ond apparatus under high flux conditions is shown in Fig. 2. This spectrum displays bands characteristic of rhodopsin and bathorhodopsin that were previously seen in steady-state resonance Raman spectra. We collected more detailed Raman spectra (Fig. 3) in the region between 800 and 1000 cm^{-1} using both a low photon flux (producing little photoalteration) and a high photon flux (producing moderate photoalteration while avoiding possible nonlinear or saturation effects). The low-flux spectrum of rhodopsin (top curve) contains a major peak that matches the distinctive 971 cm^{-1} line of unphotolyzed rhodopsin. The spectrum observed at higher flux levels (middle curve) exhibits new bands that correspond closely to the distinctive 853, 874, and 921 cm^{-1} bands of bathorhodopsin. This spectrum also exhibits a shoulder at 960 cm^{-1} that probably arises from isorhodopsin molecules produced by the subsequent photolysis of bathorhodopsin during the same 30-picosecond pulse. The high-flux spectrum of isorhodopsin (bottom curve) exhibits similar Raman bands at the bathorhodopsin positions. In addition, the 960 cm^{-1} band characteristic of isorhodopsin in this spectrum has a shoulder near 969 cm^{-1} that is probably due to rhodopsin molecules formed during the pulse.

The appearance of the low-wave-number lines of bathorhodopsin in our 30-picosecond rhodopsin spectrum shows that isomerization from 11-*cis* to a distorted all-*trans* form takes place within picoseconds of the absorption of a photon. Likewise, the picosecond Raman spectrum of photolyzed isorhodopsin shows that isomerization from 9-*cis* to a distorted all-*trans* form is also very rapid. The finding of an isorhodopsin shoulder in the high-flux rhodopsin spectrum, and vice versa, shows that these species can be interconverted in less than 30 picoseconds, probably via a common intermediate—bathorhodopsin. The resonance Raman studies reported here strongly support the hypothesis that the primary event in vision, occurring in times of picoseconds or less, is an isomerization of the retinal chromophore.

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Electrochemical Reduction of Horse Heart Ferricytochrome c at Chemically Derivatized Electrodes

Abstract. *Platinum or gold electrodes derivatized with an* N,N′*-dialkyl-4,4′-bipyridinium reagent can be used to reduce horse heart ferricytochrome c, whereas reduction does not occur at the "naked" electrodes. From 3 to 17.7 millimoles per liter, the reduction of ferricytochrome c is mass transport–limited at electrode potentials more negative than about −0.6 volt against a saturated calomel reference electrode. Data for the photoreduction of ferricytochrome c at derivatized* p*-type silicon photocathodes show directly that the rate of reduction is mass transport–limited. Use of derivatized electrodes may allow convenient manipulation and analysis of biological molecules that do not ordinarily respond at conventional electrodes.*

We report significant enhancement of the rate of electrochemical reduction of horse heart ferricytochrome c [cyt $c_{(ox)}$] by surface derivatization of Au, Pt, or *p*-type Si electrodes with reagent **1**, {*N*,*N*′-bis[3-(trimethoxysilyl)propyl]-4,4′-bipyridinium}dibromide (*1*). The cyt $c_{(ox)}$ gives a negligible response (*2*) at the "naked" (nonderivatized) electrode in the same potential regime. Large biological molecules having an electron transfer function often do not respond at conventional electrodes because the redox center—heme in cyt $c_{(ox)}$—cannot come close enough to the electrode (*3*). Such unresponsive molecules do react with

