Control of Cell Growth by Transplasmalemma Redox: Stimulation of HeLa Cell Growth by Impermeable Oxidants

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Introduction

Very little attention has been paid to energy rich redox agents, such as NADH, in contact with the interior of the plasma membrane. The energy source for plasma membrane functions such as vesicle formation and movement or transport, is always considered to be ATP derived from mitochondria or cytoplasmic glycolysis. However, the presence of high and low redox-potential compounds at the plasma membrane means that energy should be available.

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 (1, 4, 25, 26). This redox activity has been found to be related to several vital functions which include control and stimulation of cell growth (7), facilitation of iron uptake (3, 18, 20, 28) and defense against bacteria (14). In addition there is also good evidence that this redox enzyme is hormone sensitive (2, 6, 11, 13), driving amino acid transport (9), including proton release (13), and controlling adenylate cyclase activity (12). These are indications that this enzyme has an important role in the control of cellular functions.

In this study we present evidence that impermeable electron acceptors for the transplasma membrane redox system stimulate the growth of HeLa cells in a serum free medium. Insulin (30μg/ml) enhances this growth stimulation and increases the rate of oxidant reduction by cells. Impermeable oxidants, which do not interact with the electron transport system, do not stimulate growth. The coupling of proton release to this electron transport indicates that local membrane energization is affected by transmembrane electron flow and that intracellular pH may change. We propose that such activation and the increase of cytoplasmic pH can be very important in cell growth.

Materials and Methods

HeLa cells were grown in flasks with Eagle's medium containing 10% fetal calf serum, 100 u. of penicillin and 170 ug streptomycin per ml at pH 7.4 and maintained in a similar medium containing 2% fetal calf serum. Cells were prepared for study by pelleting the trypsinized suspension cultures at 27,000 g. The pellet was diluted with TD-Tris buffer (NaCl 8g/1, KCl 0.34g/1, Na_2HPO_4 0.1 g/1 and Trisma base 3g/1, pH 7.5) to a final concentration of 0.1 gm cells/ml.

Growth of HeLa cells with supplements in serum free media was carried out with cells harvested during the exponential growth phase. Insulin, ferricyanide or other oxidants can replace fetal calf serum as a growth factor for the replication of HeLa cells. Cells were grown in a serum free medium. A final concentration of 0.01-1.0 mM of ferricyanide or other oxidants and 30 μg/ml of insulin 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 (15). The colorless viable cells were counted. Cell number was determined by counting with a hemacytometer. Cell counts were obtained in duplicate with a cell counter after trypsinization.

The rate of ferricyanide reduction by HeLa cells was determined in an Aminco 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 (5), except TD-Tris buffer instead of 0.05 M sodium phosphate buffer, pH 7.0, was used. Absorbance changes were measured with the dual beam at 420 nm minus 500 nm. The extinction coefficient for ferricyanide reduction $\triangle$A420 equals 1.0 $mM^{-1}.cm^{-1}$.

The reduction rate of other oxidants were measured as described above except bathophenanthroline sulfonate (BPS) (3.3 μM) and ferric chloride (0.33 μM) were added into the assay mixture. Absorbance changes were measured with the dual beam at 535 nm minus 600 nm. The extinction coefficient for oxidant reduction was based on the formation of ferrous-BPS at $\triangle$A535 which equaled 17.6 $mM^{-1}cm^{-1}$.

Oxygen uptake was measured with an oxygen electrode in 1.3 ml TD-Tris buffer with 1 mM potassium cyanide. 0.35 μM NADH and 0.05 gm wet weight of cells were added to start the reaction.

