Biol. and Med., 1963, v114, 811.
7. Pagano, J. S., Vaheri, A., Arch. ges. Virusforsch., 1965, v17, 456.
8. Dubes, G. R., Klinger, E. A., Jr., Science, 1961, v133, 99.
9. Arlinghaus, R. B., Polatnick, J., Vande Woude, G. F., Virology, 1966, in press.
10. Bachrach, H. L., Callis, J. J., Hess, W. R., Patty, R. E., DeBoer, C. J., Hamblet, F. E., Am. J. Vet. Res., 1962, v23, 608.
11. Bachrach, H. L., Callis, J. J., Hess, W. R., Patty, R. E., Virology, 1957, v4, 224.
12. Bachrach, H. L., ibid., 1965, v25, 532.
13. Bachrach, H. L., Breese, S. S., Jr., Proc. Soc. Exp. Biol. and Med., 1958, v97, 659.
14. Holland, J. J., Bact. Rev., 1964, v28, 3.

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Toad Bladder Extract Which Binds Sodium: Role in Sodium Transport.* (31645)

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Leaf and co-workers(1) have found that the urinary bladder of *Bufo marinus* is capable of transporting sodium from the mucosal to the serosal surface against an electrochemical gradient. Susat and Vanatta(2) have reported that there is an ether-soluble compound which binds sodium (ESC-Na) which is extractable from myocardium of dogs. Preliminary experiments in this laboratory showed that sodium was present in ether extracts of the toad urinary bladder.

The hypothesis to be tested by the experiments reported is that if a compound in the extract is involved in sodium transport, then the amount of the compound will vary with some of the factors which affect the rate of sodium transport. Also, if it is involved in sodium transport, it should be present in the bladder which contains a serosal layer and also a mucosal layer of epithelial cells which are involved in the transport mechanisms. On the other hand, little or no compound would be expected to be in only serosal tissue, which does not transport sodium.

Methods. Animals. The urinary bladder of *Bufo marinus* was used in all experiments. The toads were kept in distilled water for at least 20 hours prior to use. Each was pithed and the bladder promptly dissected out for the indicated experiments.

In situ experiments were carried out by cannulating the bladder, and leaving it within the peritoneal cavity of the toad. *In vitro* experiments were carried out by dissecting the bladder free, and removing it with a small piece of gut attached. Each half bladder was then cannulated, and a small piece of the bladder attached to the gut was discarded. The bladders were filled through the cannula with the indicated mucosal solutions, the cannula stoppered, and the bladder checked for leaks. The bladder was then immersed in a beaker in approximately 20 ml of the indicated serosal solution, and incubated for the time indicated at room temperature (20-23°C), unless otherwise indicated. When either sodium-free choline Ringer's solution or potassium solutions were used the bladder was rinsed once with the sodium-free solution. At the end of the experiment a sodium analysis was carried out. There was always sodium present in an amount less that 1 mM/l. This was interpreted as coming from the wall of the bladder, and not due to a leak in the bladder.

Solutions. Regular Ringer's solution contained NaCl 114 mM, $NaHCO_3$ 3 mM, $CaCl_2$ 0.9 mM and KCl 3 mM. Choline Ringer's solution contained choline chloride 114 mM, $KHCO_3$ 3 mM and $CaCl_2$ 0.9 mM. Ringer's solutions containing sodium concentrations of 5, 15 and 40 mM were made by mixing calculated volumes of regular and choline Ringer's solutions. Sodium-free potassium

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Ringer's solution contained KCl 117 mM, $KHCO_3$ 3 mM and $CaCl_2$ 0.9 mM.

Analytical techniques. At the end of the indicated incubations, the bladders were emptied, cut loose from the cannula, and blotted free of excess moisture on filter paper. The bladders were then promptly weighed and extracted according to methods previously reported(3) with slight modifications. These modifications were that in the final steps of the analysis the separatory funnels and volumetric flasks were washed with water, and this water was analyzed on the flame photometer to assure that no sodium was present on the glassware. The water washings of the petroleum ether were likewise analyzed for sodium content to assure that the extract was not contaminated during the extraction procedure with significant quantities of water-soluble sodium.

In the experiments in which bladders were exposed to sodium-free potassium Ringer's solution, and the respective controls, the extraction procedure was altered. The bladders were emptied, blotted to remove excess solution, and filled with air. They were promptly quick frozen by immersion in liquid nitrogen and lyophilized. The dried bladders were then extracted with petroleum ether:ethanol 2:1 for 30 minutes. This extract was purified in the same manner as above.

