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Extracellular fluid volume measurements in tissues of the rainbow trout (*Oncorhynchus mykiss*) *in vivo* and their effects on intracellular pH and ion calculations

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Abstract

Extracellular fluid volume (ECFV) estimates were determined in various tissues and whole body of resting, chronically cannulated rainbow trout (*Oncorhynchus mykiss*). Fish were infused with ^{14}C-inulin, ^{3}H-polyethylene glycol (PEG, M.W. 4000), ^{3}H-mannitol, or ^{14}C-mannitol, and values of ECFV determined from tissue: plasma water distribution ratios after 6 h or 13 h equilibration. Overall, ^{3}H-PEG provided the most conservative and reliable estimates after 13 h equilibration, with ECFV values in the order: brain < white muscle < red muscle < liver < heart < gill tissue. ^{14}C-inulin yielded generally similar values to ^{3}H-PEG at 13 h, but probably overestimated ECFV in liver. ^{3}H-mannitol and ^{14}C-mannitol spaces were similar to each other and far greater than ^{3}H-PEG or ^{14}C-inulin values in most tissues. ^{3}H-mannitol values increased significantly between 6 h and 13 h, in contrast to ^{14}C-inulin. Mannitol clearly overestimated ECFV in liver and gill, and probably also heart and whole body, but may have provided more realistic estimates in brain due to better penetration of the blood-brain barrier. The Cl^-/K^+ space technique overestimated ECFV in gills, but was satisfactory in white muscle. Measurements and model calculations evaluated sources of error in intracellular pH (by ^{14}C-DMO) and ion determinations. Trapped red cells in the gills have negligible influence. Errors in ECFV are much more influential in a tissue with a high ECFV (gills) than a low ECFV (white muscle). ^{3}H-PEG is the marker of choice for intracellular pH determinations. However, even when ^{3}H-PEG is used, the potential for absolute errors in intracellular ion concentrations remains high.

Introduction

Estimates of extracellular fluid volume (ECFV) and intracellular fluid volume (ICFV) through the use of radiolabelled markers infused *in vivo* have provided insight into fluid distribution in whole fish (*e.g.*, Hickman 1972; Beyenbach and Kirschner 1976; Milligan and Wood 1986a) as well as specific tissues (*e.g.*, Houston and Mearow 1979; Milligan and Wood 1986b). ECFV estimates are also necessary for the calculation of intracellular ion concentrations (Hickman *et al.* 1964; Lutz 1972; Houston and Mearow 1979). In addition, an ECFV estimate is essential to calculate the intracellular pH (pHi) of a specific tissue (*e.g.*, Cameron and Kormanik 1982; Milligan and Wood 1986b) or mean whole body pHi (*e.g.*, Cameron 1980; Milligan and Wood 1986a) *via* the distribution of

the weak acid 5,5-dimethyloxazolidine-2,4-dione ("DMO", Waddell and Butler 1959).

There are, however, pitfalls to estimating ECFV using radiomarkers. Metabolism of the marker, penetration of intact marker and/or non-marker isotope into the ICF, and binding by a tissue all result in overestimation of ECFV (Hickman 1972; Lutz 1972; Beyenbach and Kirschner 1976; Milligan and Wood 1986a,b). Excessive marker loss at the gill and/or kidney may result in poor resolution. Spatial and temporal variations in fluid exchange between vascular and interstitial compartments may lead to artifacts (Hickman 1972; Hargens 1974; Nichols 1987). All these considerations highlight the need to critically evaluate the behavior and the equilibration time of the ECF marker in the animal or tissue of concern.

This paper expands on existing information by comparing radiomarker ECFV estimates in whole body and specific tissues of rainbow trout (*Oncorhynchus mykiss*) obtained with four commonly used radiomarkers (^{14}C-inulin, ^{3}H-PEG, ^{3}H-mannitol and ^{14}C-mannitol). For two of the radiomarkers, the influence of two commonly employed equilibration times (6 and 13 h) has also been assessed. All comparisons have been made in chronically catheterized, unanaesthetized trout held under very similar conditions of water chemistry and temperature.

Because of our particular interest in gill tissue (Wood 1991), which has a very large extracellular compartment and blood volume (Stevens 1968; Duff *et al.* 1987; Gingerich *et al.* 1987), we have quantitatively modelled the influence of the ECFV estimate on the calculation of gill pHi and ICF ions. We have also tested the Cl^-/K^+ space method for estimating ECFV in gill tissue. This technique relies on the assumption that Cl^- and K^+ are distributed between the intracellular and extracellular space according to a Donnan distribution (Conway 1957; Hickman *et al.* 1964). For comparison, a tissue of low ECFV (white muscle) has been similarily examined. The influence of trapped RBC ICF on gill intracellular ions and pHi has been evaluated.

In addition to the comparisons themselves, it is hoped that the absolute values of ECFV and ICFV, which have been tabulated for the various radiomarkers and tissues, may be of use to other workers.