Results

The impermeable electron acceptor, potassium ferricyanide, stimulates the growth of HeLa cells under the conditions of serum deprivation as shown in Figure 1. At concentrations 0.033 to 0.1 mM ferricyanide gives an optimum stimulation of growth, which shows a 2-3 fold increase in cell count over the control. At concentrations above

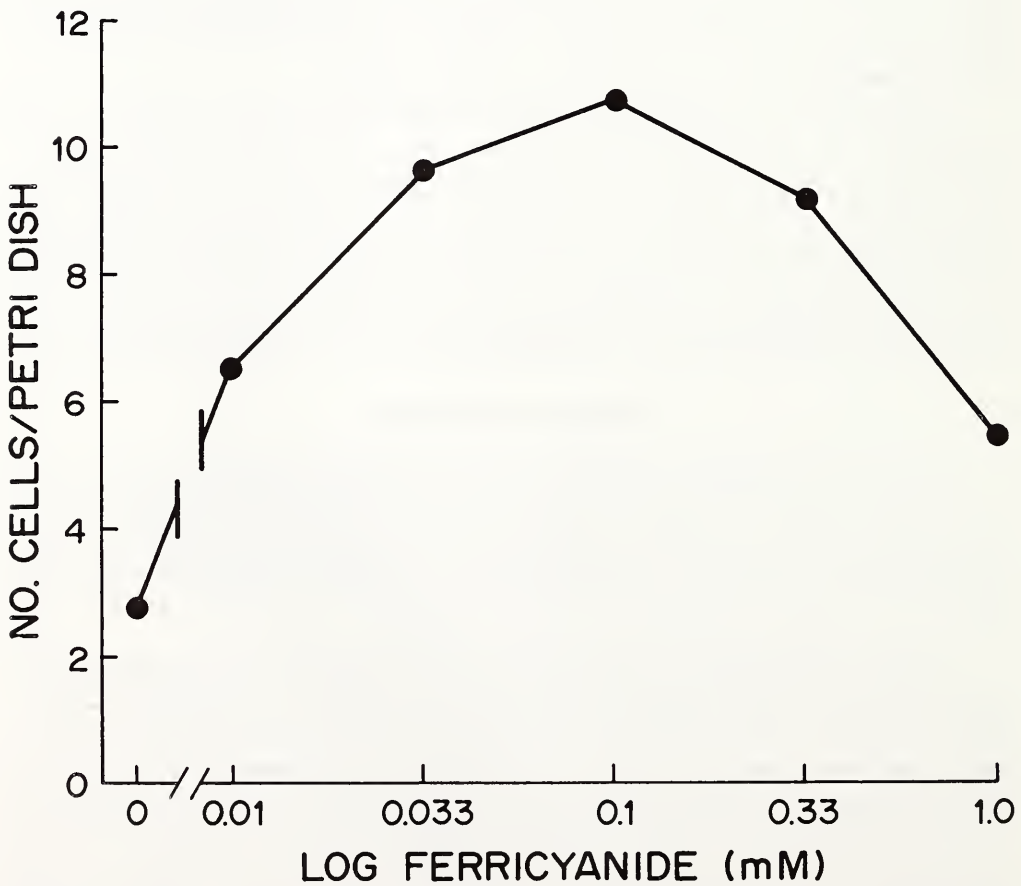

FIGURE 1. Stimulation of the growth of HeLa cells by ferricyanide. Cells were grown in a serum free medium. Cell count was determined after cells grown for 48 hr. at 37°C.

0.1 mM, ferricyanide becomes less effective. Cytotoxicity is found at ferricyanide concentrations over 1 mM which inhibits cell growth. Additive growth effects are seen with limiting levels of serum and ferricyanide up to the maximum growth with 10% of fetal calf serum. In general, lower ferricyanide (0.01 mM) requires higher serum (8%) and higher ferricyanide requires lower serum (4%) to reach maximum growth (Figure 2).

