INVESTIGATIONS ON THE EFFECT OF SOME LOCAL ANAESTHETICS AND OTHER AMINES ON THE ACTIVE TRANSPORT OF SODIUM THROUGH THE ISOLATED SHORT-CIRCUITED FROG SKIN

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SUMMARY

A study has been made of the influence of a series of local anaesthetics, which are all tertiary amines, and a series of primary, secondary and quaternary amines on the active transport of sodium across the isolated short-circuited frog skin.

The amines studied stimulate the active transport of sodium through the skin when they are added to the solution bathing the outside of the skin, and are present in ionized form in this solution.

No effect of the amines in the ionized form was observed when they were added to the solution bathing the inside of the skin.

In the form of undissociated bases, the amines inhibited the active transport of sodium through the skin, whether they were added to the outside or the inside solution.

The most reasonable explanation for the stimulating effect of the ionized amines on the active transport of sodium is that they increase the permeability to sodium of the outer membrane of the frog skin. This results in an increased influx of sodium into the epithelial cells and hence in an increased supply of sodium to the mechanism of active transport. The increase is accordingly not due to a direct effect of the ionized amine on the mechanism of active transport.

The data presented do not permit any conclusions to be drawn as to the localization of the inhibitory effect.

INTRODUCTION

As part of investigations on the mode of action of local anaesthetics[1,2] some preliminary studies were carried out on their effect on the active transport of sodium through the isolated short-circuited frog skin. These studies showed that local anaesthetics, when added to the solution bathing the outside of the skin, exert two types of action: a stimulation of the transport and a subsequent inhibition. When added to the inside solution the local anaesthetics inhibit transport without any stimulation.

KIRSCHNER[3,4] has previously shown with the same system that atropine, several curares, histamine, and pilocarpine, when added to the outside solution, stimulate

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the active sodium transport, while other drugs, tetraethylpyrophosphate, procaine, and eserine, inhibit the transport when they are added to the inside solution. From KIRSCHNER's experiments it is not possible to draw any conclusions as to the mechanism underlying the different effects of the various drugs when applied to the outside solution or the different effects of the drugs when applied to the inside or the outside solution.

The local anaesthetics studied in the preliminary experiments were all tertiary amines, and the pH employed 7.8. It seems accordingly possible that the dual action of the drug, when it is added to the outside solution, might be due to the fact that at this pH it exists partly as ionized amine and partly as undissociated base. This has been studied systematically in the present work.

It has further been investigated why the drugs act differently when applied to the inside and to the outside of the skin, and it has finally been studied in which way changes of the molecule influence the action on the skin. In addition to local anaesthetics a number of primary, secondary and quaternary amines were used in these studies.

Such studies may give information about the processes in the frog skin which underlie the active transport of sodium as well as of the action of drugs on this transport.

METHODS

Abdominal skin of *Rana esculenta* and *Rana temporaria* was used in the experiments.

The apparatus and experimental techniques used were those described by USSING AND ZERAHN[5], as modified by KOEFOED-JOHNSEN, USSING AND ZERAHN[6]. The area of skin used was 7.1 cm^2.

The Ringer solution contained 115 m*M* NaCl, 2.5 m*M* KCl, 1.0 m*M* $CaCl_2$, and was buffered to the desired pH with 2.1 m*M* sodium phosphate.

In the experiments performed at pH 10.0, the buffer used was borate instead of phosphate, and $CaCl_2$ was omitted. In all experiments, 2 I.U. of vasopressin were added to the 25 ml of Ringer solution bathing the inside of the frog skin[5].

The pH of the Ringer solution bathing the inside of the skin was 7.8 ± 0.2; the pH of he Ringer solution on the outside is stated for the individual experiments.

The experiments were carried out at room temperature, approximately 22°.

The net transport of sodium, ΔNa, was measured by the current required to short-circuit the potential across the skin[5–7]. In order to check that this current was also identical with the ΔNa when the skin was under the influence of the drugs studied, ΔNa was measured by the "double-labelling" method[8] by means of the two isotopes ^{22}Na and ^{24}Na supplied by Philips of Amsterdam, The Netherlands.

Before adding the compounds, 5 ml of Ringer solution was removed from that side on which the effect was to be studied; the compound was dissolved in this sample, which after adjustment of its pH was returned to the reservoir.

