Journal of Bioenergetics and Biomembranes, Vol. 19, No. 1, 1987

Ruthenium Ammine Complexes as Electron Acceptors for Growth Stimulation by Plasma Membrane Electron Transport

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Received July 2, 1986

Abstract

Ammineruthenium(III) complexes have been found to act as electron acceptors for the transplasmalemma electron transport system of animal cells. The active complexes hexaammineruthenium(III), pyridine pentaammineruthenium(III), and chloropentaammineruthenium(III) range in redox potential (E_0') from 305 to -42 mV. These compounds also act as electron acceptors for the NADH dehydrogenase of isolated plasma membranes. Stimulation of HeLa cell growth, in the absence of calf serum, by these compounds provides evidence that growth stimulation by the transplasma membrane electron transport system is not entirely based on reduction and uptake of iron.

Key Words: Plasma membrane; transmembrane electron transport; ruthenium complexes; cell growth.

Introduction

Hexaammineruthenium(III) and derivatives have been used as impermeable electron acceptors for isolated subcellular electron transport systems.

Tripositively charged hexaammineruthenium(III) ions have been used as selective electron acceptors for photosystem I on the outer surface of chloroplast thylakoids (Barr *et al.*, 1982). Reduction of chloropentaammineruthenium(III) in mitochondria is antimycin sensitive, which indicates reduction in the region of cytochrome *c* and not at the primary internal dehydrogenase (Clarke *et al.*, 1980). This is consistent with evidence that

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0145-479X/87/0200-0069$05.00/0

hexaammineruthenium(III) is impermeable to mitochondria (Kunz and Konstantinov, 1984). The hexaammineruthenium(III) has also been shown to be impermeable to *Rhodospirillum* chromatophore membranes (Drachev *et al.*, 1986). The plasma membrane of many cells has a transmembrane electron transport system which can reduce external impermeable oxidants such as ferricyanide or diferric transferrin on the outside of the cell (Crane *et al.*, 1985a, b). Oxidants which act as electron acceptors for the transplasma membrane electron transport have been shown to stimulate growth of melanoma (Ellem and Kay, 1983), HeLa (Sun *et al.*, 1985), and Ehrlich ascites cells (Waranimman *et al.*, 1986). In this report we describe the action of hexaammineruthenium(III) and related complexes as positively charged oxidants which can act as electron acceptors for the transplasma membrane electron transport system and stimulate the growth of serum-deprived HeLa cells. The growth stimulation by these compounds affords evidence that the stimulation of growth by oxidants is not exclusively dependent on iron compounds which have been considered to stimulate growth by supplying iron to the cell (May and Cuatrecasas, 1985; Landschulz *et al.*, 1984; Reddel *et al.*, 1985; Zwiller *et al.*, 1982) but can also reflect the capacity of the compound to act as an electron acceptor at the plasma membrane. These ruthenium complex ions offer other advantages in the study of transplasmalemma electron transport and oxidant stimulation of cell growth. In contrast to ferricyanide they are positively charged, which increases affinity to the negatively charged cell surface. They offer a wide range of redox potential (Lim *et al.*, 1972; Clarke, 1980) and electron transfer rates (Chou *et al.*, 1977). The hexaammine and pentaammine complexes lack significant absorption bands in the visible spectrum so they do not interfere with analysis of cytochromes or other redox carriers. The pentaammine pyridine complex, on the other hand, forms an absorption band on reduction with a maximum at 410 nm with a molar absorptivity of $6.5 \times 10^3\ M^{-1}\ cm^{-1}$ and is reoxidized very slowly by oxygen (Ford *et al.*, 1968; Stanbury *et al.*, 1980). Measurement of absorption at 410 nm provides a direct determination of reduction rate.

Methods

The compounds, $[Cl(NH_3)_5Ru]Cl_2$ and $[(Pyr)(NH_3)_5Ru]Cl_3$, were synthesized according to established methods (Ford *et al.*, 1968; Stanbury *et al.*, 1980) while $[(NH_3)_6Ru]Cl_3$ was obtained from the Johnson-Mathey Co. and further purified by precipitation from aqueous solution by addition of acetone followed by recrystallization from 1 M HCl.