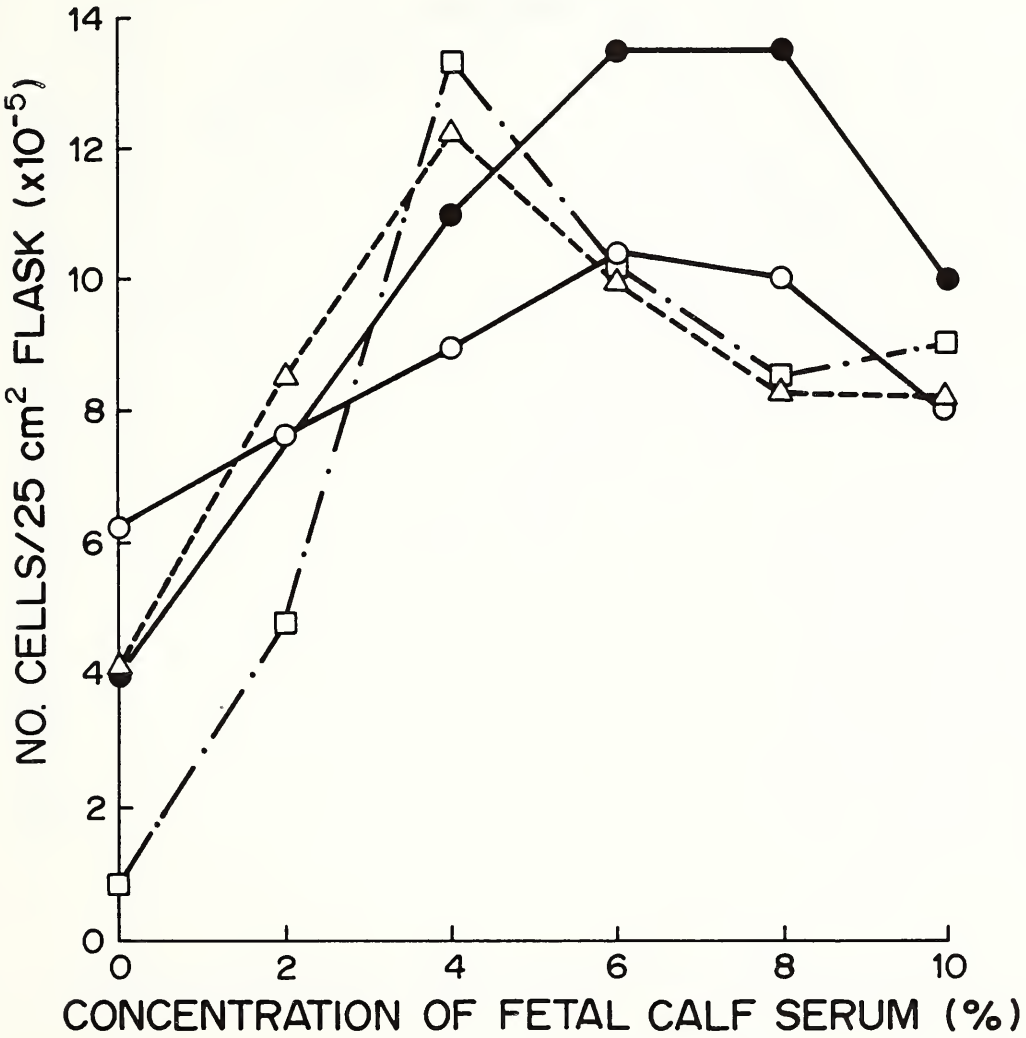

FIGURE 2. Dose-response growth curve to HeLa cells to serum supplemented with ferricyanide. ○—○—○, with ferricyanide 0.033 mM; ●—●—● with ferricyanide 0.01 mM; △—△—△, with ferricyanide 0.1 mM and —□—□, with ferricyanide 0.33 mM. 48 hr. culture.

Sodium ferricyanide stimulates growth and attachment of serum deficient HeLa cells as well as potassium ferricyanide (Figure 3). However, potassium ferrocyanide, the reduced form of ferricyanide, does not promote growth (Figure 4). The internal oxidant pyruvate does not stimulate growth either (Table 1). These results indicate that transmembrane electron flow must be involved in providing energy for cell function, since ferricyanide is extracellular and cannot itself provide nutrients for the cell.

