Br. J. clin. Pharmac. (1979), **8**, 115–124

THE CHARACTERISTICS OF THE BINDING OF 12-α-[^{3}H]-DIGOXIN TO THE MEMBRANES OF INTACT HUMAN ERYTHROCYTES: RELEVANCE TO DIGOXIN THERAPY

A.R. FORD, J.K. ARONSON, D.G. GRAHAME-SMITH & J.A. ROSE

MRC Unit and University Department of Clinical Pharmacology, Radcliffe Infirmary, Oxford OX2 6HE

1 The characteristics of the binding of 12-α-[^{3}H]-digoxin to the membranes of intact human erythrocytes are described.

2 Only one class of binding site can be demonstrated. Binding is time- and temperature-dependent, saturable and slowly reversible; it is inhibited by other cardiac glycosides and by potassium.

3 Pre-incubation with unlabelled digoxin reduces the subsequent binding of 12-α-[^{3}H]-digoxin in stoichiometric fashion.

4 The possible application of the measurement of the binding of 12-α-[^{3}H]-digoxin to the study of biochemical pharmacological events occurring during digoxin therapy is discussed.

Introduction

Plasma digoxin concentrations reflect to some extent the pharmacokinetic behaviour of digoxin in the individual patient and are of value in the diagnosis of toxicity and non-compliance. However the relationship between the concentration of digoxin in the plasma and the pharmacological and therapeutic effects of the drug is not fully understood. We have therefore been measuring some biochemical effects of digoxin on the erythrocytes of patients receiving the drug (in addition to plasma digoxin concentrations) to see how those effects compare with the effects on the heart.

It is generally agreed that the membrane-bound Na^+, K^+-ATPase is responsible for sodium and potassium transport across cell membranes (Glynn & Karlish, 1975). It is known that cardiac glycosides bind specifically to Na^+, K^+-ATPase and inhibit the enzyme in a variety of tissues including erythrocytes (Schwartz, Lindenmayer & Allen, 1975). It has been suggested that Na^+, K^+-ATPase is the pharmacological receptor for cardiac glycosides in the heart (Akera, 1977) and its presence in erythrocytes affords an accessible source of enzyme for the *in vitro* measurement of the *in vivo* effects of cardiac glycosides.

In a previous study (Aronson, Grahame-Smith, Hallis, Hibble & Wigley, 1977) we showed that ^{86}rubidium (^{86}Rb) transport by the erythrocytes of patients with atrial fibrillation fell during the early stages of digoxin therapy and that this inhibition correlated better with the fall in ventricular rate than did the plasma digoxin concentration. This inhibition of ^{86}Rb transport is presumably secondary to inhibition of the membrane-bound erythrocytic Na^+, K^+-ATPase consequent upon the binding of digoxin to the enzyme. We therefore decided to extend the earlier studies to include measurements of the capacity of patients' erythrocytes to bind [^{3}H]-digoxin *in vitro* during therapy with digoxin.

The characteristics of binding of 12-α-[^{3}H]-digoxin to the membranes of human erythrocytes *in vitro* are described in this paper. The complementary clinical studies in which the ability of patients' erythrocytes to bind 12-α-[^{3}H]-digoxin before and during therapy with digoxin has been used as a means of monitoring digoxin therapy are described in a subsequent paper.

Methods

Blood

Venous blood (10 ml) was collected into tubes containing either lithium heparin (Stayne-LH/10), 1 ml 3.8% sodium citrate or 1 ml 1% EDTA, centrifuged at 1500 *g* for 5 min and the plasma separated. The buffy coat was discarded and the red cells washed three times in 112 nM magnesium chloride solution (pH adjusted to 7.4 with Tris HCl buffer) by alternate centrifugation and resuspension at 4°C. The

0306-5251/79/080115-10 $01.00

final centrifugation was continued for 15 min in order to pack the cells as completely as possible (mean haematocrit in 25 samples after removal of last supernatant 96.8 ± 1.9%).

Incubation with 12-α-[^{3}H]-digoxin

Various amounts of both unlabelled digoxin and 12-α-[^{3}H]-digoxin were added to 25 ml Erlenmayer flasks (the final specific activity varied from 0.00179–0.0224 μCi/ng). After evaporation to dryness, which resulted in no loss of radioactivity, 4.5 ml potassium-free Ringer (Aronson *et al.*, 1977) and 0.5 ml washed erythrocytes were added. The red cell suspension was incubated at 37°C for 2 h unless stated otherwise.

Preparation of red cell membranes

After incubation the cells were separated at 4°C by centrifugation at 1500 and washed once with 8 ml 154 mM sodium chloride. The cells were then haemolysed in 8 ml phosphate buffer, pH 8 (473.5 ml 0.3 M disodium hydrogen phosphate plus 26.5 ml 0.2 M sodium dihydrogen phosphate made up to 1 l with distilled water; this stock solution diluted one in twenty with distilled water). The pellet of erythrocyte membranes was collected by centrifugation at 12500 ***g*** and washed three times with 8 ml fresh phosphate buffer by alternate centrifugation at 12500 ***g*** and resuspension. The membrane pellet was then dissolved in 3 ml of a Soluene-350 (Packard)-isopropanol mixture (1:1) and bleached with 0.5 ml 30% hydrogen peroxide. After the addition of scintillant {PCS (Amersham-Searle)-0.5N hydrochloric acid (9:1) or Instagel (Packard)-0.5N hydrochloric acid (9:1)}, the radioactivity was counted in a Wallac 81000 liquid scintillation counter after an interval of at least 6 h to allow chemiluminescence to subside. Quench correction was by the External Standard Channels Ratio method. The [^{3}H]-digoxin bound to the membranes prepared from 0.5 ml cells usually yielded counts within the range 1000–2000 counts/min.

