2.50 percent ($N = 6$). As the difference is highly significant ($P < .01$), this demonstrates that a mixture of both ions is less effective than K^+ alone in driving sodium out of the fish. This result and similar observations made for mixtures containing 5 mmole of K^+ together with 10, 25, or 50 mmole of Na^+ are indirect evidence that the K^+ influx is reduced in the presence of Na^+ ions.

In conclusion, our observations concerning the branchial sodium pump in seawater teleosts suggest that the operating mechanism is a Na-K exchange pump similar to that described for many cells (*10*). In the red cells and muscle cells an exchange diffusion mechanism also operates in parallel to the Na-K exchange pump, and part of the exchange diffusion process may be explained in terms of competition between Na^+ and K^+ for a common carrier (*11*).

One problem raised by the present investigation concerns the fate of the K^+ ions entering the gill in exchange for Na^+. Comparison of the K^+ influx and outflux in vivo for flounders in seawater shows that both fluxes are identical. Measurement of the K^+ outflux (*12*) gave a value of 145 ± 46 μmole ($N = 4$), not significantly different from that given above for the influx. The gill is obviously the major route for K^+ influx and outflux, for the renal loss of K^+ is negligible (*13*) and the drinking rate accounts for only about a 2 μmole uptake.

The main question raised by the present observations concerns the role of the Na-K activated adenosine triphosphatase activity which has been found in the teleostean gill by several authors. In *Anguilla japonica* (*14*) and *Fundulus heteroclitus* (*15*), but apparently not in *Anguilla anguilla* (*16*), the enzyme activity is higher in the seawater-adapted animals. Recent observations on many species, including the European (*17*) and American eels (*18*), confirm, however, the difference between seawater- and freshwater-adapted animals. In freshwater teleosts the branchial salt pumping mechanism exhibits high specificity for Na^+ against K^+ ions (*19*). The augmentation of the adenosine triphosphatase activity observed during adaptation to high external salinity probably coincides with the differentiation of the salt-excreting Na-K exchange and the Na-Na exchange mechanism. Furthermore, parallel variations of the enzyme activity and gill ionic turnover in seawater either after hypophysectomy (*15*) or after actinomycin D treatment (*17*) are also suggestive of an essential role of this enzyme system in sodium extrusion by the gill.

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7. To obtain the influx (in μeq hour^{-1}) of the fish the disappearance rate of external ^{42}K (in count min^{-1} hour^{-1}) was divided by the mean specific radioactivity (count min^{-1} μeq^{-1}) of potassium. Techniques and calculations are similar to those given by J. Maetz [*J. Physiol. (Paris)* **48**, 1085 (1956)] for measuring Na influx in freshwater fish.
8. The technique is outlined in R. Motais, F. Garcia Romeu, and J. Maetz (*4*) and R. Motais (*2*); Fig. 1 also illustrates an application of this procedure for different experimental conditions. Composition of artificial seawater: NaCl, 500 mmole; $CaCl_2$, 10 mmole; $MgSO_4$, 30 mmole; $MgCl_2$, 20 mmole; with or without KCl, 10 mmole.
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19. A. Krogh, *Osmotic Regulation in Aquatic Animals* (Cambridge Univ. Press, New York, 1939); J. Maetz and F. Garcia Romeu, *J. Gen. Physiol.* **47**, 1209 (1964): Table VI shows no consistent effect of the sudden increase of external K^+ concentration to one that is 50 times higher than that of Na^+ on the Na net flux of the goldfish. Recent unpublished experiments also confirm the absence of effect of K^+ on the Na^+ influx of both goldfish and eels in fresh water.
20. I thank Professor L. B. Kirschner who inspired this investigation by discussing possible models for the role of Na-K activated adenosine triphosphatase in fish gills during his sabbatical leave in my laboratory. Professor R. Motais has kindly placed unpublished data at my disposal. The technical assistance of R. Tanguy and S. Lenkauer is gratefully acknowledged.

29 July 1969

Trypsin and Papain Covalently Coupled to Porous Glass: Preparation and Characterization

Abstract. *Trypsin and papain have been covalently linked to porous glass particles. The resulting insolubilized enzymes show increased thermal stability and can be employed for extended periods of time without loss of activity.*

Insolubilized enzymes have been prepared by various methods, including polymerization onto organic polymer lattices and attachment to polymers of amino acids (*1*), coupling to cellulose (*2*) and polystyrene derivatives (*3*), and immobilization in starch (*4*) and acrylamide gels (*5*). These insoluble enzyme derivatives are either trapped in or coupled to organic carriers. Organic carriers are subject to microbial attack, and they will swell or contract depending on *p*H and other solvent conditions. Enzyme stability will be affected accordingly.

