Biochimica et Biophysica Acta, 323 (1973) 309–325

BBA 76401

ROLE OF Na^+ AND ANIONS IN THE TRIPLE RESPONSE OF ISOLATED FROG SKIN TO NOREPINEPHRINE

MARY W. PINSCHMIDT, ADDISON D. CAMPBELL and ERNST G. HUF

Department of Physiology, Medical College of Virginia, Health Science Division, Virginia Commonwealth University, Richmond, Va. (U.S.A.)

(Received March 12th, 1973)

SUMMARY

1. Isolated short-circuited frog skin in NaCl or $NaHCO_3$ solutions responded to norepinephrine ($3 \cdot 10^{-5}$ M), added to the epithelial side, with an increase in short-circuit current (s.c.c.) and open-skin potential (V) with little if any change in total skin conductance (G=s.c.c./V). Skins in Na_2SO_4 solutions gave a greatly diminished response to norepinephrine added to the epithelial side, with indication of depression of s.c.c. and V later during the experiment. Thus, the response is dependent on Cl^- or HCO_3^-.

2. Omission of Na^+ from the solution at the epithelial side (arginine chloride (outside), NaCl (inside)) resulted in a very weak norepinephrine (outside) response, suggesting that norepinephrine (outside) stimulates active inward Na^+ transport. Skins in arginine sulfate (outside), NaCl (inside); choline bicarbonate (outside), sodium bicarbonate (inside); arginine sulfate (outside), sodium bicarbonate (inside) also failed to give the marked response noted in 1.

3. When applied to the corium side, norepinephrine led to an increase in s.c.c. and G but a decrease in V by action on the glandular epithelium in the presence on the inside of NaCl or $NaHCO_3$, regardless of the presence or absence of Na^+ on the epithelial side. Flux measurements proved that active outward Cl^- transport occurred. Active outward HCO_3^- transport, suggested by the s.c.c. results, was unmeasurably small because of a 100 times greater C flux in the form of CO_2 which was equal in both directions and unaffected by norepinephrine on the corium side. Active outward Cl^- transport was greatly depressed when Na^+ on the corium side was replaced by $arginine^+$. There was good recovery of V and s.c.c. when NaCl solutions were present on both sides.

4. Ouabain ($5 \cdot 10^{-4}$ M), or propranolol ($5 \cdot 10^{-5}$ M) greatly decreased active glandular Cl^- transport following application of norepinephrine to the corium side. Diamox ($5 \cdot 10^{-3}$ M) had a small depressing effect of norepinephrine (inside) on Cl^- transport, and it depressed completely a relatively weak norepinephrine (inside) response, presumably because of a decrease in HCO_3^- transport.

5. The results are interpreted as showing functional dependency of α-inhibitory and β-stimulatory adrenergic receptor sites in frog skin epidermis on the nature of the anion present (SO_4^{2-} *vs* Cl^- and HCO_3^-), and of β-stimulatory receptor sites in the glandular epithelium on the presence of Na^+.

Abbreviations: s.c.c., short-circuit current; PD, potential difference.

INTRODUCTION

The triple action of norepinephrine refers to the fact that catecholamines with α- and β-receptor site activities can elicit in frog skin α-inhibitory, and β-stimulatory effects on Na^+ transport in the epidermis, and β-stimulatory effects on Cl^- transport in skin glands[1–11]. Recently we have published results[12] which showed that active Na^+ transport in frog skin is greatly dependent on the concentration of Cl^- in the solutions at the epidermal as well as at the corium side of short-circuited skins. From these observations the following questions arose: (1) Do hormones and other chemical agents which are known to alter the rate of transepidermal Na^+ transport in short-circuited skins depend on the presence of Cl^- to show effectiveness? (2) Does the stimulatory action of agents on Cl^- transport by the glands depend on the presence of Na^+? The work presented here is concerned with these questions. We have studied the role of Na^+, SO_4^{2-}, Cl^-, and HCO_3^- in the triple response of the skin to norepinephrine. No attempts were made to differentiate between receptors on pharmacological grounds. However, the effects of metabolic inhibitors were studied.

METHODS

Animals

All experiments were conducted at nearly constant room temperature on abdominal skins of male and female frogs (*Rana pipiens*).

Electrical measurements

The manually controlled short-circuiting technique of Ussing and Zerahn[13] was used with a few technical variations as described previously[12]. Current and skin potential (V in mV) were measured with digital multimeters (Health, model IM-102, input impedance 10^9 Ω). The skin area was 3.8 cm^2. The calomel half cells (Radiometer, K401) used in the measurements of V gave near zero asymmetry potentials in KCl solution. When tested in the various salt solutions used in this study (Table I), an asymmetry potential difference (PD) of no more than 0.8 mV was allowed. The electrodes were kept short-circuited in saturated KCl when not in use. In a number of experiments, Na^+ was omitted from the epithelial side and near zero values for V and short-circuit current (s.c.c. in μA) were found. These values, as well as conductance $G = \text{s.c.c.}/(\text{cm}^2 \cdot \text{V})$ are taken as base line values for the system as a whole. Stable base line values were soon obtained in most experiments. Our major interest, however, was in observing changes in parameters from base line values in skins treated with norepinephrine. Where both V and s.c.c. were only small fractions of unit values, G was not calculated.

Solutions

The composition of the solutions used is given in Table I. They were freshly prepared at weekly intervals. Arginine·HCl (Schwarz–Mann) was used. Arginine sulfate was prepared by titrating arginine base (Schwarz–Mann), $pK_a = 12.48$, with H_2SO_4 to give the desired solution pH. Choline bicarbonate was obtained as a 45% aqueous solution (Sigma, Grade II) and diluted to give the desired HCO_3^-

TABLE I

COMPOSITION OF SOLUTIONS

Outside refers to the epithelial side and inside refers to the corium side of the skin.

Designations used in the text	*Composition*	*pH*	*mOsM*
A NaCl (outside), NaCl (inside)	100 mM NaCl–4mM $KHCO_3$	7.8**	194
B 38 HCO_3^- (outside), 38 HCO_3^- (inside)	34 mM $NaHCO_3$–66 mM NaCl–4 mM $KHCO_3$	7.8	202
C $NaHCO_3$ (outside), $NaHCO_3$ (inside)*	100 mM $NaHCO_3$–4 mM $KHCO_3$	7.8**	210
D Na_2SO_4 (outside), Na_2SO_4 (inside)	48 mM Na_2SO_4–4 mM Na_2HPO_4–2 mM K_2SO_4	7.6	130
E arginine chloride (outside), arginine chloride (inside)	96 mM arginine·HCl–4 mM $KHCO_3$–4 mM Tris	7.7	180
F arginine sulfate (outside), arginine sulfate (inside)	96 mM arginine·H_2SO_4–2 mM K_2SO_4–4 mM Tris	7.8**	123
G choline bicarbonate (outside), choline bicarbonate (inside)	100 mM choline·HCO_3–4 mM $KHCO_3$	7.8	220

* pH values given are those at the beginning of the experiments. The values at the end of the experiments were 8.1.

