

# Energetics of Coupled Active Transport of Sodium and Chloride

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**ABSTRACT** A Clark electrode was used to measure oxygen consumption by the gall bladder, in which there is a direct and one-to-one linkage between active Na and active Cl transport.  $O_2$  uptake was reversibly depressed when Cl in the mucosal bathing solution was replaced by a poorly transported anion, such as sulfate. This effect of Cl was abolished by ouabain or in Na-free solutions. When the anion was chloride, treatment with ouabain or replacement of Na by a poorly transported cation depressed  $Q_{O_2}$  more than did replacement of Cl. However, ouabain or removal of Na also depressed  $Q_{O_2}$  in  $Na_2SO_4$  solutions, in which salt transport is minimal. It is concluded that oxygen uptake in the gall bladder consists of three fractions: 9% requires both Na and Cl, is inhibited by ouabain, and is linked to the NaCl pump; 36% requires Na but not Cl, is inhibited by ouabain, and possibly is linked to the cellular K uptake mechanism; and 55% represents basal uptake. If the extra oxygen uptake observed during transport supplies all the energy for transport, then 25 Na + 25 Cl ions are transported actively per  $O_2$  consumed; i.e., twice as many ions as in epithelia which transport only Na actively. This extra uptake is more than sufficient to supply the energy for overcoming internal membrane resistance under the experimental conditions used.

## INTRODUCTION

In the majority of epithelia secreting or absorbing salt, the coupling between cation and anion transport is electrical. For example, the active transport of sodium across frog skin (Ussing and Zerahn, 1951) and urinary bladder (Leaf, Anderson, and Page, 1958) sets up an electrical potential difference ( $\rho D$ ) which causes anions to follow passively. The observation of net sodium fluxes unaccompanied by anions in the short-circuited state, and the persistence of large  $\rho D$ 's due to the sodium pump in the presence of such non-absorbable anions as sulfate, demonstrate that the functioning of these cation transport mechanisms is independent of anions. An exception to this pattern of electrical coupling and independent transport mechanisms is provided by

the gall bladder, in which the active transport of one Na ion appears to be coupled directly and obligatorily with the active transport of one Cl ion. The existing evidence for this neutral NaCl pump consists of the following electrical observations: simultaneous transport of both Na and Cl against their electrochemical activity gradients; absence of a short-circuit current or PD associated with salt transport; absence of the PD expected for an independent Na pump in solutions of nontransported anions; and absence of the PD expected for an independent Cl pump in solutions of nontransported cations (Diamond, 1962 *a, b*; Wheeler, 1963; Dietschy, 1964; Pidot and Diamond, 1964; Diamond and Harrison, 1966). Similar electrically neutral cation-and-anion pumps have been postulated for intestine (Barry, Smyth, and Wright, 1965; House and Green, 1965) and for beetroot (Poole, 1966).

The present paper is concerned with the relationship between NaCl transport and oxygen consumption in the gall bladder. In those tissues in which the Na pump is independent of anions, there is a component of oxygen consumption which requires the presence of Na and is stoichiometrically related to Na transport (frog skin: Zerahn, 1956; urinary bladder: Leaf and Dempsey, 1960). If the concept of directly coupled Na and Cl pumps in the gall bladder is valid, this might be expected to reveal itself as a fraction of oxygen consumption requiring both Na and Cl simultaneously. From the stoichiometry one can calculate whether a NaCl pump utilizes the same or double the amount of metabolic energy as a Na pump alone. Accordingly we have measured the effect on oxygen consumption of three procedures known to inhibit transport: replacement of chloride with a poorly transported anion, replacement of sodium with a poorly transported cation, and treatment with ouabain. A preliminary account of some of this work has been given (Diamond and Martin, 1966).

#### METHODS

*Measurement of Oxygen Consumption* Oxygen uptake of rabbit gall bladder in vitro was determined by observing the rate at which oxygen tension decreased in a closed vessel (volume  $10.0 \pm 0.1$  ml) containing the gall bladder. Oxygen tension ( $p_{O_2}$ ) was measured with a Clark oxygen electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio), whose current output is directly proportional to  $p_{O_2}$ . The current output was passed across a variable resistor, the potential difference across which was recorded graphically on a Varian G-11 recorder. The electrode was calibrated by measuring its output when the vessel contained solutions equilibrated with air, 100% oxygen, or nitrogen-oxygen mixtures (40.0%, 59.3%, or 80.1%  $O_2$ , analyzed to within  $\pm 0.1\%$   $O_2$ ). With the circuit sensitivity set so that 100%  $O_2$  gave a reading of 50 mv, the voltage output was found to be a linear function (within  $\pm 3\%$ ) of oxygen tension for tensions less than 80% of atmospheric pressure. A single calibration constant of 2.03%  $O_2$ /mv, representing the mean value from electrode calibrations performed on three separate occasions during the course of the experi-

ments, could thus be used for all calculations of oxygen consumption. The calibration was checked in each experiment by measuring the electrode response in air and in 100% O<sub>2</sub>. A linear scale based on this calibration constant was superimposed on the voltage axis of the voltage-vs.-time record to convert it into a  $p_{O_2}$  axis. The solubility coefficient of oxygen was taken as 0.0244. A correction was made for barometric

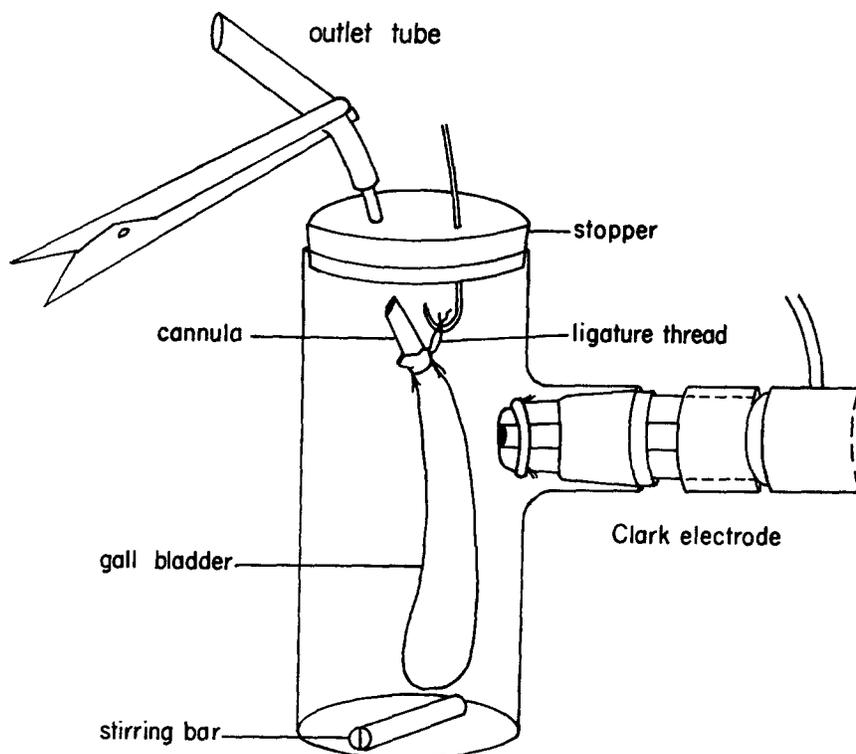


FIGURE 1. Glass vessel used for measuring oxygen consumption by the gall bladder. The gall bladder is suspended from a hook in the vessel stopper by a loop in the ligature securing its cannula. The cannula can be stoppered with a glass plug when the solutions inside and outside the gall bladder differ in composition. Air bubbles are expelled via the outlet tube (normally closed by a hemostat) when the vessel is being filled. The vessel is enclosed in a water jacket (not shown) for temperature regulation.

pressure each day. Thus if the slope of voltage output against time was found to be  $x$  mv/hr, the rate of oxygen consumption was calculated (in  $\mu\text{l O}_2/\text{hr}$ ) as  $(x \text{ mv/hr}) (2.03 \% \text{ O}_2/\text{mv}) (10.0 \text{ ml vessel volume}) (24.4 \mu\text{l O}_2/\text{ml}) \frac{(\text{barometric pressure} - P_{\text{H}_2\text{O}})}{760 \text{ mm Hg}}$

where  $P_{\text{H}_2\text{O}}$  is the water vapor pressure.