Materials and methods

Radiomarkers

^{14}C-carboxyl inulin (New England Nuclear, NEC-164A, MW = 5000–5500, 5–15 mCi/mmol) and ^{14}C-methoxy inulin (New England Nuclear, NEC-692, MW = 5000–5500, 25–100 mCi/mmol) were received as crystalline solids which were dissolved in 140 mmol NaCl and purified *via* Sephadex G50 column chromatography at 4°C shortly prior to use to exclude any lower MW products of radiolysis (Kirschner 1980). Results with these two ^{14}C-inulin compounds were combined (see Results). ^{3}H-PEG (polyethylene glycol; New England Nuclear, NET-405, MW = 4000, 2–8 mCi/mmol) was received as a crystalline solid which was dissolved in 140 mmol NaCl. ^{14}C-mannitol (New England Nuclear, NEC-314, MW = 182, 45–55 mCi/mmol) and ^{3}H-mannitol (New England Nuclear, NET-101, MW = 182, 15,000–30,000 mCi/mmol) were received in 9:1 ethanol: water which was evaporated to dryness prior to reconstitution in 140 mmol NaCl. ^{14}C-DMO (5,5-dimethyloxazolidine-2,4-dione, New England Nuclear, NEC-271, MW = 129, 40–60 mCi/mmol) was received in an ethyl acetate solution which was evaporated prior to reconstitution in 140 mmol NaCl.

Experimental animals

Experiments were performed on 48 sexually immature rainbow trout (180–320 g) obtained from Spring Valley Trout Farm, Petersburg, Ontario. The fish were acclimated to 15 ± 1°C in dechlorinated Hamilton tapwater (moderately hard water; composition as in Milligan and Wood (1986a)) for 1–2 weeks prior to experimentation, during which they were not fed. Dorsal aortic catheters (PE50), containing heparinized Cortland saline (Wolf 1963), were surgically implanted under MS222

anesthesia (Soivio *et al.* 1972). Fish were allowed to recover 48 h and held individually throughout the experiment in 10 l plexiglass boxes (McDonald 1983) each receiving a flow of about 1 l/min of dechlorinated air-saturated water at 15 ± 1°C in a 200 l semi-recirculating system (90% replacement/day).

The recovered fish were infused with a bolus of ^{3}H and ^{14}C ECF marker or a ^{3}H ECF marker and ^{14}C-DMO over a 5 min period. The ^{3}H and ^{14}C radiomarkers were infused at nominal doses of 28 and 7 uCi/ml/kg respectively with an equal volume of 140 mmol NaCl as a wash-in (total infusion volume = 4 ml/kg) followed by ~0.2 ml of heparinized Cortland saline to fill the catheter.

The equilibration times of 6 and 13 h for ECF markers were chosen for their relevance to previous studies and compatibility with equilibration times for ^{14}C-DMO (*e.g.*, Cameron (1980), and Cameron and Kormanik (1982) used 4–8 h for ^{14}C-DMO, ^{3}H-mannitol and ^{3}H-inulin; Houston and Mearow (1979), and Milligan and Wood (1986a,b) used 12 h for ^{14}C-PEG, ^{14}C-DMO and ^{3}H-mannitol.) The ^{14}C-DMO was equilibrated in the fish for 13 h in this study. After equilibration, about 0.5 ml of blood was withdrawn and discarded (to flush the catheter), then another 0.5 ml was taken. Plasma was separated *via* 2 min of centrifugation at ~9000 × G and prepared immediately for scintillation counting. The fish was then quickly killed by a blow to the head and tissues were taken for analysis, including the whole brain, sub-dorsal fin epaxial muscle, the *adductor mandibularis* red muscle from the opercular region, the ventricle of the heart, and a peripheral piece of liver (to avoid major blood vessels). Brain, heart and liver were blotted lightly on tissue paper to minimize cerebrospinal/epidural fluid or blood released during dissection. When gills were sampled they were the first organ to be removed. Arches were rinsed with water, blotted with tissue paper, and then epithelium was scraped off the filament cartilage onto a glass plate using a microscope slide, after which the tissue was homogenized with a spatula. For whole body preparation, the fish was homogenized in a blender (gills intact, minus the brain, heart, liver and less than 2 g of white and red muscle).

Sample analysis

Plasma and tissue ^{3}H and ^{14}C DPM (disintegrations per minute) were determined in sub-samples (50–100 mg) digested in glass scintillation vials with 2 ml NCS (Amersham) for less than 12 h at 40°C. Cooled digests were neutralized with 60 μl of glacial acetic acid. Organic scintillant (10 ml, OCS, Amersham) was added, samples dark-adapted and counted on a LKB counter (Rackbeta 1217) with an on-board dual label quench correction program using the external standard method. The coordinates of the program were experimentally generated using a range of quenched samples (trout tissues) in the same NCS/OCS system with known additions of ^{3}H and/or ^{14}C. Tissues were counted in duplicate except heart and brain (singular) and whole body homogenate (quadruplicate).

Plasma % H_2O was determined by refractometry (American Optical TS meter) as earlier validated for trout plasma (Audet *et al.* 1988) and tissue % H_2O by drying to a constant weight at 85°C. Whole body % H_2O was taken as 0.7308 ml/g (Milligan and Wood 1982). Hematocrit (Ht) was determined by centrifugation at 1000 × G for 5 minutes.