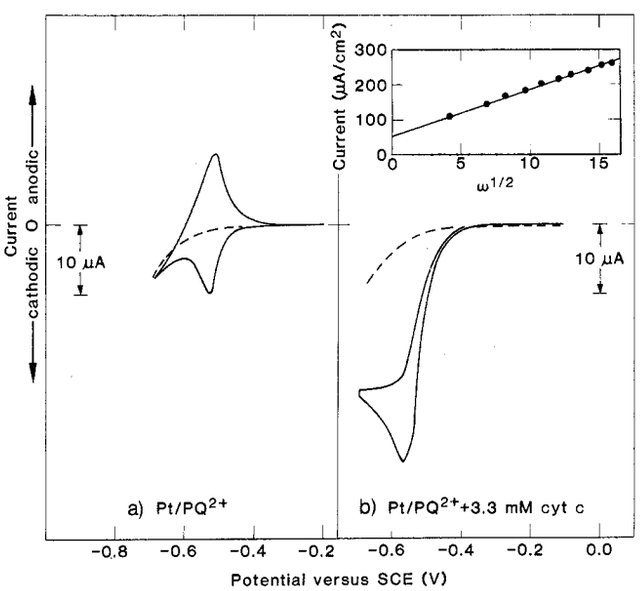

Fig. 1. Cyclic voltammetry of Pt wire electrodes at 5 mV/sec. (a) Scans of electrode (- - -) naked and (——) derivatized (2×10^{-8} mole/cm^2) with **1** in a stirred 1.0*M* $NaClO_4$ solution at *p*H 7.0 (phosphate). (b) Same conditions as in (a) after addition of 3.3 m*M* cyt $c_{(ox)}$. (Inset) Steady-state current at − 0.7 V versus SCE plotted against $\omega^{1/2}$ for a derivatized (2.1×10^{-8} mole/cm^2) rotating Pt disk electrode under the same conditions as in (b). In 1.0*M* KBr electrolyte at *p*H 7 the pretreated, but not derivatized, electrodes show no cyt $c_{(ox)}$ reduction current, but derivatized electrodes in 1.0*M* KBr behave the same way as in 1.0*M* $NaClO_4$, *p*H 7, used here.

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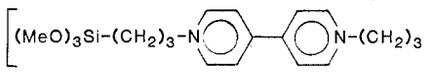
molecular reagents, and this provides a synthetic route to different redox states. Homogeneous electron transfer reagents can also be used to gain information about the redox potentials, number of electrons transferred, and mechanism of electron transfer (*3, 4*). These so-called electron transfer mediators, however, may interfere with various measurements made on the molecule of interest,

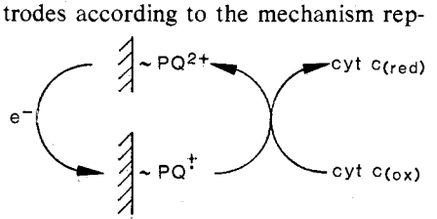


(1)

and separation and purification may be required. In the approach we used, a known mediator-redox center is immobilized on the electrode surface. Chemical derivatization of electrode surfaces has previously been shown to favorably alter the properties of electrodes (*1, 5-8*). However, the number of demonstrations of useful electrocatalysis by surface-confined mediators has been small.

The specific example of cyt $c_{(ox)}$ has been of considerable interest. In fact, its reversible reduction at a tin-doped indium oxide electrode has been reported (*9*), but it is not understood why this electrode works while others do not. The solution "catalysts" 4,4'-bipyridine and 1,2-bis(4-pyridyl)ethylene facilitate cyt $c_{(ox)}$ reduction at surfaces (*10*), presumably by adsorbing onto the electrode in a manner that precludes deleterious adsorption of the cyt c itself. Gold electrodes modified by a polymeric, nonelectroactive material derived from *N,N'*-dimethyl-4,4'-bipyridinium are apparently altered similarly with respect to the electrochemistry of spinach ferredoxin or myoglobin (*11*).

In the approach we employed we exploited the fact that the reduced form of the redox reagent *N,N'*-dimethyl-4,4'-bipyridinium, $MV^{+\cdot}$, reduces cyt $c_{(ox)}$ with a bimolecular rate constant of $> 10^8\ M^{-1}\ sec^{-1}$ (*12*). The reduction takes place at Au, Pt, and *p*-type Si electrodes according to the mechanism represented by **2**, where a surface-confined, bipyridinium-based polymeric reagent is the mediation system (*13*). The key distinctions of our findings are as follows. (i) We show direct evidence for the mechanism of electrocatalysis and provide a direct measure of the rate of cyt $c_{(ox)}$ reduction; (ii) several electrode substrates can be functionalized with **1**, including metals and semiconductors; (iii) by making deliberate variations (potential, charge, and so on) in the surface-confined reagent, the approach should be usable with other biological molecules; and (iv) the approach inherently allows easy separation of the electrode-mediator system.