The vessel (Fig. 1) was made of glass except for a rubber stopper. Care was taken to exclude air bubbles in filling the vessel by expelling them through the exhaust tube. The fluid contents were maintained at  $36.0 \pm 0.2^\circ\text{C}$  by a water jacket through which fluid was circulated by a Bronwill temperature-regulating pump. A magneti-

cally coupled stirring bar at the bottom of the vessel kept the contents stirred. As a control against artifacts due to leakage of oxygen or to bacterial contamination, the oxygen tension in the closed vessel was measured for a half-hour period without the gall bladder in each experiment, and found to remain constant within 0.4 to 1.6%. For comparison, an average gall bladder reduces the oxygen saturation in the vessel by 40 to 60% in a half-hour.

*Procedure* Rabbit gall bladders were excised, dissected, and cannulated as described previously (Diamond, 1964 *a*). The organ has the form of a sac, in which the transporting cells form a continuous layer facing the inside and a layer of connective tissue supports them on the outside. The bathing solutions adjacent to the cells and to the connective tissue are called the mucosal and serosal solutions, respectively. The cells transport NaCl and water in isotonic proportions from the mucosal to the serosal solution. To measure the rate of fluid transport, the gall bladder was cannulated in its *in vivo* orientation and filled with fluid, the cannula was plugged, and transfer of fluid from mucosa to serosa (inside to outside) was determined gravimetrically by measuring the progressive loss of weight of the gall bladder sac at 5 min intervals (Diamond, 1962 *a*, 1964 *a*). To measure oxygen consumption, the organ was everted before cannulation so that the transporting cells faced outwards. With the sac filled with about 0.5 ml fluid and the cannula plugged, a small loop in the thread by which the cannula was secured in place was used to suspend the gall bladder from a hook in the stopper of the vessel (Fig. 1). The vessel was filled with fluid preequilibrated to contain oxygen at about 60% saturation. After the vessel had been stoppered, oxygen uptake by the gall bladder was followed by measuring the fall in  $p_{O_2}$  for 15 to 40 min, which was sufficient for the gall bladder to reduce the oxygen saturation to about 20%. A straight line was fitted by eye through the resulting record of oxygen saturation against time, ignoring the first few minutes of each trace. Measurements of  $Q_{O_2}$  were related to the dry weight of the gall bladder, obtained by drying the organ overnight in an oven at 105°C.

If the effect of a given agent is reversible, it can be determined with much greater confidence and precision by using each preparation as its own control than by making comparisons between different preparations. Most of the experimental effects on oxygen uptake reported here were obtained by alternately measuring uptake by a gall bladder under some standard conditions and under altered conditions several times. Tables II and III are examples of typical experimental protocols, in which the effect of anions is determined by alternately measuring uptake in NaCl and Na<sub>2</sub>SO<sub>4</sub> Ringer's solutions. Only ouabain caused irreversible changes in oxygen uptake, and in this case repeated measurements in ouabain had to be compared to repeated measurements without ouabain obtained previously on the same gall bladder. Unless stated otherwise, experimental errors are given as standard errors of the mean. In the majority of the experiments measuring the effect of an agent on oxygen uptake, the effect of the agent on fluid transport was also checked on the same gall bladder. This was done by measuring fluid transport gravimetrically with the mucosal surface of the gall bladder facing inwards (*in vivo* orientation); removing the cannula, everting, and recannulating to measure O<sub>2</sub> uptake; reeverting and recannulating

to measure fluid transport again, etc. The eversion and reversion procedure had no detectable effect on fluid transport or oxygen uptake.

Table I gives the composition of the experimental solutions used. Solutions are frequently referred to in the text by their principal salt; e.g., "NaCl" means the Ringer solution containing mainly NaCl. Changes of bathing solution were effected by exposing the gall bladder to three consecutive washes with the new bathing solution over a 15 min period for changes in the serosal solution and over shorter periods for changes in the mucosal solution. To study the effect of ouabain, gall bladders were incubated in Ringer's solutions containing  $10^{-3}$  M ouabain for at least 40 min before measurements of fluid transport or oxygen uptake commenced. This concentration was chosen in order to inhibit transport completely and consistently, since previous

TABLE I  
COMPOSITION OF EXPERIMENTAL SOLUTIONS

	NaCl	Na <sub>2</sub> SO <sub>4</sub>	NaCH <sub>3</sub> SO <sub>4</sub>	Na isethionate	TEACl	Choline Cl	Choline sulfate
	mM	mM	mM	mM	mM	mM	mM
NaCl	142	—	—	—	—	—	—
KH <sub>2</sub> PO <sub>4</sub>	0.35	0.35	0.35	0.35	0.35	0.35	0.35
K <sub>2</sub> HPO <sub>4</sub>	2.15	2.15	2.15	2.15	2.15	2.15	2.15
CaCl <sub>2</sub>	2	—	—	2	2	2	—
MgSO <sub>4</sub>	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Glucose	11.1	11.1	11.1	11.1	11.1	11.1	11.1
Na <sub>2</sub> SO <sub>4</sub>	—	113	—	—	—	—	—
CaSO <sub>4</sub>	—	8	8	—	—	—	8
NaCH <sub>3</sub> SO <sub>4</sub>	—	—	142	—	—	—	—
Choline sulfate	—	—	—	—	—	—	113
Choline chloride	—	—	—	—	—	142	—
Tetraethyl ammonium chloride	—	—	—	—	142	—	—
Na isethionate	—	—	—	142	—	—	—

work had shown that relatively high concentrations are necessary for the ouabain effect in the gall bladder (Diamond, 1962 *a*; Dietschy, 1964).

*Effect of Gall Bladder Orientation* In two experiments oxygen uptake was measured with a gall bladder alternately in its natural orientation (serosal surface outwards) and everted (mucosal surface outwards). The average values of  $Q_{O_2}$  for these two gall bladders were, respectively, 27 and 40% lower with serosal than with mucosal surface outwards. When these same two organs were cut open so that both surfaces were in direct contact with the bathing solution in the vessel, the average values of  $Q_{O_2}$  were, respectively, 12 and 28% higher than when the mucosal surface was outwards. These differences are readily explicable in terms of the anatomy of the gall bladder. The transporting cells abut directly on the mucosal bathing solution but are separated from the serosal bathing solution by a layer of connective tissue about 300  $\mu$  thick, containing a few smooth muscle fibers. The diffusion path to the cells is thus much longer in the natural orientation than after eversion and becomes rate-limiting. All remaining experiments were carried out on everted gall bladders.

