THE UNIVERSITY OF MANITOBA

CELL GROWTH, LIPID METABOLISM, AND TEMPERATURE-SENSITIVITY IN A GLYCOSYLATION DEFICIENT, CONCANAVALIN-A RESISTANT CHINESE HAMSTER OVARY CELL VARIANT

by

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ABSTRACT

The concanavalin-A resistant glycosylation deficient, Chinese hamster ovary cell variant, C^R7, displays a variety of membrane-related abnormalities, is deficient in the biosynthesis of some membrane lipids and is temperature-sensitive for growth, being unable to proliferate at 39°C. These changes result from a single mutation of unknown nature.

C^R7 is a cholesterol auxotroph due to its inability to convert lanosterol to cholesterol. C^R7 is also deficient in its ability to regulate 3-hydroxy-3 methylglutaryl-CoA reductase, a key regulatory enzyme in the biosynthesis of cholesterol.

Growth experiments at 34°C and 39°C, the non-permissive temperature, showed that temperature-sensitive growth was relieved by mevalonate, dolichol, and dolichyl-phosphate but not by cholesterol. Other metabolites of mevalonolactone (squalene, ubiquinone, lanosterol and isopentenyladenine) were inactive in this regard.

Dolichol levels were determined in C^R7 and wild type cells at 34°C and 39°C. At 34°C (the permissive temperature for C^R7) there were no significant differences in dolichol content, but at 39°C there were differences in content and in isoprenologue distribution.

Dolichol, dolichyl-phosphate, cholesterol, and isopentenyladenine had no effect on the sensitivity of the wild type or C^R7 cells to the cytotoxic effects of concanavalin-A. Mevalonolactone or lanosterol markedly increased the resistance of C^R7 to the lectin, but had no effect on wild type cells.

A recently discovered class of mevalonate metabolites is a set of

proteins which are radiolabeled when cells are exposed to radiolabeled mevalonate. The incorporation of labeled mevalonate into proteins was examined by subjecting extracts of labeled cells to SDS-PAGE and autoradiography. Incorporation of this label into lipids was also examined. It appears that C^R7 may incorporate more label from MVA into proteins. Incorporation of label into lipids of WT and C^R7 was similar at 34°C but differed at 39°C.

Incorporation of [^{3}H]-mannose into lipid-monosaccharide; lipid-oligosaccharide and glycoprotein was examined to further characterize C^R7's behaviour. The effect of cholesterol deficiency on these processes was examined. In all instances, regardless of either cholesterol sufficiency or deficiency, or temperature, WT and Rc cell lines incorporated more label into these three compounds than did C^R7. C^R7, when grown at 39°C in the presence of mevalonate at a concentration, known to restore normal growth at 39°C, was able to respond similarly to wild type cells.

Incorporation of [^{14}C] labeled mevalonate into sterols was investigated in order to examine the products of isoprenoid synthesis. Total incorporation of label into WT, Rc, and C^R7, regardless of either temperature or of cholesterol status, was about the same. However, incorporation into individual sterols was found to be different. In C^R7, a major portion of label appeared associated with lanosterol rather than cholesterol. This reflects C^R7's apparent inability to effectively demethylate lanosterol.

The relative rate of cholesterol biosynthesis was investigated by examining the incorporation of labeled acetate into sterols determined

by digitonin precipitation. WT and Rc cells effectively responded to cholesterol deficiency and synthesized needed cholesterol. C^R7, on the other hand, can not do so. This is particularly true at the non-permissive temperature.

Total fatty acid synthesis was examined. The variant was deficient in this process relative to WT and Rc. As a result of the observation of altered fatty acid content, an examination of the rates of synthesis of saturates and unsaturates was done. A marked inability of C^R7 to make the appropriate compensatory response and modulate its fatty acid content was found.

Experiments examining DNA, RNA and protein synthesis revealed that the mutant is defective in these processes, especially at the non-permissive temperature. The addition of mevalonate to C^R7 reversed this defect.

C^R7 appears to be lanosterol demethylation defective. Desmosterol and 7-dehydrocholesterol, products of a major branch point in the cholesterol biosynthetic pathway, were shown to effectively replace cholesterol in restoring normal growth to C^R7 on LPDS-M at 34°C.

The addition of ketoconazole, a known lanosterol demethylation inhibitor, to WT cells at 39°C gave a growth response which mimicked temperature-sensitive growth. This drug amplified C^R7 temperature-sensitivity regardless of cholesterol content.

The effect of ketoconazole on the "Con-A properties" of WT and C^R7 was examined. C^R7, in the drug's presence, was found to be more resistant. WT was found to be less sensitive to the cytotoxic effects of Con-A when cultured in the presence of ketoconazole. In effect, with

respect to temperature-sensitive growth and the "Con-A response", in the presence of ketoconazole, WT behaviour became "mutant-like."

Ketoconazole inhibits lanosterol demethylation at C-14. Lanosterol demethylation at C-14 is cytochrome P-450 dependent. Cytochrome P-450 content was evaluated in WT and C^R7 cells at 34°C and 39°C. Cytochrome P-450 was detectable in WT cells at 34°C and 39°C.

In C^R7 cells, under the same conditions, cytochrome P-450 was undetectable. This finding, although preliminary, coupled with the results of the ketoconazole experiments suggests that C^R7 is lanosterol demethylation defective at C-14.

It is suggested, therefore, that abnormally large amounts of lanosterol and a dearth of cholesterol may disturb membrane function causing the phenotypic abnormalities seen in C^R7, including temperature-sensitive growth.

TABLE OF CONTENTS

TABLE OF CONTENTS (cont'd.)

TABLE OF CONTENTS (cont'd.)

LIST OF FIGURES

LIST OF FIGURES (cont'd.)

LIST OF FIGURES (cont'd.)

LIST OF FIGURES (cont'd.)

LIST OF TABLES

LIST OF TABLES (cont'd.)

LIST OF ABBREVIATIONS

ACAT	Acyl CoA:cholesterol acyltransferase
$AgNO_3$	silver nitrate
Asn	asparagine
BSA	bovine serum albumin
C	cholesterol
CHO	Chinese hamster ovary
CO	carbon monoxide
Con-A	concanavalin-A
d	days
DEAE	diethylaminoethyl-
DELIP-M	medium with delipidated serum
Dol	dolichol
Dol-P	dolichyl-phosphate
DMSO	dimethyl sulfoxide
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol bis (β-amino ethyl ether)-N,N, N^1, N^1-tetracetic acid
ER	endoplasmic reticulum
etc.	etcetera
FAME	fatty acid methyl esters
FCS	fetal calf serum
FCS-M	medium containing fetal calf serum
Fuc	fucose
Gal	galactose

LIST OF ABBREVIATIONS (cont'd.)

GalNAc	N-acetylgalactosamine
GLC	gas liquid chromatography
Glc	glucose
GlcNAc	N-acetylglucosamine
h	hours
HMG-CoA	3-hydroxy-3 methylglutaryl-coenzyme A
HPLC	high performance liquid chromatography
kDa	kilodalton
KBr	potassium bromide
	lambda (wavelength)
LDL	low density lipoprotein
LPDS-M	medium containing lipoprotein deficient serum
Man	mannose
mCi	milliCurie
mM	millimolar
α-MEM	α-minimum essential medium
oz.	ounce
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PL	phospholipid
RER	rough endoplasmic reticulum
RPE	relative plating efficiency
SDS	sodium dodecyl sulfate
Ser	serine

LIST OF ABBREVIATIONS (cont'd.)

SER	smooth endoplasmic reticulum
TCA	trichloroacetic acid
Thr	threonine
TLC	thin layer chromatography
Tris	Tris (hydroxymethyl) amino methane
μCi	microCurie
μl	microliter
μM	micromolar
V_{max}	maximum velocity

INTRODUCTION

INTRODUCTION

Mammalian cell membranes serve as vibrant liasons between the cell and its environment. Mammalian cell membranes consist of a lipid bilayer composed primarily of phospholipids and cholesterol. Proteins that have important cellular functions, such as receptors, transporters and enzymes, are embedded in the lipid bilayer. The "dynamic" state of lipids in the bilayer was described by Singer and Nicolson (1972) as one of the fundamental tenets of the fluid mosaic model of membrane structure. The plasma membrane and other cell membranes of the mammalian cell may not be accurately viewed as merely static barriers but should be seen as fulfilling vital and active roles.

Membrane fatty acid composition, phospholipid composition and cholesterol content can be modified in many different kinds of intact mammalian cells. The modifications are extensive enough to alter membrane fluidity and affect a number of cellular functions. The effects of lipid modification on cellular function are extremely complex, often varying from one type of cell to another. Indeed, they do not exert a uniform effect on all processes in a single cell line. To generalize or to predict how a given system will respond to a particular type of lipid modification is not yet possible.

Ideally, every individual component of the membrane must be synthesized and introduced into the membrane; degraded and removed from the membrane in such a manner as to not upset the function and the status of the membrane as a whole. As a consequence, the mechanisms and processes which maintain the functional integrity of the membrane and its adaptability, must be stringently regulated and controlled.

The membrane, therefore, has been recognized as playing an integral role in the regulation of many fundamental processes, and among these, is the regulation of growth and development of mammalian cells. Furthermore, cholesterol is thought to be a key participant in these processes. Indeed, cholesterol, its behaviour, and roles in cell growth are seen as playing crucial roles in normal and in pathological mammalian cell states (Yeagle, 1985). The cholesterol essential for normal cell growth can be lethal for the organism as a whole. For example, in the human population, the lethality manifests itself through the pathology of atherosclerosis.

Membrane variants of mammalian cells have been shown to be useful vehicles for the study of the structure-function relationships of the cell membrane (Stanley, 1984, 1987). Wright (1973) showed that variants resistant to the plant lectin, concanavalin-A (Con-A) could be selected in cultures of Chinese hamster ovary (CHO) cells. These variants possessed a complex pleiotropic phenotype. Ceri (1978) continued this work selecting independent Con-A resistant cell lines 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 temperature-sensitive growth, and assorted altered membrane-associated properties such as cell morphology, lectin agglutinability, cell adhesion, sensitivity to membrane active agents, Con-A binding properties and lectin-receptor mobility properties. Con-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 & Wright, 1977, 1978; Wright & Ceri, 1977). Ceri (1978)

initiated a biochemical study of the surface membranes of wild type, revertant and variant cell lines. Altered glycoprotein patterns were detected on the surface of lectin resistant cells (Ceri, 1978) and this led to a study of glycoprotein biosynthesis using surface labeling techniques. Specifically, C^R7 was found to be glycoprotein biosynthesis defective (Ceri, 1978).

It was suggested that the specific defect providing the C^R7 cell line with resistance to Con-A might be in the assembly of lipid-linked intermediates of glycosylation (Ceri, 1978; Borgford, 1984). Although the result of a single genetic lesion, C^R7 expresses many changes in membrane related phenomena. The connection between a specific defect in glycoprotein synthesis and the altered phenotype is not entirely clear.

Borgford (1984) speculated that this defect may have resulted from a deficiency in the synthesis, or turnover of dolichyl-phosphate. Borgford (1984) further speculated on the interesting circular problem of whether defective lipid metabolism is a result of a primary defect in protein glycosylation, or, whether defective glycosylation is due to a primary defect in lipid metabolism. This interrelationship between competent synthesis of glycoprotein and the regulation of lipid metabolism was explored by Borgford.

C^R7 was found to express a 10- to 20-fold greater sensitivity to the drug compactin which specifically and competitively inhibits the activity of hydroxymethylglutaryl-Coenzyme-A (HMG-CoA) reductase (Borgford, 1984). Whereas wild type and revertant cell lines up-regulated and down-regulated HMG-CoA reductase in response to changing concentrations of exogenous low-density lipoprotein (LDL), the activity of HMG-CoA reductase was apparently "fixed" in C^R7 (Borgford,

1984). The expression of the LDL-receptor was also found to be "fixed" in C^R7 (Borgford, 1984).

Under growth conditions in which the in vitro activity of HMG-CoA reductase in C^R7 was apparently 2-fold greater than that of the wild type, the two cell lines were seen to synthesize sterol at a similar rate (Borgford, 1984). This finding led to the speculation that the reductase may not be rate limiting to the synthesis of sterols in the mutant. The in vitro activity of HMG-CoA reductase may not reflect the in vivo rate of mevalonate synthesis (Borgford, 1984; Borgford et al, 1986).

Tunicamycin, a powerful inhibitor of glycosylation , suppressed the activity of HMG-CoA reductase in wild type cells, however, mutant cells were found to be insensitive to the suppression induced by tunicamycin (Borgford et al, 1986). This suppression of activity corresponded to an inhibition of the glycosylation of cellular proteins without affecting the overall rate of protein synthesis. Tunicamycin appeared to cause a specific inhibition of the synthesis of HMG-CoA reductase protein. C^R7 cells were found to be insensitive to the suppression induced by tunicamycin (Borgford, 1984; Borgford et al, 1986). These results suggested the possible existence of a glycoprotein responsible for regulating the synthesis and/or degradation of HMG-CoA reductase and perhaps other aspects of lipid metabolism, the activity of which is lost when improperly glycosylated (Borgford, 1984; Borgford et al, 1986).

The aim of this thesis, therefore, is to address some aspects of lipid metabolism and glycoprotein biosynthesis in C^R7 in an attempt to further elucidate the nature of these processes in C^R7. In addition to studying lipid metabolism and its regulation, a study of cell growth was

undertaken, more specifically, in relation to temperature-sensitive growth. This phenomenon exhibited by C^R7 is a focus of this present investigation, although not exclusively. Other than the initial observation of a temperature-sensitive growth response in C^R7 (Ceri, 1978), this altered property of C^R7 has been heretofore unexplored. Furthermore, up to this point, only a cursory examination of lipid metabolism in this cell line has occurred. Therefore a further examination of lipid metabolism in this cell line is warranted. After all, lipid is the principal component of all cell membranes. C^R7 is a membrane mutant with pleiotropic membrane altered properties. By virtue of this fact, the Con-A resistant variant, known to be defective in membrane associated biochemical processes is surely a more than adequate model system for the study of membrane biochemistry and membrane associated biochemical processes.

HISTORICAL

HISTORICAL

A cell culture system with its highly controlled yet readily manipulated milieu offers an excellent environment in which the study of biochemical and physiological processes and their regulation in mammalian cells may be undertaken, particularly with the aid of the process of "mutation."

The CHO cell line, C^R7, is as already mentioned, a Con-A resistant mutant belonging to a group of mammalian cell variants loosely identified as "membrane mutants."

The purpose of this historical is to review, although somewhat briefly, the areas of knowledge deemed relevant to the study of the C^R7 variant cell line. The subject areas to be reviewed are as follows:

(a) cell membranes and cellular functions;

(b) glycoprotein synthesis and the significance of glycosylation;

(c) biosynthesis, regulation and function of cholesterol in mammalian cells;

(d) isoprenoid biosynthesis;

(e) temperature-sensitive mammalian cell variants; and finally,

(f) lectins and lectin-resistant variant animal cells.

Cell Membranes and Cellular Functions

The cell surface membrane plays a crucial biological role, separating the internal environment of a cell from the very different biochemical milieu of the extracellular fluid. The cell surface membrane controls the inward and outward movement of many compounds. Furthermore, it helps to regulate the cellular response to a variety of external influences. Gross generalized disturbances of surface membranes would presumably be incompatible with life and suitable mechanisms must help to maintain the integrity of the fluid lipid bilayer and the function of associated enzymes, receptors and other proteins (Owen et al, 1984).

Similarities exist between the surface coat of plasma lipoproteins and the outer leaflet of mammalian cell plasma membrane: the phospholipid (PL) molecules are predominantly lecithin and sphingomyelin, and cholesterol and amphipathic polypeptides are also present. Cholesterol, virtually insoluble in water, is solubilized by phospholipids and other polar lipids. The amount of cholesterol present (expressed as a molar ratio of cholesterol (C) to phospholipid, C/PL) in plasma membranes and in lipoprotein surfaces is variable. This variability is related to the presence of glycolipids; to the preferential association of cholesterol with particular phospholipid classes and to the diminished availability of phospholipid that interacts with protein (Owen et al, 1984). Cholesterol and phospholipid molecules in a particular lipoprotein surface exchange and equilibrate with their counterparts in lipoproteins of all density classes and in cell plasma membranes (cholesterol exchanges much more rapidly than phospholipid) (Owen et al, 1984). Exchange of lipid also occurs between

the plasma membrane and membranes of intracellular organelles (Green, 1983).

If membrane lipid abnormalities occur as a consequence of changes in lipoprotein surface lipids, significant effects on cellular functions might be expected. Lipids are rarely regulatory molecules per se. However, lipids do play major roles in influencing the degree of membrane and cellular response to various internal and external stimuli by modulating changes in membrane fluidity which is largely governed by lipid composition. Cholesterol content, phospholipid classes and the length and degree of unsaturation of phospholipid fatty acyl chains are all important modulators of membrane fluidity.

Alterations in membrane lipid composition induced by abnormal lipoproteins can also cause changes in membrane fluidity and may disturb many cellular processes including membrane permeability, active transport mechanisms and the functioning of receptors and enzymes (Owen et al, 1984).

Membrane fatty acid composition, phospholipid composition and cholesterol content can be modified in many different kinds of intact mammalian cells. Such modifications can influence phagocytosis, exocytosis, immunologic and chemotherapeutic cytotoxicity, and cell growth (Spector & Yorek, 1985).

It is well established that a great many organisms alter their membrane lipid composition in response to changing temperature. Changes in phospholipid acyl chain unsaturation, phospholipid polar head group composition, and sterol content of membranes are a few of the well known responses of cells to altered temperature. These changes, in general, alter the membrane fluidity to a level more compatible with the cell's

ability to survive at the new temperature (Fulco, 1974; Ferguson et al, 1975).

Surface membranes of most eukaryotic cells are characterized by a relatively high sterol content. While the function of sterols in membranes remains speculative, studies of the effects of sterols in model lipid systems has revealed some interesting findings (Cullis et al, 1976; Demel et al, 1977; Estep et al, 1978; Mabrey et al, 1978; Kawato et al, 1978; Cullis and De Kruijff, 1978; Yeagle, 1985). These findings have indicated that sterols can have two disparate effects on phospholipid bilayers, depending upon the phase behavior of the phospholipids (De Kruijff et al, 1972). At temperatures above the gel-liquid crystalline transition, sterols have a "condensing" effect resulting in a somewhat more ordered structure than is present in the phospholipid bilayer in the absence of sterol. Conversely, at temperatures below this transition, sterol has a "disruptive" effect, causing the phospholipids to be somewhat more "fluid." In three component systems, containing sterol and two phospholipids of varying acyl chain length or degree of acyl chain unsaturation, sterols seem to interact preferentially with the lower melting component (De Kruijff et al, 1972). In three component systems, containing sterol and two phospholipids with different head groups, sterol preferentially interacts with the lipids in the order sphingomyelin, (SM) > phosphatidylcholine, (PC) > phosphatidylethanolamine, (PE) (Demel et al, 1977).

Studies of sterol effects on biological membranes also indicate that sterol can have several functions in the membrane. As an example, Mycoplasma mycoides, which possesses only a surface membrane, can adapt

to grow at low sterol concentrations (Rottem et al, 1973). Such cells have phospholipids whose acyl chains are much more saturated than those of normal cells and are also characterized by an ability to grow at lower temperatures. Employing physical measurements, Rottem et al (1973) concluded that sterol exerts a condensing effect on the membrane phospholipids of these organisms.

Work on sterol auxotrophs of mouse LM-cells has indicated that sterol depletion results in an increase in unsaturated acyl chains in plasma membrane phospholipids (Freter et al, 1979; Baldassare et al, 1979). Sterol depletion also leads to a decline in the V_{max} of 3-O-methylglucose uptake implying that the "fluidizing" effect of sterol is very important in these organisms (Baldassare et al, 1979).

Rintoul et al (1979) examined the effects of sterol depletion on plasma membrane physical properties using a fluorescent fatty acid probe. Sterol depletion to approximately 40% of the normal value in LM-cells led to an increase in the unsaturated fatty acid content of membrane phospholipids. No appreciable difference in liquid-gel transition temperatures between control and sterol depleted plasma membranes could be detected with the fluorescent fatty acid probe implying that a "disruptive" role of sterol is important in maintaining the physical properties of the membrane (Rintoul et al, 1979). In sterol depleted cells, this "disruptive" role could be partially filled by phosphatidylcholine containing a higher percentage of unsaturated acyl chains (Rintoul et al, 1979).

Baldassare et al (1979) showed that sterol depletion of two different sterol-requiring mutants of LM cells, S_1 and S_2, correlated with a dramatic rise in the 18:1 fatty acid content and a concomitant

decrease in 16:0 chains in unfractionated membrane phospholipid. Furthermore, these alterations were found to be most pronounced in isolated plasma membranes, which normally have higher sterol content than other membrane fractions.

Freter et al (1979) examined the effects of reduced cellular sterol levels on metabolism in mouse LM cells which were defective in sterol biosynthesis. Alterations in plasma membrane phospholipids reflected a substitution of lower melting phospholipids for sterols. In vivo and in vitro measurements of microsomal stearoyl-coenzyme A desaturase activity revealed a close coupling and a reciprocal relationship between cellular sterol levels and desaturase; for example, a decrease in cellular sterols to 50% of the normal content resulted in a 2-fold increase in desaturase activity (Freter et al, 1979). Garda and Brenner (1984) showed that microsomal membrane fluidification which was artificially evoked by isoamyl alcohol incorporation correlated with a decrease of Δ^9 and Δ^6 desaturation. However, cholesterol incorporation in microsomes evoked a decrease of membrane "fluidity" and a corresponding increase of the desaturation of fatty acids (Garda & Brenner, 1985). The similarity of the antagonistic responses of the desaturases evoked by two opposite changes of the membrane dynamics suggests that desaturase reactions may function as a self-regulating mechanism of the unsaturated acyl chain content. These enzymes may also exert their effects through changes of membrane "fluidity" assuming that unsaturated acids increase membrane "fluidity." A decrease in membrane "fluidity" would increase the desaturation and this would adjust the membrane "fluidity" increasing it and vice versa (Garda & Brenner, 1985). This type of viscotropic regulation is suggested to also exist in Tetrahymena

pyriformis, a protozoan ciliate (Martin et al, 1976; Skiver & Thompson, 1979). With mammalian systems, however, it is necessary to consider that the regulation of fatty acid desaturation systems is extremely complicated due in part to hormonal mechanisms that may mask simpler and more subtle fluidity-desaturase self-regulation (Brenner, 1981).

The effects of lipid modification on cellular functions are very complex, often varying from one type of cell to another and they do not exert a uniform effect on all processes in a single cell line. It is therefore not yet possible to generalize or predict how a given system will respond to a particular type of lipid modification. Many of the functional responses probably are caused directly by membrane structural changes which affect either bulk lipid fluidity or specific lipid domains. The conformation or quaternary structures of certain membrane constituents (receptors, enzymes, etc.) probably are sensitive to changes in the structure of their immediate lipid microenvironment leading to changes in activity. While this is a most likely mechanism, it must also be considered that membrane lipid compositional change may be an independent event that occurs concurrently but without any causal relationship to any functional perturbations (Spector & Yorek, 1985).

Glycoprotein Synthesis

Since C^R7 is a glycosylation defective mutant (Wright et al, 1978, 1979; Blaschuk et al, 1980a, 1980b), a brief description of glycoprotein synthesis is warranted.

Glycoproteins contain carbohydrate chains. These chains may be either N-linked (where the linkage exists between the amide group of the amino acid asparagine and the anomeric carbon of the amino sugar N-acetylglucosamine) or O-linked (where the oligosaccharide is linked to

the hydroxyl groups of serine, threonine, hydroxylysine or hydroxyproline).

Synthesis of membrane and secretory glycoproteins takes place in the rough endoplasmic reticulum (RER). Such proteins are characteristically N-linked to oligosaccharide (Struck & Lennarz, 1975). O-glycosylations take place post-translationally, after the polypeptide has left the RER.

Interestingly, very little is known about the significance of glycosylation in reference to expression of enzyme activity or function generally. The biochemical steps which lead to the formation of glycoproteins are reasonably well defined.

N-linked oligosaccharide biosynthesis involves the synthesis of a lipid-oligosaccharide precursor and the subsequent processing of this precursor into mature oligosaccharides of either "high mannose" or "complex" forms. The lipid-oligosaccharide precursor's carbohydrate portion is transfered, "en bloc," onto nascent polypeptide chains. High mannose oligosaccharide chains contain only mannose and N-acetyl glucosamine whereas complex oligosaccharides are those which contain mannose, N-acetylglucosamine plus galactose, fucose and sialic acid.

Dolichol is the lipid portion of this lipid-oligosaccharide precursor. Dolichol is actually a family of polyisoprenoid alcohols. Each member of this family has a different number of isoprene units and they are therefore of differing chain lengths. Generally, the number of isoprene units varies between 16 and 23 isoprene units, however, the a-isoprene unit is always saturated (Hemming, 1974). Dolichols are considered to be very long chain molecules and are also considered to be the membrane "anchors" upon which the oligosaccharide is assembled

(Hannover & Lennarz, 1981). Dolichyl phosphate and dolichyl pyrophosphate are two phosphorylated lipids which are substrates in glycosylation reactions.

The pathway for the assembly of the lipid-oligosaccharide is generally refered to as the "dolichol cycle" (Sharon & Lis, 1981). Carbohydrate is attached by a step-wise process onto the dolichol-phosphate precursor. The eventual removal of this carbohydrate from dolichol-phosphate ("en bloc" transfer) frees the lipid to serve as a precursor for further glycosylations. This sequence is, therefore, refered to as the "dolichol cycle." This cycle is illustrated in Figure 1.

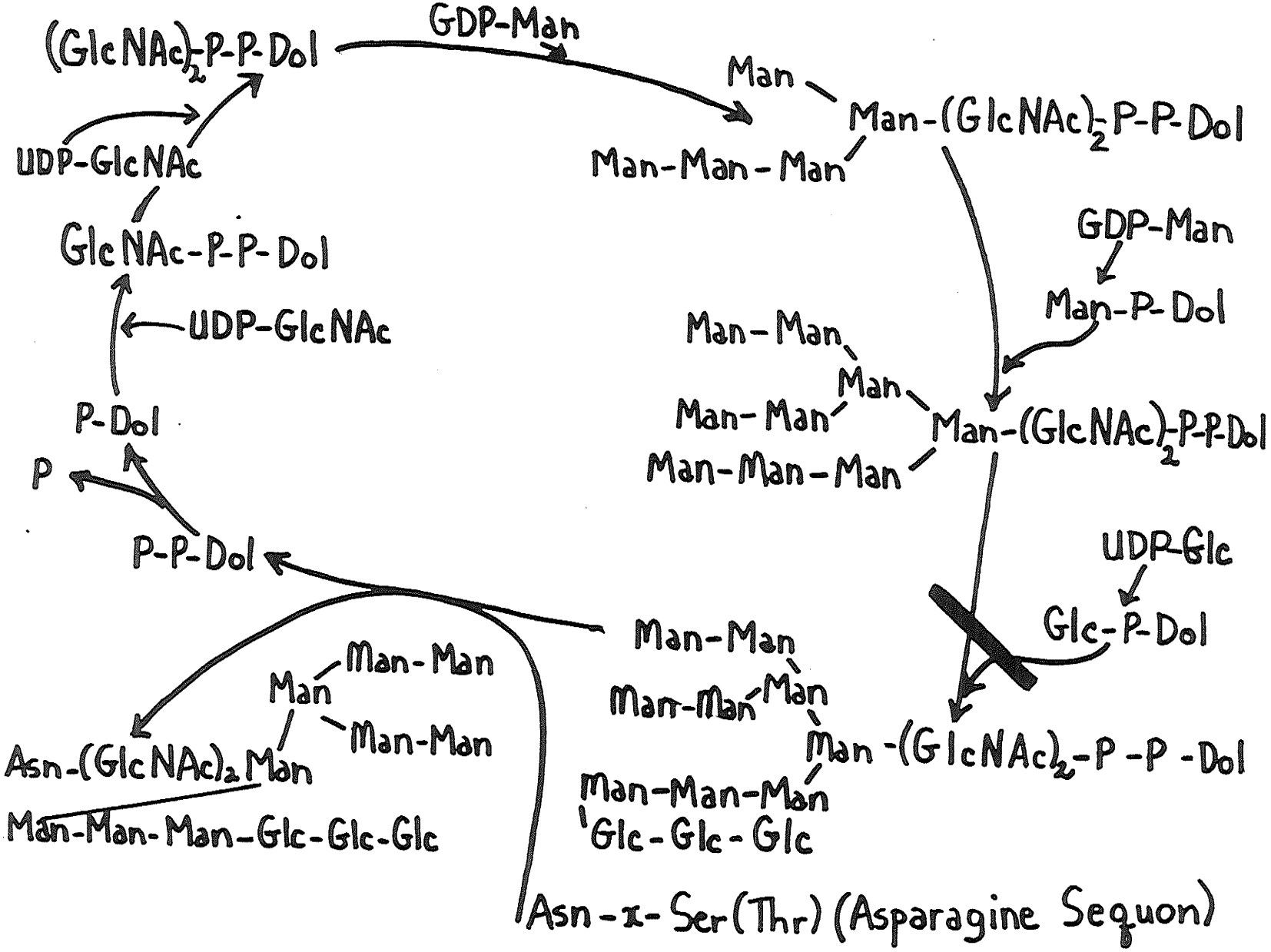
Lipid intermediates are oriented with carbohydrate moieties facing the RER's lumen. N-glycosylations take place in the lumen of the RER. Transmembrane assembly of these intermediates from nucleotide-sugar precursors found in the cytoplasm is poorly delineated at present (Hannover & Lennarz, 1981).

Figure 1 illustrates the process by which the "G-oligosaccharide" is formed. This is an oligosaccharide of the form $(Glc)_3$ $(Man)_9$ $(GlcNAc)_2$.

The examination of a number of glycosylated polypeptides revealed a tripeptide sequence Asn-X-Thr (Ser), including the oligosaccharide-linked asparagine which must be recognized by the "en bloc" transfer enzyme (Marshall, 1974). This sequence designated a "sequon" specifies, along with protein tertiary structures and other factors, the location of N-glycosylation.

Polypeptides destined to be N-glycosylated are synthesized on membrane-bound ribosomes and transported across the endoplasmic

Figure 1: A schematic representation of the dolichol cycle based on that proposed by Sharon and Lis (1981) and Turco and Robbins (1979) (from Jamieson, 1983) with a minor modification to indicate the defect in the Con-A resistant mutant B211, as according to Krag (1979).


(GlcNAc)2-P-P-Dol
GDP-Man
Man
Man-Man-Man
Man-(GlcNAc)2-P-P-Dol
UDP-GlcNAc
GlcNAc-P-P-Dol
GDP-Man
Man-P-Dol
UDP-GlcNAc
Man-Man
Man
Man-Man
Man-Man-Man
Man-(GlcNAc)2-P-P-Dol
P-Dol
P
UDP-Glc
Glc-P-Dol
P-P-Dol
Man-Man
Man
Man-Man
Man-Man
Man-Man-Man
Man-(GlcNAc)2-P-P-Dol
Asn-(GlcNAc)2 Man
Man-Man-Man-Glc-Glc-Glc
Man-Man-Man
Glc-Glc-Glc
Asn-x-Ser(Thr) (Asparagine Sequon)

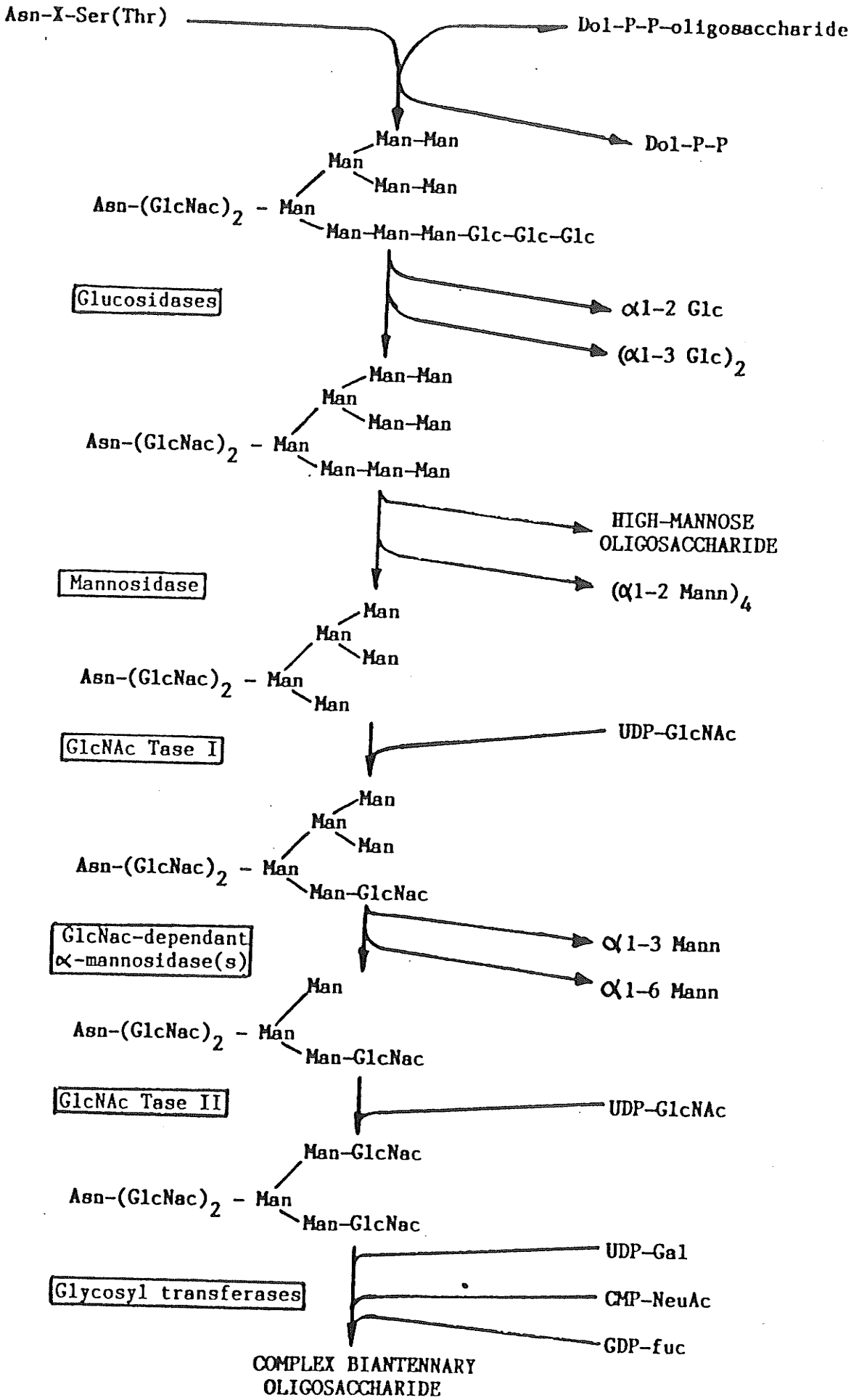
reticulum membrane. A "signal sequence" has been postulated to determine which polypeptides are to be membrane bound or exported (Blobel & Dobberstein, 1975a, 1975b). Ribosomes translating messages coding for these membrane and secretory proteins produce a nascent polypeptide. This relatively hydrophobic polypeptide, by its inherent nature, facilitates peptide and ribosome binding to the RER membrane. A docking protein and signal recognition particle also play roles.

Cytoplasmic factors are also thought to be involved in this binding process (Meyer, 1982). Once bound, a channel is formed and as translation proceeds, the nascent peptide is extruded into or through the membrane. The signal sequence is subsequently cleaved from the maturing protein.

Whether N-glycosylation is a co-translational event, occurring while the nascent peptide is still attached to the ribosome or if it is a post-translational event, occurring after the completion of translation remains a totally unresolved issue. In fact, both mechanisms may be operative. Evidence exists supporting co-translational glycosylation (Kiely et al, 1976; Rothman & Lodish, 1977; Glabe et al, 1980; Hannover & Lennarz, 1980; Hannover et al, 1982) and post-translational glycosylation (Bergman & Kuehl, 1979; Woloski & Jamieson, 1980).

Processing of N-linked oligosaccharides also occurs. Processing is characterized by both carbohydrate deletions and additions to the core oligosaccharide. A general scheme for processing sequences is illustrated in Figure 2. As a glycoprotein is processed it moves through various intracellular compartments. Processing activities are therefore spatially separated, for example, glucosidase is an RER

Figure 2: A schematic representation of the steps involved in the processing of glycoproteins (from Jamieson, 1983).


Asn-X-Ser(Thr)
Dol-P-P-oligosaccharide
Dol-P-P
Man-Man
Man
Man-Man
Asn-(GlcNac)2 - Man
Man-Man-Man-Glc-Glc-Glc
Glucosidases
α1-2 Glc
(α1-3 Glc)2
Man-Man
Man
Man-Man
Asn-(GlcNac)2 - Man
Man-Man-Man
HIGH-MANNOSE
OLIGOSACCHARIDE
Mannosidase
(α1-2 Mann)4
Man
Man
Man
Asn-(GlcNac)2 - Man
Man
GlcNAc Tase I
UDP-GlcNAc
Man
Man
Man
Asn-(GlcNac)2 - Man
Man-GlcNac
GlcNac-dependant
α-mannosidase(s)
α1-3 Mann
α1-6 Mann
Man
Asn-(GlcNac)2 - Man
Man-GlcNac
GlcNAc Tase II
UDP-GlcNAc
Man-GlcNac
Asn-(GlcNac)2 - Man
Man-GlcNac
UDP-Gal
Glycosyl transferases
CMP-NeuAc
GDP-fuc
COMPLEX BIANTENNARY
OLIGOSACCHARIDE

component (Grinna & Robbins, 1979) whereas mannosidase and transferase activities are present in the Golgi complex (Tabas & Kornfeld, 1979; Harpaz & Schachter, 1980a, 1980b; Schachter et al, 1979; Wilson et al, 1976).

The organization and function of the Golgi stacks have been greatly clarified. The stacks consist of at least three functionally and compositionally distinct compartments that operate in succession to construct the oligosaccharide chains on transported glycoproteins and to sort out proteins destined for lysosomes, the cell surface, or secretory granules (Dunphy & Rothman, 1985). These proteins are transported within the Golgi from one cisternal compartment to the next by the budding and fusion of transport vesicles (Dunphy & Rothman, 1985). The Golgi complex is where ultimate components of the plasma membrane, secretory granules and lysosomes are sorted and packaged into separate types of transport vesicles for delivery to their appropriate cellular destinations. The Golgi stack is seen as having functional as well as topological polarity.

The processing of N-linked oligosaccharide chains occurs in a stepwise manner in sequentially encountered cis, medial and trans cisternal compartments (Dunphy & Rothman, 1985).

A very interesting processing reaction is the addition of three terminal glucoses to the core oligosaccharide and their subsequent and immediate removal after the transfer of this oligosaccharide to the protein occurs. Glucose residues may direct or control the specificity of this transfer reaction. This idea is supported by the finding that oligosaccharides with such terminal additions are more rapidly transferred than those which do not bear glucose (Jurco et al, 1977).

A Golgi-located α-mannosidase, whose activity is modulated by membrane phospholipid content, may play a central regulatory role in glycoprotein processing reactions (Forsee & Schutzbach, 1981). This mannosidase is an ideal and logical candidate to play a regulatory role since its site of action is a crucial branch point between the synthesis of high-mannose and complex oligosaccharides.

Regulation of processing events may also involve phosphorylation (Sly & Fischer, 1982). Phosphorylation may act as a generalized method for controlling channeling and/or processing of glycoproteins.

A great deal is known about O-glycosylation. Several reviews of this process exist (Schachter, 1977; Schachter & Roseman, 1980). O-glycosylation of proteins is thought not to involve lipid-linked intermediates. O-glycosylation occurs after the nascent polypeptide has left the RER. The transfer of GalNac from UDP-GalNac to serine/threonine residues, which is believed to be the activity which initiates O-glycosylation, is associated with the smooth endoplasmic reticulum (SER) (Hannover et al, 1980). Later additions of carbohydrate takes place in an ordered stepwise fashion directly from nucleotide-sugar precursors. As with N-linked glycosylation, the various activities of O-glycosylation may also be spatially separated. Furthermore, later processing events, of both the N-linked and O-linked oligosaccharide chains seems to occur in the trans-Golgi (Cummings et al, 1983).

Significance of Glycosylation

The glycosylation of proteins may play a number of key roles and purposes. Cultured cells can tolerate major changes in their carbohydrate structures without a deleterious or lethal effect.

Cellular growth rate is usually not affected by these changes (Stanley, 1984). However, changes in adhesive properties and morphology have been observed to accompany certain glycosylation mutations (Gottlieb et al, 1974; Juliano, 1978; Stanley & Sudo, 1981) and such properties may be physiologically relevant in vivo.

Glycosylation mutations in animals can also induce a conditional-lethal phenotype (discussed elsewhere). Defects in intracellular trafficking are correlated with several glycosylation-defective phenotypes (Stanley, 1984). Lec 5 CHO mutants, as an example, acquire complex instead of phosphorylated oligomannosyl carbohydrates on several lysosomal enzymes and do not appropriately compartmentalize these enzymes into lysosomes (Krag & Robbins, 1982). As a result, Lec 5 mutants exhibit very low levels of intracellular lysosomal enzyme activity (Krag & Robbins, 1982).

Glycosylation may determine the susceptibility of proteins to degradation and therefore affect these proteins' rates of turnover (Leavitt et al, 1977; Olden et al, 1978). Glycosylation may influence the correct orientation of proteins in membranes and also influence the ultimate physical stability of these proteins (Pouyessegur & Yamada, 1978).

Glycosylation may afford proteins the appropriate "tag." A missing or defective tag on a protein might cause the protein to be misdirected (Sly, 1979) and this misdirection might result in inappropriate subcellular localization.

Glycosylation lesions that cause changes in the array of carbohydrates expressed at the cell surface have also been correlated with altered cellular fusion properties. A number of independently

selected lectin resistant myoblasts cannot be induced to differentiate into myotubes in culture (Sanwal, 1982; Parfett et al, 1981). A 46 K surface glycoprotein might be the fusion protein that becomes defective (Cates et al, 1984).

Several groups have shown that specific glycosylation mutations expressed in different tumor cell lines correlate with a reduction in tumorgenicity and/or a reduced ability to metastasize (Kerbel et al, 1982).

Glycosylation mutants provide the means to continue to examine the structure/function relationships of the carbohydrates associated with glycoconjugates in mammalian cells. It would appear that the process of glycosylation plays a key and central role and is truly of significance in the biological world. This is also true with respect to C^R7 which is itself a glycosylation defective variant, and as such, glycosylation and its effects may play a central role in C^R7's cellular regulation.

Biosynthesis, Regulation, and Function of Cholesterol in Mammalian Cells

Cholesterol is a small molecule first isolated from gallstones in 1784. It has played a unique role in the world of biological investigations. This is due primarily to its complex four-ring structure and its synthesis from a simple two-carbon substrate, acetate, through the action of at least 30 enzymes, many of which are still poorly understood, and its essential function in animal cell membranes.

Cholesterol is a Janus-faced molecule (Brown & Goldstein, 1986). The very property that makes cholesterol useful in cell membranes, namely its absolute insolubility in water, also makes it lethal. When cholesterol accumulates in the wrong place, it cannot be readily

mobilized (e.g.: atherosclerotic plaque development in the wall of an artery).

Animal cells are incapable of growth without cholesterol. Sterols have long been recognized as important structural components of eucaryotic cell membranes. However, procaryotic cells contain no sterol but they have been found to contain structurally and functionally similar molecules in the form of terpene derivatives (Rohmer et al, 1979).

The cholesterol requirement of mammalian cells is fulfilled in two ways, endogenously, via de novo synthesis, and exogenously, via the receptor-mediated uptake of lipoprotein. The rates of synthesis and uptake must be responsive to the immediate and individual requirements of various cells and tissues. The schematic diagram in Figure 3 summarizes the origins of cholesterol in mammalian cells.

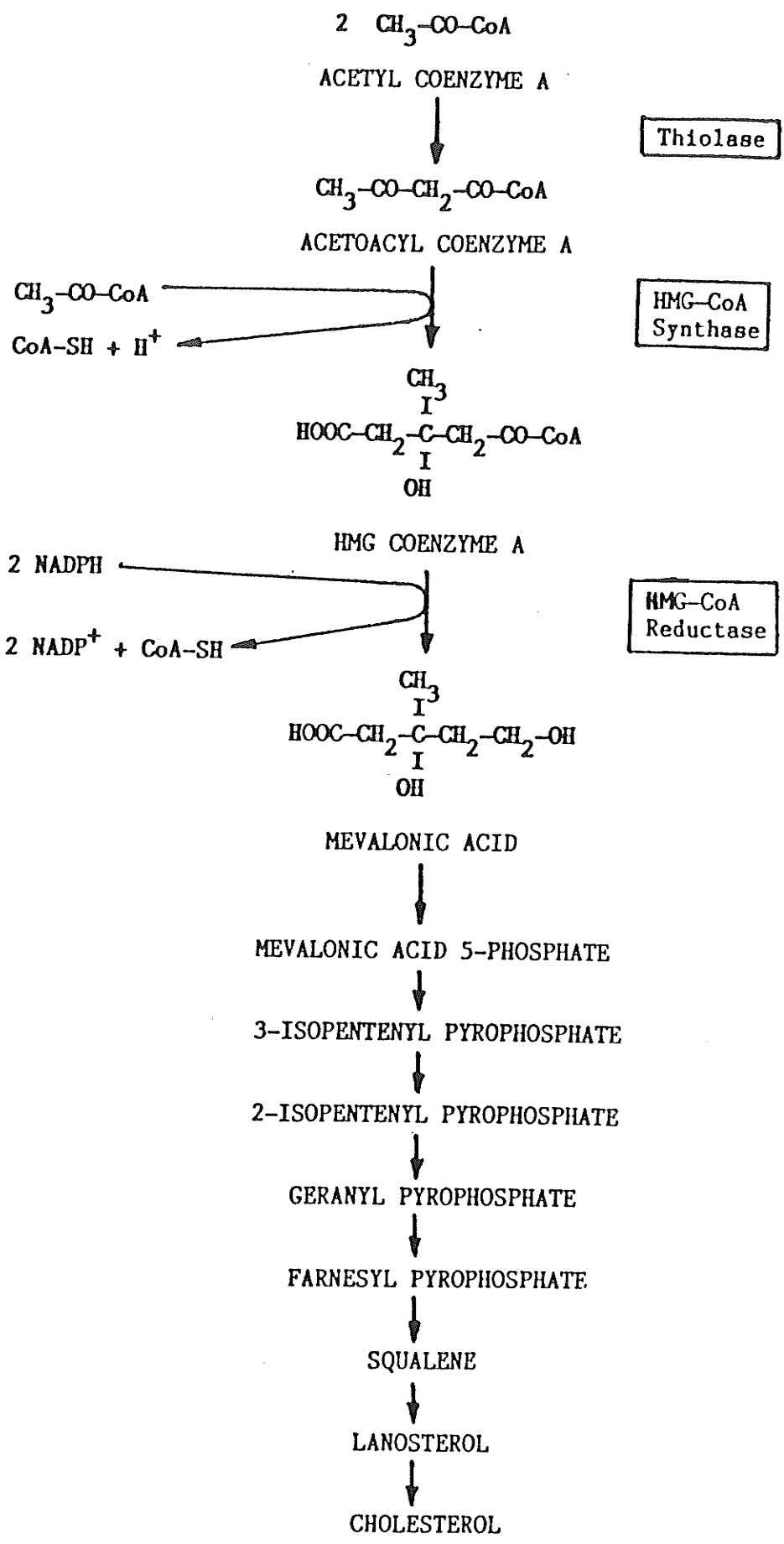
The biochemical pathway by which cholesterol is synthesized from acetate is illustrated in Figure 4. The enzyme, 3-hydroxy-3 methylglutaryl coenzyme A reductase (HMG-CoA reductase) catalyzes the first unique step in the pathway for the synthesis of cholesterol and other isoprenoids. Furthermore, it is now generally agreed that this is the most important rate-controlling enzyme of cholesterol biosynthesis in mammalian cells.

Cholesterol is imperative for the continuity of eucaryotic cells in culture or within compact tissues of organisms. An exception to this are Kc cells from Drosophila embryos maintained in culture. Silberkang et al (1983) challenged current concepts that sterols or related replacement isopentanoid molecules were required for eucaryotic membrane structure. Kc cells do not synthesize squalene and other sterols and

Figure 3: Schematic representation of the sources of cholesterol for mammalian cells (Borgford, 1984). The most significant component of low density lipoproteins is cholesterol. Furthermore, a significant portion of mevalonate goes towards the synthesis of cholesterol. Uptake and synthesis are regulated by the LDL-receptor and HMG-CoA reductase, respectively.

LDL (external)
LDL Receptor
LDL (internal)
protein
phospholipid
CHOLESTEROL
HMG-CoA
HMG-CoA Reductase
MEVALONATE
non - sterol products
ubiquinone
dolichol

Figure 4: A schematic representation of the pathway to the synthesis of cholesterol.

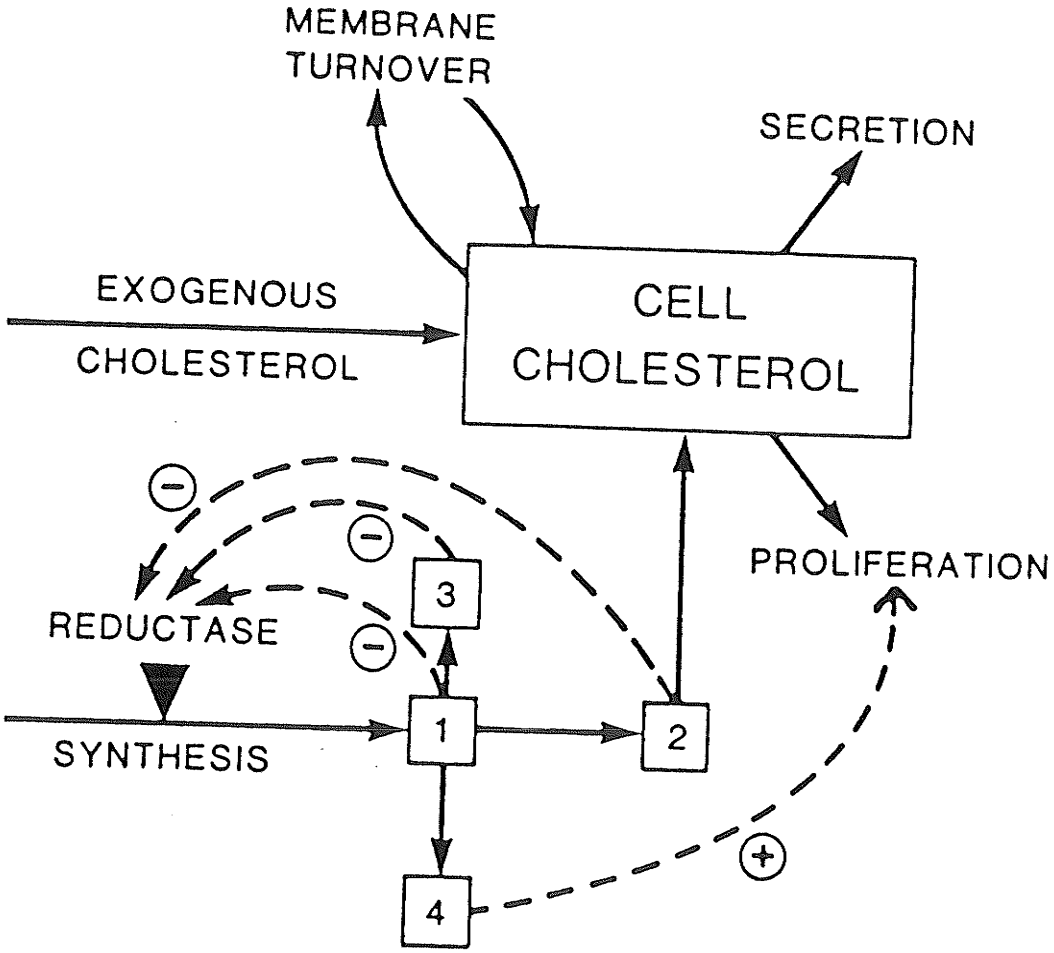

2 CH3–CO–CoA
ACETYL COENZYME A
Thiolase
CH3–CO–CH2–CO–CoA
ACETOACYL COENZYME A
CH3–CO–CoA
CoA–SH + H+
HMG–CoA Synthase
CH3
HOOC–CH2–C–CH2–CO–CoA
OH
HMG COENZYME A
2 NADPH
2 NADP+ + CoA–SH
HMG–CoA Reductase
CH3
HOOC–CH2–C–CH2–CH2–OH
OH
MEVALONIC ACID
MEVALONIC ACID 5-PHOSPHATE
3-ISOPENTENYL PYROPHOSPHATE
2-ISOPENTENYL PYROPHOSPHATE
GERANYL PYROPHOSPHATE
FARNESYL PYROPHOSPHATE
SQUALENE
LANOSTEROL
CHOLESTEROL

the incorporation of sterols into Kc cell membranes did not result in any significant alteration of total phospholipid head or acyl group composition. Similar results were shown with Schneider's Drosophila cell line I (Schneider, 1972) and with Aedes albopictus, a mosquito cell line (Singh, 1967).

Cholesterol in higher animals lends a certain rigidity to membranes at physiological temperatures (above 25-30°): other than for this reason, the reason why its need is so severe is elusive. The amount and distribution of free cholesterol in cells appears to be regulated within tight limits in keeping with its role in defining the physical state of the several kinds of membranes (Lange & Ramos; 1983). Homeostatic mechanisms have evolved to maintain the steady-state level of free cholesterol in cells. Parameters that determine steady state levels of cholesterol in cells are influx of cholesterol; efflux (secretion); net formation of membranes (cell proliferation), and de novo synthesis of cholesterol (Gibson & Parker, 1987). All must be controlled. These factors are represented in Figure 5.

The distribution of cholesterol is heterogeneous among membranes in the cell, among the many microdomains within any membrane, and in molecular form (either free, or esterified to fatty acids) (Gibson & Parker, 1987). Indeed, it is well established that the distribution of sterols among the membranes of nucleated cells is nonuniform. Plasma membranes invariably contain a high concentration of sterol (Lange et al, 1980), whereas mitochondria (Schroeder et al, 1976), endoplasmic reticulum (Colbeau et al, 1971) and other organelles (Bosman et al, 1968) have little sterol. Lange and Ramos (1983) examined the distribution of cholesterol in cultured fibroblasts, Chinese hamster

Figure 5: A diagramatic representation of the principal determinants of cholesterol homeostasis in cells. Redrawn from Gibson and Parker, 1987. Boxes numbered 1 to 3 represent sterol and nonsterol feedback signal molecules that are metabolic products of mevalonate. Number 4 is also an isoprene derivative that serves as a feedforward signal permitting cell replication (Gibson & Parker, 1987).


MEMBRANE
TURNOVER
SECRETION
EXOGENOUS
CHOLESTEROL
CELL
CHOLESTEROL
PROLIFERATION
REDUCTASE
SYNTHESIS
1
2
3
4

ovary cells, and isolated rat liver hepatocytes using the enzyme cholesterol oxidase. Most of the cholesterol was oxidized in all three cell types: 94%, 92%, and 80% in fibroblasts, in Chinese hamster ovary cells and in hepatocytes respectively. Given that cholesterol oxidase had access only to the outer surface of the cells and that cholesterol can migrate rapidly across a fixed plasma membrane, these values probably reflect the fraction of cellular cholesterol present in the plasma membrane (Lange & Ramos, 1983). The cholesterol in the plasma membrane is suggested not to be in rapid equilibrium with internal membranes and furthermore, internal organelles' cholesterol content might be overestimated due to subcellular fractions contamination by plasma membrane during their preparation (Lange & Ramos, 1983). Indeed, plasma membrane cholesterol content appears to be dependent upon simple diffusional equilibrium with plasma lipoproteins (Lange et al, 1983). Newly synthesized cholesterol enters the plasma membrane but whether it can enter the internal pool is uncertain primarily due to the fact that this pool is miniscule relative to the overwhelming pool associated with the cell surface. As a result, an active, unidirectional flux mechanism is also advocated (DeGrella & Simoni, 1982). Nevertheless, membrane replacement synthesis, (turnover), although dependent upon cholesterol availability, would not deplete cellular cholesterol. Cholesterol mobilization can be achieved through the binding to one or more kinds of sterol carrier proteins located in the cytosol (Noland et al, 1980; McGuire et al, 1984) and through the event of minute vesicles blebbing off the surfaces of membranes and fusing with other membranes (e.g.: from the cis to the trans sides of the Golgi apparatus) (Rothman, 1980) in an ordered fashion.

Kaplan and Simoni (1985) studied the transport of newly synthesized cholesterol from the RER to the plasma membrane in Chinese hamster ovary cells. They found that the process was dependent upon metabolic energy, however, the maintenance of cholesterol concentration in the plasma membrane was not an energy-requiring process. The cholesterol transport process showed a sharp temperature dependence; it ceases at 15°C, whereas cholesterol synthesis continues (Kaplan & Simoni, 1985). The site of synthesis is the rough endoplasmic reticulum, where newly synthesized cholesterol accumulates in the endoplasmic reticulum and in a low density, lipid rich vesicular fraction, suggestive of the fact that cholesterol may be transported via a vesicular system.

On the other hand, Lange and Steck (1985) suggested that cholesterol biosynthesis is not taken to completion in the endoplasmic reticulum. Digitonin treatment shifted profiles of both plasma membrane and intracellular cholesterol to higher densities. Pretreatment of intact cultured human fibroblasts with cholesterol oxidase abolished the shift of plasma membranes but not the intracellular cholesterol indicating that these two membrane pools are not entirely physically associated. Cholesterol may be concentrated for delivery to the plasma membrane by being synthesized from a sterol precursor such as lanosterol in a discrete but undefined intracellular membrane (Lange & Steck, 1985). Pretreatment of intact cells with cholesterol oxidase has been shown to abolish the digitonin shift of lanosterol but not of intracellular cholesterol suggesting that newly synthesized cholesterol and lanosterol are not in the same membrane (Lange & Muraski, 1987).

Gaylor et al (1987) used isopycnic density gradient centrifugation to study the distribution of several rat liver microsomal enzymes of

cholesterol synthesis and metabolism. All of the enzymes which were assayed in the pathway from lanosterol to cholesterol were distributed in both smooth (SER) and rough endoplasmic reticulum (RER). HMG-CoA reductase was found in both SER and RER but not associated with either plasma membrane or Golgi. Since cholesterol can only be synthesized in the presence of these requisite enzymes, Gaylor et al (1987) concluded that the intracellular site of cholesterol biosynthesis is the endoplasmic reticulum. This finding supported that of Chesterton (1968) and DeGrella and Simoni (1982) who also have demonstrated that newly synthesized cholesterol is located in the endoplasmic reticulum (ER). The enzyme acyl-CoA cholesterol acyltransferase which removes free cholesterol from the membrane by esterification was found only in the RER (Gaylor et al, 1987).

The synthesis of cholesterol in the ER as established by Gaylor et al (1987), coupled with the low cholesterol content of the RER and SER membranes suggested that cholesterol does not accumulate in ER membranes but is rapidly trafficked to its target membranes. Kaplan and Simoni (1985) and Lange and Matthies (1984) have generated evidence that trafficking of cholesterol to the plasma membrane is by vesicular means and perhaps not by membrane free means such as those mediated by nonspecific lipid transfer proteins (Billheimer & Gaylor, 1980).

The observation that the sterolgenic enzymes are located in both SER and RER while acyl-CoA cholesterol acyltransferase is restricted to the RER and is stimulated by ribosome stripping seems to indicate that sterolgenesis is closely associated with membrane protein synthesis,

making it evident that both the lipid as well as the protein aspect of membranes mature together.

Gaylor et al (1987) formulated a hypothesis which suggests a role of sterol in the biogenesis of smooth intracellular membranes and also provides a physiological function for the enzyme acyl-CoA-cholesterol acyltransferase in this process. While the entire ER membrane can carry out cholesterol biosynthesis the RER and SER contain different amounts of cholesterol. In RER cholesterol represents 0-6% of the lipid by weight while in the SER it is 10% (Amar-Costesec et al, 1974; Gaylor et al, 1987). These membranes in electron micrographs are often seen to be continuous. It would seem that the synthesis of cholesterol begins in the RER and continues even in an area of membrane devoid of ribosomes, setting up a gradient of low cholesterol, RNA rich to relatively high cholesterol, RNA poor content. It is suggested that cholesterol is no mere passenger in this transformation to SER but may be a driving force (Gaylor et al, 1987).

Acyl-CoA-cholesterol acyltransferase (ACAT) removes cholesterol from the membrane by esterifying it with a fatty acid. This is done in a manner sensitive to membrane protein biosynthesis. When ribosomes are not bound to the membrane, ACAT is stimulated. From this fact, Gaylor et al (1987) deduced that ACAT might be a protective agent that maintains RER cholesterol content low enough to not interfere with ribosome binding or other RER functions.

When the cell is fully dependent on endogenous synthesis for its cholesterol, regulatory mechanisms most likely keep cholesterolgenesis in tune with membrane protein biosynthesis. However, under conditions in which the RER cholesterol content is elevated perhaps curtailing

membrane protein biosynthesis and imperiling the cell, ACAT may serve as an important balance mechanism of the RER to regulate RER cholesterol content during periods of unregulated cholesterol insertion (Gaylor et al, 1987).

Cholesterol movements via the sterol carrier proteins or microvesicles could reach sites that are important in controlling cholesterol homeostasis. If secretion of cholesterol and/or cell proliferation increase the need for cholesterol then either/or both exogenous cholesterol and cholesterol synthesis must be enhanced to keep the cellular cholesterol level correct. As free cholesterol levels rise in cells the activity of the enzyme acyl-CoA cholesterol acyltransferase increases to divert the free cholesterol into storage. The flux of cholesterol into the cell is diminished by reducing the number of receptors for the cholesterol-rich lipoprotein LDL (low density lipoprotein), by repressing the synthesis of a specific cell surface glycoprotein (Brown et al, 1981; Goldstein, 1983). High free cholesterol levels also diminish the expressed activity of HMG-CoA reductase. All three events act to control cholesterol levels.