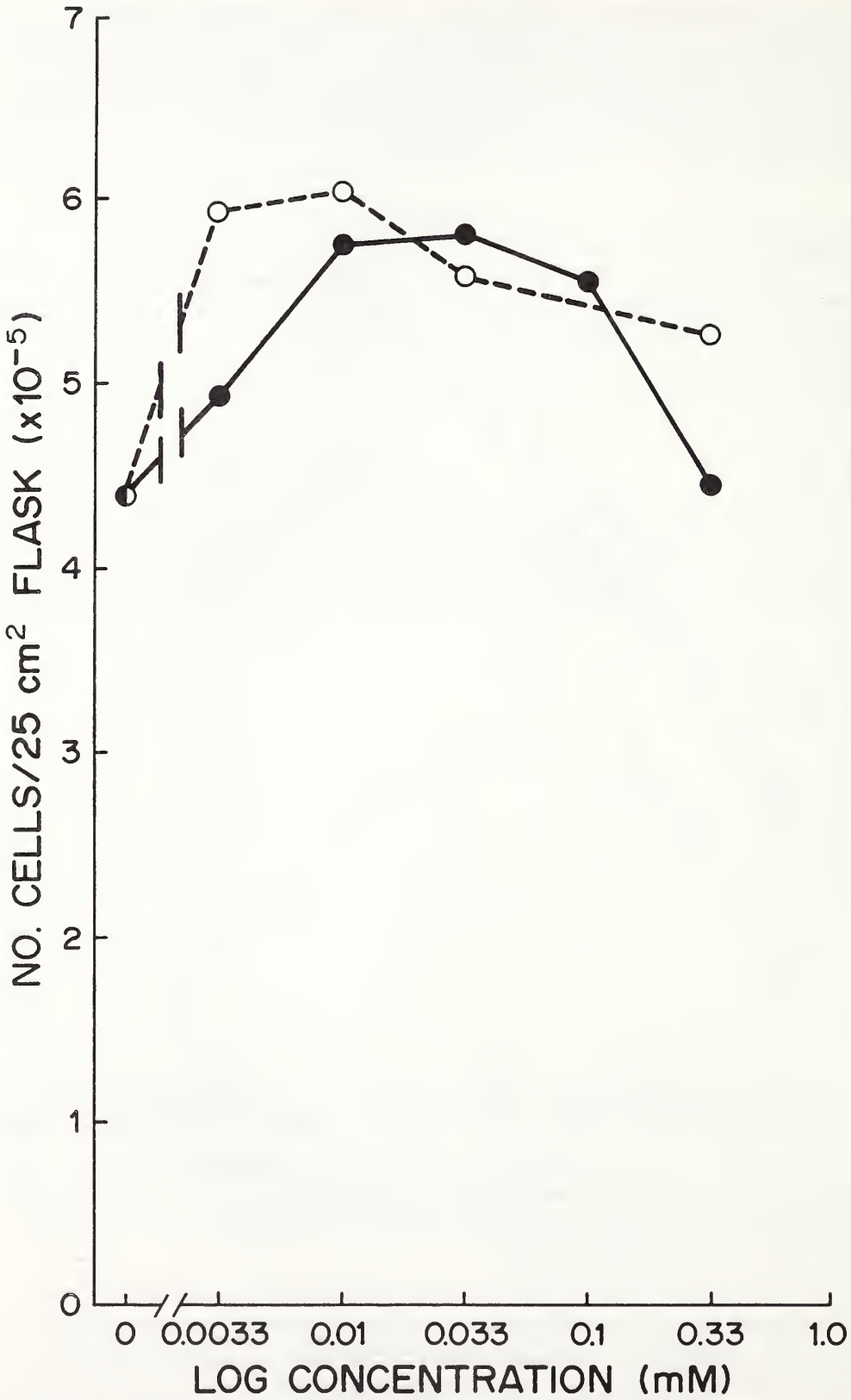

FIGURE 3. Dose-response growth curve of HeLa cells to serum free medium supplemented with potassium ferricyanide or sodium ferricyanide. ○____○____○, sodium ferricyanide and ●____●____●, potassium ferricyanide. 48 hr. culture.