** In the Diamox experiments, the pH was raised to 8.3 in control and Diamox solutions to keep the drug in solution.

concentration. The final Cl^- and HCO_3^- concentrations were checked by Cl^- titration with the Aminco Cl^- titrator and by gasometric CO_2 estimation with the Natelson Microgasometer. Tris buffer (Sigma) was added to some of the solutions used. Norepinephrine (L-arterenol bitartrate, Sigma) was always freshly prepared using the appropriate salt solution. A stock solution of 0.2 ml was then added to the chamber fluids to give the final desired norepinephrine concentration. Ouabain was purchased from Nutritional Biochemical Corporation, and Diamox (acetazoleamide) was kindly supplied by the Lederle Laboratories, Pearl River, N.Y. Propranolol (Inderal) was obtained by the courtesy of the Ayerst Laboratories, Inc., New York, N.Y.

Flux measurements

Under certain solution conditions, ^{14}C and ^{36}Cl influx and outflux, and ^{24}Na outflux measurements were performed[12,14]. The paired skins were allowed to come to non-isotopic steady state (1 h). The isotope was then added, and after 30 min three samples were taken prior to addition of norepinephrine to the corium side. Then, at 5-min intervals, 12 1-ml samples were drawn from the sink compartments and appropriate fluid replacements were made. In studies with Cl^- and HCO_3^- solutions, maintenance of ion concentrations in the chamber fluids was established by Cl^- and CO_2 analysis at the end of the experiment. $NaH^{14}CO_3$ was used for ^{14}C fluxes, and $^{24}NaCl$ for ^{24}Na fluxes. ^{36}Cl was obtained as $H^{36}Cl$. This was converted into $Na^{36}Cl$, or $K^{36}Cl$ (for the arginine experiments). The stock ^{36}Cl solutions were titrated for Cl^- concentration for application of corrections to the ion concentrations of the bathing solutions. The activities used were as follows: ^{24}Na, 100 μCi; ^{36}Cl, 50 to 60 μCi; ^{14}C, 40 μCi. ^{24}Na was counted immediately after termination of the experiment and corrections for physical decay were applied to the results. ^{14}C and ^{36}Cl (in separate experiments) were counted several weeks later in an automatic Packard liquid scintillation spectrometer.

Statistical calculations

All error values given in the following tables are S.E. Where applicable, t and P values were calculated and read from tables for paired skin experiments. Significant means $P \leqq 0.01$; not significant means $P > 0.05$.

RESULTS

(1) Consideration of norepinephrine concentration

$1 \cdot 10^{-6}$ M norepinephrine (inside) is a critical dose level. Fig. 1A shows that at this concentration there occurred a small increase in s.c.c. and V with little or no change in G. This was the case for skins in NaCl Ringer's, and for skins in Ringer's in which 34% of the NaCl was replaced by $NaHCO_3$. Previous studies have shown that the HCO_3^- concentration in skin is near 33 mmoles/l tissue water[15]. When used in concentrations of $1 \cdot 10^{-5}$ M, and $1 \cdot 10^{-4}$ M, norepinephrine (inside) greatly increased s.c.c. and G, but V decreased after a short period of increase (Figs 1B and 1C). This demonstrates clearly the occurrences of different events at different dose levels. A low dose of norepinephrine stimulates transepidermal Na^+ transport; a high dose of norepinephrine stimulates glandular Cl^- transport and inhibits epidermal transport[1-11].

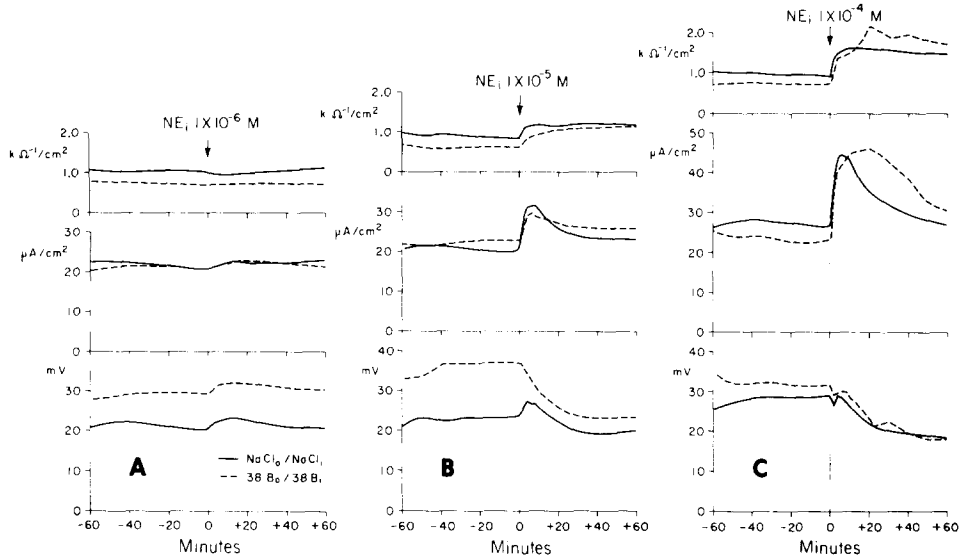

Fig. 1. Effects of norepinephrine (inside) at various dose levels. Studies on paired skins using NaCl Ringer's (A, Table I) in one half pair, and 34 $NaHCO_3$–66 NaCl Ringers's (B, Table I) in the other half pair. The procedure used in preparing this graph was similar to the one mentioned in the legend to Fig. 2. $n=4$ (A); $n=8$ (B); $n=4$ (C). S.E. at all points in time were ± 6, or less. Note: In A, increase in s.c.c. and V, with little change in G. By contrast B and C, increase in s.c.c. and G with decrease in V. NE_I, norepinephrine (inside); $NaCl_O/NaCl_I$ = NaCl (outside), NaCl (inside); $38B_O/38B_I$ = 38 bicarbonate (outside), 38 bicarbonate (inside).

