Properties of a Transplasma Membrane Electron Transport System in HeLa Cells

Iris L. Sun,[1] F. L. Crane,[1] C. Grebing,[2] and H. Löw[2]

Abstract

A transmembrane electron transport system has been studied in HeLa cells using an external impermeable oxidant, ferricyanide. Reduction of ferricyanide by HeLa cells shows biphasic kinetics with a rate up to 500 nmoles/min/g w.w. (wet weight) for the fast phase and half of this rate for the slow phase. The apparent K_m is 0.125 mM for the fast rate and 0.24 mM for the slow rate. The rate of reduction is proportional to cell concentration. Inhibition of the rate by glycolysis inhibitors indicates the reduction is dependent on glycolysis, which contributes the cytoplasmic electron donor NADH. Ferricyanide reduction is shown to take place on the outside of cells for it is affected by external pH and agents which react with the external surface. Ferricyanide reduction is accompanied by proton release from the cells. For each mole of ferricyanide reduced, 2.3 moles of protons are released. It is, therefore, concluded that a transmembrane redox system in HeLa cells is coupled to proton gradient generation across the membrane. We propose that this redox system may be an energy source for control of membrane function in HeLa cells. The promotion of cell growth by ferricyanide (0.33–0.1 mM), which can partially replace serum as a growth factor, strongly supports this hypothesis.

Key Words: Plasma membrane; electron transport; proton excretion; growth factors; ferricyanide reduction.

Introduction

Several redox systems have now been recognized in plasma membranes from various eukaryotic sources (Crane *et al.,* 1979). It was first observed by Manyai and Szekely (1954) that extracellular ferricyanide induced ATP synthesis inside erythrocytes. Subsequently it was observed that ferrocyanide induced ATP breakdown (Passow, 1963). Furthermore, Mishra and Passow (1969) later proposed that this ATP synthesis accompanied by ferricyanide

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[1]Department of Biology, Purdue University, West Lafayette, Indiana 47907.
[2]Department of Endocrinology, Karolinska Institute, Stockholm, Sweden.

reduction is due to the occurrence of a transmembrane electron flow. This transplasma membrane electron transport system, 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 (Clark *et al.,* 1981; Crane *et al.,* 1982a; Craig and Crane, 1981). Besides ATP synthesis, electron transport in this transmembrane enzyme system is accompanied by proton release from the cell (Crane *et al.,* 1982a; Dormandy and Zarday, 1965). Recently, the action of the redox enzyme has been shown to promote cell attachment and replication of melanoma cells (Ellen and Kay, 1983). The sensitivity of the enzyme to hormones also indicates that this enzyme has an important role in the control of cellular functions (Clark *et al.,* 1982; Crane *et al.,* 1982b; Goldenberg *et al.,* 1979; Goldenberg, 1982; Crane *et al.,* 1983a). Other membrane functions, such as the modification of membrane potential (Löw *et al.,* 1982), amino acid tranport (Christensen, 1979), and control of the activity of adenylate cyclase (Löw and Werner, 1979) or reduction of external ferric iron to ferrous for uptake (Bienfait *et al.,* 1982; Romheld and Marschner, 1983) have also been proposed to relate to this protonophoric transmembrane enzyme. In this study we present evidence for ferricyanide reducing activity by intact HeLa cells and we examine in detail some of the properties of this transmembrane process. The coupling of proton release to electron transport indicates that local membrane energization is affected by electron flow from internal NADH to external ferricyanide. We also show that such activation can be important in cell growth.

Materials and Methods

Growth of HeLa Cell Culture

HeLa cells were grown under an atmosphere of 5% CO_2 and 95% air at 37°C. The growth medium was Eagle's, containing 10% fetal calf serum, 100 units of penicillin, and 170 ug/ml of streptomycin, pH 7.4, and maintained in a similar medium containing 2% fetal calf serum. Confluent monolayer cultures were then prepared for study by pelleting the trypsinized suspension cultures at 15,000 g. The pellet was diluted with TD-Tris buffer (NaCl 0.14 M, KCl 5 mM, Na_2HPO_4 0.7 mM, and Trizma base 25 mM, pH 7.4) to a final concentration of 0.1 g cell wet weight/ml (g w.w./ml).

