

Evidence for a Transplasma Membrane Electron Transport System on Intact Ehrlich Lettè Ascite Tumor Cells

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Introduction

A transplasma membrane redox enzyme, which transfers electrons from reducing agents in the cytoplasm to external impermeable oxidants, such as ferricyanide, is present in all cells which have been tested (2,3,19). It was first observed by Manyai and Szekely (15) that extracellular ferricyanide induced ATP synthesis inside erythrocytes. Subsequently it was observed that ferrocyanide induced ATP breakdown (18). Furthermore, Mishra and Passow (17) later proposed that ATP synthesis accompanied by ferricyanide reduction is due to the occurrence of a transmembrane electron flow. Aside from ATP synthesis, electron transport in the transmembrane enzyme system is accompanied by proton release from the cell (5,7,19). Several vital functions have been found to be related to this redox activity. This includes control and stimulation of cell growth (8,21), energizing amino acid transport (9), inducing proton release (13) and control of the activity of adenylate cyclase (14). There is good evidence that this redox enzyme is hormone sensitive (11,13). The sensitivity of this enzyme to hormones also indicates that this enzyme can have important role in the control of cellular function. Recently, the action of the redox enzyme has been shown to promote cell attachment and replication of melanoma cells (8) and HeLa cells (20,21).

In this study it is shown that ferricyanide stimulates growth of Ehrlich Lettè Ascite cells on serum-limited media, which indicates that transmembrane redox activity can control cell growth. Other impermeable oxidants stimulate growth of Ascites cells as well; however, impermeable oxidants which do not interact with the electron transport system do not stimulate growth. Some anticancer drugs have inhibitory effects on ferricyanide reduction by Ascite cells, which correlate with decreasing cell growth.

Materials and Methods

Ascites cells were grown in flasks using RPMI-1640 culture medium (Gibco) with sodium bicarbonate (1g/500ml) and 10% fetal calf serum. Cells were incubated at an initial density of 10^5 /ml for 3-4 days at 37°C in a 5% CO₂ atmosphere (1). Cells were prepared for study by pelleting suspension cultures at 5000 rev./min. The pellet was diluted with TD-Tris buffer (NaCl 8g/l, KCl 0.34g/l, Na₂HPO₄ 0.1g/l and Trisma base 3g/l, pH 7.5) to a final concentration of 0.1 gm cells/ml.

The rate of ferricyanide reduction by Ascites cells was determined in an Amino DW-2a dual beam spectrophotometer with a linear recorder, a cuvette stirrer, and a 37° temperature controlled cuvette chamber. The assay of ferricyanide reduction was performed as described previously (4), except TD-Tris buffer was used instead of 0.05 M sodium phosphate buffer, pH 7.0. Absorbance changes were measured with the dual beam at 420nm minus 500 nm. The extinction coefficient for ferricyanide reduction at 420nm equals $1 \text{ mM}^{-1}\text{cm}^{-1}$. Reduction of cytochrome c was determined in the TD-tris buffer with 15 mg/ml cytochrome c added instead of ferricyanide. Absorption changes are measured at 550nm-540nm. Extinction coefficient is $19\text{mM}^{-1}\text{cm}^{-1}$. Reduction of hexamine ruthenium III chloride and indigotetrasulfonate was measured coupling the dye reduction to reduction of ferric ions to form ferrous bathophenan-

throline sulfonate complex as described previously (21). The effect of anticancer drugs on Ascites cell ferricyanide reduction was performed using the procedure above. Anticancer drugs were added to cells for a preincubation (3 minutes) before the assay. 0.03-0.10 grams wet weight (g.w.w.) cells were used per assay.