All enzyme assays were performed on an Aminco DW-2a dual-beam spectrophotometer. The rate of reaction for NADH-$[(NH)_6Ru]^{3+}$ reductase

and NADH-$[Fe(CN)_6]^{3-}$ reductase in plasma membranes was determined by recording the oxidation of NADH at 340 nm with a reference at 500 nm. Reduction of ferricyanide was followed at 420 nm (Sun *et al.*, 1984b). Standard assay medium for ferricyanide reduction contained 47 μM NADH, 100 μM ferricyanide, 5–10 μg protein, and 50 mM sodium phosphate buffer adjusted to pH 7.0, in a final volume of 2.8 ml. For reduction of hexaammineruthenium(III), the assay medium contained 47 μM NADH, 400 μM $[(NH_3)_6Ru]^{3+}$, 5–40 μg of protein, and either 50 mM sodium phosphate buffer at pH 7.0 or 50 mM Tris-HCl at pH 7.5, in a final volume of 2.8 ml. Extinction coefficients used in calculations of specific activities were $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ for NADH oxidation and $1.0 \times 10^{-3}\ M^{-1}\ cm^{-1}$ for ferricyanide reduction. All assays were performed at 37°C and enzyme activity is expressed in microequivalents transferred $min^{-1}\ mg^{-1}$ of protein. All ruthenium complexes were assayed in the same way.

Erythrocytes were obtained from pig blood available from a local slaughter house, and the open membrane as well as the inside-out vesicles were prepared as described by Kant and Steck (1972) and Steck and Kant (1974). The integrity of the inside-out vesicles were verified by assaying acetylcholinesterase, an enzyme exclusively located on the outside of the plasma membrane, before and after treatment with 1% detergent NP-40. The assay medium for acetylcholinesterase activity contained 0.5 mM acetylthiocholine, 0.33 mM dithiobisnitrobenzoic acid, 10–50 μg of vesicle proteins, and 0.1 M sodium phosphate at pH 7.0 in a final volume of 3 ml. Dithiobisnitrobenzoic acid solutions were prepared by dissolving 39.6 mg of this material in 10 ml of 0.1 M sodium phosphate, pH 7.0, and then adding 15 mg of $NaHCO_3$. The esterase reaction was monitored at 412 nm using a molar absorptivity of $13.6 \times 10^3\ M^{-1}\ cm^{-1}$. HeLa cells were grown on α-MEM (Gibco) without fetal calf serum as described previously (Sun *et al.*, 1985).

Reduction of ruthenium ammine complexes by HeLa cells was carried out in TD buffer (0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Trizma base, pH 7.4) with 0.1–0.4 mM ruthenium complex and 10–30 mg wet weight of cells at 37°C in a total volume of 2.8 ml. Reduction was measured by the procedure of Avron and Shavit (1963) with 6 μM bathophenanthroline disulfonate (BPS) and 3 μM ferric chloride. Absorbance at 600 nm was subtracted from 535 nm using the dual wavelength on the Aminco DW2a spectrophotometer to measure formation of ferrous bathophenanthroline disulfonate. All of this chelate was recovered in the supernatant when cells were centrifuged. Difference in extinction coefficient for 535–600 nm is $17.1\ mM^{-1}\ cm^{-1}$. For direct measurement of pentaammine pyridine ruthenium reduction, absorption at 500 nm was subtracted from 410 nm using buffer, cells and ruthenium complex (0.4 mM) only. The reduced

ruthenium complex was completely recovered in the supernatant after removal of cells by centrifugation.

Results

Since $[Fe(CN)_6]^{3-}$ and $[(NH_3)_6Ru]^{3+}$ are oppositely charged, they should respond differently to charged areas on the membrane surface and their rates of reduction vary accordingly as the ionic strength is increased (Wherland *et al.*, 1977). The effect of different salts over a range of concentrations is illustrated in Fig. 1. Potassium and sodium chloride activated ferricyanide reduction in a similar fashion and both increased the rate by a factor of 3 at 50 mM relative to the case where no salt was added. However, neither of these salts affected the rate of reduction of hexaammineruthenium(III). The addition of 5 mM $MgCl_2$ increased the rate of ferricyanide reduction by a factor of 3, but increasing concentrations inhibited the reaction. At 25 mM $MgCl_2$ the rate of ferricyanide reduction was only 70% that obtained by 5 mM.

