

# **Coupling of Solute Flows in Synthetic and Biological Membranes**

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## FOREWORD

This is one of a continuing series of reports designed to present accounts of progress in saline water conversion and the economics of its application. Such data are expected to contribute to the long-range development of economical processes applicable to low-cost demineralization of sea and other saline water.

Except for minor editing, the data herein are as contained in a report submitted by the contractor. The data and conclusions given in the report are essentially those of the contractor and are not necessarily endorsed by the Department of the Interior.

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## INTRODUCTION

The following material consists of work completed during the period of the grant. Recent preliminary results have been summarized in a recent Annual Report.

The support of The Office of Saline Water was much appreciated.

## PUBLICATIONS

1. Flux ratios and isotope interaction in an ion exchange membrane. De Sousa, R.C., Li, J.H., and Essig, A. Nature 231:44, 1971.
2. Nonequilibrium thermodynamic analysis of ion transport and membrane metabolism. Essig, A. and Caplan, S.R. *Esperienza*, In Press.
3. Salt and water transport in biological systems. Essig, A. In Biological Physics and Biomaterials Science (edited by H.E. Stanley), M.I.T. Press, Cambridge, 1971. In Press.

### Recent Abstracts:

1. Energetics of sodium transport in frog skin. I. The short-circuited state. Vieira, F.L., Caplan, S.R., and Essig, A. Third Int. Biophys. Congress. IUPAB Abstracts, 229, 1969.
2. Energetics of sodium transport in frog skin. II. The effects of electrical potential. Vieira, F.L., Caplan, S.R., and Essig, A. Third Int. Biophys. Congress, IUPAB Abstracts, 230, 1969.
3. Coupling of urea flows in the toad bladder. Lief, P.D., and Essig, A. Biophys. Soc. Abstracts, 197a, 1969.
4. Efficacy of energy utilization in active transport and muscle contraction. Essig, A., and Caplan, S.R. Fed. Proc. 29:655, 1970.
5. Flux ratios and isotope interaction in an ion exchange membrane. De Sousa, R.C., Li, J.H., and Essig, A. Biophys. Soc. Abstracts, 1971.

### Submitted for Publication:

1. Energetics of sodium transport in frog skin. I. Oxygen consumption in the short-circuited state. Vieira, F.L., Caplan, S.R., and Essig, A.
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## I. ENERGETICS OF SODIUM TRANSPORT IN FROG SKIN

### (1. Oxygen consumption in the short-circuited state. )

(Work done with F.L. Vieira and S.R. Caplan)

Sodium transport and oxygen consumption were studied simultaneously in the short-circuited frog skin. Sodium transport was evaluated from  $I_0/F$ , where  $I_0$  is the short-circuit current measured with standard Ringer's solution bathing each surface. Oxygen tension was measured polarographically. Under a variety of circumstances the rate of oxygen consumption from the outer solution exceeded that from the inner solution, the ratio being constant ( $0.57 \pm 0.09$  S.D.). Both  $I_0$  and the associated rate of oxygen consumption  $J_{RO}$  declined non-linearly with time, but the relationship between them was linear, suggesting that the basal oxygen consumption was constant. For each skin numerous experimental points were fitted by the best straight line. The intercept  $(J_{RO})_{I_0=0}$  then gave the basal oxygen consumption, and the slope  $dNa/dO_2$  gave an apparent stoichiometric ratio for a given skin. The basal oxygen consumption was about half the total oxygen consumption in a representative untreated short-circuited skin. Values of  $dNa/dO_2$  in 10 skins varied significantly, ranging from 7.1 to 30.9 (as compared with Zerahn's and Leaf and Renshaw's values of about 18). KCN abolished both  $I_0$  and  $J_{RO}$ . 2, 4-DNP depressed  $I_0$  while increasing  $J_{RO}$  4 to 5 fold. Antidiuretic hormone stimulated and ouabain depressed both  $I_0$  and  $J_{RO}$ ; in both cases apparent stoichiometric ratios were preserved.

## INTRODUCTION

Much of the work on energetics of sodium transport places great emphasis on stoichiometry. A stoichiometric ratio is generally considered to relate the rate of active sodium transport to the rate of suprabasal oxygen consumption under all conditions of operation (e.g. the short-circuited and open-circuited states). It is also often tacitly assumed that the same ratio obtains in different preparations of a given kind.

In view of the marked differences between randomly selected tissues, the assumption of a unique ratio applicable to all tissues seems unwarranted. It is also by no means evident that a unique ratio would apply to any one tissue under all experimental conditions. This would be the case only if sodium transport and metabolism were completely coupled, so that any factor operating to change one must necessarily change the other in such a way as to keep their ratio constant. Partial decoupling could in fact come about in many ways, some of which have been considered elsewhere (Rottenberg, Caplan, and Essig, 1967; Essig and Caplan, 1968). The demonstration of linear dependence between transport and metabolism is often cited as evidence for a stoichiometric relationship. However, to say that there is a linear relationship between the rate of transport and the rate of metabolism is not equivalent to saying that the rate of transport is a constant multiple of the rate of suprabasal metabolism; linearity between two flows can exist whether coupling is tight or loose.

In this paper we shall consider the relationship between the rate of sodium transport and the rate of oxygen consumption in the short-circuited frog skin exposed to identical solutions at each surface. It will be shown that even in this well-defined state the relationship between sodium transport and suprabasal oxygen consumption varies from skin to skin.

## METHODS

The studies were carried out in modified Ussing-Zerahn chambers (Ussing and Zerahn, 1951), permitting the simultaneous measurement of electrical

Fig. 1

current, transmembrane potential, and oxygen consumption (Fig. 1). Abdominal skins of Rana pipiens were mounted in a lucite chamber, exposing an area of  $7.1 \text{ cm}^2$ , and equilibrated for at least one hour before study. Each half-chamber was connected to a micro-centrifugal pump driven by a magnetic stirrer; the total volume on each side was 10.5 ml. Sodium Ringer agar "sensing" bridges were used for monitoring the electrical potential with saturated calomel half-cells, and an additional pair of bridges permitted the passage of current through silver-silver chloride electrodes. A voltage clamp was used to set the potential difference across the skin (a compensating circuit corrected automatically for the potential drop between the tips of the sensing bridges and the membrane). Electrical current was recorded continuously.

The partial pressure of oxygen in each compartment was continuously monitored by means of Clark oxygen electrodes with 0.001" Teflon membranes (Yellow Springs Instrument Company, Yellow Springs, Ohio), one of which was fitted to each micropump. Circulation at a rate of 2.3 ml/sec produced vigorous stirring at the surface of each electrode, providing stable  $pO_2$  readings. Since the response of the oxygen electrodes is very sensitive to temperature the system was provided with water jackets supplied from a constant temperature ( $25^\circ\text{C}$ ) bath. Each oxygen electrode was connected to a polarographic circuit whose output voltage was directly proportional to  $pO_2$ . When we were interested in the oxygen consumption from each solution the output voltages were recorded independently. Otherwise only the sum of the

two voltages in series was recorded, thereby reducing the error in evaluating the slopes. During the experimental periods clamping the inlet tubing isolated the solutions from air. Care was taken to avoid bubbles since these would lead to underestimation of oxygen consumption. At the beginning of each experiment the sensitivity of the circuit was adjusted so that, at the maximal pump rate, Ringer's solution equilibrated with air gave an output of 30 mV. The rate of oxygen consumption  $J_r$  ( $\mu\text{M sec}^{-1}\text{cm}^{-2}$ ) was calculated from the relation:

$$J_r = \frac{\alpha V}{E_{\text{air}} A} (P - P_{\text{H}_2\text{O}}) \frac{0.2095 \times 10^3}{760 \times 22.4} S,$$

where  $\alpha$ , the solubility coefficient of oxygen at 25°C, is 0.0271 ml O<sub>2</sub> per ml Ringer solution (expressed at S.T.P.)\*, P is the barometric pressure (mm Hg), P<sub>H<sub>2</sub>O</sub> is the water vapor pressure (mm Hg), V is the volume of a half-chamber (ml), E<sub>air</sub> is the output voltage of the polarographic circuit when each oxygen electrode is equilibrated with aerated sodium-Ringer's solution (mV), A is the membrane area (cm<sup>2</sup>), and S is the slope of the recorded plot of output voltage against time (mV sec<sup>-1</sup>), measured visually. The volume fraction of oxygen in air has been taken as 0.2095.

The response of the electrodes in the above system was tested in Ringer's solution equilibrated with air, nitrogen, and nitrogen-oxygen mixtures of 10.33 and 15.47% oxygen. As shown in Fig. 2, the response was linear for pO<sub>2</sub> values

Fig. 2

ranging from zero to that of air. This was true for each electrode, justifying the summation of the two voltages. Preliminary studies were also made of the importance of leakage and sorption of oxygen. For this purpose the system was the same as during experiments, but with a parafilm membrane in place of

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\* Computed from tables in Linke (1965).

the skin. After equilibration with air the chambers were rapidly filled with Ringer's solution equilibrated with a gas mixture of 15.47% oxygen in nitrogen. The system was then closed promptly, and the  $pO_2$  recorded for half an hour; it was found to be constant within experimental error. This shows that the solutions were adequately isolated from atmospheric air, and that desorption of oxygen from the walls of the lucite chambers could be neglected. In the following experiments the  $pO_2$  was never allowed to fall below 85% of that of air. Periodically the depleted fluids were replaced by aerated solutions, or supplemented with small volumes of oxygenated solutions sufficient to bring the  $pO_2$  to that of air. Maximal  $O_2$  consumption by each electrode in air was  $4.5 \times 10^{-2} \mu\mu \text{ mole sec}^{-1}$  (as calculated from a current of  $1.75 \times 10^{-8} \text{ A}$ ). This was negligible as compared with the rates of  $O_2$  consumption of the skins.

The standard glucose-Ringer's solution consisted of 110.0 mM NaCl, 2.5 mM  $KHCO_3$ , 1.0 mM  $CaCl_2$ , and 10.0 mM glucose, freshly prepared from concentrated stock solutions before use. The pH was 8.2 and the osmolarity  $220 \text{ mOsm l}^{-1}$ . Streptomycin sulfate (Pfizer Laboratories) was added at a concentration of  $0.1 \text{ mg ml}^{-1}$  to prevent bacterial growth. Other drugs and reagents used were antidiuretic hormone (Pitressin, Parke Davis Co.), ouabain (Sigma Chemical Co.), 2, 4-dinitrophenol (Eastman Organic Chemicals), and oxygen-nitrogen mixtures (Medical-Technical Gases, Inc., Medford, Massachusetts). Frogs (Rana pipiens) purchased from the Lemberger Company, Oshkosh, Wisconsin were kept in a moist environment at room temperature, without food, for a period of no longer than 2 weeks.

Results are presented as the mean value  $\pm$  the standard error (S.E.) if not otherwise indicated. Straight lines were fitted by the method of least squares.

## RESULTS

### 1. Oxygen uptake from the outer and inner surfaces.

In these experiments the oxygen tension of each solution was monitored continuously for 20-minute periods. The rate of oxygen consumption from the inner solution was always less than that from the outer solution; for short-circuited skins the ratio had a mean value of  $0.57 \pm 0.09$  S.D.,  $n = 77$ ; 15 skins). Typical results for four skins are shown in Table I.

Table I

The ratio was independent of total oxygen consumption, electrical potential difference across the skin, or the presence of glucose or 2, 4-DNP. As a result of different rates of oxygen consumption from the two baths an oxygen gradient developed across the membrane. Despite the progressive increase of this gradient the rates of oxygen uptake from the two baths were constant if the short-circuit current was constant. This indicates that under the conditions of these experiments the rate of diffusion of oxygen across the skin was insignificant.

### 2. Oxygen consumption and short-circuit current.

In these experiments total oxygen consumption and short-circuit current were measured simultaneously in skins exposed at each surface to identical glucose-Ringer's solutions. As is well known when the frog skin is exposed to standard sodium-Ringer's solutions the short-circuit current is equivalent to the rate of net sodium transport (Ussing and Zerahn, 1951; Leaf and Renshaw, 1957a). We have assumed that the addition of 10 mM glucose to the solutions does not alter this relationship.

In most skins, despite an equilibration period of 1 to 1.5 hours both the short-circuit current  $I_0$  and the associated rate of oxygen consumption  $J_{RO}$  declined with time, although often with superimposed fluctuations. A

representative experiment is shown in Fig. 3 (such behavior was seen both in the presence and absence of glucose). Despite the decline and fluctuation of both  $I_o$  and  $J_{ro}$ , on relating the two values for each period a linear

Fig. 3

relationship was observed. Table II gives the results for ten skins, untreated with drugs, in which there were sufficient spontaneous changes of  $I_o$  and  $J_{ro}$  to permit the demonstration of their relationship.

Table II

The existence of a linear relationship between  $I_o$  and  $J_{ro}$  permits the evaluation by extrapolation of the rate of oxygen consumption which would have obtained in the absence of short-circuit current,  $(J_{ro})_{I_o=0}$ . This quantity, which we consider to represent the basal rate of oxygen consumption, was about half the total rate of oxygen consumption in a representative freshly mounted skin. (The validity of the use of  $(J_{ro})_{I_o=0}$  as a measure of oxygen consumption unrelated to transepithelial sodium transport will be discussed below.)

A subject which has received a great deal of attention is the quantitative relationship between suprabasal oxygen consumption and active sodium transport (Ussing, 1960). Several workers have evaluated a mean ratio, based on a single experimental determination for each skin. In contrast, Table II is based on numerous experimental points for each skin; hence statistical analysis of the slopes permits the evaluation of an appropriate ratio for each. The mean slope  $dJ_{ro}/dI_o$  was  $0.854 \pm 0.344 \mu \text{ mole coul}^{-1}$ , corresponding to an average value of 14.6 sodium ions transported per molecule of oxygen consumed. However, the various skins show significantly different values.

3. Effects of antidiuretic hormone, ouabain, potassium cyanide, and 2, 4-dinitrophenol on the relationship between short-circuit current and oxygen consumption.

Since there was a linear relationship between  $I_o$  and  $J_{rO}$  as these parameters varied spontaneously, it was of interest to examine the relationship between them when they were altered by specific agents. The addition of antidiuretic hormone (ADH) to the solution bathing the inner surface of the frog skin has been reported to result in an increase of both short-circuit current and oxygen consumption (Ussing and Zerahn, 1951; Zerahn, 1956; Leaf and Renshaw, 1957a). Ouabain may be expected to cause a decrease of these parameters (Koefoed-Johnsen, 1957; Levy and Richards, 1965). Both effects can be seen in Fig. 4 which refers to the skin in which ADH produced the greatest effect. Again the relationship between  $I_o$  and  $J_{rO}$  appears to be

Fig. 4 (a and b)

linear. (The two points below the line following ouabain were obtained when the short-circuit current was still changing rapidly (see Fig. 6a). Such observations were made in only two of ten skins, and are therefore omitted from statistical calculations.)

Table III

The results of a least squares analysis of the ten skins is shown in Table III, representing combined data obtained prior to and after treatment with ADH. Similar results were found on combining these data with those obtained after the addition of ouabain; the results are shown in Table IV. An

Table IV

examination of Tables II - IV suggests that all data for a given skin lie on a single straight line. A stringent statistical analysis supported this view (see Appendix).

Figure 5 shows the apparent stoichiometric ratios for the ten skins.

Fig. 5

Again we see that although there appears to be a characteristic ratio for each, independent of the presence of ADH or ouabain, this ratio differs from skin to skin. This is most evident in considering the pooled data.

The addition of KCN not only reduced active sodium transport and the associated oxygen consumption to very low levels, but also depressed basal oxygen consumption. The effect of KCN on basal oxygen consumption is shown in Fig. 4. The addition of 2, 4-DNP caused a prompt decrease in  $I_0$  and a transient increase in  $J_{rO}$  (Fig. 6). Following blockage of active sodium transport by ouabain, only an increase in  $J_{rO}$  was observed.

Fig. 6

#### DISCUSSION

It is of interest that the rate of consumption of oxygen from the outer solution is appreciably greater than that from the inner solution, the ratio being essentially constant (Table I). This was the case in all the conditions studied. This is not in agreement with Zerahn's findings. The reason for the difference in uptake at the two surfaces is uncertain.\* However, it seems probable that the mitochondria-rich epithelial cells will consume oxygen more rapidly than the poorly cellular underlying connective tissue. Also, as suggested by Martin and Diamond (1966) for the rabbit gall bladder, the thick layer of connective tissue may constitute a more effective permeability barrier to  $O_2$  diffusion than the thinner epithelial cell layer. Similar results have recently been reported by Nellans and Finn (1970) in the toad bladder.

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\* In an attempt to minimize the contribution of bacterial contamination, all solutions were freshly prepared and contained streptomycin. However, Sharp et al. (personal communication) have recently noted continuing bacterial

As mentioned above, a progressively increasing gradient for oxygen develops, but constancy of  $J_R^O$  and  $J_R^i$  in those skins with constant  $I_o$  indicates that the rate of diffusion of oxygen across the skin is insignificant compared with the rate of oxygen consumption. In order to test this further we calculated the rate of diffusion of oxygen across an unstirred layer of water of the approximate thickness of the frog skin,  $300 \mu$ , induced by a mean difference of  $pO_2$  of 4.6 mm Hg, the largest value permitted here. The result was  $5.6 \mu\mu\text{mole sec}^{-1} \text{cm}^{-2}$ , as compared with a basal oxygen consumption of  $58.3 \mu\mu\text{mole sec}^{-1} \text{cm}^{-2}$ . Since the mean diffusion coefficient in the frog skin is presumably less than in water, and since we have ignored the effects of unstirred layers, it appears that the diffusion of oxygen across the skin is insignificant in these experiments.

Zerahn raised the question as to whether the oxygen required for transport is derived from the outside or inside solution. The observation of a constant ratio of uptake from each solution even after the abolition of sodium transport

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nucleotide turnover in toad bladders for as long as four hours after administration of streptomycin. We do not feel that the possibility of bacterial metabolism invalidates our main conclusions, for the following reasons: (i) Bacterial contamination, which should be confined predominantly to the outer layers, would be expected to vary with time. In fact no change in the ratio  $J_R^i/J_R^O$  was observed (Table I) (ii) Perturbation of  $\Delta\psi$  would be expected to change oxygen consumption related to sodium transport, but not that due to bacterial metabolism. No change in  $J_R^i/J_R^O$  was noted with large changes in  $\Delta\psi$  (Table I). (iii) If bacterial oxygen consumption were significant we would have expected to observe changes in the ratio  $dNa/dO_2$  both with the passage of time (Fig. 3) and with alteration of the rate of sodium transport by specific agents (Fig. 5). No such changes were noted.

by ouabain, and the insignificance of oxygen transport across the skin, leads us to believe that the oxygen is derived from both surfaces. In any event, it cannot be assumed that the pattern observed in vitro applied also in vivo, since in life the skin is vigorously perfused.

It is remarkable that in each skin there is a unique value of  $dNa/dO_2$  which is independent of treatment with either ADH or ouabain (Fig. 5; Tables II-IV). This is of particular interest in view of the common belief that ADH acts at an outer permeability barrier whereas ouabain acts at the pump. In terms of a formalism presented previously (Essig and Caplan, 1968) the observed results are consistent either with a change in the free energy of the metabolic driving reaction, or effects on the phenomenological coefficients of a highly coupled system.

The above findings suggest also that there are two components of oxygen consumption. One component is that associated with the process of net sodium transport, linearly related to the short-circuit current. Extrapolating to the state  $I_o = 0$  then gives us the other component, oxygen consumption apparently associated with all other cellular functions. We shall call this "basal" oxygen consumption; we do not mean to imply, however, that this oxygen consumption is necessarily unrelated to any cellular sodium transport, but only that it is unrelated to net transepithelial sodium transport. The demonstration of two functional components of oxygen consumption is reminiscent of the observations of Kidder, Curran, and Rehm (1966) in their studies of hydrogen ion secretion in bullfrog gastric mucosa. These workers interpreted relationships between oxidation-reduction states of cytochromes and active hydrogen ion secretion in terms of two parallel electron transport chains, one mitochondrial and the other extramitochondrial, the latter specifically

involved in the transfer of hydrogen ions. Similarly, Siekevitz (1965) has adduced biochemical evidence for two discrete electron transport systems, one in mitochondria and the other in the endoplasmic reticulum.

It is of interest that there appears to be a basal oxygen consumption which remains essentially constant over an extended period. The alternative hypothesis is that both basal and non-basal oxygen consumption vary linearly with short-circuit current, whether the variation in  $I_o$  is a consequence only of the passage of time or also includes the effects of ADH or ouabain. This possibility seems to us a priori much less likely than a fairly constant rate of basal consumption\*. It is noteworthy that the basal rate calculated by extrapolation of  $J_{ro}$  to  $I_o=0$  is equal to the rate of oxygen consumption observed in the presence of ouabain. This also supports the idea of near-constancy of the basal rate of oxygen consumption.