Ferricyanide Reduction by HeLa Cells

Ferricyanide reduction in HeLa cells was performed in an Aminco DW-2a dual-beam spectrophotometer equipped with a 37°C temperature-controlled chamber and a magnetic stirrer as described previously (Crane *et*

al., 1982a), except TD-Tris buffer instead of 0.05 M sodium phosphate buffer, pH 7.0, was used. Absorbance changes were measured with dual wavelength using 420–500 nm. The rates were recorded with linear recorder. A millimolar extinction coefficient of 1.0 was used for potassium ferricyanide.

Determination of Ferricyanide-Induced Proton Generation

Ferricyanide-induced proton generation was measured in a 2-ml cuvette with an Orion 701 A pH meter and a Corning glass combination electrode. Cells were suspended in a salt–sucrose solution (10 mM KCl, 10 mM NaCl, 10 mM $CaCl_2$, 0.1 M sucrose, and 5% TD-Tris buffer) to a final concentration of 0.005 g w.w./ml. The sample was stirred continuously and air was bubbled through the reaction mixture to remove CO_2. After the pH came to an equilibrium, ferricyanide (0.15–0.60 mM) was added. The proton generation was measured by the change in pH over the range 7.0–7.4.

Recovery of Ferricyanide in the Extracellular Medium following Ferricyanide Reduction by Cells

Cells (0.055 g w.w.) were suspended in 2.5 ml of TD-Tris buffer. The cell suspension was allowed to reduce ferricyanide (0.54 μmoles) for various lengths of time (10–50 min). Cells were then removed by a 2-min centrifugation in a Beckman 152 microfuge. The supernatant was used for measuring total ferricyanide with dual wavelength 420–500 nm before and after 2 mM persulfate was added.

Growth of HeLa Cells with a Supplement of Ferricyanide

Ferricyanide can replace fetal calf serum as a growth factor for the replication of HeLa cells. Cells were grown in a serum-free Eagles medium. A final concentration of 0.001–1.0 mM ferricyanide was used as a supplement 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 Mishell and Shrigi (1980). The colorless viable cells were counted.

Results

Ferricyanide Reduction in HeLa Cells

The biphasic kinetics of ferricyanide reduction by HeLa cells (fast and slow phase) is shown in Fig. 1. The rate of reduction increased in a hyperbolic
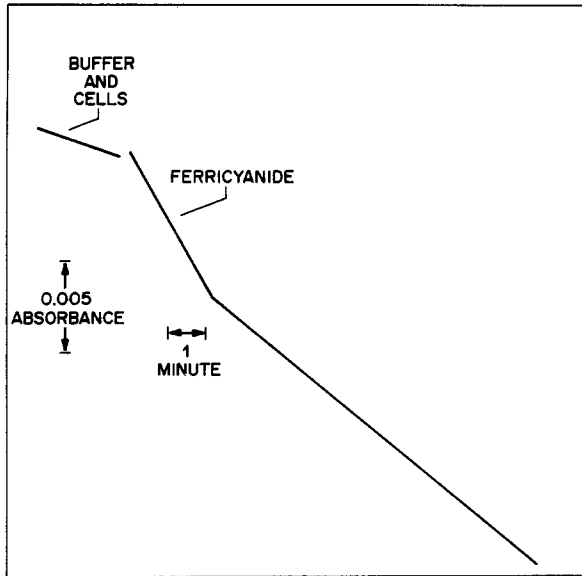
Fig. 1.   The biphasic kinetics of ferricyanide reduction by HeLa
cells (fast and slow phase).