The activity of HMG-CoA reductase, which itself is a glycoprotein, is the principal determinant of the rate of cholesterol biosynthesis. It plays a key regulatory role in cholesterol homeostasis (see Figure 5). Uptake of cholesterol-rich LDL by cells in culture by receptor-mediated endocytosis reduces HMG-CoA reductase activity (Brown et al, 1981). The synthesis of HMG-CoA reductase is diminished and its degradation increased (Faust et al, 1982). Addition of mevalonate to cell cultures has the same effect as LDL. When mevalonate content greatly exceeds its normal intracellular concentration cholesterol

synthesis is increased, reductase synthesis is impaired and degradation enhanced (Edwards et al, 1983; Sinensky & Logel, 1983). Oxygenated sterols appear naturally in small amounts as intermediates in cholesterol biosynthesis (Schroepfer, 1981; Kandutsch, 1978). The oxygenated sterol, 25-hydroxycholesterol, brings about a decline in reductase synthesis and an increase in enzyme degradation (Faust et al, 1982; Tanaka et al, 1983). The proliferation of cells in culture is impaired since formation of cholesterol and other mevalonate products is blocked (Brown & Goldstein, 1974). Compactin-resistant cells (compactin is a fungal metabolite and a competitive inhibitor of HMG-CoA reductase) have enormously high HMG-CoA reductase levels (Simoni et al, 1981; Chin et al, 1982; Luskey et al, 1983). The RER in these cells undergoes phenomenonal proliferation ("crystalloid" ER) (Chin et al, 1982; Orci et al, 1984). Adding LDL to these cells causes a reversion back to normal reductase levels and membrane morphology (Orci et al, 1984) suggesting a close correlation exists between the generation of membranes in cell proliferation and cholesterol biosynthesis (Cohen et al, 1982; Fairbanks et al, 1984).

Modulation of existing HMG-CoA reductase in cells is also as important a determinant of the rate of mevalonate production as control of reductase synthesis and degradation. The mass of HMG-CoA reductase and its activity maybe linked coordinately to other enzymes catalyzing controlled steps in lipid biosynthesis including HMG-CoA synthase (Gibson & Parker, 1987), acyl-CoA cholesterol acyltransferase, and 7 α-cholesterol hydroxylase (Scallen & Sanghvi, 1983).

HMG-CoA reductase activity has also been reported to be controlled by changes in enzyme synthesis, degradation, enzyme phosphorylation, or

undefined alterations in the catalytic activity of the enzyme. Sinensky et al (1981), Chin et al (1982), Edwards et al (1983), and Clarke et al (1983) demonstrated directly that effectors of HMG-CoA reductase enzyme activity alter the rates of enzyme synthesis, degradation, and mRNA levels.

A cytosolic protein has been shown to stimulate the activities in vitro of two microsomal enzymes, HMG-CoA reductase and methyl sterol oxidase (Gaylor & Delwiche, 1976; Spence & Gaylor, 1977). The exact role of this cytosolic protein in regulation of the reductase is unknown.

Chang and Limanek (1980) isolated mutants of CHO fibroblasts that have defective regulation of the first four enzymes of cholesterolgenesis; acetoacetyl-CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, and mevalonate kinase. All four enzymes were resistant to inhibition by hydroxysterols. It was suggested that a common cellular factor might regulate these four enzymes in normal cells (Chang & Limanek, 1980).

Cholesterol feeding results in inhibition of acetoacetyl-CoA thiolase and HMG-CoA synthase (Clinkenbeard et al, 1975) and also is known to inhibit HMG-CoA reductase activity (Rodwell et al, 1976).

Chin and Chang (1981) reported evidence for the coordinate expression of HMG-CoA reductase and the LDL receptor. Both activities were low in a mutant CHO cell and both returned to normal in parallel in a spontaneous revertant. Chang et al (1981) proposed that a protein with a rapid rate of turnover was required to mediate the effects of either 25-hydroxycholesterol or LDL on HMG-CoA reductase.

Berry and Chang (1982) characterized a sterol requiring CHO cell mutant, previously isolated by Chang et al (1977). This mutant, 215, was a cholesterol requiring CHO cell auxotroph. This variant was found to be defective in lanosterol demethylation (Berry & Chang, 1982). Furthermore, the biochemical lesion of mutant 215 was localized at the 4 α-methyl sterol oxidase enzyme system (Berry & Chang, 1982). $C^{R}7$ has been shown to be a cholesterol auxotroph (Borgford et al, 1986, Hurta & Burton, 1987).

A part of the cholesterol biosynthetic pathway previously ignored involves the following sequence of reactions: squalene→squalene 2,3 oxide→ lanosterol→cholesterol. This pathway is at present the focus of many investigators. Volpe and Obert (1982) reported the effects of 3 β-(2-diethylaminoethoxy) androst-5-en-17-one (U18666A) on the biosynthesis of cholesterol and ubiquinone in cultured C-6 glial cells. This compound was reported to inhibit cholesterol biosynthesis at the level of desmosterol reductase at low levels and of squalene synthetase at higher levels (Volpe & Obert, 1982).

Studies on the effects of U18666A on human fibroblasts and IEC-6 epithelial cells (Panini et al, 1983; Sexton et al, 1983) confirmed the observation of Volpe and Obert (1982) that acetate incorporation into cholesterol was inhibited with a corresponding increase into ubiquinone. Further studies showed that U18666A caused a remarkable enhancement of incorporation of [^{3}H] acetate into squalene-2,3-epoxide and squalene 2,3:22,23-dioxide. Panini et al (1984) showed that squalene 2,3:22,23-dioxide which accumulated when 2,3-oxidosqualene cyclase was inhibited by U18666A was a powerful inhibitor of HMG-CoA reductase. This inhibition was due to the conversion of squalene diepoxide via

cyclization catalyzed by 2,3-oxidosqualene cyclase to oxygenated derivatives of lanosterol (Panini et al, 1984). These oxygenated derivatives of lanosterol have been shown to be very powerful inhibitors of HMG-CoA reductase activity in IEC-6 cells (Panini et al, 1984).

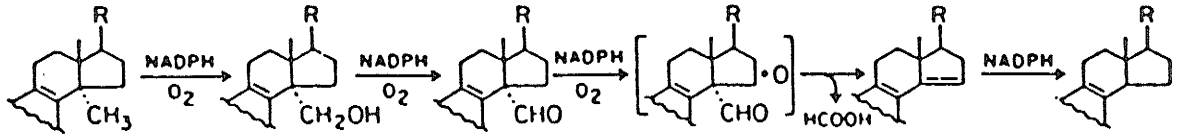
The reaction sequence between lanosterol and cholesterol involves at least 19 different enzyme reactions. Electron transport to the liver microsomal oxidases of cholesterol biosynthesis has been studied for squalene epoxidation (Ono & Bloch, 1975), 4 α-methyl sterol attack (Fukushima et al, 1981) and Δ^7-sterol-5-desaturation (Grinstead & Gaylor, 1982). Electron transport in oxidative demethylation of C-32 of lanosterol (14 α-demethylation) appears to differ from 4 α-demethylation in that 14 α-demethylation has been suggested to be cytochrome P-450 dependent (Gibbons et al, 1979) and genetically distinct (Berry & Chang, 1982) in mammals.

In both 4 α- and 14 α-demethylation sequences, three oxidative attacks appear to be followed by a lyase reaction which in turn is followed analogously by an NADPH-dependent reduction of the demethylated products (Schroepfer, 1982; Trzaskos et al, 1984). Although the steps of 14 α- and 4 α-demethylations are grossly similar, different enzymes and electron carriers appear to be required (Trzaskos et al, 1984). These processes are summarized in Figure 5a. The process of lanosterol demethylation plays a key role in the regulation of C^R_7.

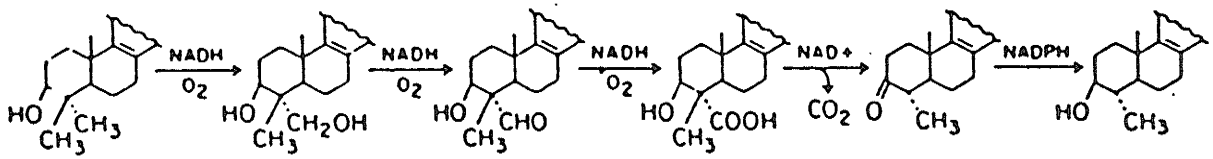
Truly, membrane elaboration requires balanced synthesis of specific protein and lipid molecules and is dependent upon feedback control from metabolic and synthetic factors. Cholesterol, being a key factor in the lipids comprising membranes, may be the most versatile feedback signal

Figure 5a: Microsomal oxidase reactions and electron transport components involved in 14 α- and 4 α-sterol demethylation. 14 α-demethylation is dependent upon a cytochrome P-450-dependent oxidase which results in removal of the 14 α-methyl group as formic acid. 4 α-demthylation is a 5-step reaction sequence involving oxidation by 4-methyl sterol oxidase, decarboxylation by 42-oic acid decarboxylase and reduction by 3-ketosteroid reductase. The 4-methyl sterol oxidase is dependent upon cytochrome b_5 and cytochrome b_5 reductase (Trzaskos et al, 1984).

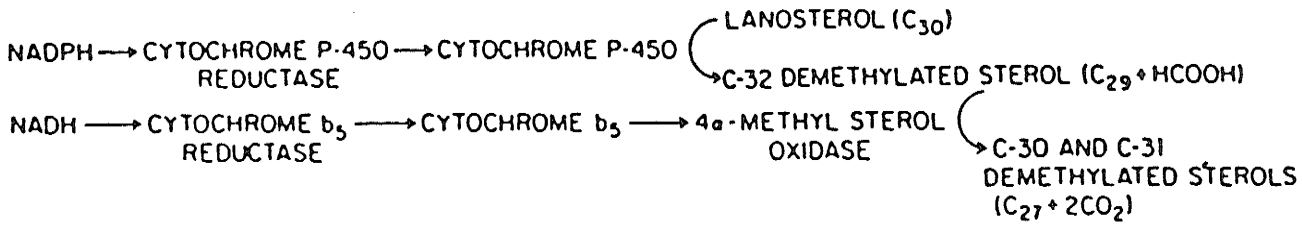
14a – DEMETHYLATION:



4a – DEMETHYLATION:



MICROSOMAL ELECTRON TRANSPORT:



and the most representative indicator of the status of membranes (Gibson and Parker, 1987).

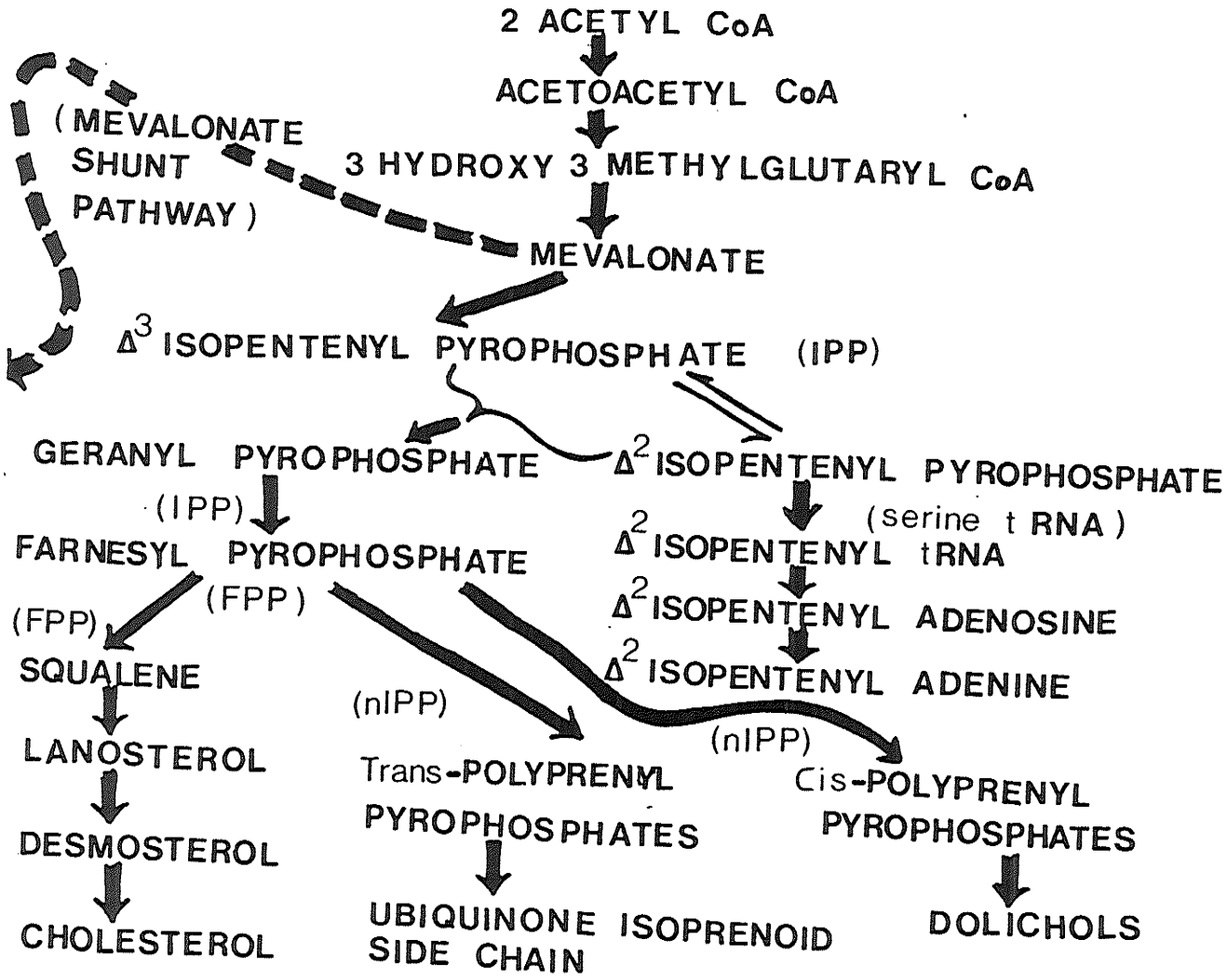
Isoprenoid Biosynthesis

The pathway of isoprenoid biosynthesis is represented in Figure 6. By examining this figure it becomes obvious that mevalonate occupies a central and critical role. Indeed, mevalonate is the precursor of three classes of molecules: (a) products that are needed for cell continuity (cholesterol in membrane formation, ubiquinone for electron transport, dolichol for glycoprotein synthesis, isopentenyl-tRNAs in protein synthesis), (b) metabolic intermediates, both sterols and nonsterols, that provide negative feedback signals that control their rates of formation by limiting the flux through HMG-CoA reductase and (c) a feed-forward, "all-is-ready" signal that permits the cell cycle to proceed (Gibson & Parker, 1987).

Isopentenyl pyrophosphate (IPP) is the basic building block of prenyl derivatives. IPP can be directed into many products in animal cells, such as cholesterol, ubiquinone, dolichol, and isopentenyl tRNA.

Ubiquinone is an important component of the electron transport chain (Crane et al, 1957). The branch point in the pathways of synthesis of cholesterol and the ubiquinone side chain occurs at the level of farnesyl pyrophosphate (FPP). In the sterol pathway, 2 FPP units condense tail to tail to form a symmetrical squalene molecule while the polyprenol pyrophosphates that make up the side chain of ubiquinone are made by the head to tail addition of IPP units in a trans linkage to FPP. Dolichols, on the other hand, are synthesized by the head to tail addition of IPP units in a cis linkage to FPP. Dolichol phosphates and pyrophosphates function as glycosylated intermediates

Figure 6: A schematic representation of the pathway of polyisoprenoid biosynthesis (from Panini et al, 1985) with modification to include the "shunt" pathway (Edmond & Popjak, 1974). The numbers in parentheses refer to the number of carbon atoms in the molecule.


2 ACETYL CoA
ACETOACETYL CoA
3 HYDROXY 3 METHYLGLUTARYL CoA
(MEVALONATE SHUNT PATHWAY)
MEVALONATE
Δ3 ISOPENTENYL PYROPHOSPHATE (IPP)
GERANYL PYROPHOSPHATE
Δ2 ISOPENTENYL PYROPHOSPHATE
(IPP)
FARNESYL PYROPHOSPHATE
(serine t RNA)
Δ2 ISOPENTENYL tRNA
Δ2 ISOPENTENYL ADENOSINE
Δ2 ISOPENTENYL ADENINE
(FPP)
(FPP)
SQUALENE
(nIPP)
(nIPP)
LANOSTEROL
Trans-POLYPRENYL PYROPHOSPHATES
Cis-POLYPRENYL PYROPHOSPHATES
DESMOSTEROL
UBIQUINONE ISOPRENOID SIDE CHAIN
DOLICHOLS
CHOLESTEROL

(Hemming, 1974; Parodi & LeLoir; 1979). Isopentenyl tRNA and its metabolites may have a role in DNA synthesis during the S-phase of the cell cycle (Quesney-Huneeus et al, 1980; Habenicht et al, 1980; Faust et al, 1980). A role in the regulation of HMG-CoA reductase is also postulated (Brown & Goldstein, 1980; Huneeus et al, 1982).

It has been demonstrated that the addition of either mevalonate or cholesterol could reverse the inhibition of cell proliferation caused by oxygenated sterols (Brown & Goldstein, 1974; Kandutsch & Chin, 1977; Cornell et al, 1977). Compactin inhibition of cell growth and proliferation could also be similarly reversed by either cholesterol or mevalonate (Goldstein et al, 1979). However, Kaneko et al (1978) and Brown and Goldstein (1980) observed that even in the presence of cholesterol, added mevalonate was needed to restore proliferation to compactin treated cells. This indicated that other isoprenoid products of mevalonate are probably also required for cell growth.

The production of mevalonic acid, in addition to serving as a precursor for the structural cholesterol requirements for cell growth, might regulate cell proliferation by playing a direct role in DNA replication (Huneeus et al, 1979). This observation is based on the finding that BHK cells synchronized by the double thymidine block procedure manifest a 10-fold increase in the activity of HMG-CoA reductase, the enzyme catalyzing mevalonate synthesis, at or just prior to the S-phase of the cell cycle (Quesney-Huneeus et al, 1983). Subsequently, isopentenyladenine, a known product of mevalonate both in procaryotic and in lower eukaryotic cells is greater than 100 times more effective than mevalonate in restoring DNA synthesis in cells in which HMG-CoA reductase, and also DNA replication is inhibited by compactin

(Quesney-Huneeus et al, 1982). A marked effect of both mevalonate and isopentenyladenine on DNA replication was observed within minutes of their addition to the cell culture suggesting that mevalonate, perhaps through the synthesis of isopentenyladenine or a related compound, zeatin, may play an initiative role in DNA replication (Quesney-Huneeus et al, 1983). Dolichol or ubiquinone were suggested as being unable to substitute for mevalonate in initiating DNA replication (Quesney-Huneeus et al, 1980).

Maltese and Aprille (1985) examined the relation of mevalonate synthesis to mitochondrial ubiquinone content and respiratory function in cultured murine neuroblastoma cells. De novo synthesis of ubiquinone (Coenzyme Q) was blocked with the aid of mevinolin, a competitive inhibitor of HMG-CoA reductase thereby suppressing the synthesis of mevalonate, an essential precursor for the isoprenoid side chain of ubiquinone. Mevalonate synthesis was demonstrated to be important for the maintenance of the intramitochondrial ubiquinone pool in cultured cells. Major changes in the ubiquinone content of the mitochondria can occur in intact cells without perturbation of respiratory function (Maltese & Aprille, 1985). However, the coincidence of decreased mitochondrial ubiquinone concentration and the inhibition of cell cycling observed in mevinolin-treated cells suggested that the availability of ubiquinone may play a role in the regulation of mitochondrial and cellular proliferation (Maltese, 1984).

James and Kandutsch (1979) examined the interrelationships between dolichol and sterol synthesis in mammalian cell cultures. Mills and Adamany (1978) demonstrated that 25-hydroxycholesterol inhibited the incorporation of acetate into dolichylpyrophosphoryl oligosaccharides

and cholesterol by similar amounts thereby suggesting that HMG-CoA reductase is the rate-limiting enzyme for the biosynthesis of both dolichol and sterol. When 25-hydroxycholesterol was added to mouse L cell cultures, [^{14}C] acetate incorporation into both dolichol and sterol decreased in a concentration dependent manner (James & Kandutsch, 1979). Comparison of the rates of sterol and dolichol synthesis from acetate indicated that situations may exist in which large fluctuations in sterol synthesis can occur while the rate of dolichol synthesis is only slightly affected. However, under conditions of substantially decreased sterol synthesis (less than 25% of control) further decrease in sterol synthesis was accompanied by a proportional decrease in dolichol synthesis (James & Kandutsch, 1979). HMG-CoA reductase was suggested to affect the rate of dolichol synthesis by altering the concentration of a substrate for an enzyme which catalyzes a rate-limiting reaction peculiar to the dolichol branch of the biosynthetic pathway. This suggests an interaction can exist by which two or more biosynthetic pathways can share common intermediates and a common regulatory enzyme while maintaining a large degree of regulatory independence (James & Kandutsch, 1979).

The relationship of dolichol synthesis to glycoprotein synthesis has also been examined. In embryonic development, dolichyl phosphate appears to control the rate of protein glycosylation since when dolichyl phosphate synthesis is inhibited by compactin, the result is an impairment of protein glycosylation (Carson & Lennarz, 1981). A significant fraction of the oligosaccharide chains synthesized during compactin treatment and transferred to protein appear to be altered so that they are more negatively charged (Carson & Lennarz, 1981).

Synthesis of more highly charged carbohydrate chains may be an attempt to compensate for the altered surface characteristics resulting from the production of underglycosylated proteins.

The influence of dolichol, dolichol esters, and dolichyl phosphate on phospholipid polymorphism and fluidity in model membranes has been examined. In biological membranes, the phospholipids are organized in a bilayer configuration (Singer & Nicholson, 1972). By using various physical methods, the influence of dolichols on lipid polymorphism and thermotropic behavior of various phospholipids in model membranes was examined. Dolichols of various chain length, polyprenols, dolichol esters, and dolichyl phosphate influence polymorphism and thermotropic behavior of phospholipids, at concentrations comparable to those found in biological membranes (Valtersson et al, 1985). The effects noted were polyprene and phospholipid type specific (Valtersson et al, 1985). The biological relevance of these effects was also examined.
Transiently formed nonbilayer lipid structures have been proposed to be intermediates in transbilayer transport of lipids and membrane fusion (Verkleij, 1984). Dolichyl phosphate can act as a carrier of sugars across the RER and this could be caused by different orientations of dolichyl phosphate in the membrane or alternatively, it could be due to the local induction of nonbilayer structures by dolichyl phosphate in the membrane (Vatersson et al, 1985). Valtersson et al (1985) suggested that possible formation of clusters of dolichol occurs in membrane bilayers at biological concentrations depending on the type of dolichol and the physical state of membranes. Furthermore, various dolichol derivatives may segregate into different pools in a membrane thereby

having structural, functional, and possibly metabolic consequences (Valtersson et al, 1985).

Watson et al (1985) investigated the nature of isoprenoid synthesis in isolated embryonic Drosophila cells (Kc cells). Mevalonate and its analogues, as well as compactin and mevinolin, modulated HMG-CoA reductase activity because they altered isoprenoid carbon flow to a sterol independent, post-isopentenyl-1-pyrophosphate regulatory signal molecule (Watson et al, 1985). Kc cells were found to shunt a significant fraction (>40%) of their post IPP-carbon to prenols for oxidative catabolism. This shunted mevalonate carbon may play a significant role in the mevalonate-mediated regulation of Kc cell HMG-CoA reductase function (Havel et al, 1986).

Much speculation and investigation has occurred regarding the identity of the nonsterol isoprenoid product(s) of mevalonate that is (are) involved in both the regulation of cell division and of HMG-CoA reductase. A coordinated regulation of cell growth and isoprenoid synthesis has been suggested (Chen, 1981; Sexton et al, 1982; Panini et al, 1982). It has not been established whether both functions are carried out by a single substance or whether several may be involved.

The search for the active nonsterol regulatory product of mevalonate metabolism undertook a new perspective from the investigation of Schmidt et al (1984). When cultured Swiss 3T3 cells in the presence of mevinolin were labeled with radioactive mevalonic acid, 40-50% of the radioactive products formed were not lipid soluble and behaved like protein. Radioactivity was associated with only a few proteins with apparent molecular weights of 13,000 to 58,000. It appeared that mevalonate was first converted to an isoprenoid compound and then was

covalently incorporated into proteins via a cycloheximide-insensitive mechanism (Schmidt et al, 1984). It was suggested that these cells formed novel metabolic products of mevalonic acid by post-translational incorporation of isoprenoids into specific cell proteins (Schmidt et al, 1984).

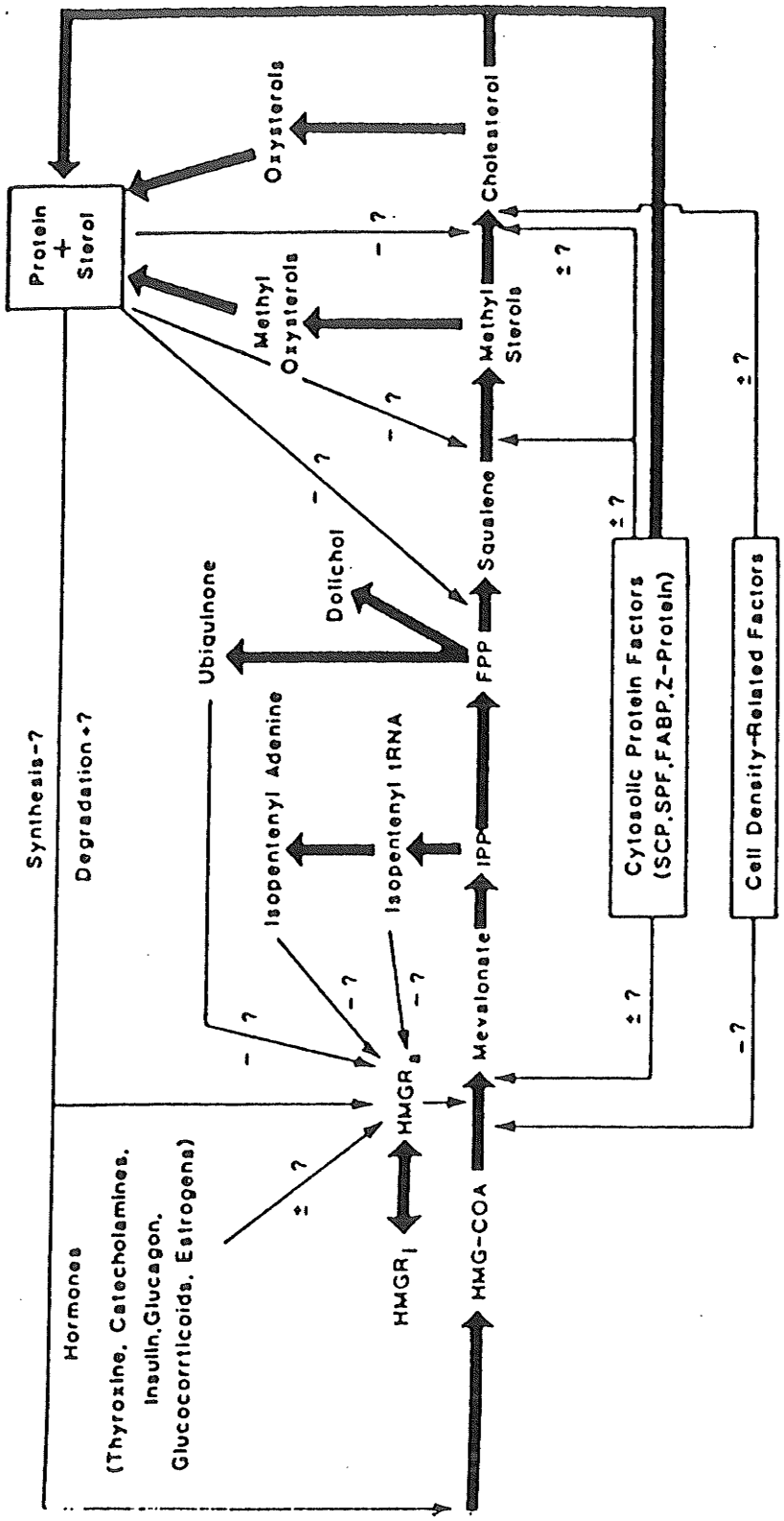
The current understanding of the factors regulating isoprenoid synthesis is summarized in Figure 7. While HMG-CoA reductase occupies a primary regulatory role, other steps in the pathway are also susceptible to regulation. By examination of Figure 7, one gains a better insight into the nature of, as well as, the complexity and intricacy of the factors which control and regulate isoprenoid biosynthesis.

Temperature-Sensitive Mammalian Cell Variants

Since C^R7 exhibits obvious temperature sensitive growth (Ceri & Wright, 1977), it is necessary to consider temperature-sensitive mutations in animal cells.

All organisms have an optimum temperature for growth and proliferation and this varies widely from species to species. A temperature-sensitive mutation can be defined as a mutation that imposes a restriction on the temperature of growth or normal function in an organism which in the wild type state does not exhibit such a restriction (Basilico, 1977). The most common temperature-sensitive mutations are heat sensitive: that is the defect is expressed only at high temperatures. A temperature-sensitive mutant is defined by two sets of conditions. A low, permissive temperature at which growth or other cell function is normal, and a high nonpermissive temperature at which either growth or a specific cell function is defective. The wild type cell behaves normally under both sets of conditions.

Figure 7: A schematic representation of the factors regulating isoprenoid biosynthesis according to Panini, Rodgers and Rudney (1985).


Protein + Sterol
Oxysterols
Cholesterol
Methyl Oxysterols
Methyl Sterols
Squalene
Dolichol
Ubiquinone
FPP
Isopentenyl Adenine
Isopentenyl tRNA
IPP
Mevalonate
HMGR$_a$
HMGR$_i$
HMG-CoA
Hormones
(Thyroxine, Catecholamines, Insulin, Glucagon, Glucocorticoids, Estrogens)
Synthesis-?
Degradation-?
Cytosolic Protein Factors (SCP, SPF, FABP, Z-Protein)
Cell Density-Related Factors
± ?
- ?
- ?
- ?
- ?
- ?
± ?
± ?
± ?
- ?
± ?

Temperature-sensitive mutations that restrict growth at low, but not at high temperature are also known. They are generally called cold sensitive mutants. Six cold-sensitive variants of Chinese hamster cells have been isolated (Farber & Unrau, 1975). One of these mutants is well characterized and shows a rapid cessation of DNA and protein synthesis upon shift from the permissive temperature (39°C) to the nonpermissive temperature (33°C). The defect of this mutant does not appear to involve ribosomal subunits assembly (Farber & Unrau, 1975).

The defect in temperature-sensitive mutants can be attributed to a single amino acid substitution in a protein (Drake, 1970); also temperature-sensitive mutants are believed to be missense mutations (Basilico, 1977). Although this has not been conclusively demonstrated for animal cell mutants there appears to be no compelling reason to doubt this assumption.

Temperature-sensitive mutations are useful vehicles for a variety of studies since they belong to the class of conditional lethal mutations, that is, mutations expressed only under conditions that the investigator can control. Thus, this type of mutation can be used to study essential functions, whose defectiveness would be lethal in a nonconditional state. They have been shown to map all over the genome of organisms studied and therefore can be used to study a variety of cell functions (Edgar et al, 1964). Finally, the transition from the permissive to the nonpermissive condition (and vice versa) can easily be accomplished by manipulating the temperature of incubation thereby permitting a direct internal control of the experiments conducted. This would allow, if desired, a sensitive and precise way of testing the involvement of a specific gene-product in determining a given phenotype.

Generally, two types of temperature-sensitive cell mutations can be distinguished. The first type is in a function that is essential for growth and cell division. Such mutants are incapable of growth at the nonpermissive temperature. The second type of mutation affects specialized or salvage functions. The temperature-sensitive mutation has no effect on growth at the high temperature, but, will inhibit or alter the expression of one or more specialized functions, resulting in a deviant but not necessarily lethal phenotype.

What follows is a brief survey of some of the kinds of temperature-sensitive mammalian cell variants. It is by no means all inclusive, and is intended to be only representative of the types and the nature of temperature-sensitive mutations investigated.

The first temperature-sensitive mutants of animal cells were isolated from BSC-1 monkey cells (Naha, 1969) and from the L line of mouse cells (Thompson et al, 1970). Well characterized temperature-sensitive mutants of animal cells isolated appear to have a defect in protein biosynthesis. One of these variants, is tsH1, a Chinese hamster ovary (CHO) cell, which ceases protein synthesis within hours of shift up to the nonpermissive temperature with concomitant polysome disaggregation (Thompson et al, 1973). This temperature-sensitive phenotype appears to be due to an inability of these cells to charge tRNA with leucine due to an ineffective leucyl-tRNA synthetase enzyme (Thompson et al, 1975; Stanners & Thompson, 1974). A related mutant, ts14, exhibits a reduced rate of protein synthesis at 39°C accompanied by a decrease in DNA synthesis. The main defect of this variant seems to be in the structure or function of the 60S ribosomal subunits at 39°C (Thompson et al, 1975).

Schroder and Hsie (1975) isolated a temperature-sensitive CHO cell variant unable to grow like the wild type at 39.5°C in minimal medium unless supplemented with hypoxanthine, thymidine, and glycine. Through further studies, an inability to form tetrahydrofolate at the nonpermissive temperature was noted (Schroder & Hsie, 1975).

A thermosensitive line (TS 111) was isolated from a suspension culture of Chinese hamster fibroblasts with a block in cytokinesis manifested at 39°C (Hatzfield and Buttin, 1975). In this cell variant, DNA and protein syntheses are not arrested but keep on at a steady rate. Giant cells form which accumulate either numerous nuclei or one big nucleus with several nucleoli and more than a hundred chromosomes (Hatzfield & Buttin, 1975).

Chinese hamster cells resistant to 6-thioguanine (a purine analogue) were isolated which exhibited temperature-sensitive growth at 39°C (Fenwick & Caskey, 1975). The specific activity of hypoxanthine-guanine phosphoribosyltransferase is at least 10 times higher in these variant cells grown at 33°C than those grown at 39°C. A missense mutation in the structural gene of this enzyme was postulated (Fenwick & Caskey, 1975).

Nigam and Brailovsky (1978) characterized a temperature-sensitive hamster cell variant, ts3, which did not proliferate at 39°C. This variant possessed a normal phenotype relative to the wild type, except ts3 possessed elevated amounts of a 250,000 daltons glycopeptide and a 200,000 daltons polypeptide, presumed to be myosin. No other alterations of major significance were observed (Nigam & Brailovsky, 1978).

A number of mammalian temperature-sensitive mutants with ostensible cell cycle specific lesions have been described. Most of these mutants are arrested in the G1 phase of the cell cycle at nonpermissive temperatures (Basilico, 1978; Cooper, 1980). Cells arrested with a G1 content of DNA are common; however, cells arrested in S or G2 are relatively rare (Melero, 1979; Ozer, 1978). Analysis of these mutants lead to the observation that the temperature-sensitive lesions affect G1 functions (Pringle, 1978; Levine, 1978). The physiology of different temperature-sensitive mutants supports the notion that different steps in G1 are affected by different mutations (Cooper, 1980). For example, the lag prior to the resumption of DNA synthesis after cells are replaced at the permissive temperature is different in different mutants (Naha et al, 1975). The longer the lag period that occurred implied that the affected function occurs "earlier" in G1. Furthermore, different mutants have different execution points (Pringle, 1978). If a cell is at a point prior to the execution point then raising the temperature will prevent DNA synthesis (Pringle, 1978). If the cell is affected at a point after the execution point, a temperature rise will not affect DNA synthesis (Pringle, 1978). However, delays in DNA synthesis might also be due to delays prior to resumption of protein synthesis (Cooper, 1980).

A mouse temperature-sensitive mutant, ts85, unable to grow at 39°C, was found to be DNA synthesis and chromosome condensation defective at this temperature (Yasuda et al, 1981). Through further investigation, this incomplete DNA replication and the defect in chromosome condensation were ascribed to a decrease in H1 histone phosphorylation (Yasuda et al, 1981).

Protein glycosylation mutants in the mouse mammary carcinoma cell line, FM3A, were selected for the ability to withstand exposure to radioactively labeled mannose at 39°C. G258 is one of these mutants which has been characterized. G258 is temperature-sensitive for cell growth (Nishikawa, 1984). The formation of lipid-linked oligosaccharides was severely inhibited in G258 cells at 39°C and G258 cells were only able to synthesize the smaller lipid-linked oligosaccharides (up to $Man_3GlcNAc_2$-PP-Dol) but were incapable of synthesizing the larger lipid-linked oligosaccharides (Nishikawa, 1984).

Ubiquitin, which is a 76 residue protein which possibly functions as a signal for attack by proteinases specific for ubiquitin-protein conjugates, occurs in eucaryotic cells either free or covalently joined to a variety of protein species. The mouse cell line, ts85, previously characterized as a cell-cycle mutant, is also temperature-sensitive in ubiquitin-protein conjugation (Finley et al, 1984). Furthermore, this effect is due to the specific thermolability of the ts85 ubiquitin-activating enzyme, E1 (Ciechanover et al, 1984). As a consequence, not only abnormal proteins, but also normal, short-lived proteins fail to be degraded efficiently in ts85 cells at the nonpermissive temperature (Finley et al, 1984).

A novel type of CHO mutant was isolated with a constitutive defect in the synthesis of lipid-linked oligosaccharides and was designated AS15-1 (Nakano & Akamatsu, 1985). AS15-1 was found to incorporate 30-fold less glucosamine into an oligosaccharide lipid fraction than the wild type; to synthesize a small amount of $Man_5GlcNAc_2$-lipid, and showed temperature sensitivity for both growth and adhesion to substratum (Nakano & Akamatsu, 1985). The constitutive defect in

oligosaccharide-lipid synthesis of the mutant AS15-1 is also responsible for all of its pleiotropic phenotypes including anomalous protein secretion. Some altered glycoproteins are perhaps unstable at the elevated temperature thereby rendering the mutant cells defective for growth and adhesion (Nakano & Akamatsu, 1985). The observation that AS15-1 is anomalous in protein secretion suggests an important role of oligosaccharide chains in secretion. In this regard, a yeast mutant, alg4-9, which was isolated as a variant with a defect in oligosaccharide-lipid synthesis (Huffaker & Robbins, 1983) has been shown to be allelic to the variant, sec 53-6, which is a temperature-sensitive mutant defective in protein synthesis (Ferro-Novick et al, 1984).

Roff et al (1986) isolated three independent CHO mutants (B3853, I223, and M311) with temperature-sensitive, pleiotropic defects in receptor-mediated endocytosis. The three mutants displayed a number of altered properties including decreased sialylation of secreted glycoproteins at 41°C and at 39°C (Roff et al, 1986). These phenotypic alterations are expressed at 41°C within 30 minutes (Roff et al, 1986).

Another mutant, tsFT20, was characterized at the cellular level and was found to lose its ability to synthesize DNA immediately after a shift to a nonpermissive temperature (Murakami et al, 1986). At 39°C, tsFT20 cells lost their colony-forming ability in one doubling time (16 hours) (Murakami et al, 1986). The cells could grow at 37°C but not above 38°C; a temperature shift (33°C to 39°C) showed that the whole S phase was temperature-sensitive, whereas G2 and M phases were not (Murakami et al, 1986). Further characterization of this murine mutant, tsFT20, revealed that it contained a heat-labile DNA polymerase

α-activity, and confirmed the notion that DNA polymerase α plays a key role in DNA replication in mammalian cells (Murakami et al, 1986).

Timchak et al (1986) described a thermosensitive lesion in a Chinese hamster cell mutant, termed G71. This temperature-sensitive, conditional-lethal lesion caused differential effects on the acidification of endosomes and lysosomes (Timchak et al, 1986). Further physiological properties of G71 were examined and it was shown that its temperature-sensitive lesion specifically affected vacuolar acidification (Marnell et al, 1986).

And finally, the regulation of polypeptide chain initiation in a CHO temperature-sensitive mutant, tsH1 was examined and shown to be defective (Clemens et al, 1987). When tsH1 cultures were shifted from 34°C to 39.5°C (nonpermissive temperature) protein synthesis was inhibited by greater than 80% due principally to a block in activity of polypeptide chain initiation factor eIF-2 (Clemens et al, 1987). An impairment of the ability of the guanine nucleotide exchange factor (GEF) was noted. The primary lesion in tsH1 cells was in their temperature-sensitive leucyl-tRNA synthetase. It was suggested that a role for eIF-2 phosphorylation and GEF activity in coupling the rate of polypeptide chain initiation to the activity of the chain elongation machinery existed (Clemens et al, 1987).

Lectins and Lectin-Resistant Variant Animal Cells

Lectin-resistant variants, particularly of Chinese hamster ovary (CHO) cells, have been isolated in many laboratories and have been invaluable in the studies of oligosaccharide and glycoprotein biosynthesis (Briles, 1982; Stanley, 1984). These variants display altered sensitivity to a variety of lectins as a result of a wide range

of abnormalities in glycoprotein biosynthesis and assembly. Mutants, such as these, are useful tools in the elucidation of biochemical pathways and processes. Animal cell mutants of this type have an advantage over other approaches in that these mutants provide stable expression of an altered phenotype. Since the first lectin-resistant animal cell mutants were initially reported (Culp & Black, 1972), studies on animal cell mutants have provided useful and novel insights into the regulation and control of biochemical processes and pathways critical for normal cellular metabolism and ultimately, mammalian cellular survival and maintenance.

Lectins (from the Latin, legere, meaning to pick or choose) are non-immunoglobulin proteins which by definition, possess an ability to bind carbohydrate structures. A number of lectins, each possessing its own unique set of properties, have been extensively studied and characterized. Lectins may elicit physiological responses, or may be mitogenic, or cytotoxic or even possess both mitogenic and cytotoxic effects depending on the concentration of lectin utilized. Indeed, lectins play a role in blood group specificity; selective agglutination of transformed cells; transient agglutination of untransformed cells, as well as a postulated involvement in tumorigenicity (Brown & Hunt, 1978).

The ability of a given lectin to bind carbohydrate structures and the affinity of a particular lectin towards an individual mammalian cell will depend not only on the nature of the cell surface carbohydrate but also on the inherent specificity of the individual lectin. As binding is a necessary condition for lectin cytotoxicity to occur, cells may be rendered resistant to a cytotoxic lectin by changes in cell surface structures, thereby, inhibiting lectin binding. Furthermore, a

"mutation" or change may structurally alter or even eliminate the required target oligosaccharide. Alternatively, membrane surfaces or associated structures may be modified in such a way as to mask, obscure or make unavailable the desired target.

A most interesting aspect of lectins, sparsely investigated, is the precise nature of the biological functions lectins may perform in the systems from which they are originally isolated. Many possible functions have been suggested, however, the foremost of these is acting as a protectant (Boyd et al, 1958).

A considerable amount of indirect evidence exists in support of a "mutational event" as a mechanism by which somatic cell variants are created. It should be noted that to date no direct definitive evidence of a specific alteration in the genetic material capable of accounting for lectin-resistant phenotypes exists. However, indirect evidence in support of the "mutational" hypothesis includes the following observations: stable variant phenotypes have been isolated and maintained in culture over many generations; selection of variants may be enhanced by the use of mutagens in the selection procedures; stable revertant phenotypes have been isolated from variant populations (Siminovitch, 1976; Wright et al, 1980). Regardless of the exact basis for lectin resistance, stable variant and revertant populations of somatic cells exist, apparently by virtue of specific inactivations and activations of the genome (Stanley, 1984; Borgford, 1984).

The CHO cells used in this study respond to the cytotoxic effects of concanavalin-A. The wild type (WT) and revertant (Rc) or (RC^R7) are sensitive to the cytotoxic effects of concanavalin-A whereas the mutant CHO cell, C^R7, is resistant to these effects.

Concanavalin-A (Con-A) was initially described in the toxic extracts of the Jack Bean (Canavalia einsformis) by Assman (1911); being first purified by Sumner and Howell (1935, 1936). Many lectins have been chemically purified, however, relatively few have been subjected to detailed structural analysis. Con-A, however, is by far the best studied lectin. Several reviews regarding Con-A structure and physical characterization exist (see Brown & Hunt, 1978).

Con-A binds a number of simple sugars and shows a high affinity for α-D-mannose residues containing unmodified hydroxyl groups at the C-3, C-4, and C-6 positions (Poretz & Goldstein, 1970). Con-A probably does not bind to mammalian membrane glycolipids (Yogeeswaran et al, 1974) since mannose is not a normal constituent of this component of animal cells. Furthermore, N-acetyl glucosamine and glucose, which Con-A binds with a less affinity than mannose do not appear to be present in the correct anomeric forms in mammalian membrane glycolipids (Goldstein & Hayes, 1978).

Con-A resistant variants exhibit a complex cellular phenotype. Investigations performed with various Con-A resistant and sensitive cell lines show that a direct correlation exists between the Con-A resistance property and the altered phenotype (Wright, 1979).

The Con-A resistant CHO cell line, designated $C^{R}7$, the focus of this study, displays a complex cellular phenotype characterized by multiple alterations in membrane-associated properties. Included in these properties are obvious temperature sensitive growth, altered cellular morphology, increased sensitivity to certain membrane-active agents, altered lectin agglutination properties, modified adhesiveness to substratum properties, and defective lectin-receptor mobility

characteristics (Ceri & Wright, 1978a). Other aberrant properties include differences in binding mechanism and amount of lectin bound per cell surface area at 4°C and altered surface glycoproteins as detected by lactoperoxidase iodination, galactose oxidase and metabolic labelling procedures (Ceri & Wright, 1978a).

Due to the nature of Con-A and its specificity for α-D-mannose residues, resistant variants might be expected to be defective in a mechanism for transferring mannose to oligosaccharide chains (Wright et al, 1980). Investigations with Con-A resistant hamster cell lines have shown that membrane preparations from such independently selected resistant lines incorporate less GDP-[^{14}C] mannose into lipid-monosaccharide, lipid-oligosaccharide and protein fractions from parental wild type cells (Krag, 1979; Krag et al, 1977; Wright et al, 1978, 1979). Furthermore, Con-A resistant mouse lymphoma cells have been shown to be defective in the biosynthesis of the oligosaccharide-lipid intermediates required for the assembly of the core region of asparagine linked oligosaccharide chains (Trowbridge et al, 1979).

Wright et al (1978, 1979) suggested that, in $C^{R}7$, a pathway for converting mannose to fucose existed. Furthermore, this particular pathway is suggested to be of greater importance in Con-A resistant cells than in WT or in Rc Con-A sensitive cell lines. In addition to this, various glycosidases (enzymes responsible for catalyzing the removal of carbohydrate residues from glycoproteins) have been examined in these CHO cells. It has been shown that significant differences in the activities of the glycosidases, which are glycoproteins themselves,

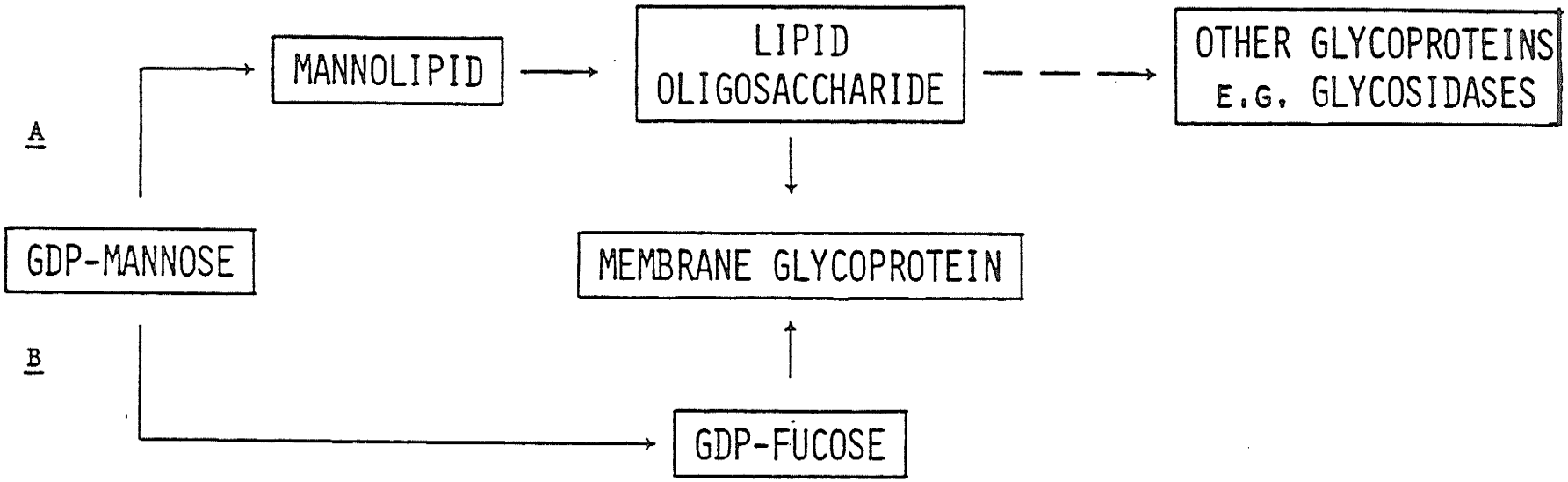
exist amongst the WT, RC^R7 and C^R7 CHO cell lines (Blaschuk et al, 1980a, 1980b).

Wright et al (1980) suggested that a single biochemical change affecting the synthesis of a particular class of glycoproteins exists thereby resulting in surface membrane component deficiencies or modifications. As a corollary of this, Wright et al (1980) suggest that the activities of glycoprotein enzymes (Blaschuk et al, 1980b) would also have wide ranging effects upon numerous biological properties of the lectin resistant cell. Figure 8 is a summary of the above mentioned differences in WT, RC^R7 and C^R7 CHO cells. Finally, the suggestion that C^R7's highly pleiotropic phenotype presumably results from a single biochemical defect is supported by the fact that a revertant cell line, in which all the altered properties reverted in concert, has been isolated from the variant C^R7 population. The locus of this defect is unknown, but it is clear that C^R7 and the closely related CHO cell variant B211 are defective in the assembly of dolichol-linked intermediates of glycoprotein biosynthesis (Cifone et al, 1979; Krag, 1979; Wright et al, 1979). C^R7 and B211 are both probably Lec 5 in the classification of Stanley (1984).

Lectins, as mentioned earlier may be used directly to isolate lectin-resistant (Lec^R) mutants, the majority of which turn out to be lectin receptor mutants expressing altered carbohydrates at the cell surface, as well as, presumably glycosylation-defective animal cell mutants (Stanley, 1984).

Animal cells contain three major classes of carbohydrate containing molecules, these being: glycoproteins, glycolipids and proteoglycans. In immortalized cells growing in tissue culture, glycoproteins probably

Figure 8: Summary of the biochemical analysis and characterization of a fundamental defect in $C^{R}7$, a Con-A resistant CHO cell (Wright & Ceri, 1977b; Wright et al, 1980).



A Functions normally in WT and RC^{R}-7; altered in C^{R}-7.

B Functions normally in WT but enhanced in C^{R}-7 at expense of pathway A.

represent the majority of the glycosylated molecules carrying carbohydrates attached to certain serine, threonine or asparagine residues of the protein portion of the molecule.

Almost all animal cell glycosylation mutants investigated to date are affected in the biosynthesis of asparagine (Asn) linked carbohydrates (Stanley, 1984).

Figures 9 and 10 illustrate oligosaccharide-dolichol biosynthesis and processing and terminal glycosylation pathways respectively. The figures also show the postulated or proven defects in a number of lectin resistant animal cell variants classified according to the Lec^R scheme of Stanley (1984). Mutants at all stages of Asn-linked carbohydrate biosynthesis have been isolated, some with lesions where inhibition of biosynthesis of the mature oligosaccharide occurs; some with lesions affecting processing reactions and others with lesions with alteration in the terminal glycosylation patterns (Stanley, 1984). These defects represent the known spectrum and complexity of carbohydrate biosynthetic pathways. Loss or acquisition of glycosyltransferase or glycosidase activities; loss of enzyme activities needed for the formation of activated sugars; loss of a sugar modifying enzyme and potential intracellular compartmentalization defects are included within this spectrum (Stanley, 1984). The schematic diagram of Asn-linked carbohydrate biosynthesis given in Figures 9 and 10 illustrates the sites at which many glycosylation mutations have been located, however, they by no means represent all the possibilities by which glycosylation mutants might arise.

C^R7, as well as B211 are probably Lec 5 variants. Possession of an altered Lec^R phenotype is the hallmark of the glycosylation mutant

Figure 9: Oligosaccharide-dolichol biosynthesis. Also indicated are the reactions at which different animal cell glycosylation mutants are blocked indicated by // and a number (Stanley, 1984).

Figure Legend:	Enzymic Change:
1	↑ UDPGn:Dol-P-Gn transferase
2	Tunicamycin-resistant UDPGn:Dol-P-Gn
3	↓ Dol-P-Man synthetase
4	Unknown
5	↓ GlcNAc-TI
6,7	Unknown

(↑= an increase; ↓= a decrease)

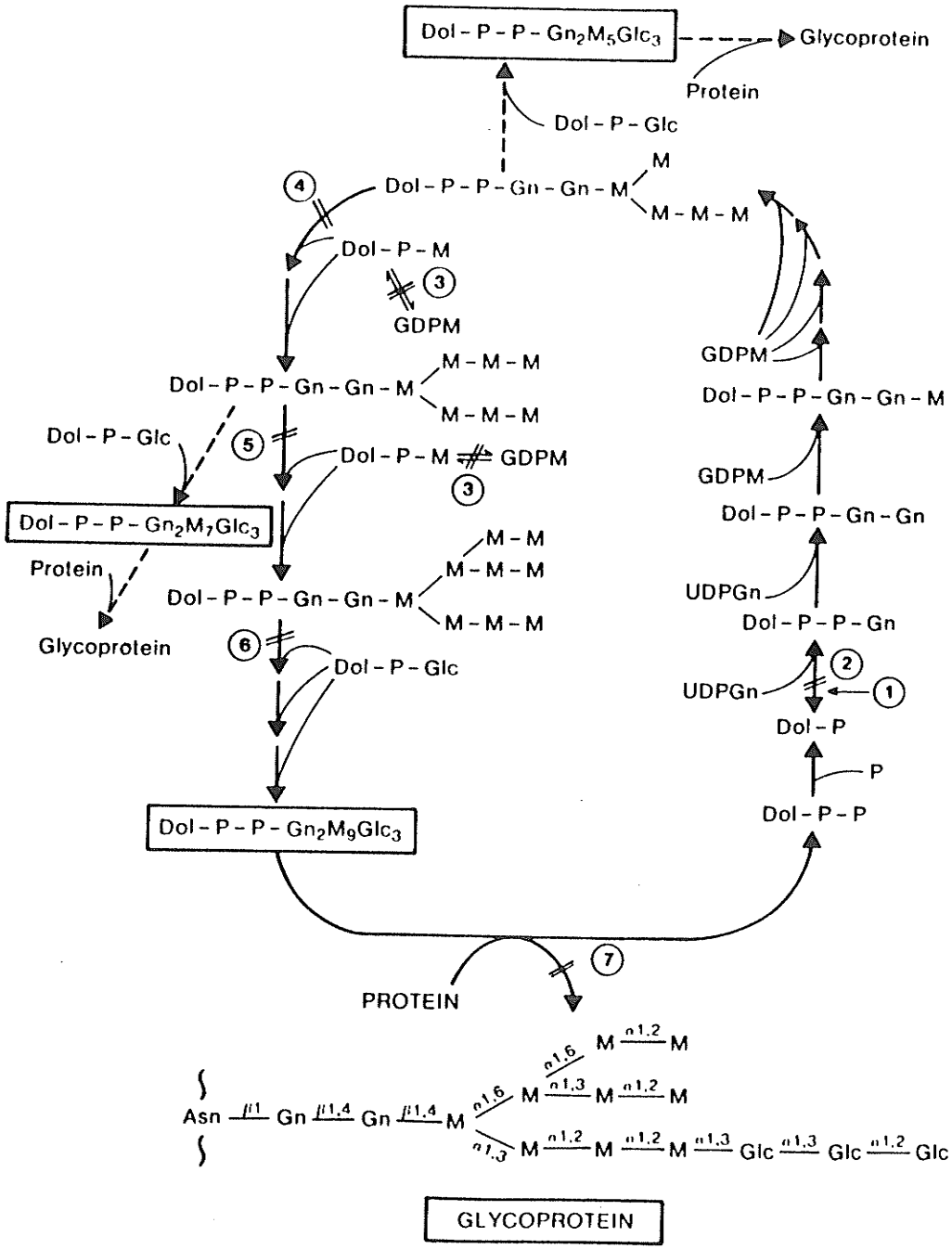
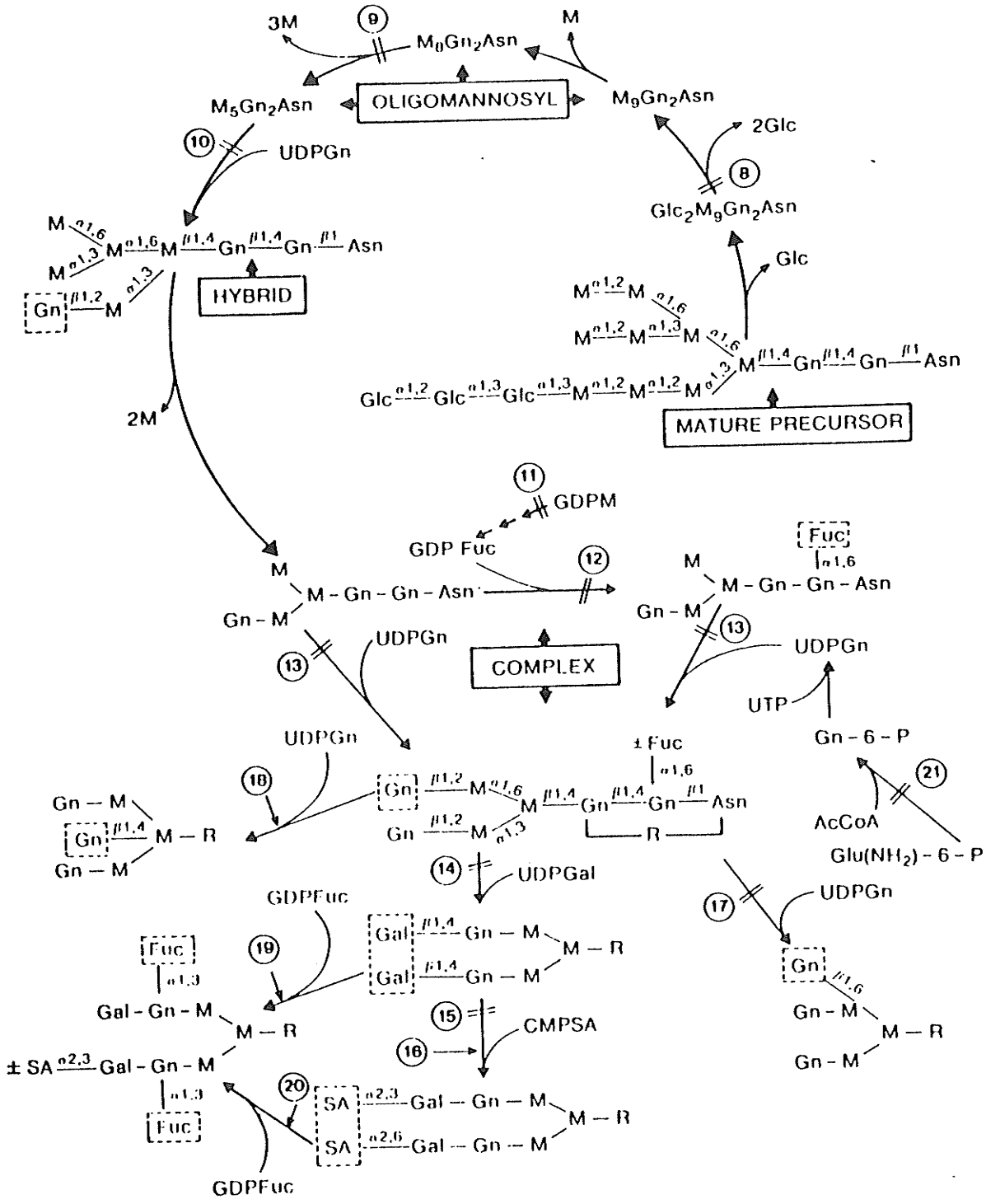

Dol-P-P-Gn2M5Glc3
Glycoprotein
Protein
Dol-P-Glc
Dol-P-P-Gn-Gn-M
M
M-M-M
4
Dol-P-M
3
GDPM
Dol-P-P-Gn-Gn-M
M-M-M
M-M-M
Dol-P-Glc
5
Dol-P-M
GDPM
3
Dol-P-P-Gn2M7Glc3
Protein
Glycoprotein
Dol-P-P-Gn-Gn-M
M-M
M-M-M
M-M-M
6
Dol-P-Glc
Dol-P-P-Gn2M9Glc3
PROTEIN
7
GDPM
Dol-P-P-Gn-Gn-M
GDPM
Dol-P-P-Gn-Gn
UDPGn
Dol-P-P-Gn
UDPGn
2
1
Dol-P
P
Dol-P-P
Asn
β1
Gn
β1,4
Gn
β1,4
M
α1,6
α1,3
α1,6
α1,3
α1,2
α1,2
α1,2
α1,3
α1,3
α1,2
Glc
Glc
GLYCOPROTEIN

Figure 10: Processing and terminal glycosylation. Also indicated are the reactions at which different animal cell glycosylation mutants are blocked as indicated by //, while those that exhibit increased or de novo expression of enzyme activity are denoted by an arrow (Stanley, 1984).

Figure Legend:	Enzymic Change:
8	↓ α glucosidase II
9	Unknown
10	↓ Glc-NAc-TI
11	↓ GDP-Man 4, 6,-dihydratase
12	Unknown
13	Unknown
14	Unknown
15	Unknown
16	Unknown
17	↓ β (1,6) GlcNAc transferase
18	↑ GlcNAc-TIII
19	↑ α (1,3) Fuc-T; α (1,3) Fuc-TII
20	↑ α (1,3) Fuc TI
21	↓ GlcNAc-6-P acetyl transferase

(↑ = an increase; ↓ = a decrease)


3M
9
M
M8Gn2Asn
M9Gn2Asn
M5Gn2Asn
OLIGOMANNOSYL
10
UDPGn
2Glc
8
Glc2M9Gn2Asn
Glc
HYBRID
MATURE PRECURSOR
2M
11
GDPM
GDP Fuc
12
Fuc
M - Gn - Gn - Asn
Gn - M
13
UDPGn
COMPLEX
UTP
Gn - 6 - P
18
UDPGn
± Fuc
21
AcCoA
Glu(NH2) - 6 - P
14
UDPGal
17
UDPGn
GDPFuc
19
15
CMPSA
16
20
GDPFuc
M - R
Asn
Gal - Gn - M
SA
Gal
Gn
Fuc
R

(Stanley, 1984). CHO mutants exhibit a dramatic change in their sensitivity to the toxicity of at least one, and often many lectins of different carbohydrate binding specificities. Lec 5 mutants are such mutants, and so is C^R7 (Ceri, 1978). C^R7 variants exhibit a modest 2- to 3-fold resistance to Con-A (Ceri, 1978) and a cross resistance to other lectins directed towards complex oligosaccharide structures have also been noted in some of these mutants (Ceri, 1978).