For gill and white muscle ion calculations, plasma Cl^- was determined with a Radiometer CMT10 chloridometer and plasma Na^+ and K^+ with a Varian AA-1275 atomic absorption flame spectrophotometer. Tissue ions were determined in a similar manner after 2 weeks digestion of dried, powdered tissue in 8% perchloric acid. When necessary for pHi calculations, whole blood extracellular pH was measured via a Radiometer G 297 capillary electrode and pHM 72 acid-base analyzer. The pHi of the red blood cells was measured by the freeze-thaw lysate method of Zeidler and Kim (1977) using the same electrode.

Calculations

In the following calculations ECFV is considered the sum of the plasma volume and interstitial fluid volume (ISFV). Plasma [ions] are considered the same as interstitial fluid [ions].

$$\text{ECFV in ml/g} = (\text{tissue DPM/g})/(\text{plasma water DPM/ml}) \quad (1)$$

$$\text{ICFV in ml/g} = (\text{tissue } H_2O \text{ in ml/g}) - (\text{ECFV in ml/g}) \quad (2)$$

It can be seen from equation (2) that a reciprocal relation exists between ECFV and ICFV when total tissue water is constant, therefore, for the sake of brevity the Results and Discussion will focus on ECFV estimates with the effect on ICFV acknowledged here.

$$\text{ICF[ion] in mmol/l} = \frac{[\text{tissue ion}] - ([\text{plasma water ion}] \times \text{ECFV}))}{\text{ICFV}} \quad (3)$$

$$Cl^-/K^+ \text{ space in ml/g} = \frac{[K^+]t[Cl^-]t - ([H_2O]t)^2[Cl^-]e[K^+]e}{[K^+]t[Cl^-]e + [Cl^-]t[K^+]e - 2[H_2O]t[Cl^-]e[K^+]e} \quad (4)$$

where the subscripts t and e represent concentrations in whole tissue (mmol/kg for ions, ml/g for H_2O) and extracellular water (mmol/l plasma water), respectively.

pHi was calculated from the distribution of ^{14}C-DMO between plasma and tissues, and the appropriate ECFV and ICFV figures, as described by Milligan and Wood (1986a,b).

Data have been expressed as means ± SEM(n) where n represents the number of different fish contributing to the mean. Significance ($p < 0.05$) was assessed using a t-test or one way ANOVA followed by a Fisher LSD test, as appropriate.

Effect of trapped RBC's on pHi and ICF[ions]

In order to assess the effects of neglecting trapped RBC's on tissue pHi and ICF[ions], estimates of the amounts of ICFV, [^{14}C-DMO] and [ions] contributed by trapped RBC's to the whole tissue values were required. Fish were infused with ^{14}C-DMO and an ECF marker (^{3}H-PEG in this case). Gill was chosen since its high amount of trapped RBC's would likely show a maximum effect on the tissue pHi and ICF [ions] calculation. Trapped gill RBC ICFV (ml/g gill tissue) was determined by measuring whole blood hematocrit (Ht) and hemoglobin (Hb, as cyanomethemoglobin, Sigma) and gill homogenate Hb. The latter was determined by homogenizing ~ 100 mg of gill homogenate in 5 ml of Drabkin's reagent with a Janke and Kunkle Ultra-Turrax, centrifuging for 15 min at ~9000 × G and then measuring the absorbance of the supernatant against appropriate cyanmethemoglobin standards at 540 nm. A correction for background absorbance was made by doing the same analysis on homogenate obtained from a gill arch perfused free of RBC's. The following equation was then applied:

$$\text{gill RBC ICFV in ml/g} = \text{gill Hb} \times (\text{Ht/whole blood Hb}) \times \text{RBC } H_2O \quad (5)$$

where RBC H_2O = 0.68 ml/g (Nikinmaa *et al.* 1987)

After direct measurement of RBC pHi (by the freeze-thaw method of Zeidler and Kim (1977)), pHe, and plasma [^{14}C-DMO], RBC[^{14}C-DMO] was calculated:

$$\text{RBC}[^{14}\text{C-DMO}] \text{ in DPM/ml ICF} = \frac{(\text{plasma}[^{14}\text{C-DMO}] \times 10^{(\text{RBC pHi} - \text{pK})} + 1))}{10^{(\text{pHe} - \text{pK}) + 1}} \quad (6)$$

RBC [^{14}C-DMO] in the gills, in DPM/g gill, was then calculated by multiplying equation (5) by equation (6). These calculations then allowed for subtraction of RBC [^{14}C-DMO] from total tissue intracellular [^{14}C-DMO] and assessment of the effect on tissue pHi. RBC [ions] were taken from Ferguson and Boutilier (1989) and used to generate gill ICF [ions] from gill homogenate (after correction for trapped ECF) in the following way;

$$\text{Gill ICF[ion] in mmol/l} = \frac{(\text{ICF[ion]} \times \text{ICFV})_{\text{homogenate}} - (\text{ICF[ion]} \times \text{ICFV})_{\text{RBC}}}{(\text{ICFV})_{\text{homogenate}} - (\text{ICFV})_{\text{RBC}}}$$

Results

There were no significant differences in any tissues (t-test, $p < 0.05$) between tissue ECFV estimates after 13 h of equilibration using ^{14}C-methoxy inulin and ^{14}C-carboxyl inulin; therefore, these data

