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Liba Goldblum
Yale University

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Lisa E. Goldblum

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A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the degree of Doctor of Medicine

Liba E. Goldblum
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Advisor-Dr. Ted Reid

Abstract

The development of completely defined, serum-free media is of importance to the study of the physiology of cultured cells. In this study, experiments aimed at developing a completely defined medium suitable for growth of a human retinoblastoma tumor cell line (Y-79) in culture were conducted. Specifically, the ability of four trace metals to enhance Y-79 cell growth was studied in serum-free medium. Addition of either cobalt, copper, chromium or selenium to a standard, serum-free medium (RPMI-1640) did not stimulate Y-79 cell growth.

Previous studies of the Y-79 cell line have demonstrated that addition of apotransferrin (3 ug/ml) to the culture medium enhances growth of Y-79 cells. Therefore, the ability of these trace metals to further stimulate growth in the presence of apotransferrin enriched medium was examined. While chromium, copper or cobalt did not increase cell number, selenium ($3x10^{-8}$-10^{-10}molar) did potentiate cell growth when added to apotransferrin enriched medium. Therefore, selenium and transferrin must act synergistically. The establishment of improved growth conditions for Y-79 cells in synthetic medium should aid in further studies of the physiology of both malignent and normal retinal cells.

Introduction

The development of completely defined, serum-free media is crucial to the successful analysis of the chemical nature of cultured cells and of the metabolites and products they excrete. Completely synthetic media are also important in determining the mechanism of action of certain factors on cultured cells. In this study, we investigated the effects of various metal ions on the growth of Y-79 cells in serum-free culture. This cell line derives from a human retinoblastoma tumor established in culture in this lab over a decade ago.(48)

Our rationale for this work came from the observation that conditioned media from Y-79 cells could support the growth of other untransformed retinal tissue which could not easily be grown in culture otherwise. Unfortunately, optimum growth of Y-79 cells required the addition of fetal calf serum to the medium. The presence of serum made it extremely difficult to isolate the growth factor, which we presumed was synthesized by Y-79 cells, enabling other neuronal tissue to grow in culture. The task became one of discovering a completely defined medium which would optimally support the growth of Y-79 cells and thereby yield conditioned medium from which we could hope to isolate a true nerve growth factor. This purified growth factor might allow us to grow and examine normal retinal cells in culture. Also, we hoped that, by elucidating the growth requirements of transformed retinal cells, i.e. Y-79 cells, we could more closely approximate the growth needs of normal retinal tissue and eventually come to a fuller understanding of the normal physiology of the retina.

Role of Selenium in tissue culture

Work by Ham in Colorado indicated that trace metals play a major role in enabling cells to grow in culture. In his efforts to develop a protein-free medium, he found that Chinese Hamster ovary and lung lines require 10^{-8}-10^{-6}M selenium (given as H_2SeO_3) for optimum growth (22). He discovered the cells' need for Selenium (Se) when he moved his lab to Boulder, Colorado and could not achieve cell growth with his protein-free medium (F-12) developed elsewhere (19). He eventually noticed that adding Se and/or L-thyroxine restored growth. Upon assaying the L-thyroxine for its Se content, present as a contaminant, he discovered that the Se alone accounted for all the observed stimulation (22). McKeehan et al. have also established Se as essential to the growth of human fetal lung cells in culture at an optimum concentration of $3x10^{-8}$M (36). The exact reasons for this Se effect are uncertain as the essential role of Se in biological systems has only recently been recognized.

Se, a group VI element under sulfur in the periodic table, was discovered by Berzalius in 1817, but for many years, few Se compounds were investigated. In the 1930's, Se was identified as a toxin in animals and extensively investigated as the cause of such animal diseases as "Blind Staggers" and "Alkali Disease" (59). However, only in the last 20 years has Se been shown to be an essential nutrient for many animals and bacteria. In 1957, Se was identified as the essential substance in Factor 3, an uncharacterized dietary supplement, which protected rats from severe necrotic degeneration of the liver (56). This observation soon led other researchers to demonstrate Se responsive diseases in over 40

species (6,36) including White Muscle Disease in lambs and calves (44), exudative diathesis in chicks and turkeys (59), and necrotic liver degeneration in rats and swine (56,9). However, to date, no human disease secondary to Se deficiency has been well documented although Keshan disease, a fatal cardiomyopathy of children occurring in a region of China where environmental Se levels are very low, is thought to be related to inadequate Se intake (72). Also, one case, resulting from prolonged total parenteral nutrition, has been reported recently (31). This dearth of people suffering from Se deficiency may perhaps be explained by the fact that most people ingest food from several geographic regions and, therefore, usually have no difficulty in consuming the presumed daily requirement of 0.75 ueqs (59).

In the last 10 years, advances have been made in the elucidation of the role of Se in biochemical processes. Several enzymes have been found to contain Se. Stadtman has elucidated two bacterial selenoenzymes, formate dehydrogenase and glycine reductase (59), and three other bacterial enzymes, nicotinic acid hydroxylase, xanthine dehydrogenase, and thiolase have recently been identified as likely selenoenzymes (60). In ovine heart and skeletal muscle, Se is incorporated into a 20,000 MW hemoprotein very similar in structure to cytochrome C. This protein is absent in the tissue of Se deficient animals suffering from White Muscle Disease (70). Finally, Se has been discovered to be an integral component of the enzyme glutathione peroxidase, (glutathione: hydrogen peroxide oxidoreductase, EC 1.11.1.9), abbreviated GSH-Px, isolated from many species, including human tissue.

GSH-Px catalyzes the reaction, ROOH + 2GSH $\longrightarrow$ ROH + GSSG +

H_20 where ROOH can be any of a variety of hydroperoxides and ROH, its corresponding alcohol. GSH is reduced glutathione: GSSG is oxidized glutathione (73). This enzyme protects cells against oxidant damage by reducing many different hydroperoxide substrates, including H_2O_2, at rates comparable to reduction of H_2O_2 by catalase (15). However, the enzyme is highly specific for GSH as a donor substrate and it will not catalyze the breakdown of hydroperoxides in the presence of other physiological hydrogen donors (60), except for one study indicating substantial reduction of linoleic acid peroxide by liver GSH-Px using cysteine and cysteamine as hydrogen donors (34).

GSH-Px was first reported present in rat and bovine erythrocytes in 1957 by Mills who noted that it protected erythrocytes from hemoglobin oxidation and hemolysis (43). Later, it was found that erythrocytes could be protected from hemolysis by adding glucose to the incubation medium. The presumed mechanism was that glucose maintained GSH levels (via the hexose monophosphate shunt) and thus, provided GSH-Px with a reducing substrate (60). Rotruck *et al.* discovered the connection between GSH and Se in 1971 when they observed that the protection from hemolysis afforded by the addition of glucose to the incubation medium was markedly decreased in erythrocytes from Se deficient rats (52). In 1973, Rotruck *et al.* identified GSH-Px as a selenoenzyme (70).

GSH-Px is an enzyme with a MW of about 80,000 although it varies somewhat from species to species and from tissue to tissue within the same species (15). It was first described in erythrocytes but has since been documented in many other cell types. Tissues highest in GSH-Px include liver, phagocytic cells,

and erythrocytes. Intermediate levels are found in heart, lung, kidney, adrenal gland, stomach mucosa, pancreas and adipose tissue. Low levels have been noted in brain, lens, testis, skeletal muscle, aorta and uterus (15). The subcellular localization of GSH-Px has indicated activity in both the cytosol and mitochondrial fractions where it is thought to protect the outer and inner mitochondrial membranes respectively from oxidation (15).

Enzyme studies using tissue from several different species indicate that the molecule is constructed of four identical subunits (MW=22,000) and contains four gram atoms of Se per mole of enzyme (46,11,12,60). It contains no heme, flavin or other metals as indicated by the gamma spectrum of the irradiated enzyme (12) and therefore, differs from all other known peroxidases. Zakowski et al. have recently shown that Se is present in the active site of the reduced enzyme in the form of selenocysteine and have proven, by sequential Edman degradation, that the selenocysteine is located within the polypeptide chain (73).

The kinetic mechanism of GSH-Px has been described by Ganther et al. as a series of three bimolecular steps (14,36) where Se participates in a set of redox reactions at the active site as follows: 1) The active site selenol(E-SeH) undergoes oxidation with peroxide to form a selenenic acid (E-Se-OH). 2) The oxidized form of the enzyme reacts with the first GSH molecule, forming a mixed selenosulfide (E-Se-SG) and a molecule of H_2O. 3) The selenosulfide linkage is cleaved by a second GSH molecule, producing oxidized glutathione and restoring the enzyme to the selenol form. An analogous set of reactions can be written in which Se cycles between selenenic (E-Se-OH) and seleninic

(E-Se-O_2H) forms. There is some evidence for the presence of different oxo-derivatives of Se during catalysis, but none of these oxidation states has been conclusively identified in the native molecule (15).

The activity of GSH-Px in vivo depends on the presence of Se. Animals maintained on Se deficient diets show rapid increases in enzyme activity when Se is reinstituted into their diets (69). Investigators working with cultured cells where Se has been found to be an essential nutrient have speculated that the growth enhancing action of Se may be due to its incorporation into the selenoenzyme, GSH-Px (36,18). More recently, Germain and Arneson have ascertained that the activity of GSH-Px in cultured mouse neuroblastoma cells is directly proportional to the concentration of selenite in the medium below $6x10^{-8}$M and levels off at concentations above this value (16). This concentration is similar to the optimum concentrations found for growth enhancement in other tissue culture work (22,2,36). Thus, the growth enhancing effects of Se may very well be secondary to its stimulation of GSH-Px synthesis and/or activity leading to protection of vital cell components from oxidation. In fact, requirements for Se in tissue culture of diploid human fibroblasts have been found to be quantitatively lower when cells are grown at lower oxygen concentrations (37). Also, the inhibitory effects of excess oxygen are much more severe when cells are marginally deficient in Se than when adequate amounts are supplied (20).

Role of Transferrin in Tissue Culture

Our lab has also noted recently that apotransferrin, in an optimum concentration of 3 ug/ml ($3x10^{-8}$M), will support the

growth of Y-79 cells in iron-free and serum-free medium (RPMI-1640). This cell line is unusual because no additional hormones or growth factors are necessary to support growth. Many labs have found that transferrin enhances growth in a variety of serum-free tissue culture systems but most have required at least insulin (24,4), and usually other growth factors as well, to sustain the cells in culture (54,41,7,10,24,49,13,55,68,45).

Transferrin is a serum protein, a B globulin, with a MW=77,000. It has a single peptide chain with 2 metal binding sites which in the past were thought to be very similar (23). However, the current notion of transferrin binding is that the A site binds most of the iron at physiological pH's and delivers the iron used by erythrocyte precursors and the placenta. The B site, employed only during situations of iron excess, carries iron to the hepatocytes (Fletcher-Huehns hypothesis) (5).

Very recently, investigators have noted the presence of specific receptors for transferrin on transformed cells. Galbraith et al. examined 18 human cell lines, including 25 transformed cell lines of malignant and lymphoblastoid origin, for their ability to bind transferrin. Less than 5% of normal cells (peripheral blood mononuclear cells and cultured diploid fibroblasts) exhibited membrane binding of transferrin whereas over 80% of the transformed cell lines demonstrated specific receptors for transferrin (13). Faulk et al. immunohistologically examined the breast tissue from 59 patients and determined that almost none (1/37) of the tissue from normal areas or benign lesions and 72.7% of the malignant specimens were positive for the presence of a transferrin receptor (10). This receptor will bind apotransferrin

as well as saturated transferrin whereas receptors found on normal erythrocyte precursors hardly bind apotransferrin, at all. Receptors with identical specificity have also been found on normal trophoblast membrane at the maternofetal interface (10). These authors conjecture that the trophoblast and the tumor may benefit from derepression of receptor synthesis for either nutritional reasons, lymphocyte modulation or escape from immunosurveillance in a manner similar to the schistosome (13).

The most obvious explanation for the increased binding of transferrin by transformed and malignant cells is a nutritional one. These rapidly growing cells probably have an increased requirement for the iron complexed with transferrin. For example, iron has been shown to be necessary for DNA synthesis and mitosis in HeLa cell culture (50) as well as cell division in cultured 3T6 cells (54). Immature leukemic cells from patients with acute leukemia will take up radioiron whereas leukocytes from healthy volunteers lack this ability (7). Iron may also be necessary for the synthesis of the electron transport chain and/or the synthesis of enzymes involved in the oxidation and reduction of critical cell products such as cis-unsaturated fatty acids and nucleic acid precursors (71). Interestingly, GSH-Px activity in erythrocytes is determined by the availability of iron during development in the bone marrow. In blood drawn during the induction of iron deficiency, younger cells had decreased enzyme activity compared to older cells. However, as iron deficient animals were refed iron, younger cells had higher enzyme activity than did older erythrocytes (51). Therefore, perhaps Y-79 and other cells exhibit increased growth in the presence of transferrin simply because of

their increased iron requirements.

However, the physiological situation is probably more complex, especially since transferrin enhances Y-79 cell growth in the apparent absence of iron. The reports, cited above, indicating that transferrin is one of a small number of proteins and growth factors necessary to support the serum-free growth of embryonal cancer cells, HeLa cells, B lymphocytes, BHK cells and 3T3 cells, suggest that transferrin may have other important roles in the physiology of many cells, a role poorly understood at the present time. Other facts about transferrin have emerged which support the concept of a wider importance for this protein. Besides its growth stimulating action, transferrin has been noted to have an antimicrobial action (5) as well as increasing the mitogenic response to phytohemagglutinin (PHA) in human lymphocytes. This enhancement of the PHA effect at 4-5 ug/ml of transferrin could not be reproduced by free iron salts ($FeCl_3$ at 10^{-15}-10^{-3}M) (66). Whatever the mechanism underlying the growth enhancing effect of transferrin, one is tempted to speculate that its in vitro action may be similar to its in vivo role since their effective concentrations are similar.

Transferrin is also known to bind many other metals besides iron (23,65) and a specific receptor for zinc, bound to transferrin, has been observed in human lyumphocytes (47). While Se is known to bind to $alpha_2$ and beta globulins and especially beta lipoprotein (6), it has not been shown to bind to transferrin. However, several investigators have used Se and transferrin in combination and found that these agents, plus other factors, could partially or completely replace serum, depending on the cell line

(18,30,29,4,41,44,37). Guilbert and Iscove noted serum sparing effects (from 30% to 2.5% fetal calf serum) in haematopoietic cell cultures grown in medium containing selenite (10^{-7}M), transferrin ($3.4x10^{-6}$M), albumin and lecithin (18). Iscove and Melchors found that murine B lymphocytes grew and matured into IgM and IgG secreting cells in serum-free culture medium containing transferrin (10^{-8}M), Se (10^{-7}M), albumin and soybean lipid. Their data are difficult to interpret because the soybean lipid used was a crude lecithin preparation (30). Hutchings and Sato developed a serum-free medium, that would support HeLa cells, containing transferrin ($5x10^{-8}$M), Se ($1.5x10^{-8}$M), as well as insulin, aldosterone, epidermal growth factor and fibroblast growth factor (29). Breitman et al. reported the growth and differenciation of human promyelocytic cells in a culture medium containing Se ($3x10^{-8}$M), transferrin ($5x10^{-8}$M) and insulin (4). Therefore, we felt it would be of interest to examine the effects of transferrin and Se, in concert, on our Y-79 retinoblastoma cell line.

