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ESTIMATION OF THE LIFESPAN OF AMILORIDE BINDING SITES IN THE MEMBRANES OF TOAD BLADDER EPITHELIAL CELLS

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SUMMARY

1. Sodium entry sites in the membranes of isolated epithelial cells prepared from bladders of toads (*Bufo marinus*) have been labelled with amiloride. The number of binding sites remained constant in suspensions for up to 100 hr.

2. In the presence of a protein synthesis inhibitor (cycloheximide, 0·5 μg/ml.) there was a decline in the density of binding sites with time. The decline in the number of binding sites was approximately exponential. Regression analysis gave a half-life of approximately 60 hr.

3. Aldosterone (5×10^{-8} M) caused a significant ($P < 0{\cdot}001$) increase (50%) in the density of amiloride binding sites. Cells which had been treated with aldosterone had populations of binding sites which declined, in the presence of cycloheximide, at rates indistinguishable from those of untreated cells.

INTRODUCTION

The extent of the transepithelial movement of sodium across a number of transporting epithelia appears to be governed, in the main, by the rate at which sodium ions penetrate the mucosal membrane of the cells and so gain access to the active transport mechanism. This is so for amphibian skins and bladders and may also be true for parts of the nephron. The flux of sodium through the mucosal membranes of epithelia will depend, in part, on the number of sites available for sodium entry.

Recently methods have been developed to measure the number of amiloride binding sites in the mucosal surface of frog skin and toad bladder (Cuthbert, 1973; Cuthbert & Shum, 1974*a*, *b*, 1975) and the results suggest they are reasonably accurate methods for determining the density of sodium entry sites. Furthermore, studies with aldosterone showed that this hormone increased the density of amiloride binding sites without affecting

the apparent affinity for this ligand (Cuthbert, Okpako & Shum, 1974; Cuthbert & Shum, 1975). The increase in the density of amiloride binding sites following aldosterone was inhibited by actinomycin D or cycloheximide.

As the actions of aldosterone result from protein synthesis via induction of a specific mRNA and aldosterone induced proteins (Scott & Sapirstein, 1975; Rossier, Wilce & Edelman, 1974) the effects of the newly synthesized protein may persist after removal of the hormone. Sharp, Komack & Leaf (1966) showed that a brief exposure to aldosterone was followed later by an increase in sodium transport. While many factors, particularly energetic considerations (Sharp & Leaf, 1966; Fanestil, Herman, Fimognari & Edelman, 1968; Lipton & Edelman, 1971; Saito, Essig & Caplan, 1973) may determine the response time to aldosterone it is clearly of interest to know the life-time of the extra amiloride binding sites which appear following aldosterone treatment.

This paper reports upon experiments designed to measure the disappearance of amiloride binding sites in isolated epithelial cells in the presence and absence of an inhibitor of protein synthesis.

METHODS

Preparation of epithelial cell preparations. Suspensions of epithelial cells were prepared from bladders of toads, *Bufo marinus*. Bladders were dissected from double-pithed animals (usually four or five) and washed in calcium-free Ringer solution (in mM): NaCl, 110; EGTA neutralized to pH 7·6 with KOH, 0·5; Tris buffer, pH 7·6, 5; and glucose, 11·1. The tissues were then incubated in the same solution containing 0·5 mg/ml. collagenase for 1 hr at room temperature. Afterwards the bladders were washed with calcium-free Ringer and incubated for a further 30 min in the calcium-free solution. The epithelial cells were then obtained by vigorous shaking and gentle scraping. The cells were washed, centrifuged (MSE Super Minor, 10 ml. tubes, 2000 rev/min for 2 min) and resuspended a total of seven times in the calcium-free solution. The resuspended cells were layered on to a sucrose solution (in mM): (sucrose, 444; NaCl, 1·1; EGTA neutralized to pH 7·6 with KOH, 0·5; Tris buffer, pH 7·6, 5; in 10 ml. tubes and centrifuged (MSE Super Minor, 2000 rev/min for 2 min). The pellet was resuspended and the procedure repeated twice. The whole procedure was designed to remove mucus (and possibly mucus cells too) from the cell suspension. The cells were finally resuspended in choline–Ringer of the following composition (in mM): choline chloride, 110; NaCl, 1·1; EGTA neutralized to pH 7·6 with KOH, 0·5; Tris buffer, pH 7·6, 5; and glucose, 1. Antibiotics (penicillin G, 1000 u./ml. and streptomycin 0·2 mg/ml.) were added to the solutions to prevent bacterial growth. The volume of the final cell suspension was adjusted so that there were about 35×10^5 cells/ml. Usually about 15 ml. cell suspension was obtained. The whole procedure took 2 hr $\pm$ 10 min to complete.

