The oxidative ability of ascorbic acid may be important in vivo; for example, in regulating biologically active proteins such as antibodies and enzymes. Additional experiments will be done in this field.

Zusammenfassung. Ascorbinsäure oxydiert die Thiolgruppen der Proteine im Plasma menschlichen Blutes schnell. Diese Fähigkeit der Ascorbinsäure mag für biologische Systeme grössere Bedeutung haben.

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Distribution of Inulin-carboxyl-C^{14} in Heart and Skeletal Muscle with Respect to in vivo and in vitro Extracellular Space Determinations

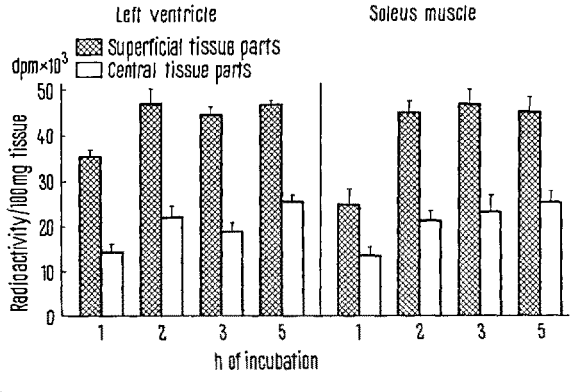
Methods for the determination of extracellular space are based on the same principle, namely the uniform distribution of an indicator throughout the entire extracellular space by the blood stream in vivo and from an incubation fluid in vitro[1]. Inulin is widely used as such an indicator since it is not metabolized and does not penetrate cells as shown by autoradiography[2] and by an osmotic gradient method[3]. In vivo experiments are generally done with nephrectomized animals; equilibrium between the serum and the extracellular space is believed to occur when the serum indicator concentration after i.v. injection falls to a constant level. After the extracellular space values obtained reach a plateau, when plotted against time, it is assumed that an equilibrium in vitro between incubation medium and extracellular space is achieved. This latter equilibration depends on the size and type of tissue as well as the nature of the indicator material and the incubation temperature[4–7].

However, it is not known whether or not these apparent equilibria indeed represent a truly uniform distribution of the indicator material throughout the extracellular compartment. Experiments were designed to verify these assumptions by measuring the distribution of inulin. Tissues exposed to inulin in vivo and in vitro were sectioned into peripheral and central parts and their concentration of radioactivity was compared with each other. With this method a direct estimation of the indicator distribution could be made.

Female golden hamsters of the London School of Hygiene strain inbred at this Institute through over 20 generations, weighing between 100 and 150 g, were used. For the in vivo experiments 8 animals were bilaterally nephrectomized under ether anesthesia and injected in the jugular vein with 0.2 ml (0.68 μc) of 100 mg% solution of inulin-carboxyl-C^{14} (New England Nuclear Corporation, Boston) in phosphate buffer (pH 7.4). Time-course studies demonstrated that the serum inulin level became constant within 60 min. After an additional period of 20 min, a piece of the left ventricle (approximately 100 mg) and the soleus muscle were excised. These tissues were washed free of any superficial radioactive contamination by 6 brief immersions in fresh Ringer's solution, then gently blotted and frozen on dry ice. The frozen tissues were sectioned with a razor blade and divided into 2 groups, a cube-like central part (15–32 mg) and a total of 6 peripheral parts (one for each face of the cube), and the radioactivity content determined separately in the following way: After weighing in tared scintillation vials these tissue pieces were digested with 0.5 ml of a toluene-soluble quarternary ammonium base (NCS, Nuclear Chicago Corporation, Chicago) at 50 °C for 20 h. Fifteen ml of scintillation fluid (4% Liquifluor in toluene, Pilot Chemicals, Watertown, Mass.) were then added to each vial. The radioactivity was measured in a refrigerated scintillation counter (Nuclear Chicago, Model 720), and corrected for background and quenching. In vitro studies were carried out on pieces of left ventricle and intact soleus muscles (cut at the tendon and left attached to the bone). Groups of 6 tissues were incubated for 1, 2, 3 or 5 h at 25 °C in Krebs-Ringer-phosphate buffer, pH 7.1, which contained 25 mg% inulin-carboxyl-C^{14} (85 μc in 100 ml), while being gassed with a mixture of 95% O_2 and 5% CO_2. The distribution of the radioactivity in the center and the surface parts was determined as described above.

The in vivo experiments revealed a nearly uniform distribution of label in the soleus and myocardium. Inside sections of myocardium contained an average of 3% less, those of the soleus muscle an average of 6% more radioactivity than the surrounding tissue. However, in the in vitro experiments no such uniformity was evident. The Figure clearly demonstrates that the central portion of these tissues contained markedly less radioactivity.

Since the pattern of the label distribution is the same in the undamaged soleus as in the myocardial tissue which



Distribution of inulin-carboxyl-C^{14} in tissues incubated in Krebs-Ringer-phosphate buffer (pH 7.1) containing 25 mg% inulin and gassed with a mixture of 95% O_2 and 5% CO_2. Each bar represents the average value of 6 experiments with standard error.

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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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Submarine Medical Center, Groton (Connecticut 06340, USA), 28 August 1967.

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[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. $0.15M$ *Tris*-Ac, pH 7.4. R. Blomfield, C. Long, E. J. Sargent and A. B. Sidle, Biochem. J. *101*, 44P (1966).
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[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.