manner with increasing ferricyanide concentrations, as indicated in Fig. 2A
for both fast and slow phases. Reciprocal plots of the rate as a function of the
ferricyanide concentration showed the apparent K_m to be 0.125 mM for the
fast rate and 0.24 mM for the slow rate. A maximum ferricyanide reducing
activity of up to 500 nmoles/min/g w.w. cells was observed for the fast rate.
Only about half of this value was reached for the slow rate. This reduction rate
was not only dependent on the ferricyanide concentration (Fig. 2A), but it was
also proportional to the cell density (Fig. 2b). To test for possible penetration
of ferricyanide into Hela cells, a ferricyanide recovery test was performed as
follows. Cell were allowed to reduce ferricyanide under normal assay condi-
tions. The total ferri-ferrocyanide content was determined in the supernatant
after cells were removed by centrifugation. Table I shows that 30 and 45%
ferricyanide was reduced after incubation of cells for 30 and 50 min,
respectively. The recovery of total ferricyanide in the supernatant was
measured by reoxidation of the reduced feerocyanide with persulfate. An
average of approximately 99% of the ferricyanide added was recovered in the
supernatant after removing the cells by centrifugation.

*The Effect of Some Inactivators on the Rate of Ferricyanide Reduction in
HeLa Cells*

Further characterization of ferricyanide reduction by intact HeLa cells
was made by applying selective inhibitors of mitochondrial activity (Table II).
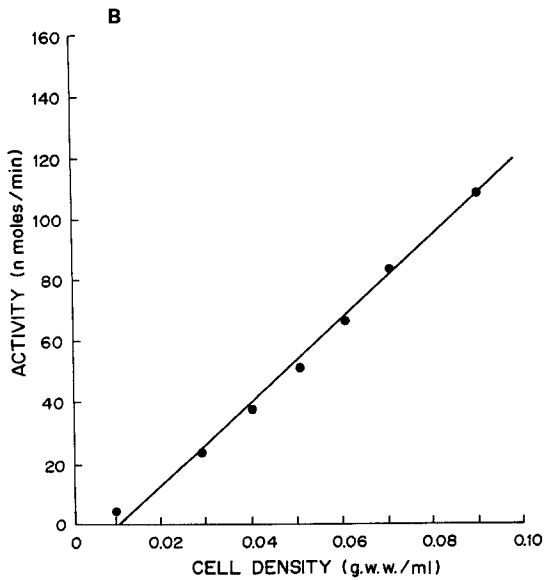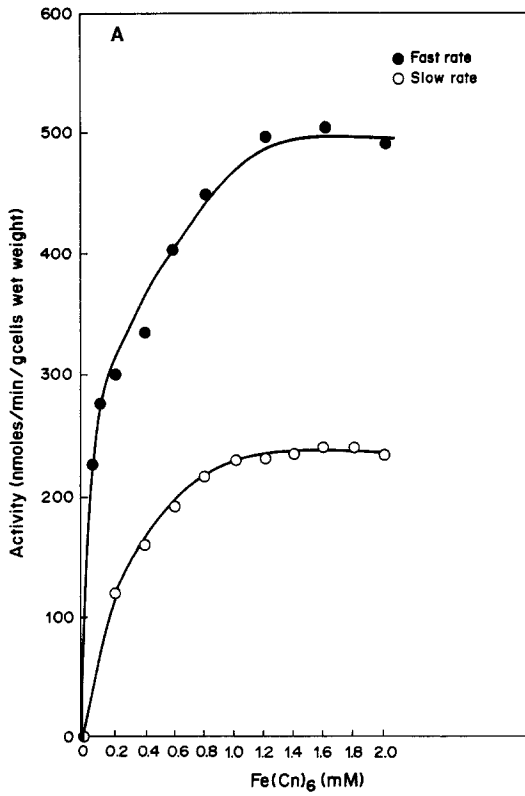
Fig. 2. Dependence of HeLa cell ferricyanide reduction on ferricyanide concentration and cell density. (A) Ferricyanide concentration; (B) cell density.

Table I. Ferricyanide Recovery in the Extracellular Medium following Ferricyanide Reduction by Hela Cells

Incubation time at 37°C (min)	Input Fe(CN)$_6$[a] (μmoles)	Fe(CN)$_6$ reduced (μmoles)	Fe(CN)$_6$ recovered in supernatant[b] (μmoles)	% Fe(CN)$_6$ recovery
10	0.54	0.09	0.538	99.6
30	0.54	0.16	0.527	97.6
50	0.54	0.24	0.54	100

[a] Fe(CN)$_6$ is potassium ferricyanide.
[b] After reoxidation of reduced ferricyanide with persulfate. Assay of ferricyanide recovery was performed as described in Materials and Methods.