Other Trace Metals

Chromium, copper and cobalt are other trace metals known to be essential in biochemical processes (39). Chromium is important for the maintenance of normal glucose tolerance (40) and at least one group has found it to enhance growth in a Chinese hamster cell line in a concentration of 10^{-9}M (22). Copper, essential for many oxidative enzymes as well as for the effective utilization of iron, has also had growth stimulating effects in vitro (10^{-7}-10^{-9}M) on human diploid fibroblasts and fetal lung cells (37), on Chinese hamster cell lines (22,27,19), and NCTC

Clone 929 Strain L cells (13). It was also present as a "contaminant" (.05 mg/L) in Takaota's studies on mouse fibroblasts, HeLa cells, monkey kidney cells, rate ascites hepatoma cells, rat thymus reticulum cells, and rat liver parenchymal cells (62,20). Cobalt is an important requirement for the activity of several enzymes, including ribonucleotide reductase and glutamate mutase, as well as for Vitamin B_{12} dependent processes. Ham has found it helpful in tissue culture of Chinese hamster cells at concentrations of 10^{-9}M (22). All of these metals are known to bind to transferrin (23) although iron will displace them if present (5). Therefore, we decided to examine the effects of the trace metals, copper, cobalt, chromium and selenium on the growth of Y-79 cells by testing them alone and in the presence of an optimum concentration of apotransferrin.

Materials and Methods

Cells and Materials

The Y-79 human retinoblastoma tumor cell line used in these studies has been in continuous cell culture in our laboratory since its establishment in 1971 after removal from the eye of a 2-1/2 year old white girl (48).

Components of the medium were obtained as follows: RPMI-1640 from Flow Laboratories; fetal bovine serum from Gibco; sodium selenite (Na_2SeO_3) from Alfa Division, Ventron Corporation; cobalt chloride ($CoCl_2 \cdot 6H_2O$) from Fisher; Chromium chloride ($CrCl_3 \cdot 6H_2O$) and copper sulfate ($CuSO_4 \cdot 5H_2O$) from J.T. Baker. Bovine albumin (fatty acid free) and Human Apotransferrin (pfs) were obtained from Sigma. The antibiotics penicillin (10,000 u/ml) and streptomycin (10,000 ug/ml) were purchased as 100x concentrated solutions in physiological saline from Grand Island Biological Company. RPMI-1640 is dissolved in deionized, doubly distilled water prepared in our laboratory.

Maintenance of Y-79 Cell Culture

The Y-79 stock cell cultures were maintained in RPMI-1640 supplemented with 5% fetal bovine serum, 3×10^{-8}M Na_2SeO_3, 100 u/ml of penicillin, 100 ug/ml of streptomycin and .01 M HEPES (N-2-hydroxyethylpiperazine-N´-2-ethane sulfonic acid). They were grown in suspension in 150 ml flasks (Costar) in a total volume of approximately 75 ml at 37°C in a moist atmosphere of 95% air and 5% CO_2. The cultures were fed twice weekly and split as needed. During feeding, approximately 10 ml of the depleted medium were left in the flasks.

Growth Experiments

Y-79 cells were spun down in 50 ml centrifuge tubes (Corning) and washed once in serum-free medium. They were resuspended by vigorous pipetting and a 0.5 ml sample was counted by Coulter Counter (standardized periodically with a hemacytometer count of cells treated with trypan blue). Cells were then divided, spun down in 15 ml centrifuge tubes (Corning), and brought up in the various experimental media. (The experimental media consisted of RPMI-1640, penicillin (100 u/ml), streptomycin (100 ug/ml), HEPES (.01M) and varying concentrations of cobalt chloride, sodium selenite, copper sulfate, or chromium chloride, with and without the addition of 3 ug/ml of apotransferrin as explained in the results.) These cells were then divided into 16x125 mm tissue culture tubes (Corning) in 1 ml aliquots with a projected final cell concentration of $5x10^5$ cells/ml, and 0.5 ml of the residual cells in the various media were counted as below to verify cell number. The tissue culture tubes were slanted at 5° and incubated at $37^{\circ}C$ in a moist atmosphere of 95% air and 5% CO_2. Cells were fed on day 4 by the following procedure: 5 ml of fresh medium were added to each tissue culture tube and the cells were spun down. 5 ml of medium was then evacuated, leaving 1 ml of medium into which the pellet was resuspended.

Counting Procedure and Data Analysis

Two tubes were randomly selected for counting on days 1,2,4, and 7. 1-1/2 hours prior to counting, bovine serum albumin was added to each sample (1 mg/ml) because this procedure was found to ensure uniformity of cell size before counting with the Coulter Counter. Each 1 ml sample was diluted with isotonic buffered

saline (Scientific Products) and counted twice on the Coulter Counter and the results corrected and averaged. Standard deviations were computed for each data point. In some cases, data were represented as a ratio of cell number at day 4 (A) to cell number at day 1 (B) in order to standardize for differences in initial cell concentration. The error of this ratio was computed via the following formula and the errors averaged.

$$S_d(A/B)=\sqrt{1/B^2(\text{var } A) + A^2/B^4(\text{var } B)}$$

Day 1, and not day 0, was chosen as the denominator for this ratio because the counting procedure for day 0 differed from that of day 1 through day 7 in number of samples averaged as well as aliquot size. In one instance, noted in the results, the ratio of cell number at day 3 to cell number at day 1 was used.

Results

I. Effect of Trace Metals on Y-79 cell growth in the Absence of Apotransferrin

In order to determine the effect of the addition of cobalt, copper, chromium, and selenium ions on the growth of Y-79 cells in culture, a variety of concentrations of each metal were tested. Figure 1 illustrates the seven day growth curve of Y-79 cells in concentrations of $CoCl_2$ ranging from 10^{-9}-10^{-6}M. In Figure 2, the ratio of cell number at day 4 to cell number at day 1 is shown. In comparison to the cells grown in RPMI-1640 without $CoCl^2$ added, there does not appear to be any enhancement of growth. Indeed, at each concentration of $CoCl_2$ tested, growth lagged behind that of the control cells. As expected, cells maintained in medium enriched with 5% fetal bovine serum outstripped all other groups in growth between day 1 and day 4.

Figure 3 and 4 show comparable results for $CuSO_4$ in concentrations ranging from 10^{-8}-10^{-6}M. There was no enhancement of Y-79 cell growth when this metal was added to the medium.

The results found when $CrCl_3$ was added, in concentrations of 10^{-10}-10^{-7}M, are displayed in Figures 5 and 6. No significant enhancement of growth was noted at any of the concentrations tested. Similarly, at 10^{-9}- $3x10^{-5}$M Na_2SeO_3, growth increments between day 1 and day 4 did not differ significantly from controls (Figures 7 and 8).

II. Effect of Trace Metals on Y-79 Cell growth in The Presence of Apotransferrin

Consistent with results found previously in our laboratory (unpublished), the addition of 3 ug/ml of apotransferrin to the

culture medium resulted in a reproducible enhancement of growth. For example, as illustrated in Figure 10, cells grown in apotransferrin-enriched medium grew better than control cells. However, growth of these cells was surpassed by that of cells grown in medium supplemented with 5% fetal bovine serum. In order to determine whether the addition of trace metals could lessen this discrepency, the effects of cobalt, copper, chromium and selenium were ascertained in the presence of apotransferrin.

Figure 9 presents the four day growth curves of cells grown in the presence of 10^{-10} - 10^{-7}M $CoCl_2$ as well as growth curves of control cells grown in either serum-free medium, serum-free medium plus apotransferrin or serum-enriched medium. Figure 10 illustrates the ratio of cell number at day 4 to cell number at day 1. When this ratio is used as a measure of growth, it becomes apparent that the addition of $CoCl_2$ does not alter the enhancement of cell growth rate produced by apotransferrin.

Figures 11 and 12 present comparable data for concentrations of $CuSO_4$ ranging from 10^{-9}- 10^{-6}M. Although 10^{-9}M $CuSO_4$ appears to enhance growth above the level produced by apotransferrin alone, suggesting that lower concentration of $CuSO_4$ may enhance growth, additional experiments including concentrations of $CuSO_4$ as low as 10^{-11}M, did not support a consistent growth effect at these lower concentrations.

The effect of $CrCl_3$ is presented in figures 13 and 14. There, too, it is evident that concentrations of this metal ion ranging from 10^{-10}- 10^{-7}M had no significant effect on growth of Y-79 cells.

In contrast to the other metals tested, Na_2SeO_3, in the

presence of apotransferrin, clearly enhanced cell growth beyond the effect produced by apotransferrin alone. This effect appears at concentrations of $3x10^{-8}$M Na_2SeO_3 and below. (Figure 16). In an attempt to define the optimum concentration of Na_2SeO_3, these experiments were repeated in the presence of lower concentrations of selenium. The effect appears to plateau at 10^{-10}-10^{-8}M selenium and by 10^{-13}M, growth enhancement disappears.

FIGURE 1

Seven day growth curve of Y-79 cells grown in the presence of RPMI-1640 + varying concentrations of $CoCl_2$ without the addition of apotransferrin. The points designated "0" and "5%" refer to those cells grown without added $CoCl_2$ or serum and those cells incubated in medium enriched with 5% fetal bovine serum respectively. Cells were fed on day 4.

KEY

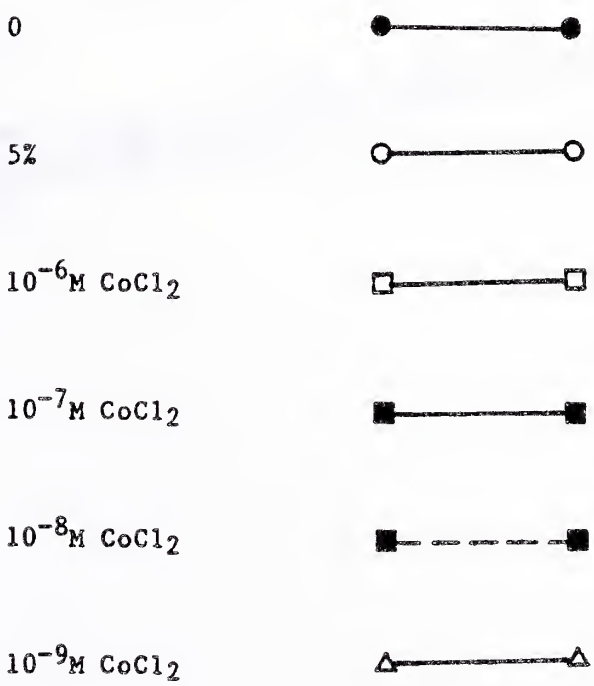


FIGURE 1

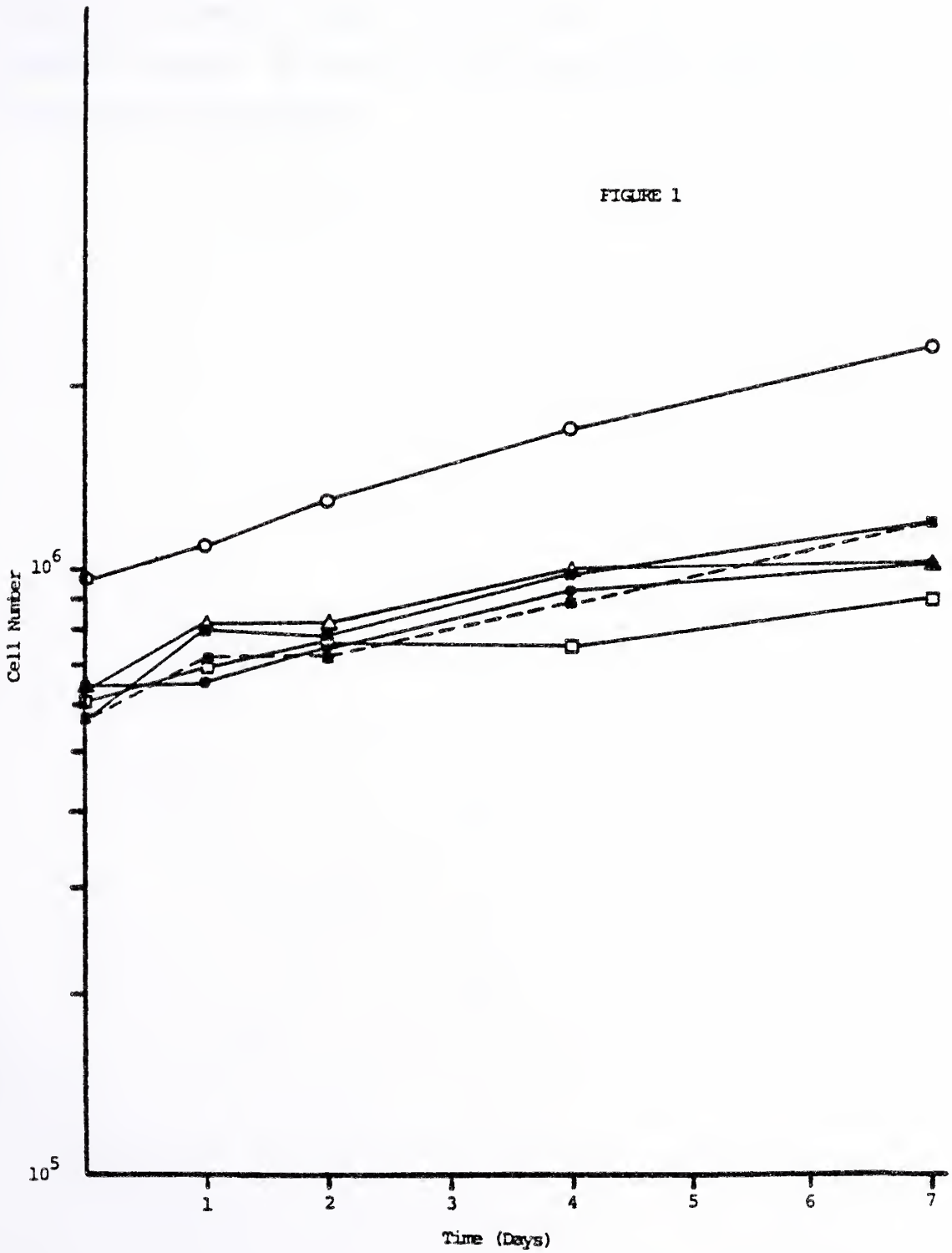


FIGURE 2

Effect of varying concentrations of $CoCl_2$ on cell growth. The ratio of cell number at day 4 to cell number at day 1 is used as a measure of growth. The effect of RPMI-1640 enriched with 5% fetal bovine serum is also shown.

