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[^{3}H]OUABAIN BINDING AND DISSOCIATION IN RABBIT COLON: EFFECT OF IONS AND DRUGS

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Abstract—^{22}Na fluxes (J) and [^{3}H]ouabain binding were studied *in vitro* in the distal portion of the rabbit colon, mounted as a membrane, separating two perspex chambers. J^{Na}_{MS} (flux of ^{22}Na from mucosal to serosal chamber) was 2.1 and J^{Na}_{SM} was 1.0 μmoles/cm^2 × hr. J^{Na}_{Net} was completely abolished by 10^{-3} M ouabain, placed in the serosal side. J^{Na}_{SM} was unaffected by ouabain. [^{3}H]Ouabain binding to the serosal surface of the colon was inhibited to the same maximal effect by 'cold' ouabain ($I_{50} = 5 \times 10^{-7}$ M), by K^+ placed in the serosal chamber ($I_{50} = 3.5$ mM) and by replacement of Na^+ with choline on the serosal side. Increased tissue concentration of Na^+ did not affect [^{3}H]ouabain binding, suggesting that extracellular rather than intracellular sodium plays a major role in cardiac glycoside binding. Various cardiac glycosides (digoxin, digitoxin, ouabain) inhibited [^{3}H]ouabain binding to the serosal side of the colon but other steroids (estriol, testosterone, desoxycorticosterone) had not effect.

Efflux of [^{3}H]ouabain, bound to the serosal side of the colon, was differentiated into dissociation of nonspecifically bound [^{3}H]ouabain (95 per cent in 60 min) and dissociation of specifically bound [^{3}H]ouabain (20 per cent in 60 min). Dissociation of specifically bound [^{3}H]ouabain was accelerated when Na was replaced by choline (50 per cent in 60 min). The dissociation rate of nonspecifically bound ouabain was unaffected by replacement of Na^+. [^{3}H]Ouabain binding to mucosal surface was unaffected by 'cold' ouabain, by increased K^+ in the medium or by replacement of Na^+. It is concluded that there is no specific binding of [^{3}H]ouabain to the mucosal surface. Omission of Ca, Mg or phosphate from the medium did not affect [^{3}H]Ouabain binding to either mucosal or serosal surfaces of rabbit colon.

The colon is the site of active sodium transport. In cells with 'symmetrical' structure, active sodium transport is transport from the intracellular to the extracellular compartment. This is true of circulating cells (erythrocytes, leucocytes, lymphocytes) or stationary cells like muscle cells or nerve cells. However, in epithelial cells, sodium transport is transcellular, thus enabling movement from a lumen to the serosal surface (intestine, kidney tubule, salivary duct epithelium). In these cells the membrane of the mucosal surface differs from that of the serosal surface.

Active transport in most cells depends on the sodium pump which acts through the enzyme Na,K-ATPase. This enzyme is specifically inhibited by cardiac glycosides and is believed to have a specific binding site for cardiac glycosides (c.g.), serving as 'receptor'. Specific binding of tritiated cardiac glycosides can, therefore, serve to identify and locate the site of the sodium pump.

The rabbit colon provides a convenient preparation where both the effect of cardiac glycosides on the sodium fluxes can be studied *in vitro* and the site of binding and, therefore, site of action of the c.g. can be identified.

The present study reports the effect of various ions and drugs on the specific and nonspecific binding of [^{3}H]ouabain to mucosal and serosal surfaces of the rabbit colon, studied *in vitro* as a membrane, mounted between two perspex chambers. The results of these experiments have been presented in a preliminary report [1].

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MATERIALS AND METHODS

Tissue preparation

Rabbits of either sex, weighing 2–3 kg, were killed by intravenous injection of pentobarbital-sodium. The abdomen was promptly opened by a midline incision and the distal colon was immediately excised, washed thoroughly with cold saline and placed in ice-cold, oxygenated Krebs' solution (composition in mM: NaCl, 118; KCl, 4.7; $MgSO_4$–$7H_2O$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 5 g/l.). The solution was constantly aerated with a mixture of 95% O_2–5% CO_2. The pH was 7.4.