The pleiotropically altered phenotype of C^R7 is similar to that expressed by independently selected Con-A resistant CHO cell mutants as described by Briles (1982). The most striking similarity between the various mutants is their characteristic temperature sensitivity. As might be anticipated, glycosylation mutations in animal cells might induce a conditional-lethal phenotype. Three distinct classes of Lec^R mutants are temperature sensitive for growth at 40°C (Stanley, 1984). Each of these mutants is affected in the biosynthesis or transfer of the oligosaccharide-lipid precursor to Asn-linked carbohydrates, more specifically, increased M_5Gn_2-P-P-Dol intermediate with normal Dol-P-Man synthetase activity; defective addition of glucose to M_9Gn_2-P-P-Dol and slow transfer of oligosaccharide to protein, respectively (Cifone et al, 1979; Krag, 1979; Krag et al, 1982; Wright et al, 1979; Tenner & Scheffler, 1979; Tenner et al, 1977; Stanley, 1984). However, other mutants affected in oligosaccharide-lipid biosynthesis (see Figure 9) do not exhibit temperature sensitive phenotypes. Temperature revertants of CHO mutants Lec 5 and Lec 9 have been selected in a single step. C^R7 was selected by a single step process (Ceri, 1978) while the revertant, RC^R7, arose spontaneously from the mutant population (Ceri, 1978). Like RC^R7, Lec 5 and Lec 9 aberrant properties all reverted in concert

(Stanley, 1984), suggesting that the temperature sensitive phenotypes of these mutants are a direct result of their respective glycosylation lesions (Stanley, 1984).

With respect to B211, Krag (1979) has demonstrated what may be the primary defect in this Con-A resistant variant, previously isolated and described by Cifone et al (1979). B211 cells showed an inability to glucosylate oligosaccharide-lipid. Deficient glucosylation in membrane preparations of this mutant seemed to be related to a reduction in the availability of dolichyl phosphate. Defective enzyme activity was also apparent. The addition of exogenous dolichyl-phosphate could enhance, to some degree, glucosylation. It was postulated that the reduced availability of the lipid substrate, in vitro was a result of an apparent accumulation of glucosylphosphoryldolichol in the dolichol cycle.

Stanley (1984) has suggested that specific lesions affecting terminal glycosylation reactions would be more likely to produce a characteristic phenotype (and by virtue of that fact, the specific defect more readily delineated). On the other hand, Stanley (1984) suggested that it might be more difficult to predict the changes in mature carbohydrate structures stemming from lesions occurring prior to the terminal glycosylation reaction(s). Lec 5 CHO cell variants represent such lesions.

Lec 5 CHO cells exhibit a markedly reduced ability to glucosylate the oligomannosyldolichol intermediate Dol-P-P-Gn_2Man_9 (Stanley, 1984). This lesion would be expected to reduce the rate of transfer of the oligomannosyl moiety to protein (Spiro & Spiro, 1982). However, once transferred, it is assumed that processing and terminal glycosylation

might be expected to occur normally. However, Lec 5 cells do not possess mature carbohydrates of normal structure since they are resistant to a number of lectins of different binding specificities. This is also true for $C^{R}7$.

Indeed, the Lec 5 phenotype is extremely complex. So much so that this complexity has led Stanley to speculate that the Lec 5 primary lesion may be a structural membrane defect that affects the function of a variety of membrane-associated glycosylation enzymes (Stanley, 1984).

METHODS AND MATERIALS

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Cells and Culture Conditions

This study employed three cell lines previously isolated and characterized (Wright, 1975; Wright & Ceri, 1977b; Ceri & Wright, 1977b). They were a wild "CO_2" type Chinese hamster ovary (CHO) cell, WT; a concanavalin A resistant mutant selected from the WT population, C^R7; and a spontaneous revertant selected from the mutant population, RC^R7, or Rc. All three cell lines have been maintained in culture continuously for a period of several years. During the course of this investigation, all cell cultures were incubated and maintained at the non-physiological temperature of 34°C. Cell cultures were also incubated at 37°C and 39°C when dictated by the type and nature of the investigation undertaken.

Cell cultures were isolated and maintained essentially as previously described (Wright, 1975; Borgford, 1984). Stock cultures were grown and maintained in alpha modification of Eagle's medium with Earle's salts and glutamine without ribosides and deoxyribosides without sodium bicarbonate (α-MEM) (Flow Laboratories). This medium was supplemented with 100 units/ml of penicillin G (Sigma) and 100 μg/ml of streptomycin sulphate (Sigma) and 2.2 mg/ml of sodium bicarbonate (Fisher Scientific Co.). Prior to use this medium was further supplemented with 10% fetal calf serum (FCS) (Gibco). This growth medium was referred to as FCS-M.

Cell lines were routinely grown on 16 oz. Brockway bottles (Brockway Glass Co. Inc.) containing 30 ml of FCS-M as stock cultures.

Growth curves, killing curves, enzyme assays and incorporation studies were conducted with cultures grown on 60 x 15 mm or 100 x 15 mm Lux tissue culture dishes (Miles Scientific) as indicated. Futhermore, unless otherwise indicated 60 x 15 mm plates received 5 ml of medium and 100 x 15 mm plates received 10 ml of medium.

Cells were removed from bottles and plates by incubation with 0.04% EDTA in Dulbecco's phosphate buffered saline (PBS) for 1 to 3 minutes. Cells were quantitated with a Coulter particle counter (Coulter Electronics Ltd.) after suitable dilution of aliquots into isotonic saline (0.15 M).

Cell lines were periodically tested for Mycoplasma contamination by routine plating techniques (Crawford, 1968) on agar plates in Hayflick's medium.

Cells were stored at -40 to -70°C as "back-up" cultures. Such cells were stored in α-MEM supplemented with 5% dimethylsulfoxide, (DMSO), Grade 1, (Sigma). Such cells were routinely reintroduced into investigations when necessary, however, only after they had been appropriately characterized as to their growth properties and response to culture in the presence of concanavalin-A.

Lipoprotein Deficient Serum

Lipoprotein-deficient serum was prepared according to the method of Brown and Goldstein (1974) essentially as previously described (Borgford, 1984). Lipoprotein-deficient serum was prepared by adjusting the specific gravity of fetal calf serum to 1.125 g/ml by the addition of potassium bromide. Approximately 88 g of KBr was added to 275 ml serum and the specific gravity determined by hydrometry. The "adjusted"

serum was distributed into 37.5 ml polyallomer centrifuge tubes (Beckman) and centrifuged in a 60 Ti rotor for either 60 hours at 175,000 x g or for 48 hours at 225,000 x g. Following centrifugation, the bottoms of the tubes were gently punctured, and the lower two-thirds of the tube was removed. This lipoprotein-deficient serum was adjusted to 275 ml with saline. The lipoprotein-deficient serum, (LPDS) was filter sterilized with a Nalgene filter unit (115 ml capacity) fitted with a 0.45 μm filter. Growth medium containing α-MEM supplemented with 10% LPDS was referred to as LPDS-M.

Preparation of Delipidized Serum

The total delipidization of fetal calf serum (FCS) was done according to the method of Rothblatt (1976). One hundred ml of FCS was treated with 175 ml each of ice cold acetone and ethanol and then was stirred for approximately 4 hours on an ice bath. Following this, the acetone-ethanol-FCS mixture was filtered on a large Buchner funnel with care such that the cake did not dry out and crack. The cake was washed with 1 litre of precooled ether in three equal lots and then sucked as dry as was possible. Following this, the cake was transfered to a large piece of filter paper, chopped up into small pieces and dried in a vacuum desiccator. It was necessary to remove the drying protein from the desiccator periodically in order to rechop it so that all the ether was properly removed. After all the ether was removed (no detectable ether odour), the protein was reconstituted to the original volume with distilled water and then filter sterilized and stored at 4°C until required. Growth medium containing α-MEM supplemented with this delipidized serum was referred to as DELIP-M.

Cell Viability

On occasion, cell viability was measured by the trypan blue dye exclusion test (Phillips, 1973) essentially as previously described (Ceri, 1978). 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 phosphate buffered saline (PBS) at pH 7.2. The viability test involved pelleting cells after three 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 5-6 minutes after cell dispersion.

Killing Curves

Killing curves were performed to determine the sensitivity of a cell population to a drug, namely concanavalin-A. The method used was as essentially described by Ceri (1978) and subsequently by Borgford (1984).

Killing curves were conducted on 100 x 15 mm Lux tissue culture dishes containing the desired concentration of concanavalin-A in 10 ml of FCS-M. Concanavalin-A (Con-A) medium was routinely prepared by the method of Wright (1973). Concanavalin-A was added to FCS-M to a final concentration of 100 μg/ml concanavalin-A. This medium was incubated at 34°C overnight and the resulting precipitate was removed by centrifugation at 10,000 x g for 40 minutes in a Sorvall RC2-B centrifuge. Wright (1973) has previously shown that when such medium was incubated at 37°C overnight less than 5% concanavalin-A is lost in

this precipitate at Con-A concentrations less than 60 μg/ml.

In such a study, each point was derived from duplicate plates. Plates were inoculated with either 300 cells per plate or 1,000 cells per plate and subsequently incubated at 34°C until colonies were visible in controls. Control plates contained no Con-A. Following a 7 to 10 day incubation period, the medium was removed and replaced with a 1:1 solution of water and ethanol containing a saturating concentration of methylene blue. After 10-20 minutes, this solution was removed and the plates were gently washed with lukewarm water. Stained colonies were enumerated using a Colony Counter (Scientifica and Cook Electronics, Ltd.).

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. In this study, the relative plating efficiency (RPE) was defined as the plating efficiency in the presence of a concentration of the drug divided by the plating efficiency in the absence of the drug. Plating efficiency was defined as the fraction of cells in a population which would give rise to colonies when plated on a solid growth surface which were made visible on staining with methylene blue.

Growth Curves

In general, growth curves were obtained by delivering an inoculum of approximately 10,000 exponentially growing cells to a series of 60 x 15 mm. Lux tissue culture dishes, containing 5 ml of FCS-M. The plates were incubated at 34°C for a period of 24 hours in this medium to allow the cells to recover. After this incubation period, a cell count was

determined and designated the "zero" time point. Simultaneously, the growth medium was changed on the remaining culture dishes to fresh FCS-M, LPDS-M, FCS-M or LPDS-M plus supplements. Lipids or other supplements were added to the cultures as complexes with bovine serum albumin (5% solution) or as ethanolic solutions. The final concentration of ethanol employed was always less than 1%. When appropriate, controls were done where the "vehicle of addition" was the only material added to the cultures. After the appropriate manipulations were completed, the cultures were returned to the desired incubation temperature either 34°C or 37°C or to 39°C, the nonpermissive temperature for growth of the mutant, $C^{R}7$.

Quantitation generally involved the removal of the entire culture with 2 ml of 0.04% EDTA-PBS solution, dilution into 38 ml of 0.15 M NaCl solution, followed by enumeration in a Coulter particle counter. Cultures were enumerated, at a minimum, three times.

Determination of Protein

Protein was determined essentially as previously described by Borgford (1984). The method used was that of Markwell et al, (1981), a modification of the method described by Lowry et al, (1951). This method was chosen as it is suited for the determination of protein in water insoluble systems, such as crude homogenates of tissue and lipoprotein fractions. Standard curves in the range of 0 to 100 μg were prepared in duplicate for each determination with "Fat Free" bovine serum albumin (BSA) (Sigma). In general, unknown samples were done in duplicate as well. Spectrophotometric measurements were performed either in a Gilford SP2400 spectrophotometer or in a Unicam SP500 Series

2 UV-Visible spectrophotometer at 660 nm.

Determination of Radioactivity

During the course of this study, radioactivity was measured in samples combined with 10 ml of Scintiverse I scintillation fluid (Fisher Scientific Co.) and counted in 20 ml glass vials. Radioactivity was evaluated in an LKB Wallac 1215 Rackbeta scintillation counter.

Preparation of Total Lipid Extracts

Cells were grown in 25 (16 oz.) Brockway bottles which contained 25 ml FCS-M. Cells were seeded at 10,000 cells per bottle and grown at 34°C for 24 hours. They were then transferred to a 37°C incubator or left at 34°C and grown until growth reached subconfluence. Cells were also grown at 39°C for period of 48 and 96 hours.

Cells were harvested with 10 ml of 0.04% EDTA in PBS per Brockway bottle, and immediately placed on ice. Subsequently, cells were pelleted by a 5 minute centrifugation in a Model CL International Clinical Centrifuge. EDTA solution was discarded and the resulting cell pellet was washed twice with ice cold PBS, and the washed cell pellets were pooled and then resuspended in 1 ml PBS.

Total lipid extracts were prepared according to the methods of Bligh and Dyer (1959) as described by Kates (1972). Pooled and washed cells suspended in 1 ml of PBS were extracted with 3.75 ml methanol-chloroform (2:1 v/v) in a 12 ml Pyrex 13, No. 8124 glass stoppered centrifuge tube overnight with intermittent shaking. The tubes were centrifuged at 1,200 x g for 20-25 minutes. The supernatant was then transferred to another glass stoppered test tube (15 ml

capacity). The cell residue was then re-extracted with 4.75 ml of methanol-chloroform-H_2O (2:1:0.8 v/v/v) for 20 minutes with intermittent shaking and then centrifuged for 15-20 minutes at 1,200 x g. Supernatant was removed and combined with the previous supernatant. These combined supernatant extracts were diluted with 2.5 ml chloroform and 2.5 ml water, mixed and centrifuged as before. The lower chloroform layer was withdrawn, diluted with an equal volume of benzene, and then brought to dryness under a stream of nitrogen gas at room temperature. The resulting residue was dissolved in 0.5 ml chloroform and stored in the freezer until used.

Cholesterol Determination

Cholesterol determinations were conducted on total lipid extracts of cells prepared by the method of Bligh and Dyer (1959). Cholesterol content was determined by an enzymatic assay (Ott et al, 1982). The reagents for the enzymatic determination of cholesterol were obtained from Sigma (Total Cholesterol Diagnostics Kit). The Sigma procedure involves use of two enzymes namely cholesterol oxidase and cholesterol esterase coupled with phenol and 4-aminoantipyrine chromogenic system described by Trinder (1969). Cholesterol esters are first hydrolyzed to free cholesterol and fatty acids by cholesterol esterase. The free cholesterol is then oxidized by cholesterol oxidase to cholest-4-en-3-one, with simultaneous production of hydrogen peroxide. The latter reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a quinoneimine dye, which has a maximum absorbance at 500 nm. The amount of colour produced is directly proportional to the concentration of total cholesterol in the sample. Quantitation

involved the comparison of sample determinations to standard curves prepared using authentic cholesterol (Sigma).

Phospholipid Determination

Phospholipid determinations were conducted on total lipids extracts of cells prepared by the method of Bligh and Dyer (1959).

Phospholipid content was assayed according to the method of Raheja et al, (1973). Quantitation involved the comparison of sample determinations to a standard curve prepared with dipalmityl phosphatidyl choline (Sigma).

Determination of Relative Percent Composition of Phospholipids

The relative percent contribution of individual phospholipids to the total phospholipid composition of the total lipid extracts of cells prepared by the method of Bligh and Dyer (1959) was determined.

Total lipid extracts were chromatographed on Redi-Plate Silica Gel G 20 x 20 cm plates (Fisher Scientific Co.) using a solvent system consisting of chloroform-methanol-acetic acid-water (25:15:4:2 v/v/v/v). Suitable standards, (phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidic acid and sphingomyelin), were also chromatographed in order to qualitatively identify the individual phospholipids. Phospholipids were identified using the Zinzadze reagent (Vaskovsky & Svetashev, 1972). Phospholipids appeared as blue spots. Such Zinzadze reagent positive spots were scraped off the plates, and the phospholipids extracted using 1 ml methanol-chloroform (2:1 v/v) and lipid phosphorus was assayed as described previously.

HMG-CoA Reductase Assay

The activity of the enzyme 3-hydroxy-3 methylglutaryl coenzyme A (HMG-CoA) reductase was determined essentially as previously described (Borgford, 1984). Tissue was homogenized as described elsewhere (Chang et al, 1981a) and the assay method employed was that of Chang et al, (1981b).

Reductase assays were conducted on monolayers growing in 100 x 15 mm Lux tissue culture dishes. Subconfluent cultures of cells were washed 3 times with 10 ml of ice cold PBS, twice with 5 ml of ice cold hypotonic Buffer K (1 mM Tris-HCl, 1 mM EGTA, and 1 mM $MgCl_2$) and then incubated 2-3 minutes with 5 ml of Buffer K at room temperature. This buffer was then removed from the culture dishes and a fresh 0.25 ml of ice cold Buffer K was added. Immediately cells were lysed and homogenates collected by rapidly scraping the culture dishes with a rubber policeman. Homogenates were transferred to Eppendorf test tubes on ice. Aliquots were taken for protein determination.

One hundred µl volumes of homogenates were preincubated with 25 µl of Buffer A (100 ml imidazole, 25 mM dithiothreitol, pH 7.4) for 10 minutes at 37°C. Following this, a 40 µl aliquot of this preincubation mixture was withdrawn and combined with 40 µl of the assay cocktail. Final concentrations in this mixture in 80 µl were: 0.245 µmol $NADP^+$; 10.4 nmol D,L-[3-^{14}C] HMG-CoA (90,000-100,000 dpm); 1.78 µmol glucose-6-phosphate; 0.073 units of glucose-6-phosphate dehydrogenase; 2.84 µmol potassium phosphate, pH 7.4; 0.64 µmol dithiothreitol; 4 µmol of potassium chloride; 1.78 µmol EDTA; and 0.8 µmol imidazole, pH 7.4. The assay was performed by incubating the reaction mixture for 60

minutes at 37°C in a shaking water bath. Following this incubation period, the reactions were terminated by the addition of a 10 µl aliquot of concentrated HCl.

Incubation of the reaction tubes overnight at 4°C allowed the mevalonate product to become lactonized. Ten µl of RS-[5-^{3}H(N)] mevalonolactone standard (usually 10,000 dpm) was added to each assay tube, the tubes were mixed using a Vortex mixer, and subsequently centrifuged for 5 minutes at 12,500 rpm in a Beckman Microfuge centrifuge at 4°C. A 40 µl aliquot was then spotted near the base of a 2.5 cm x 20 cm channel on a silica gel G thin layer chromatography (TLC) plate. Likewise, a non-radioactive mevalonolactone standard was spotted opposite assay spots in a separate channel. The TLC plates were developed in benzene/acetone (1:1). Following chromatography, the plates were allowed to air-dry in the fumehood and then the mevalonolactone spots were identified with iodine vapour. Sections corresponding in position to the non-radioactive mevalonolactone standard were scraped from the plates and their radioactivity was determined. The recovery of ^{14}C radioactivity was corrected for by the recovery of ^{3}H radioactivity. Specific activity was expressed as the quantity of product per unit of time per unit of protein (generally pmoles/minute/µg protein).

Saponification

Saponification was conducted on samples which generally contained 1 to 25 mg of tissue protein. The procedure used was essentially as described by James and Kandutsch (1979) and as previously described by Borgford (1984). Internal standards where appropriate were first added

followed by 1 ml of ethanolic KOH (final concentration 20% KOH, 66% ethanol) containing 40 µg pyrogallol. Samples were then incubated for 3 hours at 80°C in 6" screw-capped test tubes with teflon liners. At the end of this time 0.6 ml of water was added and non-saponifiable lipids were extracted with 2 x 10 ml of diethyl ether. The extract was then washed with 2 x 2 ml of 0.14 M NaCl, 2 ml of 5% sodium carbonate, 2 ml of 5% acetic acid and finally with 2 ml of sodium carbonate.

Non-saponifiable extracts were then dried under a stream of nitrogen, resuspended in an appropriate solvent and used or stored as required.

Fatty Acids

Fatty acids were prepared essentially as previously described by Borgford (1984). Fatty acids were isolated by the acidification of saponified mixtures (described previously) following the extraction of the non-saponifiable lipids. This was accomplished by the addition of 0.3 ml of 50% sulfuric acid to the extract mixture (pH was checked with pH paper). Fatty acids were then extracted with 2 x 10 ml of petroleum ether and washed with 2 x 2 ml of distilled water. Samples were dried under a stream of nitrogen and processed further as required.

Preparation of Fatty Acid Methyl Esters

Methyl esters of the total lipid extracts (or isolated fatty acids) were prepared according to the method of Nichols et al, (1965). By this method, methyl esters of both free and esterified fatty acids in the total lipid extract are prepared.

One hundred microliters of each extract was placed in suitable

vials and brought to dryness under nitrogen gas at room temperature. To these "dried" samples, 3 ml of methanol-benzene-sulfuric acid (150:75:5 v/v/v) was added. Samples were then refluxed in 6" screw capped test tubes with teflon liners for 2 hours in a 85-87°C water bath.

Following the refluxing period, the fatty acid methyl esters (FAME) were extracted with 3 x 2 ml hexane. The combined extracts were then washed with 3 x 2 ml of water. Samples were dried under a stream of nitrogen and resuspended in an appropriate solvent.

Gas Chromatography

Gas chromatography of FAME was performed on a Hewlett-Packard 5720 gas chromatograph fitted with a flame ionization detector. The column used was ¼" x 6' copper tubing packed with Silar 10C on 100/120 Chromosorb WAW (Applied Sciences). Determinations were made with a carrier gas (N_2) flow rate of approximately 40 ml/minute on a column maintained isothermally at 190°.

Fatty acid methyl esters were identified by comparison of retention times of authentic FAME standards (Applied Sciences standard H105, K103, and L203). Where the determination of relative amounts was required, integration of peak height was done manually and the contribution (% composition) of individual components of the FAME profile to the total FAME profile was calculated.

Digitonin Precipitation of Labeled Sterols

An inoculum of 60,000 cells was delivered to a series of 100 x 15 mm Lux tissue culture dishes containing 10 ml of FCS-M. Cells were grown at 34°C for 4 days. Following this growth period cells were

stressed by an exposure to 39°C for a period of 24 hours, or left at 34°C. Prior to this growth period, 10 ml of fresh FCS-M or LPDS-M was added to the culture dishes. Following this, such cells were exposed to [1-^{14}C] acetate (10 μCi/ml, 2.8 mCi/ml) for a period of 2 hours.

Following this exposure time, the medium was removed and the cells washed with 2 x 10 ml cold PBS. Cells were then removed with 1 N NaOH. (Total volume used was 0.5 ml NaOH and 50 μl of this was withdrawn to determine protein.)

Digitonin precipitates were prepared from the non-saponifiable lipid fraction of tissue. Prior to saponification 10,000 dpm of [7(n)-^{3}H] cholesterol (μCi/m mole) as internal standard and approximately 1 mg of carrier cholesterol were added. Samples were then saponified as described previously.

Non-saponifiable lipid samples were then dissolved in 1 ml of acetone-ethanol (1:1 v/v) containing 1 drop per 5 ml of 10% acetic acid. To this 0.4 ml of digitonin (0.5% in 50% ethanol) was added. Samples were then incubated at 4°C overnight.

These samples were then centrifuged at approximately 1,000 x g for 10 minutes at 4°C. The supernatant was carefully removed and the precipitate, the "digitonide", was washed with 2 x 2 ml of ice cold acetone-ether (1:2 v/v) containing 1 drop per 10 ml of 10% acetic acid. Mixtures were again centrifuged as described above and the pellets were washed with 2 ml of diethyl ether.

Following a final centrifugation of 1,000 x g for 20 minutes at 4°C, the resulting pellets were dried and resuspended in 0.5 ml of chloroform plus 0.1 ml of methanol. The resuspended pellets were then

transferred to counting vials and the tubes were washed with another volume of solvents. After the solvents had evaporated, 10 ml of scintillation fluid was added to the vials and the radioactivity was determined. Recoveries of ^{14}C-label were corrected by for the recovery of ^{3}H-internal standard.

Incorporation of Acetate into Saturated and Unsaturated Fatty Acids

An inoculum of 60,000 cells was delivered to a series of 100 x 15 mm Lux tissue culture dishes containing 10 ml of FCS-M. Cells were grown at 34°C for 3-4 days. Following this growth period cells were stressed by an exposure at 39°C for a period of 24 hours, or left at 34°C. Prior to this growth period, 10 ml of fresh FCS-M or LPDS-M was added to the culture dishes. Following this, such cells were exposed to [1-^{14}C] acetate (10 μCi/ml, 2.8 mCi/ml) for a period of 2 hours.

Following this exposure time, the medium was removed and cells washed with 2 x 10 ml cold PBS. Cells were then removed with 1 N NaOH. (Total volume used was 0.5 ml NaOH and 50 μl of this was withdrawn to determine protein.) The remaining sample was saponified, extracted and fatty acid methyl esters were prepared as previously described.

Fatty acids of varying degrees of saturation were separated by thin layer chromatography (TLC) on 5% $AgNO_3$ impregnated TLC plates using a solvent system of petroleum ether-diethyl ether (95:5 v/v) as described by Kates (1972). Following chromatography, appropriate sections were scraped from the plates and radioactivity was determined. Fatty acids were identified using parallel channels containing authentic fatty acid methyl ester standards (Applied Sciences standard H105).

Quantitation of Dolichol Levels

Dolichol was extracted from packed and washed cells using the method of Adair and Keller (1985) with minor modifications. Approximately 80-120 16-oz. Brockway bottles of both WT and $C^{R}7$ cells respectively were grown in order to gather approximately 1 g of tissue (wet weight). This was done for each of the desired conditions (see Results).

To the approximately 1 g tissue (wet weight) in a 6" screw capped test tube 1 ml 60% KOH, 2 ml 0.25% pyrogallol in methanol and 6 x 10^5 dpm of[^{3}H] dolichol were added.

[^{3}H] dolichol was previously prepared and purified in the laboratory according to the method of Keenan and Kruczek (1975). The purity of the product was subsequently re-evaluated via high pressure (performance) liquid chromatography (HPLC) prior to use in this study.

The tubes were sealed with Teflon-lined plastic caps and were placed in a boiling water bath for 2-2.5 hours, during which time complete dissolution of the tissue occured. Following incubation the samples were cooled to room temperature and then extracted with 3 x 3 ml petroleum ether.

The extracts (total volume about 9 ml) were pooled in another 6" glass tube fitted with a Teflon lined cap and then washed with 2 x 5 ml 5% acetic acid. After centrifugation for 5-10 minutes at 1,000 rpm, the upper phase was removed and taken to dryness.

The residue was dissolved in 2 ml of chloroform-methanol (2:1 v/v) and then was applied to a DEAE-cellulose column (2-3 ml packed resin in a 10 ml disposable polypropylene syringe fitted with a glass wool plug

to retain the resin). The column allows all dolichol, cholesterol and other neutral lipids to pass through while retaining dolichyl-phosphate.

The collected drop-through was treated with 0.25 volumes of 0.88% KCl, vortexed, and centrifuged. The lower phase was withdrawn, evaporated to dryness under a gentle stream of nitrogen gas and dissolved in 2 ml methanol.

The sample was then applied to a C_{18} Sep-Pak column (Waters Associates) equilibrated in methanol. The bulk of the sterols pass through the column unretarded while dolichol is retained (Wong and Lennarz, 1982). The column was washed with 10 ml methanol. Dolichol was then eluted in good yield with 10 ml HPLC grade reagent alcohol (Fisher Scientific). (Reagent alcohol is a mixture of 90% ethanol, 5% methanol and 5% isopropyl alcohol.) This alcohol fraction was then taken to dryness in preparation of the sample for HPLC analysis, and was either stored dissolved in a suitable solvent (0.5 ml) or redissolved in 0.2 ml of HPLC-grade isopropanol-methanol (90:10 v/v) (Fisher Scientific).

Dolichol content was quantitated by chromatographing standards and samples using an LKB-HPLC system employing a Hibar Pre-Packed 250-4 LiChrosorb RP-18 (5 μM) column. The solvent system which was run isocratically was isopropanol-methanol (90:10 v/v) with a flow rate of 0.70 ml/minute. The model 2140 rapid spectral detector was interfaced with an IBM-PC and the data was analyzed using Wavescan (LKB) and the series 3000 chromatography data system software (Nelson Analytical Inc.). Isoprenologue distribution was also examined and the relative contribution of each isoprenologue to the total dolichol content profile

was examined.

Incorporation of ^{14}C-Labeled Mevalonate into Sterols

The incorporation of ^{14}C-labeled mevalonate into sterols was determined essentially as described by Sexton et al, (1983). Inocula of 50,000 cells of WT, Rc, and $C^{R}7$ were each plated onto 4 individual 60 x 15 mm Lux tissue culture dishes containing 5 ml of FCS-M. After 4 days growth in FCS-M, medium was removed and replaced with either fresh FCS-M or LPDS-M as appropriate. Following a further 18 hours of incubation, the medium was again removed and replaced with the appropriate medium plus 5.9 μCi of R-[2-^{14}C] mevalonolactone (53 mCi/mmole) (Amersham). Cells were left at 34°C or transferred to 39°C for a period of 24 hours with one exception. ($C^{R}7$ was also stressed at 39°C for 36 hours, however exposure time to the label was 24 hours.)

After these incubations monolayers were washed with 3 x 5 ml of ice cold PBS. One ml of 0.1 N NaOH was then added to one of the two duplicate plates and after the monolayer had dissolved, the 1 ml was transferred to the duplicate which also contained 1 ml of 0.1 N NaOH. This plate was then scraped and the combined digests were transferred to glass tubes fitted with screw top Teflon sealed caps. The plates were each washed with 0.5 ml of 0.1 N NaOH. These washes were combined with the other 2 ml of digest. The total cell digest volume was 3 ml. From this total digest, a 0.2 ml aliquot was withdrawn for protein determination.

To the remaining digest in the tubes, pyrogallol (100 mg), ubiquinone (250 μg), cholesterol (250 μg), squalene (100 μg), lanosterol (100 μg) and 50 μl of ^{3}H-cholesterol as internal standard (approximately

25,000 dpm) were added. Then 3 ml of KOH (30% KOH in 95% methanol) was added to each tube and the tubes were heated for 2 hours at 80°C and then allowed to cool to room temperature. The saponification mixture was then diluted with 3 ml of water and subsequently extracted with 3 x 3 ml of petroleum ether. The combined petroleum ether extracts were washed with 2 x 2 ml water and subsequently washed with 2 x 2 ml of 70% ethanol. The extract was dried down under a gentle stream of nitrogen and then quantitatively transferred to preweighed storage-sample bottles and stored in the freezer resuspended in 0.5 ml of chloroform until used. Aliquots were withdrawn for the determination of radioactivity.

Non-saponifiable extracts were chromatographed in one dimension on 20 x 20 cm silica gel G TLC plates with petroleum ether-acetone (90:10 v/v) as the first solvent. Plates were then allowed to air dry before development in the same direction with chloroform as the second solvent. Plates were allowed to air dry again. Lipids were visualized by staining with p-anisaldehyde solution [p-anisaldehyde (0.5 g); concentrated H_2SO_4 (0.5 ml) and 90% ethanol (9 ml)]. The sprayed plates were heated at 100°C for 5 to 10 minutes (Dunphy et al, 1967).

Lipids were identified using suitable standards spotted on the same plates. Once identified, the incorporation of ^{14}C-labeled mevalonolactone into polyisoprenoid lipids was determined by scraping desired areas of the TLC plates into scintillation vials and determining radioactivity by scintillation counting.

[^{3}H]-Mannose Incorporation Studies

The incorporation of [^{3}H]-mannose into lipid-monosaccharide; lipid-oligosaccharide and glycoprotein was examined.

Cells (50,000 cells per plate) were delivered into 60 x 15 mm Lux tissue culture dishes containing 5 ml FCS-M. Following 4 days of growth, the medium was removed and replaced with 5 ml of fresh FCS-M or LPDS-M and growth was continued for a further 20 hours at either 34°C or at 39°C. Some of the plates received 5 ml FCS-M supplemented with mevalonolactone (10 μg/ml, 77 μM) and were grown at 39°C for a further 20 hours. Following this period of growth, cells were exposed to a four hour pulse of D-[2-^{3}H] mannose (17 μCi/ml). Following this, medium was removed and the cells were harvested. Cell monolayers were washed with 3 x 5 ml of ice cold PBS, then with 2 x 5 ml of ice cold hypotonic Buffer K (1 mM Tris-HCl, 1 mM EGTA, and 1 mM $MgCl_2$) and then incubated 2-3 minutes with 5 ml of Buffer K at room temperature. This buffer was then removed from the dishes and a fresh 0.30 ml of ice cold Buffer K was added. Immediately cells were lysed and homogenates collected by rapidly scraping the culture dishes with a rubber policeman. Cell suspensions from 2 plates were collected. An aliquot was removed for protein.

The amount of [^{3}H]-mannose incorporated into lipid-monosaccharide, 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) essentially as previously described by Ceri (1978).

This procedure involved the extraction of the lipid fraction in chloroform-methanol (2:1 v/v). The supernatant contained the lipid-monosaccharide fraction. The resulting pellet contained the lipid-oligosaccharide and glycoprotein fractions. This pellet was then

washed and further extracted with chloroform-methanol-water (1:1:0.3 v/v/v) to yield the lipid-oligosaccharide (in the supernatant) and the glycoprotein fraction (in the remaining pellet). Both lipid containing fractions were dried under a nitrogen stream and counted in Scintiverse counting fluid. The glycoprotein fraction was solubilized in 10% SDS prior to counting. All samples were done in duplicate.

Incorporation of [^{3}H]-Mevalonic Acid into Protein and Lipid

The incorporation of [^{3}H]-mevalonic acid into protein was essentially done according to the procedure of Sinensky and Logel (1985). The incorporation of label into lipid was also examined.

Cells, approximately 50,000 cells per plate, were delivered to 100 x 15 mm Lux tissue culture dishes containing 10 ml of FCS-M. Cells were grown at 34°C until cells reached near subconfluence. At this point, 2 plates of WT and of $C^{R}7$ were transferred to 39°C. Control plates were left at 34°C.

Twelve hours later, the medium was removed and replaced with 5 ml of fresh FCS-M supplemented with [2-^{3}H]-MVA (resuspended in PBS) (100 μCi/ml). The plates were then returned to their respective temperatures for a further 18 hours.

Following this period of growth, the medium was removed and the cells were harvested. Ice cold PBS (0.5 ml) was added to each plate and the monolayers were scraped off with a rubber policeman. The cell suspensions were then centrifuged using a GLC-2 Sorvall centrifuge for 5 minutes at 900 rpm. The cell pellets were resuspended in 1 ml of ice cold PBS and centrifuged again for 10 minutes at 900 rpm.

The pelleted cells were then extracted. Cells were treated with 3

x 2 ml acetone, and the resulting suspension shaken intermittently for 10 minutes. The cells were pelleted using a bench top Clinical centrifuge at 1,000 x g for 10 minutes. The pelleted cells were then extracted with 2 x 2 ml of chloroform-methanol (2:1 v/v) and then centrifuged as above. Organic extracts were pooled together and solvent was evaporated under a gentle stream of N_2 gas. The resulting lipid material was then dissolved in 0.5 ml chloroform and stored in the freezer until required.

The protein residue was redissolved in 120 µl of SDS-sample buffer (10% glycerol (w/v), 5% β-mercaptoethanol (v/v), 2.3% SDS (w/v), 0.0625M Tris-HCl, pH 6.8, .001% bromophenol blue and distilled H_2O). Two SDS-PAGE gels were run. Each contained the following: WT, 34°C; WT 39°C; C^R7 34°C; C^R7 39°C, and a lane containing a standard mixture. The gels were run for an appropriate period of time, after which time they were further processed. One gel was stained and the resulting protein profile examined. The other was autoradiographed. The autoradiogram was stored for 3 weeks at -70°C and then developed and the bands were examined.

The incorporation of label into lipids was also evaluated. An aliquot was withdrawn and counted and used to determine total incorporation of the [^{3}H] label into lipid. To examine the relative incorporation into individual lipids, samples were subjected to one dimensional TLC analysis using a two solvent system consisting of petroleum ether-acetone (90:10 v/v) as the first solvent system and chloroform alone as the second system. Lipid components were visualized with the use of standards and p-anisaldehyde as previously described in

this thesis.

Examination of DNA, RNA and Protein Synthesis

An examination of DNA, RNA and protein synthesis was done by examining the incorporation of [6-^{3}H]-thymidine, [5-^{3}H] uridine, and L-[4,5-^{3}H] leucine into DNA, RNA and protein respectively. The effect of the presence of mevalonolactone (10 μg/ml, 77 μM) on these processes at 39°C was also examined.

In all three cases, 50,000 cells were delivered to 60 x 15 mm Lux tissue culture dishes containing 5 ml FCS-M. Cells were grown at 34°C for about 4 days until the cells were near subconfluence. At this point, the medium was removed and replaced with 5 ml of either FCS-M or LPDS-M and cells were grown at either 34°C or 39°C for a further 22 hours. Some cells were also exposed to 39°C for a 22 hour period but were cultured in the presence of 5 ml of FCS-M supplemented with mevalonolactone (10 μg/ml, 77 μM). Following this 22 hour growth period, cells were exposed to the radioactively labeled compound of interest for a 2 hour pulse.

Following this, cell monolayers were washed with 2 x 2 ml of ice cold PBS. Ice cold 10% trichloroacetic acid (TCA) (500 μl) was added to each plate and the cells were removed. Each plate was then washed with fresh 500 μl of ice cold 10% TCA. This was combined with the first TCA aliquot to give a total volume of 1 ml. Samples were stored or maintained on ice during the majority of the procedures employed.

Samples were centrifuged for 15 minutes in an Eppendorf Centrifuge 5412 held in a 4°C cold room. The supernatant was carefully removed, an aliquot was used to determine radioactivity present and a sample was

stored for protein determination.

The pellet was washed with 3 x 3 ml ether in order to remove any residual TCA. After these washings, the pellet was resuspended in I N NaOH, and was allowed to remain at room temperature overnight so that the pellet could dissolve completely. Following this an aliquot was counted and the radioactivity determined was used as an indicator of the incorporation of the radioactively labeled material used and of the process under investigation. Prior to counting, an aliquot of acid was added to each of the counting vials. An aliquot was also removed and was used to determine protein.

Determination of Cytochrome P-450 Content

An attempt to determine the cytochrome P-450 content of WT and C^R7 cells was made essentially according to the procedure of Estabrook and Werringloer (1978). Approximately 80,000 cells were delivered to 50-60 Brockway bottles containing 25 ml of FCS-M. The bottles were incubated at 34°C until the cells approached subconfluence. The cells were then grown at either 34°C for a further 24 hours or were transferred to 39°C for a further 24 hours. Following this the cells were removed with 10 ml EDTA in PBS and then washed with 2 x 2 ml ice cold PBS.

Microsomes were prepared from these cells. Prior to preparing microsomes, the wet weight of the cells was determined and recorded. WT and C^R7 were both resuspended in 0.1 M potassium phosphate buffer, pH 7.5 and homogenized with a Potter-Elvejhem homogenizer. This cell suspension was also subjected to two-30 second sonic bursts with a medium sized probe using a Sonic Dismembrator Model 300 (Fisher). Cell breakage was monitored by microscopic examination of the suspension.

Once the cells were adequately broken, a microsomal suspension was prepared.

Cell suspensions were centrifuged at 10,000 x g for 20 minutes to remove cell debris. A microsomal pellet was prepared by centrifugation at 105,000 x g for 90 minutes. In the above procedure, a Beckman Model L3-50 Ultracentrifuge and a 60 Ti rotor were used. All centrifugations were done at 4°C.

The microsomal pellet was gently resuspended in 2 ml of 0.1 M potassium phosphate buffer, pH 7.5 using a hand held Potter-Elvejham homogenizer. Once resuspended, an aliquot was removed and protein determined.

The difference spectrum of cytochrome P-450 was determined using a Beckman DU-8 Spectrophotometer and a 250 µl cuvette. A spectrum from 650 nm to 400 nm was recorded. Each of the microsomal suspensions were suitably diluted prior to spectral determinations being recorded. Firstly, a base line of equal light absorbance was determined. The contents of the cuvette were then gently gassed with carbon monoxide purged of oxygen and the spectrum was recorded to determine the amount of oxyhemoglobin contamination. A few grains of sodium dithionite ($Na_2S_2O_4$) were then added to this cuvette and the difference spectrum of reduced cytochrome b_5 plus the CO adduct of reduced cytochrome P-450 was recorded. A similar amount of sodium dithionite was then added to a sample of fresh microsomal suspension (not treated with carbon monoxide gas) and the difference spectrum of the CO complex of reduced cytochrome P-450 minus the spectral contribution of reduced cytochrome P-450 was recorded. All spectral determinations were done at room temperature.

RESULTS

RESULTS

Cell Cultures

The current study employed three Chinese hamster ovary (CHO) cell lines previously isolated and partially characterized (Wright, 1975; Wright & Ceri, 1977b; Ceri & Wright, 1977b; Borgford, 1984). They were a wild type "CO_2" type Chinese hamster ovary cell, WT; a Concanavalin-A resistant mutant selected from this population, C^R7, and a spontaneous revertant selected from the mutant population, designated RC^R7 or Rc. Each of these three cell lines has been maintained in culture continuously for a period of years. WT and RC^R7 exhibited a similar cellular morphology and may be characterized as "epithelioid" in nature whereas the mutant C^R7 exhibited a "fibroblastoid" cellular morphology. It should be noted that the terms "epithelioid" and "fibroblastoid" are used rather loosely in tissue culture and often are used (as is done here) to describe the cellular appearance rather than the nature of origin of the cells. A monolayer cell which is polygonal with more regular dimensions and which grows in a discrete patch along with other cells would be regarded as "epithelioid." This describes WT and RC^R7. A monolayer cell, the length of which is usually more than twice its width would be regarded as "fibroblastoid." This would describe C^R7. Furthermore, C^R7 exhibited a more "spindle-like" shape and an irregular, less ordered growth pattern with a pronounced inclination to form microconsortia.

During this study all cultures were maintained at the nonphysiological incubation temperature of 34°C. This was done to

relieve any selective pressure associated with the temperature-sensitive nature of the Con-A resistant phenotype.

Periodically, the three cell lines were characterized. Furthermore, fresh stock cultures of all three cell lines were periodically introduced into the cells being used for the investigation, of course, only after acceptable characterization was done to verify the fidelity of each cell line. This "restocking" of the "investigation cell lines" was important to ensure that whatever observations were made would be a reflection of a "healthy" cell population and not due to a cell line of questionable status.

Periodically, killing curves were conducted with Concanavalin-A. The phenotype of each of the three cell lines was found to be stable with respect to sensitivity or resistance to this drug. D_{10} values were generally in close agreement with published data. The C^R7 cell line remained characteristically 2 to 3 fold more resistant to Con-A than the wild type cells. Revertant cells, (Rc) were found to be only marginally more resistant to Con-A than the wild type cells, having D_{10} values never greater than 1.5 fold those of wild type, or the Rc cell line exhibited similar Con-A sensitivity to that expressed by the wild type cells.

All three cell lines were routinely screened for Mycoplasma contamination and were found to be free of contamination due to Mycoplasma.

Growth Studies: Initiation of the Investigation

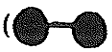
One focus of this thesis is to further examine the obvious temperature-sensitive growth behavior of C^R7 previously established

(Ceri & Wright, 1977b; Ceri, 1978). Before proceeding with this investigation it was necessary to establish that C^R7 still exhibited obvious temperature-sensitive growth at 39°C.

In this present investigation and in previous studies of the Con-A resistant phenotype, stock cultures were maintained on α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum. α-MEM is a chemically well defined supplement, whereas fetal calf serum (FCS) is not. Fetal calf serum is known to contain varying concentrations of lipoprotein-borne lipid and free lipid.

Many suitable methods exist for the removal of lipids from serum. The method of Havel et al, (1955) although incapable of eliminating free lipids from the serum, will remove the majority of lipid which is associated with lipoprotein. When the cholesterol content of serum was determined before and after removal of lipoprotein, it was shown that 98-99% of the cholesterol was removed. In absolute terms this represented less than 0.3 μg/ml of cholesterol, in the undiluted serum, or one-tenth of this concentration when provided as a supplement (10% v/v) to α-MEM. The cholesterol concentration of the lipoprotein-deficient serum was therefore always negligible. In contrast, the cholesterol content of "whole" fetal calf serum was always in excess of 500 μg/ml, or 50 μg/ml when provided as a supplement (10% v/v) to α-MEMB. Medium containing whole fetal calf serum was refered to as FCS-M. Medium supplemented with lipoprotein-deficient serum was referred to as LPDS-M.

The growth responses of all three cell lines were examined at 34°C, 37°C and 39°C. Figures 11, 12, and 13 demonstrate the growth rates of

Figure 11: Growth curves for wild type, Rc and $C^{R}7$ at 34°C. Growth curves for wild type (), Rc, (), and $C^{R}7$, (), at 34°C in medium containing 10% (v/v) of whole fetal calf serum (FCS-M). Each point represents the mean of three separate determinations. Standard deviations were 1-3% of the mean values shown.

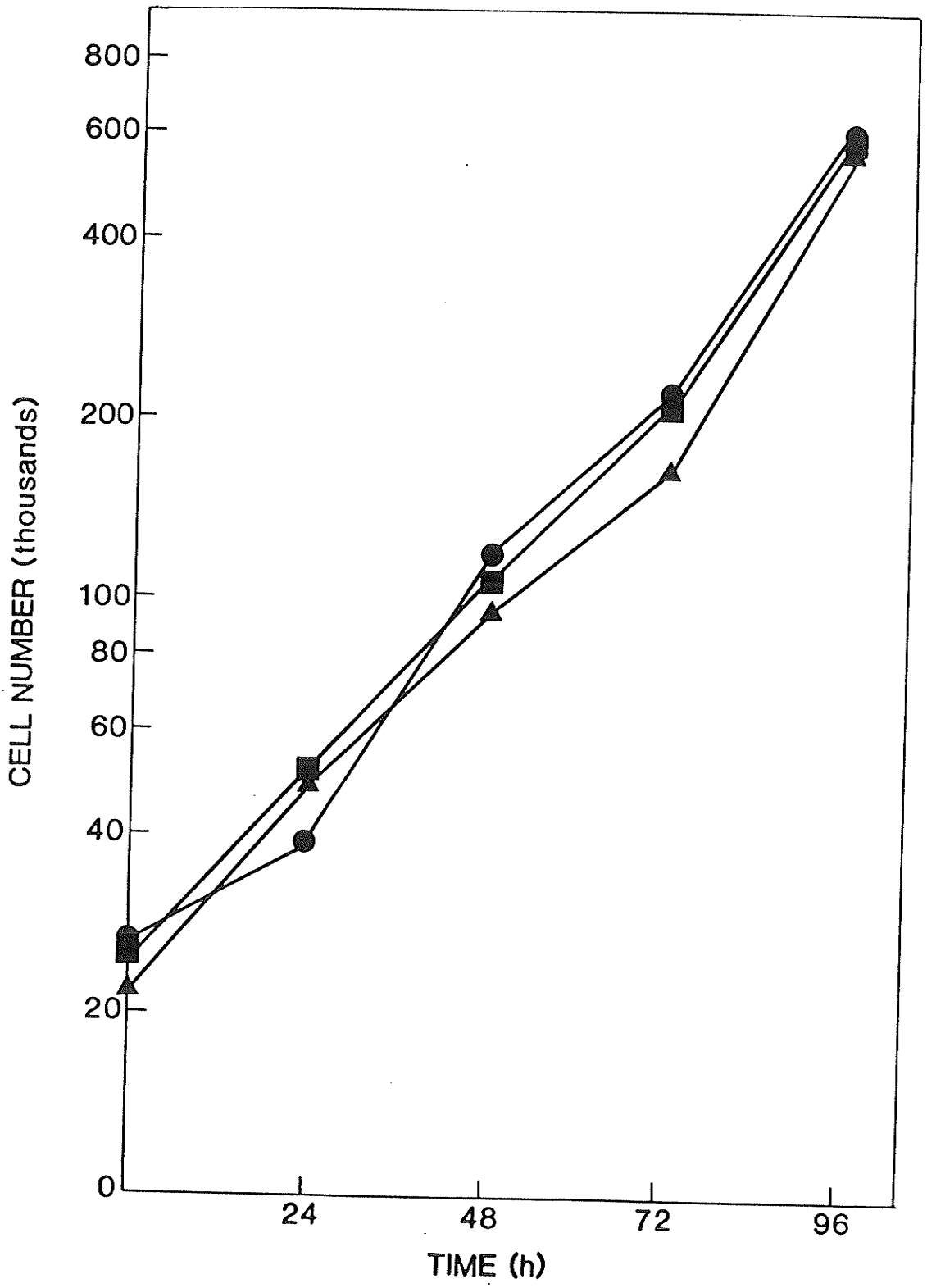
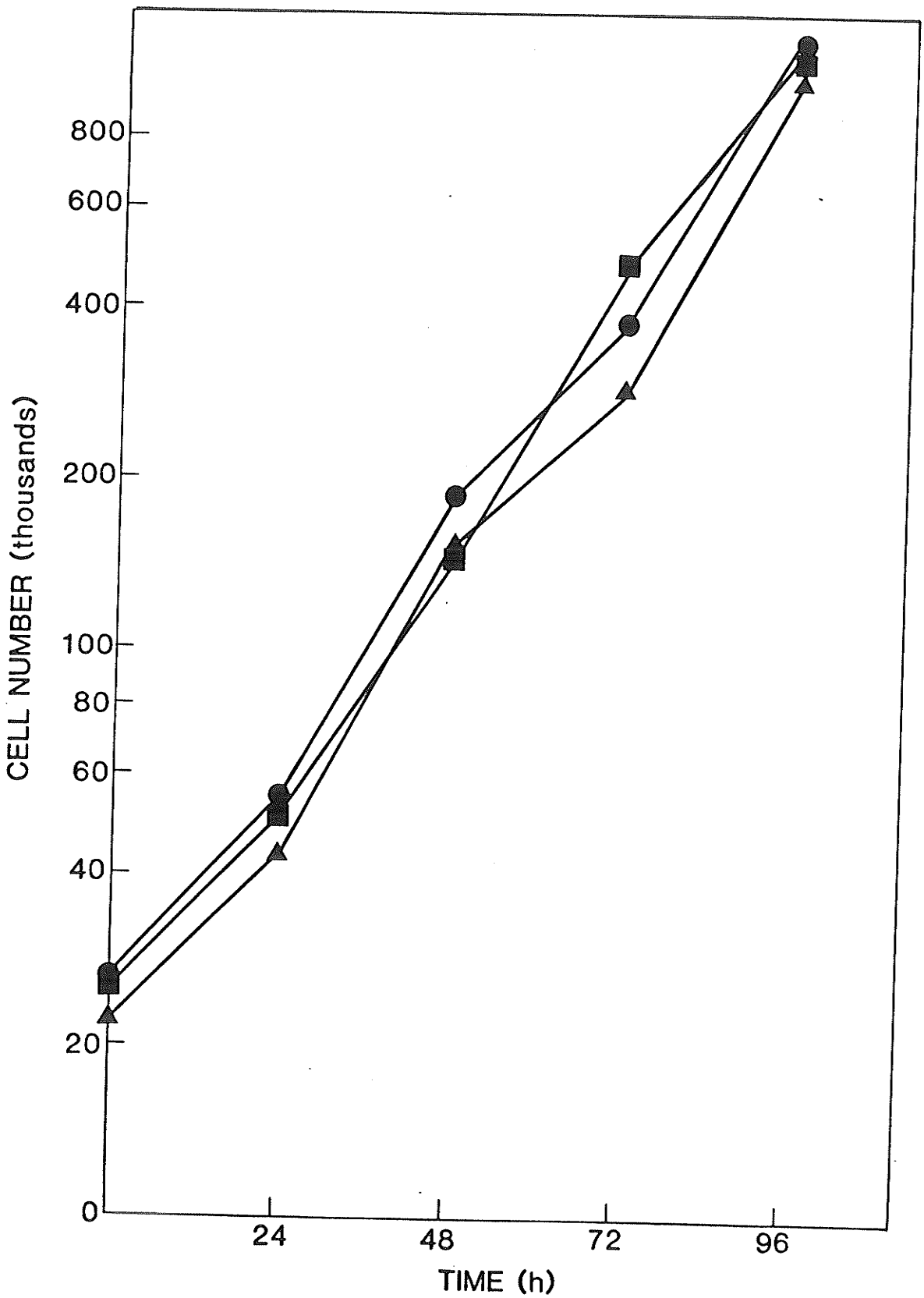

800
600
400
200
100
80
60
40
20
0
CELL NUMBER (thousands)
24
48
72
96
TIME (h)

Figure 12: Growth curves for wild type, revertant and $C^{R}7$ at 37°C.

Growth curves for wild type, (), Rc, () and $C^{R}7$, (), at 37°C in medium containing 10% (v/v) of whole fetal calf serum (FCS-M). Each point represents the mean of three separate determinations. Standard deviations were 1-3% of the mean values shown.


CELL NUMBER (thousands)
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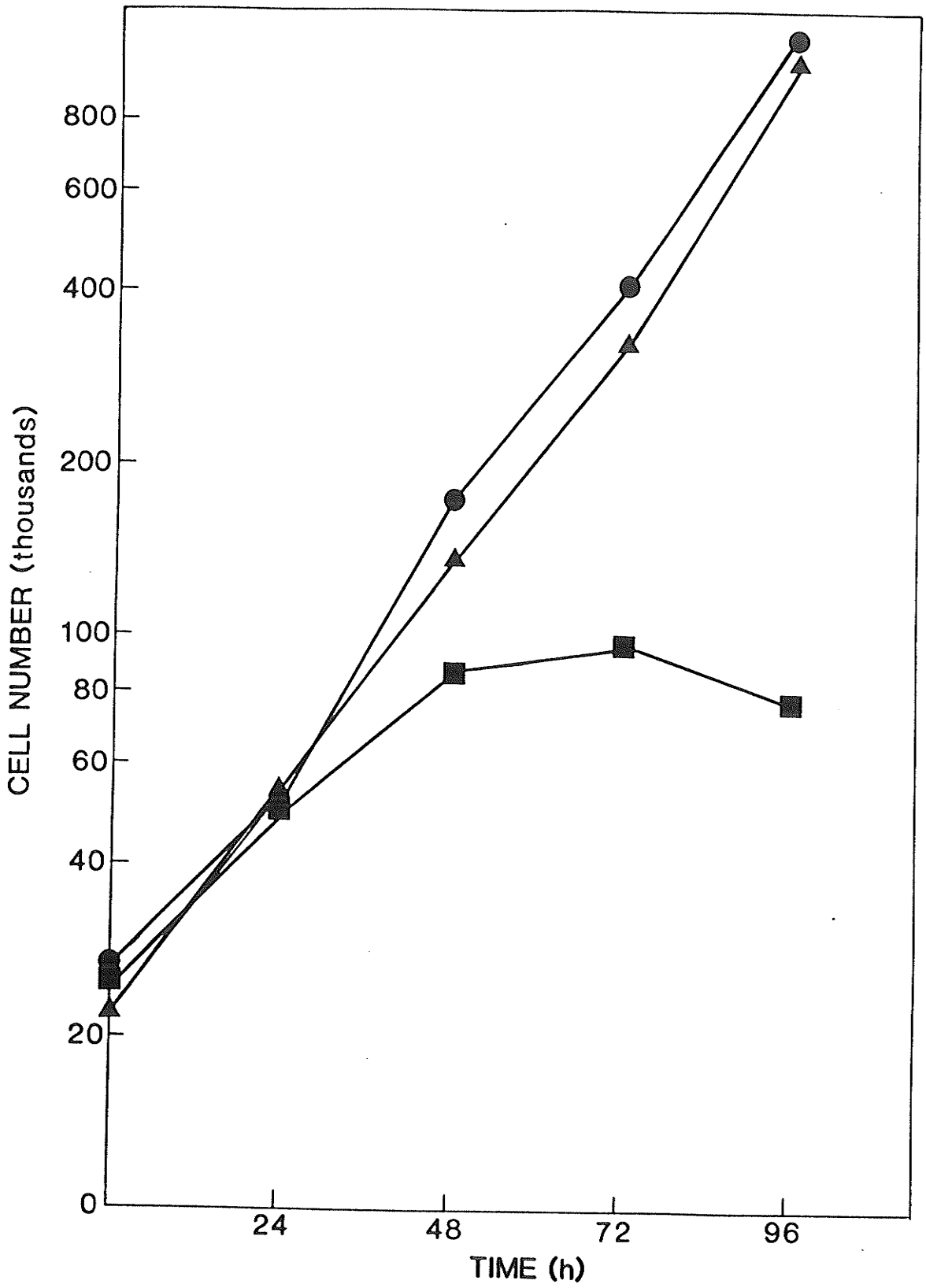
200
100
80
60
40
20
0
24
48
72
96
TIME (h)

Figure 13: Growth curves for wild type, Rc and $C^{R}7$ at 39°C. Growth curves for wild type, (), Rc, (), and $C^{R}7$, () at 39°C in medium containing 10% (v/v) of whole fetal calf serum (FCS-M). Each point represents the mean of three separate determinations. Standard deviations were 1-3% of the mean values shown.


CELL NUMBER (thousands)
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96
TIME (h)

WT, Rc, and C^R7 cell lines at 34°, 37°C, and 39°C respectively. The growth rates of all three cells were substantially the same when cultures were incubated at 34°C in medium rich in lipoprotein (FCS-M). Doubling times ranged typically from 18 to 20 hours during the log phase of growth. Similarly, at 37°C, the growth rates of all three cells were substantially the same when cultures were incubated in FCS-M. Furthermore, all three cell lines tended to exhibit a better growth response at 37°C (Figure 12) relative to that exhibited at 34°C (Figure 11) as evidenced by the cell numbers noted after 72 and 96 hours of growth respectively. Figure 13 demonstrates the growth response of WT, Rc, and C^R7 at 39°C. This temperature was previously demonstrated to be the non-permissive temperature for growth of C^R7. As evidenced in Figure 13, WT and Rc are able to proliferate and to grow well exhibiting a growth response similar to that at 37°C (Figure 12) and certainly better than that seen at 34°C (Figure 11). WT and Rc flourish at 34°C, 37°C and 39°C. However, at 39°C, when cultured in FCS-M, C^R7 is able to grow and to proliferate for only the first 36 to 48 hours (Figure 13). After 48 hours of exposure to 39°C, C^R7 failed to proliferate.

Culturing WT and Rc in FCS-M at 40°C allowed a growth response mimicking that shown at 39°C (Figure 13), however, a temperature of 41°C was shown to be non-permissive for growth for WT and Rc (data not shown). The effect of exposing C^R7 to these temperatures, namely 40°C and 41°C merely amplified the response seen at 39°C (Figure 13). In fact, these temperatures were "toxic" to C^R7 within 24 hours (data not shown).

The growth responses of WT and C^R7 were examined when cells were

cultured in the presence of medium containing 10% (v/v) lipoprotein deficient fetal calf serum (LPDS-M) as well as in the presence of medium containing 10% (v/v) and 20% (v/v) delipidated (not just lipoprotein-free) fetal calf serum (DELIP-M). WT cells, when grown at 34°C, were able to flourish regardless of the nature of the serum component (Figure 14) present suggesting that the WT can make the appropriate compensatory response to accommodate for the nutritional deficiencies which exist. On the other hand, C^R7, when cultured at 34°C, in the presence of LPDS-M clearly did not flourish after a period of 48 hours. Furthermore, this inability of C^R7 to proliferate in the presence of LPDS-M was even more dramatically illustrated when C^R7 was cultured in the presence of 10% (v/v) and 20% (v/v) of DELIP-M respectively (Figure 15).

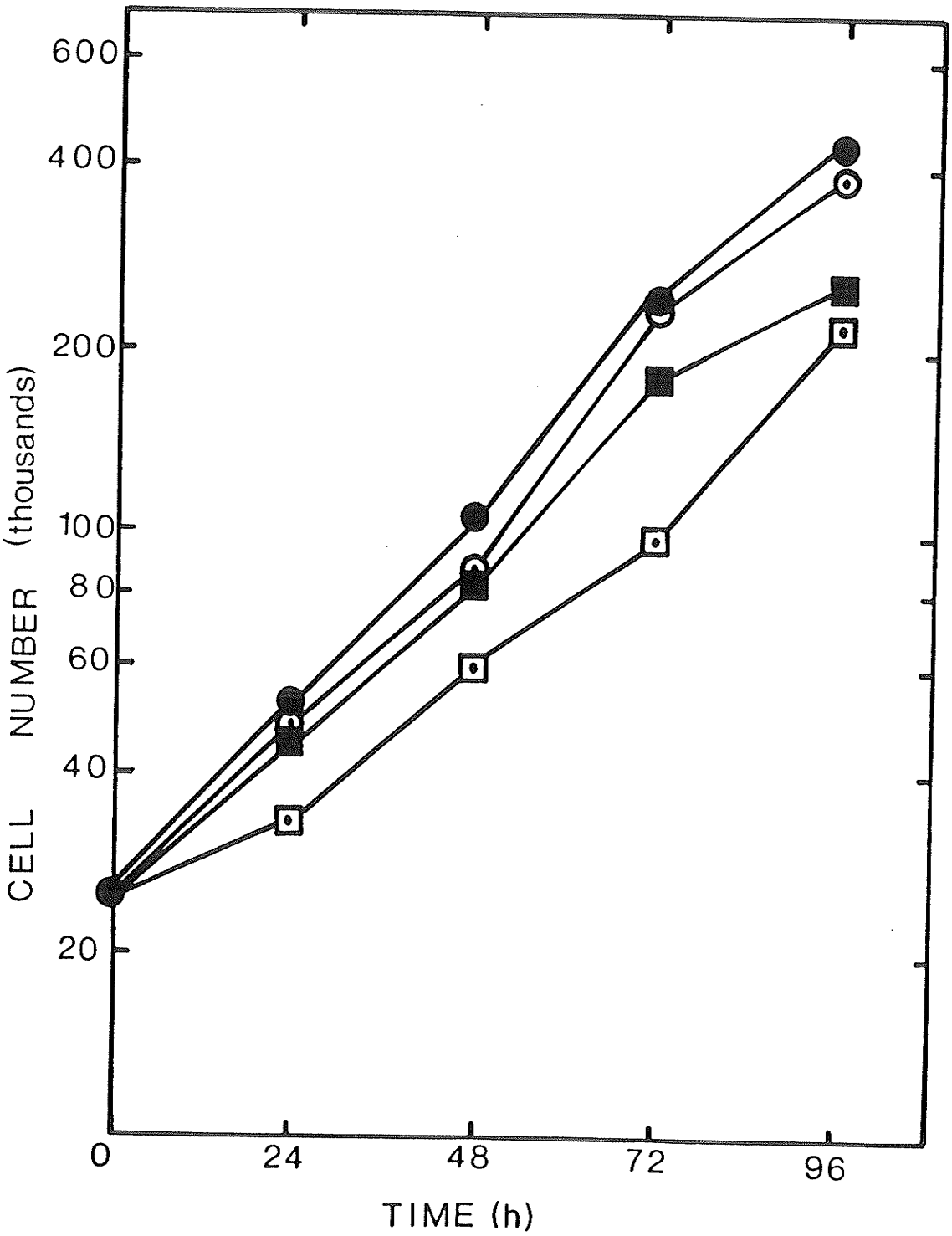
As mentioned earlier, all three cell lines grew identically at 34°C when cultured on FCS-M, with a doubling time of approximately 18-20 hours. Figure 16 shows that while WT and RC^R7 displayed identical growth characteristics (with a doubling time of 20 hours) on LPDS-M, growth of the Con-A resistant variant was not supported by this medium (Figures 15 and 16). The change in the growth rate of C^R7 on LPDS-M was not immediately apparent. The mutant remained in log phase for approximately 48 hours after the switch to LPDS-M and then abruptly stopped growing. Microscopic examination of C^R7 cells, during these first 48 hours revealed no gross morphological changes. After 48 hours in LPDS-M, C^R7 ceased division, became rounded and detached from the substratum. By 72 hours, and most certainly, by 96 hours, significant numbers of cells had become detached from their substratum and were

floating in the medium, apparently moribund. Replacement of the medium with fresh LPDS-M did not reverse this process (data not shown). However, addition of cholesterol (5 μg/ml, 13 μM, in ethanol) to LPDS-M was able to completely fulfill the growth requirement of C^R7. The final concentration of added ethanol was less than 1%. Clearly, the addition of cholesterol at the concentration of 13 μM to LPDS-M allowed growth of C^R7 with approximately the same doubling time as WT and RC^R7 (Figure 16). The mutant cells grew well in cholesterol fed cultures with a doubling time of approximately 22 hours. This is a slightly slower doubling time when compared to the FCS-M cultures which had a doubling time of approximately 18 to 20 hours. A possible explanation for this discrepancy may be the relative insolubility of cholesterol in aqueous solutions.