FIGURE 2

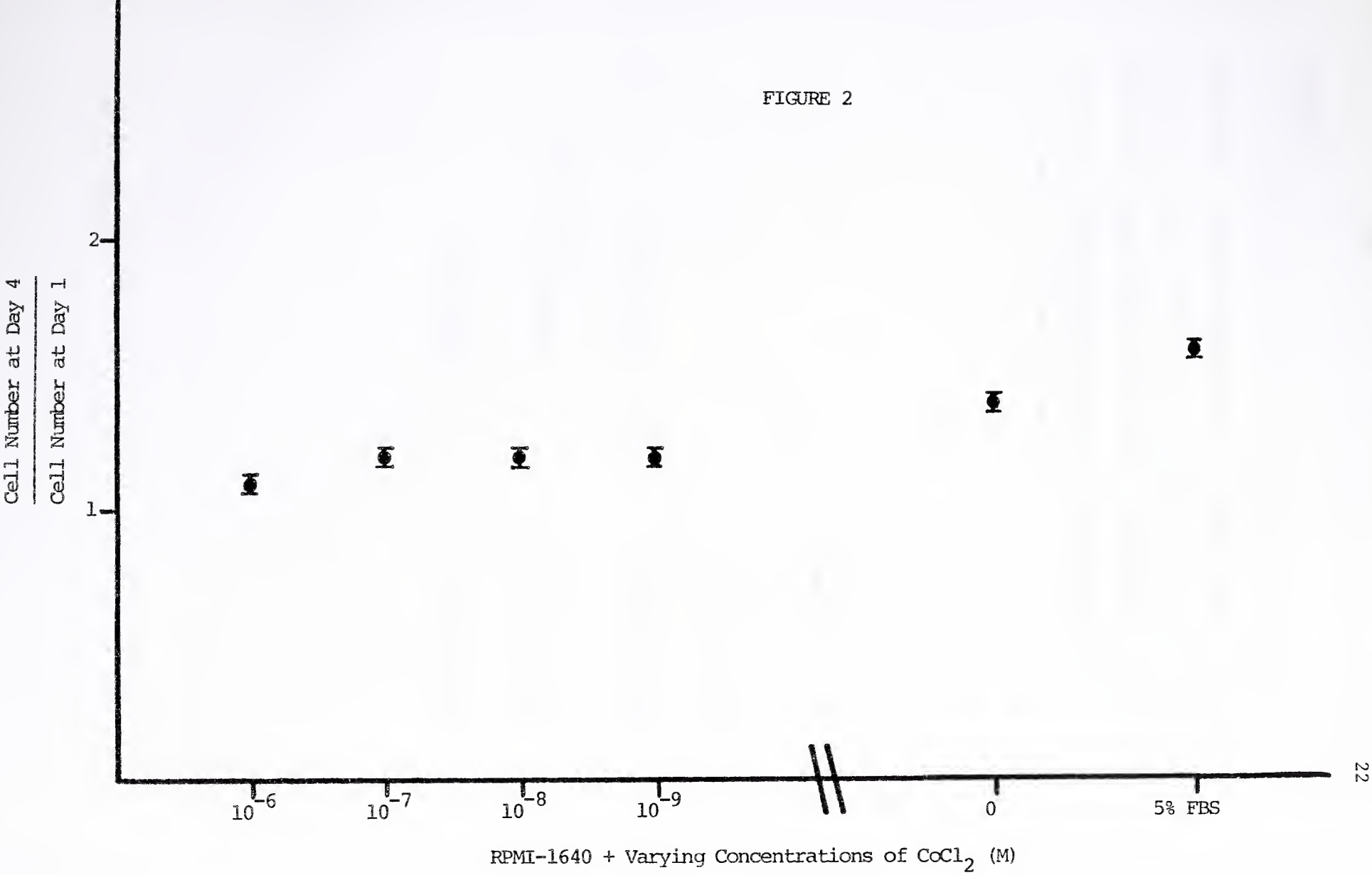

Cell Number at Day 4
Cell Number at Day 1
2
1
10^{-6}
10^{-7}
10^{-8}
10^{-9}
0
5% FBS
RPMI-1640 + Varying Concentrations of $CoCl_2$ (M)

FIGURE 3

Six day growth curve of Y-79 cells grown in the presence of RPMI-1640 + varying concentrations of $CuSO_4$ without the addition of apotransferrin. The points designated "0" and "5%" refer to those cells grown without added $CuSO_4$ or serum and those cells incubated in medium enriched with 5% fetal bovine serum respectively. Cells were fed on day 4.

KEY

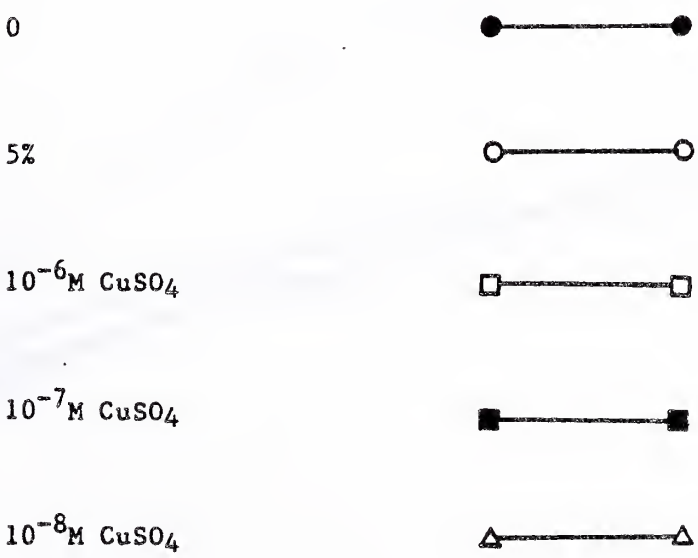


FIGURE 3

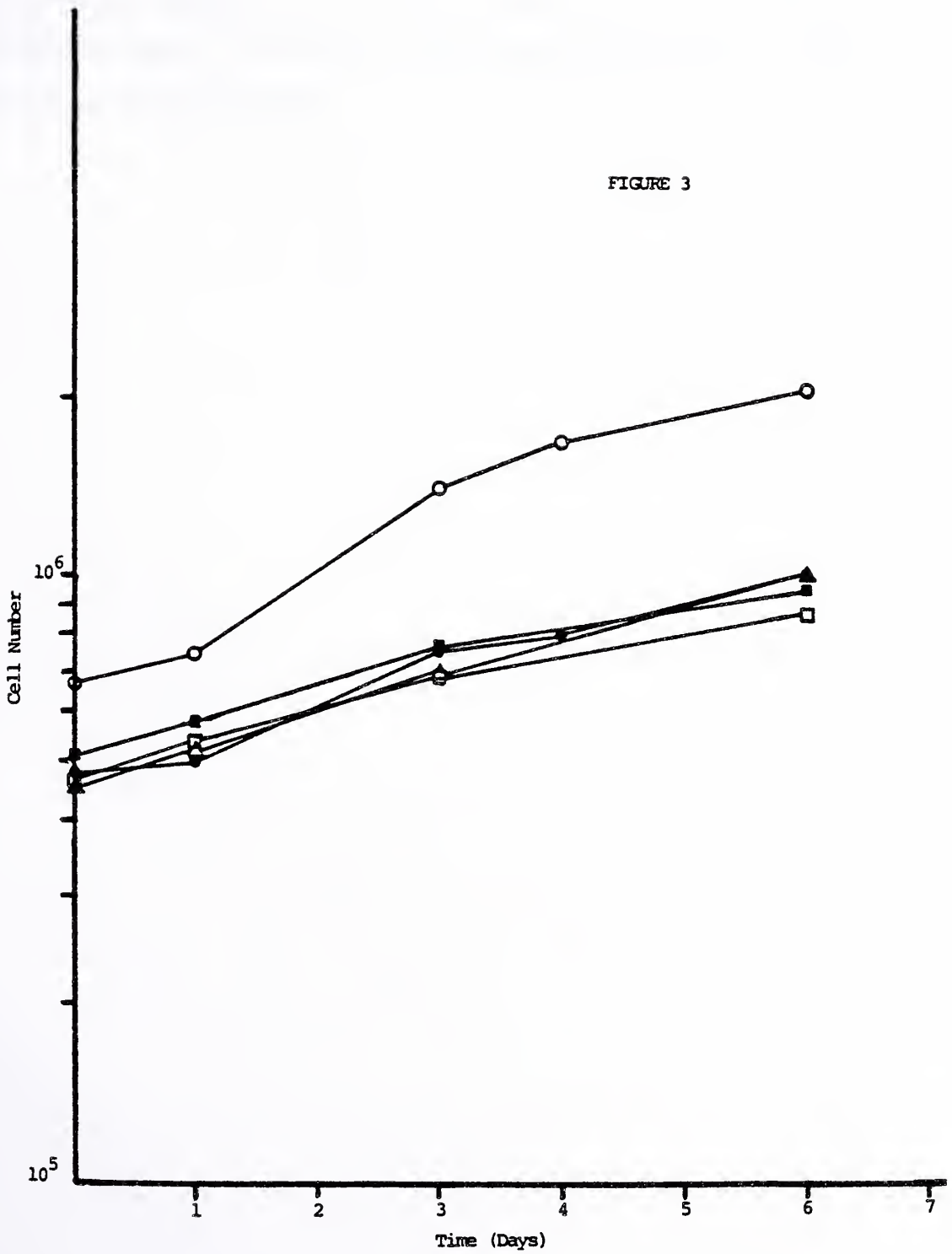


FIGURE 4

Effect of varying concentrations of $CuSO_4$ on cell growth. The ratio of cell number at day 3 to cell number at day 1 is used as a measure of growth. The effect of RPMI-1640 enriched with 5% fetal bovine serum is also shown.

FIGURE 4

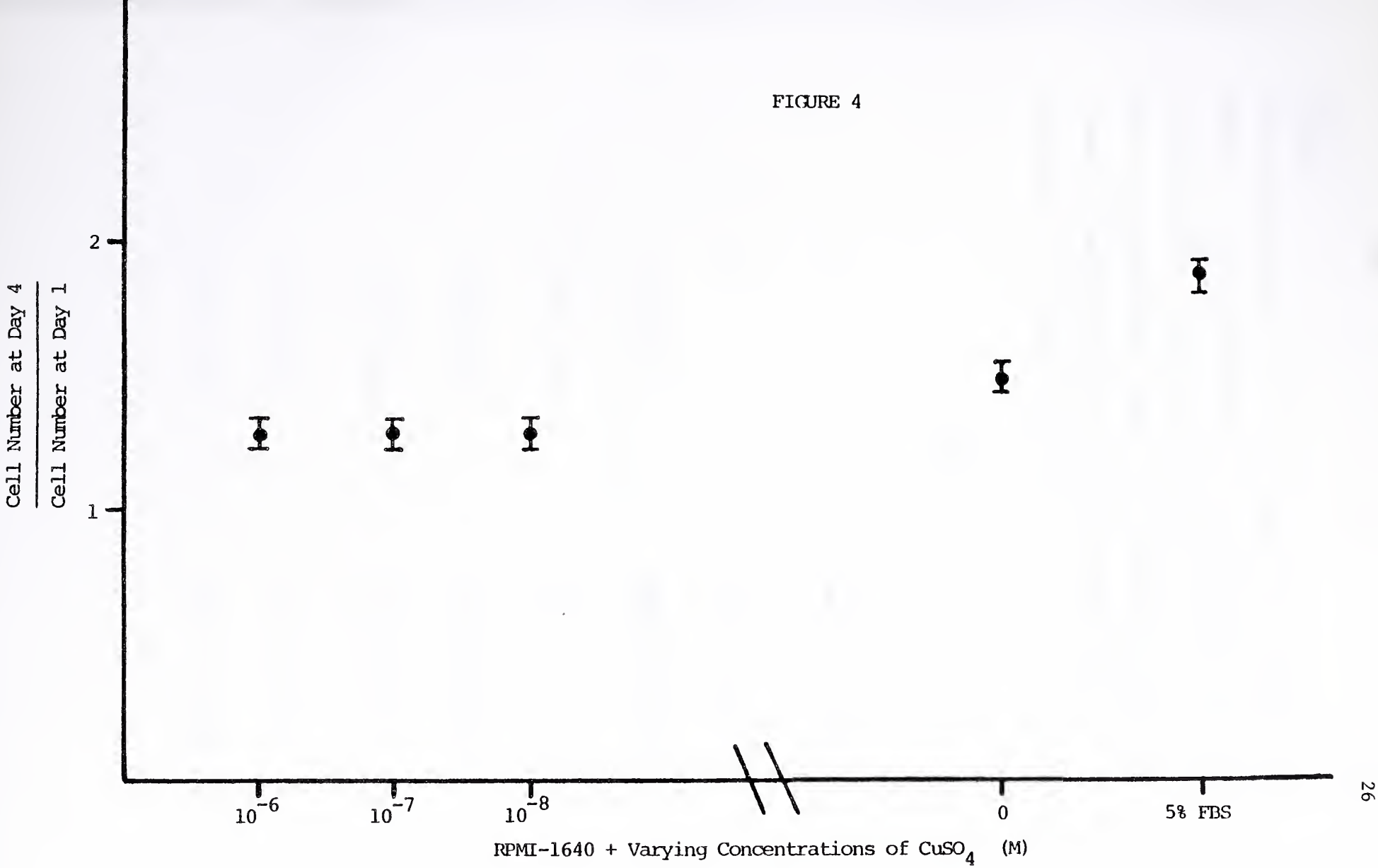

Cell Number at Day 4
Cell Number at Day 1
2
1
10^{-6}
10^{-7}
10^{-8}
0
5% FBS
RPMI-1640 + Varying Concentrations of $CuSO_4$ (M)

FIGURE 5

Seven day growth curve of Y-79 cells grown in the presence of RPMI-1640 + varying concentrations of $CrCl_3$ without the addition of apotransferrin. The points designated "0" and "5%" refer to those cells grown without added $CrCl_3$ or serum and those cells incubated in medium enriched with 5% fetal bovine serum respectively. Cells were fed on day 4.