The concentration of the undissociated base, C_B was derived from the following equations:

$$C = C_B + C_{BH^+} \tag{1}$$

$$C_B = \frac{C}{1 + \frac{C_{H^+}}{k}} \tag{2}$$

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where C is the total concentration of the dissolved drug, C_{H^+} the hydrogen-ion concentration and k the dissociation constant. The concentration of the ionized molecule, C_{BH^+}, was calculated from ref. [1].

In studies on diffusion of procaine through the skin, the procaine concentration was determined by measuring the absorption of the solution at 290 mμ in a Beckman spectrophotometer, model DU.

Procaine was used in most of the experiments, and these are therefore described in detail below. However, the effects of a series of local anaesthetics, cocaine, tropacocaine, tetracaine and nupercaine, and of certain other amines were also studied.

RESULTS

Since it appeared that the drugs studied differed in their effect on the skins of *Rana temporaria* and *Rana esculenta*, the results of the experiments on the two species are reported separately.

Rana esculenta

Addition of procaine to the outside solution

When procaine a pH 7.8 is added to the Ringer solution bathing the outside of the skin, there is an increase of the short-circuit current, followed by a decrease. Both the increase and decrease of the current depend on the procaine concentration (Figs. 1 and 2).

With 11 mM concentration (Fig. 1) the increase in the short-circuit current begins about 5 sec after the addition to the outside solution, and the slope of the curve is very steep; after 9 min the current has reached a peak and thereafter decreases slowly. With 44 mM concentration (Fig. 2) the short-circuit current increases as before, but the decrease sets in after about 30 sec, and is more pronounced than

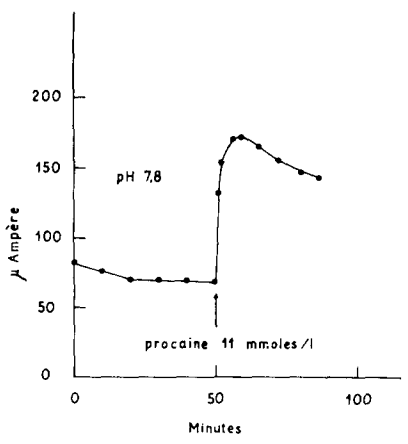

Fig. 1. The short-circuit current for an isolated frog skin before and after the addition of procaine to the Ringer solution bathing the outside of the skin. Procaine concentration 11 mM, pH 7.8.

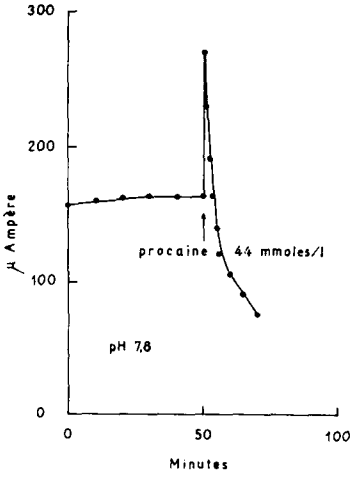

Fig. 2. The short-circuit current for an isolated frog skin before and after the addition of procaine to the Ringer solution bathing the outside of the skin. Procaine concentration 44 mM, pH 7.8.

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in the previous experiment. After a few minutes the current has fallen to a value which is lower than that recorded before the addition of procaine. The effect of procaine is reversible.

The intensity of the secondary inhibition is inconstant. In certain periods, a procaine concentration of 10 to 20 mM may thus produce a picture similar to that with 44 mM, while one similar to that with 11 mM is produced by a lower concentration.

At pH 7.8 procaine is present in the solution partially as undissociated base and partially as ionized amine. A pH shift leads to a change in the ratio of the two components. Since a pH change from 6 to 10 in the outside solution does not influence the sodium transport through the frog skin appreciably[9,10], it should be possible to study the influence of changes in the ratio of the concentration of undissociated base to that of ionized amine by varying the pH.

At a procaine concentration of 44 mM a shift in pH from 7.8 to 6.0 will give a decrease in the amount of undissociated base from 2.9 mM to 0.05 mM, while the amount of ionized amines will increase from 41.1 to 43.95 mM (pK for procaine 8.95 at 22°; *cf.* SKOU[11]).

As appears from Fig. 3, procaine in a concentration of 44 mM at pH 6.0 gives an increase in the short-circuit current which, contrary to the effect at pH 7.8 (*cf.* Fig. 2), is maintained. A shift in pH to 7.8 will give a decrease of the current.