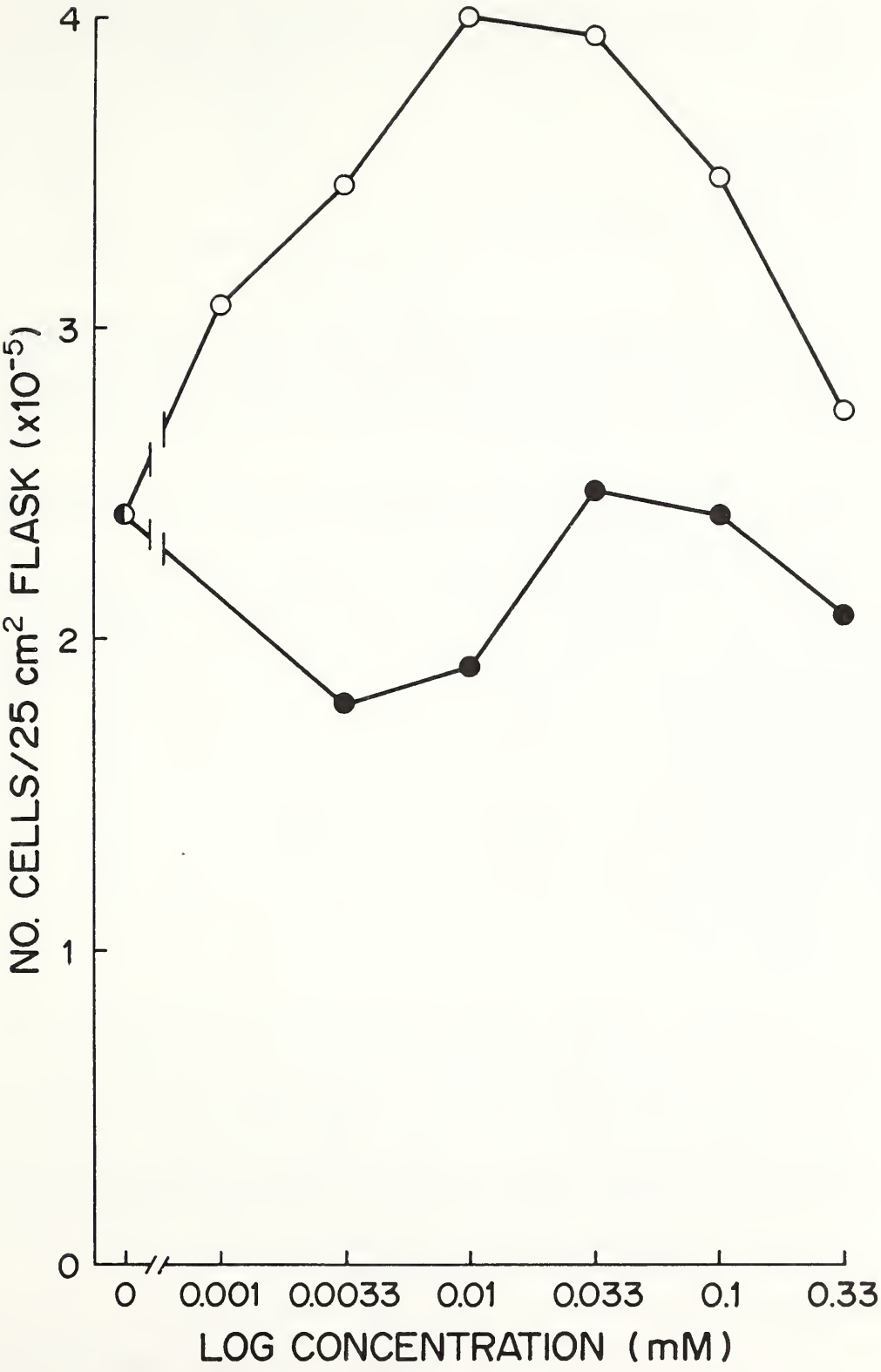

FIGURE 4. Dose-response growth curve of HeLa cells to serum free medium supplemented with potassium ferricyanide or potassium ferrocyanide. ○____○____○, potassium ferricyanide and ●____●____● potassium ferrocyanide. 48 hr. culture.

TABLE 1. Effect of pyruvate and ferricyanide on the growth of HeLa cells.

Addition	No. cells/25 cm^2 flask ($X 10^{-5}$)
Control	2.0
Pyruvate (0.01 mM)	2.1
Pyruvate (0.1 mM)	2.3
Pyruvate (1 mM)	1.4
Ferricyanide (0.033 mM) + pyruvate (0.01 mM)	3.3

Besides ferricyanide, other impermeable oxidants, such as hexaamine-ruthenium III chloride and indigotetrasulfonate, which increase oxygen uptake, also stimulate cell growth. Inactive oxidants, such as cytochrome c, do not promote cell replication (Table 2). The growth response seems to be specific for active impermeable oxidants.

TABLE 2. Effects of other impermeable oxidants on the growth and transmembrance redox system of HeLa cells.

