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Noradrenaline Induced Secretion of Nonelectrolytes through Frog Skin

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Summary. Addition of noradrenaline (4×10^{-5} M) to the inner bathing fluid in the skin of the frog *Rana esculenta* results in increased unidirectional fluxes of urea, thiourea, N-methyl-thiourea, N-N′-dimethylthiourea and mannitol. Fluxes towards the external medium (Φ_o) undergo a much greater increase than those moving in the opposite direction (Φ_i). The effect of noradrenaline on Φ_o is higher for urea and thiourea than mannitol, while its effect on Φ_o thiourea derivatives is related to lipid solubility. This phenomenon does not occur for Φ_i of the same molecules.

FCCP (10^{-6} M) pretreatment strongly inhibits the noradrenaline effect on Φ_o. In skin pretreated whith colchicine (2×10^{-5} M) both urea fluxes are increased to the same extent by noradrenaline. Noradrenaline is concluded to exert two separate effects: (1) a change in permeability in both directions; (2) a secretion of nonelectrolytes towards the external fluid. Such secretion is most probably associated with the hormone-induced secretion of fluid and electrolytes, perhaps mediated by an exocytotic mechanism.

Key words: noradrenaline, frog skin, secretion, microtubules, colchicine.

Noradrenaline is well known to produce variations in amphibian skin potential. The electrical response to noradrenaline consists of initial depolarization followed by hyperpolarization [6]. House [6] suggests that ionic movement through a transient shunt pathway in the skin glands may account for this electrical response.

Furthermore, it has also been well established that exposure to catecholamines stimulates electrolyte and protein loss from the skin of *Rana pipiens* [4], and glycoprotein and protein secretion from parotid glands in mouse [12], rabbit [5] and rat [1].

A model for the biochemical events occurring during secretion has been given by Rasmussen [8]. In this model, the first messenger, an external stimulus specific for the particular cell type, interacts with membrane-bound adenyl-cyclase to result in increased cyclic AMP synthesis within the cell. The increased concentration of cyclic AMP acts as a

second intracellular messenger, activating one or more processes or enzymes, with the subsequent setting up of a microtubule system. The secretion products are packed into vesicles bound to the microtubule system. Stimulation results in migration of complex microtubule-vesicles to the cell surface. Contact between vesicle and plasma membranes leads to fusion with subsequent discharge of vesicular content.

Preliminary observations showed noradrenaline treatment of frog skin to result in increased permeability [9].

This work, therefore, deals with the effect of noradrenaline on nonelectrolyte permeability through the frog skin, with investigation made of the nature of frog skin response to the hormone as well.

Materials and Methods

The biological "membrane" used was the isolated ventral skin of *Rana esculenta.* The incubation medium was Ringer's solution for amphibians (NaCl, 110 mM; KCl, 2 mM; $CaCl_2$, 2.5 mM buffered with Na_2HPO_4; 3.7 mM; NaH_2PO_4 1.7 mM) containing differing amounts of unlabelled test molecule.

The outfluxes (Φ_o) and influxes (Φ_i) were measured using the two symmetrical halves of the same skin in order to minimize variations among individuals. Each "membrane" was mounted between two lucite chambers containing 7 ml of incubation fluid and gassed with air at 22 ± 2 °C; the exposed area was of 2 cm^2. Measurement was carried out in the absence of concentration gradient. The skins were not short-circuited.

The labelled molecules (final activity 1 μCi/ml) were added to one of the two compartments, and once steady-state conditions were reached after 2 hr, samples were withdrawn every hour at least twice from the opposite compartment. Noradrenaline was then added to the inner compartment and other samples withdrawn at least three times. Noradrenaline concentration for all experiments, with the exception of those reported in Table 2, was 4×10^{-5} M. Colchicine experiments were carried out by adding 2×10^{-5} M of colchicine to the inner bathing solution either 4 hr before beginning the experiment or after equilibration time.

FCCP (*p*-trifluoromethoxy-carboxylcyanidephenil-hydrazone) experiments were carried out by adding FCCP 10^{-6} M to the bathing solutions at the onset of the experiments.

Radioactivity count was made with a liquid scintillation spectrometer (Packard Tri-arb 3320), with transepithelial fluxes calculated as well.

