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WATER FLUX IN ANIMALS: ANALYSIS OF POTENTIAL ERRORS IN THE TRITIATED WATER METHOD

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ABSTRACT

Laboratory studies indicate that tritiated water measurements of water flux are accurate to within -7 to +4% in mammals, but errors are larger in some reptiles. However, under conditions that can occur in field studies, errors may be much greater. Influx of environmental water vapor via lungs and skin can cause errors exceeding ±50% in some circumstances. If water flux rates in an animal vary through time, errors approach ±15% in extreme situations, but are near ±3% in more typical circumstances. Errors due to fractional evaporation of tritiated water may approach -9%. This error probably varies between species. Use of an inappropriate equation for calculating water flux from isotope data can cause errors exceeding ±100%. The following sources of error are either negligible or avoidable: use of isotope dilution space as a measure of body water volume, loss of nonaqueous tritium bound to excreta, binding of tritium with nonaqueous substances in the body, radiation toxicity effects, and small analytical errors in isotope measurements. Water flux rates measured with tritiated water should be within ±10% of actual flux rates in most situations.

INDEX TERMS

field technique; heavy water; ^{3}HHO; HTO; water balance; water turnover

INTRODUCTION

Rates of water influx and efflux in animals can be measured by injecting them with deuterium- or tritium-labeled water, and following the decline in specific activity of the isotope in body water through time. The specific activity declines because of the loss of labeled water from the animal via excretion and evaporation, and the simultaneous input of unlabeled water via oxidative metabolism, eating and drinking. This method is quite attractive to environmental physiologists because it permits measurement of water fluxes in animals that are living unrestrained in their natural habitats. The significance of such results is reflected by the several hundred research articles on animal water fluxes that have been published in the last two decades.

Unfortunately, the labeled water method involves several assumptions, which if invalid, may cause significant errors in calculated flux rates. Lifson and McClintock (28) provided a list of these assumptions. They are: (1) body water volume remains constant during the measurement period; (2) rates of water influx and efflux are constant; (3) the isotope labels only the H_2O in the body; (4) the isotope leaves the body only as H_2O; (5) the specific activity of the isotope in water lost from the animal is the same as in body water; and (6) labeled or unlabeled water in the environment does not enter the animal via respiratory or skin surfaces. Lifson and McClintock (28) summarized their theoretical and mathematical analyses of the errors that may occur if these assumptions are wrong, especially for studies employing deuterated water. We have extended these analyses by measuring the errors associated with several

of these assumptions. We give particular emphasis to errors that may occur in terrestrial field studies and to errors associated with the use of tritiated water. The first section of this paper deals with evaluations of the six assumptions. Later sections address the questions of isotope decay errors, isotope toxicity, effects of measurement errors (sensitivity analysis) and validation studies.

Open and closed systems. Water labeled with a hydrogen isotope can be used to measure water fluxes in an open system where the animal is labeled and loses isotope to an "infinite" sink, or in a closed system where either the animal or the environment is labeled initially, and the isotope eventually comes to equilibrium throughout the system. We are concerned only with the former situation, as it is the method used in field studies. The latter method is frequently used in laboratory studies of aquatic and marine animals. An equation describing isotope fluxes in closed systems is given by Potts and Rudy (40).

EVALUATION OF THE SIX ASSUMPTIONS

(1) Constant body water volume. The amount of water in an animal can remain constant through time, it can change regularly (linear or exponential increase or decrease), or it can fluctuate unevenly. In this section, we consider the former situations. Errors resulting from uneven body water fluctuations are discussed later.

The behavior of a hydrogen isotope in the body water of an animal can be described by the equation

$$k = \frac{\ln (H_1^*/H_2^*)}{t} \qquad (1)$$

where k is the fractional "turnover rate" of the isotope, H_1^* and H_2^* are initial and final specific activities of the isotope in body water, t is time elapsed (usually expressed in days) between body water samples, and ln signifies natural logarithm. The half-life ($T_{1/2}$) of the isotope in the body is

$$T_{1/2} = \frac{0.693}{k} \qquad (2)$$

where $0.693 = \ln (H_1^*/H_2^*)$ when H_2^* is half the value of H_1^*. If the animal has maintained a constant water volume throughout the measurement period, then k also represents the fractional "turnover rate" of body water, as well as the turnover rate of the isotope. If the body water volume changed, then k describes isotope turnover only, and not water flux. ($T_{1/2}$ refers only to the isotope half-life, and not the time required for turnover of half of the body water, as has been erroneously reported in the literature.) To obtain results in units of ml H_2O (kg day)$^{-1}$, we modified equation 1 to read

$$\frac{\text{ml } H_2O \text{ flux}}{\text{kg day}} = \frac{1000\ W \ln(H_1^*/H_2^*)}{M\ t} \qquad (3)$$

where W is body water volume in ml and M is body mass in g. If deuterated water (^{2}HHO or HDO) is used, H^* is usually expressed in terms of atom % excess (atom % D in body water minus atom % D in body water of an unlabeled animal). When tritiated water (^{3}HHO or HTO) is used, H^* can be counts per minute (CPM) per unit volume of body water, corrected for background CPM. It is not necessary to correct HTO values for counting efficiency or volume of water counted, as long as these factors are identical for both H_1^* and H_2^*.

For a steady-state animal (W remains constant through time), equation 3 describes both the rate of water gain and water loss, because influx = efflux = "turnover." When body water volume changes, influx ≠ efflux and the calculated "turnover rate" equals neither, but lies somewhere in between. Lifson and McClintock (28) provide equations for calculating water flux rates in animals whose water volumes change regularly with time, either in a linear or an exponential fashion. We expanded and modified these equations to convert units to those used in equation 3. When an animal's body water volume increases or decreases linearly with time,

$$\frac{\text{ml } H_2O \text{ efflux}}{\text{kg day}} = \frac{2000\ (W_2-W_1)\ \ln(H_1^*W_1/H_2^*W_2)}{(M_1+M_2)\ \ln(W_2/W_1)\ t} \qquad (4)$$

where the subscripts 1 and 2 represent initial and final values, respectively. When body water volume changes exponentially with time,

$$\frac{\text{ml } H_2O \text{ efflux}}{\text{kg day}} = \frac{2000\ W_1\ \ln(W_2/W_1)\ \ln(H_1^*W_1/H_2^*W_2)}{(M_1+M_2)\ (1-(W_1/W_2))\ t} \qquad (5)$$

In animals with linearly or exponentially changing water volumes, the rate of water influx can be calculated using the relation:

$$\frac{\text{ml } H_2O \text{ influx}}{\text{kg day}} = \frac{\text{ml } H_2O \text{ efflux}}{\text{kg day}} + \frac{2000\ (W_2 - W_1)}{t\ (M_1 + M_2)} \qquad (6)$$

Two other methods for calculating water efflux rates when W changes linearly have been published (15, 33). When $W_1 = W_2$, both of these equations are the same as equation 3. However, when $W_1 \neq W_2$, these

equations differ from each other, as well as from equation 4 thereby raising the question as to which equation should be used. Recognizing our mathematical shortcomings, we consulted a mathematician colleague (Dr. H. Strickland) about this, and he was able to prove that equation 4 is mathematically correct. Further, he pointed out that the equations of Nagy (33) and Green (15) are incorrect in part because neither includes a term to account for the rate of change in body water volume. Since the literature already contains water flux rates calculated from these incorrect equations, we wondered about the magnitude of error contained in published flux rates. To investigate this, we used fictitious but reasonable values for all parameters, and solved each equation for ml H_2O efflux $(kg\ day)^{-1}$ over a wide range of linear change in W by varying W_2. Equations 3 (steady state) and 5 (exponential change in W) were also examined to assess the error caused by using these equations in an inappropriate situation.