The cholesterol concentrations of FCS-M and LPDS-M were 130 and 0.078 μM, respectively.

Under microscopic examination, C^R7 cells exhibited "normal" C^R7 morphology when grown on cholesterol-supplemented LPDS-M, in sharp contrast to the "tattered" and "fragile" appearance seen on LPDS-M alone. Clearly, lipoproteins are necessary for the growth of the mutant in as much as they are rich sources of cholesterol. Interestingly, neither the addition of a saturated fatty acid nor the addition of an unsaturated fatty acid (as ethanolic solutions) (data not shown) would allow growth and proliferation to continue. After a period of 48 hours in culture mutant cells given only a fatty acid supplement on LPDS-M without added cholesterol stopped growing, rounded up and became moribund. Control cultures (that is, LPDS-M without supplementation of

Figure 14: Growth response of WT cells at 34°C. Growth of wild type cells at 34°C in medium containing 10% (v/v) of whole fetal calf serum (FCS-M), (); 10% (v/v) of lipoprotein-deficient serum (LPDS-M), (), and 10% (v/v) (), and 20% (v/v) of delipidated fetal calf serum (DELIP-M), (). Each point represents the mean of three separate determinations. Standard deviations were 1-3% of the mean value shown.


600
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CELL NUMBER (thousands)
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72
96
TIME (h)

Figure 15: Growth response of C^R7 cells at 34°C. Growth of C^R7 cells at 34°C in medium containing 10% (v/v) of whole fetal calf serum (FCS-M), (); 10% (v/v) of lipoprotein-deficient serum (LPDS-M), (); and 10% (v/v), (), and 20% (v/v) of delipidated fetal calf serum (DELIP-M) (). Each point represents the mean of three separate determinations. Standard deviations were 1-3% of the mean values shown.

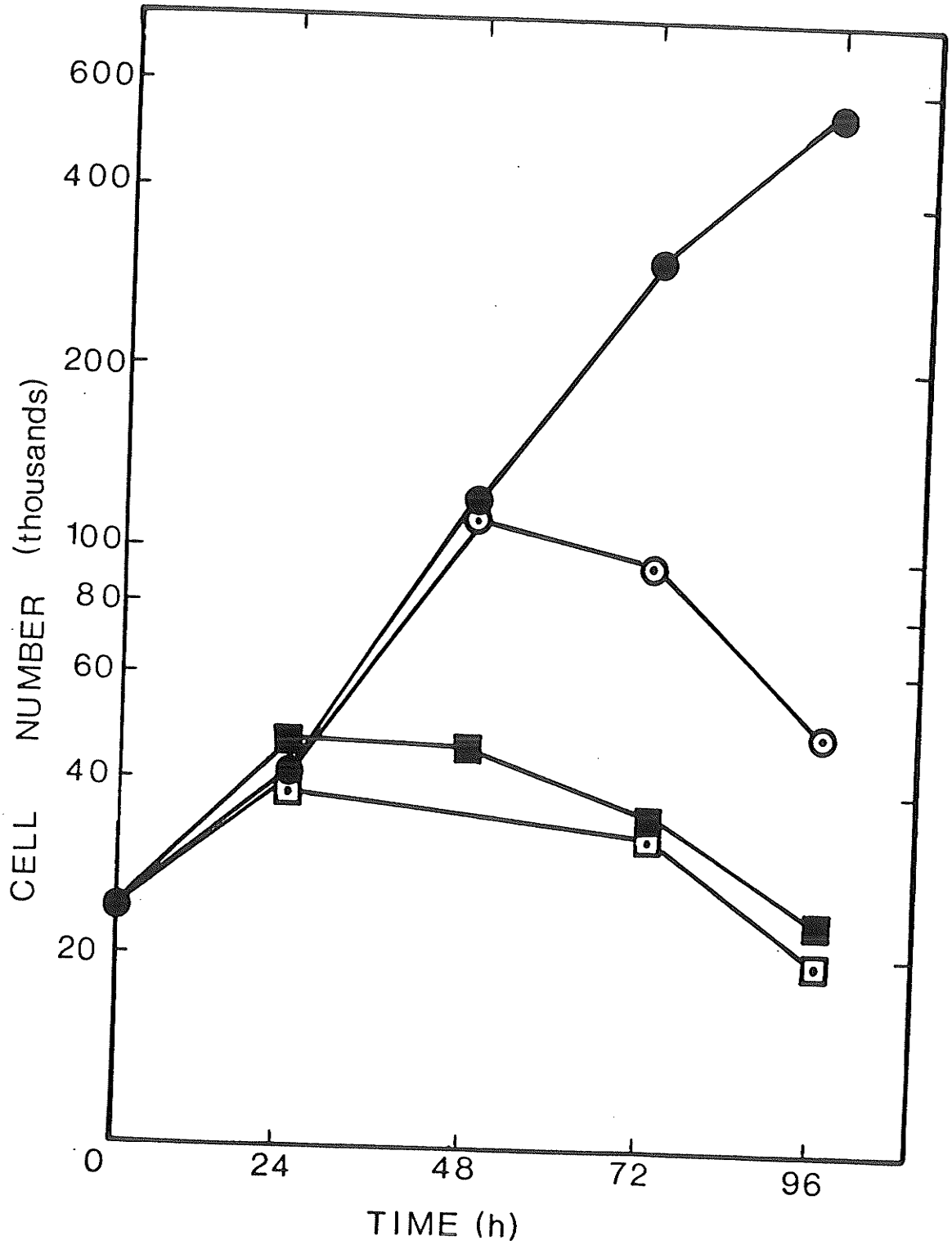
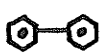
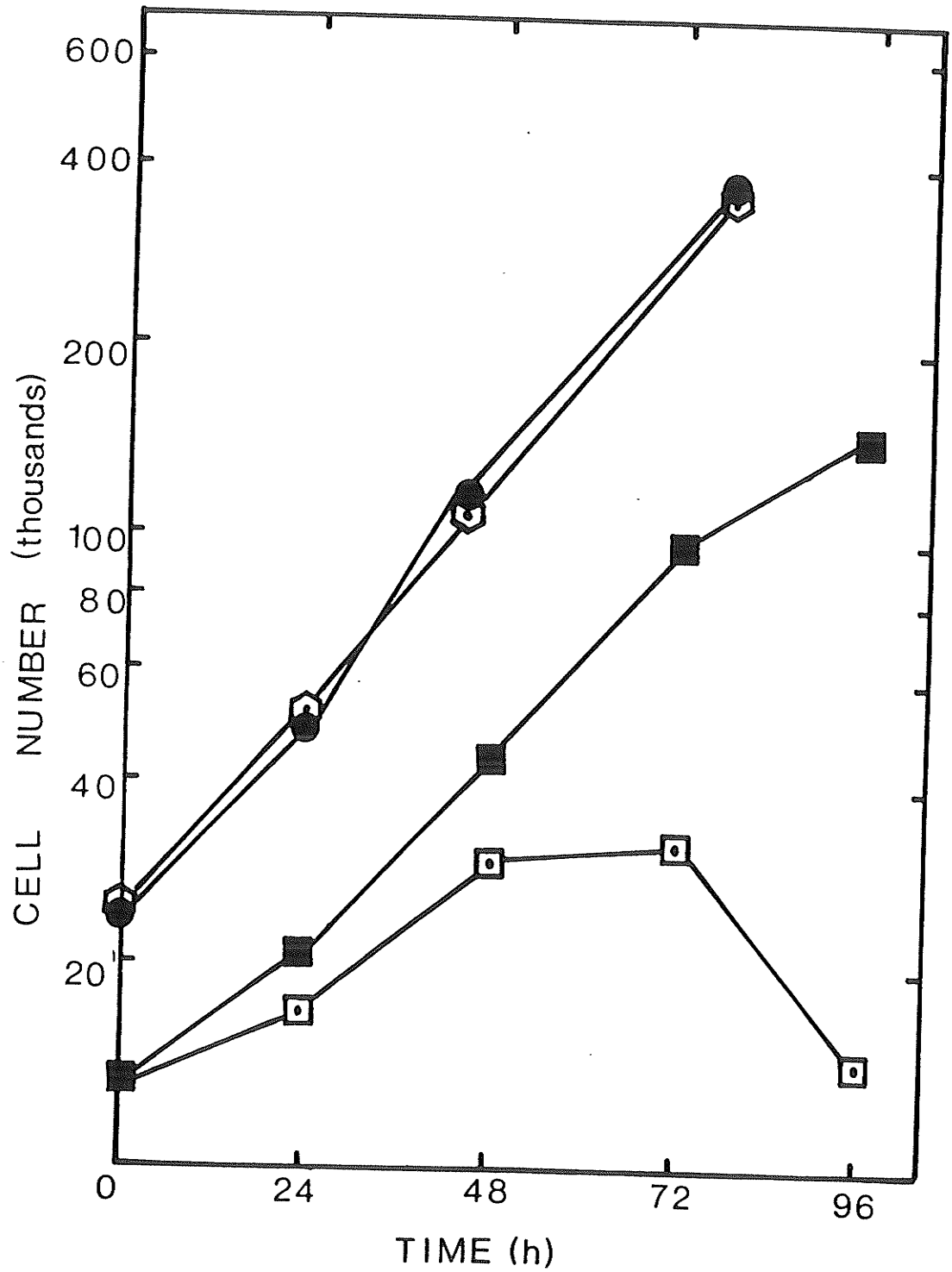

600
400
200
100
80
60
40
20
0
CELL NUMBER (thousands)
24
48
72
96
TIME (h)

Figure 16: Growth of wild type, variant and revertant cells on medium containing lipoprotein-deficient serum and the effect of cholesterol. Cells were seeded in 60 mm dishes containing 5 ml of FCS-M, and incubated 24 hours at 34°C. Cell numbers were then determined (see Methods and Materials) and the values were designated as the zero time counts. The medium was then replaced with 5 ml of LPDS-M, with or without cholesterol as indicated, and incubation continued for a further 96 hours. Cell numbers were evaluated, in triplicate, periodically. Cholesterol was added at a concentration of 13 μM as an ethanol solution (final concentration of ethanol was 0.1%). Control experiments (data not shown) showed that 0.1% ethanol did not affect growth.

Figure Legend: WT on LPDS-M, (); Rc on LPDS-M, (); C^R7 on LPDS-M, (); C^R7 on cholesterol supplemented LPDS-M, ().


600
400
200
100
80
60
40
20
CELL NUMBER (thousands)
0
24
48
72
96
TIME (h)

any kind) behaved similarly.

During the course of this study, in order to determine the effect of cholesterol deficiency and in order to differentiate endogenous lipid metabolism from the influences of exogenous lipids, it became frequently imperative to culture cells in the presence of LPDS-M. Furthermore, it became crucial to determine a suitable period of incubation in LPDS-M which allowed the cells to become roughly equilibrated, yet at the same time ensuring that this period of exposure to LPDS-M (cholesterol deficiency) was not of such a length as to render the mutant cultures moribund. Consequently, at 34°C, the permissive temperature a time of exposure of 24 hours to LPDS-M was generally used.

This "time of exposure" became a critical concern especially with respect to C^R7 when investigations were carried out at 39°C, the non-permissive temperature for growth for C^R7 (Figure 13). This was true for FCS-M and especially for LPDS-M. WT and RC^R7 grew well at 39°C in either FCS-M or LPDS-M. Therefore, with respect to C^R7, and investigations carried out at 39°C, a "time of exposure" or "stress period" of less than 48 hours was generally employed. The exact "stress period" is noted where pertinent.

Examination of Lipid Content and Composition

Mass Determination of Cholesterol and Phospholipid

Cholesterol and phospholipid are the two principal lipid components of mammalian cell membranes. The maintenance of the physical properties of the membrane, fluidity and viscosity, depend in large part upon the stoichiometric relationship of these components. Cholesterol and

phospholipid determinations were made of cells grown in FCS-M (cholesterol sufficient) and in LPDS-M (cholesterol deficient) and the molar ratios were calculated. This was done at 34°C, 37°C, and 39°C. The results are tabulated in Tables 1, 2, and 3.

At 34°C, the total cholesterol content of the mutant, with respect to cell protein was less than that of the other two cell lines, regardless of in which medium they were grown (Table 1). This, in turn, corresponded to a reduced molar ratio of cholesterol to phospholipid in C^R7, a reduction which was particularly pronounced in cells grown in LPDS-M (Table 1). Phospholipid content was essentially unchanged. The molar ratio of cholesterol to phospholipid in wild type and revertant cells was somewhat reduced as the result of incubation in LPDS-M, yet was virtually twice as high as that of the mutant (Table 1).

The findings determined for cells grown at 37°C were much the same as those determined at 34°C. The total cholesterol content of the mutant, with respect to cell protein was again less than that of WT and Rc cell lines regardless of whether cells were grown in FCS-M or in LPDS-M (Table 2). A reduced molar ratio of cholesterol to phospholipid in C^R7 was again found. This reduction was particularly pronounced in cells grown on LPDS-M (Table 2). The molar ratio of cholesterol to phospholipid in wild type and revertant cells was again somewhat reduced as the result of the incubation in LPDS-M, yet it was still virtually twice as high as that of the mutant (Table 2). Phospholipid content was similar in all three cell lines either on FCS-M or LPDS-M (Table 2).

At 39°C, similar results to those determined at 34°C and 37°C were noted, with a notable exception. The deficiencies of C^R7 were more

pronounced. The total cholesterol content of the mutant, with respect to cell protein was less than that of the other two cell lines regardless of in which medium they were grown (Table 3). This, in turn, resulted in a reduced molar ratio of cholesterol to phospholipid (C/PL) in C^R7, a reduction which is pronounced in C^R7 cells grown in FCS-M and in LPDS-M (Table 3). At 34°C and 37°C, the reduction in the (C/PL) became particularly pronounced in C^R7 cells grown in LPDS-M. Cholesterol content of WT and Rc cell lines at 39°C was found to be similar to that found at 34°C and 37°C. Furthermore, the phospholipid content of all three cell lines (exception C^R7, 39°C, FCS-M, 96 h.) at 39°C was similar.

At 39°C on FCS-M, the (C/PL) molar ratio in WT and Rc was virtually three times as high as that of the mutant (Table 3), and virtually four fold higher than that of C^R7 on LPDS-M (Table 3). Recall that at 34°C and 37°C on LPDS-M, the WT and Rc (C/PL) molar ratios were twice as high as that of the mutant. Once again, as seen at 34°C and 37°C, the molar ratio of cholesterol to phospholipid at 39°C in wild type and revertant cell lines was somewhat reduced as the result of the incubation in LPDS-M (Table 3). Nevertheless, this reduction in WT and Rc has no major effect on WT or Rc as both cell lines flourish when cultured on LPDS-M. This is not the case for C^R7 (see section on Temperature Sensitivity of C^R7 and Cholesterol Auxotrophy). Clearly, C^R7 has a problem with cholesterol synthesis/utilization.

Cellular phospholipid is a heterogeneous mixture of molecules containing fatty acids with varying degrees of saturation and unsaturation. The degree of saturation or unsaturation may change in

Table 1: Quantitation of the cholesterol and phospholipid components of WT, $C^{R}7$ and Rc cell lines for cultures grown at 34°C on either FCS-M or LPDS-M. Also given are the molar ratios of cholesterol to phospholipid, (the molecular weight of phospholipid was taken to be 730). Fifty Brockway bottles (for each cell line) were inoculated with 10,000 cells and incubated in FCS-M until subconfluent. Medium was removed and 25 bottles from each cell line received fresh FCS-M and 25 bottles from each cell line received LPDS-M. Cell cultures were incubated for a further 24 hours. Cells were then treated as described in Methods and Materials. Total lipid extracts were prepared according to Bligh and Dyer, (1959). Cholesterol and phospholipid contents were determined from aliquots of the total lipid extract as described in Methods and Materials.

Cell Line	Growth Conditions	Cholesterol Content (μg/mg cell protein)	Phospholipid Content (μg/mg cell protein)	Cholesterol-Phospholipid Molar Ratio (C/PL)
WT	34°C, FCS-M	27.0	114	0.45
Rc	34°C, FCS-M	25.0	114	0.41
C^{R}_{7}	34°C, FCS-M	15.0	85	0.38
WT	34°C, LPDS-M 24 h	22.0	116	0.36
Rc	34°C, LPDS-M 24 h	18.0	114	0.31
C^{R}_{7}	34°C, LPDS-M 24 h	11.5	120	0.18

Table 2: Quantitation of the cholesterol and phospholipid components of WT, C^R7 and Rc cell lines for cultures grown at 37°C on either FCS-M or LPDS-M. Also given are the molar ratios of cholesterol to phospholipid, (the molecular weight of phospholipid was taken to be 730). Fifty Brockway bottles (for each cell line) were inocubated with 10,000 cells and incubated at 34°C in FCS-M for a period of 24 hours, and then were transfered to 37°C and grown until subconfluent. Medium was removed and 25 bottles from each cell line received fresh FCS-M and 25 bottles from each cell line received LPDS-M. Cell cultures were incubated for a further 24 hours. Cells were then treated as described in Methods and Materials. Total lipid extracts were prepared according to Bligh and Dyer, (1959). Cholesterol and phospholipid contents were determined from aliquots of the total lipid extract as described in Methods and Materials.

Cell Line	Growth Conditions	Cholesterol Content (μg/mg cell protein)	Phospholipid Content (μg/mg cell protein)	Cholesterol-Phospholipid Molar Ratio (C/PL)
WT	37°C, FCS-M	24.0	104	0.44
Rc	37°C, FCS-M	27.5	143	0.37
C^{R}_{7}	37°C, FCS-M	18.5	114	0.31
WT	37°C, LPDS-M 24 h	23.5	123	0.31
Rc	37°C, LPDS-M 24 h	19.0	136	0.26
C^{R}_{7}	37°C, LPDS-M 24 h	9.7	104	0.18

Table 3: Quantitation of the cholesterol and phospholipid components of WT, C^R7 and Rc cell lines for cultures grown at 39°C on either FCS-M or LPDS-M. Also given are the molar ratios of cholesterol to phospholipid, (the molecular weight of phospholipid was taken to be 730). Fifty Brockway bottles (for each cell line) were inoculated with 10,000 cells and incubated at 34°C in FCS-M for a period of 24 hours, and then WT and Rc were transfered to 39°C until subconfluent. C^R7 exposure time to 39°C was 24 h and 96 h in total respectively. The C^R7 "96 h - 39°C stress period" in FCS-M used approximately 60 Brockway bottles. WT, Rc, and C^R7 were given fresh FCS-M for the final 24 hour growth period. WT and Rc cultures (25 bottles for each cell and each desired exposure time) were grown at 39°C until subconfluent, then received LPDS-M and incubated for desired exposure times. C^R7 was cultured at 34°C until subconfluent, then medium was removed, and LPDS-M added and cell cultures were then exposed to 39°C for times desired (25 bottles of C^R7 for each desired stress period were used). All cells were then treated as described in Methods and Materials. Total lipid extracts were prepared according to Bligh and Dyer, (1959). Cholesterol and phospholipid contents were determined from aliquots of the total lipid extract as described in Methods and Materials.

Cell Line	Growth Conditions	Cholesterol Content (μg/mg cell protein)	Phospholipid Content (μg/mg cell protein)	Cholesterol-Phospholipid Molar Ratio (C/PL)
WT	39°C, FCS-M	27.5	116	0.45
Rc	39°C, FCS-M	23.0	100	0.43
C^R_7	39°C, FCS-M, 24 h	10.0	124	0.15
C^R_7	39°C, FCS-M, 96 h	5.0	60	0.15
WT	39°C, LPDS-M 12 h	19.0	100	0.36
WT	39°C, LPDS-M 24 h	20.0	104	0.36
WT	39°C, LPDS-M 48 h	22.0	106	0.39
Rc	39°C, LPDS-M 48 h	27.0	140	0.36
C^R_7	39°C, LPDS-M 12 h	6.0	122	0.10
C^R_7	39°C, LPDS-M 24 h	6.5	140	0.09
C^R_7	39°C, LPDS-M 48 h	6.2	112	0.10

response to changing membrane cholesterol concentrations (Rintoul et al, 1979). C^R7 is, for all intents and purposes, a cholesterol auxotroph. This raised the issue of how the mutant regulates the fatty acid environment of its membranes.

Gas-liquid chromatography of fatty acid methyl esters (FAME) was used to determine the fatty acid composition of cellular lipids. Cells were cultured under conditions where lipoproteins were present and absent, keeping in mind that lipoproteins are a source of exogenous fatty acid as well as cholesterol.

The fatty acid content (% composition) of all three cell lines when cultured under conditions where lipoprotein was present and absent, at 34°C, 37°C, and 39°C is given in Table 4, 5, and 6 respectively.

The ratio of 16:0 to 18:1 was calculated, as was the ratio of 18:0 to 18:1 and these values are tabulated, along with (C/PL) molar ratios in Tables 7 and 8. It was expected that as the cells were removed from the presence of exogenous cholesterol a compensatory increase in unsaturated fatty acid would be observed and the index of saturation would fall. The mutant cell line, deficient in its ability to synthesize cholesterol, might be expected to have a compensatory increase in unsaturates beyond that observed in the other two cell lines when cultured in LPDS-M. Yet, instead of becoming smaller the ratio of 16:0 to 18:1, as well as the ratio of 18:0 to 18:1, rose. This rise became more apparent as the temperature changed, being most pronounced at 39°C. Interestingly, the rise in these ratios became apparent in C^R7 when C^R7 was grown at 39°C on FCS-M, (Table 7) and when C^R7 was grown on LPDS-M at 39°C, the rise in the ratio of 16:0 to 18:1 and of 18:0 to

Table 4: Gas-liquid chromatographic (GLC) determination of fatty acid methyl esters (FAME). Presented are the principal FAME of WT, Rc and $C^{R}7$ cell lines, for cultures grown at 34°C in either FCS-M or LPDS-M. The growth conditions are the same as those described for Table 1. Total lipid extracts were prepared according to Bligh and Dyer, (1959). An aliquot of the total lipid extract was taken and FAME were prepared directly, according to the method of Nichols et al, (1965) as described in Methods and Materials. Saturated and unsaturated fatty acid standards (L203, H105 and K103) used to identify and analyze FAME profiles were from Applied Science Laboratories, Inc.

Cell Line	Growth Conditions	Fatty Acid* (% Composition)									
		14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	"X"[2]	"Y"[3]
WT	34°C, FCS-M	1.08	0.22	22.14	2.44	29.96	32.45	3.25	1.11	T[1]	7.49
Rc	34°C, FCS-M	1.13	0.45	22.54	5.41	22.54	33.88	3.91	T	T	10.14
C^R_7	34°C, FCS-M	1.07	0.71	23.21	3.57	23.57	34.29	2.86	T	T	10.71
WT	34°C, LPDS-M 24 h	1.06	0.24	24.06	3.31	22.05	36.85	3.78	T	T	8.66
Rc	34°C, LPDS-M 24 h	0.45	0.45	24.68	3.13	23.09	34.98	3.92	T	T	7.02
C^R_7	34°C, LPDS-M 24 h	0.48	0.36	22.67	1.51	30.77	29.26	3.93	T	T	11.00

*Values represent relative peak areas

[1]T = trace quantities

[2]"X" determined to be 22:1

[3]"Y" determined to be 24:0

"X" and "Y" are tentative identifications based on retention times only

Table 5: Gas-liquid chromatographic (GLC) determination of fatty acid methyl esters (FAME). Presented are the principal FAME of WT, Rc and $C^{R}7$ cell lines, for cultures grown at 37°C in either FCS-M or LPDS-M. The growth conditions are the same as those described for Table 2. Total lipid extracts were prepared according to Bligh and Dyer, (1959). An aliquot of the total lipid extract was taken and FAME were prepared directly, according to the method of Nichols et al, (1965) as described in Methods and Materials. Saturated and unsaturated fatty acid standards (L203, H105 and K103) used to identify and analyze FAME profiles were from Applied Science Laboratories, Inc.

Cell Line	Growth Conditions	Fatty Acid* (% Composition)									
		14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	"X"[2]	"Y"[3]
WT	37°C, FCS-M	0.43	0.86	27.04	2.70	21.18	36.34	3.34	T[1]	T	8.22
Rc	37°C, FCS-M	1.53	0.38	28.15	2.80	21.78	34.10	3.82	T	T	7.43
C^R_7	37°C, FCS-M	0.65	0.39	24.95	2.27	26.90	31.84	3.27	T	T	9.75
WT	37°C, LPDS-M 24 h	0.49	0.39	23.45	3.10	21.72	33.60	3.45	T	T	13.79
Rc	37°C, LPDS-M 24 h	0.59	0.40	23.12	3.64	21.08	36.23	3.43	T	T	4.74
C^R_7	37°C, LPDS-M 24 h	0.54	0.27	22.64	1.75	29.11	25.69	3.14	T	T	16.85

*Values represent relative peak areas

[1]T = trace quantities

[2]"X" determined to be 22:1

[3]"Y" determined to be 24:0

"X" and "Y" are tentative identifications based on retention times only

Table 6: Gas-liquid chromatographic (GLC) determination of fatty acid methyl esters (FAME). Presented are the principal FAME of WT, Rc and $C^{R}7$ cell lines, for cultures grown at 39°C in either FCS-M or LPDS-M. The growth conditions are the same as those described for Table 3. Total lipid extracts were prepared according to Bligh and Dyer, (1959). An aliquot of the total lipid extract was taken and FAME were prepared directly, according to the method of Nichols et al, (1965) as described in Methods and Materials. Saturated and unsaturated fatty acid standards (L203, H105 and K103) used to identify and analyze FAME profiles were from Applied Science Laboratories, Inc.

Cell Line	Growth Conditions	Fatty Acid* (% Composition)									
		14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	"X"[2]	"Y"[3]
WT	39°C, FCS-M	1.29	1.03	24.14	3.45	20.17	34.74	4.83	T[1]	T	10.34
Rc	39°C, FCS-M	0.86	0.38	21.58	2.69	23.02	36.26	3.69	T	T	11.51
$C^{R}7$	39°C, FCS-M 24 h	0.44	0.44	26.34	0.15	29.99	28.16	3.80	T	T	10.61
$C^{R}7$	39°C, FCS-M 96 h	2.23	0.43	37.29	T	30.71	23.15	T	T	T	13.39
WT	39°C, LPDS-M 12 h	0.44	0.44	21.90	1.63	25.05	32.08	5.33	T	T	13.18
WT	39°C, LPDS-M 24 h	0.34	0.21	23.25	3.03	22.25	34.31	3.58	T	T	13.00
WT	39°C, LPDS-M 48 h	0.51	0.34	21.20	3.78	21.25	32.46	3.61	T	T	16.83
Rc	39°C, LPDS-M 48 h	0.47	0.24	24.51	3.09	24.37	37.12	2.76	T	T	7.37
$C^{R}7$	39°C, LPDS-M 12 h	0.78	0.47	20.51	0.63	25.24	19.87	2.53	4.73	T	25.24
$C^{R}7$	39°C, LPDS-M 24 h	0.52	0.47	29.17	0.66	28.13	22.16	3.41	T	T	11.98
$C^{R}7$	39°C, LPDS-M 48 h	1.44	0.40	24.56	0.60	32.10	18.78	2.81	T	T	16.29

*Values represent relative peak areas

[1]T = trace quantities

[2]"X" determined to be 22:1

[3]"Y" determined to be 24:0

"X" and "Y" are tentative identifications based on retention times only

Table 7: Indices of fatty acid saturation of WT, Rc and C^R7 cell lines grown on cholesterol sufficient medium. Gas-liquid chromatographic (GLC) analysis of fatty acid methyl esters (FAME) was done. Presented here are the ratio of 16:0 to 18:1 and the ratio of 18:0 to 18:1, as indices of fatty acid saturation, of WT, C^R7 and Rc cell lines, for cultures grown at 34°C, 37°C, and 39°C in FCS-M. Cholesterol-phospholipid ratios are also presented for completeness. The growth conditions employed are the same as those described for Tables 1, 2 and 3. Total lipid extracts were prepared according to Bligh and Dyer, (1959). An aliquot of the total lipid extract was taken and FAME were prepared directly, according to the method of Nichol et al, (1965). Standard FAME were from Applied Science Laboratories, Inc.

Cell Type	Growth Conditions	18:0 / 18:1	16:0 / 18:1	Cholesterol:Phospholipid[1] Molar Ratio
WT	34°C, FCS-M	0.73	0.68	0.45
Rc	34°C, FCS-M	0.69	0.68	0.41
C^R7	34°C, FCS-M	0.67	0.67	0.38
WT	37°C, FCS-M	0.58	0.75	0.44
Rc	37°C, FCS-M	0.63	0.82	0.37
C^R7	37°C, FCS-M	0.84	0.78	0.31
WT	39°C, FCS-M	0.58	0.69	0.45
Rc	39°C, FCS-M	0.63	0.60	0.43
C^R7	39°C, FCS-M (48 h)	1.06	0.94	0.15
C^R7	39°C, FCS-M (96 h)	1.33	1.61	0.15

[1]The molecular weight of phospholipid was taken to be 730.

Table 8: Indices of fatty acid saturation of WT, Rc and C^R7 cell lines grown on cholesterol deficient medium. Gas-liquid chromatographic (GLC) analysis of fatty acid methyl esters (FAME) was done. Presented here are the ratio of 16:0 to 18:1 and the ratio of 18:0 to 18:1, as indices of fatty acid saturation, of WT, C^R7 and Rc cell lines, for cultures grown at 34°C, 37°C, and 39°C in LPDS-M. Cholesterol-phospholipid ratios are also presented for completeness. The growth conditions employed are the same as those described for Tables 1, 2 and 3. Total lipid extracts were prepared according to Bligh and Dyer, (1959). An aliquot of the total lipid extract was taken and FAME were prepared directly, according to the method of Nichol et al, (1965). Standard FAME were from Applied Science Laboratories, Inc.

Cell Type	Growth Conditions	18:0 / 18:1	16:0 / 18:1	Cholesterol:Phospholipid[1] Molar Ratio
WT	34°C, LPDS-M 24 h	0.60	0.65	0.36
Rc	34°C, LPDS-M 24 h	0.66	0.72	0.31
C^R7	34°C, LPDS-M 24 h	1.05	0.77	0.18
WT	37°C, LPDS-M 24 h	0.65	0.70	0.31
Rc	37°C, LPDS-M 24 h	0.58	0.64	0.26
C^R7	37°C, LPDS-M 24 h	1.13	0.88	0.20
WT	39°C, LPDS-M 12 h	0.78	0.68	0.36
WT	39°C, LPDS-M 24 h	0.65	0.68	0.36
WT	39°C, LPDS-M 48 h	0.65	0.65	0.39
Rc	39°C, LPDS-M 48 h	0.66	0.66	0.36
C^R7	39°C, LPDS-M 12 h	1.27	1.03	0.10
C^R7	39°C, LPDS-M 24 h	1.22	1.27	0.09
C^R7	39°C, LPDS-M 48 h	1.71	1.31	0.10

[1]The molecular weight of phospholipid was taken to be 730.

18:1 corresponded to increasing exposure to LPDS-M at 39°C (Table 8). Clearly, in regard to cholesterol deficiency, C^R7 is unable to make the expected compensatory increase in unsaturated fatty acids which would be predicted as cells were removed from the presence of exogenous cholesterol.

Regarding 16:0 to 18:1 and 18:0 to 18:1 ratios in WT and Rc, when grown on FCS-M at either 34°C, 37°C, and 39°C, these values were similar. On LPDS-M, at either 34°C, 37°C, 39°C, these ratios remained unchanged or decreased marginally. Previously, WT and Rc were observed to make the expected compensatory response and increase their synthesis of and also the content of unsaturated fatty acids (Borgford, 1984; Borgford et al, 1986) and described later in this thesis. In this one instance, perhaps the LPDS-M contained sufficient residual fatty acid (from the perspective of the WT and Rc cell lines) as not to warrant an overly pronounced compensatory response on the part of the WT and Rc cell lines.

Regardless of this fact, clearly C^R7 makes an inappropriate response when faced with cholesterol deficiency (LPDS-M) and when faced with a "perceived cholesterol deficiency" (from C^R7's perspective) on FCS-M at 39°C (Tables 7 and 8).

This "perceived cholesterol deficiency" from C^R7's perspective is due to its marked inability to convert lanosterol to cholesterol (to be demonstrated later in the Results section).

The response of C^R7 on LPDS-M became increasingly apparent on LPDS-M as the growth temperature was increased from 34°C to 37°C to 39°C. And, in particular, the inappropriateness of C^R7's response to

cholesterol deficiency became most apparent as the incubation period in LPDS-M at 39°C increased (Table 8).

It should be noted that the results obtained in Tables 4, 5, 6, 7 and 8 were from experiments conducted on total lipid extracts of cells. The majority of membrane cholesterol (92% reported for CHO cells, Lange & Ramos, 1984) in mammalian cells is found in the plasma membrane. It may therefore be likely that an even more pronounced alteration in the fatty acids of the mutant would be evident in an examination of the plasma membrane lipids alone.

Phospholipids are thought to play important structural roles in the membrane (Yeagle, 1985). For this reason, the phospholipid content and composition of all three cell lines were examined. It was necessary to see if C^R7 possessed any detectable phospholipid abnormalities which could help to explain its aberrant behavior. The phospholipid content of all three cell lines, regardless of cholesterol sufficiency or deficiency, or growth temperature, was essentially the same (Tables 1, 2 and 3). The major phospholipids were found to be phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) (Tables 9 and 10).

An analysis of the relative percent composition of the major phosphatides in these cell lines was done. No appreciable difference among the three cell lines was noted regardless of the growth conditions (Tables 9 and 10). PC, PE and SM contributed approximately 50%, 30%, and 20% respectively to the overall phospholipid content.

Temperature Sensitivity of C 7 and Cholesterol Auxotrophy

As illustrated in Figure 13, when cultured in FCS-M at 39°C, C^R7

Table 9: Analysis of the relative percent composition of the major phospholipids of WT, C^R7 and Rc cell lines, for cultures grown at 34°C, 37°C and 39°C in FCS-M. The growth conditions employed are the same as those described for Tables 1, 2 and 3. Total lipid extracts of cells were prepared by the method of Bligh and Dyer, (1959). An aliquot was chromatographed using a solvent system of chloroform-methanol-acetic acid-water (25:15:4:2 v/v/v/v). Suitable standards were also chromatographed (see Methods and Materials). "Zinzadze" reagent positive material was qualitatively identified and the phospholipid was extracted using methanol-chloroform. The lipid phosphorus was assayed as described previously.

Cell Type	Growth Conditions	Relative Percent Composition of Major Phospholipids[1]		
		PC	PE	SM
WT	34°C, FCS-M	51	33	16
Rc	34°C, FCS-M	52	27	21
$C^{R}7$	34°C, FCS-M	47	29	23
WT	37°C, FCS-M	51	34	15
Rc	37°C, FCS-M	54	26	20
$C^{R}7$	37°C, FCS-M	50	34	16
WT	39°C, FCS-M	50	27	23
Rc	39°C, FCS-M	52	30	18
$C^{R}7$	39°C, FCS-M 24 h	50	33	16
$C^{R}7$	39°C, FCS-M 96 h	52	33	15

[1]PC = phosphatidylcholine, PE = phosphatidylethanolamine and SM = sphingomyelin

Table 10: Analysis of the relative percent composition of the major phospholipids of WT, $C^{R}7$ and Rc cell lines, for cultures grown at 34°C, 37°C and 39°C in LPDS-M. The growth conditions employed are the same as those described for Tables 1, 2 and 3. Total lipid extracts of cells were prepared by the method of Bligh and Dyer, (1959). An aliquot was chromatographed using a solvent system of chloroform-methanol-acetic acid-water (25:15:4:2 v/v/v/v). Suitable standards were also chromatographed (see Methods and Materials). "Zinzadze" reagent positive material was qualitatively identified and the phospholipid was extracted using methanol-chloroform. The lipid phosphorus was assayed as described previously.

Cell Type	Growth Conditions	Relative Percent Composition of Major Phospholipids[1]		
		PC	PE	SM
WT	34°C, LPDS-M 24 h	48	26	26
Rc	34°C, LPDS-M 24 h	47	31	22
C^R_7	34°C, LPDS-M 24 h	45	30	24
WT	37°C, LPDS-M 24 h	48	26	26
Rc	37°C, LPDS-M 24 h	43	32	25
C^R_7	37°C, LPDS-M 24 h	47	24	29
WT	39°C, LPDS-M 12 h	43	38	19
WT	39°C, LPDS-M 24 h	47	28	25
WT	39°C, LPDS-M 48 h	44	31	24
Rc	39°C, LPDS-M 48 h	50	32	18
C^R_7	39°C, LPDS-M 12 h	52	33	15
C^R_7	39°C, LPDS-M 24 h	43	33	24
C^R_7	39°C, LPDS-M 48 h	43	34	23

[1]PC = phosphatidylcholine, PE = phosphatidylethanolamine and SM = sphingomyelin

exhibited a temperature-sensitive growth pattern. Furthermore, this temperature-sensitive behavior became even more pronounced when C^R7 was grown in the presence of LPDS-M (Figures 17 and 19). WT grows well at 39°C regardless of cholesterol status of the medium doing well on either FCS-M or LPDS-M. The extent to which C^R7 grows on LPDS-M was shown to be directly related to the cholesterol content of the medium (Figure 18). As the amount of LPDS-M present was increased (and consequently the cholesterol content of the medium decreased) the inadequacy of C^R7 growth response was directly related to this (Figure 18).

In order to determine whether or not C^R7 was depleting at an accelerated rate, relative to WT and Rc, a component of the medium necessary for its viability and growth, the medium was replenished with fresh FCS-M and fresh LPDS-M at regular intervals. The addition of fresh FCS-M and fresh LPDS-M to C^R7 cell cultures at 39°C did not alter the temperature-sensitive growth response observed (Figure 17).

Furthermore, Figure 19 shows the behaviour of WT and C^R7 at 39°C. For WT cells, growth was normal in either FCS-M or LPDS-M, but C^R7 failed to proliferate after 48 h at 39°C regardless of which medium was employed. The addition of cholesterol to LPDS-M did not relieve temperature-sensitivity in C^R7, but merely allowed the variant to perform similarly to its behaviour in FCS-M (Figure 19). The concentration of added cholesterol was 13 μM. This amount of cholesterol was sufficient to allow C^R7 to grow "normally" on LPDS-M at 34°C (Figure 16). Thus temperature-sensitive growth and cholesterol auxotrophy are apparently not directly related.

The temperature-sensitivity of the mutant cell line at 39°C becomes

Figure 17: Effect of medium replacement on temperature sensitivity of C^R7 at 39°C. Cells were seeded in 60 mm dishes containing 5 ml FCS-M and incubated 24 h at 34°C. Cell numbers were then estimated and the values designated as zero time counts. The medium was replaced with 5 ml of FCS-M or LPDS-M as appropriate and incubation was continued a further 96 h at 39°C. During this "96 h incubation" period, some C^R7 cultures received at appropriate regular intervals fresh FCS-M and fresh LPDS-M. Cell numbers were evaluated, in triplicate, periodically.

Figure Legend: WT, FCS-M (); WT, LPDS-M (); C^R7, FCS-M (); C^R7, LPDS-M (); C^R7, regularly replaced fresh FCS-M (); C^R7, regularly replaced fresh LPDS-M, the result was the same as C^R7, LPDS-M (). Standard deviations were 1-3% of the mean values shown.

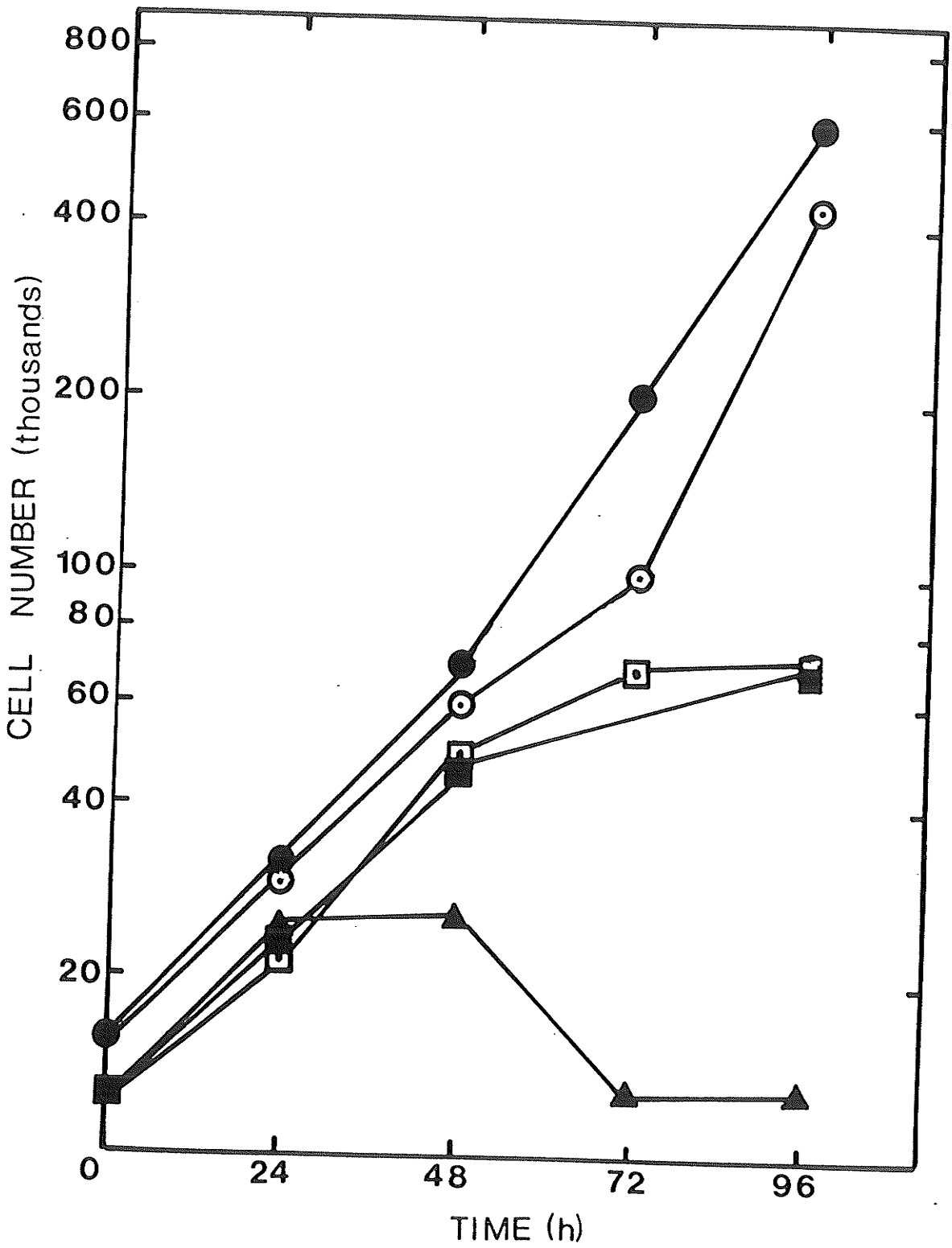

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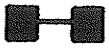
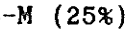
Figure 18: Effect of degree of cholesterol deficiency on C^R7 temperature-sensitive response at 39°C. WT and C^R7 (10,000 cells) were delivered to 60 mm dishes containing 5 ml FCS-M and incubated 24 h at 34°C. Cell numbers were then estimated and the values designated as zero time counts. The medium was then replaced with fresh FCS-M or LPDS-M as appropriate and in the desired proportions. Incubation was then continued a further 96 h at 39°C. Cell numbers were evaluated, in triplicate, periodically.

Figure Legend: WT, FCS-M (100%) (); C^R7, FCS-M (100%), (); C^R7, FCS-M (75%) + LPDS-M (25%), (); C^R7, FCS-M (50%) + LPDS-M (50%) (); C^R7, FCS-M (25%) + LPDS-M (75%) (); C^R7, LPDS-M (100%) (). Standard deviations were 2-4% of the mean values shown.

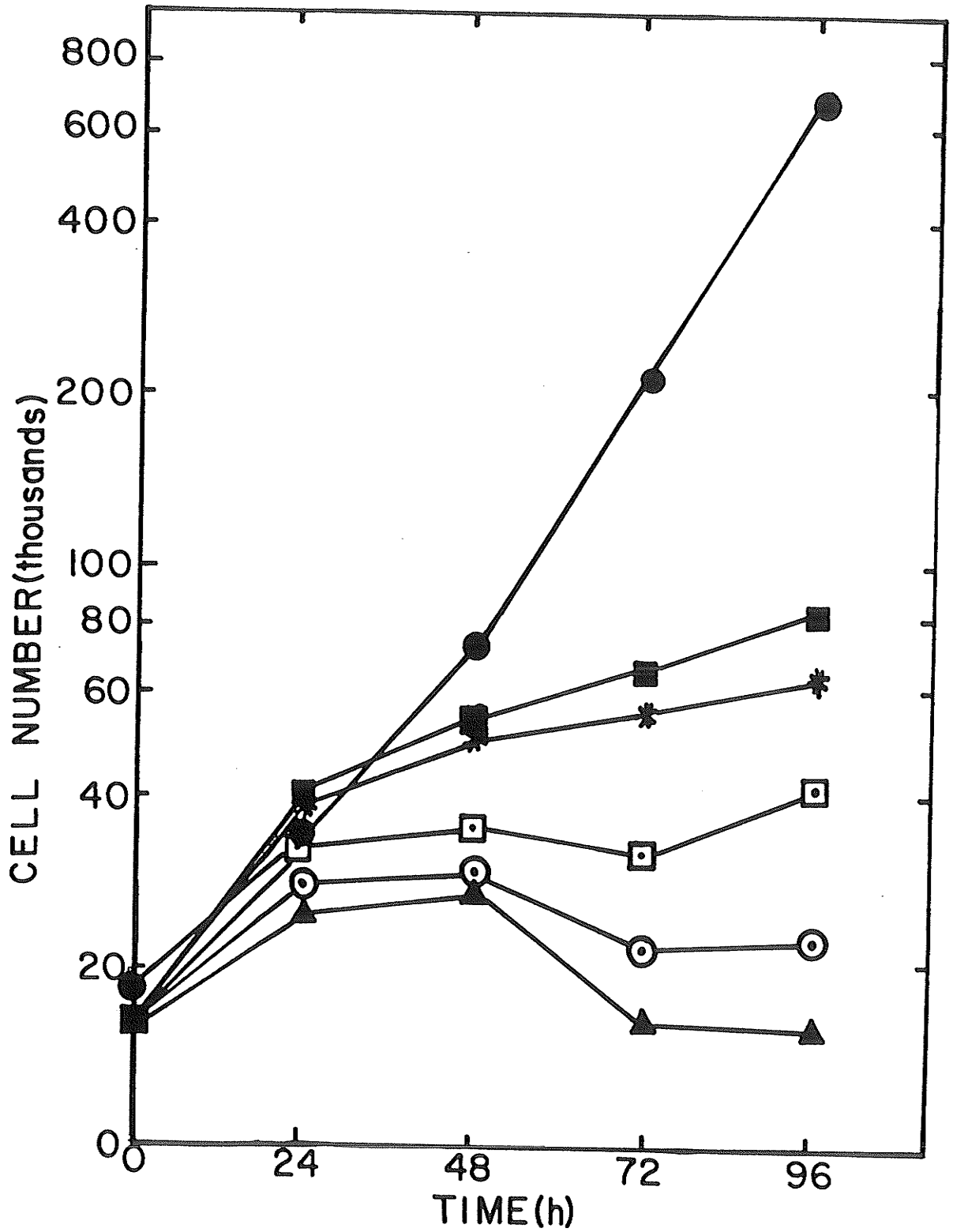

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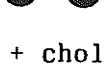
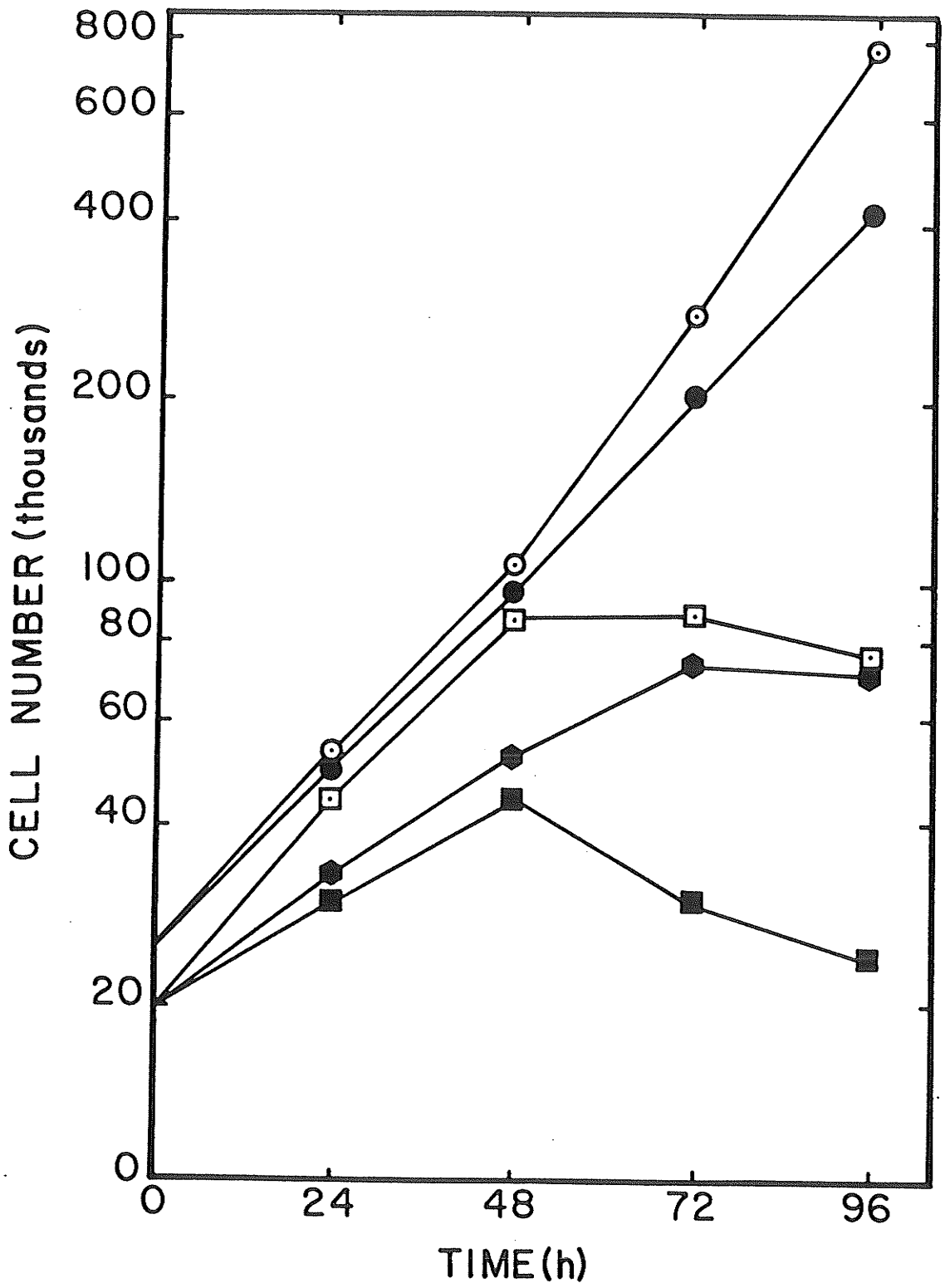
Figure 19: Temperature-Sensitivity and Cholesterol Auxotrophy. WT and $C^{R}7$ (10,000 cells) cells were seeded in 60 mm dishes containing 5 ml FCS-M and incubated 24 h at 34°C. Cell numbers were then estimated and the values designated as zero time counts. The medium was replaced with 5 ml of FCS-M or LPDS-M as appropriate with or without additions and the incubation was continued a further 96 h at 39°C. Cell numbers were evaluated, in triplicate, periodically. Cholesterol (13 μM) was added as a BSA-ethanol complex with the final concentration of ethanol in the medium being less than 1%. Control experiments showed that BSA-ethanol mixture without cholesterol had no effect on growth (see Figure 25).

Figure Legend: WT, FCS-M (); WT, LPDS-M (); $C^{R}7$, FCS-M (); $C^{R}7$, LPDS-M () and $C^{R}7$, LPDS-M + cholesterol (). Standard deviations were 1-3% of the mean values shown.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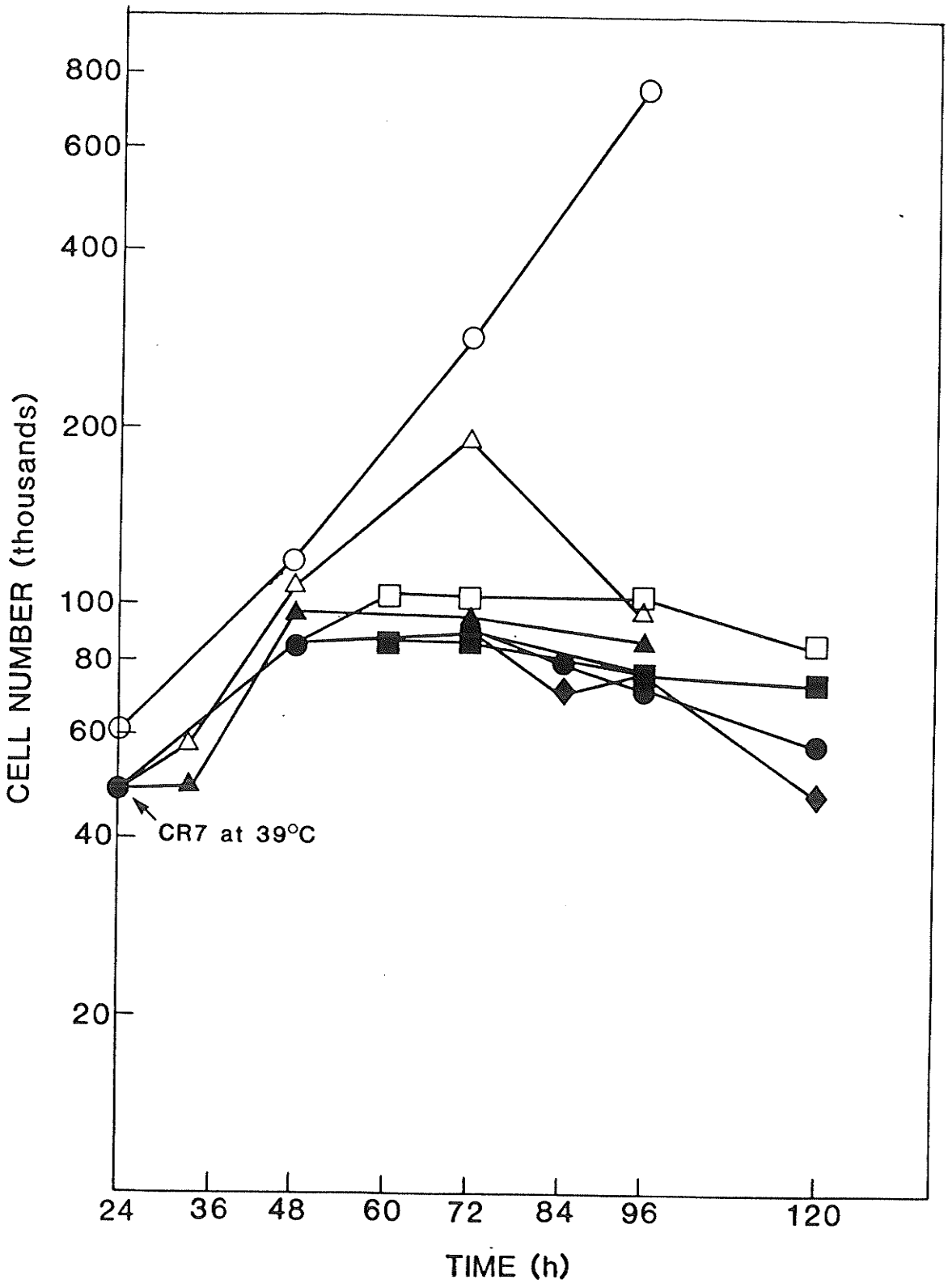
apparent some time after 24 hours incubation at the nonpermissive temperature and the effect is definitely present at 48 hours incubation and subsequent to that. What then is the effect, if any, of transferring C^R7 cells to the permissive temperatures of 34°C and 37°C following 24, 48, and 72 hours incubation at 39°C, the nonpermissive temperature? Temperature-sensitive behaviour, following culturing at 39°C, was not abolished following transfer to a permissive temperature (Figure 20) although there was some recovery in cultures returned to 34°C after short times (up to 24 h) at 39°C. With increased incubation time at 39°C prior to transfer to 34°C and to 37°C, less recovery of growth was seen (Figure 20). This may indicate that with increased exposure to 39°C, cell damage increased or the lesion(s) in C^R7 responsible for temperature-sensitivity became critical. Figure 20 also shows that C^R7 did better when returned to 34°C than at 37°C during the course of these experiments. At 37°C, cell cultures were approaching closer to the nonpermissive temperature. As a result, variant cell cultures performed less favourably due to the temperature induced cell damage already present and to the defect(s) associated with C^R7 becoming lethal.

Relief of Temperature-Sensitivity

Previous work in the laboratory has shown that C^R7 exhibits abnormalities in the metabolism of lipids which greatly influence membrane properties (Borgford et al, 1984). Further previous work in the laboratory has shown that C^R7 is defective in LDL-mediated regulation of HMG-CoA reductase, a key enzyme in the polyisoprenoid pathway (Borgford et al, 1984).

Figure 20: Growth of C^R7 at 34°C and 37°C following exposure to 39°C, the nonpermissive temperature. Cells (10,000 per plate) were seeded in 60 mm dishes containing 5 ml FCS-M and incubated 24 h at 34. Cell numbers were then estimated and the value (C^R7 - 25,173 cells) designated as the zero time count. The medium was replaced with 5 ml of FCS-M and incubation continued at 34°C (a control) and at 39°C. Following appropriate incubation at 39°C, cell cultures were returned to both 34°C and to 37°C. Cell numbers were evaluated, in triplicate, periodically. A control experiment revealed that C^R7 cell cultures incubated continuously at 39°C exhibited temperature-sensitive growth (data not shown).

Figure Legend: C^R7, FCS-M, 34°C (); C^R7, FCS-M, 24 h at 39°C prior to transfer back to 34°C, (); C^R7, FCS-M, 24 h at 39°C prior to transfer back to 37°C, (); C^R7, FCS-M, 48 h at 39°C prior to transfer back to 34°C, (); C^R7, FCS-M, 48 h at 39°C prior to transfer to 37°C, (); C^R7, FCS-M, 72 h at 39°C prior to transfer back to 34°C, () and C^R7, FCS-M, 72 h at 39°C prior to transfer back to 37°C, ().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It was reasoned, therefore, that temperature-sensitivity may be related to a non-sterol product of the polyisoprenoid pathway. Figure 21 demonstrates that C^R7 grew normally at 39°C when FCS-M was supplemented with mevalonolactone (MVA-lactone) (77 μM). Cholesterol (13 μM) had no such effect and did not influence the ability of MVA-lactone to relieve temperature-sensitivity (Figure 21). This is consistent with the knowledge that FCS-M contains substantial amounts of cholesterol, (130 μM) (Borgford et al, 1984). Figure 22 shows that, in order to relieve temperature-sensitivity of C^R7 at 39°C on LPDS-M the administration of both MVA-lactone (77 μM) and cholesterol (13 μM) in combination is required. MVA-lactone alone when delivered to C^R7 cultured on LPDS-M returned C^R7's growth response to slightly better than that on FCS-M. However, cholesterol addition to C^R7 cells on LPDS-M, merely relieved the cholesterol deficiency on LPDS-M (Figure 22). Although the administration of MVA-lactone and cholesterol in combination to C^R7 cell cultures in LPDS-M relieved temperature-sensitivity, this relief did not equal the ability of MVA-lactone or MVA-lactone in combination with cholesterol to relieve temperature-sensitivity of C^R7 on FCS-M. Due to the complex nature of the cellular response seen on LPDS-M, it is postulated that some other factor, whose identity is unknown, is required to achieve complete relief of temperature-sensitivity of C^R7 seen on LPDS-M (that is, equivalent to that seen on FCS-M).

Figure 23 shows that, at 34°C, MVA-lactone did not allow C^R7 to grow on LPDS-M. The addition of cholesterol was necessary to allow this. These data suggest that a metabolite of MVA-lactone, other than

Figure 21: Relief of temperature-sensitivity of C^R7 by MVA-lactone. WTand C^R7 (10,000 cells per plate) were seeded in 60 mm dishes containing 5 ml FCS-M and incubated 24 h at 34°C. Cell numbers were then estimated and the values designated as zero time counts. The medium was then replaced with 5 ml of FCS-M with or without additions and the incubation was continued a further 96 h at 39°C. Cell numbers were evaluated, in triplicate, periodically.