Growth studies of Ascite cells were carried out with supplements in serum-limited (with 0.5% serum) or serum-free media. Cells were harvested with EDTA solution (0.02%, pH 7.0). Ferricyanide or other impermeable oxidants can slightly replace fetal calf serum as a growth factor for the replication of Ascite cells. A final concentration of 0.0033 mM - 1.0 mM of ferricyanide or other oxidants were used as supplements for cell growth. After 2 days of incubation at 37° C cells were harvested and a cell survival count was taken immediately. Survival was determined by the eosin Y exclusion method as described by Mighell and Shrigi (16). The colorless viable cells were counted. Cell number was determined by counting with a hemacytometer.

Results

In order for cellular internal NADH to reduce the impermeable ferricyanide outside the cell, a redox system is necessary to carry electrons across the membrane. The use of intact cells, therefore, provided the best assay of the transmembrane redox enzyme activity. Figure 1 demonstrates the kinetics of ferricyanide reduction in Ehrlich

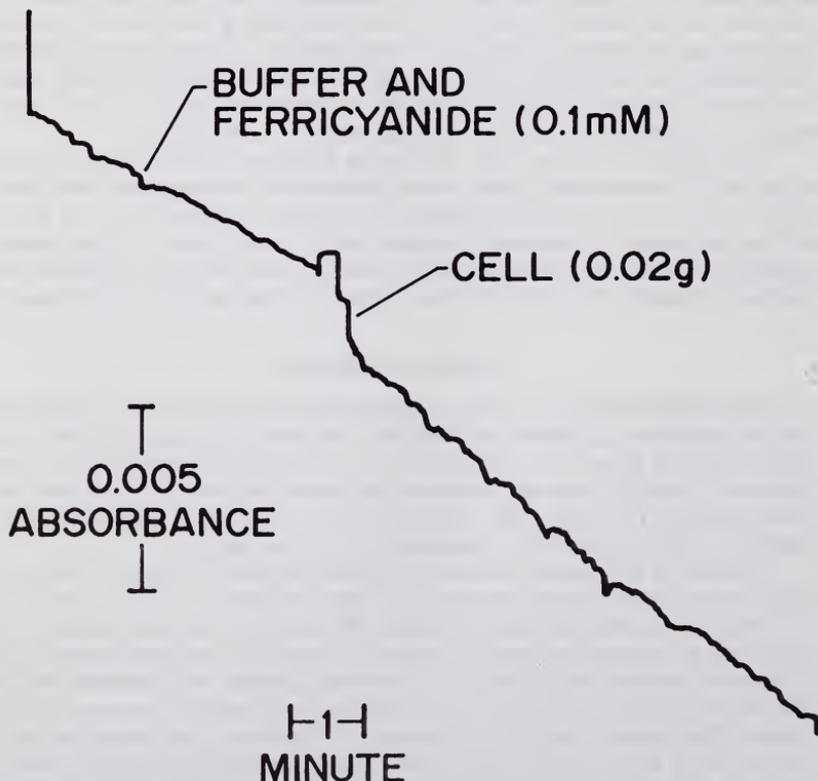


FIGURE 1. Reduction of ferricyanide by Ehrlich Lettre Ascite tumor cells. Spectrophotometer tracing of Δ absorbance 420nm minus 500nm.

Lette Ascite cells. The rate of reduction increased in a hyperbolic manner with increasing ferricyanide concentrations. The maximum ferricyanide reducing activity was reached at 60 nmoles/min/g.w.w. cells (Figure 2).