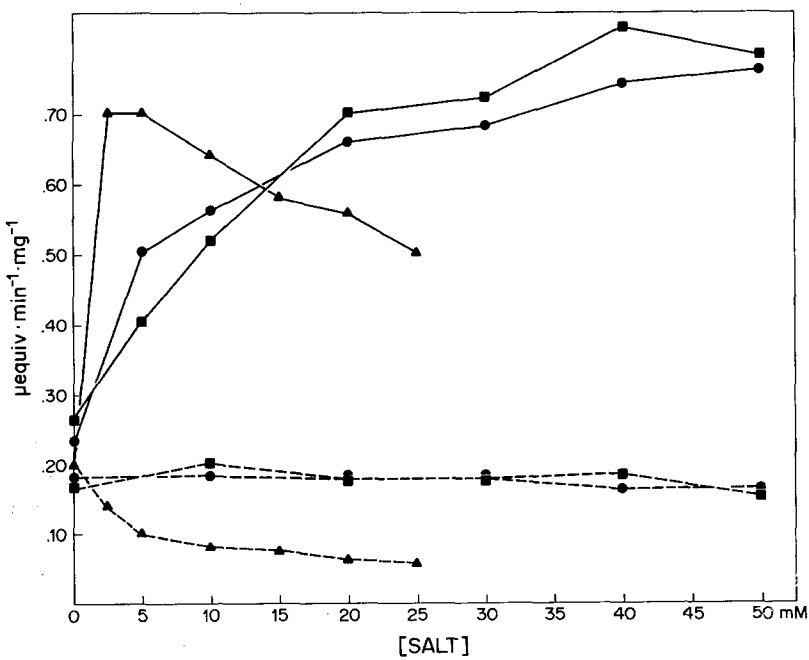

Fig. 1. Salt effects on $[(NH_3)_6Ru]^{3+}$ and $[Fe(CN)_6]^{3-}$ reductase activities. The standard assay media were used except for the buffer which was 5 mM Tris HCl, pH 7.5, for both activities. The full line represents $[Fe(CN)_6]^{3-}$ reductase activity and the dotted line $[(NH_3)_6Ru]^{3+}$ activity. (●) KCl; (■) NaCl; (▲) $MgCl_2$.

Table I. Kinetic Parameters for NADH-$[(NH_3)_6Ru]^{3+}$ and $[(Fe(CN)_6]^{3-}$ Reductase of Pig Erythrocyte Membranes

Activity	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)
$[(NH_3)_6Ru]^{3+}$ reductase	120	60
$[Fe(CN)_6]^{3-}$ reductase	1380	32

As expected on the basis of electrostatic considerations (Wherlund *et al.*, 1977), increasing concentrations of $MgCl_2$ decreased the rate of ruthenium reduction. The limiting value obtained at 25 mM $MgCl_2$ was 75% that obtained with no salt added.

Kinetic studies were done to determine if ferricyanide and hexaammineruthenium(III) reductions were catalyzed by the same enzyme. Table I shows the K_m and V_{max} obtained for these substances. At pH 7.0, in sodium phosphate buffer, the maximum rate of hexaammineruthenium reduction is nearly 10% that of ferricyanide. The rate of NADH oxidation was determined at different concentrations of ferricyanide with and without 0.2 mM $[(NH_3)_6Ru]^{3+}$ and the results are shown in double-reciprocal form in Fig. 2. At the lowest ferricyanide concentrations, the rate was elevated in the presence of Ru(III) at a concentration sufficient to saturate the enzyme. However, as the ferricyanide concentration was increased, it displaced the ruthenium complex because of its higher affinity for the enzyme. Owing to the greater V_{max} of ferricyanide, the velocity was higher when no Ru(III) was present. Such a pattern of reactivity is consistent with an enzyme acting on two different substrates with one substrate having a lower K_m and higher V_{max} (Dixon and Webb, 1979). When absorbance at 340 nm is measured with both substrates, a curved line is obtained in the double-reciprocal plot.