(2) Effects of norepinephrine (outside) vs norepinephrine (inside)

Experiments were carried out to see whether norepinephrine (outside) ($3 \cdot 10^{-5}$ M) resulted in an increase in s.c.c. and V, with little change in G when skins are bathed on both sides with NaCl or $NaHCO_3$ solutions (A and C, Table I). This was found to be the case (Figs 2 and 3, paired skins). By contrast norepinephrine (inside) resulted in an increase in s.c.c. and G, but led to a decrease in V, as already seen in Fig. 1. In the middle section of Figs 2 and 3 there are small dash marks. By connecting the appropriate ones, a base line (calculated regression line) is obtained. The areas under the s.c.c.–time curves following application of norepinephrine were then measured using a planimeter. From this, the increment in flow of electrical charge, and thus of ion flux, was obtained. The results are given in Figs 2 and 3. ($1\ \mu A/cm^2 = 3.6$ mC/(h·cm^2); 1 mC/(h·cm^2) = 0.01036 μequiv·h^{-1}·cm^{-2}). The most likely reason for the increase in s.c.c. and V with norepinephrine (outside) in stimulation of net inward Na^+ flux, rather than increase in net outward Cl^-, or HCO_3^- flux. Indeed, when applying norepinephrine (outside) to skins in solutions of arginine chloride (outside), NaCl (inside), little or no norepinephrine (outside) effect was seen (see paragraph 4 below). It will also be noted that whereas $3 \cdot 10^{-5}$ M norepinephrine (outside), and $3 \cdot 10^{-5}$ M norepinephrine (inside) gave about the same increment in net flow of charge, or net ion flux (approx. 0.1 μequiv/cm^2), norepinephrine (inside) greatly increased G, but norepinephrine (outside) altered G very little. This points to stimulation by norepinephrine of different ion flux mechanisms. To bring this in line with all the data in the literature, which is exclusively on the effects of norepinephrine (inside), it is concluded that norepinephrine (outside) stimulated

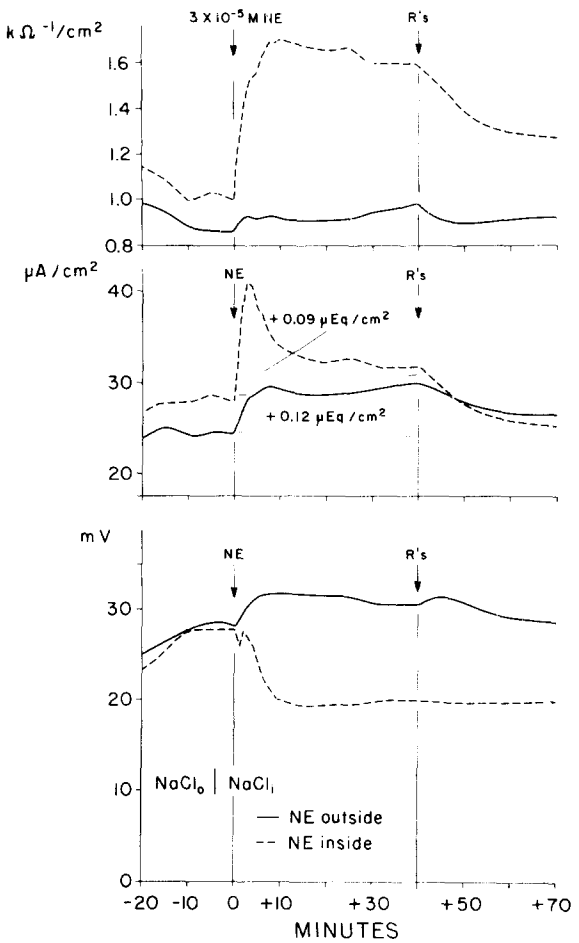

Fig. 2. Effects of norepinephrine (outside) and norepinephrine (inside). Studies on paired skins ($n=9$) in NaCl Ringer's (A, Table 1). These graphs were prepared as follows: Readings were taken from plots of the individual experiments and averages and the S.E. for each was calculated. 5 readings were taken before and after wash-out of norepinephrine, and 16 readings during the norepinephrine phase, at 1-min intervals at first. At the R's both the inside and outside solutions were replaced by fresh NaCl Ringer's. The S.E. at all points was very nearly ± 7 and ± 4 [V and s.c.c., for norepinephrine (outside)]; ± 6 and ± 4 [V and s.c.c., for norepinephrine (inside)]. NE, norepinephrine; $NaCl_o/NaCl_i$ = NaCl (outside), NaCl (inside); Eq, equiv.

active inward Na^+ transport, and norepinephrine (inside) stimulated active outward anion (Cl^-, HCO_3^-) transport. Upon closer inspection of Figs 2 and 3 one notices that norepinephrine (inside), after having first brought about the mentioned increase in s.c.c. and G, then led to a sudden decrease in these parameters, and there occurred a decrease in V subsequent to short periods of little change. This may be the result of concomitant stimulation of α-receptor sites in the epidermis leading to inhibition of net inward Na^+ transport[4,6].

(3) Cl^- dependence of stimulatory effect of norepinephrine

Fig. 4 shows results obtained when the paired skins were bathed on both sides

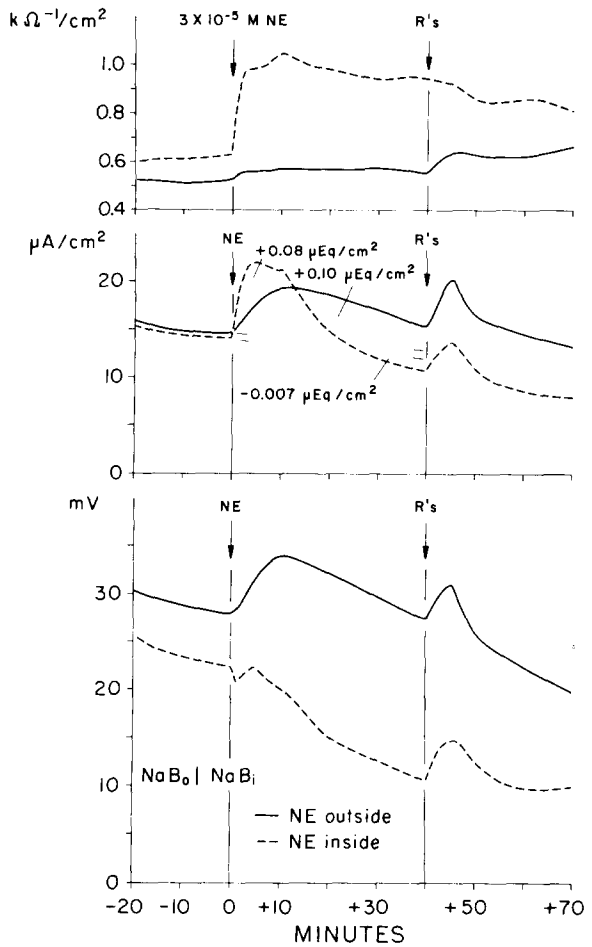

Fig. 3. Effects of norepinephrine (outside) and norepinephrine (inside). Studies were on paired skins ($n=9$) in $NaHCO_3$ solutions (C, Table I). At the R's fresh solutions were replaced on both sides of the skins. See also the legend to Fig. 2. The S.E. was ± 7 and ± 3 [V and s.c.c., for norepinephrine (outside)]; ± 3 and ± 2.5 for V and s.c.c. for norepinephrine (inside). NaB_o/NaB_i = $NaHCO_3$ (outside), $NaHCO_3$ (inside).

in sulfate Ringer's. (D, Table I). Comparing the results shown in Figs 2 and 3 with Fig. 4, there was a 70% decrease in epidermal stimulatory effect of norepinephrine (outside) when using sulfate Ringer's. When applying norepinephrine to the inside, not only was there no increase in s.c.c. (as seen in Figs 2 and 3) but there occurred a prompt and significant decrease in s.c.c. V also decreased sharply, interrupted by a brief period of rising V. G, after a small rise at the beginning returned to its pre-norepinephrine (inside) value. The decrease in s.c.c. and V seen here corroborates the notion that stimulation of inhibitory α-receptor sites in the epidermis had occurred.