Table 1. ECFV and ICFV (ml/g) in five tissues and whole body of rainbow trout using four different radiomarkers and two equilibration times

Radiomarker	^{14}C-inulin	^{3}H-PEG	^{3}H-mannitol	^{14}C-mannitol	^{14}C-inulin	^{3}H-mannitol
Equilibration time (h)	13	13	13	13	6	6
treatment	1	2	3	4	5	6
Brain ECFV	0.035 ± 0.005	0.030 ± 0.002	0.101 ± 0.004	0.125 ± 0.010	0.025 ± 0.002	0.067 ± 0.006
ICFV	0.774 ± 0.007	0.784 ± 0.006	0.732 ± 0.006	0.672 ± 0.009	0.806 ± 0.004	0.742 ± 0.009
White muscle ECFV	0.045 ± 0.004	0.054 ± 0.005	0.085 ± 0.006	0.074 ± 0.007	0.045 ± 0.004	0.064 ± 0.006
ICFV	0.750 ± 0.003	0.720 ± 0.008	0.709 ± 0.006	0.699 ± 0.015	0.749 ± 0.004	0.731 ± 0.004
Red muscle ECFV	0.092 ± 0.005	0.140 ± 0.007	0.131 ± 0.005	0.147 ± 0.012	0.076 ± 0.003	0.126 ± 0.006
ICFV	0.707 ± 0.005	0.657 ± 0.008	0.681 ± 0.007	0.651 ± 0.009	0.731 ± 0.003	0.674 ± 0.006
Heart ECFV	0.180 ± 0.013	0.170 ± 0.022	0.364 ± 0.007	0.285 ± 0.017	0.148 ± 0.006	0.279 ± 0.009
ICFV	0.624 ± 0.013	0.626 ± 0.023	0.473 ± 0.008	0.511 ± 0.015	0.686 ± 0.009	0.528 ± 0.006
Liver ECFV	0.195 ± 0.010	0.145 ± 0.009	0.862 ± 0.011	0.790 ± 0.029	0.140 ± 0.007	0.821 ± 0.010
ICFV	0.575 ± 0.011	0.630 ± 0.012	−0.084 ± 0.011	−0.014 ± 0.028	0.638 ± 0.008	−0.051 ± 0.009
Whole body ECFV	0.193 ± 0.010	–	0.278 ± 0.009	–	0.127 ± 0.004	0.287 ± 0.015
ICFV	0.538 ± 0.010	–	0.453 +0.009	–	0.603 ± 0.004	0.444 ± 0.015

Data are shown as means ± SEM. Negative ICFV occur when ECFV artifactually exceeds total tissue water content. Each treatment is numbered; respective n numbers are (1) = 11; (2) = 8, except for white muscle and brain where n = 18; (3) = 11; (4) = 8; (5) = 11; (6) = 11. In the statistical comparisons below (ANOVA plus Fisher's LSD test, $p < 0.05$), single lines underscore subsets of means from numbered treatments which are *not* significantly different from one another:

Brain ECFV 1 2 5 4 3 6
ICFV 1 2 5 4 3 6

White muscle ECFV 1 5 2 6 4 3
ICFV 1 5 6 2 3 4

Red muscle ECFV 1 5 4 3 2 6
ICFV 1 5 4 2 6 3

Heart ECFV 5 1 2 4 6 3
ICFV 5 1 2 4 6 3

Liver ECFV 1 5 2 4 6 3
ICFV 1 5 2 4 6 3

Whole body ECFV 1 5 3 6
ICFV 1 5 3 6

have been pooled. Only ^{14}C-methoxy inulin was used in experiments with 6 h of equilibration. Herein, the inulins are referred to collectively as ^{14}C-inulin, except when referring to other studies.

Results for all four radiomarkers (^{14}C-inulin, ^{3}H-mannitol, ^{3}H-PEG, and ^{14}C-mannitol, the first two at both 6 h and 13 h equilibration) in five different tissues (brain, white muscle, red muscle, heart, liver) and whole body are summarized and statistically analyzed in Table 1.

Differences among tissues

In general, ECFV values were lowest in white muscle and brain (0.03–0.13 ml/g), slightly higher in red muscle (0.07–0.15 ml/g), higher still in heart and whole body (0.17–0.36 ml/g), and greatest in liver (0.14–0.86 ml/g). As some of the latter values (determined with ^{3}H-mannitol and ^{14}C-mannitol) were higher than total tissue water, resulting in negative ICFV's, they were clearly artifactual.

Differences among radiomarkers

When the equilibration time was 13 h, almost all tissue ECFV estimates determined using ^{14}C-inulin and ^{3}H-PEG were significantly lower than ECFV estimates determined using ^{3}H- or ^{14}C-mannitol. Mannitol ECFV estimates in liver were clearly artifactual. ECFV estimates determined using ^{14}C-inulin were significantly lower in red muscle and higher in liver than estimates determined using ^{3}H-PEG, but otherwise very similar.