The oxidation rates of NADH by pig erythrocyte plasma membrane with different ruthenium complex ions serving as electron acceptors are listed in Table II. The complex $[(Pyr)(NH_3)_5Ru]^{3+}$ yielded the highest rate of electron transfer among the ruthenium complexes. The very low rate obtained with $[Cl(NH_3)_5Ru]^{2+}$ is due in part to its relatively low reduction potential (-42 mV) (Clarke, 1980). Reference to Table II reveals the general result that the rates of electron transfer increase with the reduction potentials of the oxidant as expected from the Marcus theory of electron transfer reactions (Sutin, 1973). The lack of expected correlation with the self-exchange constants of the various reactants can be attributed at least in part to the extreme sensitivity of k_{ex} for ferricyanide to the effects of different salts (Basolo and Pearson, 1967; Taube, 1970), and also, perhaps, to structural interactions at the membrane electron transfer site.

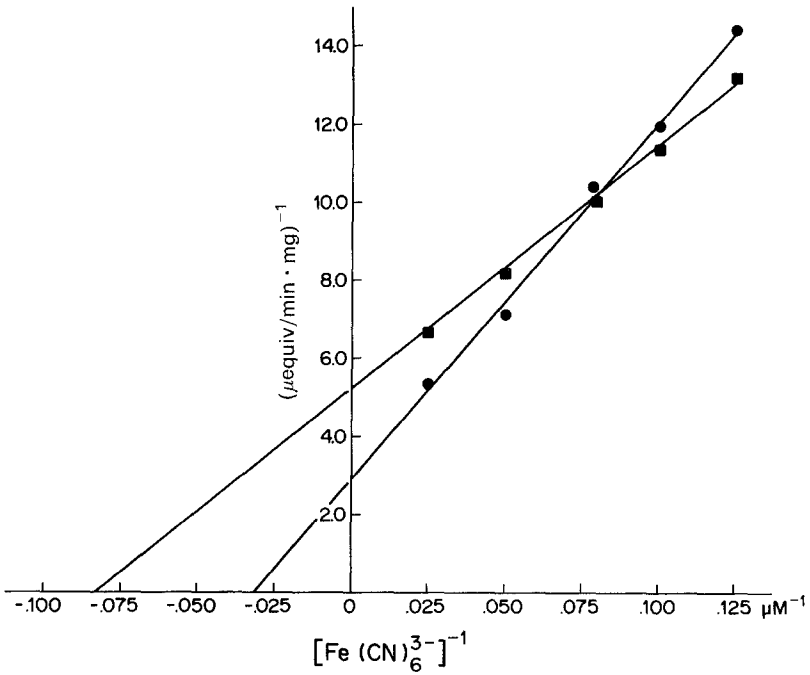

Fig. 2. Double reciprocal plots of the rate of ferricyanide reduction at 410 nm as a function of ferricyanide concentration with and without 0.2 mM $[(NH_3)_6Ru]^{3+}$. The assay media contained 47 μM NADH, 0 to 40 μM $[Fe(CN)_6]^{3-}$, 0 or 0.2 mM $[(NH_3)_6Ru]^{3+}$, 3–6 μg of red blood cell membrane proteins, and 50 mM $NaPO_4$, pH 7.0, in a final volume of 3 ml. The lines were drawn from a linear regression equation. (●) 0. mM $[(NH_3)_6Ru]^{3+}$; (■) 0.2 mM $[(NH_3)_6Ru]^{3+}$.

No significant change in the rate of reduction of $[(Pyr)(NH_3)_5Ru]^{3+}$ was observed in argon-purged solutions, so the transfer of electrons does not involve oxygen or the formation of superoxide ion (Ramasarma *et al.*, 1981). The rate of NADH oxidation was linearly proportional to the amount of protein added, and no activity was detected when the membranes were boiled for 3 min (cf. Table II).