The results, expressed as % difference from the value obtained using equation 4, are shown in Fig. 1. Use of equation 3 when the animal is not in a steady state produces large errors, as expected. The other equations yielded small errors (<5%) unless body water volume changed more than about 40% during the course of the measurement period. As this magnitude of volume change is probably uncommon in free-living animals, previously published water loss rates in non-steady state animals are probably fairly accurate, regardless of which equation (other than the steady state equation) was used, provided that body water volume changed regularly in those animals.

(2) Constant water flux rates. In most terrestrial animals, body water volumes probably do not remain constant or change regularly through time, but fluctuate in accordance with intermittent periods of feeding, drinking, defecation, urination, sweating, etc. Equations 3 through 6 are based on the assumption of constant water flux rates, and deviations from this situation will usually produce errors in calculated flux rates. A special case that is error-free occurs when water influx and efflux rates vary in parallel (increase or decrease simultaneously so that body water volume remains constant through time). For this situation, Lifson and McClintock (28) have shown mathematically that the calculated water efflux rate represents the average rate during the measurement period. This may well occur in the field. For example, it is possible that some animals urinate and defecate only while feeding. Thus, both water gain and loss rates would be high during the feeding period, low when the animal was not feeding, and body water volume would remain constant.

It is important to know how large an error may occur when flux rates vary through time. To represent an extreme but biologically feasible example, we chose the camel, which can lose up to about 35% of its body mass in water over a two-week period in summer, and then rehydrate rapidly by drinking this amount of water within a few minutes. Rates of water influx and efflux in a dehydrating camel were calculated from data given by Schmidt-Nielsen (45). These rates were then used in equations 4 and 6 to predict H_2^* in a camel that dehydrated for 14 days, and then instantaneously drank just enough water to replace all it lost. Since $W_1 = W_2$, equation 3 was used to obtain the calculated water efflux

rate for comparison with the actual efflux rate. To examine the effects of the timing of drinking on potential errors, the above calculations were repeated for a camel that drank at the beginning or in the middle of the 2 week measurement period, rather than at the end. Similarly, it is possible that a labeled field animal could be gaining water faster than it is losing it so that its water volume is increasing linearly with time, only to lose a large amount of water instantaneously sometime during the measurement period and end up having $W_2 = W_1$ at recapture. A gravid female laying a clutch of eggs or giving birth exemplifies this situation. To examine these possibilities, we simply reversed the influx and efflux rates, and repeated the three "camel" calculations described above.

Results of these experiments are shown in Table 1. The largest error (14%) occurs when water is instantaneously added at the end or withdrawn at the beginning of the measurement period. There is essentially no error when the addition or withdrawal is made in the middle of the measurement period. Thus, in field measurements, errors of this kind can be minimized by adjusting the measurement period so that drinking or elimination of large volumes occurs near the midpoint of the period. This will require detailed observation of the animal's behavior, which is not always possible. Moreover, if the animal's behavior is well-known, it would be best to sample the animal more frequently, especially just before and shortly after it drinks or voids, and use equations 4 and 6 to calculate water fluxes for each portion of the measurement period, thereby obtaining additional details about drinking rates, etc.

The above experiments show the errors due to variations in water

fluxes in extreme situations. To assess errors in a more typical situation, we compared calculated and actual water flux rates that would be found in a desert jackrabbit (34). These animals rest under shrubs during daylight hours, and feed intermittently throughout the night (5). To simplify matters, we assumed that water efflux in a jackrabbit is constant at 333 ml $(kg\ day)^{-1}$, water influx is 9 ml $(kg\ day)^{-1}$ to represent metabolic water production during the 16 hour rest period, and influx during the 8 hour feeding period is 981 ml $(kg\ day)^{-1}$. With these flux rates, W at the end of each 24 hour period is the same as at the beginning. H_2^* was predicted from equations 4 and 6 for each successive four hour period, over a span of five days. Actual flux rates were computed as the mean of individual four hour periods. Calculated flux rates were computed using equations 3, 4 and 6, as appropriate, and H_1^* was tritium specific activity at time zero for all calculations, so that each successive rate calculation represented a progressively longer period.

Results of this analysis are shown in Fig. 2. The error values shown are those for calculated water efflux rates. The errors in influx rates were nearly identical, but slightly smaller. The errors in calculated flux rates oscillated between about +3 and -2 % of actual fluxes, and were in phase with the oscillations in W. At the end of each 24 hr cycle, the error was close to zero. Thus, in field studies of many animals, errors due to unequal flux rates should be small (<5%), and can be made even smaller by carefully timing animal recaptures.

(3) Isotope labels body water only. The hydrogen atoms of water molecules disassociate rapidly and can freely exchange with rapidly

disassociable hydrogens of organic molecules, or can become associated with nonaqueous compounds in a less-rapidly exchanging mode, either by exchanging with slowly disassociating organic hydrogens or by becoming incorporated into newly synthesized molecules (6, 54). The incorporation of injected isotopic hydrogen into nonaqueous molecules can cause errors in two ways. If body water volume (W) is measured using the hydrogen isotope dilution method (36), rapid incorporation of isotope into nonaqueous compounds between the times of injection and initial sampling will lower H_1^* and yield an overestimate of W. Second, if a significant fraction of the isotope exchanges with body substances at a relatively slower rate (days), this will introduce errors in calculated water fluxes because H^* will change as a result of isotope exchange as well as from water flux through the animal.

The errors in tritiated water dilution estimates of W have been assessed in a variety of animals, and these are summarized in Table 2. HTO almost always overestimates W, and the mean error can be as high as +13%. The large range in errors found by different investigators using a single species (the white rat - Table 2) suggests that some of the error may be due to differences in techniques. However, since appreciable amounts of tritium can be found in completely dried tissues of HTO-injected animals (18, 38, 41, 48, 52), isotope incorporation into body compounds may account for much of the overestimate in W. This error is apparently independent of the amount of isotope injected. Gordon et al. (14) found that the error in rats was constant for HTO doses ranging from 0.2 to 1.5 microcuries (g body mass)$^{-0.75}$, and Tisavipat et al. (53) injected more than 2000 microcuries (g)$^{-0.75}$ into rats, but still

found an error of +12% (Table 2). In field studies, labeled animals that have been recaptured can be given a second injection in order to measure W_2 by isotope dilution. In kangaroo rats (Dipodomys), the error in this measurement was about the same as the error found upon initial injection of the same species (Table 2). If tritiated water dilution spaces are used as estimates of W in field studies, it seems advisable to collect additional animals for measurement of the error in such estimates so that they can be appropriately corrected.