A 3 cm long section of the distal colon was opened by cutting along the mesenteric border. The tissue was mounted as a membrane, separating two perspex chambers (as described by Ussing and Zerahn [2]). The exposed area of the colon was 1.77 cm^2. Twenty-eight millilitres of Krebs' solution were introduced into the chamber facing each side of the colon. The solution was oxygenated, using a mixture of 95% O_2–5% CO_2. The temperature was kept at 37°.

Determination of Na *fluxes*

Transmural fluxes of ^{22}Na from the mucosal chamber to the serosal chamber (J^{Na}_{MS}) and from the serosal chamber to the mucosal chamber (J^{Na}_{SM}), were determined by introduction of the radioisotope (0.07 μCi/ml) either to the chamber facing the serosal side or to the chamber facing the mucosal side of the colon. Preliminary experiments showed that a time lag of 15–30 min was required to reach a steady-state rate of radioisotope transfer. Therefore, sampling for the assay

of sodium fluxes was started only 30 min after the addition of the radioisotope. Samples were then taken simultaneously from the two chambers (facing mucosal and serosal surface of colon) at 5 min intervals for 40 min. ^{22}Na content in the samples of medium was measured using a Packard gamma-scintillation spectrometer. Sodium flux is expressed as μmoles/cm^2 × hr.

Effect of ouabain on sodium fluxes

Ouabain was dissolved in Krebs' solution to a final concentration of 10^{-3} M. The solution containing the ouabain was added either to one or to both chambers facing the mounted colon, 30 min before the addition of ^{22}Na. Sodium fluxes were then determined, as described above.

[^{3}H]*Ouabain binding*

The incubation medium in most of these experiments was K^+-free medium (composition in mM: NaCl, 123; $MgSO_4$–$7H_2O$, 1.2; $CaCl_2$ + 2.5; NaH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 5 g/l.). After equilibration effect of various ions on [^{3}H]ouabain binding in the colon:

(a) Na-free medium (concentration in mM: Choline–chloride, 123; $MgSo_4$–$7H_2O$, 1.2; $CaCl_2$, 2.5; NaH_2PO_4, 1.2; glucose, 5 g/l.; and Tris, 1, pH 7.4).

(b) Mg-free medium (concentration in mM: NaCl, 123; Na_2SO_4, 1.2; $CaCl_2$, 2.5; NaH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 5 g/l, pH 7.4).

(c) Ca-free medium (composition in mM: NaCl, 125; MgSO–$7H_2O$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 5 g/l., pH 7.4).

6. *Dissociation of bound* [^{3}H]*ouabain from colon.* The serosal side of the colon was exposed to [^{3}H]ouabain (5.4×10^{-6} M) for 30 min in a K^+-free medium. The tissue was then rinsed briefly in cold K^+-free Krebs' solution and mounted in the chamber as described above. Twenty-eight millilitres of the K^+-free medium were then introduced into the chamber on each side of the colon and samples of the fluid were collected at 5 or 10 min intervals for 30 min. [^{3}H]Ouabain in the tissue and in the consecutive samples of the medium

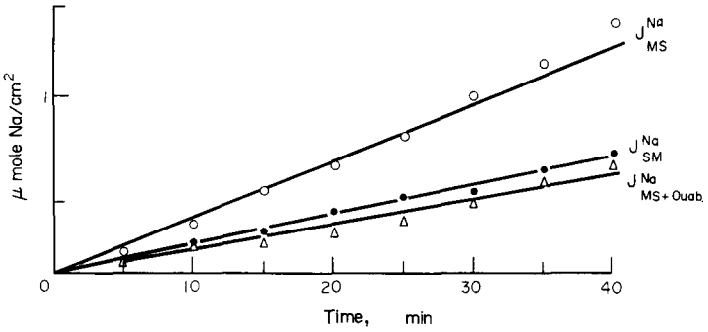

Fig. 1. Sodium fluxes through rabbit colon *in vitro*. J^{Na}_{MS}, flux from mucosa to serosa (N = 17); J^{Na}_{SM}, flux from serosa to mucosa (N = 15); J^{Na}_{MS} + Ouab., flux from mucosa to serosa in the presence of 10^{-3} M ouabain in the serosal chamber (N = 5). The flux from serosa to mucosa in the presence of ouabain coincides with the line for J^{Na}_{SM} (N = 5). Lines calculated according to the least squares method.

