5. Neither ethyl methyl sulphone nor a crude mixture containing both organic metabolites showed any marked antitubercular activity when tested *in vivo*.

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REFERENCES

- Beckmann, E. O. (1878). *J. prakt. Chem.* **17**, 439.
 Brown, H. D., Matzuk, A. R., Becker, H. J., Conbere, J. P., Constantin, J. M., Solotorovsky, M., Winsten, S., Ironson, E. & Quastel, J. H. (1954). *J. Amer. chem. Soc.* **76**, 3860.
 Canellakis, E. S. & Tarver, H. (1953). *Arch. Biochem. Biophys.* **42**, 446.
 Cantoni, G. L. (1953). *J. biol. Chem.* **204**, 403.
 Challenger, F. & Hayward, B. J. (1954). *Chem. & Ind.* p. 729.
 Challenger, F. & Liu, Y. C. (1950). *Rec. Trav. chim. Pays-Bas*, **69**, 334.
 Challenger, F. & Rawlings, A. A. (1937). *J. chem. Soc.* p. 868.
 Davies, G. E., Driver, G. W., Hoggarth, E., Martin, A. R., Paige, M. F. C., Rose, F. L. & Wilson, B. R. (1956). *Brit. J. Pharmacol.* (in the Press).
 Kushner, S., Dalalian, H., Bach, F. L., Centola, D., Sanjurjo, J. L. & Williams, J. H. (1955). *J. Amer. chem. Soc.* **77**, 1152.
 McRorie, R. A., Sutherland, G. L., Lewis, M. S., Barton, A. D., Glazener, R. & Shive, W. (1954). *J. Amer. chem. Soc.* **76**, 115.
 Pianto, E. del (1953). *Ric. sci.* **23**, 1785.
 Rose, F. L. & Spinks, A. (1948). *Biochem. J.* **43**, vii.
 Ruzicka, L., Goldberg, M. W. & Meister, H. (1940). *Helv. chim. acta*, **23**, 559.
 Schlenk, F. & Palma, R. E. de (1955). *Arch. Biochem. Biophys.* **57**, 266.
 Stokol, J. A. & Weiss, K. (1949). *J. biol. Chem.* **179**, 1049.

Ion Transport and Respiration of Isolated Frog Skin

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In the search for the mechanism of active ion transport by cells the hypothesis was early introduced that such transport might be causally linked to the transference of electrons in the oxidation-reduction reactions of the cells. The concept of oxidation-reduction reactions as sources of bioelectric phenomena in cells and tissues was first introduced by Lund in 1928. Ten years later this concept was applied to the movement of ions and secretory processes by Friedenwald & Stiehler (1938) and Stiehler & Flexner (1938). Subsequently, this hypothesis was revised and extended by a number of workers studying ion transport (Lundegårdh, 1939; Robertson & Wilkins, 1948*a, b*; Conway & Brady, 1948; Crane & Davies, 1948; Patterson & Stetten, 1949; Rehm, 1950). These views have been incorporated by Davies & Ogston (1950) into their mechanism I and by Conway (1951, 1952, 1953, 1955) who has conveniently termed this hypothesis the 'redox pump' theory of ion transport.

Basically these hypotheses state that the following two reactions occurring in aerobic metabolism are spatially separated within the cell:

Fe^{3+} and Fe^{2+} are the oxidized and reduced forms of cytochrome iron; hydrogen atoms arise from the substrate of metabolism. The electrons picked up from the hydrogen atoms in the first reaction are considered to be passed along an oxidation-reduction chain to another site in the cell where they are finally received by molecular oxygen. The hydrogen ion formed in these reactions would be available for secretion as such, or for exchange with cations outside the cell. The same reasoning can be applied to make this system transport anions. Oxidation-reduction systems which do not contain iron have been similarly considered (Patterson & Stetten, 1949).

As pointed out by Lund & Stapp (1947) and Robertson & Wilkins (1948*b*), this hypothesis has a quantitative limitation which may be tested experimentally. A maximum of four electrons, or

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univalent ions, may be transported per molecule of oxygen consumed.

In the present study this limitation was investigated by measuring simultaneously active ion transport and oxygen consumption by the isolated frog skin. Ion transport was measured electrically by the short-circuit-current method of Ussing & Zerahn (1951). Oxygen consumption was determined by a polarographic technique with vibrating platinum micro-electrodes.

The results show that, at least in this tissue, the number of ions actively transported per molecule of oxygen consumed considerably exceeds this critical value. This indicates that the 'redox' hypothesis as proposed by Conway (1954, 1955) and Lundegårdh (1954), in which oxygen is the electron acceptor, is quantitatively insufficient to account for the active transport of ions by this tissue.

METHODS

Experiments were done on the ventral-abdominal skin of male frogs (*Rana temporaria*) weighing 20–25 g., during the winter and early spring months. The frogs were stored at 4° and brought to room temperature for 1–5 days before use. They were force-fed with meal worms on removal from the refrigerator. At the start of an experiment the ventral-abdominal skin was washed with soap and water and rinsed, and the animal killed and pithed. The ventral skin was quickly dissected and, after rinsing in frog Ringer solution, mounted into a chamber similar to that described by Ussing & Zerahn (1951).

