Nonequilibrium Thermodynamic Analysis of the Coupling between Active Sodium Transport and Oxygen Consumption

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ABSTRACT Sodium transport and oxygen consumption have been simultaneously studied in the short-circuited toad skin. A constant stoichiometric ratio was observed in each skin under control condition (NaCl-Ringer's solution bathing both sides of the skin) and after block of sodium transport by ouabain. During alterations of sodium transport by removal and addition of K to the internal solution the stoichiometric ratio is constant although having a value higher than that observed in other untreated skins. The coupling between active sodium transport and oxygen consumption was studied after a theoretical nonequilibrium thermodynamic model. Studies were made of the influence of Na chemical potential difference across the skin on the rates of Na transport and oxygen consumption. A linear relationship was observed between the rates of Na transport and oxygen consumption and the Na chemical potential difference. Assuming the Onsager relationship to be valid, the three phenomenological coefficients which describe the system were evaluated. Transient increases in the rate of sodium transport and oxygen consumption were observed after a transitory block of sodium transport by removal of Na from the external solution. Cyanide blocks completely the rate of oxygen consumption in less than 2 min and the short-circuit current measured after that time decays exponentially with time, suggesting a depletion of ATP from a single compartment.

INTRODUCTION

The coupling between the rate of sodium transport and oxidative metabolism in amphibian skin has been investigated since the work of Lund and Stapp (11) when these authors correlated the electrical current flowing through a partially short-circuited skin with the number of electrons available in the oxidative metabolism. Conway (3, 4, 5) formulated his hypothesis of a "redox pump" and was able to show under experimental conditions that in

the frog skin, the rate of sodium transport is always less than four Na ions per oxygen molecule, a result which is in complete agreement with his hypothesis. On the other hand, the works of Zerahn (20, 21) and Leaf and Renshaw (10) invalidated the redox hypothesis in the preparation of amphibian skin, showing a value higher than four for the number of Na ions transported per oxygen molecule consumed by the skin.

In 1968, Essig and Caplan (6) proposed a theoretical nonequilibrium thermodynamic model for the energetics of active transport processes, considering the flow of a cation coupled to the flow of a metabolic driving reaction. Vieira et al. (18) have shown for *Rana pipiens* that the values of dNa/dO_2 varied significantly, from one skin to the other, ranging from 7.1 to 30.9 Na ions per O_2 molecule, as compared with Zerahn and Leaf and Renshaw's values of about 18. It was also shown that antidiuretic hormone stimulated and ouabain depressed both the rates of sodium transport and oxygen consumption; in both cases apparent stoichiometric ratios were preserved. In their second paper, Vieira et al. (19) were able to show a linear dependence of the rate of oxygen consumption on the electrical potential difference across the skin over a range of ± 160 mV. It was also shown that perturbations of the electrical potential difference for 10 min or more produced a "memory" effect: Accelerating sodium transport by negative clamping lowered the subsequent value of Na transport; positive clamping induced the opposite effect. Changes in the rate of oxygen consumption were in the same direction as the changes in the rate of Na transport.

The aim of the present paper is to study the coupling of Na transport and oxygen consumption in the skin of the toad *Bufo marinus ictericus*, with special emphasis on the effects of changes in the chemical component of the Na electrochemical potential difference on the rates of active transepithelial Na transport and oxygen consumption.

METHODS

Abdominal skins of *Bufo marinus ictericus* were mounted in modified Ussing-Zerahn chambers (17) permitting the simultaneous measurement of electrical current, transmembrane potential, and oxygen consumption. An area of 7.1 cm^2 of skin was exposed and equilibrated at least 1 h before study. A voltage clamp was used to adjust the voltage across the skin to zero. Electrical current was recorded continuously. Other details of methods were presented elsewhere (18).

The partial pressure of oxygen in each compartment was continuously monitored by means of Clark oxygen electrodes (Beckman Instruments, Inc., Spinco Div.). Experiments were performed in a Faraday cage. The temperature was kept in the range of 25.00 ± 0.01 °C by means of water jackets supplied from a constant temperature water bath.

The standard glucose Ringer's solution used consisted of 110.0 mM NaCl, 2.5 mM $KHCO_3$, 1.0 mM $CaCl_2$, and 10.0 mM glucose, with a pH of 8.2 and osmolality of

220 mosmol/liter. Streptomycin was added to the Ringer's solution at a concentration of 0.1 mg/ml to prevent bacterial growth. Ouabain was obtained from Sigma Chemical Co., St. Louis, Mo. Toads, captured in the vicinity of São Paulo were purchased from a local dealer (São Paulo, Brazil) and kept with free access to running tap water, without food, for a period of no longer than a month.

Unidirectional Na fluxes were measured with 24-Na added to the desired compartment and equilibrated at least 45 min before sampling. Samples were counted in an Automatic Gamma Counting System (Nuclear-Chicago Corp., Des Plaines, Ill.).

Results are presented as mean ± standard error (SE) if not otherwise indicated. Straight lines were fitted by the method of least squares.

RESULTS

1. *Evaluation of the Active Transepithelial Sodium Transport with Ringer's Solution Bathing both Sides of the Skin*

These experiments were performed to determine to what extent short-circuit current (I_o) could be used as a measurement of the active transepithelial sodium transport (J^a_{Na}). Table I shows the mean values for the influx and efflux, evaluated in paired skins, and the short-circuit current of the piece of skin where the influx was measured. Fluxes were measured in two subsequent 30-min periods for six skins. It can be noted that I_o is not statistically different from the electrically equivalent rate of net sodium flux, which is, in the present conditions, the active transepithelial sodium transport (J^a_{Na}).

TABLE I

UNIDIRECTIONAL Na FLUXES IN TOAD SKIN

Period	Influx	Efflux	Net flux	I_o	Net flux/I_o
30 min	$\mu A\ cm^{-2}$	$\mu A\ cm^{-2}$	$\mu A\ cm^{-2}$	$\mu A\ cm^{-2}$	
1	47.7±6.23	1.79±0.42	45.9±5.87*	42.8±6.94*	1.07
2	40.0±6.67	1.77±0.33	38.3±6.47*	40.3±6.63*	0.95

* Paired t test ($0.80 < P < 0.90$)

Net Na flux was compared to short-circuit current (I_o) of the skin where influx was measured. Ringer's solution bathing both sides of skin. Influx and effiux measured in paired skins of the same toad.

