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CHARACTERIZATION OF THE COMPLEX CONCANAVALIN A-RESISTANT PHENOTYPE OF CHINESE HAMSTER OVARY CELLS

BY

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In Memory of My Father

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ABSTRACT

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Three independent concanavalin A (con A)-resistant cell lines were isolated from Chinese hamster ovary (CHO) cells. C^R-7 and BC^R-2 were selected by 10 and 14 passages of wild-type clone 1 (WT-Cl I), and wild-type clone 2 (WT-Cl II) respectively, through a cyclic single step procedure. EC^R-1 was selected following a single exposure of an ethylmethane sulfonate (EMS) mutagenized wild-type, clone 3 (WT-Cl III) population to con A. All variants were selected at 34° in the presence of 40 ug/ml con A. The resistant lines showed a higher efficiency of colony formation in the presence of the lectin than did the wild-type cell lines. The variant D_{10} values were 2.5-fold higher than those of wild-type lines. Concanavalin A resistance was found to be a stable property of the variants. Studies with EMS mutagenized cells suggested that the mutagen treatment increased the frequency of con A-resistant cells in the population. The variant lines had the properties of temperature-sensitive (ts) cell lines as judged by growth studies performed on solid surfaces at the permissive (34°, 37°) and nonpermissive (39°) temperatures, and by plating efficiency experiments performed at 34° and 39°. C^R-7 cells showed ts growth properties in suspension culture and an

altered ability to incorporate DNA, RNA, and protein precursors into acid precipitable material at the non-permissive temperature. No change in the karyotype of the variants was found. A con A-resistant revertant was selected from the C^R-7 population by taking advantage of the ts growth properties of the C^R-7 cell line. The revertant (RC^R-7) was found to have a wild-type temperature growth range and to be more sensitive to con A than the parental variant C^R-7. The D_{10} value for RC^R-7 was 1.4 times that of wild-type cell lines compared to the 2.5-fold increase observed with variant populations. Experiments with pseudotetraploid cells formed by hybridizing con A-resistant and con A-sensitive cells indicated that resistance to con A behaves as a recessive trait; these hybrids (A-7, A-7B and A-7C) exhibited wild-type sensitivity to con A and wild-type growth properties at 39^o.

The independently isolated variants, C^R-7, BC^R-2 and EC^R-1 were found to share a complex pleiotropic phenotype which included several altered membrane properties. The variants showed altered cellular morphologies on solid growth surfaces, and C^R-7 showed an altered surface topography when studied with a scanning electron microscope. The con A-resistant lines showed altered levels of agglutination in the presence of con A and

phytohemaglutinin -P. The lectin-resistant lines showed an enhanced sensitivity to the membrane active agents phenethyl alcohol and sodium butyrate. The D_{10} value for each drug was approximately 2.5-fold higher with wild-type cell lines. Cell detachment studies revealed that con A-resistant cell lines adhered less tightly to growth surfaces than did wild-type cells. The binding of labelled con A to wild-type CHO cells at 4^{o} exhibited positive cooperativity. Variant cell lines exhibited altered lectin binding properties. Concanavalin A-resistant cell lines did not bind con A in a cooperative fashion and bound significantly less lectin than con A-sensitive cells per cell surface area. Studies with fluorescent labelled con A revealed reduced receptor mobility properties on con A-resistant cell lines. The revertant cell line RC^{R}-7 showed membrane-associated properties that were very similar to the wild-type parental lines. Similarly, somatic cell hybrids formed through the fusion of wild-type and lectin-resistant cells possessed membrane-associated properties that were very similar to pseudodiploid wild-type cells and control cultures of pseudotetraploid hybrid cells.

Specific cell surface labelling techniques were employed to examine the surface of con A-resistant, -sensitive and revertant cell lines. Variant cells

selected for resistance to the cytotoxic effects of con A exhibited several alterations in surface labelling patterns when compared to parental wild-type cells. The most obvious difference was the presence of an additional surface component with an apparent molecular weight of 155 000 on resistant cells that was missing from wild-type cells. In experiments with the galactose oxidase -[^{3}H]- borohydride technique the novel component accounted for 12 to 18% of the total labelled cell surface glycoprotein on resistant cells. The lacto-peroxidase catalyzed iodination of surface polypeptides and the metabolic incorporation of labelled glucosamine into membranes of resistant and sensitive cells also indicated the presence of a high molecular weight surface structure (molecular weight of 150 000 to 155 000) on resistant but not on wild-type cells. The revertant cell line showed surface labelling patterns resembling the wild-type population. In particular, the amount of label associated with the extra glycoprotein detected on resistant cells was markedly reduced on revertant cells and accounted for only 2 to 4% of the total labelled cell surface glycoprotein.

The con A-resistant cell lines were found to have reduced levels of mannose transferase activity. The incorporation of [^{14}C]- mannose into lipid, lipid

oligasaccharide, and glycoprotein fractions was found to be reduced by 4 to 5, 3 to 6 and 2 to 3-fold respectively in the three variants studied (C^R-7, BC^R-2, EC^R-1). Also, the revertant cell line (RC^R-7) incorporated mannose into all three fractions with approximately the same efficiency as wild-type cell lines.

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ABBREVIATIONS

α-mem	alpha-minimal essential medium
CHO	Chinese hamster ovary
con A	concanavalin A
EDTA	ethylene-diaminetetraacetic acid
EM	electron microscope
EMS	ethylmethane sulfonate
FCS	fetal calf serum
fl-con A	fluorescent con A
GlcNac	N-acetyl glucosamine
LCH-A	Lens culinaris A
LCH-B	Lens culinaris B
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
PBS	phosphate buffered saline
PE	plating efficiency
PEA	phenethyl alcohol
PHA	phytohemagglutinin
PPO	2,5-Diphenyloxazole
POPOP	1,4-Bis(2-(5-Phenyloxazolyl)) Benzene
RPE	relative plating efficiency
SBA	soybean agglutinin
TdR	deoxythymidine
WGA	wheat germ agglutinin

INTRODUCTION

INTRODUCTION

The surface membrane serves as a liaison between the cell and its environment. The membrane, therefore, has been recognized as playing an integral role in the regulation of growth and development of mammalian cells (Ehrlich, 1957; Weiss, 1960). The intensive study of differentiating cell systems and the in vitro study of transformed or neoplastic cells has produced a considerable body of evidence which supports the view that changes in the regulatory processes involved in the growth and differentiation of cells usually results in or from an alteration of the surface membrane (see reviews Wallach, 1972, 1975; Moscona, 1974; Barondes and Rosen, 1976; Hughes, 1976; Marchase et al. 1976; Nicolson, 1976a,b; Poste and Nicolson, 1976; Quinn,1976).

The plant lectins have proven to be powerful tools for discerning changes in surface membranes of transformed and differentiating cell systems (Bittiger and Schnebli, 1976; Nicolson, 1976a,b). Recently cell lines resistant to the cytotoxic properties of plant lectins have been selected (Wright, 1973a; Gottlieb et al. 1974; Meager et al. 1975; Stanley et al. 1975a). As lectins are known to manifest their cytotoxic effects upon cells through specific binding to the cell surface (Sharon and Lis, 1972), it might be expected that lectin-

resistant cell lines may possess altered surface membranes. Membrane variants of this type could prove useful for the study of the structure-function relationship of the cell membrane.

Wright (1973a) had previously shown that variants resistant to the plant lectin, con A, could be selected in cultures of CHO cells. These variants possessed a complex pleiotropic phenotype. This thesis is a continuation of that previous study. Independent con A-resistant cell lines were selected by a cyclic single step procedure and by exposing an EMS mutagenized population to a single passage in con A. The independent variants C^R-7, BC^R-2 and EC^R-1 were found to share a complex pleiotropic phenotype which included ts growth properties and such altered membrane-associate properties as: cell morphology, lectin agglutination, cell adhesion, sensitivity to membrane active agents, con A binding properties, and lectin receptor mobility properties. A con A-resistant revertant was selected which showed near wild-type sensitivity to con A and wild-type membrane associated properties. Concanavalin A resistance and the pleiotropic phenotype were found to be recessive in somatic cell hybrids formed through the fusion of lectin-sensitive and-resistant cell lines (Ceri and Wright, 1977a, 1978a; Wright and Ceri, 1977a,b).

The altered membrane-associated properties common to lectin-resistant cell lines prompted a biochemical study of the surface membranes of wild-type, variant, and revertant cell lines. Modifications of the plasma membrane of con A-resistant cell lines were shown by: galactose oxidase - [^{3}H]- borohydride labelling, lactoperoxidase catalyzed iodination, and metabolic labelling procedures. The revertant cell line showed wild-type like labelling patterns (Ceri and Wright, 1977c, 1978b).

The altered glycoprotein patterns detected on the surface of lectin-resistant cell lines by surface labelling procedures led to the study of glycoprotein synthesis. As con A is known to specifically interact with ∝-D mannopyranosyl and ∝-D glucopyranosyl moieties (Goldstein and Staub, 1970), the complex transferase system that adds mannose to the core area of many surface glycoproteins was studied. A marked inability to add [^{14}C]- mannose to lipid intermediates and glycoprotein fractions was found in con A-resistant cell lines. Again, the revertant was found to have near wild-type activity.

The above data is discussed both in terms of possible models for con A-resistance and with regard to the usefulness of con A-resistant cell lines in the study of the membrane's role in the regulation of growth and development.

HISTORY

HISTORY

Lectins are proteins possessing the unusual ability to agglutinate or clump a wide variety of cells. The majority of lectins have been isolated from plants and in particular from the seeds of legumes. The word lectin is derived from the Latin term, legere, to pick or choose and was first used by Boyd and Shapleigh (1954) because the proteins were specific for sugar binding.

Lectins have now been isolated from a wide variety of sources, including: bacteria (Gesner and Thomas, 1966; Gibbons et al. 1975), amoeba (Brown et al. 1975), invertebrates (Marchalonis and Edelman, 1968; Hammarström and Kabat, 1969; Müller et al. 1976), cellular slime molds (Barondes and Rosen, 1976), cells grown in tissue culture (Yamada et al. 1975; Nowak et al. 1976; Dysart and Edwards, 1977), and from embryonic tissue at specific points of development (Barondes and Rosen, 1976; Den and Malinzak, 1977). Also, a species specific lectin-like activity found on sperm cells has been described (Globe and Vacquier, 1977); this activity was referred to as "Bindin".

The specific binding properties of lectins and their unusual ability to agglutinate cells has led to an

extensive study of these unique proteins. The reader is directed to several excellent reviews on the study of lectins (Boyd et al. 1962; Sharon and Lis, 1972; Lis and Sharon, 1973; Nicolson, 1974; Chowdhury and Weiss, 1975; Bittinger and Schnebli, 1976; Sharon, 1977).

Concanavalin A Purification and Characterization:

An agglutinin was originally described in the toxic extracts of the Jack Bean (Canavalia einsformis) by Assmann (1911). This toxic substances was called concanavalin A (con A) and was first purified by Sumner and Howell (1935, 1936).

Concanavalin A was isolated in large quantities using standard protein isolation techniques (Sumner and Howell, 1935). The lectin was found to make up 2.5 to 3% by weight of the total protein in Jack Bean meal (Sumner and Howell, 1936). Affinity chromatography, utilizing Sephadex dextran beads, is now used to obtain con A in high yields (Agrawal and Goldstein, 1967). This technique has also been applied to the isolation of lectins with sugar binding specificities similar to that of con A. For example, Sephadex affinity columns have been used to purify the lentil lectins (Tiché

et al. 1970; Howard et al. 1971; Young et al. 1971) and the lectin from Pisum sativum (Entlicher et al. 1970). Elution of the lectin can be achieved by adding competing sugars (Agrawal and Goldstein, 1967) or by lowering the pH (Olson and Liener, 1967).

Considerable information is now available regarding the physical structure of con A (see reviews, Chowdhury and Weiss, 1975; Bittiger and Schnebli, 1976). Concanavalin A is made up of identical subunits of 25 500 M.W. The amino acid sequence of the polypeptide has been reported (Edelman et al. 1972). Concanavalin A contains no covalently bound carbohydrate (Le Vine et al. 1972). Concanavalin A exists as a dimer below pH 5.6 with a molecular weight of 55 000 (Becker et al., 1971). The lectin forms a tetramer having an approximate molecular weight of 112 000 from pH 5.6 to pH 7.0 and tends to form larger aggregates when the pH is greater than 7.0 (Agrawal and Goldstein, 1968; Kalb and Lustig, 1968; McKenzie et al. 1972). Each subunit contains one sugar binding site as well as a binding site for one mole each of calcium and magnesium (Yariv et al. 1968). Treatment of con A with EDTA leads to the loss of sugar binding, which can only be regained by restoring calcium and magnesium

to the system (Yariv et al. 1968; Uchida and Matsumoto, 1972). The two metal ions are not directly involved in the binding, although they are required to maintain the binding configuration (Kalb and Levitzki, 1968; Richardson and Behnke, 1976).

Edelman's group (Becker et al. 1971; Edelman et al. 1972) has proposed a model for the conformation of con A based on X-ray crystallography. This model has recently been supported by magnetic resonance studies (Fuhr et al. 1976). Evidence gained by other techniques indicates a difference in the structure between soluble and crystalline con A (Hardman and Ainsworth, 1973; Brewer et al. 1973). The exact configuration of con A remains in doubt, although circular dichroism measurements (Pflumn et al. 1971), differential U.V. spectroscopy (Hassing and Goldstein, 1970), and temperature-jump spectroscopy (Loontiens et al. 1977) reveal shifts in the molecular conformation of con A with the binding of sugars.

Goldstein (So and Goldstein, 1967 a,b) was able to show that con A binding specificity was directed primarily toward α-D Mannose or α-D Glucose residues. The C-3, C-4 and C-6 hydroxyl groups of the D-arabino-configuration are the minimal essential configuration

features required to interact with con A, while complete freedom of configuration around the C-2 position is permissable (Goldstein, 1975). Internal α-D mannose residues, found in the core of oligosaccharides, were as potent as methyl-α-D mannoside as an inhibitor of con A hemagglutination (Goldstein et al. 1973). It would then appear that con A can bind to either internal or exposed mannose residues (Goldstein, 1975).

Concanavalin A as a Biological Tool

The specificity of the interactions of plant lectins and the diverse effects they have upon cells have made these proteins powerful tools in the study of many facets of biology.

One of the first to recognize the potential of lectins was Ehrlich (see Sharon and Lis, 1972). In 1891 Ehrlich showed that mice could be immunized against the toxicity of ricin and abrin by pre-exposure to low doses of the drug. He was also able to demonstrate the neutralization of the toxin by serum from immune animals. Therefore, by the use of lectins he was able to demonstrate the fundamental properties of the immune system.

Lectins have become powerful tools in blood typing. Landsteiner (Landsteiner and Raubitschek, 1908) first revealed that differences in hemagglutination existed between different lectins and different species of erythrocytes. Blood group specific lectins, however, were not found until the late 1940s (Renkonen, 1948, 1950; Boyd and Reguera, 1949). An extensive list of lectin hemagglutination specificities can be found in a review by Toms and Western (1971).

By coupling lectins to solid supports the binding specificity of the lectins can be used in an affinity chromatography system. Concanavalin A has been bound to form poly-l-leucyl-con A and Sepharose-con A (Lloyd, 1970). These have been used in affinity chromatography systems to isolate glycoproteins, cell membranes, and even to sort cells. Examples of the uses to which con A-sepharose has been put are: to fractionate IgG and IgM (Weinstein et al. 1972), to isolate certain hormones (Dufau et al. 1972), to separate N-Glycosidic glycoproteins (Krusius et al. 1976), to isolate carcinoembryonic antigens (Brattain et al. 1975) and to isolate cellular con A receptors (Robinson et al. 1976; West and McMahon, 1977).

Other lectins have also been used in affinity chromatography systems. For example, PHA-agarose was used to isolate interferon (Dorner et al. 1973) and insulin receptors were purified on WGA-Sepharose (Cuatrecasas and Tell, 1973).

Both bound and free con A have been used to isolate cell membrane fractions (Parish and Muller, 1976, Winquist et al. 1976; Barchi et al. 1977). Affinity columns of con A-Sepharose have also been used to isolate glycolipids in liposomes (Boldt et al. 1977).

Several ingenious techniques have been developed to make use of con A in cell sorting. Concanavalin A bound to fibers has been used to select mitotically active cells in populations of intestinal epithelial cells (Edelman, 1972); these have been shown to be the stem or crypt cells which regenerate the cells of the intestinal lining (Podolsky and Weiser, 1973). Affinity columns have also been used to sort and separate different cell types grown in tissue culture (Kinzel et al. 1977).

Concanavalin A has been used to inhibit enzyme activities by binding either to the cell membrane, thereby altering required enzyme configurations or by

binding directly to some enzymes which are glycoproteins. For example, con A alters cellular ion concentrations, probably by affecting the sodium-potassium ATPase (Swann et al. 1975; Carraway et al. 1975; Negendank and Collier, 1976; Carraway and Carraway, 1976). The lectin has also been shown to inhibit 5'-Nucleotidase activity (Carraway et al. 1975; Carraway and Carraway, 1976, Kartner et al. 1977) and to alter some sugar transferase activities (Painter and White, 1976; Young et al. 1977). Concanavalin A has also been shown to affect the cyclic AMP levels of cells by acting on both the cAMP-phosphodiesterase (Luly et al. 1976) and adenylate cyclase (Michaelis and Michaelis, 1976) activities.

Concanavalin A has been useful for studies on the glycoprotein make-up of viruses. Becht (Becht et al. 1972) has scanned several virus species for lectin binding potential. Several enveloped viruses were agglutinated while non-enveloped viruses, like SV40 and polio were not clumped. Enveloped virus infectivity could be blocked by the addition of con A to virus suspensions (Zarling and Tevethia, 1971). Polio viruses which do not agglutinate, remained infectious after

con A treatment. Treating cells with con A can however, block polio infection (Okada and Kim, 1962). This may indicate that a cell surface glycoprotein may play a critical role in polio virus infectivity. However, the toxic effects of con A were not taken into consideration in this study.

Lectins have also been applied to monitoring the surface membrane changes which might accompany virus infection. Concanavalin A agglutinability has been observed to increase as viral infection proceeds and virus specific antigens appear on the cell surface (Becht et al. 1972; Poste, 1972; Penhoet et al. 1974). Changes in agglutinability can be seen prior to the insertion of virus specific proteins into the cell membrane (Tevethia et al. 1972; Post and Reeve, 1972; Poste, 1972; Poste and Reeve, 1974). Poste, (1972) has proposed that the alterations may be due to modifications on the cell surface produced by lysosomal enzymes released during virus infection.

The binding of con A to the surface of virus infected cells can lead to the inhibition of virus maturation and release (Rott et al. 1972; Stiz et al. 1977; Cartwright, 1977). Nicolson (1974) proposes that the inhibition of virus release may be due to

cross-linking of surface glycoproteins, which prevents receptor movement required to allow the insertion of viral specific proteins, and the eventual budding of the new virus progeny.

Lectins are playing a major role in the development of the field of immunology. As noted earlier lectins were one of the first molecules used in detecting the specificity of the immune system. Lectins are now being used to study the central cells of the immune system, the lymphocytes. The mitogenic activity of phytohemagglutinin towards lymphocytes was first recognized by Nowell (1960). Recently, Sharon (1976) has shown that seventeen of thirty plant lectins assayed for mitogenic activity stimulated lymphocyte DNA synthesis. The investigation of lectins as mitogens is a field of study in its own right and the reader is directed to two key reviews by Cunningham (1974) and Sharon (1976). There are, however, a few points to be made. The first is that non-mitogenic lectins are toxic to cells even if they have the same sugar specificity as a mitogenic lectin (Greene et al. 1976). This may indicate that more than lectin binding is involved in the mitogenic activity. Secondly, the Soybean agglutinin has been shown to

be mitogenic for Soybean callus cells (Howard et al. 1977). As these authors noted, the actual function of these lectins in the plants is not known and the mitogenic activity of these proteins may, in fact, be their central functions.

Lectins as Membrane Probes

The use of lectins as probes in the study of the structure-function relationship of membranes is probably the major area of lectin research.

The importance of the cell membrane in the regulation of cell growth was first recognized by Ehrlich (1957) and Weiss (1960). The involvement of the cell membrane in growth control, in neoplasia, and cell differentiation has made the field of "Membranology" (Wallach, 1972) one of the most active areas in biology.

The first concepts of the cell membrane were based essentially on static models (Danielli and Dawson, 1935; Robertson, 1959), which presumed uniformity and constancy of membranes. In contrast the current model of cell surfaces is one of a dynamic, fluid, three-dimensional mosaic (Singer and Nicolson, 1972; Singer, 1974) which would allow for the genetic and phenotypic diversity of cells.

Several markers of membrane changes in both neoplastic

cells and developing cell systems have been recognized and their roles in the regulation of growth and development are under study. Examples of such changes are: new surface antigens on developing cells (fetal antigens) and on transformed cells (tumor antigens), novel glycoproteins and glycolipids on cell surfaces, unusual cell mobility characteristics, altered patterns in cell adhesion, and changes in enzyme activities (Moscana, 1974, Nicolson, 1976a). Several excellent reviews on cell surfaces are available (Bretscher, 1974; Hughes, 1976; Quinn, 1976; Nicolson, 1976a) as are several reviews discussing membrane alterations in neoplastic or pathological states (Wallach, 1972, 1975; Nicolson, 1976b) and membrane changes in development (Curtis, 1967; Moscana, 1974; Marchase et al. 1976; Poste and Nicolson, 1976; Barondes and Rosen, 1976).

Lectins are now being used as probes of membrane changes associated with neoplasia and cellular development. For example, lectins can be used to measure changes in such membrane properties as agglutinability, distribution and mobility of receptors, and differences in the nature of lectin binding.

Aub (Aub et al. 1965a,b) first demonstrated increased agglutinability of transformed cells. Lectins agglutinated both tumor cells (Burger and Goldberg, 1967; Anzil et al.

1977) and cells transformed by virus (Benjamin and Burger, 1970; Ben-Bassat et al. 1970) to a greater degree than nontransformed cell types. Cells infected with temperature sensitive transforming viruses were found to show increased levels of lectin agglutination only at temperatures at which the virus was active (Miki and Kuivata, 1976; Marciani and Okazaki, 1976). The ability of cells to grow to high cell densities, which is indicative of increased tumorgenicity (Aaronson and Todaro, 1968a,b) has also been correlated with increased agglutinability (Pollack and Burger, 1969; Weber, 1973; Berman, 1975). However, several exceptions to this rule are known. Several examples of tumor cell lines have been described which show lower levels of agglutination than do "normal" control cells (Gantt et al. 1969; Swak and Wolman, 1972). Also, cell lines showing high growth densities have been described with agglutination properties similar to (Glimeluis et al. 1975) or lower than control lines (Hozumi et al. 1972).

Several authors have tried to relate the increase in agglutination of transformed cells to changes in the surface topography that accompanies transformation. For example, Noonan's group (vanVeen et al. 1976a,b) has related the increase in agglutinability of CHO cells in

dibutyryl cAMP to the morphological changes the drug produces on cells in culture. Willingham and Pastan (1974, 1975) have shown transformed cells possess many more microvilli, which they claim play a central role in making cells more agglutinable in the presence of con A. Ukena and Karnovsky (1977) have studied eight different cell lines with varying capacities to be agglutinated with con A. They were able to demonstrate a positive correlation between the number of microvilli on the surface of cells grown in monolayer culture and the degree of agglutination of cells by con A. They found however, the number of microvilli on the surface of cells in suspension, where agglutination studies are carried out, was approximately the same for all cell lines. They were also not able to detect changes in the number of microvilli in the presence of con A. Other groups have reported that transformed cells in suspension have fewer microvilli than nontransformed cells, and that it is the interactions along large smooth ruffles on the surface of transformed cells that are responsible for their increased agglutinability (Collard and Temmink, 1976; Oppenheimer et al. 1977). It is therefore apparent that the role of cell surface architecture in cell agglutination is still not understood.

Lectin agglutination has also been used to detect membrane changes in differentiating systems. Moscana (1974) has measured differences in con A agglutination among different cell types of chick embryos. Changes in agglutination patterns in con A, ricin, and wheat germ agglutinin have been used to monitor membrane changes in the maturation of sperm (Nicolson et al. 1977). Sperm maturation was followed from epididymal passage through ejaculation with changes in lectin receptors noted at various stages of development. Concanavalin A agglutination has also been used to follow the differentiation process in the cellular slime mold Dictyostelium discoideum (Weeks, 1973; Weeks and Weeks, 1975; Kawai and Takeuchi, 1976). Preculmination cells were found to require fifteen times the con A concentration to achieve maximal agglutination than did free living amoeba (Weeks and Weeks, 1975). Barondes' laboratory (Barondes and Rosen, 1976; Nowak et al. 1977; Kabiler and Barondes, 1977) has made use of hemagglutination techniques to demonstrate the temporal presence of lectins on the surface of preculmination slime molds, and on chick embryo tissue.

A variety of techniques have been developed to visualize the distribution and mobility of lectin binding sites on the surfaces of cells. Light microscopes fitted with ultraviolet light systems can be used to detect

con A binding sites on the cell surface tagged with fluorescein conjugated con A (Smith and Hollers, 1970). Binding sites can also be visualized in the electron microscope. For example, con A can be made electron dense with 3,3' diaminobenzidine after it has been complexed with its binding site using peroxidases (Bernhard and Avrameas, 1971). Ferritin conjugated con A can also be detected in electron micrographs (Nicolson and Singer, 1971).

Lectin receptors have been shown to be much more clustered or patched on transformed cells than "normal" cells at room temperature (Nicolson, 1971; Martinez-Palomo et al. 1972; Comoglio and Guglielmone 1972). These studies have now been applied to a large number of different cell types (see review Nicolson, 1976b), and as is the case so often with lectin research, exceptions to the rule are found (Smith and Revel, 1972; Collard and Temmink, 1974; Zagyansky et al. 1977). The clustering of con A receptors is due to the redistribution of lectin binding sites, and can be prevented by fixing the surface membrane with glutaraldehyde (Nicolson, 1973; van Blitterswijk et al. 1976). These results would indicate a greater freedom of movement of lectin binding sites on transformed cell lines.

Controversy continues as to whether or not differences exist in the number of lectin binding sites on transformed

cells as compared to "normal" cells. There is much evidence to indicate that although transformed cells are more agglutinable, they do not bind more con A than do normal cells (see review Nicolson, 1976b). Noonan and Burger (1973, 1974) have reported that differences in the number of binding sites on transformed and non-transformed cells can be detected if binding is done under conditions which prevent endocytosis and non-specific binding of con A. The calculation of cell surface areas from electron micrographs (Collard and Temmink, 1975) has revealed that estimated cell surface areas based on cells being perfect spheres may grossly underestimate true cell surface areas. Based on more accurate measurements of cell surface area obtained by electron microscopic studies Collard and Temmink (1975) have reported that transformed 3T3 cells bound seven times as much con A as did control 3T3 cells.

Differences in both the number and nature of lectin binding sites have been revealed using techniques which reduce lectin endocytosis and non-specific binding. High and low con A affinity binding sites have been found in a number of cell lines (Reisner et al. 1976; Schmidt-Ulbrich et al. 1976; Feller et al. 1977). Cooperativity of lectin binding to cell surfaces and to enzymes has

also been reported (Carraway et al. 1975; Carraway and Carraway, 1976; Bornens et al. 1976; Wright and Ceri, 1977a,b). Binding competition studies with different lectins have also revealed specificity of lectin binding sites (Gurd and Evans, 1976; Gurd, 1977).

Concanavalin A binding has been used to detect changes in surface membranes during differentiation. Differences in con A binding sites and in receptor mobility have been found during the course of differentiation of D. discoideum (Gillette et al. 1974; Monday et al. 1976; West and McMahon, 1977). The exposure of con A binding sites was seen to vary during the course of maturation of both sperm and egg cells (of different species), and to change at the time of fertilization (Yanogimachi and Nicolson, 1976; Veron and Shapiro, 1977; Nicolson et al. 1977). Changes in lectin binding sites can also be detected during various stages of embryogenesis (Moscana, 1974; Neri et al. 1975; Oppenheimer, et al. 1977).

Lectins and Somatic Cell Genetics

Recent advances in the field of somatic cell genetics (see reviews De Mars, 1974; Chu, 1974; Clements, 1975; Siminovitch, 1976) have opened a new avenue for the application of lectins to the study of the structure-function relationship of the cell membrane. Despite early

controversy regarding the nature of somatic cell mutants (Szybalski et al. 1964; Breslow and Goldsby, 1969; Mezger-Freed, 1971, 1972; Orkin and Littlefield, 1971; Harris, 1971, 1973, 1975), considerable evidence now exists that true genetic mutants can be isolated in tissue culture (Chu, 1974; De Mars, 1974; Clements, 1975; Siminovitch, 1976).

Membrane mutants have recently been selected by using drugs which act at the cell surface. Ouabain resistant cell lines, which possess an altered Na^+/K^+ ATPase activity are one example (Mayhew, 1972; Baker et al. 1974). Membrane mutants have also been indirectly selected, when drug resistance was found to be the result of altered drug permeability. Cell lines selected for resistance to actinomycin D (Bosmann, 1971) and colchicine (Till et al. 1973; Ling and Thompson, 1974) are examples of membrane mutants selected by chance rather than design. It should be noted that a permeability defect appeared to be expressed as a pleiotropic mutation and in both cases affected the permeability of a number of unrelated compounds.

The cytotoxic nature of con A was first used for the selection of revertants of virus transformed cells. Concanavalin A preferentially agglutinates transformed cells (Benjamin and Burger, 1970; Ben-Bassat et al. 1970)

and is cytotoxic for transformed cells at concentrations that do not affect "normal" control cells (Shoham et al. 1970). These properties allowed for the selection of revertants from SV40 transformed populations (Ozanne and Sambrook, 1971; Culp and Black, 1972; Wollman and Sachs, 1972; Ozanne, 1973).

Wright (1973a) was the first to use the cytotoxic properties of plant lectins for the selection of drug resistant cell lines. Several groups are now using lectins for the selection of cell variants and their work is summarized below.

Ricin Resistant Cell Lines

Ricin and abrin are potent inhibitors of protein synthesis in both whole cells and in cell free systems (Lin et al. 1970). Both lectins contain two functionally separate subunits. The B chain is responsible for the sugar specific binding, while the A chain, which is specifically taken into the cell, acts to block protein synthesis by inactivating the 60S ribosomal subunit (Olsnes and Pihl, 1973a,b; Sperti et al. 1973; Benson et al. 1975; Olsnes et al. 1976; Sandvig et al. 1976). The block in protein synthesis resulted from the enzymatic modification of the 60S subunit by the A chain of ricin. This led to the loss of the binding site for the elongation

factor EF-2 (Olsnes and Pihl, 1973a,b; Sperti et al. 1973; Benson et al. 1975; Olsnes et al. 1976; Sandvig, et al. 1976). This enzymatic modification was very rapid, with a single A chain molecule able to inactivate as many as 1,500 salt washed ribosomes per minute (Olsnes et al. 1975).

The first ricin-resistant cell lines were isolated in murine lymphomas (Hyman et al. 1974) and in Chinese hamster ovary cells (Gottlieb et al. 1974). Two classes of ricin-resistant cell lines were isolated from Chinese hamster ovary cells. The first class was found to bind less ricin and a number of other lectins due to reduced levels of sugars at the cell surface (Gottlieb et al. 1974). This class of mutant was found to possess reduced N-acetylglucosamine transferase activity which is required for the addition of sugars to the core region of glycoproteins (Gottlieb et al. 1975). This defect resulted in a shift toward lower molecular weights of cell surface glycoproteins. The second class of CHO ricin-resistant variants was found to bind equal amounts of ricin, but to have a reduced capacity to internalize ricin (Nicolson et al. 1975). Electron micrographs indicated an alteration in the microtubule network which might account for the reduced lectin incorporation.

Two similar classes of ricin-resistant BHK cell lines were isolated from methyl-N-nitro-N-nitrosoguanidine

mutagenized populations (Meager et al. 1975). Those cell lines, which bound less ricin, were again found to possess lower levels of N-acetylglucosamine transferase and to have a shift toward lower molecular weight glycoproteins at the cell surface (Meager et al. 1975; Meager et al. 1976). Ricin sensitivity could be restored to these variants by adding a ricin binding glycolipid fraction from erythrocytes to the cell growth media (Hughes and Gardas, 1976). This may indicate that glycolipid binding sites may play a critical role in ricin toxicity. Ricin-resistant variants were also found to be less adhesive (Edwards et al. 1976) which may be the result of alterations found in the surface glycoproteins (Meager et al.1976).

Mouse L cells resistant to the cytotoxic effect of ricin have also been isolated (Gottlieb and Kornfeld, 1976). The properties of the mouse L cell variants are quite distinct from those of the ricin-resistant cell lines previously discussed. One of the variant clones (clone 3) was found to contain elevated levels of sialic acid due to an increased sialyltransferase activity, which led to a shift in glycoprotein patterns toward heavier molecular weights. A second variant (clone 6) had reduced carbohydrate levels which corresponded with lower activities of the galactose and N-acetyglucosamine transferases. The glycoprotein pattern was seen to shift

toward lower molecular weights as a result of these alterations. Normal wild-type levels of ricin were bound by clone 3 cells when excess sialic acid was removed from the cell surface with neuraminidase. Drug resistance, however, only dropped to levels which were still forty to forty-five fold greater than that of wild-type cells. Clone 6 showed little difference in ricin binding following neuraminidase treatment. No changes in the mechanism of ricin endocytosis could be seen with either variant type. The authors postulate two possible explanations for these results. The first explanation involves the existence of "productive" binding sites which are responsible for the binding of the ricin which will be taken into the cell. The quantity of ricin required to kill the cell is so small that the identification of productive binding sites may prove an impossibility. A second alternative presented by the authors was the blockage of some unknown step in the complex pathway that eventually leads to ricin toxicity.

Phytohemagglutinin-Resistant Cell Lines

Unlike ricin, very little is known about the cytotoxic nature of phytohemagglutinin (PHA). Isolated from Phaseolus vulgaris, the lectin specifically binds to N-acetylgalactosamine (Borberg et al. 1966). PHA

preferentially killed transformed fibroblasts (Gail and Boone, 1972), and was toxic to transformed lymphoma cells at drug concentrations which were mitogenic to normal lymphoblasts (Dent, 1971). PHA, like ricin, contains two subunits. The binding specificty and the mitogenic activity are found on the same subunit (Allen et al. 1969; Allan and Crumpton, 1971). However, nothing is known of the toxic mechanism.

The first PHA-resistant cell lines were isolated from CHO cells by Wright (1973a). The altered sensitivity of PHA-resistant cell lines to a number of membrane active agents suggested possible membrane alterations in these cells. The PHA-resistant lines showed wild-type sensitivity to con A; however, the cytotoxic mechanism was altered since hapten inhibition of con A binding did not protect the PHA-resistant cells but did protect the wild type cells.

Chinese hamster ovary cells resistant to PHA have also been isolated by Stanley (Stanley et al., 1975a,b,c; Juliano and Stanley, 1975; Stanley and Siminovitch, 1976). The selection of PHA-resistant cell lines was carried out by the method of Wright (1973a). Two classes of PHA-resistant cell lines were isolated and separated on the basis of their sensitivities to a number of different lectins. The first class of PHA-resistant variants was

found to have a reduced N-acetylglucosamine transferase activity (Stanley et al. 1975c) similar to that seen in ricin resistant cell lines (Gottlieb et al. 1975). Further elegant studies by Schachter's group (Narasimhan et al. 1977) have shown the GlcNAc-transferase responsible for the addition of GlcNAc to core regions of the glycoprotein to be defective. No analysis of glycoprotein sugars was performed. However, changes in surface glycoproteins were demonstrated by cell surface labelling techniques (Juliano and Stanley, 1975). The second class of PHA-resistant variants had normal GlcNAc-transferase levels (Stanley et al. 1975b), but little is known of the biochemistry of these variants.

Evidence for the separation of PHA-resistant cell lines into two classes, has come from some excellent genetic studies carried out by Stanley (Stanley et al. 1975b; Stanley and Siminovitch, 1976, 1977). These studies have involved a number of other lectin resistant cell lines whose properties have not yet been reported. These lines include cells resistant to wheat germ agglutinin, ricin, the lentil lectins, and con A.

The various lectin-resistant cell lines were grouped according to their phenotype determined on the basis of the response of a particular cell line to a number of different lectins (Stanley et al. 1975b; Stanley

Siminovitch, 1976). Eight to ten distinct groups have been separated on this basis, with PHA-resistant lines belonging to two different groups (Stanley et al. 1975b). Also, cell-cell hybridization studies have been used to detect at least seven complementation groups (Stanley and Siminovitch, 1977). Again, PHA-resistant lines were found to belong to two separate groups (Stanley and Siminovitch, 1977). The genetic data obtained from the studies has shown that more than one lectin can be used to select a particular phenotype and that one lectin can be used to select more than one phenotype (Stanley and Siminovitch, 1977).

Wheat Germ Agglutinin

Kornfeld's group (Briles et al. 1977) has recently reported the isolation of WGA resistant CHO cells. Sialic acid is intimately involved in the binding of WGA to cell surfaces (Adair and Kornfeld, 1974; Cuatrecasas, 1973). The resistant line was found to have reduced levels of sialic acid in glycoprotein and glycolipid fractions. The variants were found to have normal levels of glycolipid and glycoprotein sialytransferase activities and normal amounts of glycoprotein and glycolipid receptors with in vitro assays. It is postulated that the lack of sialic acid may be due to a loss of organization of

the transferase system which is critical for optimum transferase activity (Arce et al. 1971; Wingquist and Dallner, 1976).

Concanavalin A

Concanavalin A has been intensively studied, but the mechanism of con A toxicity remains unknown. In animal studies con A has been found to associate with endoplasmic reticula and mitochondria (Tyan, 1974; Napanitaya and Tyan, 1975; Napanitaya et al. 1976). The presence of con A binding sites on eucaryotic ribosomes has been suggested (Howard and Schnebli, 1977); although no association of con A with ribosomes was observed in histological studies (Napanitaya et al. 1976). Unlike ricin, con A does not specifically block protein synthesis (Nicolson et al. 1974) but acts to prevent both DNA and protein synthesis within two hours of lectin treatment of thymocytes (Shohom et al. 1970).

Recent studies have correlated con A toxicity with the aggregation of the lectin into discrete patches or "caps" (Forsdyke, 1977; Lustig et al. 1977). Lustig (Lustig et al. 1977) has shown cell lysis to be preceeded by cap formation. Forsdyke (1977) has reported that complement played a major role in the inhibition of lymphocytes by high concentrations of con A. The

aggregation of lectin binding sites may be analogous to the aggregation of antibody binding sites required for complement binding (Medicus et al. 1976).

Wright (1973a) was the first to select con A-resistant cell lines. Mutants were selected by stepwise or cyclic single-step procedures. The con A-resistant variants were found to be temperature-sensitive for growth (Wright, 1973a, 1975). No changes in the glycosphingolipids were seen in the variant cell lines (Yogeeswaran et al. 1974). The genetic and biochemical properties of several independent con A-resistant cell lines has suggested that these lectin-resistant cells have a common pleiotropic phenotype (Ceri and Wright, 1977a; Wright and Ceri, 1977a,b; Ceri and Wright, 1978a,b). The pleiotropic phenotype involved changes in: cell morphology, lectin agglutination, cell adhesion, growth at various temperatures; reduced and altered mechanism of con A binding, drug sensitivity and defective receptor mobility. Concanavalin A-resistance was found to be recessive in somatic cell hybrids formed through the fusion of con A-resistant and con A-sensitive cell lines (Wright and Ceri, 1977b; Ceri and Wright, 1978a). The loss of con A resistance in hybrid or revertant cell lines also led to the loss of the pleiotropic phenotype (Wright and Ceri,

1977b; Ceri and Wright, 1978a). The changes in the con A-resistant phenotype have been correlated with alterations in the glycoprotein make-up of con A-resistant cell membranes (Ceri and Wright, 1978b). These changes in glycoproteins of the surface membrane may result from alterations in the mannose transferase activities of con A-resistant cell lines (Krag et al. 1977; Wright, Ceri and Jamieson, 1978).

MATERIALS AND METHODS

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1. Cells and Culture Conditions:

Chinese hamster ovary (CHO) cell lines which were originally established in Puck's laboratory (Puck et al. 1958) were used in this study. The wild-type CHO cell line was obtained from the University of Toronto, Department of Medical Biophysics.

AUX B_1, a CHO auxotroph requiring glycine, adenosine, and thymidine because of a defect in folic acid metabolism, was isolated by McBurney and Whitmore (1974). The cell line was kindly provided by Drs. Stanley and Siminovitch from the Department of Medical Genetics, University of Toronto.

H^R-100 is a CHO cell line selected for resistance to hydroxyurea (Wright and Lewis, 1974; Lewis and Wright, 1974) in our laboratory by Mr. K.A. Goodridge. The relative plating efficiency of H^R-100 in 100 µg/ml con A was approximately one compared to a value of 10^{-4} for wild-type cell lines.

With the exception of $AUXB_1$, all cell lines were grown in alpha minimal essential medium (α-mem) (Stanners et al. 1971) plus 10% (v/v) fetal calf serum (FCS) which was supplemented with Penicillin G (100 µnits/ml) and streptomycin sulfate (100 µg/ml). This was referred to as complete

growth medium. Unless otherwise noted, all cell lines were incubated at 34° in a 5% CO_2 atmosphere.

$AUXB_1$ was routinely grown in complete growth medium supplemented with thymidine (10 µg/ml) and adenosine (10 µg/ml). The medium did not have to be supplemented with glycine because the amino acid is a normal constituent of the medium.

Cell lines were routinely grown on 16 oz Brockway bottles. Cells were grown to monolayers and then removed either by trypsinization (addition of 0.05% trypsin in Dulbecco's phosphate buffered saline to cell cultures previously grown on solid surfaces) (Dulbecco and Vogt, 1954), or by incubating the cells with 0.04% EDTA in Dulbecco's phosphate buffered saline (PBS) for approximately 10 minutes. The cells were diluted in isotonic saline and counted in a particle counter (Coulter Electronics Ltd.). Approximately 10^4 cells were routinely subcultured in 40 ml of α-mem in a 16 oz Brockway bottle.

Suspension cultures of CHO cells were initiated by adding 1×10^7 cells to 200-250 ml of complete growth medium in 500 ml medium bottles. Cultures were incubated in a 34° water bath and stirred continuously by a Teflon coated magnetic stirring bar. Cultures were routinely maintained at 1 to 5×10^5 cells/ml by periodic dilution with fresh medium.

Concanavalin A medium:

Concanavalin A medium was routinely prepared by the method of Wright (1973a). Concanavalin A was added to complete growth medium to a final concentration of 40 µg/ml con A. The medium was incubated at 37^{o} overnight and the resulting precipitate was removed either by centrifugation or by filtration. Wright (1973a) has previously shown less than 5% con A is lost in this precipitate at con A concentrations less than 60 µg/ml.

Mycoplasma Assays:

Cell lines were periodically tested for Mycoplasma contamination by routine plating techniques (Crawford, 1968). Cultures are also tested for Mycoplasma by determination of uridine phosphoylase activity (Levine, 1972). All cell lines used in this study gave negative tests for Mycoplasma.

Somatic Cell Hybrids:

In order to determine the behaviour of the con A resistant trait in the presence of a wild-type genome, the C^{R}-7 cell line was hybridized with a wild-type CHO line by means of Sendai virus-mediated fusion (Kao et al. 1969). Unfortunately the con A-resistant cell lines carry no additional markers which can be used in hybrid selection. Therefore, it was necessary to construct a

con A-sensitive cell line carrying two markers which could be used for selection purposes. To this end the codominant ouabain resistant marker (Baker et al. 1974) was added to the $AUXB_1$ cell line which carries a recessive auxotrophic lesion in folic acid metabolism (McBurney and Whitmore, 1974).

The $AUXB_1$-O^R cell line was selected for growth in 2 mM ouabain (Baker et al. 1974; Mayhew, 1972) by Mr. K.A. Goodridge. The $AUXB_1$-O^R plated with an efficiency of one in 2 mM ouabain which was a concentration capable of reducing the plating of WT-Cl I to 10^{-4}. The hybridization was carried out by incubating 3×10^5 $AUXB_1$-O^R cells with an equal number of either the C^R-7 or WT-Cl I population in a 6 mm diameter well of a 96 well Linbro plastic tray containing 0.2 ml of growth medium supplemented with 0.04 mM adenosine and 0.04 mM thymidine. The cells were incubated overnight at 34^o, after which time the medium was replaced with PBS (0.2 ml) and the cells placed at 4^o for 10 minutes. The buffer was replaced with 0.2 ml of a 1:10 dilution of the stock Sendai (Lot 134-1, Connaught Med. Res. Labs) virus preparation in PBS. This was incubated an additional 10 minutes at 4^o. Next, the virus suspension was removed and warm growth medium was added. The cells were incubated for another 4 hours at 34^o after which time the cells were washed with PBS, removed with a trypsin solution, and plated in 60 mM culture dishes containing

5 ml of growth medium lacking adenosine and thymidine but supplemented with 2 mM ouabain. After 10 days a number of colonies appeared on the plates containing the selective medium; these colonies were picked with sterile Pasteur pipettes and independently grown to partial monolayers in 60 mm plates in the presence of normal growth medium. The potential hybrids were cloned, the karyotypes were examined and the cells were retested for the ability to form colonies in selective medium.

Cell Cloning:

Routinely cell suspensions were diluted to 5 cells/ml with ∝-mem and 0.2 ml of the suspension was dispensed into each well of a 96 well Linbro dish. After approximately 8-10 days incubation at 34^{o}, wells showing a single colony were trypsinized. The cells were removed and added to 60 mm plastic tissue culture dishes containing normal growth medium.

Cell Viability

Cell viability was measured by the trypan blue dye exclusion test (Phillips, 1973). This test is based upon the finding that viable cells do not take up trypan

blue whereas non-viable cells readily internalize the dye. A stock solution of trypan blue was prepared as a 0.4% solution of dye in PBS at pH 7.2. The viability test involved pelleting cells after two washes in PBS. One ml of PBS was added to the pellet and 0.1 ml of the stock dye solution added prior to the dispersion of the pellet with a Pasteur pipette. A drop of the suspension was placed on a hemocytometer and the viability counts were made 4 minutes after cell dispersion.

Plating Efficiency:

The plating efficiency (PE) of a cell population was defined as the fraction of cells in the population which would give rise to colonies when plated on a solid growth surface. To determine the plating efficiency of a population approximately 300 cells were added to a 60 mm tissue culture dish containing 5 ml of growth medium. The cells were incubated 8 to 10 days at 34°, after which time the medium was drained from the plates and the cells were stained with a saturated solution of methylene blue in 50% ethanol. Only colonies containing greater than 50 cells per colony were routinely scored. The PE was determined by dividing the number of colonies by the number of cells plated. The PE of wild-type CHO cells typically ranged between 0.75 - 0.90 when the cells were incubated at 34, 37 or 39°.

Drug Sensitivity Tests:

The sensitivity of a cell population to a drug was determined from the relative plating efficiency of the cell line in the presence of varying concentrations of the drug (Thompson and Baker, 1973). The relative plating efficiency (RPE) was defined as the PE in the presence of a concentration of the drug divided by the PE in the absence of the drug.

Growth Curves:

Growth curves were obtained by seeding 5×10^4 exponentially growing cells in 5 ml α-mem + 10% FCS in 60 mm tissue culture dishes. The plates were incubated at 34° overnight. In drug-sensitivity tests the drug was added after an initial 24 hour growth period in the absence of the drug and in temperature-sensitivity studies a sample of the cells were shifted to the non permissive temperature only after the cells were first incubated at 34° for 24 hours. Cell growth was measured by trypsinizing duplicate plates at specific time intervals, diluting the cells with isotonic saline, and estimating the cell number with the aid of an electronic particle counter (Coulter Electronics). The doubling time was determined from a plot of log cell number against the culture time in hours (Ceri and Wright, 1977a, 1978a).

Karyotype Analysis:

Cell lines exponentially growing on 16 oz Brockway bottles were blocked in metaphase by adding 0.5 µg/ml colchicine to the growth medium. The cells were incubated for one to two hours in the presence of the drug before being trypsinized and washed once with PBS. Chromosomes were then stained by the method of Rothfels and Siminovitch (1958). This involved suspending cells in hypotonic sodium citrate for 10 min. before pelleting in a conical centrifuge tube. The cell pellet was rinsed without disruption, with 1 ml of 50% acetic acid. The cells were then resuspended in a small volume of aceto-orcein stain (1% orcein in 50% acetic acid). Chromosome squashes were prepared by pressing firmly on a coverslip placed on a drop of the stained cell suspension on a microscope slide. The slide was examined with a microscope and photographs were taken of 50 to 100 metaphase cells. Enlarged prints were made and the number of chromosomes/cell counted.

Incorporation Studies:

Incorporation of (6-^{3}H) thymidine, (5-^{3}H) uridine and L-(4,5-^{3}H) leucine into acid precipitable material was measured for a 15 minute pulse in cell suspensions maintained in growth medium at 34^{o} and 39^{o}. The various labelled macromolecular precursors were added during

the pulse to a final concentration of 1.0 μCi/ml. The cells were collected by filtration onto glass fiber filters, washed with PBS, incubated in ice-cold 10% trichloroacetic acid for 10 minutes, and finally washed with 70% ethanol. Dried filters were solubilized with 1.0 ml of NCS overnight before a toluene-based scintillation fluid was added (Lewis and Wright, 1974; Ceri and Wright, 1977a).

Cell Agglutination Studies:

Cells growing exponentially in suspension culture at 34^{o} were collected by centrifugation, washed with PBS and suspended in 0.154 M NaCl solution at 2 x 10^{6} cells/ml. For the agglutination assays (Ceri and Wright, 1977a) 0.5 ml of the cell suspension and 0.5 ml of a 0.154 M NaCl soluton containing varying concentrations of con A were added to a 10 x 35 mm tissue culture dish. The con A treated suspension was slowly agitated at room temperature for 10 minutes and then examined with a light microscope for cell clumping. Agglutination was scored qualitatively on a scale of - to ++++ (no agglutination to virtually complete agglutination).

Cell Detachment Studies:

Cell adhesiveness was determined by measuring the rate of cell detachment from growth surfaces, using the method of Pouysségur and Pastan (1976). Approximately 6×10^4 cells of each cell type was plated on 60 mm culture plates and incubated overnight at 34 or 39°. To carry out cell detachment studies the medium was drained from plates, the cells were washed with PBS, and the plates were incubated at the appropriate temperature in 2 ml of PBS containing 0.03% trypsin. At various time intervals the trypsin was removed, cell aggregates were dispersed by pipetting, and the number of cells in suspension were counted with an electronic cell counter (Coulter electronics).

Concanavalin A Binding Assay:

In preparation for con A binding experiments cell lines were grown on 60 mm tissue culture plates to a cell density of approximately 1.8×10^6 cells/plate. Cultures were cooled to 0° for 5 minutes prior to con A binding in order to reduce non-specific endocytosis (Noonan and Burger, 1973, 1974; Bornens et al. 1976). The cells were washed three times with an ice cold 0.154 M NaCl solution and incubated at 4° for five minutes in PBS containing the appropriate concentration of ^3H-

labelled con A in a final volume of 2.0 ml. The cells were then washed five times in ice-cold 0.154 M NaCl and solubilized in 1 ml of 10% triton X-100 for 60 min. at 37°. The digested samples were added to a cocktail (Aquasol) and counted on a scintillation counter. The amount of non-specific binding was determined by carrying out binding studies in the presence of 0.2 M methyl α-D mannoside. The quantity of binding in the presence of the inhibitor routinely averaged 5% of the binding that occurred in the absence of the hapten; this was considered non-specific binding and was routinely subtracted from all samples (Wright and Ceri, 1977a,b).