There was no significant ferricyanide reduction, unless cells were present in the reaction mixture. Similarly, a decrease in absorbance at 420 nm was not observed, if ferricyanide was omitted. Mitochondrial inhibitors, such as 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), antimycin, and KCN showed insignificant inhibition on both rates of reduction. Azide showed a slight inhibition. Therefore, mitochondria from broken cells played no role in the reduction of ferricyanide. Ferrocyanide inhibited the slow rate of reduction significantly. A competitive inhibition is suggested (Clark *et al.,* 1981).

Effect of Glycolysis Inhibitors on HeLa Cell Ferricyanide Reduction

As an approach to identification of the source of reducing equivalents for the reduction of ferricyanide, various glycolysis inhibitors were tested (Table III). Arsenite (1.25 mM) inhibited 35% of both fast and slow rates. Iodoace-

Table II. Effect of Inhibitors on the Slow and Fast Rates of Ferricyanide Reduction in HeLa Cells[a]

Addition	Ferricyanide reduction (nmoles/min/g w.w.)	
	Fast rate	Slow rate
Control [cells + Fe(CN)$_6$]	206	113
Without cells	26	0
Without Fe(CN)$_6$	0	0
+ Rotenone (1 μM)	263	113
+ Antimycin (7.3 μM)	195	109
+ KCN (0.1 mM)	199	94
+ NaN$_3$ (0.5 mM)	131	71
+ HOQNO (1 μM)	206	105
+ Ferrocyanide (0.5 mM)	206	60

[a] The reaction mixture in each assay contains 0.05 g of cells and 0.1 mM ferricyanide.

Table III. Effect of Glycolysis Inhibitors on HeLa Cell Ferricyanide Reduction

Addition	Ferricyanide reduction (nmoles/min/g w.w.)	
	Fast rate	Slow rate
Control	169	109
+ Arsenite (1.25 mM)	113	71
+ Arsenite (2.5 mM)	120	45
+ Iodoacetamide (3.3 mM)	158	75
+ Iodoacetamide (6.6 mM)	113	30
+ Malic hydrazide (6.6 mM)	75	30

tamide showed a minor inhibition on the fast rate, whereas a strong inhibition (74%) was observed on the slow rate of ferricyanide reduction. Similarly, a strong inhibition on both fast and slow activities was seen with malic hydrazide. These results suggest that inhibiting glycolysis decreases transmembrane ferricyanide reduction rates, implying that reducing equivalents from glycolysis inside the cell are necessary for transmembrane ferricyanide reduction.

Effect of Impermeable Agents, Which Change the External Surface, on HeLa Cell Ferricyanide Reduction

To test whether ferricyanide reduction involves a site on the outer cell membrane, the effect of various impermeable agents which affect the external surface was tested (Table IV). p-Chloromercuriphenylsulfonate (PCMS) and p-chloromercuribenzoate (PCMB) caused a minor inhibition of the fast rate. However, a strong inhibition was observed on the slow rate. Stronger inhibition was seen with p-(diazoniumbenzenesulfonic acid) (DABS), trypsin, and concanavalin A, reagents capable of modifying cell surface proteins. The biphasic kinetics of ferricyanide reduction disappeared after the application of DABS, trypsin, and concanavalin A. Concanavalin A inhibited the fast rate of ferricyanide reduction but stimulated the slow rate of reduction. These results show that agents which change the external cell surface are able to affect the reduction rate. This is consistent with the idea that ferricyanide reduction takes place on the outside membrane of cells. The anticancer drug, adriamycin, at therapeutic levels also gives a strong inhibition of this transmembrane redox enzyme.