KEY

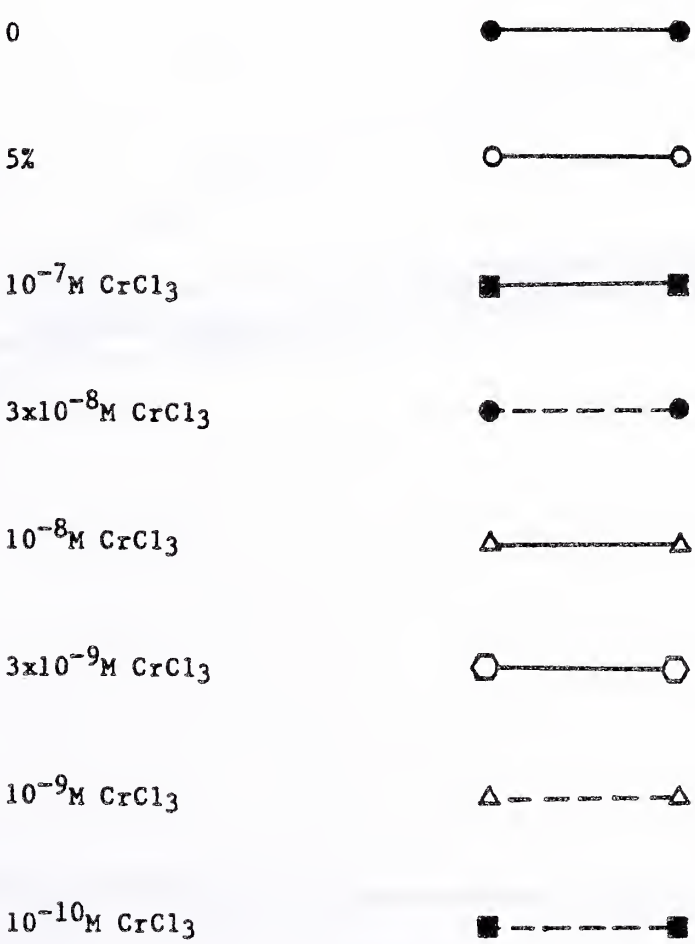


FIGURE 5

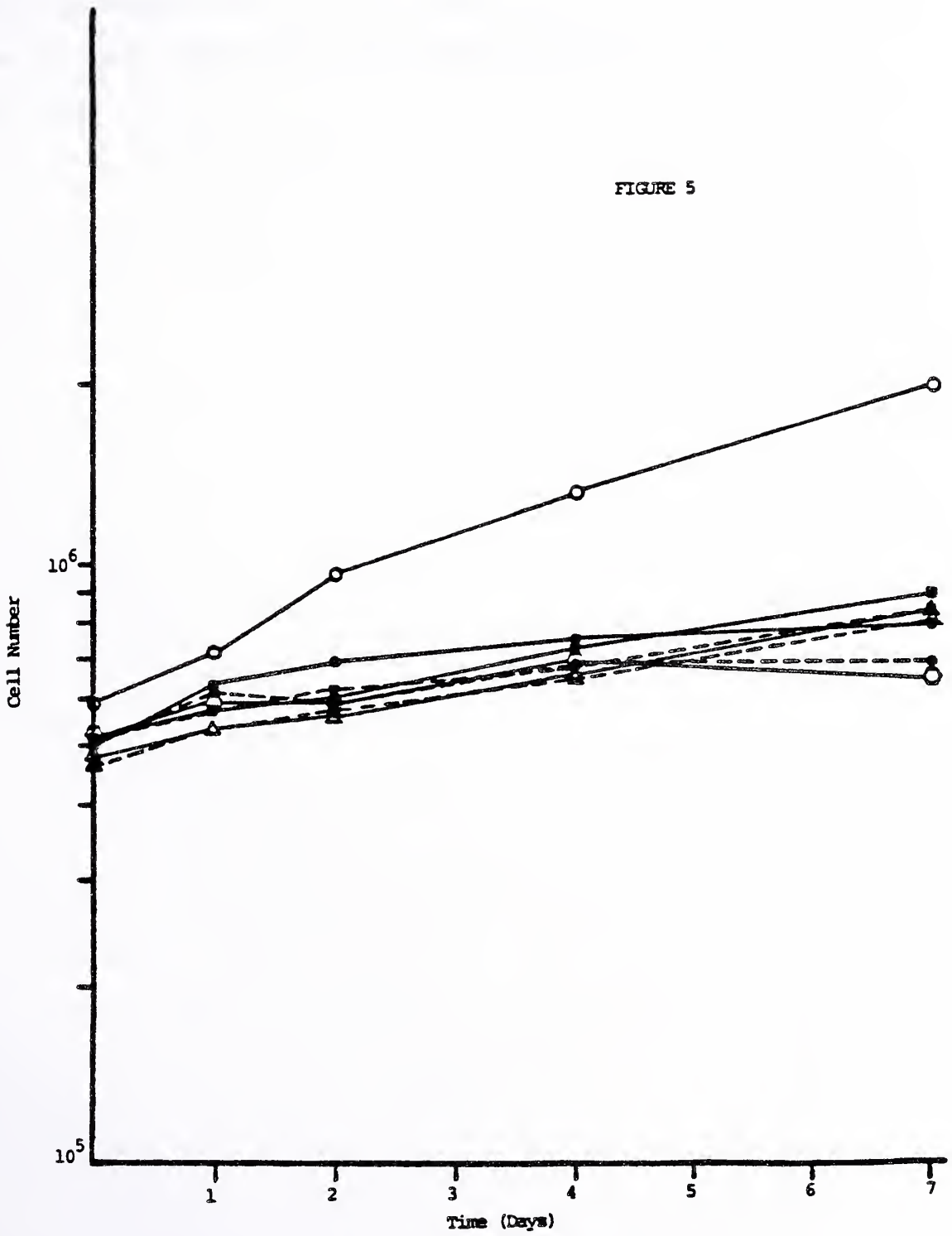


FIGURE 6

Effect of varying concentrations of $CrCl_3$ on cell growth. The ratio of cell number at day 4 to cell number at day 1 is used as a measure of growth. The effect of RPMI-1640 enriched with 5% FBS is also shown.

FIGURE 6

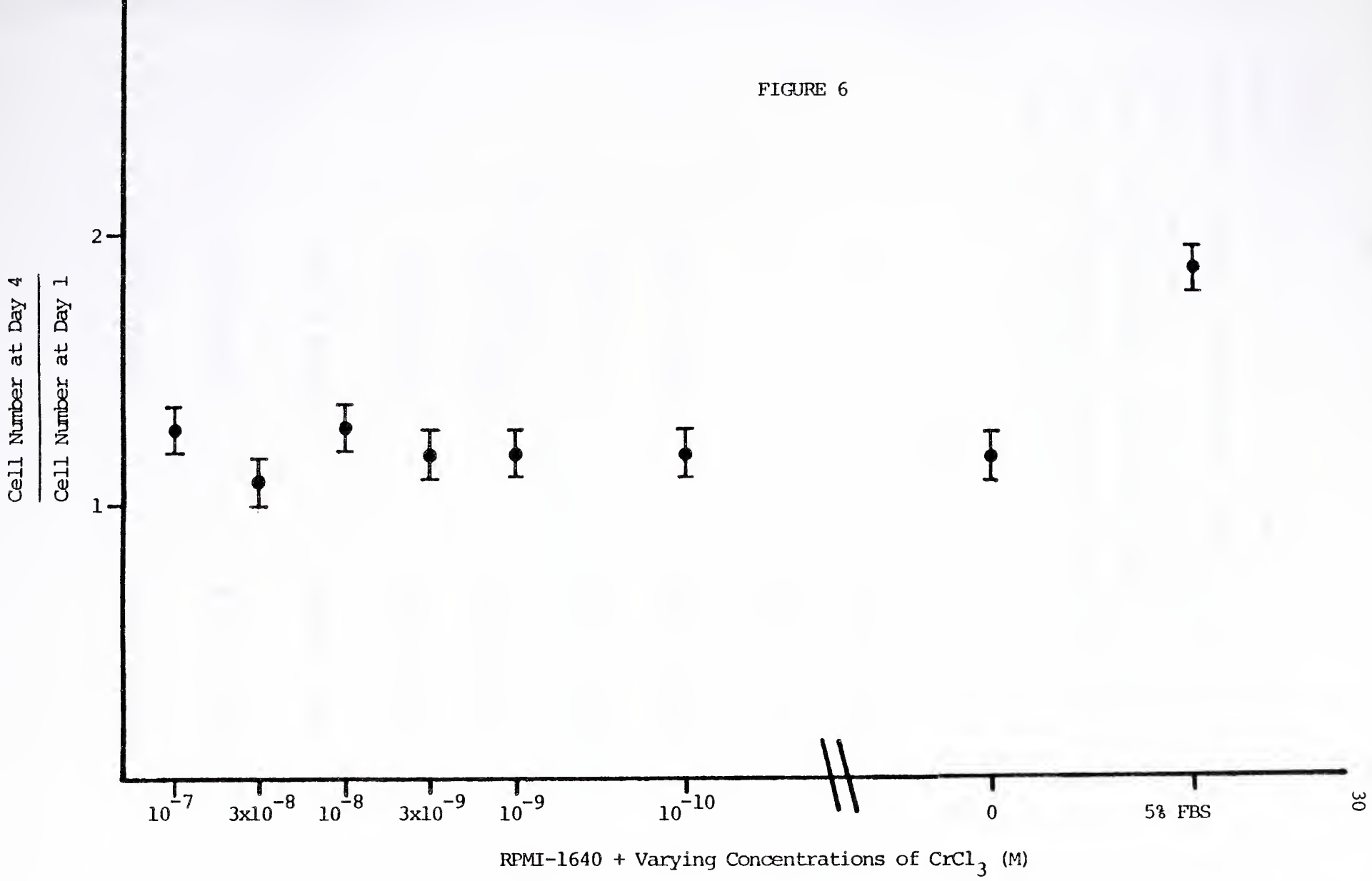

Cell Number at Day 4
Cell Number at Day 1
2
1
10^{-7}
$3x10^{-8}$
10^{-8}
$3x10^{-9}$
10^{-9}
10^{-10}
0
5% FBS
30
RPMI-1640 + Varying Concentrations of $CrCl_3$ (M)

FIGURE 7

Seven day growth curve of Y-79 cells grown in the presence of RPMI-1640 + varying concentrations of Na_2SeO_3 without the addition of apotransferrin. The points designated "0" and "5%" refer to those cells grown without added Na_2SeO_3 or serum and those cells incubated in medium enriched with 5% fetal bovine serum respectively. Cells were fed on day 4.

KEY

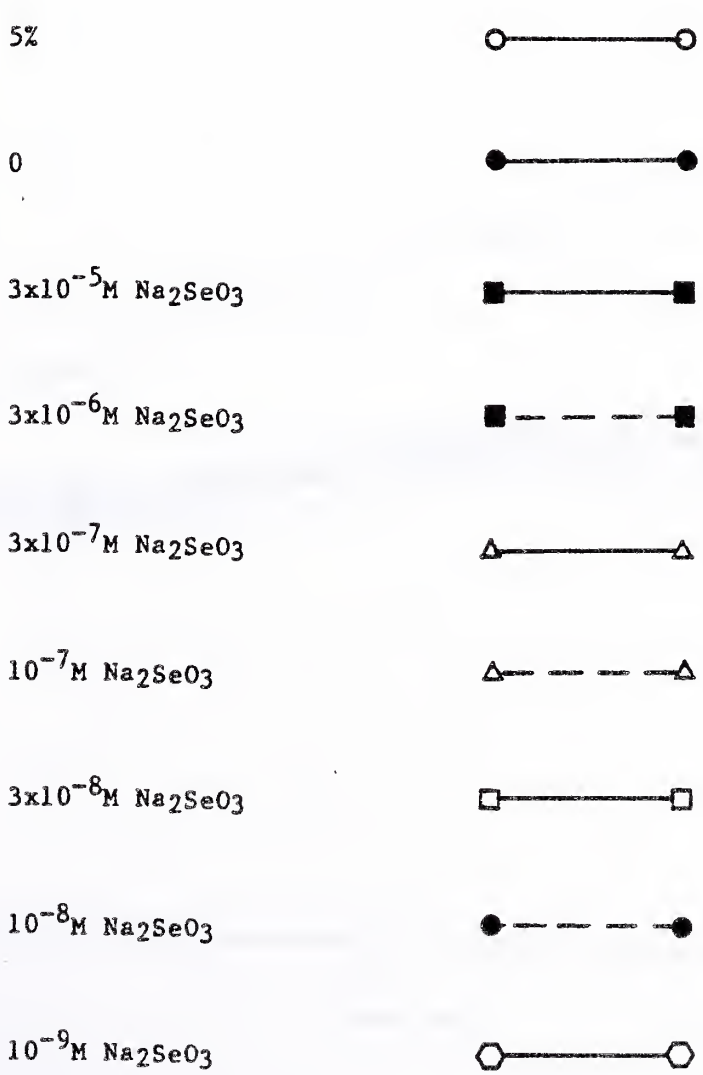


FIGURE 7

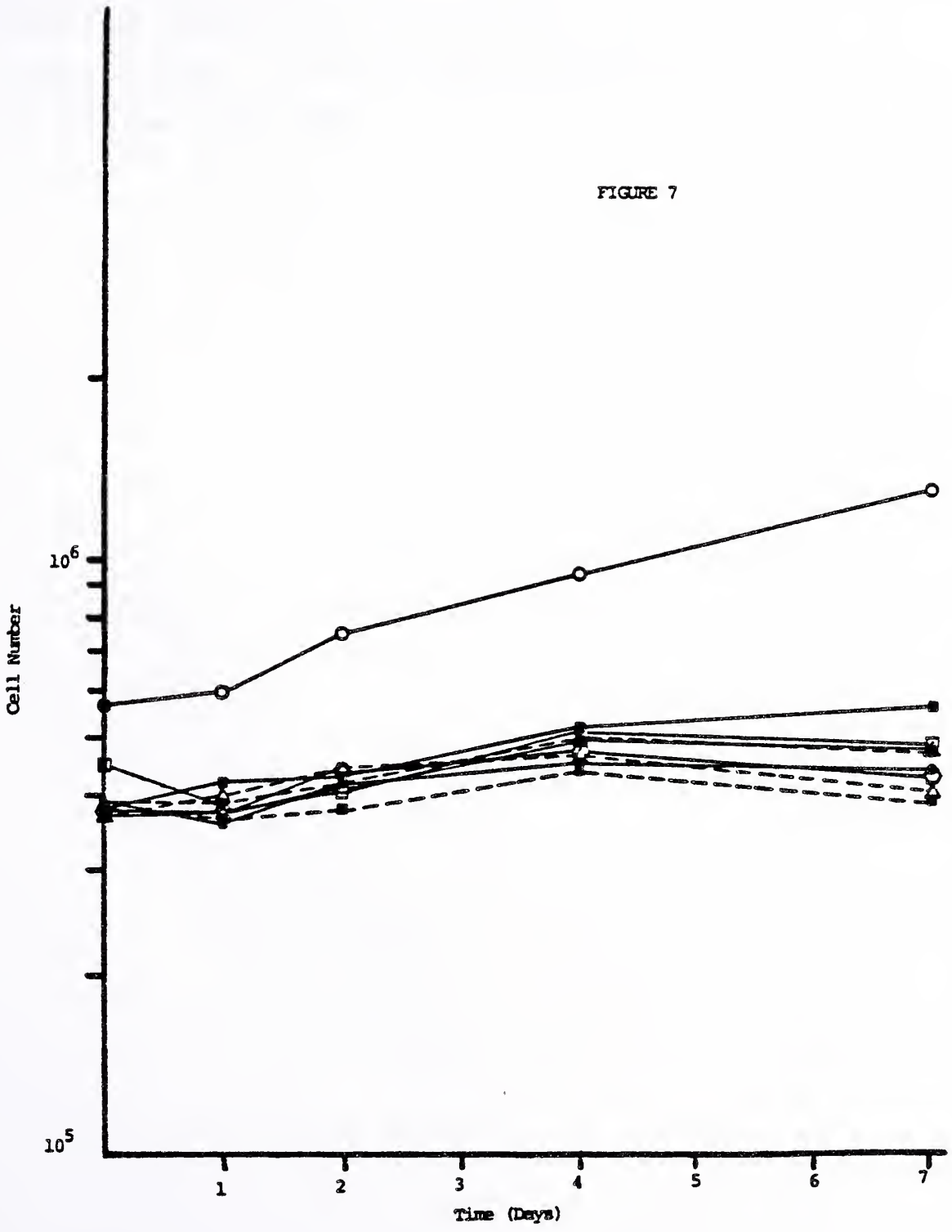


FIGURE 8

Effect of varying concentrations of Na_2SeO_3 on cell growth. The ratio of cell number at day 4 to cell number at day 1 is used as a measure of growth. The effect of RPMI-1640 enriched with 5% fetal bovine serum is also shown.