The insolubilized enzyme derivatives described here were prepared with the use of porous glass, an inorganic carrier. With the aid of an intermediate coupling agent, I have covalently bonded enzymes to many inorganic materials.

Inorganic carriers in general, and glass in particular, are not subject to microbial attack; they do not change configuration over an extensive *p*H range or under various solvent conditions; and they have a higher modulus of elasticity than organic polymers do. The enzyme-glass derivatives show increased thermal stability over enzymes insolubilized on organic carriers, and

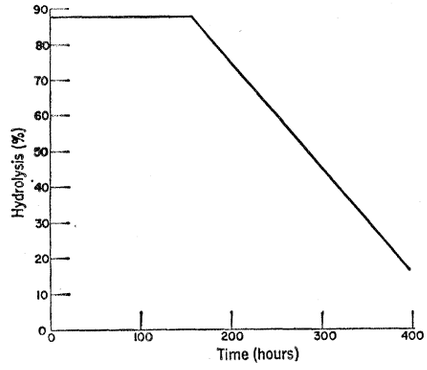

Fig. 1. Continuous trypsin assay with azo-trypsin derivative. The flow rate was maintained at 0.5 ml/min. Substrate was BAEE at 0.88 g/liter. Temperature was maintained at 23° ± 1°C. The column effluent was continuously monitored spectrophotometrically in a 1.0-cm flow-through cell at 253 nm. Percentage of hydrolysis was based on maximum optical density achieved by hydrolysis with soluble enzyme.

they are stable at room temperature over several months.

Crystalline trypsin and crude papain (*6*) were covalently coupled to a porous, 96 percent silica glass with a silane coupling agent. A sample of the porous glass [pore size, 790 ± 50 Å; surface area, 16 m^2/g (*7*)] was cleaned and then coupled to γ-aminopropyltriethoxysilane in a toluene solution (*8*).

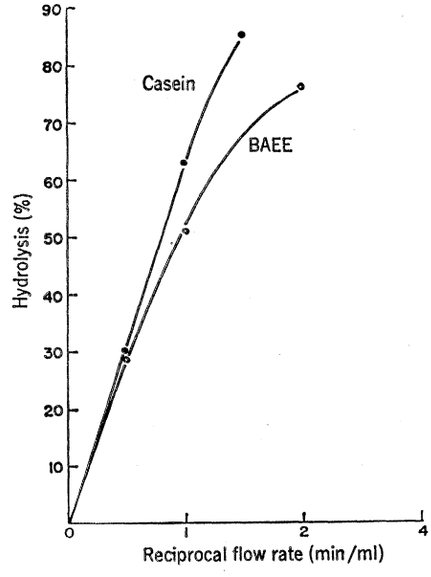

Fig. 2. Substrate hydrolysis as a function of flow rates employing a papain-glass azo-derivative. Temperature was maintained at 37° ± 1°C. Casein was employed at a concentration of 10.0 g/liter, and BAEE was used at a concentration of 0.88 g/liter. Percentage of hydrolysis was based on maximum hydrolysis achievable with soluble papain.

The aminoalkylsilane-glass derivative was either converted to an isothiocyanate derivative (*9*) or was reacted with *p*-nitrobenzoic acid to form an aminoaryl derivative (*10*). In the first case, the isothiocyanate derivative could be coupled to the trypsin by sulfonamide linkage. In the latter case, the nitro groups were reduced and covalently coupled to trypsin or papain by azo-linkage (*11*). The quantities of active enzyme coupled to these techniques were in the range 0.12 to 2.60 mg/g of glass (*12*).

The enzyme-glass derivatives were characterized for long-term stability at various temperatures. All assays were carried out by the column method. A chromatographic column was prepared and filled with an enzyme-glass derivative; the total quantity employed in a column was 1.0 g. The packed column was 1.0 cm in diameter by 5.0 cm long and had a total void volume of 2.0 ± 0.2 ml. The substrates employed were heat-denatured casein or benzoyl arginine ethyl ester (BAEE) in 0.1*M* phosphate buffer, *p*H 7.0. For the papain assays ethylenediaminetetraacetate and cysteine were added. Flow rates through the column were maintained by an LKB MiniFlow Precision Micropump. Temperature was adjusted by forcing water from a controlled temperature bath through the column jacket. The substrate reservoir was maintained in the water bath. Thus, temperature and flow rates could be altered at will.