(4) Ineffectiveness of $3 \cdot 10^{-5}$ M norepinephrine (outside) on skin glands

This was studied in paired skins (applying norepinephrine (outside), or for

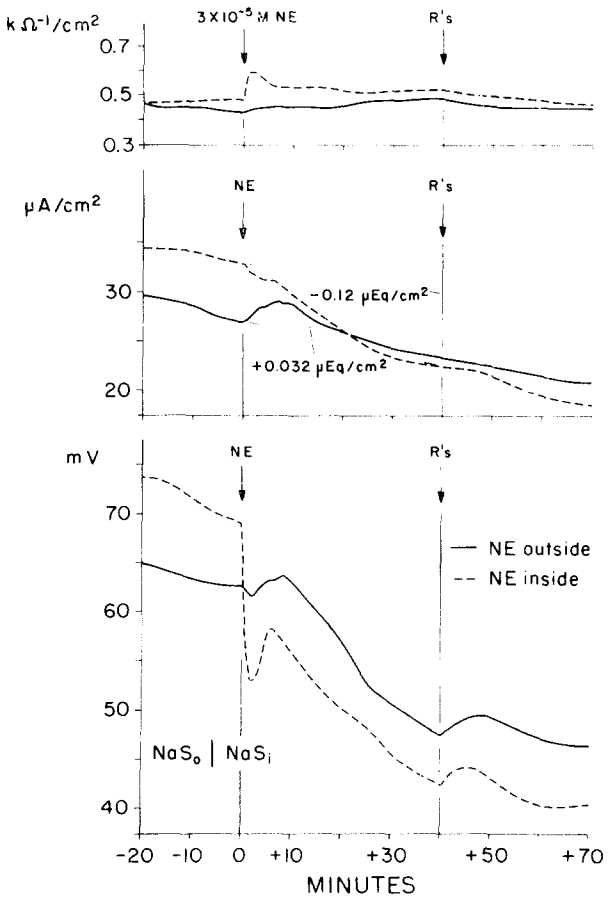

Fig. 4. Effects of norepinephrine (outside) and norepinephrine (inside). Studies on paired skins ($n=6$) in Na_2SO_4 solutions (D, Table I). At the R's fresh solutions were replaced on both sides of the skins. See also the legend to Fig. 2. The S.E. was ±10 (start) ±4 (end), and ±6 (start) an ±2 (end), [V and s.c.c. for norepinephrine (outside)]; ±6 and ±5 (start) ±3 (end), [V and s.c.c for norepinephrine (inside)]. NE, norepinephrine; $NaS_o/NaS_i = Na_2SO_4$ (outside), Na_2SO_4 (inside); Eq, equiv.

comparison norepinephrine (inside)) bathed in solutions containing NaCl, or $NaHCO_3$ on the inside (A, or C, Table I), and either arginine chloride, or arginine sulfate, or choline bicarbonate on the outside. (E, or F, or G, Table I). Thus, the stimulatory effect of norepinephrine (outside) on net inward Na^+ transport, described above, was excluded because of lack of Na^+ (outside). As expected, near zero values for s.c.c. and V were recorded prior to application of norepinephrine ($3 \cdot 10^{-5}$ M). Norepinephrine (inside) gave the responses already described in the preceding paragraphs. Norepinephrine (outside), however, had no effect. The fact that norepinephrine (inside) elicited effects on skins with $NaHCO_3$ (inside), as is the case with NaCl (inside), suggests that norepinephrine (inside) may lead to glandular active outward HCO_3^- transport, as it stimulates active outward Cl^- transport. The results with $NaHCO_3^-$ (inside), as compared to those with NaCl (inside) were less

pronounced and more variable. When G greatly increased, a brief stimulatory effect of norepinephrine (inside) on s.c.c. and V was followed by a decrease in the values for these parameters. Recovery from norepinephrine stimulation (norepinephrine wash-out) was satisfactory. Subsequent replacement of the Na^+ (outside)-free solutions by Na_2SO_4 Ringer's (terminal phase) showed good response of the epidermis to Na^+ (outside) in the series arginine sulfate (outside), NaCl (inside), but poor response in the series choline bicarbonate (outside), $NaHCO_3$ (inside) and arginine sulfate (outside), $NaHCO_3$ (inside). A typical norepinephrine (inside) response occurred also in the situation choline bicarbonate (outside), choline bicarbonate (inside). This response was rather weak. The results described here (and those of the following Section 6) are more fully presented elsewhere[29].

(5) Na^+ dependence of stimulatory effect of norepinephrine (inside)

Results of stimulation with norepinephrine (inside) of (unpaired) skins in arginine chloride (outside), arginine chloride (inside) were compared with those obtained on skins in arginine chloride (outside), NaCl (inside), and arginine sulfate (outside), NaCl (inside). The data were collected over a period of 7 months. Table II shows the results. $3 \cdot 10^{-5}$ M norepinephrine (inside) gave strong responses in terms of s.c.c. (net flows of electrical charge) and V, Series a, b, c. Although the responses were greater in the presence than in the absence of a Cl^- gradient, the difference in the values obtained in experiments in Series c and b (or a) were not statistically significant. Response to terminal provision of 110 mM Na^+ (outside) was quite good (Table III B and C controls, terminal phase). By contrast, in Series d, in which Na^+

TABLE II

Na^+ DEPENDENCE OF THE STIMULATORY NOREPINEPHRINE (INSIDE) EFFECT ON s.c.c. AND V

Recovery of s.c.c. ($\mu A/cm^2$) and V (mV) in Na^+ solutions, 1 h after solution changes. Series a, b, c with Na_2SO_4 solution at the outside, NaCl Ringer's on the inside. (a) $V = +30.8 \pm 3.3$; s.c.c. = 31.8 ± 3.8. (b) $V = +15.4 \pm 1.6$; s.c.c. = $+32.8 \pm 6.6$. Series d with NaCl Ringer's on both sides, $V = +19.5 \pm 1.6$; s.c.c. = $+23.0 \pm 2.8$. Errors are given $\pm$S.E.

