} H]digoxin molecules bound to the erythrocyte membrane. As the case when excess ouabain was added, this decrease is the result of [^{3} H]digoxin molecules dissociating from the erythrocyte membrane and not being replaced by other [^{3} H]digoxin molecules, and the rate of this decrease is determined primarily by the rate at which [^{3} H]digoxin molecules dissociate from the erythrocyte membrane. In contrast to ouabain, which exerted its effect by replacing [^{3} H]digoxin molecules that had dissociated from the glycoside-binding sites, digoxin-specific antibodies act by forming a complex with [^{3} H]digoxin molecules in the extracellular solution and thereby prevent [^{3} H]di-

gouxin molecules, which dissociate from their binding sites, from being replaced by other [³H]digoxin molecules. This complex formation also results in an increase in potassium influx and the rate of this increase, in turn, reflects the rate at which digoxin molecules are dissociating from the erythrocyte membrane. In further contrast to addition of excess ouabain, addition of digoxin-specific antiserum prevents a [³H]digoxin molecule, which leaves the intracellular fluid, from being replaced by another [³H]digoxin molecule and thereby, these antibodies also reduce the amount of [³H]digoxin that is accumulated in excess of that bound to the cell membrane. The rate of this reduction also reflects primarily the rate at which [³H]digoxin molecules pass from inside the cell across the membrane into the extracellular fluid.

ACKNOWLEDGMENTS

We are indebted to Dr. Donald H. Schmidt, Columbia University, for immunization of the sheep; to Doctors Dorothea L. Sawicki and Sam M. Beiser, Columbia University, for the gift of the 1-methyladenosine-bovine serum albumin conjugate; to B. Denise Raynor and William Stevens for their excellent technical assistance; and to Agnes Sady and Blanche Fors for typing the manuscript.

This work was supported in part by U. S. Public Health Service research grants (HL-10608 and HL-05741) and by grants-in-aid from the American Heart Association (72-853) and the New York Heart Association.

REFERENCES

- Schmidt, D. H., and V. P. Butler, Jr. 1971. Immunological protection against digoxin toxicity. *J. Clin. Invest.* **50**: 866.
- Schmidt, D. H., and V. P. Butler, Jr. 1971. Reversal of digoxin toxicity with specific antibodies. *J. Clin. Invest.* **50**: 1738.
- Curd, J., T. W. Smith, J. C. Jatton, and E. Haber. 1971. The isolation of digoxin-specific antibody and its use in reversing the effects of digoxin. *Proc. Natl. Acad. Sci. U. S. A.* **68**: 2401.
- Skelton, C. L., V. P. Butler, Jr., D. H. Schmidt, and E. H. Sonnenblick. 1971. Immunologic reversal of the inotropic effects of digoxin. *J. Clin. Invest.* **50**: 85a. (Abstr.)
- Watson, J. F., and V. P. Butler, Jr. 1972. Biologic activity of digoxin-specific antisera. *J. Clin. Invest.* **51**: 638.
- Mandel, W. J., J. T. Bigger, Jr., and V. J. Butler, Jr. 1972. The electrophysiologic effects of low and high digoxin concentrations on isolated mammalian cardiac tissue: reversal by digoxin-specific antibody. *J. Clin. Invest.* **51**: 1378.
- Hoffman, J. F., and C. J. Ingram. 1968. Cation transport and the binding of T-ouabain to intact human red blood cells. Proceedings of the 1st International Symposium on Metabolism and Membrane Permeability of Erythrocyte and Thrombocytes, Vienna. 420.
- Hoffman, J. F. 1969. The interaction between tritiated ouabain and the Na-K pump in red blood cells. *J. Gen. Physiol.* **54**: 343s.
- Baker, P. F., and J. S. Willis. 1972. Binding of the cardiac glycoside ouabain to intact cells. *J. Physiol. (Lond.)* **224**: 441.
- Gardner, J. D., and T. P. Conlon. 1972. The effects of sodium and potassium on ouabain binding by human erythrocytes. *J. Gen. Physiol.* **60**: 609.
- Dunham, P. B., and J. F. Hoffman. 1970. Partial purification of the ouabain-binding component and of Na,K-ATPase from human red cell membranes. *Proc. Natl. Acad. Sci. U. S. A.* **66**: 936.
- Erlanger, B. F., and S. M. Beiser. 1964. Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. *Proc. Natl. Acad. Sci. U. S. A.* **52**: 68.
- Butler, V. P., Jr., and J. P. Chen. 1967. Digoxin-specific antibodies. *Proc. Natl. Acad. Sci. U. S. A.* **57**: 71.
- Smith, T. W., V. P. Butler, Jr., and E. Haber. 1970. Characterization of antibodies of high affinity and specificity for the digitalis glycoside digoxin. *Biochemistry* **9**: 331.
- Davidsohn, I., and B. B. Wells. 1963. Clinical Diagnosis by Laboratory Methods. W. B. Saunders Co., Philadelphia. 73.
- Gardner, J. D., A. Lapey, A. P. Simopoulos, and E. L. Bravo. 1971. Abnormal membrane sodium transport in Liddle's syndrome. *J. Clin. Invest.* **50**: 2253.
- Sachs, J. R., and L. G. Welt. 1967. The concentration dependence of active potassium transport in the human red blood cell. *J. Clin. Invest.* **46**: 65.
- Bancroft, H. 1962. t tests for small samples. In *Introduction to Biostatistics*. Hoeber and Harper, New York. 172.
- Glynn, I. M. 1964. The action of cardiac glycosides on ion movements. *Pharmacol. Rev.* **16**: 381.
- Smith, T. W., H. Wagner, Jr., J. E. Markis, and M. Young. 1972. Studies on the localization of the cardiac glycoside receptor. *J. Clin. Invest.* **51**: 1777.
- Hoffman, J. F. 1966. The red cell membrane and the transport of sodium and potassium. *Am. J. Med.* **41**: 666.