FIGURE 8

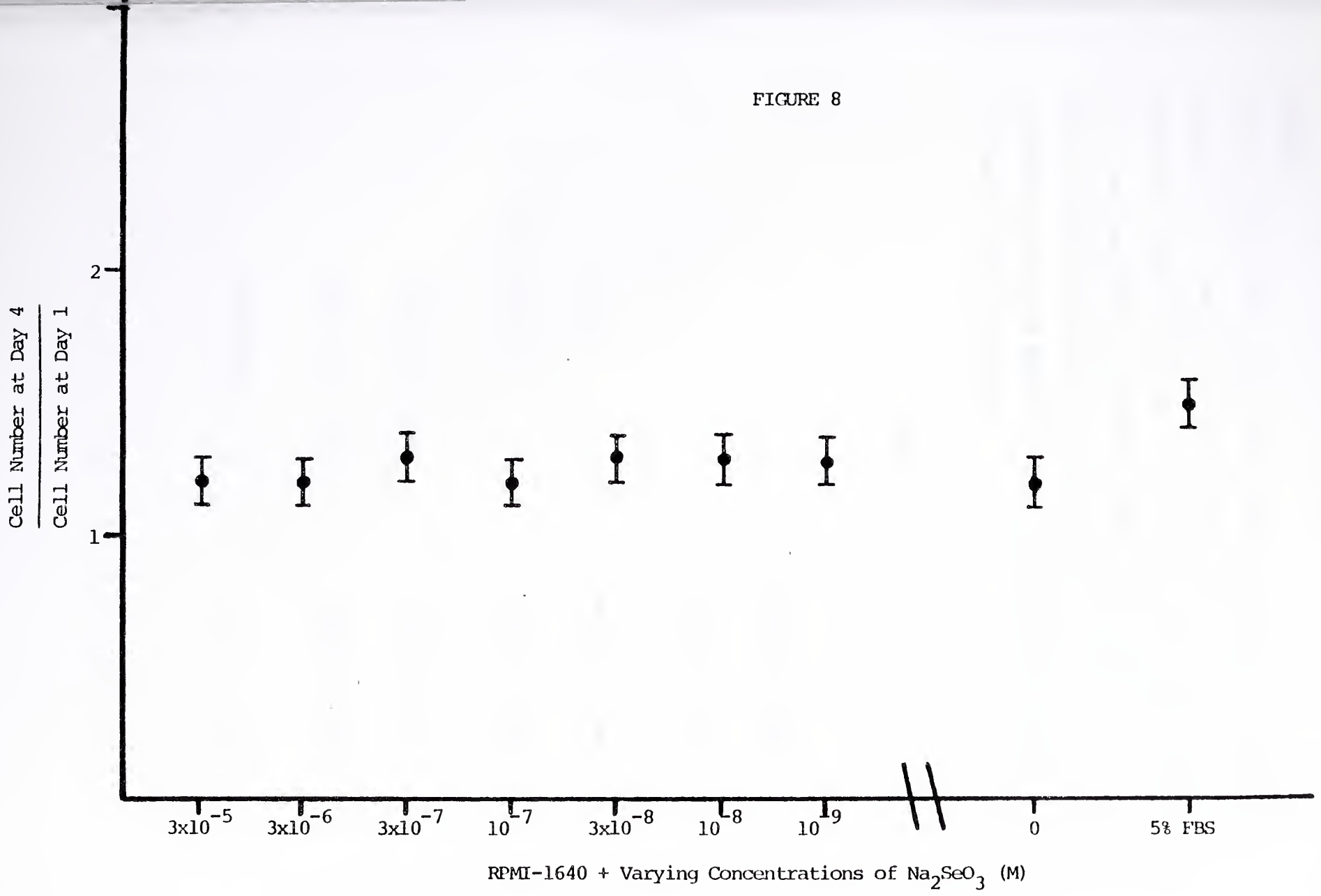

Cell Number at Day 4
Cell Number at Day 1
2
1
3x10-5
3x10-6
3x10-7
10-7
3x10-8
10-8
10-9
0
5% FBS
RPMI-1640 + Varying Concentrations of Na2SeO3 (M)

FIGURE 9

Four day growth curve of Y-79 cells grown in RPMI-1640 + 3 ug/ml of apotransferrin + varying concentrations of $CoCl_2$. The points designated "0" and "5%" refer to those cells grown without added $CoCl_2$, apotransferrin, or serum and those cells incubated in medium enriched with 5% fetal bovine serum respectively. "0 + trf" represents those cells grown in medium containing apotransferrin but devoid of $CoCl_2$ or serum.

KEY

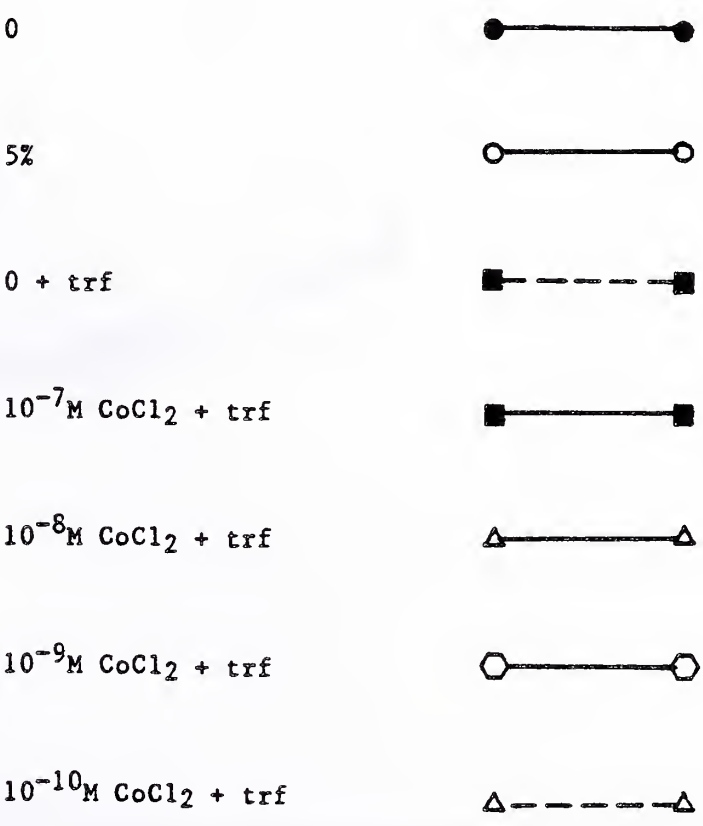


FIGURE 9

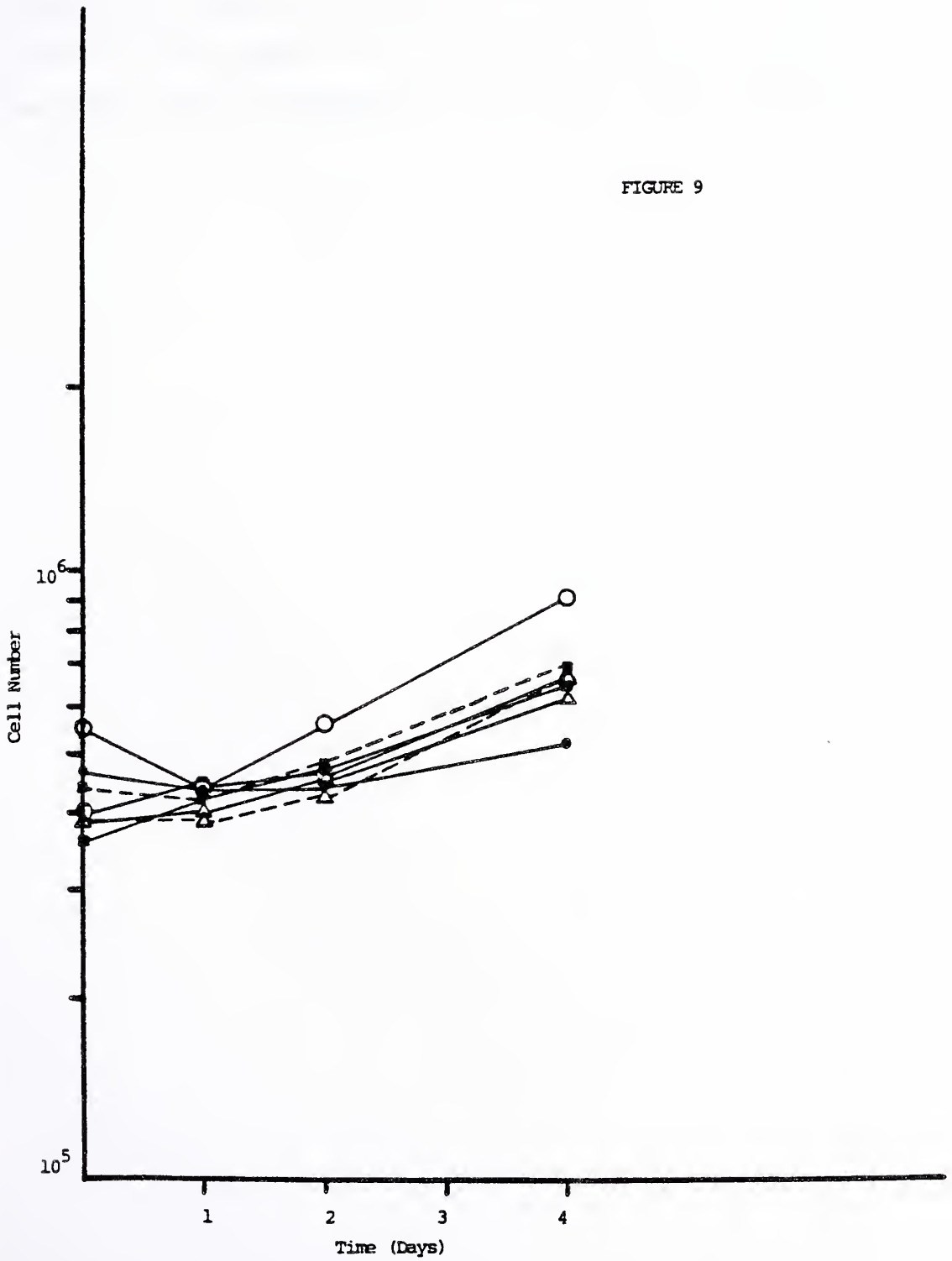


FIGURE 10

Effect of varying concentrations of $CoCl_2$ on cell growth in the presence of 3 ug/ml of apotransferrin. The ratio of cell number at day 4 to cell number at day 1 is used as a measure of growth. See figure 9 for an explanation of the symbols, "0", "5%" and "0 + trf".