The reduced form of the pyridine complex $[(Pyr)(NH_3)_5Ru]^{2+}$ exhibits a strong absorption maximum at 410 nm with a molar absorptivity of 6.5×10^3 M^{-1} cm^{-1} and is oxidized by oxygen only very slowly (Ford *et al.*, 1968; Stanbury *et al.*, 1980). This allowed independent monitoring of the rate of Ru(III) reduction, which was equal to twice the rate of NADH oxidation. This indicates that the two electrons donated by NADH are received as one electron per molecule of the ruthenium complex. The relative rates of reduction of the ruthenium complexes compared to ferricyanide (cf. Table II) show that the ruthenium ions are less reactive than $[Fe(CN)_6]^{3-}$. Nevertheless,

Table II. Oxidation Rates of NADH by Different Ammineruthenium(III) Complexes and $[Fe(CN)_6]^{3-}$ with Pig Erythrocyte Plasma Membrane

Electron acceptor	E^0 (mV)	k_{ex} (M^{-1} sec^{-1})	Reduction rate (nmol min^{-1} mg^{-1})	Relative rate
$[Fe(CN)_6]^{3-}$	300	740[a]	330	1.0
$[(Pyr)(NH3)_5Ru]^{3+}$	305[b]	4.3×10^5[c]		
Standard conditions			220	0.67
Ar purged			210	0.64
70 μg boiled membrane			0	0
$[(NH_3)_6Ru]^{3+}$	51[b]	4.3×10^3[c]	110	0.33
$[Cl(NH_3)_5Ru]^{2+}$	−42[b]		30	0.09

[a] Salt and ionic strength dependent; see Sutin (1973) and Taube (1970).
[b] See Lim *et al.* (1972) and Clarke (1980).
[c] See Table III in Chou *et al.* (1977).

$[(Pyr)(NH_3)_5Ru]^{3+}$ is a better electron acceptor for this system than cytochrome *c* or indophenol (Löw *et al.*, 1979).

The orientation of the ruthenium(III) and ferricyanide reductase activities on erythrocyte plasma membranes was studied with open membranes and inside-out vesicles (see Table III). For both electron acceptors, maximum velocities measured on inside-out vesicles were only 30 and 18%, respectively, of the corresponding rates measured on the open membranes. These results can be explained by the presence of a transmembrane NADH dehydrogenase which reacts with the impermeable intracellular NADH and the impermeable extracellular ferrycyanide or hexaammineruthenium(III). The activity of NADH cytochrome b_5 reductase on the inside of the erythrocyte membrane acting as a ferricyanide or ruthenium reductase can account for the activity of the inside-out vesicles (Kant and Steck, 1972). The transplasma membrane electron transport system can be measured with intact cells by following the reduction of the impermeable oxidants in the external media (Mishra and Passow, 1969; Dormandy and Zarday, 1965; Clark *et al.*, 1981; Crane *et al.*, 1985a). All three ruthenium complexes are reduced by HeLa cells. The reduction of pentaamine pyridine ruthenium is shown in Fig. 3. The rate of

Table III. Orientation of $[Fe(CN)_6]^{3-}$ and $[(NH_3)_6Ru]^{3+}$ Reductase Activities on Erythrocyte Membranes

Enzyme activity	Open membranes (nmol/min^{-1} mg^{-1})	Inside-out vesicles (nmol min^{-1} mg^{-1})	Ratio of activity Inside-out / Open
$[Fe(CN)_6]^{3-}$ reductase	332	99	0.30
$(NH_3)_6Ru^{3+}$ reductase	142	26	0.18
Acetylcholinesterase	878×10^3	269×10^3	0.31

Table IV. Reduction of Ruthenium Ammine Complexes by HeLa cells

Oxidant	Concentration (mM)	Rates of reduction (nmol min^{-1} g^{-1} (ww))	
		Initial rate	Slow rate
Pentaammine pyridine	0.1	100	
Pentaammine pyridine	0.4	304	
Hexaamine	0.1	16	
Pentaammine chloro	0.1	8	
Ferricyanide	0.1	210	113
Ferricyanide	0.4	330	162