Addition	No. cells/25cm^2 flask	0_2 uptake (nmoles 0_2/min/g.w.w.)*	Reduction rate (nmoles ferrous -BPS/min/g.w.w.)
Control	1.74×10^5	138	0
Hexaamine-ruthenium III chloride (0.33mM)	4.76×10^5	208	9.6
Hexaamine-ruthenium III chloride (0.1mM)	——	——	16.1
Indigotetrasulfonate (0.01mM)	8.62×10^5	173	11.8
Indigotetrasulfonate (0.1mM)	——	——	15
Cytochrome c (1.0mM)	1.65×10^5	——	0
Cytochrome c (3.0mM)	1.50×10^5	——	——

*g.w.w. indicates wet weight of cells

The application of insulin dramatically enhances the stimulating effect of ferricyanide as indicated in Figure 5. Insulin is a well known growth stimulator. At the optimum concentration (30 μg/ml), which stimulates growth, insulin also greatly increases the rate of ferricyanide reduction by HeLa cells (Table 3). Both the initial fast rate and long term slow rate of ferricyanide reduction are stimulated (27). There is a close correlation between insulin and increase in transmembrane redox enzyme activity and insulin induction of cell proliferation, as shown in Figure 6. The actual mechanism of insulin action as a growth promoter is not clear. Our results, however, indicate that electron flow through the transplamsa membrane electron transport system stimulates growth and that insulin acts to increase that flow.

Discussion

The results of Ellem and Kay for melanoma cell growth on limiting amounts of serum supplemented with ferricyanide (7) were similar to what we report here for HeLa cells. Furthermore, Mishra and Passow (16) found that reduction of extracellular ferricyanide by human erthyrocytes was accompanied by ATP formation, presumably accomplished as a result of transmembrane electron flow. Recent evidence also sug-