therefore, net sodium transport (J^{Na}_{Net}) from mucosa to serosa at the rate of 1.1 μmole/cm² × hr.

Addition of ouabain (final concentration 10^{-3} M) to the serosal compartment of the colon caused a significant decrease in J^{Na}_{Net}, this was due solely to inhibition of J^{Na}_{MS} (Fig. 1). Ouabain had no effect on J^{Na}_{SM}.

Sodium transport in the intestinal mucosa is supported by the activity of Na,K-ATPase [4]. The activity of this enzyme is inhibited by ouabain [5]. We have previously reported the activity of this enzyme in different parts of the intestine and the inhibition by ouabain [6].

[³H]*Ouabain binding to the rabbit colon*

Since ouabain inhibited net sodium transport across the colon and it also inhibits Na, K-APase of the colon mucosa, it seemed of interest to study the binding of ouabain to this preparation, both for specificity of site and for characteristics of the binding.

Ouabain, placed either in the serosal or in the mucosal compartment of the colon, showed a time-dependent binding (Fig. 2). When [³H]ouabain was added to the *mucosal* compartment, binding gradually increased with time, to reach saturation within 30 min. When placed in the serosal side, [³H]ouabain binding approach saturation only after 60 min. If [³H] ouabain was added to the mucosal compartment along with unlabelled ouabain (10^{-3} M, also in the *mucosal* compartment), there was no change in [³H]oubain binding. On the other hand, addition of unlabelled ouabain (10^{-3}) M) to the *serosal* compartment significantly decreased [³H]ouabain binding to the serosal side.

The difference in [³H]ouabain binding in the absence and presence of unlabelled ouabain is calculated as specific binding. When these values were plotted, as shown in Fig. 2 (right), it was evident that specific ouabain binding occurred only when [³H]ouabain was placed in the serosal compartment of the colon, while no specific binding was found when the glycoside was introduced to the mucosal compartment.

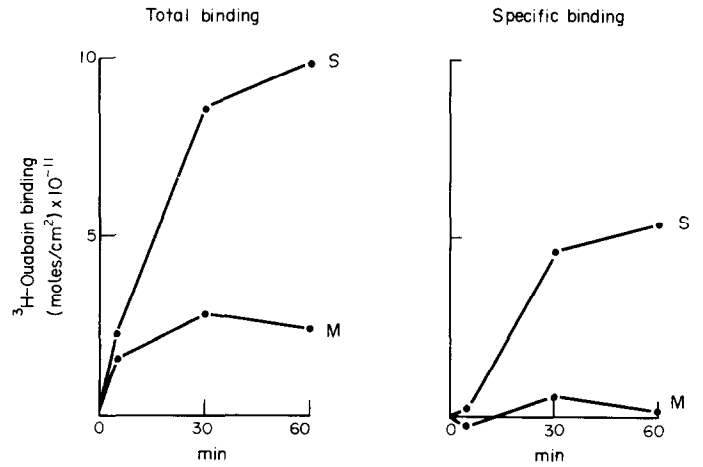

Fig. 2. Time course of [³H]ouabain binding to rabbit colon. Left side, binding in the presence of [³H]ouabain only; right side, difference between [³H]ouabain binding in the presence and absence of cold ouabain (10^{-3} M). S, binding to serosal side of the membrane (mean of 5 experiments); M, binding to mucosal side of the membrane (mean of 6 experiments).