Results

Table 1 shows the effect of noradrenaline on the unidirectional fluxes of certain nonelectrolytes through the skin of *Rana esculenta.*

Addition of the hormone results in an increase of both fluxes. Fluxes towards the external medium (Φ_o) undergo a much greater increase than those towards the internal medium (Φ_i). The effect of noradrenaline on outfluxes is related to molecular weight and lipid solubility of the

Table 1. Effect of noradrenaline on both fluxes of some nonelectrolytes across frog skin

Tested molecule	n		Φ^C	Φ^N	Δ
Urea	4	Φ_o	13.2± 1.0	42.5± 5.4	29.2±4.6
		Φ_i	16.8± 3.6	32.1± 3.0	15.3±1.6
Thiourea	7	Φ_o	21.0± 1.6	61.0± 6.0	40.0±5.1
		Φ_i	19.8± 2.6	36.1± 7.0	14.8±4.8
N-Methyl-thiourea	5	Φ_o	45.8±15.1	99.6±18.4	53.9±4.6
		Φ_i	42.1± 5.5	63.2± 6.9	21.0±6.5
N-N'-Dimethylthiourea	5	Φ_o	162.8±13.6	239.4±13.5	76.6±9.5
		Φ_i	144.4±18.5	166.5±17.6	22.1±9.6
Mannitol	8	Φ_o	4.5± 0.8	9.2± 1.3	4.4±0.8
		Φ_i	3.6± 0.4	5.4± 0.5	1.8±0.4

Control fluxes (Φ^C) were measured after the equilibration period. 4×10^{-5} M noradrenaline was then added to the inner bathing solution and new flux measurements (Φ^N) performed. $\Delta=\Phi^N-\Phi^C$. The concentration of all tested molecules was 1 mM/liter: Φ_o=flux towards the external medium and Φ_i=flux towards the internal medium expressed in nmole$\times10^{-1}$ cm^{-2} hr^{-1}. Values are mean±SE. n=number of experiments.

Table 2. Effect of differing noradrenaline concentrations on thiourea (1 mM/liter) outfluxes across the skin of *Rana esculenta*

Experimental conditions	n		Φ^C	Φ^N	Δ
Noradrenaline $4\cdot10^{-6}$ M	4	Φ_o	26.5±2.3	58.0±6.0	31.5±4.2
Noradrenaline $4\cdot10^{-5}$ M	4	Φ_o	23.6±1.5	63.3±5.7	39.7±6.5
Noradrenaline $4\cdot10^{-4}$ M	4	Φ_o	21.9±1.6	57.2±4.2	35.4±2.7

Control fluxes (Φ^C) were measured after the equilibration period. 4×10^{-6} M, 4×10^{-5} M, 4×10^{-4} M noradrenaline, respectively, were added to the inner bathing solution and new flux measurements (Φ^N) performed. $\Delta=\Phi^N-\Phi^C$. Φ_o=flux towards the external medium is expressed in nmole$\times10^{-1}$ cm^{-2} h^{-1}. Values are mean±SE. n=number of experiments.

tested molecules. It is actually higher for urea and thiourea than for mannitol and for methylated thiourea derivatives than for thiourea. Table 2 shows the response of thiourea outfluxes to the hormone to be the same at all noradrenaline concentrations tested: 4×10^{-6} M, 4×10^{-5} M, 4×10^{-4} M. Fig. 1 indicates a linear relationship between urea concentration and increased urea fluxes under noradrenaline treatment. Moreover, increase is greater for outflux than for influx. The thiourea