In order to ascertain that the chamber was free from bubbles during an experiment a transparent material was essential. Both Perspex and glass chambers were used. Fig. 1 shows a diagram of the chamber together with the accessory equipment necessary for the measurement of short-circuit current and O_2 consumption. The frog skin was mounted at *A* between the two separate halves of the chamber, which were then clamped together by the large screws at either end, making the system water and gas tight. The area of frog skin within the chambers was 3.14 cm.². Four bridges, consisting of polyethylene tubing of 1.3 mm. internal diam. filled with 2% (w/v) agar made up in 0.11 M-KCl solution, were used to measure the potential difference across the membrane and to apply the external counter-potential which reduces the membrane potential to zero during the measurements of short-circuit current. The Pt micro-electrodes were made of short lengths of Pt wire, 0.25 mm. in diam., sealed into one end of a glass tube approximately 4 cm. long. The Pt wire was cut off and polished flush with the tip of the glass tube. The tube was filled with Hg and sealed with a small brass cap through which a stainless-steel wire made contact with the Hg. This Pt micro-electrode was forced through a tightly fitting rubber stopper in the top of the chamber so that about 1.0 cm. of the glass tube projected freely into the chamber. The portion of the electrode protruding above the chamber was vibrated laterally at 50 cyc./sec. so that the platinum tip inside the chamber was displaced 3–5 mm. To ensure a constant rate and amplitude of vibration during the measurement a Mickle shaker (H. Mickle, 4, Ormond Drive,

Hampton, Middlesex, England) was converted for this purpose. The Pt micro-electrode was connected to the negative pole of a polarograph (Cambridge Polarograph, Pen-Recording Type, Cambridge Instrument Co. Ltd., Grosvenor Square, London) and the circuit was completed through an agar-0.11 M-KCl bridge of polyethylene tubing with one end in the chamber and the other dipping into the 0.11 M-KCl of an Ag-AgCl electrode. The volume of each half-chamber was determined by weighing with Hg. The volumes of the left-half chambers were 3.87 and 3.19 ml., and those of the right-half chambers 3.68 and 3.66 ml. for the Perspex and glass chambers respectively.

A rapidly moving noble-metal micro-electrode has been applied and validated for the determination of dissolved O_2 by other workers (Marsh, 1951; Longmuir, 1954; Warshowsky & Schantz, 1954; Chance & Williams, 1955). With an applied potential of 0.65 v across the Pt and Ag-AgCl electrodes, only molecular O_2 in the Ringer solution will remove electrons from the Pt cathode. The current measured through this system is proportional to the O_2 concentration.

Fig. 1. Diagrammatic sketch of the apparatus. The frog skin *A* separates the two halves of the chamber *K*, which are clamped between two set screws. A Perspex cuff *B* from the vibrator holds the platinum micro-electrodes which are connected with the polarographs *E*. The polarographic circuit is completed through a silver-silver chloride electrode *F* and a 0.11 M-KCl-agar bridge *L* with the chamber. The fluid in the chamber is circulated and aerated in the reservoirs *C* by gas entering from *D*. Two calomel electrodes *G* connected to tube potentiometer *H* are used to measure the potential across the skin. An external potential source *J* supplies the potential for short-circuiting the skin and the short-circuit current is read from the micro-ammeter *M*.

The Pt micro-electrode was vigorously vibrated to eliminate any effect of variations in the 'diffusion cone' of O_2 in the layers of medium immediately adjacent to the Pt electrode surface and also to provide adequate stirring of the medium on each side of the skin during the periods of measurement when the contents of the chamber were sealed off.

The method of Harris & Barclay (1955) was used to determine the exact relationship of O_2 tension to the diffusion current. The electrode system was placed in a glass chamber which could be evacuated, allowing measurements of O_2 tension and diffusion current to be made in a small volume of Ringer solution. Stepwise additions of 100% O_2 or room air were then introduced, the gas pressure was measured, and the diffusion current in the enclosed solution in equilibrium with the gas phase measured. Fig. 2 shows that with 100% O_2 as the gas phase a linear calibration curve was obtained. As experiments were made with room air as the gas phase, measurements were repeated with room air rather than pure O_2 . Fig. 3 shows that a similar linear calibration was obtained over this range of O_2 tensions.