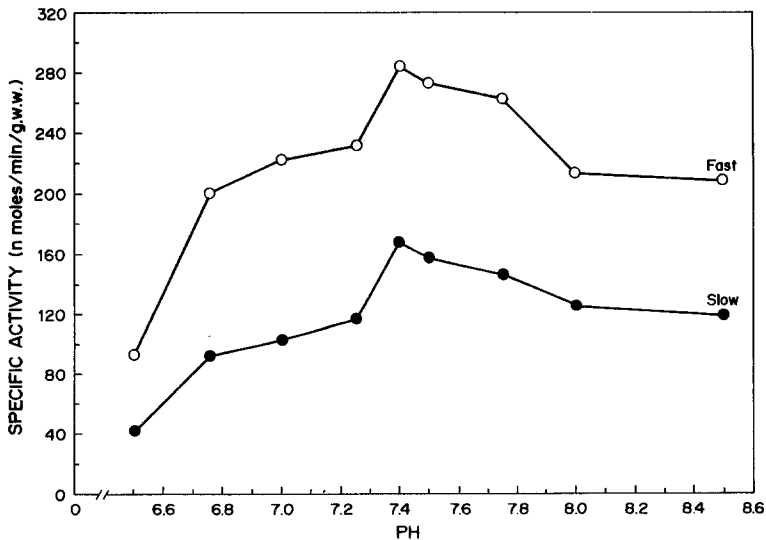### Effect of pH on Ferricyanide Reduction by HeLa Cells

To support the idea that ferricyanide reduction by Hela cells takes place on the cell surface, an experiment to test the pH dependence was performed

Table IV.   Effect of External Surface Agents on HeLa Cell
Ferricyanide Reduction

	Ferricyanide reduction (nmoles/min/g w.w.)	
Addition (final concentration)	Fast rate	Slow rate
Control I	384	103
PCMS (60 μM)	272	61
PCMB (45 μM)	295	23
DABS (0.2 mM)[a]	60	60
Trypsin (50 μg/ml)	130	130
Concanavalin A (100 μg/ml)	180	180
Control II	479	163
Adriamycin (1 μM[b])	154	60

[a]HeLa cells were incubated with DABS (0.2 mM) at 37°C for 10 min. Histidine (0.45 mM) was then added to the treated culture to remove excess DABS. Cultures were washed with TD-Tris buffer and centrifuged at 27,000 g. The pellet was resuspended in TD-Tris buffer to a final concentration of 0.1 g/ml and assayed immediately.
[b]Cells incubated with adriamycin for 3 min before addition of ferricyanide.

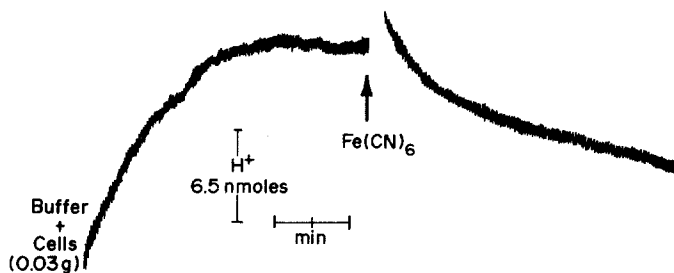**Fig. 3.**   Effect of pH on ferricyanide reduction by HeLa cells.

Fig. 4. Ferricyanide-induced proton release by HeLa cells. Assay as described in Materials and Methods.

(Fig. 3). An optimal pH of 7.4 was found for both the fast and the slow rate. A gradual decrease in both rates was seen when the pH reached above 7.5. A stronger decrease in the rate was found at pH values below 7.2. The effect of external pH on the reduction rate provides further evidence that the site of ferricyanide reduction in HeLa cells is on the outer surface.

Dependence of Ferricyanide-Induced Proton Extrusion by HeLa Cells on Cell Density and Ferricyanide Concentration

Coupling of proton release to the transmembrane redox activity was found in HeLa cells. A time course of proton release induced by ferricyanide is shown in Fig. 4. An average 2.3 nmoles of protons were extruded per nmole of ferricyanide reduced if both assays were done in the same media (salt–sucrose solution). As observed for ferricyanide reduction, the proton release depends on cell density and ferricyanide concentration (Table V). The rate of proton release also increased in a hyperbolic manner with increasing ferricyanide concentration (Fig. 5). The reciprocal plots of the rate as a function of the ferricyanide concentration showed an apparent K_m to be much lower than that of ferricyanide reduction (K_m = 0.027 mM). Different assay media (as indicated in Material and Methods) may be responsible for this difference.