Concanavalin A Receptor Mobility:

Concanavalin A receptor mobility was measured as the ability of cells to aggregate fluorescent labelled con A into discrete aggregates or "caps" as previously described (Aubin et al. 1975; Ceri and Wright, 1978a,b). In brief, cells were added to glass coverslips at 5×10^5 cells/cm^2 and incubated overnight in growth medium at 34°. The medium was removed from plates and the cells were washed twice with PBS. They were then incubated at 4° for 30 minutes in buffer containing 60 µg/ml fluorescent con A. Excess label was removed and the cells were washed twice with PBS. Capping was allowed to take place by incubating

cells in PBS for one hour at 34°. Coverslips were mounted directly on slides to dry without prior fixing of cells. A Zeiss fluorescent microscope was used to examine the slides. The percentage of cells showing tight cap formation was routinely determined by scoring at least 200 cells of each cell type. Also experiments were repeated for each cell type at least three times.

In experiments where colchicine treated cells were assayed for capping, 10^{-5} M colchicine was added to the buffers at each stage of the capping experiment.

Protein Determinations:

Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Membrane Isolation Techniques:

Cell surface membranes were isolated using the aqueous two-phase polymer system of Brunette and Till (1971). Briefly this procedure involved washing suspension grown cells in PBS and resuspending the cell pellets in a hypotonic $ZnCl_2$ (1 mM $ZnCl_2$) solution for swelling. The swollen cells were then ruptured with a tissue homoginizer and the membranes and other large cell debris

were collected by low speed centrifugation. This pellet was then resuspended in a dextran-polyethylene glycol emulsion and the purified membrane preparations were removed from the interface that forms between the dextran and polyethylene glycol after centrifugation.

Cell Surface Labelling Studies

A. Labelling of Cell Surfaces by Metabolic Incorporation

Logarithmic suspension cultures of WT-Cl I, C^R-7 and RC^R-7 cells growing in a ∝-mem supplemented with 10% fetal calf serum were labelled by the addition of 2 μCi/ml [^{3}H]- glucosamine to C^R-7 and RC^R-7 cells for one generation period (Juliano et al. 1976; Ceri and Wright, 1978b). The cells were harvested by centrifugation and washed three times with PBS. Membrane fractions were isolated by the Burnette and Till method (1971) and the protein extracted in 0.0625 M Tris-HCl pH 6.8 containing 2% SDS, 10% glycerol, and 5% 2-Mercaptoethanol (Laemmli, 1970). Gel electrophoresis was carried out on 7.5% acrylamide gels, also by the method of Laemmli (1970). The gels were run at 4 ma/tube until the tracking dye reached the bottom of the tube. The gels were fixed overnight in 50% TCA, suspended for five minutes in a 10% ethanol solution and frozen on an aluminum gel slicer pre-cooled in an ethanol-dry ice bath. The gels were sliced in 1.5 mm

sections which were digested in counting vials by adding 0.2 ml H_2O_2 to each vial and incubating the sealed vials for three hours at 70°. The vials were then cooled and counted in a liquid scintillation counter after the addition of a toluene based cocktail (Ceri and Wright, 1978b). The ^{14}C channel was set at a pulse height of 100-1000 divisions (4.5% gain) and counted a ^{14}C standard with 60% efficiency. Tritium was counted at 50-1000 divisions (60% gain) with 39% efficiency.

B. Galactose Oxidase - [^{3}H]-Borohydride Labelling

Cell surface labelling of galactose and galactosamine residues was performed by the galactose oxidase - [^{3}H]-borohydride method (Gahmberg and Hakomori, 1973; Ceri and Wright, 1977b, 1978b). Exponentially growing suspension cultures containing approximately 2 x 10^5 cells/ml in ∝-mem + 10% filtered FCS were harvested by centrifugation and washed three times in PBS. Labelling was performed as described by Gahmberg and Hakomori (1973) with approximately 20 units of galactose oxidase (by definition one unit produced a Δ A425 of 1.0/min at pH 6.0 at 25° in a peroxidase and o-tolidline system (Gahmberg and Hakomori, 1973)). The labelling period at 37° was reduced to 1.5 hours. The cells were then washed and resuspended in 1 ml of PBS containing 1 mCi of tritiated sodium borohydride for 30 min. at room temperature. The cells were washed five times and the proteins extracted by the method of Laemmli (1970). In some cases membranes were isolated by the

Brunette and Till (1971 method prior to protein extraction. The various samples were subjected to electrophoresis, the gels were sliced and the radioactivity in the samples was determined by methods previously described (see metabolic labelling).

Lactoperoxidase-Catalyzed Iodination

Logarithmic suspension cultures of WT-C1 I and C^R-7 were harvested and washed twice in PBS prior to the labelling of tyrosine and histidine residues of surface proteins by the lactoperoxidase-catalyzed iodination procedure (Sefton et al, 1973; Ceri and Wright, 1978b). Iodination was carried out in 2.0 ml of PBS containing sodium iodide (5 nmol), lactoperoxidase (15 µg), glucose (10 µM) and Na^{125} I (100 µCi). Glucose oxidase (0.025 units; 1 µM of glucose oxidized per min at 25° per unit) was added to prime the reaction. The reaction was allowed to proceed for 10 min. at 25° after which time the reaction was stopped by diluting the reaction mixture with ice-cold PBS. The cells were washed three times, and the labelling of cell surfaces was assayed as previously described (see metabolic labelling).

Mannose Transferase Assay

Cells for mannose transferase assays were grown on 100 mm tissue culture plates in alpha medium supplemented with 10% FCS. Cell lines which were being directly compared were always grown to the same cell density. Cells were harvested with PBS containing 2 mM EDTA, washed with PBS, and diluted with hypertonic Tris buffer (20 mM Tris-HCl, pH 7.4) for swelling. The cells were lysed with a tissue homogenizer and following a low speed centrifugation step, membranes were separated into a crude membrane fraction by centrifugation at 100,000 x g for one hour (Krag and Robbins, 1977). The mannose transferase assay was carried out in 20 mM Tris-HCl buffer (pH 7.4) containing 0.2 mM MgCl2, 0.2 mM $MnCl_2$, 0.15 M NaCl, 10 µM UDP-N-Actylglucosamine, and 2 µM GDP-[^{14}C]- mannose (179 mCi/mM, 5 x 10^4 cpm). Approximately 200 µg of membrane protein was added to the assays which were incubated for the desired time period in a shaking water bath at 34^{o}. The reaction was terminated by the addition of 20 volumes of chloroform: methanol (2:1 by volume). The amount of [^{14}C]-mannose incorporated into lipid, lipid-oligosaccharide, and glycoprotein was determined after the separation of these three fractions on the basis of differential solubility (Behrens et al. 1971, Waechter et al. 1973). This involved

the extraction of the lipid fraction in the chloroform methanol (2:1) which was used to terminate the reaction. The resulting pellet containing the lipid-oligosaccharide and glycoprotein was then washed and further extracted with chloroform: methanol: water (1:1:0.3 v/v) to yield the lipid oligosaccharide and glycoprotein fractions. Both lipid-containing fractions were dried over a N_2 stream and counted in a toluene based cocktail. The glycoprotein fraction was solubulized in 10% SDS prior to counting in a toluene based cocktail. All samples were done in duplicate and the results were usually within $\pm$10%.

Sources of Material

Most biochemicals and all enzymes were purchased from Sigma Chemical Co., St. Louis and all radiochemicals, with the exception of ^{3}H-concanavalin A (0.5 mCi/mg lectin) which was obtained from New England Nuclear, were purchased from Amersham/Searle Ltd.

Cell Growth Material

Material	Source
alpha-minimal essential medium	Flow Labs, Rockville
fetal calf serum	GIBCO Ltd. Or Reheis Chemical Co. Denver

Penicillin G	GIBCO Ltd.
Streptomycin	GIBCO Ltd.
Brockway bottles	Brockway Glass Co.
Culture Plates (60, 100 mm)	Lux Sci. Corp.
Linbro cloning dish #IS-FS-96-TC	Linbro Chem. Co.
Cover Slips	Corning Glass Co.
Spinner Bottles	GIBCO Ltd.

Experimental Material

Material	Source
Acrylamide	Eastman Kodak Ltd.
Colchicine	BDH Labs, To.
Concanavalin A	Biochim. Cal.
^{3}H-Concanavalin A	New England Nuclear
Flourecein bound concanavalin A	Miles-Yeda
Ethyl methane sulfonate	Eastman Kodak
NCS Tissue solubilizer	Amersham/Searle
N,N'-methylene-bisacrylamide	Eastman Kodak Ltd.
Plant lectins: lentil lectsin, SBA, and WGA	Miles Yeda
PHA-P	Difco Chem
POP	Fisher Chem. Co.
POPOP	Fisher Chem. Co.

Sendai virus Lot #134-1	Connaught Labs, To.
Toluene	Fisher Chem. Co.
Triton X-100	Baker Chem. Co.
Trypan Blue	Matheson Coleman and Bell

RESULTS

RESULTS

Section 1 Selection of Independent Concanavalin A-Resistant and -Sensitive Cell Lines

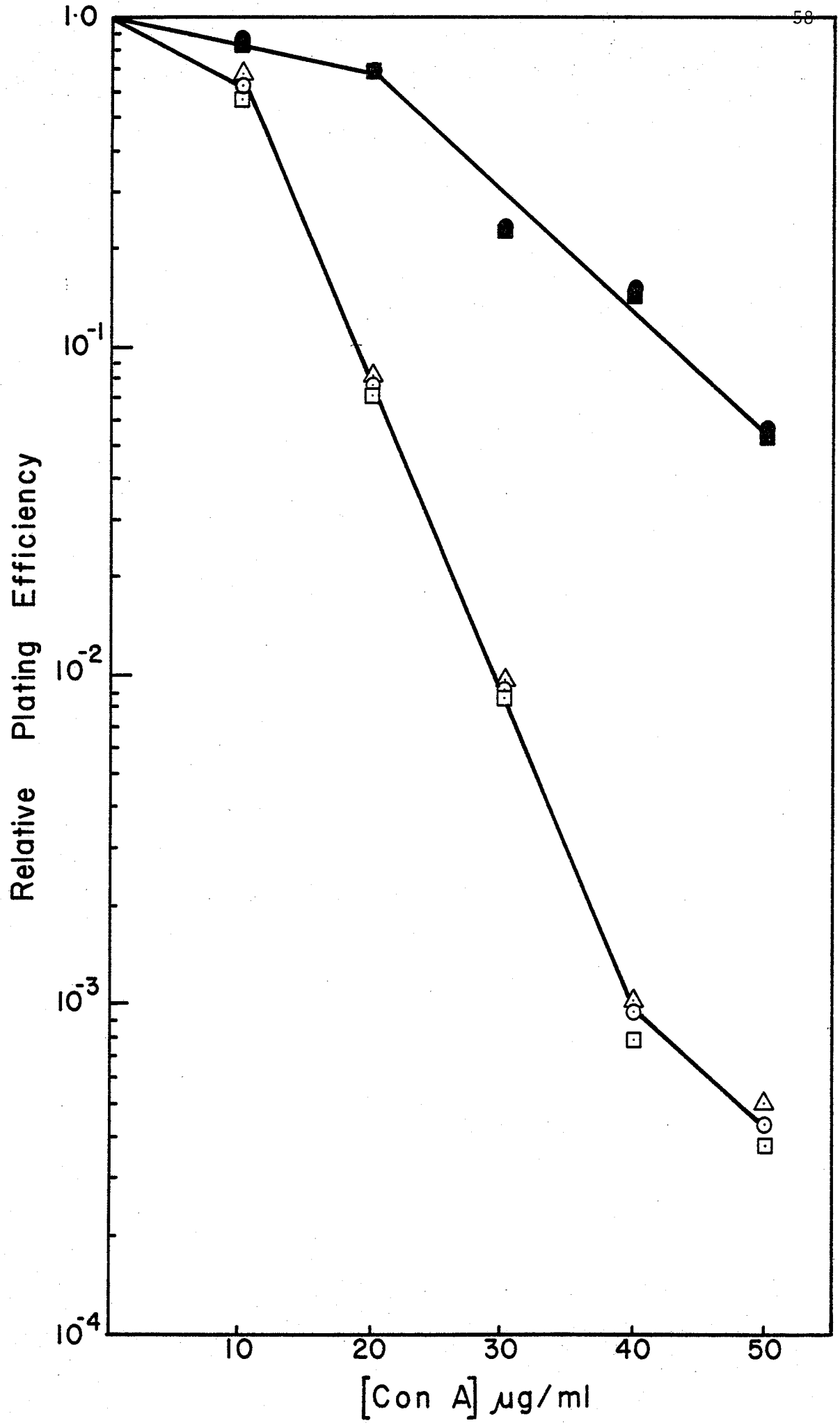
A. Selection of Independent Wild-Type Populations:

Independent variants, resistant to the cytotoxic effects of the plant lectin, con A, were required for a comparative analysis of the lectin-resistant phenotype. Therefore, three indepndent wild-type populations were isolated for variant selection by isolating independent clones of wild-type cells (see Methods section) from a heterogenous wild-type Chinese hamster ovary population, which had been in continuous culture for more than one year. Three clones were selected and designated wild-type clone 1 (WT-Cl I), wild-type clone 2 (WT-Cl II), and wild-type clone 3 (WT-Cl III).

The sensitivities of the three wild-type populations to con A were determined by standard plating techniques. Figure 1 shows the sensitivity of the three wild-type clones to con A to be nearly identical. The D_{10} value, which was defined as the drug concentration which reduces the plating efficiency of the cells to ten percent of control values, was found to be approximately 18 µg/ml con A for each of the three clones. If cytotoxicity of con A is dependent on the specific binding of the lectin

Figure 1 Effects of various concentrations of con A on the colony-forming ability of wild-type cell lines in the presence and absence of 10^{-2}M methyl ∝-D-glucose.

○, WT-Cl I; □ , WT-Cl II; △, WT-Cl III; ● , WT-Cl I + hapten; ■ , WT-Cl II + hapten.


1·0
10⁻¹
10⁻²
10⁻³
10⁻⁴
Relative Plating Efficiency
10
20
30
40
50
[Con A] µg/ml

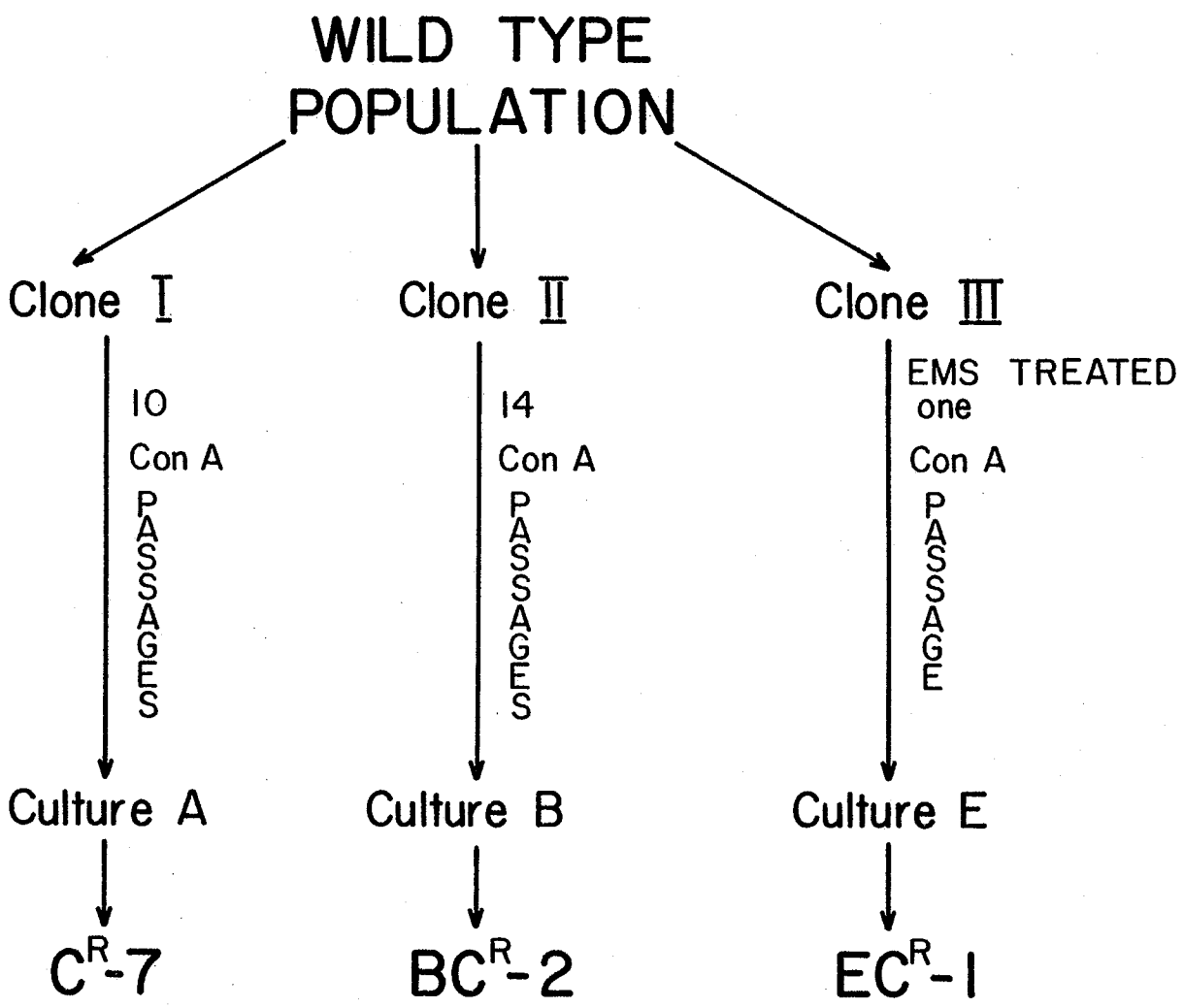
to ∝-D mannopyranosyl, ∝-D glucopysanosyl and other related sugars, then the addition of these sugars should reduce the cytotoxic effect of con A. The specificity of the drug cytotoxicity was demonstrated (Figure 1) using methyl ∝-D glucose (Wright, 1973a) to reduce con A killing.

B. Selecton of Concanavalin A-Resistant Variants:

Concanavalin A-resistant cell lines were selected from each of the three independent wild-type populations, as summarized in Figure 2.

The cyclic single-step method (Wright, 1973a, 1975; Ceri and Wright, 1977a, 1978a) was used to select lectin-resistant cell lines from WT-Cl I and WT-Cl II populations (Figure 2). Variant selection involved the following procedure: cells obtained from exponentially growing cultures of each clone were seeded at 2×10^6 cells in 16 oz Brockway bottles containing 40 ml growth medium with 40 µg/ml con A. The cells were incubated at 34^o for three to five days. The medium containing the lectin was then replaced with fresh medium lacking con A. The cells were allowed to recover and after about seven days they were removed with phosphate-buffered saline containing 2 mM ethylene-diaminetetraacetic acid, and were again incubated at a density of 2×10^6 cells/bottle

Figure 2 Summary of the selection procedures used to isolate three independent concanavalin A-resistant cell lines.


WILD TYPE POPULATION
Clone I
Clone II
Clone III
10 Con A PASSAGES
14 Con A PASSAGES
EMS TREATED one Con A PASSAGE
Culture A
Culture B
Culture E
C^R-7
BC^R-2
EC^R-1

in the presence of 40 ml of medium containing 40 µg/ml con A. This procedure of exposing the cells to medium containing lectin followed by a recovery period was performed four times. From this point on the cells were continually cultured in medium containing 40 µg/ml con A. Each time a monolayer formed the cells were removed and 2 x 10^6 cells were placed into medium containing 40 µg/ml lectin. The number of independent times the cells were exposed to medium containing con A was recorded. The selected cultures designated A and B were subcultured 10 and 14 times, respectively, (Figure 2) in the presence of con A. Thereafter, the cultures were kept in normal growth medium. Culture A was selected from WT-Cl I, while culture B was selected from WT-Cl II. The cell lines C^R-7 and BC^R-2 used in this study were cloned from cultures A and B respectively.

The WT-Cl III population was mutagenized with ethylmethane sulfonate (EMS) prior to the selection of a con A-resistant variant (Figure 2). Mutagenization was carried out by exposing 1 x 10^6 cells growing exponentially on a 100 mm tissue culture plate at 34^o to 300 µg/ml EMS. The treated cells were trypsinized, washed with PBS, re-suspended in fresh growth medium, and incubated at 34^o for 10 days to permit regrowth of surviving cells. Plating efficiencies performed on an aliquot of these cells

showed a fraction survival of colony forming ability of 0.2. The resulting mutagenized population was trypsinized and 1 x 10^6 cells were added to several 100 mm culture plates containing 25 ml of medium with 40 µg/ml con A. After the incubation in the presence of drug containing medium for twelve days one plate was observed to have a single colony. The lectin-containing medium was then replaced with fresh growth medium minus the drug and the cells were grown to a partial monolayer. The population was cloned and named EC^R-1.

The sensitivity to con A of the three independent con A-resistant cell lines was determined by plating experiments (Figure 3). The variants C^R-7, BC^R-2 and EC^R-1 appeared to be 2.5-fold more resistant to con A than were their wild-type parental lines; their D_{10} values were 45, 48 and 45 µg/ml con A as compared to 18 µg/ml for wild-type cell lines (Table I). Again, the specificity of con A cytotoxicity was demonstrated by the inhibitory effect of methyl α-D glucose (Figure 3).

An alternate procedure was used to analyze the sensitivity of cells to the cytotoxic properties of con A. Incubation of con A in growth medium containing serum results in the formation of a fine precipitate which can

Figure 3 Effects of various concentrations of con A on the colony-forming ability of con A-resistant cell lines in the presence and absence of 10^{-2}M of the hapten inhibitor methyl ∝-D glucose ●, C^R-7; ■, BC^R-2; ▲, EC^R-1; ⊙ C^R-7 + hapten ⊡ BC^R-2 + hapten. The dashed line represents the sensitivity of the wild-type population with the data taken from Figure 1.

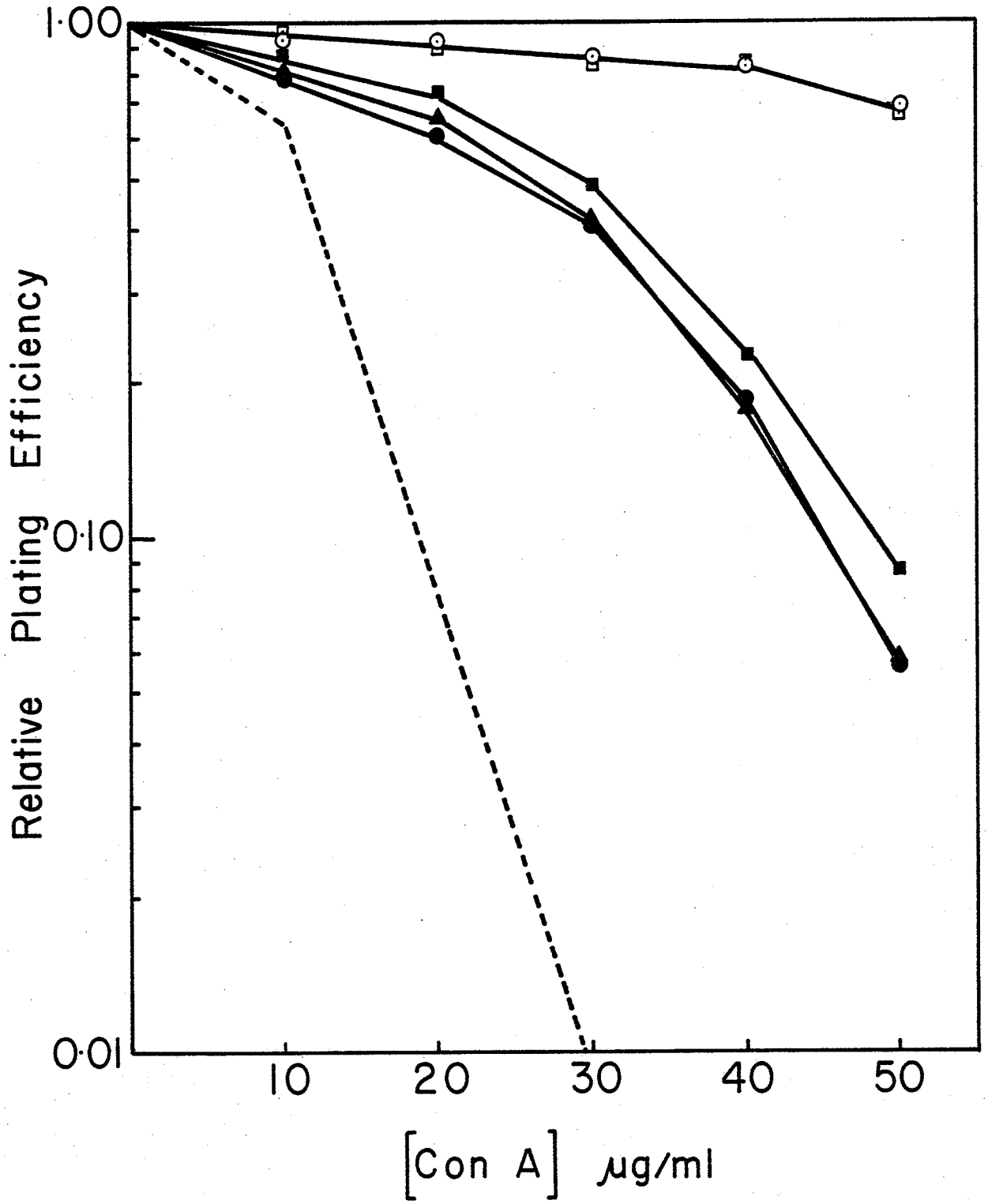

1·00
0·10
0·01
Relative Plating Efficiency
10
20
30
40
50
[Con A] µg/ml

TABLE 1

Concanavalin A Sensitivity of Wild-Type and Concanavalin A-resistant Cell Lines

Cell Line	D_{10} Value* (µg/ml con A)
WT-Cl I	18 ± 1.25
WT-Cl II	18 ± 1
WT-Cl III	18 ± 1
C^R-7	45 ± 3
BC^R-2	48 ± 4
EC^R-1	45 ± 3

*D_{10} value is defined as the concentration of drug which reduces the plating efficiency of cells to 10% of the value of control experiments. Data is derived from 5 plating experiments with each cell line.

be removed by centrifugation or filtration. Wright (1973a) has previously shown that when medium prepared at an initial concentration of 50 µg/ml con A was freed of precipitate and diluted to one-half its initial concentration, it had approximately the same effect on the plating efficiency of CHO cells as medium containing 25 µg/ml con A. It would appear, therefore, that the precipitate did not contain an appreciable proportion of con A. Furthermore, less than 5% of labelled con A added to 50 µg/ml cold con A was recovered in the resulting precipitate (Wright, 1973a). To test the possibility that the interaction between lectin and serum components may be responsible for the differential killing effect of con A for variant and wild-type cells, the cytotoxicity of con A was examined by a second procedure in the absence of serum (Ozanne and Lurye, 1974). WT-Cl I and C^R-7 cells were plated in complete growth medium for two hours. The medium was then replaced with medium minus serum but containing appropriate concentrations of con A. The plates were then incubated at 34^o for 48 hours. The medium containing drug was then removed from plates, fresh medium plus serum was added back to the plates, and the plates were incubated at 34^o to allow the growth of surviving cells. After eight

to ten days the resulting colonies were stained with methylene blue and counted. Higher concentrations of lectin were required to cause a significant reduction in colony forming ability of both wild-type and variant cells (Table II); this was probably due to the short exposure time to con A (48 hours). Longer exposure times were not possible because survival in medium lacking serum decreases dramatically after 48 hours. The C^R-7 population was found to be less sensitive to con A than WT-Cl I cells when assayed in this manner (Table II). WT-Cl I cells plated with an efficiency of about 1% after exposure to 100 µg/ml con A, whereas the plating efficiency of C^R-7 was not significantly affected by treatment with the same lectin concentration. This experiment suggests that serum components are not required for the cytotoxic action of the lectin. Furthermore, these results indicate that the small amount of serum removed as a precipitate during the preparation of medium supplemented with con A in the first method used to determine con A sensitivity (Figures 1 and 3) may not be important in the cytotoxic action of the lectin.

The stability of the con A-resistant phenotype was tested by maintaining the variant in continuous culture without exposure to the selective agents. At selected time intervals the plating efficiency of the variant was determined and compared to the plating efficiency at the

TABLE II

The sensitivity of WT-CL I and C^R-7 cells to the presence of con A in the absence of serum.

Concentration of lectin	PE* of WTCl I	PE of C^R-7
50 µg/ml	0.8	0.98
100 µg/ml	0.023	0.95
200 µg/ml	0.0014	0.60
300 µg/ml	0.0006	0.21

*PE or plating efficiency (see Methods section).

time of selection. Little change in the resistance of the variants was seen over an 18 month test period, (Table III). For example, C^R-7 which showed a relative plating efficiency of about 40% in 30 μg/ml con A when first selected showed relative plating efficiencies of 40%, 38% and 42% after 2, 12 and 18 months of continuous culture in the absence of con A.

Concanavalin A-resistant cells have previously been reported to be present in a wild-type CHO population at a frequency of approximately $1:10^5$ (Wright, 1973a, Stanley and Sminovitch, 1976). Wright (1973a) found that the mutagen, MNNG had little effect on the frequency of appearance of con A-resistant cells in a population. He also reported (Wright, 1975) that the ability to form colonies in medium containing con A significantly increased with increased exposure time to the lectin; as a gradual increase in the number and degree of resistant cells in the population probably occurs during the selection procedure. The mutagen EMS was shown to increase the mutation frequency of several other lectin-resistant cell lines (Stanley and Siminovitch, 1976). The effect of EMS on the frequency of con A-resistant cells in a population was determined by comparing the rate at which mutagenized and control cultures of WT-Cl III acquired con A-resistance in the cyclic single

TABLE III

Relative Plating Efficiences of Concanavalin A-resistant Cells After Periods of Time Without Exposure to Concanavalin A.

Cell Line	Time in Culture (months)	RPE* (30µg/ml con A)	RPE (40µg/ml con A)
C^R-7	0	40.5	18.0
C^R-7	2	40.0	16.0
C^R-7	12	39.0	18.5
C^R-7	18	41.6	17.2
BC^R-2	0	47.5	23.0
BC^R-2	6	48.8	22.1
BC^R-2	18	47.0	23.0
EC^R-1	0	42.4	18.0
EC^R-1	6	43.2	17.1
EC^R-1	18	42.0	18.3

*RPE or relative plating efficiency (see Methods section)

step selection procedure (Wright, 1975). In agreement with the previous study by Wright (1975) con A resistance in the WT-Cl III population was found to increase gradually with successive passages in the presence of lectin (Figure 4). The D_{10} value increased from 18 µg/ml before lectin exposure to 24, 31 and 45 µg/ml con A after 3, 5 and 7 passages respectively in con A. The EMS treated WT-Cl III population showed a very rapid increase in con A-resistance (Figure 5). For example, after three passages in con A the D_{10} value for EMS treated WT-Cl III was 42 µg/ml as compared to 24 µg/ml con A found with nonmutagenized control cells after the same number of passages in the lectin. The final D_{10} value obtained by WT-Cl III (45 µg/ml con A) after seven passages in the presence of the lectin was similar to the value observed with EMS treated WT-Cl III (42 µg/ml) after approximately three passages. Since significantly more passages in the presence of the lectin was required for the nonmutagenized cells to reach the same level of resistance that was achieved by the EMS treated population, it appears that EMS treatment increases the frequency of lectin-resistant cells in the population. This conclusion is also supported by the finding that the EMS treated population showed a small but significantly higher plating efficiency

Figure 4 The plating efficiency of WT-Cl III in varying concentrations of concanavalin A after successive passages in 40 µg/ml con A. △, no lectin exposure; ○, 3 passages in lectin; □, 5 passages in lectin ◇, 7 passages in lectin. The data presented is typical of that obtained in three separate experiments.

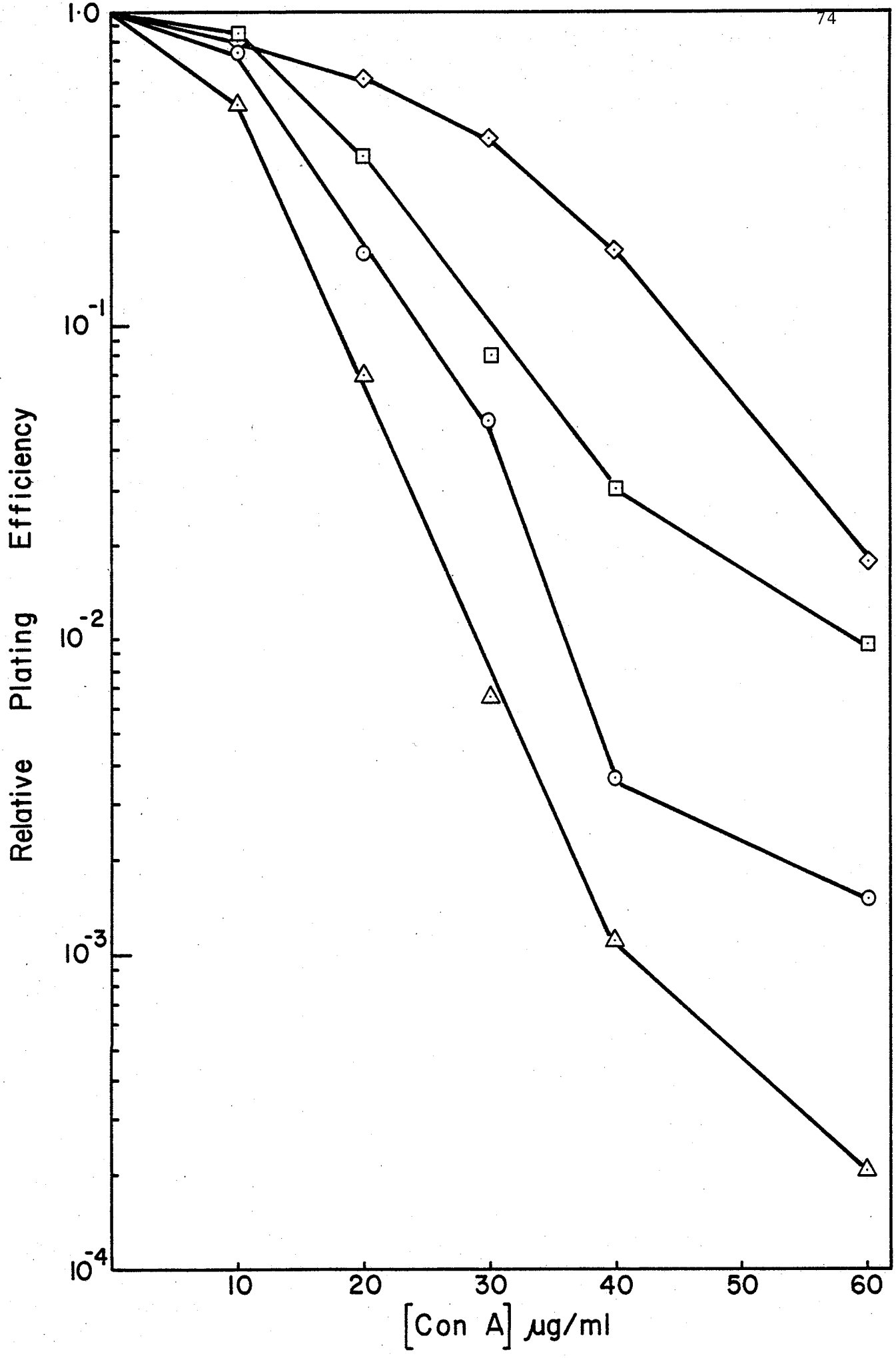
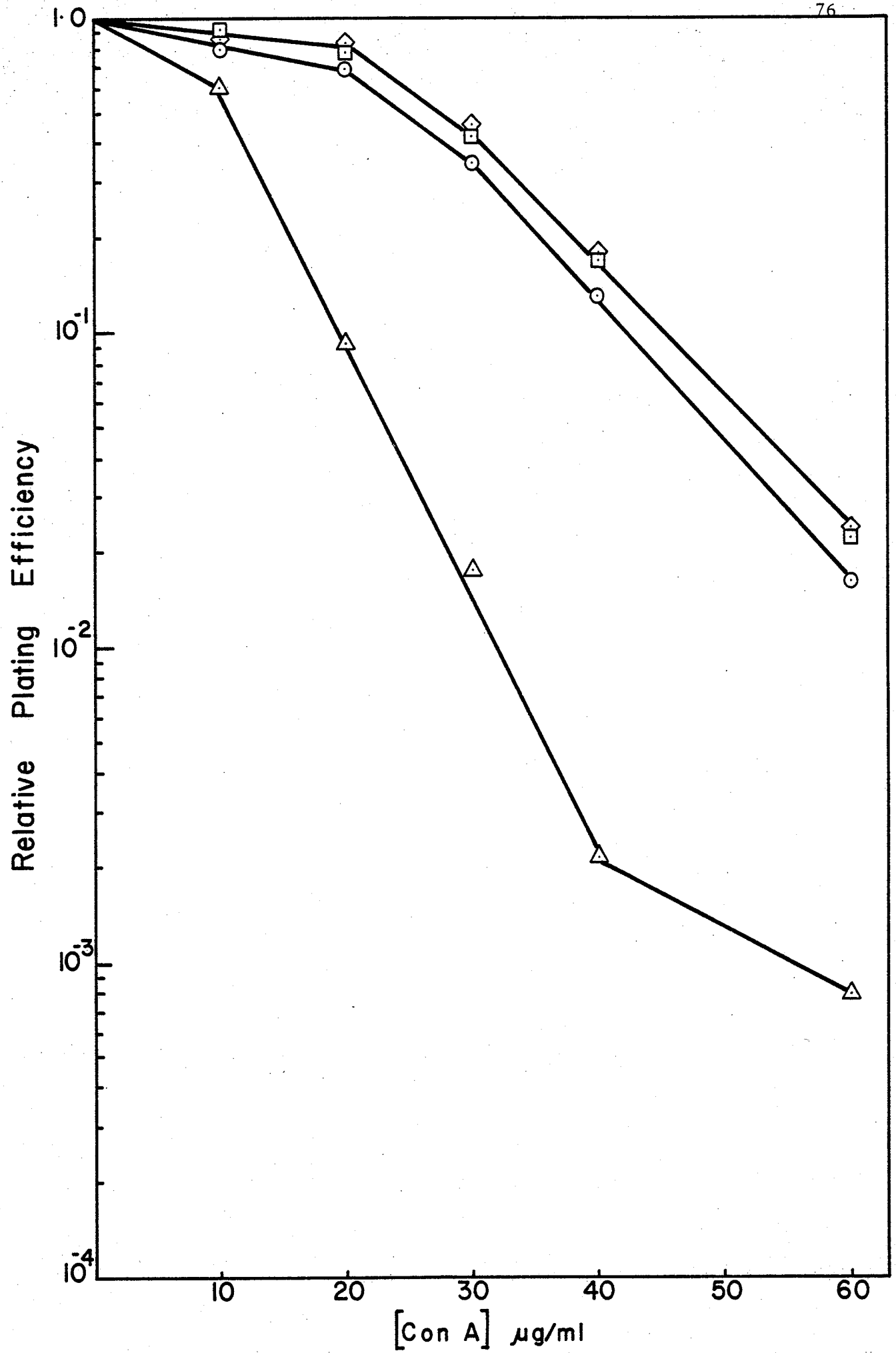

1·0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Relative Plating Efficiency
10
20
30
40
50
60
[Con A] µg/ml

Figure 5 Plating efficiency of EMS treated WT-Cl III in varying concentrations of concanavalin A after successive passages in lectin.

△, no lectin exposure; ⊙, 3 passages in lectin; ⊡, 5 passages in lectin; ◈, 7 passages in lectin. The data presented is typical of that obtained in three separate experiments.


1·0
10⁻¹
10⁻²
10⁻³
10⁻⁴
Relative Plating Efficiency
10
20
30
40
50
60
[Con A] µg/ml

(approximately 2.5-fold) than nonmutagenized cells even before any exposure to con A occurs (Figures 4 and 5).

C. Analysis of the Karyotype of Concanavalin A-Resistant Lines:

The con A-resistant variants were examined for the mean number of chromosomes per cell by arresting cells in mitosis with colchicine, staining the chromosomes, and examining chromosome squashes with the use of photomicroscopy techniques (see Methods section). The three variants and the wild-type cell lines (Cl I, Cl II and Cl III) were found to have a mean chromosome number of 21 (Figure 6). This result agrees with the pseudo-diploid chromosome number reported for CHO cells (Puck et al.1958). Chromosome squashes of WT-Cl I and C^R-7 cells are presented in Figure 7.

D. Growth Properties of Concanavalin A-Resistant Cell Lines:

Wright (1973a, 1975) had previously shown that con A-resistant cell lines possessed temperature sensitive (ts) growth properties. The variant lines C^R-7, BC^R-2 and EC^R-1 were tested for ts growth properties by shifting plates containing approximately 10^5 cells per plate from 34^o to the desired growth temperature. The respective wild-type controls were treated in an identical manner. Duplicate samples of each cell line were

Figure 6 Chromosome number distribution of WT-Cl I, C^R-7, BC^R-2 and EC^R-1 cell lines. Similar distributions were obtained with WT-Cl II and WT-Cl III.

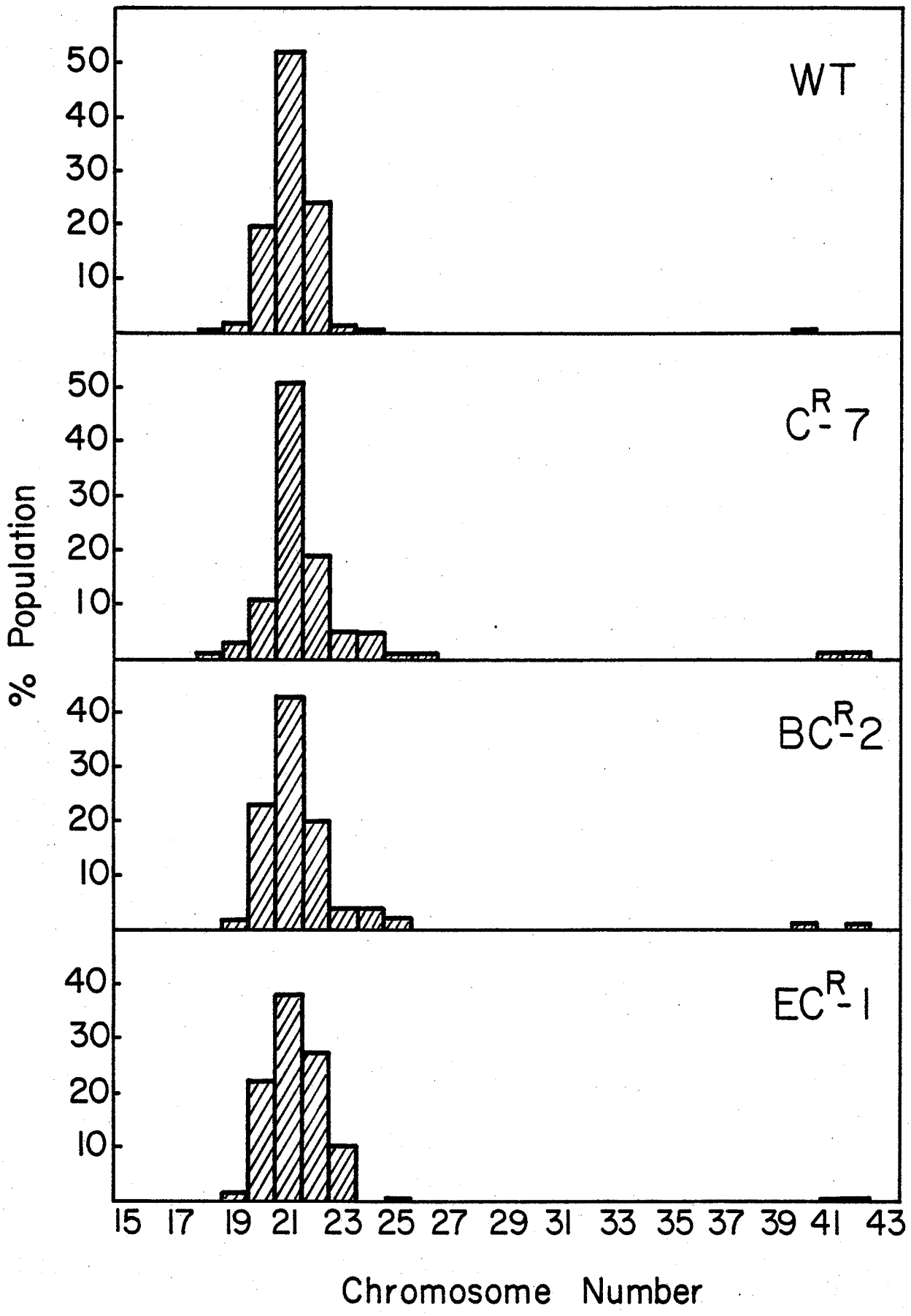
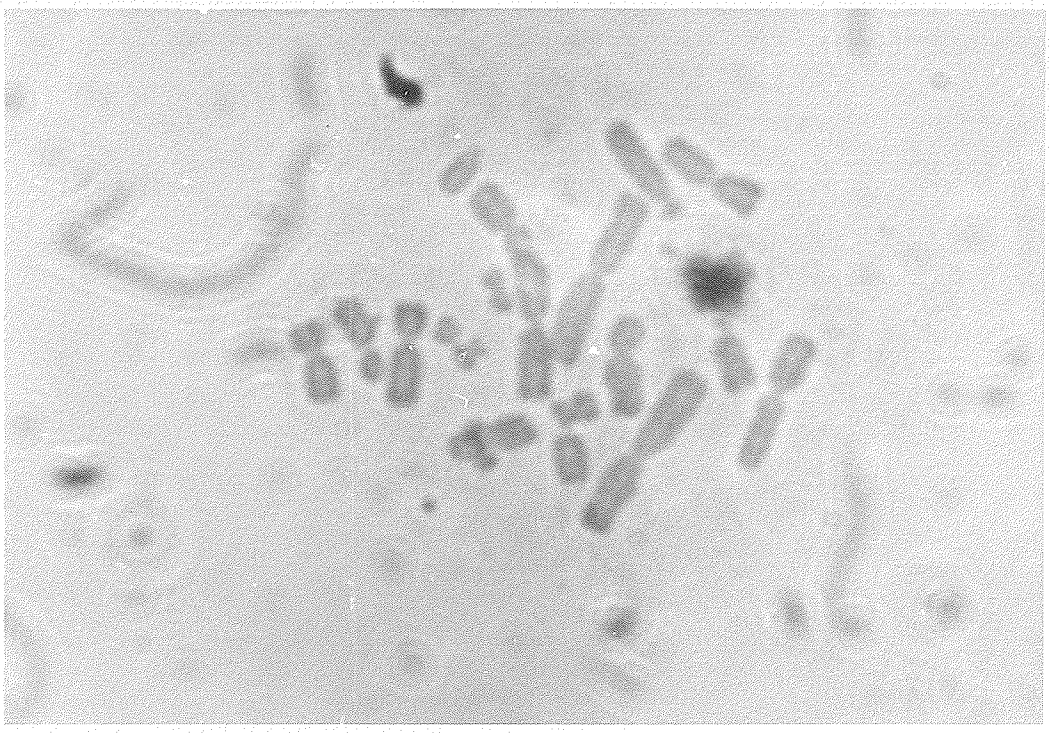
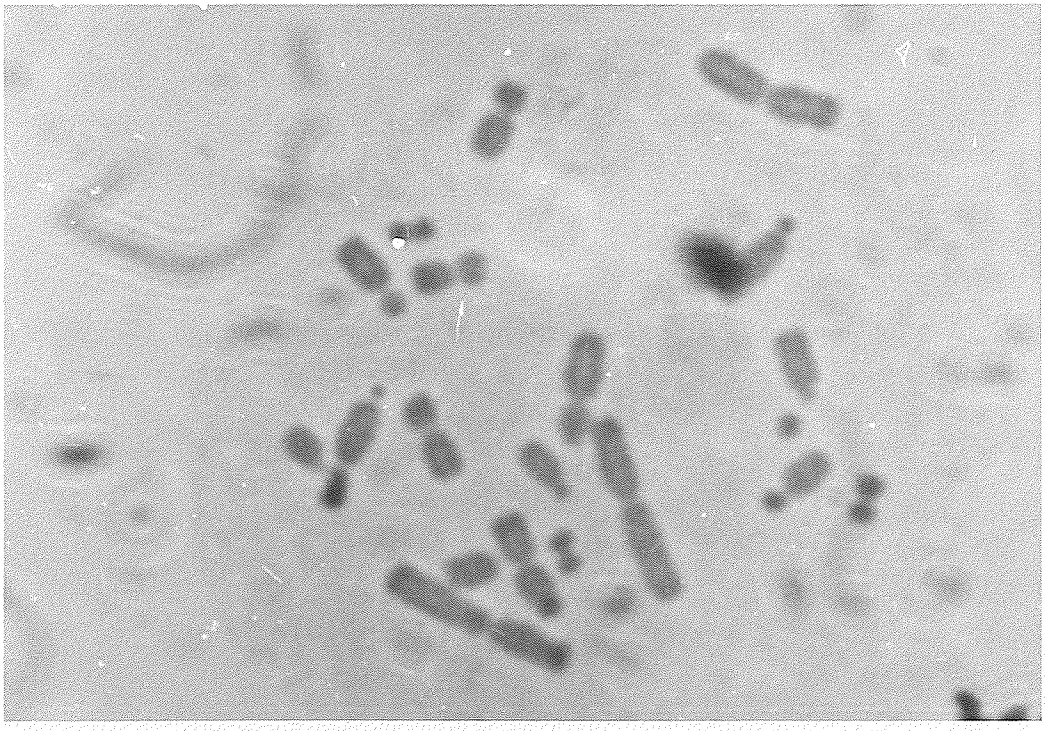

WT
C^R-7
BC^R-2
EC^R-1
% Population
50
40
30
20
10
15 17 19 21 23 25 27 29 31 33 35 37 39 41 43
Chromosome Number

Figure 7 Aceto-orcein stained metaphase cells of WT-Cl I and C^R-7.





trypsinized, diluted with isotonic saline, and counted in a cell counter (Coulter Electronics) at specific time periods. All cell lines showed good growth at 34° (Figure 8) with doubling times of 18 to 24 hours. The doubling times were observed to decrease at 37° for all cell lines. Growth rates ranged from 15 hours for wild-type cells to 22 hours for EC^R-1 cells. The doubling time for wild-type cells at 39° was approximately the same as that seen at 37°. The con A-resistant lines however, showed a marked inability to proliferate at 39°. The variant cells increased slightly in cell number within the first 24 hours (BC^R-2) to 36 hours (C^R-7, EC^R-1) of incubation and then ceased.

Temperature sensitive growth in suspension cultures was also measured with WT-Cl I and C^R-7 cells. WT-Cl I and C^R-7 cells doubled in 22 and 26 hours respectively at 34° (Figure 9). The doubling time of WT-Cl I decreased to 18 hours at 39°. The C^R-7 population increased only slightly during the first 24 hours at 39°, after which time a decrease in cell number was observed. Trypan Blue dye exclusion tests revealed that greater than 90% of WT-Cl I cells kept at 39° for 48 hours were viable while only 1% of C^R-7 cells treated in the same manner excluded the dye and were considered to be viable cells.

These results indicate that the variants exhibit ts

Figure 8 Growth curves for WT, C^R-7, BC^R-2 and EC^R-1 cell lines at 34, 37 and 39°.

