Reports

Permeability of Frog Skin to Choline

Abstract. An inward movement of choline can be demonstrated across isolated skins of *Rana pipiens* when the outside is bathed by a saline made with choline chloride. This is in contrast to an earlier report indicating that skins of *R. esculenta* are impermeable to choline. The flux was very small and independent of the active transport of sodium by the skins. For most experiments choline can be used as an "inert" replacement for sodium in this preparation.

In studying sodium movement across isolated frog skins it is frequently necessary to vary the concentration of this ion in the bathing solutions. This is often done by replacing the sodium with choline (*1*) or magnesium (*2*). Although there is little reason in many experiments to prefer either one, the use of isotonic magnesium chloride introduces a chloride asymmetry that is sometimes undesirable.

In the original work with choline an attempt was made to assess the magnitude of any choline movement across the skin by using a sensitive procedure involving acetylation followed by bioassay of the acetylcholine produced. No flux of choline could be detected across the skins of *Rana esculenta* by this method. Recently, however, the impermeability of the skin (*R. pipiens*) to choline has been questioned, and it was even suggested that the compound might be actively transported in the same direction as, and possibly in competition with, sodium (*3*). Choline is used routinely in this laboratory in experiments the design of which requires that it be virtually, if not completely, nonpenetrating. Moreover, it is being used for similar work in other laboratories (*4*). For this reason the permeability of frog skin to choline was reinvestigated, this time with C^{14}-labeled choline.

The experimental design was reasonably straightforward. Two ventral abdominal skins were set up in chambers of the type described by Ussing and Zerahn (*5*). One of these was short-circuited, and the current generated was monitored intermittently. The other preparation was not short-circuited; the skin potential was measured in this case. Both skins were from *R. pipiens*, the animals having been stored for about a month in a cold room (5°C). The labeled choline (N-methyl-C^{14}) was purified by extraction with isopropyl alcohol. The excess alcohol was removed with ether, and the choline was dried by exposure to a stream of dry air. A choline Ringer's solution was made up with nonlabeled choline treated in the same way. The isotopic compound (about 10 μc) was added, and this solution was used to bathe the outside of the skins. The inside of each skin was bathed by normal Ringer's solution. Samples of the inside solution were taken at hourly intervals, plated, dried, and counted with a gas flow tube with an ultrathin end window.

The possibility that choline and sodium might compete for the same transport mechanism was tested in the same experiment. After the inside solution had been sampled for 3 hours, 2.5 ml of normal Ringer's solution was added to the choline Ringer's in the outside chamber. This made the sodium concentration 10 mmole/lit., which nearly saturates the transport mechanism (*1*). If choline is transported by the sodium pump the choline flux should fall nearly to zero within the first minutes after the addition is made. Samples were taken for the next 2 hours and assayed as above for C^{14}.

The results of this experiment are shown in Fig. 1. Two conclusions may be drawn from the data. First, there is a movement of choline across both skins. Its magnitude is small, approximately the same as the outward *diffusion* of sodium across such preparations. The "permeability constant" for short-circuited skins in two such experiments was 8.8×10^{-4} cm/hr and 4.5×10^{-4} cm/hr. This compares with a value of 8×10^{-4} cm/hr for sodium (*1*). It might be noted that the values cited for choline probably represent upper limits for penetrability. Justification for this surmise is based in part on the tendency for choline to undergo radiolysis during prolonged storage. Any uncharged compounds produced (methanol, for example) would probably diffuse more rapidly than choline, and hence the C^{14} flux would be larger than the real choline movement. In addition, the skins used had very low diffusion resistances. The d-c resistances were about 1500 ohm cm^2 with choline Ringer's in the outside solution in contrast to the usual value of 3500 to 5000 ohm cm^2.