2. *Evaluation of the Active Transepithelial Sodium Transport with Reduced External Sodium Concentration*

These experiments were performed to evaluate the possibility of using short-circuit current (I_o), after suitable correction, as a measurement of active sodium transport across the toad skin, when external sodium concentration was reduced. The use of I_o to evaluate Na transport is necessary in the oxygen consumption experiments (Results, section 5) since the use of Na isotopes in

these experiments is impractical considering the complexity of the experimental setup. The present experiments were performed in the short-circuited state, with Ringer's solution bathing the internal surface of the skin, while in the external solution (Ringer's solution) Na ions were partially replaced by K ions in order to give the desired Na concentration. J^a_{Na} in different external Na concentrations was evaluated as follows:

$$J^a_{Na} = J^{ei}_{T-Na} - J^{ei}_{P-Na}, \tag{1}$$

where

J^{ei}_{T-Na} = total Na influx

J^{ei}_{P-Na} = passive sodium influx evaluated as

$$J^{ei}_{P-Na} = P_{Na}\,(Na)_e. \tag{2}$$

The coefficient P_{Na} evaluated in efflux experiments was considered to be constant and a very weak function of $(Na)_e$, as suggested by the results of Table II, which presents values for Na efflux in three external Na concentrations (110, 30, and 5 meq/liter). These values were not statistically different as tested by analysis of variance. The differences between I_o and J^a_{Na} were calculated for an external Na concentration of 33.7 ± 0.8 meq/liter (I_o = 53.4 ±

TABLE II

SODIUM EFFLUX AS A FUNCTION OF THREE EXTERNAL SODIUM CONCENTRATIONS

	Sodium efflux				
$(Na)_e$	66	67	68	69	70
meq/liter			*pmol cm⁻² s⁻¹*		
110	5.5	13.1	10.6	180.8	26.4
	15.8	12.2	7.8	71.9	25.8
	10.0	13.3	9.4	44.7	27.2
30	28.6	21.7	8.9	53.9	35.0
	24.4	23.3	8.9	57.5	36.9
	25.6	19.4	8.9	56.7	30.0
5	24.2	15.8	8.1	56.4	23.9
	25.0	18.3	7.8	54.4	21.9
	12.5	13.1	8.9	56.7	21.7

Analysis of variance two-way classification; $F_{columns} = 0.35$; $F_{rows} = 0.01$; $F_{row,\ column} = 0.03$.

Ringer's solution bathing internal surface. External solution was Ringer's solution in which Na was replaced by K in order to give the desired Na concentration. Experiments in short-circuited state.

7.1 μA cm^{-2}; J^a_{Na} = 51.8 ± 6.9 μA cm^{-2}; $0.05 < P < 0.10$ (n = 9) paired t test; J^a_{Na}/I_o = 0.98) and of 5.6 ± 0.4 meq/liter (I_o = 28.4 ± 4.2 μA cm^{-2}; J^a_{Na} = 30.6 ± 4.7 μA cm^{-2}; $0.025 < P < 0.05$ (n = 9) paired t test; J^a_{Na}/I_o = 1.08).

These results suggest that I_o values are reasonable measurements of J^a_{Na} in this range of concentration. Therefore, in our experiments of oxygen consumption, I_o will be used without any correction as an estimate of J^a_{Na}.

3. *Sodium Transport and Oxygen Consumption in the Short-Circuited State*

Vieira et al. (18) have shown the existence of a linear relationship between short-circuit current and oxygen consumption in *Rana pipiens* during spontaneous decline of sodium transport and after stimulation of sodium transport by antidiuretic hormone. Experiments were carried out on the toad skin to verify this relationship during spontaneous decline of I_o, during inhibition of sodium transport by ouabain and during alterations of sodium transport by removal and addition of K in the internal solution.

In freshly mounted short-circuited toad skins with Ringer's solution bathing both sides of the skin, the rates of sodium transport as measured by short-circuit current (I_o) and of oxygen consumption (J_{ro}) decline with time. After sufficient time has elapsed to permit evaluation of the relationship between these variables, ouabain was added to the internal solution to result in a concentration of 1 mM, and the rapid decline of I_o and J_{ro} was followed. Using a more refined setup than Vieira et al. (18), with less noise in the oxygen consumption recordings, we were able to obtain several measurements of J_{ro} after addition of ouabain, which permitted evaluation of the relationship between I_o and J_{ro} during the rapid decline period. The stoichiometric ratio (dNa/dO_2) between the rates of Na transport and oxygen consumption, equal to L_{Nar}/L_r (see Appendix) during the spontaneous decline (15.0 ± 2.0 Na ions/O_2 molecule) was not statistically different from the value after addition of ouabain (13.8 ± 1.5 Na ions/O_2 molecule), ($0.60 < P < 0.70$, n = 7, paired t test). Similarly, the basal rate of oxygen consumption calculated by extrapolation $(J_{ro})_{I_o=0}$ under these two conditions (25.3 ± 8.8 pmol cm^{-2} s^{-1} and 28.4 ± 4.4 pmol cm^{-2} s^{-1}, respectively) was not statistically different ($0.50 < P < 0.60$, n = 7, paired t test). Fig. 1 shows a representative experiment. Mean dNa/dO_2 and $(J_{ro})_{I_o=0}$ values were estimated by using pooled data for the two conditions, giving values of 16.3 ± 1.2 Na ions/O_2 molecule (n = 7) and 31.5 ± 6.4 pmol cm^{-2} s^{-1} (n = 7), respectively.