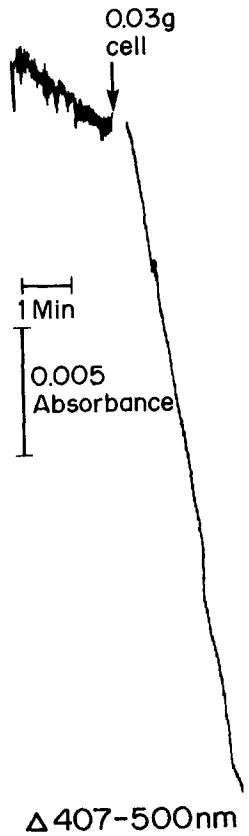

Fig. 3. Reduction of pentaammine pyridine ruthenium chloride by HeLa cells. 0.03 g (ww) cells added at arrow. 2.8 ml TD buffer, 0.4 mM ruthenium complex, 37°C. Absorbance at 500 nm subtracted from 410 nm on the dual beam. Down represents increased absorbance.

Table V. Stimulation of Oxygen Uptake by HeLa Cells with Hexaammineruthenium Chloride[a]

Addition	Oxygen uptake (nmol O_2 min^{-1} g^{-1} (ww))
Control	138
0.01 mM Hexaammine Ruthenium(III) chloride	208

[a]Oxygen uptake measured in 1.5 ml TD buffer with 50 mg (ww) cells with Gibson oxygen electrode.

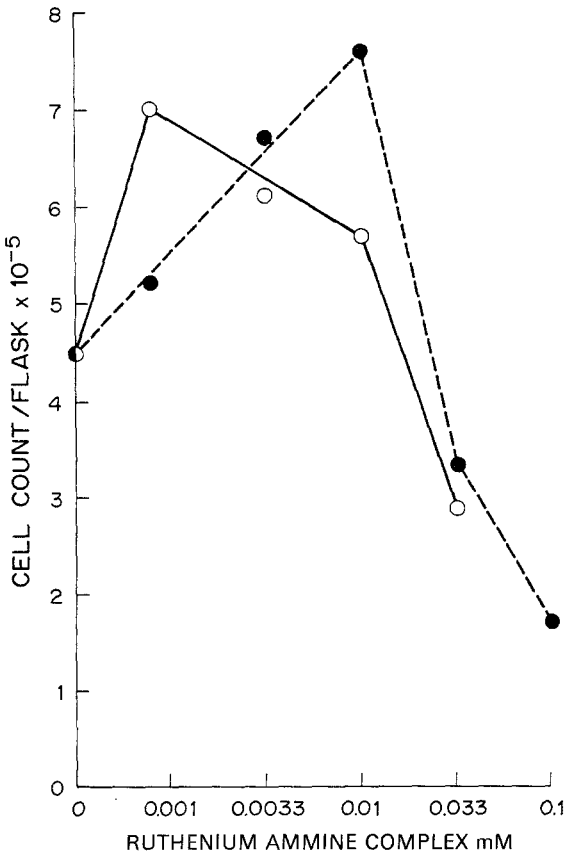

Fig. 4. Stimulation of HeLa Cell growth in the absence of fetal calf serum in α-MEM media. 48-hr culture. Cells removed by mild trypsin treatment for counting. (○) pentaammine chlororuthenium(III); (●) hexaammineruthenium(III).

reduction compares well with ferricyanide reduction and shows a steady rapid rate, rather than the initial rapid rate followed by a slower rate which is characteristic of ferricyanide reduction (Sun *et al.*, 1984b). Hexaammineruthenium and pentaamminechlororuthenium chloride show slower rates of reduction.

Since hexaammineruthenium(II) is readily oxidized by oxygen, the reduction of hexaammineruthenium(III) by HeLa cells is expected to be followed by an increase in oxygen uptake by the HeLa cells, as seen in Table V. The reduction of the $[Cl(NH_3)_5Ru]^{2+}$ may lead to loss of the chloride ion, in which case substitution into biological molecules such as proteins may occur (Margalit *et al.*, 1984). Reactions of this type have been observed with several ruthenium complexes (Srivastava *et al.*, 1979). Thus the transplasma dehydrogenase may aid in reduction and subsequent binding of certain ruthenium complexes to cells.