The second general conclusion is that choline and sodium move independently through the skin. In this experiment it can be seen that in the short-circuited skin choline influx was little changed by the initiation of sodium transport. The decrease in choline influx noted during the last period for the open-circuited skin may represent a real diminution of the flux. Initiation of a sodium influx generated the usual potential difference across the skin (40 mv, inside positive), and since this would mediate against inward cation movement some decrease in choline influx is to be expected. In neither case, however, is there any sign of interaction between the choline and sodium movements. Actually, the fact that choline movement is small and probably passive was already apparent from the results of our earlier work. It was shown that with choline Ringer's on the outside and normal Ringer's on the inside of a short-circuited skin a small *outward-directed* current developed. Since this

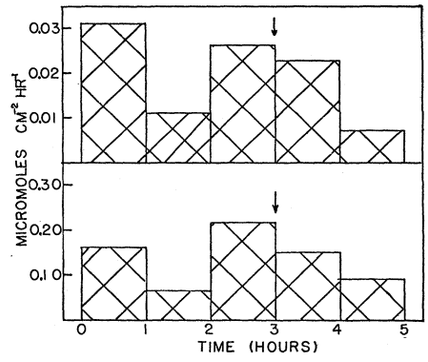

Fig. 1. Choline influx across skins of *R. pipiens*. The upper part of the figure shows the hourly movement of choline across an *open-circuited* skin. The lower portion shows the movement across a short-circuited skin. The outside of each skin was exposed to choline Ringer's during the first 3 hours. At the arrow sodium Ringer's was added to make the final sodium concentration 10 mmole/lit. Note the difference in scales on the ordinate.

Instructions for preparing reports. Begin the report with an abstract of from 45 to 55 words. The abstract should *not* repeat phrases employed in the title. It should work with the title to give the reader a summary of the results presented in the report proper.

Type manuscripts double-spaced and submit one ribbon copy and one carbon copy.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to Contributors" [*Science* **125**, 16 (1957)].

represented a difference between choline influx and sodium outflux it was obvious that choline movement, if any, must be small and probably passive.

Thus, Koblick's contention that skins of *R. pipiens* are not impermeable to choline has been confirmed. The reasons for the discrepancy between our earlier work and these experiments are unknown. Possibly a species difference is involved, since skins of *R. esculenta* were used in the older experiments. On the other hand, the magnitude of the choline flux is very small, and for most purposes the ion can be considered to be virtually nonpenetrating, especially if skins offering average resistance to diffusion are used. There is no evidence for an active choline transport across the skins tested (*R. esculenta* and *R. pipiens*) (*6*).

LEONARD B. KIRSCHNER
Department of Zoology, Washington State University, Pullman

References and Notes

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6. This work was supported by funds provided for biological and medical research by the State of Washington Initiative Measure No. 171, and by a grant-in-aid (G-4925) from the National Science Foundation.

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Isolation of Antibodies to Gelatin from Antigen-Antibody Complex by Proteolysis

Abstract. Antibodies were isolated by digestion of the gelatin-antigelatin complex with collagenase and removal of the gelatin fragments by dialysis. The pure antibody could be precipitated anew by the antigen. Antibodies to tyrosylgelatin were isolated in a similar way, but in this case peptides containing the active antigenic sites remained bound to the antibody.

Antibody preparations of high purity are a prerequisite for the study of the mechanism of antibody formation and mode of action. The purification of antibodies is based either on physicochemical procedures or on immunospecific reactions. By the second method antibodies have been purified with the help of insoluble antigens, as well as by the elution of antibody from antigen-antibody complexes by heat, strong salt solutions, alkali, or acids (*1*). Antibodies have also been purified by precipitation with slightly modified antigens, followed by dissolution in alkali or acid and selective precipitation of the modified antigen (*2*, *3*).

Enzymatic cleavage of the antigen in the antigen-antibody complex would be a very convenient method for the isolation of antibodies, if such treatment would not affect the antibody molecules. In the case of diphtheria and tetanus toxin-antitoxin floccules, digestion with pepsin or trypsin yielded preparations of antitoxins of high activity, but their molecular weights were reduced to about half the original size (*1*). In this report (*4*) we describe the isolation of pure, undamaged antibodies to gelatin by selective digestion of the antigen in the gelatin-antigelatin precipitate by collagenase. This enzyme degrades collagen and gelatin to products of an average molecular weight around 500, but seems to be inactive against other proteins (*5*).