Removal of K from the internal solution leads to a slow decline in short-circuit current of frog skin (13) (14). In this manner alterations in I_o were induced in order to study the relationship between I_o and J_{ro}. Skins bathed with normal Ringer's solution on both sides were followed until a steady state was reached. At this moment the internal solution was changed to K-free

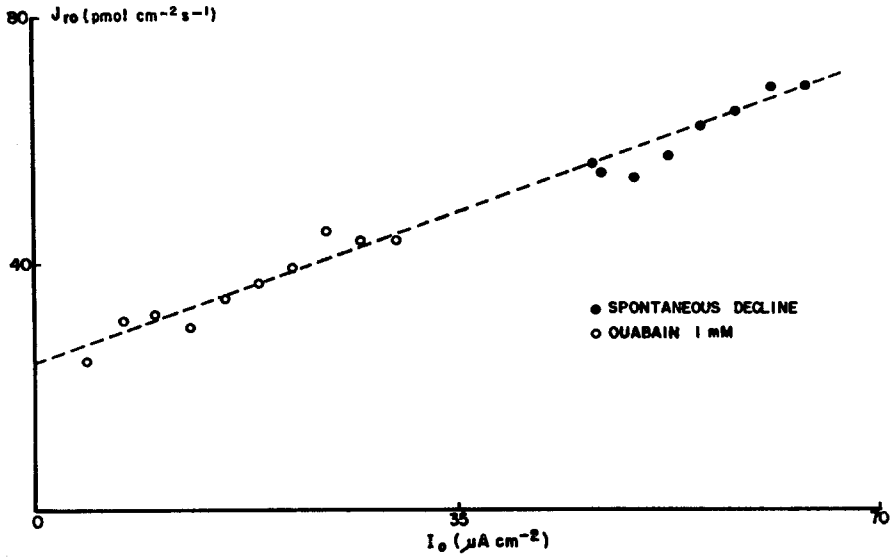

FIGURE 1. Short-circuit current (I_o) and the associated rate of oxygen consumption (J_{ro}) in control condition (Ringer's solution on both sides of skin) and after addition of ouabain to the internal solution.

Ringer's solution and I_o and J_{ro} were monitored, for a time sufficient to evaluate their relationship. Then the internal solution was again changed back to normal Ringer's solution and increases in I_o and J_{ro} were obtained. The stoichiometric ratios (dNa/dO_2) during the removal and addition of K (27.3 ± 2.5 and 28.2 ± 3.0 Na ions/O_2 molecule) were not statistically different ($0.30 < P < 0.40$, $n = 8$, paired t test.

4. *Short-Circuit Current and Oxygen Consumption in the Presence of CN^- Ions*

The rate of oxygen consumption and short-circuit current were measured in skins bathed with normal Ringer's solution on both sides. Once a steady condition was attained, KCN in a final concentration of 1 mM was added to both solutions. In less than 2 min after CN^- addition, oxygen consumption was completely reduced to zero and a decline of I_o was observed. In order to study in detail the kinetics of decline of I_o after complete block of J_{ro} experiments were carried out in which KCN was added to both internal and external solutions to give a concentration of 1 mM, and the decline of I_o was followed. Only I_o values obtained at times longer than 2 min after KCN addition were used in the calculations. An exponential decline of I_o with time was observed in all experiments. The plot of $\ln(I_o) = a + bt$ permitted the calculation of mean values for a (4.9 ± 0.2) and for b (-0.060 ± 0.006 s^{-1}) for six skins, with all the linear correlation coefficients significant at 0.01 level. In these experiments we have assumed I_o as being a reasonable evaluation of J^a_{Na}.

5. *Active Sodium Transport and Oxygen Consumption as a Function of External Sodium Concentration*

These experiments were performed to study the dependence of the rates of active sodium transport and oxygen consumption on the chemical potential difference of Na across the skin. The theoretical nonequilibrium thermodynamic model of Essig and Caplan (8) assumes a linear dependence of the active transepithelial sodium transport and oxygen consumption on the electrochemical potential difference of Na across the skin, assuming that the affinity of the chemical reaction coupled to the transporting mechanism is constant. Vieira et al. (10) have shown that a linear dependence of the rate of oxygen consumption on the electrochemical potential difference (X_{Na}) is observed when X_{Na} is altered by changing the electrical potential difference across the skin, in the absence of a Na concentration difference. In the present experiments we studied the dependence of J_{ro} and J^a_{Na} on X_{Na}, by altering the chemical component of X_{Na}, keeping the electrical potential difference across the skin equal to zero, by means of voltage clamping. These experiments were carried out with Ringer's solution bathing the internal surface, varying the Na concentration in the external solution (Na ions were replaced by K ions). The rates of active sodium transport and oxygen consumption were monitored at three different external sodium concentrations, in the range of 110-5 meq/liter. The rates of active sodium transport were estimated by short-circuit current (see Results, section 2). The stoichiometric ratio calculated under these conditions, equal to L_{Na}/L_{rNa} (see Appendix) (16.9 ± 0.8 Na ions/O_2 molecule, $n = 5$) is not statistically different from the stoichiometric ratio L_{Nar}/L_r, calculated with equal Na concentrations on both sides of the skin, (16.3 ± 1.2 Na ions/O_2 molecule), ($0.70 < P < 0.80$ [$n = 7$] t test). The slope of J^a_{Na} as function of $\ln(Na)_e$ (Appendix, Eq. A-7) permits the calculation of the phenomenological coefficient L_{Na}, equal to 0.28 ± 0.03 peq^2 cal^{-1}cm^{-2}s^{-1} in these long-term experiments.

The total substitution of Na by potassium in the external solution reduces oxygen consumption to 37.5 ± 1.2 pmol cm^{-2}s^{-1}, which is higher than the extrapolated value of 34.7 ± 1.1 pmol cm^{-2}s^{-1} ($P < 0.05$, $n = 5$, paired t test) calculated in these same experiments with changes in external Na concentration. The small short-circuit current (2.88 ± 1.06 μA cm^{-2}, $n = 5$) found when only potassium was present in the external solution might have been a consequence of passive fluxes due to the high concentration solution asymmetry across the skin. This finding suggests that P_K is greater than P_{Na} in this epithelium, and agrees with the results of Mandel and Curran (12) in frog skin.

The expected linear dependence of J^*_{ro} (suprabasal rate of oxygen consumption) on the natural logarithm of external Na concentration (internal Na

constant, see Appendix) was observed in all experiments and can be seen in Fig. 2. The calculated linear correlation coefficients for J^*_{ro} as function of $\ln(Na)_e$ are significant at the 0.01 level. The slope of J^*_{ro} as function of $\ln(Na)_e$ permits the calculation of the phenomenological cross coefficient L_{rNa}, equal to $1.68 \times 10^{-2} \pm 0.23 \times 10^{-2}$ pmol eq $cal^{-1}cm^{-2}s^{-1}$ (see Eq. A-8 in the Appendix); the intercept in turn permits the evaluation of the product L_rA, equal to 40.6 ± 5.4 pmol $cm^{-2}s^{-1}$.