It may seem surprising that sodium transport and the associated oxygen consumption frequently decline with time while basal metabolism is well maintained. It might have been expected that they would decline in parallel owing to depletion of tissue substrates. Various possibilities could explain this behavior. For example, sodium transport may well depend on metabolic intermediates, co-factors, or hormonal agents, etc., readily depleted in the in vitro preparation, which are not important for basal metabolism. This would be consistent with the fact that the electron transport system of

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\* We assume that the observed linearity is not due to the combination of two non-linear rates of oxygen consumption.

Previous data concerning the possibility of a small effect of ADH on basal oxygen consumption are conflicting (Leaf and Dempsey, 1960; Rasmussen et al., 1960; Swenson and Maffly, 1968); species differences may have contributed to this.

endoplasmic reticulum contains components lacking in mitochondria (Siekovitz, 1965).

Two metabolic inhibitors altered the regular pattern of behavior described above. The addition of 2, 4-DNP uncouples sodium transport and oxygen consumption, as shown in Fig. 6 (see also Fuhrman, 1952, and Huf et al., 1957). The increase in  $J_{\text{rO}}$  presumably reflects the characteristic effect of 2, 4-DNP on mitochondrial oxidative phosphorylation. The addition of KCN abolishes not only active sodium transport and the associated oxygen consumption, but also basal oxygen consumption, presumably by the inhibition of cytochrome oxidase (Slater, 1966).

It is pertinent to ask to what extent  $I_0$  may be supported by glycolytic metabolism. Leaf and Renshaw (1957b) noted active ion transport to occur anaerobically in frog skin at 20 to 40 per cent of the aerobic rate. However, in their study glycolytic sodium transport in the presence of oxygen may have been small since lactate production increased markedly under anaerobic conditions. The linearity noted between sodium transport and oxygen consumption in the present study indicates that if glycolytic sodium transport is appreciable it must be constant.\* Therefore, if glycolytic sodium transport were in fact appreciable the basal rate of oxygen consumption calculated by extrapolation to  $I_0 = 0$  would be erroneously low and hence smaller than the value derived from the administration of ouabain; this was not the case. For these reasons we feel that glycolytic sodium transport was small in the

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\*We have assumed that glycolytic sodium transport is not highly correlated to aerobic sodium transport: Leaf and Renshaw (1957b) found anaerobic sodium transport to be unaffected by ADH.

present studies.

Many people have considered the relationship between the rate of sodium transport and the rate of oxygen consumption in various tissues (Zerahn, 1956, 1958; Leaf and Renshaw, 1957a; Thurau, 1961; Kramer and Deetjen, 1964; Martin and Diamond, 1966). The earliest comprehensive studies involving simultaneous measurements in frog skin were carried out by Zerahn, who used the chemical method of Krogh to evaluate average rates of oxygen consumption. His experiments usually consisted in the determination of the average rate of oxygen consumption in three consecutive periods of unspecified length, two control periods bracketing an experimental period. In the experimental period the average rate of sodium transport was also evaluated. In the control periods the transepithelial active sodium transport was assumed to be eliminated by replacing the outer sodium-Ringer's solution with distilled water or magnesium-Ringer's solution. The difference between the rates of oxygen consumption in the experimental and control periods ("net oxygen consumption") was then expressed as a percentage of the rate of net sodium transport. In this way Zerahn calculated a mean value for the net percentage ("N.P.") of 22.5, i.e. 22.5 equivalents of oxygen consumed per 100 equivalents of sodium transported, and stated that "the N.P. was the same under all conditions". The factors which were varied included outer concentration of Na, electrical potential difference,  $pO_2$ , time of year, species of animal, temperature, presence or absence of posterior pituitary hormone, and methods for producing the blank. Zerahn's value corresponds to 17.8 equivalents of sodium transported per mole of oxygen consumed, as compared with the mean value of 14.6 for the untreated skins of the present study. We do not feel that there is any discrepancy between these values, since in each case the numbers are simply

means of a large number of values which vary over a considerable range. For example, using short-circuited skins of Rana temporaria with identical sodium-Ringer solutions at each surface, Zerahn found values of N.P. ranging from 14.3 to 27.8 (corresponding to  $dNa/dO_2$  values of 14.4 to 28.0 eq/mole); for Rana esculenta the values ranged from 14.9 to 34.0 (i.e.  $dNa/dO_2$  values of 11.8 to 26.9 eq/mole). In these studies the blank averaged two-thirds of the total rate of oxygen consumption and Zerahn estimated that the standard deviation of the net oxygen consumption ranged from 3 to 20 per cent. In view of the uncertainties in the chemical technique employed, and the fact that the value for each skin was derived from only a single experimental period, it was of course reasonable to emphasize only the mean value for N.P., rather than its variability.

Leaf and Renshaw (1957a), utilizing a precise polarographic technique, studied the relationship between sodium transport and oxygen consumption in the frog skin in both the absence and presence of antidiuretic hormone. Relating increments in sodium transport and oxygen consumption induced by the administration of hormone, they calculated a mean value for  $\Delta Na/\Delta O_2$  of 18.2. Again, however, the values were derived from a single experimental period, and again they varied considerably in individual skins. Excluding one grossly aberrant figure, the values ranged from 5.8 to 43.1. The present results, based on several experimental periods in each skin, show values of  $dNa/dO_2$  ranging from 7.1 to 30.9 (Fig. 5).

We feel that our results demonstrate clearly that for short-circuited skins with glucose-Ringer's solution on both sides there is no unique value for  $dNa/dO_2$ ; rather the value may differ significantly from one skin to another and possibly from one season to another. Although the reason for this

variation is unknown, certain possibilities come to mind. One possibility is recirculation of actively transported sodium (Ussing, 1966). If the magnitude of such recirculation were to differ from one skin to another, the apparent stoichiometric ratio would vary. Another possibility is variation of skin thickness. Such variation might affect sodium transport and oxygen consumption differently. Still a third possibility is variation in the state of metabolism. More generally, there are several possible sources of non-stoichiometry of the process of oxidative phosphorylation which presumably supports active transport. These have been discussed elsewhere (Rottenberg, et al., 1967).

We thank Dr. J. Gilbert and Mrs. J. Lustgarten of the Harvard Computing Center for the statistical analysis of the data.

## Legends of Figures

Figure 1. Apparatus for a simultaneous measurement of electrical parameters and oxygen consumption.

OE Clark oxygen electrodes.

PC Polarographic circuit.

R Recorder

Figure 2. Calibration of Clark oxygen electrodes.

Figure 3. Variation of short-circuit current  $I_o$  and the associated rate of oxygen consumption  $J_{rO}$  with time. The inset shows the relationship between  $I_o$  and  $J_{rO}$  of

Figure 4. Effect/antidiuretic hormone (0.25 units/ml), ouabain (1mM), and potassium cyanide (1mM) on short-circuit current  $I_o$  and the associated rate of oxygen consumption  $J_{rO}$ . All agents were added to the inner solution.

(a) Plots of  $I_o$  and  $J_{rO}$  against time.

(b) Plot of  $J_{rO}$  against  $I_o$ .

These data are from a single experiment.

Figure 5. Apparent stoichiometric ratios of short-circuited skins, calculated from  $1/F(dJ_{rO}/dI_o)$ . The bars represent 95% confidence limits.

Figure 6. Effect of ouabain (1 mM) and 2, 4-dinitrophenol (0.5 mM) on the relation between short-circuit current  $I_o$  and the associated rate of oxygen consumption  $J_{rO}$ .

## Legends to Tables

Table I. Typical rates of oxygen consumption in four skins,  $J_R^i$  and  $J_R^o$  represent uptake from the inner and outer solutions respectively.  $\Delta\psi$  is the electrical potential in the inner solution minus that in the outer solution. The duration of each period was 20 minutes; at the end of each period the solutions on each side were replaced with fresh aerated Ringer's solution. \*2, 4-DNP was added to the inner solution to give a concentration of 0.5 mM.

Table II. Analysis of the rate of oxygen consumption at short-circuit  $J_{ro}$  as a function of the short-circuit current  $I_o$  during their spontaneous decline with time (no drugs added). The data were fitted to a straight line by least squares analysis.  $(J_{ro})_{I_o=0}$  is the extrapolated rate of oxygen consumption at zero short-circuit current,  $\pm$  S.E.;  $dJ_{ro}/dI_o$  is the slope  $\pm$  S.E.;  $n$  is the number of observations; and  $r$  is the correlation coefficient. For  $n \geq 7$  a value of  $r \geq .798$  is significant at the .01 level.

Table III. Analysis of the rate of oxygen consumption at short-circuit  $J_{ro}$  as a function of the short-circuit current  $I_o$  during their spontaneous decline with time and after the administration of ADH (combined data). Symbols as in Table II. For  $n \geq 11$  a value of  $r \geq .684$  is significant at the .01 level.

Table IV. Analysis of the rate of oxygen consumption at short-circuit  $J_{ro}$  as a function of the short-circuit current  $I_o$  during their spontaneous decline with time and after the administration of ADH and ouabain (combined data). Symbols as in Table II. For  $n \geq 14$  a value of  $r \geq .623$  is significant at the .01 level.

(a)

Appendix - Statistical Analysis

Since the measured short-circuit current reflects sodium transport with high precision, we attribute all error to the measurements of oxygen consumption.

Two hypotheses were tested:

(a) The data obtained before and after treatment with ADH lie on the same straight line.

This hypothesis was tested in two ways.

(i) For each skin a curve was constructed for the untreated and ADH points considered separately. A t-test on their slopes and intercepts

gave the following results (DF indicates degrees of freedom; P, the significance level, is the probability of  $|t| \geq$  the observed value):

<u>Skin no.</u>	<u>t</u>	<u>Slope</u>	<u>DF</u>	<u>P</u>	<u>t</u>	<u>Intercept</u>	<u>DF</u>	<u>P</u>
1	.44		10	.68	-1.04		11	.32
2	.38		8	.72	1.54		9	.16
3	-.37		23	.74	.41		24	.68
4	.09		12	.94	.56		13	.60
5	-.07		7	.96	.02		8	.99
6	-.54		8	.60	.51		9	.62
7	-.41		14	.68	-.41		15	.70
8	.13		8	.92	.36		9	.74
9	.05		7	.96	.75		8	.48
10	-.03		8	.99	1.33		9	.22

(ii) Taking all skins together the significance of the difference of the mean slopes was tested:

$$\text{slope}_{\text{untreated}} - \text{slope}_{\text{ADH}} = -.01 \pm .09 \text{ (S.E.)}$$

(b)

Testing both the slopes and the angles of inclination gave the following results:

	t	DF
slopes	-.111	9
angles (rad.)	-.112	9

The above results show no significant difference between the untreated and ADH data.

(b) The data obtained after treatment with ouabain lie on the straight line obtained from the untreated + ADH data.

For each skin the average distance of the ouabain points from the untreated + ADH line was computed. The mean distance for all skins was insignificantly different from zero ( $t = -.151$ ;  $DF = 9$ ,  $P = .88$ ).

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Table I

$\Delta\psi$ mV	Oxygen Uptake $\mu\text{M sec}^{-1}\text{cm}^{-2}$			$J_R^i/J_R^o$
	$J_R^i$	$J_R^o$	$J_R$	
0	27.0	51.2	78.2	0.53
0	30.1	50.0	80.1	0.60
0	27.0	44.6	71.6	0.61
0	25.4	43.0	68.4	0.59
0	23.5	41.4	64.9	0.56
0	24.2	43.0	67.2	0.56
0	22.7	38.3	61.0	0.59
0	24.2	38.3	62.5	0.63
0	19.9	41.8	61.7	0.48
0	19.2	41.1	60.3	0.46
0	19.2	39.1	58.3	0.49
0	16.8	37.1	53.9	0.46
-100	22.7	48.1	70.8	0.47
-100	19.9	40.7	60.6	0.49
-100	18.4	38.3	56.7	0.48
0	14.1	27.4	41.5	0.51
0	13.7	28.2	41.9	0.49
0	15.2	29.3	44.5	0.52
0	15.6	31.3	46.9	0.50
0	13.7	28.5	42.2	0.48
-100	17.6	36.8	54.4	0.48
-100	16.8	34.4	51.2	0.49
-100	16.8	37.1	53.9	0.45
0	13.7	25.4	39.1	0.54
0	37.5	62.9	100.4	0.60
0	33.2	62.9	96.1	0.53
+100	24.2	35.6	59.8	0.68
+100	25.0	37.1	62.1	0.67
+100	23.5	35.2	58.7	0.67
0	37.5	56.7	94.2	0.66
0*	89.9	153.3	243.2	0.59
0*	82.5	133.3	215.8	0.62

Table II

Skin	$(J_{ro})_{I_0=0}$ $\mu\mu\text{M sec}^{-1}\text{cm}^{-2}$	$dJ_{ro}/dI_0$ $\mu\text{M coul}^{-1}$	n	r
1	$39.6 \pm 5.3$	$.774 \pm .132$	8	.923
2	$23.1 \pm 9.2$	$1.082 \pm .218$	7	.912
3	$66.0 \pm 2.1$	$.955 \pm .132$	21	.857
4	$39.9 \pm 9.5$	$.827 \pm .160$	12	.853
5	$51.2 \pm 3.4$	$.488 \pm .139$	7	.843
6	$58.6 \pm 4.1$	$.360 \pm .083$	7	.889
7	$56.2 \pm 4.0$	$.456 \pm .080$	13	.864
8	$53.6 \pm 8.1$	$1.031 \pm .295$	7	.842
9	$37.1 \pm 11.4$	$1.114 \pm .178$	7	.942
10	$56.3 \pm 7.2$	$1.452 \pm .212$	8	.942
Mean	$48.2 \pm 12.9$	$.854 \pm .344$		

Table III

Skin	$(J_{ro})_{I_0=0}$ $\mu\mu\text{M sec}^{-1} \text{cm}^{-2}$	$dJ_{ro}/dI_0$ $\mu\text{M coul}^{-1}$	n	r
1	$39.7 \pm 3.9$	$.785 \pm .088$	14	.933
2	$34.4 \pm 4.8$	$.800 \pm .098$	12	.933
3	$66.1 \pm 1.7$	$.940 \pm .091$	27	.900
4	$43.4 \pm 5.8$	$.764 \pm .091$	16	.913
5	$51.1 \pm 2.2$	$.490 \pm .068$	11	.923
6	$58.7 \pm 3.8$	$.395 \pm .060$	12	.800
7	$55.4 \pm 2.9$	$.478 \pm .048$	18	.928
8	$54.7 \pm 5.4$	$.973 \pm .171$	12	.874
9	$41.1 \pm 6.9$	$1.042 \pm .099$	11	.962
10	$60.8 \pm 4.9$	$1.283 \pm .117$	12	.961
Mean	$50.5 \pm 10.4$	$.795 \pm .280$		

Table IV

Skin	$(J_{ro})_{I_0=0}$ $\mu\text{M sec}^{-1} \text{cm}^{-2}$	$dJ_{ro}/dI_0$ $\mu\text{M coul}^{-1}$	n	r
1	40.2 $\pm$ 1.0	.774 $\pm$ .026	19	.991
2	43.4 $\pm$ 1.6	.618 $\pm$ .037	16	.976
3	66.6 $\pm$ 1.0	.919 $\pm$ .060	33	.940
4	49.5 $\pm$ 1.5	.670 $\pm$ .027	20	.986
5	50.6 $\pm$ 1.2	.504 $\pm$ .041	14	.962
6	59.7 $\pm$ 1.7	.336 $\pm$ .032	16	.943
7	52.1 $\pm$ 1.7	.528 $\pm$ .031	23	.966
8	58.9 $\pm$ 2.3	.843 $\pm$ .083	16	.938
9	32.7 $\pm$ 1.7	1.161 $\pm$ .028	15	.996
10	60.8 $\pm$ 2.4	1.284 $\pm$ .065	16	.983
Mean	51.4 $\pm$ 10.5	.764 $\pm$ .297		

Fig. 1

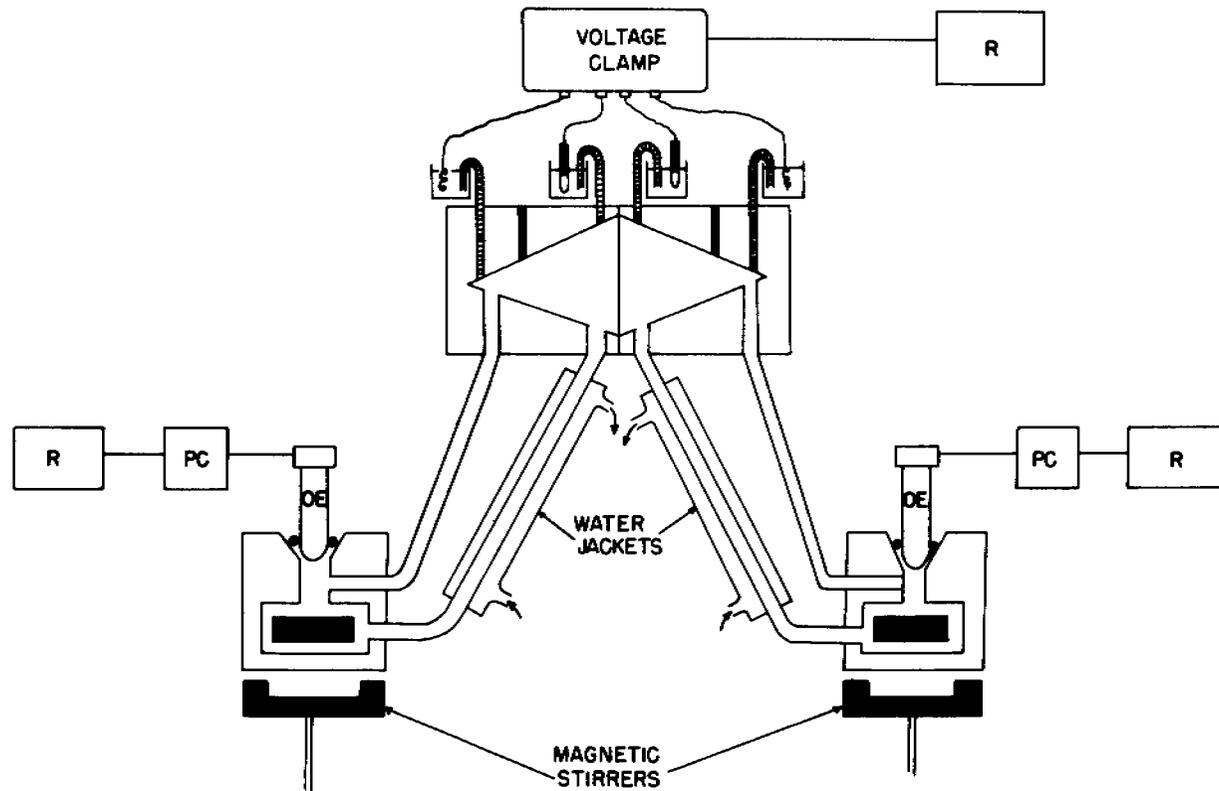


Fig. 2

CALIBRATION OF CLARK OXYGEN ELECTRODE

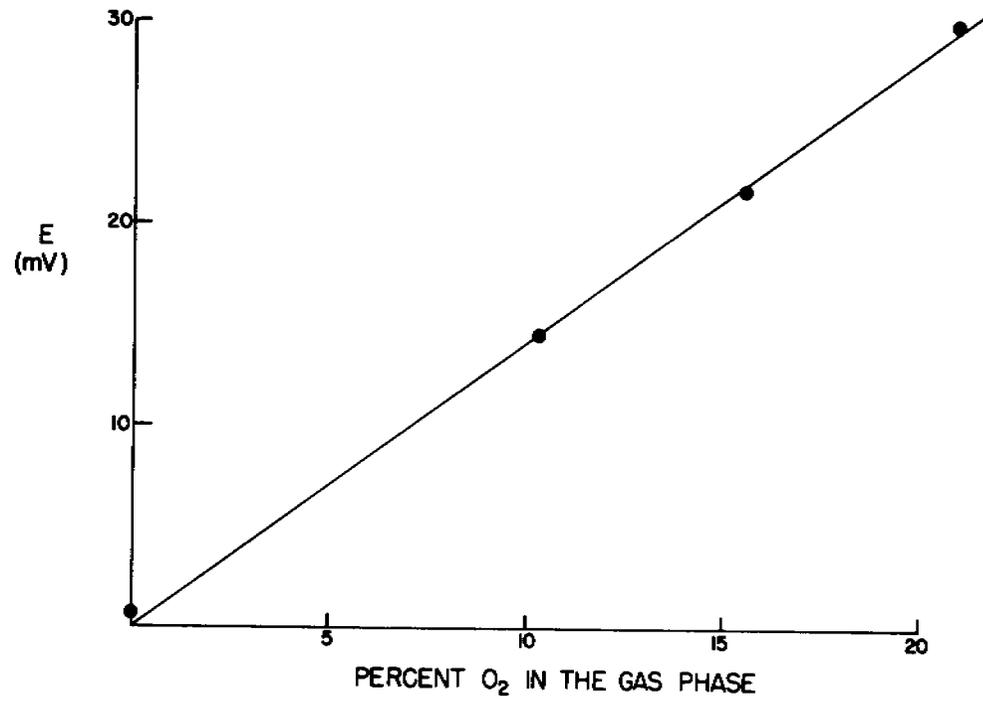


Fig. 3

VARIATION OF RATE OF OXYGEN CONSUMPTION  $J_{rO}$  AND SHORT-CIRCUIT CURRENT  $I_o$

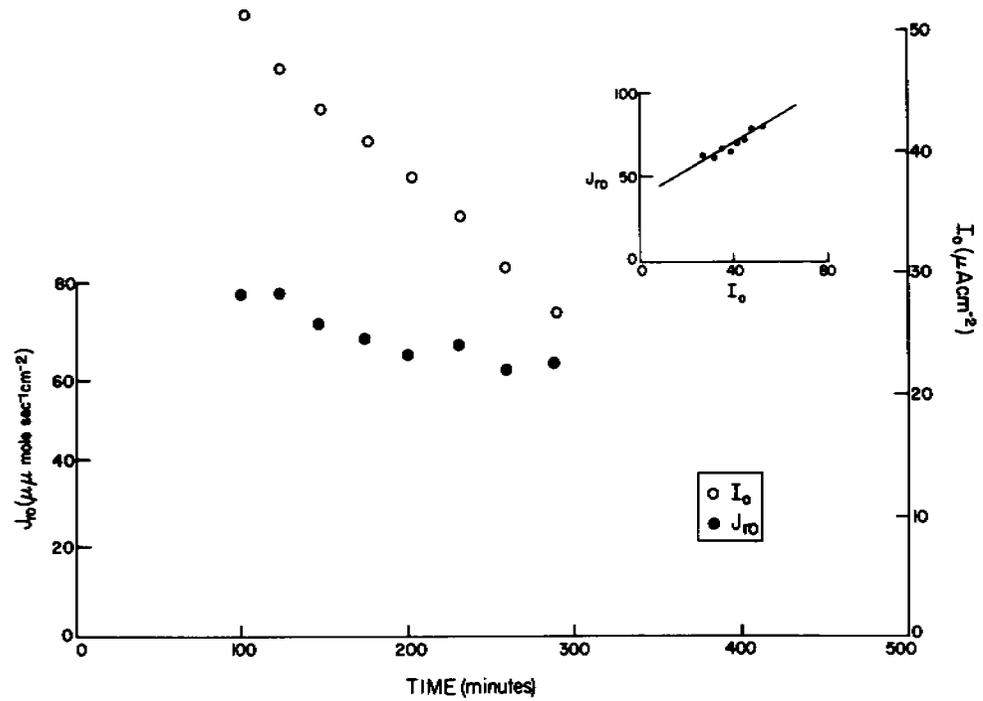


Fig. 4a

EFFECT OF ANTIDIURETIC HORMONE, OUABAIN, AND POTASSIUM CYANIDE ON RATE OF OXYGEN CONSUMPTION  $J_{rO}$  AND SHORT CIRCUIT-CURRENT  $I_o$ .