□, 34°; △, 37° and ◎ 39°.

The WT growth curve is representative of data obtained in numerous growth curves with all three wild-type cell lines.

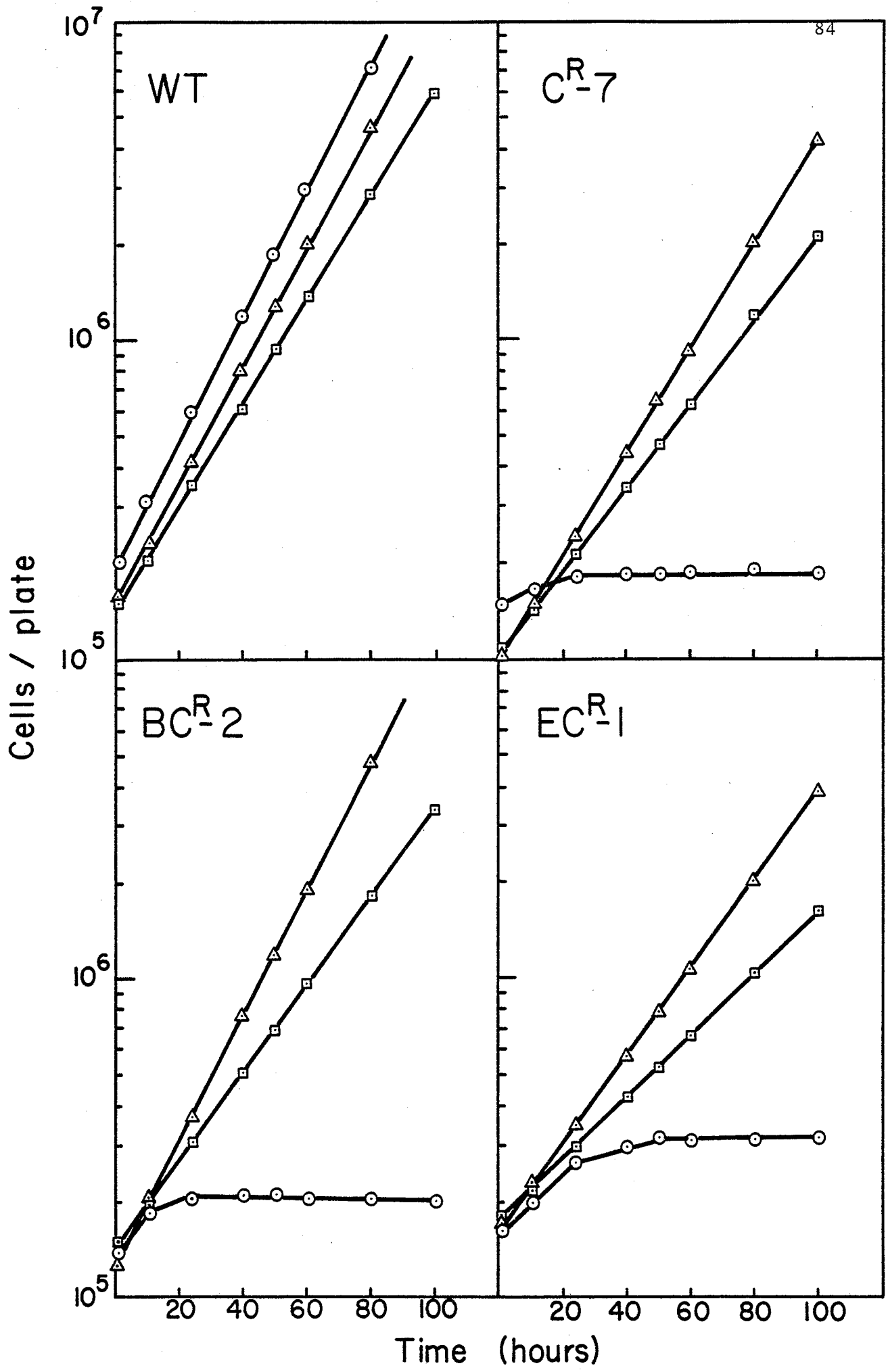
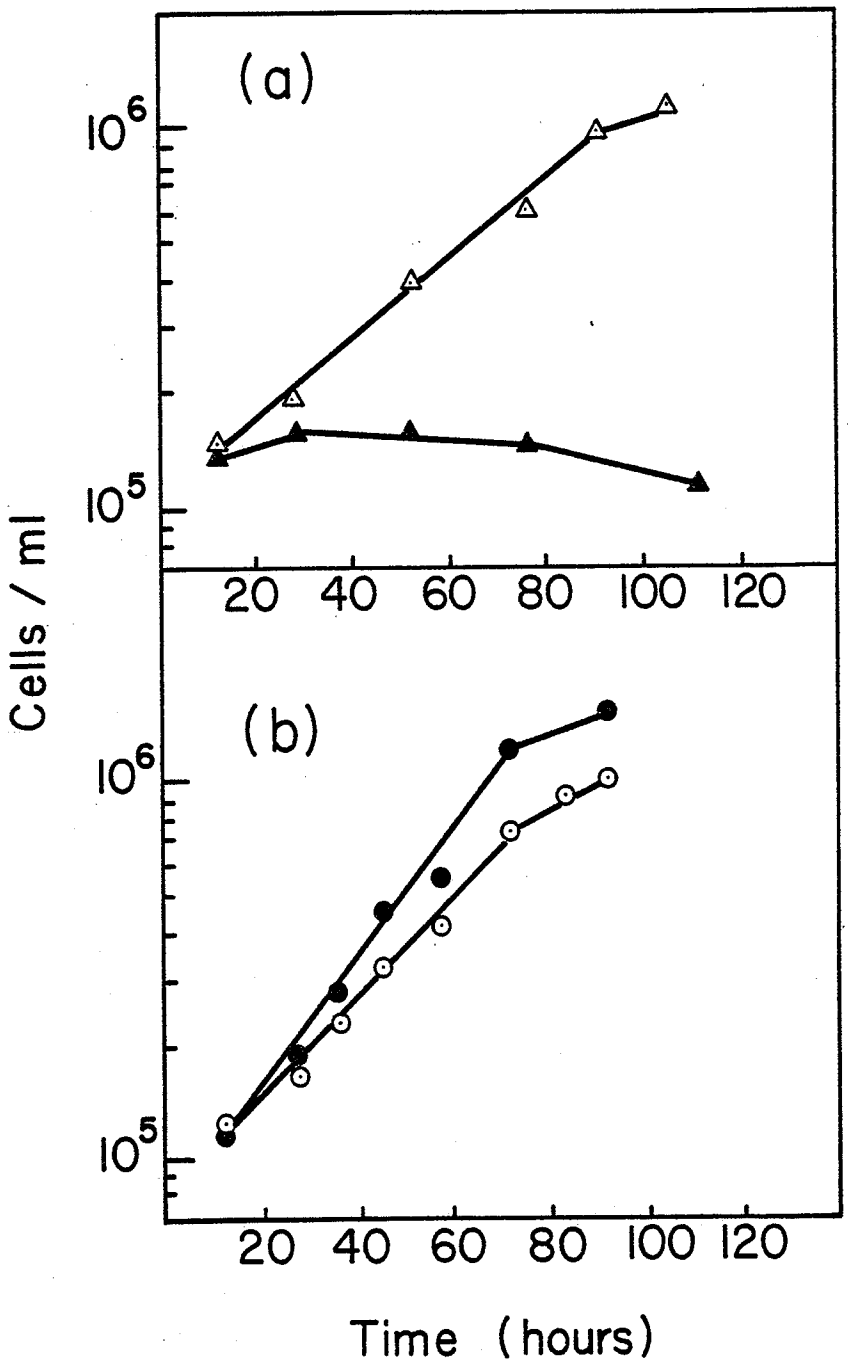

WT
C^R-7
BC^R-2
EC^R-1
Cells / plate
Time (hours)
10^7
10^6
10^5
20
40
60
80
100

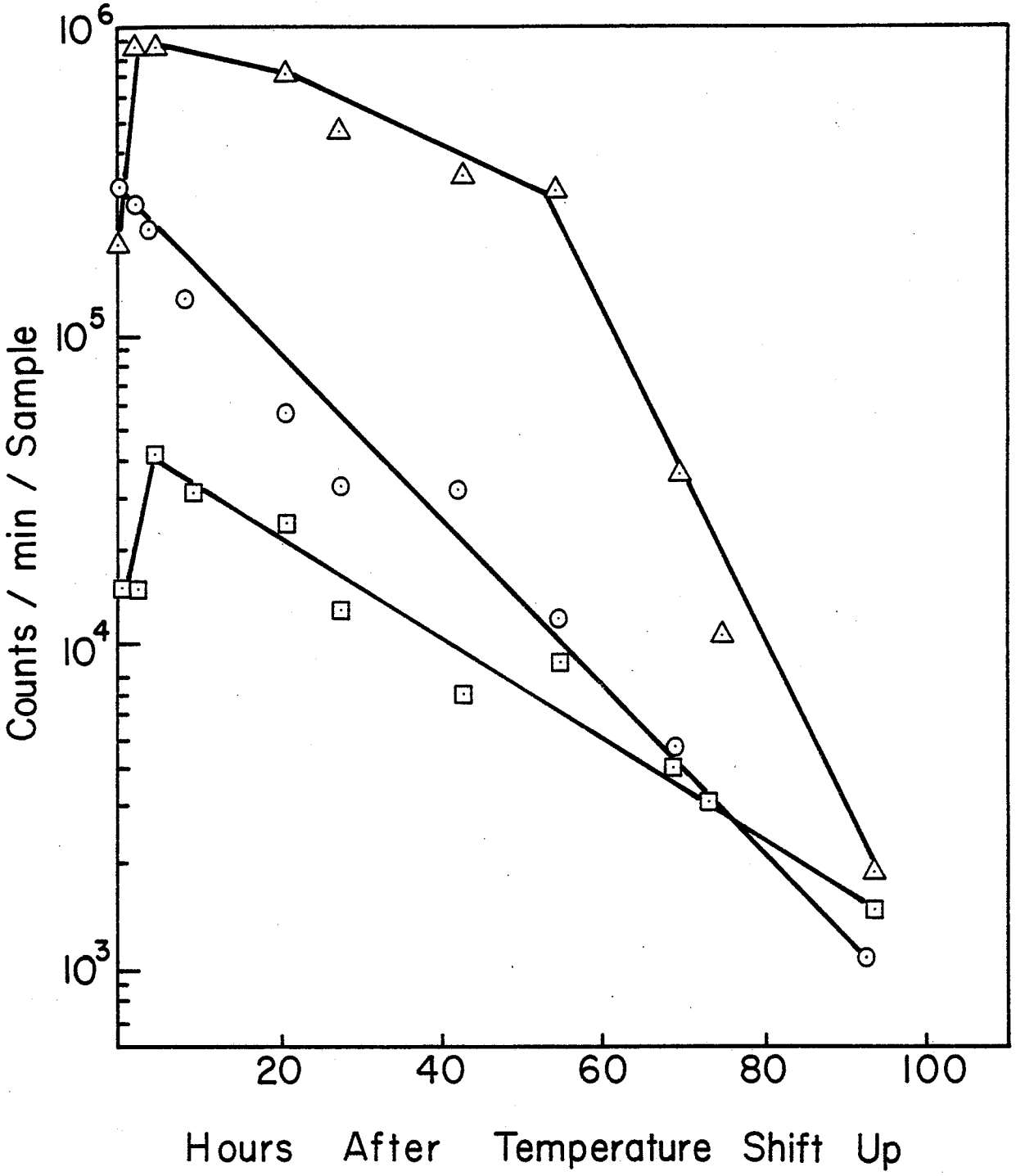
Figure 9 Growth in suspension culture of WT-Cl I and C^R-7 cell line.

△, C^R-7 at 34°; ▲ C^R-7 at 39°; ○, WT-Cl I at 34°; ●, WT-Cl I at 39°.


(a)
10^6
10^5
20 40 60 80 100 120
(b)
10^6
10^5
20 40 60 80 100 120
Cells / ml
Time (hours)

properties. The ts property of C^R-7 cells was further characterized by assaying the ability of the cell line to synthesize DNA, RNA, and protein at the non-permissive temperature. The amount of labelled precursor incorporated during a 15 minute pulse was measured in aliquots of cells taken from a spinner of C^R-7 cells shifted to 39^o. Figure 10 indicates that the temperature change produced increases in the rate of deoxythymidine (TdR) and leucine incorporated within the first few hours, followed by a marked decline in the incorporation of these precursors over a period of about four days. Incorporation of uridine declined immediately after the temperature shift. In control experiments with wild-type cells, increased rates of TdR, uridine, and leucine incorporation were observed during the first two to four hours after temperature shift-up, followed by a constant rate of precursor incorporation/cell during the four day experiment. The WT-Cl I cells showed normal doubling rates over this time span. These results suggest that the amount of labelled precursor incorporated into macromolecules by the C^R-7 population after shift up declines and resembles the reported properties of other ts mammalian cell variants maintained at non-permissive temperatures (e.g. Thompson et al. 1971).

Figure 10 Incorporation of TdR, uridine, and leucine into acid precipitable material by C^R-7 cells after shift-up to the non-permissive temperature (39°). △, TdR; ⊙, uridine, ⊡, leucine. The points represent the mean of duplicate samples from a single experiment. Similar data was obtained in two other experiments.


10^6
10^5
10^4
10^3
Counts / min / Sample
20
40
60
80
100
Hours After Temperature Shift Up

The ability to form colonies on a solid surface at 34^{o} and 39^{o} was determined by plating cells at various cell numbers in normal growth medium on 60 or 100 mm tissue culture plates and in 16 oz. Brockway bottles. The colonies were stained after ten days of growth and those containing 50 or more cells were counted (see Methods section). At permissive temperatures 76% of WT-Cl I and 81% of WT-Cl II cells formed colonies while 70% of C^{R}-7 and 62% of BC^{R}-2 cells were able to form colonies (Table IV). Both wild-type lines plated with greater than 80% efficiency at 39^{o}. The variants, however, showed a dramatic reduction in colony forming ability at the non-permissive temperature. C^{R}-7 cells were found to be more sensitive to the non-permissive temperature than were BC^{R}-2 cells (Table IV). A cell density effect on cell plating was also observed in both cell lines. C^{R}-7 cells, for example showed a plating efficiency that ranged from approximately 2×10^{-5} when 10^{5} cells were plated to 4×10^{-4} cells when 5×10^{6} cells were laid down. EC^{R}-1 also showed ts plating efficiencies (Table IV); however the ts property exhibited by this line was not characterized in the same detail as the other two variant lines. Figure 11 shows the colony forming ability of C^{R}-7 at 34 and 39^{o}.

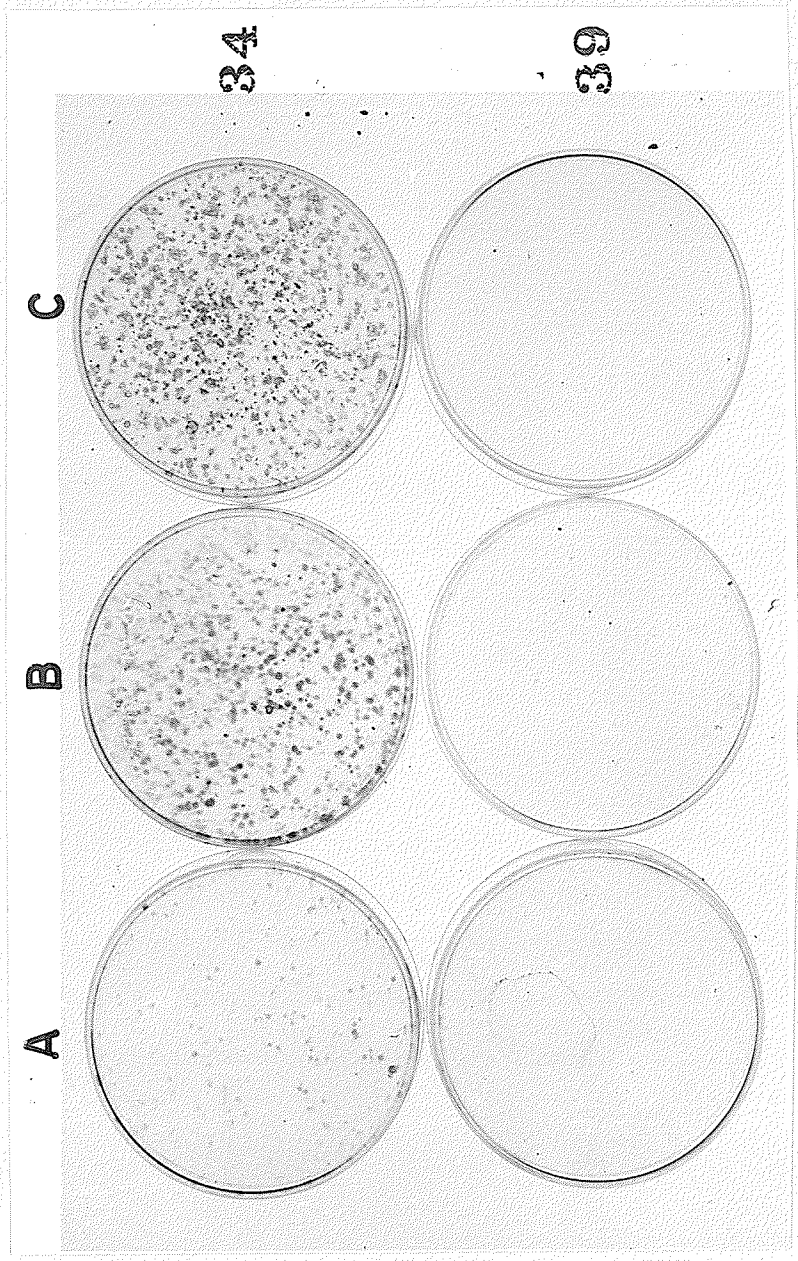
TABLE IV

Plating Efficiency at 34 and 39° at Various Cell Concentrations

Cell line	No of cells laid down	# of col @ 34	P.E. @ 34	# of col @ 39	P.E. @ 39
WT-Cl I	1×10^3	762	0.76	810	0.81
WT-Cl II	1×10^3	815	0.82	843	0.84
C^R-7	1×10^3	701	0.71	None	-
C^R-7	1×10^4	ND	ND	None	-
C^R-7	1×10^5	ND	ND	2	2×10^{-5}
C^R-7	5×10^5	ND	ND	38	7.6×10^{-5}
C^R-7	1×10^6	ND	ND	105	1.1×10^{-4}
C^R-7	5×10^6	ND	ND	2260	4.5×10^{-4}
BC^R-2	1×10^3	623	0.62	None	-
BC^R-2	1×10^4	ND	ND	None	-
BC^R-2	1×10^5	ND	ND	10	1×10^{-4}
BC^R-2	5×10^5	ND	ND	410	8.2×10^{-4}
BC^R-2	1×10^6	ND	ND	1231	1.2×10^{-3}
EC^R-1	1×10^3	720	0.72	None	-
EC^R-1	1×10^5	ND	ND	45	4.5×10^{-4}

ND - not determined

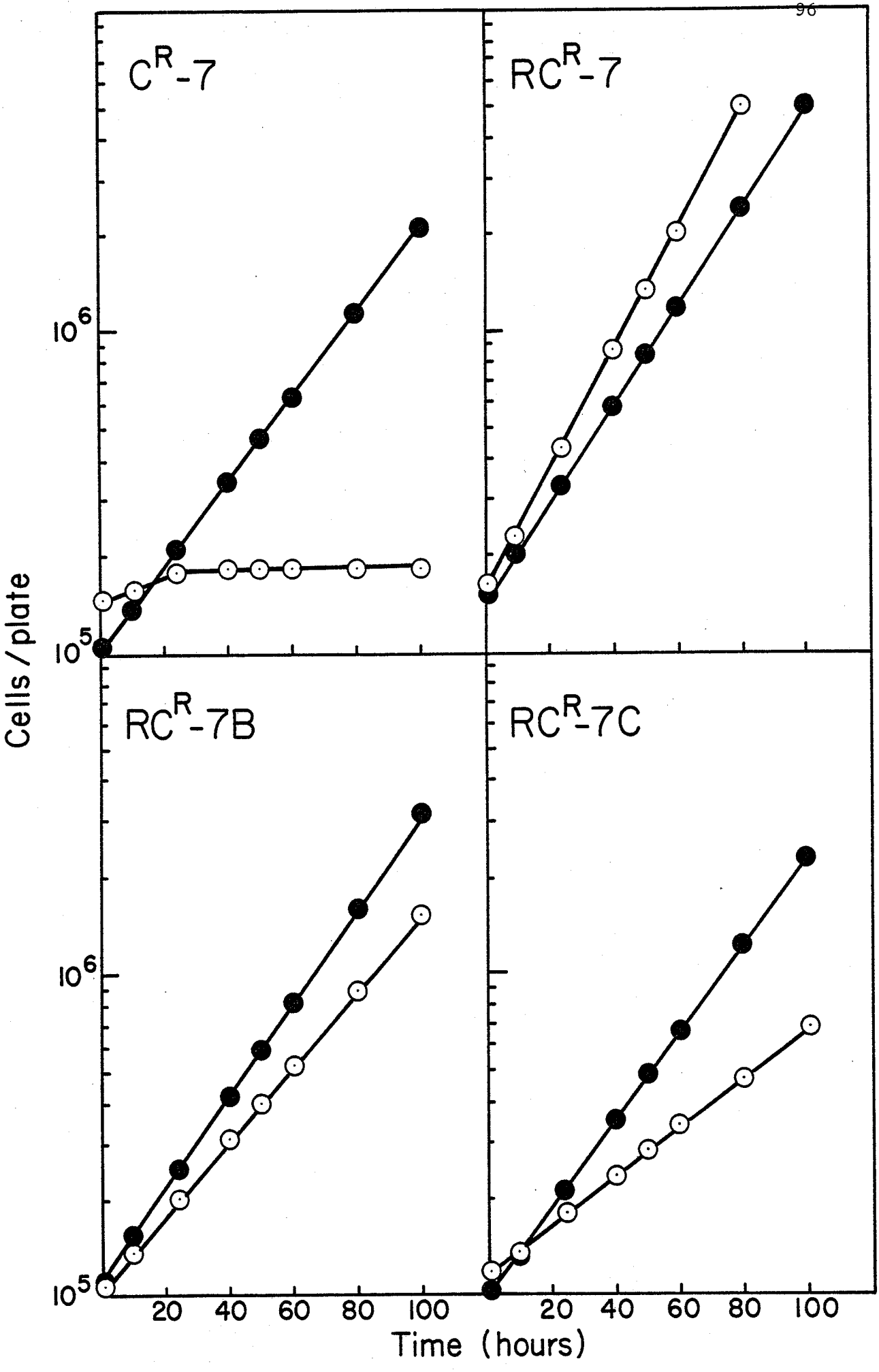
Figure 11 Plating of C^R-7 cells at 34 and 39^o.
A) 2×10^2 cells B) 2×10^3 cells c) 2×10^4 cells.


C
B
A
5
5
4
5

E. Isolation of Concanavalin A-Resistant Revertants

Concanavalin A-resistant revertants were selected from the C^R-7 population by taking advantage of the ts growth properties of this cell line (Ceri and Wright, 1977a). This was accomplished by incubating 2 x 10^6 C^R-7 cells on Brockway bottles at the non-permissive temperature. Colonies appeared after approximately 14 days at 39°. A portion of the cells from some of these colonies were removed with sterile Pasteur pipettes, added to 60 mm tissue culture plates, and grown to monolayers at 39°. The plates were used to seed Brockway bottles, which were maintained at 39°. Cloned lines designated RC^R-7, RC^R-7b, and RC^R-7c were selected from these populations (see Methods section). The ts revertant cell lines were then cultured at 34°. The growth properties of the three revertant clones were compared to the parental C^R-7 population (Figure 12). The RC^R-7 cell line showed a doubling time at 34° of 19 hours. The doubling time for RC^R-7 decreased to 17 hours at 39°. The cell lines RC^R-7B and RC^R-7C showed growth properties similar to RC^R-7 at 34°, however the doubling times of these cell lines were found to increase when incubated at the non-permissive temperature. RC^R-7B and RC^R-7C were able to proliferate at the non-permissive temperature but their growth rates were reduced when compared to the parental wild-type cells.

Figure 12 Growth curves of C^R-7, RC^R-7, RC^R-7B and RC^R-7C at 34 (●) and 39° (⊙).


C^R-7
RC^R-7
RC^R-7B
RC^R-7C
Cells / plate
10^6
10^5
20
40
60
80
100
Time (hours)

Wright (1975) has previously shown a correlation between the degree of con A-resistance and the ts growth property of variant cell lines. The revertant cell lines were tested by standard plating techniques to determine if the loss of the ts growth property was accompanied by a change in con A resistance. RC^R-7 was more sensitive to the cytotoxic effects of the lectin than was the parental variant line C^R-7(Figure 13). The D_{10} value for RC^R-7 (Table V) was 25 µg/ml con A which was intermediate between the D_{10} values of wild-type cell lines (18 µg/ml con A) and the variant C^R-7 cell line (45 µg/ml con A). The RC^R-7B and RC^R-7C cell lines were considerably more resistant to con A than were RC^R-7 cells (Figure 13). The D_{10} values approached those of con A-resistant cell lines and were approximately twice the values found with wild-type cell lines.

F. Somatic Cell Hybrids

Somatic cell hybrids were formed through the fusion of wild-type clone I or C^R-7 cells with an auxotrophic CHO cell line (McBurney and Whitmore, 1974) containing an ouabain resistant marker ($AUXB_1$-O^R) (Wright and Ceri, 1977b; Ceri and Wright, 1978a; see Methods section). The hybrid A-W was formed through the fusion of two con A-sensitive cell lines, WT-Cl I and $AUXB_1$-O^R (see Methods

Figure 13 Plating efficiencies of WT-Cl I (⊙), C^R-7 (●), RC^R-7 (◈), RC^R-7b (⊡) and RC^R-7C (△) in varying concentrations of con A.

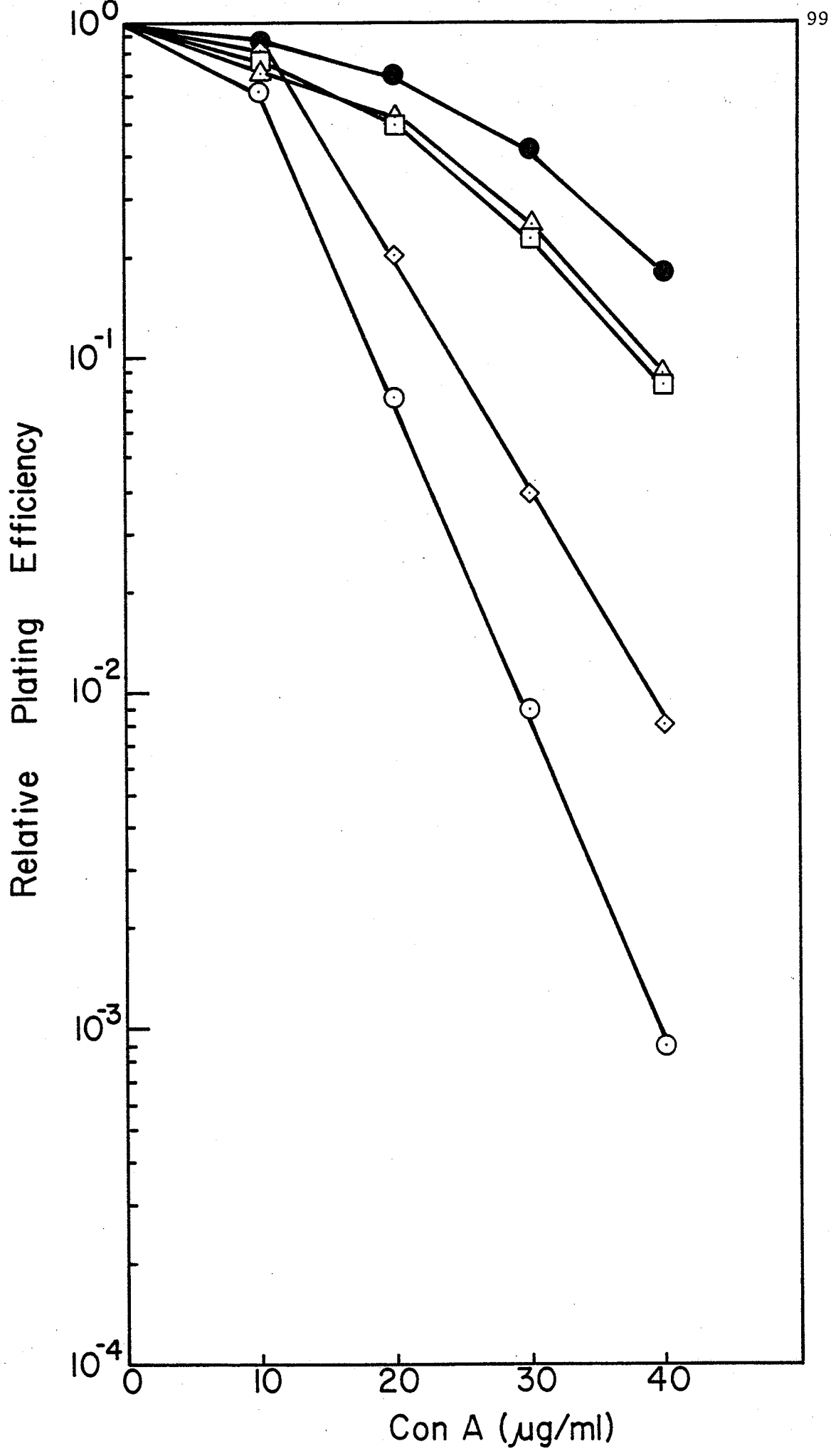

Relative Plating Efficiency
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0
10
20
30
40
Con A (μg/ml)

TABLE V

D_{10} Values* of Concanavalin A-Sensitive and Concanavalin A-Resistant Cell Lines.

Cell Line	D_{10} (µg Con A/ml)
Con A-Sensitive Lines	
WT-Cl I	18
WT-Cl II	18
WT-Cl III	18
RC^R-7	25
A-W	18
A-7	18
A-7B	25
A-7C	20
Con A-Resistant Lines	
C^R-7	45
BC^R-2	48
EC^R-1	45

* Values were obtained from a minimum of five killing curves done on each cell line.

section). It is apparent from Figure 14 that the A-W hybrid was approximately as sensitive to the toxic action of con A as were the pseudodiploid wild-type cells. Also, three other hybrid lines (A-7, A-7B, A-7C) were independently selected through the fusion of C^R-7 cells with the lectin-sensitive $AUXB_1$-O^R cell line; these hybrid lines were approximately as sensitive to the presence of con A as were the A-W hybrid cells and the pseudodiploid wild-type cells (Figure 14). The D_{10} values of con A for A-7, A-7B, and A-7C (Table V) were estimated to be 1.0, 1.4, and 1.0 times the value observed with either A-W hybrids or with wild-type cell lines. Therefore in agreement with other studies (Stanley et al. 1975c; Stanley and Siminovitch, 1976; Ceri and Wright, 1978a) the con A-resistant phenotype behaved as a recessive trait in somatic cell hybrid studies.

G. Analysis of the Karyotype of Somatic Cell Hybrids:

The mean chromosome number of the somatic cell hybrids was determined as described in the Methods section. The model chromosome number of the hybrids approached a mean value of 42, which was twice the value of a pseudodiploid cell line (Figure 15). The chromosome numbers in the hybrid cells did show some variability,

Figure 14 Plating efficiency of somatic cell hybrids A-W, A-7, A-7B and A-7C in varying concentrations of concanavalin A.

⊙, A-w; ⊡, A-7; ⬡, A-7B; △, A-7C.

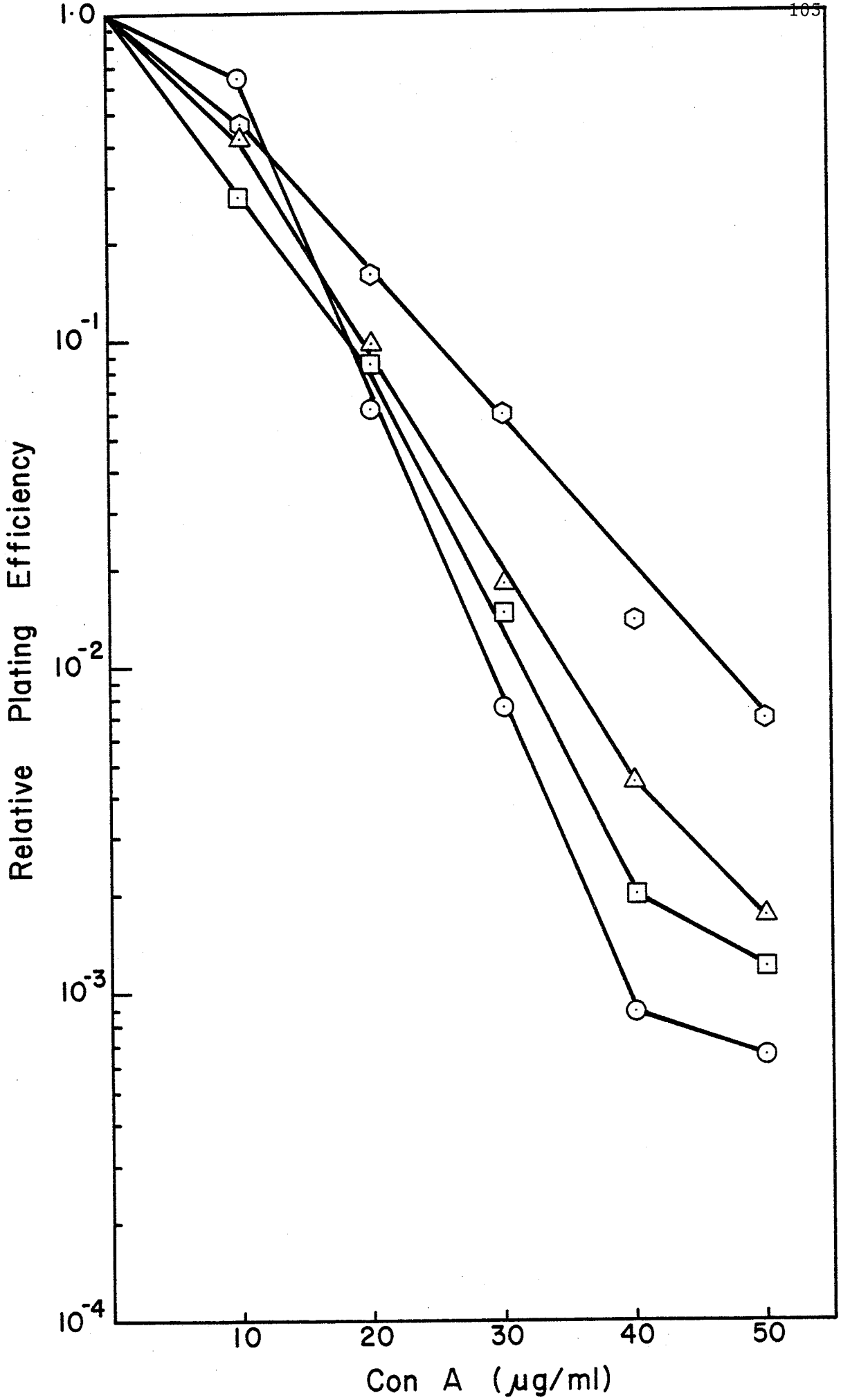
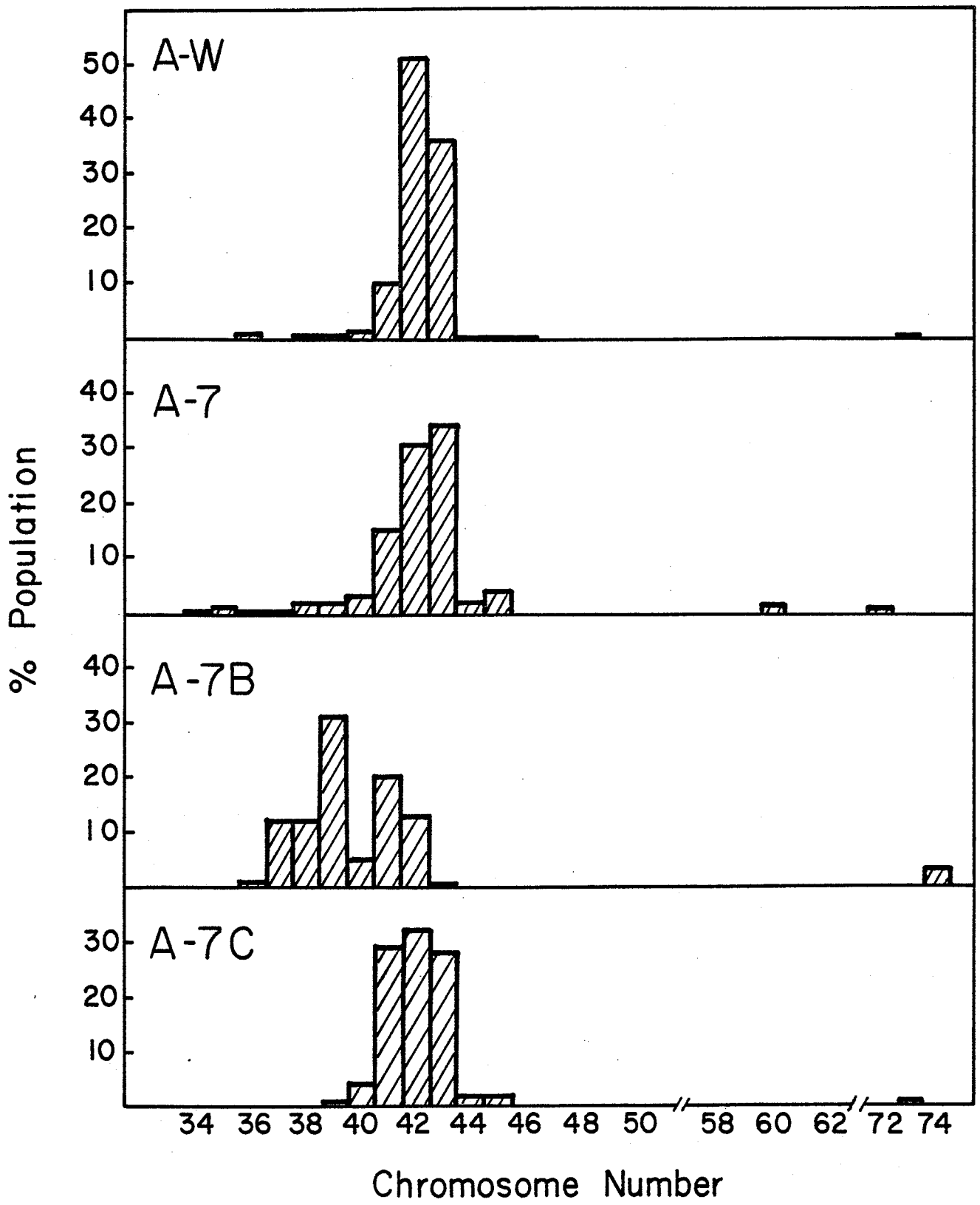

1·0
10-1
10-2
10-3
10-4
Relative Plating Efficiency
10
20
30
40
50
Con A (µg/ml)

Figure 15 Chromosome distribution in somatic cell hybrids A-W, A-7, A-7B and A-7C.

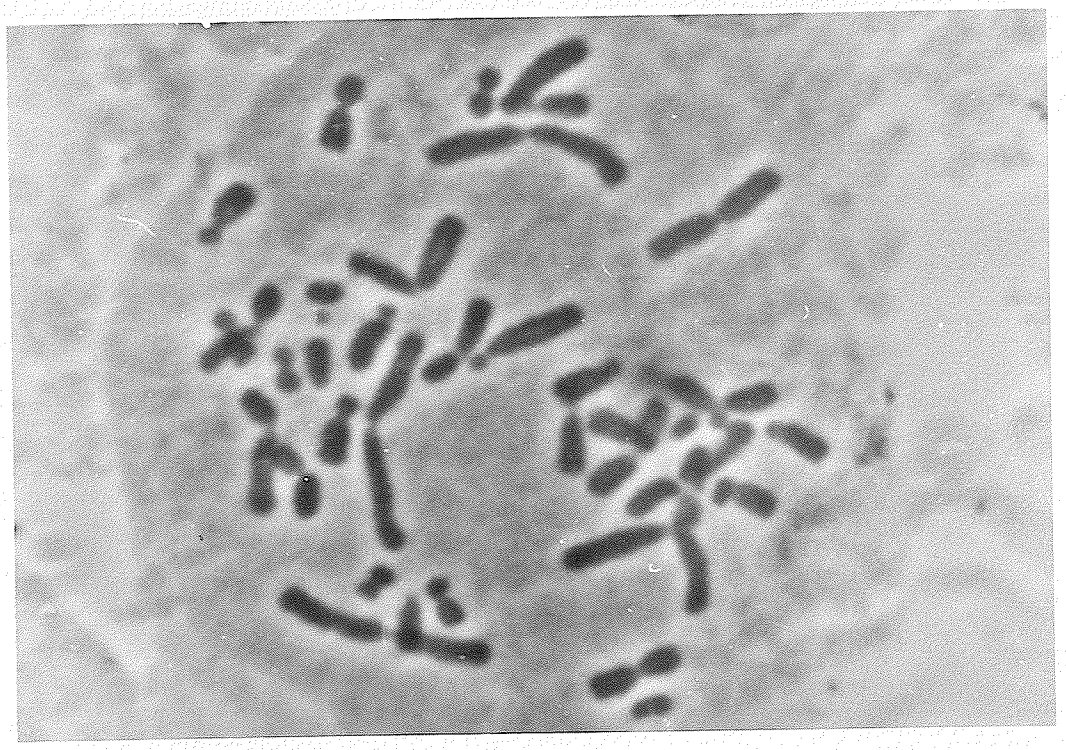

A-W
A-7
A-7B
A-7C
% Population
50
40
30
20
10
34 36 38 40 42 44 46 48 50 58 60 62 72 74
Chromosome Number

but this was consistent with the reported decrease in chromosome stability observed in hybrid CHO clones (Baker et al. 1974; Worton et al. 1977). Representative chromosome squashes of A-W and A-7 are presented in Figure 16.

H. Growth Characteristics of Somatic Cell Hybrids

Resistance to the cytotoxic properties of con A seen in the variant C^R-7, behaved as a resessive property in somatic cell hybrids (Figure 14). The growth properties and plating efficiencies of the hybrids were studied at the non-permissive temperature to determine if the ts growth properties of C^R-7 (Figure 8) also behaved as a recessive marker in hybrids. The growth responses of A-W and A-7 at 34, 37, and 39^o are shown in Figures 17a and b, respectively. The growth properties of both cell lines closely resembled those of wild-type cells (Figure 8); doubling time decreased at 37 and 39^o when compared to the growth rate at 34^o. Also, the ability of various hybrids to form colonies on solid surfaces was tested at 34 and 39^o. The hybrids (A-W, A-7, A-7B and A-7C) showed approximately the same plating efficiency at both temperatures (Table VI) It would appear therefore that hybrids formed by the fusion of con A-resistant and con A-sensitive cell lines behave

Figure 16 Aceto-orcein stained metaphase cells of hybrid lines A-W and A-7.



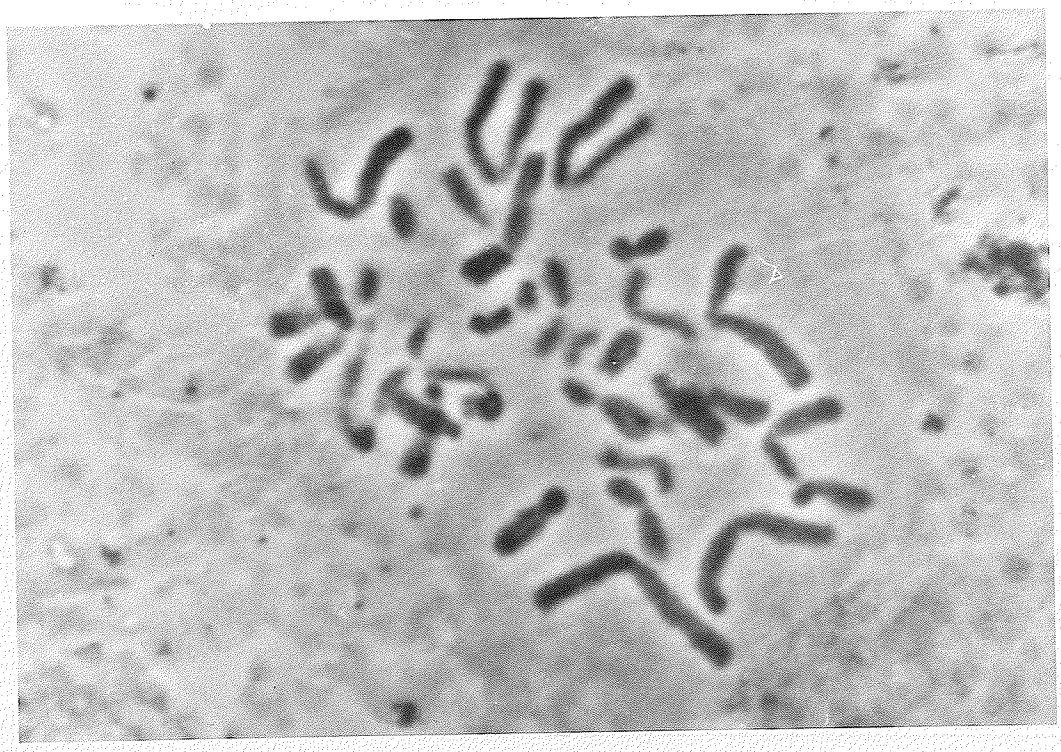


Figure 17 Growth curves of somatic cell hybrids A-W and A-7 on plates at 34 (●), 37 (△), and 39° (⊙).

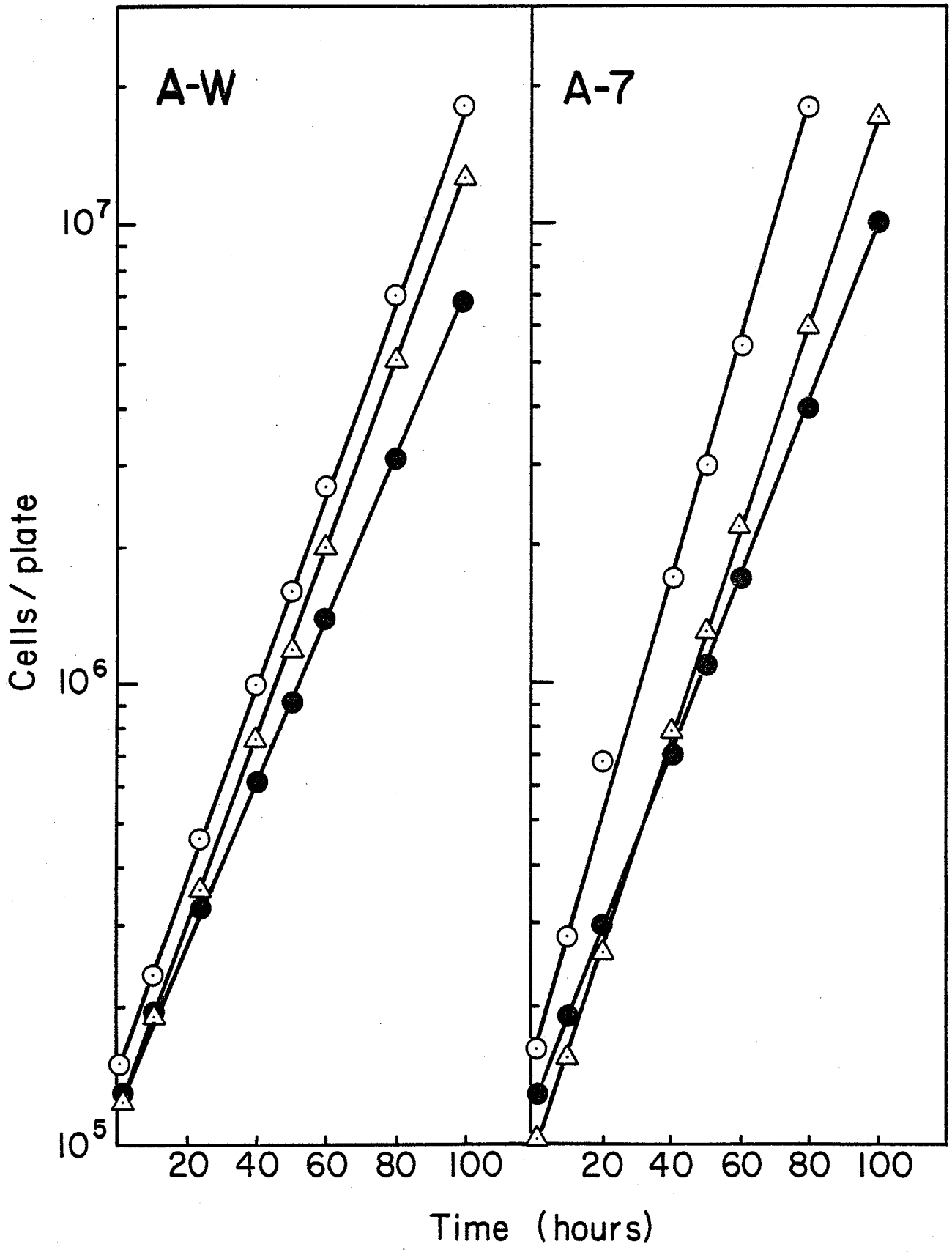

A-W
A-7
10^7
10^6
10^5
Cells / plate
20 40 60 80 100
20 40 60 80 100
Time (hours)

TABLE VI

Plating Efficiency of Hybrid Cell Lines at 34 and 39°.

Cell Line	No. of Cells Laid Down	No. of Col at 34°	PE at 34°	No. of Col at 39°	PE at 39°
A-W	1×10^3	420	.42	560	.56
A-7	1×10^3	380	.38	510	.51
A-7B	1×10^3	320	.32	470	.47
A-7C	1×10^3	280	.28	360	.36

as lectin-sensitive populations both in their response to the drug and in their ability to proliferate at 39°.

Based on the D_{10} values (Table V) the cell lines can be roughly divided into con A-resistant lines (C^R-7, BC^R-2, and EC^R-1) and con A-sensitive cell lines (WT-Cl I, II, and III, RC^R-7, A-W, and A-7). These cell lines were characterized further.