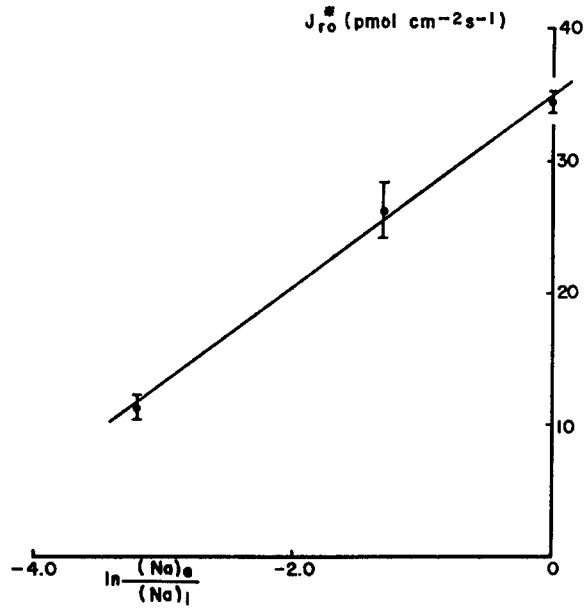

FIGURE 2. Mean J^*_{ro} values as function of external Na concentration. J^*_{ro} is equal to total oxygen consumption minus basal oxygen consumption. Internal Na concentration was kept constant.

In order to study the dependence of active Na transport on the external Na concentration, more detailed experiments were performed in eight different external Na concentrations (110.0, 73.5, 55.0, 36.6, 22.0, 15.7, 11.0, and 5.5 meq/liter); normal Ringer's solution bathed the internal surface. Active sodium transport was evaluated by short-circuit current (see Results, section 2). Fig. 3 shows a representative experiment. There is a highly linear correlation for each skin between J^a_{Na} and the natural logarithm of external Na concentration. These short-term experiments (duration of approximately 30 min) were used to calculate the direct phenomenological coefficient L_{Na}, equal to 0.45 ± 0.06 peq^2 cal^{-1} cm^{-2} s^{-1} ($n = 9$), according to Eq. A-7, of the Appendix, and the product $L_{Nar}A$, equal to 1207 ± 151 peq cm^{-2} s^{-1}, by extrapolation.

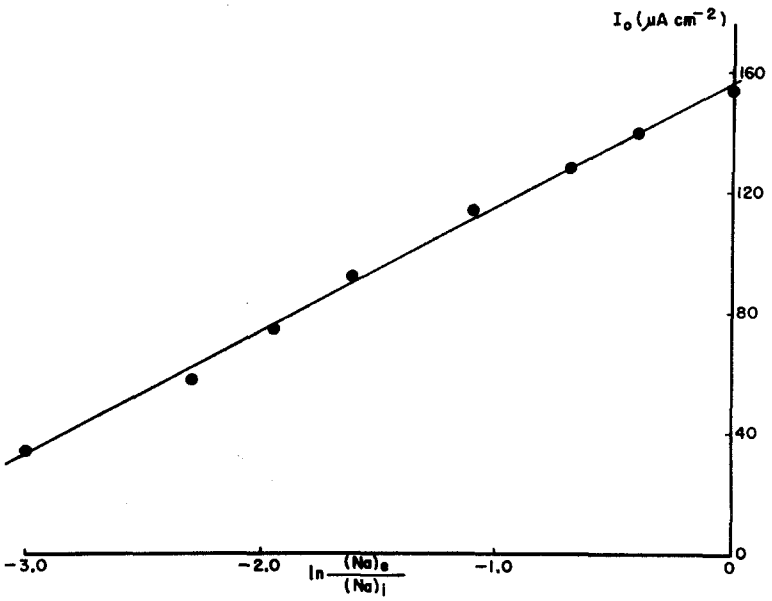

FIGURE 3. Short-circuit current (I_o) as function of external Na concentration. Internal Na concentration was kept constant.

Vieira et al. (10) have shown that perturbations of the transepithelial electrical potential difference across the skin for 10 min or more produced a "memory" effect: accelerating Na transport by negative clamping lowered the subsequent value of I_o; positive clamping induced the opposite effect. In order to study this phenomenon in more detail, experiments were performed to evaluate the dependence of J^a_{Na} and J_{ro} on the external Na concentration, before and after blocking Na transport by substituting all Na by K in the external solution. Experiments were performed in the short-circuited state with Ringer's solution on both sides and the duration of the Na transport block was 10–20 min, after which, normal Ringer's solution was replaced in the external chamber. It was observed that in the second control period the rates of Na transport and oxygen consumption were significantly higher than the values in the first control period. Fig. 4 shows one representative experiment and Table III summarizes the results.

How could this memory effect be explained in terms of phenomenological description? As seen by Eqs. A-1 and A-2 in the Appendix, this effect could be due to alterations of the phenomenological coefficients or of the affinity of the chemical metabolic driving reaction. In order to decide between these possibilities, experiments were performed measuring only J^a_{Na} as a function of external Na concentration, before and after complete block of Na transport for 20 min, by replacement of Na by K in the external solution. Oxygen con-

