

Phosphorylation of High-Molecular-Weight Membrane Protein Species in Chinese-Hamster Ovary Cells in Culture: Effect of 6-*N*,2'-*O*-Dibutyryladenine 3':5'-Cyclic Monophosphate plus Testosterone

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Growth of Chinese-hamster ovary cells in [³²P]phosphate and [³H]leucine and subsequent assay of the plasma membranes reveals phosphorylation in two protein regions corresponding to molecular weights of 280000 and 195000. Culture in the presence of the 6-*N*,2'-*O*-dibutyryl derivative of cyclic AMP plus testosterone does not stimulate [³²P]-phosphate incorporation, but determines a modification in the qualitative pattern of phosphorylation.

Growth of CHO cells* in culture in the presence of the *N*-6,2'-*O*-dibutyryl derivative of cyclic AMP plus testosterone converts the culture from one of randomly orientated cells that grow in multilayers into a monolayer that acquires strict contact inhibition (Hsie & Puck, 1971; Hsie *et al.*, 1971). That cell-surface changes are implicated in the process is supported by the fact that such contact-inhibited cells have a decreased ability to be agglutinated and 'rounded up' by plant agglutinins and specific antibodies (Hsie *et al.*, 1971).

It seems generally accepted that cyclic AMP exerts many diverse biological effects through its action on protein kinases (Kuo & Greengard, 1969*a,b*). Hence we have investigated in the present study whether the cell-surface changes occurring in CHO cells as a result of exposure to derivatives of cyclic AMP (Hsie *et al.*, 1971) involve an effect on the phosphorylation of plasma-membrane proteins.

CHO-K1 cells (Hsie & Puck, 1971) were propagated in F-12 medium (Ham, 1965) supplemented with dialysed 10% (v/v) foetal calf serum. When indicated, dibutyryl cyclic AMP and testosterone propionate were included in the cultures at concentrations of 200 μM and 15 μM respectively. Phosphorylation *in vivo* was measured by adding to the cultures carrier-free [³²P]phosphate (10–20 μCi/ml) for 48 h. In some experiments protein labelling was also carried out by adding [³H]leucine (35.5 Ci/mmol; 10 μCi/ml) to the medium mentioned above. The labelled compounds, and also Protosol and Aquasol (see below), were supplied by New England Nuclear Corp. (Boston, Mass., U.S.A.).

Plasma membranes were prepared from CHO cells by an adaptation of the Tris procedure of Warren & Glick (1969), in which the membranes were banded twice in a discontinuous gradient before use. For electrophoretic studies membrane pellets were harvested and washed twice by resuspension in

* Abbreviation: CHO cells, Chinese-hamster ovary cells.

sucrose (30%, w/v) and subsequent sedimentation by centrifugation at 6000*g* for 20 min. Membrane solubilization was achieved by incubation in medium containing (final concentrations) 10% (w/v) sucrose, 2% (w/v) sodium dodecyl sulphate, 1% (v/v) β-mercaptoethanol, 10 mM-EDTA and 50 mM-Tris-HCl buffer, pH 7.5, at 45°C for 60 min. Fractionation of 100 μg portions of the solubilized membrane proteins was immediately carried out in 10 cm × 0.5 cm neutral 5.6% polyacrylamide gels made 1% with respect to sodium dodecyl sulphate (Fairbanks *et al.*, 1971). Subsequent staining was performed in Coomassie Blue (Fairbanks *et al.*, 1971). Control experiments demonstrated that the presence of 10% (v/v) glycerol in the gels (this was included to permit a better slicing of frozen gels) did not affect the distribution of polypeptides obtained during the electrophoresis.

Analysis of radioactivity was carried out by slicing frozen gels in a hexane–solid CO₂ bath with a Metaloglass electronic gel slicer. Slices (1 mm) were incubated for 16 h at 60°C with 0.5 ml of Protosol and then their radioactivities were counted after the addition of 5 ml of Aquasol.

Examination of the spectrum of plasma-membrane polypeptides was therefore carried out under conditions favouring polypeptide disaggregation (Fairbanks *et al.*, 1971) and the migration of lipids free from protein (Carraway *et al.*, 1972). In membranes prepared either from controls or from cells grown in the presence of dibutyryl cyclic AMP plus testosterone, Coomassie Blue staining revealed over 20 different polypeptide species ranging in apparent molecular weight from 15000 to about 280000 (Fig. 1). The molecular weights of the membrane polypeptides estimated from their electrophoretic mobilities relative to standard proteins are of course tentative values, in view of the known abnormalities of certain membrane components on polyacrylamide gels (Bretscher, 1971).