Figure Legend: WT, FCS-M (); C^R7, FCS-M (); C^R7, FCS-M + cholesterol (13 μM) (); C^R7 FCS-M + MVA lactone (77 μM) (); C^R7, FCS-M + MVA lactone (77 μM) + cholesterol (13 μM) (). Cholesterol (13 μM) and MVA lactone (77 μM) were added as a BSA-ethanol complex and as an ethanolic solution respectively, with final ethanol concentration in medium being less than 1%. Standard deviations were 1-3% of the mean values shown.

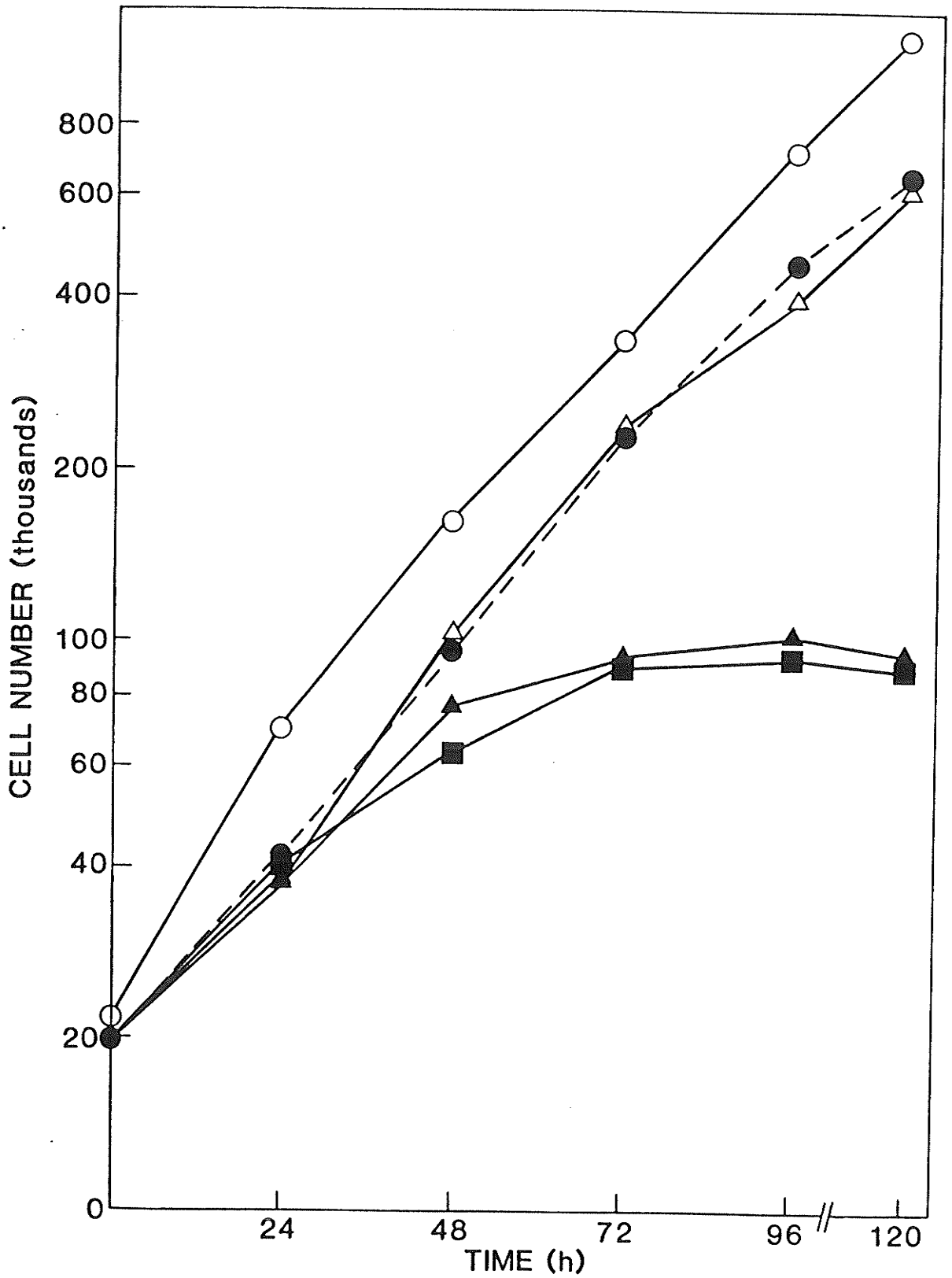
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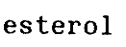
Figure 22: Effect of MVA-lactone addition on Temperature Sensitivity of C^R7 on LPDS-M. WT and C^R7 (10,000 cells) per plate) were seeded in 60 mm dishes containing 5 ml FCS-M and incubated 24 h at 34°C. Cell numbers were then estimated and the values designated at zero time counts. The medium was then replaced with 5 ml of FCS-M or LPDS-M as appropriate with or without additions and the incubation continued a further 96 h at 39°C. Cell numbers were evaluated, in triplicate, periodically. Cholesterol (13 μM) and MVA-lactone (77 μM) were added as BSA-ethanol complexes, with the final concentration of ethanol in the medium being less than 1%. Control experiments showed that BSA-ethanol mixture alone had no effect on growth (see Figure 25).

Figure Legend: WT, FCS-M (); C^R7, FCS-M (); C^R7, LPDS-M (); C^R7, LPDS-M + cholesterol (13 μM) (); C^R7, LPDS-M + MVA lactone (77 μM) (); C^R7, LPDS-M + MVA lactone (77 μM) + cholesterol (13 μM) (). Standard deviations were 1-3% of the mean values shown.

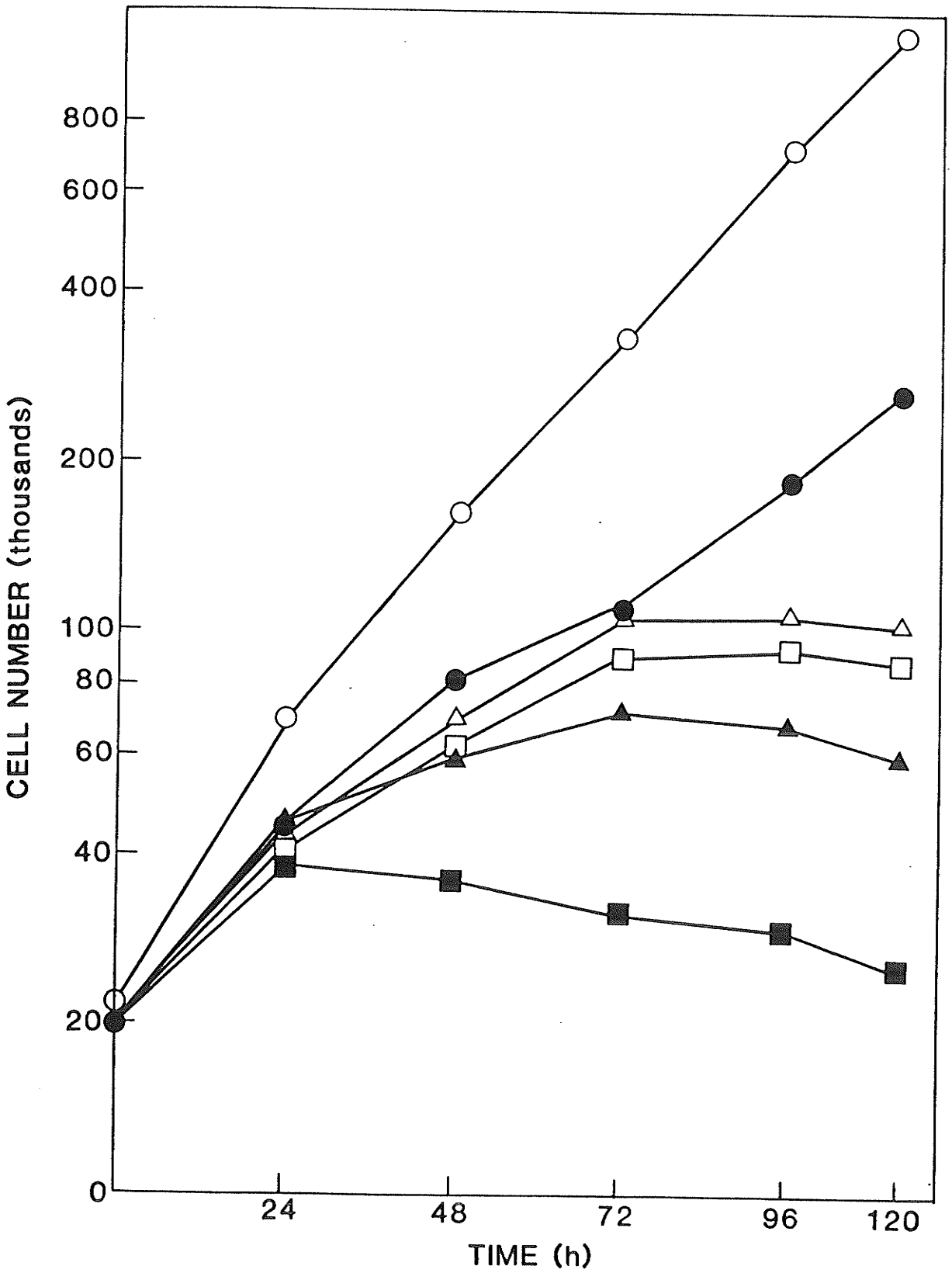

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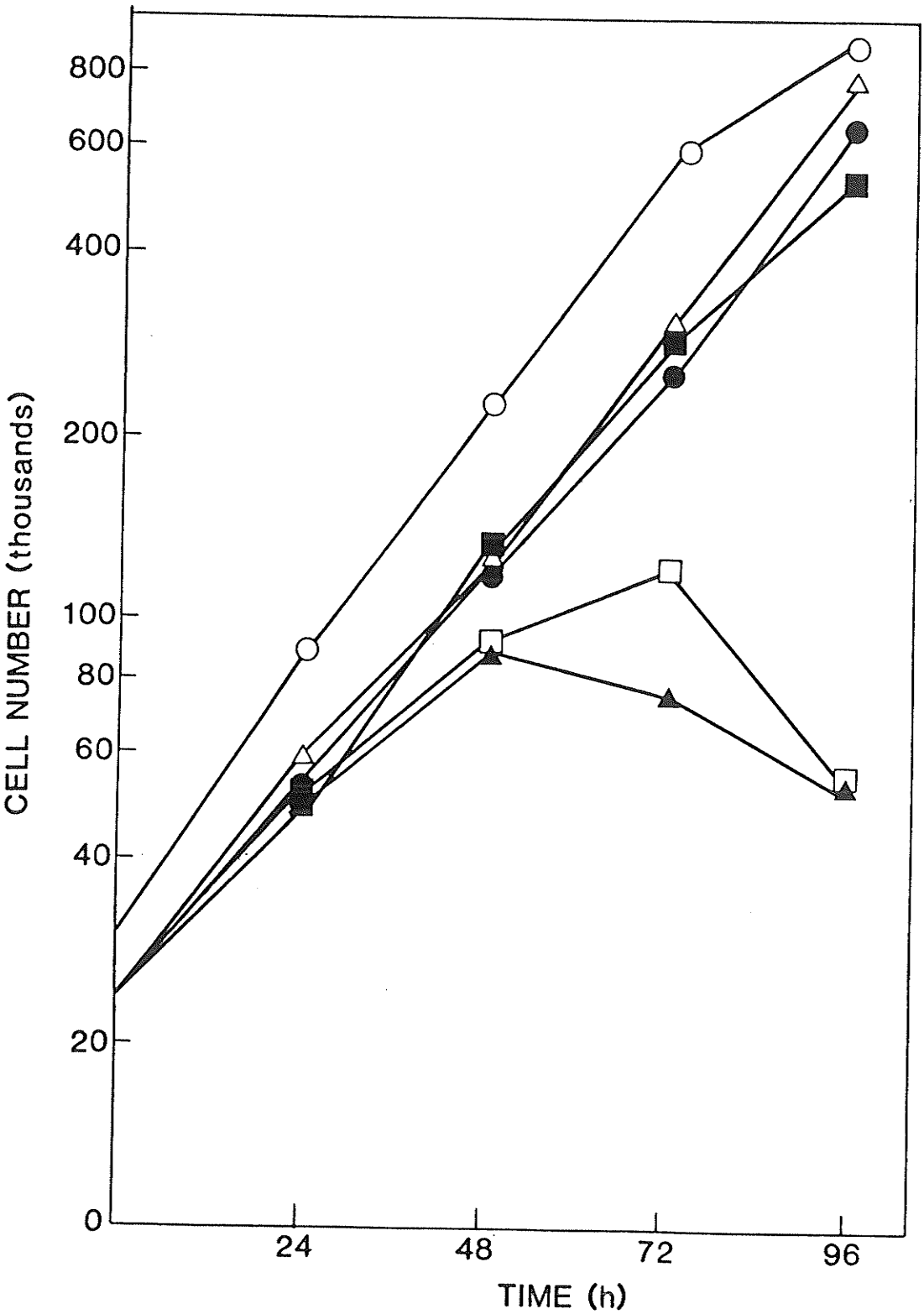
Figure 23: Failure of MVA lactone to support growth of C^R7 at 34°C onLPDS-M. WT and C^R7 cells (10,000 cells per plate) were seeded in 60 mm dishes containing 5 ml of FCS-M and incubated 24 h at 34°C. Cell numbers were then estimated and the values designated as zero time counts. The medium was replaced with 5 ml of FCS-M or LPDS-M as appropriate with or without additions and incubation was continued a further 96 h at 34°C. Cell numbers were evaluated, in triplicate, periodically. MVA-lactone (77 μM) and cholesterol (13 μM) were added as BSA-ethanol complexes with the final concentration of ethanol in the medium being less than 1%. Control experiments showed that the BSA-ethanol mixture without additions had no effect on growth.

Figure Legend: WT, FCS-M (); C^R7, FCS-M (); C^R7, LPDS-M (); C^R7, LPDS-M + MVA lactone (77 μM) (); C^R7, LPDS-M + cholesterol (13 μM) () and C^R7, LPDS-M + MVA-lactone (77 μM) + cholesterol (13 μM) (). Standard deviations were 1-3% of the mean values shown.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cholesterol, is required to relieve temperature-sensitivity. Furthermore, such relief is possible only when the growth medium contains cholesterol in amounts known to be adequate for growth at the permissive temperature (see Figures 16 and 23).

Accordingly, metabolites of MVA-lactone were tested to examine their ability to relieve the temperature-sensitivity of C^R7. Lanosterol, (46 μM); squalene, (12 μM); ubiquinone, (12 μM), and isopentenyladenine (19 μM) were unable to relieve temperature-sensitivity (Figure 24). Only dolichol (12 μM) or dolichyl-phosphate (12 μM) could relieve temperature-sensitivity of C^R7 on FCS-M. Figure 25 shows that either dolichol or dolichyl-phosphate could replace MVA-lactone in allowing C^R7 to grow in FCS-M. However, the addition of cholesterol (13 μM) together with either dolichol or dolichyl-phosphate was necessary to allow growth at 39°C in LPDS-M (Figure 26) although the growth rate did not equal that reached in FCS-M (Figure 25). Some other factor, of unknown identity, must be necessary to allow this to occur. Furthermore, dolichol or dolichyl-phosphate, like MVA-lactone (Figure 23), when administered to C^R7 cell cultures growing on LPDS-M at 34°C, was unable to restore normal growth (Figure 27). Cholesterol addition was an absolute requirement to allow normal growth of C^R7 on LPDS-M at 34°C, the permissive temperature for growth (Figure 27).

Based on this information, it is suggested that the requirement of cholesterol is associated with "growth" and that the requirement for MVA lactone or its metabolites, dolichol and dolichyl-phosphate, is more closely associated with C^R7's temperature-sensitivity.

Figure 24: Failure of some MVA-lactone metabolites to relieve temperature sensitivity of C^R7. Cells were grown and evaluated exactly as described in the legend to Figure 21.

Figure Legend: WT, FCS-M (); C^R7, FCS-M (); C^R7, FCS-M + isopentenyladenine (19 μM) (); C^R7, FCS-M + ubiquinone (12 μM) (); C^R7, FCS-M + squalene (12 μM) (); C^R7, FCS-M + lanosterol (46 μM) ().

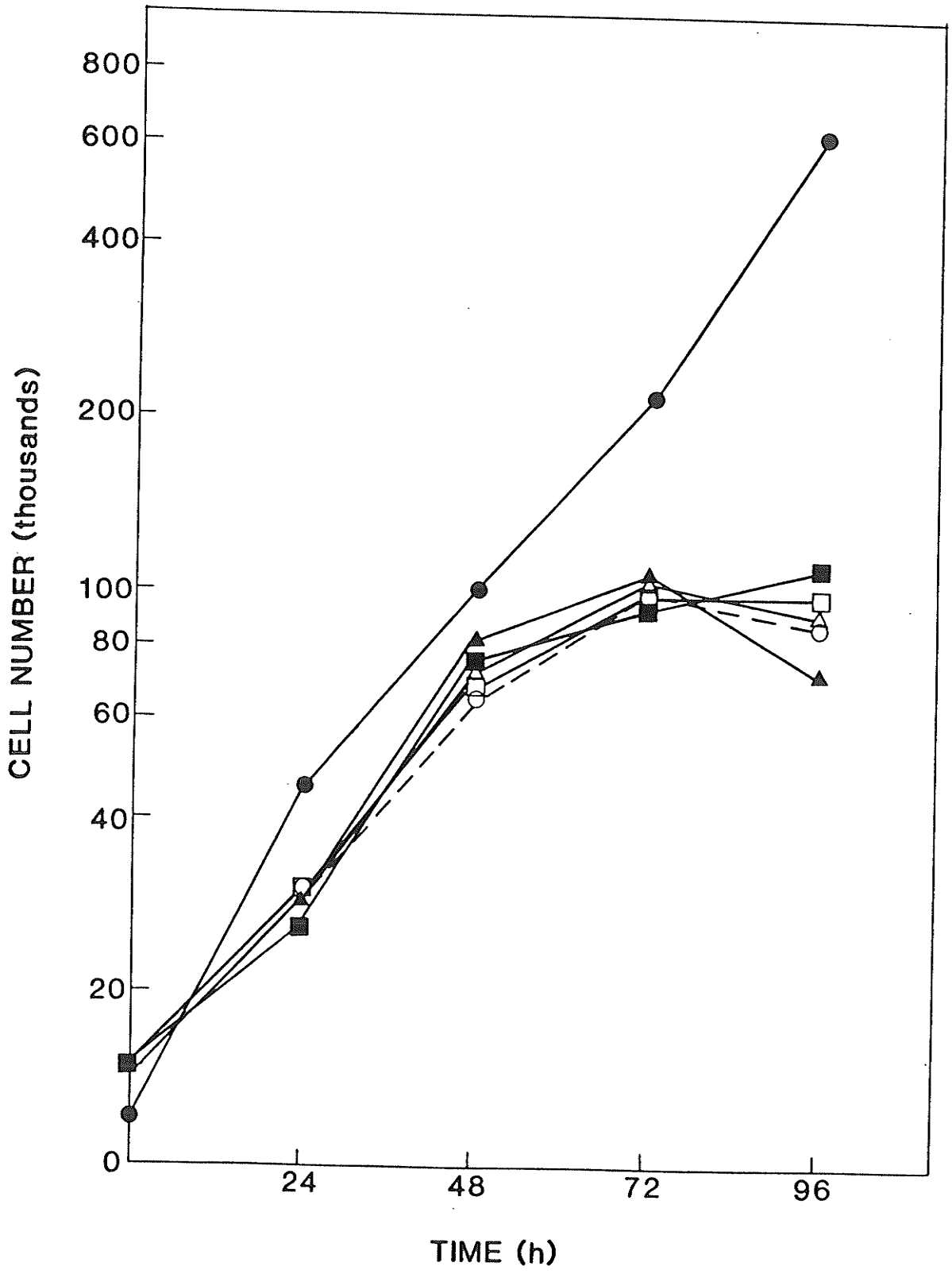
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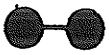
Figure 25: Relief of temperature-sensitivity of $C^{R}7$ in FCS-M by dolichol and dolichyl-phosphate. Cells were grown and evaluated exactly as described in the legend to Figure 21.

Figure Legend: WT, FCS-M (); $C^{R}7$, FCS-M (); $C^{R}7$, FCS-M + BSA-ethanol carrier (); $C^{R}7$, FCS-M + dolichol (12 μM) (); $C^{R}7$, FCS-M + dolichyl-phosphate (12μM) (). Standard deviations were 1-3% of the mean values shown.

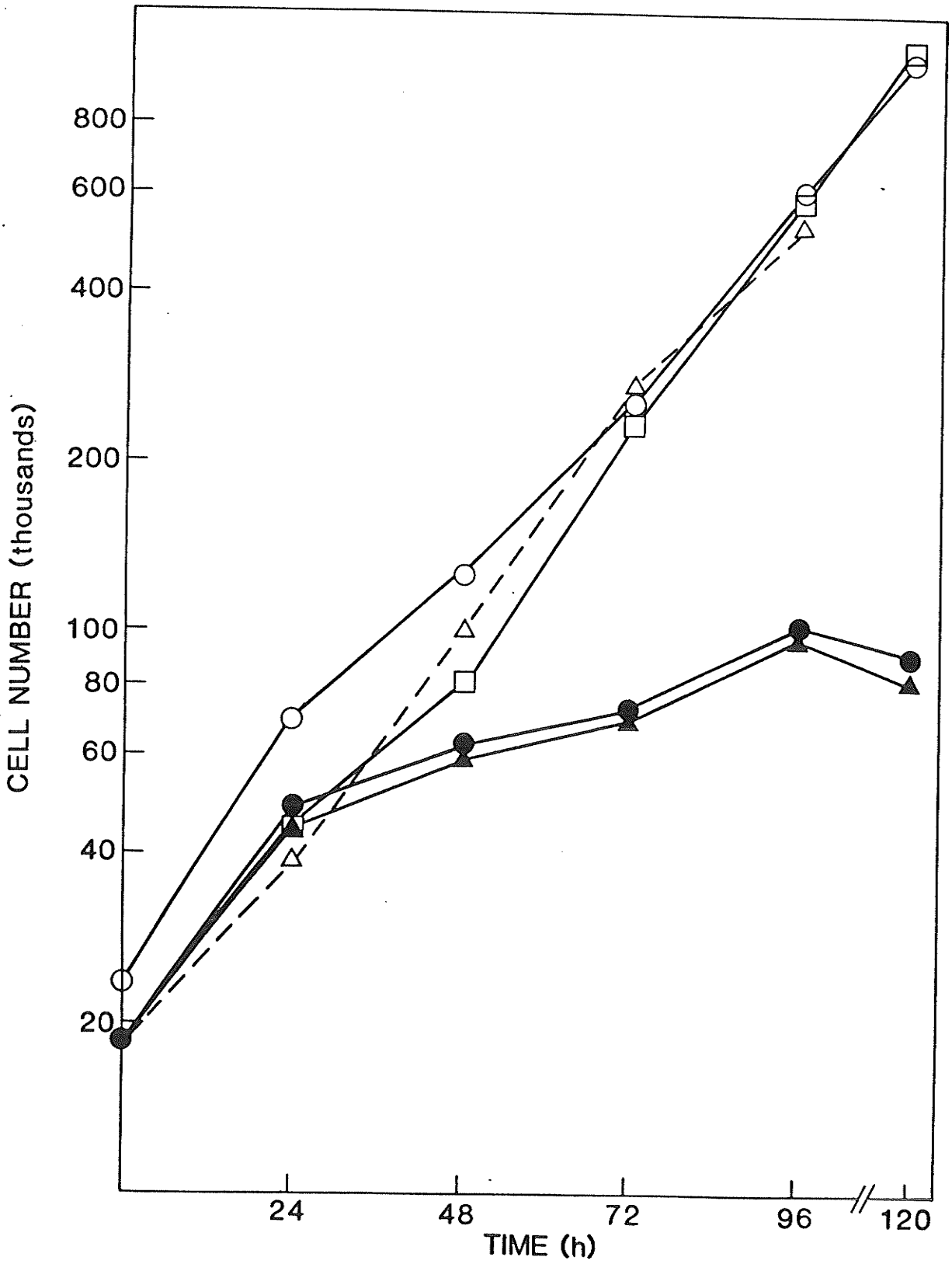

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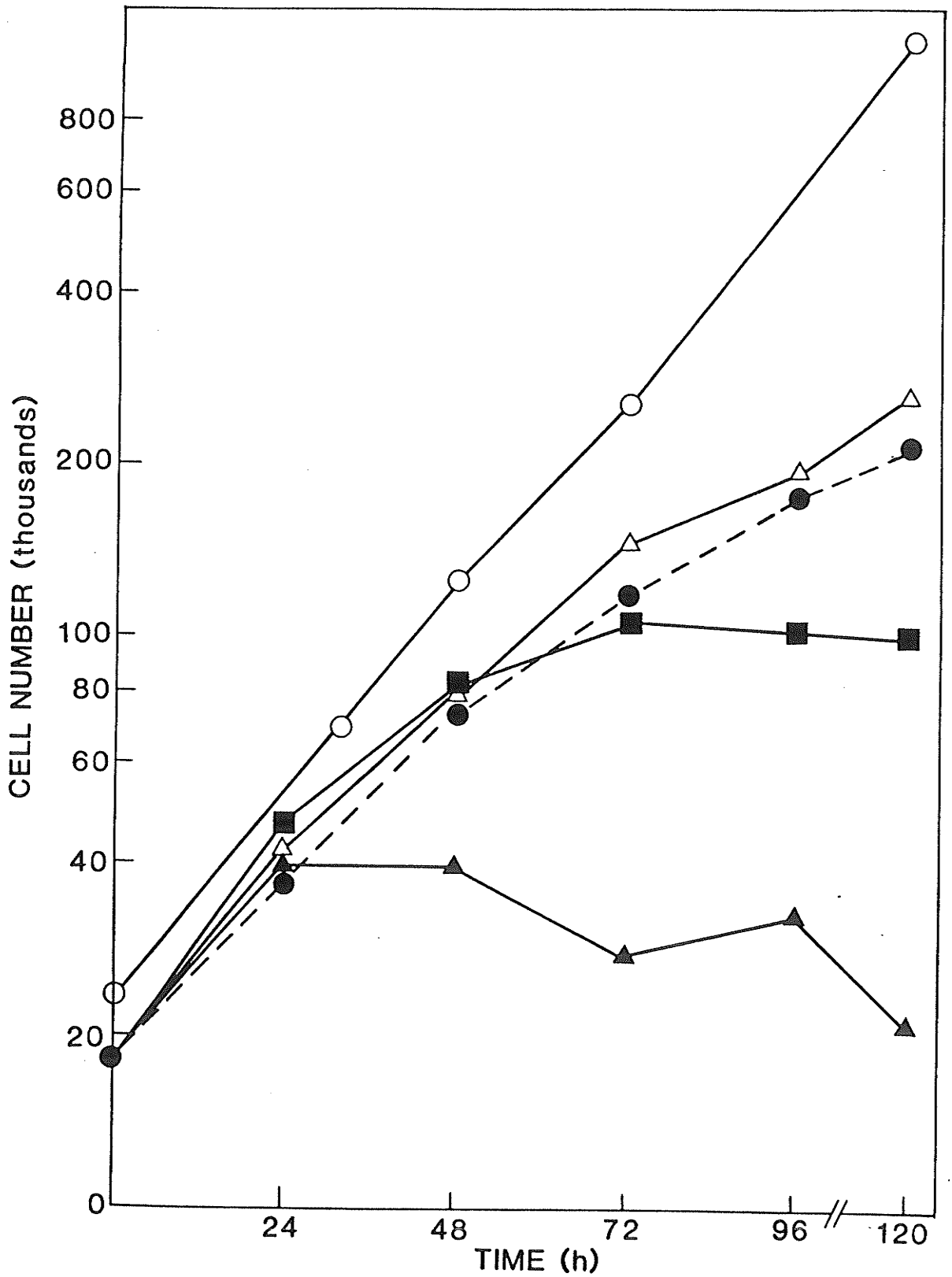
Figure 26: Relief of temperature-sensitivity of $C^{R}7$ in LPDS-M by dolichol or dolichyl-phosphate plus cholesterol. Cells were grown and evaluated exactly as described in Figure 21.

Figure Legend: WT, LPDS-M (); $C^{R}7$, LPDS-M (); $C^{R}7$, LPDS-M + dolichol (12 μM) (); $C^{R}7$, LPDS-M + dolichol (12 μM) + cholesterol (13 μM) (); $C^{R}7$, LPDS-M + dolichyl-phosphate (12 μM) + cholesterol (13 μM) (). Standard deviations were 1-3% of the mean values shown.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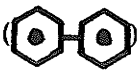
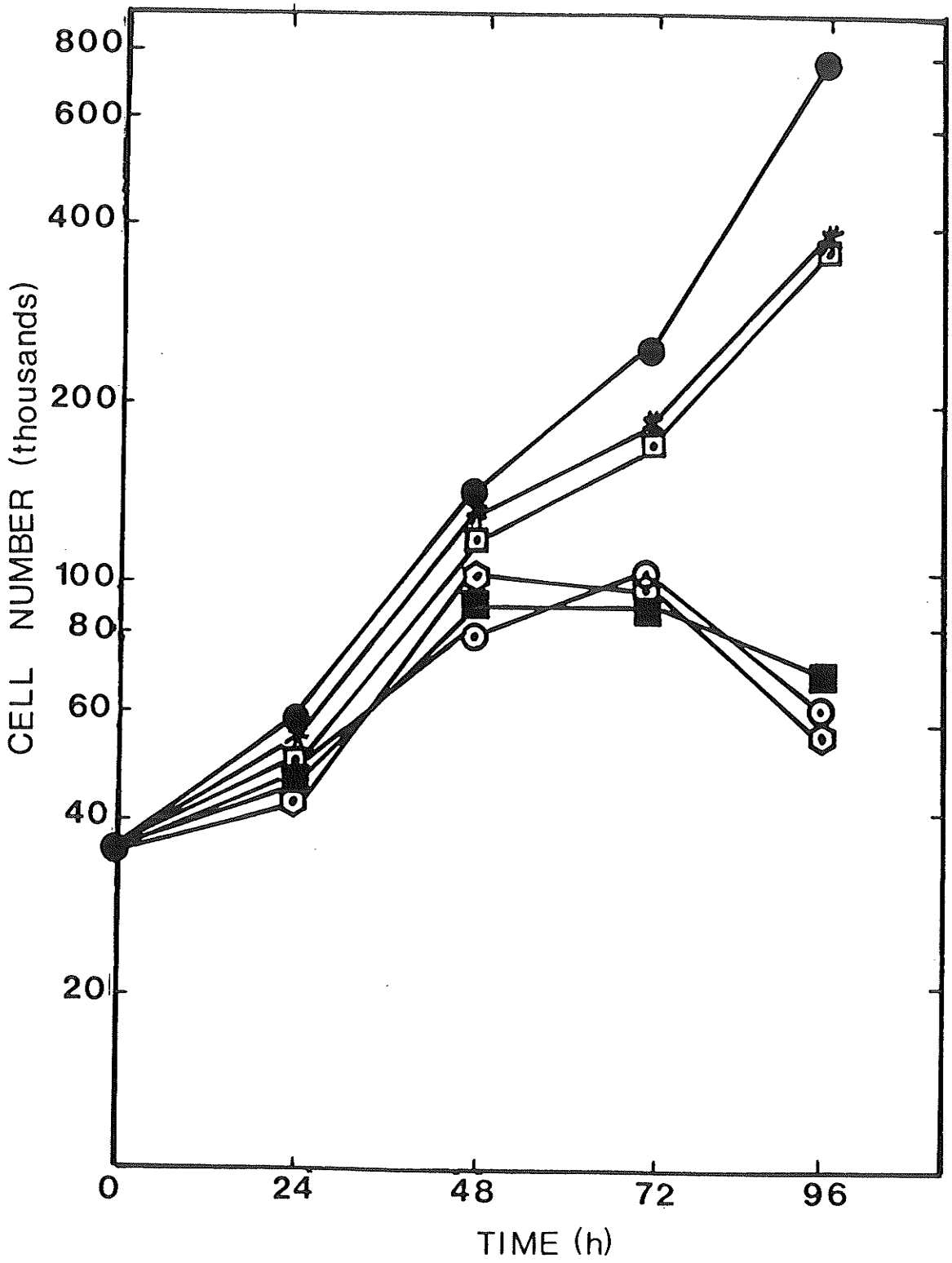
TIME (h)

Figure 27: Failure of dolichol/dolichyl-phosphate to support growth of C^R7 at 34°C on LPDS-M. Cells were grown and evaluated exactly as described in Figure 23.

Figure Legend: C^R7, FCS-M (); C^R7, LPDS-M (); C^R7, LPDS-M + dolichol (12 μM) (); C^R7, LPDS-M + dolichyl-phosphate (12 μM) (); C^R7, LPDS-M + cholesterol (13 μM) (); C^R7, LPDS-M + cholesterol (13 μM) + dolichol (12 μM) (). Standard deviations were 1-3% of the mean values shown.


800
600
400
200
100
80
60
40
20
CELL NUMBER (thousands)
0
24
48
72
96
TIME (h)

Examination of Cellular Levels of Dolichol

The finding that dolichol or dolichyl-phosphate relieved temperature-sensitivity, coupled with the knowledge that both C^R7 and the closely related cell B211 display abnormalities in dolichol metabolism, (Wright et al, 1979; Krag, 1979) prompted a further investigation to measure dolichol levels of WT and C^R7 at permissive and nonpermissive temperatures. Previous efforts within the laboratory to measure cellular dolichol levels in C^R7 had proven unsuccessful (Borgford et al, 1986) simply because an insufficient mass of cells was used. The results shown in Table 11 were obtained using a much larger quantity of cells. At 34°C dolichol levels in WT were the same in FCS-M and LPDS-M. C^R7 had a similar level of dolichol as WT when grown in FCS-M, but after 24 hour exposure to LPDS-M the dolichol level decreased by 63%. This presumably reflects the fact that LPDS-M does not support growth of C^R7. At 39°C, the dolichol levels in WT were three times higher than at 34°C. Neither the growth medium nor the time of exposure at 39°C affected these levels. Exposure of C^R7 to 39°C for 36 hours in FCS-M produced dolichol levels five-fold higher than at 34°C and about double the level in WT under the same conditions. After 48 hours in FCS-M at 39°C the levels in C^R7 declined by one-half perhaps reflecting the failure of the cells to grow at the non-permissive temperature. In LPDS-M at 39°C, no dolichol was detectable in C^R7. This is consistent with the complete failure to proliferate under these conditions illustrated in Figures 22 and 26. Furthermore, it should be noted that after 36-48 hours at 39°C or 24-36 hours in LPDS-M, C^R7 cells begin to detach from the substratum as growth slows and ceases. Measurements

Table 11: Cellular levels of dolichol in C^R_7 and WT cells. WT and C^R_7 cells were initially grown in FCS-M at 34°C for an appropriate period of time. At this point cells were subconfluent. The medium was then changed to fresh FCS-M or LPDS-M and the cells were incubated at 34°C or 39°C for the times indicated. Dolichol was determined by HPLC. Procedural losses were corrected using an internal standard of ^{3}H-dolichol (60,000 dpm). Recoveries averaged 75%. Values shown are means and standard deviations for 3-7 determinations.

Cell Line	Growth Medium	Temperature	Dolichol Content (μg/g packed cells)
WT	FCS-M	34°C	0.73 ± 0.02
C^R7	FCS-M	34°C	0.84 ± 0.09
WT	LPDS-M	34°C (24 h)	0.83 ± 0.04
C^R7	LPDS-M	34°C (24 h)	0.31 ± 0.02
WT	FCS-M	39°C (36 h)	2.32 ± 0.01
C^R7	FCS-M	39°C (36 h)	4.59 ± 0.15
WT	FCS-M	39°C (48 h)	2.12 ± 0.02
C^R7	FCS-M	39°C (48 h)	1.81 ± 0.04
WT	LPDS-M	39°C (36 h)	2.52 ± 0.03
C^R7	LPDS-M	39°C (36 h)	Undetectable

were not made beyond these times to avoid the possibility that any changes seen in dolichol levels might be the result of the poor conditions of the cells rather than preceding it.

Dolichol as isolated by the conventional lipid extraction procedures used in these studies consists of a mixture of isoprenologues with varying chain length. Therefore, an examination of the actual dolichol profiles which resulted from HPLC analysis was done. Little to no change was found in the WT dolichol profile regardless of growth conditions (Figures 28-32 inclusive). This was not so for C^R7. The dolichol profiles of C^R7 changed (Figures 33-36 inclusive). The changes manifested themselves as changes in the isoprenologue distribution within the dolichol profile observed (see Figures 33-36 inclusive). Because of the way this investigation was done, it is tempting to speculate that these changes observed in C^R7 dolichol profiles are a direct result of temperature stress. On examination of the isoprenologues present differences were noted. WT had a dolichol profile with four peaks corresponding to isoprenologues with retention times (in minutes) of 8.1, 8.6, 9.3, and 10 respectively (Table 12). On the other hand, C^R7 had a dolichol profile of four isoprenologues with retention times (in minutes) of 8.5, 9.3, 10 and 11 respectively (Table 13). The WT type dolichol profile had no detectable isoprenologue with a retention time of 11 minutes while C^R7 lacked any detectable isoprenologue with a retention time of 8.1 minutes. Figure 36a is a chromatogram of the [^{3}H] labeled dolichol internal standard used in this study to monitor recoveries.

An examination of the contribution of each of these isoprenologues

Figure 28: Profile of dolichol content. Dolichol content was determined as described in Methods and Materials. Presented here is a chromatogram at λ = 210 nm of the dolichol profile as determined by HPLC analysis of WT cells on FCS-M at 34°C.

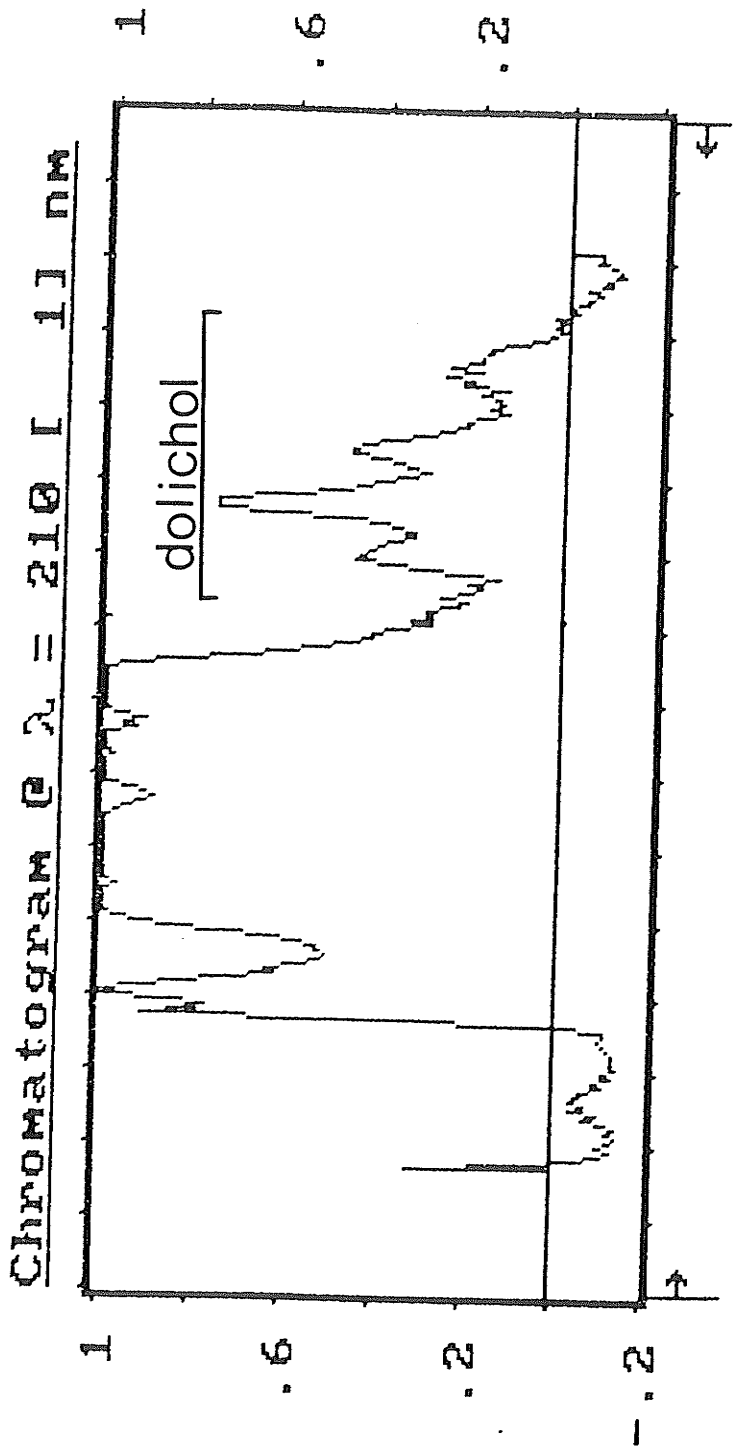

Chromatogram @ λ = 210 [11 nm
dolichol
1
6.
2.
1
6.
2.
2.
1.

Figure 29: Profile of dolichol content. Dolichol content was determined as described in Methods and Materials. Presented here is a chromatogram at λ = 210 nm of the dolichol profile of WT as determined by HPLC analysis of WT cells on LPDS-M (24 h) at 34°C.

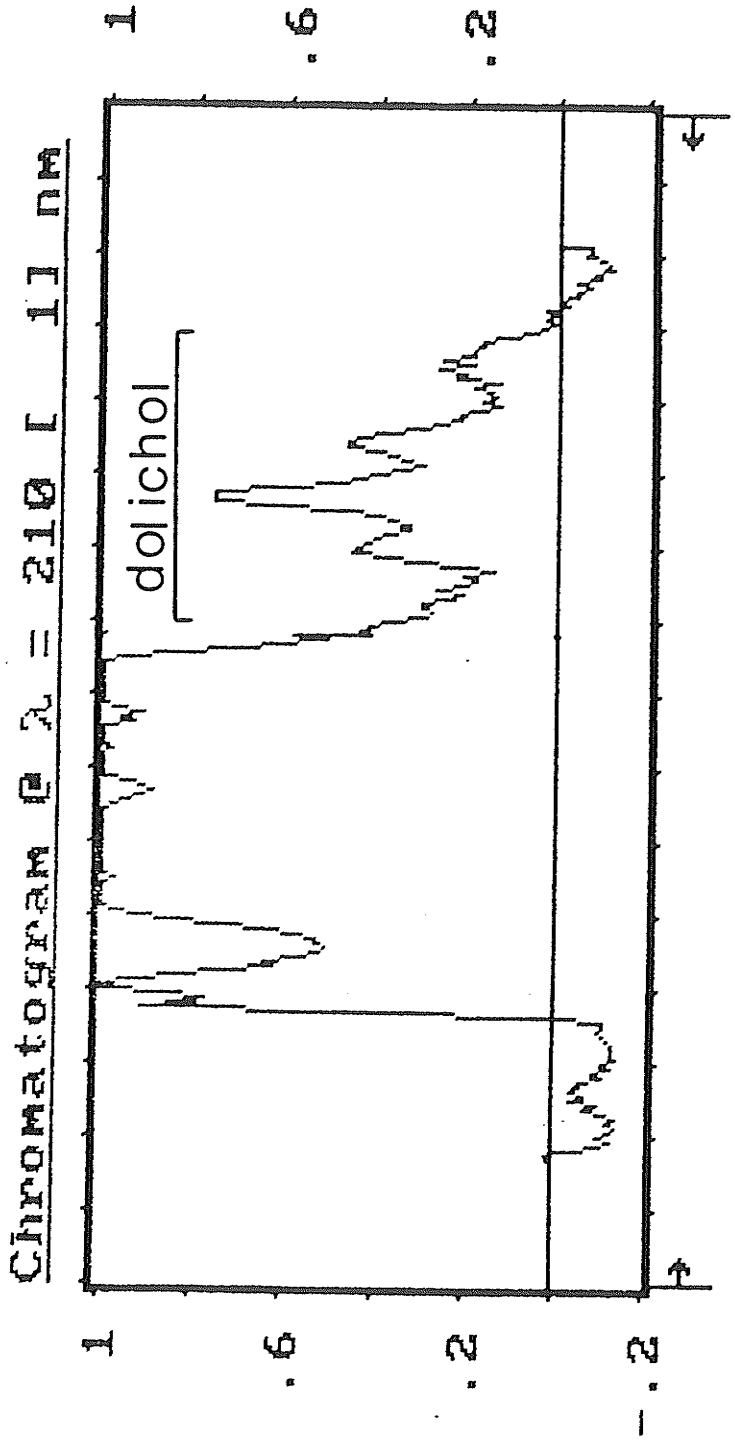

Chromatogram @ λ = 210 ± 11 nm
dolichol
1
5.
2.
2.
1
5.
2.
2.

Figure 30: Profile of dolichol content. Dolichol content was determined as described in Methods and Materials. Presented here is a chromatogram at λ = 210 nm of the dolichol profile as determined by HPLC analysis of WT cells on FCS-M at 39°C (36 h).

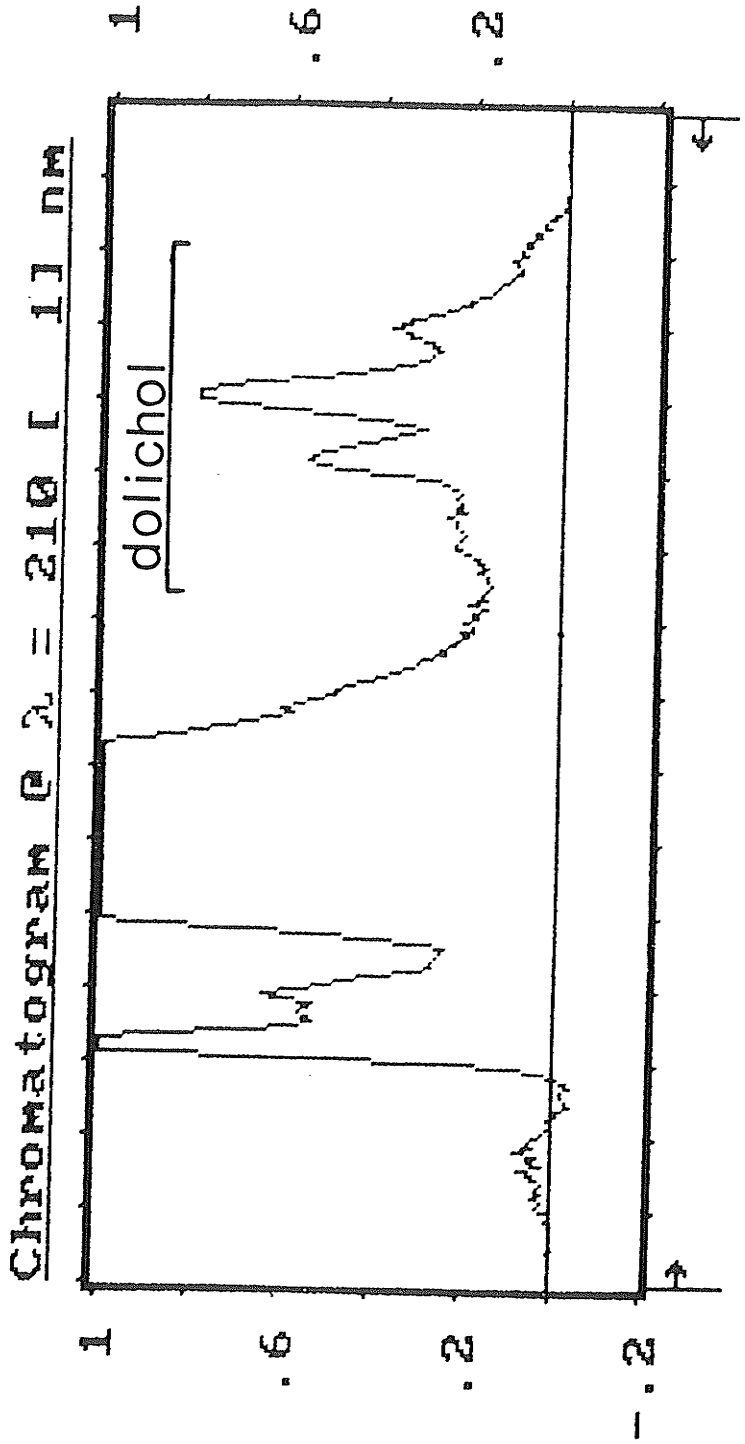

Chromatogram @ λ = 210 [11 nm
dolichol
1
.5
.2
.2
1
.5
.2

Figure 31: Profile of dolichol content. Dolichol content was determined as described in Methods and Materials. Presented here is a chromatogram at λ = 210 nm of the dolichol profile as determined by HPLC analysis of WT cells on FCS-M at 39°C (48 h).

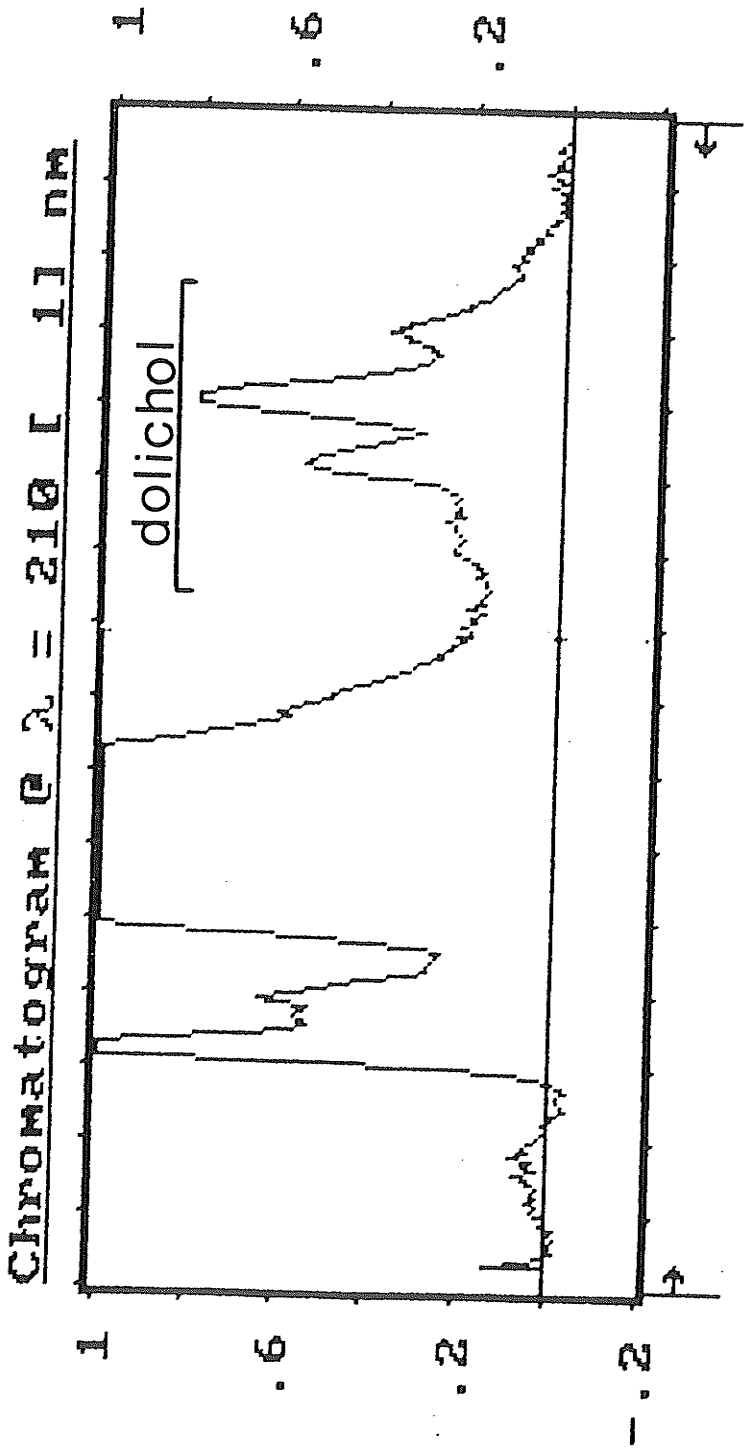

Chromatogram @ λ = 210 [11 nm
dolichol
1
.6
.2
.2
1
.6
.2

Figure 32: Profile of dolichol content. Dolichol content was determined as described in Methods and Materials. Presented here is a chromatogram at λ = 210 nm of the dolichol profile as determined by HPLC analysis of WT cells on LPDS-M at 39°C (36 h).

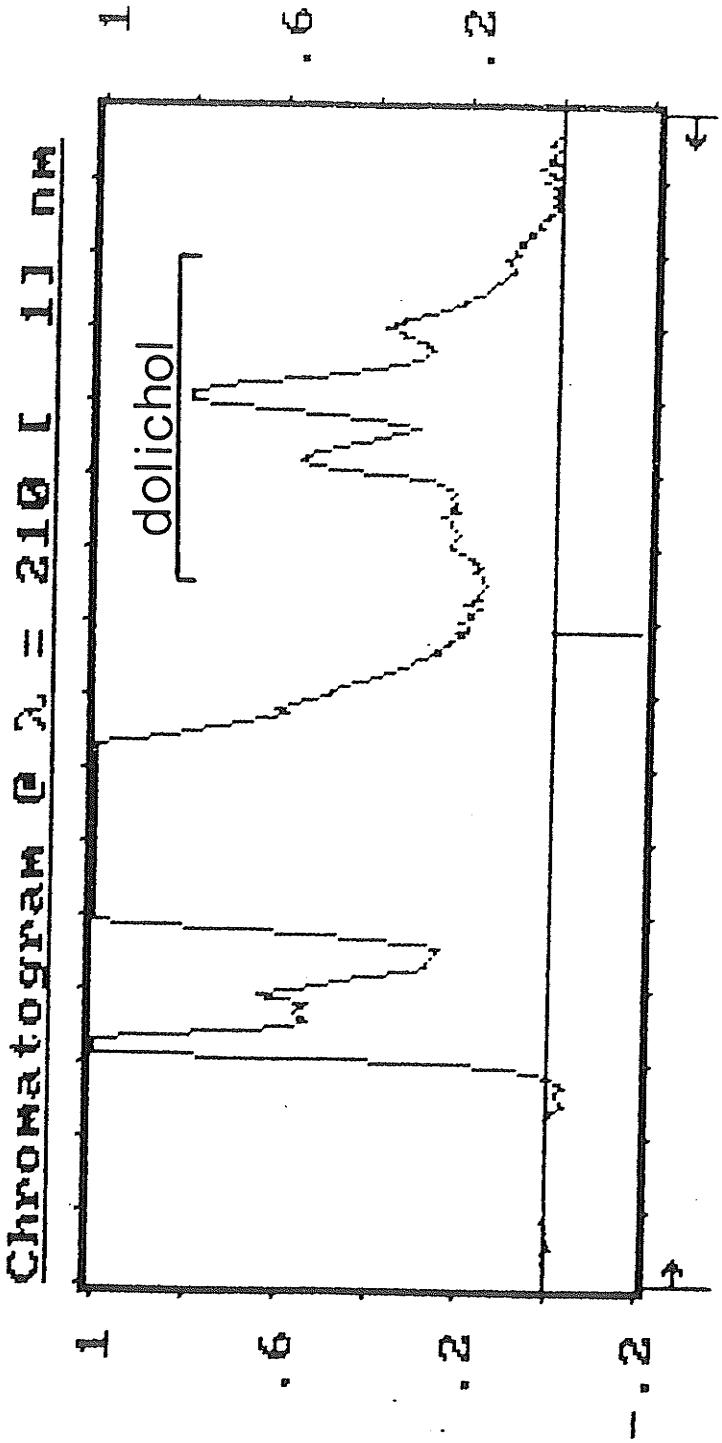

Chromatogram @ λ = 210 [11 nm
dolichol
1
6.
2.
2.
1
6.
2.
2.

Figure 33: Profile of dolichol content. Dolichol content was determined as described in Methods and Materials. Presented here is a chromatogram at λ = 210 nm of the dolichol profile as determined by HPLC analysis of C^R7 cells on FCS-M at 34°C.

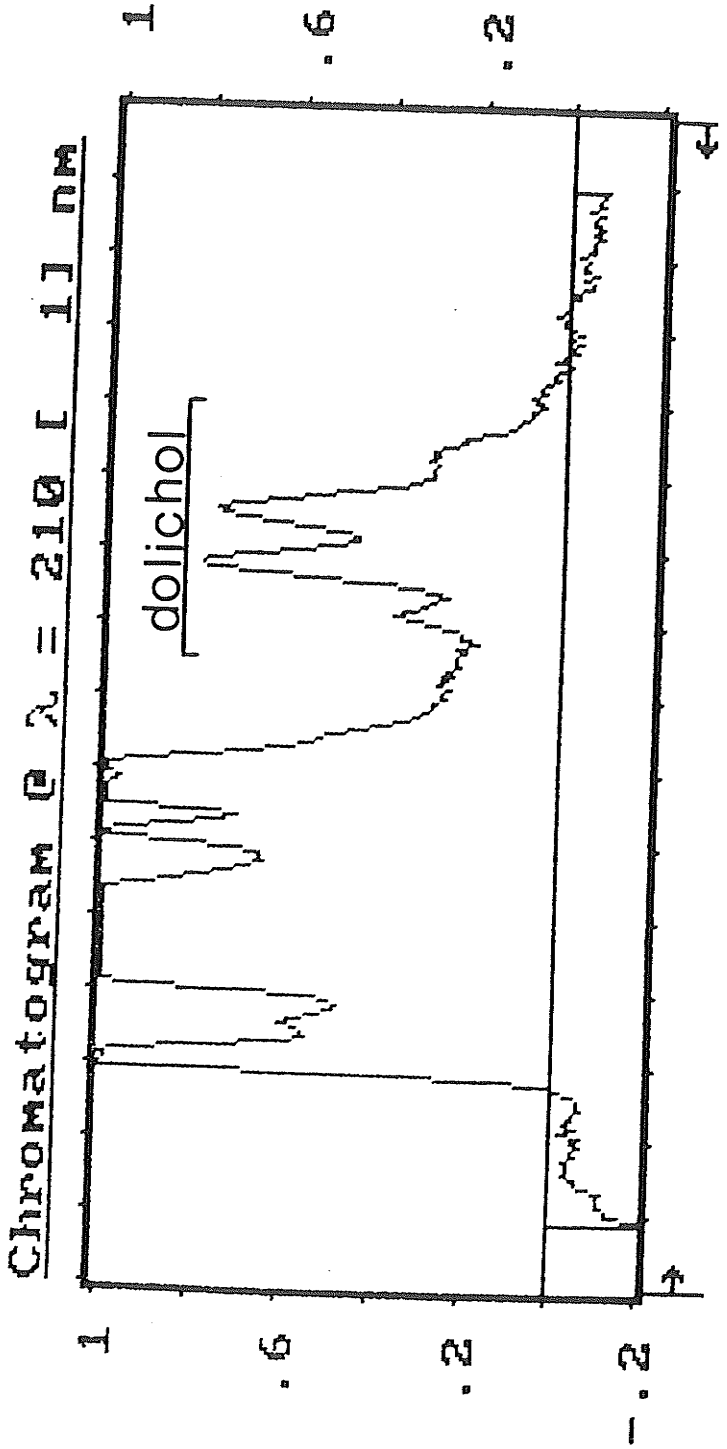
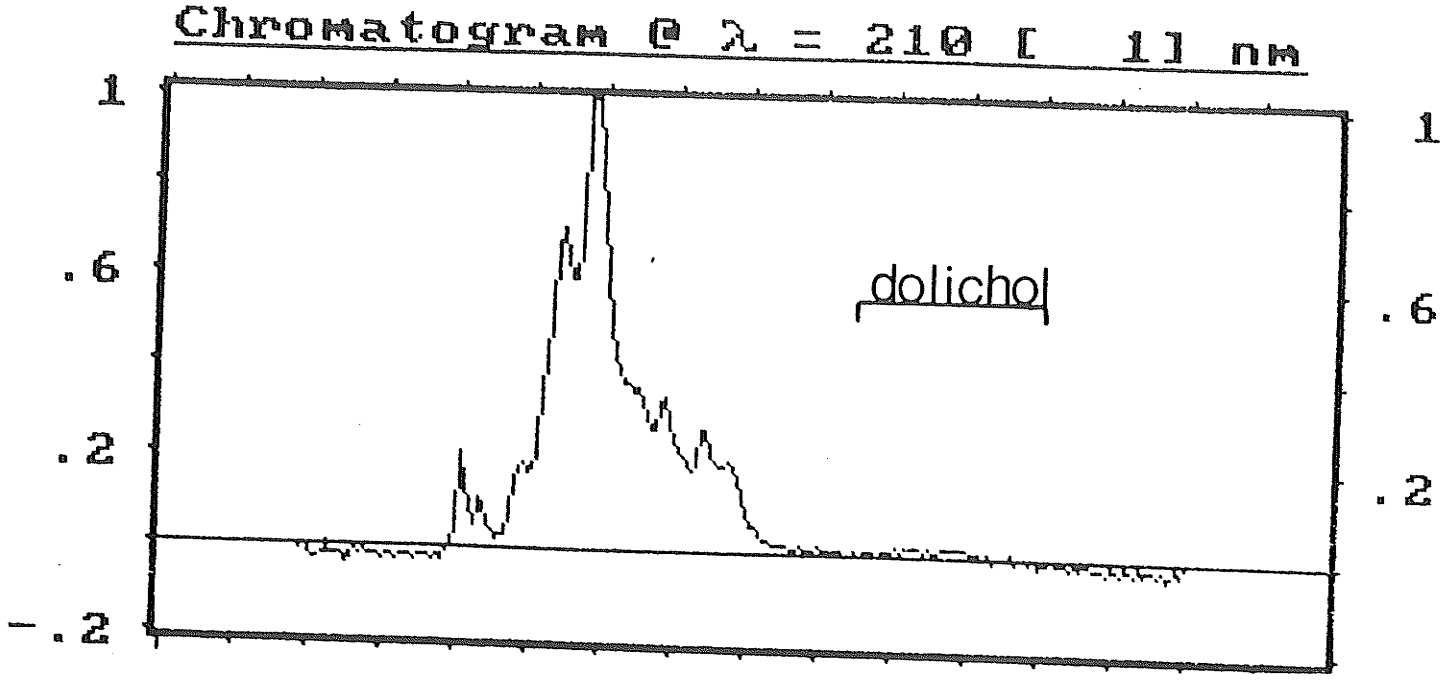

Chromatogram @ λ = 210 [11 nm
dolichol
1
6.
2.
2.
1
5.
2.
2.