Errors in HTO estimates of W can also arise from failing to wait long enough for the injected isotope to mix completely in the animal. Times required for complete mixing, as indicated by a lack of change in H^* in successive body water samples, vary with the method of HTO administration as well as between species. Intravascular injections mix more rapidly than do oral or intraperitoneal doses (49), but IV injection is not practical in all situations. Complete mixing of intravascular or intraperitoneal injections occurs in 0.5 to 3 h in animals ranging from small mammals (20, 36) to man (4, 10, 37), but up to 10 h may be necessary in large ruminants (30, 47, 49, 50), and dehydrated camels given oral doses require 18 h (29). These results indicate that there is considerable variation between species in the time required for thorough mixing of HTO. In view of this, it may be advisable to include measurements of mixing time in studies where W will be measured by hydrogen isotope dilution space.

The slow exchange of isotope with nonaqueous substances can cause an overestimate of water flux rates at the beginning of an experiment because H^* will decline from isotope binding as well as from water flux.

Lewis and Phillips (27) determined that equilibrium between aqueous and nonaqueous tritium was reached about five days after injection in growing calves, and that the bound tritium was equivalent to a volume of water amounting to 3.5% of M. In scorpions, King (24) found that H^* turnover was more rapid during the six days following injection than during the remaining 24 days. Calculated water flux rates were up to 20% higher during the first six days, apparently because tritium was being removed from body water via slow exchange with tissue hydrogen. After 25 days, the tissue-bound tritium accounted for 3.6% of all the tritium in the scorpions. An inflection in the early part of the tritium disappearance curve, as found in scorpions, has not been detected in humans (38), locusts (2), harbor seals (8), dogs (43), desert iguanas (32), and others. Thus, there appear to be at least two situations in which this phenomenon may contribute to significant errors in water flux calculations: in rapidly growing animals, where tritium is being incorporated into new tissue, and in adult animals having very low water flux rates, where the rate of tritium incorporation into nonaqueous substances becomes significant relative to the rate of tritium turnover via water flux. In field studies of such animals, errors of this kind can be minimized either by waiting several days for slow exchange to become complete before taking the initial sample of body water, or by correcting water flux calculations for tissue-bound tritium according to the equation of King (24).

Slowly exchanging tritium can also cause errors late in an experiment, when H^* becomes low, because the amount of nonaqueous tritium becomes important relative to aqueous tritium, and the rate of isotope loss from

the body becomes increasingly limited by the hydrogen exchange rate rather than by the water flux rate. This also produces an inflection in the tritium disappearance curve. Thompson (52) and Pinson and Langham (38) measured the decline in H^* in HTO injected mice over several weeks, and found that H^* declined with a constant half-life until it reached a value less than 1% of H_1^*. An increase in half-life, indicating that slowly exchanging tritium began to limit isotope turnover, occurred at a specific activity of about 30 microcuries per liter of body water. In other animals, the point at which the half-life increases probably varies with the amount of tritium injected, the species of animal used, its stage of development and the rate of water flux through the animal in relation to the rate of hydrogen exchange. The results from mice suggest that this source of error can be minimized in field studies by adjusting doses and recapture intervals so that H_2^* does not approach 1% of H_1^*.

(4) Isotope lost only in the form of water. If a significant amount of hydrogen isotope is bound to the dry matter in voided urine and feces, water fluxes will be overestimated. To investigate this, excreta from labeled animals were analyzed for bound tritium. We collected all urine and feces voided by the six kangaroo rats during the high humidity trial of the vapor input experiments (described in detail below). The excreta were oven-dried, ground and soaked in distilled water overnight so that bound tritium could exchange with hydrogens on water molecules, reversing the process by which tritium presumably became bound in the animal. Most of the bound isotope should have ended up in the water (48), which was then counted by liquid scintillation. When expressed as a percent of total isotope lost from the animals ($H_1^*W_1 - H_2^*W_2$), bound

tritium loss averaged 0.2% in feces, and 0.01% via urine. These kangaroo rats assimilated about 90% of the dry mass of their seed diet. In an herbivorous animal, rates of bound isotope excretion may be higher, because the lower digestibility of plants means higher rates of feces elimination, and because each six-carbon unit of cellulose has three exchangeable hydrogens (28). We repeated the above measurements using an herbivorous lizard (Sauromalus obesus), and found that the loss of bound tritium in urine and feces together amounted to only 0.9% of total isotope loss. Thus, errors in water flux calculations resulting from bound isotope loss are probably negligible in most animals.

(5) H^* in water lost equals H^* in body water. This assumption can be violated by either biological or isotopic fractionation. The major type of biological fractionation is nonuniform isotope specific activity in an animal's body, which can occur when mixing of body water compartments is slow in relation to rates of water gain and loss. Biological fractionation may occur when food residence time is very short. For example, in mice with diarrhea, fecal water had a deuterium specific activity only 90% of that in blood water (31). Other instances may be found in healthy animals in the field, such as the bird Phainopepla, which eats mistletoe berries and defecates the remains within 12-45 min. (55), or in animals which regurgitate food for their young, such as many bird species and wild canids. Lifson and McClintock (28) provide an equation to correct for this error, but it requires measurements of the rate and specific activity of any water that exits at a different specific activity than body water. This is usually not possible when working with free-living animals.

Isotope fractionation can occur when water evaporates from an animal,

since the heavier labeled water molecules may evaporate more slowly than unlabeled water. The ratio of the vapor pressure of deuterated water (HDO) to that of unlabeled water at saturation (the fractionation factor) is 0.93 at 20°C (25). Lifson and McClintock (28) included this ratio in an equation to correct for deuterium fractionation, and it yielded calculated flux rates that are close to actual rates. A similar equation for HTO is not available. The equilibrium vapor pressure ratio of HTO to H_2O increases with increasing temperature, and at 25°C it has been reported to be 0.77 (42) and 0.92 (46). Not knowing which of these two very different values is accurate, whether they are applicable in non-equilibrium situations, or whether they describe isotopic fractionation in animals, we measured fractionation during the course of evaporation from a physical and a biological system.