Season 1971–1972	*n*	*$3 \cdot 10^{-5}$ M norepinephrine (inside)*	
		$\Delta mC/h \cdot cm^2$	*ΔV*
Arginine sulfate (outside), NaCl (inside)			
(a) November–January	15	43.0 ± 5.5	7.3 ± 1.7
(b) February–April	15	43.8 ± 4.0	4.5 ± 0.7
Arginine chloride (outside), NaCl (inside)			
(c) October–March	12	32.7 ± 4.8*	8.3 ± 1.4**
Arginine chloride (outside), arginine chloride (inside)			
(d) March	6	5.4 ± 0.9***	2.8 ± 0.7†

* $P = 0.1$ (c *vs* b).
** $P = 0.02$ (c *vs* b)
*** $P = 0.001$ (c *vs* d).
† $0.02 > P > 0.01$ (c *vs* d).

TABLE III

STIMULATORY EFFECTS OF NOREPINEPHRINE (INSIDE)

Data on control skins and paired skins (in parentheses) treated with ouabain ($5 \cdot 10^{-4}$ M inside). Errors given are ±S.E. V in mV; s.c.c. in $\mu A/cm^2$; G in $k\Omega^{-1}/cm^2$. Figures in left column, controls; in right column, ouabain data.

Series	*(A) n=6 (ouabain)*		*(B) n=6 (ouabain)*		*(C) n=6 (ouabain)*	
	Arginine chloride (outside)	*Arginine chloride (inside)*	*Arginine chloride (outside)*	*NaCl (inside)*	*Arginine sulfate (outside)*	*NaCl (inside)*
Initial phase						
	o i	o i	o i	o i	o i	o
	+ | −	+ | −	+ | −	+ | −	+ | −	+ | −
V_0	− 3.3±1.4	(−3.3±1.5)	− 0.6±0.7	(−8.3±0.8)	+ 6.4±0.7	(+ 2.8±0
s.c.c.$_0$	− 1.8±0.5	(−2.3±0.7)	− 0.5±0.6	(−5.8±0.6)	+ 7.4±1.7	(+ 3.8±1
$3 \cdot 10^{-5}$ M norepinephrine inside						
ΔV	+ 2.8±0.7	(+2.7±0.9)	+ 5.2±0.5	(+2.8±0.4)	+ 5.4±1.5	(+ 4.2±0
$\Delta mC/cm^2 \cdot h$	+ 5.4±0.9	(+6.1±1.1)	+22.5±3.2	(+2.0±1.5)	+47.7±3.0	(+17.4±2
ΔG	+ 0.1±0.06	(+0.2±0.07)	+ 0.9±0.2	(+0.6±0.1)	+ 0.9±0.3	(+ 0.3±0
Recovery phase (norepinephrine wash-out)						
V_r	− 2.8±1.0	(−2.9±0.9)	− 0.8±0.7	(−8.3±0.9)	+ 6.0±0.6	(+ 3.7±0
s.c.c.$_r$	− 2.0±0.5	(−2.6±0.7)	− 1.1±0.8	(−8.8±0.9)	+ 8.5±1.3	(+ 3.2±0
*Terminal phase**						
V_t	+19.5±1.6	(−1.2±0.6)	+39.8±5.4	(+8.1±0.6)	+17.2±2.7	(+ 9.9±0.
s.c.c.$_t$	+23.0±2.8	(−2.5±1.9)	+32.8±6.6	(+8.0±1.2)	+35.3±1.7	(+11.4±1.
G_t	1.2±0.3	(2.0±0.8)	0.8±0.1	(1.0±0.1)	2.1±0.4	(1.2±0.

* NaCl–Ringer's on both sides (A); 55 mM Na_2SO_4 solution outside (B and C).

was lacking inside, the skins gave a very poor response to norepinephrine (inside). These skins showed satisfactory values for s.c.c. and V when the arginine chloride solutions on the two sides were replaced by NaCl–Ringer's. (Table III A control, terminal phase).

(6) Drug effects on the norepinephrine (inside) stimulatory response

All these experiments were done on paired skins, one skin serving as control. To the other skin either ouabain ($5 \cdot 10^{-4}$ M), or propranolol ($5 \cdot 10^{-5}$ M), or Diamox ($5 \cdot 10^{-3}$ M) was applied by adding the drugs to the inside solution 1 h prior to the addition of $3 \cdot 10^{-5}$ M norepinephrine to the inside. The solution conditions were as follows: arginine chloride (outside), arginine chloride (inside); arginine chloride (outside), NaCl (inside); arginine sulfate (outside), NaCl (inside), for testing ouabain effects. Arginine sulfate (outside), NaCl (inside), for testing propranolol effects. Arginine sulfate (outside), NaCl (inside); arginine sulfate (outside), $NaHCO_3$ (inside), for testing Diamox effects. It will be noted that in all cases we have avoided Na^+ on the outside of the skin to avoid possible mixed effects of norepinephrine (inside) and norepinephrine (outside) (where it might appear even if applied to the inside of the skin) on the net Na^+ influx.

Ouabain inhibited the stimulatory effect of norepinephrine (inside) on the net flow of charge ($mC/h \cdot cm^2$) in the absence and the presence of a Cl^- gradient

across the skin. 91% inhibition was seen in experiments in series arginine chloride (outside), NaCl (inside) and 64% inhibition in experiments arginine sulfate (outside), NaCl (inside) series (Table III B and C). $+\Delta V$ and $+\Delta G$ in ouabain-treated skins was also less than in control skins. From the data presented in Table II it has already been concluded that the differences in control values observed in experiments in Series B and C is not significant statistically. Inhibition might have been more complete if a somewhat higher ouabain concentration had been used. It will be noted that in the terminal phases (B and C) with provision of Na^+ (outside), the well known depressing effect of ouabain on epidermal Na^+ transport also was not complete. The small response of norepinephrine (inside) in control skins, series arginine chloride (inside), arginine chloride (outside) (Table III A), as compared to responses seen in the controls, Series B and C, has already been noted in paragraph 5. The result obtained in experiments in Series A (Table III) reveal that there appears to be an ouabain-insensitive component in the norepinephrine (inside)-stimulatory effect.