Figure 34: Profile of dolichol content. Dolichol content was determined as described in Methods and Materials. Presented here is a chromatogram at λ = 210 nm of the dolichol profile as determined by HPLC analysis of C7 cells grown on LPDS-M (24 h) at 34°C.

Figure Legend: A = actual profile; B = expanded and normalized profile.

A


Chromatogram @ λ = 210 [1] nm
1
.6
.2
-.2
dolichol

B

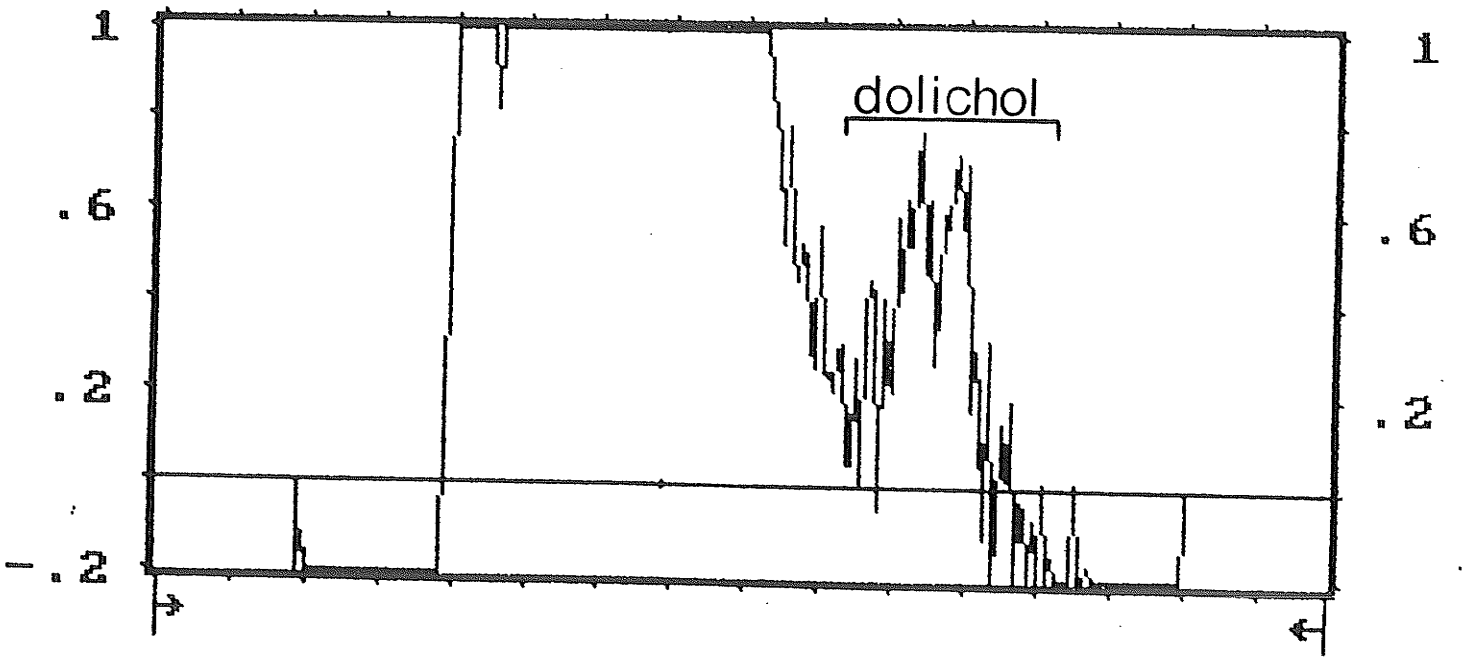

Chromatogram @ λ = 210 [1] nm
1
.6
.2
-.2
dolichol

Figure 35: Profile of dolichol content. Dolichol content was determined as described in Methods and Materials. Presented here is a chromatogram at λ = 210 nm of the dolichol profile as determined by HPLC analysis of C^R7 cells on FCS-M (36 h) at 39°C.

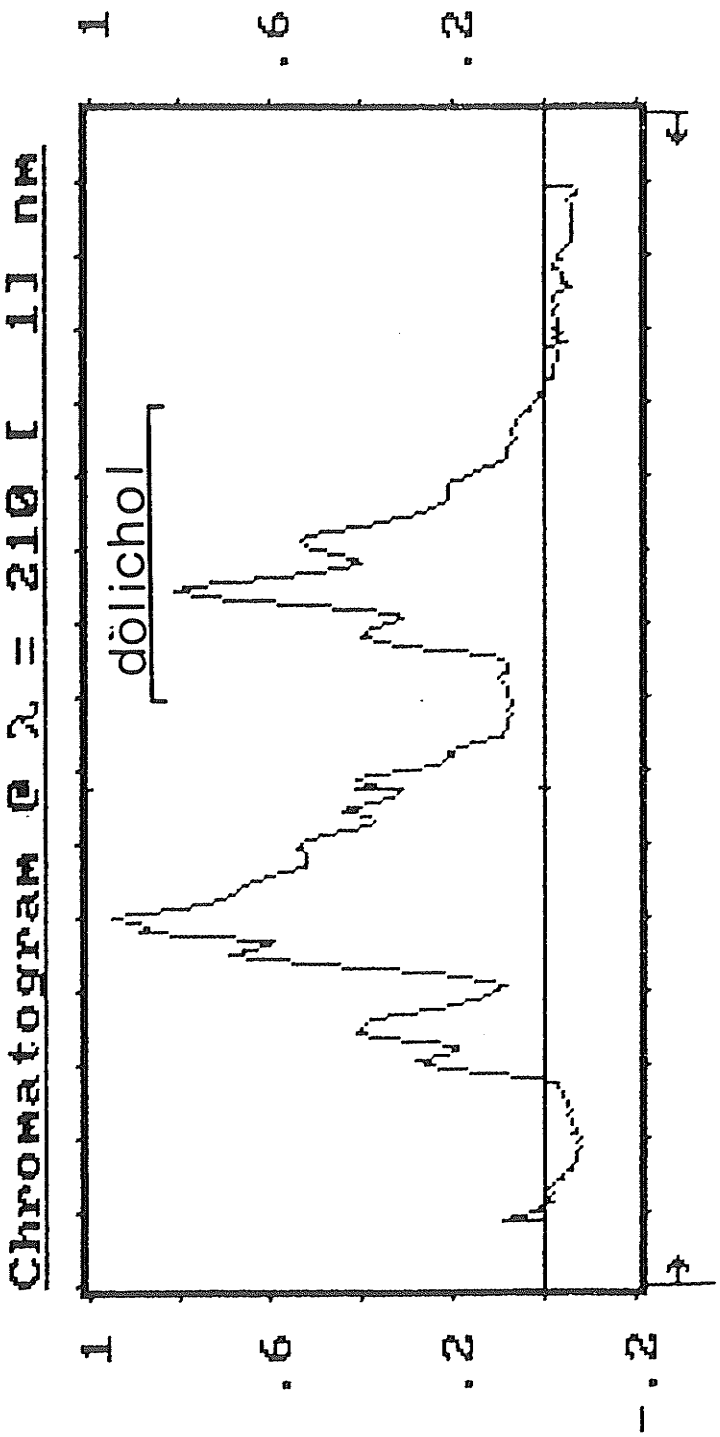

Chromatogram @ λ = 210 [1] nm
dölichol
1
.6
.2
1
.6
.2
.1

Figure 36: Profile of dolichol content. Dolichol content was determined as described in Methods and Materials. Presented here is a chromatogram at λ = 210 nm of the dolichol profile as determined by HPLC analysis of $C^{R}7$ cells on FCS-M at 39°C (48 h).

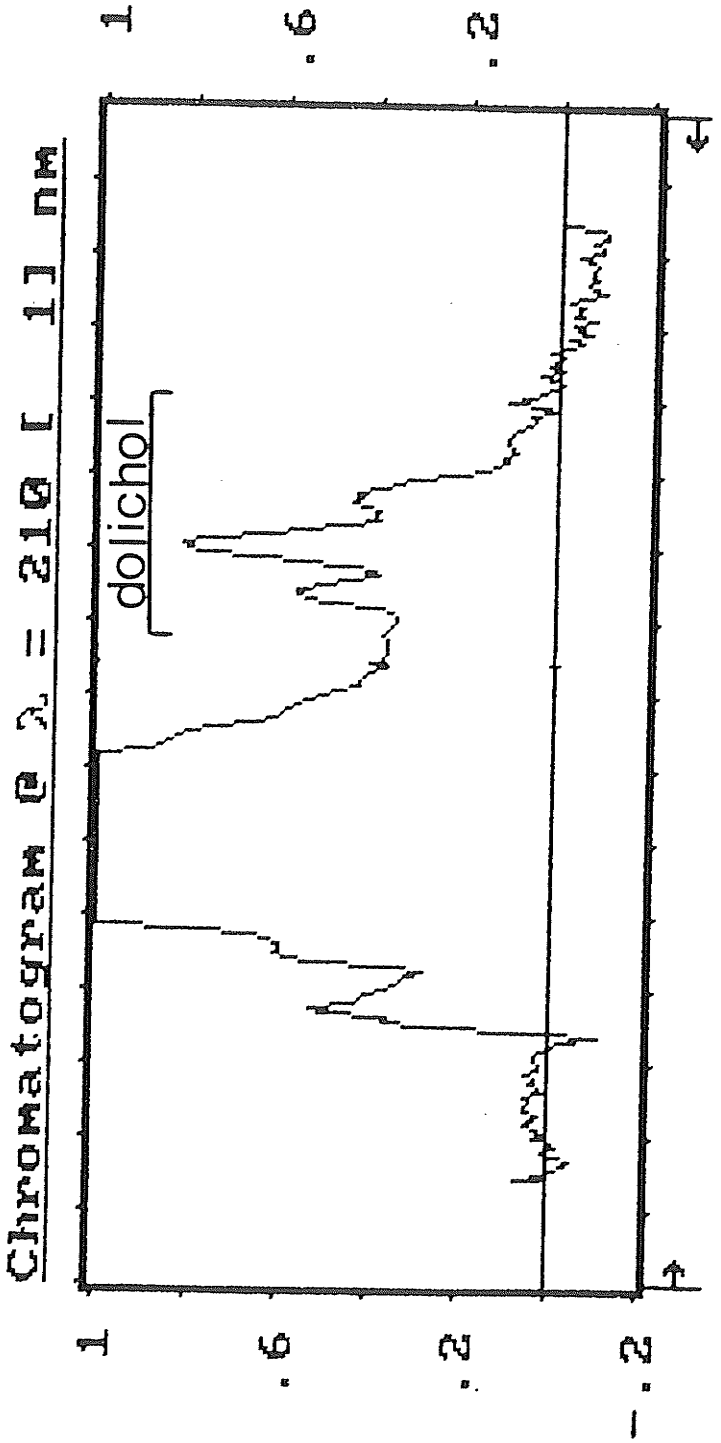
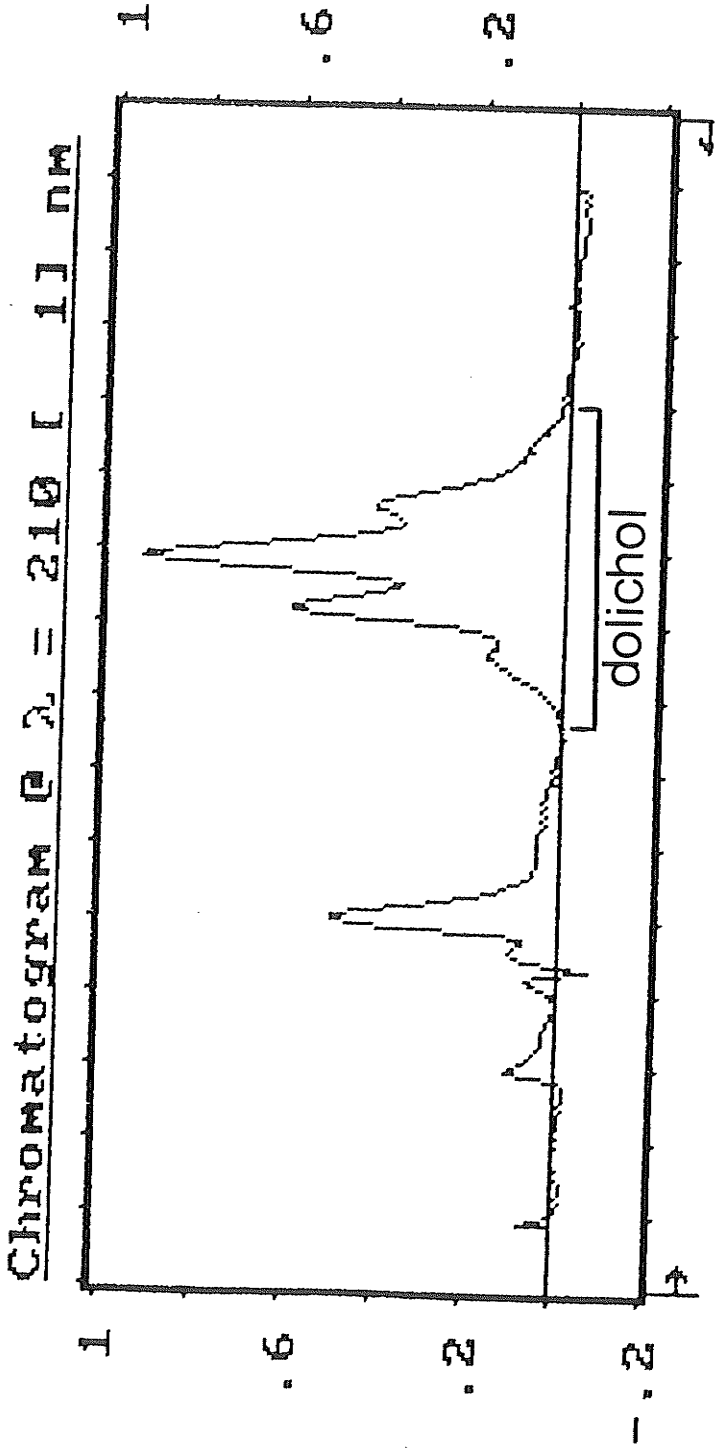

Chromatogram @ λ = 210 [11 nm
dolichol
1
.5
.2
1
.5
.2
.1

Figure 36(a): Profile of [^{3}H] labeled dolichol internal standard. Presented here is a chromatogram at λ = 210 nm of the dolichol profile as determined by HPLC analysis.


Chromatogram @ λ = 210 [11 nm
dolichol

to their respective dolichol profiles was done. No appreciable differences were found in WT cell lines. Regardless of growth conditions (temperature and cholesterol sufficiency/deficiency) employed, the relative percent composition of the individual isoprenologues of WT remained about the same (Table 12). However, regarding C^R7, marked differences in the relative percent composition of the individual isoprenologues were found when cells were grown at 34°C and 39°C (Table 13). Whether these observed changes in the distribution of the isoprenologues in the dolichol profiles of C^R7 are due to the temperature sensitive growth property of C^R7, and whether these changes have a functional role, if any, is at present, unclear.

Further Biochemical Characterization of C^R7's Behaviour

Rate of Cholesterol Synthesis at 34°C and 39°C

In order to gain a better understanding of the nature of C^R7, further biochemical characterization was undertaken. C^R7 has been shown to be a cholesterol auxotroph (Figure 16). Furthermore, the cholesterol content of mutant cells was less than wild type and revertant, regardless of growth conditions (ie. lipoprotein-rich (FCS-M) or lipoprotein-deficient (LPDS-M)) (Recall Tables 1, 2 and 3). To investigate if the mass of cholesterol in these cell lines reflects rates of sterol synthesis, cultures were incubated with radiolabeled acetate and the incorporation of this label into digitonin precipitate was measured. The results of these incorporation experiments are summarized in Figure 37.

WT and Rc both showed the ability to increase cholesterol synthesis when switched from FCS-M (cholesterol sufficient medium) to LPDS-M

Table 12: Relative percent composition of individual isoprenologues comprising the total dolichol profile of WT cells. Dolichol content was determined as described in Methods and Materials. The isoprenologue distribution of WT cells under various conditions (as indicated in table) was examined and their relative percent composition and subsequent contribution to overall dolichol content was determined as described in Methods and Materials.

Cell Line	Temperature and Growth Conditions	Relative Percent Composition of Individual Isoprenologues			
		Peak 1 8.1*	Peak 2 8.6*	Peak 3 9.3*	Peak 4 10.0*
WT	34°C, FCS-M	4.2	21.3	47.2	27.3
WT	34°C, LPDS-M 24 h	2.6	24.3	43.8	29.3
WT	39°C, FCS-M 36 h	1.9	19.4	47.7	31.0
WT	39°C, FCS-M 48 h	2.3	20.0	45.2	32.5
WT	39°C, LPDS-M 36 h	3.0	19.9	48.2	28.9

*Retention time in minutes.

Table 13: Relative percent composition of individual isoprenologues comprising the total dolichol profile of C^R7 cells. Dolichol content was determined as described in Methods and Materials. The isoprenologue distribution of C^R7 cells under various conditions (as indicated in table) was examined and their relative percent composition and subsequent contribution to overall dolichol content was determined as described in Methods and Materials.

Cell Line	Temperature and Growth Conditions	Relative Percent Composition of Individual Isoprenologues			
		Peak 1 8.5*	Peak 2 9.3*	Peak 3 10.0*	Peak 4 11.0*
C^R7	34°C, FCS-M	8.9	34.0	43.4	13.6
C^R7	34°C, LPDS-M 24 h	18.0	30.4	30.7	20.9
C^R7	39°C, FCS-M 36 h	18.0	49.2	25.9	6.9
C^R7	39°C, FCS-M 48 h	23.1	53.9	20.1	3.0
C^R7	39°C, LPDS-M 36 h	not able to determine			

*Retention time in minutes.

(cholesterol deficient medium) and also when transferred from 34°C to 39°C. C^R7 was unable to upshift cholesterol synthesis under either condition (Figure 37).

At 39°C on FCS-M (cholesterol sufficient medium) WT and Rc cell lines synthesized cholesterol at a rate equivalent to that seen for WT and Rc at 34°C on LPDS-M (cholesterol deficiency). Furthermore at 39°C on LPDS-M, WT and Rc cell lines' rates of cholesterol synthesis increased markedly.

The rate of synthesis of cholesterol by C^R7 at 39°C on LPDS-M was lower than that seen on FCS-M at 39°C, and at 34°C on both FCS-M and LPDS-M.

At 34°C on FCS-M, WT and Rc synthesized cholesterol at about the same rate as C^R7 while on LPDS-M, WT and Rc cell lines' rate of cholesterol synthesis was three fold higher than that of C^R7.

At 39°C, on cholesterol sufficient medium, the rate of cholesterol synthesis of WT and Rc cell lines was three fold higher than C^R7's rate. And, on LPDS-M at 39°C, WT/Rc rate of cholesterol synthesis was approximately ten times greater than that of C^R7 (Figure 37). Most certainly, C^R7 is defective in its ability to synthesize its own cholesterol when faced with a milieu which is cholesterol deficient (either real or perceived) or one which has an increased demand for cholesterol.

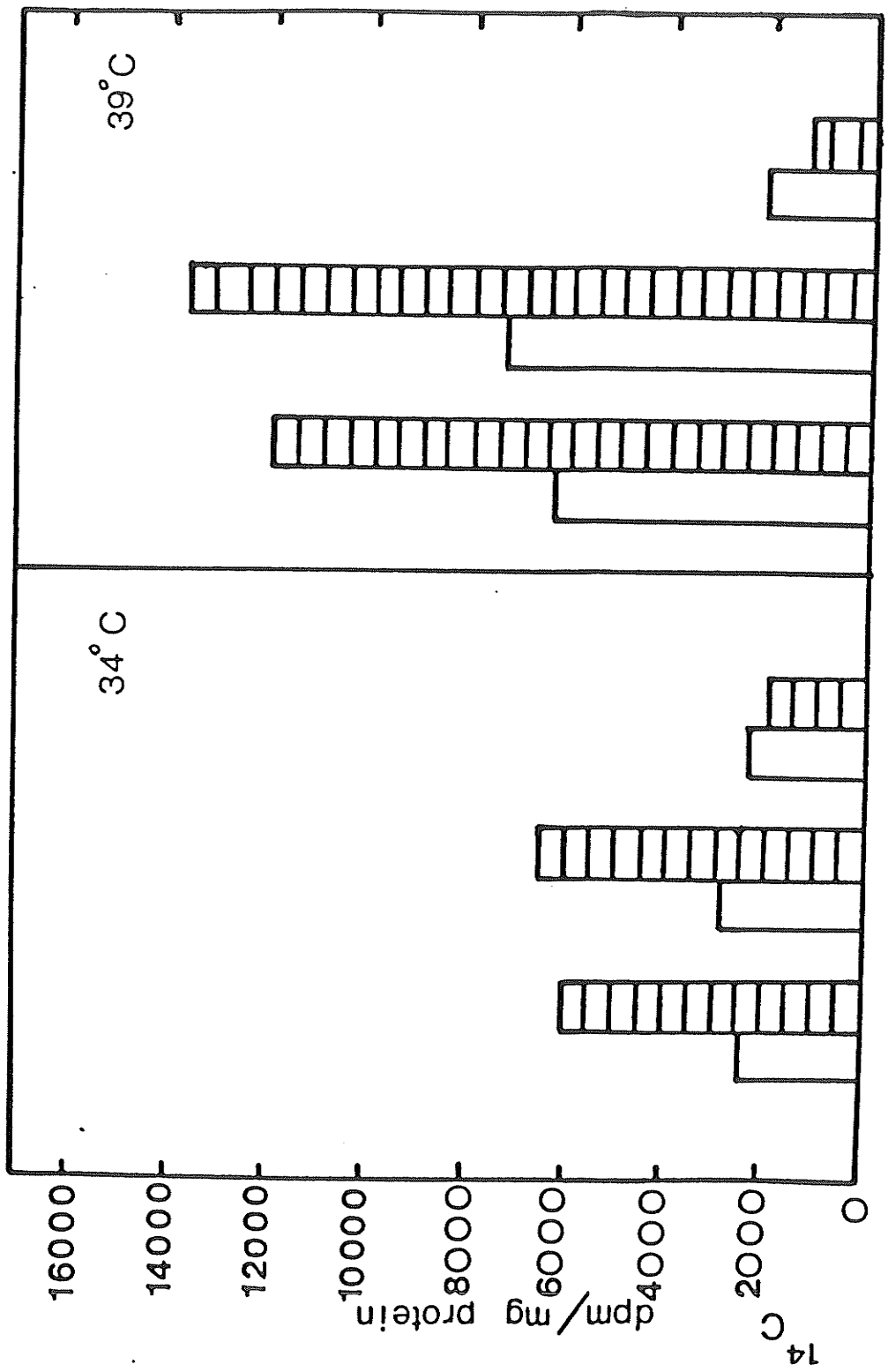
Examination of Rate of Synthesis of Fatty Acids

The observation of altered fatty acid content warranted an examination of the rate of synthesis of total fatty acids, as well as the rates of synthesis of saturates and unsaturates.

Figure 37: Incorporation of ^{14}C-labeled acetate into sterols, as determined by digitonin precipitation (2 hour incubation). Plates, 100 mm, were inoculated with 60,000 cells and then incubated for 4 days in FCS-M at 34°C. Following this growth period, medium was removed and replaced with fresh FCS-M or LPDS-M as appropriate and the cultures were incubated a further 24 h at either 39°C or 34°C. Medium was again removed and replaced with 5 ml of the appropriate medium containing 10 μCi/ml of [1-^{14}C] acetate (2.8 mCi/m mole) (Amersham). Following a 2 h incubation the medium was removed, monolayers were washed with PBS and dissolved in 0.5 ml of 1 N NaOH. Samples were saponified and incorporation into cholesterol determined as described in Methods and Materials. The incorporations illustrated represent the average of duplicates determined in two separate cultures and duplicate experiments.

Figure Legend:  - FCS-M

 - LPDS-M


16000
14000
12000
10000
8000
6000
4000
2000
0
dpm/mg protein
^{14}C
34°C
39°C
WT
Rc
Cr7
WT
Rc
Cr7
CELL TYPE

Cultures were incubated with ^{14}C-labeled acetate and the incorporation of label into various fatty acids was determined. Figure 38 summarizes the results of the total incorporation of ^{14}C-labeled acetate into FAME of cells grown on both FCS-M and LPDS-M at either 34°C or 39°C. Figure 39 summarizes the relative incorporation of ^{14}C-labeled acetate into mono-unsaturated and saturated fatty acids.

In wild type and revertant cell lines incubation at 34°C in LPDS-M caused a modest increase in the rate of total fatty acid synthesis accompanied by a relative increase in the rate of synthesis of mono-unsaturates. The synthesis of total fatty acid remained about the same in the mutant but these cells produced relatively less unsaturated fatty acid in LPDS-M at 34°C. As cells were removed from the presence of exogenous cholesterol a compensatory increase in unsaturated fatty acids would be expected. This was observed in WT and Rc but not in C^R7. This finding became more apparent when these processes were examined at 39°C.

In wild type and revertant cell lines, incubation in LPDS-M at 39°C caused a pronounced increase in the rate of total fatty acid synthesis accompanied by a marked increase in the rate of synthesis of mono-unsaturates. The synthesis of total fatty acids decreased by 50% in C^R7 on LPDS-M at 39°C relative to that seen on LPDS-M at 34°C. Relative to WT and revertant cell lines at 39°C on LPDS-M, the mutant exhibited a marked inability to produce unsaturated fatty acids, which are apparently required as evidenced by the wild type and revertant cell lines elevated responses.

The implication of these results and others presented previously in

this thesis is that the variant, C^R7, is defective in its ability to synthesize unsaturated fatty acids in addition to being defective in sterol biosynthesis.

Mutant cells deprived of lipoprotein may be maintained in culture by the addition of cholesterol alone, to the culture medium. As the mutant appears deficient in the synthesis of unsaturated fatty acids it is surprising that an unsaturated fatty acid supplement is not also necessary for its growth. The addition of fatty acid supplements to LPDS-M did not allow C^R7 to grow at 34°C (data not shown). Furthermore, the addition of fatty acid supplements to FCS-M or to LPDS-M had no ability to relieve the temperature-sensitive growth response of C^R7 seen at 39°C (data not shown). The possibility exists that there is substantial free fatty acid in the LPDS culture medium after the removal of lipoproteins, whereas negligible amounts of cholesterol remain. The concentration of the various free fatty acids in LPDS-M was determined (Borgford et al, 1986). Both unsaturated and saturated free fatty acids were apparent but it was not clear if the unsaturates were present at a concentration high enough to serve as growth supplements. Oleate, 18:1, was detected in the lipoprotein deficient serum at a concentration of 7.3 μg/ml. Oleate is present at a concentration of approximately 0.73 μg/ml in LPDS-M. This finding might be applicable to the growth response of C^R7 at 34°C on LPDS-M where the addition of cholesterol alone allows C^R7 to grow and thrive.

The discrepancy noted at 39°C is far more complex and needs further exploration.

Figure 38: Total incorporation of ^{14}C-labeled acetate into fatty acid methyl esters of cells grown on FCS-M or LPDS-M at either 34°C or 39°C. Cell growth parameters and methodology were as described in Methods and Materials. The incorporations illustrated represent the average of duplicate determinations from duplicate cultures.

Figure Legend:  - FCS-M

 - LPDS-M

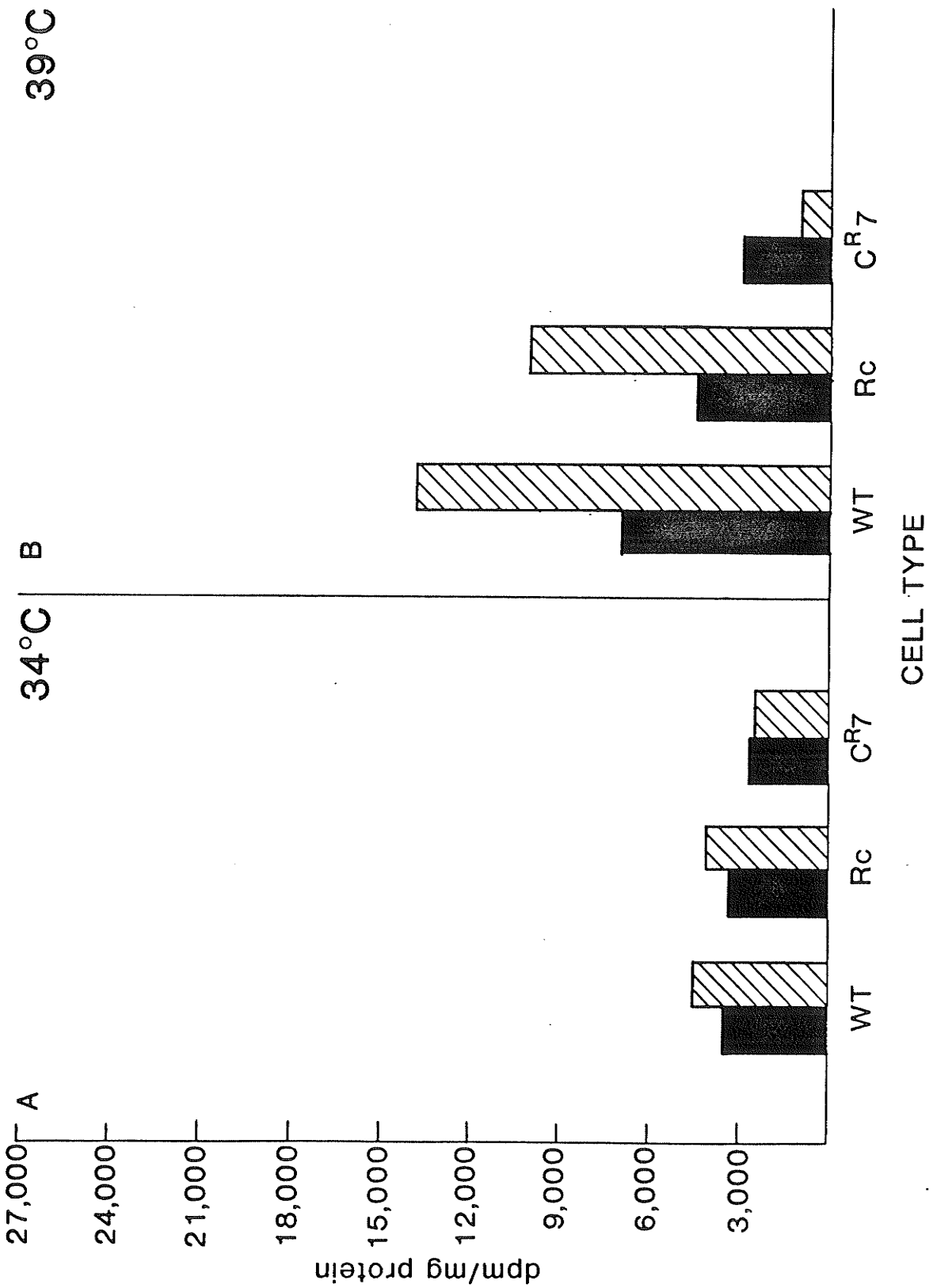
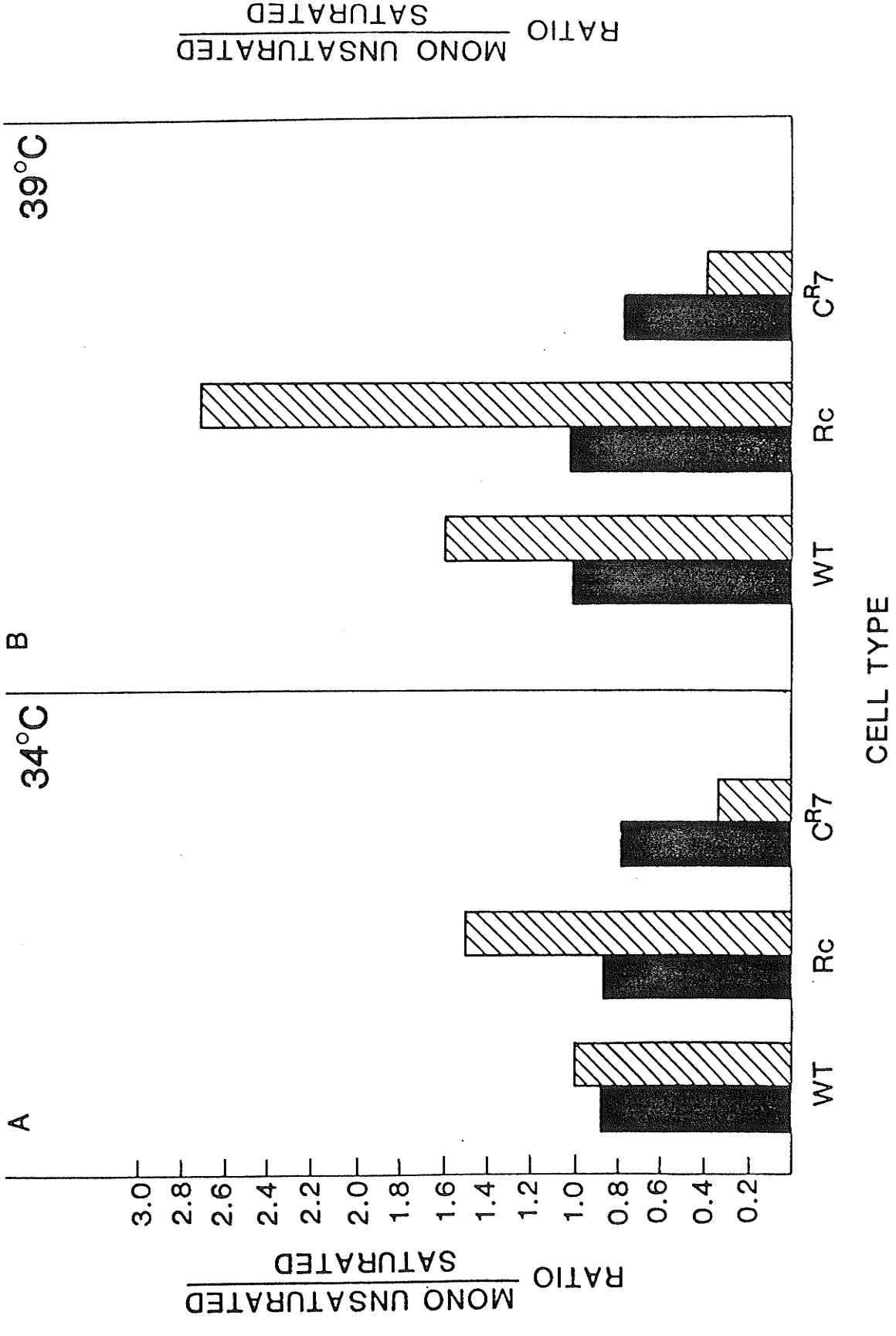

A
B
34°C
39°C
dpm/mg protein
27,000
24,000
21,000
18,000
15,000
12,000
9,000
6,000
3,000
WT
Rc
C^R7
CELL TYPE

Figure 39: Relative incorporation of ^{14}C-labeled acetate into mono-unsaturated and saturated fatty acids. Parameters of cell growth and FAME preparation were as described in Methods and Materials. Fatty acids of varying degrees of saturation were separated by thin layer chromatography (TLC) on 5% $AgNO_3$ impregnated silica gel plates. TLC plates were developed with petroleum ether-diethyl ether (95:5). Following chromatography, appropriate sections were scraped from the plates and radioactivity was determined. Fatty acids were identified using parallel channels containing authentic fatty acid methyl ester standard (H105) (Applied Sciences). The incorporations illustrated represent the average of duplicates determined in two separate cultures.

Figure Legend:  - FCS-M

 - LPDS-M


RATIO
MONO UNSATURATED
SATURATED
39°C
34°C
A
B
WT
Rc
C^R7
CELL TYPE
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2

Examination of HMG-CoA Reductase Levels

HMG-CoA reductase catalyzes the first unique step in the pathway for the synthesis of cholesterol and other isoprenoids, and it is now generally agreed that this is the most important rate-controlling enzyme of cholesterol biosynthesis in mammalian cells.

Since C^R7 was unable to upshift cholesterol synthesis under conditions where this was required (switching from FCS-M to LPDS-M and when transferred from 34°C to 39°C), an examination of HMG-CoA reductase enzyme activity was warranted. The findings of this examination are presented in Table 14.

All three cell lines expressed similar reductase enzyme activity at 34°C on FCS-M. This is a consistent finding despite the fact that it is contrary to a previous observation. Borgford (1984) found that reductase activity of C^R7 at 34°C on FCS-M was at least two fold greater than either WT or Rc cell lines' reductase activity.

At 34°C, on LPDS-M (24 h) WT and Rc reductase activities rose sharply, increasing 5-fold over reductase activities seen when these cells were grown on FCS-M. However, the reductase activity of C^R7 increased only very slightly. Wild type and revertant were seen to incorporate much more label into sterol during growth on LPDS-M (Figure 37) reflecting the higher reductase levels. But, despite a slight increase in reductase activity, C^R7 incorporated less label into sterol on LPDS-M than on FCS-M at 34°C (Figure 37).

Reductase activities of all three cell lines were found to be twice those recorded at 34°C on FCS-M when reductase levels were determined at 39°C when cells were grown on FCS-M (cholesterol-sufficient medium). WT and Rc cell lines were found to incorporate at least 3 times more label

into sterol at 39°C (FCS-M) when compared to incorporation levels at 34°C (FCS-M). Therefore, an increase in reductase activity at 39°C (FCS-M) is quite consistent. However, this is not true for C^R7. In C^R7, the increased reductase activity was not reflected in a similar increase in incorporation of label into sterol (Figure 37).

Furthermore, at 39°C on LPDS-M, wild type and revertant had increased reductase activity, roughly corresponding once again with increased cholesterol synthesis (Figure 37). C^R7 enzyme activity was markedly reduced and is consistent with marginal cholesterol synthesis rates. Wild type and revertant cell reductase activities were determined to be 5 to 6 times greater than those of C^R7 at 39°C on LPDS-M (Table 14).

An examination of HMG-CoA reductase enzyme activity of WT and C^R7 cell lines at 39°C on FCS-M revealed some interesting findings (Table 15). With increased incubation at 39°C (FCS-M) the level of reductase enzyme activity of WT remained virtually unchanged. However, reductase activity of C^R7 increased as the length of incubation at 39°C on FCS-M increased (up to 48 hours) and then sharply declined at 60 h. Recall that the nonpermissive temperature for growth of C^R7 is 39°C on FCS-M. These elevated reductase enzyme activity levels do not translate to expected elevated rates of cholesterol synthesis in C^R7. This is a very interesting finding and will be addressed in the Discussion.

Examination of Mannose Incorporation into Lipid-Monosaccharide, Lipid-Oligosaccharide and Glycoprotein

One important use of dolichol is its role in glycoprotein

Table 14: HMG-CoA reductase activity in WT, Rc and $C^{R}7$ at 34°C and 39°C. Plates, 100 mm, were inoculated with cells (50,000 cells per dish) and incubated at 34°C in FCS-M until all cultures were subconfluent. Medium was removed and fresh FCS-M or LPDS-M as appropriate was added and cultures were incubated a further 24 hours at either 34°C or 39°C. After this incubation period cell monolayers were washed and HMG-CoA reductase activity was determined as described in Methods and Materials. Results are expressed in terms of the specific activity of HMG-CoA reductase and represent the average of duplicate determinations on two separate cultures.

Cell Line	Growth Conditions	HMG-CoA reductase specific activity (p moles min^{-1} μg^{-1})
WT	34°C, FCS-M	0.14 ± 0.03
Rc	34°C, FCS-M	0.13 ± 0.03
C^R7	34°C, FCS-M	0.13 ± 0.04
WT	34°C, LPDS-M 24 h	0.68 ± 0.07
Rc	34°C, LPDS-M 24 h	0.61 ± 0.06
C^R7	34°C, LPDS-M 24 h	0.22 ± 0.04
WT	39°C, FCS-M 24 h	0.25 ± 0.02
Rc	39°C, FCS-M 24 h	0.22 ± 0.03
C^R7	39°C, FCS-M 24 h	0.27 ± 0.03
WT	39°C, LPDS-M 24 h	0.82 ± 0.07
Rc	39°C, LPDS-M 24 h	0.63 ± 0.06
C^R7	39°C, LPDS-M 24 h	0.14 ± 0.03

Table 15: HMG-CoA reductase activity in WT, and C^{R} 7 at 39°C on FCS-M. Plates, 100 mm, were inoculated with 200,000 cells per plate and incubated at 34°C for 2-3 days. Medium was removed and replaced with 10 ml fresh FCS-M and cells were incubated at 39°C, the nonpermissive temperature, for the periods indicated. After each incubation period cell monolayers were washed and HMG-CoA reductase activity was determined as described in Methods and Materials. Results are expressed in terms of the specific activity of HMG-CoA reductase and represent the average of duplicate determinations on duplicate cultures.

Cell Line	Growth Conditions	HMG-CoA reductase specific activity (p moles min^{-1} μg^{-1})
WT	39°C, FCS-M 24 h	0.25 ± 0.03
C^R7	39°C, FCS-M 24 h	0.22 ± 0.01
WT	39°C, FCS-M 36 h	0.20 ± 0.01
C^R7	39°C, FCS-M 36 h	0.24 ± 0.03
WT	39°C, FCS-M 48 h	0.23 ± 0.01
C^R7	39°C, FCS-M 48 h	0.35 ± 0.01
WT	39°C, FCS-M 60 h	0.22 ± 0.05
C^R7	39°C, FCS-M 60 h	0.12 ± 0.05

synthesis, where it is a component of lipid-monosaccharides and lipid-oligosaccharides which are ultimately transferred to glycoproteins. It has long been clear that C^R7 is defective in glycoprotein synthesis--it was selected as a Con-A resistant variant. As well, glycoproteins are thought to be involved in thermal stability of cells, so the glycosylation deficiencies of C^R7 might be involved in its temperature-sensitivity. Therefore a measurement of the ability of C^R7, WT and a Rc cell line to incorporate labeled mannose into lipid-monosaccharide, lipid-oligosaccharide and glycoprotein at both 34°C and 39°C in both FCS-M and LPDS-M was carried out.

Figure 40 shows that C^R7 incorporated much less [^{3}H] mannose into all three compounds at 34°C than did wild type and a revertant cell line. This is consistent with a reduced ability to use dolichol. At 39°C, all three cell lines incorporated less mannose into lipid-oligosaccharide and glycoprotein than at 34°C, but incorporation into lipid-monosaccharide was about the same. Once again, C^R7 incorporated much less than WT or Rc.

There were similar findings when the experiment was repeated only in the presence of LPDS-M which are illustrated in Figure 41. As in Figure 40, where cells were grown on FCS-M, Figure 41 shows that C^R7 incorporated much less labeled mannose into all three compounds at 34°C than WT and Rc. At 39°C, all three cell lines incorporated less mannose into lipid-oligosaccharide and glycoprotein than at 34°C, but incorporation into lipid-monosaccharide was about the same with C^R7, with a marginal increase noted with WT and Rc. Once again, C^R7 incorporated much less label than WT or Rc. This was especially true

Figure 40: [^{3}H]-mannose incorporation study at 34°C and 39°C on FCS-M (cholesterol sufficient medium). Cells (50,000 cells per plate) were delivered to 60 mm culture dishes containing 5 ml of FCS-M and grown at 34°C for 4 days. The medium was then removed and replaced with fresh FCS-M and growth was continued for a further 20 hours at either 34°C or 39°C. Cells were then exposed to a 4 hour pulse of D-[2-^{3}H] mannose (17 μCi/ml) (Amersham). Following this, cell monolayers were washed and processed as described in Methods and Materials. The amount of [^{3}H]-mannose incorporated into lipid-monosaccharide, lipid-oligosaccharide and glycoprotein was determined as described in Methods and Materials. Results presented are the average of duplicate determinations on 2 separate cell cultures per cell line.

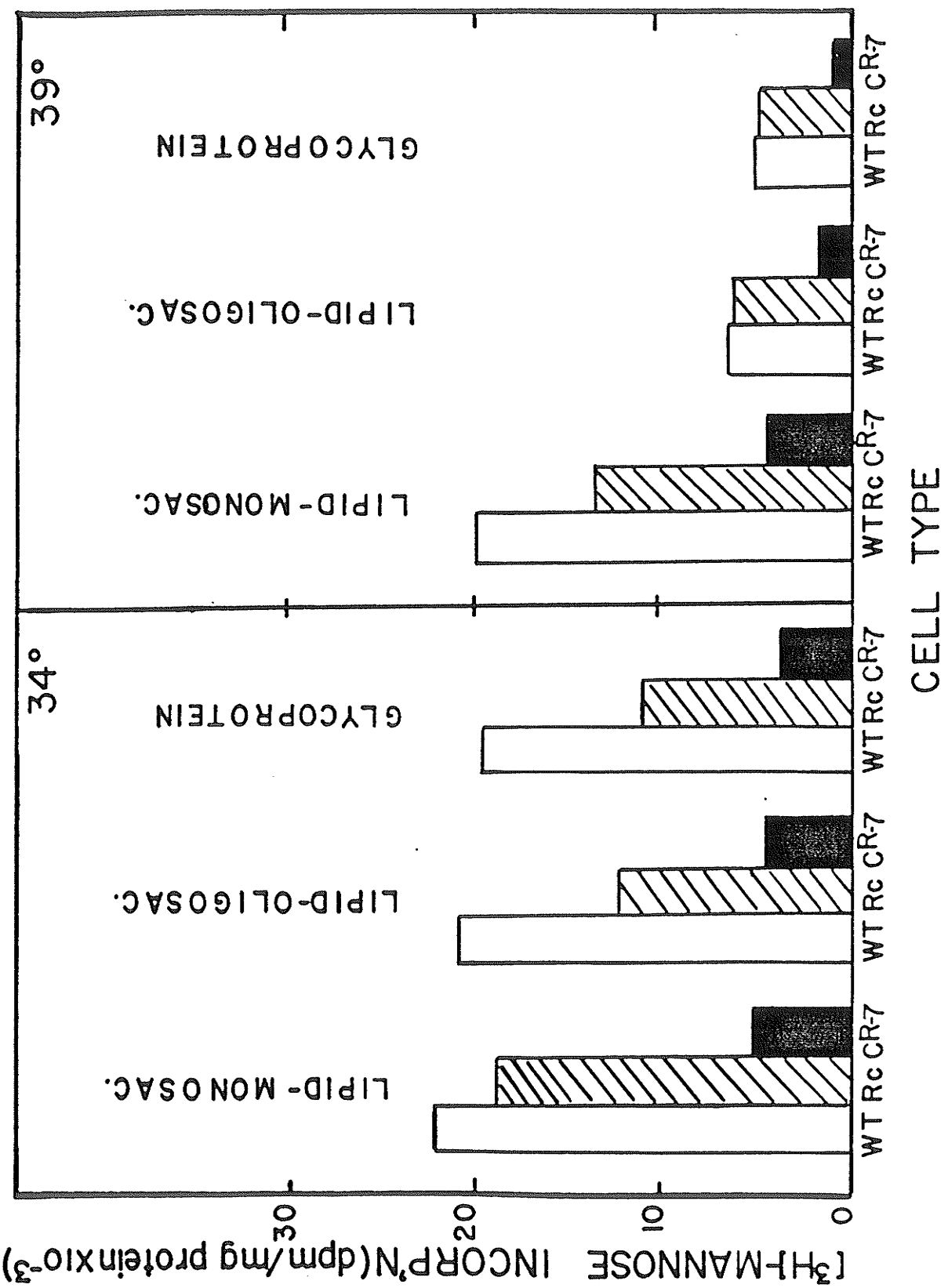
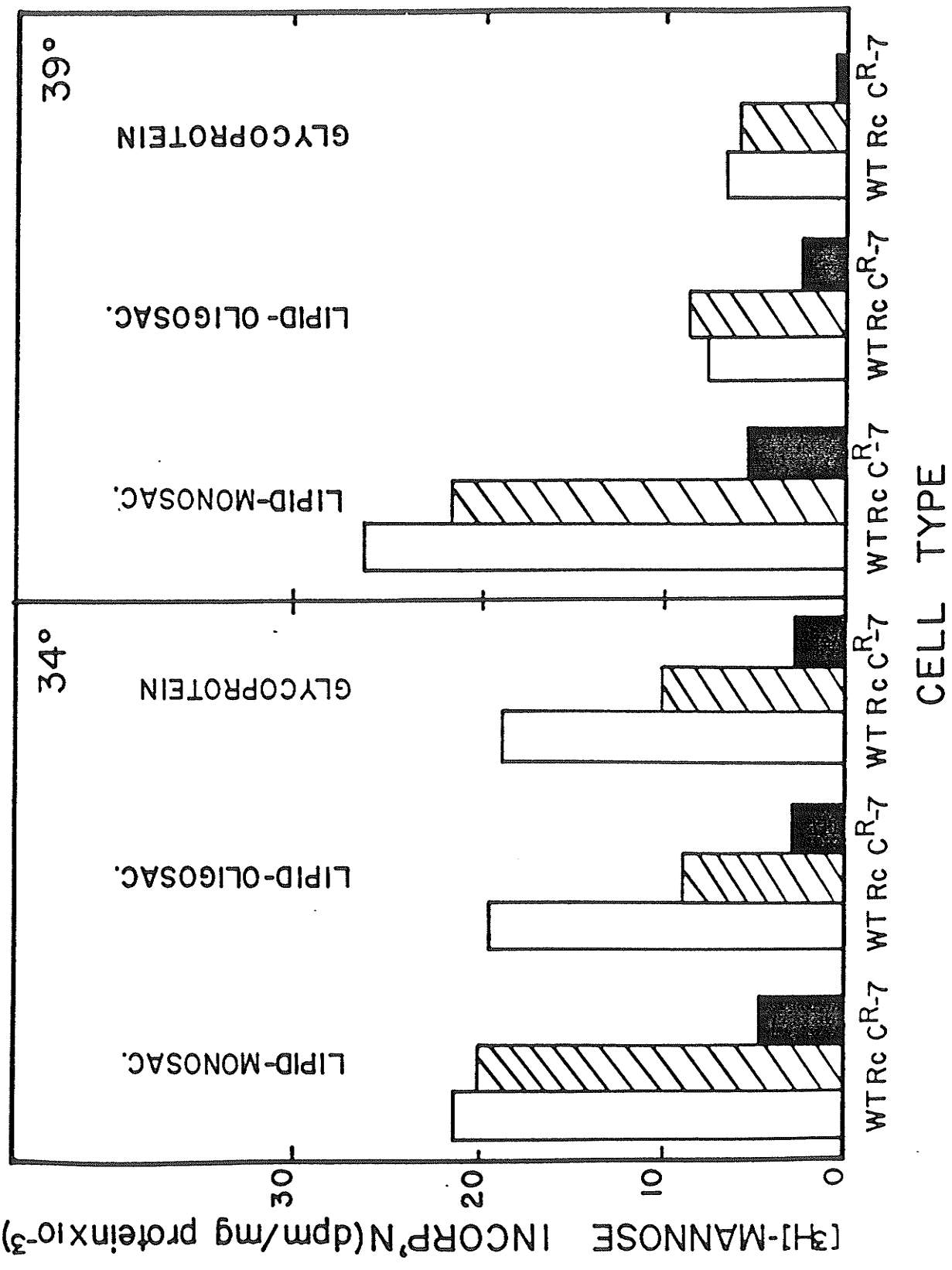

[3H]-MANNOSE INCORP'N (dpm/mg protein x10-3)
0
10
20
30
34°
39°
LIPID-MONOSAC.
LIPID-OLIGOSAC.
GLYCOPROTEIN
WT Rc CR-7
CELL TYPE

Figure 41: [^{3}H]-mannose incorporation study at 34°C and 39°C on LPDS-M (cholesterol deficient medium). Cells (50,000 cells per plate) were delivered to 60 mm culture dishes containing 5 ml of FCS-M and grown at 34°C for 4 days. The medium was removed and replaced with 5 ml fresh LPDS-M and growth was continued for a further 20 hours at either 34°C or 39°C. Cells were then exposed to a 4 h pulse of D-[2-^{3}H] mannose (17 μCi/ml) (Amersham). Following this, cell monolayers were washed and treated as described in Methods and Materials. The amount of [^{3}H]-mannose incorporated into lipid-monosaccharide, lipid-oligosaccharide and glycoprotein was determined as described in Methods and Materials. Results presented are the average of duplicate determinations on 2 separate cell cultures per cell line.


[3H]-MANNOSE INCORP'N (dpm/mg protein x 10-3)
0
10
20
30
34°
39°
LIPID-MONOSAC.
LIPID-OLIGOSAC.
GLYCOPROTEIN
WT Rc cR-7
CELL TYPE

for incorporation into glycoproteins.

Figure 42 shows that C^R7, when grown at 39°C on FCS-M supplemented with mevalonolactone (77 μM), was able to incorporate mannose into lipid-monosaccharide, lipid-oligosaccharide and glycoprotein at a level comparable to that of WT. The previous deficit of C^R7 regarding these processes was overcome when mevalonate was present. Recall mevalonolactone (77 μM) (Figure 21) restored normal growth properties to C^R7 at 39°C on FCS-M abolishing temperature-sensitive growth.

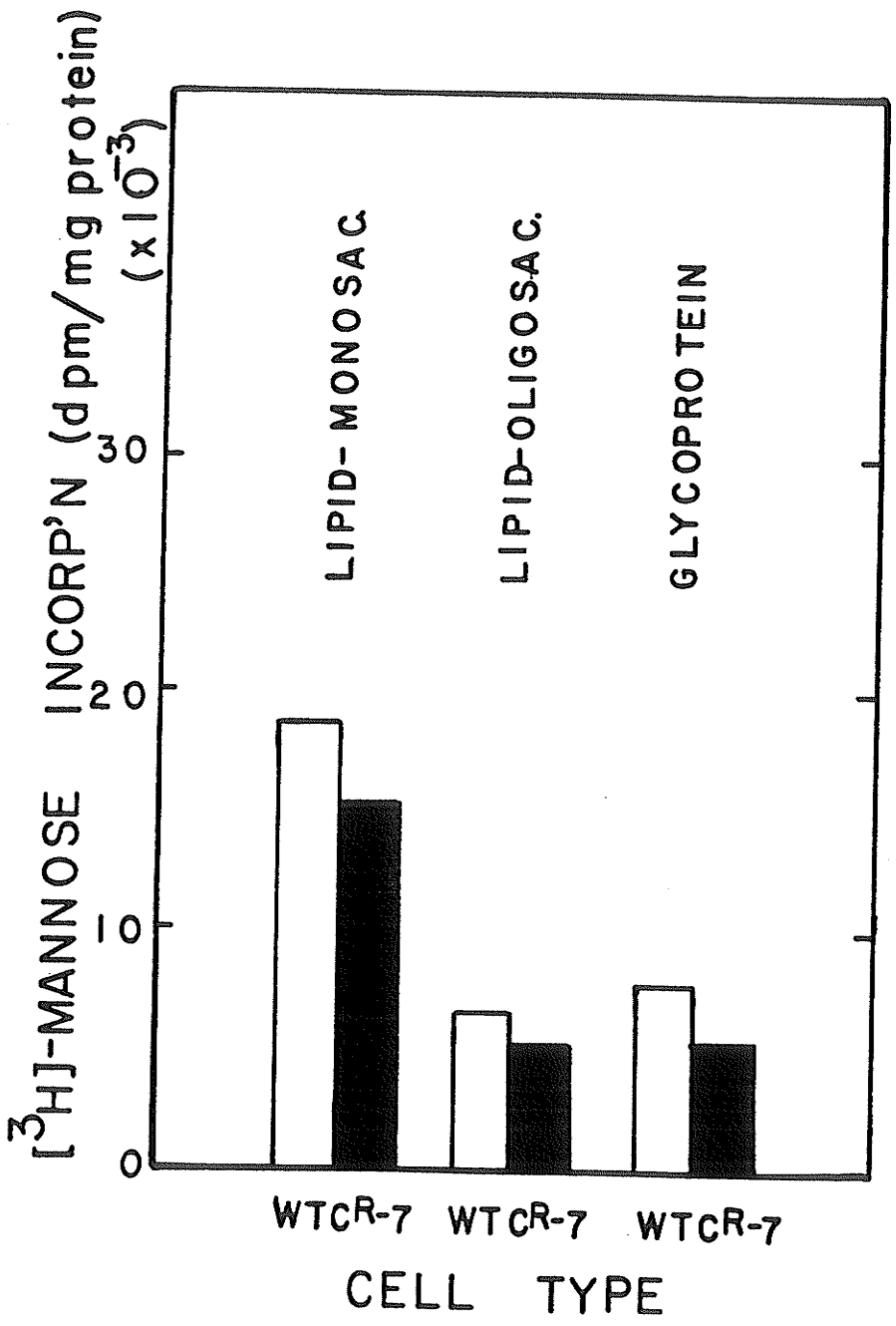
Based on these findings, no obvious relationship exists between cholesterol status and lipid-monosaccharide, lipid-oligosaccharide and glycoprotein synthesis.

DNA, RNA and Protein Synthesis: The Effect of Mevalonate Addition on C^R7

DNA synthesis was examined in all three cell lines by monitoring the incorporation of [6-^{3}H] thymidine into DNA at 34°C, 39°C, and at 39°C when C^R7 was cultured in the presence of MVA-lactone (77 μM). This concentration of MVA-lactone relieved temperature sensitive growth of C^R7 cells.

Figure 43 shows the results of these incorporation studies. At 34°C, "DNA synthesis" as measured by uptake of labeled thymidine is essentially the same regardless of whether the cells were grown on FCS-M or LPDS-M. At 39°C, the uptake of label by wild type and revertant cell lines was essentially unchanged from that measured at 34°C. Again, cholesterol sufficiency or cholesterol deficiency had no noticeable effect on this process in either WT or Rc. However, this was not true for the mutant.

Figure 42: [^{3}H]-mannose incorporation study at 39°C on FCS-M supplemented with MVA-lactone. WT and $C^{R}7$ (50,000 cells per plate) were delivered to 60 mm culture plates containing 5 ml of FCS-M and grown at 34°C for 4 days. The medium was removed and WT cell cultures received 5 ml fresh FCS-M and the $C^{R}7$ cell cultures received 5 ml fresh FCS-M supplemented with MVA-lactone (77 μM) and growth was continued at 39°C for a further 20 hours at 39°C. Cell cultures were then exposed to a 4 h pulse of D-[2-^{3}H] mannose (17 μCi/ml) (Amersham). Following this, cell monolayers were washed and processed as described in Methods and Materials. The amount of [^{3}H]-mannose incorporated into lipid-monosaccharide, lipid-oligosaccharide and glycoprotein was determined as described in Methods and Materials. Results presented are the average of duplicate determinations on 2 separate cell cultures per cell line.


[3H]-MANNOSE INCORP'N (dpm/mg protein) (x10-3)
0
10
20
30
LIPID-MONOSAC.
LIPID-OLIGOSAC.
GLYCOPROTEIN
WTCR-7
WTCR-7
WTCR-7
CELL TYPE

At 39°C on FCS-M, WT and Rc had a 5-fold greater rate of uptake of labeled thymidine than did C^R7. This was also evident on LPDS-M. C^R7 was able to incorporate labeled thymidine 5 times greater at 34°C than it was at 39°C regardless of cholesterol status. However, when C^R7 was cultured at 39°C in FCS-M supplemented with MVA-lactone (77 μM), C^R7 cells were able to synthesize DNA as measured by uptake of labeled thymidine at a rate comparable to that seen in WT at 39°C on FCS-M.

RNA synthesis was also examined in all three cell lines by monitoring the incorporation of [5-^{3}H] uridine into RNA at 34°C, 39°C, and 39°C with C^R7 cultured in the presence of MVA-lactone (77 μM). Figure 44 shows the results of these incorporation studies. At 34°C, "RNA synthesis" as measured by uptake of label is approximately the same in all three cell lines, regardless of whether FCS-M or LPDS-M was present. A somewhat decreased rate of uptake of label by all three cell lines was seen at 34°C on LPDS-M. The most pronounced decrease was seen, however, in C^R7. At 39°C on FCS-M, "RNA synthesis" of WT and Rc cell lines was similar to that seen at 34°C on FCS-M. This was not the case with C^R7. The rate of uptake of label by C^R7 was three fold less than that of WT and Rc (at 39°C, FCS-M) and at least four times less at 39°C on FCS-M than it (C^R7) was at 34°C on FCS-M.

At 39°C on LPDS-M, a pronounced increase in uptake of label was seen in both WT and revertant, however, this again was not true for C^R7. In fact, WT and Rc cell lines' "rate" of uptake was at least four

Figure 43: DNA synthesis in WT, Rc and $C^{R}7$ cell cultures. The incorporation of [6-^{3}H]-thymidine into DNA was examined at 34°C, 39°C and 39°C in the presence of MVA-lactone. 50,000 cells of each cell line were delivered to individual 60 mm tissue culture dishes containing 5 ml FCS-M. Cell cultures were grown at 34°C for about 4 days until the cells approached subconfluence. At this point, medium was removed and replaced with 5 ml of either FCS-M or LPDS-M as appropriate and cell cultures were grown at either 34°C or 39°C for a further 22 hours. $C^{R}7$ cell cultures were also exposed to 39°C for a 22 hour period but were cultured in the presence of 5 ml of FCS-M supplemented with MVA-lactone (77 μM). Following this 22 hour growth period, cells were exposed to [6-^{3}H]-thymidine (Amersham) for a 2 hour pulse. Following this, cell monolayers were washed and removed with ice cold TCA and then processed further as described in the Methods and Materials. Values shown are means of duplicate determinations on two separate cultures per cell line.