In a control experiment, gelatin (U.S.P. granular, Fisher) was digested with collagenase (*6*) (4 units of collagenase per milligram of gelatin) in 0.05*M* tris buffer, *p*H 7.4, containing 0.005*M* $CaCl_2$, at 25°C, for 3 hours. The reaction mixture was subjected to exhaustive dialysis against water at 2°C for 3 days, and the solution both inside and outside the dialysis bag were hydrolyzed (6*N* HCl, 110°C, 24 hr). All the hydroxyproline of the original gelatin was found (*7*) on the outside, while none remained in the dialysis bag. This shows that the gelatin was split quantitatively into dialyzable fragments. When human γ-globulin was treated with collagenase under similar conditions but in phosphate buffer, no dialyzable peptides were split off (Kjeldahl nitrogen analysis). It appeared, in fact, that the γ-globulin molecules were completely unchanged by the enzyme, as the material sedimented at the same rate before and after treatment with collagenase in tris buffer ($S_{20} = 6.6$, at a 0.17 percent concentration in 0.15*M* NaCl; Spinco model E ultracentrifuge, at 56,100 rev/min), and the area enclosed by the gaussian curve in the sedimentation pattern remained constant.

Rabbit antiserum to gelatin was prepared and the antibodies were precipitated with gelatin, according to Maurer (*8*). The precipitate resulting from the addition of 0.65 mg of gelatin to 13 ml of antiserum was washed repeatedly with 0.15*M* NaCl. It contained 0.25 mg of antigen (33 μg of hydroxyproline in the hydrolyzate) and 1.53 mg of antibody (derived from the extinction at 280 mμ of the neutralized solution of the precipitate in 0.1*N* NaOH). A suspension of this precipitate in 2 ml of 0.05*M* tris buffer, *p*H 7.4, containing 0.005*M* $CaCl_2$ was treated with collagenase (3 units), at 25°C. After 3 hours the remaining insoluble material

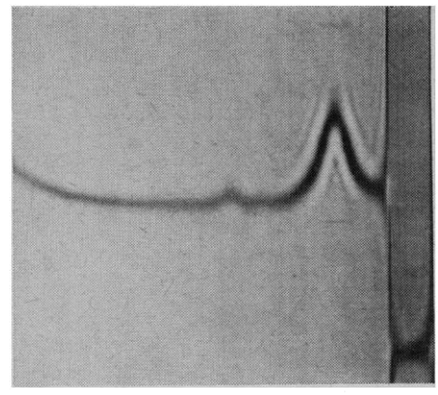

Fig. 1. A sedimentation pattern, in a Spinco model E ultracentrifuge, of antityrosylgelatin (0.17 percent in 0.15*M* NaCl), at 20°C. The photograph was taken 16 minutes after full speed (56,100 rev/min) was attained.

was centrifuged off. It contained 26 percent of the gelatin in the original precipitate (65 μg of gelatin, as calculated from hydroxyproline data). The supernatant fluid was subjected to exhaustive dialysis against the buffer, at 2°C. After 3 days the contents of the dialysis bag were brought up to 3.5 ml. The fact that hydroxyproline was found in the hydrolyzate of this solution, demonstrated that no gelatin-split products were present. The solution contained 80 percent of the antibody in the original precipitate (0.35 mg of antibody per milliliter, as calculated from the extinction at 280 mμ). Only one spot was detected on paper electrophoresis, migrating identically with the γ-globulin in rabbit normal serum. The material showed a sedimentation constant of S_{20} equal to 7.14 in the ultracentrifuge [rabbit γ-globulin has a sedimentation constant S_{20} approximately equal to 7 (*1*)]. The purified antibody could be precipitated (*8*) anew by the addition of gelatin.

Gelatin is a weak antigen; it yields antisera of low antibody titer, and the precipitin reaction takes a prolonged time. Attachment of tyrosine peptides to gelatin converts it into a relatively powerful antigen (*9*). In view of this enhancement of antigenicity, the possibility of obtaining pure antibodies from the system polytyrosylgelatin-antipolytyrosylgelatin was also investigated. A gelatin derivative enriched with only 2 percent tyrosine (*10*) ("tyrosylgelatin") served as antigen in this experiment. It had been shown previously (*9*) to be a strong antigen in comparison to gelatin. Upon digestion with collagenase and exhaustive dialysis (conditions similar to the control experiment with gelatin) tyrosylgelatin was split into fragments that dialyzed out completely, as followed spectrophotometrically (293.5 mμ, *p*H 13), as