J. exp. Biol. 148, 495–499 (1990)
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SHORT COMMUNICATION

FISH HEPATOCYTES ACCUMULATE HEPES: A POTENTIAL SOURCE OF ERROR IN STUDIES OF INTRACELLULAR pH REGULATION

By PATRICK J. WALSH

Division of Biology and Living Resources, Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149–1098, USA

Accepted 22 September 1989

Since their introduction in 1966, the so-called ‘Good’s’ hydrogen ion buffers (e.g. Hepes, Pipes, etc.; Good *et al.* 1966; Good and Izawa, 1972) have been used in many experimental systems. Their popularity is widespread because they appear to fit many of the criteria for ideal biological buffers, including appropriate pKa values, high aqueous solubility, minimal complexation with metal ions, and low membrane permeability (Good *et al.* 1966; Good and Izawa, 1972). More recently, the fit of Good’s buffers to these criteria has come under closer scrutiny. Under some circumstances, particularly in the *in vitro* study of metal ion–metalloprotein interactions (Nakon and Krishnamoorthy, 1983) and in studies of free-radical production (Grady *et al.* 1988), their use may lead to spurious results.

Low membrane permeability of these buffers has been predicted based on their high molecular weight and zwitterionic characteristics. However, to my knowledge, no direct studies have been performed to test this property of Good’s buffers. In at least one line of investigation, high permeability and accumulation of buffer by cells *in vitro* could have important implications. Studies of the mechanisms of intracellular pH (pHi) regulation in general (for reviews, see Roos and Boron, 1981; Walsh and Milligan, 1989) and of the mechanisms of the effects of temperature on pHi (for review, see Reeves, 1985) have employed *in vitro* cell preparations bathed in Good’s buffers. If these buffers were to accumulate to a significant degree, measurements of intrinsic intracellular buffering capacity would be artificially increased. Also, since many of these buffers have pKa–temperature coefficients similar to those of the histidine-imidazole group (approximately -0.014 and $-0.017\,\mathrm{pKa\,^{\circ}C^{-1}}$, respectively), any significant accumulation could obscure true pHi responses to temperature changes. For these reasons, I have studied the extent of uptake of one of Good’s buffers (Hepes) by toadfish hepatocytes *in vitro*.

Viable hepatocytes were isolated from the gulf toadfish *Opsanus beta* by

Key words: Hepes, ‘Good’s’ buffers, hepatocytes, toadfish, *Opsanus beta*, buffering capacity, intracellular pH.

previously published methods (Walsh, 1987), except that in some cases Hepes was omitted from the perfusion, washing and resuspension solutions. pH was maintained at 7.7 in all solutions by the addition of $5\,mmol\,l^{-1}$ $NaHCO_3$ and by continuous gassing with 0.25 % CO_2 in air; incubation temperature was 24°C. At various intervals following exposure to [^{14}C]Hepes, hepatocytes were separated from their media by previously applied methods (Cornell, 1980; Walsh, 1986, 1987). Briefly, 1 ml of cell suspension was layered on top of 0.4 ml of bromododecane in a microcentrifuge tube, and cells were pelleted by centrifugation for 1 min at 13 000 *g*. A small amount of extracellular water remained as a contaminant in the pellet, and this was quantified by the use of ^{14}C-labelled polyethylene glycol (Amersham). Intracellular water content was quantified by drying the pellets to constant weight (overnight at 60°C). ^{14}C activity was quantified by liquid scintillation counting of dispersed pellets and samples of supernatants in Aqueous Counting Scintillant (ACS) (Amersham). [^{14}C]Hepes [1-(2-hydroxy[1,2-^{14}C]ethyl)piperazine-4-(ethane-2-sulphonic acid)] (code CFQ 3667, batch RJC/C5/84/1, specific activity $2.1\,mCi\,mmol^{-1}$) was synthesized by Amersham Corporation (Oakville, Ontario and Arlington Heights, IL) and radiochemical purity was 97 %, as determined by thin-layer chromatography. All other chemicals were purchased from Sigma Chemical (St Louis, MO).

Toadfish hepatocytes were isolated under three conditions of prior Hepes exposure, and approximately $20\,mg\,cells\,ml^{-1}$ was incubated with $10\,mmol\,l^{-1}$ total Hepes and $0.25\,\mu Ci\,ml^{-1}$ [^{14}C]Hepes. As illustrated in Fig. 1, significant

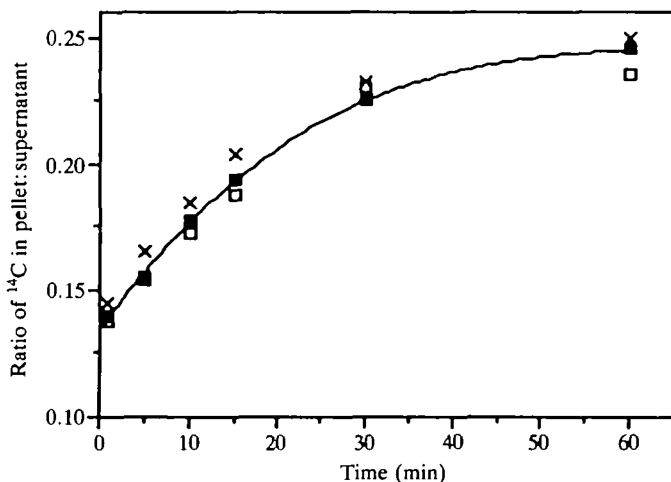

Fig. 1. Time course of ^{14}C uptake by toadfish hepatocytes exposed to [^{14}C]Hepes in a total [Hepes] of $10\,mmol\,l^{-1}$. Points are means of two hepatocyte preparations (i.e. fish) each, prepared three different ways before exposure: perfused and washed in $10\,mmol\,l^{-1}$ Hepes (□); perfused and washed in $0\,mmol\,l^{-1}$ Hepes (×); perfused in $10\,mmol\,l^{-1}$ and washed in $0\,mmol\,l^{-1}$ Hepes (■). Points are ratios of raw data: (disints $min^{-1}\,mg^{-1}$ pellet wet mass)/(disints $min^{-1}\,mg^{-1}$ supernatant mass), uncorrected for extracellular fluid content and cell water content.