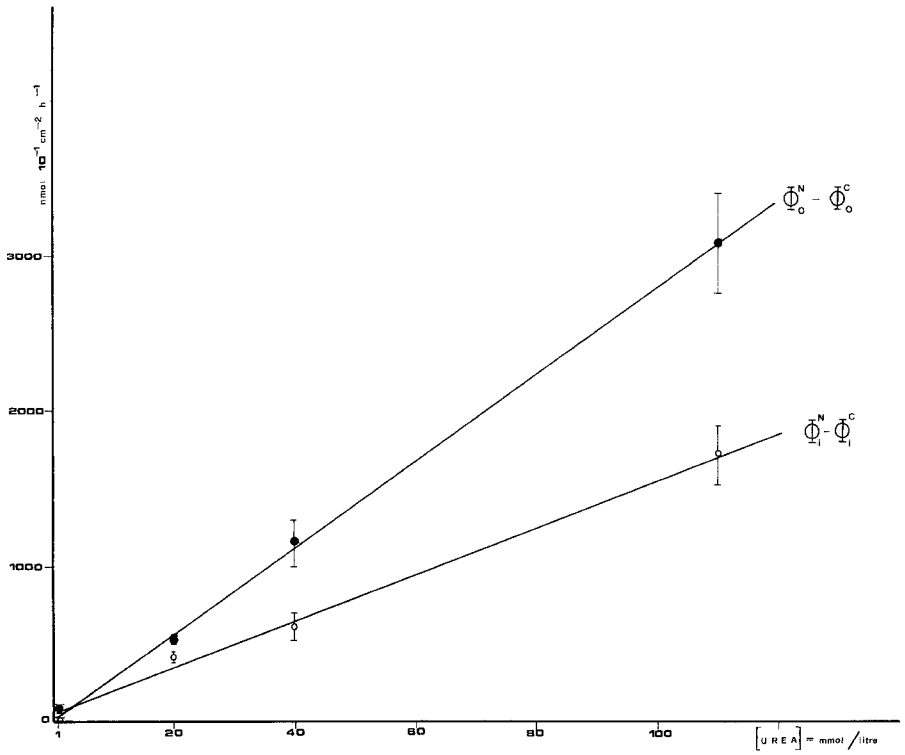

Fig. 1. Effect of noradrenaline on urea fluxes at different urea concentrations. Each point represent the mean of nine experiments ± SE. $\Phi_o^N - \Phi_o^C$ and $\Phi_i^N - \Phi_i^C$ are the differences between outfluxes (Φ_o^N) or influxes (Φ_i^N) after noradrenaline treatment and control outfluxes (Φ_o^C) or influxes (Φ_i^C). Regression lines obtained by the least squares method

pattern differs from that of urea (Fig. 2). In fact, the noradrenaline elicited flux increase is not a linear function of molecule concentration; rather at high thiourea concentrations, the hormone effect tends to decrease.

Table 3 shows a marked decrease in the effect of noradrenaline on both urea fluxes in the presence of thiourea 110 mM as compared to that found without thiourea (Table 1).

The residual hormonal effect is virtually the same on both urea fluxes. Thus, high thiourea concentrations seem to affect the response mechanism of frog skin to noradrenaline. However, this effect cannot be due to simple disruption of skin architecture brought on by the increased osmolarity of the bathing fluids because: (a) Φ_o^C of urea in the presence of 110 mM thiourea (Table 3), do not differ from the Φ_o^C reported in Table 1 without treatment; (b) Φ_o/[thiourea] and Φ_i/[thiourea] (thiourea concentration 110 mM, *see* Fig. 2), are virtually equal to that reported