Section 2 Altered Membrane-Associated Properties of Concanavalin A-Resistant Cell Lines

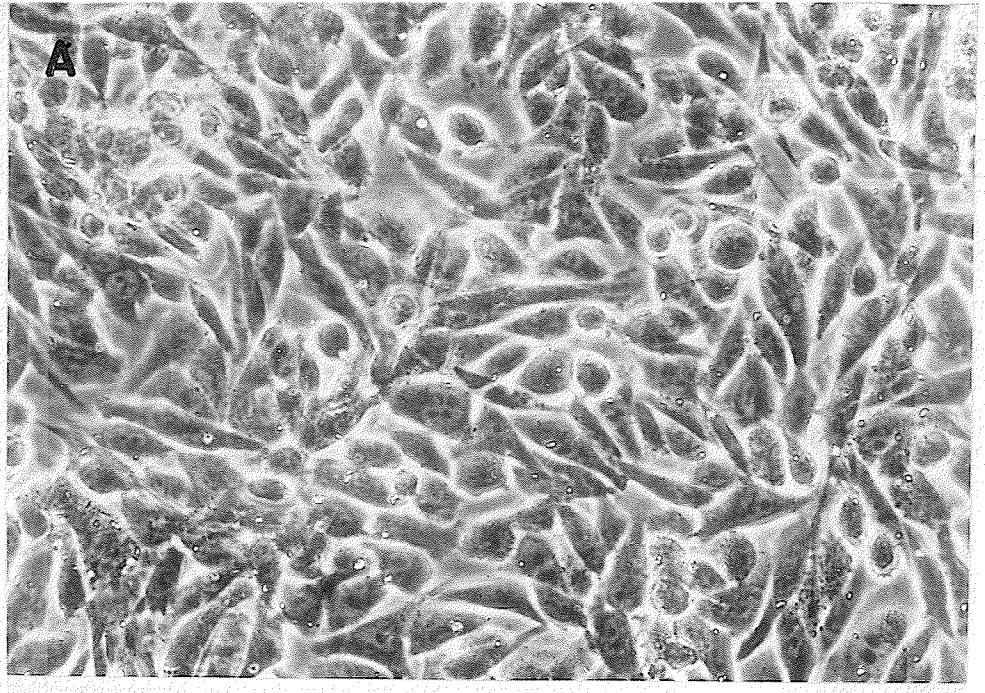
Concanavalin A causes a wide variety of effects upon mammalian cells in culture by binding to specific carbohydrate moieties on the cell surface (Sharon and Lis, 1972). Therefore, it might be expected that cells resistant to the cytotoxic properties of con A would possess alterations at the level of the plasma membrane. The cell lines previously discussed have been separated into lectin-sensitive and lectin-resistant cell lines (Table V). These cell lines were further studied for possible alterations in membrane-associated properties.

A Morphology:

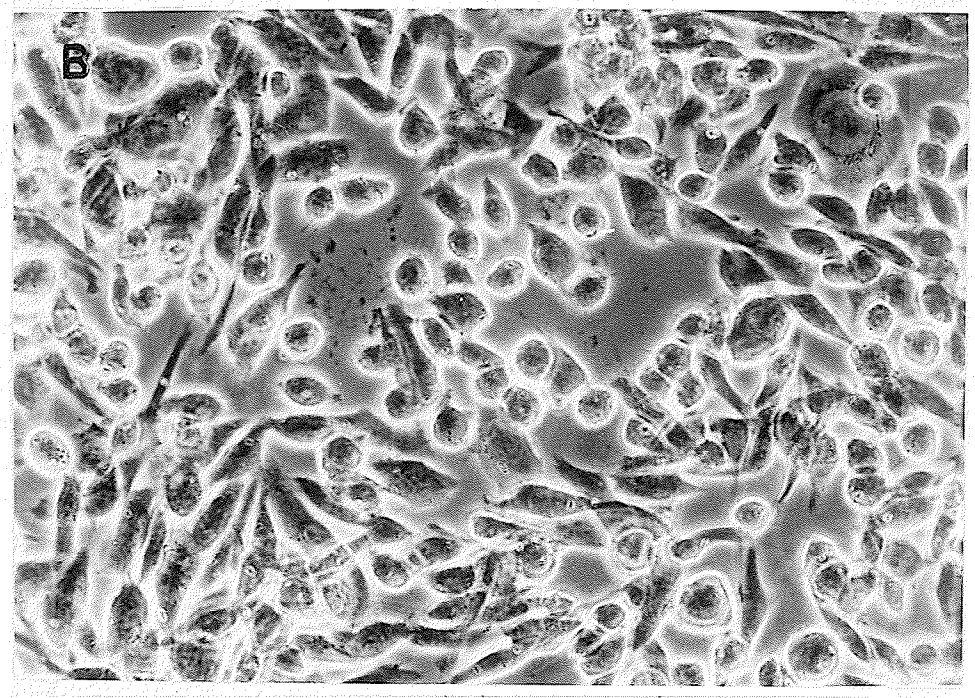
Morphology differences may reflect actual changes in the composition or conformation of cell surface structures, since it is known that modifications to the cell surface usually lead to changes in morphology (Wright, 1973b; Wright et al. 1973; Hanneberry et al. 1975).

The morphologies of the three wild-type clones (Figure 18) are similar, showing a tight growth pattern with little evidence of cells aggregating to form clumps.

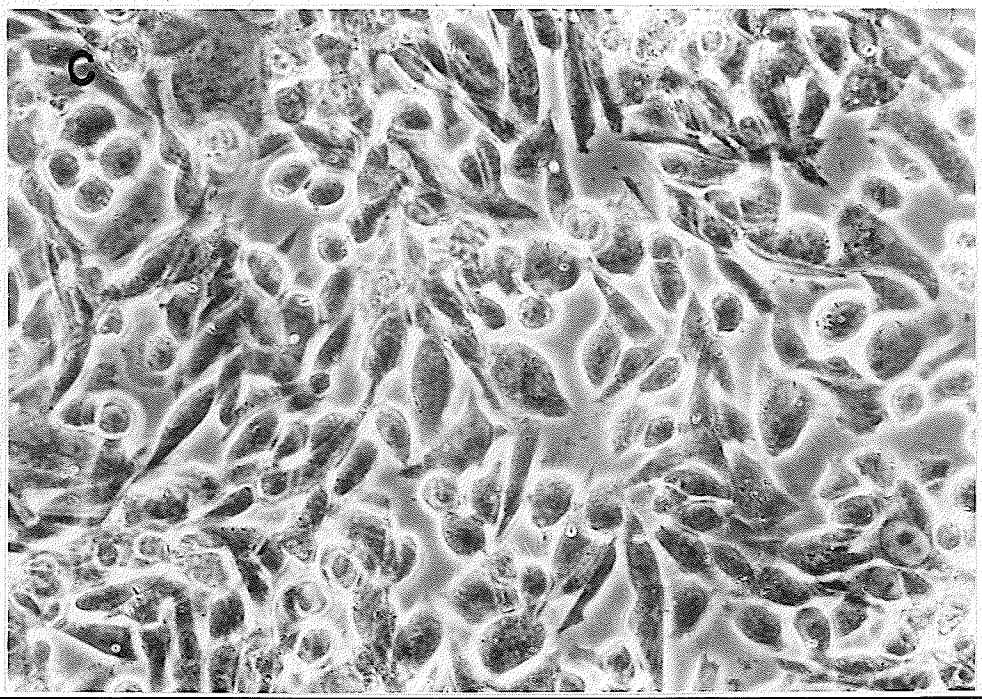
Figure 18 Morphology of near confluent cultures of WT-Cl I (a), WT-Cl II (b), and WT-Cl III (c) grown on plates at 34°. Magnification: 160 X.



A



B

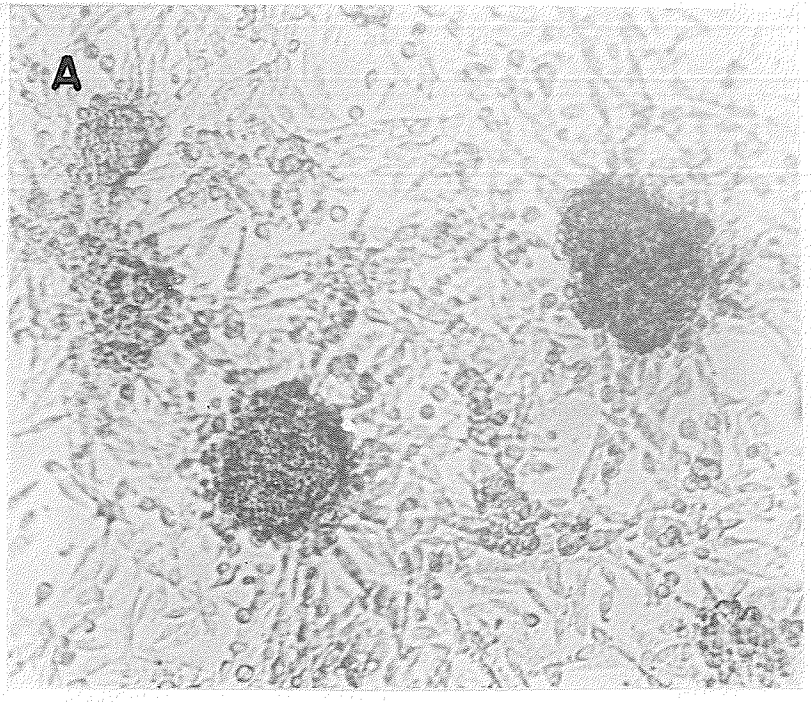


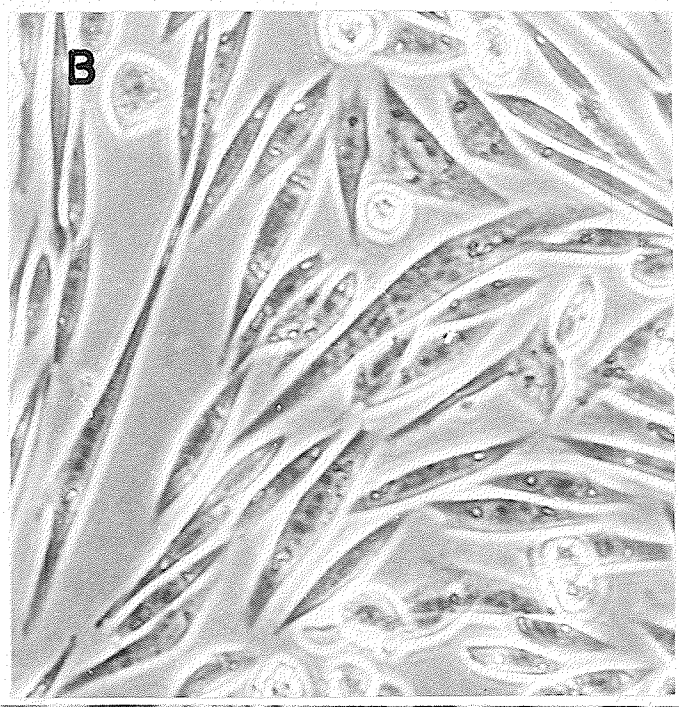
C

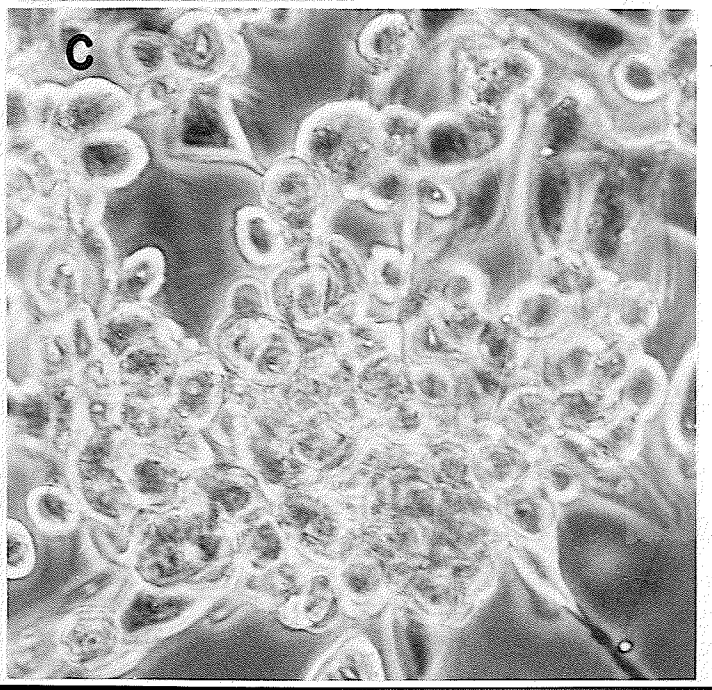
Although the morphology of each of the three variant cell lines is distinct, a marked difference between the variants and the parental wild-types is obvious (Figure 18 and Figure 19). C^R-7 (Figure 19a) cells formed large clumps or spheres of cells which appeared loosely attached to the cells on the substratum. The cells on the substratum tended to grow in a more disorganized criss-cross pattern that that observed with wild-type cells. Similar to C^R-7, the EC^R-1 cells (Figure 19c) grew in a more disorganized criss-cross fashion. Large multicellular clumps or spheres of cells were present; however, cell aggregation was less pronounced in EC^R-1 than in C^R-7. The appearance of BC^R-2 (Figure 19b) cultures was quite different from cultures of either the wild-type or the other two lectin-resistant cell lines. BC^R-2 cells did not form large multicellular clumps, and the shape of the individual cells was distinctly more "fibroblast-like" when compared to parental wild-type cells.

There is a considerable controversy concerning the role played by surface topography on the interaction of con A with cells (Willingham and Pastan, 1974, 1975; Collard and Temmik, 1975; Oppenheimer et al. 1977a, b). Pastan's group (Willingham and Pastan, 1974, 1975) believes that the presence of an increased number of

Figure 19 Morphologies of near confluent cultures of the variant cell lines C^R-7 (a), BC^R-2 (b) and EC^R-1 (c). Magnification 160 X, 600 X, and 320 X respectively.

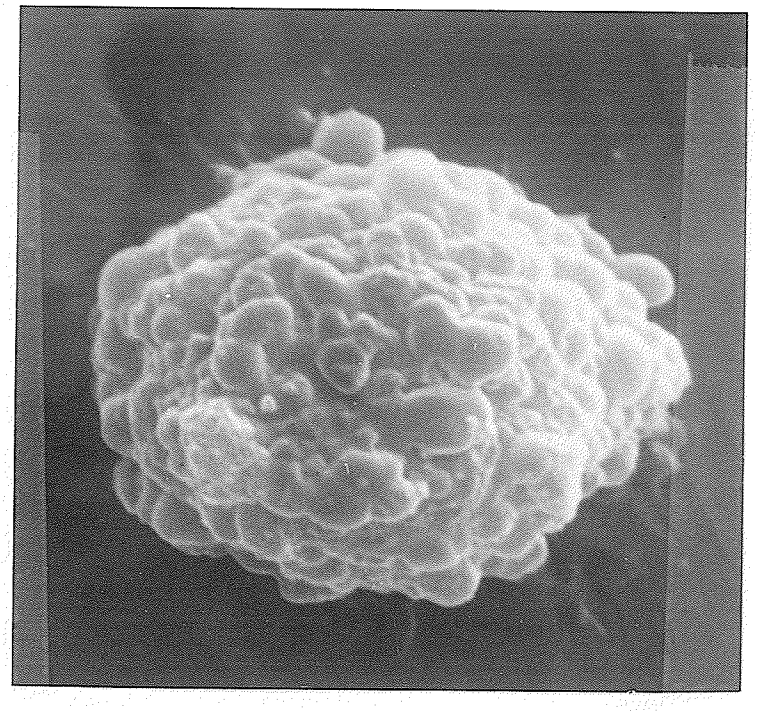

A


B

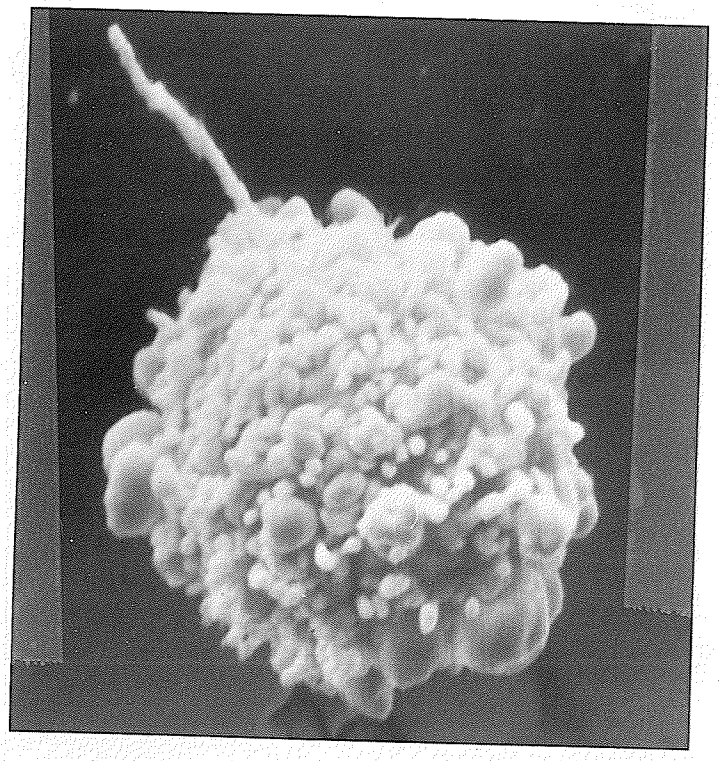

C

microvilli is responsible for increased con A agglutination of transformed cells. Other groups (Collard and Temmink, 1975; Oppenheimer et al. 1977a, b) have implicated large smooth surface areas in the agglutination of transformed cells by con A. Preliminary studies on the surface topography of WT-Cl I and C^R-7 cells were carried out following the procedures of Collard and Temmink (1975). Coverslip cultures, seeded with 5 x 10^5 cells, were incubated overnight in complete growth medium. The cells were washed with PBS and fixed 5 min. in 2.5% gluteraldehyde. Cells were then dehydrated in successive baths of increasing ethanol concentration. The dehydrated specimens were critical-point dried and coated with gold prior to being studied in a Phillips scanning electron microscope. Duplicate fields of each cell type were prepared on three separate occasions. Typical surface morphologies were as shown in Figure 20. The WT-Cl I cells were found to possess a relatively smooth surface with large blibs and surface rills (Figure 20 a). The surface of C^R-7 was rougher in comparison with many more small blibs and microvilli present (Figure 20b) Although these results are preliminary and restricted to just WT Cl I and C^R-7 they do indicate possible interesting differences in the

Figure 20 Scanning electron micrographs of a) WT-Cl I and b) C^{R}_{-7} cells grown on coverslips at 34^{o}. Magnification: 30,000 X.



A



B

surfaces of con A-resistant and sensitive cell lines.

B Cell Agglutination Properties:

Lectins, by definition, possess the ability to agglutinate cells, and these molecules are often used as tools or probes in investigations concerning cell surfaces (Sharon and Lis, 1972; Nicolson, 1974). The results of con A agglutination experiments with con A-resistant and sensitive cell lines are shown in Table VIII. Higher concentrations of con A were required to agglutinate the variant populations. For example, in the presence of 500 µg/ml con A virtually 100% of the wild-type cells were agglutinated, whereas only 50% of the variants C^R-7 and EC^R-1 were clumped. It should be noted that BC^R-2 cells consistently formed many small clumps when suspended in buffer without lectin; therefore, although BC^R-2 cells appeared to resemble C^R-7 and EC^R-1 in these studies, it was not possible to estimate agglutination accurately.

The revertant cell line RC^R-7 behaved like a wild-type population in con A sensitivity tests (Figure 13). It also showed an agglutination profile which was indistinguishable from the original parental wild-type cell line (Table VII).

Both wild-type - wild-type (A-W) and wild-type -

TABLE VII

Cell agglutination in the presence of concanavalin A.

Cell line	Con A conc. (μg/ml) 0	50	250	500	750	1000
wild-types (I, II, & III)	-	-	++	++++	++++	++++
C^R-7	-	-	-	++	+++	++++
EC^R-1	-	-	-	++	+++	++++
RC^R-7	-	-	++	++++	++++	++++
A-W	-	+	+++	++++	++++	++++
A-7	-	+	+++	++++	++++	++++

BC^R-2 cells consistently form many small clumps of cells when suspended in buffer minus lectin. Although BC^R-2 cells appeared to resemble C^R-7 and EC^R-1 cells in these studies it was not possible to estimate agglutination accurately. Data presented was obtained from at least three agglutination experiments with each cell line.

variant (A-7) hybrids were more easily agglutinated in the presence of con A than con A-resistant cell lines. The hybrids, in fact, were consistently found to agglutinate at slightly lower concentrations of the lectin than pseudodiploid wild-type or revertant cell lines. The mechanism responsible for the increased agglutinability of the hybrid cells is not understood.

Pretreatment of the cell surface with trypsin can lead to a change in the agglutination patterns of cells (see review Schnebli, 1976). As seen in Table VIII higher concentrations of con A were required to agglutinate both WT-Cl I and C^R-7 cells after treatment with 0.15% trypsin for 15 min. at room temperature; however C^R-7 cells still required higher lectin concentrations to show agglutination than did wild-type cells.

Clearly, con A-resistant cells are less easily agglutinated in the presence of con A than are con A-sensitive cells. In contrast to these observations, the con A-resistant cell lines were found to agglutinate more readily with the lectin Phytohemagglutinin-P, (PHA) than did wild-type cells (Table IX). For example, in the presence of 64 µg/ml PHA, con A-resistant cells exhibited essentially 100% agglutination, whereas the parental wild-type lines showed less than 50% clumping.

TABLE VIII

Concanavalin A agglutination after trypsin treatment.

A) No Trypsin Treatment.

Cell line	0	50	250	500	750	1000
WT-Cl I	-	-	++	++++	++++	++++
C^R-7	-	-	-	++	+++	++++

B Trypsin Treatment

WT-Cl I	-	-	+	++	++++	++++
C^R-7	-	-	-	+	++	++++

Data is representative of two separate experiments.

TABLE IX

Cell agglutination in the presence of Phytohemaglutinin-P.

Cell line	PHA-P Concentration (µg/ml) 0	16	32	64	128	320
Wild-type (Cl I & III)	-	-	-	++	++++	++++
C^R-7	-	-	-	++++	++++	++++
EC^R-1	-	-	++	++++	++++	++++

BC^R-2 cells consistently form many small clumps of cells when suspended in buffer minus lectin. Although BC^R-2 cell appeared to resemble C^R-7 and EC^R-1 cells in these studies it was not possible to estimate agglutination accurately.

These studies indicate that the lectin agglutination properties of the con A-resistant variants are distinctly different from the sensitive cell lines.

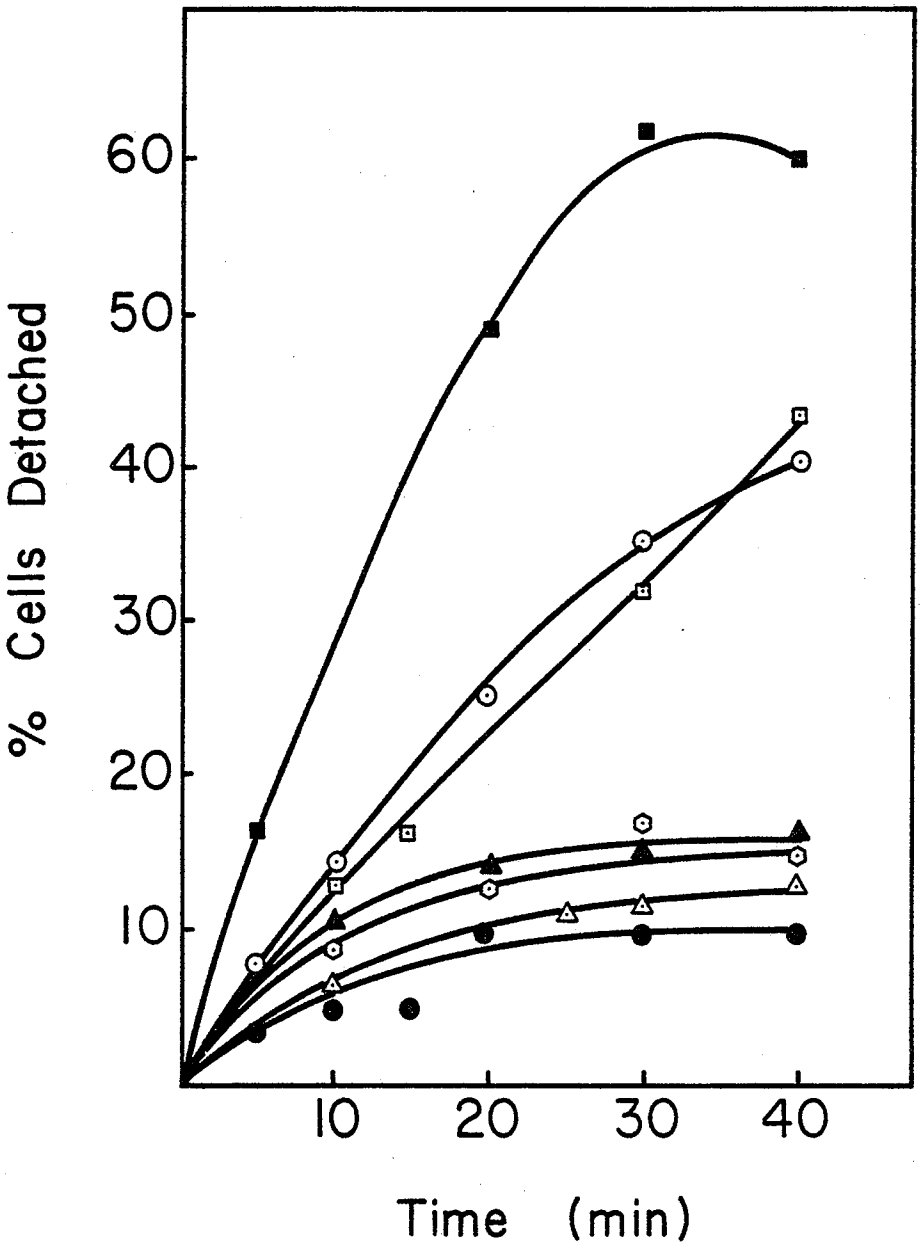
C Cell Adhesion:

The ability of cells to adhere to a substratum is a property of the cell membrane. Cell variants with reduced ability to attach to growth surfaces have been selected (Pouysségur and Pastan, 1976; Hynes, 1976; Klebe et al. 1977). Adhesion to solid surfaces can be quantitated by measuring the rate at which cells are removed from a solid growth surface with a trypsin solution (Pouysségur and Pastan, 1976).

The rate at which con A-resistant variants detached in the presence of 0.03% trypsin (see Methods section) was much greater than the rate observed with wild-type cell lines (Figure 21). For example, after 40 min of trypsin treatment approximately 60% of C^R-7 cells and 40% of BC^R-2 and EC^R-1 cells had detached, while only 10% of wild-type clones I, II, or III had lifted from the plates (Figure 21).

The RC^R-7 cell line showed greater cell adhesion than did the C^R-7 line from which it was derived. While 60% of C^R-7 cells detached after 40 min. only about 15%

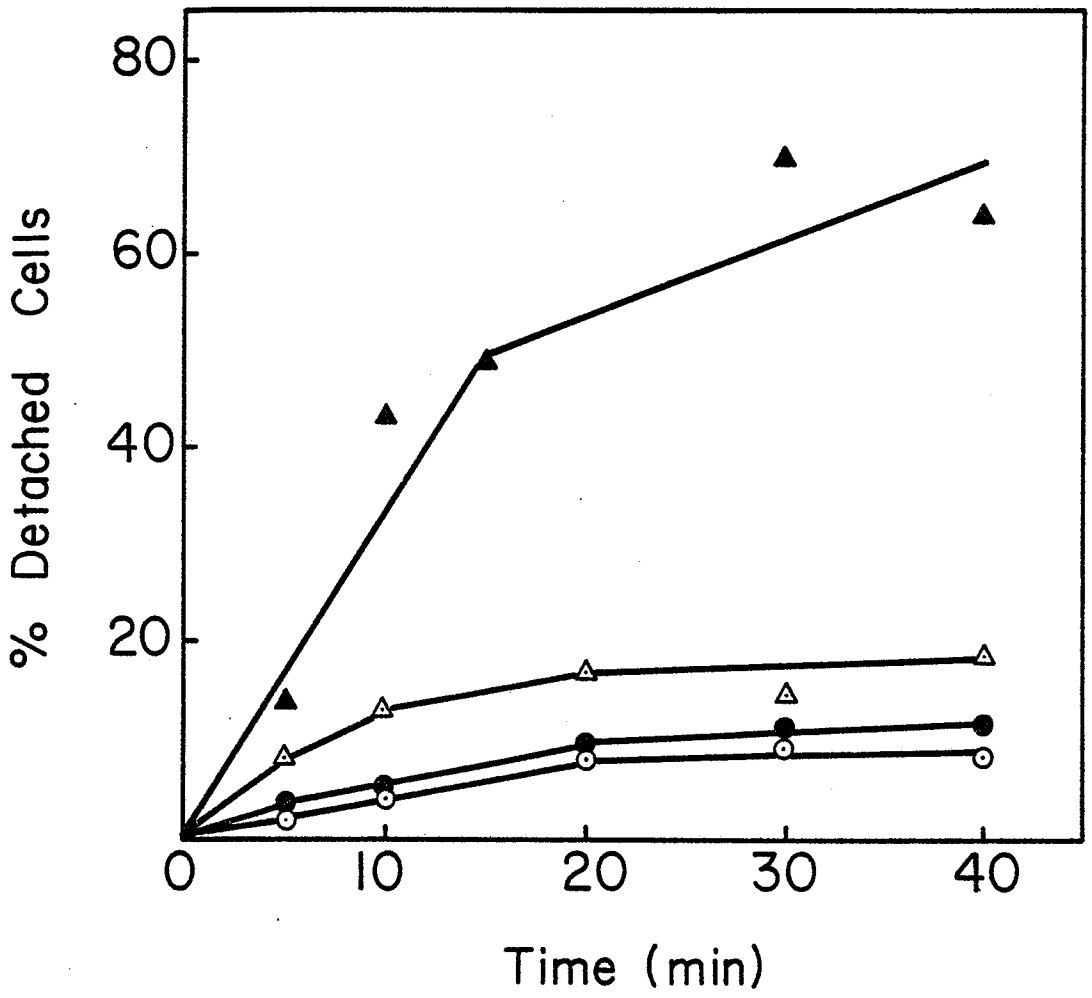
Figure 21 Kinetics of detachment of wild-type (●), C^R-7 (■), BC^R-2 (⊙), EC^R-1 (⊡), RC^R-7 (▲), A-W (△), and A-7 (⬡) cells from a plastic tissue culture plate with PBS containing 0.03% trypsin. Cells were incubated at 34° for 24 hours in normal growth medium prior to experiment. Points represent the mean of duplicate points from a single experiment. Similar data was obtained in three separate experiments.


% Cells Detached
60
50
40
30
20
10
10
20
30
40
Time (min)

of RC^R-7 cells detached after identical treatment (Figure 21). The reduced cell adhesion property observed with C^R-7 was not expressed in the hybrid A-7, which was formed through the fusion of a C^R-7 cell with a wild-type cell. The A-7 detachment kinetics were very similar to those observed with the pseudo-diploid wild-type cells and with the wild-type - wild-type (A-W) hybrid cell line (Figure 21).

Since con A-resistant cells show ts growth properties the detachment kinetics of C^R-7 and WT-Cl I cell lines were compared when cells were incubated for 24 hours at 34^o or 39^o. Figure 22 shows the detachment kinetics of C^R-7 cells grown at 34 and 39^o to be markedly different. Approximately 50% of the C^R-7 cells grown at 34^o lifted from the growth surface after 15 minutes in the trypsin solution while only 15% of the C^R-7 cells maintained at 39^o detached under the same conditions. A very slight decrease in the numbers of wild-type cells released after growth at 39^o was also observed (Figure 22). These results point to a difference in adhesive properties between con A-resistant and con A-sensitive populations; furthermore this cell surface property may be expressed as a ts function in variant cells.

Figure 22 Detachment kinetics of wild-type (●) and C^R-7 (▲) cells maintained at 34° and wild-type (⊙) and C^R-7 (△) cells maintained at 39° for 24 hours before detachment assay in 0.03% trypsin. Points represent the mean of duplicate values from a single experiment. Data is typical of two individual experiments.


80
60
40
20
% Detached Cells
0
10
20
30
40
Time (min)

D Sensitivity to Membrane Active Agents:

Variants selected for resistance to one drug often exhibit altered responses to a number of unrelated drugs (Bosman, 1971; Wright, 1973a; Ling and Thompson, 1974; Wright and Lewis, 1974; Ceri and Wright 1977a, 1978a). Previously, Wright (1973a) had reported that con A-resistant CHO cell lines showed "collateral sensitivity" to a number of membrane active agents. Sodium butyrate alters the surface membrane of cells (Wright, 1973b; Simmons et al. 1975). The mode of action of the drug appears to be through the modification of glycosyl-transferases (Fishman et al. 1974). Phenethyl alcohol (PEA) has also been shown to be a membrane active agent (Wright et al. 1973; Nunn, 1977). The treatment of CHO cells with 0.1% PEA for 8 hours resulted in significant changes in the glycoprotein pattern of the cell surface (Figure 23, Ceri and Wright, 1977b), as determined by galactose oxidase -[^{3}H]- borohydride labelling (see Methods section).

Drug sensitivity was assayed by determining the plating efficiency of cells in the presence of varying concentrations of sodium butyrate or PEA. Figure 24 shows data from a typical plating experiment with WT-Cl I and C^R-7 cells plated in the presence of various concentrations of phethyl alcohol (Figure 24a) or sodium

Figure 23 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of CHO cells pretreated with 0.1% PEA (A) and untreated control cells (B). Galactose oxidase -[^{3}H]- borohydride surface labelling was performed as described in Methods except cells were labelled from plates instead of from suspension cultures.

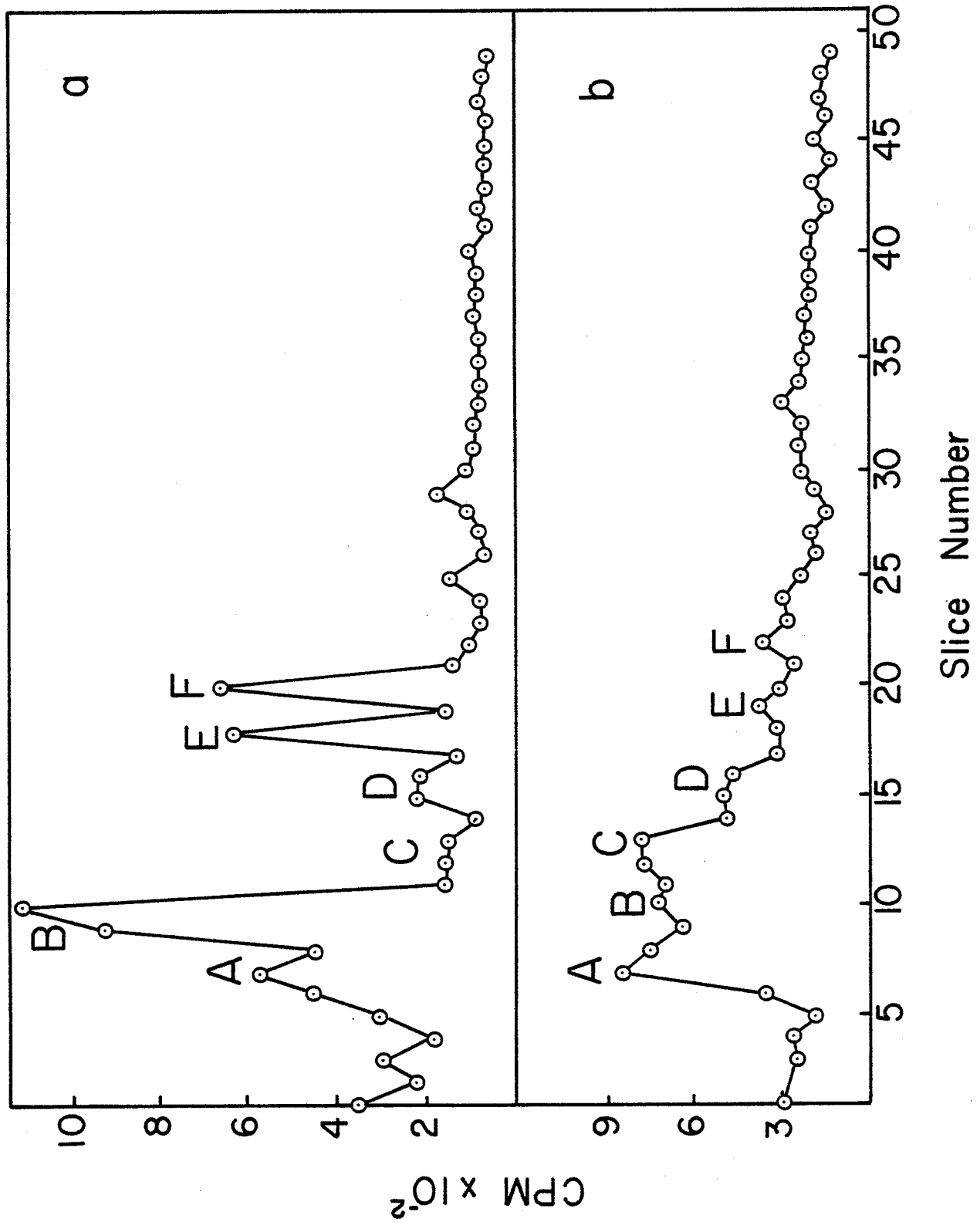

a
b
A
B
C
D
E
F
CPM x 10⁻²
Slice Number

Figure 24 Relative Plating Efficiency of WT-Cl I (⊙) and C^R-7 (△) cells in varying concentration of Phenethyl alcohol (a) and sodium butyrate (b).

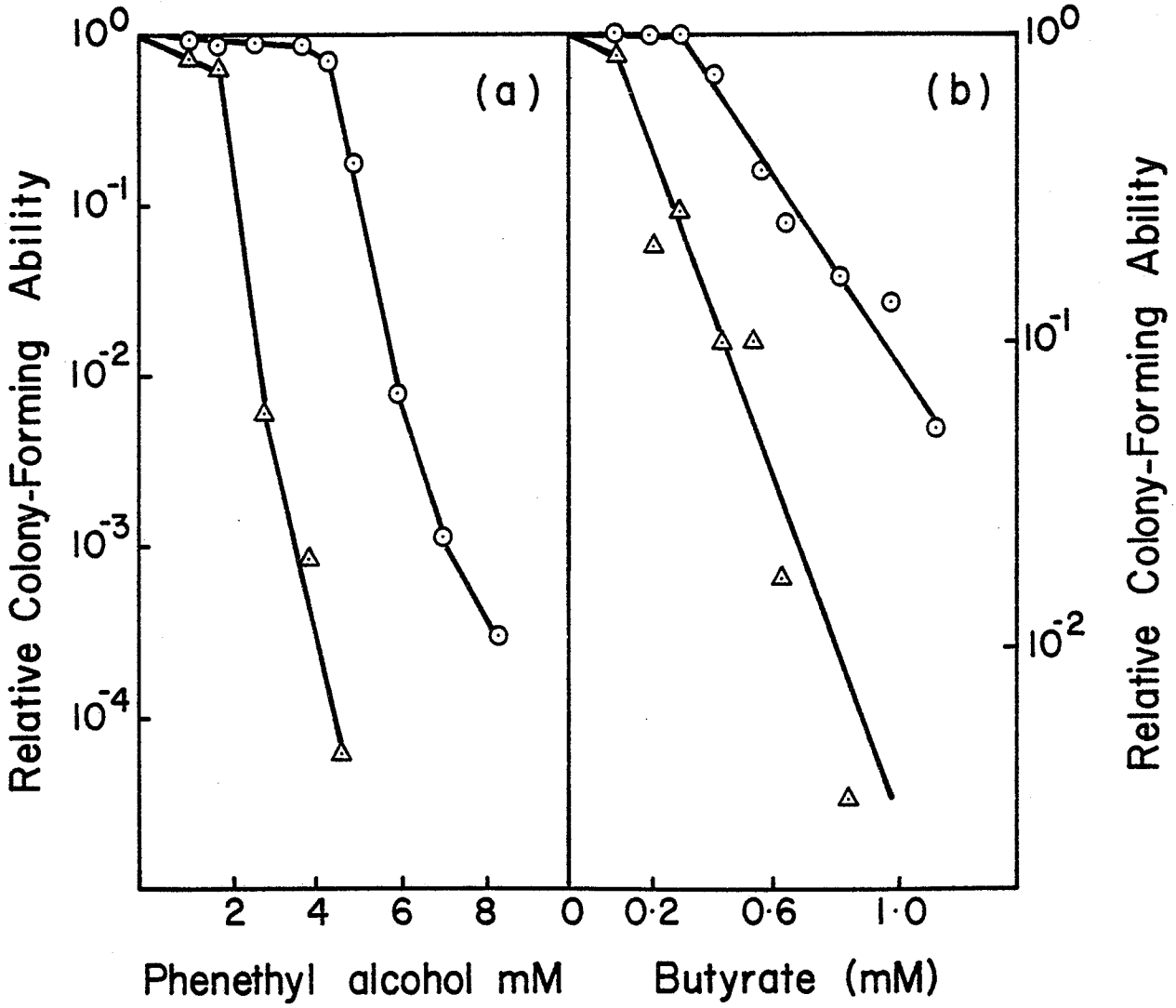

Relative Colony-Forming Ability
10^0
10^-1
10^-2
10^-3
10^-4
(a)
(b)
2
4
6
8
0
0·2
0·6
1·0
Phenethyl alcohol mM
Butyrate (mM)
Relative Colony-Forming Ability
10^0
10^-1
10^-2

TABLE X

Sensitivity of con A-resistant and sensitive lines to the membrane active agents Phenethyl Alcohol and Sodium Butyrate.

Cell Line	D_{10} (mM; Phenethyl Alcohol)	D_{10} (mM; Sodium Butyrate)
WT-Cl I	5.1	0.74
WT-Cl II	5.1	0.74
WT-Cl III	5.1	0.73
C^R-7	2.1	0.34
BC^R-2	2.0	0.33
EC^R-1	1.8	0.35
RC^R-7	4.6	0.70
A-W	4.2	0.70
A-7	4.0	0.68

The plating efficiency of each cell line was determined by plating varying numbers of cells in the presence of various concentrations of each of the drugs. The concentration of drug that reduced colony-formation to 10% of controls was defined as the D_{10} for that particular drug. Data was obtained from duplicate plating experiments performed with each drug on all cell lines.

butyrate (Figure 24b). The D_{10} values of C^R-7 and WT-Cl I cell lines in PEA were found to be 2.1 and 5.1 mM, while in sodium butyrate the D_{10} values were 0.34 and 0.74 mM respectively. The D_{10} values of the other con A-sensitive and -resistant cell lines were calculated in a similar manner and are presented in Table X. The resistant cell lines BC^R-2 and EC^R-1 behaved similarly to the C^R-7 cell line and were 2 to 2.5 times more sensitive to the drugs than the parental wild-type cells. The revertant and hybrid cell lines, which behaved as wild-type populations in con A sensitivity tests, also showed "wild-type" levels of sensitivity toward PEA and sodium butyrate.

These results agree with those of a previous report (Wright, 1973a) which showed con A-resistant cells were significantly more sensitive to a number of membrane active agents.

E Sensitivity to Plant Lectins

Lectin-resistant cell lines often show altered sensitivities toward other plant lectins (Wright, 1973a; Stanley et al. 1975b; Stanley and Siminovitch, 1976). The relative sensitivities of C^R-7 and WT-Cl I cells toward the lentil lectins (LCH-A, LCH-B), soybean agglutinin (SBA), wheat germ agglutinin (WGA) and phyto-

TABLE XI

Relative Sensitivity of C^R-7 Cells to Plant Lectins

Lectin	Relative Sensitivity of C^R-7 Cells
LCH-A	R
LCH-B	R
SBA	S
WGA	S
PHA-P	S

R: C^R-7 cells plated at an efficiency of at least 75% at lectin concentrations which reduced WT-Cl I plating efficiency to less than 1%.

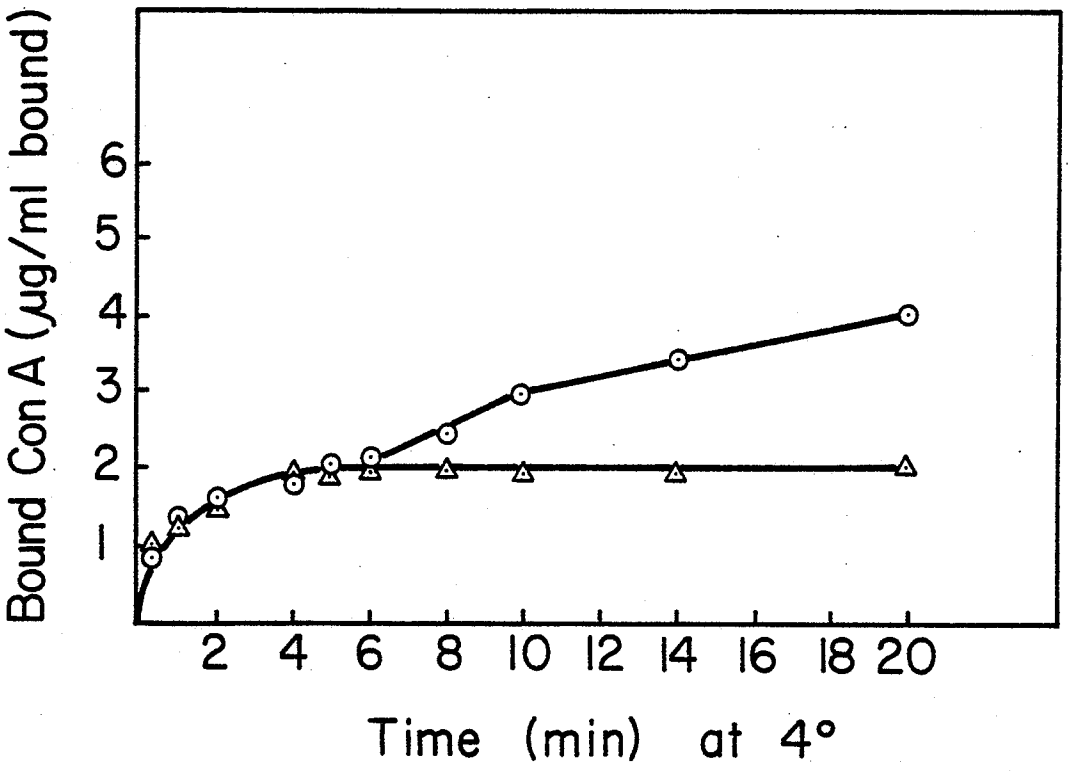
S: C^R-7 and WT-Cl I plated at approximately the same efficiency at all lectin concentrations tested.

hemagglutinin-P (PHA-P) were assayed by plating cells in varying concentrations of the drug. C^R-7 cells were found to be cross-resistant to the lentil lectins but both cell lines showed similar sensitivities to the other lectins tested (Table XI). It should be noted that con A and the lentil lectins bind specifically to mannose while SBA, WGA, and PHA-P show binding specificities for GalNAc, GLcNAc, and GalNAc respectively (Sharon and Lis, 1972).

F Concanavalin A Binding Studies:

The resistance of the variant cell lines to the cytotoxic effects of con A and the reduced agglutinability of these cell lines may result from a reduction in the ability of variant cells to bind con A. Wright (1973a) has previously tested for differences in con A binding at room temperature with lectin-resistant cell lines, but was unable to demonstrate significant changes. It is now clear that it is very important to block endocytosis of labelled con A during binding studies (Norman and Burger, 1973, 1974). The method previously employed by Wright (1973a) did not prevent endocytosis, and subtle differences in con A binding may have been masked. Preliminary binding studies were carried out with wild-type cells (Noonan and Burger, 1974) to determine if saturating binding

Figure 25 Time course concanavalin A binding study at 4° with WT Cl I cells pretreated 40 min with 10 mM sodium azide (△) and untreated WT-Cl I(⊙) cells. The experiment was carried out in the presence of 100 µg cold concanavalin A.


Bound Con A (µg/ml bound)
1
2
3
4
5
6
2
4
6
8
10
12
14
16
18
20
Time (min) at 4°

could be achieved under conditions in which endocytosis was likely to be inhibited. A time course binding study was carried out at 4^{o} in the presence and absence of 10 mM of the metabolic inhibitor sodium azide (Figure 25). It was observed that in the absence of the inhibitor saturation binding was not obtained; however when cells were pre-treated for 40 minutes with 10 mM sodium azide and then assayed in the presence of the inhibitor, saturation binding occurred after approximately five minutes. This time period was routinely used in all binding studies.

The diameters of each cell type was determined in order to compare the quantity of con A bound per cell surface area. An average of 200 cells of each cell type were measured with a light microscope fitted with a micrometer. The average cell diameter was used to calculate the cell surface area, assuming the cells were smooth spheres (Collard and Temmink, 1975). The values for the cell surface areas are presented in Table XII.

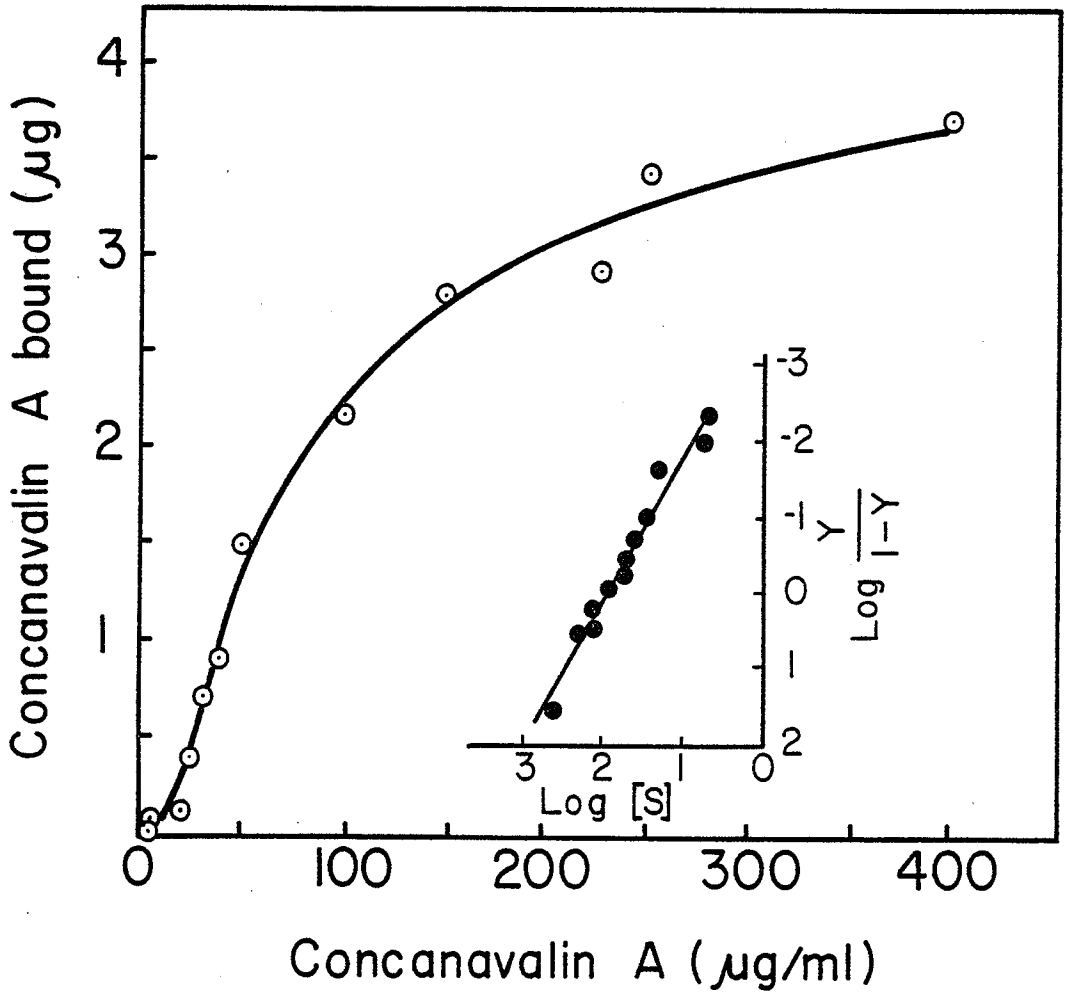
The binding of labelled con A to wild-type CHO cells as a function of lectin concentration is shown in Figure 26. Apparently, the binding curve obtained was not hyperbolic and suggested that con A was binding to cells with positive cooperativity. When the data was analyzed for possible cooperative effects by means of a

TABLE XII

Cell surface areas of concanavalin A-resistant and concanavalin A-sensitive cell lines.