After mounting the frog skin in the chamber each half of the chamber and its aeration reservoir were filled with about 10 ml. of frog Ringer solution (112 mM-NaCl, 1.88 mM-KCl, 2.38 mM- $NaHCO_3$ and 0.89 mM- $CaCl_2$). Care was taken to eliminate all gas bubbles from the chamber. The bathing medium was aerated with room air or other appropriate gas mixture through the bubbling and mixing system described by Ussing & Zerahn (1951). After a period of equilibration with room air, during which a constant O_2 diffusion current was recorded by the polarograph, the inlets and outlets of the chamber were quickly clamped off with small surgical arterial bulldog clamps. The linear decrease in the diffusion current indicates the drop in O_2 concentration in the enclosed volume of fluid in contact with the frog skin during the period of measurement. The 'zero' value for the O_2 diffusion current was obtained by bubbling cylinder N_2 into the chamber to wash out all traces of O_2 from the bathing solution. Essentially all O_2 was first removed from the cylinder N_2 by bubbling the latter through a vanadyl sulphate solution as recommended by Meites & Meites (1948). The quantity of O_2 consumed by the skin was calculated from the known values for the volume and temperature of fluid in each chamber, the solubility of O_2 in Ringer solution and the linear relation between O_2 concentration and diffusion current. Because of the relatively slow rate of diffusion of O_2 through the skin simultaneous measurements of O_2 disappearance were made separately in the medium bathing each side of the skin.

In order to assess the reproducibility of the O_2 measurement by this technique air was mixed with N_2 in a large cylinder to give a final O_2 concentration in the range of that measured in these studies. After each period of measurement the chamber fluid was equilibrated with gas from this cylinder and measurements were made on the O_2 concentration in this cylinder mixture. A total of 131 such measurements was made during the course of the experiments and the coefficient of variation for a single measurement of O_2 concentration was found to be 0.84%. There was no difference between the measurements made simultaneously, but separately, on each side of the skin.

The temperature of the bathing solution was 23–28° in these experiments and was measured before and after each 15–20 min. period. No substrate was added to the Ringer solution. To minimize any O_2 consumption due to the

unavoidable presence of bacteria on the frog skin the ventral abdominal skin was washed with soap and water immediately before its dissection and the duration of the experiments was kept down to 2 hr. from the time of mounting the skin. In the last 74 periods of measurement crystalline sodium penicillin (165 units/ml. of Ringer solution) and streptomycin calcium chloride (0.1–0.2 mg./ml. of Ringer solution) were contained in the bathing medium.

Ussing & Zerahn (1951) have shown that, with the same medium on either side of the skin, when an external potential is applied across the isolated frog skin so as to reduce the spontaneous membrane potential to zero, the current

Fig. 2. Calibration of vibrating platinum micro-electrode for oxygen tensions from 0 to 100%. The oxygen diffusion current measured polarographically is shown on the ordinate with oxygen tension on the abscissa. Oxygen tension was determined by placing the electrode assembly into an evacuated container, adding increments of 100% oxygen and measuring the pressure in the closed system after each addition.

Fig. 3. Calibration of vibrating platinum micro-electrode for oxygen tensions over the working range from 0 to 21%. The technique is the same as for Fig. 2 except that increments of room air were added instead of 100% oxygen. Note that the currents measured in this curve are higher than those obtained with 100% oxygen. Differences in electrical resistance of various electrode assemblies, different amplitudes of vibration of the platinum electrode, etc., will alter the magnitude of current measured. The only point of importance to the present study is that during any period of observation a linear relationship between current and oxygen concentration applies, as is shown here.

measured in the external circuit just equals the active Na transport through the skin. Table 1 shows the equality of net Na transport and short-circuit current through the isolated ventral skin of *Rana temporaria* in 14 separate periods of 60 min. each. Four frogs were used. The techniques of measurement are those described by Ussing (Levi & Ussing, 1949; Ussing & Zerahn, 1951). The results confirm a much larger number of similar measurements made in Ussing's laboratory (Ussing & Zerahn, 1951; Fuhrman, 1952; Ussing, 1954) and by Linderholm (1952). Because of this equality of short-circuit current and active Na transport, the former has been used as the measure of the latter in these experiments. Readings of short-circuit current were made directly from a micro-ammeter at intervals of 2.5 min. during each period and averaged to obtain the mean ion transport during the period.

When posterior-pituitary preparations were tested for their effect on O_2 consumption and short-circuit current they were added to the Ringer solution bathing the inside of the skin after at least two control periods had been obtained. The posterior-pituitary extract used was Pitressin (Parke, Davis and Co. Ltd., Batch No. LS 588 F), of which 2 units was added. Dried whale posterior-pituitary powder (generously made available by Professor H. H. Ussing) and pure Vasopressin (A-V-N-3) and Oxytocin (O-N-3, generously supplied by Professor V. du Vigneaud) were also used. Approximately 1 mg. of the dry powder or 1 unit of the pure hormones was added to the solution bathing the inside of the skin.

Manometric measurements were made of O_2 consumption of frog skin with the standard Warburg apparatus. The ventral-abdominal skins of 15 frogs were divided sagittally into two halves. Each half, with wet weight of about 0.4 g., was placed in a separate flask containing 4.0 ml. of frog Ringer solution without added substrate and 1 unit of Pitressin (0.05 ml.) in the side arm of one flask. After 20 min. for equilibration the Pitressin was tipped into the medium and measurements were made of O_2 uptake at 5 min. intervals for two consecutive 1 hr. periods.