At pH 10.0 and a total procaine concentration of 3.2 mM we have the same concentration of undissociated base in the solution, 2.9 mM, as at 44 mM and pH 7.8; but the concentration of ionized amine is now only 0.3 mM against 41.1 mM. The result of adding procaine to a concentration of 3.2 mM to the outside solution at pH 10.0 is shown in Fig. 4. There is a sudden decrease in the short-circuit current, and, unlike at pH 7.8, this is not preceded by an increase in the current.

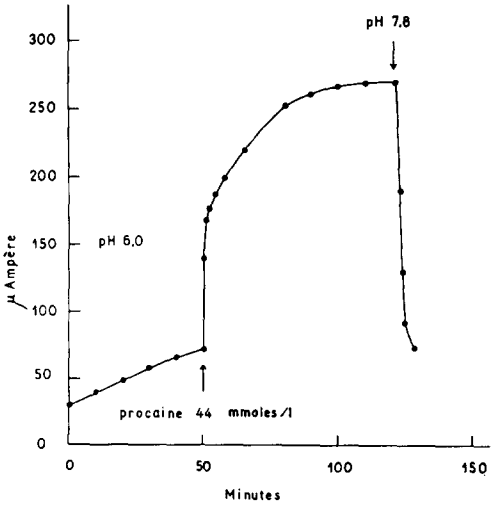

Fig. 4. The short-circuit current for an isolated frog skin before and after the addition of procaine to the Ringer solution bathing the outside of the skin. Procaine concentration 3.2 mM, pH 10.0.

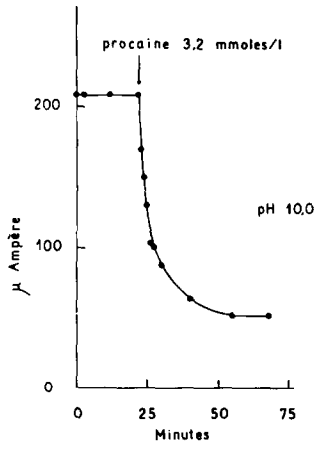

Fig. 3. The short-circuit current for an isolated frog skin before and after the addition of procaine to the Ringer solution bathing the outside of the skin. Procaine concentration 44 mM, pH 6.0.

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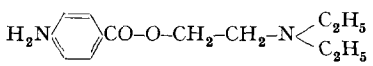
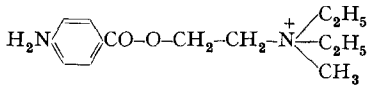
By introducing a methyl group at the tertiary nitrogen in procaine, the drug is transformed into a quaternary compound, *i.e.*, a compound which resembles procaine, but is ionized in aqueous solution at pH 6–10.



Procaine



Quaternary "procaine"

As appears from Fig. 5, addition of this quaternary "procaine" to the outside fluid of the skin to a concentration of 44 m*M* at pH 7.8 results in an increase in the short-circuit current without any secondary decrease. The behaviour of the current is the same when the compound is added to the same concentration at pH 6.0 or pH 10.0.

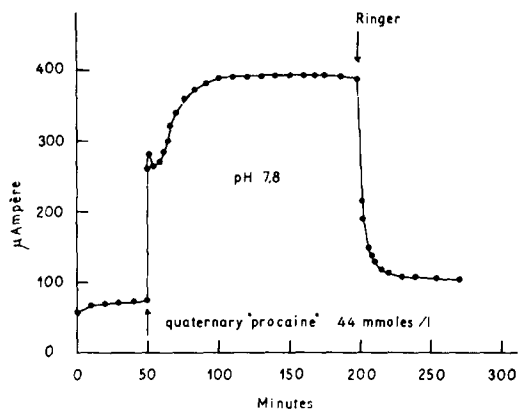


Fig. 5. The short-circuit current for an isolated frog skin before and after addition of quaternary "procaine" to the Ringer solution bathing the outside of the skin. Concentration of quaternary "procaine" 44 m*M*, pH 7.8.

The results obtained in the experiments with quaternary "procaine" show that the differences in the response of the skin to the addition of procaine at various pH levels is not due to an effect on the skin of the change in pH but is due to the difference in the concentrations of undissociated base and ionized amine in the solution. Moreover, the experiments show that the stimulant effect is due to the presence of ionized amine in the solution, whereas the inhibition is due to the presence of procaine as undissociated base in the solution.