Propranolol decreased net flow of charge by 82%, and $+\Delta E$ and $+\Delta G$ were also diminished. This confirms results in the literature[2-4,6,10,11], except that in the present studies inhibition was seen when NaCl (outside) Ringer's was replaced by arginine sulfate (outside) solution. Epidermal response to Na^+ (outside) at the end of the experiments was satisfactory in control and propranolol-treated skins. Diamox, at the dose used ($5 \cdot 10^{-3}$ M) had a small depressing effect on the norepinephrine (inside) response, looking at the change in net flow of electrical charge in experiments in series arginine sulfate (outside), NaCl (inside). The statistical significance of differences in responses of control and treated skins, however, is marginal ($0.05 > P > 0.03$). There was no statistically significant difference between control and treated skins in the $+\Delta V$ and $+\Delta G$ responses elicited by norepinephrine (inside). Epidermal responses to Na^+ (outside) was satisfactory both in control and Diamox-treated skins. On the other hand, in experiments in series arginine sulfate (outside), $NaHCO_3$ (inside), Diamox abolished the small norepinephrine (inside) responses, except for a small remaining $+\Delta V$ response. Epidermal response to Na^+ (outside) tested at the end of the experiments was poor in control and treated skins, as has already been noted in paragraph 4 for solution conditions choline bicarbonate (outside), $NaHCO_3$ (inside), and arginine sulfate (outside), $NaHCO_3$ (inside).

(7) Fluxes

Table IV summarizes the results on some flux studies. They show: (1) When using arginine chloride (outside), NaCl (inside), approximately 0.24 μmole $NaCl \cdot cm^{-2} \cdot h^{-1}$ diffused in the outward direction. Norepinephrine (inside) stimulated outflux of NaCl, with Cl^- flux exceeding Na^+ flux such that the average s.c.c. was fully accounted for. In other words, there occurred an active outward Cl^- transport. (2) In the absence of Na^+ (inside) (arginine chloride (inside), arginine chloride (outside)), no active outward Cl^- transport was detectable by the flux method, but a relatively small s.c.c. was noticeable, as if a small Cl^- transport had occurred. Participation of a not recognized (metabolic) anion cannot be excluded. (3) When using choline bicarbonate (outside), $NaHCO_3$ (inside), a relatively large carbon flux, equal in both directions and unaffected by norepinephrine (inside) occurred and a small s.c.c. was noticeable, as if a very small amount of HCO_3^- had been actively transported after giving norepinephrine (inside). The value for unidirectional carbon

TABLE IV

AVERAGE INFLUX, OUTFLUX AND NET FLUX DATA ON Cl^-, Na^+ AND CARBON

Data were obtained on 4 pairs of skins for each solution condition, J_i, J_o and J_n, μequiv·cm^{-2}·h^{-1}. N = 12 (pre-norepinephrine (inside)). N = 48 (post-norepinephrine (inside)). Average s.c.c. obtained by graphic integration of the corresponding 1-h s.c.c.-time curve. 1 μA/cm^2 = 0.0373 μequiv·cm^{-2}·h^{-1}. J_i (influx); J_o (outflux); J_n (net flux).

	J_i^{Cl}	J_o^{Cl}	J_n^{Cl}	J_o^{Na}	$J_n^{Cl} - J_o^{Na}$	
					by av. flux	*by av. s.c.c.*
Arginine chloride (outside), NaCl (inside)						
Pre-norepinephrine (inside)	0.24 ± 0.03	0.45 ± 0.05	0.21 ± 0.06 **	0.26 ± 0.05	−0.05 ± 0.08	
Post-norepinephrine (inside)	0.38 ± 0.04	1.24 ± 0.10	0.86 ± 0.11 **	0.67 ± 0.06	0.19 ± 0.12	
ΔJ	0.14 ± 0.05 *	0.79 ± 0.11 **	0.65 ± 0.12 **	0.41 ± 0.08 **	0.24 ± 0.14 ***	0.23 ± 0.05
Arginine chloride (outside), arginine chloride (inside)						
Pre-norepinephrine (inside)	0.35 ± 0.05	0.47 ± 0.10	0.12 ± 0.11 *			
Post-norepinephrine (inside)	0.49 ± 0.05	0.59 ± 0.09	0.10 ± 0.10 *			
ΔJ	0.14 ± 0.07 *	0.12 ± 0.13 *	−0.02			0.07 ± 0.02 ***
	J_i^{C}	J_o^{C}	J_n^{C}	J_o^{Na}		$J_n^{C} - J_o^{Na}$
Choline bicarbonate (outside), $NaHCO_3$ (inside)						
Pre-norepinephrine (inside)	10.8 ± 1.0	10.9 ± 1.1	−0.1	0.19 ± 0.02		
Post-norepinephrine (inside)	10.1 ± 1.0	10.0 ± 1.4	−0.1	0.27 ± 0.04		
ΔJ	−0.7 *	−0.9 *	0	0.08 ± 0.04 *		0.07 ± 0.01 ***

* No significant difference

** Significant difference

*** Dividing by 0.01036 gives 23.2 and 6.8 mC·cm^{-2}·h^{-1}, respectively, comparable to the values given in Table IIc and IId.

flux is in fair agreement with the value given by Smith *et al.*[14] (7.6 μmoles$\cdot$cm$^{-2}\cdot$h^{-1}) for skins in $NaHCO_3$ (outside), $NaHCO_3$ (inside), attributable to CO_2 diffusion. Thus, here again, as in the case of arginine chloride (outside), arginine chloride (inside) the s.c.c. method appears to be more sensitive (but not ion specific) than the flux method, especially in the case of ^{14}C fluxes in view of the large $^{14}CO_2$ fluxes. If indeed norepinephrine (inside) stimulated net active outward HCO_3^- flux of the order of 0.07 μequiv$\cdot$cm$^{-2}\cdot$h^{-1}, the data indicate that CO_2 flux is approximately 100 times greater than net HCO_3^- flux. (4) Comparing Cl^- and HCO_3^-, these studies show that whereas active outward Cl^- transport can be initiated by norepinephrine (inside) provided Na^+ (inside) is present, only very little HCO_3^- seems to be actively transported even in the presence of Na^+ (inside). That some norepinephrine (inside) initiated transport does occur, as suggested by s.c.c. measurements, is supported by the fact that the glandular secretions from norepinephrine-treated frogs have a high alkalinity[7,15,16].

DISCUSSION

Epidermal effects of norepinephrine (outside)

Reviewing the pertinent literature[1–11] it appears that little attention has been given to testing the effects of norepinephrine (outside), *i.e.* norepinephrine applied to the epidermal, not to the corium side of the skin. Norepinephrine ($3\cdot10^{-5}$ M) when applied to the epidermal side of the skin leads to an increase in open skin potential ($V=\psi$ (inside)$-\psi$ (outside)) and inward short-circuit current (s.c.c./cm^2) in the presence of Na^+ (outside), provided that either Cl^-, or HCO_3^- was also present. Changes in total skin conductance (G=s.c.c./cm$^2\cdot V$) were minimal (Figs 2 and 3). Other anions, except SO_4^{2-}, have not been studied. In the absence of Cl^- and HCO_3^- but in the presence of SO_4^{2-} (Fig. 4), norepinephrine (outside) nearly failed to elicit its stimulatory effect on the epidermis. On the other hand, the s.c.c. and V time course indicates that both s.c.c./cm^2 and V decreased 10 min after applying norepinephrine (outside).