A: 34°C, FCS-M; LPDS-M
B: 39°C, FCS-M; LPDS-M
C: 39°C, FCS-M supplemented with MVA-lactone (77 μM)

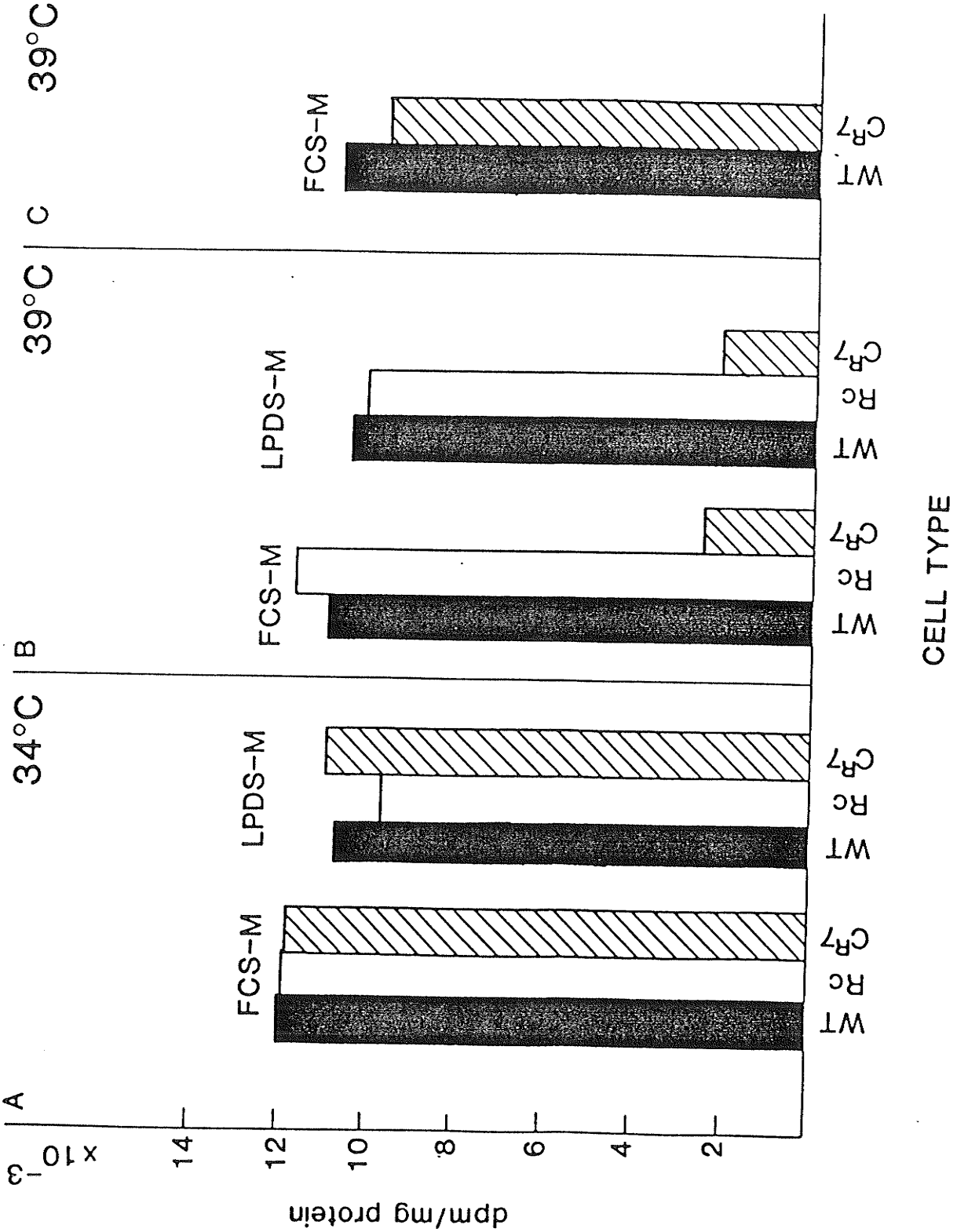
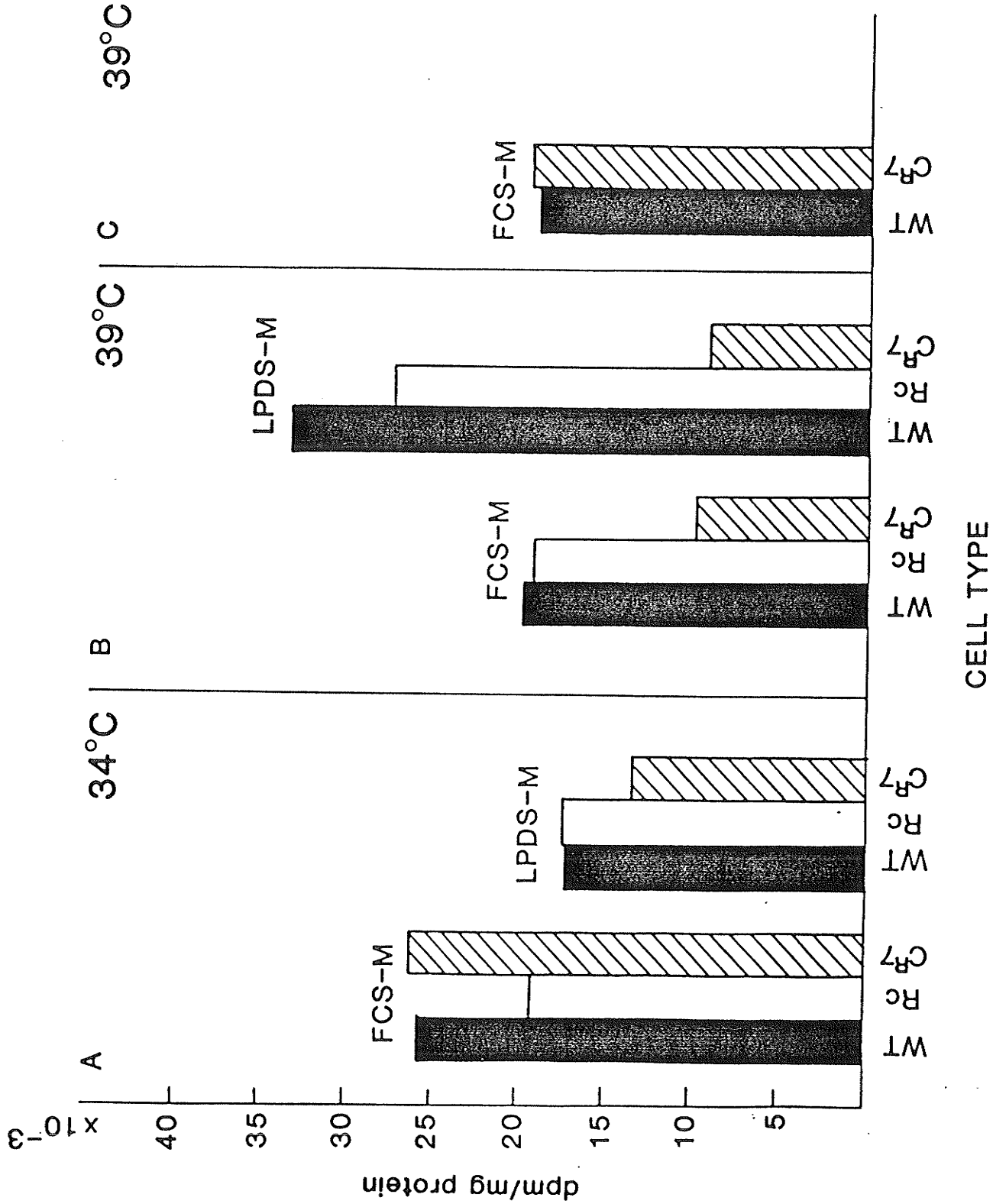

A
B
C
34°C
39°C
39°C
FCS-M
LPDS-M
FCS-M
FCS-M
WT
Rc
CR7
dpm/mg protein
x10-3
2
4
6
8
10
12
14
CELL TYPE

Figure 44: RNA synthesis in WT, Rc and C^{R} 7 cell cultures. The incorporation of [5-^{3}H]-uridine into RNA was examined at 34°C, 39°C and 39°C in the presence MVA-lactone. Cell growth conditions were as described in Figure 43. Cell cultures were exposed to a 2 hour pulse of [5-^{3}H]-uridine. Incorporation into RNA determined as described in Methods and Materials. Values shown are means of duplicate determinations on two separate cultures per cell line.

A: 34°C, FCS-M; LPDS-M
B: 39°C, FCS-M; LPDS-M
C: 39°C, FCS-M supplemented with MVA-lactone (77 μM)


dpm/mg protein
x 10⁻³
40
35
30
25
20
15
10
5
A
34°C
B
39°C
C
39°C
FCS-M
LPDS-M
FCS-M
LPDS-M
FCS-M
WT
Rc
Cβ7
CELL TYPE

fold greater than that of the variant.

When C^R7 was grown at 39°C on FCS-M supplemented with MVA-lactone (77 μM), C^R7 and WT cell lines had comparable rates of RNA synthesis equal rates of uptake) (Figure 44).

Finally, an examination of protein synthesis in all three cell lines was undertaken. The incorporation of L-[4,5-^{3}H]-leucine into protein was examined at 34°C, 39°C, and 39°C in the presence of MVA-lactone (77 μM). Figure 45 summarizes the results of these investigations. "Protein synthesis" as measured by the uptake of labeled leucine into protein was found to be essentially the same for all three cell lines when cultured at 34°C on FCS-M. Furthermore, on LPDS-M at 34°C, WT and Rc expressed similar levels of uptake of label as on FCS-M, but in C^R7, uptake of label was reduced.

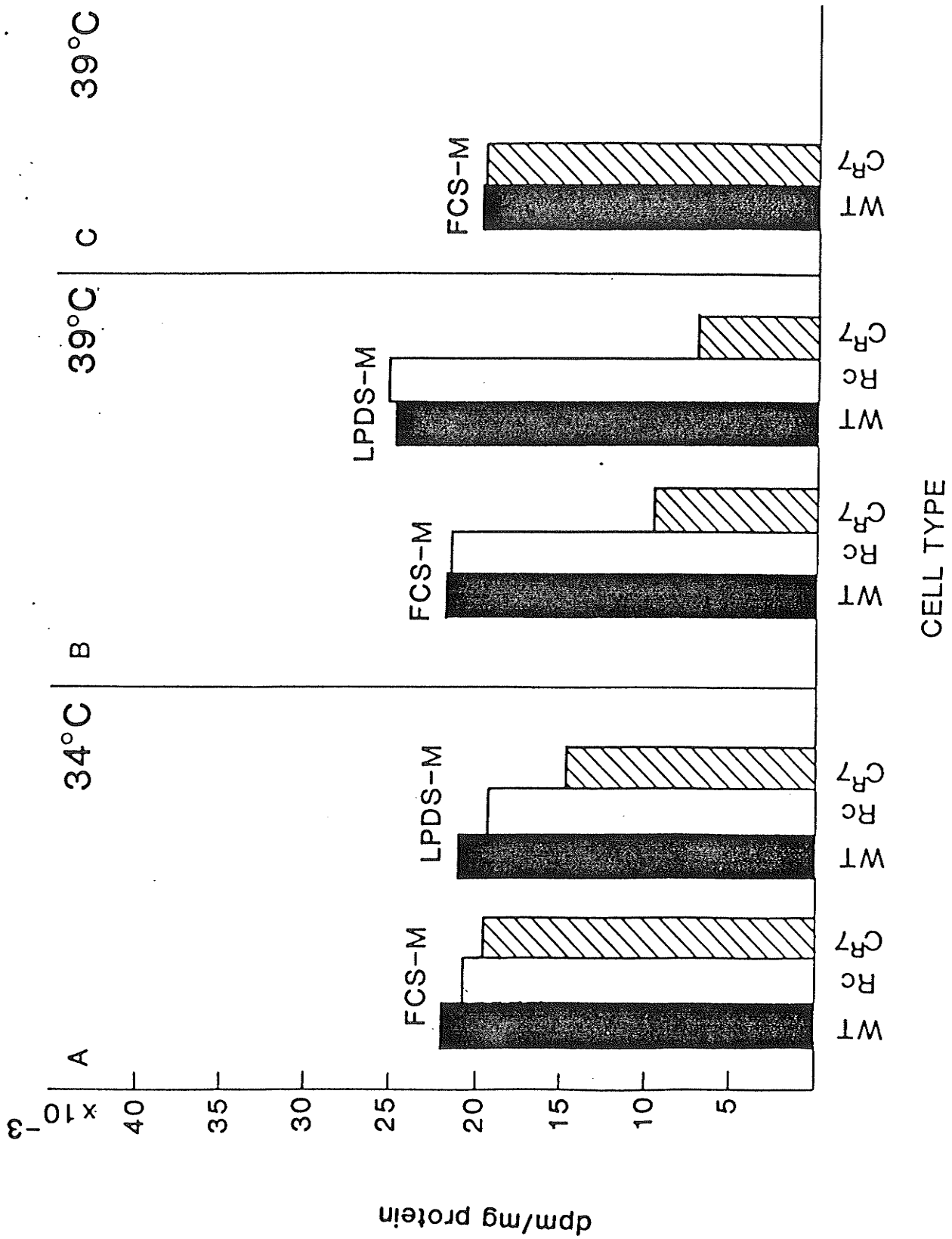
At 39°C on FCS-M, a slight increase in the rate of uptake of label by WT and Rc cells (relative to that seen at 34°C on FCS-M) was observed. However, the uptake of label by WT and Rc cells was at least two fold greater than that of C^R7. Furthermore, the uptake of label by C^R7 cells at 39°C (FCS-M) was about 2-fold less than that at 34°C (FCS-M).

Also at 39°C on LPDS-M, WT and Rc cells were able to incorporate slightly more label than at 39°C on FCS-M or at 34°C regardless of whether FCS-M or LPDS-M was present. This difference was probably not significant. With respect to variant cells, at 39°C (LPDS-M), WT and Rc cells incorporated at least four times more label than C^R7.

Furthermore, a substantial decrease of uptake of label by C^R7 cells was seen at 39°C (LPDS-M) relative to that seen at 34°C (LPDS-M). This

Figure 45: Examination of protein synthesis in WT, Rc, and $C^{R}7$ cell cultures. The incorporation of L-[4,5,-^{3}H]-leucine into protein was examined at 34°C, 39°C and 39°C in the presence of MVA-lactone. Cell growth conditions were as previously described in Figure 43. Cell cultures were exposed to a 2 hour pulse of L-[4,5,-^{3}H]-leucine (Amersham). Incorporation of label into protein was done as described in Methods and Materials. Values are shown the means of duplicate determinations on two separate cultures per cell line.

A: 34°C, FCS-M; LPDS-M
B: 39°C, FCS-M; LPDS-M
C: 39°C, FCS-M suplemented with MVA-lactone (77 μM)


dpm/mg protein
x 10⁻³
40
35
30
25
20
15
10
5
A
34°C
B
39°C
C
39°C
FCS-M
LPDS-M
FCS-M
LPDS-M
FCS-M
WT
Rc
CR7
CELL TYPE

finding was most obvious when C^R7 was in a situation of increased demand for cholesterol, either in LPDS-M or at 39°C. Interestingly, when C^R7 was cultured at 39°C on FCS-M supplemented with MVA-lactone (77μM), the deficit between levels of uptake between C^R7 and WT (on FCS-M) was alleviated. Clearly, based on these findings and others presented previously, mevalonate (MVA-lactone) plays a pivotal role in the regulation of the mutant.

Incorporation of [^{3}H]-Mevalonic Acid into Protein and Lipid

Sinensky and Logel (1985) examined the incorporation of labeled mevalonic acid into proteins of a CHO somatic sell mutant which is blocked in one of the mevalonate-biosynthesizing enzymes (3-hydroxy-3 methylglutaryl-coenzyme A synthase). Lavel was found to be incorporated into a number of "terpenylated" proteins. These labeled proteins were hypothesized to be "trigger" proteins. Proteins required for the transition from a quiescent to the proliferative state define restriction points in the cell cylce (Pardee, 1974) and have been referred to as "trigger" proteins (Alberts et al, 1983).

In light of the intriguing role mevalonate plays in C^R7, it was decided to investigate whether or not labeled mevalonate could be incorporated into proteins in these cell lines. The incorporation of label into lipids was also examined.

Table 16 shows the incorporation of [^{3}H]-mevalonic acid into protein of WT and C^R7 cells grown at 34°C and 39°C on FCS-M. Both cell lines incorporated approximately the same amount of label into proteins at 39°C, while at 34°C, C^R7 cells incorporated slightly more label into

Table 16: Incorporation of [^{3}H]-mevalonic acid into protein. The incorporation of [2-^{3}H]-MVA (resuspended in PBS) (100 μCi/ml) into proteins of WT and $C^{R}7$ cell cultures was essentially done according to the procedure of Sinensky and Logel, (1985) and as described in Methods and Materials. Presented here is the total label incorporated into cell protein for a given experiment. A aliquot was withdrawn and radioactivity determined.

Cell Line	Growth Conditions	Total Incorporation of [^{3}H]-Mevalonic Acid into Cell Protein (dpm/mg cell protein)
WT	34°C, FCS-M	4,848
C^R7	34°C, FCS-M	8,016
WT	39°C, FCS-M	11,400
C^R7	39°C, FCS-M	12,960

total protein than did wild type cells.

Figure 46 illustrates the nature of the proteins found in these cell lines as determined on an SDS-PAGE gel. No obvious difference in the nature of the kinds of proteins isolated from wild type and mutant cells was found at either 34°C or 39°C.

However when this gel was examined for the incorporation of label (done by autoradiography) differences in incorporation were noted. Figure 47 illustrates the incorporation of label into protein of wild type and mutant cells. Although the autoradiogram is of a quality less than ideal, on careful examination, differences between the two cell lines are evident.

Wild type cells were found to incorporate label into two proteins (a "doublet") at both 34°C and 39°C with apparent masses of 23 and 24 kDa. On the other hand, $C^{R}7$ cells at 34°C incorporated label into a doublet with apparent masses of 23 and 24 kDa, but at 39°C, mutant cells were found to incorporate labeled mevalonate into several proteins with apparent masses of 14 kDa, 20 kDa, 23 kDa and 24 kDa (the doublet), 36 kDa and 42 kDa respectively.

Sinensky and Logel (1985) found label incorporated into proteins of a MeV-CHO cell mutant of approximate molecular weights of 43,000 and a doublet at 23,000 and 24,000.

In addition an examination of the incorporation of label into lipid was also done. Table 17 shows that both cell lines incorporated approximately the same amount of label into total non-saponifiable lipid at both 34°C and 39°C, although the amount incorporated at 39°C was greater (almost two fold greater) than that incorporated at 34°C. This

Figure 46: SDS-PAGE of cellular proteins labeled with [2- ^{3}H]-MVA. The incorporation of [2-^{3}H]-MVA (resuspended in PBS) (100 μCi/ml) into protein of WT and $C^{R}7$ cell cultures was essentially done according to the procedure of Sinensky and Logel, (1985) and as described in Methods and Materials. Presented here is an SDS-PAGE gel showing the proteins extracted from these cell lines.

Figure Legend: Lane 1, WT, 34°C; lane 2, WT, 39°C, lane 3 standard mixture consisting of bovine albumin, egg albumin, glyceraldehyde 3-P dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor, and α-lactalbumin; lane 4, $C^{R}7$, 34°C and lane 5, $C^{R}7$, 39°C. Standard (3.5 mg) was dissolved in UMS sample buffer (1.5 ml) and 25 μl was chromatographed. Cell protein samples were redissolved in 120 μl of SDS-sample buffer (for constituents see Methods and Materials) and 25 μl was chromatographed. Gels were chromatographed for an appropriate period of time and protein bands visualized by Coomassie blue staining. SDS-PAGE was carried out as described by Laemmli, (1970).

(Laemmli, U.K. (1970), Nature, 227, 680-685).

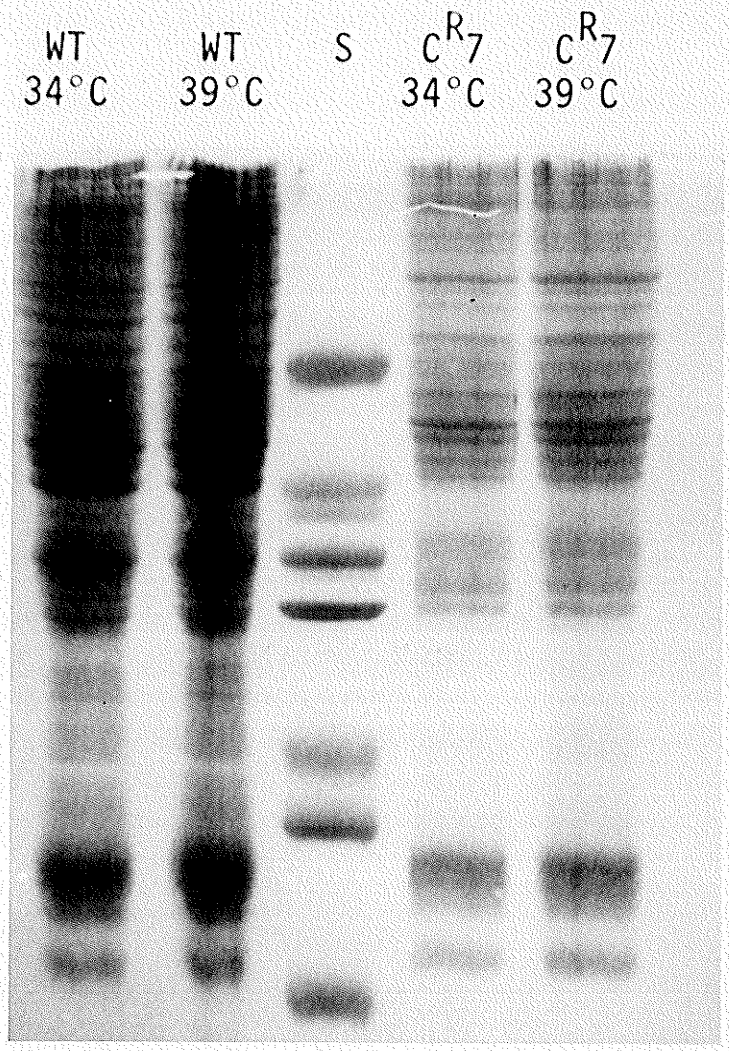

WT
34°C
WT
39°C
S
$C^{R}7$
34°C
$C^{R}7$
39°C

Figure 47: Radioautogram of SDS-PAGE gel showing proteins labeled with [2-^{3}H]-MVA. The incorporation of [2-^{3}H]-MVA (resuspended in PBS) (100 μCi/ml) into protein of WT and $C^{R}7$ cell cultures was done essentially according to the procedure of Sinensky and Logel, (1985) and as described in Methods and Materials. Presented here is a photograph of the developed autoradiogram of the SDS-PAGE gel illustrated in Figure 46, showing the nature of ^{3}H-MVA label incorporated into protein.

WT WT
34° 39°

C^R7 C^R7
34° 39°

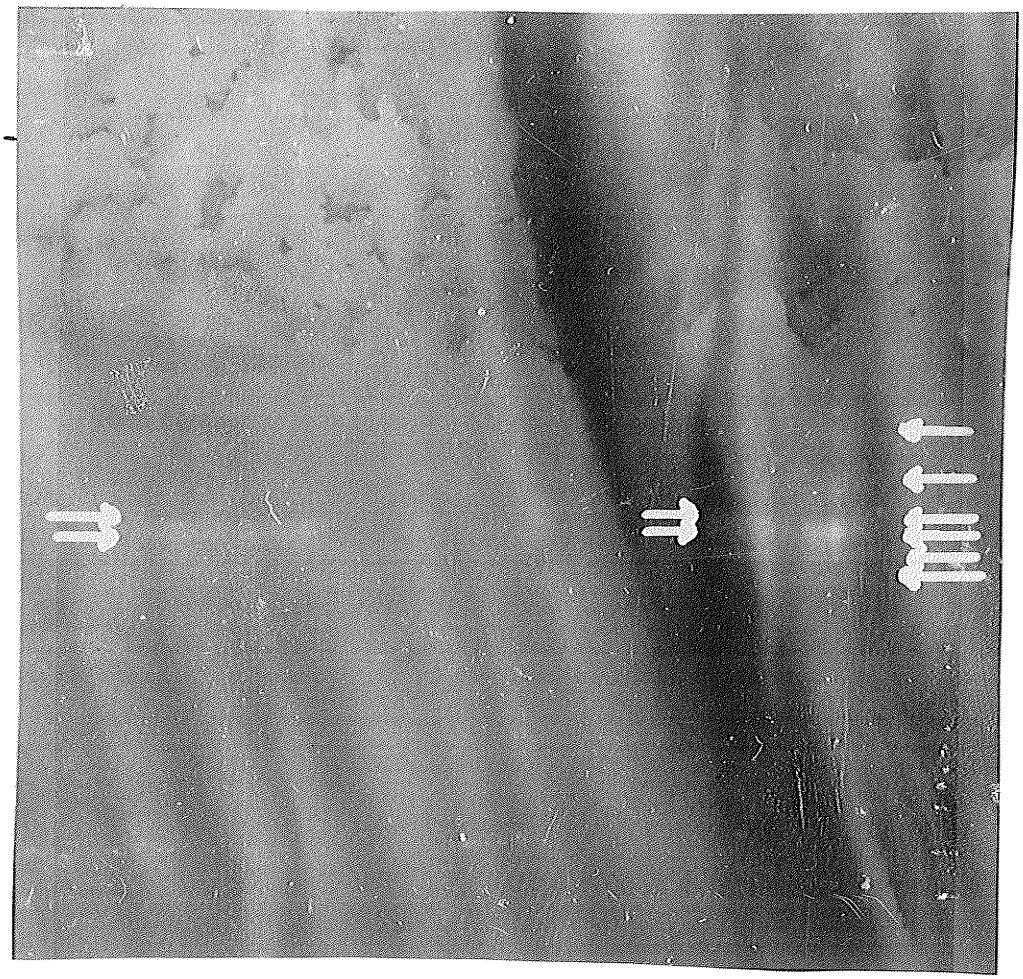


Table 17: Incorporation of [^{3}H]-mevalonic acid into non-saponifiable lipid. During the procedures examining the incorporation of [^{3}H]-mevalonic acid into protein done essentially according to the method Sinensky and Logel (1985) and as described in Methods and Materials, the incorporation into lipids was also examined. Presented here is the recovery of radioactivity in non-saponifiable lipids of WT and $C^{R}7$ cells incubated at 34°C and 39°C on FCS-M in the presence of [2-^{3}H]-mevalonic acid (Amersham). The values given are the results of single determination.

Cell Line	Growth Conditions	Total Incorporation of [^{3}H]-Mevalonic Acid into Non-saponifiable Lipids (dpm/mg cell protein)
WT	34°C, FCS-M	72,230
C^R_7	34°C, FCS-M	124,064
WT	39°C, FCS-M	184,213
C^R_7	39°C, FCS-M	215,432

was true for both the wild type and the variant.

Table 18 shows that incorporation of labeled mevalonic acid into individual non-saponifiable lipid components of wild type and mutant cell lines grown at 34°C and 39°C on FCS-M. At 34°C, the major portion of the label appeared in cholesterol for both WT and C^R7. However, at 39°C on FCS-M, once more the majority of label in WT appeared associated with cholesterol, but this was not the case for the variant. In the mutant, the label appeared predominantly in lanosterol as opposed to cholesterol (Table 18).

It would appear from the preceding findings that mevalonate plays an important, if not, a critical role in the survival and growth of C^R7. Since the addition of MVA-lactone (77 μM) restored parameters such as DNA, RNA, and protein synthesis to normal and relieved temperature-sensitive growth of C^R7, one of the membrane-associated properties in C^R7, could the addition of MVA-lactone have a similar effect on another property? The property chosen for investigation was Con-A resistance, which is also a membrane phenomenon.

Con-A Resistance and C^R7

Figure 48 shows that adding MVA-lactone (77 μM) increased the Con-A resistance of C^R7, rather than making it Con-A sensitive. Since the addition of MVA-lactone restored normal growth to C^R7 at 39°C (similar to WT) it was reasoned that it might also confer a "wild type" Con-A response to C^R7--that is--C^R7 would become Con-A sensitive. This was not the case. MVA-lactone addition had no effect on WT cells.

Since dolichol/dolichyl-phosphate could substitute for MVA-lactone in restoring normal growth to C^R7 at 39°C, their effect on Con-A

Table 18: Incorporation of [^{3}H]-mevalonic acid into individual non-saponifiable lipid components of wild type and $C^{R}7$ cell lines grown at 34°C and 39°C on FCS-M (cholesterol sufficient medium). To examine the relative incorporation into individual lipids, non-saponifiable lipid fractions were subjected to one dimensional TLC analysis using a two solvent system consisting of petroleum ether and acetone (90:10 v/v) as the first solvent system with chloroform alone as the second solvent used. Lipid components were identified with the aid of authentic lipid standards and were visualized with p-anisaldehyde as described previously. The values given are the results of duplicate determinations.

Relative incorporation of [^{3}H]-mevalonic acid into individual non-saponifiable lipids
(expressed as a percentage recovery of total [^{3}H] label)

Cell Line	Growth Conditions	1 Origin	2 ?	3 ?	4 Ubiquinone	5 ?	6 Cholesterol	7 Lanosterol	8 ?	9 Squalene
WT	34°C, FCS-M	5.2	1.4	1.1	4.8	17.6	53.9	6.9	1.4	7.7
C^R7	34°C, FCS-M	0.8	3.3	-	13.9	-	46.2	3.1	28.5	4.2
WT	39°C, FCS-M	4.4	0.6	0.4	1.5	-	72.4	6.4	1.2	13.1
C^R7	39°C, FCS-M	1.9	5.8	-	14.6	-	29.7	42.7	-	5.3

? unidentified components

- undetectable

Note: The numbers 1-9 inclusive refer to spot numbers as they appeared on the TLC plate.

resistance was examined. Figure 49 shows that dolichol (12 μM) and dolichyl-phosphate (12 μM) had no appreciable effect on C^R7 or WT Con-A response. C^R7 was Con-A resistant and WT was Con-A sensitive.

Cholesterol (13 μM) and isopentenyladenine (19 μM) were unable to substitute for MVA-lactone and increase Con-A resistance of C^R7 (see Figures 50 and 51). No effect was seen with wild type.

Figure 52 shows that adding lanosterol (46 μM) to FCS-M increased the Con-A resistance in C^R7, to an even greater extent than MVA-lactone was able to do. There was no effect on WT.

Con-A killing curves were also done at 39°C on FCS-M. C^R7 is temperature-sensitive for growth at 39°C on FCS-M but this was relieved in the presence of MVA-lactone. Figure 53 illustrates that C^R7 remains more resistant to Con-A at 39°C than WT. (Although no adequate control can be done for C^R7 at 39°C). Figure 54 shows that when grown at 39°C on FCS-M supplemented with dolichol (at a concentration capable of allowing normal growth), C^R7 possessed a Con-A response similar to WT, that is, it was Con-A sensitive but still less sensitive to Con-A than WT.

Incorporation of Mevalonate into Sterols

C^R7, as shown, was unable to upshift cholesterol synthesis when required and has a known inability to normally regulate HMG-CoA reductase activity (Borgford, 1984), it was decided to determine the incorporation of labeled mevalonolactone--the product of HMG-CoA reductase--into cholesterol and other polyisoprenoids. In this approach to examine the products of isoprenoid synthesis, cultures of wild type,

Figure 48: Effect of MVA lactone on the Con-A resistance of $C^{R}7$. Con-A killing curves were determined as described in Methods and Materials. The data shown was obtained using an inoculum of 1,000 cells. The same result was obtained using an inoculum of 300 cells (data not shown). Points represent the mean of duplicate or triplicate plates from a single experiment. Similar data was obtained in six separate experiments.

Figure Legend: WT, control (■—■) WT + MVA-lactone (77 μM) (▲—▲); $C^{R}7$, control (○—○); $C^{R}7$ + MVA-lactone (77 μM) (●—●). MVA-lactone was added as either an ethanolic solution or as an ethanol-BSA complex. Final concentration of ethanol was always less than 1%. Control cultures contained the desired concentration of Con-A plus the vehicle by which the compound of interest was added. Standard deviations were 1-3% of the mean values shown.

Growth Temperature: 34°C.

Type of Medium Used: FCS-M.

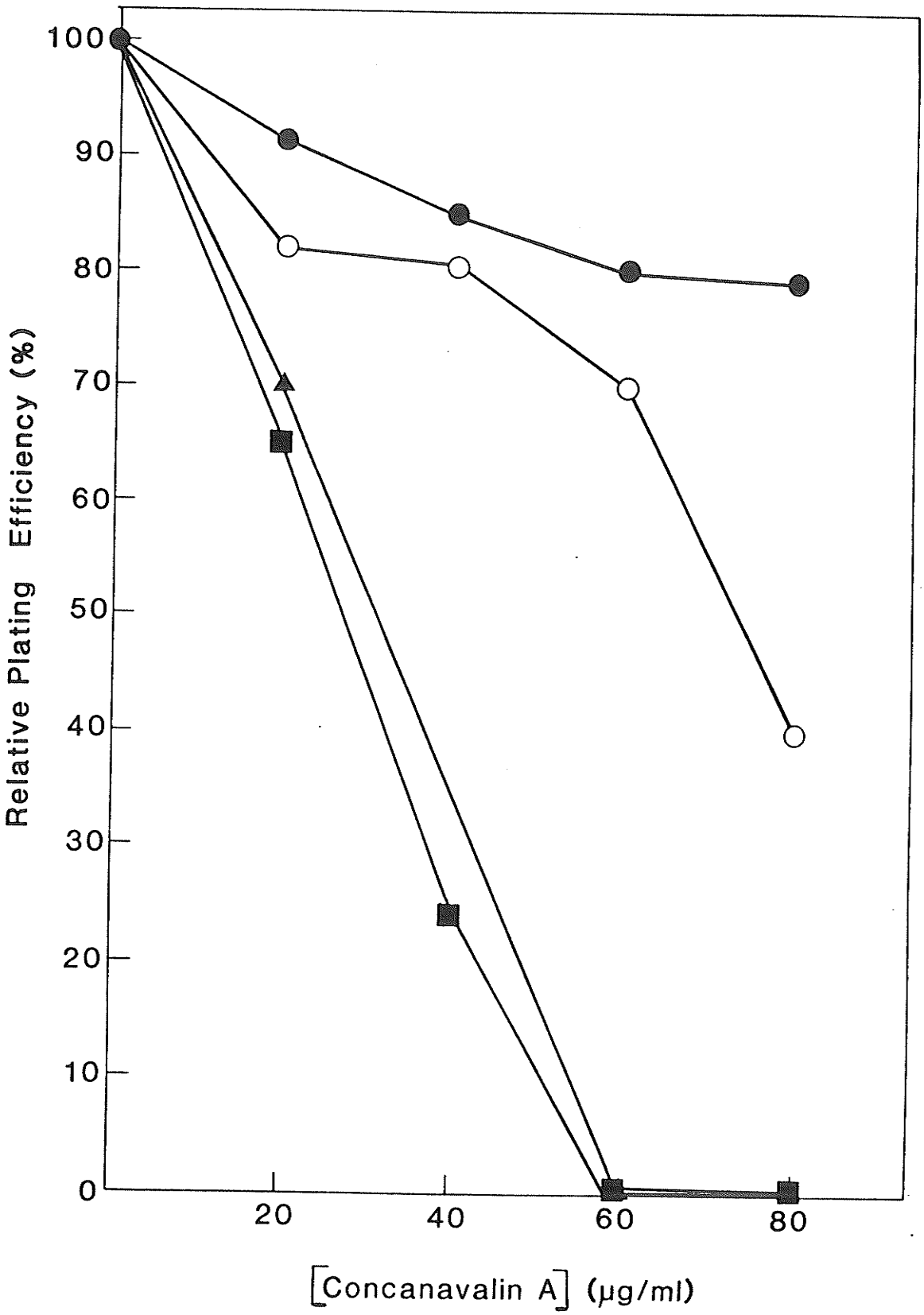

100
90
80
70
60
50
40
30
20
10
0
Relative Plating Efficiency (%)
20
40
60
80
[Concanavalin A] (μg/ml)

Figure 49: Effects of dolichol and dolichyl-phosphate on Con-A resistance of $C^{R}7$. Con-A killing curves were determined as described in Methods and Materials. The data shown was obtained using an inoculum of 1,000 cells. The same result was obtained using an inoculum of 300 cells (data not shown). Points represent the mean of duplicate plates from a single experiment. Similar data was obtained in four separate experiments.

Figure Legend: WT, control (); WT + dolichol (12 μM) (); $C^{R}7$, control (); $C^{R}7$ + dolichol (12 μM) (); $C^{R}7$ + dolichyl-phosphate (12 μM) (). WT + dolichyl-phosphate not done. Dolichol and dolichyl-phosphate were added as BSA-ethanol complexes. BSA-ethanol solution alone had no effect on Con-A response of either $C^{R}7$ or WT. Standard deviations were 1-3% of the mean values shown.

Growth Temperature: 34°C.

Type of Medium Used: FCS-M.

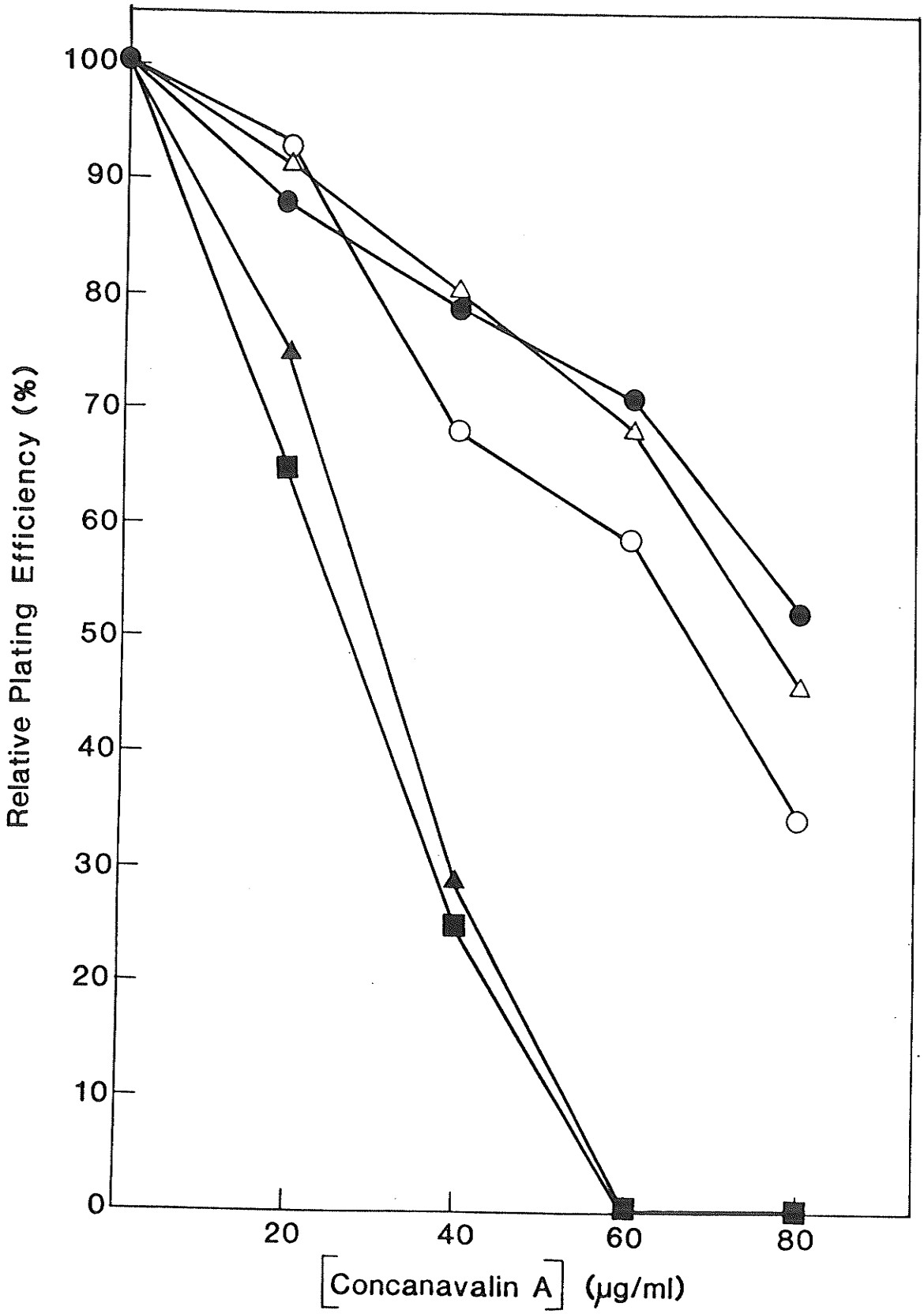
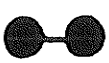

Relative Plating Efficiency (%)
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
[Concanavalin A] (μg/ml)

Figure 50: Effect of cholesterol on Con-A resistance of $C^{R}7$. Con-A killing curves were determined as described in Methods and Materials. The data shown was obtained using an inoculum of 1,000 cells.

Figure Legend: WT, control (); WT + cholesterol (13 μM) (); $C^{R}7$, control (); $C^{R}7$ + cholesterol (13 μM) (). Cholesterol was added as a BSA-ethanol complex. BSA-ethanol solution alone had no demonstratable effect on Con-A response of either WT or $C^{R}7$ (data not shown).

Points shown represent the mean of duplicate plates from a single experiment. Similar data was obtained in two separate experiments. Standard deviations were 1-3% of the mean values shown.

Growth Temperature: 34°C.

Type of Medium Used:FCS-M.

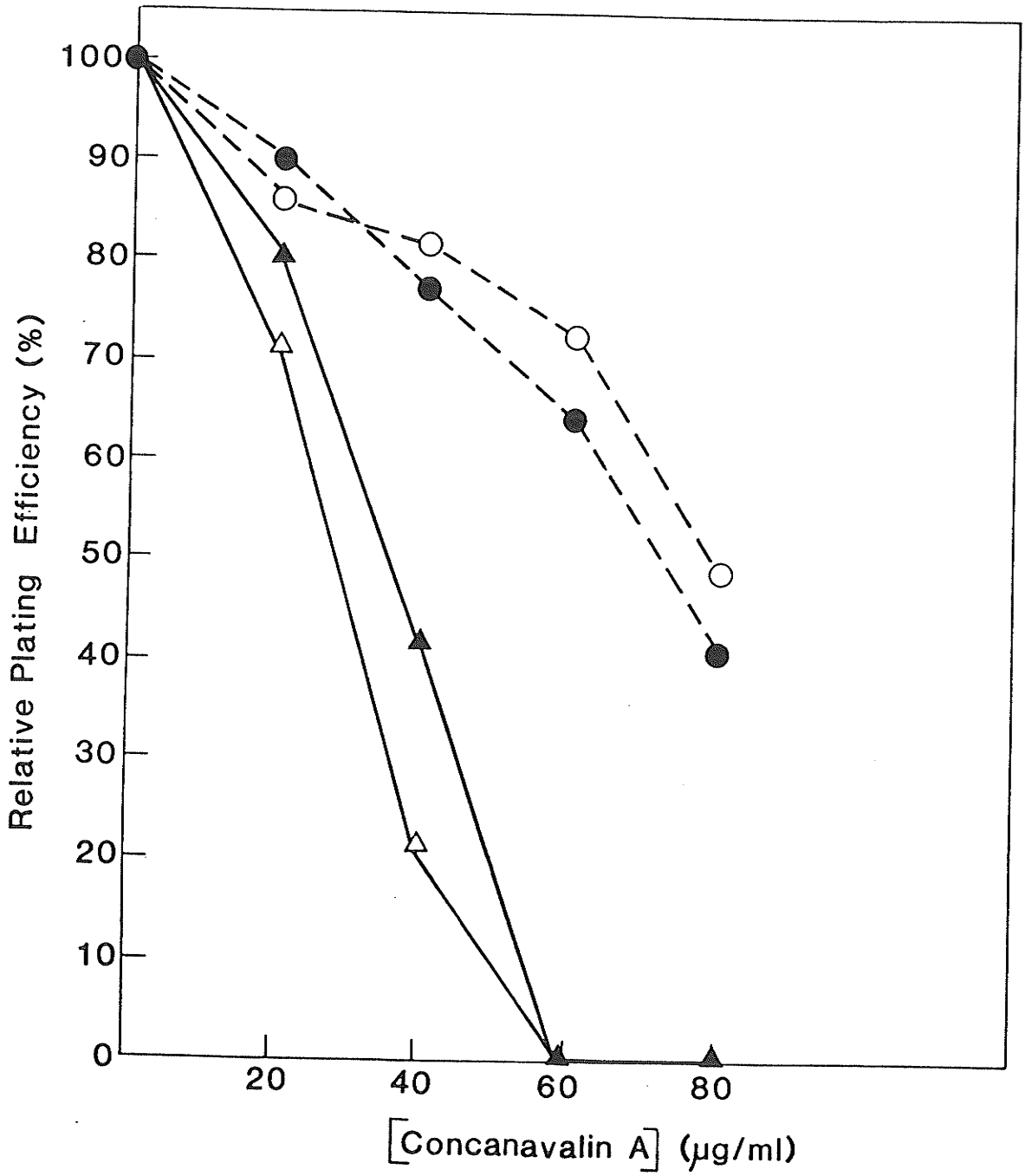

Relative Plating Efficiency (%)
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
[Concanavalin A] (μg/ml)

Figure 51: Effect of isopentenyladenine (IPA) on Con-A resistance of C^R7. Con-A killing curves were determined as described in Methods and Materials. The data shown was obtained using an inoculum of 1,000 cells. IPA was added as a BSA-ethanol complex. Control experiments showed that the addition of this complex alone had no effect on WT or C^R7's response to Con-A.

Figure Legend: WT, control (); WT + IPA (19 μM) (); C^R7, control (), C^R7 + IPA (19 μM) (). Points shown represent the mean of duplicate plates from a single experiment. Similar data was obtained in two separate experiments. Standard deviations were 2-4% of the mean values shown.

Growth Temperature: 34°C.

Type of Medium Used: FCS-M.

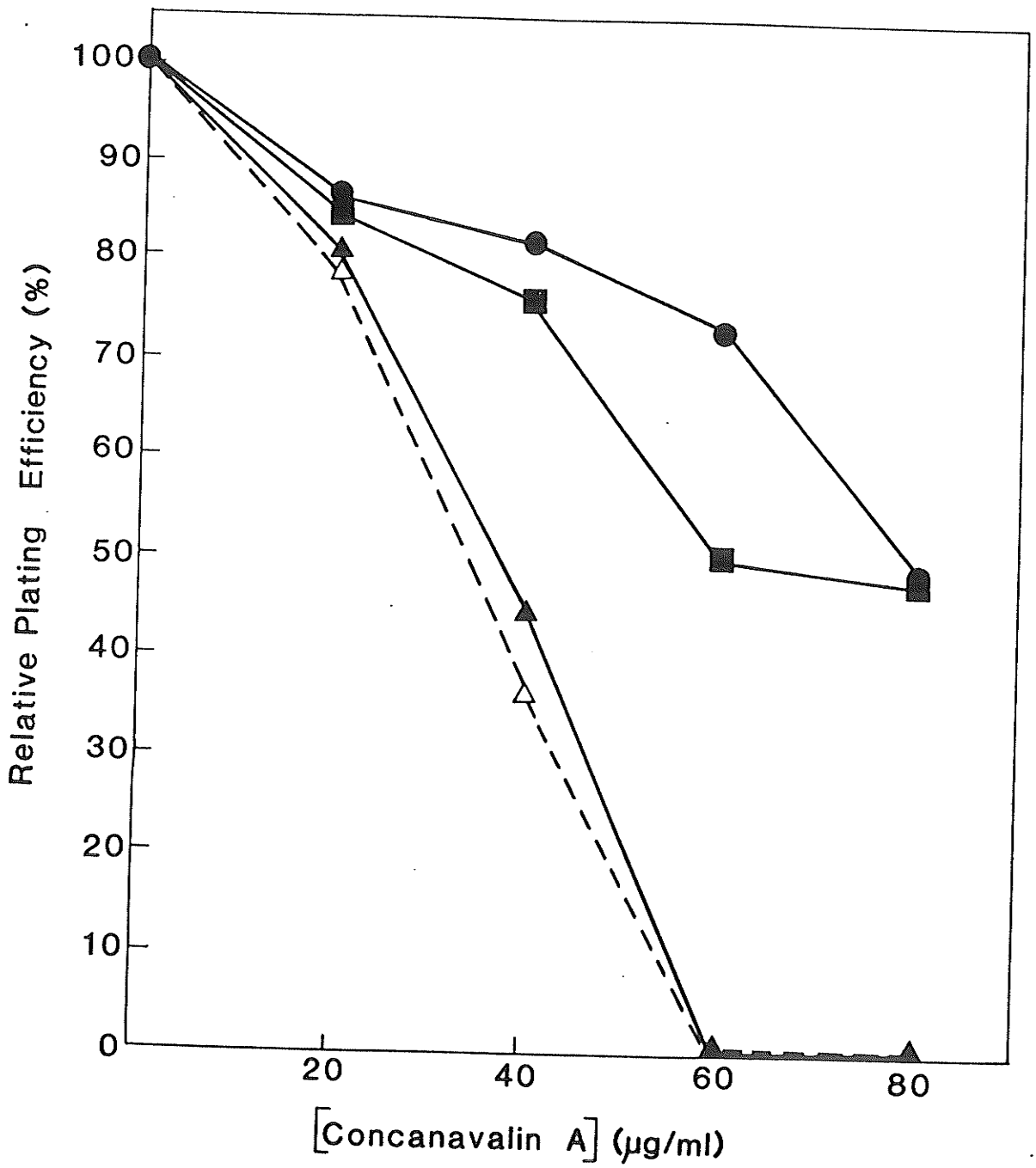
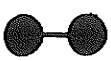
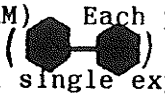

Relative Plating Efficiency (%)
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
[Concanavalin A] (µg/ml)

Figure 52: Effects of MVA lactone and lanosterol on Con-A resistance of $C^{R}7$. Con-A killing curves were determined as described in Methods and Materials. The data shown was obtained using an inoculum of 1,000 cells. The same result was obtained using an inoculum of 300 cells (data not shown). MVA lactone was added as an ethanolic solution and lanosterol was added as a BSA-ethanol complex. Control experiments showed that neither ethanol alone nor the BSA-ethanol complex had any effect on WT/$C^{R}7$ response to Con-A.

Figure Legend: WT, control (); WT + MVA lactone (77 μM) (); WT + lanosterol (46 μM) (); $C^{R}7$ control (); $C^{R}7$ + MVA-lactone (77 μM) (); $C^{R}7$ + lanosterol (46 μM) (). Each point shown represents the mean of duplicate plates from a single experiment. Similar data was obtained in three separate experiments. Standard deviations were 1-3% of the mean values shown.

Growth Temperature: 34°C.

Type of Medium Used: FCS-M.

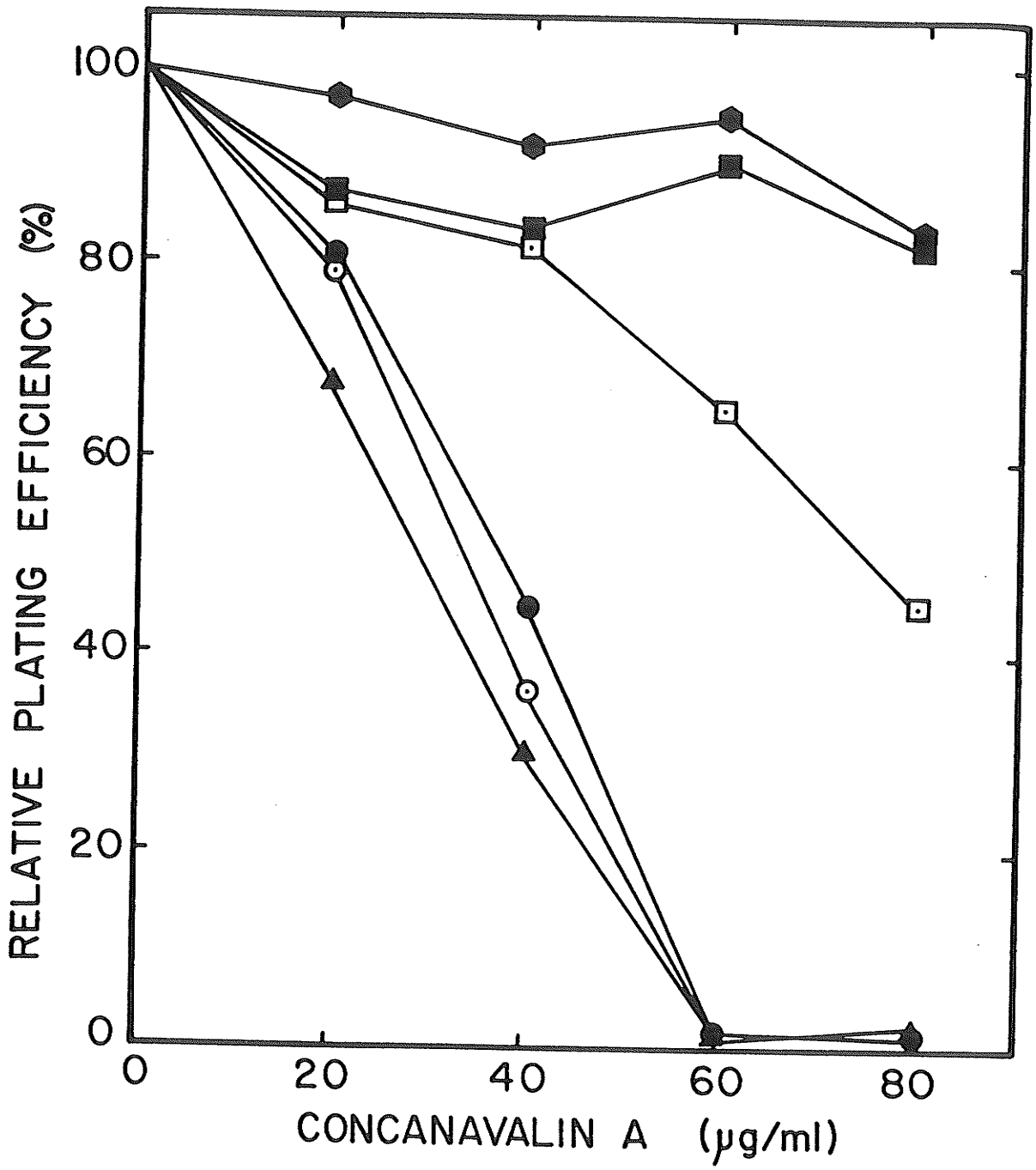

RELATIVE PLATING EFFICIENCY (%)
100
80
60
40
20
0
0
20
40
60
80
CONCANAVALIN A (µg/ml)

Figure 53: Concanavalin-A killing curve of WT and C^R7 at 39°C. Con-A killing curves were performed as described in Methods and Materials except that the incubation temperature was 39°C and not the usual incubation temperature of 34°C. The data shown was obtained using an inoculum of 1,000 cells.

Figure Legend: WT (▲–▲); C^R7 + MVA-lactone (77 μM) (added as a BSA-ethanol complex) (●–●). Each point shown represents the mean of duplicate plates from a single experiment. Similar results were obtained in two separate experiments. Standard deviations from the mean were 2-4% of the values shown.

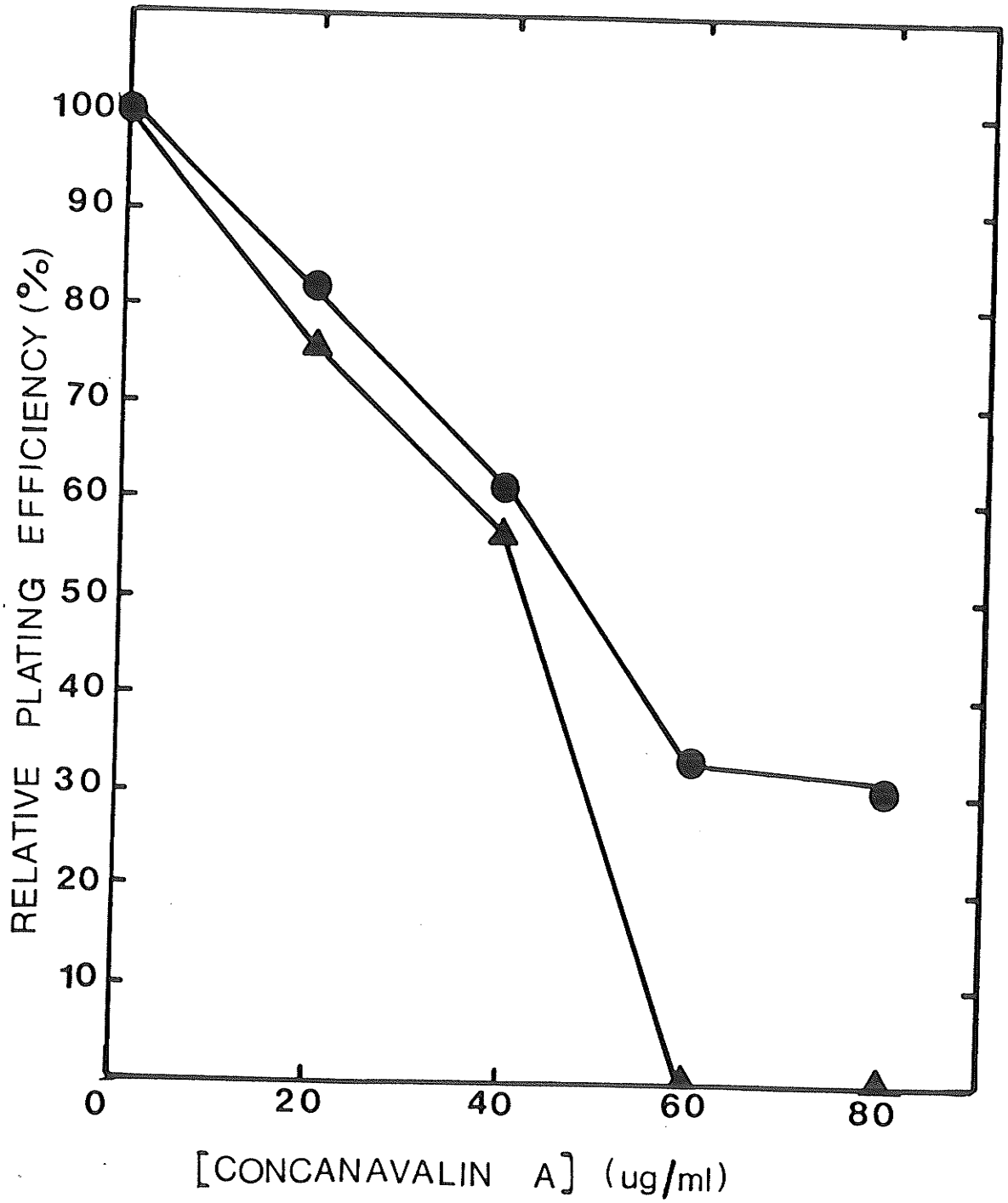
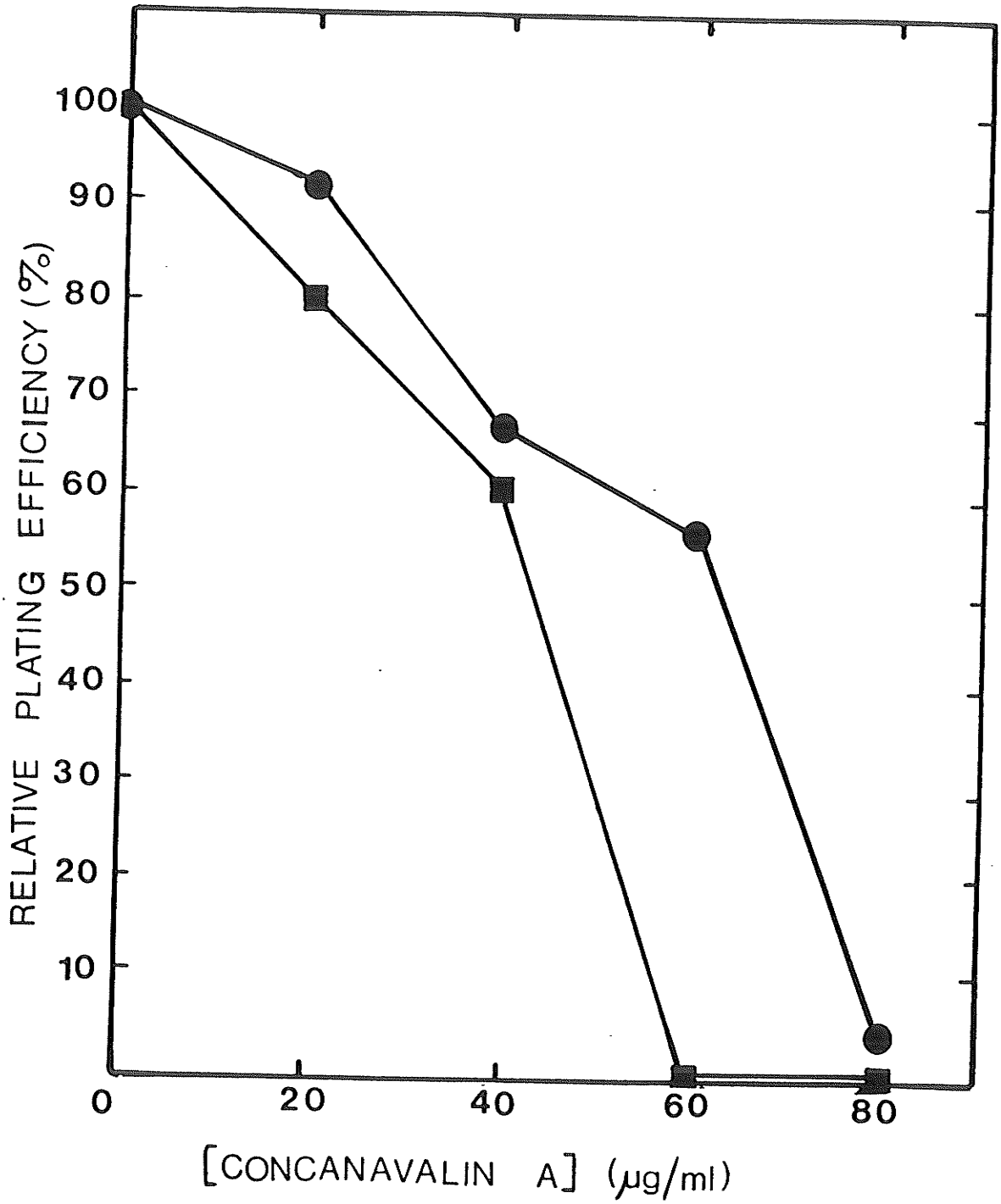

RELATIVE PLATING EFFICIENCY (%)
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
[CONCANAVALIN A] (ug/ml)

Figure 54: Con-A killing curves of C^R7 at 39°C done in the presence of dolichol. Con-A killing curves were performed as described in Methods and Materials except the incubation period was at 39°C not 34°C. The data shown was obtained with an inoculum of 1,000 cells.

Figure Legend: WT (); C^R7 + dolichol (12 μM) as a BSA-ethanol complex (). Each point represents the mean of duplicate plates from a single experiment.


RELATIVE PLATING EFFICIENCY (%)
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
[CONCANAVALIN A] (μg/ml)

revertant and mutant cells were incubated with radiolabeled mevalonate. The metabolic products of this incubation were identified by TLC following saponification. The total amount of label incorporated into non-saponifiable fractions was relatively high in all three cell lines whether the cells were grown on FCS-M or LPDS-M (Tables 19 and 20). Although it was found that all three cell lines incorporate similar amounts of mevalonate into non-saponifiable products, they were found to incorporate significantly different amounts into individual sterol components (Tables 21(a) and 21(b)).