At pH 6.0, the increase in the short-circuit current is rectilinear on addition of procaine in concentrations up to 20 m*M*, and at pH 10.0 the decrease in the current is rectilinear up to procaine concentrations of about 2 m*M*.

Addition of procaine to the inside solution

In the experiments so far reported, procaine was added to the solution bathing the outside of the skin. If procaine is added to the solution bathing the inside of

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the skin, at pH 7.8, the short-circuit current is inhibited, and, unlike the conditions observed on addition of procaine to the outside fluid, this inhibition is not preceded by an increase in the short-circuit current (Fig. 6; *cf.* Fig. 2).

An increase or a decrease in the pH of the inside solution will produce such pronounced changes in the sodium transport through the skin that it is impossible to study the effect of a change in the ratio of undissociated base and ionized amine. However, it was found that quaternary "procaine" did not influence the short-circuit current when it was added to the inside solution. It is therefore reasonable to assume that the inhibitory effect of procaine from the inside, just as that from the outside, is due to the fact that procaine is present in the solution as undissociated base, whereas procaine as ionized amine has no effect when it is added to the inside of the skin.

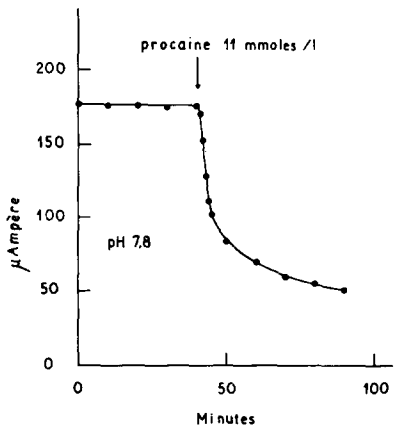

Fig. 6. The short-circuit current for an isolated frog skin before and after the addition of procaine to the Ringer solution bathing the inside of the skin. Procaine concentration 11 m*M*, pH 7.8.

Measurements of the net transport of sodium

If the sodium in the outside solution is replaced by Mg in iso-equivalent concentration, there is no transport, and there is only a small negative short-circuit current owing to the loss of sodium from the inside Ringer solution. Under these circumstances addition of procaine to the outside solution does not give an increase in the short-circuit current. The increase in current produced by procaine depends accordingly on the presence of sodium in the outside solution.

In the isolated short-circuited skin, the short-circuit current is identical with the net transport of sodium, ΔNa[5-7]. In order to study if this is so also when the skin has been treated with procaine, the net transport of sodium was measured directly, and compared with the short-circuit current. ΔNa was expressed in $\mu A/cm^2$, so that it was directly comparable with the short-circuit current. The results of a representative experiment are shown in Table I, and in Table II are listed the mean values obtained in the experiments in which procaine was added to the outside solution to concentrations of 11 and 44 m*M* at pH 7.8, and to a concentration of 44 m*M* at pH 6.0.

After addition of procaine in a concentration of 44 m*M* to the inside solution

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TABLE I

REPRESENTATIVE EXPERIMENT SHOWING THE INFLUENCE OF PROCAINE ON THE ACTIVE TRANSPORT OF SODIUM AND ON THE SHORT-CIRCUIT CURRENT

The sodium influx was measured by labelling the outside sodium with ^{22}Na and the sodium efflux by labelling the inside sodium with ^{24}Na.

Treatment	*Experimental period (min)*	*Influx ($\mu A/cm^2$)*	*Efflux ($\mu A/cm^2$)*	*ΔNa ($\mu A/cm^2$)*	*Short-circuit current ($\mu A/cm^2$)*
Procaine 44 m*M* inside, pH 7.8	60	4.0	1.1	2.9	6.6
After washing, control, pH 7.8	60	18.4	1.4	17.0	17.4
Procaine 11 m*M* outside, pH 7.8	15	24.3	0.3	24.0	30.6
—	60	30.7	1.4	29.3	33.7
—	60	37.0	1.3	35.7	33.9
—	65	30.4	0.9	29.5	30.7

TABLE II

THE INFLUENCE OF PROCAINE ON THE ACTIVE TRANSPORT OF SODIUM AND ON THE SHORT-CIRCUIT CURRENT

The figures shown are mean values from experiments in which procaine was added to the outside solution. ΔNa is the net transport of sodium/cm^2, *i.e.* the difference between the influx and efflux of sodium. The influx and efflux were measured by labelling the outside sodium with ^{22}Na and the inside sodium with ^{24}Na.