(2)

Horse heart ferricytochrome c, of molecular weight 12,300 (*3, 14*), was the highest purity material available (Sigma Chemical Co., type VI). Ultraviolet-visible absorption spectroscopy was used to confirm its purity at the maximum absorption wavelength λ_{max} of 526 nm (extinction coefficient $\epsilon = 11{,}000\ M^{-1}\ cm^{-1}$) in H_2O/phosphate buffer, *p*H 7.0 (*14*). As expected (*2, 3*), cyt $c_{(ox)}$ was not reducible at naked Au or Pt electrodes even at potentials as much as 0.7 V more negative than the formal potential, $E°$[cyt $c_{(ox)}$/cyt $c_{(red)}$] = 0.02 V against a saturated calomel electrode (SCE) (*4, 15*). It was also not reducible at an illuminated, naked, *p*-type Si semiconductor photocathode, although it should be on thermodynamic grounds (*16*). We knew (*1*) that Au, Pt, or *p*-Si could be derivatized with **1** to yield a durable, surface-confined electroactive *N,N'*-dialkyl-4,4'-bipyridinium system $(PQ^{2+/+\cdot})_{surf}$ with $E°$ $(PQ^{2+/+\cdot})_{surf} = -0.50$ V versus SCE, and attempted to use such derivatized electrodes to effect cyt $c_{(ox)}$ reduction. The solution reductant *N,N'*-dimethyl-4,4'-bipyridinium, $MV^{+\cdot}$, reduces cyt $c_{(ox)}$ with a rate constant of $> 10^8\ M^{-1}\ sec^{-1}$ (*12*), and $E°(MV^{2+/+\cdot}) = -0.69$ V versus SCE in aqueous solution (*17*). Thus electrodes coated with the $(PQ^{2+/+\cdot})_{surf}$ system should mediate the reduction of cyt $c_{(ox)}$, provided the surface-confined reductant can penetrate cyt $c_{(ox)}$ as the $MV^{+\cdot}$ species does and provided the ~ 200-mV smaller driving force from the $(PQ^{2+/+\cdot})_{surf}$ system is not critical.

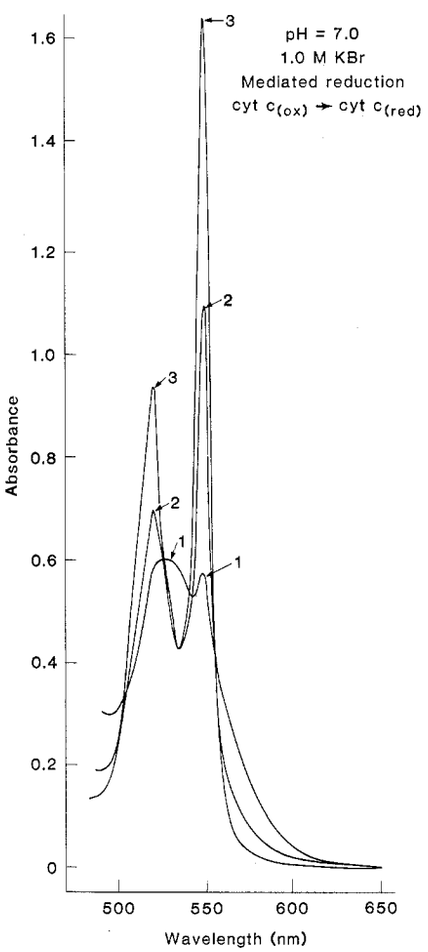


Fig. 2. Spectrophotometric analysis of electrochemical reduction of 2.2 m*M* cyt $c_{(ox)}$ in 1.00 ml of a stirred 1.0*M* KBr solution, *p*H 7.0 (phosphate). A 1.0-cm^2 (total area) Pt foil derivatized with **1** (2.7×10^{-8} mole/cm^2) was used as the cathode and was potentiostated at −0.6 V versus SCE. (Curve 1) Analysis of a solution (25 μl diluted to 1.0 ml) containing a small amount of cyt $c_{(red)}$. (Curve 2) After 0.12 C was passed, absorbance at 550 nm: calculated, 1.17; found, 1.8. (Curve 3) After 0.20 C was passed, absorbance at 550 nm: calculated, 1.63; found, 1.64. The electrode was unchanged after the experiment, and current due to H_2 evolution was negligible for this electrode.