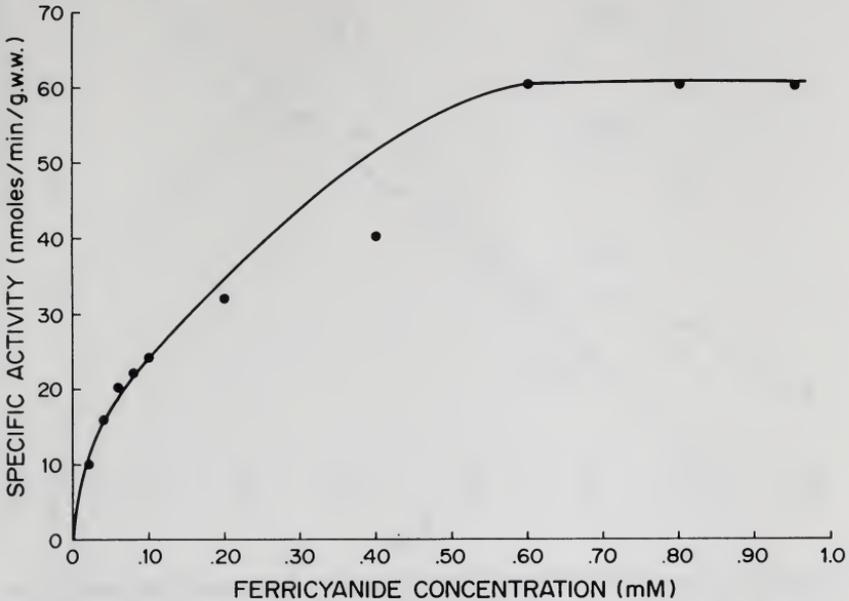


FIGURE 2. Dependence of Ehrlich Lette Ascites cells' ferricyanide reduction on ferricyanide concentration.

Lineweaver-Burke reciprocal plots of the rate as a function of the ferricyanide concentration showed an apparent K_m to be 0.08 mM and a V_{max} to be 50 nmoles/min/g.w.w., as indicated in Figure 3.

Potassium ferricyanide, the impermeable electron acceptor, stimulates the growth of Ascite cells under conditions of serum limited (0.5%) media, as shown in Figure 4. The optimum growth stimulation is at a concentration of .01 mM of ferricyanide, which shows about a 1.9 fold increase in cell count over the control. At concentrations above 0.1 mM, ferricyanide became toxic to the cells, as shown by the reduction of cell counts.

Reduction of other impermeable oxidants such as hexamine-ruthenium III chloride and indigotetrasulfonate were also observed as shown in Table 1.

Cells supplemented with hexamine-ruthenium III chloride (0.033) show an increase of 20% in cell count over the control, while it is 13.6% when supplemented with indigotetrasulfonate (0.033). These impermeable oxidants increase oxygen uptake, which also stimulates cell growth. Inactive oxidants such as cytochrome C do not promote cell replication (Table 2).

Coupling of proton release to the transmembrane redox activity was found in Ascites carcinoma (Table 3). An average of 3.3 nmoles of protons were extruded per nmole of ferricyanide reduced, if both assays were done in the same media (sucrose-salt solution).

Anticancer drugs, such as bleomycin, adriamycin and cis-diamine-dichloro-platinum