radioactivity could be detected in pellets of toadfish hepatocytes, and these values increased until they levelled off at about 60 min. Ratios of pellet to supernatant ^{14}C at 60 min were corrected for extracellular fluid volume (18.2±1.5%, mean ±S.E.M., N=3) and intracellular water content (65.0±2.0%). Then, using the specific activity of Hepes (uncorrected for radiochemical impurities), the maximum accumulation of Hepes by the cells was calculated to be 1.08 mmol l^{-1} intracellular water under these conditions.

To determine whether the amount of Hepes accumulated was concentration dependent, unlabelled Hepes concentration was varied from 0 to 50 mmol l^{-1} at a constant level of [^{14}C]Hepes (0.25 μCi ml^{-1} or 0.119 mmol l^{-1} at the above-stated specific activity). No significant variation was observed in the *ratio* of ^{14}C in the pellet to that in the supernatant (uncorrected ratio = −0.0001[Hepes]+0.2212, r=0.96, N=10). As in the first experiment, calculated total Hepes accumulation by the cells was about 10% of the extracellular concentration over the range of concentrations. In these experiments, hepatocytes may have accumulated radiochemical impurities (3% of the total radioactivity): a possibility supported by an experiment in which the presence or absence of unlabelled Hepes had no effect upon the ratio. However, our limited data do not allow a precise estimate of the extent of the accumulation of impurities. Because of this limitation, our calculated Hepes accumulation by hepatocytes should be recognized as an *upper limit*.

Our results indicate that toadfish hepatocytes accumulate a small but measurable quantity of Hepes. However, it is important to place this value in perspective. We have measured the non-bicarbonate buffering capacity of toadfish hepatocytes (in suspension medium containing 10 mmol l^{-1} Hepes) as −42.80±6.18 mmol pH $unit^{-1}$ l^{-1} (mean±S.E.M., N=7) by the NH_4Cl prepulse method using 2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and dimethyloxazoladine dione (DMO) as pHi indicators (Walsh, 1989). If one adds buffering from bicarbonate (2.3[HCO_3^-]; Burton, 1978), a total buffering capacity of approximately −49 mmol pH $unit^{-1}$ l^{-1} is obtained, and this value agrees well with previously published values for fish tissues (e.g. Heisler, 1986; Milligan and Wood, 1986, 1987). Thus, accumulation of 1.08 mmol l^{-1} Hepes under these conditions leads to an overestimate of total buffering capacity of only 2.3%.

What are the potential effects of accumulated Hepes on the temperature coefficient of intracellular buffers? Unfortunately, there are no studies on the proportion of intracellular buffers that are imidazole-like in fish liver. However, Heisler and Neumann (1980) have measured this proportion to be approximately 57%, 70% and 74% for red muscle, heart muscle and white muscle, respectively, of the larger spotted dogfish (*Scyliorhinus stellaris*). Assuming that a mean of these values (67%) is approximately correct for toadfish hepatocytes, then 1.08 mmol l^{-1} Hepes accumulation at 10 mmol l^{-1} external Hepes concentration would increase the imidazole-like, temperature-sensitive buffering capacity by about 3.8%, again a relatively small proportion. In a prior study of the effects of temperature on pHi of hepatocytes from American eels (*Anguilla rostrata*), we used an external Hepes concentration of 25 mmol l^{-1} (Walsh and Moon, 1983).

Assuming that the calculations and logic applied to the toadfish hepatocytes extend to the eel hepatocytes, then imidazole-like, temperature-sensitive buffering capacity was probably artificially elevated, but by only 9.4 %. It is reasonable to conclude, then, that the results of Walsh and Moon (1983) were little biased by the addition of Hepes to the external medium.

In summary, toadfish hepatocytes accumulate only a small amount (up to 10 % of extracellular concentration) of Hepes when incubated for the short periods (up to 60 min) characteristic of many *in vitro* experiments. The percentage of uptake does not change with Hepes concentration over the range 0.1 to 50 $mmol\,l^{-1}$ leading to the accumulation of progressively larger amounts of Hepes. Given the relatively small accumulation of Hepes and the small changes in intracellular buffering capacity associated with incubations of hepatocytes in 10 $mmol\,l^{-1}$ Hepes, I recommend that this concentration be used as a maximum where possible in future experiments.

This research was supported by grants to PJW from the Natural Sciences and Engineering Research Council of Canada (A0144) (while he was a faculty member at the University of Ottawa) and the National Science Foundation (DCB-8608728). I am grateful to Jimbo Luznar for providing the toadfish. I also wish to thank Drs Norbert Heisler and Fred White for first suggesting that Hepes accumulation by cells *in vitro* might be a potential source of bias in temperature/intracellular pH studies.

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