Figure 1 shows representative data for a Pt/$(PQ^{2+/+\cdot})_{surf}$ cathode compared to a naked Pt cathode for cyt $c_{(ox)}$ reduction. The naked electrode shows negligible current attributable to cyt $c_{(ox)}$ reduction over the potential regime scanned. But the Pt/$(PQ^{2+/+\cdot})_{surf}$ cathode shows cyt $c_{(ox)}$ reduction when the electrode potential is negative enough for some $(PQ^{+\cdot})_{surf}$ to be present. Spectrophotometric analyses and integration of current show that the current efficiency for cyt $c_{(ox)} \rightarrow$ cyt $c_{(red)}$ is 100 ± 5 percent (Fig. 2). The cyt $c_{(red)}$ has a distinctive ultraviolet-visible absorption spectrum with $\lambda_{max} = 550$ nm ($\epsilon = 28{,}000\ M^{-1}\ cm^{-1}$) (*9*). Complete reduction of 2.2 m*M* cyt $c_{(ox)}$ in H_2O/1.0*M* KBr at *p*H 7.0 (phosphate buffer) was observed for a Pt electrode bearing 2.7×10^{-8} mole of the $(PQ^{2+/+\cdot})_{surf}$ system per square centimeter (*18*). The Pt/$(PQ^{2+/+\cdot})$ electrodes are thus durable enough to carry out cyt $c_{(ox)}$ reduction on a synthetic scale. Similar behavior was observed with pretreated Au electrodes derivatized with **1** (*18*).

Data for a rotating Pt/$(PQ^{2+/+\cdot})_{surf}$ disk electrode show that the limiting current at −0.7 V versus SCE for a 3.3 m*M* cyt $c_{(ox)}$ solution is directly proportional to the square root of the rotational velocity ω up to the highest value of ω for our motor, which is rated at 2000 rev/min (Fig.

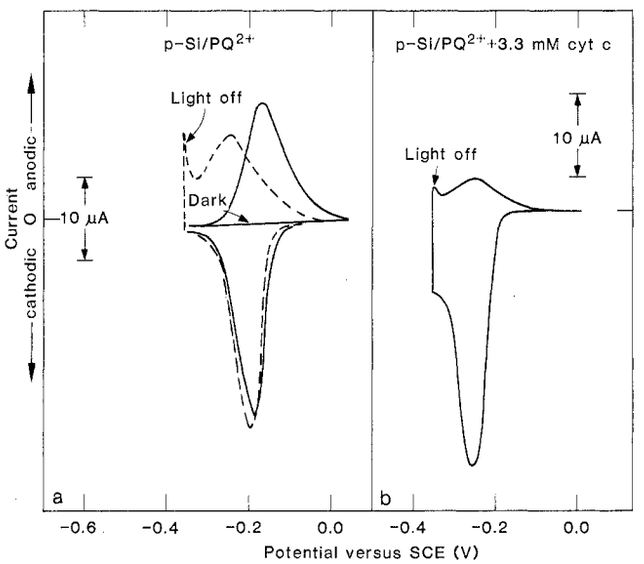


Fig. 3. Cyclic voltammetry at 5 mV/sec: *p*-type Si electrodes in stirred 1.0*M* $NaClO_4$ solution, *p*H 7.0 (phosphate). (a) Electrode derivatized (2.8×10^{-8} mole/cm^2) with **1** scanned (——) in light and in dark and (- - -) with light blocked at the cathodic limit. Illumination was at 632.8 nm from a 5-mW He/Ne laser expanded to provide ~ 50 mW/cm^2 over the entire electrode surface. (b) Same conditions as in (a) but with 3.3 m*M* cyt $c_{(ox)}$ added. Scan with light blocked at the cathodic limit shows diminished anodic current compared to (a) and a larger cathodic current due to mediated cyt $c_{(ox)}$ reduction.