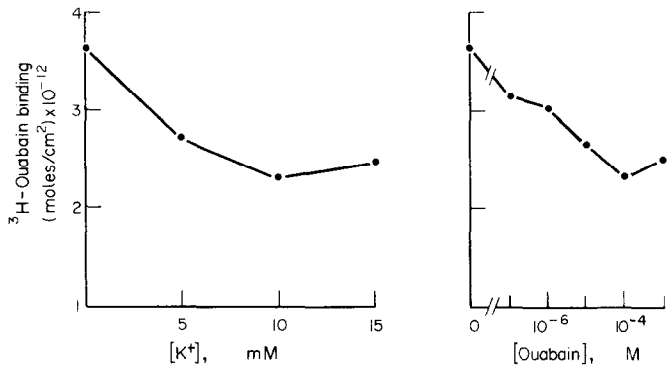

Fig. 3. Effect of K^+ and of ouabain on [^{3}H]ouabain binding to the rabbit colon. Each point is the mean of 5 experiments. It is evident that the maximal effect of K^+ is equal to that of cold ouabain.

Characteristics of specific [^{3}H]ouabain binding to rabbit colon

Increasing the concentration of K^+ in the serosal compartment from 0 to 15 mM caused a gradual decrease of binding of [^{3}H] ouabain placed in the same compartment. The maximal effect of K^+ was found at 10 mM K^+ (Fig. 3, left). The I_{50} * for K^+ was 3.5 mM. Changes of K^+ concentration in the mucosal compartment had no effect on the binding of [^{3}H]ouabain placed in the serosal compartment. The maximal effect of serosal K^+ on [^{3}H]ouabain binding was equal to that obtained by the addition of unlabelled ouabain (10^{-3} M) to the serosal compartment, as seen in Fig. 3 (right). The I_{50} for inhibition of [^{3}H]ouabain binding by unlabelled ouabain was 5×10^{-7} M.

On the other hand, addition of 10 mM K^+ to the *mucosal* compartment had no effect on the binding of [^{3}H]ouabain placed in the same compartment (Fig. 4).

The specificity of ouabain binding was next assessed by studying the effect of the addition of various unlabelled steroids, together with [^{3}H]oubain, on the binding of [^{3}H]ouabain.

Figure 5 shows that the addition of other unlabelled cardiac glycosides (digoxin, digitoxin) to the serosal compartment resulted in a decrease of [^{3}H]ouabain binding in the same compartment. However, steroids unrelated to cardiac glycosides, e.g. desoxycorticosterone acetate, estriol and testosterone, had no effect on [^{3}H]ouabain binding in the serosal compartment (Fig. 5).

The effect of ions on [^{3}H]ouabain binding to rabbit colon

The absence or presence of Mg^{2+}, Ca^{2+} or Pi, in either the serosal or the mucosal compartment or in both compartments, did not significantly affect specific [^{3}H]ouabain binding to the rabbit colon. Replacement of sodium with choline in the *serosal* compartment significantly reduced the spedific [^{3}H]ouabain binding but did not affect nonspecific [^{3}H]ouabain binding (Fig. 6). Replacement of sodium with choline in the *mucosal* compartment had no effect on [^{3}H]ouabain binding in the mucosal compartment (Fig. 6). Omission of sodium from the mucosal compartment also had no effect on [^{3}H]ouabain binding in the serosal compartment (not shown in figure).

The effect of tissue sodium concentration on [^{3}H]ouabain binding to rabbit colon

It has been reported that the presence of sodium ions is essential for ouabain binding to microsomal membrane fractions [7]. Since in our experiments the replacement of sodium in the serosal compartment reduced the specific [^{3}H]ouabain binding, one possibility to explain this observation could be a change in intracellular sodium concentration that secondarily affects ouabain binding. To assess this possibility we have compared [^{3}H]ouabain binding at increasing tissue sodium concentrations. Figure 7 shows that specific [^{3}H]ouabain binding to the serosal compartment was