FIGURE 10

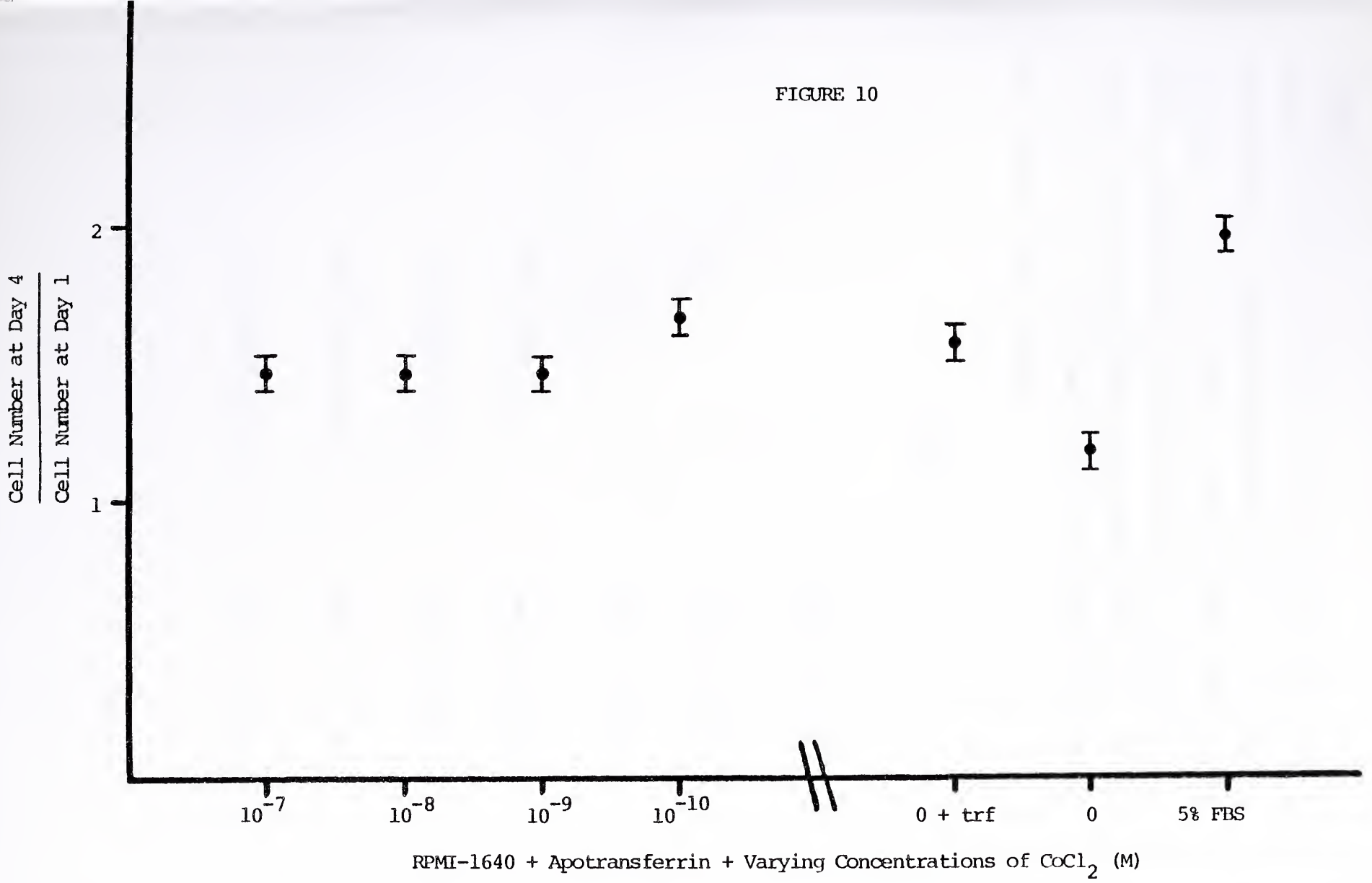

Cell Number at Day 4
Cell Number at Day 1
2
1
10^{-7}
10^{-8}
10^{-9}
10^{-10}
0 + trf
0
5% FBS
RPMI-1640 + Apotransferrin + Varying Concentrations of $CoCl_2$ (M)

FIGURE 11

Four day growth curve of Y-79 cells grown in RPMI-1640 + 3 ug/ml of apotransferrin + varying concentrations of $CuSO_4$. The points designated "0" and "5%" refer to those cells grown without added $CoCl_2$, apotransferrin, or serum and those cells incubated in medium enriched with 5% fetal bovine serum respectively. "0 + trf" represents those cells grown in medium containing apotransferrin but devoid of $CuSO_4$ or serum.

KEY

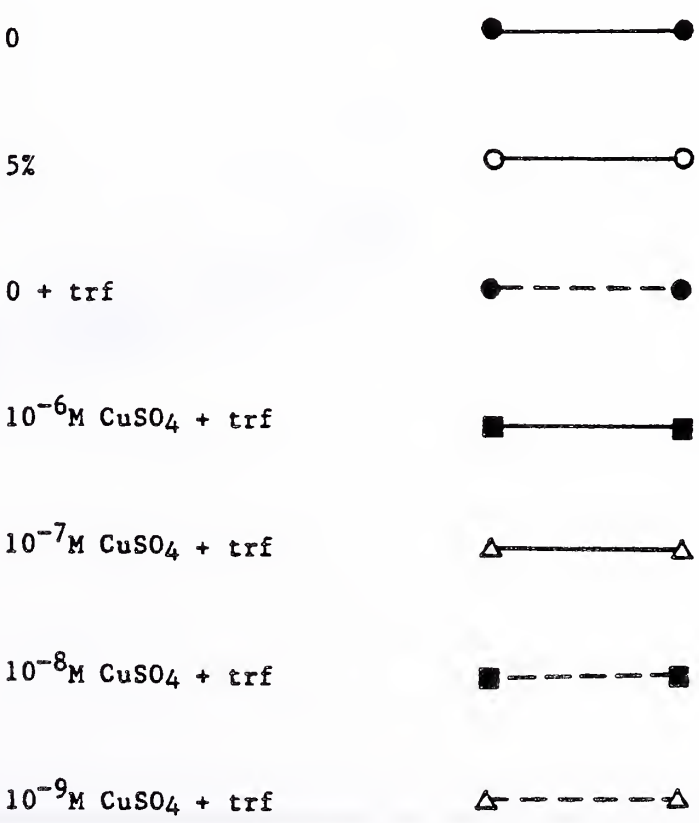


FIGURE 11

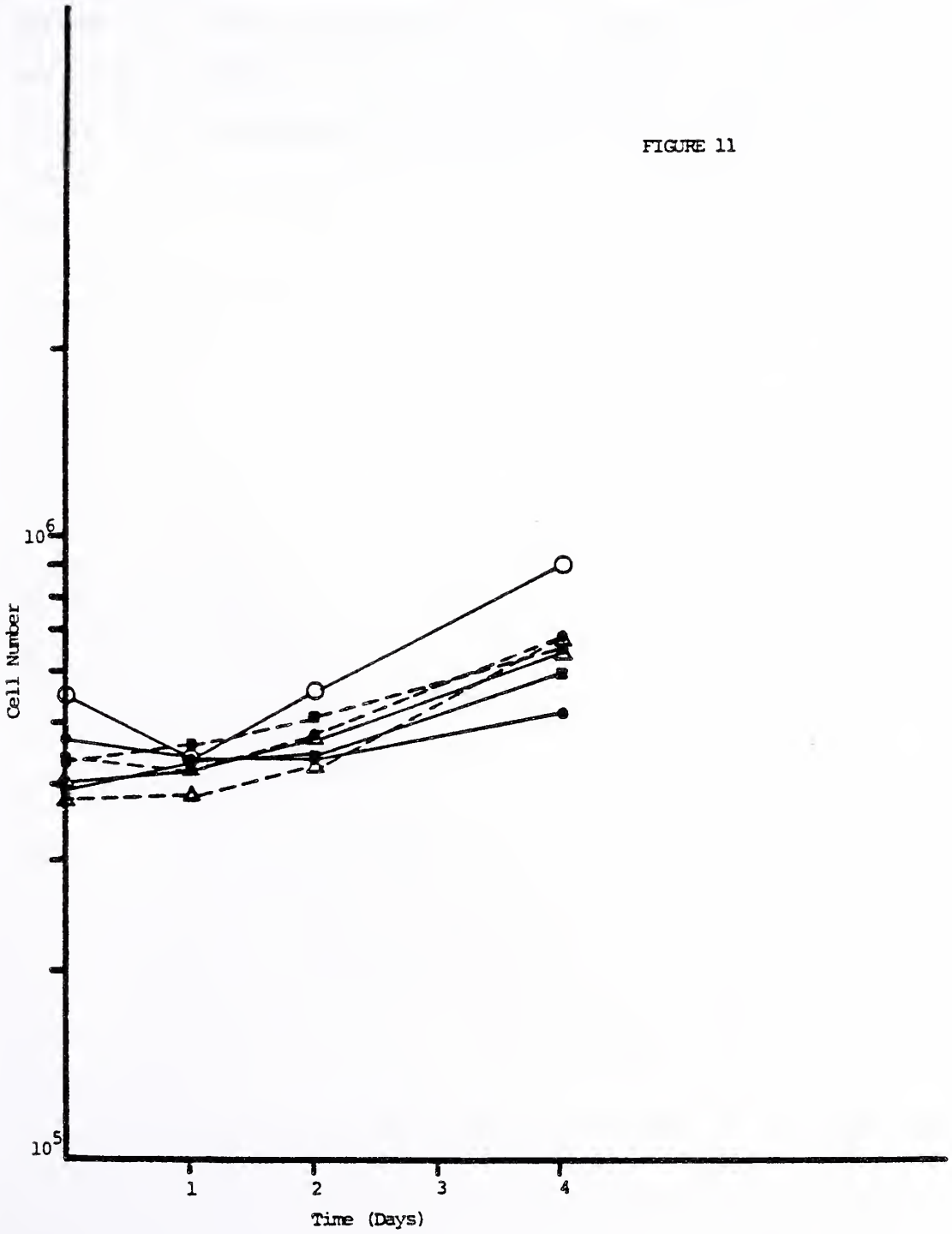


FIGURE 12

Effect of varying concentrations of $CuSO_4$ on cell growth in the presence of 3 ug/ml of apotransferrin. The ratio of cell number at day 4 to cell number at day 1 is used as a measure of growth. See figure 11 for an explanation of the symbols, "0", "5%", and "0 + trf".

FIGURE 12

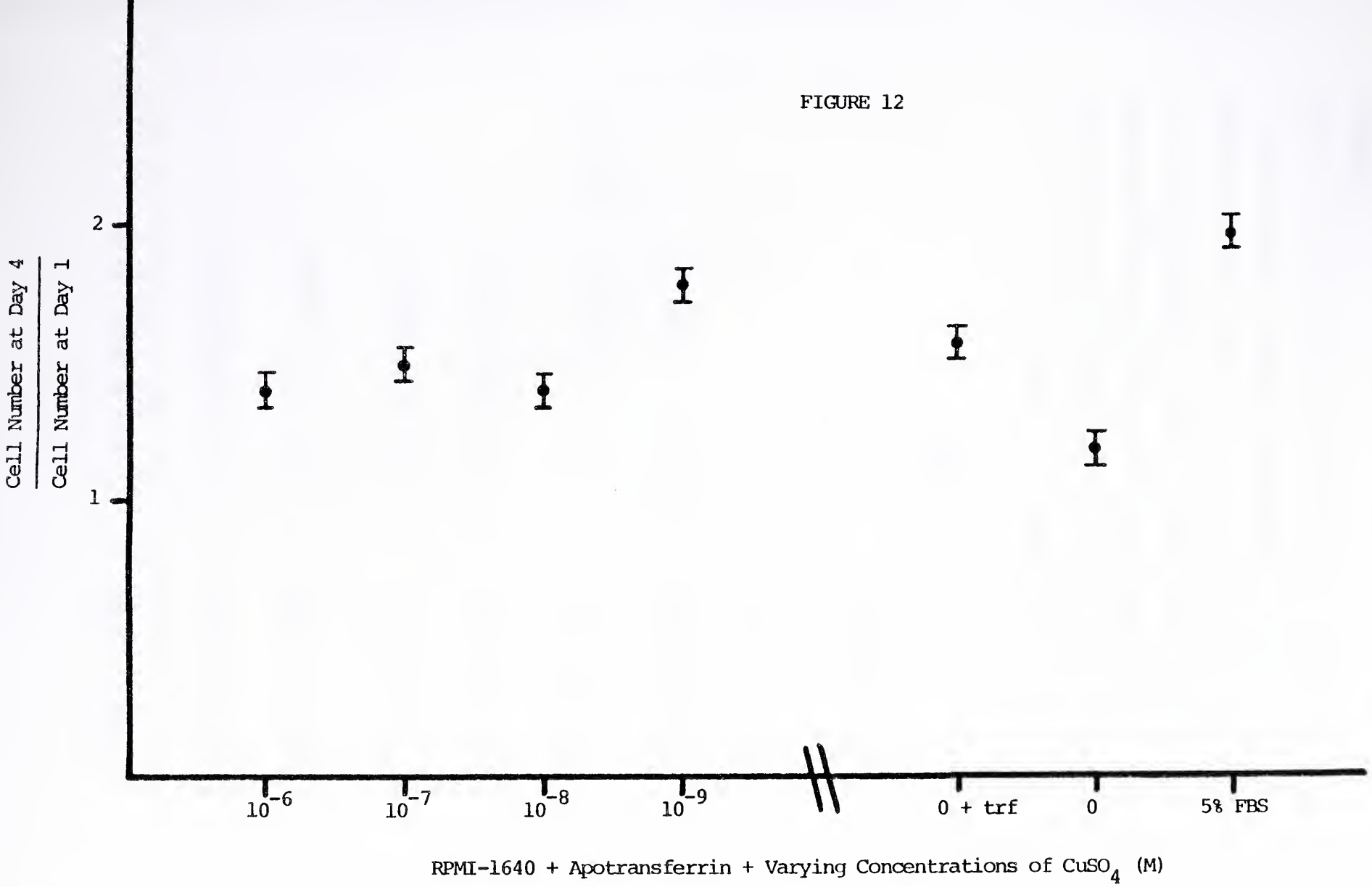

Cell Number at Day 4
Cell Number at Day 1
2
1
10^{-6}
10^{-7}
10^{-8}
10^{-9}
0 + trf
0
5% FBS
RPMI-1640 + Apotransferrin + Varying Concentrations of $CuSO_4$ (M)

FIGURE 13

Seven day growth curve of Y-79 cells grown in RPMI-1640 + 3 ug/ml of apotransferrin + varying concentrations of $CrCl_3$. The points designated "0" and "5%" refer to those cells grown without added $CrCl_3$, apotransferrin, or serum and those cells incubated in medium enriched with 5% fetal bovine serum respectively. "0 + trf" represents those cells grown in medium containing apotransferrin but devoid of $CrCl_3$ or serum. Cells were fed on day 4.

