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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Active ion transport was measured by the short-circuit technique of Ussing & Zerahn (1951) with current readings made at intervals of 2.5 min. during the experiments. The short-circuit current was measured with a Weston d.c. milliammeter, model 622 (Weston Electrical Instrument Corp., Newark, New Jersey, U.S.A.), having full-scale deflexion of $100\mu\text{A}$ at full sensitivity and an accuracy of 0.5% of full-scale reading. Measurements of ionic fluxes have not yet been done under anaerobic conditions to validate the equality of short-circuit current and active sodium transport that applies aerobically (Ussing & Zerahn, 1951). The principle that short-circuit current represents active ion transport must, however, apply although it possibly measures something less than the total active ion transport engaged in by the skin under these conditions.

The skins were mounted in a chamber similar to that described by Ussing & Zerahn (1951) and gassed through the bubbling and mixing device they describe. Volumes of 10.0 or 12.0 ml. of frog Ringer solution (112 mM-NaCl, 1.88 mM-KCl, 2.38 mM- NaHCO_3 and 0.89 mM- CaCl_2) were used as the bathing medium on each side of the skin and samples were removed as desired by pipette. No substrate was added to the medium but 0.1 mg. of calcium streptomycin and 165 units of sodium penicillin were contained in each ml. The medium was equilibrated with room air, 'oxygen-free' N_2 (British Oxygen Co.) or 100% CO (Imperial Chemical Industries, Ltd., guaranteed to contain less than 0.1% of O_2). The N_2 and CO were further deoxygenated by bubbling through a double train of vanadyl sulphate solution as recommended by Meites & Meites (1948). The O_2 tension in the medium bathing each side of the skin was continuously recorded by two vibrating Pt micro-electrodes assembled and operated as previously described (Leaf & Renshaw, 1957).

The effect of neurohypophyseal hormones was tested by addition of 2 units (0.10 ml.) of Pitressin (Parke, Davis and Co. Ltd., Batch no. LS588F) to the medium bathing the inside of the skin.

Lactic acid production by the skins was measured in the bathing medium during five of the short-circuit current experiments and in four additional experiments on isolated skins in which the ventral skin of each animal was divided in half. One half was incubated in 5.0 ml. of frog Ringer anaerobically with N_2 for 1 hr. while the other half was similarly incubated with room air or 100% O_2 . In the latter experiments the medium and the skins were separately analysed for lactic acid. The skins were dropped into liquid air, pulverized in a chilled mortar and extracted with 1-2 ml. of cold 10% trichloroacetic acid. Lactic acid determinations were made on this extract and washings of the residue. Lactic acid was measured by the method of Barker & Summerson (1941). Dry weight of skins was obtained after drying overnight at 105° .

RESULTS

Fig. 1 shows the characteristic pattern of ion transport by the isolated frog skin when exposed to anaerobic conditions. The short-circuit current drops rapidly, as oxygen is removed, to a level 20-50% of that in the presence of oxygen and becomes quite stable at this lower level for 1-2 hr. On re-exposure to air there is a rapid return toward the pre-

anaerobic levels of ion transport. If the anaerobiosis is prolonged much beyond this period of time the ion transport is no longer returned by oxygen to control rates; the skin has suffered some irreversible change from protracted anoxia.

As shown in Fig. 1, changing from pure nitrogen to pure carbon monoxide has no further effect on short-circuit current. In none of the nine experiments in which carbon monoxide was substituted for the nitrogen during anaerobic incubation was there any additional effect attributable to it.

The application of neurohypophyseal hormones to the skin was found to have no effect during anaerobic incubation in eight experiments as shown in Fig. 1, and had only a questionable and slight effect in two other experiments in which it was tested. This is in contrast to the prompt and large stimulation of ion transport it characteristically produces under aerobic conditions (Ussing & Zerahn, 1951).