Mathematical methods

Statistical analysis was carried out using methods described in Snedecor & Cochran (1967) and probit analysis as described by Finney (1971). Results are expressed as mean ± 1 s.d. Erythrocytic binding of 12-α-[^{3}H]-digoxin is expressed as pg bound/0.5 ml cells; digoxin concentrations are expressed in ng/ml of the final total incubation mixture. For Scatchard analysis, the amount of [^{3}H]-digoxin bound is expressed as moles of digoxin bound/l incubation fluid to facilitate calculation of dissociation constants.

Results

The results of the binding assay were affected neither by the solution used to wash the cells (isotonic sodium chloride, isotonic magnesium chloride or potassium-free Ringer) nor by the anticoagulant used (lithium heparin, sodium citrate or EDTA).

The degree of red cell [^{3}H]-digoxin binding was unaffected by changes in the haematocrit of the cell suspension within the range 5–20%; the incubations were usually carried out at haematocrit of 10% as described above.

The within-assay coefficient of variation was 5% (twelve measurements on the same sample of red cells). The between-assay coefficient of variation (separate samples from the same individual assayed in duplicate on eight occasions) was 4%.

1. *Cellular distribution of [^{3}H]-digoxin*

It should be stressed that the described technique measures [^{3}H]-digoxin bound to red cell membranes; this is only a small proportion of the total amount of [^{3}H]-digoxin which accumulates in the cell. As an illustration of this, in an experiment on cells from a normal individual the amount of erythrocytic [^{3}H]-digoxin binding to the membranes at a digoxin concentration of 100 ng/ml was 2.5 ng/0.5 ml cells and the intracellular accumulation (measured by counting the radioactivity present in the haemolysate) was 65 ng/0.5 ml cells (i.e. of the total amount accumulated in the cell only 3.7% was bound to the membrane).

2. *Characteristics of the binding of digoxin to erythrocytes*

(a) *Saturability* [^{3}H]-digoxin binding to red cells was saturable, the amount of bound digoxin increasing with increasing initial concentrations of digoxin in the medium and reaching a plateau at a digoxin concentration of about 100 ng/ml (Figure 1).

(b) *Prevention of binding by ouabain* [^{3}H]-digoxin binding was reduced by excess (10^{-3}M) ouabain to 13% (± 7%) of the radioactivity bound in the absence of ouabain at a digoxin concentration of 100 ng/ml. The results which follow refer to the total amount of membrane-bound [^{3}H]-digoxin.

(c) *Rate of [^{3}H]-digoxin binding: dependence on concentration and temperature* The time-course of [^{3}H]-digoxin binding was dependent on the initial digoxin concentration in the medium. Binding was more rapid and equilibrium reached more quickly at high digoxin concentrations (Figure 2).

The rate of digoxin binding was also temperature-

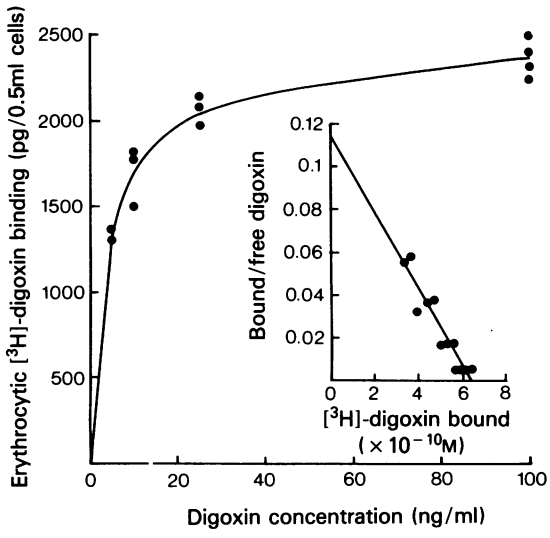


Figure 1 The relationship between the binding of [^{3}H]-digoxin to the membranes of erythrocytes from a normal subject and the initial digoxin concentration in the medium. Inset: The same data presented as a Scatchard plot. The maximum binding was 2470 pg/0.5 ml cells (6.32×10^{-10}M) and the apparent dissociation constant (K_D) was 5.55×10^{-9}M.