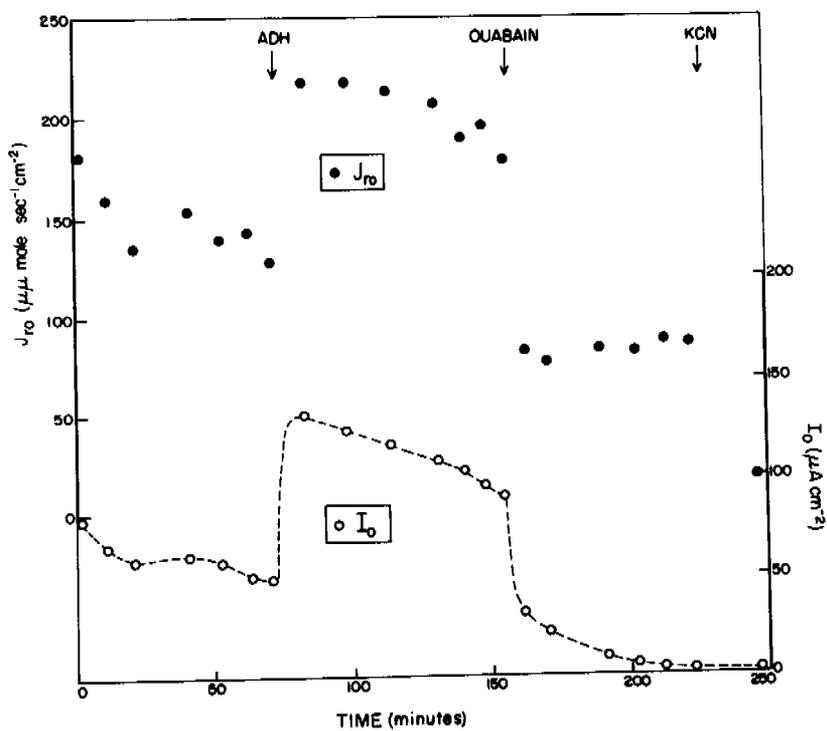


Fig. 4b

EFFECT OF ANTIDIURETIC HORMONE, OUABAIN, AND POTASSIUM CYANIDE ON THE RELATIONSHIP BETWEEN RATE OF OXYGEN CONSUMPTION  $J_{rO}$  AND SHORT-CIRCUIT CURRENT  $I_0$

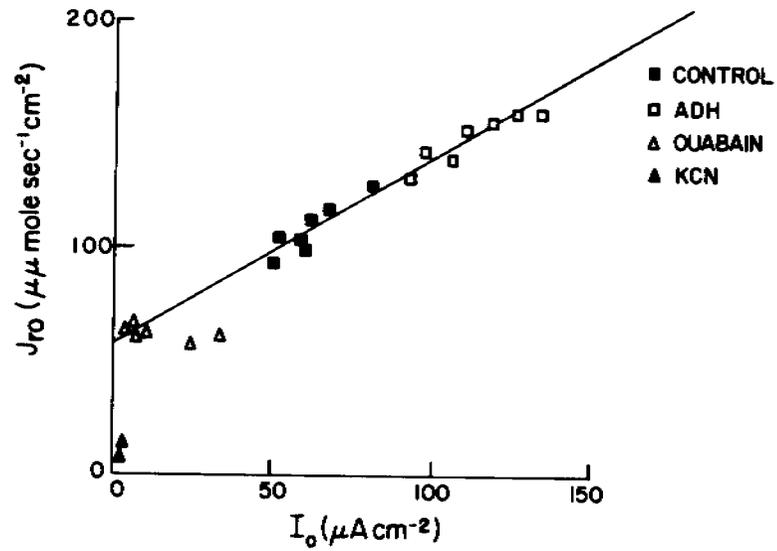


Fig. 5

*APPARENT STOICHIOMETRIC RATIOS OF FROG SKINS*

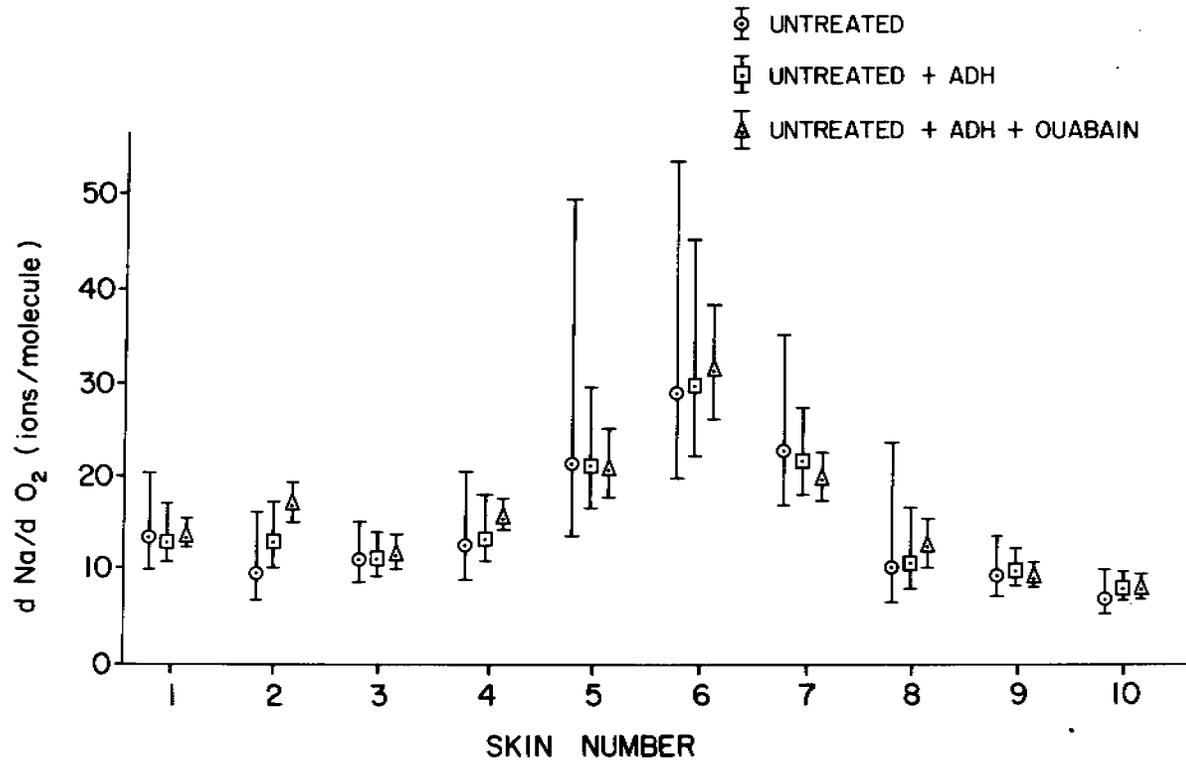
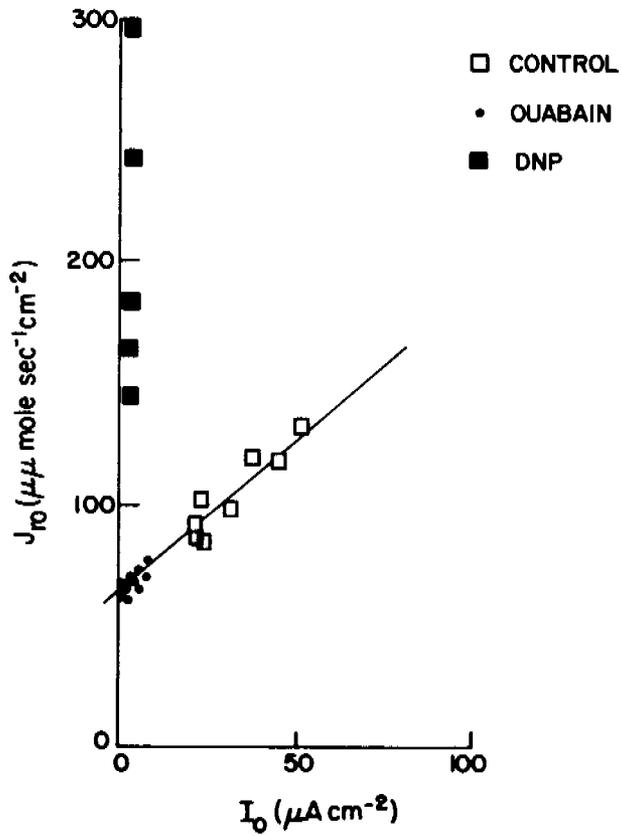


Fig. 6

EFFECT OF OUABAIN AND 2,4-DNP  
ON THE RELATIONSHIP BETWEEN RATE  
OF OXYGEN CONSUMPTION  $J_{rO}$  AND SHORT-  
CIRCUIT CURRENT  $I_0$



## II. ENERGETICS OF SODIUM TRANSPORT IN FROG SKIN

### (2. The effects of electrical potential on oxygen consumption)

(Work done with F.L. Vieira and S.R. Caplan)

Studies were made of the dependence of the rate of oxygen consumption  $J_r$  on the electrical potential difference  $\Delta\psi$  across the frog skin. Following the abolition of sodium transport by ouabain the basal oxygen consumption was independent of  $\Delta\psi$ . In fresh skins  $J_r$  was a linear function of  $\Delta\psi$  over a range of at least  $\pm 70$  mV. Treatment with aldosterone stimulated the short circuit current  $I_o$  and the associated rate of oxygen consumption  $J_{rO}$  and increased their stability; linearity was then demonstrable over a range of  $\pm 160$  mV. Brief perturbations of  $\Delta\psi$  ( $\pm 30$  to 200 mV) did not alter subsequent values of  $I_o$ . Perturbations for 10 or more minutes produced a "memory" effect both with and without aldosterone: accelerating sodium transport by negative clamping lowered the subsequent value of  $I_o$ ; positive clamping induced the opposite effect. Changes in  $J_{rO}$  were more readily detectable in the presence of aldosterone; these were in the same direction as the changes in  $I_o$ . The linearity of  $J_r$  in  $\Delta\psi$  indicates the validity of analysis in terms of linear nonequilibrium thermodynamics—brief perturbations of  $\Delta\psi$  appear to produce no significant effect on either the phenomenological coefficients or the free energy of the metabolic driving reaction. Hence it is possible to evaluate this free energy.

## INTRODUCTION

Many people have considered the energetics of ion transport in epithelia (e.g. Ussing and Zerahn, 1951; Zerahn, 1956, 1958; Ussing, 1960; Heinz and Patlak, 1960; Patlak, 1961; Martin and Diamond, 1966; Civan, Kedem, and Leaf, 1966). In general these studies have followed those of Ussing, who treated the system in terms of its electrical analogue. This led him to introduce the concept of "the electromotive force of the active sodium transport",  $E_{Na}$ . Two methods are used for the evaluation of  $E_{Na}$ : (1) determination of the electrochemical potential difference of sodium required to reduce its flux to zero, (2) measurement of the flux ratio at short-circuit. As was stressed by Ussing and others, the  $E_{Na}$  determined experimentally should be regarded only as an effective potential, reflecting the mode of evaluation. In particular, leak must decrease its magnitude. Furthermore, the common use of the flux ratio to evaluate  $E_{Na}$  requires the questionable assumption that the movements of abundant and tracer species of sodium ions in the active transport pathway are independent (Ussing, 1960). In any event equivalence of the values of  $E_{Na}$  obtained by the above two techniques would require that the rate of metabolism be independent of the electrical potential difference across the membrane (Kedem and Essig, 1965; Blumenthal and Kedem, 1969).

In view of the above considerations several authors have attempted a more comprehensive thermodynamic treatment. Since the systems are not at equilibrium, such a treatment must necessarily be based on the use of nonequilibrium thermodynamics (for example, Kedem, 1961; Hoshiko and Lindley, 1967; Essig and Caplan, 1968). In these studies linear relations were assumed between the rates (transport and metabolism) and the forces (electrochemical potential difference of sodium and free energy of the

metabolic driving reaction). Such linearity, if it existed, would greatly simplify thermodynamic analysis and would also be of fundamental theoretical significance (Prigogine, 1961). In the present paper we examine the question of whether linearity does in fact obtain between oxygen consumption and electrical potential difference over a significant range.

## GLOSSARY

A	affinity of metabolic reaction
$E_{Na}$	electromotive force of sodium transport
F	Faraday constant
I	electrical current density
$I_0$	electrical current density in the short-circuited state ( $\Delta\psi = 0$ )
$J_{Na}$	rate of sodium transport per unit area
$J_r$	rate of oxygen consumption at any given value of $\Delta\psi$ per unit area
$J_{r0}$	rate of oxygen consumption in the short-circuited state ( $\Delta\psi = 0$ ) per unit area
$L_{Na}, L_{Na^+}, L_r$	phenomenological conductance coefficients
$X_{Na}$	negative electrochemical potential difference of sodium
$\Delta\psi$	electrical potential difference: potential in the inner solution minus that in the outer solution. (Inner and outer refer to the intact animal.)

## METHODS

Most of the methods used in this study were as described in detail in Part I (Vieira, Caplan, and Essig, in preparation). In summary, sodium transport and oxygen consumption were measured in skins (Rana pipiens) mounted in standard lucite chambers. Sodium transport was measured by the short-circuit technique of Ussing and Zerahn (1951). Oxygen consumption was measured by a polarographic method using two oxygen electrodes. A voltage clamp permitted setting the potential difference across the skin at values ranging from 200 to -200 mV, with a compensating circuit providing automatic correction for the potential drop between the voltage-sensing agar-bridge tips and the membrane. Fresh standard glucose-Ringer's solution (110.0 mM NaCl, 2.5 mM KHCO<sub>3</sub>, 1.0 mM CaCl<sub>2</sub>, and 10.0 mM glucose; pH 8.2 and osmolarity 220 mosm l<sup>-1</sup>) was used. Streptomycin sulfate was added in a concentration of 0.1 mg ml<sup>-1</sup> to prevent bacterial growth. In the standard protocol oxygen consumption was studied 1.5 hours after mounting the skin. In the aldosterone-aged preparations, d-aldosterone-21-acetate was added to the inner solution in 10 μl of methanol to give a final concentration of 10<sup>-6</sup>M. The system was then aerated for 18 hours. At this time the solutions were replaced by fresh glucose-Ringer's solution and aldosterone was added to the inner solution as above. After 1 to 1.5 hours the air bubbler was detached and the oxygen electrode-micropump system was attached as shown in Fig. 1 of Part I. Thereafter, in order to maintain oxygen-tension at near physiological levels, both solutions were replaced at approximately 25 minute intervals by fresh aerated glucose-Ringer's solution containing 10<sup>-8</sup>M aldosterone. Streptomycin sulfate was obtained from Pfizer Laboratories,

ouabain from Sigma Chemical Company, and d-aldosterone-21-acetate was kindly provided by Dr. Maurice Pechet.

Results are presented as the mean value  $\pm$  the standard error (S.E.) if not otherwise indicated. Straight lines were fitted by the method of least squares.

## RESULTS

### 1. Effect of the electrical potential difference on the rate of oxygen consumption.

Two different kinds of preparation were used. (a) Fresh skins were studied after 1 to 1.5 hours of incubation in glucose-Ringer's solution. (b) Aldosterone-aged skins were studied after 18 hours of incubation in glucose-Ringer's solution containing aldosterone.

#### a) Fresh skins.

Studies were made of the dependence of the rate of oxygen consumption  $J_r$  on the electrical potential difference  $\Delta\psi$  imposed across the skin. The magnitude and sequence of the perturbations of  $\Delta\psi$  were varied in different experiments. As shown in Fig. 7 a change in  $\Delta\psi$  resulted promptly in a change in the rate of oxygen consumption, normally within less than 30

Fig. 7

seconds. Usually a new steady state value was reached within less than 2 minutes. Thus, to ensure stationarity  $J_r$  was evaluated 4 to 6 minutes following change of  $\Delta\psi$ .

Typical relationships between  $J_r$  and  $\Delta\psi$  are shown in Fig. 8 where each plot represents data obtained within a single 25 minute interval. (During

Fig. 8

this time the skin was stable, as shown by the fact that repeated

determinations at a given value of  $\Delta\psi$  resulted in closely similar values of  $J_r$ .) Positive perturbations of  $\Delta\psi$ , which decreased the rate of sodium transport, reduced the rate of oxygen consumption and vice-versa. Characteristically the relationship between  $J_r$  and  $\Delta\psi$  was linear over a range of at least  $\pm 70$  mV, occasionally  $\pm 100$  mV.

The addition of ouabain to the inner solution ( $10^{-3}$  M) regularly abolished sodium transport almost completely within 30 to 45 minutes, and reduced the rate of oxygen consumption to the basal level (Part I). In these circumstances  $J_r$  was no longer dependent on  $\Delta\psi$ , as can also be seen in Fig. 8. This demonstrates that the influence of the electrical potential difference on the rate of oxygen consumption is mediated through an effect on the active transport process.

It is of course desirable to examine the relationship between  $J_r$  and  $\Delta\psi$  in greater detail over a large range. To do so several determinations of  $J_r$  must be made, occasionally requiring as long as four hours. During these measurements appreciable spontaneous decline of sodium transport and metabolism may occur. Since such instability interferes with the determination of the relationship between  $J_r$  and  $\Delta\psi$  it is useful to monitor  $I_o$  and  $J_{ro}$ ; in a stable preparation both would be nearly constant for long periods. However, in forty-four untreated skins studied over an extended period only one was stable, and this at a low level of sodium transport.

b) Aldosterone-aged skins.

In order to obtain more comprehensive data a more stable preparation is desirable. We had observed that following several hours of incubation in glucose-Ringer's solution  $I_o$  was quite stable; however, its magnitude

was small. Since it has been noted that aldosterone increases the rate of sodium transport in epithelia (Crabbé, 1961; Sharp and Leaf, 1964; Porter and Edelman, 1964; Nielsen, 1969; Voûte et al., 1969) we used it for this purpose. Prolonged exposure to  $10^{-6}$  M aldosterone in glucose-Ringer's solution resulted in stability, with a significantly larger value of  $I_0$  than in an untreated skin from the same animal. Furthermore,  $J_{rO}$  was comparable in magnitude to the rate of oxygen consumption in freshly mounted skins and was also relatively stable. Although the rates of sodium transport and oxygen consumption decreased with time, this effect was much smaller than in the untreated skins. Eleven aldosterone-treated skins were studied extensively; of these only one was unstable.