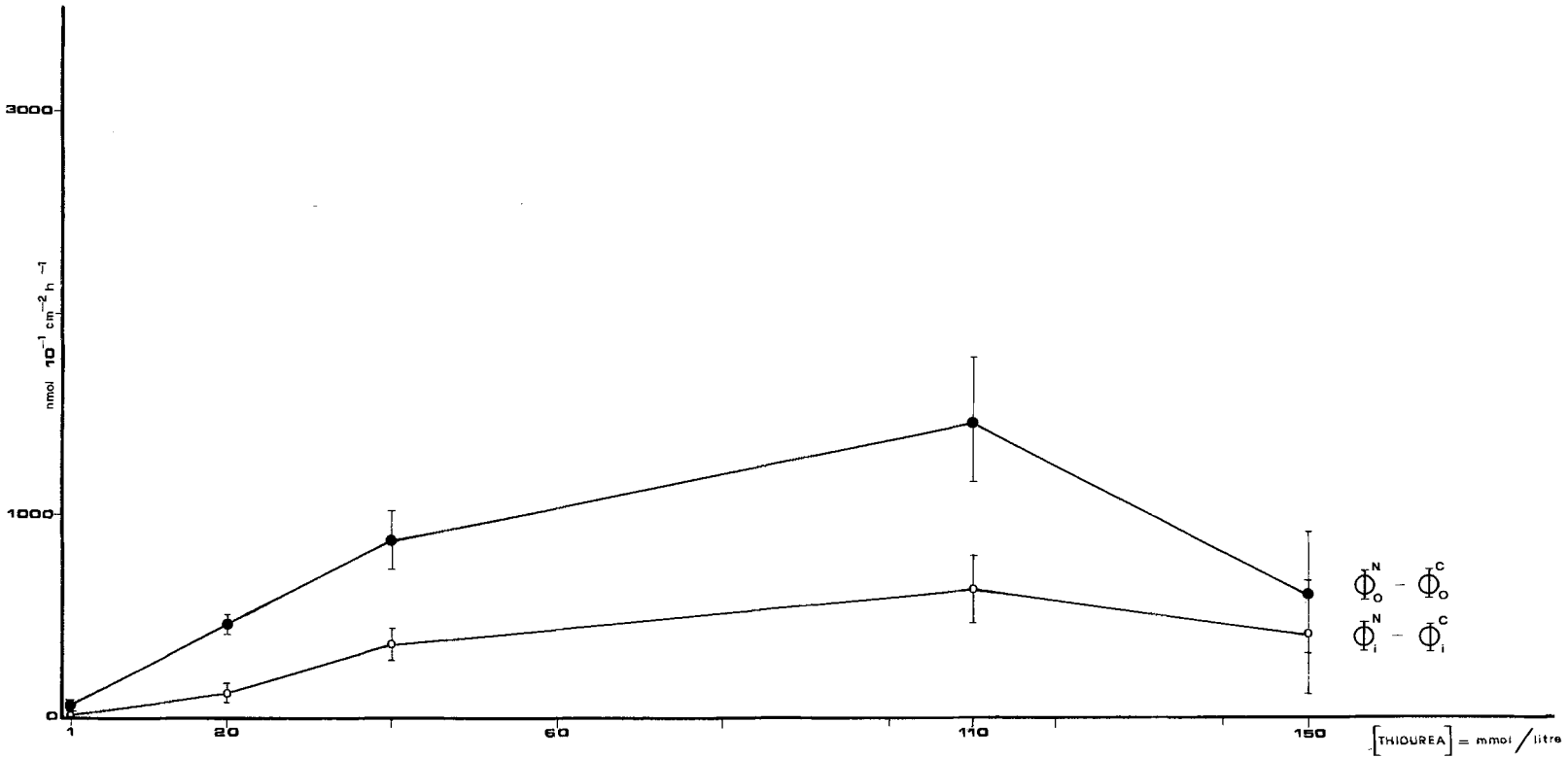

Fig. 2. Effect of noradrenaline on thiourea fluxes at different thiourea concentrations. Each point represents the mean of eight experiments ± SE. $\Phi_o^N - \Phi_o^C$ and $\Phi_i^N - \Phi_i^C$ are the differences between outfluxes (Φ_o^N) or influxes (Φ_i^N) after noradrenaline treatment and control outfluxes (Φ_o^C) or influxes (Φ_i^C)

Table 3. Effect of high thiourea concentration on the response of both urea (1 mM/liter) fluxes to noradrenaline across frog skin

Tested molecule	n		Φ^C	Φ^N	Δ
Urea	6	Φ_o	19.4±3.1	24.7±3.1	5.3±0.9
		Φ_i	16.3±2.0	20.2±1.7	3.9±1.0

Thiourea 110 mM/liter was added to both inner and outer medium at the onset of experiments. Control fluxes (Φ^C) measured at the end of the equilibration period. 4×10^{-5} M noradrenaline then added to the inner solution and new flux measurements (Φ^N) performed. $\Delta=\Phi^N-\Phi^C$. Φ_o=flux towards the external medium, and Φ_i=flux towards the internal medium expressed in nmole $\times 10^{-1}$ cm^{-2} hr^{-1}. Values are mean±SE. n=number of experiments.

