had cut surfaces, damaged cells cannot be considered as a cause of this phenomenon. Nor can the freezing and cutting techniques be a factor because a uniform distribution of label was found in the in vivo tissues which were treated in an identical manner. Finally, this difference between the inner and outer portions of the incubated tissues also cannot be a normal variation in the extracellular space, since no such variation was observed in the in vivo studies. The failure of the indicator to penetrate to the center of the tissues in vitro may be a function of time, for its radioactivity appears to continue to increase throughout the 5 h of incubation (see Figure).

Investigators have relied upon the time-course plateau of extracellular space values in vitro as a criterion of equilibrium. The present work, testing the indicator distribution, clearly points out the fallacy of that assumption and therefore casts serious doubts upon the validity of the in vitro approach to extracellular space measurements[8].

Zusammenfassung. Gleichmässige Indikatorverteilung im Gewebe ist Ausdruck für Konzentrationsausgleich zwischen Blutstrom bzw. Inkubationsmedium und Extrazellulärraum. Es wurde ein zentraler Block aus einem Gewebsstück herausgeschnitten und die Konzentration von Inulin-carboxyl-C^{14} darin mit der im peripheren oberflächlichen Gewebe verglichen. Im Myokard des linken Ventrikels und im Soleusmuskel fand sich in vivo eine gleichmässige Verteilung, in vitro hingegen nicht; selbst bei einer Inkubationsdauer von 5 h in einem als optimal anzusehenden Milieu. Damit ist die Methode, wahre Extrazellulärräume in vitro zu messen, fragwürdig. Hingegen hat sich die Echtheit von in-vivo-Bestimmungen bestätigt.

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Bio-Research Institute, Cambridge (Mass. 02141, USA), 18 September 1967.

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Carbonic Anhydrase Activity of Erythrocyte Ghosts

The paper by ENNS[1] on the facilitation of carbon dioxide transport across membranes by carbonic anhydrase (CA), is noteworthy in view of our rather poor understanding of the mode of action of this enzyme system in the regulation of ionic balance in spite of many studies on the application of CA inhibitors to the control of tissue electrolyte levels[2]. The relationships of the carbonic anhydrases to membrane transport not only of carbon dioxide and bicarbonate but also of sodium and potassium and other tissue components deserve further serious considerations.

In his report, however, ENNS attributed an increased rate of CO_2 transfer by erythrocyte ghosts to the carbonic anhydrase content of these structures. Previous unavailing efforts to localize CA in erythrocytes prompted me to make a further attempt to find these enzymes in the cell membranes or ghosts.

Membranes were produced first from rat and then from guinea-pig and human erythrocytes both by lysing washed cells in a weakly buffered aqueous solution containing cysteine and EDTA and subsequently washing 2–4 times with this solution and then with $^1/_3$ isotonic NaCl[3] and by successively treating other batches of cells with decreasing concentrations of a buffered isotonic solution[4].

As illustrated by the data of the Table, sufficient washing of the membranes in dilute NaCl solution to remove hemoglobin (Hb) detectable at 540 nm in the extracts as cyanomethemoglobin removed all but traces of the activity in each of the experiments. Although the assay procedure for CA[5] was more sensitive than that for Hb, requiring sometimes 1 or 2 additional washes to remove the residual CA activity, I conclude that the CA's are no more a part of erythrocyte membranes than the Hb.

Whether carbonic anhydrase activity remained attached to the ghosts employed by ENNS or whether another component of his preparations accounted for their ability to speed CO_2 transport remains a point of considerable significance[6].

Comparison of removal of Ca and Hb from human erythrocyte

Supernate from washing step No.	% original Hb	% original CA
2	1.10	4.99
4	0.23	0.13
6	–[a]	< 0.03

[a] Light scattering $>$ 540 absorption.

Zusammenfassung. Durch wiederholtes Waschen mit hypotonen Lösungen gelingt es, mit dem Hämoglobin auch die Carboanhydrase vollständig aus Erythrocytenstromata zu entfernen.

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[1] T. ENNS, Science *155*, 44 (1967).

[2] G. DE STEVENS, *Diuretics* (Academic Press, New York 1963).

[3] Based on method of E. T. DUNHAM and I. M. GLYNN, J. Physiol. *156*, 274 (1961), the hemolyzing and washing solution contained $10^{-4} M$ cysteine, $10^{-3} M$ EDTA (ethylenediamine tetraacetate) and $10^{-4} M$ *Tris*-Ac (*Tris*(hydroxymethyl) aminomethaneacetate) at a final pH of 7.4.

[4] The cells were treated with 1:5, 1:6, 1:7 and 1:8 dilutions (1 vol. NaCl mix plus 4 vol. H_2O, etc.) of the following: $4 \times 10^{-4} M$ cysteine and $2 \times 10^{-3} M$ EDTA in 9 vol. 0.88% NaCl plus 1 vol. $0.15 M$ *Tris*-Ac, pH 7.4. R. BLOMFIELD, C. LONG, E. J. SARGENT and A. B. SIDLE, Biochem. J. *101*, 44P (1966).

[5] D. V. TAPPAN, M. J. JACEY and H. M. BOYDEN, Ann. N.Y. Acad. Sci. *121*, 589 (1964).

[6] The opinions expressed herein are those of the author and do not reflect the views of the Navy Department or the Submarine Medical Center.