With the use of the more stable preparation linearity was demonstrable even in long term experiments over a large range of  $\Delta\psi$ . Figs 9 a and 10 a show the results of two such experiments. As is seen, linearity was

Figs. 9 and 10

observed over a range of  $\pm 160$  mV. The corresponding plots of  $I_0$  and  $J_{rO}$  against time in Figs. 9 b and 10 b indicate the stability of these preparations.

In these studies we avoided several positive or negative periods in succession, as for example, 0, -40, -80, -120, -160, -200, followed by the positive values, in order to prevent systematic effects of polarity, as will be discussed in section 2 below. Providing that this precaution is taken, the sequence of electrical perturbations had no effect on the demonstration of linearity. In the first skin (Fig. 9a) the sequence was 0, 40, 0, -40, 0, ..., 0, -160 mV; in the second (Fig 10a) it was 0, 40, -40, 0, ..., 0, 200, -200 mV, this sequence being followed twice in succession.

## 2. The "memory" effect.

Brief perturbations of  $\Delta\psi$  ( $\pm$  30 to 200 mV for a few seconds) did not alter the subsequent values of  $I_0$  and  $J_{R0}$ . Longer perturbations (here for 10 or more minutes) produced a "memory" effect: positive clamping, which slowed active sodium transport, transiently increased the subsequent values of  $I_0$  and  $J_{R0}$ ; negative clamping induced the opposite effect. The effect was more pronounced with higher magnitudes of  $\Delta\psi$ . The phenomenon was observed in both fresh and aldosterone-aged skins, but because the effects were small they were more readily demonstrated in stable preparations. Normally the memory effect is more clearly observed in  $I_0$  than in  $J_{R0}$  owing to the greater precision in measuring  $I_0$ . Figure 11 shows the memory effect on  $I_0$  in three different skins treated with aldosterone. When large perturbations of  $\Delta\psi$  were employed ( $\pm$  160 mV for some 15 ~ 20 minutes) effects on both  $I_0$  and  $J_{R0}$  were observed. Figure 12 shows the changes in  $I_0$  and  $J_{R0}$  between successive short-circuited states; each value of  $I_0$  and  $J_{R0}$  was determined 4 to 6 minutes after returning to the short-circuited state. In this case a positive correlation between the two effects was seen.

Fig.12

In Fig 11 a short-circuited state was interposed between each positive and negative period. In other studies each positive period was immediately followed by a negative period of the same duration and magnitude of potential; in these cases the memory effect was almost completely abolished. The compensating effect of sequential positive and negative periods was seen also in the rate of oxygen consumption, as shown for example by Fig 10b.

## DISCUSSION

### 1. General considerations.

A linear relationship between the rate of oxygen consumption and the electrical potential difference is interesting per se, but takes on added significance in attempts to understand the fundamental mechanisms of active transport. In principle any consistent relationship between flows and forces would be of value in correlating behavior in a variety of conditions. Obviously a linear relationship would have the greatest utility.

Accordingly, several authors have attempted to consider active transport from the viewpoint of linear nonequilibrium thermodynamics. Kedem (1961) showed that the formalism permits the correlation of results of different measurements. Hoshiko and Lindley (1967) extended the methods of Kedem in single salt and bi-ionic systems and outlined procedures for the evaluation of the requisite 10 or 15 coefficients. Essig and Caplan (1968) treated the transport of a single cation driven by a single metabolic reaction, and showed that a composite system comprising a "pump", a series barrier, and a leak pathway may be described by linear equations, providing that each element shows linearity. In the present study we have carried out initial experiments with the aim of determining the extent to which active sodium transport and the associated oxidative metabolism in the frog skin may in fact be analyzed in the framework of a linear nonequilibrium thermodynamic model.

In order to analyze data in terms of this model it is necessary that certain preliminary requirements be satisfied. Firstly, data must be obtained in the steady-state, which means that all pertinent parameters

must be constant with time. This requirement can of course only be approximated. In order to determine the extent to which the steady-state requirement is satisfied, we monitored intermittently the rates of sodium transport and oxygen consumption in the short-circuited state. (It has been shown that both in the absence of aldosterone and in the steady state following administration of aldosterone the short-circuit current in frog skins is electrically equivalent to net sodium transport (Ussing and Zerahn, 1951; Nielsen, 1969).) In freshly mounted skins  $I_0$  and  $J_{r0}$  were stable only rarely. In general, both declined appreciably with time, and in addition showed spontaneous fluctuations. Skins which were aged in the presence of aldosterone and glucose were usually much more stable than fresh skins. A second requirement is that a new steady state be reached promptly after perturbation of  $\Delta\psi$ . This condition was clearly satisfied, since constant values of  $J_r$  were established within 2 minutes of the electrical perturbations, whereas measurements were made after 4 to 6 minutes. Another requirement is that the results of variation of  $\Delta\psi$  must be specific, reflecting intrinsic changes in the function of the active transport system. Specificity was shown by the insensitivity of  $J_r$  to  $\Delta\psi$  after sodium transport had been blocked by ouabain. It is also necessary that electrical clamping be harmless in the voltage range and for the duration employed. This was demonstrated in stable preparations by the return of  $J_{r0}$  and  $I_0$  to their previous values after electrical perturbations, provided that the memory effect was small or compensated by two periods of identical length and opposite polarity.

As discussed in Part I the rate of oxygen consumption after the blockage of active sodium transport by ouabain is a good estimate of the basal rate of oxygen uptake unrelated to transepithelial sodium transport. Therefore the results obtained following administration of ouabain indicate that the basal rate of oxygen consumption was unaffected by changes in the electrical potential difference across the skin.

## 2. Linearity.

The linear current-voltage relationship commonly observed in the frog skin may well reflect linearity of the intact sodium transport system, but is not completely convincing because of the undefined

contribution of artifactual leak pathways introduced by the mounting procedure. However, studies in the toad bladder restricted to tissues in which two-thirds of the total conductivity was by way of the active transport pathway showed a linear relation between current and potential difference (Essig and Lief, in preparation).

The present study enables us to make precise statements about the relationship between  $J_r$  and  $\Delta\psi$  in frog skin. Although we did not always observe linearity, in those cases where the skin was stable, as shown by constancy of  $J_{r0}$  with time, linearity was impressive over a wide range. In the absence of aldosterone linearity was demonstrable over a range of  $\pm 70$  mV; after incubation with aldosterone linearity was demonstrable over as much as  $\pm 160$  mV in stable skins.

## 3. Phenomenological description.

The observation of linearity indicates that the phenomenological coefficients characterizing the system and the affinity  $A$  of the metabolic driving reaction must be nearly invariant on perturbation of  $\Delta\psi$ . The alternative possibility, that the coefficients and/or the affinity may

vary so as to produce the observed linearity, seems unlikely.\* Accordingly, the behavior of the system may be described by standard equations of nonequilibrium thermodynamics, viz:

$$J_{Na} = L_{Na} X_{Na} + L_{Nar} A \quad (1)$$

$$J_r = L_{Nar} X_{Na} + L_r A \quad (2)$$

Here  $J_{Na}$  represents net sodium flux,  $X_{Na}$  is the negative electrochemical potential difference of sodium across the skin, and the L's are phenomenological coefficients. With identical solutions at each surface  $X_{Na} = -F\Delta\psi$ . In writing the cross-coefficient as  $L_{Nar}$  in both equations (1) and (2) the validity of the Onsager reciprocal relation has been assumed.\*\*

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\*It is conceivable that A might be a linear function of  $\Delta\psi$  (Essig and Caplan, 1968). However, since the memory effect evaluated at short-circuit is small it would seem that A could not be a strong function of  $\Delta\psi$ .

\*\*Since A appears to be constant in these studies, we cannot rule out the existence of higher order terms in the affinity. For the present purposes this is immaterial; all of our conclusions are unaffected by this consideration.

In a previous publication (Essig and Caplan, 1968) the phenomenological equations were presented in terms of resistance coefficients rather than conductance coefficients for reasons cited. In the present context the conductance formulation is intuitively more meaningful.

Although the Onsager relation has been widely tested, its validity for active transport is as yet unknown. In a model system in which the enzymatic hydrolysis of an amide results in current flow simulating active transport the relation was found to hold (Blumenthal et al., 1967).

#### 4. Evaluation of the affinity.

The apparent validity of equations (1) and (2) justifies the estimation of an equation derived previously (Essig and Caplan, 1968). For linear systems obeying the Onsager reciprocal relation between phenomenological coefficients

$$A = - \frac{I_o}{(\partial J_r / \partial \Delta\psi)_A} \quad (3)$$

Values of A calculated in this way are given in Tables I and II.

#### Tables I and II

These affinities represent the negative free energy of the oxidative metabolic reaction which "drives" sodium transport. It is to be emphasized that the values presented are those appropriate for the skins under the conditions obtaining at the time of their observation, and are not to be confused with the often cited negative free energies evaluated under standard conditions.

Table I shows the results of replicate studies in 5 untreated fresh skins. The mean affinity value,  $44.2 \pm 13.2$  kcal/mole of oxygen, is to be compared with the value of some 116 kcal/mole cited for glucose oxidation under physiological conditions (Davies and Ogston, 1950). Table II shows the results of studies in 7 aldosterone-treated skins, each representing a period of some 2 hours or less. The high values of the correlation coefficients indicate linearity. The mean affinity value,  $107.1 \pm 51.5$  kcal/mole of oxygen, is higher than in the untreated skins. Since adequate control studies were not performed we cannot conclude that aldosterone was responsible for this difference.

It is to be expected that with the passage of time the affinity will decrease at a rate determined by substrate utilization, and a rapid

decline in the affinity may well account for a rapid decrement in  $I_0$  and  $J_{r0}$  as was sometimes observed. However, since the techniques employed here for the evaluation of A necessitated the use of stable preparations, and since the studies of oxygen consumption were carried out for only limited periods, we were not able to demonstrate the time-dependence of A. In the studies shown in Table V, the short-circuit current varied more than the affinity. This may possibly reflect variations in membrane permeability.

##### 5. Significance of the "memory" effect.

In view of the evidence above for near-constancy of the affinity it is of interest that positive clamping, which slows active sodium transport, transiently increases subsequent values of  $I_0$  and  $J_{r0}$ , and that negative clamping induces the opposite effects. These phenomena might of course reflect transient effects of the electrical potential on tissue permeability coefficients or rate constants, but this seems unlikely, particularly in view of the small magnitudes of the perturbations which we often employed. Another possibility is that changes in the rates of transport resulting from perturbations of  $\Delta\psi$  might slightly alter the concentrations of metabolic intermediates so as to induce the small changes observed. Such behavior is consistent with a model for the active transport system discussed previously (Essig and Caplan, 1968, Appendix I). It might seem as though short-term variations of A resulting from perturbations of  $\Delta\psi$  would invalidate our attempts to evaluate an affinity characteristic of each skin. However, the sequence of perturbations was chosen so as to minimize the memory effect. Furthermore, the clear demonstration of the memory effect requires longer perturbations than were employed in the

studies of  $J_r$  vs.  $\Delta\psi$ .

## 6. Concluding remarks.

Considerable interest attaches to the mechanisms whereby various substances alter the rate of sodium transport (Porter and Edelman, 1964; Sharp and Leaf, 1964; Sharp et al., 1966; Fanestil et al., 1967). In principle such substances may act by effects on (1) permeability, (2) coupling between transport and metabolism, or (3) modification of the affinity of the metabolic driving reaction. The present means of evaluating the affinity may be useful in differentiating between these possibilities.

The efficiency of active transport is often evaluated in terms of "the calorific value of 1 eq. of oxygen" (Zerahn, 1958). It seems more appropriate to utilize the free energy of the metabolic driving reaction measured in vivo rather than an enthalpy derived from bomb calorimetry.

As mentioned above, equivalence of the two methods used for the evaluation of the "electromotive force" of sodium transport  $E_{Na}$  requires constancy of the rate of metabolism. However, the present study shows that  $J_r$  varies markedly with  $\Delta\psi$ .

We thank Dr. J. Gilbert and Mrs. J. Lustgarten of the Harvard Computing Center for the statistical analysis of the data.

Figure 7. Change in the rate of oxygen consumption with perturbation of the electrical potential difference  $\Delta\psi$ . The rate of oxygen consumption is calculated from the slopes of the curve as described in Part I. The dashed lines indicate times of alteration of  $\Delta\psi$ . The calculations were based upon the steady-state slopes during the periods indicated by the arrows.

Figure 8. Dependence of the rate of oxygen consumption  $J_r$  on the electrical potential difference  $\Delta\psi$  during several 25-minute periods of study of a single skin. The plot in open circles represents data obtained following the elimination of active sodium transport with ouabain.

Figure 9. (a) Dependence of the rate of oxygen consumption on the electrical potential difference  $\Delta\psi$  in the presence of aldosterone.  
(b) Plots of  $I_o$  and  $J_{rO}$  versus time. These indicate the long-term stability of the preparation. The sequence of perturbations of  $\Delta\psi$  was chosen so as to avoid "memory" effects (see Results, section 2).

Figure 10 (a) Dependence of the rate of oxygen consumption on the electrical potential difference  $\Delta\psi$  in the presence of aldosterone.  
(b) Plots of  $I_o$  and  $J_{rO}$  versus time (see Legend, Fig. 3b).

Figure 11 Variation of the short-circuit current following perturbation of the electrical potential difference  $\Delta\psi$ . This study was carried out in the presence of aldosterone.

Figure 12 Effect of extended electrical potential perturbations on the subsequent values of short-circuit current  $I_o$  and rate of oxygen consumption  $J_{rO}$ . The change in oxygen consumption is plotted against the change in short-circuit current. The perturbations last some 15 to 20 minutes.

Table V. Values of  $I_0$ ,  $-\partial J_r / \partial \Delta \psi$ , and the affinity A calculated from equation (3) (untreated skins). Mean values are given  $\pm$  S.E.

Table V I. Values of  $I_0$ ,  $J_{r0}$ ,  $-\partial J_r / \partial \Delta \psi$ , and the affinity A calculated from equation (3) (aldosterone-treated skins). The observations were made at 6 minute intervals;  $n_0$  is the number of simultaneous determinations of  $I_0$  and  $J_{r0}$ . Perturbations of potential were made in 40 mV steps ranging from -160 to +160 mV. Mean values are given  $\pm$  S.E.; n is the total number of observations and r the correlation coefficient. For  $n \geq 11$  a value of  $r \geq 0.684$  is significant at the 0.01 level.

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TABLE - V

	$I_0$ ( $\mu\text{A cm}^{-2}$ )	$\Delta\psi$ (mV)	$-\partial J_r / \partial \Delta\psi$ ( $\mu\text{mole sec}^{-1} \text{cm}^{-2} \text{mV}^{-1}$ )	A ( $\text{kcal mole}^{-1}$ )	Mean A ( $\text{kcal mole}^{-1}$ )
I	63.4	-30,30,0	.364	41.6	$48.3 \pm 2.9$
	60.6	-30,30,0	.293	49.4	
	40.8	-60,60,0	.234	41.7	
	26.1	-90,90,0	.119	52.3	
	26.1	-90,90,0	.111	56.4	
II	59.2	-30,30,0	.345	41.0	$50.9 \pm 2.9$
	70.4	30,-30,0	.299	56.2	
	45.1	-60,60,0	.217	49.7	
	56.3	60,-60,0	.224	60.0	
	35.2	-90,90,0	.187	45.1	
	35.2	90,-90,0	.156	53.8	
III	63.4	0,-30,0	.306	49.5	$42.1 \pm 2.8$
	54.9	-60,-30,0	.371	35.4	
	52.1	0,30,60	.338	36.8	
	59.4	60,30,0	.299	47.5	
	52.8	60,0,-60	.306	41.3	
IV	66.9	0,50,0	.370	43.2	$57.1 \pm 6.1$
	65.1	0,-50,0	.223	69.9	
	57.0	-50,0,50	.293	46.5	
	52.8	-50,0,50	.280	45.0	
	51.7	50,0,-50	.211	58.6	
	52.8	-50,0,50	.159	79.5	
V	20.7	0,-50,0,50	.246	20.1	$22.7 \pm 2.9$
	17.6	0,-100,0,100	.223	18.9	
	13.8	0,50,0,-50	.105	31.3	
	12.7	0,100,0,-100,-100	.147	20.7	
Mean	$46.6 \pm 3.4$		$.247 \pm .016$		$44.2 \pm 13.2$

TABLE V I

Skin	$I_o$ ( $\mu A \text{ cm}^{-2}$ )	$J_{ro}$ ( $\mu\mu\text{mole sec}^{-1}\text{cm}^{-2}$ )	$n_o$	$-\partial J_r / \partial \Delta\psi$ ( $\mu\mu\text{mole sec}^{-1}\text{cm}^{-2}\text{mV}^{-1}$ )	n	r	A ( $\text{kcal mole}^{-1}$ )
VI	$32.1 \pm 1.1$	$81.9 \pm 1.0$	4	$0.156 \pm 0.010$	20	0.963	$49.2 \pm 3.8$
VII	$116.5 \pm 1.1$	$88.8 \pm 1.3$	4	$0.137 \pm 0.013$	20	0.928	$203.7 \pm 16.2$
VIII	$96.3 \pm 0.4$	$86.6 \pm 1.2$	5	$0.179 \pm 0.012$	21	0.957	$128.5 \pm 8.6$
IX	$99.6 \pm 1.3$	$115.5 \pm 1.4$	6	$0.233 \pm 0.017$	14	0.969	$102.2 \pm 6.4$
X	$51.1 \pm 0.6$	$90.2 \pm 3.3$	4	$0.212 \pm 0.041$	11	0.865	$57.5 \pm 10.9$
XI	$74.0 \pm 0.5$	$86.3 \pm 0.7$	5	$0.191 \pm 0.008$	21	0.985	$92.4 \pm 3.9$
XII	$85.8 \pm 0.8$	$84.7 \pm 0.9$	4	$0.177 \pm 0.009$	20	0.976	$115.9 \pm 6.0$
Mean	$79.3 \pm 29.4$	$90.6 \pm 11.3$		$.184 \pm .032$			$107.1 \pm 51.5$

CHANGE IN THE RATE OF OXYGEN CONSUMPTION WITH  
PERTURBATION OF THE ELECTRICAL POTENTIAL DIFFERENCE  
 $\Delta \psi$  IN THE FROG SKIN

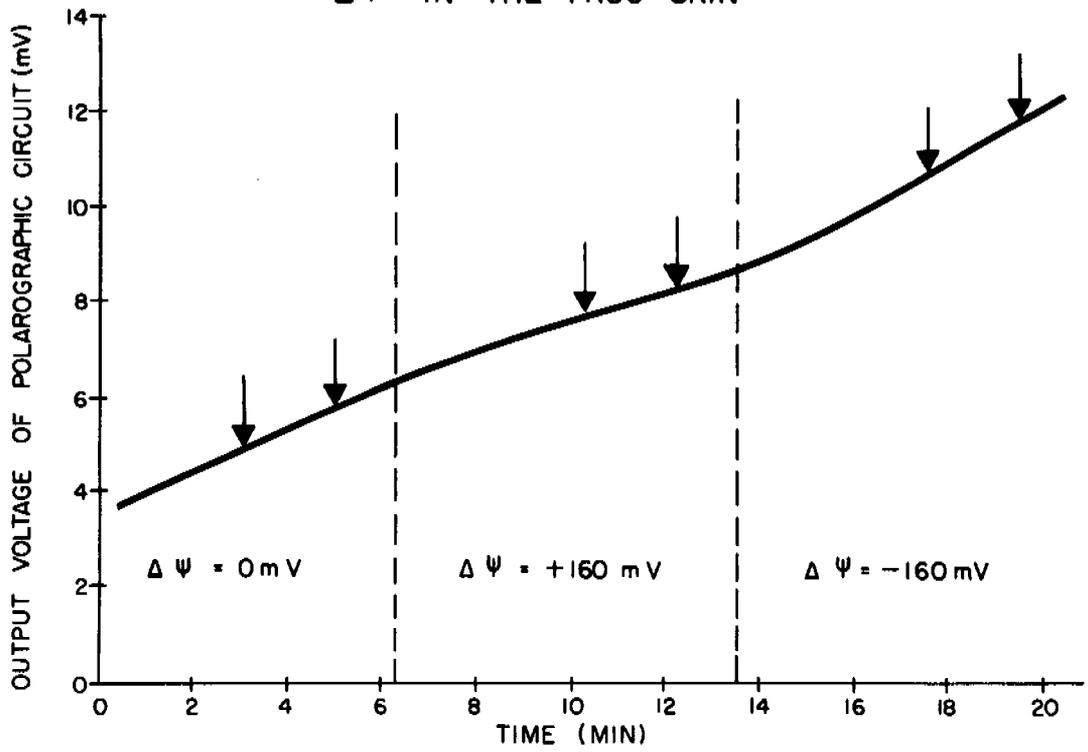


Fig. 7

DEPENDENCE OF THE RATE OF OXYGEN CONSUMPTION  $J_r$  ON THE ELECTRICAL POTENTIAL DIFFERENCE  $\Delta\psi$ ; EFFECT OF OUABAIN