Cell line	Surface Area (μM^2)
C^R-7	1150
BC^R-2	1145
EC^R-1	1120
WT-Cl I	850
WT-Cl II	854
WT-Cl III	850
RC^R-7	1010
A-W	1110
A-7	1510
H^R-100	940

Figure 26 ^{3}H-Labelled con A bound per 1.8 x 10^{6} wild-type cells at various concentrations of lectin. Inset: data presented in the form of a Hill plot. Hill coefficient was calaculated to be 1.8.


Concanavalin A bound (μg)
4
3
2
1
0
100
200
300
400
Concanavalin A (μg/ml)
-3
-2
-1
0
1
2
Log Y/1-Y
3
2
1
0
Log [S]

Hill plot (Hill, 1913), it became obvious that a significant amount of lectin was bound to the cells in a cooperative manner (Figure 26, see inset); the Hill coefficient was calculated to be 1.8.

When the binding of labelled con A to C^R-7 cells was examined as a function of lectin concentration (Figure 27) the binding curve appeared to be hyperbolic. The data from Figure 27 was analyzed by a Hill Plot and a Hill coefficient of 1.0 was calculated, indicating a lack of cooperativity in the binding of labelled con A to the variant cells (Figure 27, see insert).

The data in Figures 26 and 27 were also analyzed by Scatchard plots (Scatchard, 1949); the data is shown in Figure 28. Clearly, the con A was bound to wild-type cells in a cooperative fashion, as judged by the non-linearity of the Scatchard plot. Conversely a linear Scatchard plot, which indicates lack of cooperativity, was obtained when the lectin-resistant cells were incubated in the presence of labelled con A. By extrapolation to the abscissa the Scatchard representation provides an estimate of the amount of lectin bound per culture at saturation binding. Although it is difficult to extrapolate a non-linear Statchard plot to the abscissa it was still possible to estimate that

Figure 27 ^{3}H-Labelled con A bound per 1.8 x 10^{6} variant cells at various concentrations of lectin. Inset: data presented in the form of a Hill plot.

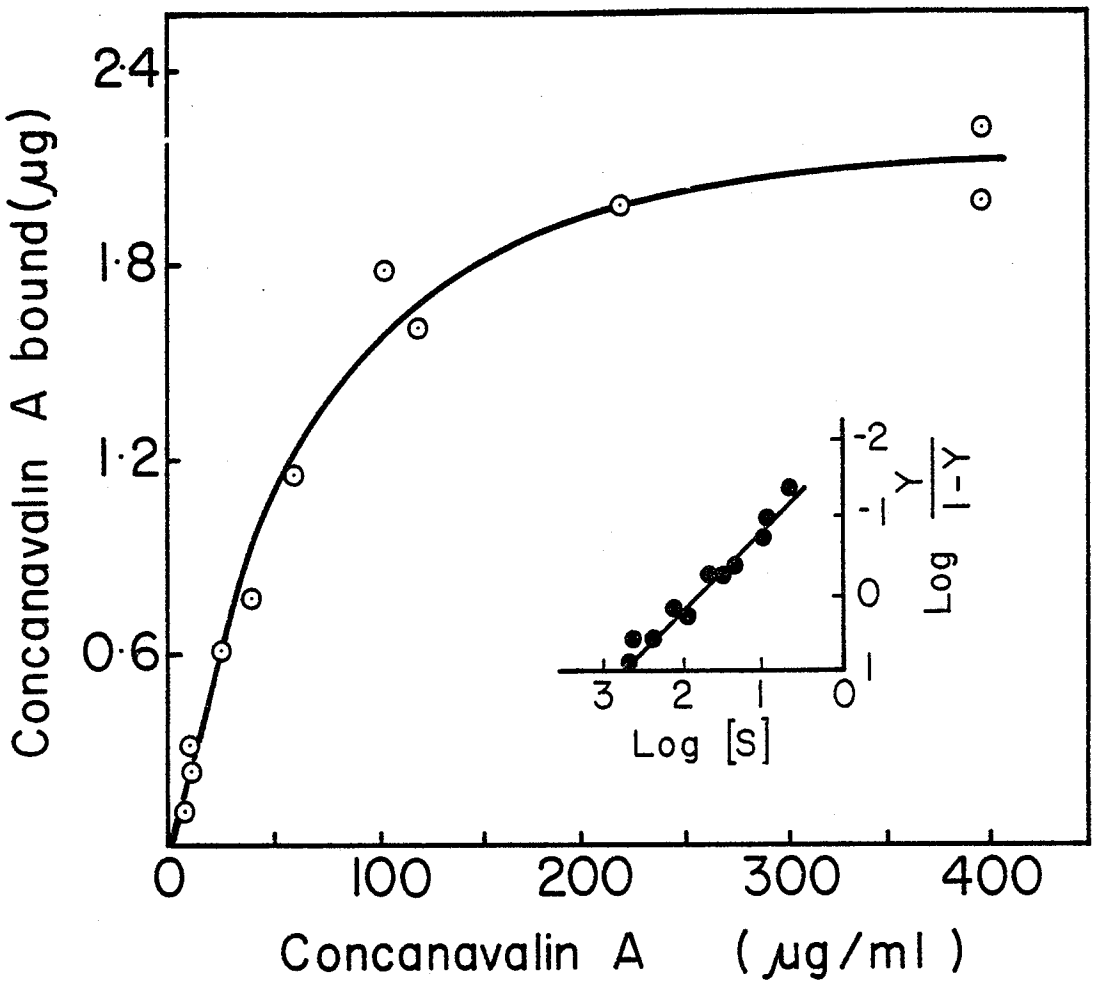
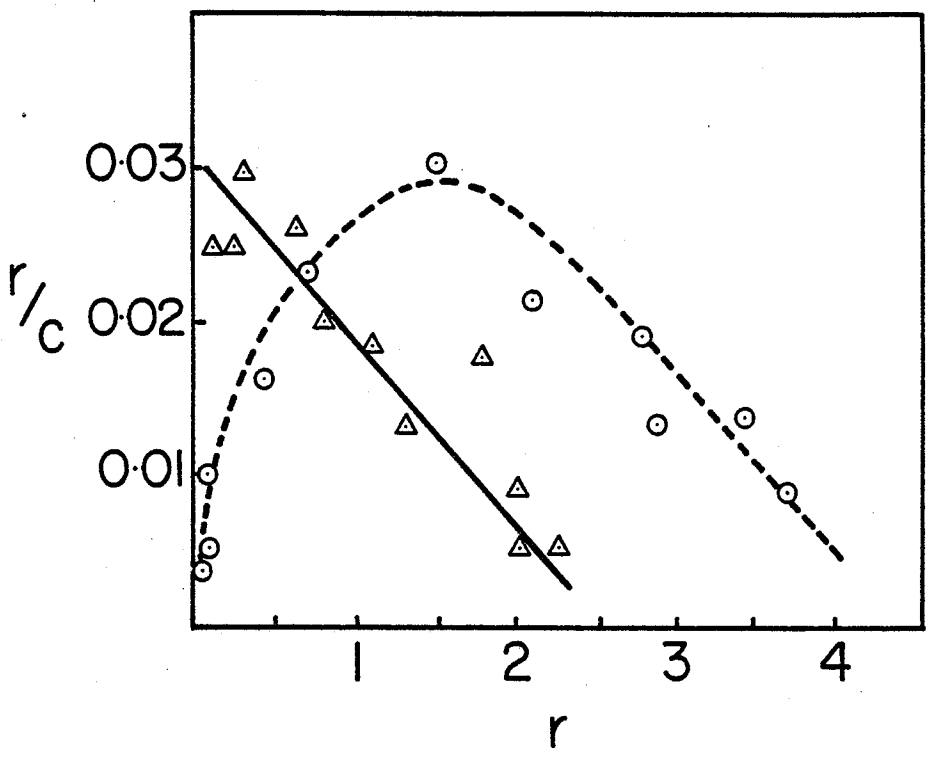

2·4
1·8
1·2
0·6
Concanavalin A bound(μg)
0
100
200
300
400
Concanavalin A (μg/ml)
-2
-1
0
1
Log Y/1-Y
3
2
1
0
Log [S]

Figure 28 Data from Figure 26 (○, wild-type cells) and Figure 27 (△, variant cells) plotted according to Scatchard's equation: $r/c=nK - rK$ where, r represents the amount of lectin bound, c is free lectin concentration, n is the amount of lectin bound at saturation and, K is the apparent association constant for lectin-receptor site binding. Each point represents the average of duplicate points from a single experiment; similar data has been obtained in 3 separate experiments.


0·03
0·02
0·01
r/c
1
2
3
4
r

approximately 4.5 µg/ml con A was bound/1.8 x 10^6 wild-type cells at saturation binding. Assuming a molecular weight of 110 000 for con A (Sharon and Lis, 1972) an estimated 1.6 x 10^4 con A molecules of con A were bound/μm^2 of wild-type surface area, which was calculated at 854 μm^2 (Table XII). As indicated in Table XIII identical results were obtained with all three wild-type cell lines (Cl I, Cl II and Cl III). By extrapolating the Scatchard plot obtained with C^R-7 (Figure 27) it was determined that 2.5 µg/ml con A bound to 1.8 x 10^6 C^R-7 cell at saturation binding. The surface area of the C^R-7 cell was estimated at 1150 μm^2 which meant that 6.5 10^3 molecules of lectin were bound/μm^2 surface area of variant cells at saturation binding.

The wild-type cell line was therefore capable of binding approximately 2.5 times more lectin/surface area than was the C^R-7 population.

Similar differences in the ability to bind con A were found when the other independently isolated variants were studied. When the binding of ^{3}H-con A to BC^R-2 (Figure 29) and EC^R-1 (Figure 30) was examined as a function of lectin concentration, the binding curves obtained appeared to be hyperbolic. When the binding data from these variants was analyzed by Hill plots

Figure 29 ^{3}H-labelled con A bound/1.8 x 10^{6} BC^{R}-2 cells at various concentrations of lectin. The inset in the upper part shows a Hill plot of the data. The Hill coefficient which represents the slope of this plot was calculated to be 1.0. The inset at the bottom presents the data according to Scatchard's equation: r/c=nk-rk where, r, represents the amount of lectin bound, c, is the free lectin concentration, n, is the amount of lectin bound at saturation and, k, is the apparent association constant for lectin: receptor site binding. Each point represents the average of duplicate points from a single experiment; similar data has been obtained in 3 separate experiments.

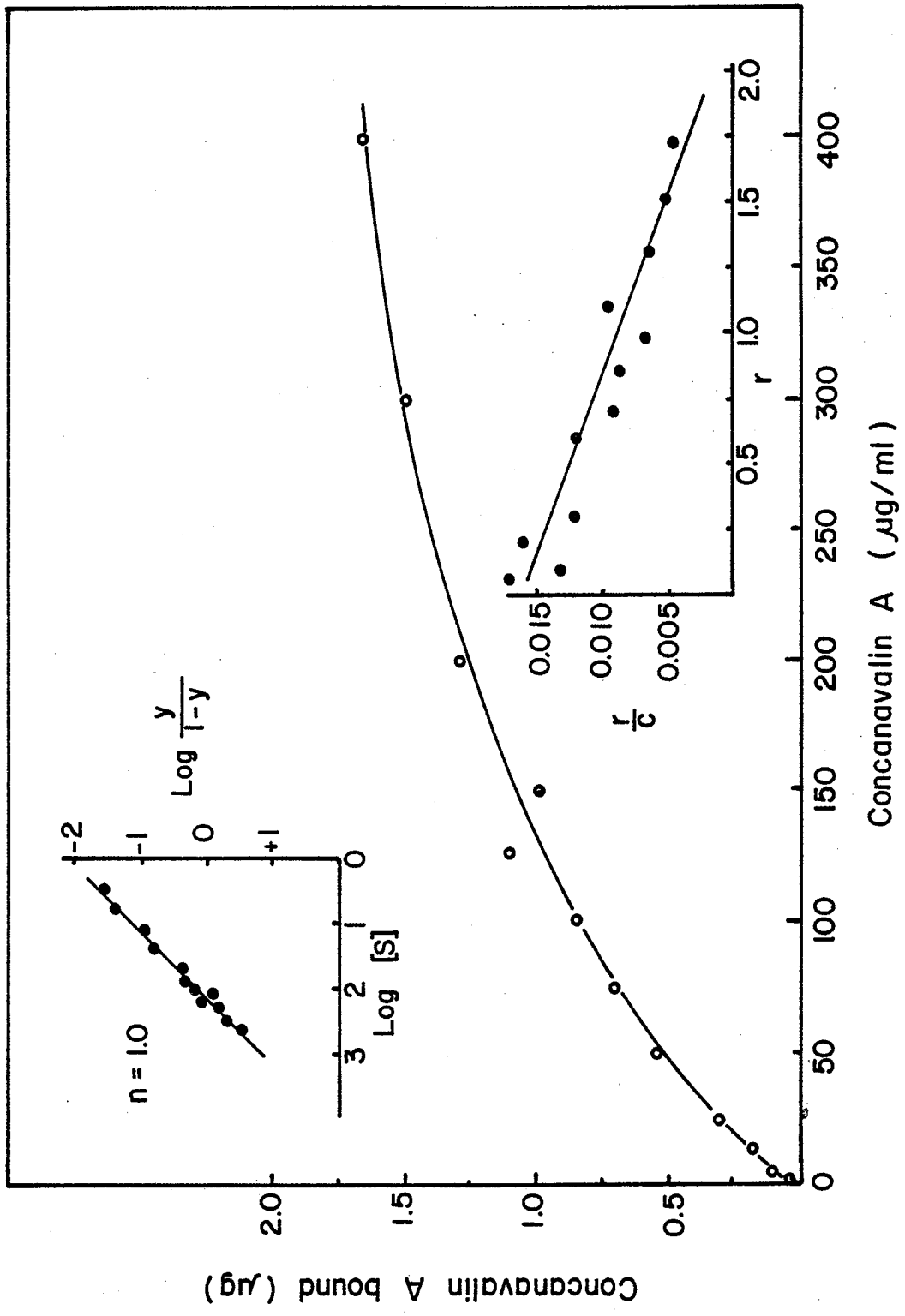
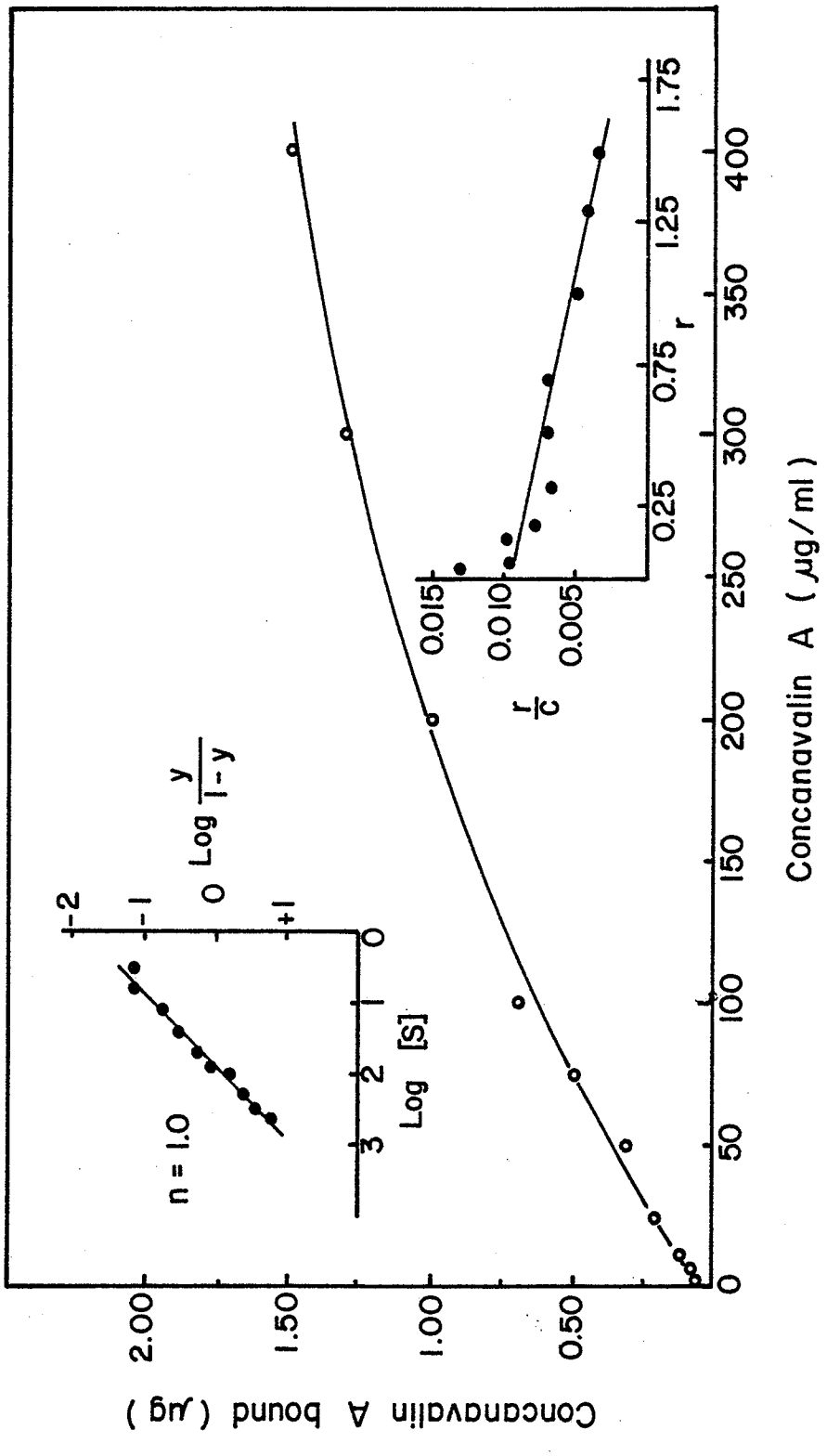

Concanavalin A bound (µg)
2.0
1.5
1.0
0.5
0
50
100
150
200
250
300
350
400
Concanavalin A (µg/ml)
n = 1.0
Log y/(1-y)
-2
-1
0
+1
Log [S]
3
2
1
0
r/c
0.015
0.010
0.005
r
0.5
1.0
1.5
2.0

Figure 30 ^{3}H-labelled con A bound/1.8 x 10^{6} EC^{R}-1 cells at various concentrations of lectin. The inset in the upper part shows a Hill plot of the data. The Hill coefficient was calculated to be 1.0. The inset at the bottom presents the data according to Scatchard's equation . Each point represents the average of duplicate points from a single experiment; similar data has been obtained in 2 separate experiments.


Concanavalin A bound (µg)
2.00
1.50
1.00
0.50
0
50
100
150
200
250
300
350
400
Concanavalin A (µg/ml)
n = 1.0
Log [S]
3
2
1
0
-2
-1
0
+1
Log y/(1-y)
r/c
0.015
0.010
0.005
0.25
0.75
1.25
1.75
r

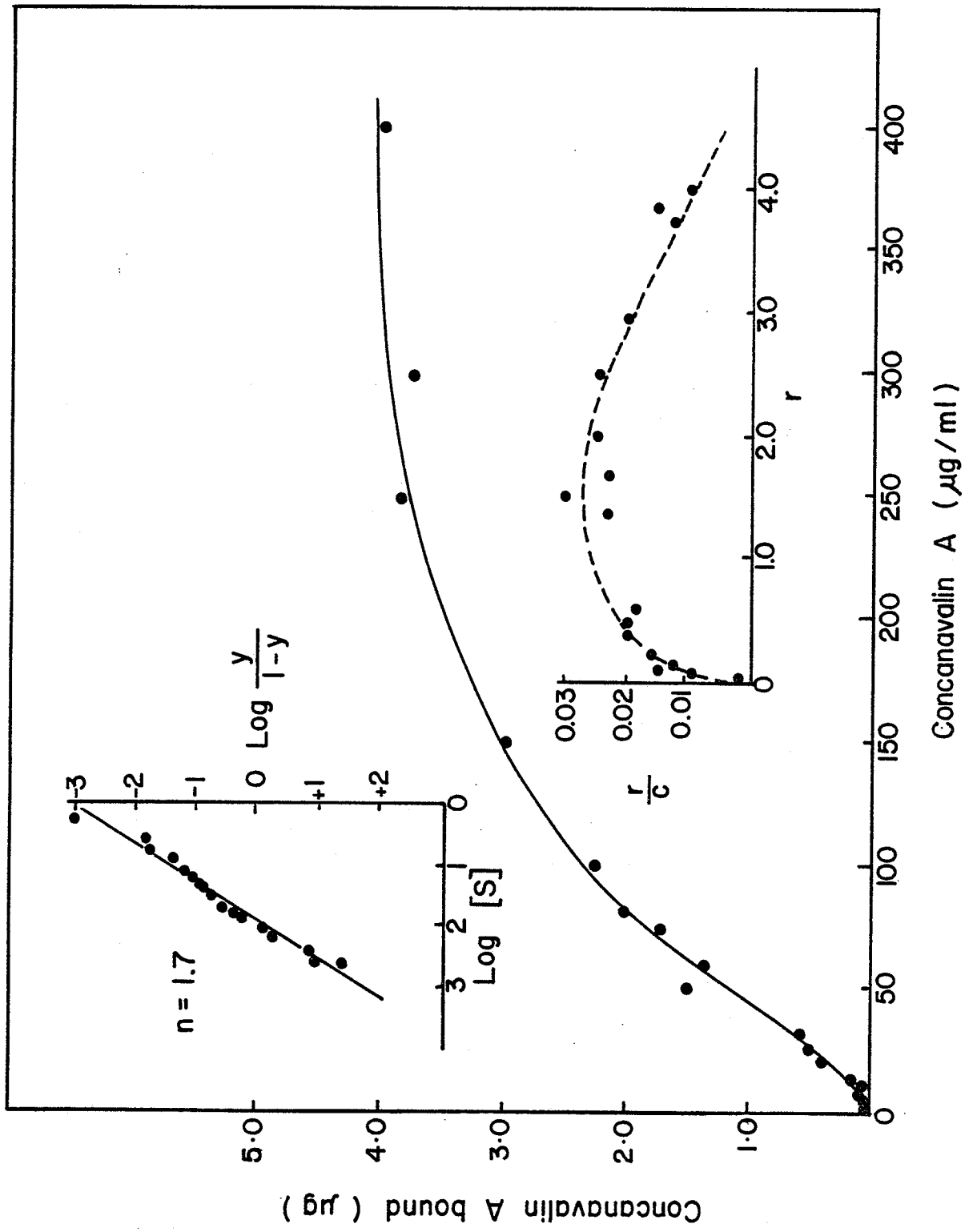
(Figures 29 and 30; see insets) a Hill value of 1.0 was obtained, indicating that the labelled con A was probably binding to non-interacting lectin sites at the surface of these con A-resistant variants. Scatchard plots (Figures 29 and 30; see insets) of the binding curves were linear, again indicating the lack of a cooperative binding mechanism in the mutant cell line. By extrapolation of the Scatchard plot to the abscissa it was determined that BC^R-2 and EC^R-1 bound approximately 2.1 and 2.3 µg/ml con A/1.8 x 10^6 cells respectively (Figures 29 and 30; see insets). These values represent 6.3 x 10^6 molecules and 6.9 x 10^6 molecules of con A bound/variant cell surface. The surface areas of BC^R-2 and EC^R-1 were calculated to be 1145 μm^2 and 1120 μm^2, therefore at saturation binding BC^R-2 bound 5.5 x 10^3 molecules and EC^R-1 bound 6.2 x 10^3 molecules of con A/ μm^2 of surface area. It is clear, therefore, that con A-resistant lines bind con A in a non-cooperative fashion and bind a maximum of 5.5 to 6.5 x 10^3 molecules of lectin/μm^2 of cell surface area.

As an additional control for the differences in binding mechanism between wild-type cells and those lines selected for their resistance to con A, binding studies were also carried out with a cell line selected for resistance to the toxic action of an unrelated drug, hydroxyurea.

The cell line H^R-100 was selected in our laboratory by Mr. K.A. Goodridge by using previously published selection procedures (Wright and Lewis, 1974; Lewis Wright, 1974). The H^R-100 cell line is highly resistant to the toxic effects of hydroxyurea and exhibited a wild-type sensitivity to con A.

The binding of labelled con A to H^R-100 cells as a function of lectin concentration is shown in Figure 31. The binding curve obtained appeared to be sigmoidal and suggested that con A was binding with positive cooperativity. Analysis of the binding curve by the Hill plot (Figure 31, see inset) indicated a significant amount of lectin was bound to the cells in a cooperative manner. The Hill coefficient was calculated to be 1.7. The non-linearity of the Scatchard plot (Figure 31, see inset), also indicated that the lectin bound to H^R-100 cells in a cooperative fashion. As with the wild-type population, it was difficult to extrapolate the non-linear Scatchard plot to the abscissa, however it was possible to estimate that approximately 5.2 µg/ml con A was bound per 1.8×10^6 cells at saturation. This represents approximately 1.6×10^7 molecules of lectin bound per cell. If the cell surface area of 940 μm^2 for H^R-100 is taken into consideration it then represents 1.7×10^4 molecules of lectin bound/μm^2 surface area. Therefore it is apparent that H^R-100,

Figure 31 ^{3}H-labelled con A bound/1.8 x 10^{6} $H^{R}100$ cells at various concentrations of lectin. The inset in the upper part shows a Hill plot of the data. The Hill coefficient was calculated to be 1.7. The inset at the bottom presents the data according to Scatchard's equation. Each point represents the average of duplicate points from a single experiment; similar data has been obtained in 3 separate experiments.


Concanavalin A bound (µg)
Concanavalin A (µg/ml)
n = 1.7
Log y/1-y
Log [S]
r/c
r

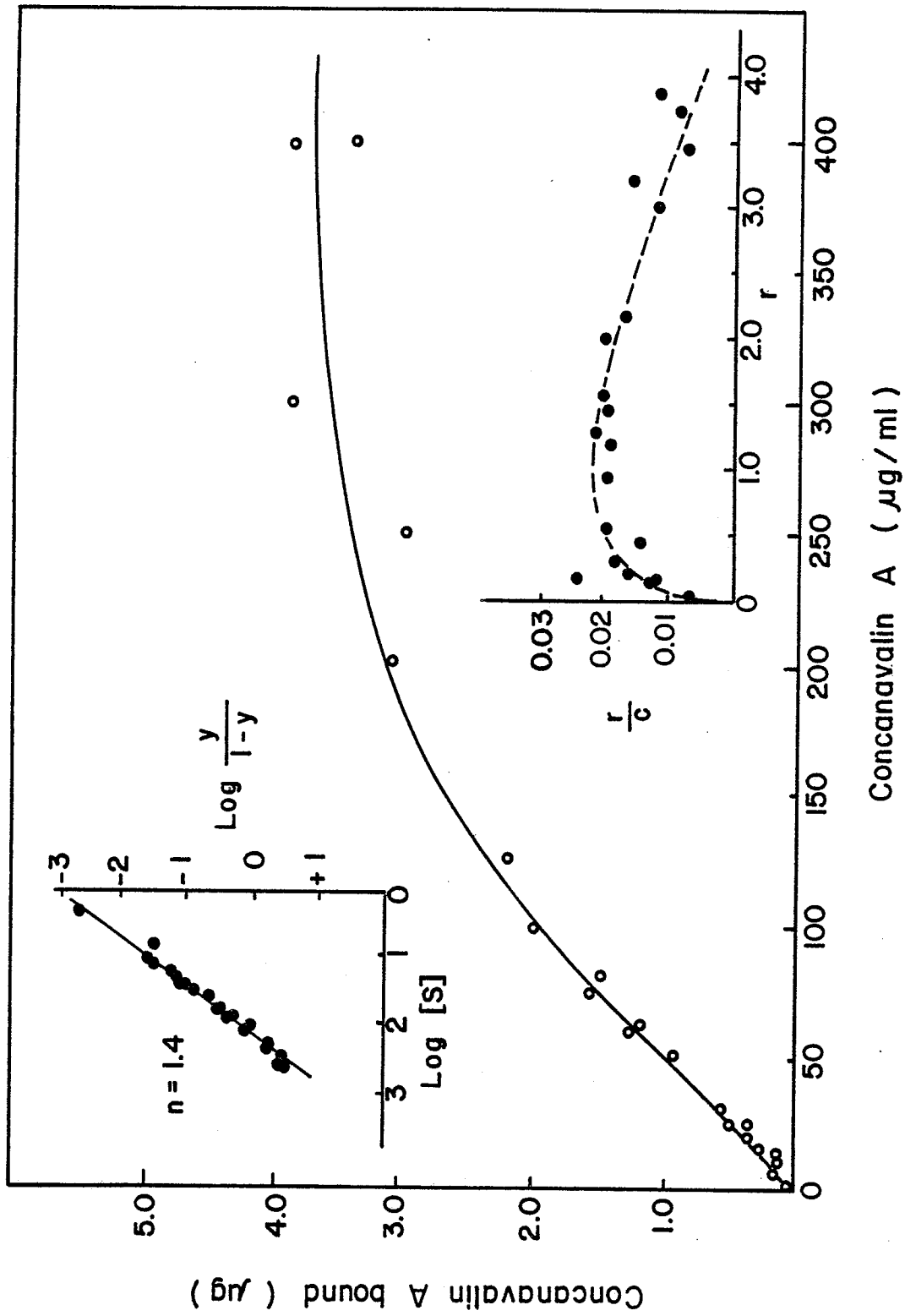
a variant selected for resistance to a drug other than con A, exhibited a binding mechanism similar to that found for the 3 independent wild-type population studied, and bound approximately the same amount of con A/surface area as wild-type cells.

The binding of labelled con A to RC^R-7 cells was studied to determine if the return to con A-sensitivity also resulted in a return to a cooperative binding mechanism.

The binding of labelled con A to RC^R-7 cells as a function of lectin concentration is shown in Figure 32. The binding was not hyperbolic; when the data was analysed for possible interactions between binding sites by the Hill and Scatchard plots (Figure 32; see insets) it was clear that the lectin bound to the intact cells in a cooperative manner. The Hill coefficient was calculated to be 1.4 as compared to 1.8 with wild-type cells and 1.7 for H^R-100 cells (Table XIII). This indicates that the interactions between con A binding sites in the revertant may not be quite as strong as the cooperative interactions observed in the wild-type case.

An estimate of the amount of bound con A derived from the non-linear Scatchard plot revealed that about

Figure 32 ^{3}H-labelled con A bound/1.8 x 10^{6} RC^{R}-7 cells at various concentrations of lectin. The inset in the upper part shows a Hill plot of the data. The Hill coefficient was calculated to be 1.4. The inset at the bottom presents the data according to Scatchard's equation. Each point represents the average of duplicate points from a single experiment; similar data has been obtained in 3 separate experiments.


Concanavalin A bound (µg)
5.0
4.0
3.0
2.0
1.0
0
50
100
150
200
250
300
350
400
Concanavalin A (µg / ml)
n = 1.4
-3
-2
-1
0
+1
Log y / 1-y
3
2
1
0
Log [S]
0.03
0.02
0.01
r / c
0
1.0
2.0
3.0
4.0
r

5 μg/ml con A bound per 1.8×10^6 cells. This value corresponds to 1.5×10^7 molecules of lectin bound per cell, and when the cell surface area (1010 μm^2) was considered, it was calculated that approximately 1.5×10^4 molecules of lectin was bound/μm^2 surface area.

These results which are similar to the data obtained with wild-type cells strongly suggest that a return to a con A-sensitive phenotype (RC^R-7 cells) was accompanied by a change to a 'wild-type' mechanism of con A binding.

Both hybrid cell lines A-W, formed by the fusion of 2 lectin-sensitive cell lines, and A-7, formed by the fusion of a lectin-sensitive and a lectin-resistant cell line, showed wild-type sensitivity to con A (Table V). Binding studies were carried out to determine if the hybrid cells showed a 'wild-type' binding mechanism for con A.

The results of lectin binding studies are shown in Figures 33 and 34. Clearly the binding data obtained with the two hybrid cell lines was similar. An analysis of the binding data by Hill and Scatchard plots (Figures 33 and 34, see insets) indicated that con A bound to both hybrid lines with positive cooperativity. The interactions between con A binding sites in the pseudo-tetraploid cells, however appeared to be weaker than those

Figure 33 ^{3}H-labelled con A bound/1.8 x 10^{6} A-W cells at various concentrations of lectin. The inset in the upper part shows a Hill plot of the data. The Hill coefficient was calculated to be 1.3. The inset at the bottom presents the data according to Scatchard's equation. Each point represents the average of duplicate points from a single experiment; similar data has been obtained in 3 separate experiments.

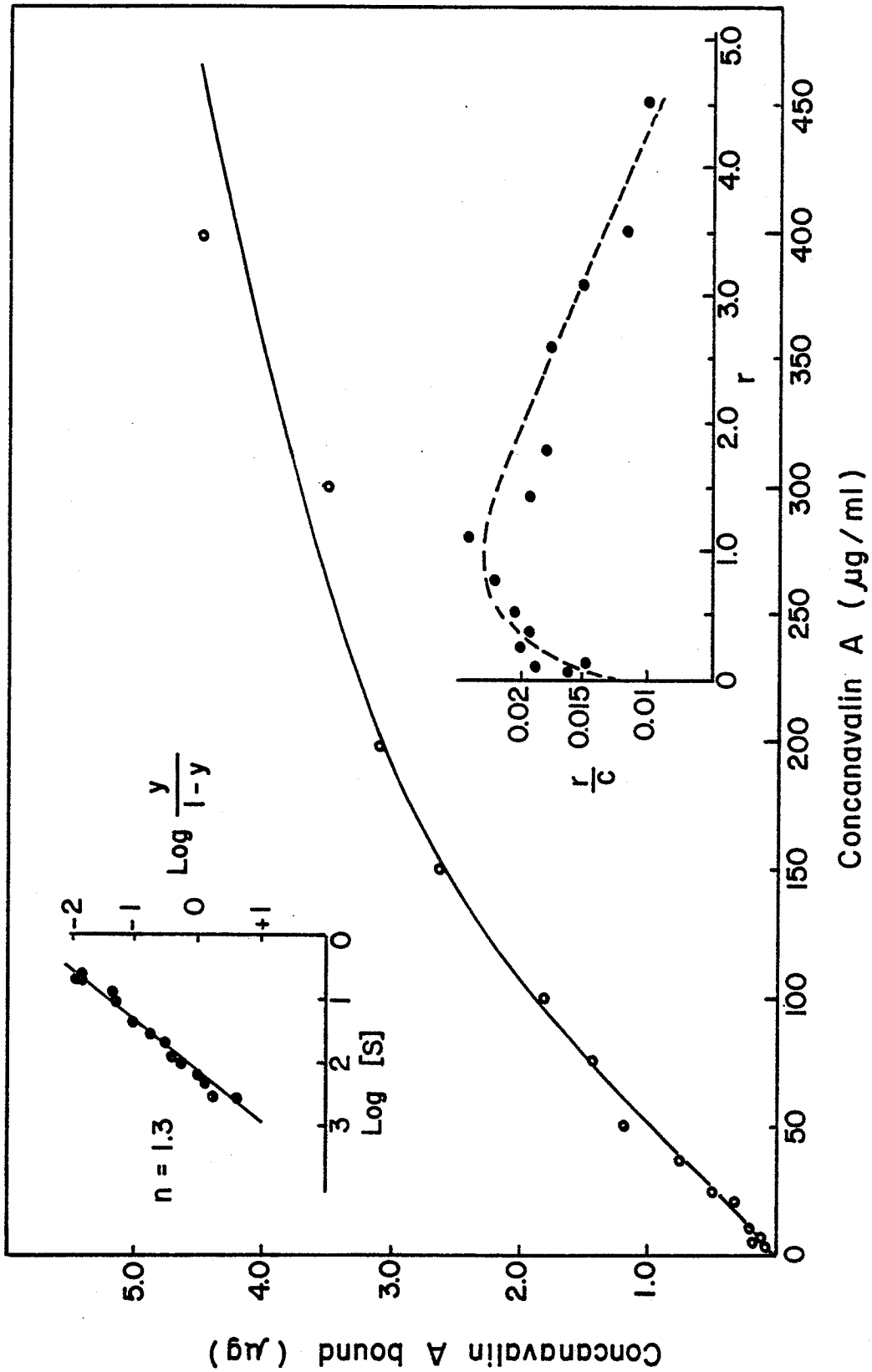
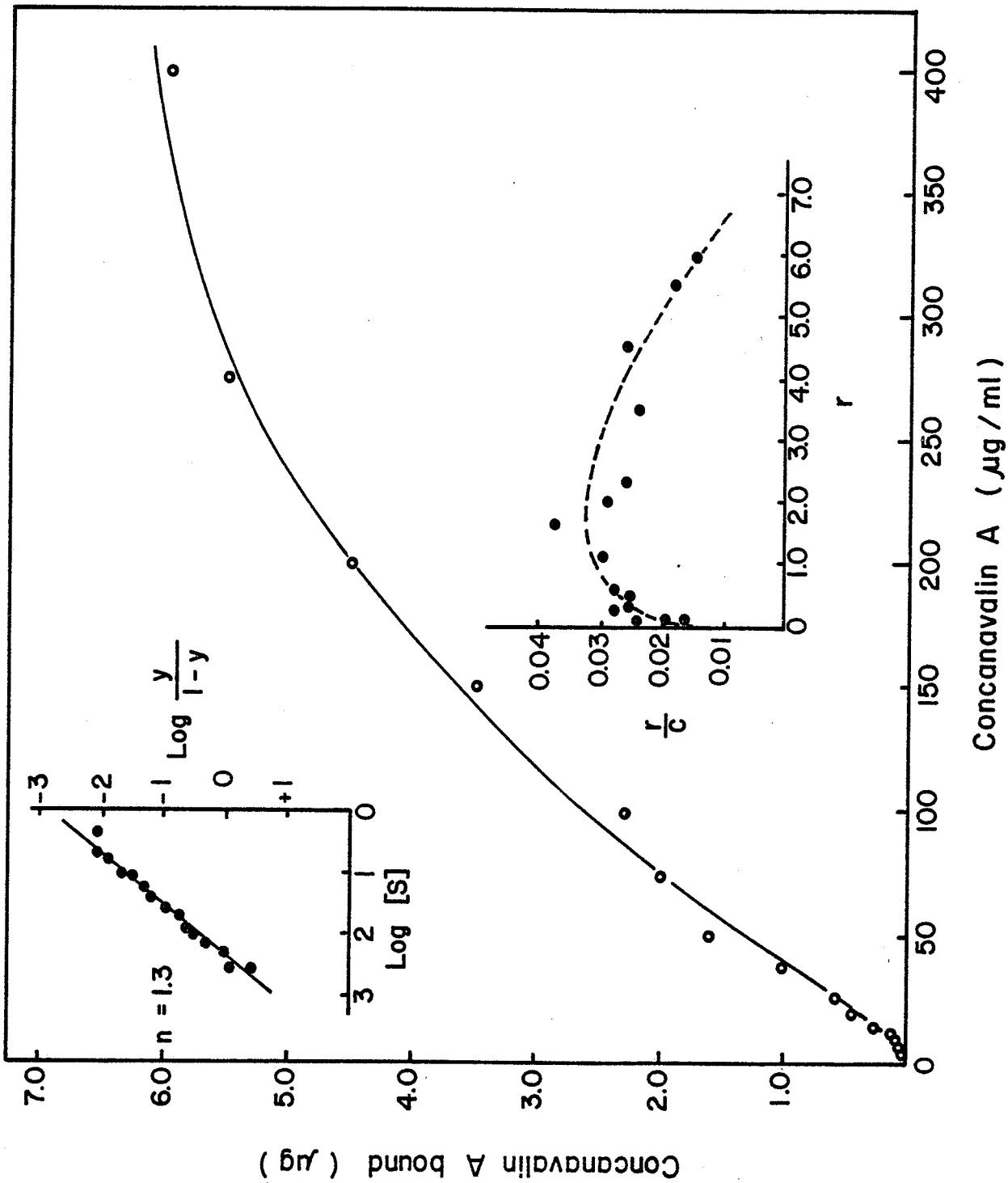

Concanavalin A bound (µg)
Concanavalin A (µg/ml)
n = 1.3
Log [S]
Log y/(1-y)
r/c
r

Figure 34 ^{3}H-labelled con A bound/1.8 x 10^{6} A-7 cells at various concentrations of lectin. The inset in the upper part shows a Hill plot of the data. The Hill coefficient was calculated to be 1.3. The inset at the bottom presents the data according to Scatchard's equation. Each point represents the average of duplicate points from a single experiment; similar data has been obtained in 2 separate experiments.


Concanavalin A bound (µg)
Concanavalin A (µg / ml)
n = 1.3
Log [S]
Log y / 1 - y
r / c
r

of the pseudodiploid cells. For example, the Hill coefficients (Figures 33 and 34; see insets) for both A-W and A-7 were 1.3 as compared to 1.8 for wild-type cells. The reason for these weaker interactions is not yet known.

Once again the lack of linearity with Scatchard plots (Figures 33 and 34, see insets) made it difficult to accurately determine the maximum amount of lectin bound. It was possible, however, to estimate that approximately 4.4 and 8.0 μg/ml of con A bound to 1.8×10^6 cells of A-W and A-7 hybrid lines, respectively. These values represent 1.7×10^7 molecules of con A bound/cell surface. When the cell surface areas of A-W (1110 μm^2) and A-7 (1510 μm^2) were considered, it was calculated that approximately 1.5×10^4 and 1.6×10^4 molecules of con A were bound per μm^2 cell surface area of A-W and A-7 cells respectively. Again these estimates of con A bound/cell surface area closely reflect the values found with the wild-type cells as summarized in Table XIII.

The con A-resistant cell lines were found, therefore, to bind con A by an altered mechanism when compared to wild-type cell lines, and to bind significantly less con A than wild-type cells.

G. Concanavalin A Recpetor Mobility:

Alterations in the surface membrane of mammalian cells

TABLE XIII

Summary of Concanavalin A Binding Experiments.

Cell Lines	Hill coefficient	Concanavalin A molecules/μm^2 surface area ($\times 10^{-3}$)
a) Concanavalin A-Resistant Lines		
C^R-7	1.0	6.5
BC^R-2	1.0	5.5
EC^R-1	1.0	6.2
b) Concanavalin A-Sensitive Line		
Wild-type*	1.8	16.0
H^R-100	1.7	17.0
RC^R-7	1.4	15.0
A-W	1.3	15.0
A-7	1.3	16.0

* The same results were obtained with WT-Cl I, WT-Cl II, and WT-Cl III populations.

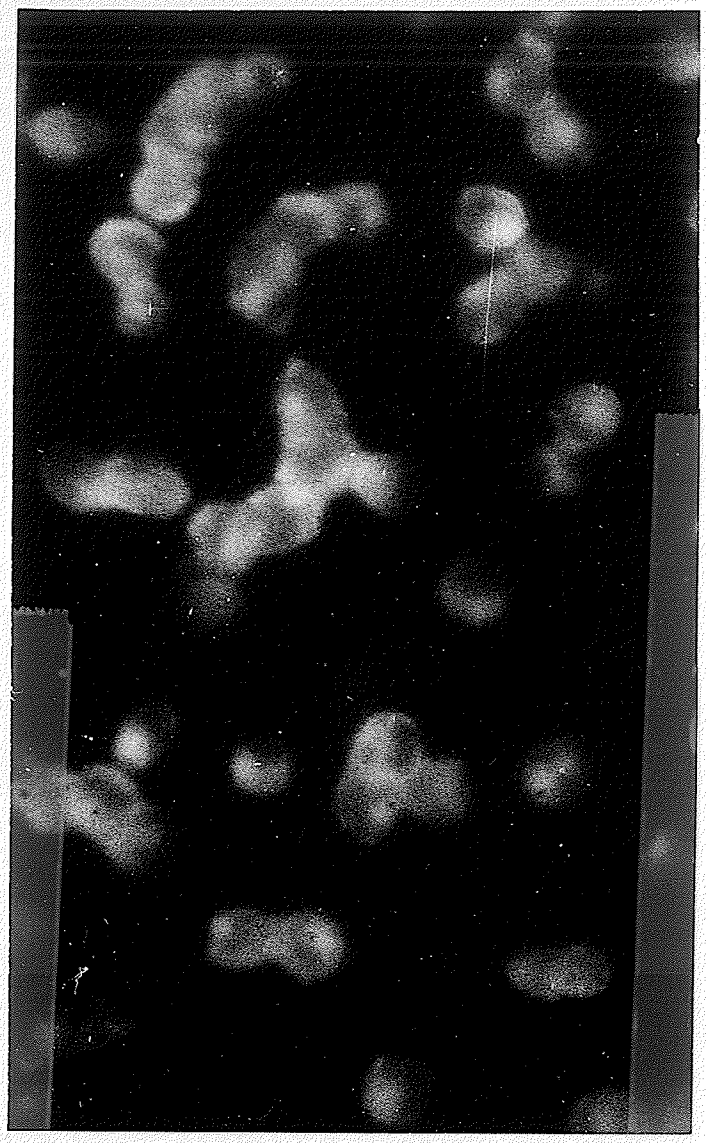
result in important changes in the fluidity and receptor mobility properties of these membranes (Rutishauser et al. 1974; van Veen et al.1976; Albrecht-Buehler and Chen, 1977). The mobility of con A receptors on the surface membrane can be visualized by binding fluorescent conjugated con A (fl-con A) to cells at 4^{O} and then shifting them to normal growth temperatures to observe the redistribution of lectin binding sites (see Methods section).

The ability of CHO cells to aggregate fl-con A into tight aggregates or "caps" has previously been reported (Aubin et al. 1975; Storrie, 1974).

Typical cap formation with wild-type CHO cells is shown in Figure 35. Experiments with WT-Cl I, WT-Cl II and WT-Cl III cells indicated that greater than 95% of the cells produced tight caps after incubation for 1 hour at 34^{O} following fluorescent con A binding (Figure 36).

A significant difference in cap forming ability was observed when con A-resistant cells were analyzed by this technique. Most of the lectin resistant cells showed a more random distribution of label over the cell surface, with some patching observed at contact points between adjacent cells (Figure 37). Approximately 10, 15 and 25% of the C^{R}-7, BC^{R}-2 and EC^{R}-1 populations exhibited an ability to form caps (Figure 36). Increasing the incubation period to more than 3 hours did not increase

Figure 35 Cap formation on parental wild-type CHO cells. Experiments were performed as described in Methods section. More than 200 cells of the wild-type Cl I, Cl II, and Cl III) were examined in each of 3 separate experiments.
Magnification: a) 240 X; b) 700 X.


A

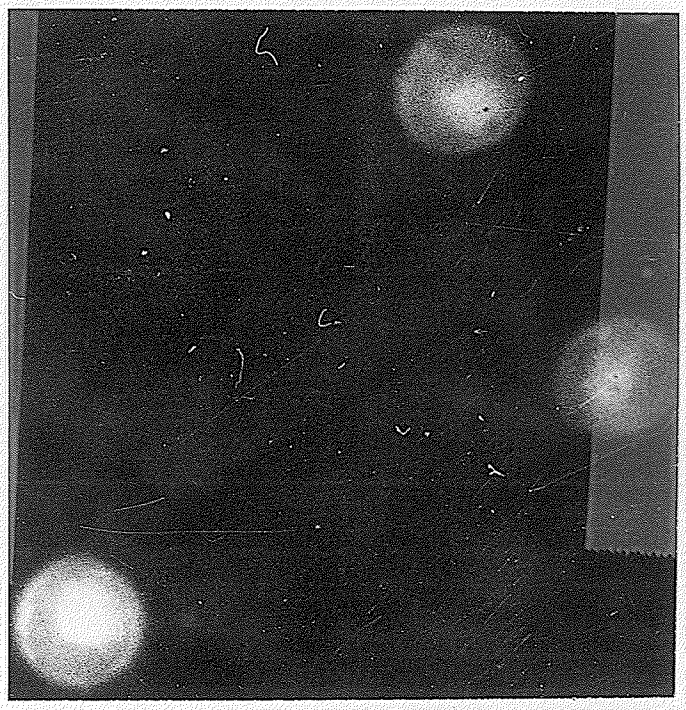

B

Figure 36 Percent cap formation on wild-type (A), RC^R-7 (B), A-W (C), A-7 (D), C^R-7 (E), EC^R-1 (F) and BC^R-2 (G) populations. Experiments were performed as described in Methods section. More than 200 cells of each cell line were examined in each of three separate experiments.

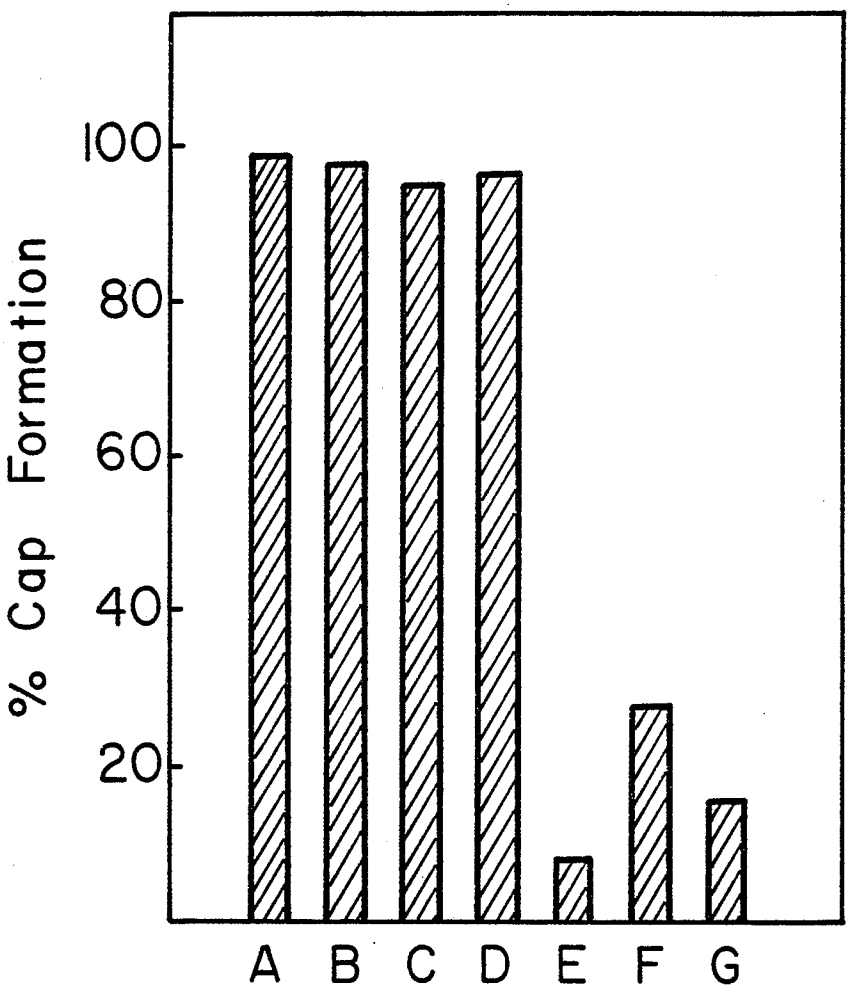
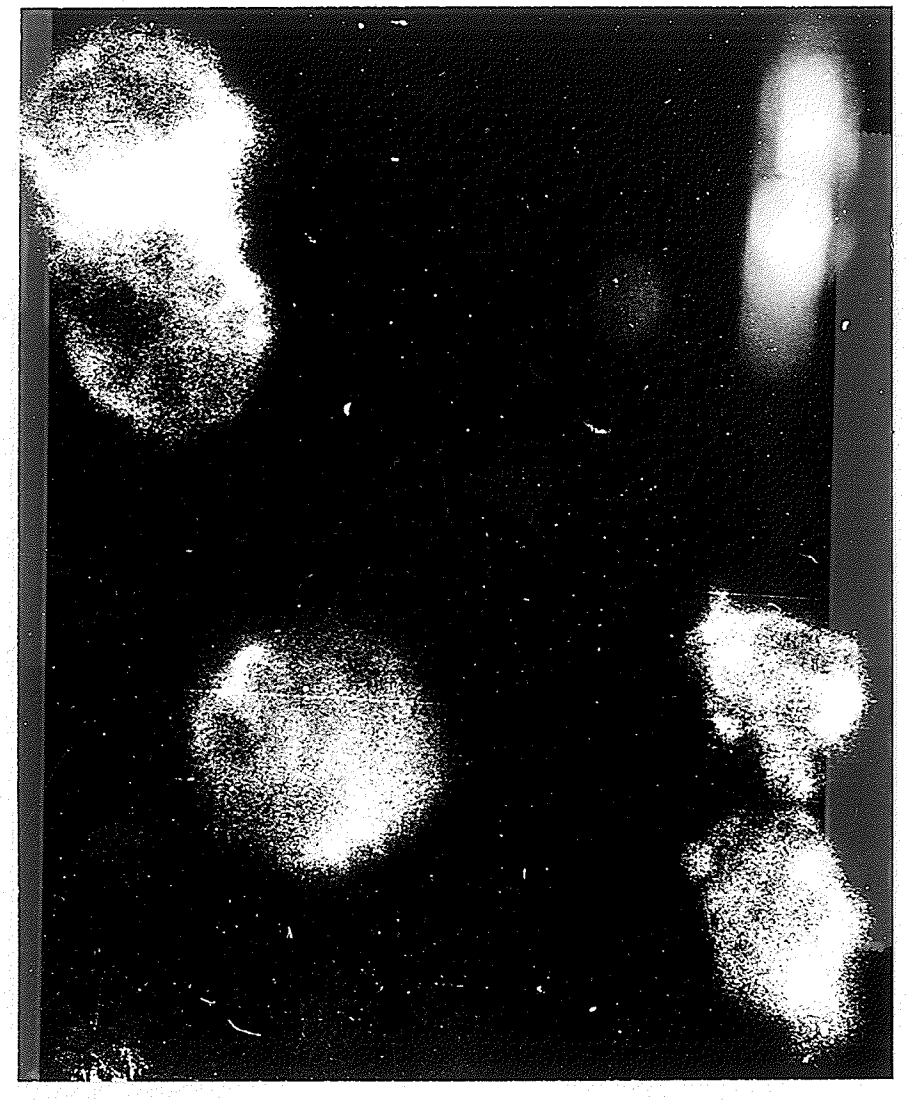

% Cap Formation
100
80
60
40
20
A
B
C
D
E
F
G

Figure 37 Typical lectin distribution on variant cell line (C^R-7). Experiments were performed as described in Figure 35. Magnification; 1,600 X.



the proportion of variant cells which showed tight caps.