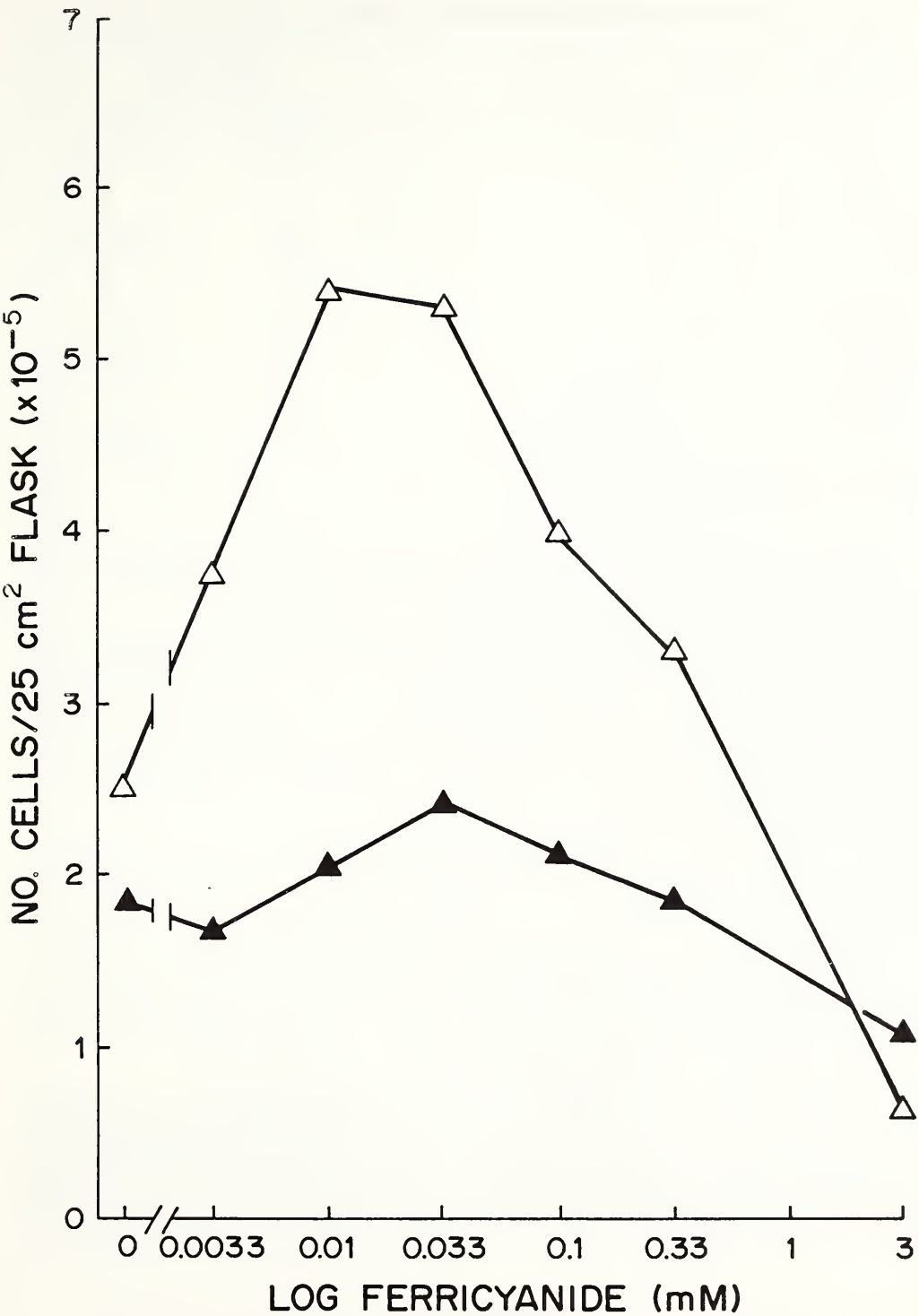

FIGURE 5. Dose response curve to HeLa cells to serum free medium supplemented with ferricyanide and insulin. ____▲____▲____▲, without insulin, ____△____△____△ with insulin (30 ug/ml). 48 hr. culture.

gests that the electron flow is more important for cell transition from G_1 to S phase than is the production of ATP therefrom (19). Therefore, there is considerable evidence

TABLE 3. The effect of insulin on ferricyanide reduction by HeLa cells.

Concentration of ferricyanide	Specific activity (nmoles/min/g.w.w.)	
	Fast rate	Slow rate
0 mM	0	0
0 mM + I	0	0
0.0033 mM	101	52
0.0033 mM + I	208	134
0.01 mM	145	40
0.01 mM + I	221	154
0.033 mM	214	87
0.033 mM + I	314	158
0.1 mM	259	94
0.1 mM + I	334	175
0.33 mM	278	118
0.33 mM + I	475	250

I indicates insulin (30 μg/ml)

that the transmembrane electron flow plays an important role in the control of cellular function. The fact that four of the most used anticancer drugs can inhibit transmembrane redox enzyme activities (21-24) further supports this idea.

It has been shown in several cell types that increase of cytoplasmic pH (alkalinization) is associated with cell division (8-17). We have previously shown that ferricyanide induced proton release from HeLa cells (25) in concentrations that coincide with the concentration, which gives the maximum growth stimulation. The basis for redox stimulation of growth is not quite clear yet. However, it is possible that ferricyanide induced proton release across the membrane would increase the pH of the cytoplasm and thus increase cellular mitosis.

Transferrin can act as an electron acceptor for the transmembrane redox system (Sun and Crane, unpublished). Part of the growth stimulatory effects of transferrin may be based on an oxidant effect at the cell surface. However, stimulation of growth by an oxidant is not limited to iron compounds such as ferricyanide or transferrin. The growth of HeLa cells is also stimulated by hexaamine-ruthenium III chloride, a trivalent cation and by indigotetrasulfonate (Table 2). The use of a series impermeable indigo sulfonates with different redox potential shows that extracellular oxidants with a redox potential $E'_{7,0}$ above -125 mV can stimulate growth (27).

The mode of action for insulin as a growth stimulator is unknown. Our results that the insulin stimulation of ferricyanide reduction correlates with its promotion of cell growth plus the evidence that insulin increases proton release from the cell induced by ferricyanide (Sun and Crane unpublished) suggest that the activation of the redox system and a stimulation of a redox driven proton pump would be a basis for insulin action as a growth factor.