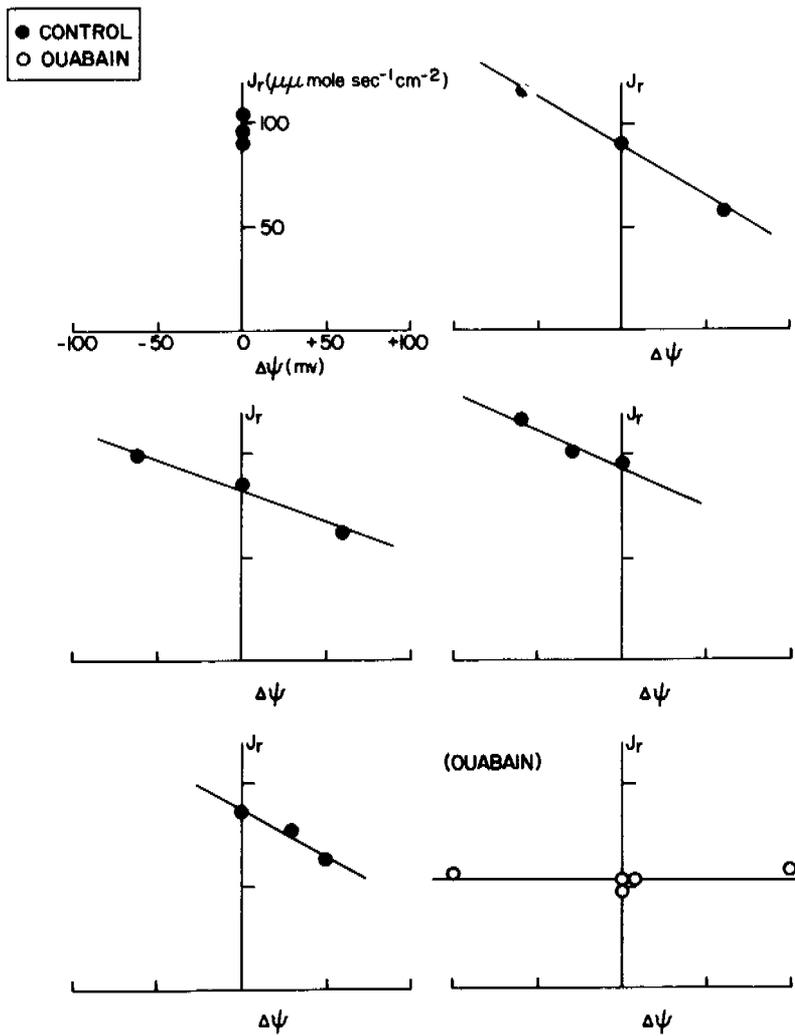


Fig. 8

DEPENDENCE OF THE RATE OF OXYGEN  
CONSUMPTION  $J_r$  ON THE ELECTRICAL  
POTENTIAL DIFFERENCE  $\Delta \Psi$  IN THE  
ALDOSTERONE-TREATED FROG SKIN

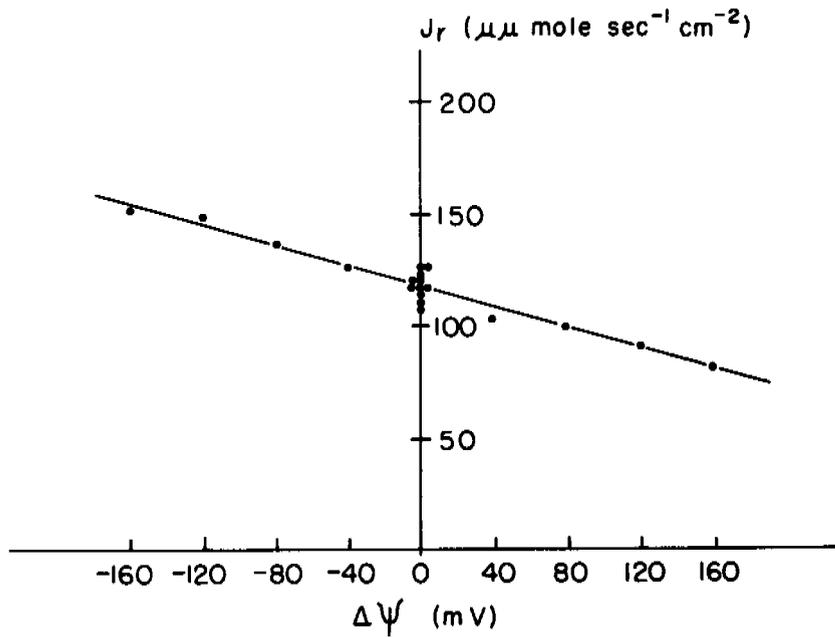


Fig. 9 A

VARIATION OF  $J_{r0}$  AND  $I_0$  FOLLOWING  
 PERTURBATION OF  $\Delta \Psi$  - ALDOSTERONE  
 TREATED FROG SKIN

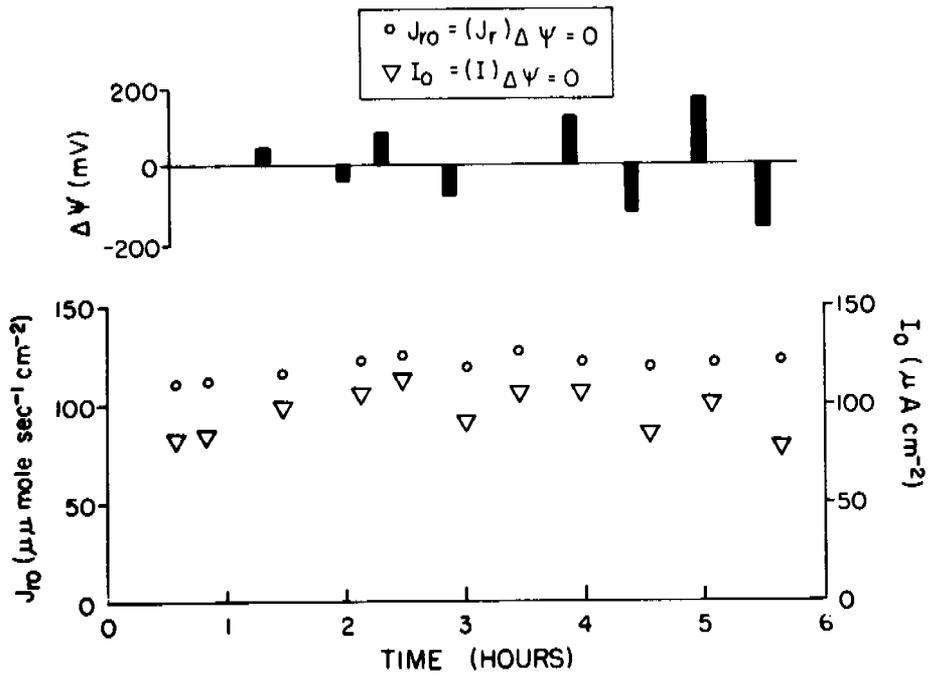


Fig. 9 B

Fig. 10 a

DEPENDENCE OF THE RATE OF OXYGEN  
CONSUMPTION  $J_r$  ON THE ELECTRICAL  
POTENTIAL DIFFERENCE  $\Delta \Psi$  IN  
THE ALDOSTERONE-TREATED FROG SKIN

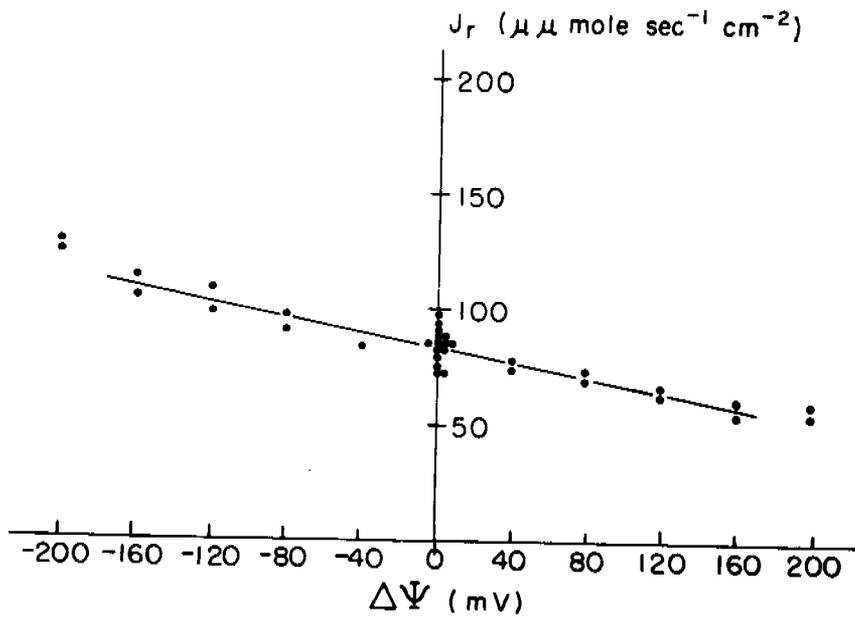
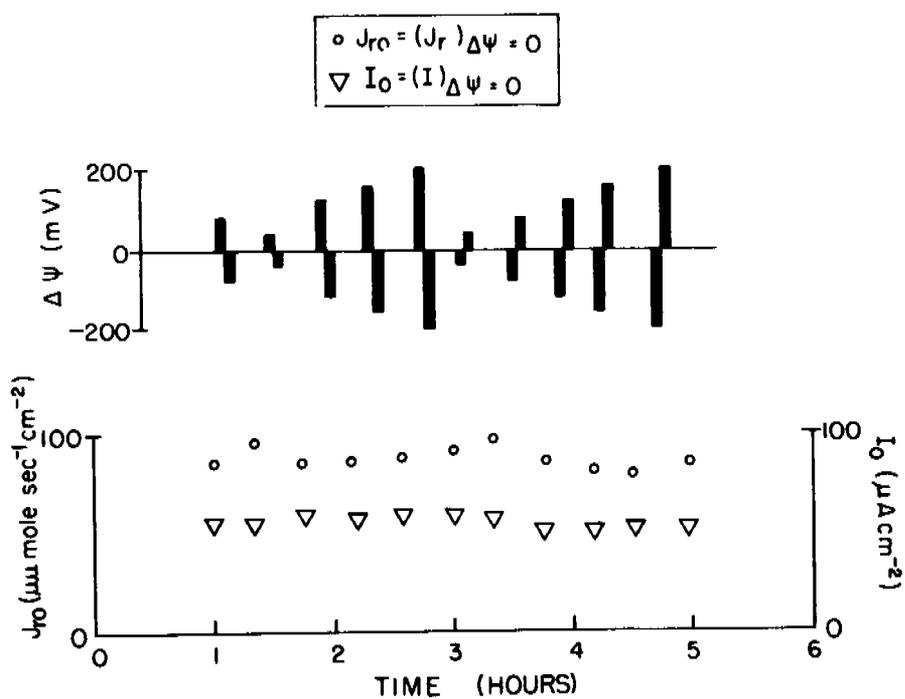


Fig. 10 b

VARIATION OF  $J_{r0}$  AND  $I_0$  FOLLOWING  
PERTURBATION OF  $\Delta\psi$  - ALDOSTERONE  
TREATED FROG SKIN



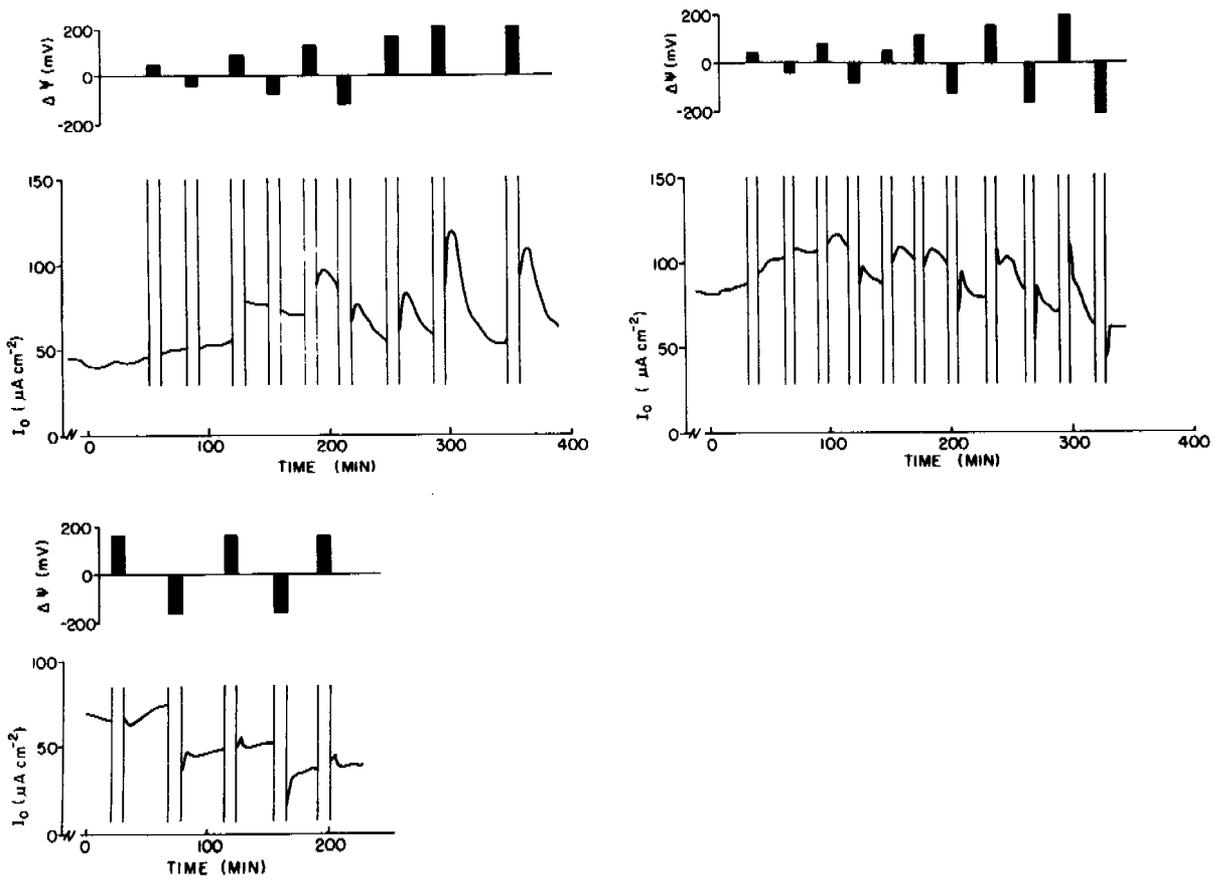


Fig. 11

EFFECT OF ELECTRICAL POTENTIAL PERTURBATIONS  
 ON SUBSEQUENT VALUES OF SHORT-CIRCUIT  
 CURRENT  $I_0$  AND OXYGEN CONSUMPTION  $J_{r0}$ .

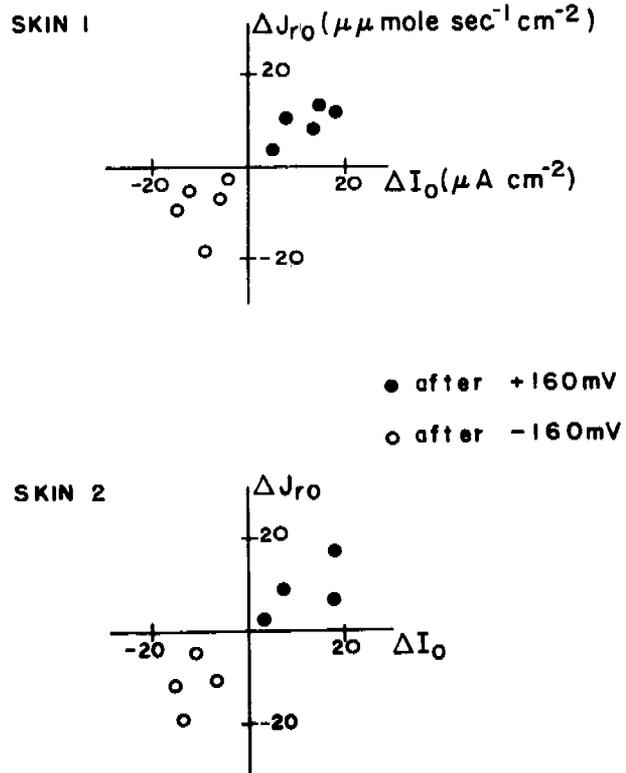


Fig. 12

### III. Nonequilibrium Thermodynamic Analysis of Ion Transport and Membrane Metabolism

(Work done with S.R. Caplan)

Many people have considered the energetics of ion transport across epithelial membranes. In general these studies have been based on those of Ussing (1), who wrote in terms of the work required to accomplish the transport of one equivalent of sodium. This was considered to comprise three components: "(a) the work required to overcome the concentration gradient ..., (b) the work required to overcome the potential gradient ..., (c) the work required to overcome the internal sodium resistance of the skin ..."; this latter quantity was evaluated from the "flux ratio" or ratio of the "unidirectional" fluxes of sodium. The total work was thus evaluated (per mole of sodium) as

$$W = RT \ln (c_i/c_o) + F E + RT \ln (M_{in}/M_{out}), \quad (1)$$

where  $M_{in}/M_{out}$  represents the flux ratio. Since the skin was treated in terms of an electrical analogue, Ussing spoke also of "the electromotive force of active sodium transport,"  $E_{Na}$ . This was obtained by dividing the work  $W$  by the Faraday.

Two general methods were employed to evaluate  $W$  and  $E_{Na}$ : (a) One was to determine the electrochemical potential difference adequate to reduce the rate of net sodium transport (and hence  $\ln M_{in}/M_{out}$ ) to zero. This was accomplished by manipulation of the concentration difference or electrical potential difference across the membrane, or by the substitution of impermeant for permeant anions. (b) Another method was to expose the skin to identical solutions at each surface, nullify the spontaneous electrical potential difference, and measure the flux ratio under these "short-circuit" conditions.

As was pointed out by Ussing, these methods permit the evaluation only of an apparent  $E_{Na}$ , since they do not take into account the influence of leak pathways. In addition, on the basis of the work of Hodgkin and Keynes in poisoned squid axons, as well as on the basis of it was appreciated that "exchange diffusion", there are fundamental difficulties in attempts to utilize the flux ratio to evaluate energetic parameters (1-3).

We would raise additional considerations. For one, it is to be questioned whether it is appropriate to include a dissipative term in an expression for the work accomplished by a process; this is not customary in the thermodynamic analysis of heat engines or other energy converting systems. Also, it would seem that in considering a frog skin in terms of an electrical analogue there is the danger of assuming a constant and stoichiometric relationship between the rates of transport and metabolism under all conditions of operation (4). Such complete coupling is in fact found with perfect electrochemical cells. However, in biological systems partial decoupling would be expected to occur frequently, consequent to either the breakdown of metabolic intermediates, or the dissipation of electrochemical potential gradients by way of leak pathways (5). In such circumstances an expenditure of metabolic energy would be required to maintain an electrochemical potential difference across a tissue even in the absence of a net flow. This is in seeming conflict with the formulation underlying equation (1), which implies that in the absence of net flow  $W$  would equal zero. It may be objected that equation (1) must be considered as providing only a minimal value for the necessary rate

of expenditure of metabolic energy, but if so the formulation loses some of its value as a means of analyzing the energetics of the transport system with precision.

Still other attempts at analyzing the energetics of sodium transport systems have emphasized the evaluation of the efficiency. This has generally been defined as the quotient of  $\frac{thc}{}$  rate of performance of work, evaluated as described above, and the rate of expenditure of metabolic energy. This latter quantity was taken to be the product of the rate of supra-basal oxygen consumption and the "calorific value" of 1 gram equivalent of oxygen. The definition of an efficiency function in this manner casts further doubt on the appropriateness of the inclusion of a dissipative term in the expression for work. Classically one would expect that an increase in dissipation would decrease the efficiency of a process. In addition to this objection and the others raised above, one might question evaluating available energy from calorimetry, rather than from measurements of free energy.