The revertant cell line RC^R-7 and the hybrid cell lines A-W and A-7 exhibited wild-type membrane properties in previous studies (Ceri and Wright, 1977a, 1978a; see previous studies this thesis). Their ability to aggregate lectin receptors was also found to be similar to the wild-type cell lines (Figure 36). RC^R-7 was found to aggregate fl-con A into tight caps (Figure 38) in approximately 95% of cells kept one hour at 34^O (Figure 36). Similarly, the hybrid cell lines capped fl-con A (Figure 36). Perhaps it should be noted that the RC^R-7 and hybrid cell lines also showed a cooperative con A binding mechanism (Table XIII).

The regulation of surface receptor movement may be accomplished through a cytoskeleton system located below the surface membrane (Edelman, 1974, 1976). The disruption of the microtubule formation with colchicine led to the loss of cap formation and the random distribution of fl-con A on the surface of various CHO cell lines (Aubin et al. 1975). The effect of colchicine on con A-resistant and -sensitive cell lines was studied to determine if the difference in capping in the variant cell lines involved altered microtubules. The cell lines were pre-treated with colchicine as previously described by Aubin (Aubin et al. 1975). The lectin distribution on colchicine treated WT-Cl I seen in Figure 40 typifies the results obtained with the other cell lines

Figure 38 Cap formation in RC^R-7 cell lines. Experiments were performed as in Figure 35.
Magnification: 1,400 X.

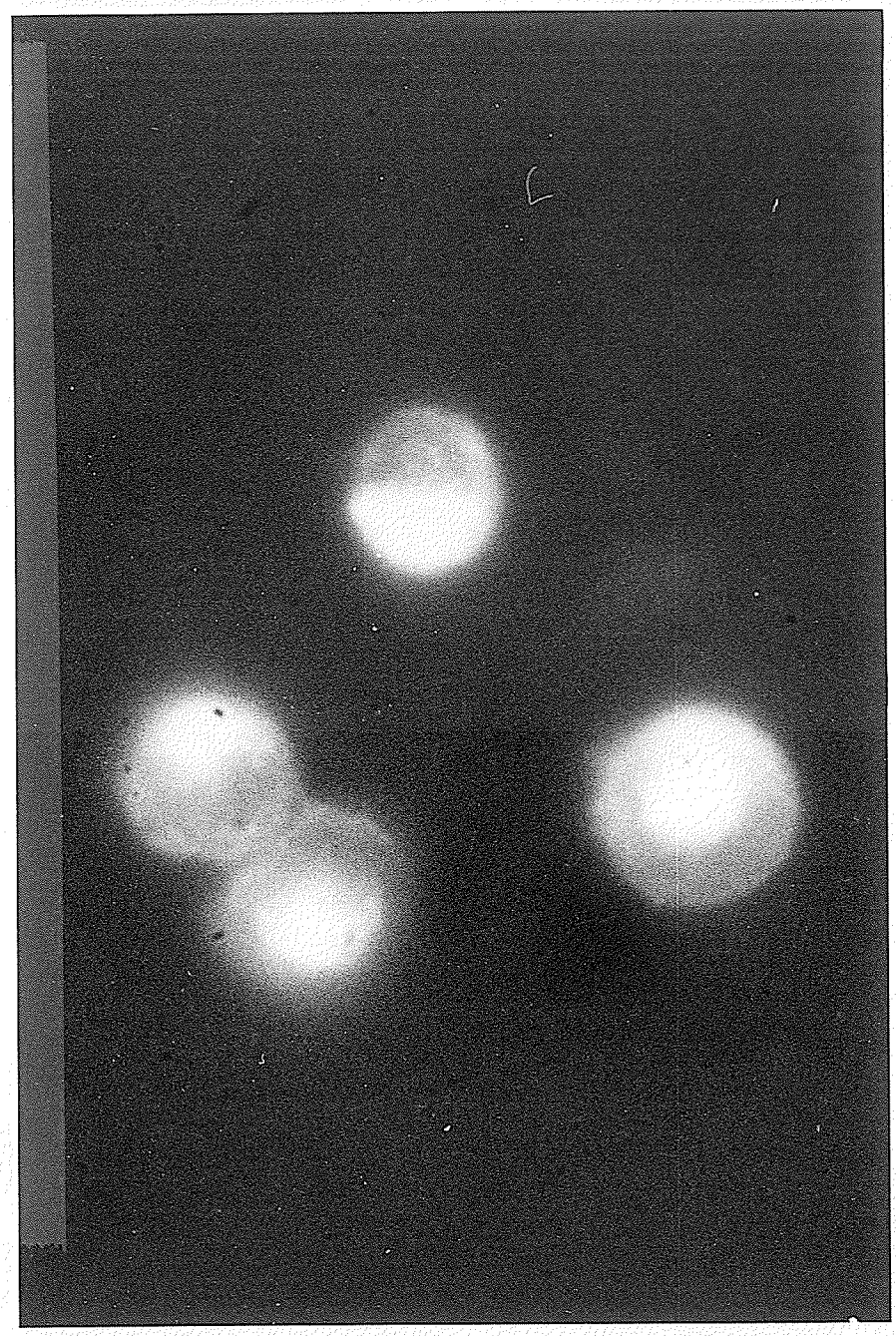


Figure 39 Cap formation in the hybrid cell line A-7.

Experiment performed as in Figure 35.

Magnification: 1,000 X.

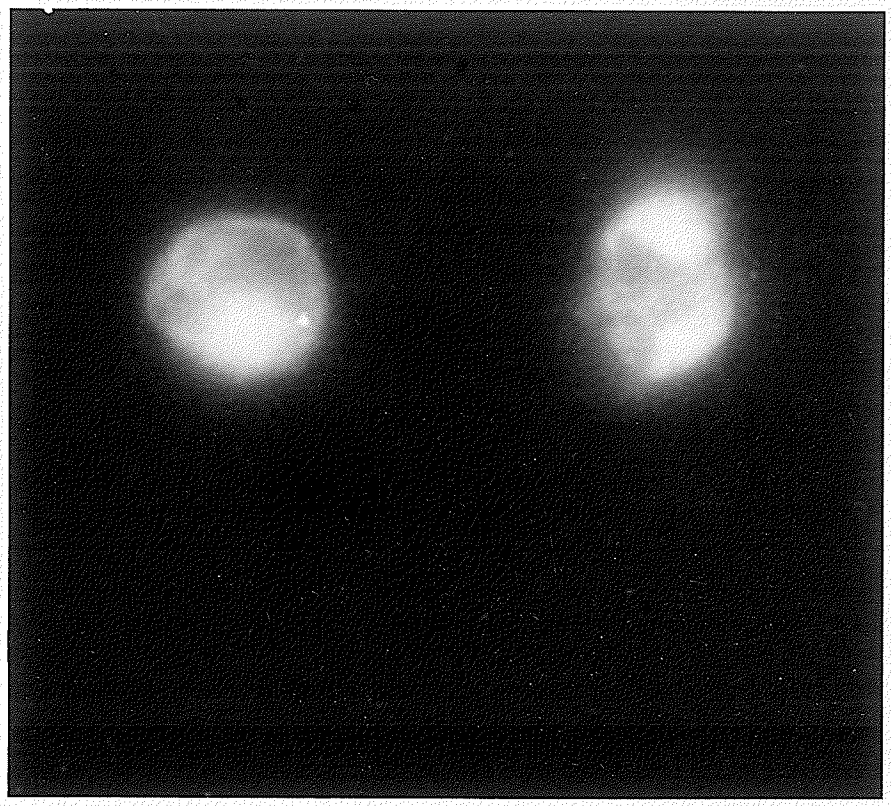
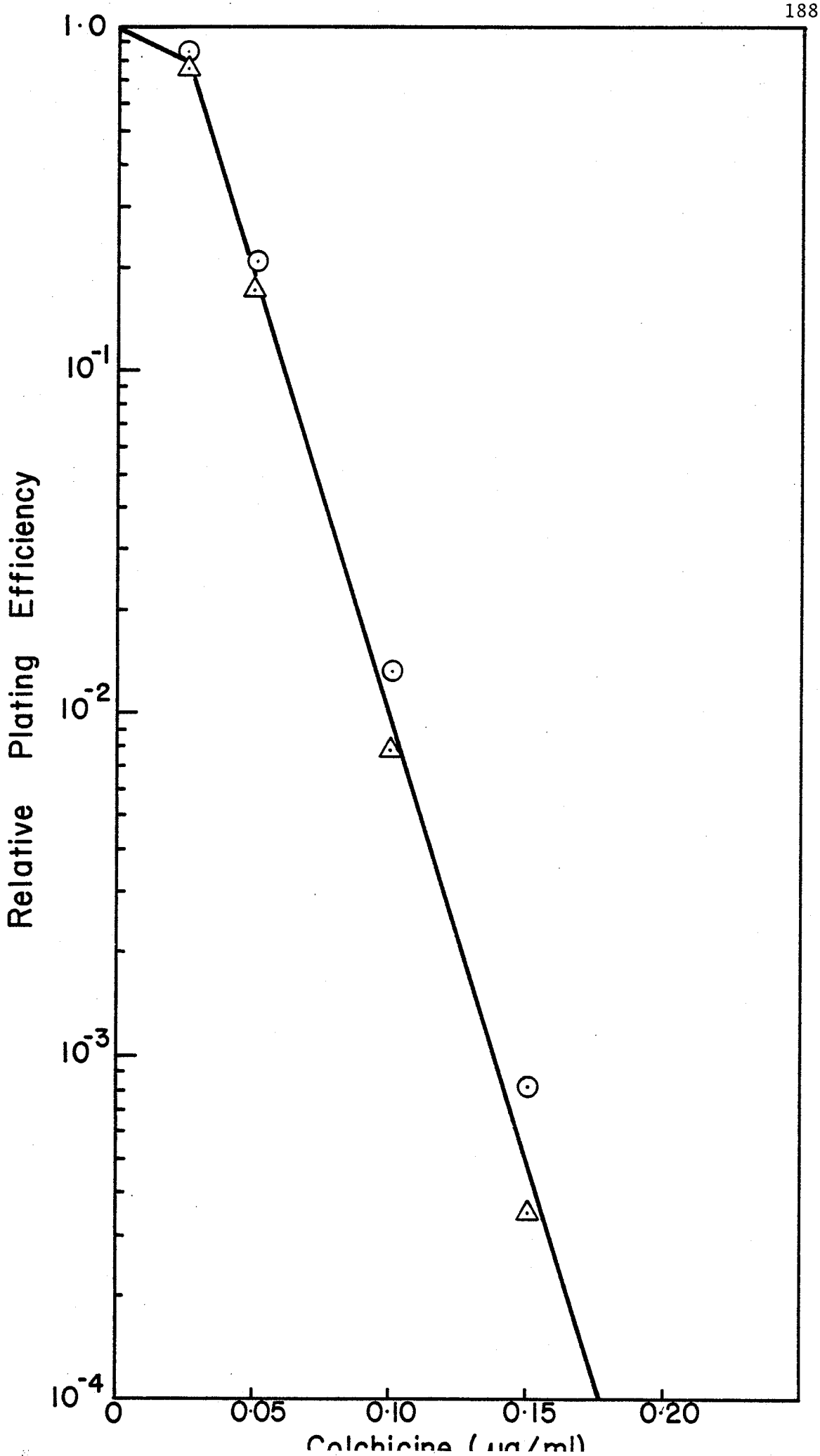


Figure 40 Cap formation of WT-Cl I cells treated with 10^{-5} M colchicine. Experiments were performed as in Figure 35. Magnification: 750 X.

(WT-Cl II and III, C^R-7, BC^R-2, EC^R-1, A-W, and A-7). The lectin appeared evenly distributed over the entire cell surface. These results suggest that the differences in capping previously observed with the con A-resistant cell lines probably do not involve obvious changes in the microtubule system. This point is supported by the observation that WT-Cl I and C^R-7 cells exhibited similar sensitivities to colchicine toxicity in plating efficiency experiments performed in the presence of colchicine (Figure 41).

The lack of cooperativity in con A binding observed with con A-resistant cell lines (Table XIII) may in part be due to the loss of lectin receptor mobility of these cell lines (Figure 36). Perhaps receptor site mobility is an important part of the apparent cooperative lectin binding mechanism observed with con A-sensitive cell lines. To test this point the binding of labelled con A to WT-Cl I cells was characterized after the mobility of surface components was blocked by first fixing the cell surface for 60 minutes with 3.0% gluteraldehyde (Nicolson, 1972; Van Blitterswijk et al. 1976). When the data was analyzed (Figure 42), the binding curve appeared to be hyperbolic; this result suggested that the binding of lectin occurred without positive cooperativity. Further analysis of the data by Hill (1913) and Scatchard (1949) replots (Figure 42;

Figure 41 Plating efficiency of WT-Cl I (⊙) and C^R-7 (△) cells in varying concentrations of colchicine. Points represent the mean of duplicate points from a single experiment. Similar results were obtained in two separate experiments.


1·0
10⁻¹
10⁻²
10⁻³
10⁻⁴
Relative Plating Efficiency
0
0·05
0·10
0·15
0·20
Colchicine (μg/ml)

see insets) again indicated the lack of cooperative lectin binding. Therefore, the gluteraldehyde treatment, which results in the loss of receptor mobility of wild-type cells (Nicolson, 1976a) also leads to the loss of cooperative binding of con A (Figure 42). This suggests that the loss of receptor mobility on con A-resistant cell lines may be at least partly responsible for the loss of the cooperative con A binding mechanism.

H. Concanavalin A-Resistant Phenotype

Independent con A-resistant cell lines share a complex phenotype which is not observed with con A-sensitive cell lines (Table XIV). The results presented in this section supports the view that con A is an excellent selective agent for obtaining mammalian cells with altered membrane-active properties and provides convincing evidence that the altered cellular properties exhibited by the lectin-resistant cell lines are directly related to con A resistance.

Figure 42 ^{3}H-labelled con A bound to 8×10^{6} gluteraldehyde fixed WT-Cl I cells at various concentrations of lectin. The inset in the upper part shows a Hill plot of the data. The Hill coefficient was calculated to be 1.0. The inset at the bottom presents the data according to Scatchard's equation. Each point represents the average of duplicate points.

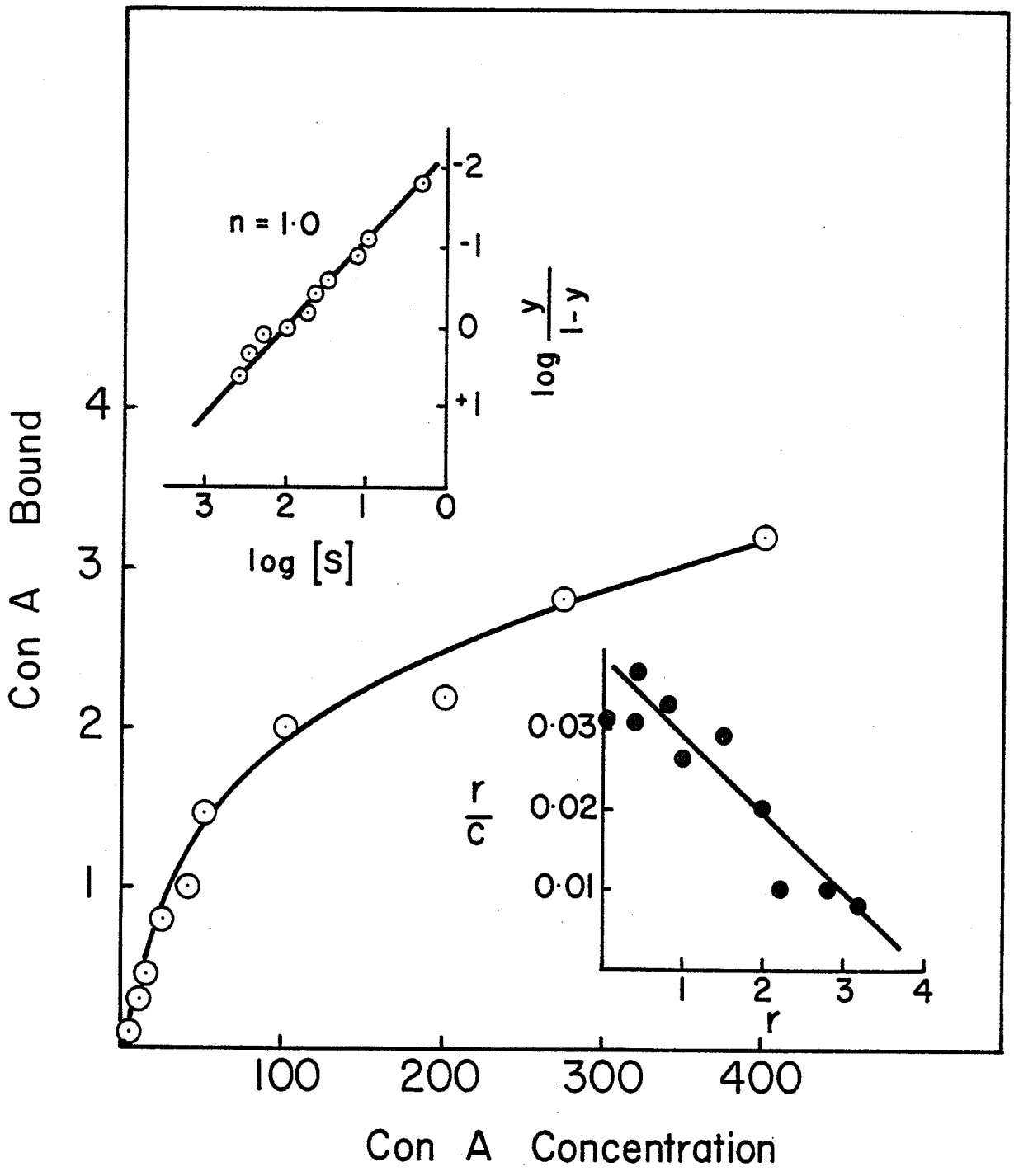

Con A Bound
Con A Concentration
100
200
300
400
1
2
3
4
n = 1·0
log [S]
3
2
1
0
-2
-1
0
+1
log y/1-y
r/c
0·03
0·02
0·01
r
1
2
3
4

TABLE XIV

Pleiotropic Changes of the Concanavalin A-Resistant Phenotype

Membrane Property	Cell Lines					
	WT	C^R-7	BC^R-2	EC^R-1	RC^R-7	A-7
Altered morphology on solid surfaces (in comparison to WT-clones)		+	+	+		
Relative cell adherence[1]	1	0.44	0.66	0.61	0.97	0.94
D_{10} PEA	5.1mM	2.1mM	2.0mM	1.8mM	4.6mM	4.0mM
D_{10} NaButyrate	.73mM	.34mM	.33mM	.35mM	.70mM	.68mM
Cooperativity of lectin binding	+ve	None	None	None	+ve	+ve
Con A bound (molecules/ μm^2 surface area) ($X10^{-3}$)	16	6.5	5.5	6.2	15	16
Receptor mobility (% capping)	>90	10	15	25	>90	>90
Agglutination in 500 µg/ ml Con A	100%	50%	ND	50%	100%	100%

[1]Cell detachment in 0.03% trypsin after 40 min. at 34° relative cell number attached to plates following treatment.
ND - not done

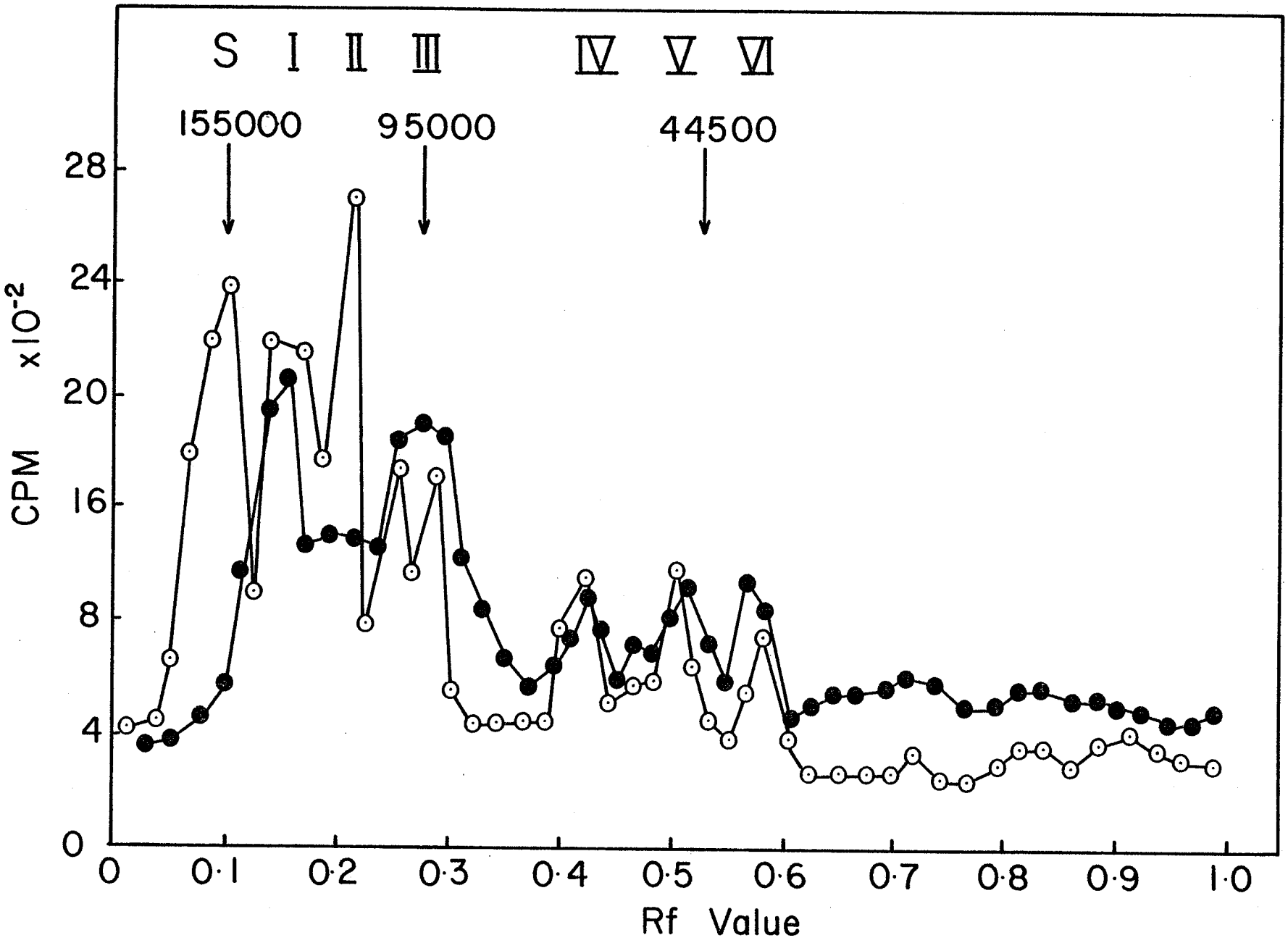
Section 3 Surface Membrane Alterations on Concanavalin A-Resistant Cell Lines

Many of the altered cellular properties exhibited by con A-resistant cell lines should be due to changes in the surface carbohydrate-containing structures (Ceri and Wright, 1977a, 1978a). Cell surface labelling techniques were used to demonstrate glycoprotein changes on the surface of ricin-resistant (Gottlieb et al. 1975), PHA-resistant (Juliano and Stanley, 1975) and WGA-resistant (Briles et al. 1977) cell lines. Since con A is known to interact with cell surface glycoproteins (Sharon and Lis, 1972) there was a very good possibility that con A-resistant cell lines would also show obvious changes in surface glycoproteins. To test this possibility three distinct surface labelling techniques were used to examine the surfaces of wild-type, con A-resistant and revertant cell lines.

A Galactose Oxidase - [^{3}H] - Borohydride Labelling

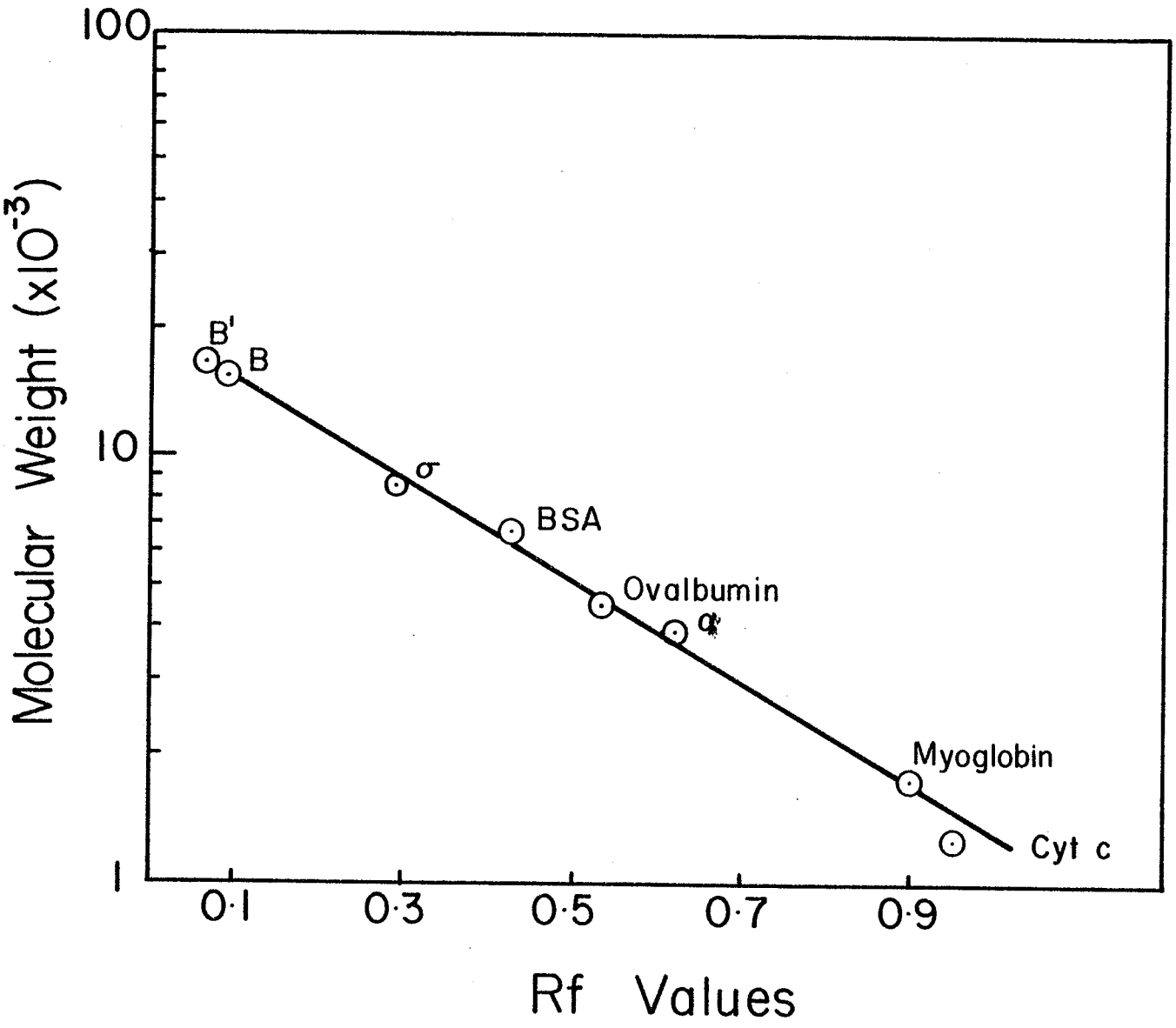
Galactose and galactosamine residues at the cell surface were labelled using the galactose oxidase - [^{3}H] - borohydride method (Gahmberg and Hakomori, 1973; Ceri and Wright, 1977b; Ceri and Wright, 1978; see Methods section). The results of the galactose labelling of WT-Cl I

Figure 43 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of wild type (●) and C^{R}-7 (⊙) preparations labelled by the galactose-oxidase-[^{3}H]-borohydride technique as described in the Methods section. The gels contained 7.5% acrylamide. Bovine serum albumin, oval bumin, myoglobin and cytochrome c with molecular weights of 68 000, 44 500, 17 800 and 12 400 respectively were used as standards. Also included were the E. coli RNA polymerase β', β, σ and α subunits (kindly provided by Dr. C.T. Chow) with molecular weights of 165 000, 155 000, 95 000 and 39 000 respectively. The molecular weights indicated by the arrows represent the relative positions of the β' and σ subunits and ovalalbumin respectively. The data is typical of seven separate labelling experiments.


S I II III IV V VI
155000
95000
44500
CPM x10-2
28
24
20
16
8
4
0
0 0·1 0·2 0·3 0·4 0·5 0·6 0·7 0·8 0·9 1·0
Rf Value

and variant C^R-7 are shown in Figure 43. Labelled glycoprotein peaks I, II, III, IV, V, and VI with apparent molecular weights of 140 000, 120 000, 95 000, 67 000, 50 000, and 40 000 were found on both wild-type and the variant cell line. The molecular weights were determined by comparing the distance a particular peak moved in a gel with the distance that known molecular weight markers migrated in the gel. The molecular weight markers that were used included bovine serum albumin (68 000), ovalbumin (44 500), myoglobin (17 800) and cytochrome c (12 400). Also included were the E. coli RNA polymerase β^1, β, and σ subunits (kindly provided by Dr. C.T. Chow) with molecular weights of 165 000, 155 000, 95 000, and 39 000 respectively. A typical standard curve indicating the relative positions of the various molecular weight markers is shown in Figure 44. In six separate experiments peak II appeared as a well defined sharp peak with C^R-7 cells whereas this peak was observed to be more broadly labelled with wild-type cells. Also a single broad peak III, with an apparent molecular weight of 95 000, labelled with the parental wild-type population appeared as two separate peaks with apparent molecular weights of 105 000 and 90 000 with C^R-7 cells. However a very

Figure 44 Molecular weight standard curve in 7.5% polyacrylamide gels. Points represent the β', β, σ and ∝ subunits of E. coli polymerase and bovine serum albumin, ovalbumin, myoglobin, and cytochrome c with molecular weights of 165 000, 155 000, 95 000, 39 000, 68 000, 44 500, 17 800, and 12 400 respectively.


100
10
1
Molecular Weight (x10^{-3})
B'
B
σ
BSA
Ovalbumin
α
Myoglobin
Cyt c
0·1
0·3
0·5
0·7
0·9
Rf Values

obvious difference between wild-type and resistant cells was the presence of a labelled peak, designated S, with an apparent molecular weight of 155 000 on C^R-7 cells which was absent from WT-Cl I. The results from six separate experiments have indicated that the S peak on C^R-7 cells accounted for 12 to 17% of the total labelled cell surface glycoprotein (Table XV).

Although surface labelling procedures label the surface membrane in the majority of the cell population, it has been reported that they may also label internal sites in a small subpopulation of nonviable cells (Juliano and Behar-Bonnelier, 1975). The altered cell surface labelling patterns obtained with C^R-7 cells are probably not due to the labelling of internal sites of a small sub-group of variant cells because the labelled patterns obtained from protein extractions from either whole cells or from purified membrane preparations (see Methods) were very similar (Figure 45).

The galactose-oxidase - [^{3}H] - borohydride labelling patterns obtained from comparisons of BC^R-2 with its parental line WT-Cl II (Figure 46) and from EC^R-1 with its parental line WT-Cl III (Figure 47) were essentially similar to those of WT-Cl I and C^R-7. Again, the same major peaks I to VI were present in all parental and

Figure 45 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell extracts of C^R-7 (⊙) and extracts of surface membrane preparations of C^R-7 (●) labelled as described in Figure 43.

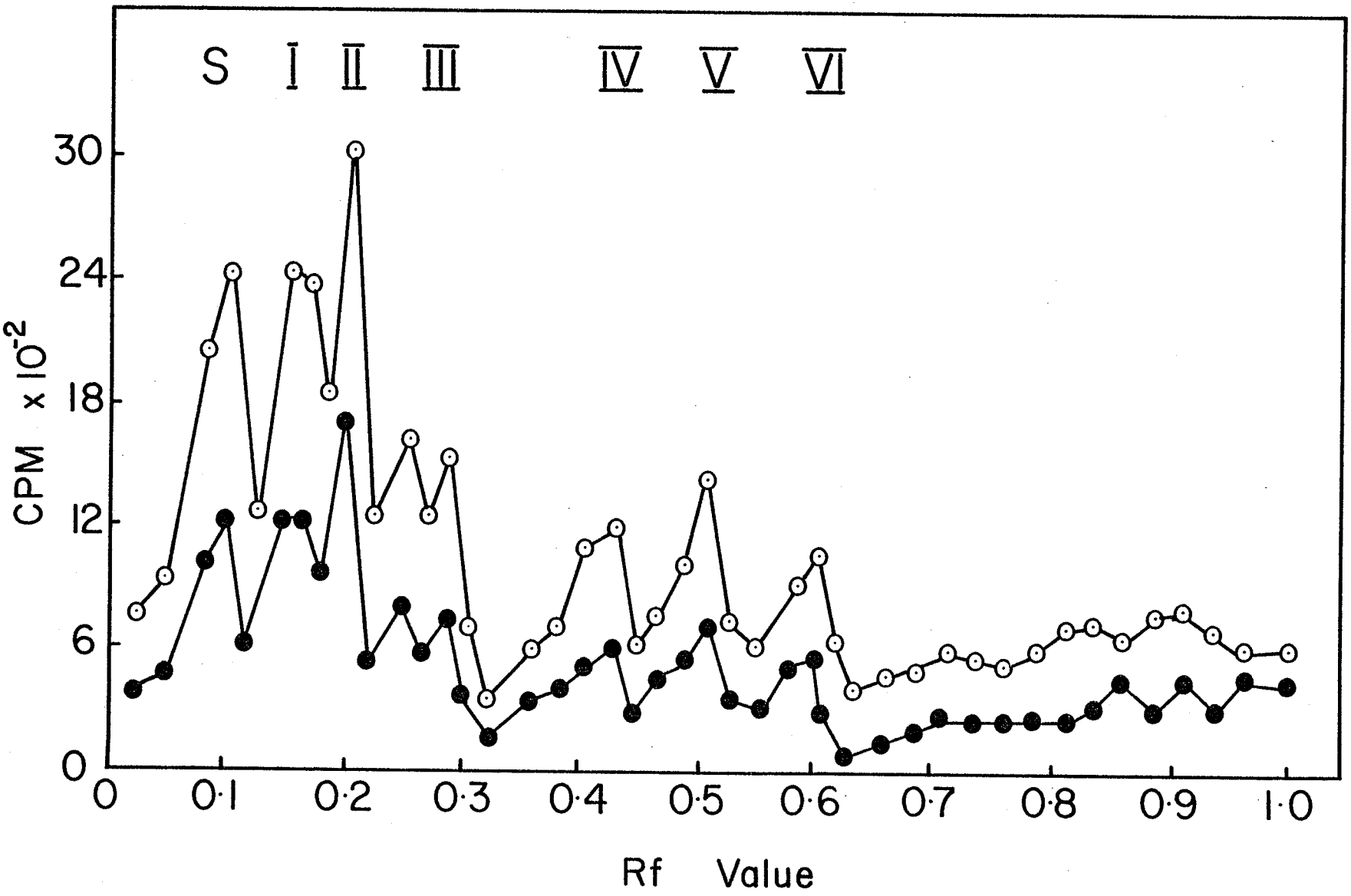

S
I
II
III
IV
V
VI
30
24
18
12
6
0
CPM x 10-2
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Rf Value

Figure 46 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of WT-Cl II (●) and BC^{R}-2 (⊙) preparations labelled as described in Figure 43. Data is typical of three separate labelling experiments.

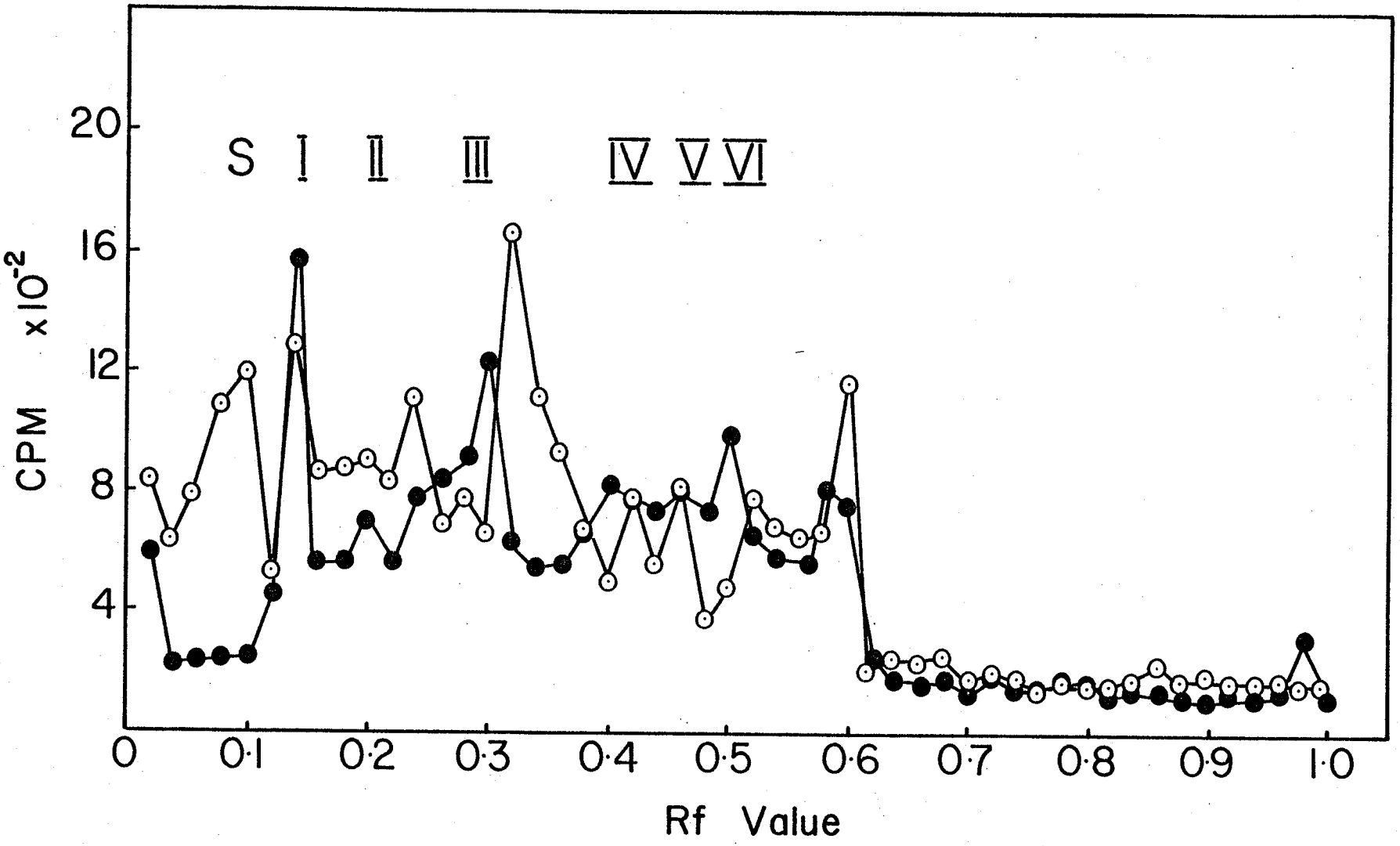
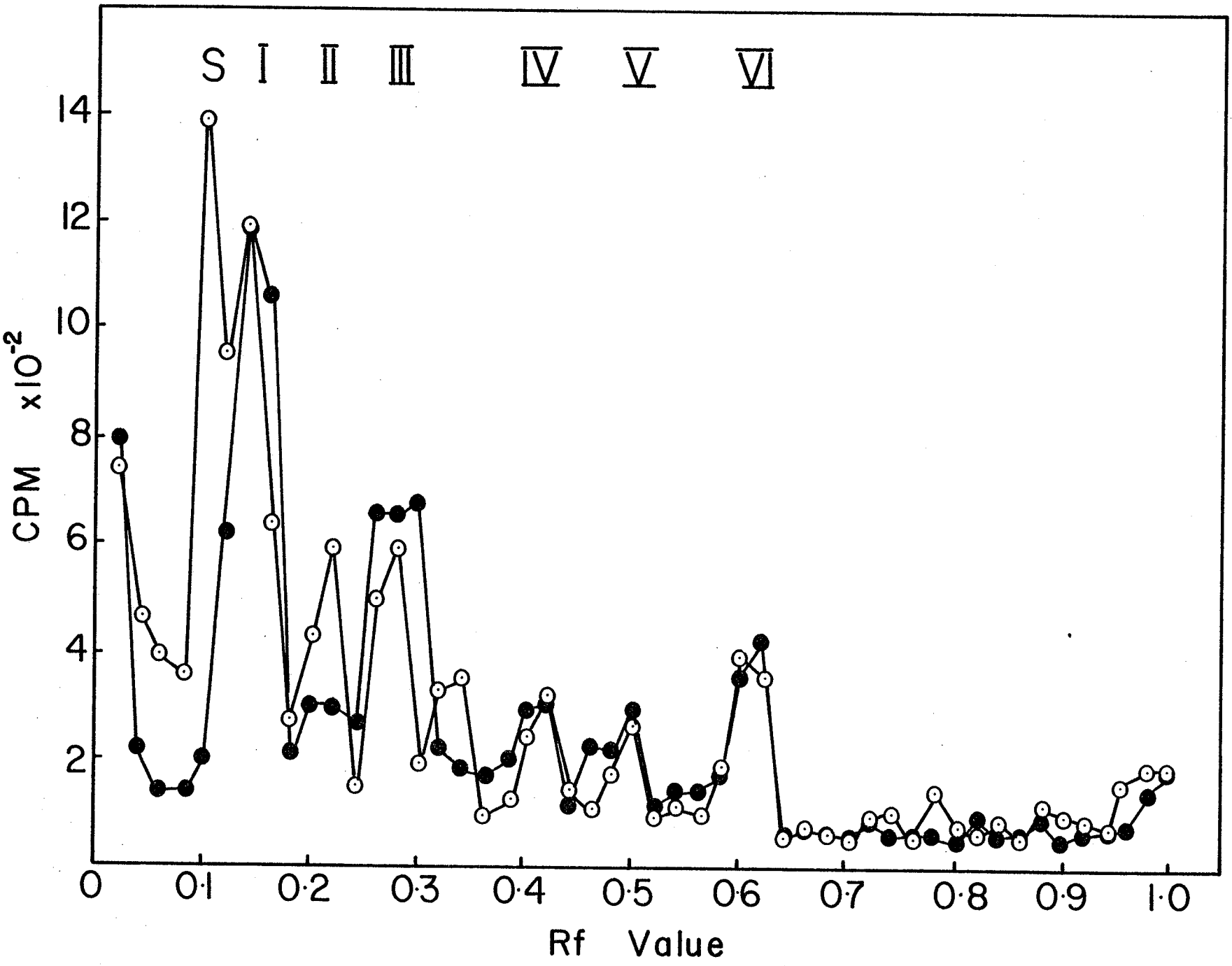

S
I
II
III
IV
V
VI
20
16
12
8
4
CPM x10-2
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Rf Value

Figure 47 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of WT-Cl III (●) and EC^{R}-1 (⊙) preparations labelled as described in Figure 43. Data presented is typical of two labelling experiments.


S
I
II
III
IV
V
VI
14
12
10
8
6
4
2
CPM x10-2
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Rf Value

variant cell lines. Subtle differences in the proportions and exact molecular weights of the peaks do exist between the three independent wild-type populations. Such variances in the cell membrane glycoprotein labelling patterns have previously been noted between subpopulations of CHO cell lines by other workers too (Juliano and Stanley, 1974; Juliano et al. 1976). As with C^R-7 cells the major difference in labelling patterns between con A-resistant and wild-type cells was the presence of the S peak (molecular weight 155 000) only on the surface of con A-resistant cell lines (Figures 46 and 47). Furthermore it is apparent from Table XV that the proportion of cell surface label associated with the S glycoprotein was approximately the same for the three independently selected variant cell lines.

B Lactoperoxidase - ^{125}I Labelling

A second cell surface labelling procedure was used to study the surface membrane proteins. The amino acids, tyrosine and histidine, in the surface polypeptides of WT-Cl I and C^R-7 cells were labelled with ^{125}I using the lactoperoxidase procedure (Sefton et al. 1973; Ceri and Wright, 1978b). Figure 48 indicates that there are some significant differences in the labelling patterns obtained with these two cell lines. For example, in

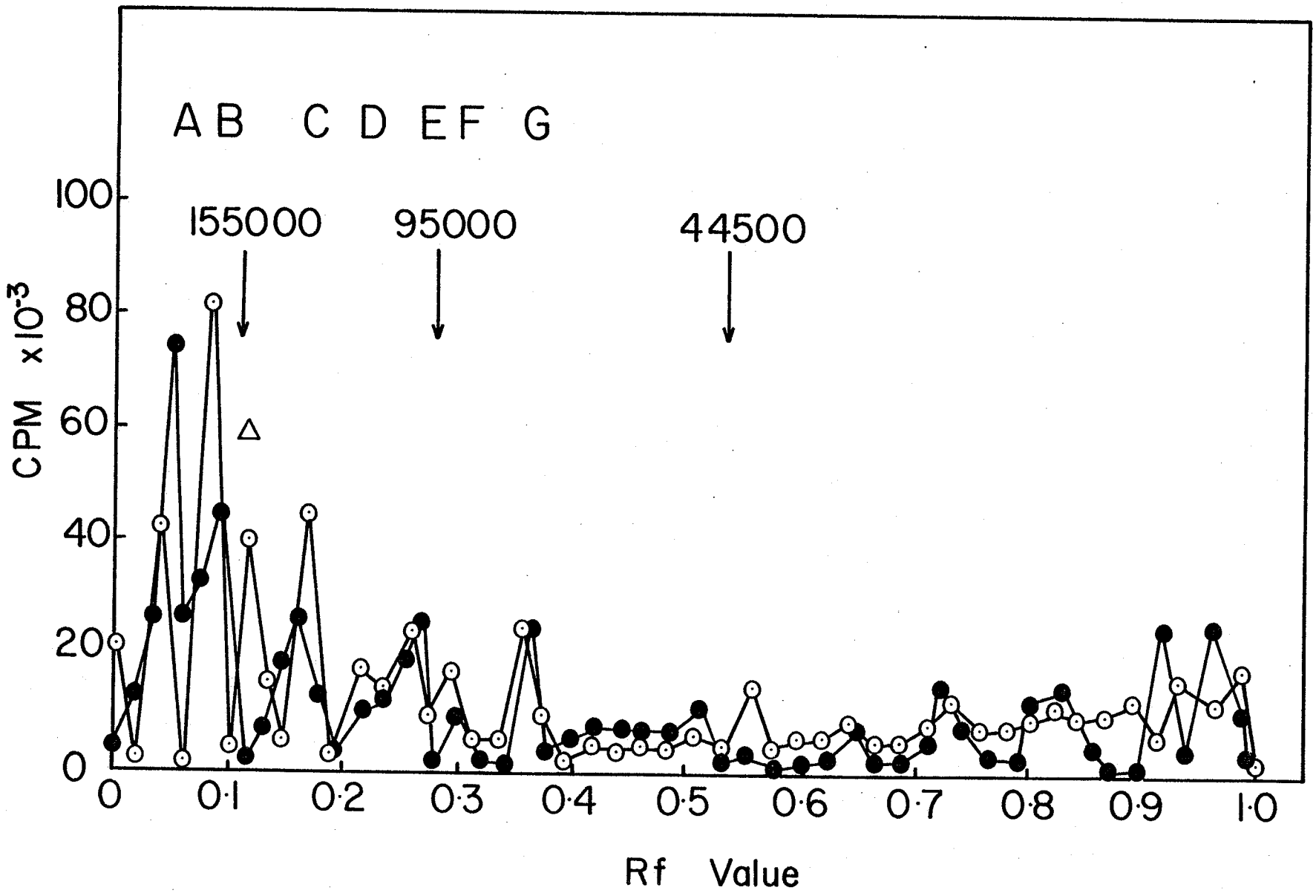
TABLE XV

Percent of cell surface label associated with the S peak

Cell Line	S as a % of total label
C^R-7	12 to 17
BC^R-2	15 to 18
EC^R-1	15 to 17

The data was obtained from five labelling experiments with C^R-7 and two labelling experiments with BC^R-2 and EC^R-1.

Figure 48 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of wild type (●) and C^R-7 (⊙) preparations labelled by the lactoperoxidase catalyzed iodination technique as described in the Methods section. The gels contained 7.5% acrylamide. The molecular weight standards that were used in these experiments are described in Figure 43. (Δ) locates the position of a labelled peak on C^R-7 cells which is missing from wild-type cells. Similar data was obtained in two labelling experiments.