## RESULTS

1. *Effect of  $p_{O_2}$  upon Oxygen Consumption* Since the method of measuring oxygen uptake depends upon observing the rate of fall of oxygen tension ( $p_{O_2}$ ) in the vessel, it is necessary to establish the range over which oxygen consumption is independent of  $p_{O_2}$ . Accordingly the rate of oxygen con-

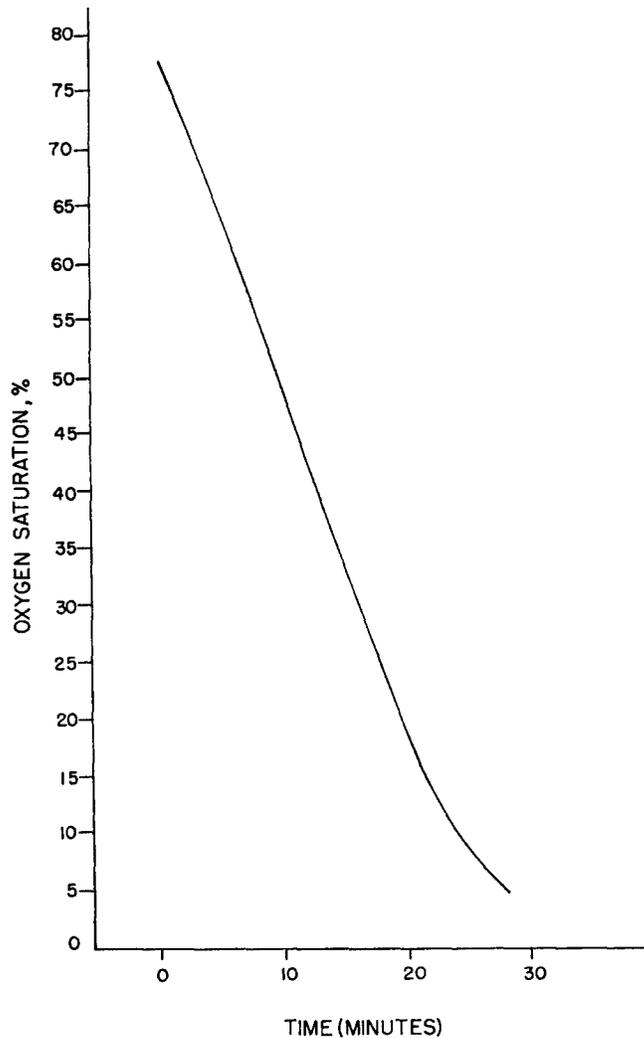


FIGURE 2. Oxygen consumption by rabbit gall bladder as a function of oxygen saturation. The ordinate is the  $O_2$  saturation (measured as the current output of the Clark electrode times a calibration constant) in the closed vessel containing an everted gall bladder, both of whose surfaces are bathed by NaCl Ringer's solution. The vessel was initially filled with fluid equilibrated with 77%  $O_2$ .  $O_2$  consumption by the gall bladder (rate of decrease of vessel  $O_2$  saturation) falls off only at saturations around 10%.

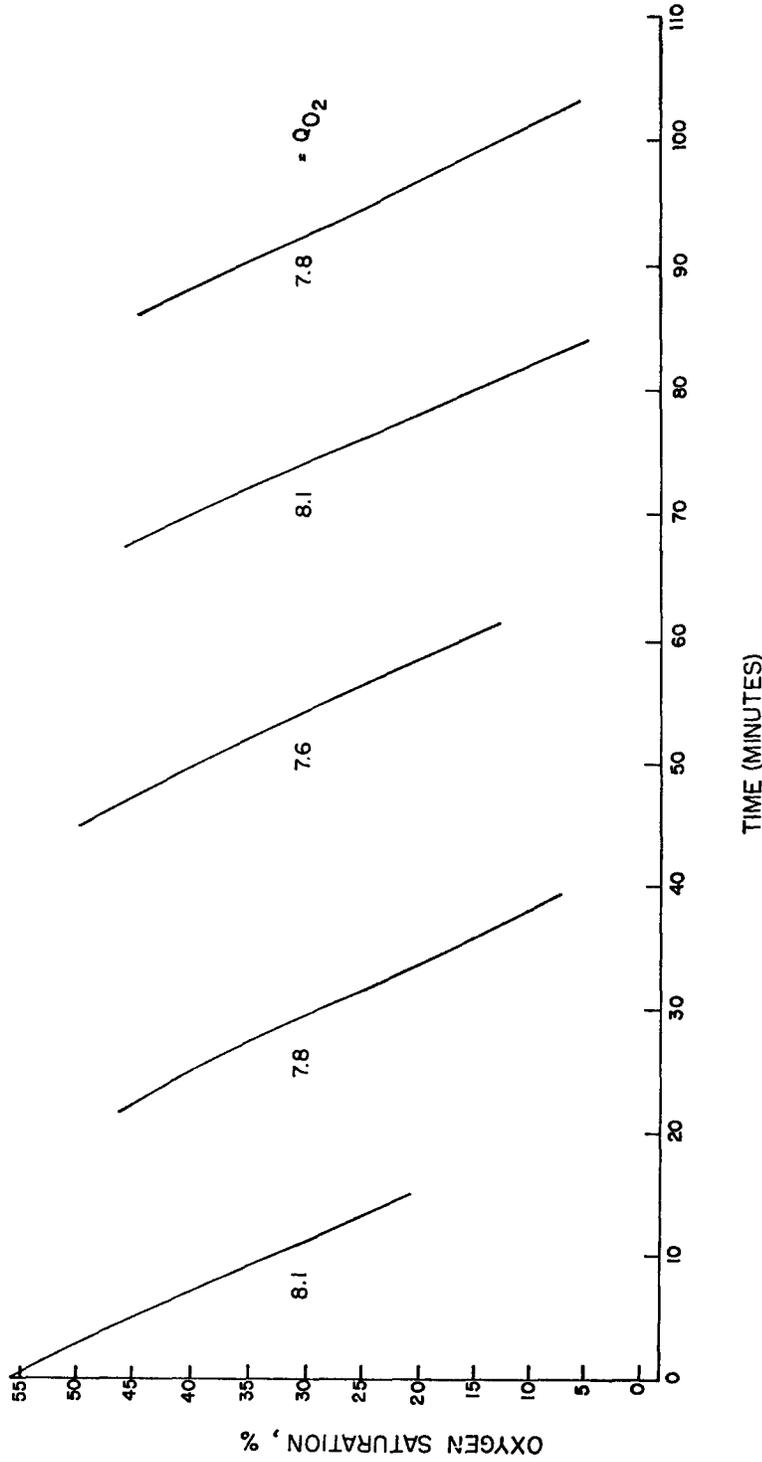


FIGURE 3. Reproducibility of oxygen consumption determinations. ( $Q_{O_2}$ ) in  $\mu l O_2/mg, hr$ , calculated from the slope of the curve as explained under Methods. Both the mucosal and serosal bathing solutions were NaCl Ringer's solution. The number next to each curve is the oxygen uptake

sumption was followed continuously as a gall bladder reduced oxygen saturation from 80% to a low value. As seen in Fig. 2, which depicts the results of one of four such experiments, the slope of a graph of oxygen saturation against time becomes nonlinear only at saturations below 10%. Since the slope is directly proportional to the rate of oxygen consumption, uptake is independent of saturations between 75 and 10%. The same result was obtained in the other three experiments. Hence experimental measurements of uptake were terminated before the saturation had fallen below 10%, and generally were taken in the range 60 to 20%.