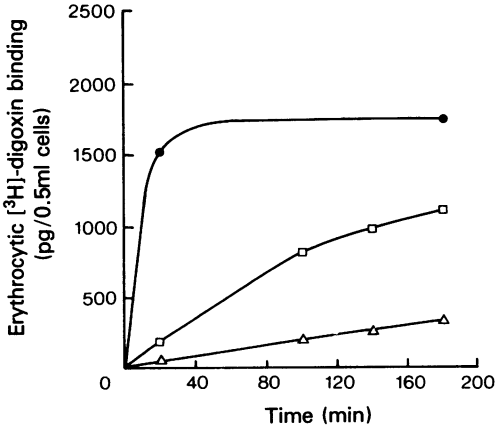


Figure 2 The time course of binding of [^{3}H]-digoxin to erythrocytic membranes at different ligand concentrations (△1ng/ml; □ 5ng/ml; ● 100ng/ml).

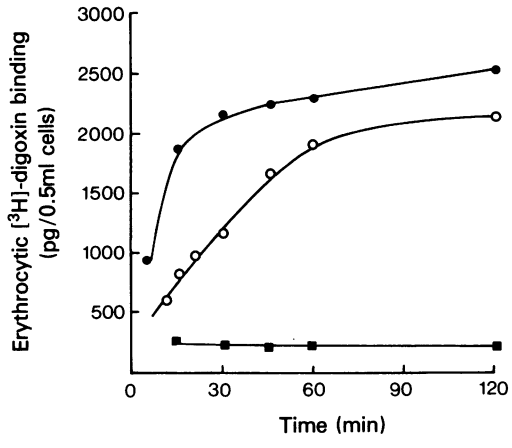


Figure 3 The time course of binding of [^{3}H]-digoxin to erythrocytic membranes at different temperatures (● 37°C; ○ 27°C; ■ 5°C).

dependent (Figure 3). Binding was virtually abolished at 5°C and the rate of binding was reduced at 27°C compared with 37°C (Q_{10} of the initial rate of binding = 3).

(d) *Dissociation of the binding of* [^{3}*H*]*-digoxin* [^{3}H]-digoxin already bound to erythrocytes was not displaced by further incubation with high concentrations of unlabelled digoxin (Figure 4) or with 10^{-3}M unlabelled ouabain (not illustrated).

The dissociation of bound [^{3}H]-digoxin from the membranes of intact red cells was studied in red cells which had been incubated with [^{3}H]-digoxin (100 ng/ml) for 2 h at 37°C. The cells were washed three times in ice-cold isotonic saline and resuspended in potassium-free Ringer containing excess unlabelled ouabain (10^{-3}M) in order to prevent the rebinding of [^{3}H]-digoxin after dissociation. The amount of [^{3}H]-digoxin bound to the red cells was measured at various times thereafter (Figure 5). The dissociation was slow ($T_{\frac{1}{2}} = 17$ h) at 37°C and was inhibited at 5°C.

(e) *The kinetics of* [^{3}*H*]*-digoxin binding to erythrocytes* Although bound [^{3}H]-digoxin cannot be displaced appreciably during a 2 h incubation with unlabelled drug, the reversibility of the binding reaction is demonstrated by the measurable (albeit slow) dissociation of the bound radioligand (Figure 5).

If the binding reaction is described by the following equation:

$$[^3\text{H}]\text{-Digoxin} + \text{Receptor} \rightleftharpoons \underset{\text{complex}}{\text{Receptor-}[^3\text{H}]\text{-Digoxin}}$$

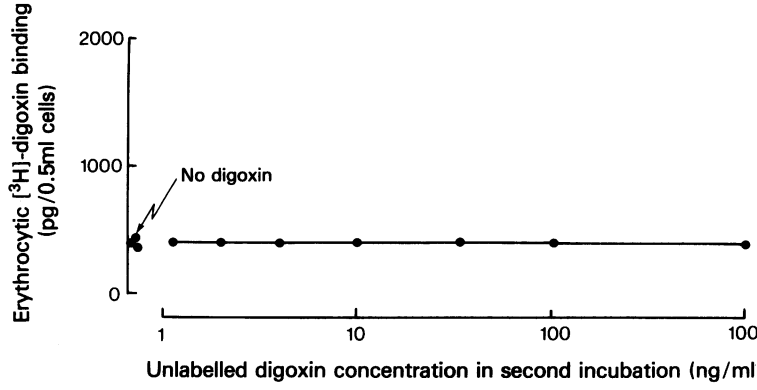


Figure 4 Attempted displacement of [^{3}H]-digoxin from red cells by unlabelled digoxin. Red cells from a normal subject were incubated for 2 h with 2 ng/ml [^{3}H]-digoxin and then washed three times with ice-cold 154 mM NaCl. They were then incubated in potassium-free Ringer containing various concentrations of unlabelled digoxin for 2 h prior to preparation of the membranes for counting.