To learn more about the sources of energy for the skin during anaerobiosis, estimations of lactate were made on the fluid bathing both sides of the skin in five experiments. Table 1 shows the lactate accumulation on the inside and outside of the skins during aerobic and anaerobic incubation as well as the simultaneous anaerobic ion transport. Results are expressed as $\mu\text{mole}/3.14\text{ cm.}^2$ of skin and the individual dry weights of this area of skin are recorded. It is evident that lactate is formed both

Fig. 1. A comparison of aerobic and anaerobic active ion transport by the isolated frog skin. The rate of active ion transport is indicated on the ordinate as the short-circuit current in $\mu\text{A}/\text{cm.}^2$ of skin. The actual area of skin used was 3.14 cm.^2 . Time is the abscissa. After an initial period of aeration, the medium bathing the skin was equilibrated with 100% nitrogen. At 23 min. the polarographic tracing indicated that the medium was 'oxygen free'. The rate of ion transport dropped rapidly initially in the absence of oxygen but then became stable. Altering the gas phase to 100% carbon monoxide produced no further effect. Addition of 2 units of Pitressin to the medium bathing the inside of the skin was without effect on ion transport anaerobically. Re-aeration after 70 min. without oxygen returned the rate of ion transport toward control levels.

Table 1. *Lactic acid formation and anaerobic ion transport by isolated frog skin*

The area of skin was 3.14 cm.². Inside and outside refer to medium bathing inside and outside of frog skin.

Expt. no.	Time (min.)	Lactic acid (μ moles)		Time (min.)	Lactic acid (μ moles)		Anaerobic ion transport (μ equiv.)	Dry wt. (mg.)
		Outside	Inside		Outside	Inside		
1	25	0	0.10	73	0	0.24	1.67	15.0
2	28	0.08	0.37	75	0.06	0.11	1.47	17.5
3	25	0.03	0.15	47	0.02	0.38	1.55	17.4
4	53	0.05	0.10	55	0.04	0.14	1.05	19.5
5	29	0.06	0.15	60	0.04	0.10	0.97	12.2

Table 2. *Aerobic and anaerobic lactic acid formation by isolated frog skin*

Expt.	Lactic acid (μ mole/100 mg. of wet skin/hr.)		
	In air	In O ₂	In N ₂
1	0.05	—	0.42
2	0.10	—	0.33
3	—	0.20	0.30
4	—	0.22	0.41

aerobically and anaerobically and that under both conditions it accumulates more in the medium bathing the inside of the skin.

To test the possibility that the formation of lactate aerobically was due to insufficient aeration of the skin when in the short-circuit current chamber, four additional experiments were done in test tubes fitted with a bubbling device so that one half of a skin could be gassed with pure nitrogen and the other half with air or 100 % oxygen. Table 2 shows that, even in 100 % oxygen, lactate is formed by this tissue, but that anaerobically the rate of lactate formation is greater than aerobically. The anaerobic rate was up to eight times higher than the aerobic rate. Results are expressed as μ moles of lactic acid/100 mg. wet wt. of skin/hr. The quantities of lactic acid found in the skin were small compared with that in the medium and the values presented are the totals for skin and medium.

DISCUSSION

Francis (1934) studied the effect of oxygen removal on the membrane potential of isolated frog skin and stated that: 'If the rate of respiration falls off there is no reserve material or auxiliary process by which the potential can be maintained in spite of oxygen lack.' Lund & Stapp (1947) noted, however, that a current could be drawn for several hours from the partially short-circuited frog skin under presumably anaerobic conditions, but they made no comment regarding this finding.

The present study emphasizes that the inhibition of active ion transport during oxygen lack is only partial, and for a time at least, is reversible. The

results show that ion transport can continue anaerobically; they are compatible with the view that anaerobic glycolysis can supply the required energy for this active process. This finding possibly links the mechanism of ion transport in human red blood cells, which are known to be dependent upon glycolytic supplies of energy (Danowski, 1941; Harris, 1941; Maizels, 1951) with the study of ion transport by other tissues in which aerobic-energy sources have been thought to be essential for active transport. It is likely that a requirement for the ion-transporting mechanism is a supply of adenosine triphosphate, and it makes no difference whether this is provided by aerobic or anaerobic metabolism.

The lack of demonstrable anaerobic ion transport by some other tissues which have been tested (Turner, Eggleston & Krebs, 1950; Mudge, 1951*a, b*; Whittam & Davies, 1953) is probably dependent upon at least two factors: (1) the insufficient supply of energy anaerobically by some tissues, notably kidney and brain; (2) the insensitivity of most methods of measuring active ion transport, which do not permit a distinction between a marked degree and complete suppression of active transport.