A beaker containing 5 ml of water and 5 μCi of tritium was placed in an airtight dessicator at 23°C along with some anhydrous $CaSO_4$ to absorb water vapor. H^* and W in the beaker were measured periodically during the seven days it took for all the water to evaporate away. The measured increase in H^* was compared with the increase expected if the fractionation factor was 0.77 and 0.92, as calculated with the equation (22)

$$H^* = H_1^* (W)^{F-1} \qquad (7)$$

where W is the proportion of W_1, remaining in the beaker at a given time, and F is the fractionation factor. To assess the behavior of HTO as it evaporates from an animal, we replaced the beaker with a toad (Bufo marinus, with a full urinary bladder) that had been injected with HTO, and repeated

the above measurements until the toad died of dehydration. The toad did not urinate or defecate, so all of its water loss was by evaporation. The beaker and toad experiments were each done three times. To make the results of beaker and toad experiments comparable, it was necessary to correct the toad H^* values for metabolic water production. Metabolic rates of the toads were measured over three 12-hour periods during the experiment by determining the decline in oxygen concentration in the dessicator, using a Beckman E2 analyzer. The mean rate of O_2 consumption was converted to H_2O production using the factor 0.53 ml H_2O formed per liter O_2 consumed (for fat metabolism). Then, equations 4 and 6 were solved for H_2^* at each t to determine the reduction in H^* due to metabolic water production alone, and the difference between these values and H_1^* was added to observed H^* values to correct them for metabolic water production.

The results of these experiments were plotted on logarithmic coordinates (to transform them into straight line relationships) and regression lines were calculated using the least squares method (Fig. 3). For the beaker experiments, the correlation coefficient is statistically significant ($r = 0.992$, $P < 0.01$), and the slope of the line indicates a fractionation factor of 0.907 (95% confidence interval = 0.900 - 0.913), which is close to that found in an equilibrium situation at 25°C by Sepall and Mason (46). The correlation coefficient for the toad experiments is also significant ($r = 0.77$, $P < 0.01$), but the slope is lower and corresponds to an "effective" fractionation factor (defined below) of 0.953 (95% confidence interval = 0.942 - 0.964). As the 95% confidence intervals for the beaker and toad experiments do not overlap, the F values

for these two systems are significantly different. According to equation 32 of Lifson and McClintock (28), an F value of 0.953 yields a 4.7% underestimate of water efflux rate in an animal whose entire water loss is by evaporation only. For an F value of 0.91 (beaker), the underestimate is 9%.

The term "effective" fractionation is intended to distinguish between fractionation during evaporation from animals versus inanimate systems, for the following reason. Many animals have skin glands which secrete drops of body fluid onto the skin surface. While water is evaporating from a drop, isotopic fractionation should occur in accordance with physical principles. However, eventually the entire drop will evaporate and the specific activity of isotope in the total evaporate will be the same as in body water (assuming no isotopic exchange occurs between water in the drop and body water during the course of evaporation). Therefore, evaporation via this avenue will have no effect on H^* in the animal. This could be thought of as another form of biological fractionation. This "bulk flow" of water may also occur in non glandular areas of skin to some degree. If water moves across skin through very narrow channels that retard mixing of water at the evaporating surface with water at the beginning of the channel, then H^* at the evaporating surface will become higher and higher as fractional evaporation proceeds, and HTO will evaporate more rapidly. H^* at the surface could, in theory, become sufficiently high that H^* in evaporated water would eventually equal that in the animal, thereby completely eliminating errors due to isotopic fractionation effects at the skin. If this phenomenon occurs in animals, then the effective F value for different animals should vary in accordance

with those factors that affect mixing of water in skin, such as cutaneous blood flow, skin thickness and skin microstructure. This phenomenon may occur to some degree during evaporation at respiratory surfaces, but since pulmonary epithelium is thin and highly perfused by blood, a large gradient in H^* would not be expected. If this is true, then differences in the ratio of pulmonary to cutaneous evaporation should influence the total HTO fractionation error occurring in different species.

Pinson and Langham (38) found that the tritium specific activities in sweat, insensible perspiration, exhaled water vapor, urine and blood in HTO-labeled humans were the same, within the error of measurement (< 3%). However, extremely large fractionation effects have been reported for other animals. Siri and Evers (48) measured H^* in body water and in water vapor collected simultaneously in downstream cold traps, and reported F values ranging from 0.78 to 0.96 in humans and 0.35 to 0.55 in pigeons. Using a similar method, Hatch and Mazrimas (18) found F values of 0.44 in kangaroo rats and 0.64 in mice. Haines et al. (17) also found that water vapor collected in cold traps from HTO-injected rodents had a relatively low H^* compared to that in body water, but the cause was dilution of evaporated body water by unlabeled water absorbed to fur. We suspect that the large fractionation effects found by Siri and Evers (48) and by Hatch and Mazrimas (18) were also partly due to water absorbed to fur or feathers. Moreover, our experiences with downstream cold traps indicate that it is difficult to keep them free of contamination from other sources of unlabeled water.

(6) No water input via skin and lungs. At the interface between air and liquid water (or a liquid-containing surface such as skin or lung), water

molecules are continuously moving from the liquid to the vapor state. The rate at which this occurs (unidirectional efflux rate) is independent of the amount of water vapor in the air. Water molecules in the vapor state are also condensing into the liquid continuously, but the condensation rate (unidirectional influx rate) is directly proportional to the vapor pressure and temperature just above the surface. In dry air, the unidirectional influx rate is zero, and in saturated air, it equals the unidirectional efflux rate. Animal physiologists are interested primarily in net evaporation rate (the difference between the unidirectional influx and efflux rates), because this relates directly to the water balance status of the animal. HTO measures unidirectional fluxes rather than net evaporation, and the difference between these is therefore considered to be an "error" in many water balance studies. [In some arthropods that can increase their body mass by absorbing vapor from unsaturated air, HTO has been used specifically to measure unidirectional influx and efflux (9).] Similarly, if liquid water contacts the skin of an animal, water molecules can exchange across the integument, thereby changing H^* even though the animal may show no net change in its water balance status.

The magnitude of this type of error depends primarily on two factors: the rate of ambient water input across skin and lungs, and the specific activity of tritium in the ambient water. The input of water vapor via skin should be directly related to the ambient humidity and the permeability of the skin to water. In this regard, Pinson and Langham (38) found that when the forearm of a human was exposed to either HTO vapor (at saturation) or liquid, the labeled water entered the body at about the same rate as the forearm lost water by evaporation (as determined in dry air).

The input of water via lungs should be related to respiratory minute volume (the product of breathing rate and tidal volume) and the water content of the inspired air, providing that the proportion of inhaled water vapor that condenses into the liquid phase in the lungs is constant. Humans inhaling HTO vapor retained nearly 99% of the isotope in the respiratory system (38), indicating that essentially all of the water vapor in inspired air mixes with, and is replaced, by body water.

The specific activity of the isotope in ambient water can have a great influence on the errors resulting from water input via skin and lungs. If H^* in incoming water equals H^* in the animal, then no error is introduced, because H^* in the animal would not change (28). If ambient water is unlabeled, then H^* in the animal will be reduced and calculated water fluxes will be too high. Alternatively, if ambient H^* is higher than in the animal, water flux rates measured with HTO will be underestimates.