Labelling of cell suspension with amiloride. In all the experiments reported in this paper labelling was carried out at one of two amiloride concentrations, either 22 or 50 nM. All estimates reported are the means of at least seven measurements. The procedure was identical to that described previously by us (Cuthbert & Shum, 1975).

Briefly, 100 μl. aliquots of cells were placed in 300 μl. microfuge tubes and mixed with [^{14}C]amiloride (specific activity 54 mc/m-mole) to give a final concentration of 22 or 50 nM. After exactly 2 min the tubes were centrifuged in a Beckman 152 microfuge at 15,000 *g* for 2 min. During this time the cells passed into a silicone fluid layer at the base of the tube. After removing the aqueous layer by aspiration the tips of the tubes were clipped off into scintillation vials and the radioactivity measured by liquid scintillation spectrometry. At the same time other tubes were processed similarly except the cells were exposed to amiloride at a concentration of 2 or 5 μM but with a specific activity of only 0·54 mc/m-mole. The mean difference between the radioactivity entrained into the silicone layer at the two specific activities was taken as the amount of displaceable ligand. Sufficient counts were accumulated so that the counting errors were less than 2 %. The difference between the mean counts retained at high and low specific activities was tested for significance using a Student's *t* test.

Throughout the paper the density of amiloride binding sites is expressed as sites/μm^2. The assumptions made are that the mean diameter of the cells is 20 μm and that one sixth of the cell surface is exposed to the mucosal face. These assumptions, of course, do not affect the meaning of the results as the sites/μm^2 can be converted to sites/cell simply by multiplying by 260 (i.e. the estimated area of the mucosal face in μm^2).

Suppose the mean difference in the radioactivity retained by an aliquot of cells at high and low specific activities is x disintegrations/min, d.p.m., then the number of sites/μm^2 is given by d where

$$d = \frac{x \text{ d.p.m.} \times 6{\cdot}02 \times 10^{23} \text{ molecules mole}^{-1}}{y \times 260\ \mu\text{m}^2 \times 2{\cdot}22 \times 10^{12} \text{ d.p.m. c}^{-1} \times 54 \text{ c.mole}^{-1}}$$

and where y is the number of cells in an aliquot. Cell counts were measured using a standard haemocytometer.

Incubation of cell suspensions. During the experiments cell suspensions were kept in small conical flasks (20 ml.). The flasks were immersed in a water-bath at 22° C and gently shaken. Each time aliquots of cells were taken for labelling as described above, a further aliquot was taken for counting in the haemocytometer and for microscopic examination. The cells remained free in suspension for at least 100 hr, probably as a result of the lack of divalent cations in the bathing fluid. The cells were spherical in shape and no changes in physical appearance were noted during the experiments. The cells did not appear to be dividing as the cell count did not increase with time. Over the period 6–100 hr the cell density in suspensions decreased by approximately 10 %, whether or not cycloheximide was present. As we had no reason to believe that receptor molecules present in cell fragments resulting from the dissolution of some 10 % of the cells would not bind amiloride, calculations were based on the number of cells present in the aliquots at the start of the experiments. If this supposition is untrue the density of binding sites will be underestimated by approximately 10 % at 100 hr. When cells were incubated for 120–140 hr in the presence of cycloheximide there was a dramatic fall in the number of intact cells to 30–40 %. At this time clumping of cells and cell debris occurred and it was no longer possible to centrifuge the cells from the aqueous to silicone fluid layers. Consequently all the results in this paper were obtained with suspensions incubated for periods up to 100 hr only. It should be noted that the concentration of cycloheximide used in these experiments (0·5 μg/ml.) was sufficient to block the effect of aldosterone on sodium transport (Fanestil & Edelman, 1966) and sufficient to prevent the increase in amiloride binding site density following aldosterone (Cuthbert & Shum, 1975), although protein synthesis in bladders is not abolished but reduced to 40 % of

normal (Fanestil & Edelman, 1966). Presumably this minimal protein synthesis is sufficient to maintain cell integrity for 100 hr, after which rapid dissolution occurs at around 120 hr.