RESULTS

Relationship between ion transport and oxygen uptake. The observed relationship of active ion transport to total oxygen consumption by the isolated frog skin is shown in Fig. 4 as a frequency distribution graph of the number of sodium ions transported per molecule of oxygen consumed in the 120 periods studied. In only five periods was this ratio found to be 4.0 or less. It is apparent that in

the majority of experimental periods the number of ions transported per molecule of sodium exceeds the upper limit of 4.0 required by the 'redox' hypothesis, as recently formulated by Lundegårdh (1954) and Conway (1953, 1954, 1955). The mean value for this ratio is 6.82 and the standard error of the mean is ± 0.16 , indicating that even the mean of this series of observations significantly exceeds a value of 4.0.

Evaluation of experimental error. An attempt has been made to evaluate the magnitude of possible errors which may enter into the determination of this ratio. The coefficient of variation of a single polarographic measurement of oxygen concentration was 0.84% (a standard deviation of 0.16 for a series of 131 measurements on a standard gas mixture containing 19.25% of oxygen). As the oxygen consumption in a usual 15–20 min. period

Fig. 4. Relationship of ion transport to total oxygen consumption by the isolated frog skin. The figure is a frequency distribution graph of the results of 120 separate periods of measurements made simultaneously of short-circuit current and oxygen consumption. The ordinate is the frequency at which any given ratio of the number of sodium ions transported/molecule of oxygen consumed was obtained. The values of this ratio form the abscissa. The great majority of values for the ratio are above 4 and thus incompatible with the unmodified 'redox' hypothesis of ion transport.

Table 1. Comparison of net sodium flux and short-circuit current of isolated frog skin

Sodium influx was measured with ^{22}Na and outflux with ^{24}Na according to Levi & Ussing (1949). Mean net Na flux is influx minus outflux. Short-circuit current was measured directly as described in text. Values in columns 3–6 are expressed as $\mu\text{equiv. of Na/cm.}^2/\text{hr.}$

No. of periods	Duration of each period (min.)	Mean Na influx	Mean Na outflux	A Mean net Na flux	B Mean short-circuit current
14	60	1.54	0.125	1.42	1.48

Mean difference (B – A) = $+0.059 \mu\text{equiv./cm.}^2/\text{hr.}$; s.e. mean difference = $\pm 0.035 \mu\text{equiv./cm.}^2/\text{hr.}$; hence means do not differ significantly.

was 2–3 % of the original 21 % present, the error in a single measurement of oxygen consumption may be expected to be ± 5 –8 %. The volume of the chambers is known by calibration to an accuracy of approximately 0.4 % and this source of error is therefore negligible. To exclude the possibility that oxygen was diffusing from the Perspex into the Ringer solution, giving thereby falsely low measurements of oxygen consumption, additional measurements were made in a glass chamber. The mean and standard error of the ratio of ions transported to molecules of oxygen consumed in 13 periods with the glass chamber was 6.56 ± 0.45 as compared with 6.85 ± 0.17 for the 107 measurements made with the Perspex chambers. As these two series are not statistically different the possibility of a significant diffusion of oxygen from the heavy-walled Perspex chamber is excluded, and the results have been pooled together.

As the inlet and outlet tubes to the chamber could not be clamped off flush with the chamber some error was introduced by oxygen diffusing from the volume of fluid within the narrow tubing into the chamber. The radius of each opening was 1 mm. and the volume of fluid occupying this dead space was found by calibration with mercury to be 2.9 and 4.7 % of the volume of the Perspex and glass chambers respectively. Thus if all the oxygen contained in this trapped volume of fluid were available to the frog skin an error of this magnitude would be introduced.

Another potential source of error was the presence of air bubbles trapped in the chambers during measurements. A careful inspection of the transparent chamber was made with a bright beam of light before each period to exclude the presence of any bubbles, and frequent checks were made during the course of an experiment. Nevertheless the possibility that a very small gas bubble might have been present on some occasions cannot be excluded. By injecting air from an Agla micrometer syringe (Burroughs Wellcome and Co., London) into the closed chamber it was found that a bubble of $2 \mu\text{l}$. was readily noted and was considerably larger than any bubble that could have escaped detection during an experiment. Hence it can be safely stated that an error no larger than 1 % could have been introduced into the oxygen measurement by the presence of undetected gas bubbles in the chamber.

The errors in the measurement of short-circuit current, though difficult to assess, cannot be more than ± 5 %. This figure is supported by the results in Table 1, if equality of net sodium flux and short-circuit current is accepted and the variations of the results are assigned equally to experimental error in the isotope results and in the short-circuit current measurements.

From the above considerations the coefficient of

variation for a single determination of the ratio of ions transported to molecules of oxygen consumed would be approximately ± 9 %, with the error in the mean value of the 120 measurements being less than ± 1 %. The ratio might have been overestimated by an additional maximal error of 6 % because of the possible dead-space and gas-bubble error discussed above.