2. *Reproducibility* In the experiment illustrated in Fig. 3, the rate of oxygen consumption was measured five consecutive times in the same gall bladder exposed to the same set of experimental conditions (NaCl Ringers'

TABLE II  
EFFECT OF ANION SUBSTITUTION IN THE MUCOSAL SOLUTION

Time	Serosa	Mucosa	$Q_{O_2}$
<i>p.m.</i>			$\mu\text{l O}_2/\text{mg, hr}$
2:04-2:21	Na <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	12.0
2:30-2:44	Na <sub>2</sub> SO <sub>4</sub>	NaCl	12.7
3:03-3:19	Na <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	12.0
3:27-3:44	Na <sub>2</sub> SO <sub>4</sub>	NaCl	13.0
4:01-4:17	Na <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	12.3
4:23-4:39	Na <sub>2</sub> SO <sub>4</sub>	NaCl	12.9
5:00-5:15	Na <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	12.0

Each row represents consecutive measurements of oxygen uptake in the same gall bladder, with the mucosal solution alternately Na<sub>2</sub>SO<sub>4</sub> and NaCl Ringer's solutions (see Table I for composition). From 11:31 a.m. to 12:07 p.m. the rate of water transport was determined gravimetrically in NaCl Ringer's solution as 12.4  $\mu\text{l H}_2\text{O}/\text{mg, hr}$ , and from 12:57 to 1:57 p.m. in Na<sub>2</sub>SO<sub>4</sub> Ringer's solution as 2.71  $\mu\text{l H}_2\text{O}/\text{mg, hr}$ .

solution as both the mucosal and serosal bathing solutions). The five resulting measurements of the fall in oxygen saturation are qualitatively and quantitatively similar. From the slopes of these curves,  $Q_{O_2}$  was computed to have an average value of 7.9  $\mu\text{l O}_2/\text{mg dry wt., hr}$  in this gall bladder, with a standard deviation of  $\pm 0.2 \mu\text{l}$  ( $\pm 2.5\%$ ). This standard deviation includes the error in reading off the slope of the curves. All experimental effects to be described were well outside this limit of reproducibility and involved average changes in  $Q_{O_2}$  of 9 to 45% from a base level determined in the same gall bladder.

3. *Effect of Anions* The first method used to determine the effect of a change in transport rate upon oxygen uptake was to measure  $Q_{O_2}$  when chloride in the bathing solution was replaced by the poorly absorbable anion sulfate. In three experiments the rate of fluid transport was measured gravimetrically in both Na<sub>2</sub>SO<sub>4</sub> and NaCl Ringer's solution for the same gall

bladder, and the transport rate in sulfate was found to be on the average only 19% of that in chloride. The transport rate in sulfate Ringer's solution was determined for a total of five gall bladders and had an average value and standard error of  $1.71 \pm 0.40 \mu\text{l H}_2\text{O}/\text{mg, hr}$ , 20% of the average value for ten gall bladders in chloride Ringer's solution ( $8.75 \pm 0.69 \mu\text{l H}_2\text{O}/\text{mg, hr}$ ). Similarly, in fish gall bladder (Diamond, 1962 *a*) sulfate Ringer's solutions were absorbed at a rate only 17% of that for chloride Ringer's solutions.

Table II illustrates the results of an experiment in which the effect of sulfate-chloride substitution on the fluid transport rate and on oxygen uptake was determined for the same gall bladder. First, the rate of fluid transport was

TABLE III  
ASYMMETRY OF ANION SUBSTITUTION EFFECT

Serosa	Mucosa	$Q_{O_2}$
		$\mu\text{l O}_2/\text{mg, hr}$
NaCl	NaCl	11.3
NaCl	Na <sub>2</sub> SO <sub>4</sub>	9.5
Na <sub>2</sub> SO <sub>4</sub>	NaCl	11.6
Na <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	9.9
Na <sub>2</sub> SO <sub>4</sub>	NaCl	12.3
Na <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	9.9
Na <sub>2</sub> SO <sub>4</sub>	NaCl	12.1
Na <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	9.9

Each row represents consecutive measurements of oxygen uptake, all on the same gall bladder.

measured for 36 min with NaCl Ringer's solution as both the mucosal and serosal bathing solutions, and found to be  $12.4 \mu\text{l H}_2\text{O}/\text{mg, hr}$ . Next, both the mucosal and serosal solutions were changed to Na<sub>2</sub>SO<sub>4</sub> Ringer's solutions, and fluid transport was measured for 60 min at the lower rate of  $2.71 \mu\text{l H}_2\text{O}/\text{mg, hr}$ . Finally, with Na<sub>2</sub>SO<sub>4</sub> Ringer's solution still on the serosa, oxygen uptake was measured while the mucosal bathing solution was alternately Na<sub>2</sub>SO<sub>4</sub> and NaCl Ringer's solution. As seen in Table II, NaCl reversibly stimulates  $Q_{O_2}$  relative to the value in Na<sub>2</sub>SO<sub>4</sub>, and this effect is observed for seven consecutive changes of solution.

The symmetry of the anion effect was tested in the experiment of Table III, where all four possible combinations of NaCl or Na<sub>2</sub>SO<sub>4</sub> as the mucosal or serosal bathing solutions were tested. NaCl Ringer's solution as the mucosal bathing solution stimulates oxygen uptake, regardless of whether the serosal solution is NaCl or Na<sub>2</sub>SO<sub>4</sub>. Correspondingly, when Na<sub>2</sub>SO<sub>4</sub> is the mucosal solution, replacement of serosal Na<sub>2</sub>SO<sub>4</sub> with NaCl fails to stimulate oxygen uptake. Evidently it is only the anion on the mucosal side which affects  $Q_{O_2}$ . Since the direction of fluid transport is from mucosa to serosa, it is also the anion in the mucosal solution which determines the fluid transport rate.

For 25 gall bladders the average value of  $Q_{O_2}$  with NaCl Ringer's solution as the mucosal bathing solution and either NaCl or  $Na_2SO_4$  Ringer's solution as the serosal bathing solution was  $11.0 \pm 0.5 \mu\text{l O}_2/\text{mg, hr}$ . The corresponding figure for 15 gall bladders with  $Na_2SO_4$  as the mucosal bathing solution was  $9.6 \pm 0.5 \mu\text{l O}_2/\text{mg, hr}$ , 12.7% lower. However, because of the variation between different preparations, a more direct comparison is between values for NaCl and  $Na_2SO_4$  obtained on the same gall bladder. In ten gall bladders  $Q_{O_2}$  was measured alternately with NaCl or  $Na_2SO_4$  as the mucosal bathing solution, as in Table II, and the average depression of  $Q_{O_2}$  produced by replacement of mucosal Cl with  $SO_4$  was  $9.1 \pm 1.6\%$ . While this change is not large, it can be elicited repeatedly and consistently in the same prepa-