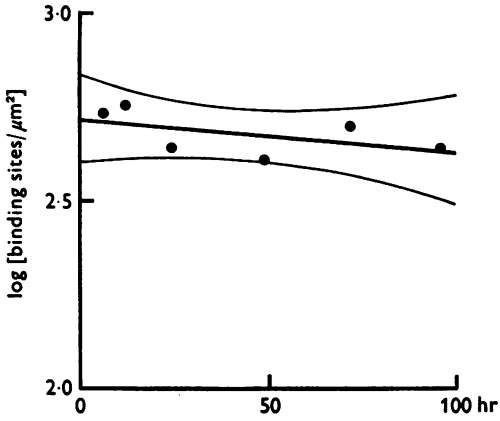

Fig. 1. Log [binding sites/μm^2] *versus* time for a cell suspension in the absence of cycloheximide. Concentration of amiloride was 50 nM. Each point is the mean of at least seven observations. Statistically significant amounts of binding ($P = 0{\cdot}05$ or less) were obtained at all times. The straight line is the calculated regression line. The curved lines are 95 % confidence limits.

TABLE 1. Binding site density *versus* time: characteristics of the regression lines

Experiment	Binding sites/μm^2 at $t = 0$ (± S.E. of mean)	log [sites/μm^2]/hr	
		Slope	95 % confidence limits
1	518 (569–472)	−0·0009	−0·003 to +0·0012
2	437 (483–396)	−0·0019	−0·004 to +0·0003

Measurements made using 50 nM amiloride (54 mc/m-mole). Experiment 1 is illustrated in Fig. 1. Binding sites at $t = 0$ were estimated by extrapolation, binding measurements were made between 6 and 110 hr following tissue isolation.

RESULTS

The density of amiloride binding sites has been investigated during two periods, 0–7 and 6–100 hr, zero time referring to the time at which the bladders were removed. The reason for this is that it was found previously (Cuthbert & Shum, 1975) that the binding site density fell rather abruptly in the first 3–4 hr, after which the density remained stable. Slightly different protocols were needed for the two periods under investigation and the results are presented separately in this section.

The behaviour of binding sites during the period 6–100 hr

Initial experiments were designed to establish that the site density remained constant with time after isolation. If this is so then it may be assumed either that the preparation and incubation of the cells did not affect the turn-over of binding sites, or alternatively there is no turn-over. Indeed it was found that the binding site density remained constant as illustrated in Fig. 1. The slope of the regression line relating density *versus* time was not significantly different from zero. A second experiment identical in protocol to the first gave similar results. The data for both experiments are given in Table 1. The site density at zero time was obtained by extrapolation and used simply for the purposes of comparison. Although in neither of the experiments was there any significant change in binding site density with time, if the slope of the regression line shown in Fig. 1 is assumed to be real then the apparent half-life of the binding sites is approximately 14 days.

The next set of experiments was identical to that already described with the exception that a protein synthesis inhibitor, cycloheximide (0·5 μg/ml.) was added to the cell suspension immediately before the first binding measurement. Fig. 2 shows a single result from one of five experiments. In the presence of cycloheximide the binding site density fell with time, approximately two-thirds of the binding sites disappearing within 100 hr. Regression lines relating number of binding sites *versus* time were calculated for each experiment. In all experiments the correlation was more significant with the semilog plots compared to that with linear scales. The half-lives for five semilog regression lines are given in Table 2; the mean value was found to be approximately 54 hr. The possible significance of the half-time values is discussed at the end of this paper. For comparison Fig. 2*A* shows the same data as in Fig. 2*B* plotted on an arithmetic scale.

Our next series of experiments were designed to see if a similar pattern of decline was observed for cells which had been pre-treated with aldosterone some hours before the addition of cycloheximide. We have shown previously (Cuthbert & Shum, 1975) that aldosterone increases the number of amiloride binding sites in epithelial cells, and that this increase is blocked by cycloheximide or actinomycin D.

In the experiments reported here it was decided to use a lower concentration of aldosterone than had been used previously, so it was necessary to establish that an increase in binding site density also occurred with these conditions.