This suggests that the main action of norepinephrine (outside) was that it increased the active Na^+ transport potential, E_a^{Na}. Applying the electrical equivalent circuits proposed by Ussing and Zerahn[13], and by Linderholm[17,18], one has to consider the following relationships:

$$\text{s.c.c.} = E_a^{Na}\cdot G_a^{Na} \text{ (short-circuited skin, } V_0=0)$$

$$V_0 = E_a^{Na}\cdot G_a^{Na}/(G_a^{Na}+\Sigma G_p) \text{ (open-circuited skin, s.c.c.}=0)$$

$$G_T = G_a^{Na}+\Sigma G_p = \text{s.c.c.}/V_0$$

G_a^{Na}=conductance for Na^+ in the active transport pathway; ΣG_p=sum of conductances for all passively moving ions in the shunt pathway. V_0=skin potential. If norepinephrine (outside) increased E_a^{Na} only, s.c.c./$V_0=G_T$ would remain constant, as was nearly the case in our experiments. If norepinephrine (outside) would also lead to an increase in G_a^{Na}, then ΣG_p would have to decrease by the same amount to give constant G_T. ($G_T=G_a^{Na}+\Sigma G_p$). This is a less likely event. The assumption is made, here, that E_a^{Na} does not vary with V_0. The data of Linderholm support this, but not unequivocally. He also reported that G_a^{Na} and G_p^{Cl} changed little

when the skins, with Ringer's solutions on both sides were short-circuited. Additional experiments are needed to explain the effects of norepinephrine (outside) on the epidermis.

It may be assumed that the described effects of norepinephrine (outside) are the result of stimulation of β-stimulatory, and α-inhibitory receptor sites in the epidermis, the existence of which has been postulated by several investigators who have tried to unravel glandular from epidermal responses by the use of chemical adrenergic blockers while applying norepinephrine (inside)[2–10], or by using stripped epidermis and applying norepinephrine to both sides of the epidermal membrane[11]. We have not yet tested whether elicitation of the epidermial stimulatory action of norepinephrine (outside) requires presence of Cl^- (inside) only, or of Cl^- (outside) only, or Cl^- (inside) and Cl^- (outside). The rate of active inward Na^+ transport itself (irrespective of norepinephrine effects) is altered by Cl^- (inside) and Cl^- (outside) by different mechanisms[12]. In the presence of Cl^- (inside), when osmotic balance and hence epidermal cell volume is maintained, net rate of active inward Na^+ transport and V are increased. Presence of Cl^- (outside) can both stimulate and inhibit active Na^+ transport for unknown reasons. Thus, anions such as Cl^- and HCO_3^-, as compared to SO_4^{2-}, conceivably could make β-adrenergic stimulatory receptor sites more accessible so that norepinephrine (outside) stimulates V and s.c.c. Absence of Cl^- or HCO_3^- from the outside solution may uncover α-adrenergic inhibitory receptor sites. These two types of receptor sites need not be located in the same epithelial strata. The possibility that Na^+ and Cl^- (or HCO_3^-) cross some barrier as a pair cannot completely be ruled out, if subsequently Cl^- (or another anion) would return to the source side if the s.c.c.–Na^+ equivalence rule is obeyed, which is mostly (but not always) the case. It should be mentioned, here, that the permeability of the whole skin to HCO_3^- is very low, as compared to Cl^- permeability[14]. This does not exclude the possibility that HCO_3^- penetrates into the skin without crossing it. In other words, for co-actions of Cl^- and HCO_3^- on Na^+ transport it may not be necessary that these anions have to permeate across the whole short-circuited skins.

Glandular effects of norepinephrine (inside)

The work of Koefoed-Johnsen *et al.*[1], Seldin and Hoshiko[19], Campbell *et al.*[16], Watlington[2–6], Lindley[20], Watlington and Huf[7] makes it very likely that the norepinephrine (inside) effects described in this study are of glandular and not of epidermal origin. To remove further doubts, in all experiments with intent solely to stimulate the glandular anion transporting mechanism, Na^+-depleted solutions were placed on the outside of the skin. Solutions used were either arginine chloride (outside); arginine sulfate (outside) or choline bicarbonate (outside), and (on the inside) arginine chloride (inside); or NaCl (inside); or choline bicarbonate (inside); or $NaHCO_3$ (inside). By omitting Na^+ (outside) and in some cases also Cl^- (outside) it would seem highly unlikely that norepinephrine (inside) ($3 \cdot 10^{-5}$ M), some of which probably penetrates the skin and appears in the outside solution, could have had any significant stimulatory or inhibitory effect on the Na^+ and Cl^- transporting systems in the epidermis; Na^+ and Cl^- outside concentrations, as the case must have been near zero. By doing this one has to consider, of course, the fact that ion concentration gradients are set up with the possibility of changing diffusional fluxes

under the influence of norepinephrine (inside). However, the increase in $V=\psi$ (inside) $-\psi$ (outside) and in inward s.c.c. seen when applying norepinephrine (inside) to skins under the conditions arginine sulfate (outside), NaCl (inside) and arginine chloride (outside), NaCl (inside) could not have been the result of increasing passive outflux of Na^+. Also, in the latter case, passive net outflux of Cl^- would not occur in short-circuited skins. The same holds for the HCO_3^- experiments. Thus, in either case one must consider that the increment in net flow of charge is caused by active outward anion transport *via* the glands. The results of flux measurements (Table IV) give proof of this in the case of Cl^- (arginine chloride (outside), NaCl (inside)) but not for HCO_3^- (choline bicarbonate (outside), $NaHCO_3$ (inside)). CO_2 flux is at least 100 times greater than a possible (and likely) net HCO_3^- outflux in the norepinephrine (inside) stimulated skin (Results, section 7). In this situation data on HCO_3^- fluxes are, of course, difficult to obtain. House[21] has shown that in toad skin a relatively weak component of the hyperpolarising effect of norepinephrine (inside) [$4\cdot10^{-5}$ M; $MgSO_4$ (outside), NaCl (inside)] stems from an increase of the inner epidermal border permeability for K^+. For the present we have neglected this seemingly minor contribution.

We have observed that in the controls (initial and recovery phase) and solution conditions arginine chloride (outside), arginine chloride (inside); choline bicarbonate (outside), choline bicarbonate (inside); arginine chloride (outside), NaCl (inside); choline bicarbonate (outside), $NaHCO_3$ (inside), V_0 varied between ±4 mV. These include small asymmetry potentials between the carefully selected pairs of calomel electrodes. Only with solutions arginine sulfate (outside), NaCl (inside), and arginine sulfate (outside), $NaHCO_3$ (inside), V_0 values of $+5$ to $+9$ mV (outside negative), and -6 to -8 mV, respectively, existed during the initial phase and the recovery phase. This indicates the existence of small Cl^- diffusion potentials in the former, and Na^+ diffusion potentials (low HCO_3^- permeability) in the latter case.