Table V. Dependence of Ferricyanide-Induced Proton Extrusion of HeLa Cells on Cell Concentration[a]

Weight of cells (mg w.w.)	Proton release (nmoles/min)	Protein release (specific activity, nmoles H^+/min/g w.w.)
10	0.34	34
20	0.67	33.5
30	0.95	32
50	1.85	37

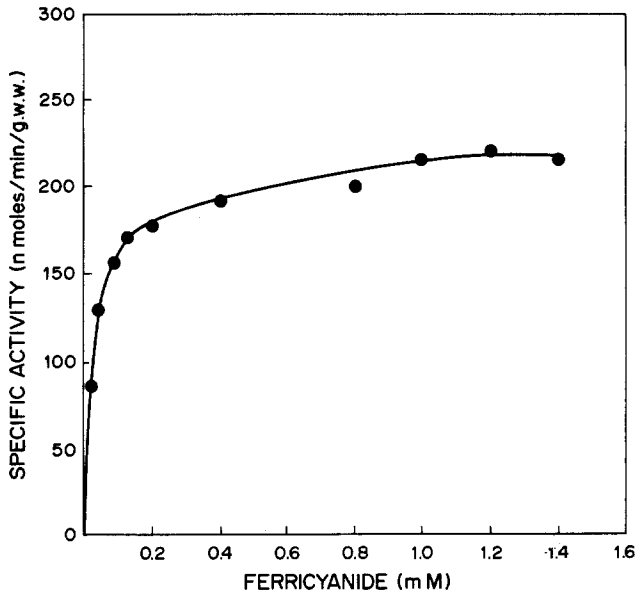[a]w.w. = wet weight. Concentration of ferricyanide in the assay is 0.1 mM.

Fig. 5. Dependence of ferricyanide-induced proton generation by
HeLa cells on ferricyanide concentration.

Replacement of Serum to Stimulate Cell Growth by Ferricyanide

The addition of potassium ferricyanide to serum-free media stimulated growth of HeLa cells. Significant stimulation was seen at concentrations 0.01–0.1 mM (Fig. 6). There was an insignificant effect on growth by ferrocyanide at the same concentration range. Cytotoxic effects were observed with ferricyanide concentrations above 0.1 mM. Since ferricyanide is impermeable to cells and yet acts as a growth factor, transmembrane electron flow, with the accompanying proton movement, can be the basis for stimulation of cell division (Ellem and Kay, 1983).

Discussion

Transmembrane redox enzymes involved in protonophoric electron transport can be coupled to the energy-transducing adenosine triphosphatases, which are known to support the transport of ions, amino acids, and the precursors of RNA and DNA into the cell (Martonosi, 1982). In the plasma membrane redox enzymes are hormone sensitive and involved in proton transfer (Löw et al., 1979). Therefore, these enzymes can be involved in the control of the growth and development of cells.
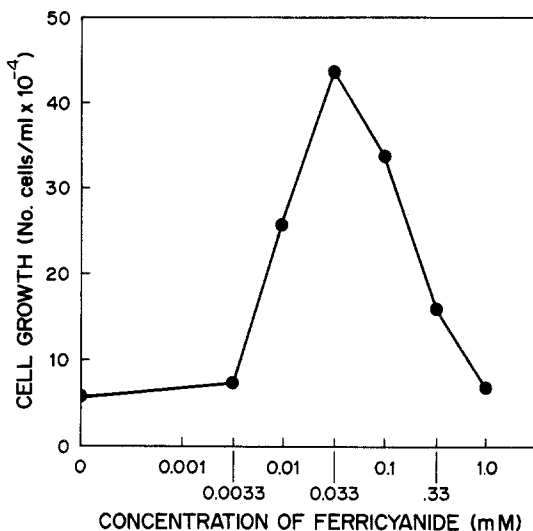
Fig. 6. Dose–response curves of HeLa cells to ferricyanide in serum-free media.