Treatment	*Number of experiments*	*ΔNa ($\mu A/cm^2$)*	*Short-circuit current ($\mu A/cm^2$)*	*ΔNa—short-circuit current ($\mu A/cm^2$)*
Control, pH 7.8	6	27.9	27.3	0.4
Procaine 11 m*M*, pH 7.8	5	42.3	44.7	—2.4
Procaine 44 m*M*, pH 7.8	4	9.6	14.3	—4.7
Control, pH 6.0	3	32.3	31.9	0.4
Procaine 44 m*M*, pH 6.0	3	62.6	59.4	3.2

(Table I) it was not possible to obtain a steady state; the current fell throughout the experiment. The same was the case after adding procaine in a concentration of 44 m*M* at pH 7.8 to the outside solution (Table II). Taking this into consideration, the difference obtained in these periods was in fact small enough to indicate that there is agreement between ΔNa and the short-circuit current. In the other periods shown in the tables there is good agreement.

The changes in short-circuit current observed after addition of procaine to the skins thus represent changes in the net transport of sodium.

During the experiment, procaine passes through the frog skin. Thirty min after the addition of procaine to the outside solution to a concentration of 44 m*M* at pH 7.8, the concentration of procaine in the inside solution is 0.06 m*M*. The penetration is slower at lower pH levels. Thus, at the same outside concentration but at pH 6.0, the inside concentration is only 0.001 m*M* after 30 min. Quaternary "procaine" does not pass through the skin, and apparently procaine is therefore capable of crossing the skin only in the form of undissociated base.

The effect of a number of other local anaesthetics, cocaine, tropacocaine, tetra-

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caine and nupercaine, was also studied. Like procaine, all these drugs are tertiary amines, and it was found that they all had both a stimulating and an inhibitory effect on the short-circuit current. In order to exert a stimulating effect they must all, again like procaine, be present in the solution bathing the outside of the skin as ionized amine, while in order to bring about inhibition they must be present either in the inside or the outside solution as undissociated base.

However, the stimulating effect is not an exclusive property of tertiary and quaternary amines. All the primary and secondary amines studied also produce an increase in the short-circuit current when they are added to the outside solution. The primary amine amphetamine and the secondary amine metamphetamine thus give a stimulation of the same order of magnitude as procaine. In order to exert a stimulating effect these substances, like tertiary amines, must be present in the ionized form, while as undissociated bases they inhibit the short-circuit current. Diethylaminoethanol is one of the two compounds which are derived from procaine by splitting the ester bond, and it is the fraction containing the tertiary amine. This compound has only a slight stimulating effect. Only when diethylaminoethanol is combined with paraaminobenzoic acid to form procaine is vigorous stimulation obtained.

The stimulation exerted by tetramethylammonium is so slight that it is difficult to decide if there is any effect at all, while that of tetraethylammonium is more distinct, although still slight. In the same molar concentrations as procaine, acetylcholine has a stimulating effect equal to about ¼ of that of procaine.

Rana temporaria

Only in a minority of the experiments did addition of procaine to the outside solution result in an increase in the short-circuit current when skins of *Rana temporaria* were used, and the increase occurred more slowly and was smaller than that observed with skins of *Rana esculenta*. Most of the experiments showed either no effect or, when the experiments were performed at a pH at which some of the procaine was found as undissociated base, an inhibition. In some cases in which it was not possible to stimulate the current with procaine from the outside, stimulation was obtained when the skins were pretreated on the inside with procaine, which was washed off immediately afterwards.

In skins of *Rana temporaria*, KIRSCHNER[4] was unable to induce any stimulation of the short-circuit current by addition of atropine to the outside solution. Since both procaine and atropine stimulate the short-circuit current in skins of *Rana esculenta*, the cause of the absence of effect of atropine can scarcely be that atropine and procaine differ in their effect on skins of *Rana temporaria*; it would be more reasonable to seek the explanation in the capricious way in which skins of *Rana temporaria* respond.

Whether the drug is added to the outside or the inside solution, the inhibitory effect of procaine as undissociated base on skins of *Rana temporaria* is much stronger than that observed in skins of *Rana esculenta*.