A B C D E F G
155000
95000
44500
CPM x10-3
100
80
60
40
20
0
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Rf Value

experiments with C^R-7 cells an enhancement of label associated with peak B was routinely noted when comparisons were made with the wild-type cell line. Also, in comparisons between wild-type and C^R-7 cells, peak C normally moved a little slower and peak B slightly faster in the wild-type preparations. Of particular interest was the presence of a labelled peak with a molecular weight of approximately 150 000 which was seen only on C^R-7 cells (see, Δ). The extra protein peak observed with C^R-7 cells may correspond to the glycoprotein component found to be present on the variant cells but missing from wild-type cells in the galactose-oxidase - [^{3}H] - borohydride experiments (Figures 43, 45, 46). Although this extra protein peak may not represent the same structure labelled by the previous procedures, the similarities in apparent molecular weight estimates suggest that they may be part of the same glycoprotein.

C Metabolic Labelling

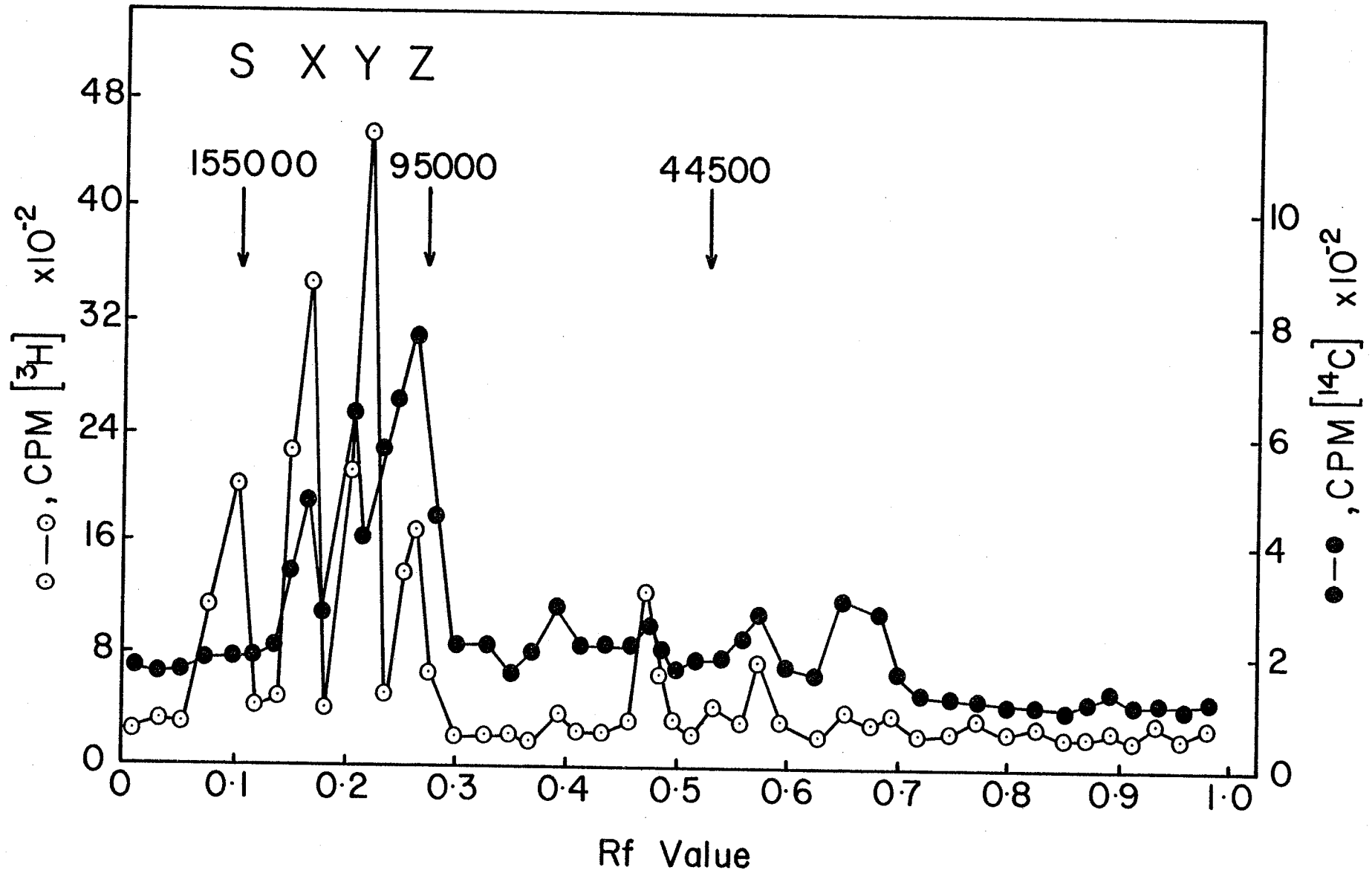
Cell surfaces were also examined by the metabolic incorporation of labelled glucosamine followed by membrane isolation and SDS gel electrophoresis (Juliano et al. 1976; Ceri and Wright 1978b). Again, clear differences in the labelling patterns of wild-type and

con A-resistant cells were observed (Figure 49). These differences in the labelled peaks support the findings of the previous experiments; there are significant modifications in the glycoprotein component of the resistant cells. For example, with this labelling procedure it was routinely observed that the prominent peak designated Y moved a little faster in experiments with membranes from variant cells compared to wild-type cells. It is also important to note that similar to the findings provided by the two previous surface labelling techniques the metabolic incorporation study revealed that the lectin-resistant cells contained a labelled peak with an apparent molecular weight of approximately 155 000, which was missing from the labelling pattern of wild-type cells. Furthermore, the results of the metabolic incorporation experiments firmly suggest that the extra cell surface component, S, observed in previous cell surface labelling experiments probably resulted from a cellular biosynthetic alteration in resistant cells rather than the alternative possibility of an unusual conformational change in variant cells leading to an increase in surface labelling activity.

D Labelling of a Concanavalin A-Resistant Revertant

The surface labelling of a revertant cell line,

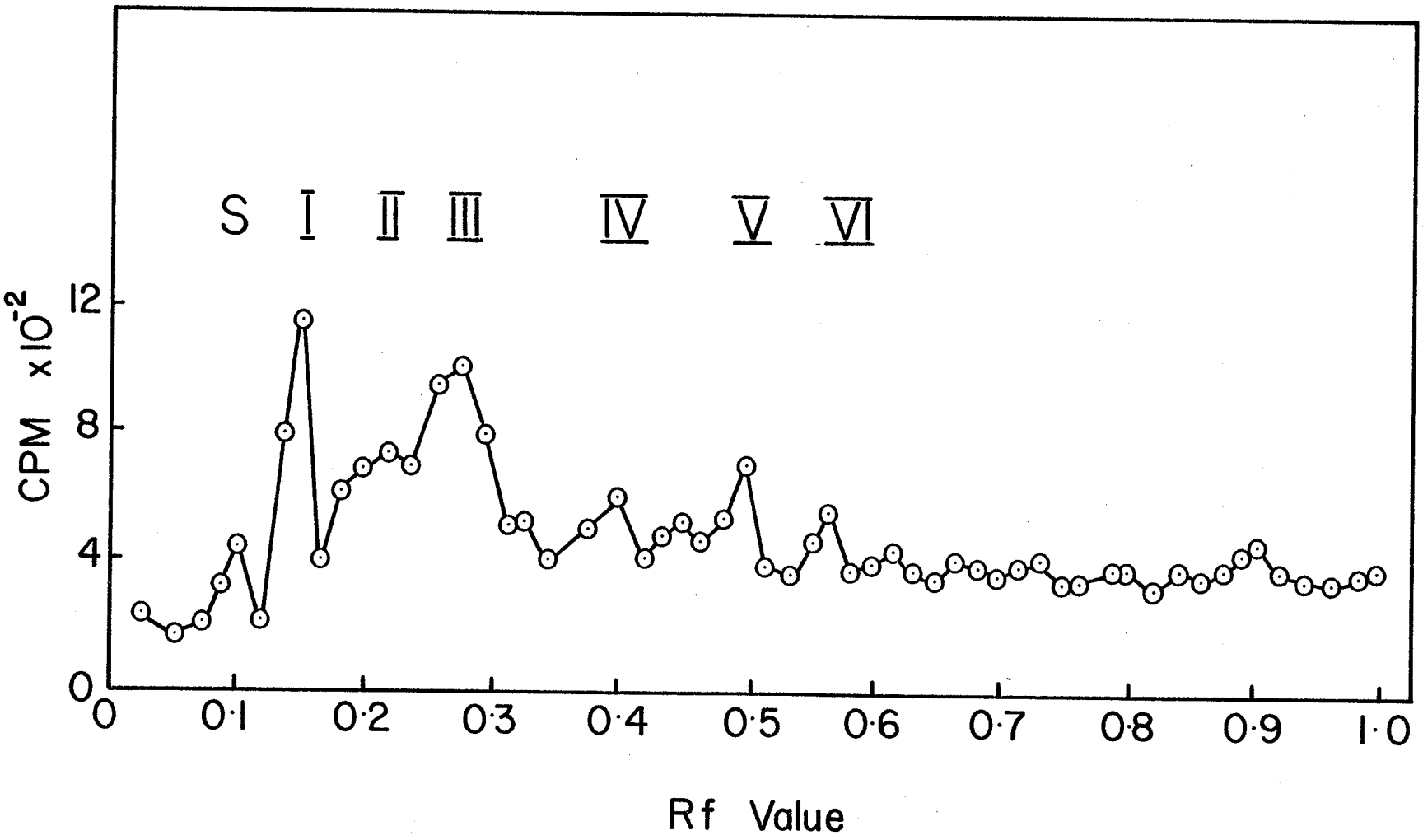
Figure 49 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of wild-type membrane preparations (●) labelled by the incorporation of ^{14}C-glucosamine and C^{R}-7 membrane preparations (⊙) labelled by the incorporation of ^{3}H-glucosamine performed as described in the Methods section. Similar data was obtained in two other labelling experiments.


S X Y Z
155000
95000
44500
o—o, CPM [3H] x10-2
●—●, CPM [14C] x10-2
Rf Value
0
8
16
24
32
40
48
2
4
6
8
10
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0

RC^R-7, was carried out to determine if reversion to con A-sensitivity was accompanied by a reversion to a 'wild type' glycoprotein surface labelling pattern. The labelling of the RC^R-7 line with the galactose-oxidase - [^{3}H] - borohydride method yielded a pattern very similar to that of wild-type and variant cell lines (Figure 50). Glycoprotein peaks I to VI with apparent molecular weights ranging from 140 000 to 40 000 were present on the cell surface. However, the glycoprotein pattern obtained with RC^R-7 cells shares some of the characteristics normally observed with either the wild-type or C^R-7 cell lines (Figure 43). For example, peaks II and III resemble the broad peaks obtained with the wild-type population. Also, the novel glycoprotein with an apparent molecular weight of 155 000, only observed on con A-resistant cell lines, was found at the surface of RC^R-7 cells, but at a very reduced level. In three separate experiments in which RC^R-7 cells were labelled, the novel glycoprotein peak accounted for two to four percent of the total labelled cell surface glycoprotein; this represents only about ten to twenty percent of the label normally associated with the extra peak in con A-resistant cell lines (Table XV).

RC^R-7 cells were also labelled by the incorporation

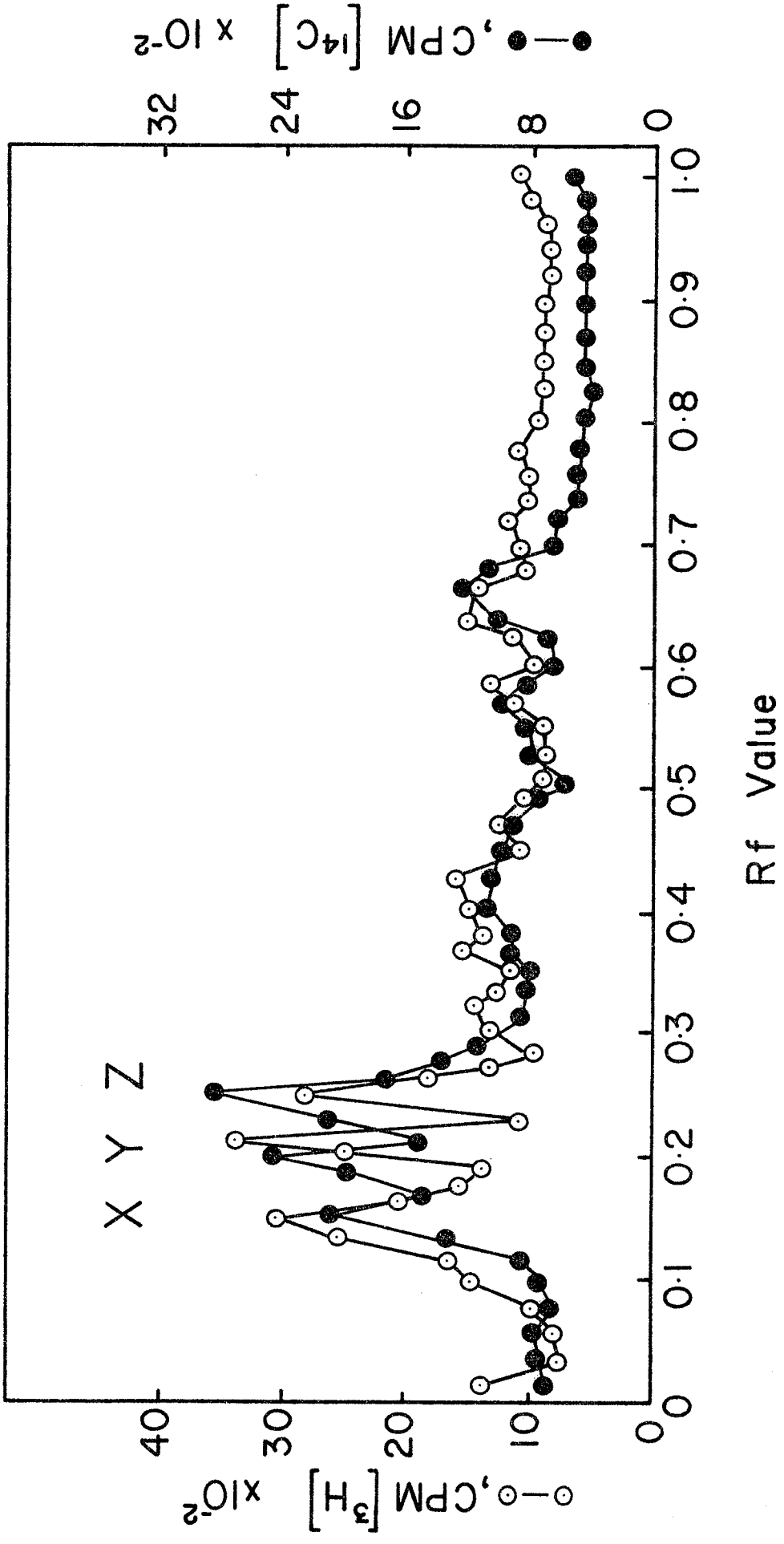
Figure 50 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of RC^R-7 preparations labelled by the galactose-oxidase-[^{3}H]-borohydride technique as described in Figure 43. WT-Cl I and C^R-7 control patterns are seen in Figure 43. Similar data was obtained in two labelling experiments.


S
I
II
III
IV
V
VI
CPM x10-2
12
8
4
0
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Rf Value

of labelled glucosamine. SDS gel electrophoresis of glucosamine labelled membranes of WT-Cl I and RC^R-7 cells are presented in Figure 51. The patterns of the two cell lines were very similar, but again the RC^R-7 pattern was intermediate to the wild-type and C^R-7 patterns. For example, like the labelling pattern with the C^R-7 membranes, the peak designated Y moved a little slower in the preparation of wild-type cells as compared to the revertant. Also, in agreement with the galactose oxidase - [^{3}H] - borohydride labelling procedure the novel 155 000 molecular weight protein was present in RC^R-7 cells but at much reduced levels. The S peak appears as a small but distinct shoulder on the first major peak of RC^R-7 cells but is missing from the labelling profile observed with wild-type cells labelled by the same procedure (Figure 51).

The cell surface labelling studies reported in this section are consistent with the point of view that cell lines selected for con A-resistance possess alterations in the glycoprotein composition of the plasma membrane.

Figure 51 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of wild-type membrane preparations (●) labelled by the incorporation of ^{14}C-glucosamine and RC^{R}-7 membrane preparations (⊙) labelled by the incorporation of ^{3}H-glucosamine performed as described in the Methods section. Similar data was obtained in two other labelling experiments.


●—●, CPM [14C] x 10-2
32
24
16
8
0
○—○, CPM [3H] x10-2
40
30
20
10
0
X Y Z
0·1 0·2 0·3 0·4 0·5 0·6 0·7 0·8 0·9 1·0
Rf Value

Section 4 Altered Mannose Transferase Activity of Concanavalin A-Resistant Cell Lines

Resistance to a number of lectins has been attributed to altered mechanisms of cell surface glycosylation leading to surface sugar changes and reduced levels of lectin binding (Gottlieb and Kornfeld, 1975; Stanley et al. 1975a). Altered glycosylation of these variants has been determined by carbohydrate analysis (Gottlieb et al. 1974) and by cell surface labelling methods (Gottlieb et al. 1975; Juliano and Stanley, 1975). Altered glycosyltransferase activities have been demonstrated in PHA, WGA, and ricin-resistant cell lines. For example, resistance of some ricin and PHA-resistant cell lines has been attributed to very low levels of GlcNAc transferase activity (Gottlieb et al. 1975; Meager et al. 1975; Stanley et al. 1975c). Also sialyltransferase changes have been associated with ricin-resistant mouse L cells (Gottlieb and Kornfeld, 1976) and WGA-resistant CHO cell lines (Briles et al. 1977).

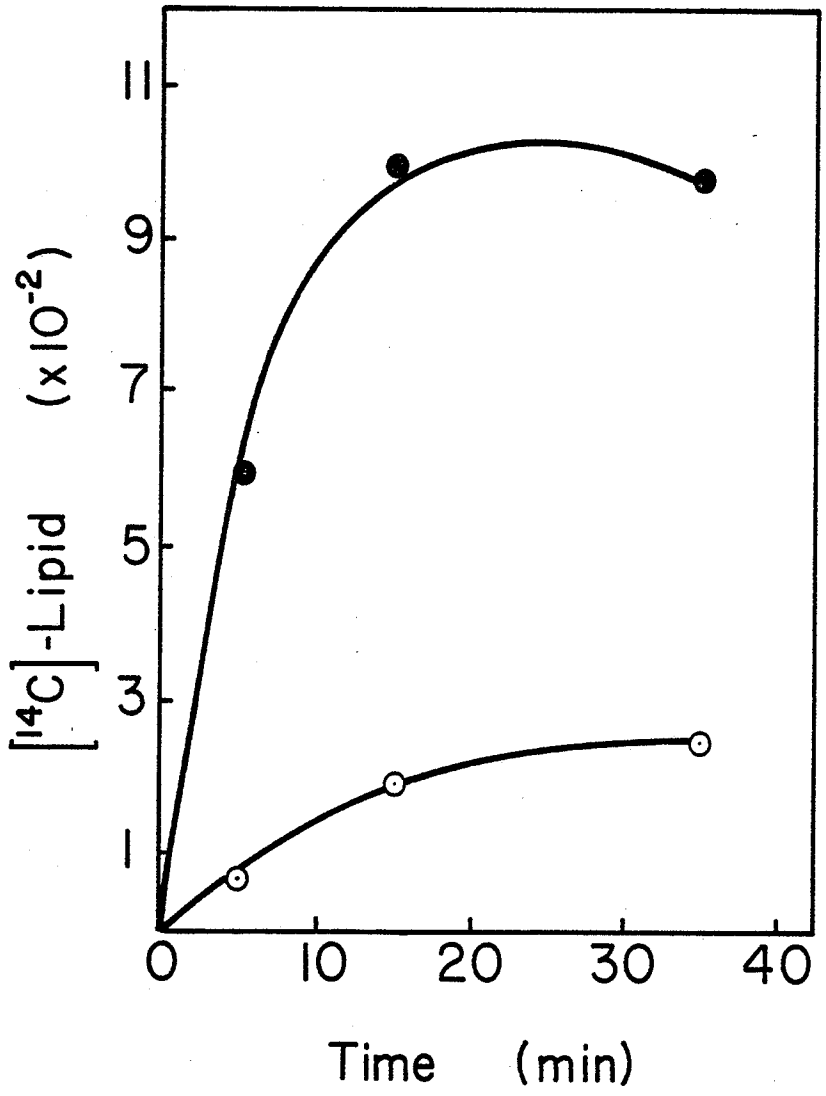
Surface labelling experiments with con A-resistant cell lines (Ceri and Wright, 1978b; results section 3 of this thesis) have strongly suggested that the variants contained significant modifications in cell surface glycoproteins. These changes suggest that the variants

may possess altered glycosyltransferase activity. The specificity of con A for ∝-D mannopyranosyl and ∝-D glucopyranosyl moieties (Goldstein and Staub, 1970) led to the study of the mannose transferase system in con A-resistant and sensitive cell lines.

The addition of mannose to form the core region of glycoproteins involves the transfer of mannose from GDP-mannose to lipid carriers, which include mannosylphosphyoryldolichol and an oligosaccharide-pyrophosphoisoprenol lipid (see reviews Lucas and Waechter, 1976; Waechter and Lennarz, 1976). A terminal glucose residue must be added to the oligosaccharide-lipid prior to the transfer of the core region to the polypeptide (Robbins et al. 1977). Each step of the complex transferase system was assayed by measuring the incorporation of [^{14}C]-mannose into lipid monosaccharide, lipid oligosaccharide, and glycoprotein fractions following the methods of Behrens (Behrens et al. 1971) and Waechter (Waechter et al. 1973; see methods section).

The incorporation of ^{14}C-mannose into the lipid monosaccharide fraction of WT-Cl I and C^R-7 is shown in Figure 52. Both WT-Cl I and C^R-7 incorporated ^{14}C mannose into the lipid-monosaccharide fractions; however, the level of incorporation was 4- to 5-fold higher with

Figure 52 Incorporation of [^{14}C]-mannose into the lipid monosaccharide fraction of WT-Cl I (●) and C^R-7 (⊙) cell lines. Cell lines were grown to a final concentration of 2 x 10^6 cells/plate. Points represent the mean of duplicate points from a single experiment. Similar results were obtained in three other experiments.


[14C]-Lipid (x10-2)
11
9
7
5
3
1
0
10
20
30
40
Time (min)

WT-Cl I than with C^R-7 membrane extracts. A marked difference in the amount of ^{14}C-mannose incorporated into the lipid-oligosaccharide fraction was also seen (Figure 53). The level of mannose incorporation into lipid oligosaccharide was almost 6-fold greater in wild-type membrane fractions. In spite of the much reduced levels of ^{14}C-mannose incorporated into the lipid fraction with C^R-7 membrane preparations, the levels of mannose incorporated into glycoprotein showed only a 2-fold difference between C^R-7 and WT-Cl I preparations (Figure 54).

The ability to incorporate ^{14}C-mannose into lipid-monosaccharide, lipid-oligosaccharide, and glycoprotein fractions was also compared in the con A-resistant variants BC^R-2 and EC^R-1 and their respective wild-type lines, WT-Cl II and WT-Cl III. Similar results to those observed with WT-Cl I and C^R-7 were obtained with these two variant lines (Figures 55 and 56). For example, the incorporation of ^{14}C-mannose into the lipid-monosaccharide fraction was approximatley 5-fold lower in both BC^R-2 (Figure 55a) and EC^R-1 (Figure 56a) in comparison to the parental wild-type populations. Both cell lines also showed a 4-fold reduction of [^{14}C]-mannose incorporated into the lipid oligosaccharide fractions as compared to wild-type levels (Figures 55b and 56b). Also

Figure 53 Incorporation of [^{14}C]-mannose into the lipid oligosaccharide fraction of WT-Cl I (●) and C^R-7 (⊙) cell lines. Cells were grown to a final concentration of 2 x 10^6 cells/plate. Points represent the mean of duplicate points from a single experiment. Similar results were obtained in three other experiments.

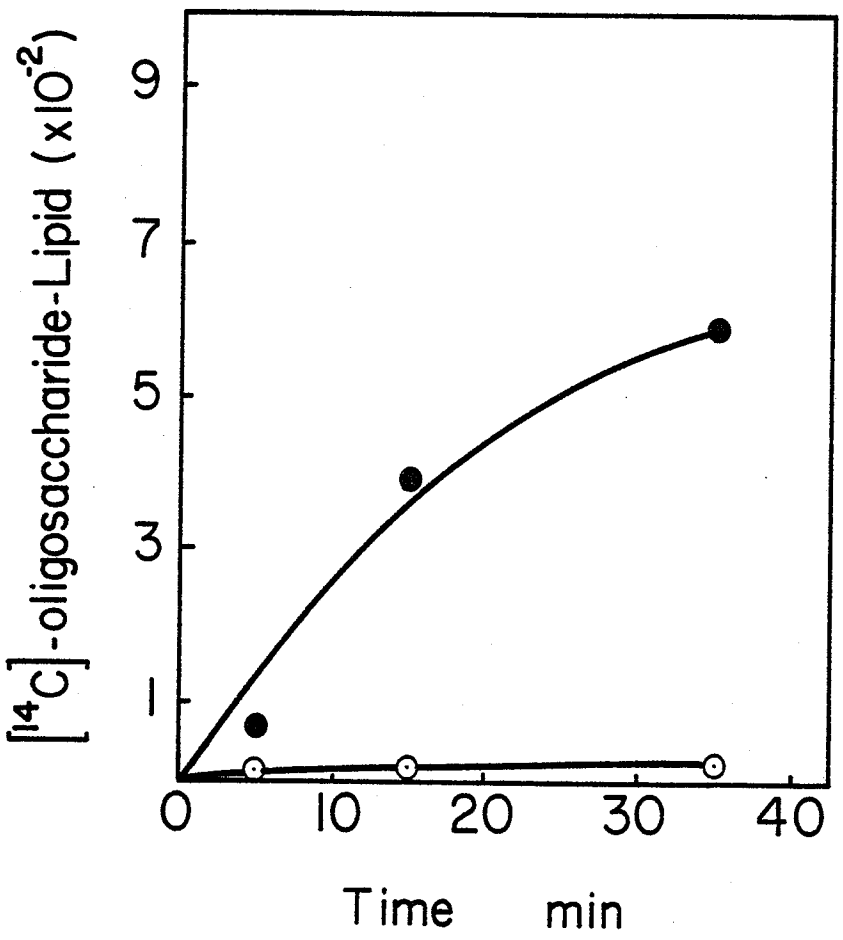

[14C]-oligosaccharide-Lipid (x10-2)
9
7
5
3
1
0
10
20
30
40
Time min

Figure 54 Incorporation of [^{14}C]-mannose into the glycoprotein fraction of WT-Cl I(●) and C^{R}-7 (⊙) cell lines. Cells were grown to a final concentration of 2 x 10^{6} cells/plate. Points represent the mean of duplicate points from a single experiment. Similar results were obtained in three other experiments.

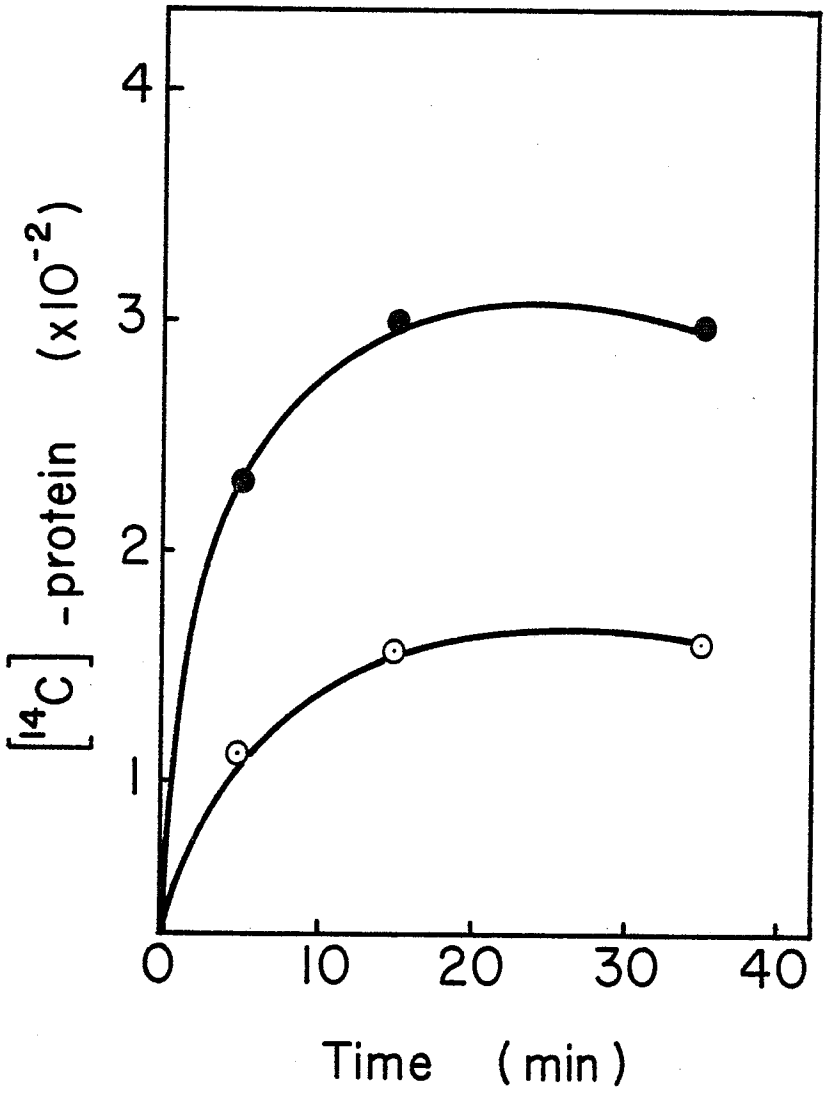

[14C] -protein (x10-2)
4
3
2
1
0
10
20
30
40
Time (min)

Figure 55 Incorporation of [^{14}C]-mannose into lipid (a), lipid oligosaccharide (b) and glycoprotein fractions of WT-Cl II (△) and (c) BC^{R}-2 (⊙) cell lines. Cells were grown to a final concentration of 6 x 10^{5} cells/plate. Points represent mean of duplicate points of a single experiment. Similar results were obtained in two other experiments.

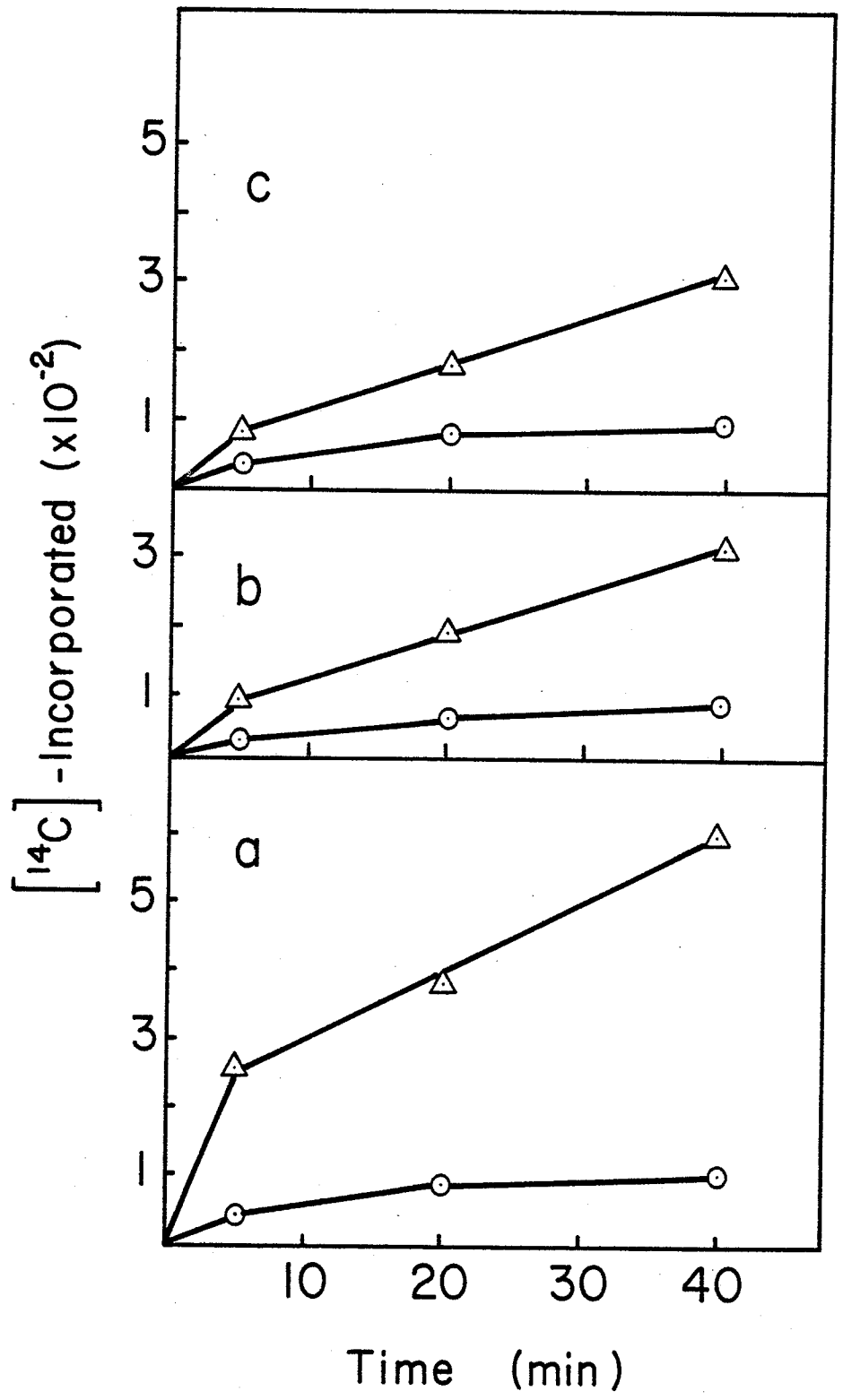
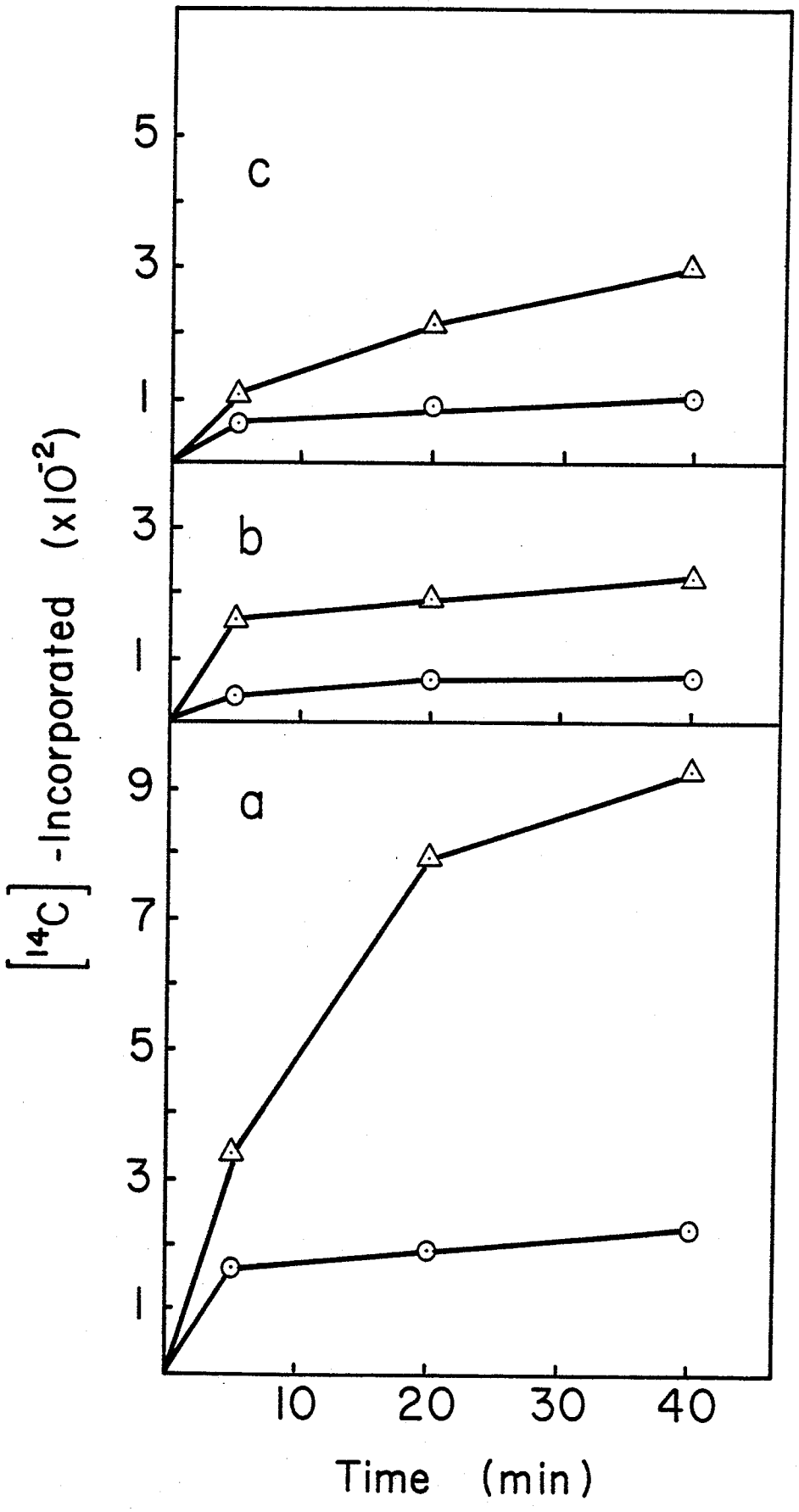

c
b
a
5
3
1
5
3
1
10
20
30
40
Time (min)
[14C]-Incorporated (x10-2)

Figure 56 Incorporation of [^{14}C]-mannose into lipid (a), lipid oligosaccharide (b), and glycoprotein (c) fractions of WT-Cl III (△) and EC^{R}-1 (◎) cell lines. Cells were grown to a final concentration of 6 x 10^{5} cells/plate. Points represent mean of duplicate points of a single experiment. Similar results were obtained in two other experiments.

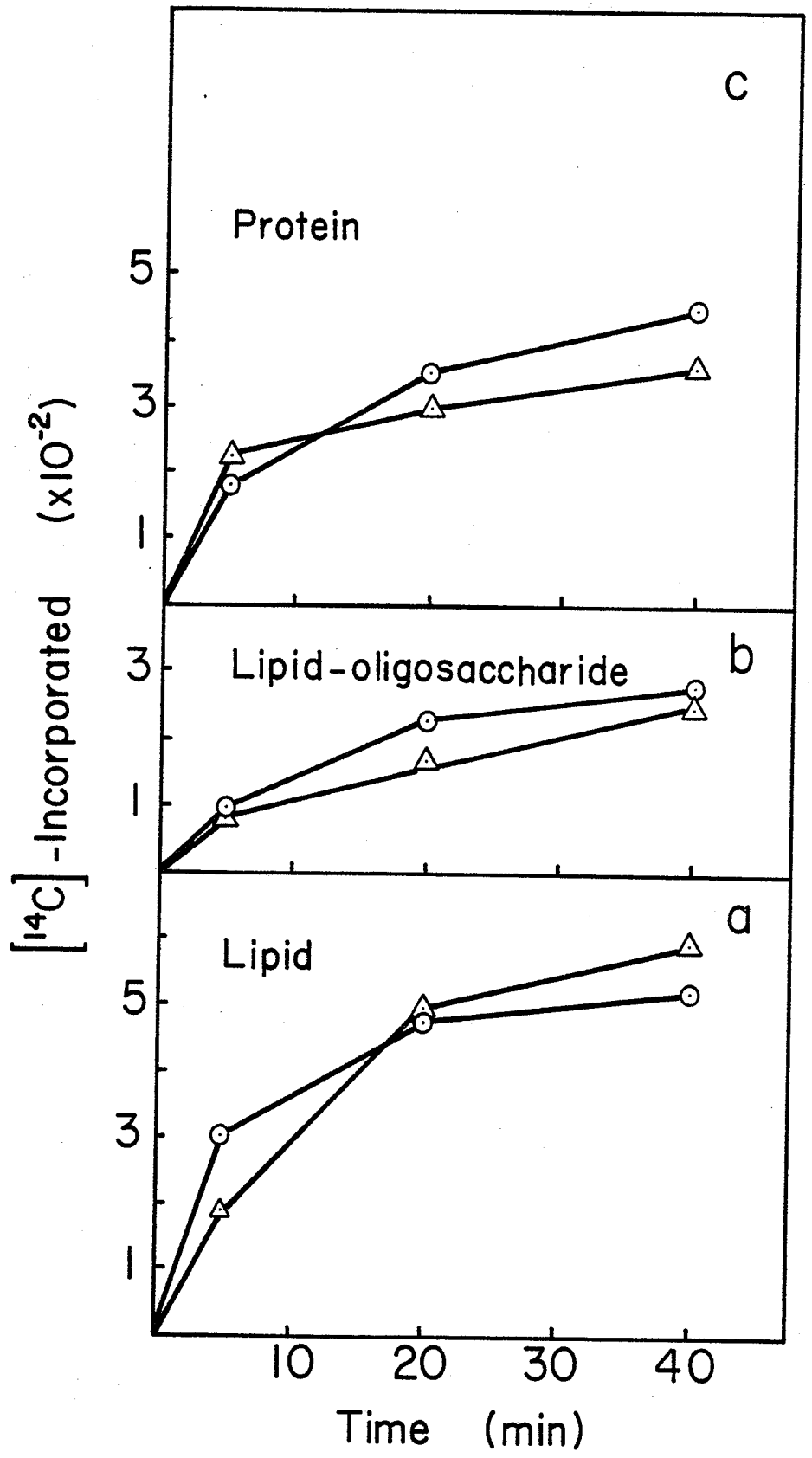

c
b
a
5
3
1
3
1
9
7
5
3
1
10
20
30
40
Time (min)
[14C] -Incorporated (x10-2)

the incorporation of labelled mannose into the glycoprotein fraction of these 2 variants was reduced to about a third when compared to the incorporation of label into the glycoprotein fraction of the respective wild-type lines (Figures 55c and 56c).

Therefore it would appear that the con A-resistant lines possess a lesion in the complex glycosyl transferase system required for the addition of mannose to the core region of glycoproteins. The mannose transferase activity of RC^R-7 cells was also assayed and compared to WT-Cl I populations to determine if the return to con A sensitivity (Table V) and near con A-sensitive surface labelling (Figures 50 and 51) also resulted in a return to near normal transferase activities. Figure 57 reveals that the levels of ^{14}C-mannose incorporated into lipid, lipid-oligosaccharide, and glycoprotein fractions were almost identical in RC^R-7 and WT-Cl I preparations. It would appear that the loss of con A resistance was accompanied by the return to normal levels of mannose transferase activity.

It is apparent that the con A-resistant phenotype involves an altered mannose transferase activity. The return to normal lectin sensitivity is accompanied by normal mannose transferase activity.

Figure 57 Incorporation of [^{14}C]-mannose into lipid (a), lipid oligosaccharide (b) and glycoprotein (c) fractions of WT-Cl I (△) and RC^{R}-7 cell lines. (⊙) Cells were grown to a final concentration of 6 x 10^{5} cells/plate. Points represent mean of duplicate points of a single experiment. Similar results were obtained in two other experiments.


c
Protein
5
3
1
b
Lipid-oligosaccharide
3
1
a
Lipid
5
3
1
[14C]-Incorporated (x10-2)
10
20
30
40
Time (min)

DISCUSSION

DISCUSSION

A. Selection of Concanavalin A-Resistant Cell Lines

Concanavalin A is an excellent selective agent for obtaining lectin-resistant mammalian cell lines (Wright, 1973a, 1975; Ceri and Wright, 1977a, 1978a; Figures 2 and 3). Although the three variant cell lines described in this thesis were isolated from independent wild-type cell lines by different selection procedures, the D_{10} values (45, 48 and 45 μg/ml con A for C^R-7, BC^R-2 and EC^R-1) for each variant line was similar. The con A-resistant cell lines exhibited a 2.5-fold increase in resistance to the lectin when the D_{10} values of variant and wild-type cell lines were compared. Concanavalin A-resistant cell lines have also been isolated by Baker's laboratory (Krag et al. 1977) by using methods previously described by our laboratory (Wright, 1973a, 1975; Ceri and Wright, 1977a). Although very little is known about these variants, Stanley (Stanley et al. 1975b) has indicated that the con A-resistant cell lines isolated by Baker were approximately 1.7-fold more resistant to con A cytotoxicity than the parental wild-type CHO cells.

In general, alterations in phenotype can be brought about by one of two fundamental processes. A variant phenotype can result from a true mutation, that is a stable change in the base sequence of the DNA molecule, or a phenotypic change might be accomplished by a shift in the genetic expression of a particular genotype (Ceri and Wright, 1977a; Harris, 1975; Mezger-Freed, 1972). It is becoming more and more clear that most stable phenotypic variants selected in cell culture are the result of actual mutations as opposed to epigenetic events (see review by Siminovitch, 1976).

A number of genetic guidelines have been proposed (Chu, 1974; De Mars, 1974; Siminovitch, 1976) to determine whether or not a particular variant phenotype results from a mutational or epigenetic event. The con A-resistant trait was found to be stable for almost two years in cell culture (Table II); revertants have been selected that are con A-sensitive (Figure 13); and the proportion of lectin-resistant cells in a population was increased after mutagen treatment (Figures 4 and 5). These findings are consistent with the view that the con A-resistant phenotype is the result of a mutational event.

Mammalian cells selected for resistance to the lectins PHA, WGA, SBA and LCA have stable phenotypes and the

proportion of cells in a population which exhibits resistance to these lectins is increased by EMS treatment (Stanley et al. 1975b; Stanley and Siminovitch, 1977; Wright, 1973a). Furthermore, con A-resistance was found to behave as a recessive trait in somatic cell hybrids (Figure 14). Similarly, other lectin-resistant cell lines (WGA, SBA, LCH and con A-resistant) were also found to be recessive in hybrid studies (Stanley et al. 1975b; Stanley and Siminovitch, 1977). Although these observations do not entirely rule out a possible stable epigenetic mechanism they provide strong support for the idea that resistance to most lectins is a result of a mutational event.

B. Pleiotropic Phenotype of Concanavalin A-Resistant Cell Lines

The three independent con A-resistant cell lines were found to possess similar alterations in some of their fundamental biological properties (Wright et al. 1976; Ceri and Wright, 1977a,c, 1978a; Wright and Ceri, 1977a,b; Results section 1 and 2 this thesis). Among the altered properties exhibited by con A-resistant variants was an inability to proliferate at 39°. The selection of con A-resistant cell lines was first

performed at 34^{o} (Wright, 1973a) because it was postulated that con A-resistance could result from membrane changes that would not significantly affect the proliferation of cells at the selection temperature (34^{o}) but would alter the growth characteristics of the cells at some other non-permissive temperature. Growth studies with con A-resistant cell lines on plates previously revealed a general correlation between lectin resistance and altered growth properties at 39^{o} in over 200 clones studied (Wright, 1973a, 1975). The variant cell lines used in this study also showed ts growth properties (Figure 8, Table III). The range of temperatures over which the cells are sensitive appears to be narrow, as all variant lines showed good growth at 37^{o}. The ts property of the C^{R}-7 cell line was also tested in suspension culture. This cell line showed a marked inability to incorporate DNA, RNA and protein precursors into acid precipitable material in temperature shift-up experiments (Figures 9 and 10). Furthermore, Stanley (Stanley et al. 1975b) reported that con A-resistant cell lines used in her study of lectin resistance also showed ts growth properties. Unfortunately very little data on the cellular properties of these variants has been reported.

Lectin-resistance was also accompanied by obvious modifications to some cellular membrane-associated properties. For example, con A-resistant cell lines contained marked changes in cellular morphology when cultured on plates (Figure 19). Altered cellular morphology has been used as an indicator of membrane changes induced by chemical modification with membrane active agents (Wright, 1973b; Wright et al. 1973; Fishman et al. 1974; Hanneberry et al. 1975; Van Veen et al. 1976 a,b; Ceri and Wright, 1977b). Electron microscopy studies (Figure 20) also suggested that important cell surface changes occur on con A-resistant cells, and although these studies were of a preliminary nature, they indicate that the variants should be very useful in future studies on determining a possible role of surface topography in the interaction of con A with the cell (eg. Collard and Temmink, 1975).

Cell agglutination properties are often used to demonstrate membrane changes during normal cellular differentiation and in the transformation of normal cells to a neoplastic state (see review Nicolson, 1974). The con A-resistant cell lines showed a marked decrease in agglutinability by con A (Table VII). Also,pretreatment of cells with trypsin did not alter the relative

differences in agglutination between wild-type and variant cell lines. It is interesting to note that the con A-resistant cell lines were more agglutinable than the wild-type population by the lectin PHA (Table IX). These results indicate changes exist in the cell surface carbohydrate and/or carbohydrate-containing structures on con A-resistant variants.

The ability of cells to adhere to growth surfaces is a property of the plasma membrane (see review Hynes, 1976). The altered cellular adhesive properties of the variant cell lines (Figure 21) further suggests that con A-resistant variants possess altered surface membranes. This view is supported by a recent study on cell adhesion of membrane variants selected for resistance to PHA and colchicine (Juliano, 1978). The adhesive property of the C^R-7 cell line was found to be dependent upon the temperature at which the cells were previously maintained (Figure 22). C^R-7 cells placed at the non permissive temperature for 24 hours prior to detachment studies were more tightly bound to the substratum. This is an important observation since it indicates that a ts change occurs at the cellular membrane of con A-resistant cells. This membrane change may be involved in the ts growth properties exhibited by con A-

resistant cell lines (Figures 8 and 9).

Surface membrane modifications often lead to changes in the sensitivity to various drugs (Bosmann, 1971; Biedler et al. 1975). For example, cell lines selected for resistance to colchicine are usually cross-resistant to a number of unrelated drugs (Bech-Hansen et al. 1976), and contain significant changes in cell surface glycoproteins (Ling, 1975; Juliano et al. 1976; Juliano and Ling, 1976). Concanavalin A-resistant variants exhibit collateral sensitivity to a number of unrelated agents (Till et al. 1973; Wright, 1973a; Ceri and Wright, 1977a, 1978a). In this study con A-resistant cell lines were shown (Figure 24, Table X) to exhibit an increased sensitivity to the membrane-active agents PEA (Wright et al. 1973) and sodium butyrate (Wright, 1973b). The relative sensitivity of one of the con A-resistant cell lines, C^R-7, to several lectins was also examined (Table XI). The variant cells were cross-resistant to the lectins that share the same sugar binding specificity as con A (LCH-A and LCH-B) but exhibited a 'wild-type' sensitivity to lectins which do not bind the same sugars as con A (WGA, SBA, PHA). This data suggests that con A cytotoxicity was dependent upon the binding of α-D mannopyranosyl or related structures and

that there was a defect in the availability of these particular residues on con A-resistant cells. Similar results with PHA were previously reported by Wright (1973a). However the con A-resistant cell lines used by Stanley (Stanley et al. 1975b) exhibited an increased resistance to PHA and several other lectins as well.

The binding of con A to the cell surface of intact cells was carried out at 4^{o} to reduce endocytosis and non specific binding (Noonan and Burger, 1973, 1974; Bornens et al. 1976). These studies revealed that con A-resistant cell lines bound less lectin/surface area and bound the lectin by a different mechanism than wild-type cells (Figures 26 to 34; Wright and Ceri, 1977a,b). Lectin-sensitive cell lines bound approximately 2.5 times more con A/unit area than con A-resistant cells. Also, analysis of the data showed that con A-sensitive cells bound con A with positive cooperativity whereas the resistant cell lines appeared to be binding to independent non-interacting receptor sites at the cell surface. The lack of cooperativity in lectin binding with con A-resistant cells may be due to alterations in cell surface structures which prevent or modify the ability of the variant cell membrane to undergo the

conformational changes detected with con A-sensitive cells. A reduction in the amount of lectin bound to the surface of resistant cells as compared to lectin-sensitive cells suggests that the con A-resistant cell surface either contains carbohydrate modifications or that some potential receptor sites exposed on con A-sensitive cells are present but not available for con A binding with lectin-resistant cells due to the alterations at the variant cell surface. Some recent lectin binding studies from other laboratories have also suggested that membrane conformation changes can occur (Reisner et al. 1976; Bornens, et al. 1976; Rutishauser et al. 1974). Furthermore, Stanley and Craven (1977) have recently reported that WGA-resistant cell lines exhibited altered cooperative effects in the binding of WGA.