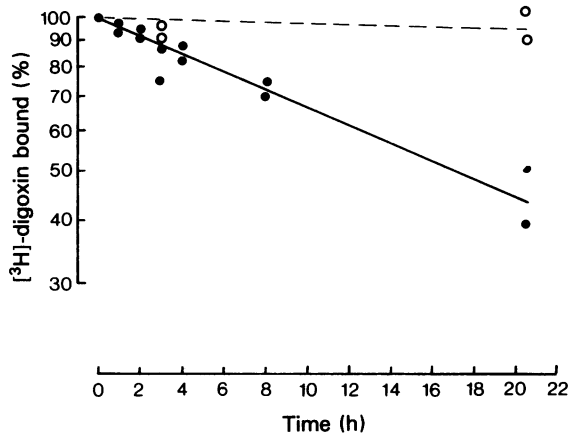


Figure 5 The dissociation of bound [^{3}H]-digoxin from the membranes of intact erythrocytes in a potassium-free Ringer solution containing excess unlabelled ouabain. (○ 5°C; ● 37°C). The amount of [^{3}H]-digoxin bound at any time is expressed as a percentage of the amount bound at the beginning of the incubation.

then the Law of Mass Action predicts that if the plot of bound drug/free drug against bound drug measured at equilibrium (after Scatchard, 1949) yields a straight line then it can be assumed that a single class of binding sites is present. The intercept on the ordinate of such a plot is equal to B_{max}/K_D and the slope of the line is $-1/K_D$ where B_{max} is the maximum amount bound and K_D is the apparent dissociation constant.

Plotting the data for [^{3}H]-digoxin binding to erythrocytes in this way yielded straight lines (see inset to Figure 1) thus suggesting the presence of a single class of binding sites. The maximum amount of [^{3}H]-digoxin bound as calculated from the Scatchard plots is compared with the measured amount of [^{3}H]-digoxin bound at a digoxin concentration of 100 ng/ml in Figure 6. The regression line is not significantly different from the line $y=x$ suggesting that the amount bound at a digoxin concentration of 100 ng/ml gives a good prediction of the maximum [^{3}H]-digoxin binding.

(f) *The effect of potassium on [^{3}H]-digoxin binding* [^{3}H]-digoxin binding to erythrocytes was inhibited by the addition of 4 mM KCl to the Ringer solution (Figure 7). Potassium in this concentration increased the apparent dissociation constant (K_D) by a factor of approximately five, without altering the calculated maximum amount of [^{3}H]-digoxin bound (the intercepts on the abscissa of the Scatchard plot do not differ significantly).

(g) *[^{3}H]-digoxin binding to red cells in the presence of other cardiac glycosides* The presence of other cardiac glycosides in the incubation medium inhibited the binding of 12-α-[^{3}H]-digoxin. Increasing concentrations of inhibitor glycoside were associated with a progressive reduction in [^{3}H]-digoxin binding. The data are shown plotted in probit scale in Figure 8.

The concentration of inhibitor required to reduce [^{3}H]-digoxin binding by 50% (IC_{50}) can be related to the apparent dissociation constant of the inhibitor for

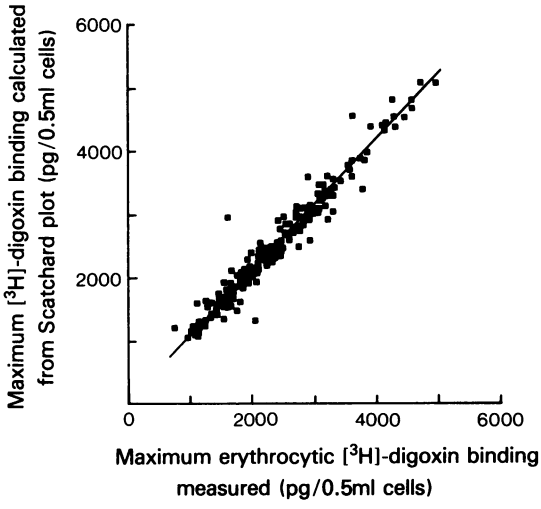


Figure 6 The relationship between the maximum [^{3}H]-digoxin binding calculated from Scatchard plots and the [^{3}H]-digoxin binding measured at a digoxin concentration of 100 ng/ml ($r=0.97$, $P<0.001$). The 189 subjects included normal volunteers, patients not taking digoxin and patients on digoxin therapy.

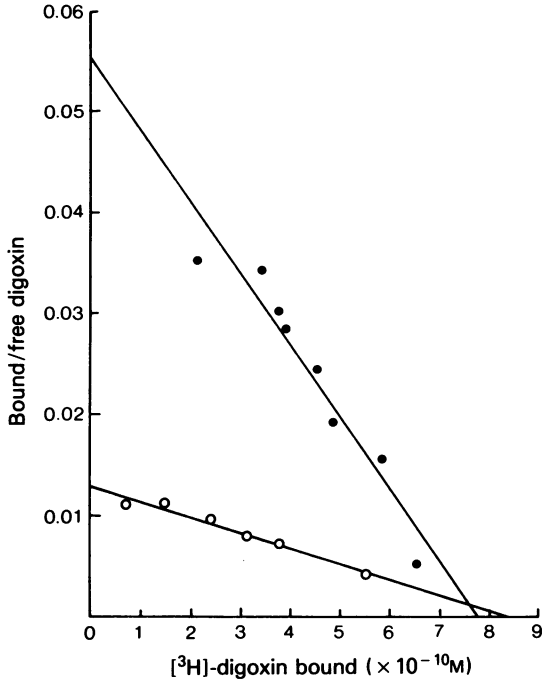


Figure 7 The binding of [^{3}H]-digoxin to normal erythrocytes in the presence (○) and absence (●) of 4 mM KCl. The values of maximum binding were 3181 pg/0.5 ml and 2936 pg/0.5 ml respectively (not significantly different) and the apparent dissociation constants were 5.5×10^{-8}M and 1.1×10^{-8}M respectively.

the glycoside-binding site (K_I) (Cheng & Prusoff, 1973):

$$K_I = IC_{50}\left(1+\frac{[^3H]\text{-digoxin}}{K_D}\right)$$

The values of K_I for unlabelled digoxin, ouabain and dihydrodigoxin were 9.1×10^{-9}M, 3.5×10^{-9}M and 3.3×10^{-7}M respectively.