The most rigorous test of molecular weight effects was when ^{3}H-PEG and ^{14}C-mannitol were equilibrated for 13 h simultaneously in the same fish. ECFV estimates were significantly lower via ^{3}H-PEG in all tissues except red muscle, where there was no difference. In 3 of 5 tissues, ECFV estimates via the two different mannitols at 13 h were significantly different. However, the absolute differences were small and did not occur in a consistent manner. ECFV estimates via ^{3}H-mannitol were significantly lower in brain but higher in heart and liver compared to estimates via ^{14}C-mannitol. When the equilibration time was 6 h, ECFV was significantly lower via ^{14}C-inulin than via ^{3}H-mannitol in all tissues analysed, consistent with the 13 h results.

Effects of equilibration time

Only ^{14}C-inulin and ^{3}H-mannitol were equilibrated for both 6 h and 13 h. For ^{14}C-inulin, the ECFV values were marginally lower at 6 h than at 13 h in all tissues, but the difference was significant only for liver. However, for ^{3}H-mannitol, all ECFV estimates except red muscle were significantly lower at 6 h than at 13 h. Despite this clear time-dependancy of the ^{3}H-mannitol ECFV estimate, ^{3}H-mannitol values at 6 h were significantly greater than those via either ^{14}C-inulin or ^{3}H-PEG at 13 h for most tissues. The only exceptions were white and red muscle, and even here the 6 h ^{3}H-mannitol estimates were greater than the 13 h ^{14}C-inulin estimates. Furthermore, even at 6 h, the ^{3}H-mannitol value for liver was clearly too high.

Measurements on gill tissue and white muscle

^{3}H-PEG, ^{3}H-mannitol, ^{14}C-mannitol (all at 13 h) and Cl^-/K^+ space were used to estimate ECFV in gill tissue (Table 2). The ^{3}H-PEG estimate (about 0.22 ml/g) was by far the lowest, approximately half the values yielded by the ^{3}H-mannitol and Cl^-/K^+ space methods. The ^{14}C-mannitol estimate was intermediate.

In white muscle, the Cl^-/K^+ space estimate was about 64% of the ^{3}H-PEG estimate and lower than any of the radiomarker ECFV estimates for white muscle in Table 1.

Tables 3 and 4 summarize the various measurements made simultaneously in the plasma, whole blood, gill tissue and white muscle of resting rainbow trout. These data were used to calculate the Cl^-/K^+ space estimate of ECFV in gill tissue and white muscle (Table 2), and to assess the influence of trapped RBC's on calculated gill parameters. Measured RBC pHi was 7.311 ± 0.012 (n = 8), and RBC ICF ions were $[Na^+]$ = 31.8, $[Cl^-]$ = 34.3

Table 2. ECFV and ICFV estimates in gill tissue by four different methods and in white muscle by two different methods

	ECFV (ml/g)	ICFV (ml/g)
Gill tissue		
^{3}H-PEG (n = 18)	0.218 ± 0.018	0.631 ± 0.021
^{3}H-mannitol (n = 8)	0.440 ± 0.011*	0.419 ± 0.011*
^{14}C-mannitol (n = 8)	0.344 ± 0.030*	0.493 ± 0.032*
Cl^-/K^+ space (n = 8)	0.424 ± 0.012*	0.413 ± 0.013*
White muscle		
^{3}H-PEG (n = 8)	0.036 ± 0.003	0.736 ± 0.006
Cl^-/K^+ space (n = 8)	0.023 ± 0.002*	0.749 ± 0.005

Data are shown as mean ± SEM; *significantly different from respective ^{3}H-PEG estimate; Note: 1. All radiomarkers equilibrated 13 h. 2. For gill tissue, the ^{3}H-mannitol and Cl^-/K^+ space estimates were not significantly different from one another. The ^{14}C-mannitol estimate was significantly different from all others. 3. For white muscle, the ^{3}H-PEG estimates were not significantly different from values obtained on a separate group of fish by ^{3}H-PEG in Table 1.

and $[K^+]$ = 142.5 mmol/l (Ferguson and Boutilier 1989). Based on Hb and Ht measurements, trapped whole blood accounted for about 0.27 ml/g of the volume of the gill tissue (gill whole blood volume = gill Ht/whole blood Ht), but trapped RBC ICFV amounted to only about 0.04 ml/g. When this small volume of RBC ICFV was taken into account in calculations otherwise based on ^{3}H-PEG distribution, gill tissue pHi (7.49 ± 0.02, n = 8) increased by only 0.01 unit and ICF [ions] ($[Na^+] = 28.9 \pm 4.6$, $[Cl^-] = 43.5 \pm 2.5$ and $[K^+] = 118.3 \pm 6.8$ mmol/l, n = 8) changed 2% or less. Owing to the much smaller trapped RBC ICFV in white muscle relative to gill tissue (*cf.*, Gingerich and Pityer 1989), the effect of trapped RBC ICFV on white muscle pHi and ICF [ions] would be minuscule.