Because of the various shortcomings of the above formulations it has seemed to us appropriate to analyze the energetics of active transport in terms of nonequilibrium thermodynamics. Such an analysis of the kinetics of isotope flows was carried out in collaboration with Professor Ora Kedem (6). We consider a system of identical elements across which a cation moves as a consequence of its electrochemical potential gradient and/or coupled flows. We assume, however, that there is no coupling between the flows of the abundant and the

tracer forms of the test species ("isotope interaction"). For such a system the formulation shows that

$$\underline{RT} \ln f = X - \int_0^{\Delta x} \sum_{+j} J_j dx. \quad (2)$$

Here  $f$  is the flux ratio and the integral across the membrane represents the contribution of coupled flows  $J_j$  of all species, as well as that of metabolism ("active transport") to the flux of the test species. Thus, in the absence of isotope interaction the flux ratio evaluates the forces promoting net transport. In the presence of isotope interaction, however, the situation is quite different, since now

$$\underline{RT} \ln f = (R_+^{ax}/R_+^a)(X - \int_0^{\Delta x} \sum_{+j} J_j dx). \quad (3)$$

Here  $R_+^a$  is the resistance to net flow by way of the active transport pathway, whereas  $R_+^{ax}$  is the isotope exchange resistance determined from a tracer experiment. In the presence of isotope interaction  $R_+^a \neq R_+^{ax}$  and hence the flux ratio no longer quantifies the forces producing net transport. Furthermore, it was shown that both in the absence and presence of isotope interaction the flux ratio is exquisitely sensitive to the existence of "leak" pathways.

The application of the above formulation to a system comprising parallel arrays of active and passive "leak" pathways permits a comparison of the  $E_{Na}$ 's which would be obtained by the two means described above:

$$(a) \quad F(E_{Na})_a = - (X)_{J=0} = - \frac{R_{+r}^a}{R_+^a/R_+^p + 1} (J_r)_{J=0}. \quad (4)$$

(Here  $R_{+r}^a$  is the phenomenological resistance coefficient for coupling of cation transport and metabolism, and  $R_+^p$  is the resistance coefficient of the passive pathway.)

$$(b) \quad F(E_{Na})_b = (\underline{RT} \ln f)_{X=0} = - \frac{\overline{R_+^{ax}} R_{+r}^a}{R_+^a} (J_r)_{X=0}. \quad (5)$$

where  $\overline{R}_+^X$  is an "apparent exchange resistance". It is seen that in order for the two techniques to give the same value for  $E_{Na}$  three conditions must be satisfied: (a) absence of leak, (b) absence of isotope interaction, and (c) constancy of metabolism. (Under these circumstances  $R_p \rightarrow \infty$ ,  $\overline{R}_+^X = R_+^a$ , and  $(J_r)_{J=0} = (J_r)_{X=0}$ .) Such a combination of circumstances would seem most unlikely.

The deficiencies of an isotope kinetic treatment of the energetics of a system led us to make a more general thermodynamic analysis of active transport (7). In this we follow the original formulation of Kedem (8) and a subsequent more comprehensive analysis of coupled two-flow systems (4). We assume a two-flow process with the rate of active cation transport  $J_+^a$  and that of metabolism (oxygen consumption)  $J_r$  each a linear function of both the electrochemical potential difference of sodium,  $-X$ , and the affinity (negative free energy)  $A$  of the oxidative driving reaction. Thus, assuming the validity of the Onsager reciprocal relations,

$$J_+ = L_{+}^a X + L_{+r}^a A, \quad (6)$$

and

$$J_r = L_{+r}^a X + L_{rr}^a A. \quad (7)$$

Several features of this formulation are worth comment. It is clear that if such simple linear relations are appropriate the analysis of the energetics of an active transport system will be much facilitated, but it may be doubted that the assumption of linearity is realistic. As is well known, for simple chemical reactions in solution the rate of reaction is a linear function of the affinity only very near equilibrium, i.e., only if  $A \ll RT$ . However, as has been emphasized by Prigogine, biological reactions are often made up of so large a number of reactions

in series that, although the "global" affinity  $A$  of the over-all reaction may be large, the "local" affinity  $A_j$  of each component reaction may be  $\ll RT$  (9). In this case the rate  $J_{rj}$  of each component reaction will be a linear function of its affinity  $A_j$ ; if in addition the  $L_j$ 's are insensitive to the concentrations of the metabolites the steady state rate of over-all reaction  $J_r$  will be a linear function of  $A$ . A more detailed analysis has shown that for enzymatic systems obeying Michaelis-Menten kinetics the  $L_j$ 's may indeed be independent of concentration and, therefore, complex reactions may show linearity in a range of impressively large values of the affinity (10). Furthermore, if an active transport mechanism can be described by equations (6) and (7), a similar linear formulation is applicable to the experimentally observable transport system comprising both the active mechanism and any associated leak pathways (7).

As the examination of equations (6) and (7) makes clear, a fixed stoichiometric ratio between  $J_+$  and  $J_r$ , applicable whatever the values of  $X$  and  $A$ , will be found only for a very special relationship between the phenomenological coefficients. As was indicated above, in general such complete coupling between transport and metabolism would not be expected; even if the cation pump mechanism were itself completely coupled, the presence of the passive pathway necessary for continuing salt transport would partially decouple the composite system.

If the transport system is linear and the phenomenological coefficients and affinity are insensitive to variation of the electrical potential difference  $\Delta\Psi$ , the affinity can be evaluated, being given by

$$A = - \frac{(\partial(\Delta\Psi))}{\partial J_r} A \quad (FJ_+ X=0) \quad (8)$$

We note that if  $A$  is near-constant on variation of  $\Delta\Psi$ ,  $J_r$  cannot be constant, and hence the two techniques for the evaluation of  $E_{Na}$  discussed would in fact lead to discrepant results, as anticipated.

Further consideration of the nonequilibrium thermodynamic approach (4) suggests the utility of an efficiency function defined in terms of the dissipation function:

$$\eta = - \frac{J_+ X}{J_r \Lambda} \quad (9)$$

As is seen, this efficiency function is unambiguous, being the quotient of an output (external work) and an input of metabolic energy. Note that the input of metabolic energy here is evaluated from an actual free energy of reaction appropriate to the tissue under study. With the aid of isotope techniques the rate of electrochemical work could be evaluated accurately, and combined with measurements of  $J_r$  and of  $A$ , determined as described above. Hence this efficiency function should provide <sup>a/</sup>useful and precise criterion for the analysis of the energetics of an active transport system in a variety of circumstances.

It is now pertinent to consider various experimental results bearing on the validity of the above formulations. We consider first some experiments in highly permselective anion exchange membranes prepared by the adsorption of a quaternary ammonium polyelectrolyte (PVBT) on collodion. As demonstrated by the studies of Gottlieb and Sollner in such PVBT membranes (11), the exchange resistance  $R^X$  determined by tracer isotope studies may be appreciably less than the resistance to net flow  $R$ . Hence these membranes permit a convenient test of the validity of the general flux ratio formulation discussed above. This was done by determining the

flux ratios of chloride across membranes exposed at each surface to 0.1 M KCl. The flux ratios were varied by inducing electrical potential differences ranging from 10 to 75 mV (12). Since electroosmotic flow is insignificant the force promoting net transport in this passive system is simply  $X = -F\Delta\psi$ . According to the original formulations neglecting the possible influence of isotope interaction (equation (2)), the flux ratio would be given by  $f = \exp(X/RT)$ . As is seen from Figure 1, this is far from the case. According to the modified flux ratio formulation incorporating the contribution of isotope interaction (equation (3)), it would be expected that  $f = \exp[(R^X/R)(X/RT)]$  (6). The agreement with the experimental results is excellent, indicating the validity of the general formulation. These results emphasize that it cannot be assumed in general that the determination of the flux ratio will evaluate the forces promoting transport, even in a homogeneous system.

Other aspects of the above formulations were tested in two epithelial tissues which carry out vigorous active transport of sodium, the frog skin and the toad bladder. Previous studies in the frog skin have emphasized the ratio between sodium transport and supra-basal oxygen consumption, citing a value of some 16 to 20 mEq.  $\text{Na}^+$  per mole  $\text{O}_2$  (13,14). Since these studies presented only a single value for each skin it was not clear whether the variability observed represented experimental limitations or true differences between different skins. In the present studies  $(J_r)_{\Delta\psi=0}$  was determined repeatedly as the short-circuit current  $I_0$  declined spontaneously with time. The results showed clearly that the ratio  $d\text{Na}/d\text{O}_2$  varies significantly from one skin

to another; the values ranged from 8 to 31 mEq.  $\text{Na}^+$  per mole  $\text{O}_2$  (15).

Despite the common observation of Ohm's law behavior <sup>in /</sup> epithelial tissues the relation between the rate of active transport  $J_+^a$  and the electrical potential difference  $\Delta\psi$  has been unclear owing to the poorly defined contribution of leak pathways. In order to clarify this issue the current-voltage relationship was studied in toad bladders in which the analysis of tracer potassium fluxes had demonstrated very low permeability of leak pathways (16). In nine tissues in which some two thirds of the conductivity was attributable to the active pathway the current-voltage relationship was highly linear, indicating that the rate of active sodium transport is in fact a linear function of  $\Delta\psi$ . This result suggests strongly that the phenomenological coefficients of the active transport system are constant. Furthermore, this result suggests that the affinity of some intermediary reaction sequence which is driving active transport is insensitive to perturbation of  $\Delta\psi$  (equation (6)).

Studies of the relationship between the rate of oxygen consumption  $J_r$  and the electrical potential difference  $\Delta\psi$  were carried out in the frog skin (17). (Control studies demonstrated that when sodium transport was abolished by the administration of ouabain  $J_r$  was unaffected by the perturbation of  $\Delta\psi$ .) In order to promote vigorous and stable active sodium transport the tissues were exposed overnight to aldosterone and glucose. Under these conditions it was possible to demonstrate linear relationships between  $J_r$  and  $\Delta\psi$ , when the latter

was varied over a range of  $\pm 160$  mV (Figure 2). Again, this result suggests strongly that the phenomenological coefficients and the affinity of an intermediary reaction sequence of the active transport system are insensitive to perturbation of  $\Delta\psi$  (equation (7)).

Fig. 14

This being the case it was possible to use equation (8) to evaluate the affinity. Values in fresh skins incubated in glucose plus aldosterone ranged from some 23 to 99 kcal/mole oxygen, as indicated in Table I. These values are to be compared with a free energy of glucose oxidation of 116 kcal/mole under physiological conditions (18). We would emphasize that the affinity evaluated in this manner represents a negative free energy of the driving reaction pertinent to the tissue studied under the experimental conditions employed. As such it is a relevant parameter for the analysis of the energetics of the transport system and should therefore not be confused with free energies of reaction determined under standard conditions differing greatly from the physiological.

Table VII

The possibility of a linear thermodynamic analysis of the active transport system should permit a comprehensive study of its energetics. Eventually it may lead to differentiation between effects on the phenomenological coefficients and effects on the free energy of the metabolic driving reaction. This may prove useful in clarifying the mode of action of drugs and hormones which influence the rate of active sodium transport.

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TABLE VII

SKIN	AFFINITY (kcal/mole O <sub>2</sub> )	S.D.
1	38.2	34.2
2	48.3	6.5
3	50.9	7.1
4	42.1	6.2
5.	57.1	14.9
6	22.7	5.7
7 <sup>a</sup>	46.8	4.3
8 <sup>a</sup>	51.2	3.2
9 <sup>a</sup>	98.9	5.8

## LEGENDS TO FIGURES AND TABLE

Figure 1 3      Theoretical and observed flux ratios in PVBT collodion membranes. The membranes were exposed at each surface to 0.1 M KCl. Flux ratios of chloride were measured at  $\Delta\Psi = 10, 25, 50, 75$  mV.

Figure 1 4      Dependence of the rate of oxygen consumption  $J_r$  on the electrical potential difference  $\Delta\Psi$  in the aldosterone-treated frog skin. Following a change of  $\Delta\Psi$  a new steady state value of  $J_r$  was reached within less than 2 minutes. Thus, to ensure stationarity  $J_r$  was evaluated 4 to 6 minutes following the change of  $\Delta\Psi$ .

Table V I I      Determination of the affinity A of the metabolic driving reaction. A was calculated from equation (8). The superscripts <sup>a</sup> indicate skins treated with aldosterone.

Fig. 14

DEPENDENCE OF THE RATE OF OXYGEN  
CONSUMPTION  $J_r$  ON THE ELECTRICAL  
POTENTIAL DIFFERENCE  $\Delta\psi$  IN THE  
ALDOSTERONE-TREATED FROG SKIN

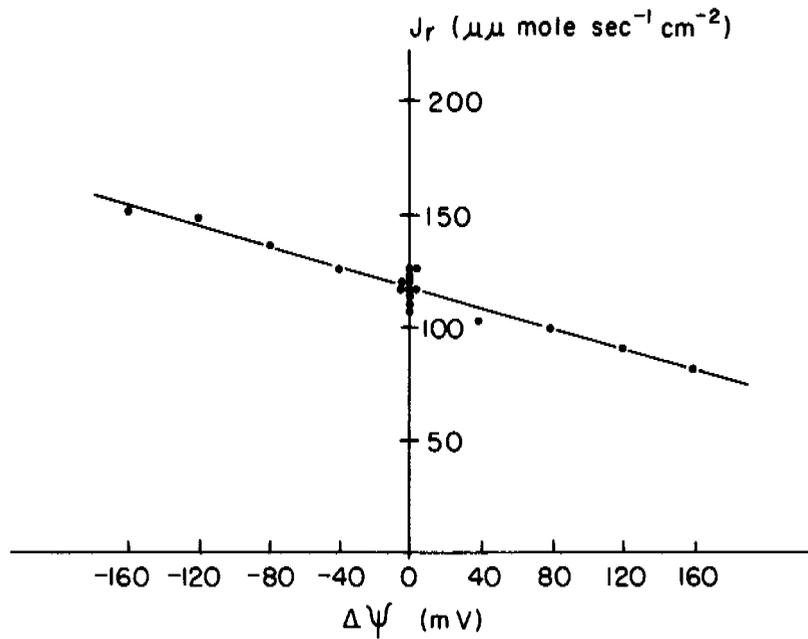
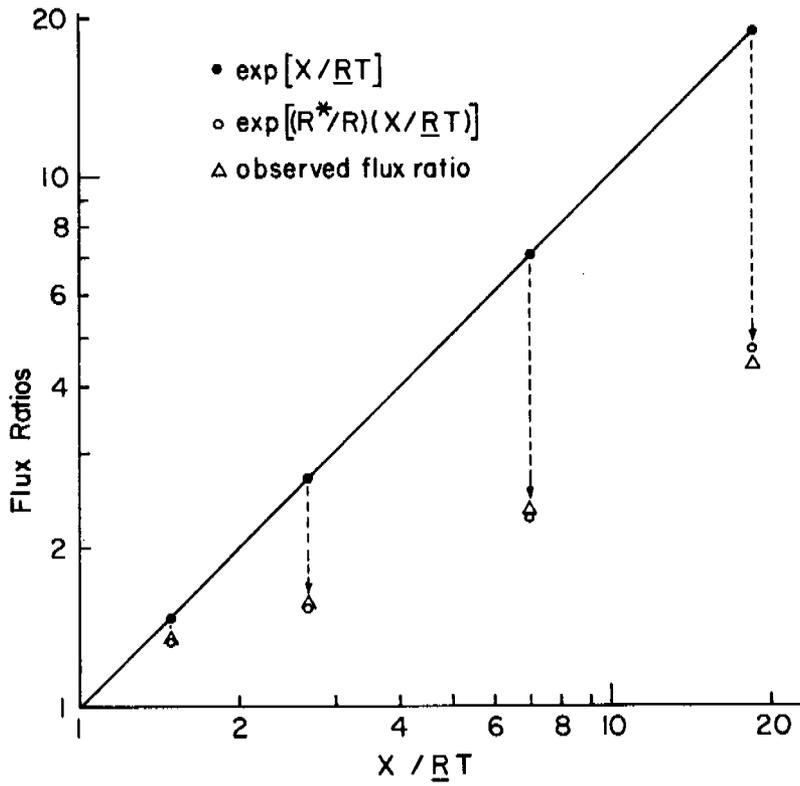


Fig. 13

Theoretical and Observed Flux Ratios  
in PVBT Collodion Membranes



#### IV. FLUX RATIOS AND ISOTOPE INTERACTION IN AN ION EXCHANGE MEMBRANE

(Work done with R.C. de Sousa and J.H. Li)

##### Abstract

Flux ratios were evaluated in membranes with apparent discrepancies between tracer exchange and electrical resistance. The results substantiate an earlier thermodynamic formulation, indicating that anomalous tracer permeability coefficients and flux ratios are attributable to coupling of isotope flows ("isotope interaction"). The effects observed seem unlikely to involve the carrier mechanisms usually invoked in biology.

Despite the widespread use of radioactive tracers in the study of permeation of biological tissues, it is well known that the permeability coefficient derived from tracer exchange may differ appreciably from that derived from net flow<sup>1-4</sup>. This is the case, for example, for water flow in the frog skin and toad bladder<sup>1-3</sup> and for sugar transport in red blood cells<sup>4</sup>.

A similar ambiguity attends the use of the "flux ratio" to evaluate the forces promoting transport. According to the original formulations of Ussing<sup>2,5</sup> and Teorell<sup>6</sup>, for simple passive flow across a membrane the ratio of "unidirectional fluxes" is given by

$$f = \exp (X/\underline{RT}), \quad (1)$$

where  $X$  is the negative electrochemical potential difference of the test species, and  $\underline{R}$  and  $T$  are the gas constant and absolute temperature respectively. Deviations from this relation (i.e., "abnormality" of the flux ratio) were considered to indicate additional forces such as those of solvent drag or active transport. Again, although the flux ratio equation has been useful in characterizing the forces which influence net transport, important exceptions have been noted<sup>1,2,7,8</sup>. One of the clearest is the case of potassium flow in the poisoned squid axon, where the flux ratio is markedly abnormal despite the apparent absence of either solvent drag

or active transport<sup>7</sup>.

Although the above difficulties have long been appreciated and have been explained in mechanistic terms, a unifying treatment had been lacking. In order to provide such a general formulation Kedem and Essig examined the theory of isotope flows in terms of nonequilibrium thermodynamics<sup>9</sup>. In this view both of the above anomalies can be attributed quite generally to coupling between the flows of the abundant and tracer species ("isotope interaction"). The formulation is particularly simple if net flow of the test species is the result only of its electrochemical potential difference, so that the phenomenological resistance coefficient for net flow is given simply by

$$R = X/J. \quad (2)$$

In the presence of isotope interaction, R is unequal to the exchange resistance  $R^X$ , where

$$R^X = (-RT \Delta\mu/J^X)_{J=0}. \quad (3)$$

(Here J and  $J^X$  are the net flow and tracer flow respectively and  $\Delta\mu$  is the difference in specific activity across the membrane.) Furthermore, with isotope interaction the flux ratio is abnormal even in the absence of solvent drag and active transport, being given by

$$f = \exp [(R^X / R) (X/RT)]. \quad (4)$$

In the absence of isotope interaction  $R^X = R$ , and equation (4) reduces to equation (1).

Although the broad validity of the assumptions of the above formulation suggests its general usefulness, it has not been tested experimentally. An opportunity to do so was provided by Gottlieb and Sollner's recent observations that collodion membranes activated with certain quaternary ammonium polyelectrolytes show tracer exchange rates greater than predicted from their electrical resistance<sup>10</sup>. The present study demonstrates that the flux ratio in these membranes is "abnormal",

according to equation (1), and that its value is predicted quantitatively by the modified expression of equation (4).

Membranes were prepared by adsorption of polyvinyl benzyl trimethyl ammonium chloride (PVBT, Dow Chemical Co. ) on a collodion matrix, as described previously <sup>10,11</sup>. Their usefulness for the present purpose is enhanced by their near-ideal permselectivity, permitting the evaluation of net anion flow from electrical current. The membranes were exposed at each surface to 0.1 M KCl. R was evaluated from the electrical resistance and  $R^X$  from the self-diffusion of Cl-36 (Table VIII). In several membranes it was possible to reproduce the effect noted by Gottlieb and Sollner, with values of  $R^X/R$  as low as 0.42.

Membranes with unequal values of  $R^X$  and R permitted the testing of the modified flux ratio equation. To do this, unidirectional fluxes were evaluated from two series of measurements of tracer flow, one with and one against the electrical potential gradient. As is seen, the flux ratio  $f$ , abnormal according to equation (1), is closely predicted by equation (4). An unanticipated finding was that not all PVBT-collodion membranes showed a significant difference between  $R^X$  and R.\* In these membranes, which served as useful controls, the flux ratio was "normal".\*\*

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\* Dr. M. Gottlieb has also noted this variability in PVBT-collodion membranes (personal communication). The factors accounting for the variability of  $R^X/R$  are as yet undefined.

\*\* It is believed that electroosmotic flow is insignificant in the membranes employed here <sup>10</sup>. If appreciable, the values of  $R^X/R$  would be smaller than reported, but the fundamental formulation (equation (29) of reference (9)) remains valid.

The present results substantiate the thermodynamic formulation presented previously, and therefore support the view that anomalous tracer permeability coefficients and flux ratios are due to coupling of isotope flows (in this case negative coupling)<sup>9</sup>. In biological membranes flux ratios which lie between  $\exp(X/RT)$  and unity as observed here are usually attributed to exchange diffusion by means of mobile carriers<sup>2</sup>. However, other mechanisms are plausible on theoretical grounds<sup>12-14</sup>. The unlikelihood of carriers traversing the synthetic membranes studied here suggests that such carriers may not in fact be the mechanism of abnormal flux ratios in biological membranes.

