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Bipyridine-Induced Synthesis of Lactate Dehydrogenase and Aldolase

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The addition of 2,2′-bipyridine to a strain of mammalian cells cultured *in vitro* caused an increased synthesis of lactate dehydrogenase (LDH) and of aldolase. The effects of bipyridine were prevented by the concomitant addition of cobaltous ions, whereas addition of ionic iron caused an increased stimulation of the LDH and aldolase activities compared to that obtained with bipyridine alone. The increased synthesis of LDH concerned the H and M sub-unit to about the same extent.

Variations in lactate dehydrogenase (LDH) and aldolase activities in cultured animal cells related to variations in oxygen tension have been repeatedly demonstrated.[1–6] Similar variations in activity have been observed after exposing cells to certain chelating agents and consequently it has been proposed that the effect of oxygen upon these enzymes is mediated by some metal.[3]

In view of this, the effects of a number of metal ions and chelating agents on the regulation of LDH and aldolase were tested on an established cell strain (Chang liver), in which these enzymes are known from previous works to respond to changes in oxygen tension.[4,5]

MATERIALS AND METHODS

Cells and tissue culture procedures. Chang liver cells, originally obtained from Microbiological Ass., Bethesda, Maryland, U.S.A., were cultured in suspension in a slightly modified [7] Eagle medium, supplemented with 10 % horse serum. The stock cultures were propagated by regular dilution with fresh medium twice a week. Though care was taken to standardize the culture methods, the LDH and aldolase activities of the stock cultures varied. Comparable experiments were therefore always started with cells derived from the same stock culture. At the start of each experiment the cells were sedimented by centrifugation and transferred to fresh medium at a cell density of about 0.5×10^6 cells/ml. In all experiments reported here the cultures were continuously exposed to an atmosphere containing 95 % air and 5 % carbon dioxide.

Analytical procedures. LDH activity was determined by the method of Stambaugh and Post [8] and aldolase activity by a modification [5] of the method of Sibley and Lehninger.[9] The nitrogen content of the homogenates was measured by a micro-Kjeldahl

method and the glucose content of the medium was assayed with a glucose oxidase reagent (Kabi, Sweden).

Chemicals. Actinomycin D was purchased from Merck, Sharp and Dohme Laboratories, U.S.A., 2,2′-bipyridine and 1,10-phenanthroline from Merck, Germany. Other chelating agents used were obtained from Hopkin and Williams Ltd., Great Britain.

RESULTS

Cells cultured in the presence of 2,2′-bipyridine ($0.5 \times 10^{-4} - 10^{-4}$ M) showed an increased activity of LDH compared to the control cultures. The increase concerned the H and the M sub-unit to about the same extent (Fig. 1). The presence of bipyridine in the medium at the concentrations used to induce an increase in LDH activity also inhibited cell growth (measured as increase in cell number) (Fig. 4). When cells were exposed to bipyridine with an equimolar amount of ionic iron added, the LDH activity showed a greater increase than with bipyridine alone (Fig. 1). The difference became evident during the last part of the experimental period. The presence of ionic iron did not affect the growth inhibition produced by bipyridine. Iron was added as ferrous succinate, ferrous chloride, ferric chloride, and as ferrous complex of bipyridine with similar results.

In cells that had been incubated in a medium containing 10^{-4} M bipyridine for about 24 h, then collected by centrifugation and resuspended in fresh medium without any additions, the LDH activity continued in increase for the next 24 h, and then slowly started to decrease towards the initial activity. Also the reversal concerned both types of sub-units to about the same extent (Fig. 2). Parallel to the decrease in LDH activity the cells regained their ability to grow.

Exposure to bipyridine also caused an increased aldolase activity. When ionic iron was added in addition to bipyridine, the increase in activity became markedly greater (Fig. 3). The alterations of aldolase activity were also similar to those observed for LDH after change of culture medium.

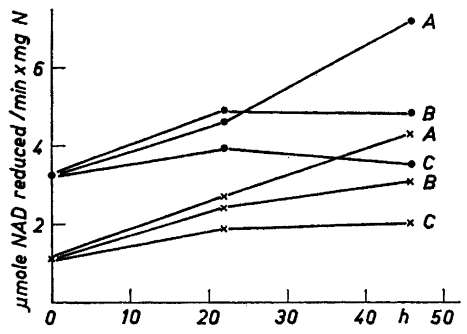

Fig. 1. Activity of the H (●) and M (×) sub-units. Culture A was exposed to 10^{-4} M bipyridine and 10^{-4} M Fe^{2+}, culture B to 10^{-4} M bipyridine, and culture C received no additions.

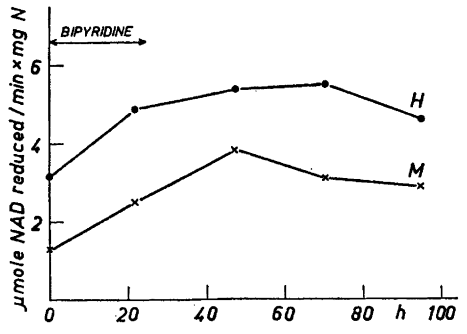

Fig. 2. Activity of the H and M sub-units in a culture exposed to 10^{-4} M bipyridine for the first 24 h of the experimental period and then transferred into fresh medium.

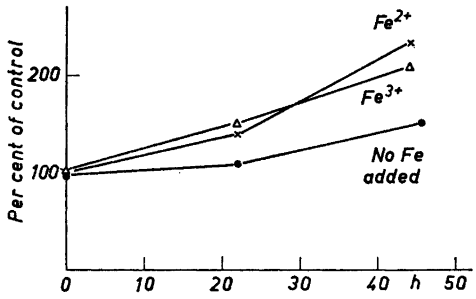

Fig. 3. Effect of equimolar amounts of ionic iron and bipyridine on aldolase activity. The cells were exposed to 0.75×10^{-4} M bipyridine and the activity is calculated as per cent of untreated control cultures.