To assess the errors due to cutaneous and respiratory input of water vapor, we compared HTO-measured water influxes with water influxes determined by material balance, using kangaroo rats (Dipodomys merriami, 30 to 40 g). These rodents can maintain a constant body mass on seeds alone (no drinking water) thus simplifying water input measurements. Also, their low water requirements facilitate detection of any errors. The first experiment was designed to investigate the effects of unlabeled ambient water vapor at various relative humidities. Six animals were housed individually in large glass tubes (6 x 30 cm) containing hardware cloth platforms and stoppers at both ends. Air was bubbled through unlabeled, distilled water held at various temperatures in a water bath to provide different humidities, and then metered into the chambers at 300 ml $(min)^{-1}$. This flow rate was

sufficient to keep the H^* in chamber water vapor lower than 10% of that in the animals, as determined by counting water collected in downstream cold traps. The rats were offered husked oat seeds that had equilibrated with the relative humidities used in each experiment, and food consumption was determined as the difference between the dry masses of seeds offered and seeds uneaten. Preformed water input was calculated from feeding rate and measured water contents of the seeds, and metabolic water production was determined from the difference between energy ingested and energy voided as urine and feces, using published values for the carbohydrate, fat and protein assimilated from oats (56) and the metabolic water yields of these substances (11). Preformed and metabolic water influxes were added to yield total water influx rates. The animals were given intraperitoneal injections of HTO (1.1 μCi per g body mass) in order to measure influx by the decline in H^* in successive blood samples. Each experiment lasted 8-12 days. Since all the animals maintained constant body masses, influx rates were calculated using equation 3.

The results of these experiments are shown in Table 3. HTO overestimated actual water influx rates at all humidities tested, and the error increased with increasing humidity. For field applications, expressing the error in units of ml H_2O $(kg\ day)^{-1}$ is probably better than using percent error, because percent error should vary with feeding rate and diet water content, but the absolute error should not. We conclude that significant overestimates can occur in HTO-determined water fluxes when animals are in moist, unlabeled air or where their skins are wetted by unlabeled liquid water. The magnitude of this error probably varies between animals in accordance with variations in such properties as skin permeability, surface

to volume ratios, and mass-specific respiratory minute volumes.

The errors resulting from input of water vapor of the same or higher H^* than in body water were examined in a single experiment using kangaroo rats. The methods described above were used , except that the air stream was bubbled through water containing HTO, such that H^* in the water vapor in the chambers was constant and near the mean H^* in the animals during the first two days of the experiment. H^* in the animals dropped progressively below ambient H^* as they continued to feed through the subsequent two measurement periods (Fig. 4). Humidity in the chambers averaged 20.9 mg H_2O (l air)$^{-1}$, and air flow rates averaged 60 ml (min)$^{-1}$.

The results (Table 4) indicate that there was essentially no error when H^* in ambient water vapor is about the same as in the animal. When H^* in ambient vapor exceeded that in the animal, water fluxes were underestimated, with the error increasing as the difference between isotope specific activities inside and outside the animals increased. Using deuterated water in white mice, McClintock and Lifson (31) found that water fluxes were underestimated by 14% when voided HDO was allowed to reenter the animals.

In field studies, significant amounts of HTO might be found in the air in burrows, dens, closed nests, etc., where water lost from a labeled animal could accumulate. If all the water vapor around an animal had recently evaporated from that animal, then there should be little error in water flux measurements. H^* in ambient vapor might exceed that in the animal when an animal returns to a burrow it had occupied earlier (when its

H^* was higher), or when an animal with a low H^* enters a burrow containing another animal with a higher H^*.

It is difficult to predict the H^* in ambient vapor (and the resulting error in flux measurements) that a fossorial animal would encounter in the field. To determine this empirically, we constructed an artificial burrow in the laboratory by surrounding a hardware cloth tube with soil collected near kangaroo rat burrows in the Mojave Desert. Then, we introduced a labeled kangaroo rat, and compared water influx rates measured via HTO and the balance method as above. The animal was free to forage on the surface of the dirt filled box for the weighed seeds we provided. Humidity and temperature in the burrow were measured using a Thunder Scientific model HSP-100 system, Samples of burrow water vapor were collected periodically using a cold probe placed close to the animal, and analyzed for H^*. The soil, collected between 15 and 100 cm depth in midsummer , was very dry, and burrow humidity stabilized at 14.9 mg H_2O $(1\ air)^{-1}$ with the rat inside. To examine the influence of soil moisture, we wetted the soil to field capacity (soaked then completely drained) and repeated the above measurements. Burrow humidity stabilized at 21.7 mg H_2O $(1\ air)^{-1}$ during this experiment.

H^* in water vapor from both dry and moist burrows was less than 10% of H^* in the animals (Table 5). HTO overestimated water influx rates in both situations. The error was large in the moist burrow, and about as would be predicted from Table 3. In the dry burrow, the error was much lower but still significant. The comparatively low H^* in burrow water vapor indicates that labeled water lost from the animal rapidly exchanges with unlabeled soil water, even in relatively dry soil. This suggests that water flux measurements in burrowing animals will more likely include overestimates due to unlabeled vapor influx than underestimates resulting

from labeled vapor influx. The influx of ambient water vapor appears to be the largest potential error in field measurements, and it seems unavoidable.

Lifson and McClintock (28) provide an equation which corrects for the input of labeled or unlabeled water vapor. However, its use requires knowledge of the specific activity of ambient water vapor and the rate of evaporative water loss. These are difficult to measure in field situations, but laboratory results from appropriate experiments might be of value in correcting field data.

Summary of evaluations of assumptions. Assumption (1) - constant body water volume. Equations are available for calculating accurate water flux rates from HTO turnover in animals whose water volume (W) remains constant, changes linearly or changes exponentially through time. Use of an inappropriate equation can cause very large errors. Assumption (2) - constant water flux rates. The above equations assume water fluxes are constant through time. When fluxes are not constant, these equations yield errors as high as 15% in extreme but biologically feasible situations. Under more realistic circumstances, errors are around 3%, and can be eliminated by appropriate adjustment of sampling times. Assumption (3) - tritium labels body water only. Some tritium from injected HTO becomes bound to nonaqueous compounds in the body. If tritium dilution space is equated with body water volume, errors ranging from -6 to 13% of W can occur. The slow exchange of tritium between water and nonaqueous compounds during a measurement period can cause errors in calculated water fluxes, but these errors can be avoided by appropriate adjustment of sampling times. Assumption (4) - tritium leaves animals only in the form of water. In kangaroo rats and lizards, the efflux of tritium that was bound to dry matter

in urine and feces was insignificant (<1% of total tritium efflux). Assumption (5) - The specific activity in water leaving a labeled animal is the same as that in the animal's body water. In some situations, food may not remain in an animal's digestive tract long enough for labeled body water to equilibrate with water in the digesta. This will cause underestimates in calculated flux rates. Tritiated water is heavier than unlabeled water, and evaporates more slowly. This can cause underestimates of as much as 10% in calculated water fluxes, depending on the proportion of total water loss that is due to evaporation and on the nature of water movement across the skin. In toads, the underestimate due to fractional evaporation was less than 5%. Assumption (6) - water in the environment does not enter animals across their skin or lung surfaces. Water does enter animals via their skin and lungs. The entry of water vapor by these routes can cause errors greater than ±50%, depending on ambient humidity, specific activity of tritium in water vapor around the animal, the rate of liquid water flux through the animal, surface to volume ratio, respiratory minute volume, etc. This phenomenon is apparently the largest unavoidable source of error in HTO measurements of water flux.