Discussion

Behavior of the ECFV labels

It is clear from the present study that measurements of ECFV in trout tissues (and therefore indirectly ICFV) are relative rather than absolute values dependent on the choice of marker type and equilibration time. This conclusion agrees with that of several previous studies of fish tissues (*e.g.*, Beyenbach and Kirschner 1976; Houston and Mearow 1979; Cameron 1980). In general, differences appeared to be correlated with molecular weight, with mannitol (MW = 182) penetrating tissues to a greater absolute extent than PEG (MW = 4000) and inulin (MW = 5000 – 5500) (Table 1). ^{3}H-mannitol and ^{14}C-mannitol provided generally similar ECFV estimates in most tissues (absolute differences were small and inconsistent), suggesting that differences in lability or counting accuracy of the two labels were not important complicating factors. ^{3}H-PEG and ^{14}C-inulin provided much smaller ECFV estimates than either of the mannitol radiomarkers in most tissues and whole body. The ^{3}H-PEG and ^{14}C-inulin values were usually, but not always similar; ^{3}H-PEG ECFV's were significantly higher in red muscle but lower in liver.

Differences amongst radiomarkers may be explained by greater penetration of the ISF, greater penetration of the ICF, binding of the markers in the tissues, or metabolism of the markers (Lutz 1972; Hickman 1972; Beyenbach and Kirschner 1976; Milligan and Wood 1986a,b). The first factor will clearly improve the accuracy of the ECFV estimate, while the other three factors will contribute to overestimation of the true value. Mannitol grossly overestimated ECFV in the liver after only 6 h of equilibration. Kirschner (1980) found that intact rainbow trout gills were 5 times more permeable to ^{3}H-mannitol than to ^{14}C-inulin, so mannitol diffusion into the ICF was one likely cause. However, as the mannitol-based ECFV estimate in liver was greater than total tissue water, metabolism and/or binding of mannitol must also occur in the liver. The same may be true in at least two other tissues, for we have indirect evidence from pHi and ICF [ion] studies in both heart (R.S. Munger and C.M. Wood, unpublished; Milligan and Wood 1986b) and gills (see below) that the mannitol-based ECFV estimate in these tissues is impossibly high. These sites of overestimation undoubtedly contributed to the much higher whole body ECFV values found with mannitol at both 6 h and 13 h (Table 1). In the channel catfish, Cameron (1980) similarly found higher whole body ECFV estimates with ^{14}C-mannitol than with

Table 3. Plasma, whole gill tissue and whole white muscle parameters determined simultaneously in resting rainbow trout

	Plasma	Gill tissue	White muscle
Na^+ (mmol/kg)	152.1 ± 0.5	56.5 ± 1.1	7.1 ± 0.4
Cl^- (mmol/kg)	136.9 ± 0.7	60.9 ± 1.6	5.6 ± 0.3
K^+ (mmol/kg)	2.8 ± 0.0	69.5 ± 1.6	96.5 ± 1.9
Water (ml/g)	0.969 ± 0.003	0.837 ± 0.003	0.772 ± 0.006

Data are shown as mean ± SEM, n = 8.

Table 4. Whole blood and gill tissue parameters determined simultaneously in resting rainbow trout

	Whole blood	Gill tissue
Ht (ml RBC/ml)	0.253 ± 0.017	0.066 ± 0.003
Hb (g Hb/ml)	0.062 ± 0.009	0.019 ± 0.001
Hb/Ht (g Hb/ml RBC)	0.281 ± 0.012	–
Trapped whole blood (ml/g)	–	0.271 ± 0.026
Trapped RBC ICFV (ml/g tissue)	–	0.045 ± 0.002

Data are shown as mean ± SEM, n = 8.

^{3}H-inulin, though the differences were not as pronounced.

The question, therefore, arises whether mannitol is a reliable ECFV marker in any tissue of the rainbow trout. In general, the answer would be appear to be no, inasmuch as virtually all the 13 h estimates with ^{3}H-mannitol were significantly greater than 6 h estimates (Table 1), suggesting progressive penetration, binding, or metabolism. Furthermore, ^{3}H-mannitol estimates, even at 6 h, were significantly greater than 13 h ^{14}C-inulin or ^{3}H-PEG estimates for virtually all tissues. The one exception may be brain, where PEG and inulin values seemed exceptionally low, and mannitol estimates more reasonable, in light of the visibly higher content of cerebrospinal and epidural fluids in this tissue. Large molecules are poorly permeable across the blood-brain barrier (Cserr *et al.* 1978), though our recent study of the seawater skate brain indicates that even mannitol is restricted to some extent (Wood *et al.* 1990).

Less than ideal behavior in the liver may also be a problem with ^{14}C-inulin. This conclusion is based on the observations that: (i) liver was the only tissue in which the ECFV estimate with ^{14}C-inulin increased significantly between 6 h and 13 h; (ii) liver was the only tissue in which the ^{14}C-inulin ECFV was larger than the ^{3}H-PEG ECFV at 13 h (Table 1). The work of Hickman (1972) on southern flounder, of Hickman *et al.* (1972) on winter flounder, and of Schmidt-Neilsen *et al.* (1972) on winter flounder and American eels, all provide indirect support for this conclusion, based on comparisons of various inulin and PEG radiomarkers. Furthermore, these studies suggest that the problem may extent to other metabolically active tissues such as kidney, which was not sampled in the present investigation. Similarily, Beyenbach and Kirschner (1976) concluded in seawater-adapted rainbow trout that ^{14}C-PEG (MW not given) "was more effectively confined to the extracellular space" than was ^{3}H-methoxy inulin (though note the differences in isotopes from the present study). All these studies indicate that PEG is a more reliable ECFV marker than inulin for metabolically active tissues such as liver. However, it must be noted that Houston and Mearow (1979), using ^{14}C-PEG with 12 h equilibration in freshwater rainbow trout, reported ECFV values for liver more than twice those found with ^{3}H-PEG in the present study. They concluded that ^{14}C-PEG was not a suitable ECFV marker for liver tissue. The reason for this difference is unknown.