The analysis for sodium and potassium was capable of detecting 0.003 meq/l, or in the dilutions used, 0.03 microequivalents/sample of either cation. At the concentration range usually encountered, the reproducibility was $\pm$ 1% for Na, and $\pm$ 2% for K, the latter being due to the greater relative instrument error at the lower concentrations encountered.

Statistics. The methods of analysis of the difference of the mean and the analysis of the mean difference were Student's "t" test as described by Fisher(4).

Experimental. Bladders and serosal tissue. Bladders from 10 toads were analyzed for the ESC-Na content. Serosal tissue was stripped from the wall of the peritoneal cavity and was placed in Ringer's solution, then extracted and analyzed for its ESC-Na content. The average and standard error of 10 analyses in each series was 6.69 $\pm$ 1.40† μeq/g for bladders and 0.33 $\pm$ 0.15 μeq/g for serosal tissue.

Sodium-free Ringer's solution. Bladders of 20 toads were exposed *in situ* and each bladder cannulated. Ten of the bladders were filled with Ringer's solution and 10 with choline Ringer's solution. The incubation was carried out for a total of 60 minutes, with the mucosal solutions changed after the first 15 minutes. At the end of the 60-minute period, each bladder was removed and the ESC-Na content determined. The average ESC-Na content was 7.86 $\pm$ 1.16 μeq/g for bladders filled with Ringer's solution and 3.52 $\pm$ 0.49 μeq/g for those filled with choline Ringer's solution ($p = <0.01$).

The *in situ* experiments were repeated *in vitro*. Two series of 10 determinations each were made; the control with regular Ringer's solution on both surfaces averaged 9.35 $\pm$ 1.09 μeq/g, and the experimental series with choline Ringer's solution on the mucosal surface, regular Ringer's solution on serosal surface averaged 5.97 $\pm$ 0.48 μeq/g ($p = <0.05$).

A repeat of the control series averaged 7.49 $\pm$ 0.75 μeq/g; and an experimental series with choline Ringer's on the serosal surface, regular Ringer's on the mucosal surface averaged 3.69 $\pm$ 0.51 μeq/g ($p = 7 \times 10^{-5}$).

Sodium 5, 15 and 40 mM. Frazier(5) showed that as the concentration of sodium in the mucosal media increased from virtually zero to 117 mM, the rate of sodium transport was increased up to a concentration of about 30 mM, after which further increases in sodium concentration did not further increase the rate of sodium transport. For this reason, the effects of 5, 15, and 40 mM sodium concentrations in Ringer's solution bathing the mucosal surface were studied. One half bladder was incubated with regular Ringer's solution on both sides, and the other half bladder was incubated with the indicated concentration. ESC-Na concentration was determined on each half bladder and the mean difference calculated. The difference of a total of 10 such pairs was determined at each of the 3

† Average $\pm$ standard error.

concentrations. The average differences (experimental — control) of the experiments were: Na 5 mM — 3.11 ± 0.99 μeq/g, ($p = <0.02$); Na 15 mM — 3.41 ± 1.57 μeq/g, ($p = <0.05$); and Na 40 mM — 0.37 ± 0.92 μeq/g, ($p = <0.35$). The differences between the 5 mM and 15 mM experiments were not significant ($p = >0.80$).

Thus it appeared that the effect of mucosal sodium concentration showed similar concentration dependence for the effect on ESC-Na content of the bladder wall as for the reported effects on sodium transport.

Effect of cold on ESC-Na content. Half bladders were prepared as described above and incubated with regular Ringer's solution on the serosal surface and choline Ringer's solution on the mucosal surface for 15 minutes at room temperature. This incubation was continued in the control half bladder, but the experimental half was placed in the cold (0-2°C) with chilled Ringer's solution on the serosal surface and chilled choline Ringer's solution on the mucosal surface. After 15 minutes in the cold, the experimental half bladder was filled with chilled regular Ringer's solution and kept in the cold for 30 minutes and the control half bladder was filled with regular Ringer's solution at room temperature. At the end of this time the half bladders were extracted.

The ESC-Na content of the control half bladders averaged 12.09 μeq/g and of the cold incubated half bladders 7.37 μeq/g. The mean difference with its standard error was 4.72 ± 1.73 μeq/g ($p = <0.05$).