TABLE IV  
EFFECT OF OUABAIN ON ANION SUBSTITUTION EFFECT

Time	Bathing solution	$Q_{O_2}$
<i>p.m.</i>		$\mu\text{l O}_2/\text{mg, hr}$
12:02-12:22	$Na_2SO_4$	8.2
12:40-12:57	NaCl	9.4
3:07-3:33	$Na_2SO_4 + 10^{-3} \text{ M ouabain}$	6.1
3:48-4:15	NaCl + $10^{-3} \text{ M ouabain}$	6.2

Each row represents consecutive measurements of oxygen uptake in the same gall bladder. In each period the mucosal and serosal bathing solutions were identical. From 11:15 to 11:55 a.m. the rate of water transport was determined gravimetrically in  $Na_2SO_4$  Ringer's solution as  $0.75 \mu\text{l H}_2\text{O}/\text{mg, hr}$ , and from 1:13 to 1:55 p.m. in NaCl Ringer's solution as  $11.28 \mu\text{l H}_2\text{O}/\text{mg, hr}$ .

ration and is more than three times the standard deviation of repeated measurements of  $Q_{O_2}$  in NaCl Ringer's solution. None of the ten gall bladders tested failed to show this depression of  $Q_{O_2}$  by replacement of Cl with  $SO_4$ . The same effect was elicited with two other anions, methyl sulfate and isethionate, which resemble sulfate in being poorly absorbed (methyl sulfate: Diamond, 1962 *a*; isethionate: Wheeler, 1963). Thus, in two gall bladders replacement of mucosal NaCl with sodium isethionate reversibly depressed  $Q_{O_2}$  by an average of 6.7%, while replacement of mucosal NaCl with  $NaCH_3SO_4$  produced a depression of 8.0% in one gall bladder.

This depression of  $Q_{O_2}$  produced by replacement of mucosal Cl with poorly absorbed anions could either represent a specific stimulatory effect of Cl on basal oxygen consumption, or else it could be related to the inhibition of fluid transport. Two methods were used for deciding on the correct interpretation. The first method, illustrated by the experiment of Table IV, consists of observing whether the cardiac glycoside ouabain, which abolishes active transport of NaCl in the gall bladder (Diamond, 1962 *a*; Dietschy, 1964; Diamond and Tormey, 1966) also abolishes the dependence of  $Q_{O_2}$

upon the mucosal anion. First, the rate of fluid transport was determined gravimetrically in  $\text{Na}_2\text{SO}_4$  Ringer's solution as  $0.75 \mu\text{l H}_2\text{O}/\text{mg, hr}$ . Second, oxygen uptake was measured and found to be 12.5% lower in  $\text{Na}_2\text{SO}_4$  ( $8.2 \mu\text{l O}_2/\text{mg, hr}$ ) than in  $\text{NaCl}$  ( $9.4 \mu\text{l O}_2/\text{mg, hr}$ ). Next, the rate of water transport in  $\text{NaCl}$  Ringer's solution was measured as  $11.28 \mu\text{l H}_2\text{O}/\text{mg, hr}$ , 15 times higher than the previously determined rate in  $\text{Na}_2\text{SO}_4$ . Finally, ouabain was added, and the difference between measurements of  $Q_{\text{O}_2}$  in  $\text{Na}_2\text{SO}_4$  and  $\text{NaCl}$  now disappeared ( $6.1$  vs.  $6.2 \mu\text{l O}_2/\text{mg, hr}$ , respectively).

The average value of  $Q_{\text{O}_2}$  for nine gall bladders in  $\text{NaCl}$  Ringer's solution with ouabain added was  $6.0 \pm 0.4 \mu\text{l O}_2/\text{mg, hr}$ . For six gall bladders in

TABLE V  
ANION SUBSTITUTION EFFECT IN PRESENCE OF OUABAIN

$\text{Na}_2\text{SO}_4 + \text{ouabain}$	$Q_{\text{O}_2}$	$\text{NaCl} + \text{ouabain}$	$\frac{Q_{\text{O}_2, \text{Na}_2\text{SO}_4 + \text{ouabain}}}{Q_{\text{O}_2, \text{NaCl} + \text{ouabain}}}$
	$\mu\text{l O}_2/\text{mg, hr}$		
	4.4	4.5	0.98
	7.1	7.2	0.99
	6.1	6.2	0.98
	5.8	5.8	1.00
Average			0.99

In each gall bladder  $Q_{\text{O}_2}$  was measured several times alternately in  $\text{NaCl}$  Ringer's solution +  $10^{-3} \text{ M}$  ouabain and in  $\text{Na}_2\text{SO}_4$  Ringer's solution +  $10^{-3} \text{ M}$  ouabain. The mucosal and serosal solutions were identical in any experimental period. The experiment was performed on four gall bladders, and each row gives average values of  $Q_{\text{O}_2}$  for a different gall bladder.

$\text{Na}_2\text{SO}_4$  Ringer's solution with ouabain added,  $Q_{\text{O}_2}$  was virtually the same:  $6.2 \pm 0.4 \mu\text{l O}_2/\text{mg, hr}$ . A more exact comparison is possible from repeated alternate measurements of  $Q_{\text{O}_2}$  on the same gall bladder in  $\text{Na}_2\text{SO}_4$  and  $\text{NaCl}$  after addition of ouabain. As seen in Table V, anion substitution in ouabain-treated gall bladders affects  $Q_{\text{O}_2}$  on the average by only 1%, less than the standard deviation of replicate determinations ( $\pm 2.5\%$ ).

The second method for determining whether the dependence of  $Q_{\text{O}_2}$  upon mucosal anions reflects the dependence of transport upon mucosal anions involved replacement of sodium in both bathing solutions with the poorly absorbed cation choline. Wheeler (1963) and Dietschy (1964) have shown that fluid transport ceases if choline chloride is substituted for  $\text{NaCl}$  in the bathing solutions. In two experiments with choline sulfate as the serosal bathing solution,  $Q_{\text{O}_2}$  was measured when choline chloride and choline sulfate were repeatedly alternated as the mucosal bathing solution. Table VI shows that the difference between the average values of  $Q_{\text{O}_2}$  for choline chloride or cho-

line sulfate on the mucosa is on the average zero. Hence the dependence of  $Q_{O_2}$  upon the mucosal anion disappears after replacement of Na by choline.

Thus, the experiments on anion substitution show that there is a fraction of the total oxygen consumption which requires the presence of a transportable anion in the mucosal bathing solution, requires the simultaneous presence of sodium, and is inhibited by ouabain.

4. *Effect of Cations* Experiments analogous to those described in the preceding section for anion substitutions were carried out for cations, by replacing Na with the cations choline or tetraethyl ammonium (TEA). Diamond (1962 *a*) found TEA to be poorly transported by the gall bladder. We checked this in one experiment and found that the rate of fluid transport

TABLE VI  
ANION SUBSTITUTION EFFECT IN ABSENCE OF SODIUM

Choline sulfate	$Q_{O_2}$	Choline chloride	$\frac{Q_{O_2}, \text{ choline sulfate}}{Q_{O_2}, \text{ choline chloride}}$
	$\mu\text{l } O_2/\text{mg, hr}$		
5.0		4.9	1.02
4.4		4.5	0.98
Average			1.00

In each gall bladder  $Q_{O_2}$  was measured several times alternately with choline chloride or choline sulfate Ringer's solution as the mucosal bathing solution. The serosal solution remained choline sulfate throughout. The experiment was performed on two gall bladders, and each row gives average values of  $Q_{O_2}$  for a different gall bladder.

in TEACl was 80% below the average rate in NaCl. The evidence that choline chloride is poorly transported was cited on p. 305.