Effect of posterior-pituitary preparations. Ussing & Zerahn (1951) have shown that extracts of mammalian posterior-pituitary glands increase active sodium transport and short-circuit current through the isolated frog skin. The effects of such extracts on oxygen consumption and short-circuit current are shown in Table 2. Only the experimental periods just before and just after addition of the neurohypophyseal preparation to the solution bathing the inside of the skin are presented here. Not more than 30 min. elapsed between these two consecutive periods. In all instances neurohypophyseal extract resulted in the expected increase in ion transport and in all but one experiment with the whale powder a simultaneous increase in oxygen consumption was observed. This one exceptional experiment also showed only a small effect of the hormone preparation on ion transport. The increases in both oxygen consumption and sodium transport are highly significant ($P < 0.001$, t test). If the one exceptional experiment as well as the very high value obtained in Expt. 17, Table 2, are omitted, the ratio of the increment in sodium transport to the increment of oxygen consumption yields a mean value of 18.2 sodium ions transported/molecule of oxygen consumed, with a standard error of ± 2.3 .

A complete analysis of variance of the results for the two periods before and the two periods after exposure to neurohypophyseal hormones indicate that there was no significant change in oxygen consumption by the skin with time. However, to eliminate completely this possibility, as well as to provide a check by an independent method, a series of measurements were made of oxygen consumption by conventional manometric techniques in a Warburg apparatus. Paired observations with and without the addition of Pitressin were made on the two halves of the ventral-abdominal skin of 15 frogs. As observations were made for two periods of 1 hr. on each half-skin, 30 simultaneous paired comparisons were obtained. In every instance the half-skin to which Pitressin had been added showed a higher oxygen consumption/mg. dry wt. than its control half. The mean increase was 23 % (Table 3).

A comparison of the polarographic and manometric measurements of the effect of neurohypophyseal preparations on oxygen consumption by the isolated frog skin is given in Table 3. Results are expressed as Q_{O_2} (μl . of O_2 consumed/mg. dry wt.). The polarographic data refer only to the period

before and that immediately following addition of the neurohypophyseal preparation; the results include those with Pitressin, whale posterior-pituitary powder and pure vasopressin and oxytocin. The manometric results were obtained only with Pitressin. The slightly higher rates of oxygen consumption both before and after addition of neurohypophyseal preparations found with the polarographic method are probably the result of slight

stretching and perhaps of short-circuiting the skin during the polarographic measurements. By both methods, however, a significant and similar increase in oxygen consumption resulted from the neurohypophyseal preparations. The higher Q_{O_2} values obtained by the polarographic method support the view that this method does not underestimate oxygen consumption and thereby give falsely high ratios of ion transport to oxygen consumption.

Table 2. *Effect of neurohypophyseal preparations on oxygen consumption and ion transport*

Expt.	Oxygen consumption $\mu\text{mole/cm.}^2/\text{hr.}$			Sodium transport $\mu\text{equiv./cm.}^2/\text{hr.}$			$\Delta\text{Na}/\Delta\text{O}_2$
	Before	After	ΔO_2	Before	After	ΔNa	
Effects of Pitressin							
1	0.331	0.447	0.116	2.51	3.22	0.71	6.12
2	0.454	0.535	0.081	1.50	3.18	1.68	20.7
3	0.291	0.386	0.095	1.29	2.45	1.16	12.2
4	0.226	0.318	0.092	1.43	2.40	0.97	10.5
5	0.326	0.386	0.060	2.26	3.76	1.50	25.0
6	0.250	0.304	0.054	1.34	2.55	1.21	22.4
7	0.290	0.405	0.115	1.56	2.58	1.02	8.9
8	0.308	0.336	0.028	2.55	3.54	0.99	35.4
9	0.429	0.486	0.057	2.16	3.80	1.64	28.8
10	0.153	0.180	0.027	0.64	1.29	0.65	24.1
Effects of whale posterior-pituitary powder							
11	0.199	0.142	-0.057	0.96	1.46	0.50	—
12	0.131	0.267	0.136	1.24	2.09	0.85	6.3
13	0.248	0.284	0.036	1.68	2.74	1.06	29.4
14	0.350	0.468	0.118	2.61	3.73	1.12	9.5
15	0.218	0.250	0.032	1.90	3.28	1.38	43.1
16	0.244	0.262	0.018	1.43	2.04	0.61	33.9
Effects of pure oxytocin							
17	0.233	0.238	0.005	0.98	1.75	0.77	154
18	0.215	0.253	0.038	1.32	1.54	0.22	5.8
19	0.270	0.381	0.111	1.56	2.68	1.12	10.1
20	0.261	0.314	0.053	1.61	2.14	0.53	10.0
21	0.242	0.309	0.067	1.70	2.57	0.87	13.0
22	0.302	0.336	0.034	1.78	2.34	0.56	16.5
Effects of pure vasopressin							
23	0.234	0.304	0.070	1.51	2.34	0.83	13.0
24	0.270	0.314	0.044	1.67	2.38	0.71	16.1
Means	0.270	0.329	0.060	1.63	2.58	0.94	18.22
S.E.M.	—	—	± 0.0089	—	—	± 0.075	± 2.3
P	<0.001			<0.001			

Table 3. *Comparison of polarographic and manometric measurements of oxygen consumption by frog skin*

	No. of expts.	Mean temp. (°)	Q_{O_2}		Mean diff.	S.E.M. diff.	P
			Before	After			
Polarographic	24	25.6	1.42	1.72	0.30	0.050	<0.001
Manometric	15	25.3	1.00	1.23	0.23	0.030	<0.001

Increased O_2 consumption after addition of neurohypophyseal preparation: 21% by polarographic measurement; 23% by manometric measurement.