Table 4. Effect of noradrenaline on thiourea fluxes in media of different osmolarity

Experimental conditions	n		Φ^C	Φ^N	Δ
A	6	Φ_o	24.4±3.1	29.9±2.8	5.5±1.3
		Φ_i	25.4±1.6	29.3±1.5	3.8±1.9
B	5	Φ_o	24.2±2.3	26.9±2.2	2.6±0.3
		Φ_i	23.9±1.4	25.2±1.2	1.3±1.3

Thiourea 110 mM/liter was added to both inner and outer medium at the onset of experiments. In *A* the bathing fluids were Ringer's solution plus thiourea 110 mM, in *B* total osmolarity of Ringer's solution was unchanged (110 mM thiourea substituted for 55 mM NaCl). Control fluxes (Φ^C) measured at the end of the equilibration period. 4×10^{-5} M noradrenaline then added to the inner solution and new flux measurements (Φ^N) performed. $\Delta=\Phi^N-\Phi^C$. Φ_o=flux towards the external medium and Φ_i=flux towards the internal medium expressed in nmole $\times 10^{-1}$ cm^{-2} hr^{-1}/[thiourea]. Values are mean±SE. n=number of experiments.

Table 5. Effect of colchicine on the response of both urea (1 mM/liter) fluxes to noradrenaline across frog skin [preincubation time with colchicine=4 hr (*A*) and without preincubation (*B*)

Tested molecule		n		Φ^C	Φ^N	Δ
Urea	(A)	4	Φ_o	10.5±2.2	32.8±1.4	22.3±1.6
			Φ_i	8.4±1.7	29.2±3.0	20.7±3.4
	(B)	5	Φ_o	13.2±0.3	40.6±1.9	27.4±2.1
			Φ_i	12.5±1.0	29.3±2.6	16.9±1.7

2×10^{-5} M colchicine was added to the inner bathing solution before (*A*) or after (*B*), the equilibration period, after which control fluxes (Φ^C) were measured. 4×10^{-5} M noradrenaline was then added to the inner solution and new flux measurements (Φ^N) were taken. $\Delta=\Phi^N-\Phi^C$. Φ_o=flux towards the external medium, and Φ_i=flux towards the internal medium are expressed in nmole $\times 10^{-1}$ cm^{-2} hr^{-1}. Values are mean±SE. n=number of experiments.

Table 6. Effect of FCCP (*p*-Trifluoromethoxy-carboxylcyanidephenyl-hydrazone) on the response of thiourea (1 mM/liter) outfluxes to noradrenaline across frog skin[a]

Tested molecule	n		Φ^C	Φ^N	Δ
Thiourea	5	Φ_o	48.9±4.7[b]	57.3±2.0	13.3±1.1

[a] 10^{-6} M FCCP was added to the bathing solution at the onset of experiments. Control fluxes (Φ^C) were measured after equilibration period. 4×10^{-5} M noradranaline was then added to the inner side and new flux measurements (Φ^N) were performed. $\Delta=\Phi^N-\Phi^C$. Φ_o = flux towards the external medium is expressed in nmole $\times 10^{-1}$ cm^{-2} hr^{-1}. n = number of experiments. Values are mean ± SE.

[b] The higher control thiourea 1 mM/liter outflux compared to that reported in Table 1, was due to a seasonal variability.

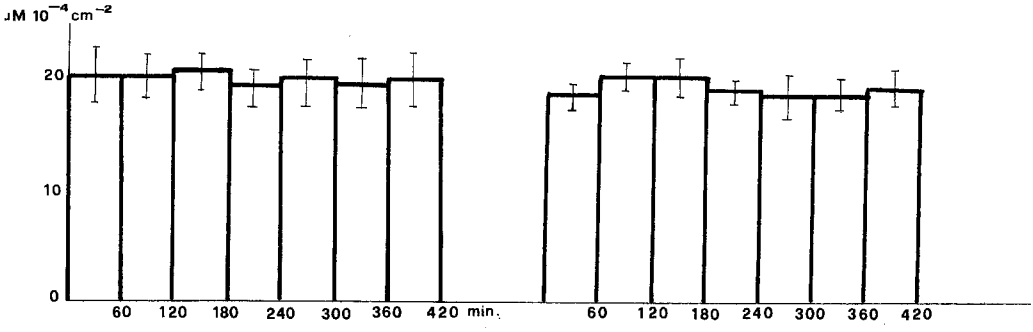

Fig. 3. Urea 1 mM outfluxes across frog skin in the presence (*B*) and absence (*A*) of colchicine 2×10^{-5} M. Number of experiments: group *A*, n=7; group *B*, n=6

in Table 1 as Φ^C; (c) the noradrenaline effect on thiourea 110 mM fluxes, in media with unchanged total osmolarity, is equal to that found at higher osmolarity (Table 4).