Table VII illustrates an experiment in which the effects of choline chloride and NaCl upon  $Q_{O_2}$  were tested in the same gall bladder. When the mucosal bathing solution is choline chloride, oxygen uptake is the same whether the serosal solution is choline chloride or NaCl. However, replacement of choline chloride on the mucosa by NaCl stimulates  $Q_{O_2}$ . Oxygen uptake in NaCl bathing solutions after treatment with ouabain was found to be the same as in choline chloride without ouabain.

For nine gall bladders the average value of  $Q_{O_2}$  in choline chloride Ringer's solution was  $5.8 \pm 0.7 \mu\text{l } O_2/\text{mg, hr}$ , 53% of the average value for gall bladders in NaCl Ringer's solution. In five gall bladders, when  $Q_{O_2}$  was determined repeatedly and alternately in NaCl and choline chloride for the same gall bladder,  $Q_{O_2}$  in choline chloride was on the average  $55 \pm 6\%$  of its value in NaCl. In two gall bladders oxygen uptake in TEACl was 56% of

its value in NaCl for the same gall bladder. None of the seven gall bladders tested failed to show this decrease in  $Q_{O_2}$  upon replacement of Na with choline or TEA. The depression in  $Q_{O_2}$  produced by substitution of a poorly transported cation for Na (about 45%) is thus much greater than the depression produced by substitution of a poorly transported anion for chloride (9%).

In sulfate solutions removal of Na also depressed oxygen uptake. For two gall bladders in choline sulfate  $Q_{O_2}$  was measured as 5.3 and 6.0  $\mu\text{l O}_2/\text{mg}$ , hr, respectively, considerably lower than the value for any gall bladder in  $\text{Na}_2\text{SO}_4$ . The average of these two values (5.65) is 59% of the average  $Q_{O_2}$  for 15 gall bladders in  $\text{Na}_2\text{SO}_4$ . Since salt transport is minimal in  $\text{Na}_2\text{SO}_4$ , most of the Na-free effect on oxygen consumption cannot be related to salt transport.

TABLE VII  
EFFECT OF CATION SUBSTITUTION

Serosa	Mucosa	$Q_{O_2}$
		$\mu\text{l O}_2/\text{mg, hr}$
NaCl	NaCl	12.0
Choline Cl	NaCl	13.2
NaCl	Choline Cl	8.7
NaCl	NaCl	13.5
Choline Cl	Choline Cl	8.7

Each row represents consecutive measurements of oxygen uptake in the same gall bladder, with the bathing solutions as indicated.

5. *Effect of Ouabain* In bathing solutions containing sodium, addition of  $10^{-3}\text{M}$  ouabain produced a large decrease in  $Q_{O_2}$ , regardless of whether the anion was chloride or the poorly transported sulfate. Table IV illustrated the inhibitory effect of ouabain in NaCl and  $\text{Na}_2\text{SO}_4$ . For nine gall bladders the average value of  $Q_{O_2}$  in NaCl after addition of ouabain was  $6.0 \pm 0.4 \mu\text{l O}_2/\text{mg, hr}$ , 55% of the average value for 25 gall bladders in NaCl without ouabain. In six gall bladders  $Q_{O_2}$  in NaCl after addition of ouabain was on the average  $57 \pm 4\%$  of the value in the same gall bladder before addition of ouabain (Table VIII). The effect of ouabain in  $\text{Na}_2\text{SO}_4$  was similar but not quite as large. The average value of  $Q_{O_2}$  for six gall bladders in  $\text{Na}_2\text{SO}_4$  after addition of ouabain was  $6.2 \pm 0.4 \mu\text{l O}_2/\text{mg hr}$ , 65% of the average value for ten gall bladders in  $\text{Na}_2\text{SO}_4$  without ouabain. In five gall bladders  $Q_{O_2}$  in  $\text{Na}_2\text{SO}_4$  after addition of ouabain was on the average  $72 \pm 4\%$  of the value in the same gall bladder before addition of ouabain (Table VIII). As apparent from Table VIII, there was no instance in which ouabain failed to depress  $Q_{O_2}$  in either NaCl or  $\text{Na}_2\text{SO}_4$ .

In sodium-free solutions the effect of ouabain was smaller. The  $Q_{O_2}$ 's of

two gall bladders in TEACl were depressed 13 and 37%, respectively, by ouabain, while the  $Q_{O_2}$  of one gall bladder in choline chloride was depressed 18%. Not only is the effect of ouabain in Na-free solution smaller on a percentage basis than in solutions containing sodium, but also it must be borne

TABLE VIII  
EFFECT OF OUABAIN

NaCl	$Q_{O_2}$	NaCl + ouabain	$\frac{Q_{O_2}, \text{NaCl} + \text{ouabain}}{Q_{O_2}, \text{NaCl}}$
$\mu\text{l } O_2/\text{mg, hr}$			
11.7		7.9	0.68
13.6		6.9	0.51
8.0		4.5	0.56
9.4		6.2	0.66
9.9		4.1	0.41
10.4		6.4	0.62
Average			0.57
$\text{Na}_2\text{SO}_4$		$\text{Na}_2\text{SO}_4 + \text{ouabain}$	$\frac{Q_{O_2}, \text{Na}_2\text{SO}_4 + \text{ouabain}}{Q_{O_2}, \text{Na}_2\text{SO}_4}$
7.2		4.4	0.61
9.0		7.1	0.79
8.0		6.4	0.80
8.2		6.1	0.74
9.9		6.4	0.65
Average			0.72

In each gall bladder  $Q_{O_2}$  was measured several times without ouabain, then after addition of  $10^{-3}$  M ouabain. The mucosal and serosal solutions were identical in any experimental period. The experiment was performed on 11 gall bladders, and each row gives average values of  $Q_{O_2}$  for a different gall bladder.

in mind that  $Q_{O_2}$  in the absence of ouabain is 45% smaller in a Na-free than in a Na-containing solution. Hence the absolute magnitude of the decrease in  $Q_{O_2}$  produced by ouabain in Na-free solutions is only one third to one fourth of the decrease in Na-containing solutions.

#### DISCUSSION

1. *Division of Total Oxygen Consumption into Components* The results suggest that oxygen consumption of the gall bladder consists of three fractions, as illustrated in Fig. 4.