With casein substrate the column effluent was collected in a fraction collector, precipitated with trichloroacetic acid, and examined spectrophotometrically at 280 nm. With BAEE the effluent was continuously monitored spectrophotometrically at 253 nm in a flow-through cell (1.0 cm).

A trypsin-glass derivative prepared by azo-linkage was continuously assayed over a period of 347 hours at 23°C with BAEE as substrate. To observe any losses in enzyme activity initial activity was set below 100 percent by adjusting the flow rate. No activity was lost during the first 158 hours. By 270 hours, the enzyme-glass derivative retained 50 percent activity. At the conclusion of the experiment, 16.5 percent activity remained (Fig. 1). For both casein and BAEE the percent hydrolysis observed with a papain-glass derivative was inversely proportional to flow rate (Fig. 2).

Table 1 shows the values of the temperature coefficient (Q_{10}, ratio of reaction rates at $T°$ plus 10°C and at the initial temperature $T°$) for the trypsin and papain-glass derivatives. Thermal inactivation of both trypsin and papain in their soluble forms occurs within 30 minutes at approximately 60°C (*13*). Higher temperatures increase the rate of denaturation. The thermal inactivation curves for the trypsin-glass derivatives prepared by azo- and sulfonamide-linkages are similar. Irreversible denaturation begins at 53° and 50°C, respectively, when held at those temperatures for 30 minutes. However, the papain-glass derivative was extremely stable. When continually assayed at 88°C, no detectable denaturation occurred for at least 80 minutes. At this temperature, hydrolysis of the casein substrate was so rapid that hydrolysis was almost complete, even at a concentration of 3 percent. During this 80-minute period, 2.60 mg of active enzyme covalently coupled to porous glass hydrolyzed approximately 7.5 g of casein. During the time between 80 and 120 minutes, the activity decreased 60 percent.

Table 1. Temperature coefficient (Q_{10}) values for enzyme-glass derivatives.

Substrate (g/liter)	Temperature for Q_{10} (°C)	Q_{10}
	Papain (Azo)	
BAEE 0.16	35–45	1.81
Casein 10.0	35–45	1.83
Casein 20.0	50–60	2.83
Casein 20.0	60–70	3.04
	Trypsin (Azo)	
Casein 10.0	35–45	2.77
	Trypsin (Sulf)	
Casein 10.0	35–45	1.75

The increased thermal stability of the papain bound to glass cannot be wholly explained by increased rigidity, because the trypsin bound to glass lost activity at 50° to 53°C. However, the high Q_{10} values for the trypsin-glass derivative do indicate some increased thermal stability.

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Artificial Placenta: Two Days of Total Extrauterine Support of the Isolated Premature Lamb Fetus

Abstract. *A premature lamb fetus was totally sustained by extracorporeal perfusion with the use of a silicone-membrane blood oxygenator and parenteral nutritional support. The fetus remained in a metabolically stable state lasting several days.*

The fetus is an unstable subject for isolated physiological and biochemical study (*1*). Data on the fetal metabolic state have previously resulted from analyses of blood obtained from an indwelling catheter placed in an umbilical artery or vein (*2*). Data so obtained on oxygen saturation of arterial blood, oxygen consumption, and lactate in the plasma differ from measurements obtained at cesarean section or delivery (*3*).

To study the fetus in a controlled, stable environment, we devised a total support system for isolated fetal perfusion (*4*). The system consisted of (i) a silicone membrane lung which had a constant volume of 70 ml and a gas-exchange area of 0.4 m^2 (the lung was in the form of a spiral coil), (ii) a continuously infused (8.4 ml/hour) nutritional support, and (iii) a thermoregulated (40° to 41°C) bath of synthetic amniotic fluid (*5*).

Using spinal anesthesia and clean but not sterile surgical techniques, we exposed fetal umbilical cords by cesarean section from Hampshire and Corriedale ewes of known gestation age. After the fetus was given heparin (500 U.S.P. units per kilogram), we cannulated one umbilical artery to the level of the renal artery. The cannulas were made to our specifications: outside diameter, 2.6 mm; inside diameter, 2.1 mm; segmented polyurethane reinforced with steel spring wire, 0.10 mm in diameter (*4, 6*). Umbilical arterial blood drained into an expandable silicone reservoir bag 35 cm below the fetus. Blood was then pumped by a large-bore, occlusive roller pump at 26 ml per revolution through the membrane lung and returned to the fetal umbilical vein by a wire-reinforced cannula (3.8 mm outside diameter, 3.3 mm inside diameter). After partial bypass was instituted, the second umbilical artery was similarly cannulated, and the fetus was maintained completely separate from the ewe. After cannulation, the fetus was transferred to the bath of synthetic amniotic fluid (Fig. 1).