The view that ion transport was specifically dependent upon aerobic sources of energy has given rise to the oxidation-reduction hypothesis of active transport in which the transference of electrons from substrate to molecular oxygen via the oxidation-reduction reactions of the cytochrome system is considered to be the essential feature of the transporting mechanism. Evidence has been recently provided that the oxidation-reduction hypothesis is insufficient to account for observed rates of ion transport by frog skin (Zerahn, 1956; Leaf & Renshaw, 1957). The continued transport by this tissue in the absence of oxygen, and even in the presence of 100 % carbon monoxide, provide an even more forceful demonstration that active ion transport is not dependent upon oxidations involving molecular oxygen. Although the initial decrease in ion transport rate that occurs when oxygen is removed from the skin could be the result of cessation of electron transport through the

cytochrome system, a second ion-transporting mechanism would then have to be postulated to explain the anaerobic transport. It seems simpler at present, therefore, to assume that the higher rates of ion transport that occur aerobically than anaerobically are due to the more efficient energy-producing reactions in aerobic than in anaerobic metabolism, and that high-energy phosphate compounds produced by either anaerobic or aerobic pathways of metabolism are utilizable by a single ion-transporting mechanism which, for a given ion, may be similar in different tissues.

From the figures in Table 1 for active ion transport and simultaneous anaerobic lactate formation one can calculate the number of univalent ions transported per molecule of lactic acid formed. Values of 3.9–8.6 are obtained, which would mean some two to six univalent ions transported per high-energy phosphate bond synthesized, assuming glycogen to be the substrate metabolized. However, this calculation is tentative, since it is based on only a few measurements and since the total lactate production of this complex tissue must be used rather than just that part of it which supplies energy for the ion-transport process.

It has been shown that the increased ion transport produced aerobically in this tissue by neurohypophyseal hormones is associated with an increased oxygen uptake (Leaf & Renshaw, 1957). The absence of an effect of these hormones anaerobically in this tissue suggests that the action of the hormones on ion transport is somehow associated with their ability to increase the availability of aerobic-energy sources for operation of the ion-transport mechanism.

SUMMARY

1. Active ion transport by the isolated frog skin has been measured electrically by the method of Ussing & Zerahn (1951) under aerobic and anaerobic conditions.

2. In the presence of 100 % nitrogen or 100 % carbon monoxide ion transport was found to continue for over an hour, although at a lower rate (about 20–40 %) than in the presence of oxygen.

3. Under anaerobic conditions an increase in lactic acid formation was demonstrated which is compatible with the view that the energy for active ion transport anaerobically is supplied by glycolysis. In five experiments values were obtained indicating that two to six univalent ions were transported per high-energy phosphate bond synthesized anaerobically.

4. The stimulatory effect of neurohypophyseal hormones on ion transport in this tissue is absent during anaerobiosis.

5. The results conflict with the oxidation-reduction hypothesis of ion transport in which molecular oxygen is the final electron acceptor and support the view that the ion transport in this tissue is dependent upon high-energy phosphate compounds which can be supplied by either aerobic or anaerobic metabolic pathways.

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REFERENCES

- Barker, J. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
 Danowski, T. S. (1941). *J. biol. Chem.* **39**, 693.
 Francis, W. L. (1934). *J. exp. Biol.* **11**, 35.
 Francis, W. L. & Pumphrey, R. J. (1933). *J. exp. Biol.* **10**, 379.
 Harris, J. E. (1941). *J. biol. Chem.* **141**, 579.
 Leaf, A. & Renshaw, A. (1957). *Biochem. J.* **65**, 82.
 Lund, E. J. & Stapp, P. (1947). *Bioelectric Fields and Growth*, p. 268. Ed. by Lund, E. J. Austin: University of Texas Press.
 Maizels, H. (1951). *J. Physiol.* **112**, 59.
 Meites, L. & Meites, T. (1948). *Analyt. Chem.* **20**, 984.
 Mudge, G. H. (1951a). *Amer. J. Physiol.* **165**, 113.
 Mudge, G. H. (1951b). *Amer. J. Physiol.* **167**, 206.
 Turner, C., Eggleston, L. V. & Krebs, H. A. (1950). *Biochem. J.* **47**, 139.
 Ussing, H. H. & Zerahn, K. (1951). *Acta physiol. scand.* **23**, 110.
 Whittam, R. & Davies, R. E. (1953). *Biochem. J.* **55**, 880.
 Zerahn, K. (1956). *Nature, Lond.*, **177**, 937.