DISCUSSION

Previous workers have evaluated the ratio of the number of ions transported by various tissues per molecule of oxygen consumed by that tissue. Robertson & Wilkins (1948*b*) studied the relationship between the increase in oxygen consumption of well-washed plant tissues when placed in salt solutions and the salt accumulation by the tissue. They interpreted their results to be consistent with the 'redox' hypothesis of Lundegårdh (1939, 1940) for active accumulation of anions by plants, although occasional ratios above 4.0 were reported.

Crane & Davies (1948, 1951) compared the hydrochloric acid production of the isolated frog gastric mucosa and the increase of oxygen consumption of that tissue after addition of histamine to the medium. Ratios for moles of hydrochloric acid formed to moles increase of oxygen uptake as high as 11.5 were obtained in individual experiments. On the assumption that the increase in the rate of respiration of the mucosa after histamine provides the energy for acid secretion, very high thermodynamic efficiencies were calculated for the secretory process. When the rate of acid production was compared, however, with the total oxygen consumption by the tissue, ratios were found lower than the average figure of 2.2 obtained by Teorell (1949). So long as it is unproven that the resting respiration of the whole mucosa cannot become available in part for the secretory process when histamine is added, these findings are not conclusive evidence for the existence of ratios above 4.0 in a tissue in which the ratio of ion transport to total oxygen consumption is as low as 2.0. This objection has been countered with the argument that the acid-secreting cells comprise only about 5% by dry weight of the gastric mucosa (Crane & Davies, 1951), so that the ratios of acid formation to oxygen consumption by the cells actually involved in acid secretion must considerably exceed the ratio obtained for the total mucosa.

Davenport (1952) and Davenport & Chavre (1953) compared the rates of acid secretion and oxygen consumption of frog gastric mucosa and of mouse stomachs and showed that the efficiency of acid secretion, i.e. the slope of the regression line of moles of acid produced to moles of oxygen consumed, was actually variable. He concludes from his studies on frog and mouse gastric mucosae: 'we have never found the molar ratio of acid secreted to oxygen consumed to be significantly greater than 4'.

The other attempts to determine this ratio have been made on the frog skin. Lund & Stapp (1947) compared the rate of flow of current through the partially short-circuited skin with the number of electrons made available by the oxygen consumption of the skin. This ratio of output of electricity in

coulombs to the coulomb equivalents of total oxygen consumption expressed as a percentage gave values close to 100% in a few experiments (equivalent to slightly less than 4.0 for the ratio as calculated in this paper). They realized that their figures represented minimal values for this ratio because the current measured was dependent upon the external resistance of the circuit used. This experimental difficulty became surmountable by the introduction of the completely short-circuited skin preparation (Ussing & Zerahn, 1951) used in the present study.

In 1952 Linderholm made calculations of this 'coulomb' efficiency for the short-circuited skin and stated: 'the quantity of electricity transferred with the sodium ions in the active transport is found to be 70 to 90 per cent of the quantity of electricity transferred by electrons in the oxidation processes'. Linderholm's data have been restated by Conway (1954, 1955) to give a ratio of 3.28 sodium ions transported per molecule of oxygen consumed. Conway (1954, 1955) emphasizes these results to support his 'redox pump' theory (Conway, 1951). However, Linderholm's results are open to criticism on two points. His calculations were made from mean values for oxygen consumption and for ion transport in separate experiments rather than from simultaneous measurements of both values in the same skin. Of greater significance is the fact that he apparently worked with skins of very low short-circuit current. Thus his mean figure for the short-circuit current in 10 skins was $19 \mu\text{A}/\text{cm}^2$ or $0.71 \mu\text{equiv.}/\text{cm}^2/\text{hr}$. This is to be compared with mean short-circuit currents of 1.63 and $2.58 \mu\text{equiv.}/\text{cm}^2/\text{hr}$. (Table 2, before and after neurohypophyseal preparations respectively) in the present study. Though the corresponding mean oxygen consumption of 0.27 and $0.33 \mu\text{mole}/\text{cm}^2/\text{hr}$. in the present study was greater than the mean figure of $4.8 \mu\text{l.}/\text{cm}^2/\text{hr}$. or $0.21 \mu\text{mole}/\text{cm}^2/\text{hr}$. found by Linderholm, the ion transport was so much greater as to yield the high ratios reported here. Every attempt was made in the present study to secure lively, healthy young frogs which would yield high short-circuit currents. That our measurements of ion transport are not excessively high is seen in the finding by others of similar rates of ion transport by isolated frog skins (Ussing & Zerahn, 1951; Fuhrman 1952; Ussing, 1954). In frog skins showing such low rates of electrical activity as those studied by Linderholm it may be expected that the residual oxygen consumption not related to ion transport becomes a more sizeable fraction of the total oxygen consumption of the skin, thereby depressing the ratio of ion transport to oxygen consumption. In the present study all low values for this ratio were obtained from skins showing low rates of ion transport. A further indication of increasing efficiency of