ISOTOPE DECAY

Being a radionuclide, tritium disappears as a result of radioactive decay, with a half-life of about 12.3 years. This will cause a decline in H^* in an animal in addition to that caused by water flux. One way to solve

this problem is simply to store all samples from an experiment and analyze them together. This way, the isotope in the samples and in the animal are decaying at the same rate, thereby cancelling out the error. If samples are not analyzed simultaneously, the decay error can be corrected using the equation (33)

$$\ln H^*_{2C} = \ln H^*_1 + k_d t_a - \ln (H^*_1/H^*_2) \qquad (8)$$

where H^*_{2C} is the corrected H^*_2, k_d is the rate of tritium decay (1.53×10^{-4} / day) and t_a is the time elapsed between analyses of H^*_1, and H^*_2 (not the time between taking the samples). Because the decay rate of tritium is so low, this correction does not become important until t_a is longer than several weeks.

ISOTOPE TOXICITY

Isotopically labeled water is heavier than unlabeled water, and can have adverse effects on animals. For example, D_2O in body water concentrations between 1 and 25% depresses metabolism and growth, apparently by slowing biochemical reactions, and chronically higher concentrations in endotherms are usually fatal (32, 44, 51). In tritiated studies, HTO is normally used at concentrations of about one HTO molecule per ten billion H_2O molecules of body water, so the "heaviness" of water in HTO-labeled animals should not disrupt the organism, but the radiation dose delivered by tritium might. The magnitude of the radiation dose an animal receives depends on the half-life of tritium in the organism and the amount of tritium administered. The latter usually depends on body size (as

this influences the volume of body fluid that can be removed for radioassay) as well as on counting statistics. For animals weighing between 10 g and 1 kg, we usually inject about 1 μCi (g body mass)$^{-1}$. The maximum noninjurious dose suggested for HTO tracer experiments in mammals is 100 μCi (g body mass)$^{-1}$ (12). However, the maximum permissible body burden for humans has been set much lower, from 1 to 3.7 mCi per individual, or about 0.02 μCi (g body mass)$^{-1}$ (38, US National Bureau of Standards Handbook No. 69).

The total radiation dose (D, in rads) an animal receives from a single injection of HTO can be calculated with the equation (19)

$$D = 0.406\ C_1^*\ T_{1/2} \qquad (9)$$

where C_1^* is the initial specific activity of tritium in the animal in μCi (g body mass)$^{-1}$ and $T_{1/2}$ is half life of tritium in the body. For a 1 kg mammal injected with 1 μCi/g and having a predicted half-life of about 4 days (43), D = 1.6 rads. This is less than 1% of the dose needed to induce acute effects, such as diarrhea and radiation death, and less than 10% of the threshold for chronic effects (e.g., impaired fertility and cancer) in mammals (39). Thus, prudent use of HTO can avoid complications due to isotope toxicity.

SENSITIVITY ANALYSIS

It is important to know the size of errors that can occur in calculated flux rates from analytical errors in the measurements of $\dot{H}^*$, M, W and t. We assessed this by solving equation 3 (steady state) with fictitious but realistic data. Then, we changed one value by 1%, solved the

equation again, and compared the results. This was repeated for each measured value, and over a wide range of combinations of values.

When measured values of M, W or t are either too high or too low by 1%, the resulting error in the calculated flux rate will be close to 1%. Errors resulting from inaccurate measurement of H^* are more complicated, because the size of the flux rate error is related to the difference between H_1^* and H_2^*. If H_2^* is 90% of H_1^*, then a ±1% error in either H_1^* or H_2^* yields a flux rate error of about ±10% (Fig. 5). The lower H_2^* becomes relative to H_1^*, the lower the flux rate error and at one isotope half-life, the error is about 1.5%. When equations 4 and 5 (W changing linearly through time) were examined as above, the resulting errors followed the same patterns as seen with equation 3, including the high sensitivity to errors in H^* shown in Fig. 5. However, the errors in water influx and water efflux rates were not equal. The one containing the largest error depended on whether W was increasing or decreasing. In field studies, errors of this kind can be minimized by waiting for at least one half-life before recapturing HTO-labeled animals.

VALIDATION STUDIES

The accuracy of the labeled water method has been tested in many species by comparison with the balance method. Lifson and McClintock (28) summarized the validation studies done with deuterated water: mean errors ranged from -8 to +5% in rats and mice. However, all the HDO studies were done in metabolism chambers utilizing dry air, which minimizes the error due to water vapor influx thereby favoring validity (26). All of the validation studies involving HTO were done in labora-

tory settings where ambient air was not dry. Nevertheless, a similar range of errors (-7 to +4%) has been found in mammals (Table 6), although greater errors occurred in some reptiles.

These results are both encouraging and surprising. On the basis of the large errors from water vapor input measured in kangaroo rats (Tables 3 and 5), we expected the absolute errors in other species to correlate with absolute humidity in ambient air. It may be that kangaroo rats are unusual in this regard, and that the magnitudes of the different types of errors vary between species, cancelling each other out to different degrees. For example, different species could show different degrees of fractional evaporation of HTO, which acts to cancel vapor input errors. The rate of air movement around an animal could affect the error from cutaneous water vapor input, through its influence on boundary layer thickness. Also, very small animals have high surface to volume ratios, and cutaneous vapor input may be relatively large (a possible explanation for the large error found in Uta lizards). These results indicate that HTO provides a reasonably accurate (± 10%) measure of water flux rates in most species tested thus far. However, there appears to be sufficient variation between species and sufficient unpredictability in the different causes of errors that we suggest: if in doubt, validate.

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Table 1. Errors in calculated values of ml H_2O flux $(kg\ day)^{-1}$ when the flux rates vary linearly through time, but $W_2 = W_1$ because of instantaneous water uptake or loss.

	% error in calculated flux rate		
	Point in measurement period of instantaneous water gain or loss		
	beginning	middle	end
Loss > gain, volume declining linearly, instantaneous water gain	-4.7	+1.4	+14.4
Gain > loss, volume increasing linearly, instantaneous water loss	+14.4	+1.4	-4.7

See text for details of calculations

Table 2. Errors in estimated total body water volume (W) by HTO dilution space, as compared with measurements of W by drying to constant mass.