DISCUSSION

Stimulation

As appears from the experiments of KIRSCHNER[3,4] and those reported here, a

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number of widely different drugs are capable of stimulating the active transport of sodium across the isolated frog skin. Features which are common to all these drugs are (1) that they are amines, (2) that they must be added to the solution bathing the outside of the skin, and (3) that they must be present in ionized form in order to exert this effect.

The stimulating effect seems to be independent of whether the drugs are primary, secondary, tertiary or quaternary amines. The effects of tertiary procaine and of quaternary "procaine" were of the same order of magnitude, and so were the effects of the primary amine, amphetamine, and the secondary amine, metamphetamine.

On the other hand, the effect seems to depend on the molecule to which the amino group is linked. It was found that the effect of the quaternary amine, acetylcholine, was much less than that of the quaternary "procaine".

KOEFOED-JOHNSON AND USSING[12] showed that the potential across frog skin varies with the concentrations of sodium and potassium on the two sides of the skin in such a manner that it must be assumed that frog skin contains two functional membranes: an outer membrane, which is specifically permeable to sodium but not to the other cations, and which probably corresponds to the surface of the epithelial cells, and an inner membrane, which is specifically permeable to potassium, and which is probably situated at the inward-facing membrane of the stratum germinativum. These authors assume that the active transport of sodium is localized to the innermost of these two membranes, while no active transport occurs through the outer membrane. If transport through the skin is to take place, sodium must be transported passively through the outer membrane into the epithelial cells, from which active transport takes place through the inner membrane. If this is correct, there must be an electrochemical gradient for sodium from the outside solution into the epithelial cells.

KOEFOED-JOHNSON AND USSING's hypothesis has been supported by work performed by ENGBÆK AND HOSHIKO[13], who showed that the total potential across the epithelial layer is the sum of two or more potentials.

It has been shown by OTTOSEN *et al.*[14] that in the short-circuited frog skin the potential difference between the bathing solution and the inside of the epithelial cells is zero or small. We do not know the concentration of sodium inside the epithelial cells, but it is presumably low. The short-circuited frog skin with Ringer solution on the outside should therefore have a high electrochemical gradient for passive transport of sodium from the outside into the cells.

In experiments with giant axons, HODGKIN AND KEYNES[15] showed that the active transport of sodium out of the nerve increased proportionally to the intra-axonal concentration of sodium. If, similarly, the active transport of sodium from the epithelial cells of the frog skin to the inside fluid is proportional to the intracellular sodium concentration, an increase in the influx of sodium from the outside solution into the epithelial cells must result in an increase in active transport.

It is the ionized amines that are capable of increasing the active transport of sodium, but only when they are added to the outside solution. Since the ionized amines do not pass through the skin, their point of attack is presumably the outermost of the two membranes in the frog skin, and their effect may consist in increasing the permeability of this membrane to sodium. Since there is an electrochemical gradient for sodium from the outside solution to the epithelial cells, an increase in the permeability of the membrane to sodium will lead to an increase in the sodium

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influx and hence in the sodium concentration in the epithelial cells. As mentioned above, this must be assumed to produce an increase in the active transport of sodium.

This hypothesis about the effect of the ionized amines may be supported by the fact that quaternary amines are able to increase the permeability to sodium of the membranes of the muscle end plate (*cf.* RIKER[16]) and of the electroplax from *Electrophorus electricus*[17,18].

Inhibition

The amines studied inhibit the active transport of sodium when they are present as undissociated base either in the outside or the inside solution.

In the undissociated form, amines are able to penetrate into the epithelial cells. Depending on the pH, a certain fraction of the undissociated base will become ionized, and the amine will thus be present in the cells both as undissociated base and in the ionized form.

A similar reversible inhibition of the active transport of sodium can be produced by a non-ionized drug, *e.g.* butyl alcohol. There are certain points of resemblance between local anaesthetics as undissociated base and butyl alcohol (lipoid solubility and ability to penetrate into and change the physical properties of lipoid-containing interfaces[1]); it might therefore be reasonable to assume that it is the undissociated base of a local anaesthetic agent which exerts the inhibitory effect.

It is not possible, on the basis of these experiments, to decide whether the inhibitory effect on the sodium transport is due to an inhibition of the process that is responsible for the active transport or to an inhibition of the passive transport of sodium, for example, through the outer membrane of the frog skin.

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