^{3}H-PEG also appeared to be the most realistic ECFV marker in gill tissue in the present study, though inulin was not evaluated (Table 2). As in other tissues, ^{3}H-mannitol and ^{14}C-mannitol gave extremely high values (Table 2) which were clearly artifactual. When used to calculate intracellular parameters, mannitol spaces yielded negative ICF concentrations for some ions and impossibly low pHi values (see below). Interestingly, Cl^-/K^+ space estimates of ECFV in gills were as high as the

mannitol values, indicating that the basic assumptions behind the calculation (*i.e.*, that ECF $[K^+][Cl^-]$ = ICF $[K^+][Cl^-]$ according to a Donnan distribution; Conway 1957) were violated in this tissue. However the same problem did not apply in white muscle; here the Cl^-/K^+ space was lower than the ECFV estimate with any radiomarker, even ^{3}H-PEG (Table 2). The Cl^-/K^+ space is generally considered to provide a reasonable ECFV estimate in skeletal muscle; the present value was similar to that reported previously in rainbow trout white muscle (Hickman *et al.* 1964; Houston and Mearow 1979).

Surprisingly, whole body ECFV estimates with ^{3}H-mannitol were the same at 6 h and 13 h equilibration (Table 1) despite increases with time seen in most individual tissues, especially liver. ^{14}C-inulin yielded much lower estimates which increased significantly between 6 h and 13 h. The whole body mannitol values determined by homogenization in the present study were similar to values determined previously with mannitol in rainbow trout by kinetic (Milligan and Wood 1982) and net retention methods (Milligan and Wood 1986a). The lower whole body inulin spaces at 13 h by homogenization were also similar to values determined previously with inulin in trout by kinetic models (Nichols 1987). The choice of marker appears to be a more important consideration than the method in estimating whole body ECFV.

In general, we conclude that ^{3}H-PEG is the most reliable and conservative of the markers tested for most tissues in rainbow trout. ^{14}C-inulin provides generally comparable values, though its use should be avoided, or the equilibration time shortened (*i.e.*, 6 h rather than 13 h) for metabolically active tissues such as liver. The need to purify inulin by chromatography also makes it less suitable. ^{3}H- and ^{14}C-mannitol probably overestimate ECFV in most tissues. Their use is generally not recommended, though they may provide more realistic estimates in brain tissue, and in situations where there is a need for rapid equilibration (< 6 h). The Cl^-/K^+ space technique, which does not involve a radiolabel, works in white muscle but not in gill tissue; for other tissues, it should be critically evaluated against a conservative marker such as ^{3}H-PEG.

ECFV effects on pHi and ICF [ions] calculations

Model calculations were performed to assess the influence of errors in ECFV on pHi and ICF [ions] for the tissues with the highest (gills) and lowest (white muscle) extracellular spaces. Representative electrolyte and ^{14}C-DMO distribution data from actual experimental fish were used to generate curves in Figs. 1, 2, and 3, at a range of assumed ECFV values. The mean ECFV estimates at 13 h by ^{3}H-PEG (minimum) and ^{3}H-mannitol (maximum) were plotted as points of reference. The influence of trapped RBC's on gill pHi and ICF [ions] was removed mathematically, though as noted earlier, the actual correction was very small.

The model calculations demonstrate that estimation of pHi by the distribution of ^{14}C-DMO between ECF and ICF is relatively insensitive to ECFV when the ECFV is small (Fig. 1). Thus the 60% increase in white muscle ECFV between ^{3}H-PEG and ^{3}H-mannitol changed pHi by only 0.06 units. However, when the absolute ECFV is large, the pHi calculation becomes critically dependant on the ECFV estimate. A comparable 60% increase in ECFV in gill tissue would have lowered pHi by 0.40 units, and the actual 100% difference between ^{3}H-PEG and ^{3}H-mannitol values lowered gill pHi by 1.3 units to ridiculously low values (Fig. 1). Note, however that the observed ^{3}H-PEG space for gill tissue is on the "plateau" of the curve, where the pHi estimate is much less dependant on ECFV. Similar patterns were observed for other tissues with higher ECFV (liver, heart; R.S. Munger and C.M. Wood unpublished). These conclusions are in general accord with those of Hinke and Menard (1978) who conducted a comparable sensitivity analysis for pHi determination in barnacle muscle fibres. ^{3}H-PEG would appear to be the ECFV marker of choice for ^{14}C-DMO determinations of pHi in fish.