ion transport with high rates of ion transport is seen from the effects of neurohypophyseal preparations. Whereas these preparations stimulated an increase in both ion transport and oxygen consumption the former was affected relatively more than the latter so that a significant increase in the ratio of ion transport to oxygen consumption was obtained. This ratio had a mean value of 6.29 before and 8.00 after addition of neurohypophyseal preparations, giving a mean difference of 1.71 with a standard error of the mean difference of ± 0.32 and $P < 0.001$.

The present results indicate that even when compared with the total oxygen consumption of the skin the number of ions transported per molecule of oxygen consumed significantly exceeds 4.0. A similar result with the same tissue has recently been obtained by Zerahn (1956). As the frog skin contains many cells in addition to the basal-epidermal cells thought to be the site of the active ion transport (Ussing, 1949) it would seem probable that the true ratio of ion transport to oxygen consumed by cells involved in the transport would be considerably higher than the values reported here. An attempt to obtain this true ratio was made by comparing the increase of ion transport after addition of neurohypophyseal preparations with the concomitant increment of oxygen consumption. Table 2 shows the ratios so calculated ($\Delta\text{Na}/\Delta\text{O}_2$). The experimental error propagated into the calculation of these ratios is, of course, considerable; the standard deviation for a single ratio being about 50% of the ratio. In addition, the calculation of such a ratio may be open to the same sort of criticism that Davenport has made of this type of calculation for the gastric mucosa. Nevertheless, the mean value of 18 probably gives the best estimate possible at present of the actual stoichiometric relationship which exists between ion transport and metabolism in the fraction of cells of the tissue which are engaged in the transport process.

Davies & Ogston (1950) have modified the 'redox' hypothesis of ion transport to yield maximum ratios of 12 for the number of univalent ions transported to molecules of oxygen consumed (their mechanism II). More recently Davies (1956) has proposed a scheme consistent with even higher ratios of ion transport to tissue respiration. The present study suggests that an upper limit of 12 may be actually too low for this tissue, but a definite statement cannot be made from these results.

Though the interpretation of a ratio calculated from the increment of ion transport and oxygen consumption stimulated by neurohypophyseal preparations is of questionable validity, the results clearly indicate that ion transport and oxygen consumption are both increased in this tissue by some factor or factors contained in posterior-

pituitary gland of mammalian origin. Both pure oxytocin and pure vasopressin in the large amounts used (1 unit) were found to stimulate ion transport and oxygen consumption. No attempt, however, has been made to evaluate the minimal amount of each compound which is effective. The stimulation of ion transport by crude posterior-pituitary preparations has previously been established (Ussing & Zerahn, 1951) but the effect on oxygen consumption constitutes a new finding.

No inconsistencies are encountered when the energy requirements for the high rates of ion transport are related to the energy available from aerobic metabolism in this tissue. The total energy production of the skin before and after the addition of neurohypophyseal preparations may be calculated from the mean oxygen consumption figures of Table 2, and the factor 0.116 calories/ μ mole of oxygen consumed (Davies & Ogston, 1950). The minimal energy requirement for sodium transport is obtainable from the mean current figures of Table 2 and an approximate value of the potential of the sodium pump calculated from the sodium influx and outflux figures of Table 1, according to Ussing & Zerahn (1951). Although the calculations utilize results from different experiments, they yield an order of magnitude for the minimal energy requirement for the ion transport through the short-circuited frog skin. This was calculated to be 8 and 10% of total metabolism before and after neurohypophyseal preparations respectively, and 22% for the increment of ion transport and oxygen consumption stimulated by the hormonal preparation.

The results of this study clearly indicate that the unmodified 'redox' hypothesis of ion transport in which molecular oxygen is the final electron acceptor is insufficient to account for the observations reported here on the short-circuited frog skin. It would seem premature to attempt to generalize the results obtained in this tissue to the problem of ion transport by other cells or tissues.

SUMMARY

1. Simultaneous measurements of active ion transport and oxygen consumption have been made on the isolated frog skin. The short-circuit current was used as the measure of ion transport (Ussing & Zerahn, 1951) and oxygen consumption was measured polarographically with vibrating platinum micro-electrodes.

2. The ratio of the number of sodium ions transported per molecule of oxygen consumed was found to range from 2 to 13 (120 periods of measurement) with a mean of 6.82 and a standard error of the mean of ± 0.61 . In all but five periods the value of this ratio was above 4.0.