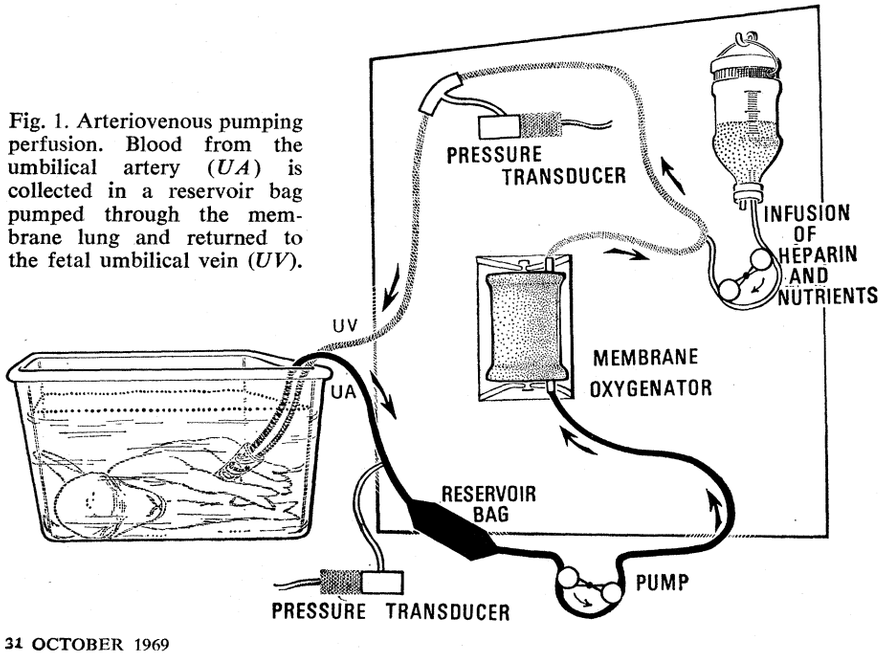

Fig. 1. Arteriovenous pumping perfusion. Blood from the umbilical artery (*UA*) is collected in a reservoir bag pumped through the membrane lung and returned to the fetal umbilical vein (*UV*).

The extracorporeal circuit and one membrane lung were primed with 240 ml of packed, adult-sheep red cells (washed with Ringer solution containing lactate) with 2000 U.S.P. units of heparin added. If two membrane lungs in series were used (fetal weight > 3 kg), the circuit was primed with 310 ml of sheep red cells. All extracorporeal tubing was made of silicone rubber with tetrafluoroethylene connectors. During perfusion, heparin (150 U.S.P. units per kilogram per hour), antibiotics (penicillin, chloramphenicol, and sodium colistimethate), and nutrients (glucose, amino acids, and vitamins) were continuously infused.

Biochemical and physiological effects of prolonged perfusion in a 3.05-kg male Hampshire fetus of 125 days gestation age (term, 147 days) are illustrated in Fig. 2. The *p*H of the blood was stabilized in the range of 7.40 ± 0.05 with P_{CO_2} between 30 and 50 mm-Hg. No buffers were added. Oxygen consumption, monitored continuously by spirometry of the membrane oxygenator gas supply, averaged 6.0 ± 0.5 cm^3 kg^{-1} min^{-1} with fetal rectal temperature varying between 39.2° ± 0.5°C. When the rate of flow in the artificial placenta was 70 to 100 ml kg^{-1} min^{-1}, oxygen saturation in the umbilical artery was between 55 and 75 percent. To compensate for early metabolic acidosis, we increased initial blood flow through the membrane oxygenator up to 150 ml kg^{-1} min^{-1}. Fetal blood pressure in the aorta was measured by occluding umbilical arterial inflow for 3 seconds, and thereby we avoided another arterial cannulation in a heparinized fetus.

Fluid volume was initially adjusted with packed, washed, adult-sheep red cells to maintain an umbilical arterial pressure of 85 mm-Hg. No further infusions of red cells or plasma were necessary. Hemoglobin fell from 16 g/100 ml to 8 g/100 ml in 55 hours, an amount consistent with sampling losses of 265 ml. This volume was replaced by Ringer solution containing lactate. Free hemoglobin in the plasma decreased from an initial 100 mg/100 ml