Fig. 1. Observation of $^{13}$C label in $^1$H n.m.r. spectra

(a) The lactate methyl resonance in successive spectra obtained following the addition of D-[1-$^{13}$C]glucose to an erythrocyte suspension. The central peak (B) is from $^{13}$C-labelled lactate and the satellites (A) from $^{13}$C-labelled lactate. (b) The alanine methyl resonance in successive (2 min) spectra obtained following the addition of alanine aminotransferase to a mixture of 10 mM DL-$^{13}$C alanine and 10 mM pyruvate.

are observed. The exchange of a $^{13}$C label between the methyl groups of alanine and pyruvate, catalysed by the enzyme alanine aminotransferase, is shown in Fig. 1(b). The exchange can be used to measure enzyme activity in vitro and in situ. In addition to obtaining fractional labelling, observation of $^{13}$C in the proton n.m.r. spectrum has the added advantage that it is more sensitive than direct observation using $^1$C n.m.r. Although resolution is poorer in $^1$H n.m.r. we have recently developed a heteronuclear spin echo experiment which improves the resolution and further enhances the sensitivity of the technique.


Association of aldolase with the membranes in concentrated human erythrocyte lysates

ROBERT J. SIMPSON, KEVIN M. BRINDLE and IAIN D. CAMPBELL
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

There has been considerable interest in the binding of enzymes to the cytoplasmic membrane of the human erythrocyte (Ottaway & Mowbrary, 1977; Gillies, 1982). Aldolase is typical of these enzymes in that it binds at low ionic strength but is eluted by conditions resembling those expected to exist within the erythrocyte (Strapazon & Steck, 1976, 1977). Recently, indirect experiments with intact erythrocytes have indicated that a significant proportion of this enzyme is bound in situ in the intact cell (Tsai et al., 1982). This result implies that the interaction of aldolase with human erythrocyte membranes is in some way different in intact cells from that expected from the work in vitro. We have studied the binding of aldolase to membranes in concentrated erythrocyte lysates, which may be expected to resemble the intact cell in their composition.

Human erythrocytes were stored in citrate/phosphate/dextrose at 4°C for 10–25 days before use. The cells were washed twice in 5 vol. of 5 mM-sodium phosphate (pH 7.4)/0.15 M-NaCl, suspended in 10 vol. of Krebs–Ringer buffer and incubated for 1 h at 37°C. The cells were then washed twice in Krebs–Ringer buffer. After the final wash, the cells were centrifuged for 5 min in an Eppendorf type 5412 centrifuge. This yields samples with haematocrits exceeding 99%. These cells, contained in 1.5 ml capped Eppendorf tubes, were frozen twice in liquid N$_2$ and thawed at 37°C. This process yields lysates in which the cell contents are completely exposed to the cell exterior (Simpson et al., 1982a,b). Assays were essentially as described by Beutler (1975). The membrane volume was determined by diluting the lysate with 9 vol. of water and centrifuging for 15 min in a Hawksley microhaematocrit centrifuge.

When packed erythrocyte lysates are subjected to centrifugation (Beckman I.5.50b, SW27 head with 38.5 ml buckets), the membranes accumulate at the top, as demonstrated by the data from membrane volume and acetylcholinesterase assays (Table 1). Other enzymes and haemoglobin sediment to degrees related to their molecular weight (Table 1) while the low molecular weight peptide glutathione shows no tendency to accumulate in any fraction (data not shown). Full (100%) recovery of all components could be obtained (compared with
Table 1. Relative distribution of erythrocyte components in centrifuged lysates

<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular weight</th>
<th>Distribution ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
</tr>
<tr>
<td>Membrane volume</td>
<td>&lt;0.033</td>
<td>0.10</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>0.034</td>
<td>0.12</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>1.5</td>
<td>1.26</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>1.8</td>
<td>—</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>2.4</td>
<td>1.40</td>
</tr>
<tr>
<td>Aldolase</td>
<td>—</td>
<td>1.40</td>
</tr>
<tr>
<td>Fumarase</td>
<td>2.5</td>
<td>—</td>
</tr>
<tr>
<td>Catalase</td>
<td>2.9</td>
<td>—</td>
</tr>
</tbody>
</table>

The results are given as a percent of the total quantity of the component. The distribution ratio is the quantity in the bottom fraction divided by the quantity in the top fraction.