1) (*19*). Since $\omega^{1/2}$ is proportional to the rate of mass transport (*19, 20*), we conclude from the strict linearity of the plot that the heterogeneous electron transfer rate constant, k_{het}, is mass transport-limited and is ≥ 0.06 cm sec^{-1}. Considering that negligible current passes at the naked electrode, we conclude that the $(PQ^{2+/+\cdot})_{surf}$ mediates the reduction. A very significant increase in reduction rate is found, albeit at a potential considerably more negative than $E°$ for the cyt c redox system.

We obtained data for a rotating Pt/$(PQ^{2+/+\cdot})_{surf}$ disk electrode as a function of cyt $c_{(ox)}$ concentration (3 to 17.7 m*M*), and plots of limiting current (recorded at −0.7 V versus SCE) against $\omega^{1/2}$ were linear in all cases. The slopes of the plots were directly proportional to the concentration of cyt $c_{(ox)}$.

Since no cyt $c_{(ox)} \rightarrow$ cyt $c_{(red)}$ reduction current is found until there is a detectable $(PQ^{2+})_{surf} \rightarrow (PQ^{+\cdot})_{surf}$ reduction current, we suggest that the cyt $c_{(ox)}$ reduction proceeds by the sequence:

$$(PQ^{2+})_{surf} \rightarrow (PQ^{+\cdot})_{surf} \quad (1)$$

$$(PQ^{+\cdot})_{surf} + \text{cyt } c_{(ox)} \xrightarrow{k_2} \text{cyt } c_{(red)} + (PQ^{2+})_{surf} \quad (2)$$

Assuming that the concentration of $(PQ^{+\cdot})_{surf}$ exposed to the solution of cyt $c_{(ox)}$ is ~ 10^{-10} mole cm^{-2} (*21*) and knowing that $k_{het} \geq 0.06$ cm sec^{-1}, we conclude that $k_2 \geq 6 \times 10^8$ mole^{-1} cm^3 sec^{-1} or $\geq 6 \times 10^5$ M^{-1} sec^{-1}. This is consistent with the rate constant $> 10^8$ M^{-1} sec^{-1} for cyt $c_{(ox)}$ reduction by MV^+.

Data for cyt $c_{(ox)}$ reduction at an illuminated *p*-type Si/$(PQ^{2+/+\cdot})_{surf}$ photocathode provide direct evidence that Eqs. 1 and 2 can account for the reduction of cyt $c_{(ox)}$ (Fig. 3). Reduction does not take place at *p*-type semiconductors in the dark, but on illumination at an energy equal to or greater than the semiconductor band gap, reduction can occur at electrode potentials more positive than those at a reversible electrode [such as Pt or Au for $(PQ^{2+/+\cdot})_{surf}$] (*1, 22*). The reduction of $(PQ^{2+})_{surf}$ on *p*-type Si occurs at a potential up to 550 mV more positive than on Pt or Au (*1*). But oxidation of $(PQ^{+\cdot})_{surf}$ on *p*-type Si does not require light (Fig. 3), and the positive potential sweep in the dark can be used to monitor the concentration of photogenerated $(PQ^{+\cdot})_{surf}$ after some time, t_i, in the presence of variable concentrations cyt $c_{(ox)}$. When no cyt $c_{(ox)}$ is present, the $(PQ^{+\cdot})_{surf}$ goes only to $(PQ^{2+})_{surf}$ by electrode oxidation (Fig. 3). But when cyt $c_{(ox)}$ is present, the oxidation produces $(PQ^{2+})_{surf}$ and cyt $c_{(red)}$, and a lower $(PQ^{+\cdot})_{surf}$ concentration is detected after t_i in the positive potential sweep. Thus the ability to photogenerate $(PQ^{+\cdot})_{surf}$, the inability to generate $(PQ^{+\cdot})_{surf}$ in the dark, and the ability to oxidize $(PQ^{+\cdot})_{surf}$ in the dark allow us to directly monitor reaction according to Eq. 2 on *p*-type Si by measuring the time dependence of disappearance of $(PQ^{+\cdot})_{surf}$ in the presence of cyt $c_{(ox)}$. Similar experiments have been described for semiconductor photoelectrodes that respond to two stimuli (light and potential) (*23*). Analysis of the data again shows $k_2 \geq 6 \times 10^5$ M^{-1} sec^{-1}. Thus the data are consistent with the chemistry shown in Eqs. 1 and 2 being the only mechanism for cyt $c_{(ox)}$ reduction at electrodes derivatized with **1**. An alternative mechanism for cyt $c_{(ox)}$ reduction would be one that need not proceed through generation of $(PQ^{+\cdot})_{surf}$ and its interaction with cyt $c_{(ox)}$. Such a mechanism appears to occur in the reduction of spinach ferredoxin or myoglobin at Au electrodes modified by a polymeric, nonelectroactive material derived from *N,N'*-dimethyl-4,4'-bipyridinium (*11*) or in cyt $c_{(ox)}$ reduction catalyzed by 4,4'-bipyridine (*10*).