Low concentrations of ferricyanide have been shown to stimulate growth of HeLa cells in the absence of fetal calf serum, and this stimulation has been related to the transmembrane electron transport (Sun *et al.*, 1984c, 1985). Low concentrations of the rutheniumammine complexes also stimulate HeLa cell growth in the absence of serum (Fig. 4). The response is similar to the stimulation found with ferricyanide. Higher concentrations inhibit growth similar to inhibitions seen with higher concentrations of ferricyanide (Ellem and Kay, 1983; Sun *et al.*, 1984c).

Discussion

Diferric transferrin or other iron compounds are well known to be required for growth of cells (Barnes and Soto, 1980; May and Cuatrecasas, 1985; Reddel *et al.*, 1985) and it is considered that the growth stimulation is based on supply of iron to the cell. Two mechanisms of iron uptake have been proposed. For iron compounds in general the ferric form may be reduced at the cell surface for uptake of the less charged ferrous form through plasma membrane transport carriers. For diferrric transferrin bound to the transferrin receptor endocytosis exposes the bound iron to the acidic environment of the endocytic vacuole for release of the iron and return of the apotransferrin to the surface (Thorstensen and Romslo, 1984; Octave *et al.*, 1983; Nunez *et al.*, 1983; Morgan, 1983). Although the impermeability and stability of ferricyanide makes it an unlikely source of iron, and it has been shown not to supply iron to L1210 cells (Basset *et al.*, 1986), the growth stimulation by ferricyanide is always subject to the interpretation that it serves as an iron source (Ellem and Kay, 1983; Sun *et al.*, 1985). Since desferroxamine can inhibit cell growth and can remove iron from cells, it is clear that iron is

needed for cell growth (Basset *et al.*, 1986). There is evidence, however, that transferrin and the transferrin receptor may have a role in growth stimulation beyond iron supply (May and Cuatrecasas, 1985). We have shown that the transplasma membrane electron transport system acts as an NADH diferric transferrin reductase in connection with the transferrin receptor to reduce diferric transferrin at the cell surface (Crane *et al.*, 1985b; Löw *et al.*, 1986; Navas *et al.*, 1986). We have also presented preliminary evidence that impermeable redox agents other than iron compounds can stimulate HeLa cell growth (Sun *et al.*, 1984). It is clear that the ruthenium complexes can act as electron acceptors at the cell surface and can stimulate growth without acting as an iron supply. Optimum growth effects are observed in the range 0.001–0.01 mM which is lower than optimum ferricyanide concentrations (Sun *et al.*, 1985). Thus the growth stimulation by the ruthenium ions provides evidence that transplasma membrane electron transport has a role in growth stimulation beyond any role in ferric iron reduction. The uptake of Ru from ^{103}Ru-labelled transferrin by tumors may also be dependent on the release of Ru(II) following reduction of Ru(III)-transferrin complexes (Som *et al.*, 1983).

Of course the natural diferric transferrin can serve as both electron acceptor and iron source. Since the hexaammine and pentaammine chlororuthenium(II) ions are rapidly oxidized by oxygen, a small amount of either ion is sufficient for growth stimulation and oxygen would be the final electron acceptor. This recycling of an autooxidizable external acceptor may also be a factor in growth stimulation by ferrous salts since these are readily oxidized in solution (Rudland *et al.*, 1977; Young *et al.*, 1979). When transferrin is present, it provides an even more facile system for oxidation of ferrous iron even at low oxygen tension (Gaber and Aisen, 1970). Ferrous iron produced by reduction of diferric transferrin by the electron transfer system at the cell surface would be rapidly complexed to the newly formed apotransferrin and reoxidized by oxygen to diferric transferrin. The transferrin–iron combination thus provides a link to oxygen for the transmembrane electron transport system which will allow the following reactions to provide a continuous flow of electrons across the plasma membrane even with low concentrations of iron:

$$2e^- + \text{diferric transferrin} \rightarrow 2Fe^{2+} + \text{apotransferrin}$$

$$2Fe^{2+} + \text{apotransferrin} + 2H^+ + \tfrac{1}{2}O_2 \rightarrow \text{diferric transferrin} + H_2O$$

Acknowledgment

This work was supported by PHS grant CA36761 and Career Award GM K621839 (FLC). Work at Boston College was conducted under PHS grant GM26390.

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