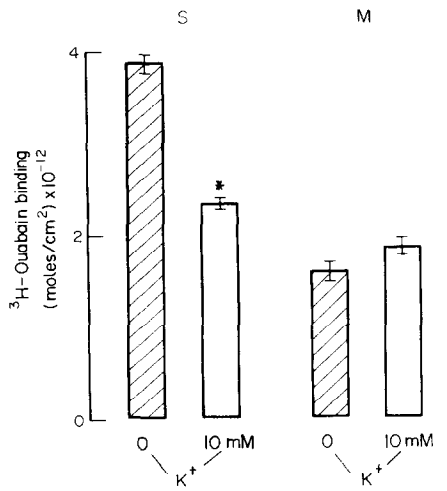

Fig. 4. Effect of K^+ on [^{3}H]ouabain binding to serosal and mucosal sides of the rabbit colon. S, binding of [^{3}H]ouabain to the serosal side of the rabbit colon; M, binding of [^{3}H]ouabain to the mucosal side of the rabbit colon. Each column is the mean of 6 experiments. Vertical bars = S.E. *$P < 0.01$ for the difference between [^{3}H]ouabain binding in the presence and absence of K^+.

* Concentration necessary for 50 per cent inhibition of specific binding of [^{3}H]ouabain.

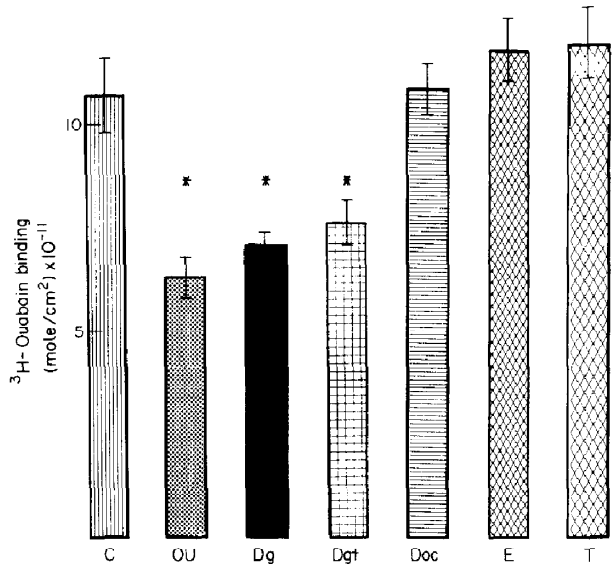

Fig. 5. Effect of various cardiac glycosides and steroid hormones on [^{3}H]ouabain binding to the serosal side of the rabbit colon. C, control (N = 8); Ou, effect of 10^{-3} M ouabain on [^{3}H]ouabain binding (N = 5); Dg, effect of digoxin (2.2×10^{-4} M) on [^{3}H]ouabain binding (N = 5); Dgt, effect of digitoxin (9×10^{-5} M) on [^{3}H]ouabain binding (N = 4); Doc, effect of desoxy-corticosterone acetate (5×10^{-5} M) on [^{3}H]ouabain binding (N = 5); E, effect of estriol (10^{-4} M) on [^{3}H]ouabain binding (N = 5); T, effect of testosterone (10^{-4} M) on [^{3}H]ouabain binding (N = 5). Vertical bars = S.E. *P < 0.02 compared to [^{3}H]ouabain binding in control (C).

unchanged in spite of substantial differences of intracellular sodium concentration. (The intracellular sodium concentration calculated after subtraction of extracellular space sodium was increased by 114 per cent during the prolonged incubation.)

Dissociation of [^{3}H]*ouabain from rabbit colon*

Figure 8 shows that the dissociation rate of nonspecifically bound [^{3}H]ouabain from rabbit colon was rather fast (95 per cent within 60 min). The dissociation of specifically bound [^{3}H]ouabain in the serosal compartment was much slower (20 per cent within 60 min).

When dissociation of [^{3}H]ouabain from the colon was carried out in a sodium-free medium (sodium was replaced with choline), there was no change in the dissociation rate of nonspecifically bound [^{3}H]ouabain, while the dissociation rate of the specifically bound glycoside was substantially increased to 55 per cent within 60 min (Fig. 8).