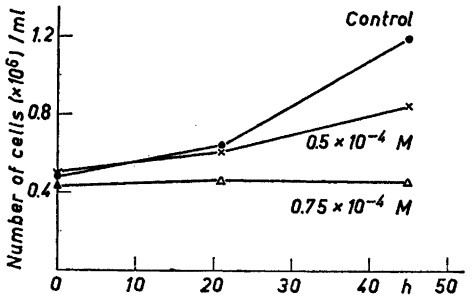

Fig. 4. Effect of bipyridine concentration on cell growth.

The changes in enzyme activities induced by bipyridine alone, as well as with ionic iron added, were prevented by the concomitant addition of actinomycin D (0.2 μg/ml) indicating that the increased activities may be ascribed to synthesis of new enzyme protein.

The cell samples collected from cultures containing bipyridine showed a faint pink coloration which was more intense in cells from cultures that also had received ferrous or ferric ions. The coloration was not removed when the cells were washed in a balanced salt solution. Cobaltous ions, added in equimolar amounts prevented the effects of bipyridine on growth, on enzyme activities, and on the development of coloration. On the other hand, the addition of equimolar amounts of cupric ions did not influence the effects of bipyridine. Exposure to ferrous (10^{-4} M), cupric (0.5×10^{-4} M), or cobaltous (10^{-4} M) ions in the absence of bipyridine did not cause any significant changes in LDH or aldolase activity.

Similar results were obtained when the cells were exposed to 1,10-phenanthroline (0.5×10^{-4} M). Also when phenanthroline was used the stimulation of enzyme synthesis was enhanced by the addition of ferrous ions. Other chelating agents tested, *i.e.* ethylenediaminetetraacetic acid (EDTA) (10^{-4}—2×10^{-3} M), diethylenetriaminepentaacetic acid (5×10^{-4} M), 1,2-diaminocyclohexane *N,N,N',N'*-tetraacetic acid (2×10^{-4} M), *N,N*-dihydroxyethylglycine (2×10^{-4} M), nitrilotriacetic acid (2×10^{-4} M), cupferron (10^{-4} M), and dimethylglyoxime (10^{-4} M) did not cause any changes in LDH or aldolase activity.

Although bipyridine affected enzymes involved in glucose metabolism, the rate of glucose uptake from the medium did not differ significantly from that of the control cultures.

DISCUSSION

The addition of ferrous, cupric, or cobaltous ions to the medium in the absence of bipyridine did not bring about any notable changes in aldolase or

LDH activity. However, extrapolating from results of experiments on rabbits, in which administration of cobalt produced effects on the kidney LDH isoenzyme pattern and erythropoietin production similar to those resulting from reduced local oxygen tension caused by severe anaemia,[10] it was presumed that the presence of cobaltous ions would influence LDH synthesis in Chang cells. The effects of cobalt on rabbit kidney do not, however, seem to be directly applicable to the LDH and aldolase regulation of these cells.

The reported increase in LDH and aldolase activities induced by bipyridine is consistent with the results of Goodfriend *et al.* working with a strain of monkey heart cells.[3] However, in contrast to the monkey heart cells, Chang liver cells do not respond to EDTA with any changes in activity of these two enzymes. In fact the highest dose of EDTA used (2×10^{-3} M) was toxic and brought about cell death in 24—48 h. The toxic effect of EDTA may possibly be due to lower toleration of cells cultured in suspension than of cells in monolayer cultures or simply to differences between the two cell strains.

The addition of bipyridine also inhibited growth of the cultures (Fig. 4), an effect that may possibly be due to chelation of metal ions essential to some metabolic activities of the cells. This growth inhibition, as well as the effects on LDH and aldolase syntheses caused by bipyridine, were completely neutralized by the concomitant addition of cobaltous ions, which presumably combine with bipyridine to a complex with no activity in these cells. In contrast to this, the addition of equimolar amounts of ferrous ions, which could also be expected to bind bipyridine similarly to cobaltous ions, did not prevent the growth inhibition, but markedly potentiated the action of bipyridine on LDH and aldolase syntheses. The addition of cupric ions did not modify the effects of bipyridine on enzyme activities or on growth.

The pink coloration observed in the collected cell samples is apparently similar to the coloration which is reported to develop on the surface of yeast cells exposed to phenanthroline.[11] It is more pronounced when ionic iron has been added to the medium and is probably a ferrous complex of bipyridine. As it is not removed when the cells are washed in a balanced salt solution, the complex is present either within the cell or in some way bound to its surface. The increased intensity of the coloration after addition of ferric ions shows that the cells are able to reduce these to ferrous ions.

The effects of bipyridine or bipyridine combined with ferrous ions were not identical to those obtained after exposing cells to an anoxic atmosphere. There were certain important differences: In the present experiments the synthesis of the H sub-unit was more stimulated than that observed after anoxic conditions, whereas the amplitude of the increased synthesis of the M sub-unit was of the same order as provoked by anoxia. Furthermore, though exposure to bipyridine induced changes in the activities of enzymes involved in the glucose metabolism, the rate of glucose uptake was not, in contrast to cells kept under anoxic conditions, different from that of the control cultures. These differences may suggest that the processes affected by bipyridine are not altogether identical with those affected by anoxia. The facts, that the action of bipyridine is enhanced by ionic iron but not by the other ions tested and that the changes in enzyme activities always occur together with the development of a pink coloration of the cells may suggest that the active

principle is a ferrous complex of bipyridine. It seems possible that this complex acts on the enzyme forming system directly and not necessarily by chelation of a metal mediating the effects of oxygen.

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