(h) *The effect of preincubation with unlabelled digoxin upon the subsequent binding of [^{3}H]-digoxin* Figure 9 shows the effect of preincubating erythrocytes from a normal subject with various concentrations of unlabelled digoxin for 2 h. After this preincubation, the cells were washed three times with 154 mM sodium chloride and the binding of [^{3}H]-digoxin measured at a concentration of 100 ng/ml. The amount of [^{3}H]-digoxin bound diminished with increasing concentrations of unlabelled digoxin used in the preincubation, the minimum [^{3}H]-digoxin binding occurring at a preincubation digoxin concentration of 100 ng/ml. This minimum amount of [^{3}H]-digoxin bound was also approximately equal to the amount bound in the presence of 10^{-3}M ouabain after preincubation in the absence of digoxin. Thus incubation with unlabelled digoxin prevents the subsequent binding of [^{3}H]-digoxin.

Figure 10 shows the effect of incubating cells in media containing lower concentrations of unlabelled digoxin (0, 2 or 5 ng/ml) for 2 h, washing the cells three times and then incubating them with various concentrations of [^{3}H]-digoxin. Preincubation with either 2 ng/ml or 5 ng/ml of unlabelled digoxin reduced the maximum amount of [^{3}H]-digoxin bound. These reductions in [^{3}H]-digoxin binding were equal to the amount of [^{3}H]-digoxin bound at 2 ng/ml and 5 ng/ml after preincubation in the absence of digoxin. Figure 10 shows the same data presented as a Scatchard plot. The slopes of the lines are not significantly different and thus the dissociation constant has the same value for each binding curve.

(i) *Binding of [^{3}H]-digoxin to cells of normal individuals* In ten normal individuals the maximum erythrocytic [^{3}H]-digoxin binding was 2528 (±392) pg/0.5 ml red cells and the dissociation constant 9.4 (±2.26) $\times10^{-9}$M. The estimated number of molecules of digoxin bound per cell (calculated from Avogadro's constant and the mean corpuscular volume) was 339±52.

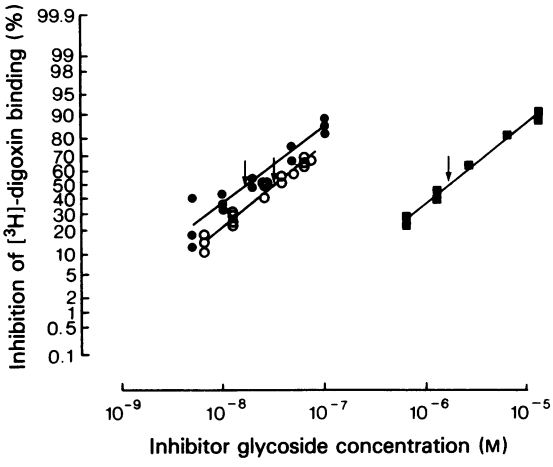


Figure 8 The inhibition of erythrocytic [^{3}H]-digoxin binding by cardiac glycosides (● ouabain; ■ dihydrodigoxin; ○ digoxin). The percentage inhibition of [^{3}H]-digoxin binding shown in probit scale is plotted against the concentrations of inhibitor glycoside. The arrows indicate the concentrations at which 50% inhibition occurred.

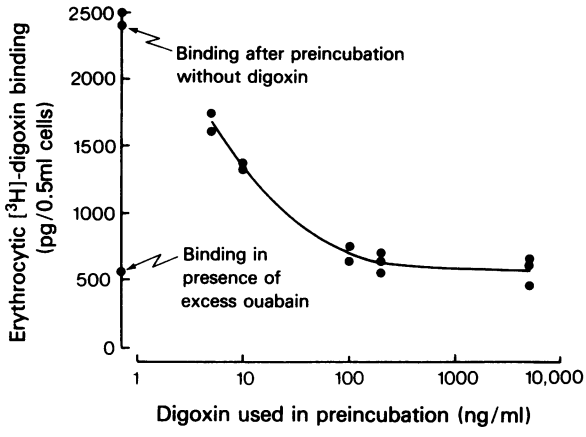
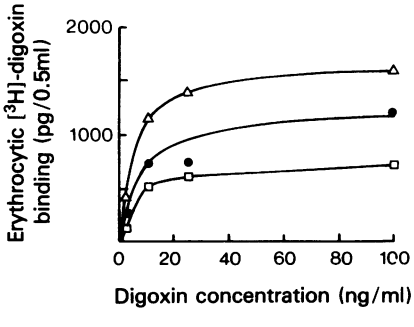


Figure 9 The effect of preincubation with various concentrations of digoxin on the subsequent maximum binding of [^{3}H]-digoxin. Cells from a normal subject were incubated with various concentrations of unlabelled digoxin for 2 h, washed three times with ice cold 154 mM NaCl and then incubated with 100 ng/ml [^{3}H]-digoxin for 2 h. On the ordinate are shown the values of [^{3}H]-digoxin binding both in the absence and presence of excess unlabelled (10^{-3}M) ouabain after a preincubation in the absence of digoxin.