We thank Dr. M. Gottlieb and Dr. K. Sollner for samples of membranes and helpful advice and D. Nadel for skillful technical assistance.

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Membrane	$\Delta\psi$ (mv)	$(10^7 R^X$ Kcal-cm <sup>2</sup> sec-mole <sup>-2</sup> )	$R^X/R$	exp (X/ <u>RT</u> )	f	exp ( $\frac{R^X X}{R \underline{RT}}$ )
D	10	14.1	0.71	1.48	1.34	1.32
D	25	14.1	0.69	2.65	2.05	1.96
A	25	14.8	0.44	2.65	1.56	1.53
A	50	14.8	0.42	7.02	2.33	2.26
K	50	35.9	0.57	7.02	3.17	3.01
H	50	73.0	0.60	7.02	3.02	3.22
H	75	64.9	0.61	18.59	4.96	5.89
H	75	42.0	0.53	18.59	4.48	4.69
F	10	18.8	1.04	1.48	1.50	1.50
F	10	18.1	0.97	1.48	1.51	1.47
E	25	113	1.00	2.65	2.80	2.68
E	50	108	1.07	7.02	7.88	8.01

Table VIII Theoretical and observed flux ratios in PVBT-collodion membranes. Exp (X/RT) is the flux ratio predicted by equation (1), f is the observed flux ratio, and  $\exp(\frac{R^X X}{R \underline{RT}})$  is the flux ratio predicted by equation (4). These quantities were evaluated as follows: Membranes in lucite chambers were exposed at each surface to 0.1 M KCl. The "outer" solution (left side) contained high specific activity KCl<sup>36</sup>. The electrical potential difference ( $\Delta\psi = \psi^{in} - \psi^{out}$ ) was measured by means of calomel half cells and 3 M KCl-agar bridges apposed to the membranes. Ag-AgCl electrodes and a voltage clamp were used to set  $\Delta\psi$  as desired. The D. C. electrical resistance  $R$  of 1 cm<sup>2</sup> of membrane was determined from Ohm's law, transiently setting the potential at  $\pm 10$  mv. Since the chloride transport number was  $>0.99$ , the phenomenological resistance coefficient was taken as  $R=F^2R$ , where F is the Faraday. Tracer fluxes  $J^X$  and "unidirectional fluxes"  $J^X/\Delta\rho$  were determined in three series of experiments, with  $\Delta\psi$  zero, positive, and negative respectively.

Following each series (three or four 10-15 minute periods) cold solutions were replaced. At zero potential difference  $J = 0$ , permitting the calculation of  $R^x$  from equation (3). The flux ratio was calculated from  $f = \text{influx/outflux}$ , taking the mean value of  $(J^x/\Delta\rho)_{\Delta\psi +}$  as the influx and that of  $(J^x/\Delta\rho)_{\Delta\psi -}$  as the outflux. This means of determining the outflux is experimentally simpler than reversal of the hot and cold sides. The use of equation (28) of reference (9), which incorporates the effect of backflux precisely, did not alter  $f$  significantly.

## V. Coupling of urea flows in the toad bladder

( Work done with P.D. Lief )

### INTRODUCTION

Because of its importance in the renal concentrating system, the mechanism of urea transport is of considerable interest. Amphibian epithelia are useful model systems and have directed attention to three types of interactions.

First, interaction of urea with the membrane, as evaluated by tracer urea permeability in the frog skin and toad bladder, has allowed speculation as to the mechanism of permeation (1,2). Second, the interaction of urea with solvent water (solvent drag) has supported the thesis that urea and water share a common pathway across these tissues (3,4). A third type of interaction, that of urea with other solute particles such as manitol or sucrose, has been observed in the frog skin and toad skin (4-8). This effect has appeared to be relatively non-specific, having been demonstrated for various solute pairs.

One case of solute-solute interaction, that in which the tracer and abundant species are chemically identical, is of special interest. Several investigators have touched upon such interaction for urea, but their findings and interpretations are not completely consistent. In the toad skin, Ussing described a net movement of tracer urea induced by a gradient for abundant urea in the absence of a tracer gradient. This was attributed to solvent drag caused by the abundant urea, directed anomalously due to specific peculiarities of tissue structure (4). More recently, while describing the effects of abundant hypertonic urea upon toad bladder, Urakabe, Handler and Orloff /commented that they did not observe evidence of interaction with tracer urea (9).

The special case of identical solute coupling (or "isotope interaction") is of fundamental importance in biology, since tracer substances are widely used to estimate both the permeabilities of parent species and the forces promoting transport. As has been shown, moreover, demonstration of coupling can give insights into the nature of membranes and of solute permeation of those membranes. Whereas interpretations of interaction between chemically different solutes are necessarily somewhat ambiguous, clearly both tracer and abundant urea will traverse the same pathway. Urea-urea coupling has been <sup>observed in specialized</sup> ~~synthetic~~ systems, but the degree of interaction is small in dilute aqueous solutions (10). In the present study we investigated isotope interaction and related phenomena in the urinary bladder of the toad.

## METHODS

I. Biological Membranes - General Methods: Bufo marinus toads from the Dominican Republic (National Reagents, Bridgeport, Connecticut) were kept on damp paper without feeding and sprinkled with tap water daily. After the animals were doubly pithed the bladders were removed, rinsed in 50 ml. of sodium Ringer's solution (NaR) <sup>(Na 113.5, Cl 116.9, K 4.0, HCO<sub>3</sub>, 2.4, Ca 1.8 meq/L.)</sup> three times to remove endogenous antidiuretic hormone and, for coupling studies, mounted in standard Ussing-Zerahn lucite chambers of 7.54 cm<sup>2</sup> area, fitted with 3.0 molar KCl agar bridges. Open circuit potential <sub>/a</sub> was determined with Keithley VTVM potentiometer and the transmembrane potential was controlled with a voltage clamp. Membranes were short-circuited throughout except for brief periods for the determination of electrical resistance.

When employed, antidiuretic hormone (Pitressin, Parke Davis) (ADH) was added to the serosal bath to give a concentration of some 130 mU/ml.

Studies of volume flow ( $J_v$ ) were performed in a modified chamber in which one side was completely sealed except for a calibrated horizontal pipette; stirring was accomplished by magnetic rotors.

II. Selection of Paired Tissues: Both hemibladders from a single toad were removed, rinsed, mounted in identical chambers, and bathed in NaR. Following 15-30 minutes of equilibration the open circuit potential was measured. All pairs in which either hemibladder had a value of less than 15.0 mv were discarded. Membranes which were retained were shortcircuited, and after an additional 15 to 30 minutes, the electrical resistance (R) was determined by rapidly varying the transmembrane potential from -10.0 to +10.0 mv and observing the resulting change in steady-state current. Prior studies have shown that the current-voltage relationship is linear in this range in the toad bladder (11) permitting the calculation of R from Ohm's law. All pairs in which either membrane had a resistance of less than 300 ohms were discarded (except for certain preliminary experiments). Membranes which satisfied the above criteria were returned to the short-circuited state and utilized in one of the following protocols.

III. Protocols - Toad Bladder:

A. Preliminary Experiments: Tracer C-14 urea (New England Nuclear Corporation, Boston, Massachusetts) was added to both mucosal (outer) baths or to both serosal (inner) baths of a pair. Non-radioactive urea was added to the mucosal bath or to the serosal bath of one hemibladder of each pair to produce a concentration of 0.3 M urea. Following equilibration, samples for measurement of radioactivity were taken at 0, 30, and 60 minutes.

Specimens of unmounted tissue were taken initially for determination of per cent tissue water; at the conclusion of the experiment, the tissue contained within the chamber was taken for determination of tissue water and labelling by C-14. Specimens were weighed immediately on a Mettler balance to within 10  $\mu$ g, then dessicated at 95°C for 24 hours and weighed again following return to room temperature. Specimens for determination of C-14 labelling were pulverized and dissolved in measured volumes of 10 per cent trichloroacetic acid according to the technique of Leaf et al (2).

B. Coupling of Flows - 0.3 M Urea: Tracer C-14 urea was added to the mucosal bath of one and to the serosal bath of the other hemibladder of a pair. After 15 minutes of equilibration, initial, 30 and 60 minute samples were taken. Following this control period, non-radioactive urea was added either to both mucosal or to both serosal baths to produce a concentration of 0.3 M urea (hypertonic period). After 15 minutes of re-equilibration initial, 30 and 60 minute samples were again obtained. Following this, ADH was administered. After waiting 10 minutes for the onset of peak effect, initial, 30 and 60 minute samples were again taken, constituting the hypertonic-ADH period.

C. Coupling of Flows - Long Control Period: This protocol was similar to that of III-B, but with a 120 minute control period, and a 90 minute hypertonic period, and no hypertonic-ADH period.

D. Coupling of Flows - 0.1 M Urea: The protocol was identical to that of III-B except that the concentration of abundant urea was 0.1 M.

E. Estimation of Reflection Coefficient for Urea: Unpaired membranes mounted in the volume flow chamber were bathed with 0.3 M urea in NaR at one surface (either mucosal (series I) or serosal (series II)) and 0.2 M sucrose in NaR at the other surface. Measurements of

volume flow were carried out at 5 minute intervals for 15 to 30 minutes. Following the addition of ADH, measurements of  $J_V$  were continued for another 15 to 30 minutes.

The added solute concentration of the serosal bath was then raised to 0.3 M sucrose in series I or 0.45 M urea in series II and  $J_V$  was measured for a final 15 to 30 minutes.

F. Attempts to Abolish Water Flow: This protocol was identical to that of III-B except that in the hypertonic period sucrose was added to give a concentration of 0.2 M in the /opposite solutions to those containing 0.3 M urea.

IV. Analysis of C-14 Activity: For each flux period 100-500  $\mu$ l samples were taken from each bath and placed in sealed vials containing 15.0 ml of liquid scintillation fluid (17). Samples were counted on the day of the experiment whenever possible, or at latest within 96 hours. If not counted promptly samples were stored in a cool, dark place; such samples showed no change in counts for periods of up to four weeks. Samples were counted in a Packard Tri-Carb liquid Scintillation Counter at settings with a counting efficiency of greater than 95 per cent. Quenching by NaR, 0.3 M urea, 0.2 M sucrose, or ADH was negligible.

V. Analysis of Data:

Tissue water was expressed as  $\% \text{ TW} = (1 - \frac{\text{Dry wt.}}{\text{Wet wt.}}) \times 100$ .

Tissue labelling was expressed as  $\% \text{ TI} = \frac{\text{counts per minute per ml tissue H}_2\text{O}}{\text{counts per minute per ml 'hot' bath}} \times 100$ .

Tracer permeability was evaluated as  $-J^X/\Delta c^X$ , where  $J^X$  is the tracer flux per unit area per unit time, and  $-\Delta c^X$  is the concentration difference of the tracer across the membrane. Placement of radioactivity in the outer or inner solution permitted the determination of inward or outward tracer permeability, denoted  $\vec{J}^X/\Delta c^X$  or  $\overleftarrow{J}^X/\Delta c^X$  respectively.

Statistical analyses were carried out as described by Snedecor<sup>12</sup>. Means were compared by analysis of variance. Slopes and intercepts were calculated by the method of least squares. Comparison of slopes and intercepts was accomplished by analysis of covariance; p values  $> .05$  were considered insignificant and were not reported.

## RESULTS

### I. Distribution of Permeability Data

Preliminary inspection of the values for urea permeability ( $-J^*/\Delta c^*$ ) showed a skewed distribution of the data (Figure 15a). Conversion to logarithms produced a more normal distribution (Figure 15b). Normal equivalent deviate analysis confirmed this impression. Consequently, all values of ( $-J^*/\Delta c^*$ ) were converted to logarithms for statistical analysis, and all average values cited are geometrical means.

### II. Preliminary Experiments - Effects of Hypertonic Urea.

Bidirectional tracer permeabilities were determined in 12 pairs of hemibladders in which one hemibladder was treated with 0.3 M urea in either the mucosal (M) or the serosal (S) bath, with the other serving as a control. Results are shown in Table IX. The addition of urea to the mucosal bath resulted in bidirectional tracer permeabilities. Conversely, in 5 of 6 hemibladders the addition of urea to the serosal bath resulted in decreases of tracer permeability. The effects on tracer permeability paralleled the effects on electrical conductivity ( $1/\kappa$ ). In the one anomalous experiment in which there was a rise in  $\vec{J}^x/\Delta c^x$  in the presence of hypertonic serosal urea there was also a rise in electrical conductivity.

The water content of these 24 hemibladders was  $80.18 \pm 0.77\%$ , a value similar to that of Leaf and Hays (2).

Effects on C-14 tissue labelling are shown in Table X. Hypertonic urea resulted in an increase in labelling from the mucosal bath whether ~~(T) or (X)~~ added to either M or S. There were no consistent effects on labelling from the serosal bath.

Because of the marked and inconsistent effects of hypertonic urea on intrinsic membrane permeability these experiments provided no evidence concerning possible interaction between abundant and tracer urea flows.

III. Coupling of Flows - 0.3 M Urea: In order to investigate the possibility of interaction of urea flows we carried out experiments in which the hemibladders from a single animal were treated identically with respect to abundant urea, one membrane then being used for the determination of inward tracer permeability and the other for outward tracer permeability. In the presence of coupling to the flow of abundant urea it would be expected that the two would be affected differently. In order to interpret such differences, however, it was necessary to ascertain the relationship between the two tracer permeabilities in the absence of abundant urea.

A. Control Period - Data from 32 experiments prior to the addition of hypertonic urea are shown in Figure 16.

Figure 14

Over a wide range of values ( $1.07-234 \times 10^{-7} \text{cm-sec}^{-1}$ ) there was an impressive correlation between the inward and outward tracer permeabilities of paired hemibladders (correlation coefficient = 0.94;  $p < .001$ ). Plotting the logarithms of these data gives the least squares line  $y = 1.015x - 0.033$ . Since the intercept differs insignificantly from zero

this justifies the calculation of a line passing through the origin and the evaluation of a corrected slope. Both the original slope and the corrected slope (0.995) differ insignificantly from 1.000, indicating absolute equality of the two tracer permeabilities. This permits the construction of a ratio of tracer permeabilities  $r = (\bar{J}^{\bar{x}}/\Delta c^x) / (\bar{J}^{\bar{x}}/\Delta c^x)$  for each pair of hemibladders, which provides a convenient means of analysis of the data.

In eight experiments the effect of time was investigated. The corrected slope in the first hour was 0.995, and in the second hour was 0.982. The two values differed insignificantly from each other and from 1.000.

B. Mucosal Hypertonicity - In 11 experiments, following the control period, non-radioactive urea was added to the mucosal baths of both hemibladders to produce a concentration of 0.3 M. Increases in the bidirectional tracer permeabilities and conductivities were noted, as in the preliminary experiments. However, the comparison of  $r$  in the control and hypertonic periods permitted the evaluation of coupling.

#### Figure 17

These data are presented in Figure 17. The (geometric) mean of  $r$  increased significantly from 0.832 (mean  $\log r = 0.087 \pm 0.037$ ), ( $p < .001$ ) to 1.22 (mean  $\log r = 0.080 + 0.075$ ) in the control period. These results indicate coupling of flows of abundant and tracer urea. At the end of this period, ADH was added. This resulted in marked increases in both tracer permeabilities for urea, as expected. In addition, there was a further significant increase in  $r$ , to a mean value of 1.82 (mean  $\log r = 0.261 \pm 0.056$ ) ( $p < 0.001$ ) in the hypertonic -ADH period. Again this is consistent with coupling of flows.

C. Serosal Hypertonicity: In 12 experiments following the control period urea was added to the serosal bath to a concentration of 0.3 M. As in the preliminary experiments, decreases in tracer permeabilities and conductivities were noted. Examination of  $r$  again

permitted the evaluation of coupling (Figure 18)

### Figure 18

Mean  $r$  decreased significantly from 1.09 (mean  $\log r = 0.037 \pm 0.038$ ) in the control period to 0.585 (mean  $\log r = -0.233 \pm 0.049$ ) in the hypertonic period ( $p < .001$ ). Again, the subsequent addition of antidiuretic hormone produced the expected increases in tracer permeabilities. However, the mean value of  $r = 0.644$  ( $\log r = -0.191 \pm 0.035$ ) was not significantly different in the hypertonic-ADH period. Again, the results were consistent with the coupling of flows of abundant and tracer urea both in the absence and presence of ADH.

It should be noted that in each case the above observed effects on  $r$  were demonstrated in the face of opposing influences of volume flow and solvent drag.

#### IV. Coupling of Flows - 0.1 M Urea.

In contrast to the preceding studies with 0.3 M urea, the effects of 0.1 urea<sup>/M</sup> on tracer permeabilities and electrical conductivities were not consistent. Furthermore, no significant change of  $r$  was noted in the hypertonic or hypertonic-ADH periods, either in 4 experiments with mucosal hypertonicity or in 4 experiments with serosal hypertonicity. Thus we were unable to demonstrate coupling at this concentration of urea.

V. Attempt to Abolish Volume Flow: As noted in Section III, effects on  $r$  were demonstrated despite opposing influences of volume flow and solvent drag. Therefore, in order to demonstrate the extent of isotope interaction more precisely, an attempt was made to study coupling while abolishing the volume flow resulting from the concentration gradient of urea.

It has been reported previously that in the toad bladder the reflection coefficient for urea is 0.67 and that for sucrose is 1.0 ( ). Accordingly, 0.2M sucrose should approximately balance the osmotic effect of 0.3 M urea. This was tested by exposing the tissues to 0.3 M urea in NaR at either surface, and to 0.2M sucrose in NaR at the other. Volume flow was reduced from an expected value of some  $200 \mu\text{l}/\text{cm}^2\text{-hr.}$  for 0.3M urea/NaR ( ) to undetectable levels (less than  $10 \mu\text{l}/\text{cm}^2\text{-hr.}$ ). When additional solute was later added to either bath volume flows increased promptly, and in proportion to the new osmotic gradient, thus sustaining the validity of the previous negative observations.

Since 0.2 M sucrose essentially abolished the water flow induced by 0.3 M urea, determinations of inward and outward tracer permeabilities were carried out in 10 pairs of membranes, utilizing these balanced solutions. However, effects on tracer permeabilities and conductivities were erratic. Further, no consistent effects on  $r$  were observed in either the hypertonic or the hypertonic-ADH periods in any group of experiments with balanced tonicity.

#### DISCUSSION

In the presence of appreciable isotope interaction a tracer permeability coefficient will fail to quantify the permeability for net flows ( ). Coupling of abundant and tracer urea flows in the toad bladder is slight, and of no importance in this regard. However, the demonstration of coupling is of interest for two reasons; first, as an illustration of a technique which allows the precise demonstration of small effects <sup>/on</sup> tissue permeability, and second, in its implications as to the mechanism of urea permeation of the toad bladder.

It might be anticipated that urea-urea interaction would be difficult to demonstrate. In an analysis of data from the literature Curran, Taylor and Solomon pointed out that at the concentrations which we employed, effects of isotope interaction in nonselective membranes would be hidden by the error involved in estimating unidirectional fluxes (10). Our preliminary experiments in single membranes confirmed the fact that changes in tracer permeability with time, whether spontaneous or induced by hypertonic urea, were far greater than attributable to coupling. We therefore abandoned this approach for one permitting the comparison of bidirectional tracer flows.

Ideally, to minimize non-specific effects one would simultaneously evaluate both flows in a single membrane. While this has been done previously in studies of urea in the toad bladder (2), it requires the use of  $N^{15}$  urea and mass spectrometry, which were unavailable to us. Instead, therefore, we used two closely paired membranes exposed to tracer urea at opposite surfaces. Close pairing meant that both hemibladders were from a single toad; that they were removed, handled and mounted at about the same time; <sup>/that</sup> they were screened according to the same electrical criteria; and that they were treated identically (including exposure to abundant solute and ADH) throughout the experiment. By careful attention to these details, we were able to obtain an excellent correlation between simultaneously determined bidirectional permeability coefficients in paired tissues (Figure 16) even when the control observations were extended to two hours.

An explanation of how paired tissues can distinguish intrinsic tissue permeability effects from effects attributable to coupling is shown in Figure 19. The upper panel shows the equivalence of control bidirectional tracer permeabilities. The addition of

hypertonic solute to the same side of each membrane (center panel) may cause large increases (or decreases) in tracer flows, but in the absence of coupling, the effects in each tissue should be the same, and the equivalence of the two permeabilities would be maintained. In contradistinction, in the presence of coupling the two flows will be affected differently as illustrated in the lower panel. Hence, in spite of marked changes in both tracer permeabilities, coupling is identified and quantified by a change in the value of  $r$  from 1. It should be pointed out that it is possible to demonstrate coupling even if the control value of  $r$  differs from 1, although less conveniently. This will be discussed in the next paper ( ).