3. These results invalidate for frog skin the 'redox' hypothesis of active ion transport as formulated by Conway (1953, 1954, 1955) and Lundegårdh (1954) according to which no more than four univalent ions can be transported per molecule of oxygen consumed.

4. Crude neurohypophyseal preparations which are known to augment the active transport of sodium by the isolated frog skin are shown to increase oxygen uptake by about 20 %. Both pure oxytocin and pure vasopressin similarly increase ion transport and oxygen consumption by this tissue.

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REFERENCES

- Chance, B. & Williams, G. R. (1955). *Nature, Lond.*, **175**, 1120.
- Conway, E. J. (1951). *Science*, **113**, 270.
- Conway, E. J. (1952). *The Biochemistry of Gastric Acid Secretion*. Springfield, U.S.A.: Thomas.
- Conway, E. J. (1953). *Int. Rev. Cytol.* **2**, 419.
- Conway, E. J. (1954). *Symp. Soc. exp. Biol.* no. 8, p. 297.
- Conway, E. J. (1955). *Int. Rev. Cytol.* **4**, 377.
- Conway, E. J. & Brady, T. G. (1948). *Nature, Lond.*, **162**, 457.
- Crane, E. E. & Davies, R. E. (1948). *Biochem. J.* **43**, xlii, xliii.
- Crane, E. E. & Davies, R. E. (1951). *Biochem. J.* **49**, 169.
- Davenport, H. W. (1952). *Fed. Proc.* **11**, 715.
- Davenport, H. W. & Chavre, V. J. (1953). *Amer. J. Physiol.* **174**, 203.
- Davies, R. E. (1956). *Symposium on Active Transport* (in the Press). Madison: University of Wisconsin.
- Davies, R. E. & Ogston, A. G. (1950). *Biochem. J.* **46**, 324.
- Friedenwald, J. S. & Stiehler, R. D. (1938). *Arch. Ophthalm., N.Y.*, **20**, 761.
- Fuhrman, F. A. (1952). *Amer. J. Physiol.* **171**, 266.
- Harris, H. & Barclay, W. R. (1955). *Brit. J. exp. Path.* **36**, 592.
- Levi, H. & Ussing, H. H. (1949). *Nature, Lond.*, **164**, 928.
- Linderholm, H. (1952). *Acta physiol. scand.* **27**, suppl. 97.
- Longmuir, I. S. (1954). *Biochem. J.* **57**, 81.
- Lund, E. J. (1928). *J. exp. Zool.* **51**, 265, 291, 327.
- Lund, E. J. & Stapp, P. (1947). *Bioelectric Fields and Growth*, p. 235. Ed. by Lund, E. J. Austin: University of Texas Press.
- Lundegårdh, H. (1939). *Nature, Lond.*, **143**, 203.
- Lundegårdh, H. (1940). *Ann. agric. Coll. Sweden*, **8**, 233.
- Lundegårdh, H. (1954). *Symp. Soc. exp. Biol.* no. 8, p. 262.
- Marsh, G. A. (1951). *Analyt. Chem.* **23**, 1427.
- Meites, L. & Meites, T. (1948). *Analyt. Chem.* **20**, 984.
- Patterson, W. B. & Stetten, D., jun. (1949). *Science*, **109**, 256.
- Rehm, W. S. (1950). *Gastroenterology*, **14**, 401.
- Robertson, R. N. & Wilkins, M. (1948a). *Nature, Lond.*, **161**, 101.
- Robertson, R. N. & Wilkins, M. (1948b). *Aust. J. sci. Res.* **1**, 17.
- Stiehler, R. D. & Flexner, L. B. (1938). *J. biol. Chem.* **126**, 603.
- Teorell, T. (1949). *Experientia*, **5**, 409.
- Ussing, H. H. (1949). *Acta physiol. scand.* **17**, 1.
- Ussing, H. H. (1954). *Ion Transport Across Membranes*, p. 3. Ed. by Clarke, H. T. New York: Academic Press Inc.
- Ussing, H. H. & Zerahn, K. (1951). *Acta physiol. scand.* **23**, 110.
- Warshowsky, B. & Schantz, E. J. (1954). *Analyt. Chem.* **26**, 1811.
- Zerahn, K. (1956). *Acta physiol. scand.* **36**, 300.

The Anaerobic Active Ion Transport by Isolated Frog Skin

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Francis & Pumphrey (1933) and Francis (1934) found that maintenance of a membrane potential by isolated frog skin was dependent upon the presence of oxygen. Largely as a result of these early studies, as well as from the use of inhibitors of metabolism, it has become accepted that active ion transport by the isolated frog skin is entirely dependent upon oxidative sources of energy. The

present results demonstrate that some active ion transport does, in fact, continue under completely anaerobic conditions though at a slower rate (about 20–40 %) than in the presence of oxygen.

METHODS

Experiments were done on the ventral skin of 15 male frogs (*Rana temporaria*) during the late spring months. The frogs were stored and prepared as previously described (Leaf & Renshaw, 1957).

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