The effect of the ECFV estimate on ICF [ion] calculations (Figs. 2, 3) is qualitatively similar to the effect on pHi (Fig. 1). However the magnitude of the error depends on whether the intracellular electrolyte is present in low or high concentration. Thus for Na^+ and Cl^-, which exhibit very low levels in the white muscle and high levels in ECF (Table 3),

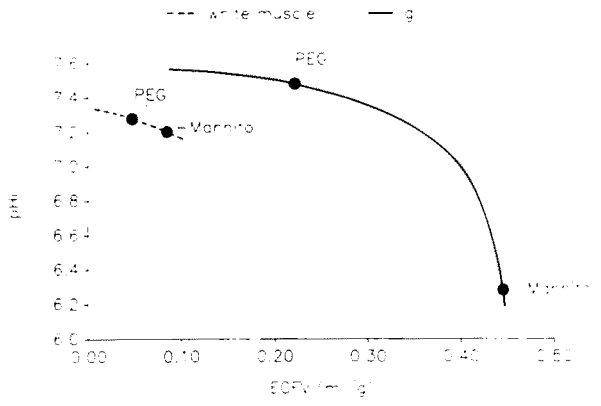

Fig. 1. Model calculations demonstrating the effect of the ECFV measurement on the intracellular pH (pHi) of two tissues, as calculated from the measured distribution of ^{14}C-DMO between plasma and tissue. White muscle has an ECFV in a very low range, and gill tissue has an ECFV in a very high range. For each, the mean ECFV estimates at 13 h by ^{3}H-PEG (minimum) and ^{3}H-mannitol (maximum) are plotted as points of reference.

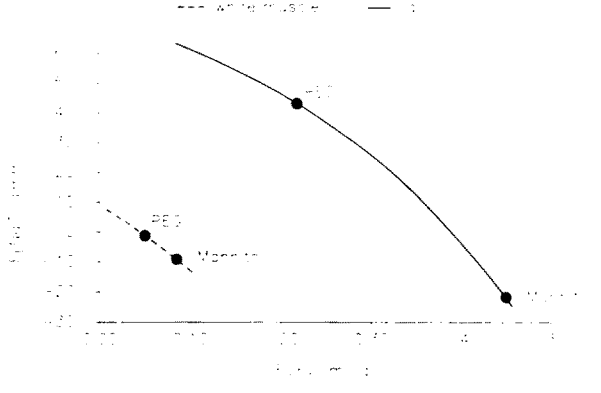

Fig. 2. Model calculations demonstrating the effect of the ECFV measurement on the intracellular sodium concentration (ICF [Na^+]) of two tissues, as calculated from the measured concentrations in plasma and tissue. White muscle has an ECFV in a very low range, and gill tissue has an ECFV in a very high range. For each, the mean ECFV estimates at 13 h by ^{3}H-PEG (minimum) and ^{3}H-mannitol (maximum) are plotted as points of reference.

use of ^{3}H-mannitol space yields negative ICF concentrations, and even small variations in the ^{3}H-PEG space will have a large influence (Fig. 2). In gill tissue, [Na^+] and [Cl^-] are relatively higher (Table 3), which would attenuate this influence if the ECFV were small. However, since the ECFV estimates are much larger, the critical dependance of ICF concentration on the ECFV estimate persists.

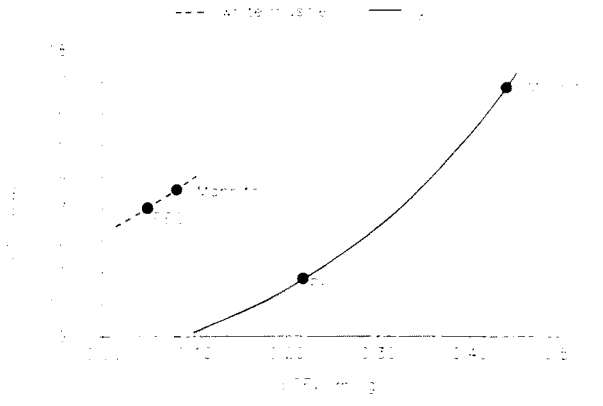

Fig. 3. Model calculations demonstrating the effect of the ECFV measurement on the intracellular potassium concentration (ICF [K^+]) of two tissues, as calculated from the measured concentrations in plasma and tissue. White muscle has an ECFV in a very low range, and gill tissue has an ECFV in a very high range. For each, the mean ECFV estimates at 13 h by ^{3}H-PEG (minimum) and ^{3}H-mannitol (maximum) are plotted as points of reference.

Notice that there is no "plateau" region, so the potential for error is very large.

For K^+, which is present in low concentration in the ECF and high concentration in the tissues (Table 3), ECFV error has only a small influence in white muscle, and the ^{3}H-PEG and ^{3}H-mannitol estimates are very similar (Fig. 3). However in gills, where both the absolute spaces and their variation between radiolabels are larger, the ICF [K^+] varies more than 60 mmol/l between the ^{3}H-PEG and ^{3}H-mannitol estimates. Space-dependant variations of similar magnitude are evident in the ICF [ion] data of Houston and Mearow (1979) on rainbow trout. In conclusion, ^{3}H-PEG again appears to be the best ECFV marker for ICF [ion] measurements, but the large potential for error must be kept in mind for all radiolabels and tissues. Relative trends will be more reliable than absolute values.

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