Ferricyanide reduction by HeLa cells is a saturable process, which requires cells and is proportional to cell concentration (Fig. 2B). Lack of inhibition by most mitochondrial inhibitors indicates that mitochondria from broken cells do not cause significant ferricyanide reduction (Table II). The rate of ferricyanide reduction in HeLa cells is not affected by the rate of ferrocyanide oxidation, since the rate of this oxidation in HeLa cells is negligible. Based on the effect of glycolysis inhibitors (Table III), the source of reducing equivalents for the reduction of ferricyanide has been identified as NADH. The HeLa cells seem to depend on glycolysis to contribute reducing agents for transmembrane ferricyanide reduction.

The cause of the biphasic enzyme kinetics of ferricyanide reduction observed in this study is not clear. The fact that agents such as DABS (Berg, 1969) and concanavalin A, which react with external sites on the membrane, eliminate the biphasic response suggests that surface properties of the membrane control the activity. Since the biphasic effect is not eliminated by agents (PCMS, PCMB) which react with thiols, the rapid rate is not based entirely on oxidation of exposed thiols on the surface of the cell.

Adriamycin is an antitumor drug which has been shown to act at sites on the plasma membrane (Tritton and Yee, 1982; Tökes et al., 1982). We have shown that adriamycin can inhibit both the fast and slow rate of ferricyanide reduction by SV40 virus-transformed rat embryo liver cells (Sun et al., 1983). Low concentrations of adriamycin also inhibit the ferricyanide reduction by Hela cells, so the redox system can be a site for adriamycin action as an antitumor drug.

Evidence has been presented for the coupling of proton release to the transmembrane redox activity in HeLa cells (Fig. 4). Similar results have been reported from yeast cells (Crane *et al.,* 1982a) and plant cells (Crane *et al.,* 1983b). The data concerning the effect of pH (Fig. 3) on Hela cells are also consistent with the view that reduction of extracellular ferricyanide is accompanied by proton extrusion from the cell into the extracellular fluid. In general, the rate is less affected by pH values above 7.4 than below. Thus, at pH values below neutrality the rate is more strongly inhibited. Similar results have been demonstrated with isolated perfused rat liver (Clark *et al.,* 1982) and yeast cells (Crane *et al.,* 1982a). The transmembrane proton movement which accompanies electron transport may be a factor in controlling the activity of the enzyme. The proton release by the transmembrane enzyme may also stimulate cell division, since alkalinization of the protoplasm has been related to mitogenesis (Gerson *et al.,* 1982).

The impermeable electron acceptor, ferricyanide, which can accelerate electron transport through the plasma membrane, increases growth of melanoma cells when serum levels are limiting (Ellem and Kay, 1983). Our results indicate that stimulation of cell growth can even occur in the serum-free but ferricyanide-supplemented media (Fig. 5). It would appear that the serum, which is necessary for cell growth, contains factors which can stimulate transmembrane electron transport. It is not known what these factors may be. However, transferrin, which is a component of serum, has been shown to replace serum in the growth of many cells (Hutchings and Sato, 1978). Since transferrin can act as an electron acceptor for the transmembrane dehydrogenase, the effect of fericyanide on growth may be to replace transferrin as an acceptor for electron flow across the plasma membrane. The fact that maximum growth stimulation by ferricyanide occurs at low ferricyanide concentrations, equivalent to concentrations which saturate redox-induced proton release, but below concentrations which give maximum electron transport, suggests that the growth stimulation is based on proton extrusion. At 0.1 mM ferricyanide, log phase HeLa cells will reduce ferricyanide at 100 nmoles/min/g w.w. of cells. At an average concentration during growth of 1 $\times$ 10^5 cells per flask (or 0.001 g of cells) ferricyanide would be reduced at a rate of 0.1 nmoles/min. At 400 nmoles per 4 ml of media (25 cm^2 flask), it would take 4000 min to reduce the ferricyanide or approximately 3 days. The inhibitory effects of higher ferricyanide concentrations may be based on greater electron transport, which would deplete supplies of internal reductants.

Institute for General Medical Research. The cooperation and advice of Dr. D. J. Morré and Dr. L. Jacobsen of the Purdue Cancer Center was most helpful.

We dedicate this paper to David E. Green because in many ways this never would have been done except for his inspiration, influence, and training. We believe it fits well into his constant quest for new ideas and new interpretations of old ideas.

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