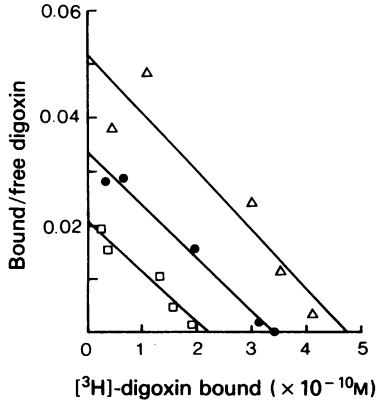


Figure 10 The effect of preincubation with low concentrations of unlabelled digoxin on the subsequent binding of various concentrations of [^{3}H]-digoxin (△ no digoxin; ● 2ng/ml digoxin; □ 5ng/ml digoxin). Erythrocytes from a normal subject were preincubated with 0, 2 or 5 ng/ml unlabelled digoxin for 2 h then washed three times with ice-cold 154 mM NaCl. The cells were then incubated with various concentrations of [^{3}H]-digoxin for 2 h prior to preparation of the membranes for counting. On the left the relationship between [^{3}H]-digoxin binding and the digoxin concentration of the medium in the second incubation is shown for the three groups of cells. On the right the data are plotted after Scatchard (1949). The values of maximum [^{3}H]-digoxin binding were 1850 pg/0.5 ml cells, 1330 pg/0.5 ml cells and 850 pg/0.5 ml cells for the control cells, those preincubated with 2 ng/ml digoxin and those preincubated with 5 ng/ml unlabelled digoxin respectively. The corresponding values for the dissociation constants were 9.4×10^{-9}M, 10.2×10^{-9}M and 10.7×10^{-9}M respectively and these do not differ significantly.

Table 1 Values of maximum glycoside binding, K_D and K_I for normal human erythrocytes in different studies

Source	*Ligand (Concentration used M)*	*Maximum binding*	*K_D (in the absence of K^+) $\times 10^{-9}$ M*	
(1) Ellory & Keynes (1969)	12-α-[^{3}H]-digoxin (10^{-5})	0.39(±0.08) pmol/mg protein ~200 molecules/cell		
(2) Hoffman (1969)	[^{3}H]-ouabain (6×10^{-9})	~200 molecules/cell		
(3) Baker & Willis (1972)	[^{3}H]-ouabain (10^{-5})	260 molecules/cell		
(4) Gardner & Conlon (1972)	[^{3}H]-ouabain (10^{-10} to 10^{-5})	18.8(±1.7) pmol/ml cells ~1200 molecules/cell	K_D ouabain K_I ouabain digoxin digitoxin	6.25 2.96 (±0.49) 3.46 (±0.65) 4.94 (±0.91)
(5) Gardner, Kiino, Swartz & Butler (1973)	[^{3}H]-ouabain (10^{-9} to 10^{-5}) [^{3}H]-digoxin (10^{-9} to 10^{-5})	18.3(±2.4) pmol/ml cells ~1000 molecules/cell	K_D ouabain K_D digoxin	7.8 (±0.9) 15.0 (±1.3)
(6) Erdmann & Hasse (1975)	[^{3}H]-ouabain (up to 4×10^{-8})	228(±28) molecules/cell	K_D ouabain	2.8 (±0.05)
(7) This paper	12-α-[^{3}H]-digoxin (1.3×10^{-7})	2528(±392) pg/0.5 ml cells 6.47(±1.00) pmol/ml cells 339(±52) molecules/cell	K_D digoxin K_I digoxin K_I ouabain dihydrodigoxin	9.4 (±2.3) 9.1 3.5 330 (3.3×10^{-7})

In thirty-eight patients not taking cardiac glycosides the maximum [^{3}H]-digoxin binding was 2677 ($\pm$ 797) pg/0.5 ml cells and the dissociation constant 10.1 ($\pm$ 3.0) $\times 10^{-9}$M. These values are not significantly different from those of the normal individuals.

Discussion

Characteristics of the binding of [^{3}H]-digoxin

The binding of cardiac glycosides to their putative receptor, Na^+, K^+-ATPase, has been studied in detail in several different tissues and species by a number of workers (for references see Schwartz *et al.*, 1975). Glycoside binding to human red cells (see Table 1 for references) has previously been shown to possess the following characteristics:

(a) time and temperature dependency
(b) saturability
(c) reversibility
(d) a single class of binding sites
(e) inhibition by potassium
(f) inhibition by other cardioactive glycosides.

Those findings have been confirmed in this study. The binding of 12-α-[^{3}H]-digoxin to the membranes of intact red cells was found to be saturable (Figure 1), time and temperature dependent (Figure 3) and inhibited by the presence of other cardioactive glycosides (Figure 8) or of potassium (Figure 7). The reversibility of the binding reaction was demonstrated by the measurable dissociation of the bound radioligand (Figure 5). Scatchard analysis yielded a straight line which is compatible with the presence of a single class of binding sites.