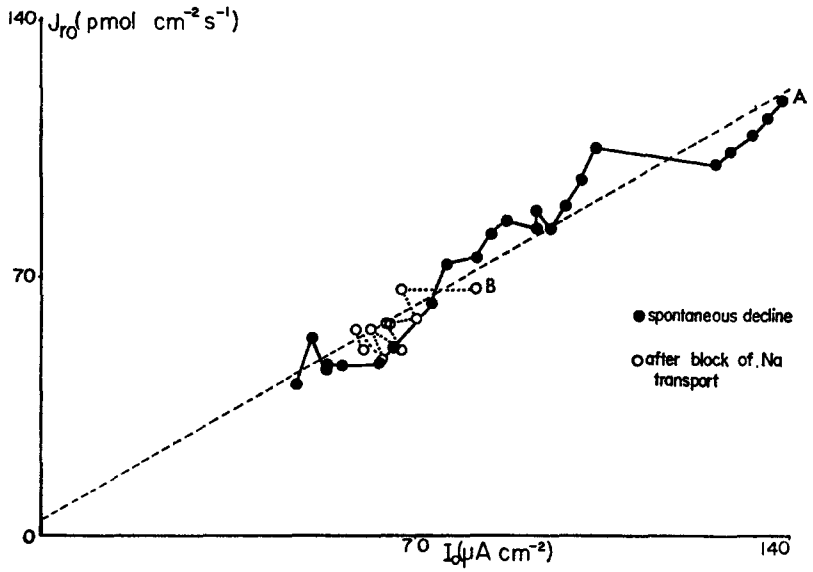

FIGURE 4. Simultaneous memory effect in short-circuit current (I_o) and oxygen consumption (J_{ro}). Solid circles correspond to a control period before block of Na transport. Open circles correspond to a control period after block of Na transport. Na transport was blocked by substituting Na with K in the external solution. Initial point of each period is indicated by letters (A, before block of Na transport and B, after block of Na transport).

TABLE III

MEMORY EFFECT IN SHORT-CIRCUIT CURRENT (I_o) AND RATE OF OXYGEN CONSUMPTION (J_{ro}) AS A CONSEQUENCE OF Na TRANSPORT BLOCK BY COMPLETE SUBSTITUTION OF Na BY K IN THE EXTERNAL SOLUTION

Skin	I'_o	I''_o	ΔI_o	J'_{ro}	J''_{ro}	ΔJ_{ro}
	μA/cm²	μA/cm²	μA/cm²	pmol/cm² s	pmol/cm² s	pmol/cm² s
8	48.1	82.0	33.9	40.9	67.2	26.3
9	58.7	51.7	−7.0	59.5	65.8	6.3
10	61.5	93.3	31.8	58.9	77.5	18.6
12	12.3	27.6	15.3	36.6	54.4	17.8
14	21.2	43.8	22.6	26.6	63.6	37.0
16	13.4	25.4	12.0	30.0	55.1	25.1
17	17.0	20.2	3.2	43.7	36.0	−7.7
Mean	33.2*	49.1*	16.0	42.3‡	59.9‡	17.6
SE	8.3	10.8	5.6	4.9	5.0	5.5

* Paired t test ($0.01 < P < 0.05$). ‡ Paired t test ($P < 0.01$).

I_o = short-circuit current; J_{ro} = rate of oxygen consumption. I'_o and J'_{ro} are last measured values of I_o and J_{ro} before substitution of Na by K in the external solution. I''_o and J''_{ro} are the first values of I_o and J_{ro} in control conditions after return Na in the external solution. $\Delta I_o = I''_o - I'_o$; $\Delta J_{ro} = J''_{ro} - J'_{ro}$.

sumption was not measured in these experiments in order to reduce the duration of the second control period and, therefore, to be able to detect transitory alterations of the phenomenological coefficient L_{Na} or the product $L_{Nar}A$. Fig. 5 shows a representative experiment. Mean values for L_{Na} and $L_{Nar}A$ before and after block of Na transport are respectively: $L_{Na\,(before)} = 0.43 \pm 0.06$ peq^2 cal^{-1} cm^{-2} s^{-1}; $L_{Na\,(after)} = 0.48 \pm 0.05$ peq^2 cal^{-1} cm^{-2} s^{-1} ($0.60 < P < 0.70$, $n = 10$, paired t test). $L_{Nar}A_{(before)} = 102.7 \pm 13.6$ μA cm^{-2}; $L_{Nar}A_{(after)} = 117 \pm 11.7$ μA cm^{-2} ($P < 0.01$, $n = 10$, paired t test).

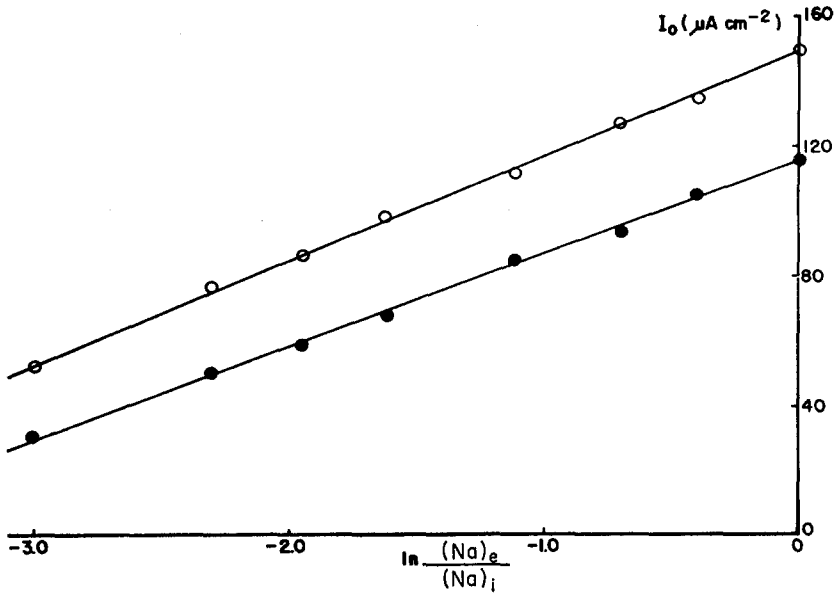

FIGURE 5. Short-circuit current (I_o) as a function of external Na concentration before (solid circles) and after (open circles) block of Na transport by substitution of Na by K in the external solution. Internal Na concentration was kept constant.

DISCUSSION

The behavior of biological systems performing transport of molecules across a barrier can be described by phenomenological equations of nonequilibrium thermodynamics. Essig and Caplan (6) have proposed a theoretical model for the active transport of a single cation coupled to a chemical reaction. The adequacy of this model to sodium transport was partially tested by Vieira et al. (18) (19) in the skin of the frog *Rana pipiens*. They have shown that there is in short-circuited state a linear relationship between the rates of sodium transport and oxygen consumption, and that the rate of oxygen consumption is a linear function of the transepithelial electrical potential difference.