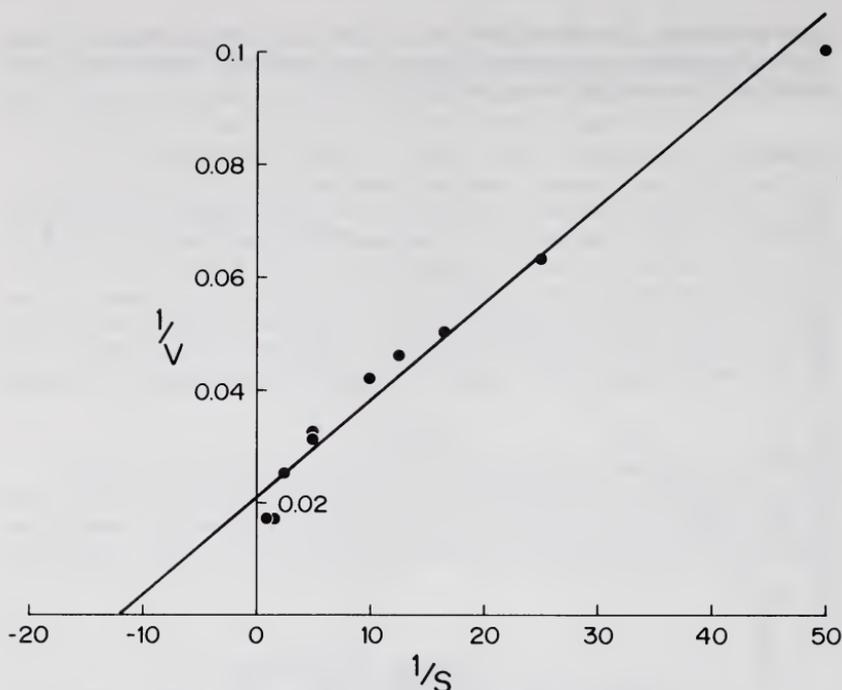


FIGURE 3. Lineweaver-Burke plot of relation between ferricyanide concentration and the rate of ferricyanide reduction by Ascites cells.

II (cis-platin), partially inhibited (above 50%) the ferricyanide reduction by Ascites tumor cells (Table 3).

Discussion

The results indicate that stimulation of cell growth can occur in serum-free or serum-limited media supplemented by ferricyanide, an electron acceptor of transmembrane redox system. It would appear that serum, which is necessary for cell growth, must contain factors which can stimulate transmembrane electron transport. Transferrin, for example, which is a component of serum, has been shown to replace serum in the growth of many cells (20). Transferrin can also act as an electron acceptor for the transmembrane dehydrogenase (Crane, unpublished). The effect of ferricyanide on growth could be to replace transferrin as an acceptor for the electron flow across the plasma membrane. The inhibitory effects of higher ferricyanide concentrations may be based on greater electron transport, which would deplete supplies of internal reductants.

It has been previously shown that ferricyanide induced proton release across the membrane, which would increase the pH of the cytoplasm in HeLa cells (3,19). The release of protons from the cell, which accompanies the redox activity, may be related to control of cell division. It is possible that ferricyanide stimulates cell growth through the action described above, for alkalization of the protoplasm has been shown to relate to mitogenesis (10). Other impermeable oxidants, which can accept electrons from the plasma membrane system, also give some growth stimulation, whereas oxidants which do not accept electrons are inactive. The basis for stimulation of growth