Effect of potassium Ringer's solution. Nine half bladders were incubated for 30 minutes with regular Ringer's solution on the serosal side, and sodium-free potassium Ringer's solution on the mucosal side. The paired half bladders were used as control experiments and were incubated with regular Ringer's solution on both sides. Eight of the nine experiments showed a reduction in ESC-Na, and the average change in ESC-Na for the 9 experiments was — 7.52 ± 1.97 μeq/g ($p = <0.01$). Part of the sodium was replaced by potassium, the average change being 3.78 ± 0.86 μeq/g ($p = <0.01$). Of the 8 showing a decrease in sodium the change in the sum of ESC-Na plus ESC-K showed a decrease of 5.81 ± 1.78 ($p = <0.02$), thus indicating that there was a real effect of the lowered sodium concentration in the mucosal media, and not simply a replacement of the Na in the extract by K.

Discussion. Ussing(6) reviews 6 different theories regarding sodium transport. Three of these theories suggest that there is an interaction of the sodium with some molecular component of the cell. If one accepts this, it is likely that this interaction can be expressed as a chemical reaction, the equilibrium of which is dependent upon the concentrations of substances reacting and the resultant product. It is argued that the effect of a virtual zero concentration of sodium in the mucosal media limits this reaction at the site of uptake, and there results a reduction in the quantity of the product, which is the sodium in combination with the molecular component of the cell. Since the ESC-Na content of the bladder is reduced by this procedure, the results are consistent with the theory that ESC-Na is the substance which is the product of the interaction hypothecated in the proposed models.

Similarly, the release of the sodium on the serosal side of the epithelial cell would again be a chemical reaction dependent upon the concentrations of the compounds on both sides of the membrane. If the concentration on the serosal side were reduced, the equilibrium conditions of this reaction would result in a reduction of sodium in the compound. The results of this experiment are also compatible with the theory that the ESC-Na compound is the transport substance.

At this point, an alternate theory as to the significance of ESC-Na concentration changes must be considered. The theory is that the complex which binds sodium is not involved in transport but is formed during the extraction process. Then the sodium-free Ringer's solution might reduce the total sodium content of the bladder wall and during the extraction process the amount of bound sodium might come to reflect this change in total sodium content.

The first argument against this point is a comparison of the sodium and potassium found in the extract. If the compound

binding cations in the extract were formed during the extraction process, it would be expected to bind sodium as readily as potassium. If the low sodium content of the mucosal or serosal solutions lowered the sodium content of the bladder wall, and hence of the extract, the potassium contained in the bladder wall should be unchanged, and hence the ratio of sodium to potassium changed in favor of more potassium binding. For example, the potassium bound in the extract in the *in situ* experiments was 2.31 μeq/g in the control series, and 1.27 μeq/g in the series in which choline Ringer's solution was placed in the bladder. In every series in which the ESC-Na was lowered by the low sodium solutions, the potassium in the extract was also lowered instead of raised as would be expected.

A second argument against the effect being introduced during the extraction process comes from a series of failures to produce anticipated results. When I first attempted to repeat the *in situ* experiments *in vitro*, I met with failure. Bladders which were incubated at room temperature in a beaker with Ringer's solution on the outside and choline Ringer's solution on the mucosal surface did not show a significant reduction in ESC-Na. After 3 series of 10 experiments each, with failures each time, I finally noted that in my technique the bladders were being removed from the beakers at the end of incubation, opened, dried on filter paper, and placed in a weighing bottle. They were then taken to an analytical balance in another room, weighed, and returned, at which time the extraction procedure was begun.

The possibility was readily apparent that in the time from incubation until extraction the mucosal surface of the bladder was contaminated with sodium Ringer's solution from the serosal surface, and ESC-Na could then be formed. A change in technique to one of promptly weighing each bladder on a Roller-Smith balance and beginning the extraction procedure promptly produced the results reported.

Since the time interval between the blotting of the bladder on filter paper and the extraction would not influence the total sodium content of the wall of the bladder, this observation was against the second interpretation given above.