The present work, carried out on the skin of the toad *Bufo marinus ictericus*

is a further experimental test of the linear nonequilibrium thermodynamic model proposed by Essig and Caplan (6). It shows that the rates of active sodium transport and oxygen consumption are linear functions of the chemical potential difference of Na across the skin when changing the external Na concentration. Assuming the validity of Onsager's reciprocal relationship, this linearity permits the calculation of the three phenomenological coefficients which describe the system.

In order to study explicitly the coupling of active sodium transport to metabolism, preliminary experiments were performed to evaluate to what extent the rate of active sodium transport could be measured by short-circuit current in the skin of the toad *Bufo marinus ictericus*. When equal Na concentrations bathe both sides of the skin, results of section 1 (Results) clearly demonstrate that short-circuit current is a valid measurement of the active transepithelial Na transport, since the rate of active sodium transport calculated from isotope measurement is identical to the rate of sodium transport measured by short-circuit current. In the experiments with lower sodium concentration in the outer solution, short-circuit current was shown to be a reasonable evaluation of the rate of transepithelial active sodium transport, in the range of 5–110 meq/liter, according to section 4 of Results.

According to Eqs. A-1 and A-2, the active transepithelial sodium transport (J^a_{Na}) and the related oxygen consumption J^*_{ro} (total oxygen consumption minus basal oxygen consumption) are linear functions of the two related forces, the negative transepithelial sodium electrochemical potential difference (X_{Na}) and the affinity of the metabolic driving reaction (A). X_{Na} is easily controlled in the experiments by proper values of the electrical potential difference and sodium concentration difference across the skin; on the other hand, A is not accessible to experimental control and may be evaluated only in some cases (19).

With X_{Na} equal to zero, changes in J^a_{Na} are accompanied by proportional variations of J^*_{ro} and a constant stoichiometric ratio (L_{Nar}/L_r) is maintained for a given skin, independently of the level of Na transport. Such behavior was observed during the spontaneous decline of sodium transport with time and after inhibition of J^a_{Na} by ouabain. Such constancy of the stoichiometric ratio for a given skin was also observed by Vieira et al. (18) in the *Rana pipiens* during spontaneous decline and after stimulation of Na transport by antidiuretic hormone. Their observations also suggested that during inhibition by ouabain, the stoichiometric ratio is maintained. During decline and recovery of the short-circuit current by removal and addition of potassium to the internal solution, the stoichiometric ratio remained unchanged, but these values (27.3 ± 2.5 and 28.2 ± 3.0 Na ions/O_2 molecule, respectively) are significantly higher than the mean value of 16.3 ± 1.2 Na ions/O_2 molecule, for pooled data obtained during spontaneous decline and after addition of oua-

bain ($P < 0.01$). We have no explanation for this difference. It is pertinent, therefore, at this point to look at the constancy of the stoichiometric ratio (L_{Nar}/L_r) during the rapid fall of the short-circuit current and oxygen consumption, in the course of ouabain action. We assumed that short-circuit current is a fair measurement of the rate of transepithelial active sodium transport after addition of ouabain, but no direct proof of this assumption was obtained in view of the technical difficulties imposed by the rapid decline in sodium transport. The finding of a constant stoichiometric ratio is compatible with ouabain blocking a progressively greater number of apparent transporting units with time, without altering their intrinsic stoichiometric ratio. The behavior of the transport system under the inhibitory action of ouabain is markedly different from that observed while under the effect of dinitrophenol observed in *Rana pipiens* (18) where this inhibitor was shown to "uncouple" [1] the sodium transport system from the metabolic driving reaction system.

The influences of sodium chemical potential difference on the rate of active sodium transport and oxygen consumption were studied by altering the external sodium concentration maintaining constant internal sodium concentration (NaCl-Ringer's solution). It has been shown by several authors that the rate of transepithelial sodium transport in amphibian skin is a nonlinear function of the external sodium concentration. Kirschner (9) found that short-circuit current across frog skin rose by increasing sodium concentration, reached a maximum at about 35 meq/liter of external Na and then decreased as the concentration was raised further. Cereijido et al. (1) interpreted the saturation of the rate of sodium transport in *Rana pipiens* with increasing external Na concentration as due, in part, to changes in the permeability of the outer membrane rather than to saturation of the transporting system ("pump") itself. Cirne and Malnic (2) observed that short-circuit current in the toad skin of *Bufo paracnemis* and *Bufo ictericus* could be fitted in the range of 5–50 meq/liter external Na concentration by Michaelis-Menten kinetics. In our experiments, the rates of Na transport and oxygen consumption were logarithmic functions of the external Na concentration in the range of 5–110 meq/liter. The same results could also be fitted by Michaelis-Menten kinetics, as exemplified in Fig. 6 *a* and *b*, for a representative experiment. Certainly, this is a consequence of the small range of Na concentrations used in these experiments.

Considering the coupling of Na transport and oxygen consumption shown in these experiments and the linear dependence of oxygen consumption with the electrical potential difference across the skin in *Rana pipiens* (19), we may

[1] "Uncouple" is used here with the particular meaning of disconnecting the transport system from the metabolic driving reaction, without the usual biochemical implication and without specifying any particular point in the metabolic driving reaction chain.

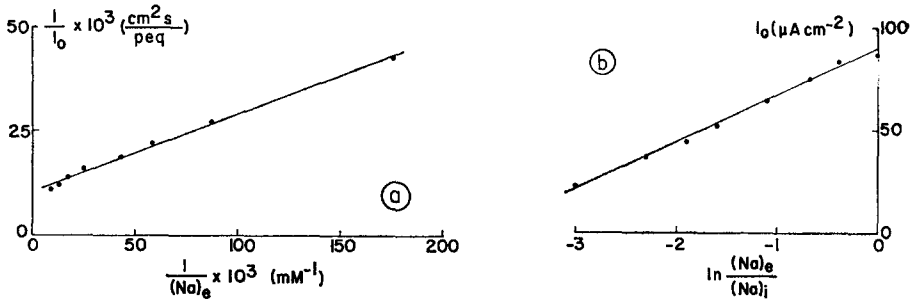

FIGURE 6. Rate of Na transport (as measured by short-circuit current) as a function of external Na concentration. (*a*) Lineweaver-Burk plot of $1/I_o$ as a function of $1/(Na)_e$. (*b*) I_o as a function of ln $(Na)_e$. Internal Na concentration was kept constant.

assume that the rate of sodium transport could also be a linear function of the electrical potential difference across the skin. In the toad bladder, Saito et al. (14) have shown a linear dependence of the rate of active sodium transport on the electrical potential difference. On the other hand, Mandel and Curran (13) observed in *Rana pipiens* that active Na transport displays saturation as a function of applied potential and both the level of saturation and the potential at which it is achieved are functions of the Na concentration in the external solution. Reasons for these differences are still obscure.