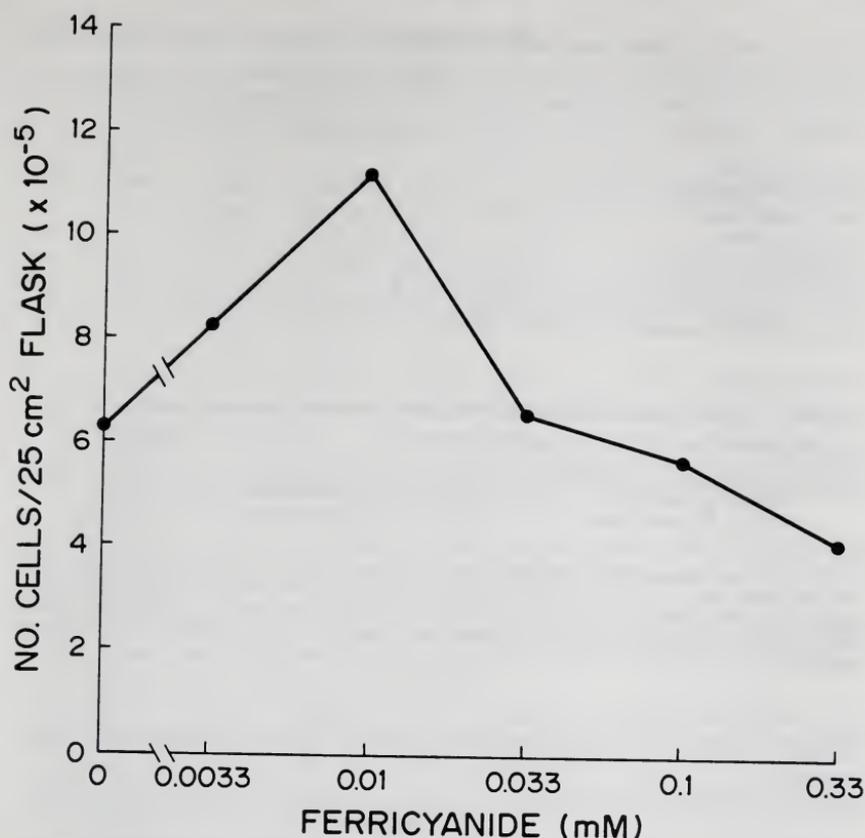


FIGURE 4. Effect of ferricyanide concentration on the growth of Ehrlich Lettre Ascites cells in serum-limited media with 0.5% serum.

by these oxidants may be the same as the basis for the ferricyanide stimulation of cell growth.

Transmembrane redox enzymes involved in protonophoric electron transport can be coupled to the energy transducing adenosine triphosphatases which are known to support several vital functions, such as transport of amino acids into the cells (15). Control of amino acid transport can be another way that these enzymes can be involved in the control of the growth and development of cells (9).

TABLE 1. Redox Activity of Ehrlich Lettre Ascites Carcinoma Cells

External Electron Acceptor	Rate of reduction Specific activity (nmoles/min/gww)*
Potassium ferricyanide (0.1mM)	22.5
Hexamine-ruthenium III chloride (0.1mM)	4.4
Indigo-tetrasulfonate (0.1mM)	4.1

*gww indicates gram wet weight of cells.

TABLE 2. Effect of other Impermeable Oxidants on the Growth of Ehrlich Ascites Cells.

Addition	Cell Count No. cells/25cm ² flask
Control (no addition)	12,000
Hexamine-ruthenium III Chloride (0.033 mM)	15,000
Indigotetrasulfonate (0.033mM)	14,200
Cytochrome C (0.033mM)	11,000

Cells were grown in a serum free media for 48 hr.

TABLE 3. Comparison of Ferricyanide-induced Proton Release and the Rate of Ferricyanide Reduction by Ehrlich Lettre Ascite Cells.

Rate of proton release (nmoles of H ⁺ /min/g.w.w.)	Rate of ferricyanide reduction (nmoles ferricyanide/min/g.w.w.)	H ⁺ /e ⁻
52	15.6	3.3

The redox activity and the rate of ferricyanide induced proton assay were both assayed in a sucrose-salt solution (sucrose 0.1M, NaCl 10 mM, KCl 10 mM, CaCl₂ 10mM). Ferricyanide concentration was 0.1mM. Proton release was measured by following change in pH of the external solution with a pH meter and glass electrode as previously described (19).

TABLE 4. Effect of Anticancer Drugs on Ferricyanide Reduction by Ehrlich Lettre Ascites cells.

Addition	Ferricyanide Reduction Specific Activity (nmoles/min/g.w.w.)	% Inhibition
Control	103	—
Bleomycin (50ug/ml)	60	42
Bleomycin (75ug/ml)	37.5	64
Adriamycin (10 ⁻⁶ M)	90	13
Adriamycin (10 ⁻⁷ M)	90	13
Adriamycin (5 × 10 ⁻⁷ M)	37.5	64
Adriamycin (10 ⁻⁶ M)	52.5	50
Cis-platin (5 × 10 ⁻⁷ M)	75	28
Cis-platin (10 ⁻⁶ M)	30	71

Anticancer drugs such as bleomycin and adriamycin inhibit the growth of HeLa cells (22,23). If the proton pumping redox system in the plasma membrane functions to stimulate cell division, then inhibitors of this enzyme should inhibit growth of the cells. It has been shown that these drugs inhibit the growth of other cells at concentrations which inhibit the proton pumping redox system (19,20,22,23).

Acknowledgments

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