A third argument against the view that the concentration of the compound is affected during the extraction process and not in life is supported by the experiments in which the bladders were incubated in the cold. I interpret the results of these experiments to support the view that ESC-Na is involved in the transport process. I believe that during the incubation with a sodium-free media at room temperature the ESC-Na content of both half bladders would be reduced. The experimental bladder was then placed in the cold so that metabolic processes supporting the transport system were either severely compromised or completely stopped. When the sodium Ringer's solution was introduced onto the mucosal surface, the half bladder at room temperature reformed ESC-Na in the process of sodium transport, and the bladder in the cold did not. Diffusion of sodium would occur into the fluids of both half bladders during this time. At the end of the period, the bladders were extracted at room temperature, and the difference in ESC-Na observed is more probably due to a difference which existed in the intact bladder than to a difference which developed during the extraction process.

The question is raised as to whether the reduction of the ESC-Na concentration observed with the sodium-free choline Ringer's solutions might be due to the replacement of the sodium by choline on the reacting groups. It was shown in our laboratory that the extracts obtained under these conditions did in fact contain choline. However, the experiments with the potassium Ringer's solutions clearly indicate that the process is not an entire replacement of the sodium by another cation, although a part of the reduction in sodium concentration may be by this mechanism. Also, if it were merely the presence of choline in the bladder wall during the extraction procedure which reduced the ESC-Na concentration, then a reduction in ESC-Na would not be expected in the experiment with the chilled Ringer's solution.

Frazier *et al*(5) report that the sodium concentration at the mucosal surface is rate-

limiting up to a concentration of 30 to 60 mM. The shape of the curve is such that the exact point is not easily determined. The fact that the ESC-Na content of the bladder wall is lowered by mucosal concentrations of 5 and 15 mM, but not by concentration of 40 mM, is again an argument in favor of the theory that ESC-Na plays a part in the transport process.

The finding of much larger quantities of ESC-Na in bladder wall than in serosal tissue again favors the theory that ESC-Na is a molecular substance involved in the process of transporting sodium against an electrochemical gradient.

Summary. There is in the urinary bladder of *Bufo marinus* an ether soluble compound which binds sodium (ESC-Na). The theory that this compound is in some manner involved in the transport of sodium across the wall of the bladder is supported by the following observations: 1. The content of ESC-Na in the bladder wall is 9.35 μeq/g, while that of serosal tissue, which does not transport Na, is 0.33 μeq/g. 2. The quantity of ESC-Na is reduced by placing a sodium-free solution at either the serosal or mucosal surface. The significance of these findings is discussed. 3. There is a similarity between the effect of increasing the sodium concentration of the mucosal fluid on reduction of ESC-Na concentrations and on rate of sodium transport. 4. The possibility that ESC-Na did not exist as such in the body has been considered. Instead it might be formed after death of the bladder and/or during the extraction process. Data are presented which make this latter possibility unlikely. 5. It is concluded that the data presented support the hypothesis that ESC-Na is a substance which plays a part in the sodium transport process.

1. Leaf, A., Anderson, J., Page, L. B., J. Gen. Physiol., 1958, v41, 657.

2. Susat, R., Vanatta, J. C., Proc. Soc. Exp. Biol. and Med., 1965, v119, 534.

3. ———, ibid., 1962, v109, 317.

4. Fisher, R. A., Statistical Methods for Research Workers, 6th ed., Oliver & Boyd, London, 1936.

5. Frazier, H. S., Dempsey, E. F., Leaf, A., J. Gen. Physiol., 1962, v45, 529.

6. Ussing, H. H., Handbuch der Experimentellen Pharmakologie, Springer-Verlag, Göttingen, 1960, 68.

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Properties of Staphylococcus Phage UC-18: A Comparison with the International Phage Series. (31646)

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Numerous investigators(1,2,3,4) have reported the experimental use of staphylococcus phages in an attempt to reduce the number of "untypable" cultures. Some of these phages have proved useful in typing cultures which generally show inhibition patterns with Group III phages of the international basic series. One phage (UC-18) has been especially valuable in characterizing cultures from hospital-acquired infections and it has been suggested that this phage be added to the basic series (5). A study of the properties of this phage would be useful before addition to the basic series is proposed formally. This is a report of the properties (morphology, serology, thermal inactivation, buoyant density and burst size) of phage UC-18. A comparison is made on the basis of these properties between phage UC-18 and the phages comprising the basic series.

Materials and methods. Phage UC-18 was furnished by Dr. P. B. Smith (Communicable Disease Center, Atlanta, Ga.). Trypticase soy agar and broth were used for propagation and experimental procedures.

Density gradient centrifugation in cesium