Bladders were dissected from toads and divided into pairs of hemibladders. The control group of hemibladders was treated with solvent (1% alcohol) while the test group was treated with aldosterone (5×10^{-8} M)

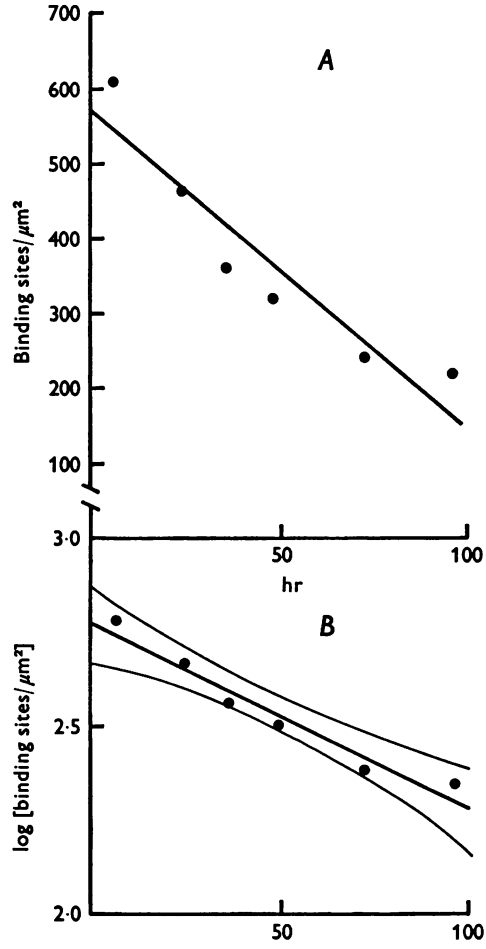

Fig. 2. Regression lines for the number of binding sites *versus* time in the presence of cycloheximide (0·5 μg/ml.) plotted: *A*, on arithmetic; and *B*, semilogarithmic scales. Concentration of amiloride was 50 nM. Statistically significant amounts of amounts of binding ($P = 0{\cdot}05$ or less) were detected at all times. Straight lines are regression lines, and curved lines show the 95 % confidence limits. From *B*, $t_{\frac{1}{2}} = 60{\cdot}1$ hr.

for 4 hr in high-sodium Ringer. After this the cells were harvested as before; cycloheximide, 0·5 μg/ml., was added to each cell suspension and the labelling procedure carried out. Between twenty-five and thirty-three pairs of measurements were made on each cell suspension, rather than the more usual seven pairs. The labelling protocol took approximately 3 hr, corresponding to the period 6–9 hr after removal of the bladders. Aliquots

of cells were taken in turn from test and control suspensions so that actual times of labelling during the 6–9 hr period were evenly distributed for the two suspensions. Three experiments of this type were carried out. In a further two experiments a slightly modified protocol was used. A cell suspension was prepared from several bladders which was then divided equally into two: one half was treated with aldosterone (5×10^{-8} M) and the other with solvent. After 4 hr cycloheximide (0·5 μg/ml.) was added to

TABLE 2. Binding site density *versus* time in the presence of cycloheximide (0·5 μg/ml.); characteristics of regression lines

Experiment	Binding sites/μm^2 at $t = 0$ ($\pm$ S.E. of mean)	log [sites/μm^2]/hr		$t_{\frac{1}{2}}$ (hr)
		Slope	95 % confidence limits	
1	597 (643–554)	−0·0050	−0·0066 to −0·0034	60·1
2	415 (502–343)	−0·0049	−0·0074 to −0·0024	61·5
3	1042 (1311–828)	−0·0093	−0·0131 to −0·0055	32·4
4	552 (617–494)	−0·0052	−0·0079 to −0·0024	57·9
5	582 (658–515)	−0·0053	−0·0105 to −0·0001	56·8

Measurements made using 50 nM amiloride (54 mc/m-mole). Experiment 1 is illustrated in Fig. 2.

both suspensions and the labelling protocol carried out during a 3 hr period starting 6 hr after the bladders were removed. Antibiotics (penicillin, 1000 u./ml. and streptomycin, 0·5 mg/ml.) were also added to the cell suspensions in these experiments so that the constitutions of the aliquots used in the binding studies were similar in all respects to those used for the 100 hr experiments. This eliminates discrepancies which arise if antibiotics interfere with the response to aldosterone or modify amiloride binding. The results of five experiments are presented in Table 3.