If there was weak binding such that 5% of the aldolase in an uncentrifuged lysate was membrane bound then the distribution ratio for aldolase in experiments (b), (c) and (d) would have been 1.3, 1.4 and 1.14 respectively. Thus we can say that less than 5% of aldolase is associated with the membrane in concentrated erythrocyte lysates.

Hydrostatic pressures generated during ultracentrifugation have been known to disrupt protein-protein interactions (Beaufay & Amar-Costescu, 1976). In experiments (b)–(d) (Table 1), however, the maximum hydrostatic pressure generated is restricted to 52 atm (5269 kPa) by the use of short sample columns (0.5 cm for a 1 ml sample).

To conclude, these data do not provide support for the proposal that aldolase is bound in significant quantity to the membrane in the human erythrocyte. The report that this enzyme is bound to the membrane in intact cells does not, therefore, appear to have any thermodynamic basis.


Hormonal regulation of Ca\textsuperscript{2+} efflux from liver mitochondria

TIMOTHY P. GOLDSTONE and MARTIN CROMPTON

Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

It is thought that \(\alpha\)-adrenergic agonists promote hepatic glycogenolysis by increasing the cytosolic free \(\text{Ca}^{2+}\) and thereby activating phosphorylase \(b\) kinase (for review, see Williamson et al., 1981). The source of the hormonally sensitive \(\text{Ca}^{2+}\) is disputed, however. Particular controversy surrounds the possible release of mitochondrial \(\text{Ca}^{2+}\) during adrenergic stimulation (Foden & Randle, 1978; Blackmore et al., 1979).

On the other hand, it is generally considered that the stimulation of glycogenolysis by glucagon and \(\beta\)-adrenergic agonists may be accounted for by changes in the tissue concentrations of cyclic AMP.

Recent studies, however, indicate that infusion of liver with the \(\beta\)-adrenergic agonist isoprenaline causes an activation of a Na\textsuperscript{+}-dependent system for mitochondrial Ca\textsuperscript{2+} efflux, and that this activation is stable and persists through the mitochondrial isolation procedure. Phenylephrine, an \(\alpha\)-adrenergic agonist, did not affect the release of mitochondrial Ca\textsuperscript{2+} (Goldstone & Crompton, 1982). In the present work these findings have been extended by comparing the effects of infusion with glucagon and exogenous cyclic AMP with those induced by isoprenaline.

Livers were perfused as reported previously (Goldstone & Crompton, 1982); the medium used was Krebs–Henseleit (1922) bicarbonate buffer containing 10 mm-lactate plus 1 mm-pyruvate at 37°C at a flow rate of 40 ml/min. After perfusion for 30 min, the right lobe was clamped off, removed and homogenized. The remaining lobes were perfused for a further 2 min with isoprenaline, glucagon, cyclic AMP or phenylephrine, which were introduced into the perfusion medium. The treated lobes were then removed and homogenized in a manner identical with that used for the control lobe. Mitochondria from control and treated homogenates were prepared as reported previously (Goldstone & Crompton, 1982). The mitochondria were suspended finally in a medium containing 210 mm-mannitol, 70 mm-sucrose and 10 mm-Tris/HCl, pH 7.4. Measurement of Ca\textsuperscript{2+} efflux was performed exactly as described previously (Goldstone & Crompton, 1982).

Each test determination with mitochondria from treated lobes was accompanied by a control with mitochondria from untreated lobes of the same liver. Na\textsuperscript{+}-dependent efflux refers to Ca\textsuperscript{2+} efflux in the presence of Ruthenium Red plus 6 mm-Na\textsuperscript{+}, minus the efflux in the presence of Ruthenium Red alone (Na\textsuperscript{+}-independent efflux).

Table 1. Effect of infusion with hormones and cyclic AMP on the rate of mitochondrial Ca\textsuperscript{2+} efflux

Control and treated mitochondria were isolated from perfused livers as outlined in the text. The initial rates of Ca\textsuperscript{2+} efflux from mitochondria preloaded with 7 mmol of Ca\textsuperscript{2+}/mg of protein were measured in the presence of 2 mmol of Ruthenium Red/mg of protein and in the presence and absence of 6 mm-Na\textsuperscript{+}. Results are means ± S.E.M. for the numbers of assays shown in parentheses.