Alterations in cell surface structures can lead to changes in surface fluidity and receptor mobility characteristics of cells (Albrecht-Buehler and Chen, 1977; Rutishauser et al. 1974; Van Veen et al. 1976 a,b). Therefore, the receptor mobility properties of lectin-resistant and -sensitive cell lines were examined. The mobility of lectin receptors was greatly reduced on con A-resistant cell lines (Figure 36). Although more than 90% of the wild-type cells aggregated fl-con A into discrete

caps less than 10, 15 and 25% of C^R-7, BC^R-2 and EC^R-1 lines respectively, were able to form caps. There did not seem to be obvious changes in the microtubule arrangement in the variant cells since lectin-resistant and sensitive cell lines exhibited similar plating efficiencies in the presence of colchicine (Figure 41) and the distribution of fl-con A on lectin-sensitive and resistant cell lines were very similar after colchicine treatment (Figure 40). Also, an examination of tubulin with fluorescent antibody techniques did not reveal any modifications in the arrangement of microtubules in the variant cell lines (D. Brown, Univ. Ottawa, personal communication). It seems possible that lack of cooperativity in the binding of con A to resistant cells may be due, at least in part, to the reduced mobility of lectin receptor sites on variant cell lines. This point is supported by the observation that immobilization of wild-type cell surfaces with gluteraldehyde (Figure 42) leads to a non-cooperative mechanism of lectin binding and to a small reduction in the amount of con A bound/cell surface area.

It is clear from the discussion presented so far that con A is an excellent selective agent for obtaining lectin-resistant cell lines with altered membrane-associated properties. The results presented in this thesis indicate

that the interesting cellular properties exhibited by the variant cell lines are directly related to the con A-resistant phenotype and are not due to a trivial property of the selection technique (Ceri and Wright, 1978a). This statement is supported by the following observations; 1) three con A-resistant cell lines selected from independent wild-type clones by single step (EC^R-1) or cycling (C^R-7, BC^R-2) methods possess changes in growth and membrane-associated properties; 2) the selection of revertant cells (RC^R-7) which exhibited a near 'wild-type' sensitivity to con A cytotoxicity also showed growth and membrane-associated properties that were very similar to parental wild-type cells; 3) somatic cell hybrids formed through the fusion of wild-type and lectin-resistant cells exhibited a con A-sensitive phenotype, and possessed growth and membrane-associated properties that were very similar to pseudodiploid wild-type cells and control cultures of pseudotetraploid hybrid cells; 4) important differences in the mechanism of lectin binding and the amount of lectin bound/cell surface area are found to exist between con A-resistant and -sensitive cell lines. These results also support the view that a single pleiotropic mutation was responsible for the complex phenotype exhibited by the various con A-resistant cell lines.

Perhaps it should be noted that the studies reported in this thesis do not rule out the possibility of eventually isolating a distinctly different class of con A-resistant variant which would possess another unique set of altered cellular properties since it is reasonable to expect that more than one type of cellular change could lead to a con A-resistant phenotype (Wright, 1975; Stanley et al. 1975b; Stanley and Siminovitch, 1977).

C. Altered Surface Membranes of Concanavalin A-Resistant Cell Lines

The results discussed above clearly indicate that altered membrane properties are associated with con A-resistance. The altered membrane properties of the con A-resistant cell lines probably result from alterations to the glycoprotein and perhaps to the glycolipid components of the plasma membrane as the carbohydrate moieties of these structures are involved in lectin binding. The biochemical characterization of the cell surface membrane by specific cell surface labelling techniques clearly show alterations in the glycoprotein composition of the variant cell lines when compared to parental wild-type lines (Ceri and Wright, 1978b; results

section 3, this thesis). Although there were several changes in the galactose oxidase -[^{3}H]-borohydride labelling patterns of con A-resistant cell lines, the obvious difference between variant and wild-type cells was the presence of an additional surface component, with an approximate molecular weight of 155 000 on lectin-resistant cells which was missing from wild-type cells. This novel protein designated, S, accounted for 12 to 17, 15 to 18, and 15 to 17% of the label associated with the surface of C^R-7, BC^R-2, and EC^R-1 cells respectively. Comparisons of labelling patterns from whole cell extracts and purified membrane preparations indicated that the S peak was not the result of labelling non-surface components. Furthermore, surface labelling by lactoperoxidase iodination, and metabolic incorporation techniques confirmed the presence of an additional component of an approximate molecular weight of 150 000 to 155 000 on the surface of variant cells. This would suggest that the glycoprotein resulted from altered glycoprotein synthesis. If the altered surface labelling patterns observed with the three independent variants are involved in the expression of the complex lectin-resistant phenotype, it would be expected that a revertant cell line would exhibit labelling

patterns similar to the patterns observed with wild-type cells. This expectation was realized in the surface labelling studies carried out with the RC^R-7 cell line. In particular, it is interesting to note that the label associated with the S glycoprotein was greatly reduced and accounted for only 10 to 20% of the label associated with the peak on C^R-7 cells.

Alterations in cell surface glycoproteins have also been observed in ricin, PHA and WGA-resistant cell lines (Gottlieb et al. 1975; Juliano and Stanley, 1975; Meager et al. 1976: Gottlieb and Kornfeld, 1976; Briles, et al. 1977). These changes have involved either a decrease in glycosylation of the surface glycoproteins, which correlates with a reduced GlcNAc transferase activity (Gottlieb et al. 1975; Stanley et al. 1975c; Meager et al. 1976), or an altered sialic acid content resulting from changes in the sialyltransferase system (Gottlieb and Kornfeld, 1976; Briles et al. 1977). The altered glycoprotein patterns observed with the con A-resistant cells are obviously very different from those obtained with cell lines selected for resistance to other lectins.

Mammalian cells selected for resistance to actinomycin D or colchicine have also exhibited interesting changes

in surface glycoproteins (Bosman, 1971; Ling, 1975; Juliano et al. 1976). For example, some recent studies with colchicine-resistant CHO lines have shown the presence of a novel glycoprotein with a molecular weight of approximately 170 000 at the surface of colchicine-resistant but not wild-type cells (Juliano et al. 1976; Juliano and Ling, 1976). Perhaps there are some important similarities between the mechanism responsible for the surface changes reported for these variants and the con A-resistant cell lines described in this thesis. Noonan's laboratory (Van Veen et al. 1976a,b) has suggested that novel surface components may be involved in membrane changes observed with CHO cells grown in the presence of dibutyryl-cAMP . They suggest that these novel proteins may be involved in regulating membrane fluidity through a mechanism resembling the anchorage hypothesis proposed by Edelman's laboratory (Edelman, 1974, 1976; McCain et al. 1977). Anchored lectin receptors have been implicated in the altered con A capping properties of transformed cell lines (Lotem et al. 1976). Also, the involvement of the LETS (large external transformation sensitive) protein in modulating membrane fluidity has been demonstrated (Chen et al. 1976; Albrecht-Buehler and Chen, 1977).

Similar studies with the S glycoprotein are currently being attempted in our laboratory. For example, it would be interesting to determine if the addition of a purified preparation of S glycoprotein to wild-type cells would alter the lectin receptor mobility or con A binding characteristics of these cells (eg. Lipkin and Knecht, 1976).

Alterations to membrane lipid fractions have been reported for cells resistant to lectins and other drugs (Hughes and Gardas, 1976; Gottlieb and Kornfeld, 1976; Whatley et al. 1976; Ng et al. 1977). A preliminary study of the glycosphingolipids of con A-resistant and wild-type cells cultured in this laboratory failed to show significant changes (Yogeeswaran et al. 1974). However, a more comprehensive study on the lipids (neutral, phospholipid, and glycolipid) of the variant and parental wild-type cell lines is now in progress in our laboratory.

D. Mannosyl Transferase Alterations in Concanavalin A-Resistant Cell Lines

The alterations to the glycoprotein fractions of ricin, PHA, and WGA-resistant cell lines were associated with altered glycosyltransferase activities (Gottlieb et al. 1975; Juliano and Stanley, 1975; Meager et al.

1976; Briles et al. 1977). The specificity of con A for α-D mannopyranosyl moieties prompted a study of the mannosyl transferase system in the lectin-resistant variants. The con A-resistant cell lines all showed a marked inability to incorporate [^{14}C]-mannose into lipid, lipid-oligosaccharide, and glycoprotein fractions. A 3 to 5-fold reduction in the incorporation of mannose into lipid and lipid-oligosaccharide fractions was observed, while the incorporation into glycoprotein was reduced 2 to 3-fold. A revertant cell line, which possessed near wild-type surface labelling patterns (see Section 3; Results), was also found to possess mannosyl transferase activities which closely resembled those of wild-type cell lines. Very similar results were recently reported by Krag's group (Krag et al. 1977) for con A-resistant cells selected by procedures previously described by our laboratory (eg. Wright, 1973a, 1975). In both studies the level of [^{14}C]-mannose incorporated into glycoproteins on variant cells was unexpectedly high considering the marked inhibition of mannose incorporation into the lipid fractions. Since GDP-mannose can be converted to GDP-fucose by a multienzyme epimerase reaction (Sharon, 1975) glycoproteins were isolated from wild-type and C^R-7 cells following incorporation of label from GDP-[^{14}C]-mannose and extraction of lipid linked intermediates. Hydrolysis

of the glycoproteins was carried out by Dr. J.C. Jamieson (Department of Chemistry); the label was present mainly as mannose and fucose and the amount of label in fucose was greater in glycoproteins isolated from C^R-7 cells than glycoproteins isolated from wild-type cells (Wright, Ceri, and Jamieson, 1978). These experiments suggest that the unexpectedly high levels of [^{14}C] label incorporated into variant cell surface glycoproteins may be due, at least in part, to a greater than 'wild-type' conversion of mannose to fucose (which is transferred to cell surface glycoproteins without a lipid intermediate step). Perhaps it should be noted that detailed attempts to determine cell surface sugar composition from variant and wild-type cells is currently underway (Blaschuk, Wright and Jamieson).

Recent collaborative studies between our laboratory and Dr. Jamieson's laboratory have shown that con A-resistant cell lines also contain important changes in glycosidase activities (Blaschuk, Wright and Jamieson, 1978). Although the work is still in progress it is clear that changes in these enzyme activities would ultimately lead to important modifications in cell surface carbohydrate content. It is clear that the alterations in the biosynthesis of surface glycoproteins

on con A-resistant cell lines is more complex than the changes reported in ricin and PHA-resistant cell lines (Gottlieb et al. 1975; Stanley et al. 1975c). It appears that con A-resistant cells contain an alteration to an early step in the regulation of glycoprotein biosynthesis. An altered glycoprotein synthesis regulatory mechanism has also been suggested for WGA-resistant cell lines (Briles et al. 1977).

E. Conclusion

The changes in several different enzyme activities associated with the biosynthesis of surface glycoproteins in the lectin-resistant cell lines suggests that the primary alteration in con A-resistant cells is probably located in a mechanism that regulates the levels of these enzyme activities. Such a change would directly lead to altered cell surface glycoproteins and to modifications in various cell surface-associated properties in variant cell lines.

Although the cytotoxic property of con A was first described in 1911 (Assimann, 1911) the mode of action that eventually leads to cell death remains unknown (Nicolson, 1974; Fordsdyke, 1977; Lustig et al. 1977). Lectins like con A interact with mammalian cell surface oligosaccharide chains (Bernhard and Avrameas, 1971;

Goldstein and Misaki, 1970). Concanavalin A specifically binds to ∝-D mannopyranosyl and ∝-D glucopyranosyl groups (Goldstein and Staub, 1970). These sugars are involved in the binding of con A to CHO cell surfaces (Figures 26 to 34) and in the cytotoxic response observed when CHO cells are cultured in the presence of the lectin (Figures 1 and 3). These results are supported by studies carried out with different cell lines by many laboratories (see reviews, Sharon and Lis, 1972; Nicolson, 1974). However, it has also been observed that lectins, including con A, enter the cell after short periods of incubation at 37^{o} (Noonan and Burger, 1974; Hyman et al. 1974) and probably bind to various intracellular organelles (Napanitaya et al. 1976). It should also be noted that addition of con A to cultured somatic cells eventually leads to significant reduction in DNA and protein synthesis but the mechanism involved in this inhibitory process is not understood (Nicolson, 1976a). The lack of information concerning a mode of action for con A killing with mammalian cells in culture makes it very difficult to identify which of the membrane property changes are most likely to play an important role in con A-resistance.

Undoubtedly the cellular membrane and many fundamental membrane-associated properties are altered in variant cell lines. Also, these changes are observed with

independent con A-resistant cell lines and are not found with wild-type, revertant, or hybrid cell lines, which exhibit greater sensitivity to con A cytotoxicity (Table V). It is clear that the cell surface alterations described in this thesis are an important part of the general con A-resistant phenotype. However, it is not yet clear which of the many changes is/are directly responsible for the lectin-resistant property and which of the alterations are by-products of the pleiotropic mutation.

It could be suggested that the reduction in lectin binding with con A-resistant cells at 4^{o} would lead to a resistant phenotype; the variants, which show a 2.5-fold increase in resistance also bind approximately 2.5-fold less con A/cell surface area as compared to wild-type cells. However, it should be noted that significant differences in lectin binding are not observed when experiments are carried out at room temperature (Wright, 1973a) where endocytosis and non-specific binding can occur (Noonan and Burger, 1974). Also preliminary studies on con A permeability has indicated that reduced but significant amounts of the lectin can enter variant cells after an exposure to labelled con A for approximately 1 hour at room temperature (Wright,

unpublished data). Therefore it is likely that the interesting modifications observed in con A-binding experiments with variant cell lines play a minor role in determining the resistant phenotype.

Recently studies on con A cytotoxicity have shown that an inverse relationship exists between the ability of cells to cap fl- con A and their sensitivity to the cytotoxic property of the lectin (Forsdyke, 1977; Lustig et al. 1977). These investigations suggest that the reduced ability of con A-resistant cells to aggregate lectin receptors into discrete caps (Figure 36) may be important in determining the lectin-resistant property. Although the altered mechanism responsible for the reduced capping on variant cells is not known some recent studies with the LETS protein suggest a possible mechanism (Albrect-Buehler and Chen, 1977). These studies have shown that the presence of an extra glycoprotein on the surface of a cell may lead to alterations in the local fluidity properties on the cell surface. This suggests that the novel glycoprotein, S, found on the surface of con A-resistant cells but not con A-sensitive cells may play an important role in changing the surface mobility properties of lectin receptors on the variant cell lines; this alteration could lead to a reduction in lectin capping and

cytotoxicity (Forsdyke, 1977; Lustig et al. 1977).

Although it cannot be determined which of the many cell surface modifications is/are directly involved in allowing the variant cell to proliferate in normally cytotoxic concentrations of con A, the data presented in the thesis clearly supports the initial hypothesis, that cell lines selected for resistance to con A possess altered cell membranes and important changes in membrane-associated properties. The isolation and characterization of lectin resistant cell lines was undertaken in order to establish a biochemical and genetic system to study some of the key cell functions associated with the cellular membrane. It is interesting to note (Table XVI) that the altered cellular properties observed with independently isolated con A-resistant cell lines closely resemble many of the fundamental changes found to be associated with malignant transformation or reported to occur during normal differentiation of mammalian cells (Moscana, 1974; Marchase et al. 1976; Nicolson, 1976a,b). It is clear that con A-resistant variants provide a novel opportunity for investigations into membrane structure-function relationships.

TABLE XVI

Correlation of Membrane Alteration in Con A-Resistant, Neoplastic, and Differentiating Cell Systems.

	Membrane-Associated Property	Lectin-Resistant Cells	Neoplastic[1] Cells	Differentiating[2] Cells
1	Modified Adhesion	+	+	+
2	Altered Mobility of Surface Components	+	+	+
3	Altered Agglutination Properties	+	+	+
4	Altered Enzymes	+*	+	+
5	New Antigens	ND	+	+
6	Modified Glycoproteins	+	+	+
7	Modified Glycolipids	in progress	+	+
8	Altered Lectin Binding	+	+	+
9	Altered Permeability or Transport	ND	+	+

*Altered glycosidase activities have been found in the variants as well as altered secretion patterns of these enzymes (Blaschuk, Wright and Jamieson, 1978).

[1]See reviews Nicolson, 1976a,b.

[2]See reviews Moscana, 1974; Marchase et al. 1976.

ND - not determined.

REFERENCES CITED

REFERENCES CITED

Aaronson, S.A. and Todaro, G.J. 1968a. Science 162 1024-1026.

Aaronson, S.A. and Todaro, G.J. 1968b. J. Cell. Physiol. 72:141-148.

Adair, W.L. and Kornfeld, S. 1974. J. Biol. Chem. 249: 4696-4704.

Agrawal, B.B.L. and Goldstein, I.J. 1967. Biochim. Biophys. Acta 147:262-271.

Agrawal, B.B.L. and Goldstein, I.J. 1968. Arch. Biochem. Biophys. 124:218-229.

Arce, A., Maccioni, H.J. and Caputto, R. 1971. Biochem. J. 121:483-493.

Albrecht-Buehler, G. and Chen, L.B. 1977. Nature. 266: 454-456.

Allan, D. and Crumpton, M.J. 1971. Biochem. Biophys. Res. Commun. 44:1143-1146.

Allen, L.W., Svenson, R.H. and Yachnin, S. 1969. Proc. Natl. Acad. Sci. U.S. 63:334-341.

Anzil, A.P., Stavron, D. Blinzinger, K. and Osterkamps, U. 1977. Eur. J. Cancer 13:627-632.

Assimann, F. 1911. Arch für die Gesante Physiol. 137: 489-510. cited in Sumner, J.B. and Howell, S.F. 1936. J. Bacteriol. 32:227-237.

Aub, J.C., Sanford, B.H. and Cote, M.N. 1965a. Proc. Natl. Acad. Sci. U.S. 54:396-399.

Aub, J.C., Sanford, B.H. and Wang, L. 1965b. Proc. Natl. Acad. Sci. U.S. 54:400-402.

Baker, R., Brunette, P., Mankovitz, R., Thompson, L., Whitmore, G., Siminovitch, L. and Till, J. 1974. Cell 1:9-21.

Barchi, R.L., Borilla, E. and Wong, M. 1977. Proc. Natl. Acad. Sci. U.S. 74:34-38.

Barondes, S.H. and Rosen, S.D. 1976. Neuronal Recognition. Ed. S.H. Barondes. Plenum Press, New York, pp. 331.

Bech-Hansen, N.T., Till, J.E. and Ling, V. 1976. J. Cell. Physiol. 88:23-32.

Becht, H., Rott, R. and Klenk, H.D. 1972. J. Gen. Virol. 14:1-8.

Becker, J.W., Reeke, G.N. and Edelman, G.M. 1971. J. Biol. Chem. 246:6123-6125.

Behrens, N.H., Parodi, A.J. and Leloir, L.F. 1971. Proc. Natl. Acad. Sci. U.S. 68:2857-2860.

Ben-Bassat, H., Inbar, M. and Sachs, L. 1970. Virol. 40:854-859.

Benjamin, T.L. and Burger, M.M. 1970. Proc. Natl. Acad. Sci. 67:929-934.

Benson, S., Olsnes, S., Pihl, A., Skorve, J. and Abraham, K.A. 1975. Eur. J. Biochem. 59:573-580.

Berman, L.D. 1975. Int. J. Cancer 15:973-979.

Bernhard, W. and Avrameas, S. 1971. Exp. Cell Res. 64:232-236.

Biedler, J.L., Riehm, H., Peterson, R.H.F. and Spengler, B.A. 1975. J. Natl. Cancer Inst. 55:671-680.

Bittiger, H. and Schnebli, H.P. 1976. Concanavalin A as a Tool. J. Wiley and Sons, New York. pp. 639.

Blaschuk, O., Wright, J.A. and Jamieson, J.C. 1978. Can Fed. Biol.Soc. (in the press).

Boldt, D.H., Speckart, S.F., Richards, R.L. and Alving, C.R. 1977. Biochem. Biophys. Res. Commun. 74:208-214.

Borger, H., Woodruff, J., Hirschhorn, R., Gesner, B., Miescher, P. and Silber, R. 1966. Science 154: 1019-1020.

Bornens, M., Karsenti, E. and Avrameas, S. 1976. Eur. J. Biochem. 65:61-69.

Bosmann, H. 1971. Nature 233:566-569.

Boyd, W.C. and Reguera, R.M. 1949. J. Immunol. 62: 333-339.

Boyd, W.C. and Shapleigh, E. 1954. J. Lab. Clin. Med. 44:235-237.

Boyd, W.C., Bhatia, H.M., Diamond, M.A. and Matsubara, S. 1962. J. Immunol. 89:463-470.

Brattain, M.G., Jones, C.M., Pittman, J.M. and Pretlow, T.G. 1975. Biochem. Biophys. Res. Commun. 65: 63-67.

Breslow, R. and Goldsby, R. 1969. Exp. Cell Res. 55: 339-346.

Bretscher, M.S. 1974. Perspectives in Membrane Biology (Ed. Estrada, O.S. and Gitler, C.) Academic Press, New York. p. 3-24.

Bretton, R., Wicker, R. and Bernhard, W. 1972. Int. J. Cancer 10:397-409.

Brewer, C.F., Sternlicht, H., Marcus, D.M. and Grollman, A.P. 1973. Biochemistry 12:4448-4457.

Briles, E.B., Li, E. and Kornfeld, S. 1977. J. Biol. Chem. 252:1107-1116.

Brown, R.C., Bass, H. and Coombs, J.P. 1975. Nature 254:434-435.

Brunette, D.M. and Till, J.E. 1971. J. Membrane Biol. 5:215-224.

Burger, M.M. and Goldberg, A.R. 1967. Proc. Natl. Acad. Sci. U.S. 57:359-366.

Carraway, C.A., Jett, G. and Carraway, K.L. 1975. Biochem. Biophys. Res. Commun. 67:1301-1306.

Carraway, K.L. and Carraway, C.H. 1976. J. Supranol. Structure 4:121-126.

Cartwright, B. 1977. J. gen Virol. 34:249-256.

Ceri, H. and Wright, J.A. 1977a. Exp. Cell Res. 104: 389-398.

Ceri, H. and Wright, J.A. 1977b. Can. J. Microbiol. 23:835-839.

Ceri, H. and Wright, J.A. 1977c. Can. Fed. Biol. Soc. 20:64A.

Ceri, H. and Wright, J.A. 1978a. Exp. Cell Res. In the press.

Ceri, H. and Wright, J.A. 1978b. Exp. Cell Res. In the press.

Chen, L.B., Gallimore, P.H. and McDougall, J.K. 1976. Proc. Natl. Acad. Sci. U.S. 73:3570-3574.

Chowdhury, T.K. and Weiss, A.K. 1975. Concanavalin A. Plenum Press, New York. pp. 360.

Chu, E.H.Y. (1974). Genetics 78:115-132.

Clements, G.B. 1975. Advances in Cancer Research (Klein, G. and Weinhouse, S. Eds.) Academic Press, New York. 21:273-90.

Collard, J.G. and Temmink, J.H.M. 1974. Exp. Cell Res. 86:81-86.

Collard, J.G. and Temmink, J.H.M. 1975. J. Cell Sci. 19:21-32.

Collard, J.G. and Temmink, J.H.M. 1976. J. Cell Biol. 68:10o-112.

Comoglio, P.M. and Guglielmore, R. 1972. FEBS Letts. 27:256-258.

Crawford, Y. 1968. A Laboratory Guide to Mycoplasma of Human Origin. Naval Medical Research Unit, no. 4, 2nd Edition.

Cuatrecassas, P. 1973. Biochemistry 12:1312-1323.

Cuatrecasas, P. and Tell, G.P.E. 1973. Proc. Natl. Acad. Sci. U.S. 70:485-489.

Culp, L.A. and Black, P.H. 1972. J. Virol. 9:611-620.

Cunningham, B.A., Wang, J.L., Gunther, G.R., Reeke, J.N. Jr. and Becker, J.W. 1974. Cellular Selection and Regulation In the Immune Response (Ed. Edelman, G.) Raven Press, New York. p. 177-197.

Curtis, A.S.G. 1967. The Cell Surface: Its Molecular Role in Morphogenesis. Academic Press, New York. 405 pp.

Danielli, J.F. and Davson, H. 1935. J. Cell Comp. Physiol. 5:495-508.

DeMars, R. (1974) Mutation Research 24:335-364.

Den, H. and Malinzak, D.A. 1977. J. Biol. Chem. 252: 5444-5448.

Dent, P.B. 1971. J. Natl. Cancer Inst. 46:763-768.

Dorner, F., Scrib, M. and Weil, R. 1973. Proc. Natl. Acad. Sci. U.S. 70:1981-1985.

Dufau, M.L., Tsuruhara, T., and Catt, K.J. 1972. Biochim. Biophys. Acta 278:281-292.

Dulbecco, R. and Vogt, M. 1954. J. Exp. Med. 99:167-182.

Dysart, J. and Edwards, J.G. 1977. FEBS Letts. 75: 96-100.

Edelman, G.M., Cunningham, B.A., Reeke, G.N., Becker, J.W., Waxdal, M.J. and Wang, J.L. 1972. Proc. Natl. Acad. Sci. U.S. 69:2580-2584.

Edelman, G.M. 1974. Control of Proliferation in Animal Cells (Eds. Clarkson, B. and Boserga, R.) Cold Spring Harbor Laboratory, New York 1:357-378.

Edelman, G.M. 1976. Science. 192:218-226.

Edwards, J.G., Dysart, J. and Hughes, R.C. 1976. Nature. 264:66-68.

Ehrlich, P. 1957. Paul Ehrlich: Gesammelte Arbeiten, Heidelberg, Springer Verlag, Vol II. 1970. cited in Wallach, D.F.H. 1972. The Plasma Membrane: Dynamic Perspectives, Genetics, and Pathology. The English Universities Press Ltd. New York. pp. 189.

Elkin, N.M. and Whitmore, G.F. 1967. The Radiobiology of Cultured Mammalian Cells. Gordon and Beach, New York. p. 594.

Entlicher, G., Koster, J.V. and Kocourek, J. 1970 Biochim. Biophys. Acta 221:272-281.

Feller, M., Richardson, C., Behnke, W.D. and Gruenstein, E. 1977. Biochem. Biophys. Res. Commun. 76:1027-1035.

Fishman, P.H., Simmons, J.L., Brady, R.O., and Freese, E. 1974. Biochem. Biophys. Res. Commun. 59:292-299.

Forsdyke, D.R. 1977. Nature 267:358-360.

Fuhr, B.J., Barber, B.H. and Carver, J.P. 1976. Proc. Natl. Acad. Sci. U.S. 73:322-326.

Gahmberg, C.G. and Hakomori, S. 1973. J. Biol. Chem. 248:4311-4317.

Gail, M. and Boone, C. 1973. Exp. Cell Res. 70:33-40.

Gantt, R.R., Martin, J.R. and Evans, V.J. 1969. J. Natl. Cancer Inst. 42:369-373.

Gesner, B.M. and Thomas, L. 1966. Science 151:590-591.

Gibbons, R.A., Jones, G.W. and Sellwood, R. 1975. J. Gen. Microbiol. 86:228-240.

Gillette, M., Dengler, R. and Filosa, M. 1974. J. Exp. Zool. 190:243-251.

Glabe, C.G. and Vacquier, V.D. 1977. Nature 267:836-837.

Glimelius, B., Nilsson, K. and Pontén, J. 1975. Int. J. Cancer 15:888-896.

Goldstein, I.J. and Misaki, A. 1970. J. Bact. 103:422-425.

Goldstein, I.J. and Staub, A.M. 1970. Immunochemistry 7:315-318.

Goldstein, I.J., Reichert, C.M., Misaki, A. and Gorin, P.A.J. 1973. Biochim, BIophys. Acta 317:500-504.

Goldstein, I.J. 1975. Concanavalin A. (Ed. Chowdhury and Weiss.) Plenum Press, New York. pp. 35-53.

Gottlieb, C., Skinner Sr., A.M. and Kornfeld, S. 1974. Proc. Natl. Acad. Sci. U.S. 71:1078-1082.

Gottlieb, C., Baenziger, J. and Kornfeld, S. 1975. J. Biol. Chem. 250:3303-3309.

Gottlieb, C. and Kornfeld, S. 1976. J. Biol. Chem. 251:7761-7768.

Greene, W.C., Parker, C.M. and Parker, C.W. 1976. J. Biol. Chem. 251:4017-4025.

Gurd, J.W. and Evans, W.H. 1976. Can. J. Biochem. 54: 477-480.

Gurd, J.W. 1977. Biochemistry 16:369-374.

Hammarström, S. and Kabat, E.A. 1969. Biochemistry 8 2696-2705.

Hardman, K.D. and Ainsworth, C.F. 1972. Nature New Biol. 237:54-55.

Hardman, K.D. and Ainsworth, C.F. 1973. Biochemistry 12:4442-4448.

Harris, M. 1971. J. Cell. Physiol. 78:177-184.

Harris, M. 1973. Genetics Suppl. 73:181-185.

Harris, M. 1975. J. Cell. Physiol. 86:413-430.

Hassing, G.S. and Goldstein, I.J. 1970. Eur. J. Biochem. 16:549-556.

Hill, A.V. 1913. Biochem. J. 7:471-480.

Howard, I.K., Sage, H.J., Stein, M.D., Young, N.M., Leon, M.A. and Dyckes, D.F. 1971. J. Biol. Chem. 246: 1590-1595.

Howard, J., Sannon, L., Oki, S. and Murashige, T. 1977. Exp. Cell Res. 107:448-450.

Howard, G.A. and Schnebli, H.P. 1977. Proc. Natl. Acad. Sci. U.S. 74:818-821.

Hozumi, M., Miyake, S., Mizunoe, F., Sugimura, T., Irie, R.F., Koyama, K., Tomita, M. and Ukita, T. 1972. Int. J. Cancer 9:393-401.

Hughes, R.C., and Gardas, A. 1976. Nature 264:63-66.

Hughes, R. 1976. Membrane Glycoproteins: a review of structure and function. Butterworths, London. pp. 357.

Hyman, R., Lacorbiere, M., Staverek, S. and Nicolson, G. 1974. J. Natl. Cancer Inst. 52:963-969.

Hynes, R.O. 1976. Biochim. Biophys. Acta 458:73-107.

Juliano, R.L. and Stanley, P. 1975. Biochim. Biophys. Acta 389:401-406.

Juliano, R., Ling, V. and Graves, J. 1975. J. Supramol. Structure. 4:521-526.

Juliano, R.L. and Ling, V. 1976. Biochim. Biophys. Acta 455:152-162.

Juliano, R.L. L978. J. Cell Biol. 76:43-49.

Kalb, A.J. and Lustig, A. 1968. Biochim. Biophys. Acta 168:366-367.

Kartner, N., Slavik, M. and Riordon, J.R. 1977. Can. Fed. Biol. Soc. 20:172A.

Kawai, S. and Takeuchi, I. 1976. Develop. Growth and Different. 18:311-317.

Kinzel, V., Richards, J. and Kübler, D. 1977. Exp. Cell Res. 105:389-400.

Klebe, R.J., Rosenberger, P.G., Naylor, S.L., Burns, R.L., Novak, R. and Kleinman, H. 1977. Exp. Cell Res. 104:119-125.

Kobiler, D. and Barondes, S. 1977. Dev. Biology 60: 326-330.

Krag, S.S., and Robbins, P.W. 1977. J. Biol. Chem. 252: 2621-2629.

Krag, S.S., Cifone, M., Robbins, P.W. and Baker, R.M. 1977. J. Biol. Chem. 252:3561-3564.

Krusius, T., Finne, J. and Rauvala, H. 1976. FEBS Letts. 71:117-120.

Laemmli, U.K. 1970. Nature 227:680-685.

Landsteiner, K. and Raubitschek, H. 1908. Zbl. Bakt. 45:660 cited in Sharon and Lis 1972. Science 177:949-959.

Levine, D., Kaplan, M.J. and Greenaway, P.J. 1972. Biochem. J. 129:847-856.

Levine, E., Thomas, L., McGregor, D., Hayflick, L. and Eagle, H. 1968. Proc. Natl. Acad. Sci. U.S. 60" 583-589.

Levine, E. 1972. Exp. Cell Res. 74:99-109.

Lewis, W.H. and Wright, J.A. 1974. Biochem. Biophys. Res. Commun. 60:926-933.

Lin, J.Y., Kao, W.Y., Tserng, K.Y., Chen, C.C. and Tung, T.C. 1970. Cancer Res. 30:2431-2433.

Ling, V. 1975. Can. J. Genetics and Cytology. 17:503-515.

Lipkin, G. and Knecht, M.E. 1976. Exp. Cell Res. 102: 341-348.

Lis, H. and Sharon, N. 1973. Annu. Rev. Biochem. 42: 541-574.

Lloyd, K.O. 1970. Arch. Biochem. Biophys. 137:460-468.

Loontiens, F.G., Clegg, R.M., Van Landschoot, A. and Jovin, T.M. 1977. Eur. J. Biochem. 78:465-469.

Lotem, J., Vlodavsky, I. and Sachs, L. 1976. Exp. Cell Res. 101:323-330.

Lowry, O., Rosebrough, N., Farr, A. and Randall, R. 1951. J. Biol. Chem. 193:265-275.

Luly, P., Scapin, Ferretti, E. and Tomasi, V. 1976. FEBS Letts. 64:209-213.

Lucas, J.J. and Waechter, C.J. 1976. Mol. Cell Biochem. 11:67-78.

Lustig, S., Ascher, O., Fishman, P., Djaldetti, M. and Pluznik, D.H. 1977. J. Cell Biol. 75:388-397.

McBurney, M.W. and Whitmore, G.F. 1974. Cell 2:173-182.

McClain, D.A., D'Estestachio, P. and Edelman, G.M. 1977. Proc. Natl. Acad. Sci. U.S. 74:666-670.

McKenzie, G.H., Sawyer, W.H. and Nichol, L.W. 1972. Biochim. Biophys. Acta 263:283-293.

Marchalonis, J.J. and Edelman, G.M. 1968. J. Mol. Biol. 32:453-465.

Marchase, R.B., Vosbeck, K. and Roth, S. 1976. Biochim. Biophys. Acta 457:385-416.

Marciani, D.J. and Okazaki, T. 1976. Biochim. Biophys. Acta 455:849-864.

Martinez-Palomo, A., Wicker, R. and Bernhard, W. 1972. Int. J. Cancer 9:676-683.

Mayhew, E. 1972. J. Cell. Physiol. 79:441-452.

Meager, A., Ungkitchanukit, A., Nairn, R. and Hughes, R.C. 1975. Nature 257:137-139.

Meager, A. Ungkitchanukit, A. and Hughes, R.C. 1976. Biochem. J. 154:113-124.

Medicus, R.G., Schreiber, R.D., Gatze, O. and Muller-Eberhard, H.J. 1976. Proc. Natl. Acad. Sci. U.S. 73:612-616.

Mezger-Freed, L. 1971. J. Cell Biol. 51:742-751.

Mezger-Freed, L. 1972. Nature 235:245-246.

Michaelis, E.K. and Michaelis, M.L. 1976. Life Sci. 18:1021-1030.

Miki, T. and Kuwata, T. 1976. J. gen. Virol. 33: 25-30.

Monday, R., Jaffe, R. and McMahon, D. 1976. J. Cell Biol. 71:314-319.

Mascona, A.A. 1974. The Cell Surface in Development. John WIley and SOns, Toronto. pp. 334.

Müller, W.E., Müller, I. Zahn, R.K. and Kurelec, B. 1976. J. Cell Sci. 21:227-241.

Napanitaya, W. and Tyan, M.L. Annual Proceedings Electron Microscopy Society of America (Bailey, G.W. ed.) cited in Napanitaya et al. 1976. Proc. Soc. Exp. Biol. Med. 153:213-219.

Napanitaya, W., Hanker, J. and Tyan, M. 1976. Proc. Soc. Exp. Biol. Med. 153:213-219.

Narasimhan, S., Stanley, P. and Schachter, H. 1977. J. Biol. Chem. 252:3926-2933.

Negendank, W.G. and Collier, C.R. 1976. Exp. Cell Res. 101:31-40.

Neri, A., Roberson, M., Connolly, D.T. and Oppenheimer, S.B. 1975. Nature 258:342-344.

Ng, S.K., Rogers, J. and Sanwal, B.D. 1977. J. Cell. Physiol. 90:361-374.

Nicolson, G.L. 1971. Nature New Biol. 233:244-246.

Nicolson, G.L. and Singer, S.J. 1971. Proc. Natl. Acad. Sci. U.S. 68:942-945.

Nicolson, G.L. 1972. Nature. N.B. 239:193-197.

Nicolson, G. 1974. Int. Rev. Cytol. 39:89-190.

Nicolson, G.L., Robbons, J.C. and Hyman, R. 1975. J. Supranol. Struct. 4:15-26.

Nicolson, G.L. 1976a. Biochim. Biophys. Acta 457: 57-108.

Nicolson, G.L. 1976b. Biochim. Biophys. Acta 458: 1-72.

Nicolson, G.L., Usui, N., Yanagimachi, R., Yanagimachi, H. and Smith, R.L. 1977a. J. Cell Biol. 74:950-962.

Nicolson, G.L., Oppenheimer, S.B. and Giotta, G.J. 1977b. J. Cell Biol. 75:209A.

Noonan, K.D. and Burger, M.M. 1973. J. Biol. Chem. 248:4286-4292.

Noonan, K.D. and Burger, M.M. 1974. Methods Enzymol. 32:621-625.

Nowak, T.P., Haywood, P.L. and Barondes, S.H. 1976. Biochem. Biophys. Res. Commun. 68:650-657.

Nowell, P.C. 1960. Cancer Res. 20:462-468.

Okada, Y. and Kim, J. 1962. Virol. 50:507-515.

Olsnes, S. and Pihl, A. 1973a. Eur. J. Biochem. 35: 179-185.

Olsnes, S. and Pihl, A. 1973b. Biochemistry 12:3121-3126.

Olsnes, S., Fernandez-Puentes, C., Carrasco, L. and Vazquez, D. 1975. Eur. J. Biochem. 60:281-288.

Olsnes, S., Sandvig, K., Refsnes, K. and Pihl, A. 1976. J. Biol. Chem. 257:3985-3992.

Olson, M.O.J., and Liener, I.E. 1967. Biochemistry 6:105-111.

Oppenheimer, S.B., Bales, B.L., Brenneman, G., Knapp, L., Lesin, E.S., Neri, A. and Pollock, E.G. 1977. Exp. Cell Res. 105:291-300.

Orkin, S. and Littlefield, J. 1971. Exp. Cell Res. 69: 174-180.

Ozanne, B. and Sambrook, J. 1971. Biology of Oncogenic Viruses (Ed. Silvestri, L.) North Holland, Amsterdand pp. 248-257.

Ozanne, B. 1973. J. Virol. 12:79-89.

Ozanne, B. and Lurye, M. 1974. Control of Proliferation in Animal Cells (Eds. Clarkson, B. and Baserga, R.) Cold Spring Harbor Lab. 1:177-190.

Painter, R.G. and White, A. 1976. Proc. Natl. Acad. Sci. U.S. 73:837-841.

Parish, R.W. and Müller, U. 1976. FEBS Letts. 63: 40-44.

Penhoet, E., Olsen, C., Carlson, S., Lacorbiere, M. and Nicolson, G.L. 1974. Biochemistry 13:3561-3566.

Pflumn, M.N., Wang, J.L. and Edelman, G.M. 1971. J. Biol. Chem. 246:4369-4375.

Phillips, H.J. 1973. Tissue Culture Methods and Applications. (Eds. Kruse, Jr., P.K. and Patterson, Jr., M.K.). Academic Press, New York. 406-408.

Podolsky, D.K. and Weiser, M.M. 1973. J. Cell Biol. 58: 497-500.

Pollack, R.E. and Burger, M.M. 1969. Proc. Natl. Acad. Sci. U.S. 62:1074-1076.

Poste, G. 1972. Exp. Cell Res. 73:319-328.

Poste, G. and Reeve, P. 1972. Nature New Biol. 237: 113-114.

Poste, G. and Reeve, P. 1974. Nature New Biol. 247: 469-471.

Poste, G. and Nicolson, G.L. 1976. The Cell Surface (Ed. Poste, G. and Nicolson, G.L.) North-Holland Publishing Co. New York. pp. 766.

Pouysségur, J.M. and Pastan, I. 1976. Proc. Natl. Acad. 73:544-548.

Puck, T.T., Cieciura, S. and Robinson, A. 1958. J. Exp. Med. 104:427-434.

Puck, T.T., Cieciura, S. and Robinson, A. 1958. J. Exp. Med. 108:945-956.

Quinn, P.J. 1976. The Molecular Biology of Cell Membranes. United Park Press, Baltimore, pp 229.

Reisner, Y., Lis, H. and Sharon, N. 1976. Exp. Cell Res. 97:445-448.

Renkonen, K.O. 1948. Ann. Med. Exp. BIol. Fenn. 26: 66. cited in Sharon, N. and Lis, H. 1972. Science 177:949-959.

Renkonen, K.O. 1950. Ann. Med. Exp. BIol. Fenn. 28: 45. cited in Sharon, N. and Lis, H. 1972. Science 177:949-959.

Richardson, C.E. and Behnke, W.D. 1976. J. Mol. Biol. 102:441-452.

Robbins, P.W., Krag, S.S. and Liu, T. 1977. J. Biol. Chem. 252:1780-1785.

Robertson, J.D. 1959. Biochem. Soc. Sym. 16:3-29.

Robinson, J.M., Smith, D.F., Davis, E.M., Gilliam, E.B., Capetillo, S.C. and Walborg, E.F. Jr. 1976 Biochem. Biophys. Res. Commun. 72:81-88.

Rott, R., Becht, H., Klenk, H.D. and Scholtissek, C. 1972. Z. Naturforech B 27:227-238.

Rutishauser, U., Yahara, I. and Edelman, G.M. 1974. Proc. Nat. Acad. Sci. 71:1149-1153.

Sandvig, K., Olsnes, S. and Pihl, A. 1976. J. Biol. Chem. 251:3977-3984.

Scatchard, G. 1949. Ann. N.Y. Acad. Sci. 51:660-672.

Schmidt-Ulbrich, R., Wallach, D.F.H. and Hendricks, J. 1976. Biochim. Biophys. Acta 443:587-600.

Schnebli, H.P. 1976. Concanavalin A as a Tool. (Eds. Bittiger, H. and Schnebli, H.P.) John Wiley and Sons Ltd. London. pp 249-255.

Sefton, B.M., Wickus, G.G. and Burge, B.W. 1973. J. Virol. 11:730-735.

Sharon, N. and Lis, H. 1972. Science 177:949-959.

Sharon, N. 1976. Mitogens in Immunology. (Eds. Oppenheim, S. and Rosenstreich, A.) Academic Press, New York. 31-41.

Sharon, N. 1975. Complex Carbohydrates. Addison-Wesley Publishing Co. Reding Mass. pp. 466.

Sharon, N. 1977. Scient. American. 236:108-119.

Shohan, J., Inbar, M. and Sacks, L. 1970. Nature. 227:1244-1246.

Siminovitch, L. 1976. Cell 7:1-35.

Singer, S.J. and Nicolson, G.L. 1972. Science 175: 720-731.

Singer, S.J. 1974. Adv. Immunol. 19:1-68.

Smith, C.W. and Hollers, J.C. 1970. J. Reticuloendothel. Soc. 8:458-459.

Smith, S.B. and Revel, J-P. 1972. Develop. Biol. 27: 434-441.

So, L.L. and Goldstein, I.J. 1967a. J. Immunol. 99: 158-163.

So, L.L. and Goldstein, K.J. 1967b. J. Biol. Chem. 242:1617-1622.

Sperti, S., Montanaro, L., Mattioli, A. and Stirpe, F. 1973. Biochem. J. 136:813-815.

Stanley, P., Caillibot, V. and Sminovitch, L. 1975a. Somat. Cell Genet. 1:3-26.

Stanley, P. Caillibot, V. and Siminovitch, L. 1975b. Cell 6:121-128.

Stanley, P., Narashmhan, S., Siminovitch, L. and Schachter, H. 1975c. Proc. Natl. Acad. Sci. U.S. 72:3323-3327.

Stanley, P. and Siminovitch, L. 1976. In Vitro. 12: 208-215.

Stanley, P. and Siminovitch, L. 1977. Somatic Cell Genet. 3:391-405.

Stanley, P. and Carver, J.P. 1977. Proc. Natl. Acad. Sci. U.S. 74:5056-5059.

Stanners, C.P., Eliceiri, G.L. and Green, H. 1971. Nature N.B. 230:52-54.

Stitz, L., Reinacher, M. and Becht, H. 1977. J. Gen. Virol. 34:523-530.

Storrie, B. 1974. J. Cell Biol. 62:247-252.

Sumner, J.B. 1919. J. Biol. Chem. 37:137-142.

Sumner, J.B. and Howell, S.F. 1935. J. Immunol. 29:133-134.

Sumner, J.B. and Howell, S.F. 1936. J. Bacteriol. 32:227-237.

Swak, A. and Wolman, S.R. 1972. In Vitro. 8:1-5.

Swann, A.C., Daniel, A., Albers, R.W. and Koval, G.J. 1975. Biochim. Biophys. Acta 401:299-306.

Szybalski, W., Ragni, G. and Cohn, N. 1964. Symp. Inter. Soc. Cell Biol. 3:209-221.

Tevethia, S.S., Lowry, S., Rawls, W., Melnick, J. and McMillan V. 1972. J. Gen. Virol. 15:93-97.

Thompson, L.H., Mankovitz, R.M., Baker, J., Till, J.E., Siminovitch, L. and Whitmore, G.F. 1970. Proc. Natl. Acad. Sci. 66:377-384.

Thompson, L.H., Mankovitz, R., Baker, R.M., Wright, J.A, Till, J.E., Siminovitch, L. and Whitmore, G.F. 1971. J. Cell Physiol. 78:431-439.

Tichá, M., Entlicher, G., Kostir, J.V. and Kacourek, J. 1970. Biochim. Biophys. Acta 221:282-289.

Till, J., Baker, R.M., Brunette, D., Ling, V., Thompson, L., and Wright, J.A. 1973. Fed. Proc. 32:29-33.

Toms, G.C. and Western, A. 1971. Chemotaxonomy of Leguminosae. Eds. Harborne, B., Bouler, D. and Turner, B.L. Academic Press, New York. p. 367.

Tyan, M.L. 1974. Proc. Soc. Exp. Biol. Med. 146:1163-1164.

Uchida, T. and Matsumato, T. 1972. Biochim. Biophys. Acta 257:230-234.

Ukena, T.E. and Karnovsky, M.J. 1977. Exp. Cell Res. 106:309-325.

Van Blitterswijk, W.J., Wallborg, Jr., E.F., Feltkamp, C.A., Hilkmann, H.A.M. and Emmelot, P. 1976. J. Cell Sci. 21:579-594.

Van Veen, J., Roberts, M. and Noonan, K.D. 1976a. J. Cell Biol. 70:204-216.

Van Veen, J., Noonan, K.D. and Roberts, R.M. 1976b. Exp. Cell Res. 103:405-413.

Veron, M. and Shapiro, B.M. 1977. J. Biol. Chem. 252:1286-1292.

Vosbeck, K. and Roth, S. 1976. J. Cell Sci. 22: 657-670.

Waechter, C.J. and Lennarz, W.J. 1976. Annu. Rev. Biochem. 45:95-112.

Waechter, J.C., Lucas, J.J. and Lennarz, W.J. 1973. J. Biol. Chem. 248:7570-7579.

Wallach, D.F.H. 1972. The Plasma Membrane: Dynamic Perspectives, Genetics and Pathology. English University Press Ltd. New York. pp. 186.

Wallach, D.F.H. 1975. Membrane Molecular Biology of Neoplastic Cells. Elsevier Scientific Publishing Co. Amsterdam. pp 525.

Wollman, Y. and Sachs, L. 1972. J. Membrane Biol. 10:1-10.

Warren, J.R. and Kowalski. M.M. 1977. Exp. Cell Res. 107:462-466.

Weber, J. 1973. J. Cell Physiol. 81:49-54.

Weeks, G. 1973. Exp. Cell Res. 76:476-470.

Weeks, C. and Weeks, G. 1975. Exp. Cell Res. 92:372-382.

Weinstein, Y., Givol, D. and Strausbauch, P.H. 1972. J. Immunol. 109:1402-1404.

Weiss, P. 1960. Proc. Natl. Acad. Sci. U.S. 46:993-1000.

West, C.M. and McMahon, D. 1977. J. Cell Biol. 74: 264-273.

Whatley, R., Ng, S.K.-C., Rogers, J., McMurray, W.C. and Sanwal, B.D. 1976. Biochem. Biophys. Res. Commun. 70:180-185.

Willingham, M.C. and Pastan, I. 1974. J. Cell Biol. 63:288-294.

Willingham, M.C. and Pastan, I. 1975. Proc. Natl. Acad. Sci. U.S. 72:1263-1267.

Wingquist, L. and Dallner, G. 1976. Biochim. Biophys. Acta 436:399-412.

Winquist, L., Eriksson, L., Dallner, G. and Ersson, B. 1976. Biochem. Biophys. Res. Commun. 68:1020-1026.

Worton, R., Ho, C. and Duff, C. 1977. Somatic Cell Genet. 3:27-45.

Wright, J.A. 1973a. J. Cell Biol. 56:666-675.

Wright, J.A. 1973b. Exp. Cell Res. 78:456-460.

Wright, J.A., Ceri, H. and Lewis, W.H. 1973. Nature New Biol. 244:84-86.

Wright, J.A. and Lewis, W.H. 1974. J. Cell.Physiol. 83:437-440.

Wright, J.A. 1975. Can. J. Microbiol. 21:1650-1654.

Wright, J.A., Lewis, W.H., Goodridge, K.A. and Ceri, H. 1976. Can. J. Genet. and Cytol. 18:577A.

Wright, J.A. and Ceri, H. 1977a. FEBS Letts. 78:124-126.

Wright, J.A. and Ceri, H. 1977b. Biochim. Biophys. Acta 469:123-136.

Wright, J.A., Ceri, H. and Jamieson, J.C. 1978. Can. Fed, Biol. Soc. (in the press).

Yamada, K.M., Yamada, S.S. and Pastan, I. 1975. Proc. Natl. Acad. Sci. U.S. 72:3158-3162.

Yanagimachi, R. and Nicolson, G.L. 1976. Exp. Cell Res. 100:249-257.

Yariv, J., Kalb, A.J. and Levitzki, A. 1968. Biochim. Biophys. Acta 165:303-305.

Yogeeswaran, G., Murray, R.K. and Wright, J.A. 1974. Biochem. Biophys. Res. Commun. 56:1010-1016.

Young, N.M., Leon, M.A., Takahashi, T., Howard, I.K. and Sage, H.J. 1971. J. Biol. Chem. 246:1596-1601.

Young, M.E.M., Moscarello, M.A. and Roidren, J.R. 1976. J. Biol. Chem. 251:5860-5866.

Zagyansky, Y., Benda, P. and Biconte, J.C. 1977. FEBS Letts. 77:206-208.

Zarling, J. and Tevethia, S. 1971. Virol 45:313-316.