In these experiments the binding site density during 6–9 hr was somewhat lower than expected; however, the animals used for these experiments were from a different batch than those used for the earlier experiments. In each experiment aldosterone caused an increase in binding site density. The inevitability of large S.E. of the mean with low binding site densities does not allow the difference in individual experiments to achieve significance. However, the data can be properly regarded as a series of paired observations as, in each experiment, the two suspensions were derived from the same tissues. Application of the t test for paired observations showed that the mean increase in binding site density with aldosterone (50 %) was highly significant ($P < 0·001$).

In two further experiments bladders were divided into control and test

TABLE 3. The effect of aldosterone (5×10^{-8} M) on binding site density

Experiment	Binding sites/μm^2		
	Aldosterone	Control	% increase
1	321 ± 93 (25)	189 ± 74 (24)	70
2	326 ± 70 (34)	215 ± 89 (28)	52
3	283 ± 61 (33)	215 ± 72 (33)	32
4	375 ± 83 (27)	255 ± 79 (28)	47
5	531 ± 153 (27)	355 ± 159 (28)	50

In experiments 1–3 the cells were isolated after paired tissues were exposed either to aldosterone or solvent. In experiments 4–5 isolated cell suspensions were prepared and divided into two, one portion being treated with aldosterone and the other with solvent alone.

Binding was measured during the period 6–9 hr following isolation of the tissue. Amiloride concentration was 50 nM (54 mc/m-mole).

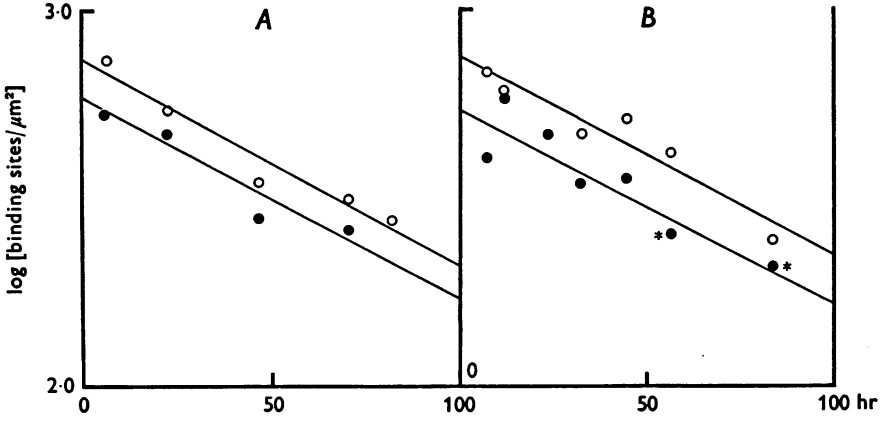

Fig. 3. *A* and *B*, show results from two identical experiments. Regression lines for the relationship between log (binding sites/μm^2) and time. Open circles show results for aldosterone (5×10^{-8} M) treated suspensions and filled circles for control suspensions. Details of the characteristics of the regression lines are given in Table 4. Amiloride concentration was 50 nM. Statistically significant amounts of binding ($P = 0{\cdot}05$ or greater) were obtained except at points indicated by an asterisk.

halves. The test group were treated with aldosterone (5×10^{-8} M) in high-sodium Ringer for 4 hr, while the control group were treated with solvent. At the end of the incubation the cells were harvested from both groups, antibiotics (penicillin 1000 u./ml. and streptomycin 0·5 mg/ml.) and cycloheximide (0·5 μg/ml.) were added and the density of binding sites

TABLE 4. Binding site density *versus* time in the presence of cycloheximide (0·5 μg/ml.); the effects of aldosterone (5×10^{-8} M); characteristics of regression lines

Experiment	Control suspension			Aldosterone (5×10^{-8} M) treated suspension		
	Slope log [sites/μm^2]/hr	95 % confidence limits of slope	$t_{\frac{1}{2}}$ (hr)	Slope log [sites/μm^2]/hr	95 % confidence limits of slope	$t_{\frac{1}{2}}$ (hr)
1	−0·0052	−0·0079 to −0·0024	57·9	−0·0053	−0·0076 to −0·0030	56·7
2	−0·0053	−0·0105 to −0·0001	56·8	−0·0055	−0·0081 to −0·0029	54·7

Measurements made using 50 nM amiloride (54 mc/m-mole).

estimated over the period 6–100 hr. Semilog plots from the results of both experiments are shown in Fig. 3. Details of regression analysis are given in Table 4. The half-times for the decay of binding site density were approximately the same in both experiments, whether or not the cells had been treated with aldosterone. From these results it seems that the extra binding sites appearing after aldosterone treatment have similar decay times to those normally present.