Of the three chemical inhibitors used (ouabain, Diamox and propranolol, a β-adrenergic blocker) applied to the inside prior to giving norepinephrine (inside), propranolol and ouabain greatly, and Diamox moderately ($0.05>P>0.03$) decreased the s.c.c. (flow of charge) when NaCl (inside) was used. Inhibition by Diamox of active inward Cl^- transport at the epidermal level has recently been described by Erlij[22]. Comparing the actions of propranolol and Diamox with the actions of ouabain, when using NaCl (inside) and arginine chloride (outside), or arginine sulfate (outside), an important difference was noted. In the ouabain studies, but not in the propranolol and Diamox studies, there existed a difference in the initial and recovery phase potential values (V_0; V_r) for control and ouabain-treated skins ($0.03>P>0.01$), indicating that an increase in permeability for Na^+ had occurred. This would be in agreement with the observations of Biber *et al.*[13]. Increase in leakiness of the skins for Na^+ could, at least in part, diminish the increment in the rise of s.c.c. when applying norepinephrine (inside). This factor could not have entered in the actions of propranolol and Diamox; there were no statistically significant differences in the V_0 and V_r values. Diamox also decreased the norepinephrine (inside)-initiated small rise in s.c.c. when $NaHCO_3$ (inside) was used. The mode of action of the inhibitors is unknown.

Attention is called to the results obtained in Series A (Table III) with arginine chloride (outside), arginine chloride (inside). Norepinephrine (inside) still produced

a small increase in s.c.c. and V, but there was no difference between the controls and ouabain-treated skins, although active inward Na^+ transport, when changing to NaCl (outside), NaCl (inside), was completely abolished in the ouabain-treated skins, but not in the controls. Either there is an ouabain-insensitive Cl^- outflux component in the norepinephrine (inside) response, or norepinephrine (inside) has other, as yet unrecognized effects on electrolyte distribution in glandular epithelium. Many drugs, including ouabain lead to loss of K^+ and gain of Na^+ in skin[24–26].

The results given in Table II and Table IV were of particular interest to us. They show that the response of the glandular epithelium to norepinephrine (inside) by activating β-adrenergic receptor sites[2–7,9], *i.e.* active outward Cl^- transport, is strongly dependent on the presence of Na^+ (inside). A non-competitive activation by Na^+ of an active Cl^--transporting mechanism in frog cornea epithelium has been described by Zadunaisky[27]. Kristensen[28] found that net inward Cl^- transport in frog skin (*R. temporaria*) which can be inhibited by Diamox ($0.8 \cdot 10^{-3}$ M) is largely, but not entirely independent of Na^+ transport. Comparing the effect of norepinephrine (outside) on epidermis with the norepinephrine (inside) effect on glandular epithelium, it is interesting to note that stimulation of presumably, active inward Na^+ transport by norepinephrine (outside) is dependent on Cl^-, and stimulation of active outward Cl^- transport by norepinephrine (inside) is dependent on Na^+ (inside). Additional studies are needed to elucidate the mechanism(s) of these ionic interactions in Na^+ and Cl^- transport.

ACKNOWLEDGEMENTS

This research was supported by N.I.H. Grants 5 KO6 GM 16687-10 and GM 03545-19.

REFERENCES

1 Koefoed-Johnsen, V., Ussing, H. H. and Zerahn, K. (1952) *Acta Physiol. Scand.* 27, 38
2 Watlington, C. O. (1965) *J. Clin. Invest.* 44, 1108
3 Watlington, C. O. (1967) *Med. Coll. Va. Quart.* 3, 157
4 Watlington, C. O. (1968) *Am. J. Physiol.* 214, 1001
5 Watlington, C. O. (1968) *Comp. Biochem. Physiol.* 24, 965
6 Watlington, C. O. (1969) *Biochim. Biophys. Acta* 193, 394
7 Watlington, C. O. and Huf, E. G. (1971) *Comp. Gen. Pharmacol.* 2, 295
8 Bastide, F. and Jard, S. (1968) *Biochim. Biophys. Acta* 150, 113
9 McAfee, R. D. (1970) *Biochim. Biophys. Acta* 203, 104
10 Fassina, G., Fiandini, G., Carpenedo, F. and Santi, R. (1971) *Comp. Gen. Pharmacol.* 2, 27
11 Rajerison, R. M., Montegut, M., Jard, S. and Morel, F. (1972) *Pflügers Arch.* 332, 313
12 Huf, E. G. (1972) *Acta Physiol. Scand.* 84, 366
13 Ussing, H. H. and Zerahn, K. (1951) *Acta Physiol. Scand.* 23, 110
14 Smith, T. C., Hughes, W. D. and Huf, E. G. (1971) *Biochim. Biophys. Acta* 225, 77
15 Friedman, R. T., LaPrade, N. S., Aiyawar, R. M. and Huf, E. G. (1967) *Am. J. Physiol.* 212, 962
16 Campbell, J. P., Aiyawar, R. M., Berry, E. R. and Huf, E. G. (1967) *Comp. Biochem. Physiol.* 23, 213
17 Linderholm, H. (1952) *Acta Physiol. Scand.* 27, Suppl. 97, 1
18 Linderholm, H. (1954) *Acta Physiol. Scand.* 31, 36
19 Seldin, J. P. and Hoshiko, T. (1966) *J. Exp. Zool.* 163, 111

20 Lindley, B. D. (1969) *J. Gen. Physiol.* 53, 427
21 House, C. R. (1971) *J. Physiol. London* 218, 305
22 Erlij, D. (1971) *Phil. Trans. R. Soc. Lond.* B. 262, 153
23 Biber, T. U. L., Chez, R. A. and Curran, P. F. (1966) *J. Gen. Physiol.* 49, 1161
24 Huf, E. G., Doss, N. S. and Wills, J. P. (1957) *J. Gen. Physiol.* 41, 397
25 Zerahn, K. (1969) *Acta Physiol. Scand.* 77, 272
26 Nagel, W. and Dörge, A. (1971) *Pflügers Arch.* 324, 267
27 Zadunaisky, J. A. (1972) *Biochim. Biophys. Acta* 282, 255
28 Kristensen, P. (1972) *Acta Physiol. Scand.* 84, 338
29 Pinschmidt, M. W. (1973) Ph.D. Thesis, School of Basic Sciences and Graduate Studies, Virginia Commonwealth University, Richmond, Va.