Values for maximium glycoside binding and apparent dissociation constant

The values of maximum [^{3}H]-digoxin binding and the apparent dissociation constant (K_D) are compared with the values quoted by other workers for digoxin and other cardiac glycosides in Table 1. One would expect that the maximum number of binding sites would be the same for all glycosides assuming that one mole of receptor binds one mole of glycoside and that the different glycosides have the same receptor binding site. One would not necessarily expect the apparent dissociation constants for the different glycosides to be the same.

With respect to our figures for maximum binding there was good agreement with the data of Ellory & Keynes (1969), Hoffman (1969), Erdmann & Hasse (1975) and Baker & Willis (1972). Gardner and his co-workers (Gardner & Conlon, 1972; Gardner, Kiino, Swartz & Butler, 1973) quote values for maximum glycoside binding three times higher than our values. They note that their values are considerably higher than the figures quoted by other workers and attribute this to 'differences in the method of extracting bound radioactivity from the cells, in the composition of the liquid scintillation mixture and the procedure used to correct for variable counting efficiency'. They used 10% perchloric acid to extract [^{3}H]-ouabain from washed red cells or [^{3}H]-digoxin from filtered red cell membranes and state that perchloric acid gives a higher percentage extraction of labelled glycoside than Bray's solution as used by Hoffman (1969).

In our method the membranes are dissolved in Soluene 350-isopropanol and the method does not depend on extraction of the radioactivity. We have compared our method with the extraction of [^{3}H]-digoxin from washed erythrocyte membranes by 10% perchloric acid and find that the latter does not result in a greater yield of bound counts/min than the former. We cannot explain the high values of maximum binding obtained by Gardner and his co-workers.

The values obtained in these studies of K_D for [^{3}H]-digoxin binding and of K_I for the inhibition of that binding by unlabelled digoxin are of similar magnitude (9.4×10^{-9}M and 9.1×10^{-9}M respectively, Table 1). The values of K_D (15.0×10^{-9}M) quoted by Gardner *et al.* (1973) and of K_I (3.46×10^{-9}M) quoted by Gardner & Conlon (1972) are within the same order of magnitude as our values. In both our studies and those of Gardner and co-workers, incubation times have not exceeded 3 h in experiments involving the measurement of K_D or K_I. However, we have also

Table 2 Values for K_D and K_I of [^{3}H]-digoxin binding and the IC_{50} of ^{86}Rb uptake for three different glycosides in normal human erythrocytes

Glycoside	*K_D (M)*	*K_I (M)*	*IC_{50} for ^{86}Rb Uptake (M)*
Digoxin	9.4×10^{-9}	9.1×10^{-9}	1.24×10^{-8}
Ouabain	—	3.52×10^{-9}	3.7×10^{-9}
Dihydrodigoxin	—	3.3×10^{-7}	7.5×10^{-7}

carried out experiments with incubation times of 12 h and have found that under these conditions maximum binding occurs at digoxin concentrations of less than 10 ng/ml (compare Figure 1) although the *value* for maximum binding is unchanged. Data from these experiments suggest that the value of K_D is $1-2 \times 10^{-9}$M. Nevertheless because the value of maximum binding at a digoxin concentration of 100 ng/ml occurred at 2 h and was no different from that which occurred after longer incubations with lower digoxin concentrations we have carried out our clinical studies (Ford, Aronson, Grahame-Smith & Carver, 1979a,b) using incubation times of two hours. It is not in any case clinically practicable to use longer incubation times particularly in view of the fragility of red cells under these circumstances.

Binding of [^{3}H]-digoxin in the presence of other cardiac glycosides

[^{3}H]-digoxin binding was inhibited by unlabelled digoxin, ouabain and dihydrodigoxin and the values of the K_I for these compounds are given in Table 2. The value of K_I for ouabain was in good agreement with the values of K_I or K_D obtained by other workers (Table 1). In addition, experiments have been carried out in which the inhibition of ^{86}rubidium uptake by normal human erythrocytes by these glycosides was measured. The concentrations of glycoside required to produce 50% inhibition (IC_{50}—Aronson & Grahame-Smith, 1977) are shown in Table 2 and the values are in good agreement with the values of K_I for the corresponding glycoside.

Preincubation with unlabelled digoxin

Figures 9 and 10 show that preincubation with various concentrations of unlabelled digoxin diminishes or prevents the subsequent binding of [^{3}H]-digoxin. This seems likely to be due to occupation of binding sites by the unlabelled digoxin so that these sites are unavailable to [^{3}H]-digoxin in the second incubation. The evidence which supports this is as follows. Firstly, bound 12-α-[^{3}H]-digoxin is resistant to washing and to attempted displacement by unlabelled glycoside. Secondly, in experiments of the type shown in Figure 9 the minimum [^{3}H]-digoxin binding occurred when the concentration of unlabelled digoxin used in the preincubation was 100 ng/ml; this is the concentration at which saturation of digoxin-binding sites occurs (Figure 1). Lastly, in experiments of the type shown in Figure 10, the reductions in maximum [^{3}H]-digoxin binding after preincubation with either 2 ng/ml or 5 ng/ml unlabelled digoxin were equal to the amounts of [^{3}H]-digoxin bound at these concentrations after a preincubation in the absence of digoxin.