KEY

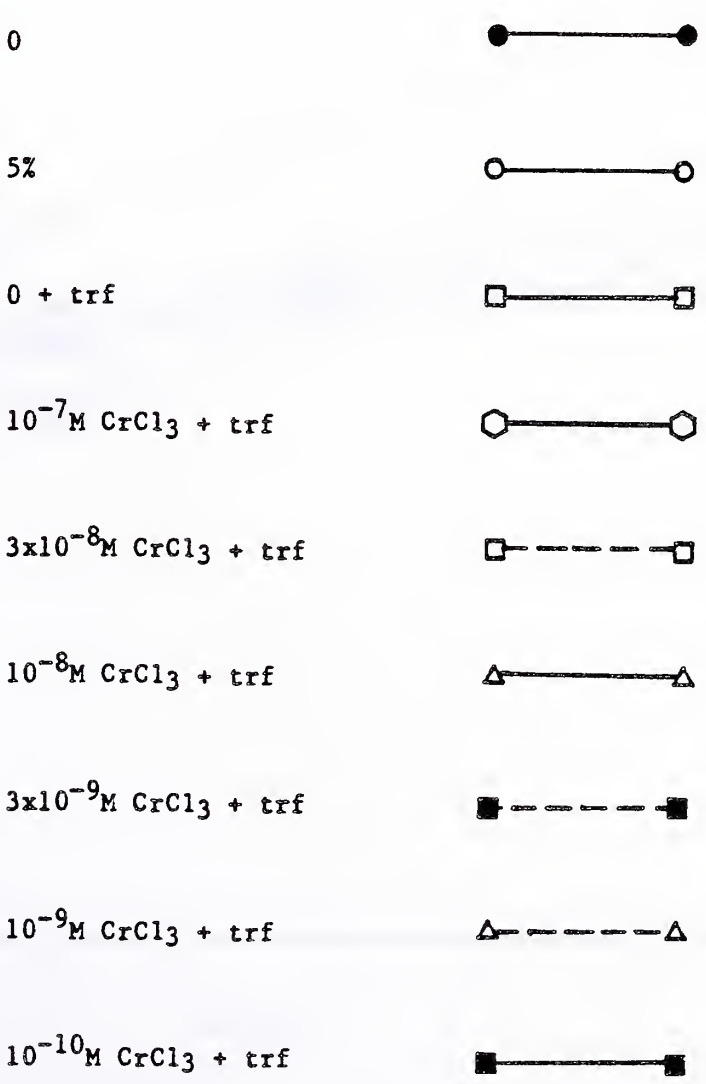


FIGURE 13

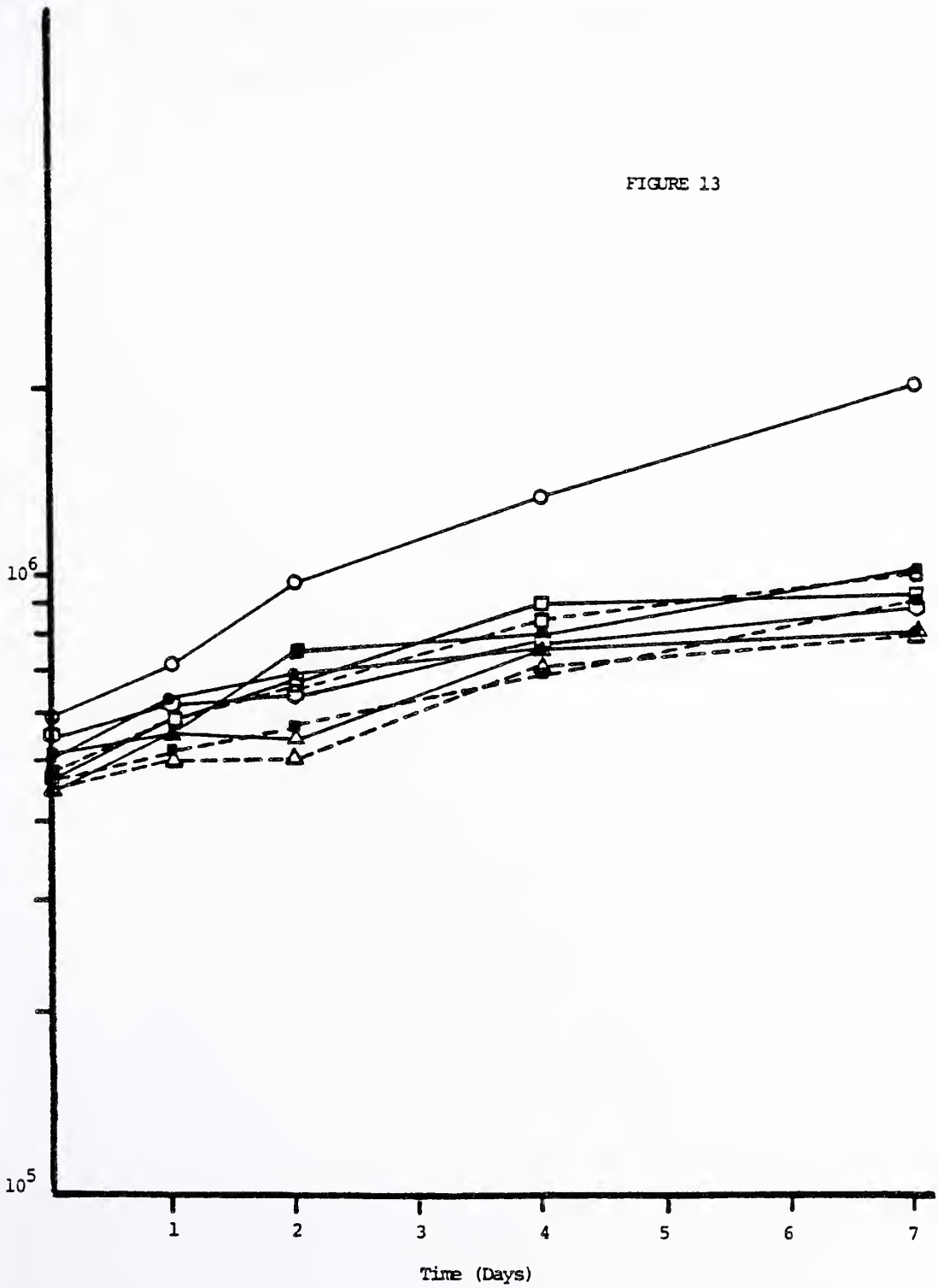


FIGURE 14

Effect of varying concentrations of $CrCl_3$ on cell growth in the presence of 3 ug/ml of apotransferrin. The ratio of cell number at day 4 to cell number at day 1 is used as a measure of growth. See figure 13 for an explanation of the symbols, "0", "5%", and "0 + trf".

FIGURE 14

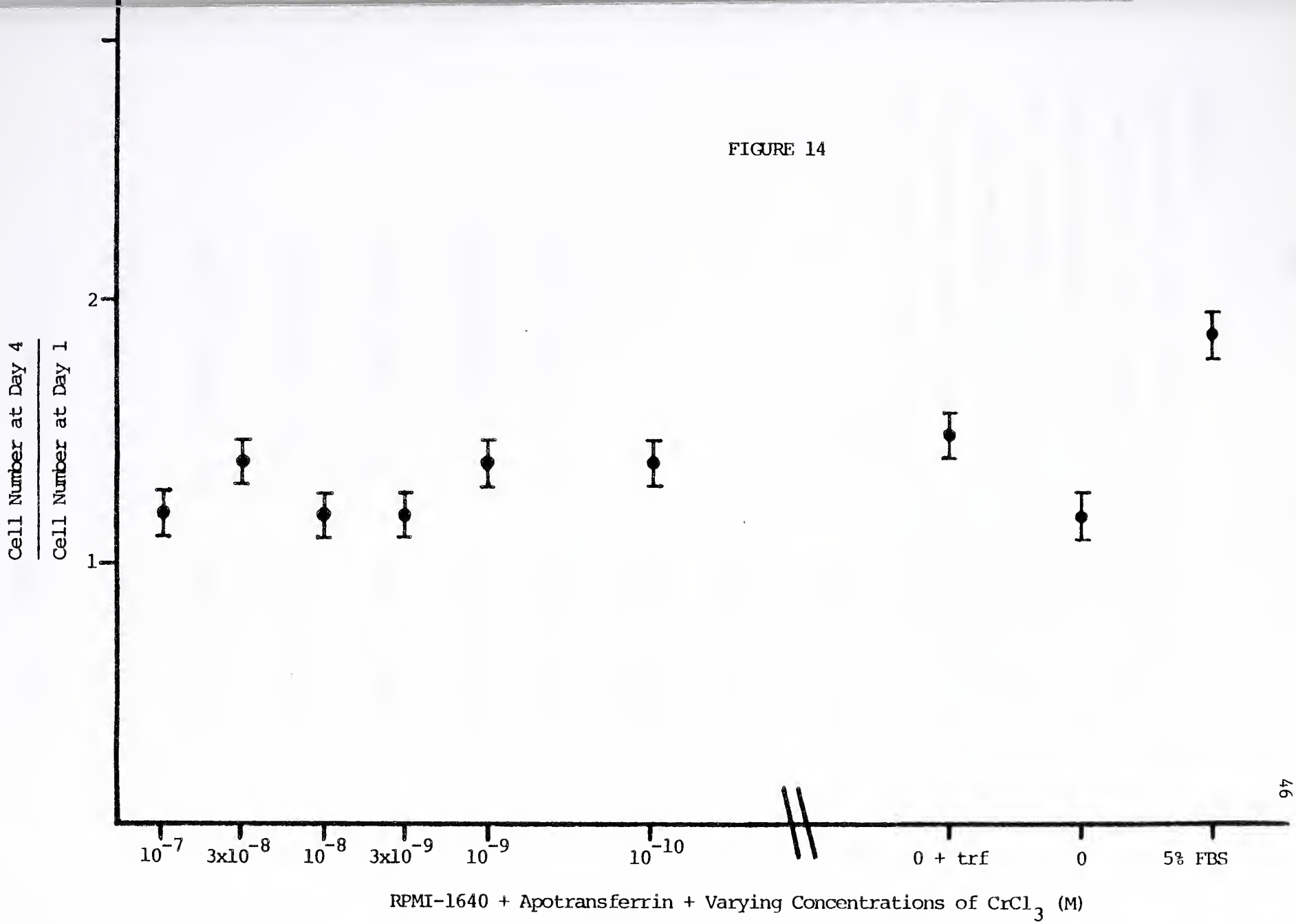

Cell Number at Day 4
Cell Number at Day 1
2
1
10^-7
3x10^-8
10^-8
3x10^-9
10^-9
10^-10
0 + trf
0
5% FBS
RPMI-1640 + Apotransferrin + Varying Concentrations of CrCl3 (M)

FIGURE 15

Seven day growth curve of Y-79 cells grown in RPMI-1640 + 3 ug/ml of apotransferrin (trf) + varying concentrations of Na_2SeO_3. The points designated "0" and "5%" refer to those cells grown without added Na_2SeO_3, trf, or serum and those cells incubated in medium enriched with 5% fetal bovine serum respectively. "0 + trf" represents those cells grown in medium containing trf but devoid of Na_2SeO_3 or serum. Cells were fed on day 4.