The behaviour of binding sites in the period 2–7 hr

In our previous study (Cuthbert & Shum, 1975) we reported that the binding site density fell rapidly in the first few hr after cells were isolated. We were unable then to resolve the reason for this but considered it may have been the result of tissue disruption. Accordingly we were careful to perform binding studies at fixed times after the tissues had been removed and the cell suspension prepared.

We have shown with another tissue, frog skin, that binding sites can be made to appear and disappear at suitable transepithelial potentials (Cuthbert & Shum, 1976), and perhaps latent binding sites can also be uncovered by tissue disruption. As aldosterone increases binding site density (Cuthbert & Shum, 1975) it might do this by exposing latent sites in the membrane, and perhaps oppose the initial rapid decay in binding site density seen in control preparations.

The experiments reported here were designed to see if aldosterone affected the initial rapid reduction in binding site density, and if it did not do so what other factors may be involved.

Customarily we have exposed cells to aldosterone for 4 hr before measuring amiloride binding, which of course is not possible when binding is measured at 2 hr. Consequently aldosterone was given by injection into the dorsal lymph spaces (0·8 μmole in 0·8 ml. 1% alcohol) 2 hr before the animals were killed. Binding was measured at between 2 and 7 hr in cells isolated from aldosterone treated toads and the results compared with our earlier data (Fig. 4*A*).

The solution in which binding measurements were made in the series of experiments reported in this paper contained 1 mM glucose, whereas glucose was absent in experiments reported earlier. A further experiment was carried out with cells from aldosterone treated toads in which glucose was absent from all the preparative solutions. These results too are pictured in Fig. 4*A*. While the three sets of results are not strictly comparable, as the cells were derived from different tissues, all three groups of data show that there is an initial sharp reduction in binding site density in the few hours after cell separation.

To make sure that the presence or absence of glucose did not affect the

results a final experiment was performed in which bladders were divided into halves. One group was processed in the absence of glucose, while the other group was treated in the presence of glucose. In this instance the results from the two groups are comparable as they derive from the same

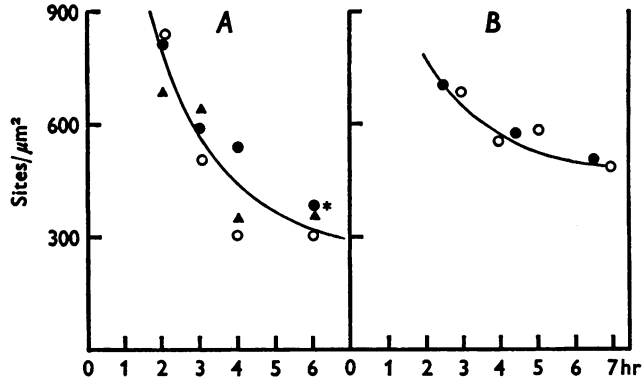

Fig. 4. Relation of number of binding sites to time measured between 2 and 7 hr following isolation of the tissue. All points represent significant amounts of binding ($P < 0{\cdot}05$) except point marked by asterisk ($P < 0{\cdot}1$). Amiloride concentration was 22 nM. In *A* results of three separate experiments are represented: the cell suspensions were treated with aldosterone (5×10^{-8} M), triangles; aldosterone (5×10^{-8} M) and glucose (1 mM), filled circles; open circles represent previously published data (Cuthbert & Shum, 1975) when both aldosterone and glucose were absent. In *B* results from two suspensions derived from the same tissue in the absence, open circles, or presence, filled circles, of glucose (1 mM) are shown.

common tissue pool. Fig. 4*B* shows the results of this experiment. It took rather longer to prepare two batches of cells so that the first binding measurements were made rather later than for the experiments illustrated in Fig. 4*A*. Thus the early part of the curve has probably been missed. Clearly the presence or absence of glucose makes no significant difference to the binding site density.

DISCUSSION

In the absence of a protein synthesis inhibitor there was no significant change in the amount of amiloride bound to cell suspensions during a period of 100 hr. This result, considered with those obtained in the presence of cycloheximide, suggests that the isolated cell preparation we have developed is viable, at least in terms of the turn-over of amiloride binding sites.