DISCUSSION

Sodium fluxes, J_{MS}^{Na}, J_{SM}^{Na} and J_{Net}^{Na} across the rabbit colon *in vitro* were studied. The results reported in this paper on rabbit distal colon confirm previous observa-

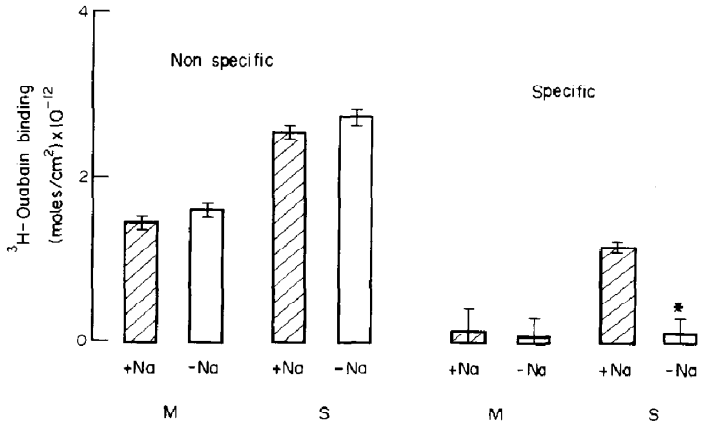

Fig. 6. Effect of Na^+ on specific and nonspecific [^{3}H]ouabain binding to mucosal and serosal sides of the rabbit colon. M, binding to mucosal side; S, binding to serosal side. $-Na^+$, sodium replaced by choline. Each column is the mean of 6 experiments. Vertical bars = S.E. *P < 0.01 compared to binding in the presence of Na^+.

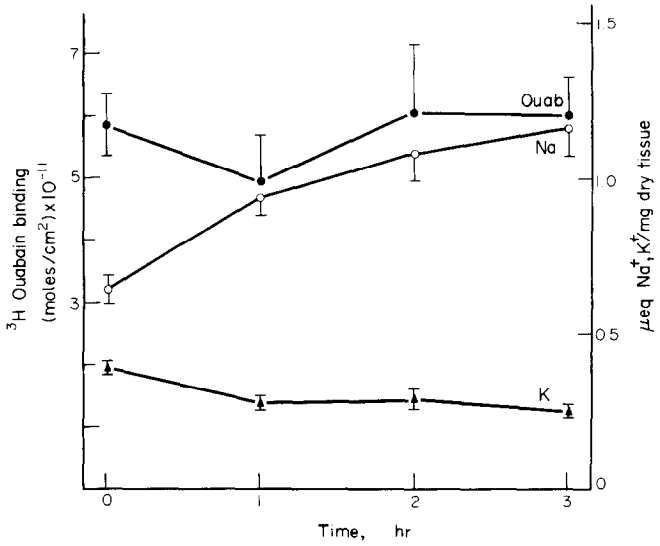

Fig. 7. Effect of increasing tissue sodium concentration on [^{3}H]ouabain binding to rabbit colon. Rabbit colon was preincubated *in vitro* in a K-free, sodium medium to cause progressive loading of Na^+ in tissue. At different time periods pieces of colon were removed and specific [^{3}H]ouabain binding was assessed. Abscissa denotes duration of preincubation. Ouab, [^{3}H]ouabain binding (N = 6). Na, sodium concentration in tissue (N = 8). Each value in the successive determinations was significantly higher than the initial value ($P < 0.001$). K, K^+ concentration in tissue (N = 8). Vertical bars = S.E. Ordinate on right, tissue concentration of sodium and of potassium.