In summary, the mediated reduction of horse heart ferricytochrome c with electrodes modified with **1** has been described. The mechanism for mediation involves reduction of the surface-confined reagent, which then interacts with ferricytochrome c. The observed rate of mediated reduction is mass transport-limited and a heterogeneous electron transfer rate constant ≥ 0.06 cm/sec is found. Electrodes are sufficiently durable to reduce ferricytochrome c in concentrations near its solubility limit without deterioration of their electrocatalytic activity. Although the mediated electron transfer involving ferricytochrome c and $(PQ^{2+/+\cdot})_{surf}$ is extremely rapid, determination of the formal potential for the metalloprotein from this interface is precluded by the large driving force for reaction (~ 500 mV). To obtain potentiometric data for metalloproteins from a derivatized electrode, it is necessary to have a broad distribution of surface-confined molecules with different redox potentials, all of which are capable of rapidly equilibrating the protein and surface potentials.

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18. In a typical experiment we use 0.5 to 1.0 ml of 1 to 3 m*M* cyt $c_{(ox)}$ in buffered aqueous solution (*p*H 5 to 7) and an Au or Pt electrode with an exposed area of ~ 0.5 cm^2 derivatized with the $(PQ^{2+/+})_{surf}$ system at 10^{-9} to 10^{-8} mole/cm^2. Electrolyte solutions are kept under Ar, since $(PQ^{+\cdot})_{surf}$ and cyt $c_{(red)}$ are sensitive to air. Derivatization of Au or Pt is effected by reacting pretreated [M. S. Wrighton, M. C. Palazotto, A. B. Bocarsly, J. M. Bolts, A. B. Fischer, L. Nadjo, *J. Am. Chem. Soc.* **100**, 7264 (1978)] surfaces with 1 to 3 m*M* CH_3CN solutions of **1** (*1*) under N_2 for 3 to 24 hours at 298 K. Electrochemistry was carried out with a three-electrode configuration in a two-compartment cell, using a saturated calomel electrode as reference and Pt as the counterelectrode. Cyclic voltammetry and other electrochemical measurements were carried out with a PAR model 173/175 potentiostat/programmer with a model 179 digital coulometer. Spectral measurements were made with a Cary 17 ultraviolet-visible-near infrared spectrophotometer.
19. Procedures for rotating disk electrodes are described in S. Piekarski and R. N. Adams, in *Physical Methods of Chemistry*, A. Weissberger and B. Rossiter, Eds. (Wiley, New York, 1971), part 2A, chap. 7.
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21. Electrodes with the $(PQ^{2+/+})_{surf}$ system at ~ 10^{-8} mole/cm^2 have generally been used. But since cyt $c_{(ox)}$ is mass transport-limited, it responds only to the $(PQ^{2+/+})_{surf}$ material available at the outermost surface. We assume that the coverage in this "monolayer" is ~ 10^{-10} mole/cm^2. We have consistently observed the same current density for cyt $c_{(ox)}$ reduction with electrodes having coverage in the range 7×10^{-10} to 3×10^{-8} mole/cm^2.
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22 September 1980

Insulin as a Potent, Specific Growth Factor in a Rat Hepatoma Cell Line

Abstract. *A line of rat hepatoma cells in culture which, in response to serum starvation, become arrested in the early G_1 phase of growth, can be stimulated by insulin alone to enter the cell cycle and traverse S phase. A half-maximum response is observed at 30 to 70 picomolar concentrations and the maximum response is essentially identical to that found with optimum serum concentrations.*