Applications of the technique

The technique described above allows the maximum *in vitro* erythrocytic [^{3}H]-digoxin binding to be measured giving a measure of the number of sites available for binding. The experiments in which erythrocytes were preincubated with unlabelled digoxin and then subsequently exposed to [^{3}H]-digoxin show that there is a finite number of glycoside-binding receptors. These receptors could be occupied either with unlabelled digoxin in the first incubation or by [^{3}H]-digoxin in the second incubation. If the maximum [^{3}H]-digoxin binding for cells which had not been exposed to digoxin were known, then following exposure to unlabelled digoxin one could assess the occupancy of the glycoside receptors by measuring the reduction in *in vitro* [^{3}H]-digoxin binding.

If this were to apply to digoxin bound *in vivo* to the erythrocytes of patients receiving the drug, then one would expect that during treatment there would be a reduction in the amount of [^{3}H]-digoxin that could be bound *in vitro* to these cells. Because digoxin binds to erythrocyte membranes, is not easily washed off and results in stoichiometric occupancy of receptor sites the binding technique allows the maximum *in vitro* erythrocytic [^{3}H]-digoxin binding, and thus the number of cardiac glycoside binding sites, to be measured. The results of such measurements in patients on digoxin therapy are described in subsequent papers.

References

AKERA, T. (1977). Membrane adenosinetriphosphatase: a digitalis receptor? *Science*, **198**, 569–574.

ARONSON, J.K. & GRAHAME-SMITH, D.G. (1977). Monitoring digoxin therapy: II. Determinants of the apparent volume of distribution. *Br. J. clin. Pharmac.*, **4**, 223–227.

ARONSON, J.K., GRAHAME-SMITH, D.G., HALLIS, K.F., HIBBLE, A. & WIGLEY, F. (1977). Monitoring digoxin therapy: I. Plasma concentrations and an *in vitro* assay of tissue response. *Br. J. clin. Pharmac.*, **4**, 213–221.

BAKER, P.F. & WILLIS, J.S. (1972). Binding of the cardiac glycoside ouabain to intact cells. *J. Physiol. Lond.*, **224**, 441–462.

CHENG, Y. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmac.*, **22**, 3099–3108.

ELLORY, J.C. & KEYNES, R.D. (1969). Binding of tritiated digoxin to human red cell ghosts. *Nature, Lond.*, *221*, 776.

ERDMANN, E. & HASSE, W. (1975). Quantitative aspects of ouabain binding to human erythrocyte and cardiac membranes. *J. Physiol. Lond.*, **251**, 671–682.

FINNEY, D.J. (1971). *Probit Analysis* 3rd Ed. Cambridge: Cambridge University Press.

FORD, A.R., ARONSON, J.K., GRAHAME-SMITH, D.G. & CARVER, J.G. (1979a). Changes in cardiac glycoside receptor sites, ^{86}rubidium uptake and intracellular sodium concentrations in the erythrocytes of patients receiving digoxin during the early phases of treatment of cardiac failure in regular rhythm and of atrial fibrillation. *Br. J. clin. Pharmac.*, **8**, 125–134.

FORD, A.R., ARONSON, J.K., GRAHAME-SMITH, D.G. & CARVER, J.G. (1979b). The acute changes seen in cardiac glycoside receptor sites, ^{86}rubidium uptake and intracellular sodium concentrations in the erythrocytes of patients during the early phases of digoxin therapy are not found during chronic therapy: Pharmacological and therapeutic implications for chronic digoxin therapy. *Br. J. clin. Pharmac.*, **8**, 135–142.

GARDNER, J.D. & CONLON, T.P. (1972). The effects of sodium and potassium on ouabain binding by human erythrocytes, *J. gen. Physiol.*, **60**, 609–629.

GARDNER, J.D., KIINO, D.R., SWARTZ, T.J. & BUTLER, V.P. (1973). Effects of digoxin-specific antibodies on accumulation and binding of digoxin by human erythrocytes. *J. clin. Invest.*, **52**, 1820–1833.

GLYNN, I.M. & KARLISH, S.J.D. (1975). The sodium pump. *Ann. Rev. Physiol.*, **37**, 13–55.

HOFFMAN, J.F. (1969). Invited discussion. The interaction between tritiated ouabain and the Na-K pump in red blood cells. *J. gen. Physiol.*, **54**, 343s–350s.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660–672.

SCHATZMANN, H.J. (1953). Herzglykoside als Hemmstoffe für den aktiven Kalium-und Natriumstransport durch die Erythrocyten-membran. *Helv. Physiol. Acta*, **11**, 346–354.

SCHWARTZ, A., LINDENMAYER, G.E. & ALLEN, J.C. (1975). The sodium-potassium, adenosine triphosphatase: pharmacological, physiological and biochemical aspects. *Pharmac. Rev.*, **27**, 3–134.

SNEDECOR, G.W. & COCHRAN, W.G. (1967). *Statistical Methods*, 6th edition. Iowa: Iowa State University Press.

(Received September 26, 1978)