Examination of the ³²P radioactivity profiles for

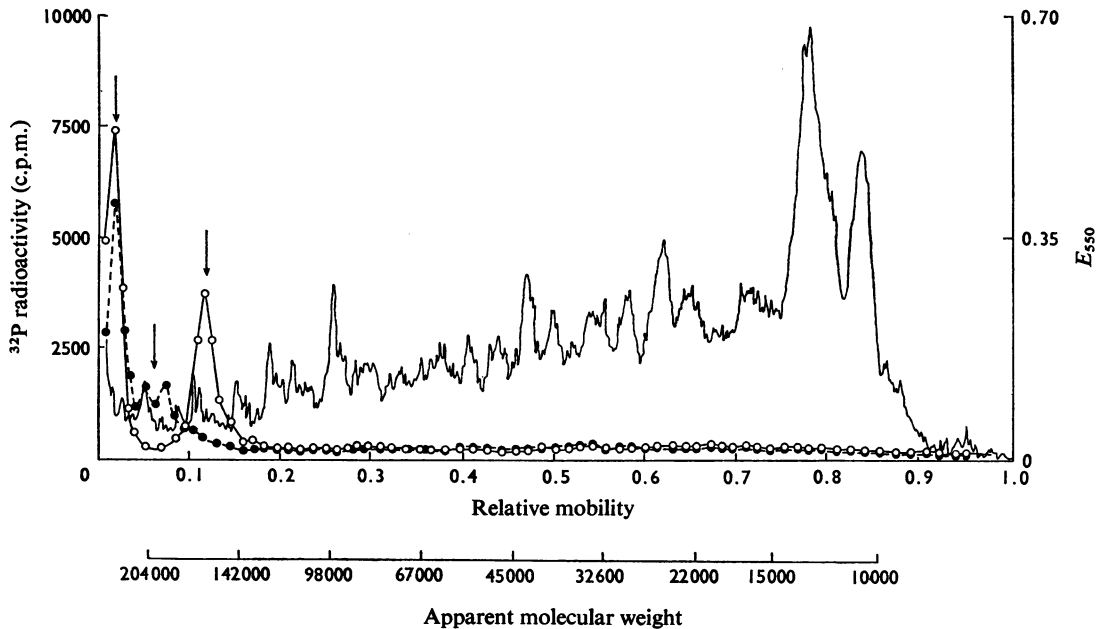


Fig. 1. Distribution of protein staining and phosphorylation in electrophoretograms of membranes from CHO cells

Cells were labelled with [^{32}P]phosphate and membranes were prepared and dissociated as indicated in the text. For electrophoresis $100\mu\text{g}$ of membrane protein was run in 1% sodium dodecyl sulphate–polyacrylamide gel. Apparent molecular weights were estimated from the migration of several protein standards (γ -globulin, albumin, ovalbumin and myoglobin) obtained from Schwarz–Mann Biochemicals (Orangeburg, N.Y., U.S.A.) and subjected to identical dissociation and parallel electrophoresis. The arrows indicate areas of the gels referred to in the text. \circ , [^{32}P]Phosphorylation of membrane proteins from control cells; \bullet , [^{32}P]phosphorylation of membrane proteins from cells grown in the presence of dibutyryl cyclic AMP plus testosterone; —, protein staining pattern (E_{550}) with Coomassie Blue for membranes from cells grown in the presence or in the absence of dibutyryl cyclic AMP plus testosterone.

detection of phosphorylated polypeptides revealed always a major radioactive peak associated with a minor protein component present both in control cells and in cells grown in the presence of dibutyryl cyclic AMP. This band had a very slow mobility, corresponding to an apparent molecular weight of about 280000, and contained about 70% of the total protein-bound ^{32}P in membranes both from control cells and from those grown in the presence of dibutyryl cyclic AMP plus testosterone. However, whereas control membranes showed only one other phosphorylated species with an apparent molecular weight of 195000, membranes from cells treated with dibutyryl cyclic AMP plus testosterone showed two additional ^{32}P -labelled polypeptides, of apparent molecular weights 250000 and 225000 (Fig. 1). The patterns of phosphorylation into the plasma-membrane components of CHO cells grown in the presence or in the absence of dibutyryl cyclic AMP plus

testosterone resemble preliminary results obtained for the erythrocyte membrane, in which phosphorylation also was found to involve three bands representing the polypeptide species of highest molecular weights (Duffy & Schwartz, 1972).

To test whether the phosphorylation in the CHO-cell plasma membranes, as above reported, indeed corresponded to phosphoproteins, membranes were exposed to ribonuclease, deoxyribonuclease and Pronase (Strand & August, 1971) before electrophoresis in the sodium dodecyl sulphate–polyacrylamide gels. Although nuclease treatment produced no significant effect, after Pronase treatment ($100\mu\text{g}/\text{ml}$) at 37°C for 30min it was found that on subsequent electrophoresis the ^{32}P radioactivity had migrated with material of very low molecular weight. Also, exposure to 0.1M-NaOH for 15min at 90°C caused the disappearance of essentially all the ^{32}P associated with the membranes.