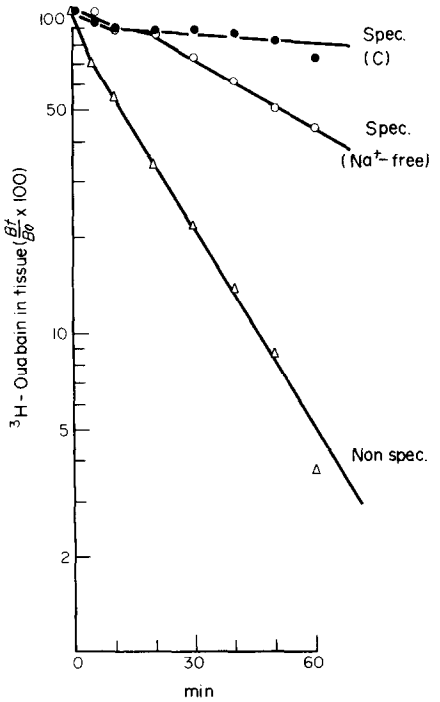

Fig. 8. [^{3}H]ouabain dissociation from rabbit colon. Non spec., dissociation of non-specifically bound [^{3}H]ouabain; spec. (c), dissociation of specifically bound [^{3}H]ouabain (control), spec. (Na^+-free), Dissociation of specifically bound [^{3}H]ouabain into Na^+-free medium. Dissociation of non-specifically bound [^{3}H]ouabain into Na-free medium coincided with the line for the dissociation into control medium. Each point is the mean of 5 experiments. Ordinate, amount of [^{3}H]ouabain at time (t) as a per cent of the amount in the tissue at the begining of the experiment. The initial rate (first 10 min) was faster than the following period in all experiments. The points from 10 min onwards fall on a line, calculated according to the method of least mean squares.

tions, in several other species, indicating that net sodium transport in the colon proceeds from mucosa to serosa [8–12]. J^{Na}_{Net} from mucosa to serosa was completely abolished by ouabain. Since ouabain also inhibits its Na, K-ATPase activity, [7, 13–15] a correlation between sodium transport in the rabbit colon and ouabain-sensitive ATPase is suggested. A different situation is observed in kidney cortex, where two mechanisms for Na transport can be distinguished, one dependent on Na, K-ATPase, the other, also proceeding in the presence of inhibitors of this enzyme [16, 17]. Attempts to identify sodium transport in the rabbit colon, sensitive to inhibitors such as ethacrynic acid [17], have shown no evidence for such transport (Albin and Gutman, unpublished observations).

Most of the studies on cardiac glycoside binding have been carried out on isolated membranes *in vitro* [25–27]. The studies on isolated membranes or on the purified enzyme indicated that the receptor for ouabain binding is Na, K-ATPase. However, the procedure of isolation and purification of membrane fractions may induce conformational changes of the enzyme, which could affect the binding characteristics of Na, K-ATPase for cardiac glycosides, compared to those in the intact tissue. Therefore, studies on tissues or isolated cells may give a better characterisation of the binding properties of the glycoside to the enzyme in the intact membrane.

Furthermore, the outer membrane of red cells or cardiac cells is generally homogeneous, while the mucosa of rabbit colon has an asymmetric structure, where the luminal and serosal sides of the cells have different functions.

In the intact colon, unlabelled ouabain was found to inhibit [^{3}H]ouabain binding only in the serosal compartment, indicating that specific ouabain binding is present only in the serosal side of the cells (Fig. 2). The

specificity of ouabain binding to the serosal membrane was also supported by the inhibition of [^{3}H]ouabain binding by the addition of other cardiac glycosides to the medium, while addition of various other steroids had no effect (Fig. 5). This point was of interest because in another study in our laboratory, on [^{3}H]ouabain binding to kidney slices, various steroid hormones inhibited the binding of ouabain to cortical but not to medullary slices (Neufeld and Gutman, to be published). These findings are similar to the observations on cardiac glycoside binding to isolated heart and to the isolated Na,K-ATPase [13, 28, 29].

Several investigators have shown that potassium inhibited [^{3}H]ouabain binding to isolated Na,K-ATPase [20, 21] and to red blood cell ghosts [30]. Concomitantly with inhibition of ouabain binding there was also inhibition of the inotropic effect of ouabain [13]. We have observed a similar effect of K^+ on [^{3}H]ouabain binding in the rabbit colon (Fig. 4).