Animal	M g	Error % of W	Reference
Mammals			
Mouse (Calomys)	18	+5.2	Holleman & Dieterich (21)
Mouse (Peromyscus)	19	+2.3	Holleman & Dieterich (21)
Mouse (Mus)	20?	+3.7	Siri & Evers (48)
Mouse (Antechinus)	26	+6.2	Nagy et al. (35)
Vole (Microtus)	28	+0.1	Holleman & Dieterich (21)
Rat (Dipodomys)	44	+2.1	Nagy (unpubl.)
" reinjection*	39	+3.1	Nagy (unpubl.)
Mouse (Acomys)	57	+2.3	Holleman & Dieterich (21)
Lemming (Lemmus)	64	-5.7	Holleman & Dieterich (21)
Rat (Rattus)	227	+1.7	Culebras et al. (7)
" "	150-250	+6.5	Foy & Schnieden (13)
" "	200?	+6.4	Siri & Evers (48)
" "	162	+12.0	Tisavipat et al. (53)
" "	70-190	+9.7	Gordon et al. (14)
Guinea-pig (Cavia)	500?	+1.6	Siri & Evers (48)
Rabbit (Oryctolagus)	1300	+3.1	Green & Dunsmore (16)
" "	3200	+2.8	Pace et al. (36)
Sheep (Ovis)	41000	+3.0	Smith & Sykes (49)

Bird			
Pigeon (*Columba*)	300?	+2.5	Siri & Evers (48)
Reptiles			
Anole (*Anolis*)	1.2	+4.0	Nagy & Rand (unpubl.)
Chuckwalla (*Sauromalus*)	167	+3.9	Nagy (unpubl.)
Arthropods			
Beetle (*Eleodes*)	1.0	+13.1	Bohm & Hadley (1)
Scorpion (*Hadrurus*)	6	+6.0	King (24)

Values are the overestimate (+) or underestimate (-) contained in the HTO space value, expressed in terms of percent of W.

*Reinjection of animals previously labeled with HTO.

Table 3. Error in HTO measured water influx rate resulting from input of unlabeled water vapor via lungs and skin in kangaroo rats.

	Absolute humidity, mg H_2O (l air)$^{-1}$				
	3.8	6.8	10.4	16.8	19.8
Water influx rate, ml (kg day)$^{-1}$					
HTO method	48.9	52.3	57.6	74.0	89.3
	(2.2)	(2.7)	(2.6)	(3.0)	(7.7)
Balance method	45.6	46.0	47.0	50.2	62.6
	(3.7)	(4.3)	(1.9)	(3.0)	(9.9)
Difference	+3.3*	+6.3**	+10.6**	+23.9**	+26.8**
	(2.7)	(4.1)	(1.8)	(3.5)	(4.1)
% error in HTO method	+7.7	+14.3	+22.7	+47.9	+44.2
	(6.2)	(9.4)	(4.1)	(8.5)	(12.5)

Values are means of six animals with SD in parentheses.

*P <0.05, and **P <0.005 (t-test), as compared with a difference of zero.

Table 4. Error in HTO-measured water influx rate resulting from input via skin and lungs of water vapor at the same specific activity as in the body (0-2 days) and at progressively higher specific activities (3-8 and 9-16 days).

	Time interval after injection		
	0-2 days	3-8 days	9-16 days
Water influx rate, ml $(kg\ day)^{-1}$			
HTO method	57.2	44.1	30.1
	(9.1)	(4.7)	(8.9)
Balance method	56.3	55.4	70.1
	(5.0)	(4.0)	(2.5)
Difference	+0.9	-11.3*	-40.1*
	(11.7)	(4.2)	(9.1)
% error in HTO method	+1.6	-20.2	-57.1
	(22.3)	(6.8)	(12.9)

Values are means of six kangaroo rats, with SD in parentheses.

*P <0.005 (t-test)

Table 5. Error in HTO-measured water influx rate in kangaroo rats living in an artificial burrow in dry and moist soil.

	Dry burrow, 14.9 mg H_2O (l air)$^{-1}$	Moist burrow, 21.7 mg H_2O (l air)$^{-1}$
Water influx rate, ml (kg day)$^{-1}$		
HTO method	55.1 (2.0)	91.0 (1.8)
Balance method	46.7 (1.7)	59.7 (0.4)
Difference	9.4* (0.3)	31.3* (2.2)
% error in HTO method	20.1 (0.1)	52.4 (4.0)
$\frac{H^*\text{ in vapor}}{H^*\text{ in animal}} \times 100$	9.5	1.5

Values are means of two animals with SD in parentheses.

*$P < 0.005$ (t-test)

Table 6. Summary of HTO validation studies in animals.

Animal	M_b g	Absolute Humidity mg H_2O (l air)$^{-1}$	Error in HTO method ml (kg day)$^{-1}$	%	Reference
Mammals:					
Kangaroo rat (Dipodomys)	35	19.8	+26.8	+44.2	from Table 3
Squirrel (Ammospermophilus)	96	8-12	+1.1	+0.5	W. H. Karasov (pers. comm.)
Gopher (Thomomys)	125	18	-3.0	-3.2	R. D. Gettinger (pers. comm.)
Marsupials (Perameles)	977				
(Macrotis)	1081	6-10		-7	Hulbert and Dawson (23)
(Isoodon)	1468				
Jackrabbit (Lepus)	1800	6-12	+1.7	+1.1	Nagy et al. (34)
Rabbit (Oryctolagus)	1800	<20	+2.0	+3.6	Green and Dunsmore (16)
Monkey (Alouatta)	5600	20	-3.0	-4.0	Nagy and Milton (unpubl.)
Reindeer (Rangifer)	79600	<9.4	-0.2	-0.1	Cameron et al. (3)
		<3.4	-1.5	-2.5	
		<1.1	+0.5	+1.3	

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Reptiles					
Lizard (Uta)	3	12	+6.1	+29.1	Nagy (unpubl.)
Lizard (Sauromalus)	167	4	-0.3	-2.0	Nagy (33)
Tortoise (Gopherus)	520	12	+1.3	+11.5	Nagy (unpubl.)

Errors are shown as the over- or underestimate in ml (kg day)$^{-1}$ and as percent of total influx rate.

FIGURE LEGENDS

Fig. 1. Percentage error in calculated rates of water efflux resulting from the use of inappropriate equations. Errors are shown as functions of the total percent change $[100\ (W_2-W_1)/W_1]$ in body water volume (W), with W changing linearly through time.

Fig. 2. Errors in calculated water efflux rates resulting from cyclic variation in body water volume in a jackrabbit. Changes in body mass were assumed to be due only to changes in body water volume, which cycled because rates of water influx were not constant through time. See text for calculation methods.