The ability of insulin to act as a growth factor has been investigated in a variety of cell types in culture including chick embryo fibroblasts (*1*), human fibroblasts (*2*), mouse 3T3 fibroblasts (*3*), rat liver cells (*4*), and baby hamster kidney cells (*5*). In these studies at least one of three limitations was noted. First, supraphysiological concentrations of insulin, ranging from 20 n*M* to 1 μM and higher, were required for a maximum response (*1*, *4*, *5*). Second, although in some of these reports a response was observed with concentrations as low as 10 n*M*, in no instance was a maximum effect obtained at this concentration. Indeed, even at significantly greater concentrations the response to insulin was not comparable to that found with optimum concentrations of serum (*2*-*4*). Third, in most of the reports, controls indicating the biological specificity of the response for insulin are lacking (*1*, *4*, *5*). This last criticism is particularly important when one considers the supraphysiological concentrations usually used and the report that an apparent growth effect could still be obtained by insulin preparations that had been autoclaved (*6*). This suggests that higher concentrations of insulin can produce "growth effects" in the absence of biologically active insulin molecules. In view of the observations that one or more of these three limitations applies to virtually all studies of the possible role of insulin as a growth factor, it is clear that the significance of insulin as a physiologically meaningful growth factor remains in doubt.

We have been using a clone of the H4-EII-C3 rat hepatoma cell line (H35) originally described by Pitot *et al.* (*7*). This

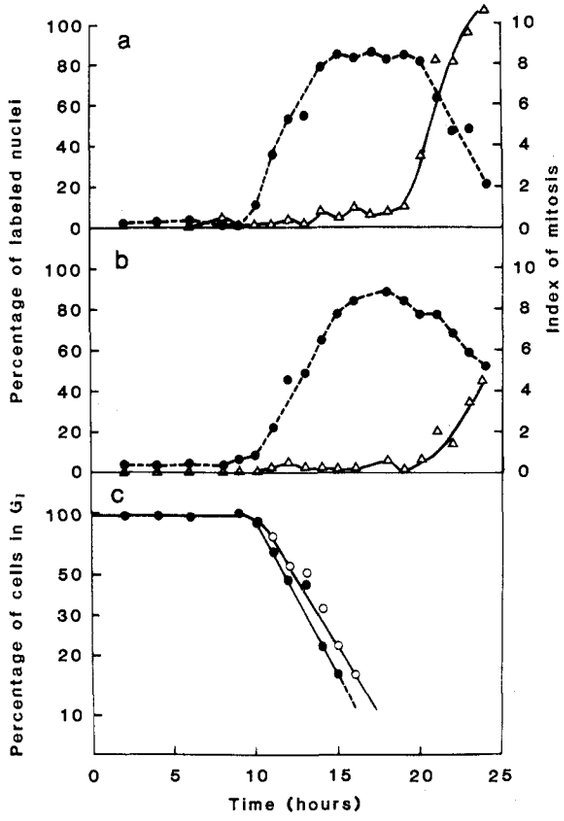

Fig. 1. Cells were grown in Dulbecco's modified Eagle's medium with 5 percent calf serum and 5 percent fetal calf serum in an atmosphere with 10 percent CO_2 at 37°C. Two days after subculture into 60-mm dishes the cells were washed once with balanced salt solution and fresh serum-free medium (SFM) was added. Cells remained in SFM for approximately 72 hours and then the medium was changed to (a) serum-containing medium or (b) SFM plus 10^{-9}*M* insulin. Every hour for 30 minutes, the cells were exposed to [^{3}H]thymidine (NEN, 0.5 μCi/ml, 0.2 μM). At the end of the labeling period, the cells were washed, fixed and dried, and then examined by autoradiography with NTB2 (Eastman Kodak). After development, the cells were stained with Giemsa (Harleco) and the dishes scored for the percentage of labeled nuclei and the mitotic index, with at least 1000 cells per dish being counted. The mitotic index is a reflection of cells counted in all phases of mitosis. Symbols: in (a) and (b), ●, percentage of labeled nuclei; △, index of mitosis; in (c), ●, 10 percent serum-containing medium; ○, serum-free medium containing 10^{-9}*M* insulin.

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