The concentration of cycloheximide used in our experiments is sufficient

to prevent the increase in sodium transport brought about by aldosterone in isolated bladders (Fanestil & Edelman, 1966) although protein synthesis is inhibited by only 60%. We assume that this concentration of inhibitor is also sufficient to prevent the synthesis of the 'normal' sodium entry sites, if indeed these are different from those formed under the influence of aldosterone. It may be that both result from protein synthesis controlled by the same gene, and it is perhaps pertinent to note that in several tissues where amiloride has powerful blocking activity sodium transport is increased by aldosterone. This is true of the kidney, amphibian skins and bladders, salivary duct and the colon (for references see Cuthbert, 1974).

We have used only two concentrations of amiloride in these experiments, 22 and 50 nM. Reference to our original binding curve (Cuthbert & Shum, 1975) shows that at the lower concentration practically all the displaceable amiloride is bound to high affinity sites with properties consistent with those of the sodium entry sites. The occupancy at a concentration of 22 nM is around 50%. To increase the chances of detecting statistically significant binding after the site density had fallen to low values we used 50 nM amiloride, which gives an occupancy of approximately 80%. However, at this concentration some 30% of the displaceable binding is not to high affinity sites.

We chose to fit most of our data to semilogarithmic regression lines, but do not attach particular significance to this. The fit was better on semilogarithmic scales than linear ones, and attempts at more complex fits would be unjustified with the present data. Furthermore, we did not subtract the expected amount of non-specific binding when using 50 nM amiloride, as there is no reason *a priori* for believing this would remain constant after cycloheximide.

From Table 2 it would appear that the half-life for the binding site is around 60 hr, excepting for a single experiment when a half-life of approximately half this value was obtained. Without attaching precise mathematical significance to the meaning of half-life it is reasonable to conclude that the density of the sodium entry sites falls to low values ($< 25\%$ of normal) within 120 hr.

The results with aldosterone confirm an earlier report (Cuthbert & Shum, 1975) that this hormone increases the binding site density. We cannot know from these experiments whether the binding sites, presumed equivalent to sodium entry sites, are indeed functional. However, simultaneous binding and transport studies on intact frog skin treated with aldosterone suggest that the new sites are permitting sodium entry (Cuthbert *et al.* 1974).

We are unable to detect any difference in the rate of loss of binding sites in tissues treated with aldosterone compared to control tissues. An impor-

tant consequence of this is that the effects of a single exposure to aldosterone may be expected to persist for at least 120 hr, as far as the presence of extra entry sites is concerned. It is just possible that aldosterone could alter the rate at which preformed sites decayed. It would, however, require an unusual coincidence if the over-all disappearance rate of a mixed population of aldosterone induced and pre-formed sites equalled that of a single population of pre-formed sites uninfluenced by aldosterone.

It is of interest to compare the behaviour of bladders from a functional aspect (sodium transport) in response to aldosterone with the behaviour of the entry sites. It is known that substrate depleted bladders will respond to pyruvate many hr after exposure to hormone (Sharp & Leaf, 1964). Yet when a protein synthesis inhibitor is added after aldosterone at a time when the increase in transport becomes apparent then the response is inhibited within 10–12 min (Sharp & Leaf, 1966). These authors conclude the half-life of the protein is only 40 min. However, as stated in the Introduction the response to aldosterone probably involves energetic considerations as well as a modification of the entry step. Thus it is probably irrelevant to compare half-lives based on transepithelial sodium movement with direct estimates of ligand binding to entry sites.

There would seem to be three ways in which aldosterone induced protein(s) affect the appearance of new entry sites. First, aldosterone induced protein may constitute new entry sites. Binding of α-bungarotoxin to nicotinic receptors in developing myotubes is inhibited to cycloheximide, but not for the first $2\frac{1}{2}$ hr. In this sytsem there appears to be a pool of precursor material awaiting incorporation into the membrane (Hartzell & Fambrough, 1973). The precision of the binding data with amiloride is insufficiently accurate to know if this is true too for bladder cells. Second, aldosterone induced protein might be an enzyme which uncovers latent sites in the membrane. Such an agent need not necessarily have a long half-life.

Finally it is possible that aldosterone induced protein is an agent which prevents the normal break-down of sodium entry sites. This possibility can probably be excluded from the results presented in this paper.

We are still unable to explain the rapid decline in binding site density which occurs in the first few hr following isolation. However, this phenomenon does not appear to be influenced by aldosterone, or the presence of substrate.

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