Fig. 3. Fractional evaporation of HTO from a beaker (dots) and a toad (circles), as indicated by the increase in tritium specific activity in the liquid phase during evaporation. The dashed lines represent the increase expected if the fractionation factor (F) = 0.77 (42) or 0.92 (46)

Fig. 4. Change in tritium specific activity (H^*) through time in kangaroo rats exposed to tritiated water vapor that was held at a constant H^* (bubbler).

Fig. 5. Error introduced into calculated flux rate (equation 3) as a result of an analytical error of 1% in H_1^* or H_2^*. The flux error will be positive or negative depending on the polarity of the error in H^*.

Fig. 1

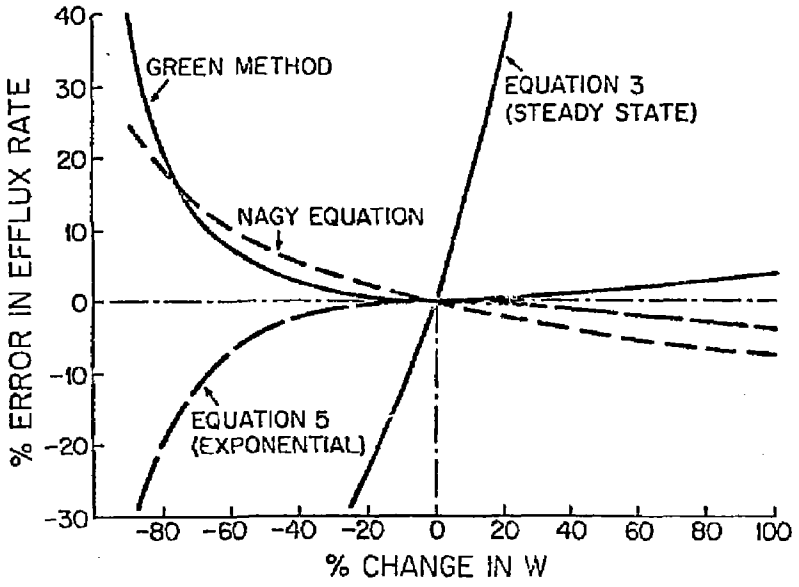
GREEN METHOD
EQUATION 3
(STEADY STATE)
NAGY EQUATION
EQUATION 5
(EXPONENTIAL)
% ERROR IN EFFLUX RATE
% CHANGE IN W
40
30
20
10
0
-10
-20
-30
-80
-60
-40
-20
0
20
40
60
80
100

Fig. 2

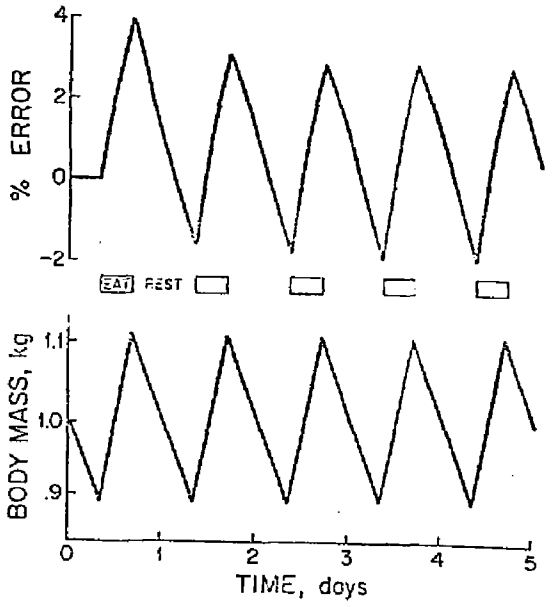
% ERROR
4
2
0
-2
EAT
REST
BODY MASS, kg
1.1
1.0
.9
0
1
2
3
4
5
TIME, days

Fig. 3

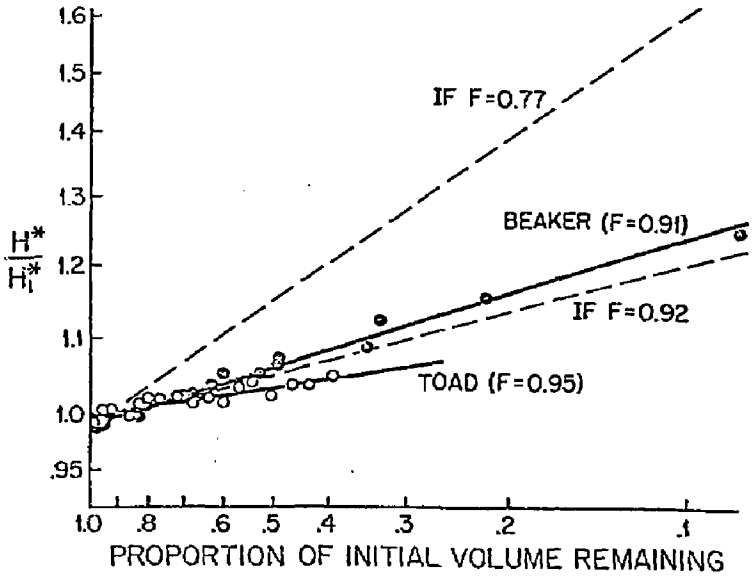
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.95
H*/H1*
IF F=0.77
BEAKER (F=0.91)
IF F=0.92
TOAD (F=0.95)
1.0
.8
.6
.5
.4
.3
.2
.1
PROPORTION OF INITIAL VOLUME REMAINING

Fig. 4

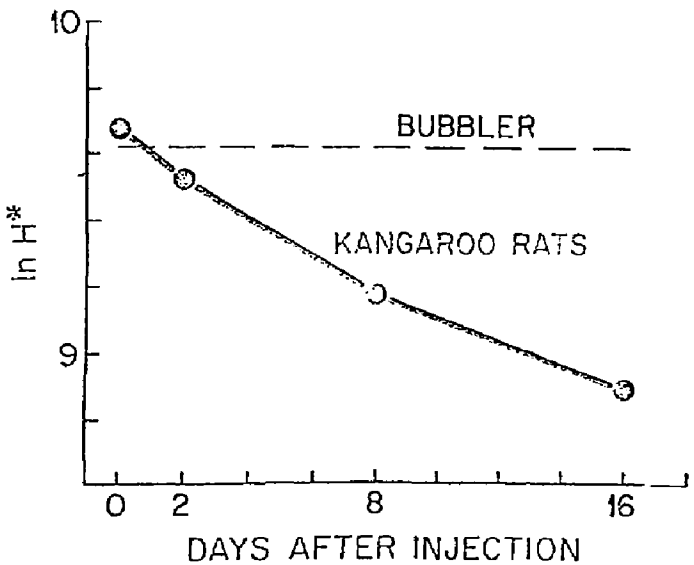
10
9
ln H*
BUBBLER
KANGAROO RATS
0
2
8
16
DAYS AFTER INJECTION

Fig. 5