The presence of Na^+ ions had been reported as essential for ouabain binding, either to the intact heart [32] or to the isolated Na,K-ATPase [8, 33]. Our observations corroborate these earlier results (see Fig. 6).

The mechanism of the effect of sodium on ouabain binding is, however, not clear. One line of reasoning is that sodium is required for the phosphorylation of Na,K-ATPase by ATP and that it is only the phosphorylated enzyme that binds cardiac glycosides [34, 35]. If this were the case, then intracellular sodium concentration should play a critical role, since an increase of intracellular sodium activates the enzyme and enhances phosphorylation of the enzyme by ATP [14, 34, 35]. However, our experiments showed no effect of changes of tissue sodium concentration on [^{3}H]ouabain binding (Fig. 7). It seems, therefore, that it is the presence of extracellular sodium at the site of binding that is essential for specific ouabain binding.

The role of extracellular sodium in [^{3}H]ouabain binding has recently also been reported in frog skin [36]. In this report, a possible role for intracellular sodium is suggested, based on inhibition of [^{3}H]ouabain binding by the addition of amiloride to the outer surface of the skin. However, in this study the concentration of [^{3}H]ouabain used (10^{-6} M) could already have caused inhibition of the sodium pump by itself and, therefore, have affected binding. In addition, no distinction was made of *specific* [^{3}H]ouabain binding and finally, to reduce sodium on the external surface of the skin, no replacement by other ions was used but water as a bathing medium, thus creating a drastic osmotic gradient. Interpretation of the data, therefore, is rather ambiguous.

On the other hand, the report by Bodemann and Hoffman [30] on [^{3}H]ouabain in erythrocytes provides evidence that increased intracellular sodium did not enhance [^{3}H]ouabain binding, in line with our results in rabbit colon.

A possible explanation for the conflicting reports on the role of intracellular sodium in cardiac glycoside binding may be provided by the recent work of Fricke and Klaus [37, 38]. These authors have studied isolated heart membranes for cardiac glycoside binding and found evidence for two different effects: one at a low Na concentration ($K_{0.5}$ = 3–5 mM) and the other at a much higher concentration (in the case of ouabain binding, $K_{0.5}$ for Na = 64 mM). It may be suggested that the effect at low Na concentrations was due to activation of the enzyme Na,ATPase by intracellular sodium, while the effect at high Na concentrations is the one observed in our study and in others, due to extracellular sodium and directly affecting the binding of ouabain [30, 36].

The importance of extracellular sodium for ouabain binding is also supported by the rate of ouabain dissociation: in the absence of sodium, dissociation of specifically bound [^{3}H]ouabain was accelerated (Fig. 8). This phenomenon is different from that observed in binding to membrane fractions, where addition of sodium can accelerate ouabain dissociation [39]. The effect of sodium on dissociation of ouabain from isolated membranes was explained as a change induced by Na in the conformation of the phosphorylated enzyme [40]. The observations in our experiments may, therefore, indicate a different type of effect.

Furthermore, the presence of sodium affects specific ouabain binding only in the serosal compartment and does not affect the nonspecific ouabain binding at the mucosal surface, thus supporting the conclusion that the ouabain receptor is located in the serosal surface of the colon.

The lack of effect of Ca^{2+}, Mg^{2+} and Pi in the incubation medium on specific ouabain binding does not completely rule out a possible role for these ions in the binding process. Inorganic phosphate is present intracellularly and this Pi may modulate ouabain binding. Omission of Pi from the incubation medium does not eliminate intracellular Pi. Similar reasoning may be developed for the other ions. However, the experiments do preclude any major role for these ions in the external medium on cardiac glycoside binding.

In conclusion, the present report shows that specific ouabain binding (indicated by saturation and inhibition by other cardiac glycosides) is confined to the serosal side of the colon. This binding is inhibited by potassium and *depends* on the presence of sodium in the extracellular space. The dissociation of specifically bound [^{3}H]ouabain is accelerated in the absence of sodium. Intracellular sodium does not seem to be crucial for specific binding.

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