According to the theoretical model of Essig and Caplan (6) a linear dependence should be expected to occur between the two flows, J^a_{Na} and J^*_{ro} and one of the forces, the sodium chemical potential difference across the skin, if the other force (the affinity A) is constant. The results of our experiments are in full agreement with the theoretical postulates, since a linear dependence was demonstrable between both flows, J^a_{Na} and J^*_{ro}, and the logarithm of the external Na concentration in the range of 5–110 meq/liter, as seen in Figs. 5 and 6. The observed linearity of the two flows with the force X_{Na} is a fair indication of the constancy of the other force, the affinity A during the experimental period. Nevertheless, it is not conclusive proof, since in a more sophisticated system, with a regulatory mechanism, the affinity could be a linear function of X_{Na}. Assuming the constancy of A, the two phenomenological coefficients L_{Na} and L_{rNa} were evaluated experimentally (Results, section 5) according to Eqs. A-7 and A-8 (Appendix). Mean values of 0.45 ± 0.06 peq^2 cal^{-1} cm^{-2} s^{-1} and 0.28 ± 0.03 peq^2 cal^{-1} cm^{-2} s^{-1} were obtained for L_{Na} in short-term and long-term experiments, respectively (Results, section 5). For L_{rNa} a mean value of $1.7 \times 10^{-2} \pm 0.2 \times 10^{-2}$ peq mol cal^{-1} cm^{-2} s^{-1} was calculated (Results, section 5). The difference between L_{Na} values obtained in long-term and short-term experiments could be an indication of a decline of the phenomenological coefficients with time.

From the experiments during spontaneous decline of J^a_{Na} and J^*_{ro},[2] Eq. A-11 leads us to the experimental evaluation of L_{Nar}/L_r (equal to the stoichiometric ratio under zero Na electrochemical potential difference), on the assumption that in these long-term experiments (several hours) the decline of J^a_{Na} and J^*_{ro} is a consequence of a decline of the affinity. The experimentally calculated ratio (L_{Nar}/L_r) (see Results, section 3) was shown to be 15.0 ± 2.0 eq mol^{-1}. On the basis of Onsager's reciprocal relationship, a value of 1.1 × 10^{-3} pmol^2 cal^{-1} cm^{-2} s^{-1} could be calculated for the phenomenological coefficient L_r.

The two mean stoichiometric ratios L_{Nar}/L_r (calculated with X_{Na} equal to zero) and L_{Na}/L_{rNa} (evaluated in the presence of changes of X_{Na}), permitted evaluation of an average value for the degree of coupling (q), between the two flows, according to Kedem and Caplan (8):

$$q = \frac{L_{Nar}}{\sqrt{L_{Na} \cdot L_r}}.$$

A mean value of 0.94 was obtained with coefficients calculated in long-term experiments, which in turn permits the calculation of the maximum efficiency for the transporting system, according to (8):

$$\eta_{max} = \frac{q^2}{(1 + \sqrt{1 - q^2})^2},$$

and equal to 50%. This value should be compared with an efficiency in the range of 40 to 50% calculated by Ussing (16) on the basis of Zerahn's results (20). The value of this comparison is to show that this biological transport system works not too far from its maximal possible efficiency. Obviously, this is not a precisely valid comparison, considering that the efficiency calculated by Ussing (16) was based on an estimate of the work (W) performed by the skin through the relation

$$W = RT \ln (M_{in}/M_{out}),$$

(where $[M_{in}/M_{out}]$ is the flux ratio), and on the calorific value of equivalent of oxygen as being 25,000 cal.

W is the work required to overcome the internal resistence of the transport system. In Ussing's condition this was the only kind of work, since the skin

[2] J^*_{ro} is the suprabasal rate of oxygen consumption obtained by the difference between the total rate of oxygen consumption minus the basal rate of oxygen consumption, calculated by extrapolation, $(J_{ro})_{I_o = 0}$. In all experiments $(J_{ro})_{I_o = 0}$ was remarkably constant during all the experimental periods.

was bathed by identical solutions on both sides and also in short-circuited state.

It is interesting to compare, at this point, the action of two inhibitors, ouabain and cyanide on the coupling of oxygen consumption and sodium transport in the toad skin. As indicated above (Results, section 3) ouabain blocks sodium transport and oxygen consumption without altering the ratio L_{Nar}/L_r, indicating that the stoichiometric ratio is not altered by ouabain. On the other hand, cyanide, which also inhibits J^o_{Na} and J_{ro}, works in a different way, "uncoupling" [1] the rate of sodium transport from the rate of oxygen consumption. In less than 2 min J_{ro} is reduced to zero, which is an indication of a block of aerobic ATP synthesis. However, short-circuit current is still present for at least 60 min after addition of cyanide. If we assume that ATP production was completely blocked after 2 min and I_o was maintained by an ATP pool present in the skin, we could write, keeping in mind the constancy of the stoichiometric ratio L_{Nar}/L_r for a given skin:

$$\frac{d(ATP)}{dt} = -k\,(ATP) \tag{3}$$

and

$$I_o = -k_1 \frac{d(ATP)}{dt} = k\,k_1\,(ATP). \tag{4}$$

Integrating Eq. 3

$$(ATP) = (ATP)_{t=0}\,e^{-kt}. \tag{5}$$

Therefore,

$$I_o = k\,k_1\,(ATP)_{t=0}\,e^{-kt}. \tag{6}$$

The experimental results show a logarithmic dependence of I_o on time ($\ln I_o = a + bt$). This behavior strongly suggests that the transport system works at the expense of an ATP pool which seems to be progressively depleted with time and that the ATP pool related to Na transport is apparently not compartmentalized within the cells, since its decay follows a one-compartment kinetics. These observations do not invalidate the possibilities that the ATP pool related to transport could be separated from other (nontransport) ATP pools. As already mentioned in Results, these conclusions are based on the assumption that I_o in presence of KCN is still a reasonable measurement of the rate of Na transport. Preliminary experiments in our laboratory using a Na isotope kinetics with sampling at each minute indicate that this assumption can be considered a valid one. The linear dependence of ln (I_o) on time and the fact that I_o falls to less than 5% of the initial level in about 60 min after addition of cyanide is consistent with a minor role of anaerobic metabolism in sustaining sodium transport under these conditions.