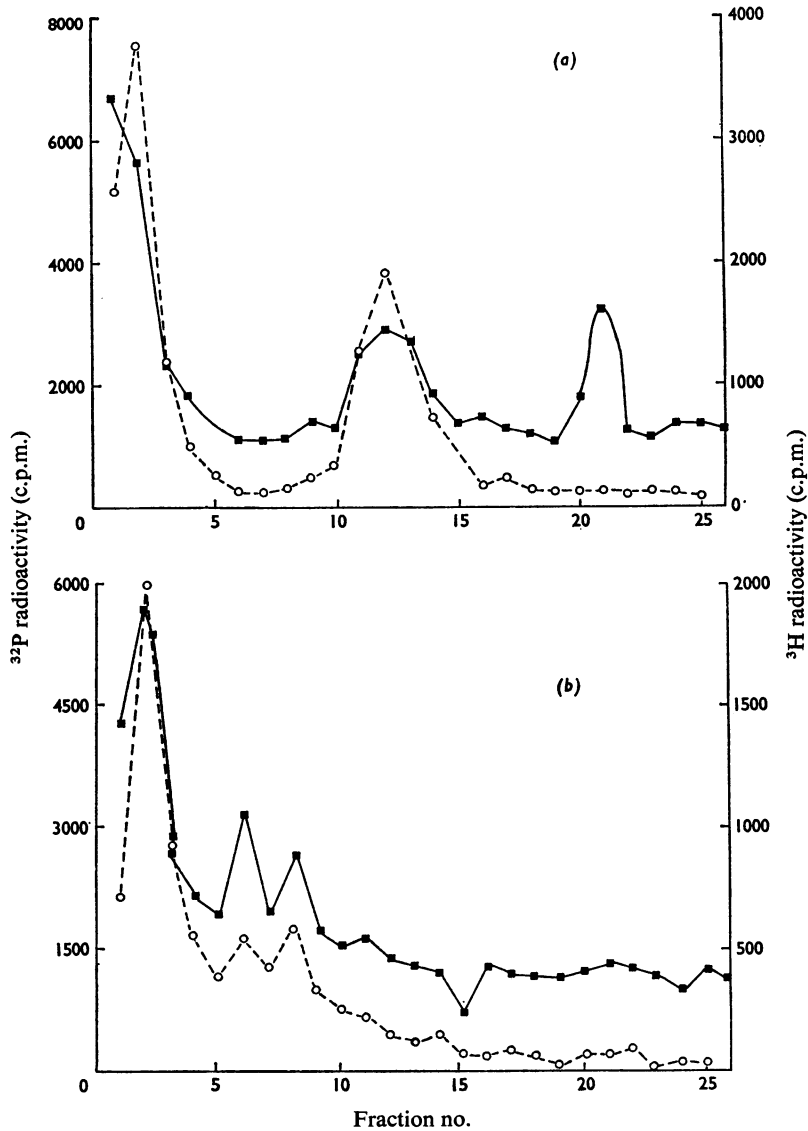


Fig. 2. Relative distribution of phosphorylation and protein radioactivity in membranes from CHO cells

Cells were grown in the presence of [^3H]leucine and [^{32}P]phosphate and membranes were prepared for electrophoresis and counting of radioactivity as indicated in the text. Only the sections of the gels containing the early migrating polypeptides were assayed for radioactivity to a length of 3 cm from the origin because other experiments (see Fig. 1) always demonstrated the slow migration of phosphorylated proteins. (a) Membranes from control cells; (b) membranes from cells grown in the presence of dibutyryl cyclic AMP plus testosterone. \circ , ^{32}P radioactivity; \blacksquare , ^3H radioactivity.

Similarly, after the labelling of CHO cells in the presence or in the absence of dibutyryl cyclic AMP in medium containing [^3H]leucine and [^{32}P]phos-

phate, gel electrophoresis showed (Fig. 2) that the peaks containing ^{32}P radioactivity also contained the radioactive amino acid. Some ^3H -labelled protein

material did not enter the gel, and this may be due to incomplete dissociation of the membranes. This was especially the case in controls, suggesting that somehow growth in the presence of dibutyryl cyclic AMP affects the electrophoretic behaviour of CHO-cell plasma membranes.

The results presented indicate that phosphorylation *in vivo* of plasma-membrane proteins occurs selectively on high-molecular-weight peptide species that appear to be a minor proportion of the membrane components of CHO cells. No significant differences were obvious in the overall qualitative membrane protein pattern in that no major additions or alterations were evident between treated and untreated cells, as detected by Coomassie Blue staining. However, growth in the presence of dibutyryl cyclic AMP plus testosterone did promote reproducible alterations in the qualitative pattern of the phosphorylated polypeptides, and these may reflect some of the significant changes occurring in CHO cells as a result of growth in the presence of dibutyryl cyclic AMP plus testosterone.

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