(a) FRACTION LINKED TO FLUID TRANSPORT (9% OF TOTAL) Let us begin with the experiments involving anion substitution, which are the sim-

plest to interpret. These experiments revealed a fraction of oxygen uptake which requires a transportable anion in the mucosal solution; which is indifferent to the anion (Table III) and cation (Table VII) composition of the serosal solution; which requires the presence of sodium, presumably only in the mucosal solution; and which disappears after treatment with ouabain. The requirements of this fraction are thus identical with the requirements for the mucosa-to-serosa fluid transport elucidated by previous experiments

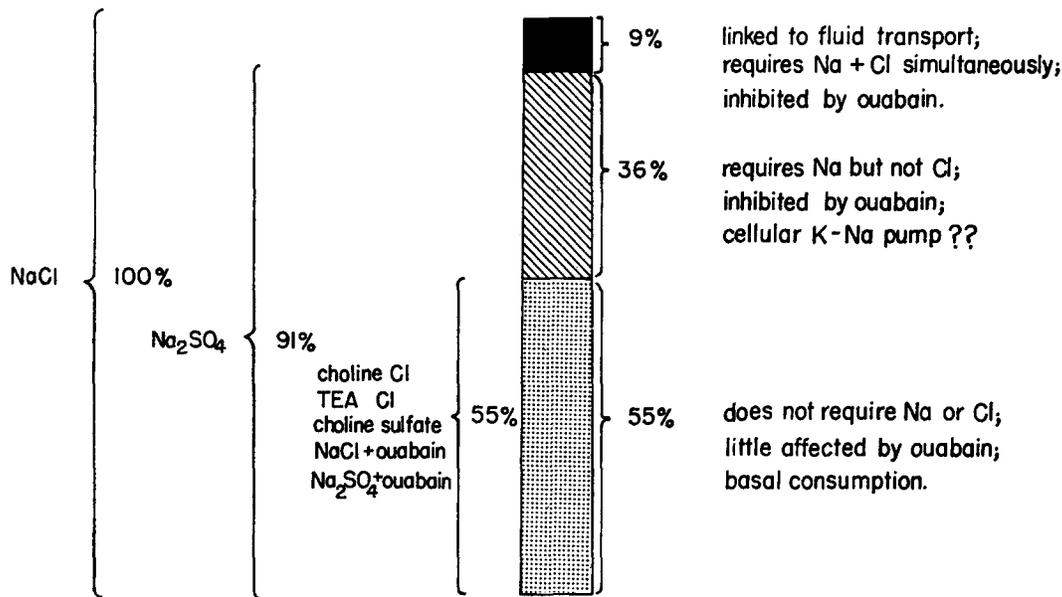


FIGURE 4. Breakdown of total oxygen uptake of the gall bladder into fractions (see Discussion for details). The brackets on the left indicate which fractions are present in each Ringer's solution.

(Diamond, 1962 *a*). The absence of an anion effect on  $Q_{O_2}$  in choline chloride (i.e. as compared to choline sulfate), indicating that this fraction requires the simultaneous presence of Na and Cl and cannot be activated by either alone, parallels the electrical finding that there is no short-circuit current developed in  $Na_2SO_4$  or choline chloride Ringer's solutions. The active transport mechanism can therefore function only by conveying Na and Cl together, rather than one species independently, as in most other epithelia. Presumably the link between salt transport and oxygen uptake is that transport involves the breakdown of ATP to ADP, the concentration of which has been shown by Chance and Williams (1956) to control the rate of mitochondrial respiration. Similarly, Whittam (1961) has demonstrated the control of oxygen uptake in kidney and brain slices by an ATP-hydrolyzing transport system.

(b) FRACTION REQUIRING Na BUT NOT Cl (36% OF TOTAL) It seems at first paradoxical that replacement of Na by a nontransported cation, or treatment with ouabain in NaCl Ringer's solution, should reduce  $Q_{O_2}$  by 45%, whereas replacement of Cl causes only a 9% reduction. One is tempted to identify 45% of the oxygen uptake with NaCl and water transport because both are sodium-dependent and ouabain-sensitive. Two facts, however, compel one to resist this temptation: the fact that replacement of chloride by sulfate inhibits transport almost completely while reducing  $Q_{O_2}$  only by 9%; and the fact that in  $Na_2SO_4$ , removal of Na or addition of ouabain eliminates an additional 36% of the total oxygen consumption, even though transport was already minimal in  $Na_2SO_4$ . One must therefore conclude that 36% of the total oxygen uptake is in a separate fraction which is not directly related to NaCl transport and does not require chloride but shares the properties of sodium dependence and ouabain sensitivity with the fluid transport mechanism. Treatment with ouabain or removal of Na would thus eliminate both this fraction and the fluid transport fraction, reducing  $Q_{O_2}$  by  $36 + 9 = 45\%$ , while removal of Cl would eliminate only the NaCl transport fraction (9%).

Further experiments would be necessary to establish the origin of this 36% fraction. One possible explanation arises from the observation that the epithelial cells of the gall bladder resemble other cells in maintaining the intracellular concentration of potassium much higher than in the bathing medium. If this potassium pool is maintained by the conventional sodium-dependent, ouabain-sensitive uptake mechanism found in many other cells (Skou, 1964), its energy requirements might represent the 36% fraction of  $O_2$  uptake. Whittam (1961) obtained evidence in support of this interpretation for quite similar findings in brain and kidney, where a fraction representing 40% of the  $O_2$  uptake is eliminated by ouabain or by replacement of sodium with choline. Some evidence in fact suggests that the only primary effect of ouabain in the gall bladder is to eliminate this potassium uptake mechanism, and that the resulting disruption in intracellular ionic balance then inhibits NaCl transport secondarily (Diamond, 1964 *a*). While such an explanation for the 36% fraction is still an unproven hypothesis, it suffices to say for our present purposes that this fraction is not directly associated with NaCl transport since it persists in the absence of NaCl transport.

(c) BASAL CONSUMPTION (55% OF TOTAL) This is the oxygen uptake remaining in NaCl or  $Na_2SO_4$  after ouabain treatment, in choline sulfate, in choline chloride, or in TEACl. Oxygen consumption in solutions of the two last named salts is slightly further depressed by ouabain, possibly reflecting an effect of high concentrations of ouabain on metabolism. This fraction must account for all the other energy requirements of the gall bladder.

2. *Ratio of Ions Transported per Oxygen Consumed* This ratio may be computed from the decrease in oxygen consumption and in ion transport caused

by replacing mucosal chloride with sulfate. As in computations of Na/O<sub>2</sub> ratios for other epithelia (Zerahn, 1956; Leaf and Dempsey, 1960; Lassen and Hess Thaysen, 1961; Lassen, Munck, and Hess Thaysen, 1961), we assume that basal oxygen consumption is not diverted to supply energy for salt transport and that only the extra oxygen consumption observed on replacing sulfate with chloride is utilized (see p. 312 for discussion of this assumption).

Removal of mucosal chloride causes the rate of fluid transport to drop from an average of 8.75 to 1.71  $\mu\text{l}/\text{mg}$ , hr; i.e., by 7.04  $\mu\text{l}/\text{mg}$ , hr. The absorbed fluid is a virtually isotonic NaCl solution (Diamond, 1962 *a*, *b*). Since 156 mM NaCl is isotonic to the Ringer solution used in these experiments, the decrease in salt transport is  $(7.04 \times 10^{-6}) (156 \times 10^{-3}) = 110 \times 10^{-8}$  moles NaCl/mg, hr.

On the other hand, this decrease in ion transport is associated with a decrease in  $Q_{\text{O}_2}$  by 9.1%; i.e., by  $(0.091) (11.0) = 1.00 \mu\text{l O}_2/\text{mg}$ , hr. This volume contains  $(1.00 \times 10^{-6})/22.4 = 4.46 \times 10^{-8}$  moles O<sub>2</sub>.

Thus, per mole O<sub>2</sub> consumed there is transported actively  $110 \times 10^{-8} / 4.46 \times 10^{-8} = 24.6$  moles NaCl, or 49.2 moles of ions. If one takes the number of high energy phosphate bonds formed per half-molecule O<sub>2</sub> as 3, then  $49.2/6 = 8$  ions (4 Na + 4 Cl) would be transported per high energy phosphate split.