The results presented in Fig. 4 and Table III show that a block of Na transport by removal of Na from the external solution induces a subsequent increase in J^a_{Na} and J_{ro}, when Na is returned to the external solution. This behavior, and the previous results of Vieira et al. (19), (showing that accelerating Na transport by negative clamping lowers the subsequent value of I_o and positive clamping induces the opposite effect) could be interpreted according to Eqs. A-1 and A-2, as a result of an alteration of the phenomenological coefficients L_{Nar} and L_r or of the affinity of the metabolic driving reaction or both. The example of Fig. 4 indicates that the ratio L_{Nar}/L_r is maintained after blocking Na transport, which could be a consequence of a constancy of L_{Nar} and L_r or due to a proportional increase of these two coefficients, although, in the present experiments it is impossible to decide for one of the three possibilities. The experiments (Results, section 5 and Fig. 5) clearly demonstrate that the memory effect in Na transport is due to an increase in the product $L_{Nar}A$, without significant alteration of L_{Na}. It would be interesting to know whether the increase in $L_{Nar}A$ is a consequence of an increase in L_{Nar}, in A, or in both. On speculative basis, it is most probable that the memory effect is due to an increase in A, which would result from an accumulation of metabolic intermediates, probably not only ATP, since, in terms of the oxidative phosphorylation regulatory mechanism, an increase in an ATP pool related to transport (after Na transport block), would result in an increase in the rate of sodium transport although accompanied by reduction in the rate of oxygen consumption. Therefore, it might be expected for example that accumulation of ATP would cause an increase in the NADH/NAD ratio and that with rapid lowering of ATP concentration after the release of block the high NADH/NAD ratio would then cause rapid oxygen consumption.

APPENDIX

Essig and Caplan (6) have developed a theoretical nonequilibrium thermodynamic description of active transport of a cation and its coupling to a metabolic driving reaction. The necessary equations derived from the paper of Essig and Caplan will be presented in terms of conductance coefficients.

$$J^a_{Na} = L_{Na} X_{Na} + L_{Nar} A \qquad \text{(A-1)}$$

$$J^*_{ro} = L_{rNa} X_{Na} + L_r A \qquad \text{(A-2)}$$

J^a_{Na} represents net active sodium flux, J^*_{ro} is the rate of suprabasal oxygen consumption, X_{Na} is the negative electrochemical potential difference of sodium across the skin, A is the affinity of the metabolic driving reaction and L's are phenomenological coefficients. The validity of the Onsager reciprocal relationship has been assumed: ($L_{Nar} = L_{rNa}$).

Writing explicitly X_{Na} as

$$X_{Na} = RT \ln(Na)_e - \ln(Na)_i - F\Delta\psi, \tag{A-3}$$

where $(Na)_e$ and $(Na)_i$ are Na concentrations in the external and internal bathing solutions; $\Delta\psi$ is the electrical potential difference across the skin equal to $\psi i - \psi e$; R, T, and F have their conventional meanings, we have in the short-circuited state ($\Delta\psi = 0$):

$$X_{Na} = RT(\ln(Na)_e - \ln(Na)_i). \tag{A-4}$$

Introducing (A-4) into (A-1) and (A-2) we have

$$J^a_{Na} = L_{Na}\, RT\, (\ln(Na)_e - \ln(Na)_i) + L_{Nar}A, \tag{A-5}$$

$$J^*_{ro} = L_{rNa}RT\, (\ln(Na)_e - \ln(Na)_i) + L_rA. \tag{A-6}$$

Eqs. A-5 and A-6 indicate that both fluxes are linear functions of $\ln(Na)_e$ and at constant $(Na)_i$ the L's could be evaluated assuming a constant value of A.

$$L_{Na} = \frac{1}{RT}\left[\frac{\Delta J^a_{Na}}{\Delta \ln (Na)_e}\right](Na)_i, A, \tag{A-7}$$

$$L_{rNa} = \frac{1}{RT}\left[\frac{\Delta J_{ro}}{\Delta \ln (Na)_e}\right](Na)_i, A. \tag{A-8}$$

The stoichiometric ratio between active sodium transport and oxygen consumption can be evaluated in the two conditions below: (*a*) When $(Na)_e = (Na)_i$ and $\Delta\psi = 0$ Eqs. A-5 and A-6 reduce to

$$J^a_{Na} = L_{Nar}A, \tag{A-9}$$

$$J^*_{ro} = L_rA, \tag{A-10}$$

and

$$\frac{J^a_{Na}}{J^*_{ro}} = \frac{L_{Nar}}{L_r}, \tag{A-11}$$

where J^*_{ro} is the rate of oxygen consumption associated to active sodium transport which can be evaluated by the difference between total oxygen consumption (J_{ro}) and basal oxygen consumption $(J_{ro})_{I_o=0}$. This last quantity can be evaluated experimentally (see Results). Eqs. A-11 and A-8 can be used to evaluate L_r under the condition of validity of Onsager relationship. (*b*) When $\Delta\psi = 0$ and Na concentration is reduced in the external solution,

Eqs. A-7 and A-8 lead to:

$$\frac{L_{Na}}{L_{rNa}} = \left[\frac{\Delta J^{a}_{Na}}{\Delta J_{ro}}\right] (Na)_i, A. \qquad (A\text{-}12)$$

The degree of coupling (8) defined as

$$q = \frac{L_{Nar}}{\sqrt{L_{Na} \cdot L_r}}, \qquad (A\text{-}13)$$

can be calculated by A-11 and A-12.

This work was supported by a grant from Fundação de Amparo à Pesquisa do Estado de São Paulo (Biológicas 70/1447).
We are grateful to Doctors G. Malnic, A. Essig, and P. F. Curran for careful reading and criticism of our manuscripts.

Received for publication 4 October 1973.

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