Colchicine is well known to exert disruptive effects on the microtubules involved in the cellular action of such hormones as vasopressin within the cells [10].

Table 5 shows the effect of noradrenaline on urea fluxes to be symmetrical in skin preincubated with colchicine. Furthermore, the effect is very similar to that found for influxes in the absence of colchicine (Table 1). In other words, colchicine may decrease the response of outfluxes but has no effect on noradrenaline elicited influx increase. Colchicine added immediately after the equilibration period, (i.e., without preincubation) (Table 5), fails to influence the noradrenaline effect previously reported (Table 1). Decreased hormone effect on thiourea

outfluxes results when the frog skin is preincubated with 10^{-6} M FCCP (Table 6). Indeed, the increased outflux results are very similar to that of influxes without FCCP (Table 1). The effects of colchicine and FCCP are not due to tissue damage. In fact, as reported in Fig. 3, colchicine treatment for 7 hr does not increase urea 1 mM fluxes. Not even FCCP increases skin permeability [9]. Therefore in our experimental conditions, these drugs do not elicit the formation of any leak pathway.

Discussion

The present study was undertaken in order to investigate the effect of noradrenaline on nonelectrolyte permeability in frog skin.

Unidirectional nonelectrolyte fluxes with different molecular weight and lipid solubility appear to increase after noradrenaline stimulation (Table 1). The greater effect on the outfluxes as compared to influxes could be accounted for by the existence of a secretory phenomenon. Indeed, this view is in agreement with the data of Campbell and Huf [4] and House [6]. In fact, it has been reported that in amphibian skin catecholamines produce an increase in active sodium influx and chloride outflux acting both on epithelial and gland cells. Therefore, a possible explanation for our results (Table 1) could be that noradrenaline elicits a secretory phenomenon towards the external medium responsible for increased outflux. The behavior of methylated thiourea derivatives is in agreement with this hypothesis. In fact, it can be assumed that there is a larger cellular pool available for the secretion by increasing lipid solubility. In accordance with this view, a lower outflux enhancement was found for mannitol, a hydrophilic molecule with a higher molecular weight.

In agreement with the view that noradrenaline elicits a secretory phenomenon in frog skin epithelium, increased influx can be accounted for by the incorporation of secretion granule membrane into the apical membrane of the gland cells [1]. This incorporation may, in turn, be responsible for the change in membrane surface and thus for the rate of nonelectrolyte permeation across it. However, hormone effect on influxes is quite similar for thiourea and its methylated derivatives, i.e., unrelated to lipid solubility. A close correlation between lipid solubility and influx enhancement following noradrenaline is to be expected if the effect on influx is due to an incorporation of secretion granule membrane into gland cell membrane. Therefore, this hypothesis may be ruled out, with the suggestion that hormone treatment has two different effects:

(a) symmetrical increase of permeability unrelated to the lipid solubility of test molecules; and (b) asymmetrical transport towards external medium related to the lipid solubility of nonelectrolytes.

The fact that, during the hormone action, the unstirred layers do not play a significant role in the differing increase of both fluxes must be also considered. Indeed, this factor is known to be virtually nonexistent in frog skin for such molecules as amides [11]. On the other hand, previously reported effects of noradrenaline on urea fluxes were not modified by vigorously stirring the bathing fluids (M. Svelto, *unpublished results*).