By means of this analysis, in our studies with 0.3 M urea, coupling was shown with consistency. Regardless of whether solute was added to the mucosal bath (enhancing permeability) or the serosal bath (depressing permeability), positive solute-solute interaction was demonstrated.

From inspection of Figures 17 and 18 it is seen that, prior to the addition of ADH, the ratio  $r$  was affected more by the addition of abundant urea to the serosal bath than to the mucosal bath. A possible explanation for this observation may be formulated if the membrane is considered to have two discrete pathways for urea permeation, the first, a non-selective leak pathway the second a selective, ADH sensitive channel. Both the studies of Curran et al (10) and our observations in large-pore synthetic membranes indicate that coupling is unlikely to be significant in a non-selective aqueous channel. If so, conditions which increase the relative contribution of the non-selective channel might be expected to obscure coupling effects. Conversely, a relative reduction of the

/pathway

permeability of the non-selective should facilitate the demonstration of coupling.

In the present studies, the effect of leak was minimized by rejection of membranes of low initial resistance, and by careful handling of the tissues. After addition of hypertonic urea to the mucosal medium the electrical resistance fell precipitously, suggesting the opening of large leaks; in these membranes coupling effects were small.

On the other hand, in membranes in which urea was added to the serosal baths the electrical resistance remained unchanged or rose, ruling out the existence of large leaks; here the observed coupling was greater. The inverse relationship between the magnitude of passive leak pathways (as indicated by the electrical resistance) and the magnitude of coupling is striking, and lends support to the concept that coupling does not occur in the non-selective pathway. It is of interest that following the administration of ADH, in contrast to the findings in its absence,  $r$  was more markedly affected by hypertonicity in the mucosal bath than in the serosal bath. This might possibly be a consequence of solvent drag. Studies of Bentley in the toad bladder indicate that, for a given osmotic gradient, volume flow in the direction  $M \rightarrow S$  is greater than in the direction  $S \leftarrow M$  (15, 16).  
/therefore be expected to be greater with hypertonic  $S$  than with hypertonic  $M$ .  
Since solvent drag would directly oppose and obscure coupling effects, the experimental data are consistent with this formulation.

An attempt was made to minimize water flow and solvent drag by the use of osmotically balanced solutions in the hope that the true magnitude of coupling would be thereby revealed. Unfortunately, the effects of addition of solute to both bathing solutions were inconsistent. These failures underscored the necessity for careful pairing of tissues in order to illustrate a small effect.

Further difficulties were encountered in the studies utilizing a lower concentration of ~~max~~ urea, 0.1M. Here, despite closely similar behavior of paired tissues, coupling could not be demonstrated. Since technical and methodologic errors were not apparent, we concluded that the magnitude of coupling was too small to detect by the present techniques.

In summary, a method is presented which has allowed the demonstration of isotope interaction. The effect is small, and requires the presence of a relatively large driving force of the abundant solute. Coupling of solute flows can be shown to occur in face of the opposing effects of solvent drag. Our data suggest that the specific urea-urea interaction probably does not occur in aqueous channels of synthetic membranes or in the non-specific leak pathway of the toad bladder, rather it seems to occur in a selective pathway, presumably non-aqueous in part.

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TABLE IX

A.	<u>Control Period</u>	<u>0.3M Urea to M</u>
	$(\vec{J}^*/\Delta c^*) (10^{-7} \text{ cm sec}^{-1})$	
	25.2	46.2
	6.7	57.6
	18.3	66.7
Mean	16.7 $\pm$ 5.4	56.8 $\pm$ 5.9
	$(\overleftarrow{J}^*/\Delta c^*) (10^{-7} \text{ cm sec}^{-1})$	
	7.2	71.3
	9.5	39.0
	4.3	67.4
Mean	7.0 $\pm$ 1.5	59.2 $\pm$ 10.2
B.	<u>Control Period</u>	<u>0.3 M Urea to S</u>
	$(\vec{J}^*/\Delta c^*) (10^{-7} \text{ cm sec}^{-1})$	
	19.4	94.7
	93.5	54.3
	3.2	1.8
Mean	38.7 $\pm$ 27.8	50.3 $\pm$ 26.9
	$(\overleftarrow{J}^*/\Delta c^*) (10^{-7} \text{ cm sec}^{-1})$	
	68.3	25.8
	11.7	8.2
	5.4	3.0
Mean	28.5 $\pm$ 20.0	12.3 $\pm$ 6.9

TABLE X

## A. Labeling from the mucosal bath (%)

	<u>Control Tissue</u>	<u>0.3M Urea to M</u>
	5.6	7.7
	4.8	12.1
	4.6	8.9
Mean	5.0 $\pm$ 0.3	9.6 $\pm$ 1.3

	<u>Control Tissue</u>	<u>0.3M Urea to S</u>
	5.0	7.7
	7.2	5.1
	3.2	15.4
Mean	5.1 $\pm$ 1.2	9.4 $\pm$ 3.1

## B. Labeling from the serosal bath (%)

	<u>Control Tissue</u>	<u>0.3M Urea to M</u>
	76.9	77.1
	145.7	137.6
	75.1	77.4
Mean	99.2 $\pm$ 23.2	97.4 $\pm$ 20.1

	<u>Control Tissue</u>	<u>0.3M Urea to S</u>
	67.5	67.7
	82.5	87.0
	84.0	94.3
Mean	78.0 $\pm$ 5.3	83.0 $\pm$ 7.9

Fig. 15 a

### DISTRIBUTION OF PERMEABILITY COEFFICIENTS

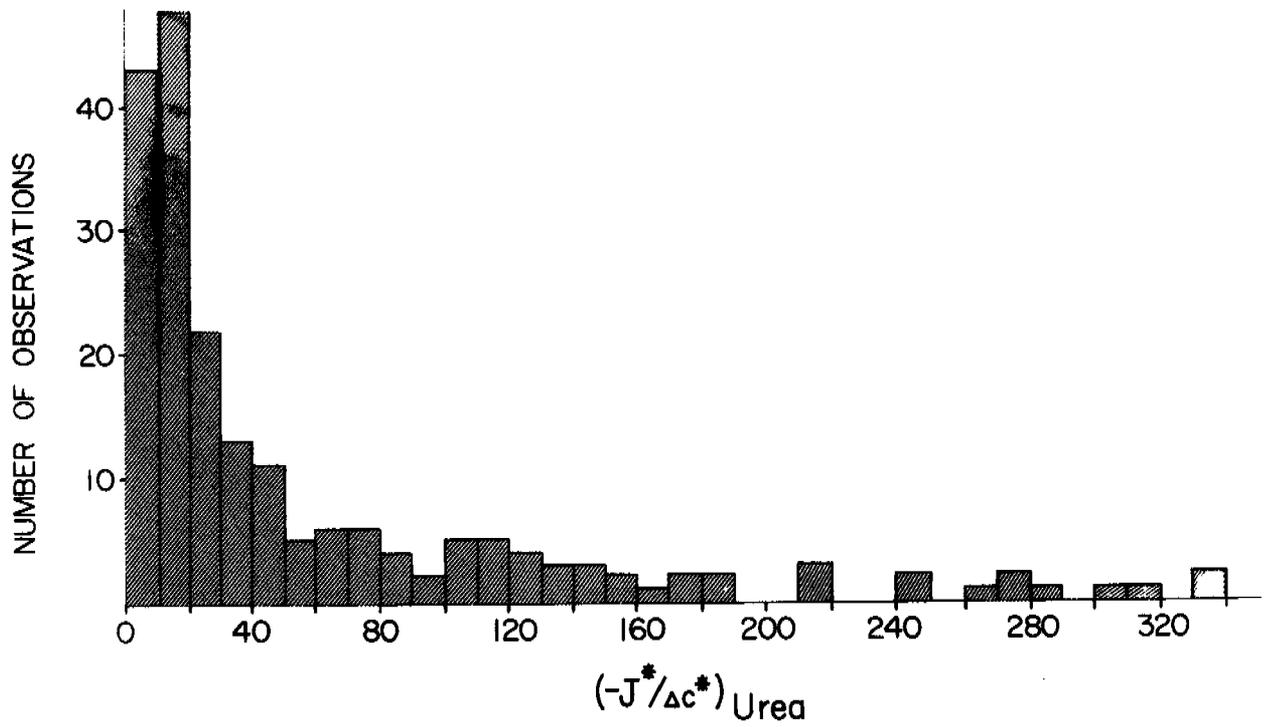
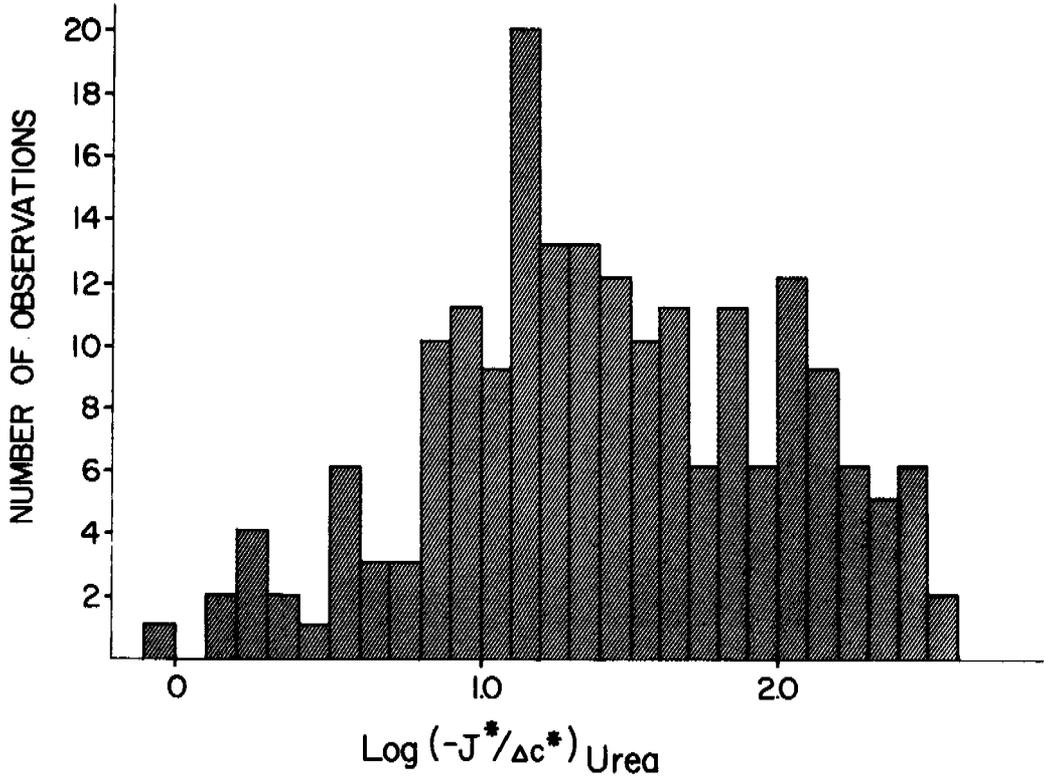


Fig. 15 b

### DISTRIBUTION OF PERMEABILITY COEFFICIENTS



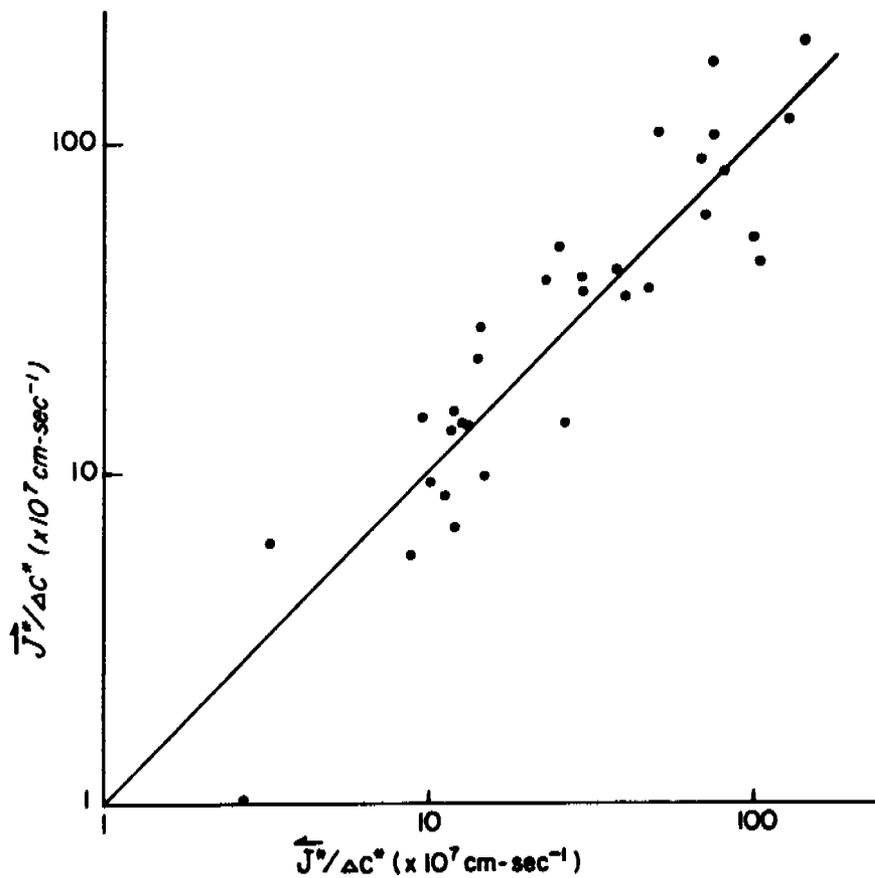


Fig. 16

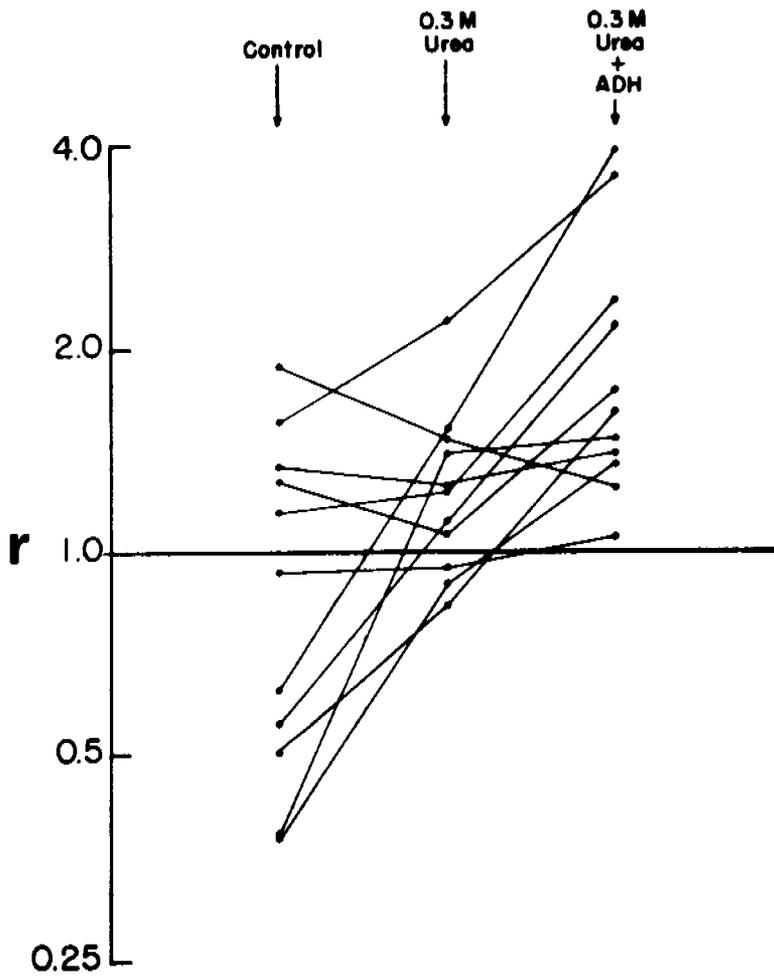


Fig. 17

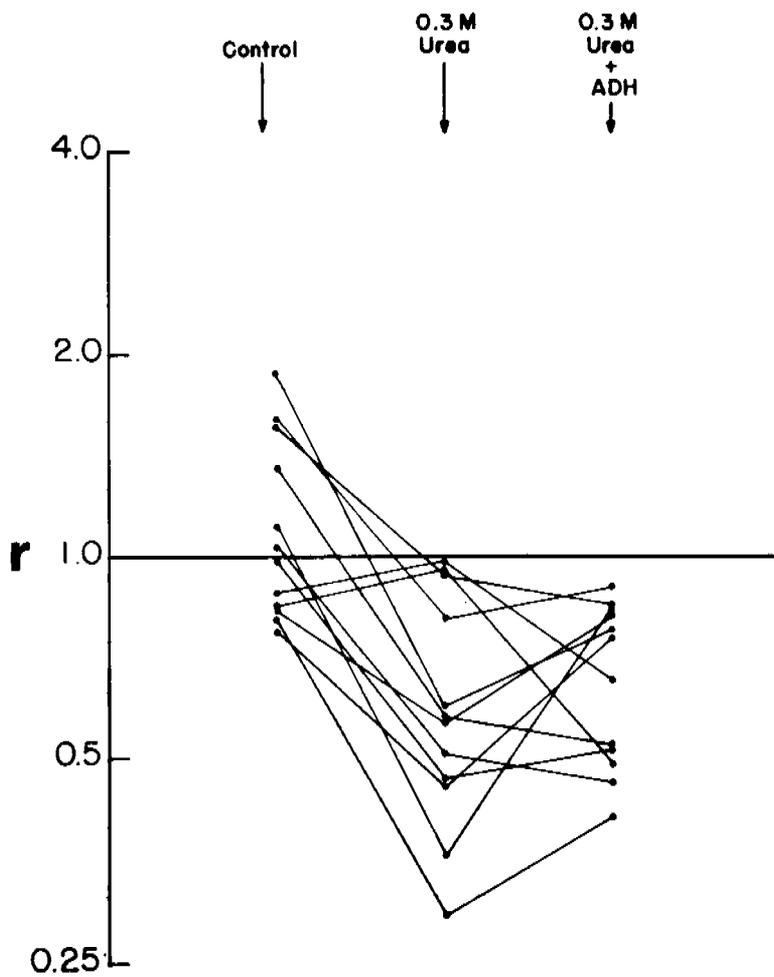


Fig. 18

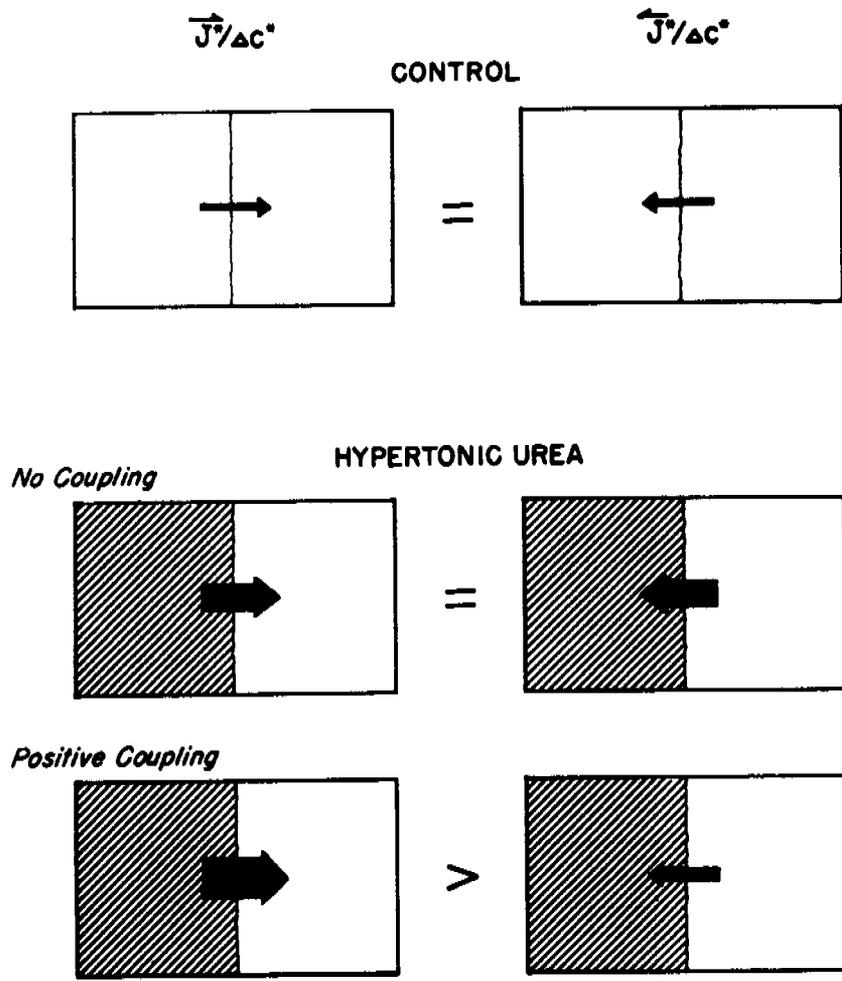


Fig. 19