KEY

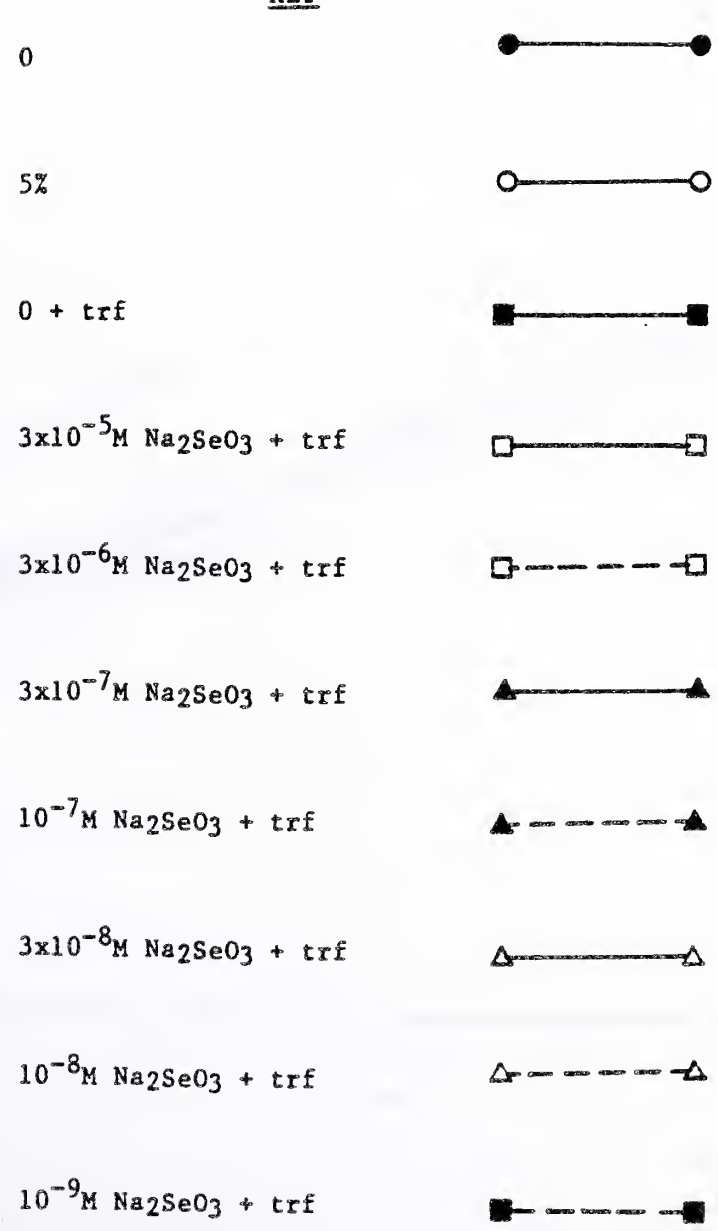


FIGURE 15

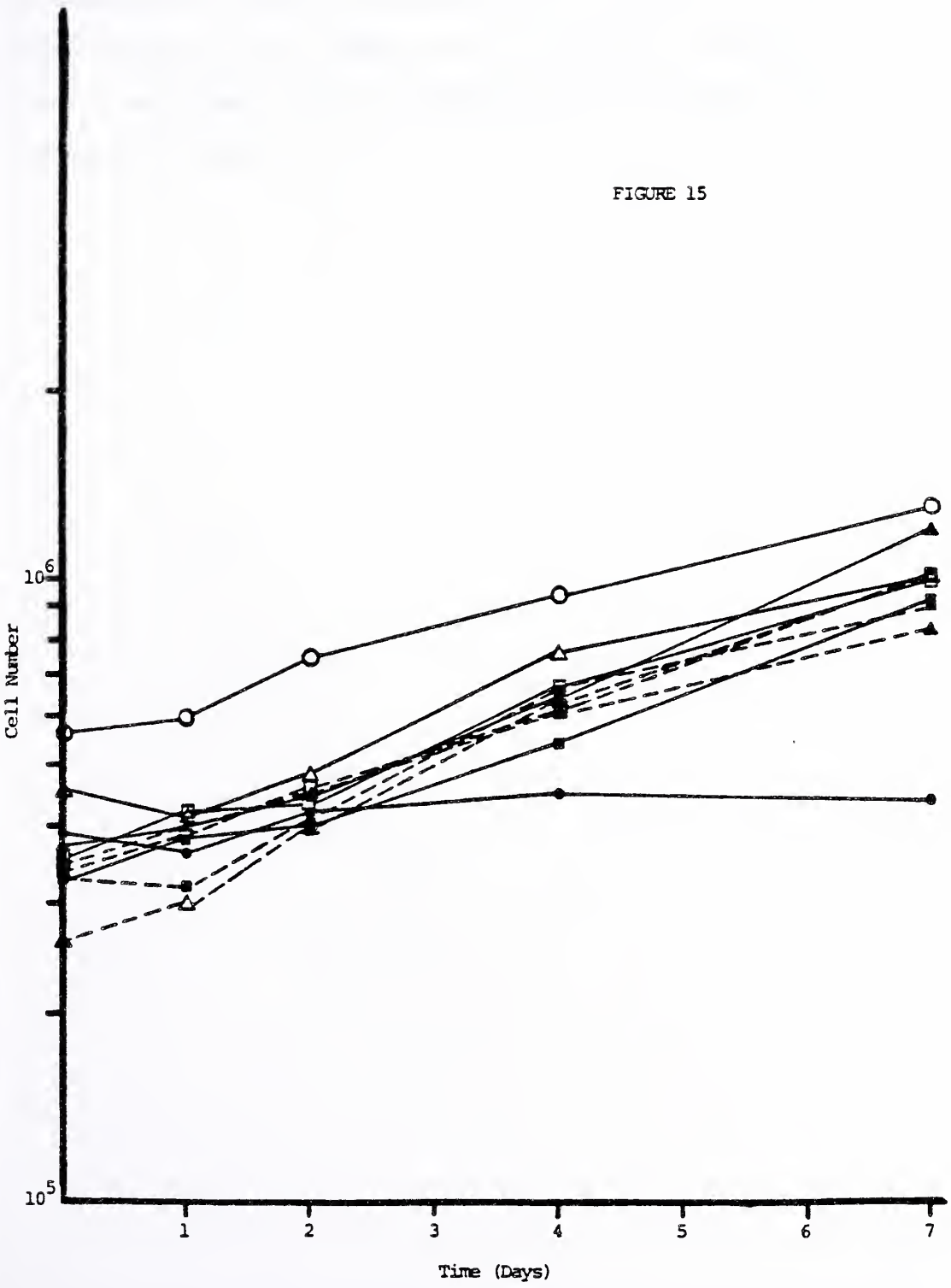
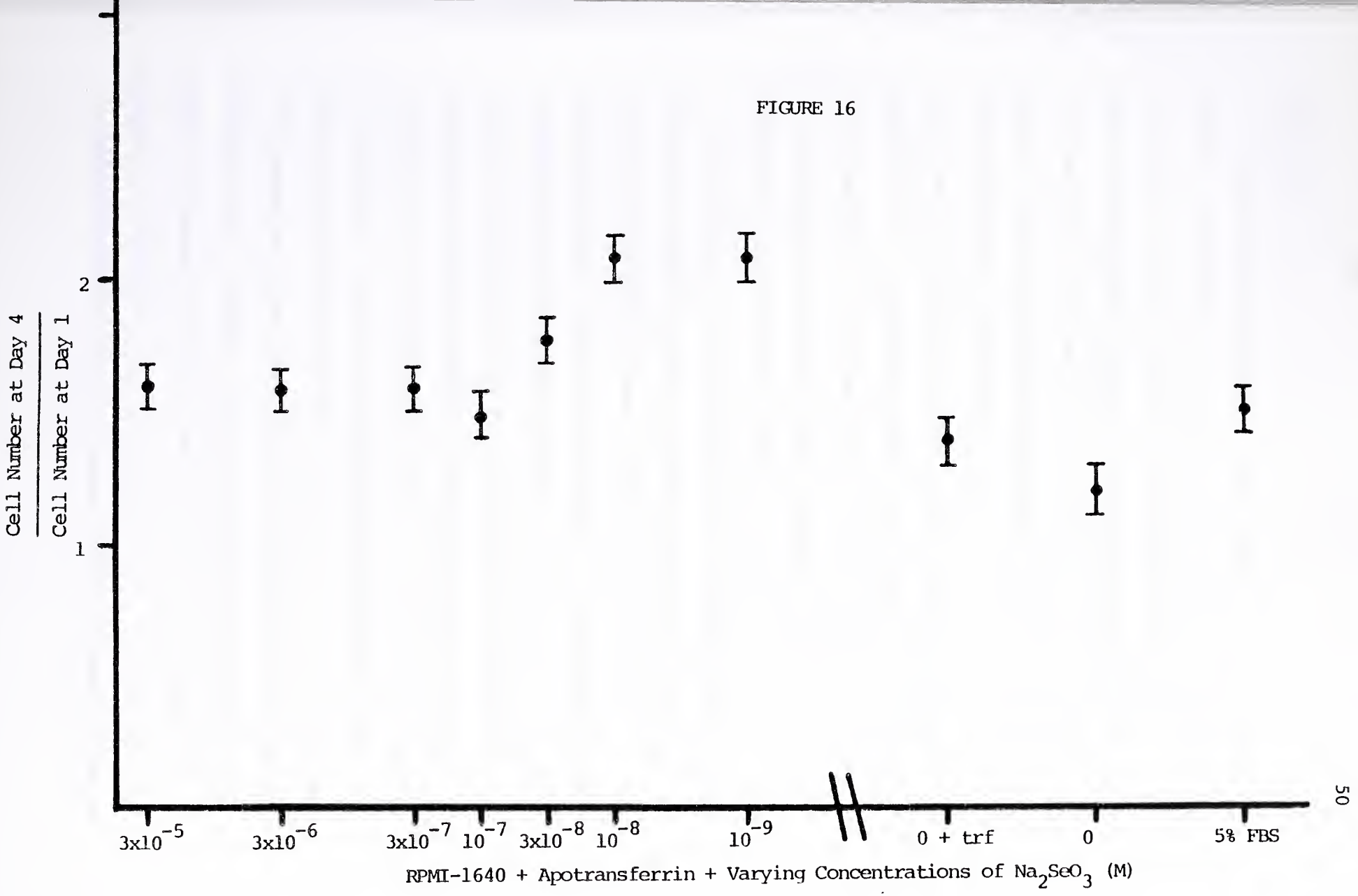


FIGURE 16

Effect of varying concentrations of Na_2SeO_3 on cell growth in the presence of 3 ug/ml of apotransferrin. The ratio of cell number at day 4 to cell number at day 1 is used as a measure of growth. See figure 15 for an explanation of the symbols, "0", "5%", and "0 + trf".

FIGURE 16


Cell Number at Day 4
Cell Number at Day 1
2
1
3x10^-5
3x10^-6
3x10^-7
10^-7
3x10^-8
10^-8
10^-9
0 + trf
0
5% FBS
RPMI-1640 + Apotransferrin + Varying Concentrations of Na2SeO3 (M)

Discussion

These studies indicate that none of the four trace metals, tested in the absence of trasnferrin, were active in enhancing Y-79 cell growth. In fact, the cobalt and copper ions, at the concentrations tested, appeared to be somewhat inhibitory for the first four days of the experiment. Perhaps, a trial of lower amounts of these ions might be justified in light of Ham's use of 10^{-8}M $CuSO_4 \cdot 5H_2O$ and 10^{-9}M $CoCl_2 \cdot 6H_2O$ as optimum concentrations in his protein-free, synthetic medium, MCDB-301 (22). The ineffectiveness of the addition of cobalt may also be secondary to the presence of a minimal amount of vitamin B_{12} (0.005 mg/L) in RPMI-1640. Also, perhaps, the Y-79 cell line is unable to utilize inorganic cobalt or copper.

The inability of chromium chloride to stimulate growth was not unexpected in light of present knowledge regarding the role of chromium in biological systems. Chromium has been studied extensively because it has long been known to be necessary for the maintenance of normal glucose tolerance in animals (31). However, chromium is biologically active in the form of an incompletely characterized group of complexes collectively known as "glucose tolerance factor". This factor is thought to operate by complexing with insulin and its tissue receptor and thereby facilitating insulin-receptor binding (40,38). Therefore, it is not surprising that chromium has never been found to be effective in the absence of insulin (39). Perhaps, a trial of chromium in the presence of insulin may unmask a growth potentiating effect of this metal in culture of Y-79 cells.

Another difficulty remains, however. While inorganic chromium

is an effective dietary supplement for animals, the active form, the so-called glucose tolerance factor, has never been completely isolated. It is thought to contain trivalent chromium complexed with nicotinic acid, glutamic acid, glycine, cysteine and traces of other amino acids (64). However, synthetic complexes of these molecules have been found to be less effective in lowering plasma concentrations of glucose and triglycerides than the glucose tolerance factor extracted from brewer's yeast (67). Therefore, adding inorganic chromium or even synthetic chromium complexes, as above, to Y-79 cell medium, even in the presence of adequate amounts of insulin, may not lead to enhanced growth (above that caused by insulin alone). Observation of the effect may have to await purification and characterization of the glucose tolerance factor.

The failure of selenium to enhance growth is also not surprising because Y-79 cell stock cultures are maintained in medium containing $3x10^{-8}$M Na_2SeO_3. It probably takes far longer than the length of our experiments (7 days) for cells to become selenium depleted. Indeed, in the one available case report of a patient on total parenteral nutrition who developed selenium deficiency, the patient did not become symptomatic until about two years after beginning his course of hyperalimentation (31). Also, the in vitro studies of Rotruck et al. demonstrated that rat erythrocytes only become abnormally susceptible to hemolysis 36-39 days after the rats begin receiving selenium deficient diets (52). Several years ago, our lab did some preliminary experiments which indicated that selenium enhanced the growth of Y-79 cells, and since that time, Na_2SeO_3 has been added to the stock medium in

an optimum concentration of $3x10^{-8}M$. Perhaps, the lack of effect seen now is simply due to the longer time course necessary to deplete the stock cells of already present selenoenzymes, such as glutathione peroxidase (GSH-Px). Another trial of selenium with cells that had been grown in the absence of this metal for a more prolonged period would be useful to both confirm selenium's ability to enhance Y-79 cell growth as well as to elucidate the time course necessary to deplete the cells of selenium.

The mechanism of the growth enhancing effect of transferrin remains unclear. Young et al. have noted a growth stimulating effect of transferrin on SV3T3 cells at an optimum concentration of $10^{-8}M$ but this effect is dependent on the presence of 100 ng/ml of biotin (71). RPMI-1640 contains 200 ng/ml of biotin and perhaps, the effect of transferrin on Y-79 cells is also biotin dependent. Young et al. postulate that this effect may be secondary to unrelated metabolic events as their lab has also noted that biotin lowers the level of lactate secreted by SV3T3 cells (71). It would be interesting to see if the growth enhancing effect of transferrin on Y-79 cells is retained when biotin is deleted from the medium.

We must also consider the possibility that Y-79 cells are producing a growth factor(s) which interacts with transferrin to provide a stimulus for cells to proliferate. Similar factors have been observed by Delarco and Todaro in transformed mouse cell lines and they term these agents "sarcoma growth factors" (8). This hypothesis is attractive in light of the growth permissive effect of medium, conditioned by Y-79 cells, on untransformed retinal cells.

The presence of transferrin receptors on the surfaces of human lymphoblastoid and other malignant cell lines has been noted by Galbraith et al. (13). They hypothesize that transferrin receptors are used by these cells to bind host protein and thereby hide cell-specific antigens from the host immune system. This immunosurveillance theory is probably not an important consideration under tissue culture conditions. However, tumor receptors for transferrin could still be important intermediaries for transferrin's growth effect in vitro. Therefore, a demonstration of the presence of apotransferrin receptors on the surface of Y-79 cells would certainly be of interest.

It is unlikely that the growth effect of transferrin can be explained simply in terms of supplying the iron requirement of Y-79 cells since apotransferrin was used in all of our experiments. However, it is possible that traces of iron salts contaminated our transferrin preparation and provided Y-79 cells with a critical element. We are planning to assay our apotransferrin for the presence of iron to help clarify this possibility. It would also be interesting to see if growth effects could be duplicated with other organic iron compounds such as hemoglobin or transferrin from other species, or even, inorganic iron salts.

Perhaps, transferrin plays a role in transporting other crucial metallic cations besides iron. The consistently greater growth achieved by cells incubated in serum enriched medium compared to cells grown in transferrin enriched medium might be explained by the presence of these trace metals in serum. Perhaps, the growth enhancing effect of transferrin could be explained, and indeed, increased to the levels achieved by cells grown in the

presence of serum by adding certain trace metals to the medium. Our studies were aimed at examining several likely candidates, including copper, cobalt, chromium and selenium.

As shown in the results, copper, cobalt and chromium had no significant effects on Y-79 cell growth. These observations were somewhat surprising since these three essential metals are all known to bind to transferrin (23) and therefore, one might conjecture that transferrin could increase their availability to cells. The unlikely possibility also exists that these metals, required in trace amounts, are already present as contaminants in saturating amounts, and therefore, no effect is seen with the addition of even larger amounts.

The most intriguing observation of this study is the effect of selenium and transferrin on the growth of Y-79 cells. Although transferrin alone had a small growth enhancing effect on Y-9 cells, the addition of selenium and transferrin resulted in far greater growth than that achieved by cells grown in transferrin alone. The explanation for this effect is unclear. However, a related observation comes from the work of Rodvien et al. (51). They noted that rabbit erythrocyte GSH-Px activity was markedly decreased by the induction of iron deficiency anemia whereas anemia secondary to massive phlebotomy or administration of intraperitoneal phenylhydrazine did not lead to changes in enzyme activity. Also, during the induction of iron deficiency, reticulocytes had lower enzyme activity than the older erythrocytes while during the iron repletion phase, the opposite was observed. Other enzymes, such as glutathione reductase, maintained constant activities throughout the experiments. They conjecture that iron

may be necessary for the synthesis of GSH-Px or perhaps, that this enzyme requires an iron containing protein as a coenzyme or electron carrier. Therefore, in our studies, transferrin may be acting in an iron transport capacity, providing Y-79 cells with enough iron to incorporate the available selenium into GSH-Px and thus protect the cell membranes from oxidation. However, as the extent of selenium binding to transferrin is unknown, we cannot be certain that transferrin does not play a more direct role. Perhaps, it enables the cell to transport selenium more efficiently, thereby making it more available for GSH-Px synthesis, itself stimulated by the increased concentration of selenium (16).

While selenium alone enhances growth when added to Y-79 cells which have been incubated in selenium deficient medium for a prolonged period (past unpublished experiments), our current experiments do not reveal a growth enhancing effect of selenium when it is added alone to cells previously maintained in selenium enriched medium. Therefore, as discussed earlier, it is probable that certain selenoproteins are not seriously depleted within one week of incubation in selenium free medium. However, the enhancement of growth seen when cells are incubated in the presence of selenium and apotransferrin occurs within several days of the beginning of the experiment (Figures 15 and 16). Therefore, it remains unclear that this effect of selenium is, in fact, dependent on its incorporation into selenoproteins. Alternatively, selenium could be incorporated into different selenoproteins in the presence of apotransferrin. Perhaps, transferrin increases selenium's availability to Y-79 cells and/or its intracellular concentration enabling them to synthesize other important

selenoenzymes. Of course. tranferrin, itself, acting as a growth factor, could be directly responsible for activating a selenium dependent system. Further experiments should include observations of the growth effect of apotransferrin in cells that have been depleted of selenium over a prolonged period in order to ascertain that transferrin is active alone as a growth promoting agent. Also, elucidation of a connection between transferrin and selenium systems will depend on further studies of transferrin and selenium binding as well as GSH-Px activity in the Y-79 cell line.

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