The linear relationship between flux increase after noradrenaline and urea concentration (Fig. 1), is in agreement with the hypothesis that noradrenaline can elicit a "secretory" phenomenon in frog skin. In fact increasing the cellular pool available for secretion results in a linear increase of the hormone effect on outfluxes. Moreover, a linear function between increased influx and molecular concentration is to be expected for a change in membrane permeability in gland and/or epithelial cells. A different pattern is shown by thiourea (Fig. 2). In fact, at high thiourea concentrations a lower and almost symmetrical increase of both fluxes occurs. This could resemble an inhibitory phenomenon of some specific mechanism. However, thiourea derivatives are known to behave as metabolic inhibitors [2], a phenomenon which may, in turn, be responsible for the behavior shown by the noradrenaline effect at high thiourea concentrations. In order to test this hypothesis, study has been made of the hormone effect on urea 1 mM fluxes in the presence of 110 mM thiourea. Under the above mentioned conditions, noradrenaline fails to increase urea fluxes to the same extent previously detected (Table 1). It seems most likely, therefore, that thiourea transport does not display a true saturation mechanism. We may thus conclude that noradrenaline-elicited nonelectrolyte secretion is not complicated by any type of interaction between test and "carrier" molecules. Microtubules have been reported to form the structural element along which secretory granule movement takes place [10]; they are also intimately involved in granule motion. In either case, the alteration of microtubular structure would disrupt the structural fromework of the system and lead to granule migration inhibition.

Colchicine, which exerts a disruptive effect on microtubules interacting with microtubule protein [12–13], has been widely used as a tool for investigation of the role of microtubules in cell function. In order to test whether microtubules are involved in noradrenaline-induced nonelec-

trolyte "secretion", experiments on frog skin (Table 5) were carried out. It is noteworthy that both urea unidirectional fluxes are increased to the same extent by the hormone, after 4 hr of colchicine preincubation. This increase is quite similar to that found for urea influxes in the absence of colchicine (Table 1).

These experiments support the idea that microtubules play a role in the action of noradrenaline on nonelectrolyte "secretion".

On the other hand, the fact that influx increase is not modified by colchicine preincubation strongly suggests that the nature of hormonal effect on influxes is completely different from that found for the "secretory" phenomenon.

Moreover, the effect of colchicine cannot be due to interaction with the noradrenaline receptor. In fact, colchicine added just after the equilibration times was found to be incapable of exerting an inhibitory effect. It is well known that colchicine binding to the tubulin extracted in porcine brain approaches the maximum value in about 4 hr. This time span seems similar to that required to obtain colchicine inhibition of the response to noradrenaline (Table 5).

Since the energy supply could be a limiting factor for a secretory process [3], the frog skin was incubated in Ringer's solution containing FCCP 10^{-6} M. The presence of metabolic uncoupler (Table 6), strongly inhibits the noradrenaline effect on thiourea outfluxes. In fact, outfluxes decrease to the same value reported in Table 1 for influxes.

These results, therefore, give further evidence that the higher effect of noradrenaline on the outfluxes might be due to a secretory process. This secretory process may be an epiphenomenon of the hormone-elicited secretion of fluid and electrolytes.

Two considerations support this view:

(1) No specificity is exhibited by nonelectrolyte "secretion"; in fact, all the molecules tested in the present work show asymmetrical transport under noradrenaline treatment.

(2) None of the nonelectrolytes tested exhibit a true saturation kinetic under noradrenaline treatment.

On the other hand, the hypothesis that noradrenaline induces an olocryne nonelectrolyte secretion may be ruled out. In fact, this process might result in loss of tissue selectivity as regarding nonelectrolyte permeability. However, if the ratio Φ_o urea/Φ_o mannitol, is taken as a tool for skin selectivity, no reduction is found after noradrenaline treatment.

Finally the secretion cannot be due to a drag effect exerted by water bulk flow through water-filled pores since the extent of nonelectrolyte

secretion is related to lipid solubility. Thus exocytosis may be seen as responsible for the hormone-elicited asymmetrical nonelectrolyte transport. According to this hypothesis the extent of nonelectrolyte "secretion" should be related to the permeation rate of these molecules through two membranes: (a) the plasma membrane of secretory cells; and (b) the membrane of intracellular vesicles prior to the exocytotic process.

Certain preliminary results of this work have been presented at the 5th International Biophysic Congress, Copenhagen, 1975.

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