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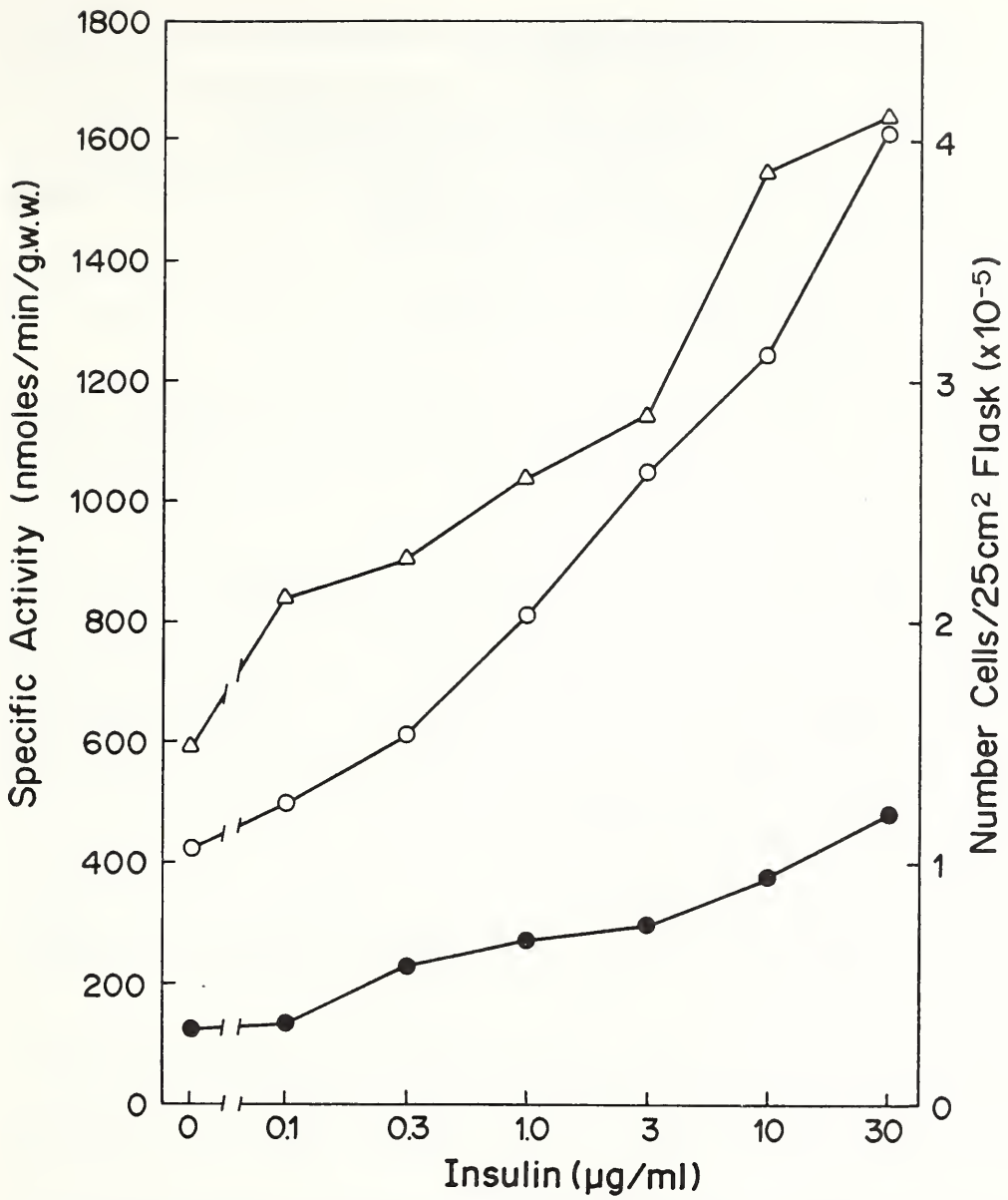

FIGURE 6. The correlation between insulin promotion of cell growth and insulin stimulation of transmembrane redox enzyme activities. ○____○____○, slow rate of HeLa cells ferricyanide reduction; ●____●____● fast rate of HeLa cells ferricyanide reduction; △____△____△, cell growth. Left ordinate indicates the specific activity of ferricyanide reduction by HeLa cells. Right ordinate indicates the stimulation of cell growth under the condition shown in the figure.

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