For comparison the following figures have been reported for four other epithelia, all of which transport sodium actively but chloride passively: frog skin, 16 to 20 sodium ions per O<sub>2</sub> (Zerahn, 1956); urinary bladder, Na/O<sub>2</sub> = 19 (Leaf and Dempsey, 1960); dog kidney, Na/O<sub>2</sub> = 28 (Lassen, Munck, and Hess Thaysen, 1961); and rabbit kidney, Na/O<sub>2</sub> = 25 (Lassen and Hess Thaysen, 1961). Assuming again a P/O ratio of 3, these results would yield a Na/P ratio of 2.7–4.7. Baker (1965) found a Na/P ratio of 2.7–4.0 for Na transport by crab nerve, Baker and Shaw (1965) obtained Na/P = 3 for Na transport by squid nerve, and Glynn (1962) calculated Na/P = 3 for red blood cell.

Thus, the gall bladder, in which both Na and Cl transport are active, pumps approximately twice as many ions *actively* per extra O<sub>2</sub> consumed as do epithelia which transport only Na actively. In other epithelia, however, a sodium active transport mechanism sets up an electrical potential difference favoring chloride movement, and this electrical coupling ensures the passive transport of one chloride ion to maintain electroneutrality for every sodium ion undergoing net transport across the membrane actively. Hence, the sum of ions transferred *actively* and *passively* for each O<sub>2</sub> consumed is the same in other epithelia as in the gall bladder. From the standpoint of thermodynamics the total osmotic work of salt transport would be the same for an independent Na pump and a neutral NaCl pump, since the potential difference  $\Delta V$  set up by the former would increase the work to transport Na and decrease the

work to transport Cl by the same amount ( $F \Delta V$  per mole, where  $F$  is the Faraday). It was previously suggested (Diamond, 1962 *a*) that the NaCl pump of the gall bladder consists of a carrier on which is located both a Na-binding site and a Cl-binding site and which can cross the membrane only when both binding sites are occupied. The measurements of oxygen uptake reported here might be taken to mean that the splitting of a molecule of ATP drives a complete cycle of net transport involving about four such double carriers, just as ATP would drive about three to four sodium carriers in other epithelia. In frog sartorius muscle a quite different type of evidence, namely, measurements of tracer effluxes, also suggests that Na ions are actively transported across the membrane in groups of three (Keynes and Swan, 1959; Keynes, 1965).

While this *relative* comparison makes it clear that it costs no more energy to drive the NaCl carrier of the gall bladder than to drive the Na carriers of other tissues, the need for caution in interpreting *absolute* values of Na/O<sub>2</sub> ratios for all epithelia should be stressed. The significance of these values rests upon the assumption that all energy for Na or NaCl transport comes from the extra oxygen uptake observed during transport and that there is no diversion of basal uptake. This assumption is made plausible by several indirect lines of evidence: the constancy of the ratio when the rate of ion transport is controlled by varying the external resistance (Ussing, Kruhoffer, Hess Thaysen, and Thorn, 1960); the fact that the ratio is approximately the same in several epithelia with Na pumps, although these pumps account for quite different percentages of the total O<sub>2</sub> uptake; and the fact that ratios of Na transport to splitting of high energy phosphate bonds determined directly in nerve and erythrocytes are close to those calculated in epithelia on the above assumption. This evidence still does not constitute direct proof that the assumption is valid, and one must maintain an open mind on this point at present. The following section shows at any rate that the NaCl/O<sub>2</sub> value calculated for the gall bladder is energetically reasonable.

3. *Work Performed in Transport* The work performed by a transport mechanism may be divided into two terms: (*a*) So called "osmotic" work, representing the reversible expenditure of energy required to move a substance against gradients of chemical potential. For an ion the osmotic work per mole is the electrochemical potential difference across the gall bladder as given by the expression

$$\bar{\mu}_s - \bar{\mu}_m = RT \ln \frac{\gamma_s C_s}{\gamma_m C_m} + zF\Delta V \quad (1)$$

where subscripts *m* and *s* refer to the mucosal and serosal bathing solutions, *C*'s are concentrations,  $\gamma$ 's single ion activity coefficients,  $\Delta V$  the electrical potential of the serosal with respect to the mucosal solution, *z* the valence, *F*

the Faraday,  $R$  the gas constant, and  $T$  the absolute temperature. (b) Work representing the irreversible expenditure of energy required to transfer a substance through a membrane against the internal resistance of the membrane. This component of work exists even in the absence of any concentration gradient or electrical potential difference. Mechanical analogues of these two terms would be the work against gravity required to move a weight up an inclined plane, and the work against friction required to move a weight along any surface, whether inclined or level. An expression proposed for the work against internal membrane resistance per mole substance transferred is:

$$RT \ln \frac{M_{ms}}{M_{sm}} \quad (2)$$

where  $M_{ms}$  is the one way tracer flux from mucosa to serosa, and  $M_{sm}$  the back-flux from serosa to mucosa (Ussing, 1949; Heinz, 1956). The larger the one way fluxes upon which net active transport is superimposed, the smaller will be this resistive term.

The work performed by the gall bladder under the conditions of our experiments may be estimated as follows. When the gall bladder is transporting NaCl between identical bathing solutions, the electrical potential difference is generally less than 1 mv because of the one-to-one linkage between Na and Cl transport (Diamond, 1962 *b*; Wheeler, 1963; Dietschy, 1964; Pidot and Diamond, 1964). Thus,  $\Delta C = 0 = \Delta V$ , and the only work is against the internal resistance of the membrane. Wheeler (1963) measured tracer fluxes across rabbit gall bladder and expressed them as partial conductivities related to net weight. His results may be recalculated to yield the following estimates for serosa-to-mucosa fluxes: Na, 2.95  $\mu\text{mole/mg dry wt., hr.}$ ; Cl, 2.48  $\mu\text{mole/mg dry wt., hr.}$  Superimposed upon this flux in the direction mucosa-to-serosa is the net flux due to active transport. Since fluid transport proceeded in our experiments at an average rate of 8.75  $\mu\text{l/mg dry wt., hr.}$  and consists of isotonic (156 mM) NaCl, the mucosa-to-serosa active flux for either Na or Cl is  $(8.75)(0.156) = 1.37 \mu\text{mole/mg dry wt., hr.}$  The total work, which is the sum of work against internal resistance for Na and Cl, is therefore:

$$RT \ln \frac{M_{ms}^{\text{Na}}}{M_{sm}^{\text{Na}}} + RT \ln \frac{M_{ms}^{\text{Cl}}}{M_{sm}^{\text{Cl}}} = (617 \text{ calories/mole}) \left( \ln \frac{2.95 + 1.37}{2.95} \right. \\ \left. + \ln \frac{2.48 + 1.37}{2.48} \right) = 508 \text{ calories/mole NaCl}$$

One mole of  $\text{O}_2$  was found to be consumed for 24.6 moles NaCl transported and yields about 100,000 calories. The energy available per mole NaCl transported is therefore  $100,000/24.6 = 4060$  calories, eight times greater than that required.

This calculation of work against internal resistance should be regarded only as an estimate, because of uncertainties about equation 2 and the origin of the passive fluxes in rabbit gall bladder. However, this estimate suffices to indicate that the oxygen uptake which we observed is more than adequate to supply the energy requirements of transport under our experimental conditions.

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