Table 21(a) shows that in WT and Rc cells ^{14}C-label accumulated predominantly in cholesterol in cultures grown in both FCS-M and LPDS-M. The variant, C^R7, accumulated almost equal amounts of this label in lanosterol and cholesterol when cultured in FCS-M whereas more than twice as much appeared in lanosterol compared to cholesterol when cultured in LPDS-M. These findings were noted at 34°C, the permissive temperature for all three cell lines.

Table 21(b) shows the results found at 39°C. C^R7 is temperature-sensitive for growth at this temperature. Once again, wild type and revertant cell lines accumulated ^{14}C-label predominantly in cholesterol in cultures grown in both FCS-M and LPDS-M. The mutant accumulated almost equal amounts of label into lanosterol and cholesterol when cultured in FCS-M, but approximately nine times as much label appeared in lanosterol than in cholesterol when cultured in LPDS-M. Furthermore, a significant amount of label appeared associated with ubiquinone when C^R7 was cultured in LPDS-M at 39°C (Table 21(b)).

Table 22 illustrates the ratio of label incorporated into

Table 19: Recovery of radioactivity in non-saponifiable lipid fractions of WT, Rc, and $C^{R}7$ cells incubated at 34°C and 39°C on FCS-M plus 5.9 μCi of R-[2-^{14}C] MVA-lactone (53 mCi/m mole) (Amersham). The values given are the results of single determinations. Recoveries averaged 93%.

Cell Line	Growth Conditions	Total Incorporation of ^{14}C-MVA-Lactone into Non-saponifiable Lipids (dpm/mg cell protein)
WT	34°C, FCS-M	14,504
Rc	34°C, FCS-M	12,206
$C^{R}7$	34°C, FCS-M	12,784
WT	39°C, FCS-M (24 h)	16,497
Rc	39°C, FCS-M (24 h)	12,640
$C^{R}7$	39°C, FCS-M (24 h)	25,851
$C^{R}7$	39°C, FCS-M (36 h)	15,591

Table 20: Recovery of radioactivity in non-saponifiable lipid fractions of WT, Rc, and $C^{R}7$ cells incubated at 34°C and 39°C on LPDS-M plus 5.9 μCi of R-[2-^{14}C] MVA-lactone (53 mCi/m mole) (Amersham). The values given are the results of single determinations. Recoveries averaged 82%.

Cell Line	Growth Conditions	Total Incorporation of ^{14}C-MVA-Lactone into Non-saponifiable Lipids (dpm/mg cell protein)
WT	34°C, LPDS-M (24 h)	12,191
Rc	34°C, LPDS-M (24 h)	19,756
$C^{R}7$	34°C, LPDS-M (24 h)	10,587
WT	39°C, LPDS-M (24 h)	14,918
Rc	39°C, LPDS-M (24 h)	15,032
$C^{R}7$	39°C, LPDS-M (24 h)	16,333

Table 21(a): Incorporation of ^{14}C-labeled mevalonolactone into individual lipid components of WT, Rc, and $C^{R}7$ cell lines incubated at 34°C on FCS-M and LPDS-M. To examine the relative incorporation into individual lipids, non-saponifiable lipid fractions were subjected to one dimensional TLC analysis using petroleum ether-acetone (90:10 v/v) as the first solvent system followed by chromatography using chloroform as the second solvent system. Lipid components were visualized and identified with the aid of p-anisaldehyde and authentic lipid standards respectively and as described previously. The results given are the results of duplicate analyses.

Cell Line	Growth Conditions	Percentage Recovery of ^{14}C-label								
		1 Origin	2 ?	3* Q	4 ?	5 Cholesterol	6 ?	7 Lanosterol	8 ?	9 Squalene
WT	34°C, FCS-M	2.4	10.1	3.7	-	67.2	3.3	10.0	2.3	1.0
Rc	34°C, FCS-M	5.1	8.3	3.2	-	70.0	3.0	6.5	2.2	1.7
C^R7	34°C, FCS-M	14.7	5.4	16.2	-	27.3	5.2	20.1	2.5	8.6
WT	34°C, LPDS-M (24 h)	2.8	-	10.5	-	57.8	4.5	5.8	3.5	14.8
Rc	34°C, LPDS-M (24 h)	2.3	-	9.8	-	56.3	-	4.8	24.5	2.3
C^R7	34°C, LPDS-M (24 h)	1.9	-	25.1	-	19.7	-	46.4	2.5	4.4

? unidentified

- undetectable

*Q = ubiquinone

Numbers 1-9 inclusive correspond to spots which appeared on the TLC plate.

Table 21(b): Incorporation of ^{14}C-labeled mevalonolactone into individual lipid components of WT, Rc, and $C^{R}7$ cell lines incubated at 39°C on FCS-M and LPDS-M. Protocol employed is as described in Methods and Materials and in the description accompanying Table 20.

Cell Line	Growth Conditions	Percentage Recovery of ^{14}C-label								
		1 Origin	2 ?	3 Q	4 ?	5 Cholesterol	6 ?	7 Lanosterol	8 ?	9 Squalene
WT	39°C, FCS-M (24 h)	1.2	4.0	11.3	4.7	61.3	4.7	4.7	2.5	5.6
Rc	39°C, FCS-M (24 h)	2.6	4.6	8.0	8.7	66.5	2.3	3.3	1.9	2.1
C^R_7	39°C, FCS-M (24 h)	3.2	-	7.8	-	34.4	13.4	27.9	3.1	10.2
C^R_7	39°C, FCS-M (36 h)	7.0	2.3	11.0	3.5	30.4	7.0	27.1	7.6	3.5
WT	39°C, LPDS-M (24 h)	0.8	-	10.1	-	78.7	2.4	6.3	-	1.7
Rc	39°C, LPDS-M (24 h)	3.2	-	5.9	-	55.4	5.4	5.4	12.3	12.4
C^R_7	39°C, LPDS-M (24 h)	2.2	-	30.8	-	6.3	-	52.8	2.1	5.8

? unidentified

- undetectable

Numbers 1-9 inclusive correspond to spot position as the lipid components appeared on the TLC plate.

Table 22: Incorporation of ^{14}C-mevalonolactone into cholesterol and lanosterol of WT, Rc, and $C^{R}7$ cell lines grown at 34°C and 39°C in the presence of FCS-M and LPDS-M. A ratio of ^{14}C incorporation is presented. Incorporation of ^{14}C into lanosterol plus cholesterol constituted 60-80% of the total incorporation into non-saponifiable lipids (average being 69%).

Cell Line	Growth Conditions	Ratio of ^{14}C Incorporation (lanosterol/cholesterol)
WT	FCS-M, 34°C	0.15
Rc	FCS-M, 34°C	0.10
$C^{R}7$	FCS-M, 34°C	0.83
WT	LPDS-M, 34°C (24 h)	0.11
Rc	LPDS-M, 34°C (24 h)	0.09
$C^{R}7$	LPDS-M, 34°C (24 h)	2.50
WT	FCS-M, 39°C (24 h)	0.09
Rc	FCS-M, 39°C (24 h)	0.06
$C^{R}7$	FCS-M, 39°C (24 h)	0.91
$C^{R}7$	FCS-M, 39°C (36 h)	0.83
WT	LPDS-M, 39°C (24 h)	0.10
Rc	LPDS-M, 39°C (24 h)	0.10
$C^{R}7$	LPDS-M, 39°C (24 h)	8.38

lanosterol over cholesterol. It is clear from this data that in C^R7, the majority of radiolabeled mevalonate appears in lanosterol. This was especially so in LPDS-M and at 39°C. Wild type and revertant incorporated mevalonalactone primarily into cholesterol, very little label appeared in lanosterol. C^R7 is unable to adequately convert lanosterol to cholesterol and this defect is accentuated when the cholesterol demand is high--ie: in LPDS-M or at 39°C.

Furthermore, if this conclusion is correct, C^R7 should be able to grow on LPDS-M supplemented with intermediates of the pathway between lanosterol and cholesterol. Figure 55 shows that this was true for desmosterol and 7-dehydrocholesterol.

Desmosterol (13 μM) and 7-dehydrocholesterol (13 μM) allowed C^R7 to grow normally at 34°C on LPDS-M (Figure 55).

These results led to the speculation that the presence of unusually large amounts of lanosterol in cellular membranes, coupled with the relative absence of cholesterol, might be responsible for the phenotypic changes in membrane-related properties that are characteristic of C^R7.

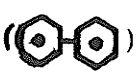
Lanosterol Demethylation as a Determinant of "Mutant" or "Wild Type" Behaviour

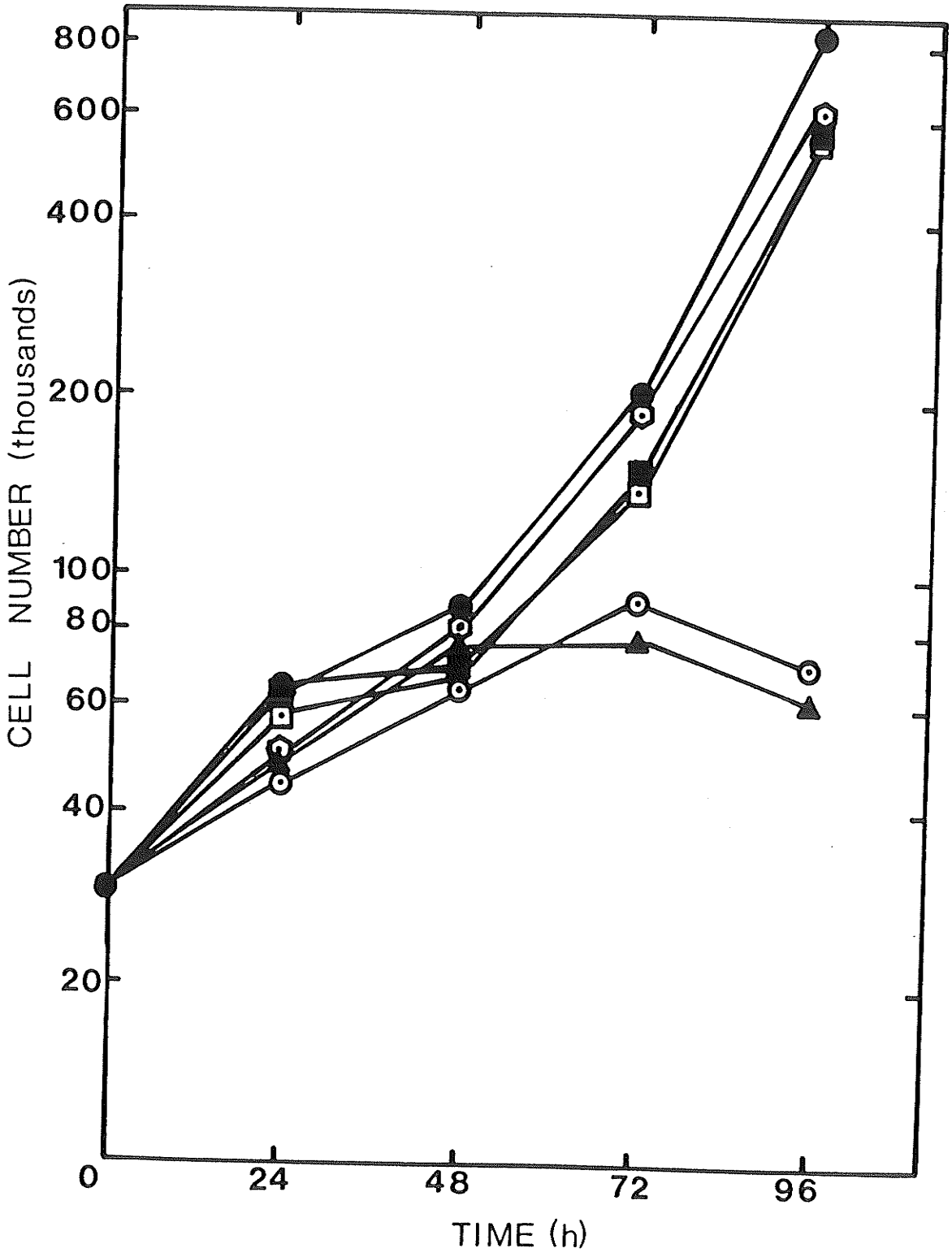
Effects of Addition of Ketoconazole

Ketoconazole is a substituted imidazole that is an orally effective antimycotic agent displaying activity against a wide variety of pathogenic fungi (Borelli et al, 1979). Ketoconazole is a drug that inhibits the cytochrome P-450 dependent step of C-14 demethylation of lanosterol (Van den Bossche et al, 1980).

It was reasoned that treatment of WT cells with ketoconazole should

Figure 55: Effect of addition of desmosterol and 7-dehydrocholesterol on growth of $C^{R}7$ at 34°C. $C^{R}7$ (10,000 cells plate) were delivered to 60 mm dishes containing 5 ml FCS-M and incubated 24 h at 34°C. Cell numbers were then estimated and the values designated as zero time counts. The medium was removed and then replaced with 5 ml of FCS-M or LPDS-M as appropriate with or without additions and incubation was continued a further 96 h at 34°C. Cell numbers were evaluated, in triplicate, periodically. Desmosterol and 7-dehydrocholesterol were added as a BSA-ethanol complex, with the final concentration of ethanol in the medium being less than 1%.

Figure Legend: $C^{R}7$, FCS-M (); $C^{R}7$, FCS-M + BSA complex (); $C^{R}7$, LPDS-M + desmosterol (13 μM) (); $C^{R}7$, LPDS-M + 7-dehydrocholesterol (13 μM) (); $C^{R}7$, LPDS-M + BSA complex (); $C^{R}7$, LPDS-M (). Standard deviations were 1-3% of the mean values shown.


800
600
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CELL NUMBER (thousands)
24
48
72
96
TIME (h)

cause accumulation of lanosterol. Thus WT might be induced to behave in a C^R7 mutant-like way.

Figure 56 shows that wild type cells grew normally at 34°C in the presence of ketoconazole (20 μM). Thus the drug is not cytotoxic at this concentration. The drug became cytotoxic to WT at concentrations greater than 40 μM (data not shown).

In LPDS-M, however, WT cells seemed to behave like C^R7 in that proliferation ceased after 72 hours. Ketoconazole presumably prevented cholesterol synthesis and LPDS-M does not supply cholesterol. Therefore, growth would be expected to cease.

Figure 57 shows that at 39°C, 20 μM ketoconazole induced WT to behave in a mutant-like manner, in that it failed to proliferate, even in FCS-M. That is, WT behaved in a temperature-sensitive fashion in the presence of 20 μM ketoconazole.

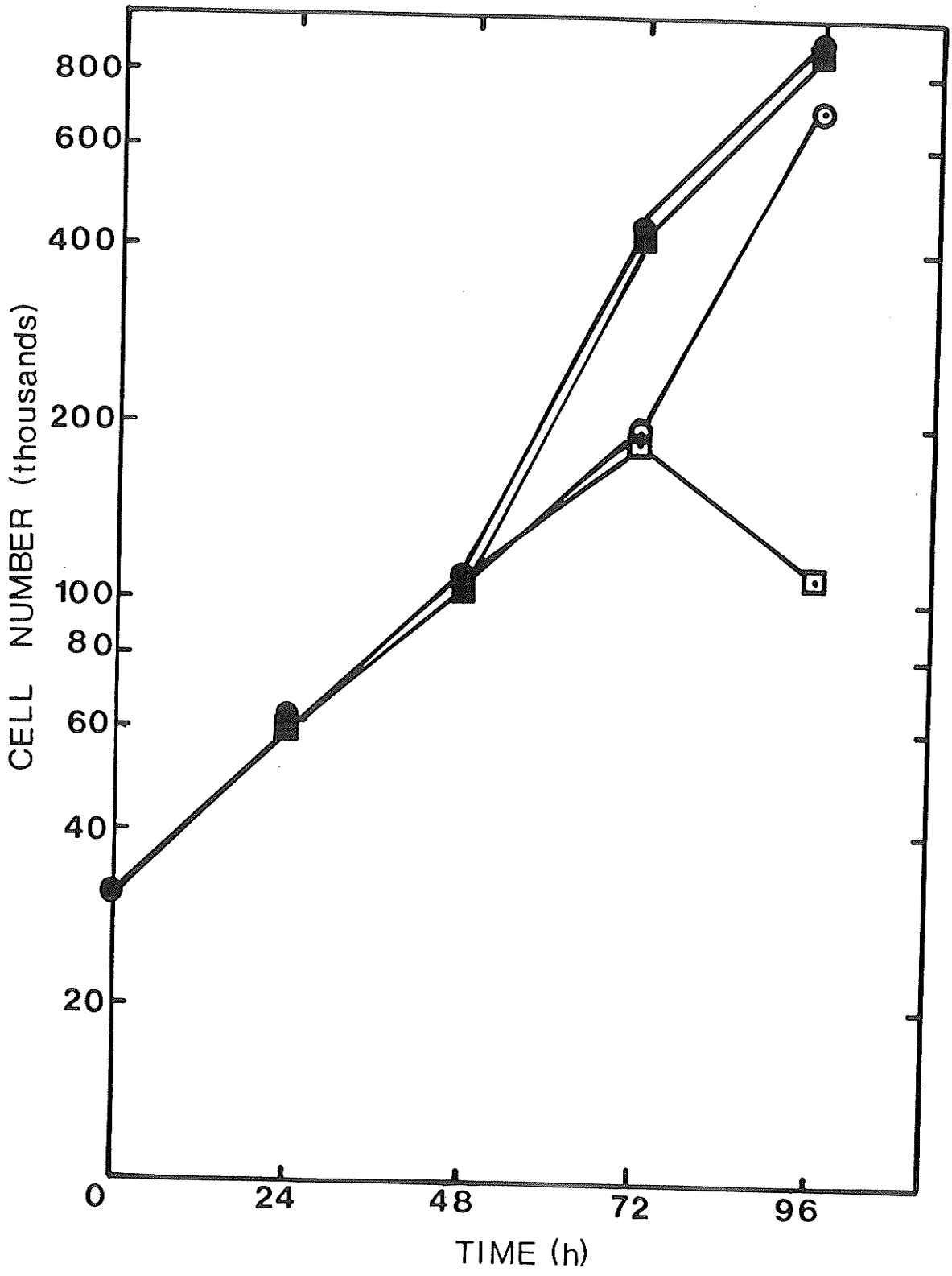
Figure 58 shows that in the presence of ketoconazole C^R7 fails to thrive on FCS-M at 34°C. This response is akin to growth on LPDS-M. Furthermore, C^R7's inabilty to grow on LPDS-M is accentuated in the presence of ketoconazole.

Figure 59 shows that the presence of ketoconazole has the ability to make C^R7 grow less well at 39°C whether C^R7 is cultured on FCS-M or LPDS-M. This is as expected. After all, C^R7 is a mutant--and a cholesterol auxotroph.

Figure 60 shows that as the concentration of ketoconazole increases (and presumably the extent of lanosterol demethylation at C-14 decreases) the greater is the magnitude of the temperature-sensitive-like growth response of WT cells at 39°C on FCS-M. That is, in the

Figure 56: Effect of ketoconazole on growth of WT at 34°C. WT cells were seeded in 60 mm dishes containing 5 ml FCS-M and incubated 24 h at 34°C. Cell numbers were then estimated and the values designated as zero counts. The medium was then replaced with 5 ml FCS-M or LPDS-M with or without additions and incubation was continued a further 96 h at 34°C Ketoconazole (20 μM) was added as an alcoholic solution with the final concentration of ethanol in the medium being 1%.

Figure Legend: WT, FCS-M (); WT, FCS-M + ketoconazole (20 μM) (); WT, LPDS-M (); WT, LPDS-M + ketoconazole (20 μM) (). Standard deviations were 1-4% of the mean values shown.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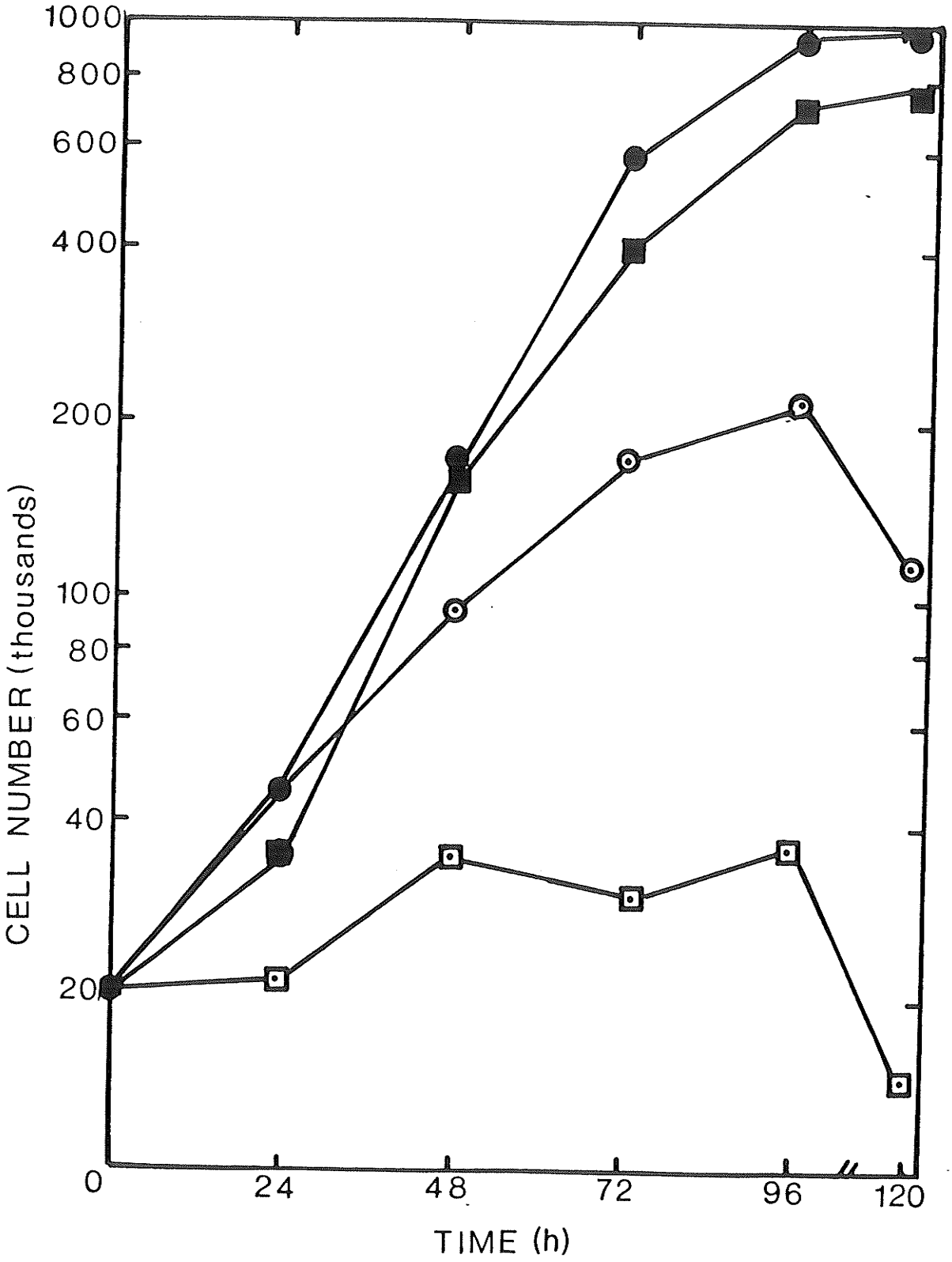
CELL NUMBER (thousands)
24
48
72
96
TIME (h)

Figure 57: Effect of ketoconazole on growth of WT at 39°C. Protocol followed as described in Figure 56 except that the further 96 h incubation was done at 39°C.

Figure Legend: WT, FCS-M (); WT, FCS-M + ketoconazole (20 μM) (), WT, LPDS-M () and WT, LPDS-M + ketoconazole (20 μM) (). Standard deviations were 2-4% of the mean values shown.


1000
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CELL NUMBER (thousands)
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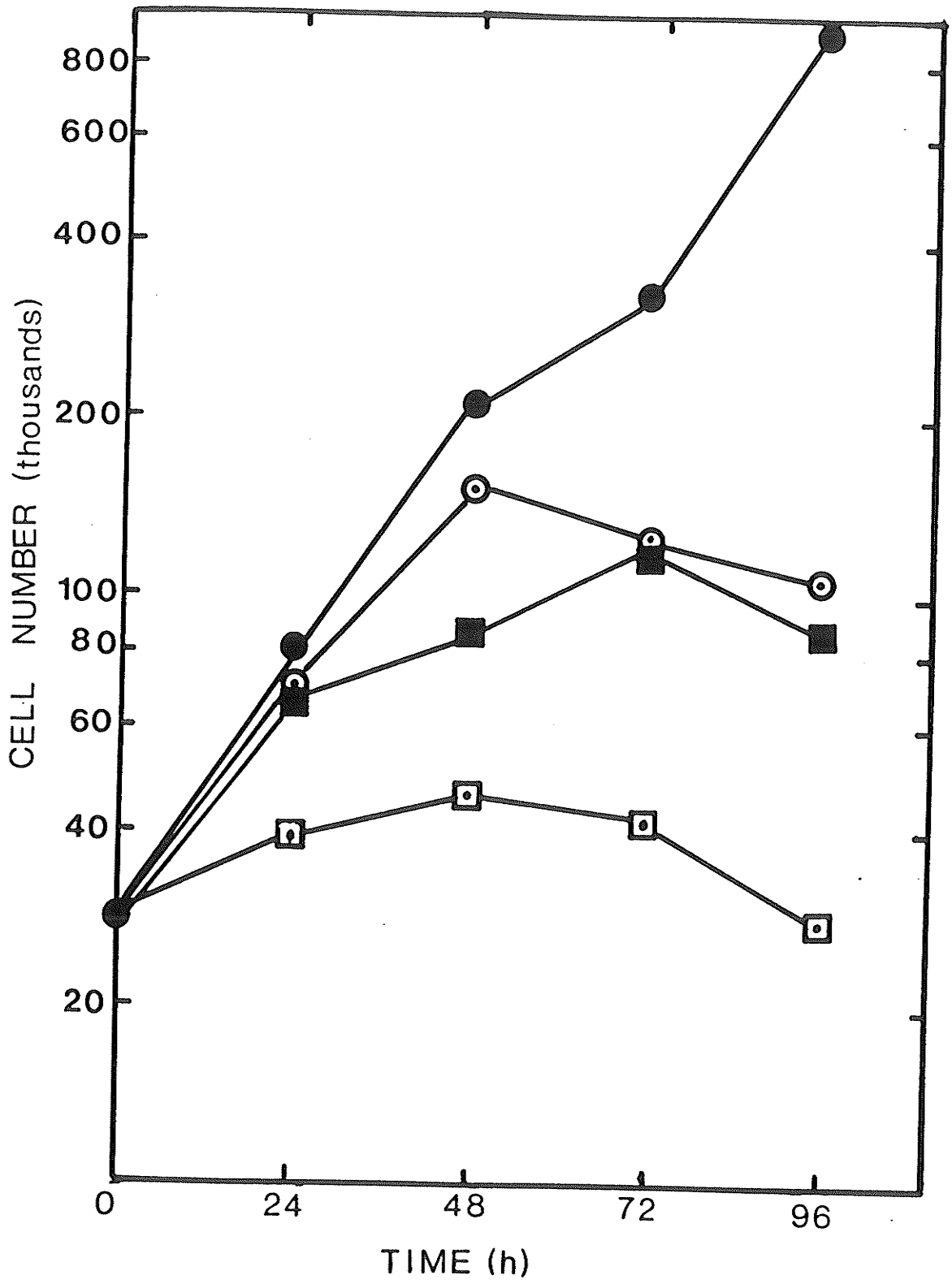
120
TIME (h)

Figure 58: Effect of ketoconazole on growth of C^R7 at 34°C. C^R7 cells were seeded in 60 mm dishes containing 5 ml FCS-M and incubated 24 h at 34°C. Cell numbers were then evaluated and the values designated as zero counts. The medium was replaced with 5 ml FCS-M or LPDS-M with or without additions and incubation was continued a further 96 h at 34°C. Ketoconazole (20 μM) was added as an ethanolic solution (final ethanol concentration being 1%).

Figure Legend: C^R7, FCS-M (); C^R7, FCS-M + ketoconazole (20 μM) (); C^R7, LPDS-M (); C^R7, LPDS-M + ketoconazole (20 μM) (). Standard deviations were 1-3% of the mean values shown.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CELL NUMBER (thousands)
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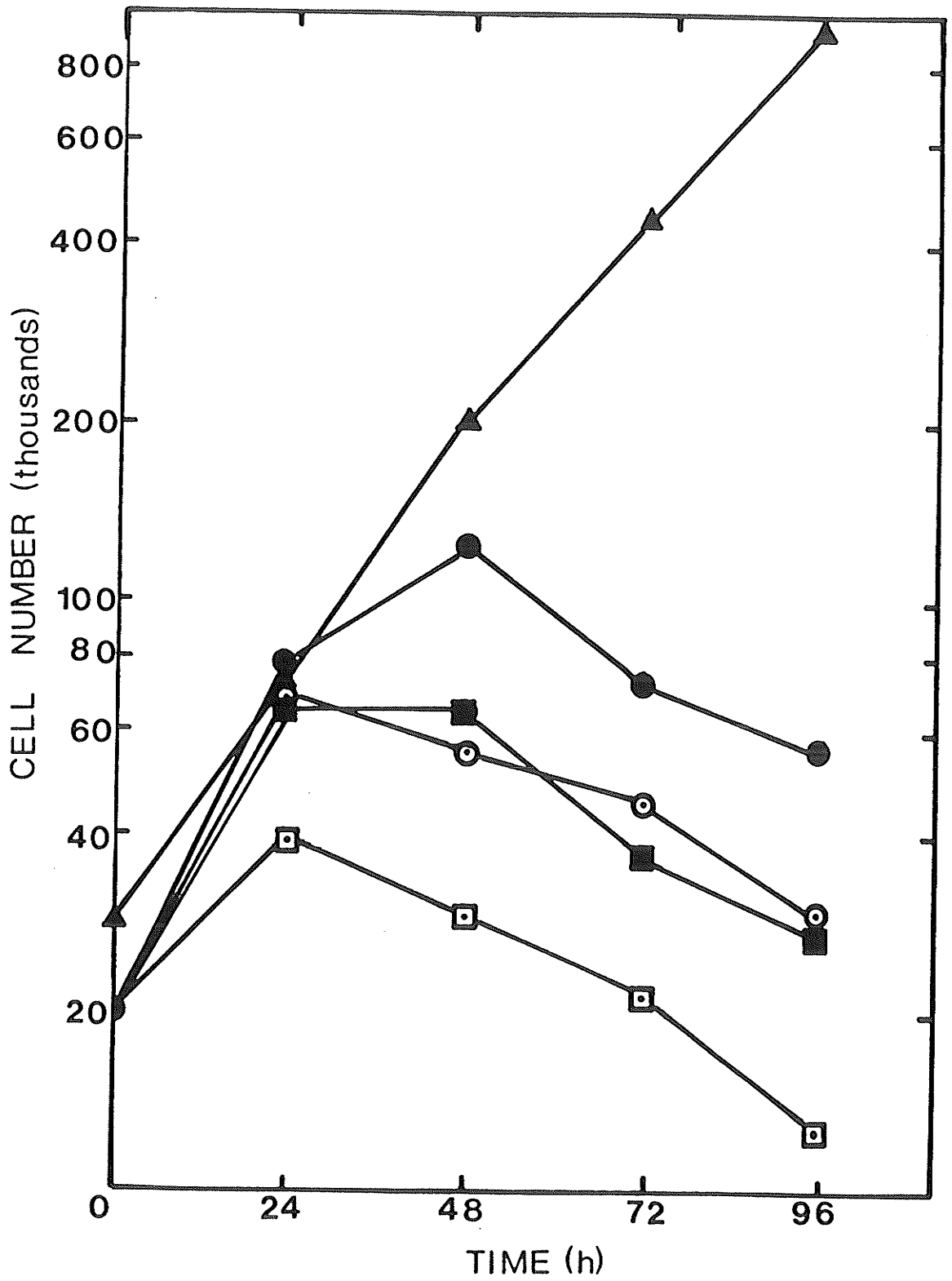
TIME (h)

Figure 59: Effect of ketoconazole on growth of $C^{R}7$ at 39°C. Protocol followed as described in Figure 58, except further 96 h incubation period was carried out at 39°C.

Figure Legend: WT, FCS-M (); $C^{R}7$, FCS-M (); $C^{R}7$, FCS-M + ketoconazole (20 μM) (); $C^{R}7$, LPDS-M (); $C^{R}7$, LDSM + ketoconazole (20 μM) (). Standard deviations were 2-4% of the mean values shown.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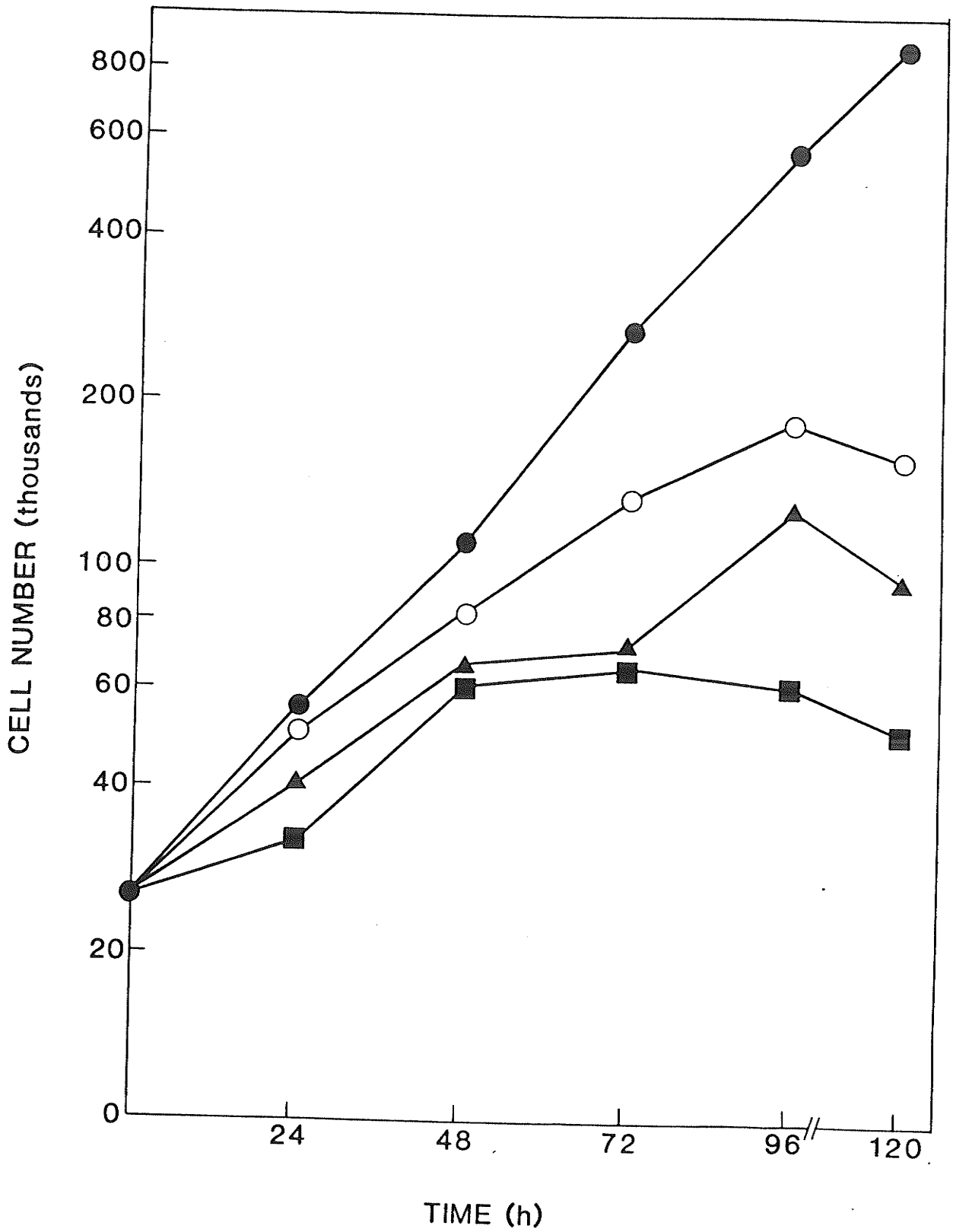
CELL NUMBER (thousands)
TIME (h)

Figure 60: Growth of WT in presence of ketoconazole at 39°C. Cells were plated out into 60 mm dishes (10,000 cells per plate) containing 5 ml FCS-M and incubated 24 h at 34°C. Cell numbers were then estimated and the values designated as zero time counts. The medium was removed and replaced with FCS-M + appropriate amount of ketoconazole and incubation was continued a further 120 h at 39°C. Cell numbers were evaluated, in triplicate, periodically. Ketoconazole was added as an ethanolic solution.

Figure Legend: WT, FCS-M (); WT, FCS-M + ketoconazole (25 μM) (); WT, FCS-M + ketoconazole (40 μM) (); WT, FCS-M + ketoconazole (60 μM) (). Standard deviations were 2-4% of the mean values shown.


CELL NUMBER (thousands)
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TIME (h)

presence of increased concentration of ketoconazole, WT grows less well.

The effect of ketoconazole addition on the Con-A resistance property of WT and C^R7 cell lines was investigated. Figure 61 shows that the administration of ketoconazole (25 μM) increased the Con-A resistance of both WT and C^R7 cell lines. This data was obtained using an inoculum of 1,000 cells. Figure 62 shows unequivocally that the administration of ketoconazole (25 μM) increased the Con-A resistance of both WT and C^R7 cell lines. This data was obtained using an inoculum of 10,000 cells.

In Figure 62, it is clear that, at least so far as the response to Con-A is concerned, wild type cells have become "mutants," behaving exactly like C^R7.

Examination of Cytochrome P-450 Levels in Wild Type and Mutant Cells

Results to this point led to the speculation that the presence of unusually large amounts of lanosterol in the cellular membranes, coupled with the relative absence of cholesterol, is responsible for the phenotypic changes in the membrane properties that are characteristic of C^R7. Ketoconazole-induced lanosterol accumulation caused the wild type to effectively mimic the behavior of the mutant, thereby exhibiting some of the aberrant properties of C^R7. Furthermore, these results suggest that C^R7 is probably defective in lanosterol demethylation at C-14. Since C-14 demethylation is a cytochrome P-450 linked process, an attempt to quantitate the amount of cytochrome P-450 in wild type and mutant cells was initiated.

The presence of cytochrome P-450 was demonstrated in wild type cells grown at either 34°C or 39°C on FCS-M (data not shown). But no

Figure 61: Effects of ketoconazole on Con-A response of $C^{R}7$ and WT. Con-A killing curves were determined as described in Methods and Materials. The data shown was obtained using an inoculum of 1,000 cells. Ketoconazole was added as an ethanol solution (final medium concentration of ethanol was 1%). Values shown are the averages of duplicate plates of a single experiment. Similar results were achieved in three separate experiments.

Figure Legend: $C^{R}7$ (); $C^{R}7$ + ketoconazole (25 μM) (); WT (); WT + ketoconazole (25 μM) (). Standard deviations were 1-4% of the mean values shown.

Temperature of Growth: 34°C.

Type of Medium Used: FCS-M.

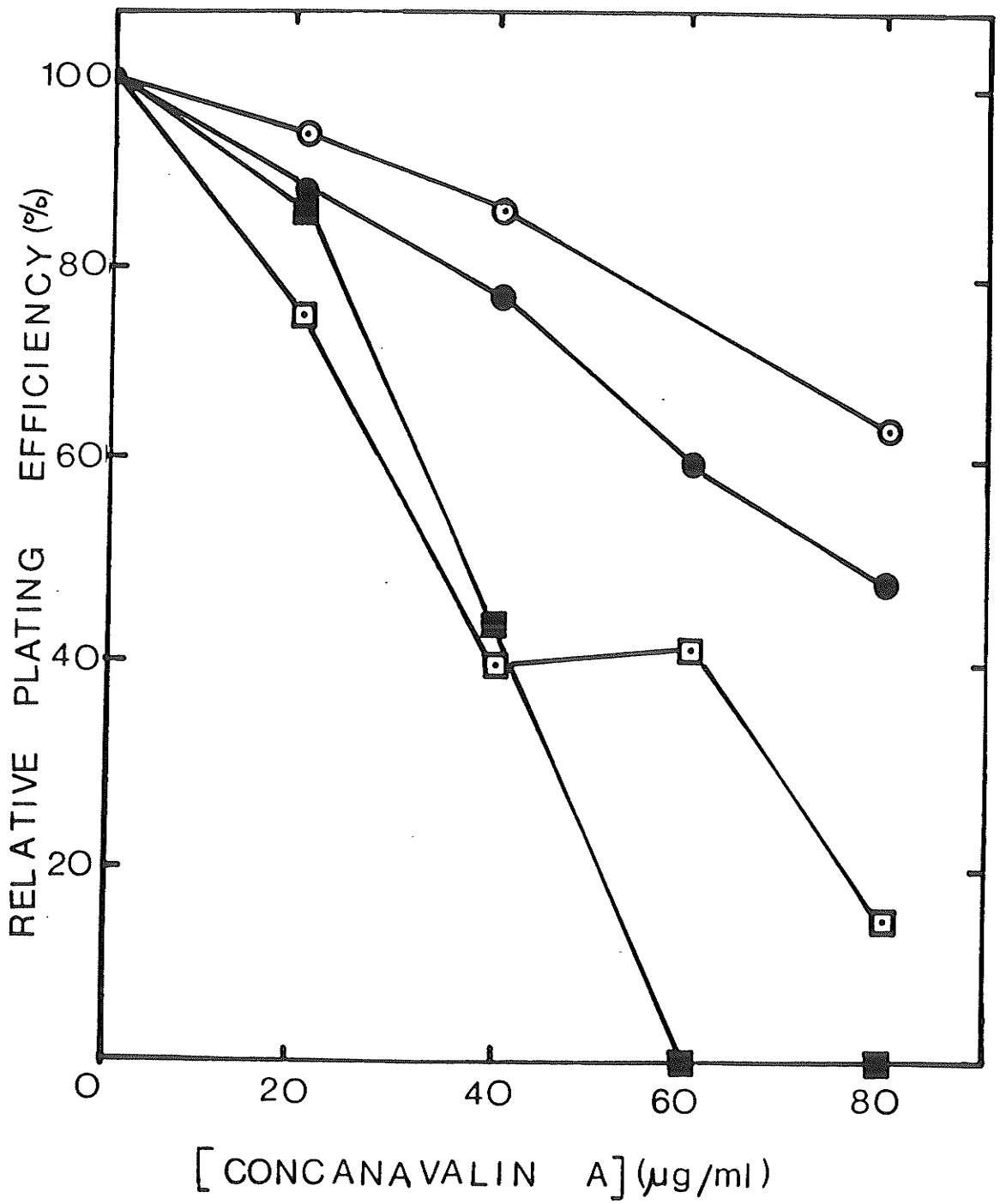

100
80
60
40
20
0
RELATIVE PLATING EFFICIENCY (%)
0
20
40
60
80
[CONCANAVALIN A] (μg/ml)

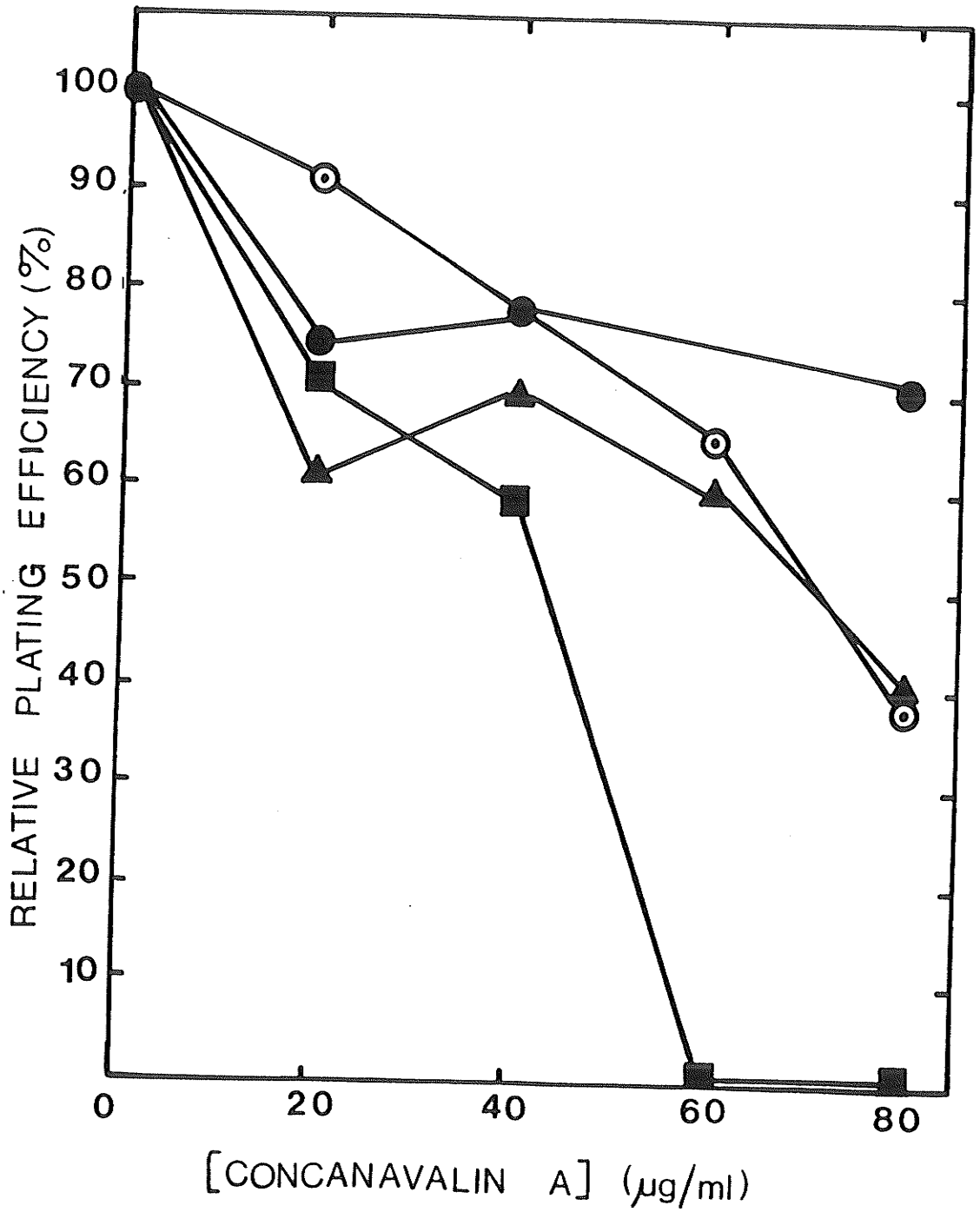
Figure 62: Effect of ketoconazole on Con-A response of WT and $C^{R}7$.

Con-killing curves were determined as described in Methods and Materials and in Figure 61. The data shown was obtained using an initial inoculum of 10,000 cells. Values shown are the averages of duplicate plates of a single experiment.

Figure Legend: $C^{R}7$ (), $C^{R}7$ + ketoconazole (25 μM) (); WT (); WT + ketoconazole (25 μM) ().

Temperature of Growth: 34°C.

Type of Medium Used: FCS-M.


RELATIVE PLATING EFFICIENCY (%)
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
[CONCANAVALIN A] (μg/ml)

cytochrome P-450 was detectable in mutant cells, either at 34°C or at 39°C. Protein content and number of cells used were comparable for both wild type and mutant cells. The amount of cytochrome P-450 detectable in wild type was very small. The fact that cytochrome P-450 was detectable in wild type cells but not in C^R7 suggests that C^R7 may indeed contain less cytochrome P-450 than the wild type cells. By virtue of this fact, cytochrome P-450 mediated processes may be defective in C^R7. However, a more detailed examination of the levels of cytochrome P-450 in these cells is necessary in order to evaluate the validity of this idea. Unfortunately, such an examination may prove to be difficult and problematic.

DISCUSSION

DISCUSSION

C^{R} 7--Its Abnormal Biochemistry and Its Failure to Convert Lanosterol to Cholesterol

Since the first demonstration that cells of an animal can grow outside the body as single cells (Harrison, 1907), the use of cell culture techniques to study various aspects of biology and medicine has become a more than adequate methodology for scientific investigations.

The mammalian plasma membrane is intimately involved in the regulation of many important, and at times, critical cellular functions. The surface of a cell participates in the regulation of intracellular communication, cell growth, the immune response, and in neoplastic conditions (Wallach, 1975; Nicolson, 1976; Wright, 1979; Stanley, 1984; Stanley, 1987).

Lectins, a group of proteins (other than immunoglobulins) which bind to specific cell surface carbohydrates and agglutinate many types of cells, are often used as tools or probes to investigate architectural features of cell surfaces and provide some valuable insights into the function of membrane carbohydrate-containing structures (Lis & Sharon, 1973; Briles, 1982; Stanley, 1984).

A considerable number of lectin-resistant variants, particularly of Chinese hamster ovary (CHO) cells, have been isolated in several laboratories and have proved useful in studies of oligosaccharide and glycoprotein biosynthesis (Briles, 1982; Stanley, 1984). These variants display altered sensitivity to a variety of lectins as a result of a wide range of abnormalities in glycoprotein biosynthesis and assembly.

The selection of Con-A resistant mammalian cell mutants many years ago had as a prime objective the selection of cells which were altered

in membrane-related phenomena. Con-A resistant Chinese hamster ovary cell lines have been isolated in a number of laboratories (Wright, 1973, 1975; Cifone & Baker, 1976; Cifone et al, 1979; Briles et al, 1978; Briles, 1982; Stanley, 1984). All of the isolates described showed a striking phenotypic similarity and are refered to as glycosylation-membrane mutants.

The concanavalin-A resistant CHO cell line designated C^R7 displays a complex phenotype characterized by multiple alterations in membrane-associated properties including temperature-sensitive growth, altered morphology, adhesiveness, lectin-binding and receptor mobility and altered membrane glycoproteins detectable by surface labeling (Wright et al, 1980). This highly pleiotropic phenotype presumably results from a single biochemical defect as a revertant cell line (RC^R7 or Rc), in which all altered properties revert in concert, has been isolated from the variant C^R7 population. The locus of this defect is unknown.

The Con-A resistant CHO cell variants C^R7, B211 and CAR-F are phenotypically similar and may be considered as examples of the Lec 5 phenotype (Briles, 1982; Stanley, 1984). The complexity of this phenotype has made it difficult to pinpoint its biochemical basis, but it is clear that these variants are deficient in the assembly of lipid-linked oligosaccharides and express aberrant mature surface glycoproteins (Cifone et al, 1979; Krag, 1979; Wright et al, 1979; Krag & Robbins, 1982). The temperature-sensitive growth properties of these cells is consistent with this deficiency since it is probable that carbohydrate components of glycoproteins stabilize their functional conformations. The complexity of the Lec 5 phenotype has led Stanley to

speculate that the primary lesion in Lec 5 may be a structural membrane defect that affects the function of a variety of membrane-associated glycosylation enzymes (Stanley, 1984).

Indirect support for this idea has been provided by work from this laboratory which showed that C^R7 exhibits abnormalities in the metabolism of lipids which greatly influence membrane properties. C^R7 is auxotrophic for cholesterol because it fails to adequately convert lanosterol to cholesterol (to be discussed later), and is defective in the binding and internalization of low-density lipoprotein (LDL) and in LDL-modulated regulation of HMG-CoA reductase, the key regulatory enzyme of polyisoprenoid synthesis. It is also compromised in its ability to synthesize fatty acids, in particular, unsaturated fatty acids (Borgford & Burton, 1982; Borgford et al, 1986).

Cell growth, temperature sensitivity and altered lipid metabolism were areas of concern which this present investigation addressed. All three cell lines, wild type, a revertant cell, and the mutant, C^R7, were found to grow well at 34°C and 37°C when cultured on FCS-M (medium which contained whole fetal calf serum, a somewhat undefined supplement containing both lipoprotein-borne lipids and free lipids). FCS-M may also be defined as cholesterol sufficient medium. Wild type and revertant cell types were also able to thrive at 39°C in FCS-M. However, 39°C was found to be a non-permissive temperature for growth of the variant (Figure 13), in agreement with the earlier results of Ceri (1978).

Some methods for the removal of lipids from serum require extraction with organic solvents. Since the majority of serum lipids are lipoprotein-borne, it is possible to effectively eliminate them, in

a less disruptive manner, by removing all serum lipoprotein. Having removed lipoproteins from the serum, cholesterol was found to be present in negligible amounts.

The variant cell line, C^R7 cannot be maintained on LPDS-M (cholesterol deficient medium) at any temperature (Figure 15, 16, 17 & 18). Wild type and revertant cell lines can be maintained as these cell lines are able to initiate the appropriate compensatory response, and synthesize cholesterol when the demand presents itself (Figure 37). C^R7 can not do this (Figure 37). But the growth requirements of the mutant are apparently fulfilled completely by the addition of cholesterol (13 μM) to LPDS-M (Figure 16). These results suggest that the mutant is unable to sufficiently synthesize cholesterol, an observation consistent with the hypothesis that these cells are somehow defective in their expression of HMG-CoA reductase. HMG-CoA reductase, a transmembrane glycoprotein of endoplasmic reticulum (Chin et al, 1984), has been identified as the rate limiting enzyme of sterol biosynthesis (Chang, 1983). The cholesterol auxotrophy of the mutant is also consistent with the finding that it is probably lanosterol demethylation defective (Tables 20, 21 & 22). Other mammalian cells characterized as cholesterol auxotrophs have been found to be lanosterol demethylation defective (Berry & Chang, 1982).

The inability of C^R7 to be maintained on LPDS-M (cholesterol deficient medium) was further amplified when the mutant was cultured in the presence of DELIP-M (medium containing "completely" delipidated serum) (Figure 15). In view of this response it is surprising that C^R7 has not a requirement for a specific fatty acid supplement in order to proliferate on LPDS-M. This is underscored by the fact that C^R7 is

defective in the synthesis of unsaturated fatty acids (Borgford et al, 1986; Figure 39).

It should be noted however that C^R7 does make fatty acids, both saturated and unsaturated (Figures 38 & 39). The defective synthesis of fatty acids and especially unsaturated fatty acids, which occurs in C^R7 is not lethal, but may have important membrane effects especially under certain conditions, such as, an increase in the growth temperature and the accompanying stresses.

In this respect, C^R7 differs from a CHO cell variant isolated by Chang and co-workers (Limanek et al, 1978; Chin & Chang, 1981, 1982).

The defect in cholesterol biosynthesis in the mutant becomes obvious by comparison of the cholesterol content of the mutant with that of both wild type and revertant, and in particular, by examining the cholesterol-phospholipid ratios as the growth temperature is increased (Tables 1, 2 & 3). This deficit exhibited by the variant is related to decreased cholesterol and not to phospholipid content (Tables 1, 2 & 3). Phospholipids are thought to fulfill a structural function in membranes (Yeagle, 1985). Since C^R7 is a membrane mutant an examination of phospholipid content of the cells was warranted. Phospholipid content of all three cell lines was about the same, irrespective of growth conditions and temperature (Tables 1, 2 & 3). Furthermore, no difference, amongst the three cell types, in the kind and content of the major phosphatides, irrespective of growth conditions and temperature (Tables 9 & 10) was found. PC (approximately 50%), PE (approximately 30%) and SM (approximately 20%) were found to be the major phosphatides (values quoted are relative percent composition). Kimelberg and Papahadjopoulos (1972) examined the phospholipid composition of

mammalian cells and found the major phosphatides and their relative percent composition to be: phosphatidylcholine (PC) (65%); phosphatidylethanolamine (PE) (18%) and sphingomyelin (16%). This accounted for greater than 85% of the total phospholipid in these cells.

The fatty acid composition of these cells was also examined revealing that the mutant contains less unsaturated fatty acid under both growth conditions (FCS-M or LPDS-M) than the wild type and revertant. Furthermore, as the temperature was increased this became more apparent (Tables 7 & 8). It was not clear, however, from this observation if the altered indices of unsaturation resulted from altered rates of synthesis. The lipoprotein of FCS-M is an undefined source of fatty acid, as well as cholesterol, and LPDS-M may contain varying quantities of free fatty acids. Therefore the mass determination does not necessarily indicate the precise nature of biosynthetic defects.

It would be expected, however, that the removal of cholesterol (lipoprotein) from the growth medium would cause mammalian cells to "up-regulate" endogenous synthesis of sterol and alter the rates of synthesis of specific fatty acids. It is presumed that altered rates of fatty acid synthesis would help to maintain the fluidity characteristics of the cell membranes. Cholesterol is known to make relatively liquid membranes more ordered; and relatively crystalline membranes more fluid. It is a little unclear as to what would be the expected response to the depletion of cholesterol in this study. It has been shown elsewhere that depletion of cholesterol from animal cell membranes induces a compensatory increase in the synthesis of unsaturated fatty acids, which have a fluidizing/liquefying effect on the membrane environment (Rintoul, 1979; Silbert & Baldassare, 1979).

Wild type and revertant cells responded to the removal of cholesterol from the medium, as expected, with the required compensatory response, that is, with an increase in the synthesis of cholesterol (Figure 37) and unsaturated fatty acids (Figure 39). This was not true for C^R7. Incorporation studies revealed that the mutant cell is truly defective in the adaptive synthesis of cholesterol but also found the mutant to be defective in the synthesis of total fatty acids and also in the synthesis of unsaturated fatty acid (Figures 38 & 39; Tables 7 & 8). This defect in the mutant became more apparent as the temperature was changed from that which was permissive for growth (34°C) to that which was not (39°C), and also was related to cholesterol status of the medium. This may indicate a common regulation of fatty acid and cholesterol synthesis. On the other hand, the altered rates of synthesis may be completely independent consequences of defective glycoprotein synthesis in C^R7.

The results of incorporation experiments with ^{14}C-labeled acetate demonstrated little difference between the rate of sterol synthesis in the three cell lines when they were cultured in FCS-M (Figure 37). This is quite consistent with the determined activity of HMG-CoA reductase. All three cell lines had similar reductase activities at 34°C on FCS-M. This is contrary to a previous observation made in the laboratory. Borgford (1984) determined that HMG-CoA reductase, supposedly the rate limiting enzyme in this synthesis, was two fold greater in the mutant than in either the wild type or revertant under this growth condition. It was suggested that the flux of metabolites through the polyisoprenoid pathway were being diverted towards the synthesis of non-sterol lipids such as dolichol and ubiquinone (Borgford, 1984).

However, at 39°C, HMG-CoA reductase levels were double those seen at 34°C on FCS-M in all three cells (Table 14). This corresponded to an at least two fold increase in the rate of synthesis of sterol in both WT and revertant cells yet this was not the case for the mutant (Figure 37). In fact on FCS-M, at 39°C, the reductase enzyme activities of WT remained constant whereas those in the mutant were found to increase as the exposure time at 39°C on FCS-M increased, up to a point (Table 15) after which reductase enzyme activity sharply decreased. A greater demand for cholesterol at 39°C exists (supported by the elevated reductase activities of all three cell lines) (Table 14). Why this should be is not quite clear. Perhaps, the increased cholesterol demand is necessary to maintain membrane integrity or is related to slightly elevated growth rates or related to the synthesis of new membranes. Whatever the reason, this greater demand for cholesterol cannot be met by C^R7. And, an eventual decrease in reductase activity results. The increasing reductase enzyme activities seen at 39°C on FCS-M may be interpreted as the mutant's compensatory response, although ultimately futile, to a perceived cholesterol deficiency. FCS-M has a healthy complement of cholesterol. The eventual decrease in reductase activity reflects, in part, a response to cellular death.

Furthermore, on LPDS-M at either 34°C or 39°C, WT and Rc HMG-CoA reductase enzyme activity levels greatly increase (more so at 39°C) (Table 14) in order to compensate for the cholesterol deficiency. C^R7 activity levels do not reflect a similar response. Indeed at 39°C on LPDS-M enzyme activity in C^R7 is half that seen at 34°C on LPDS-M.

C^R7 has been shown to be apparently able to synthesize a functional LDL receptor in addition to a catalytically functional reductase

(Borgford, 1984). In wild type cells the ability to regulate the levels of reductase and the LDL receptor activities are coordinately expressed, whereas in the mutant these elements appeared to be coordinately "fixed" in expression (Borgford, 1984). As mentioned earlier, the mass determination of lipid in the three cell lines revealed a marked deficiency in the cholesterol content of the mutant which corresponded to an inappropriate molar ratio of cholesterol to phospholipid (Tables 1, 2 & 3). This observation is consistent with defective expression of HMG-CoA reductase and the LDL receptor.

The paradox of a high reductase activity without increased synthesis of sterols may be explained if the specific activity of reductase determined in C^R7 at 39°C on FCS-M does not truly reflect the endogenous synthesis of mevalonate. Another possible explanation is defective lanosterol demethylation in the mutant (to be addressed later).

Since small amounts of mevalonate are an absolute requirement for the growth of all mammalian cells (Popjak et al, 1985), it can be said that the mutant cells are capable of synthesizing at least marginal amounts of this key intermediate of the polyisoprenoid pathway. Whereas enzyme activity and sterol synthesis are proportional in the wild type cell line, there is no correlation between the two in the mutant. C^R7 was previously found to be extremely sensitive to compactin, a competitive inhibitor of HMG-CoA reductase (Borgford, 1984). Borgford (1984) suggested that this sensitivity to compactin was likely due to the small amount of endogenous mevalonate synthesis in these cells, despite the fact the HMG-CoA reductase activity in vitro is relatively high. Furthermore, the possibility of "incorrect" intracellular

targetting of the reductase in the mutant may also be pertinent. Future investigations into the subcellular localization/distribution of the reductase in these cells may yield useful information.

Furthermore, the activity of enzymes preceding HMG-CoA reductase in the polyisoprenoid pathway may be rate-limiting to the synthesis of sterols in C^R7. This possibility remains to be investigated.

The defect in C^R7 has at least two major consequences for sterol metabolism. The defect affects the ability of the mutant cells to synthesize mevalonate without altering the _in vitro_ characteristics of the reductase. It also affects the ability of the mutant to up-regulate and down-regulate the expression of the reductase and by implication, the LDL receptor. The ability to synthesize mevalonate may be a prerequisite to the normal regulation of sterol metabolism.

Defective lipid metabolism in the Con-A resistant mutant extends beyond the regulation of cholesterol metabolism. Chang and co-workers (Limanek _et al_, 1978; Chin & Chang, 1981, 1982) isolated and characterized a Chinese hamster ovary cell mutant that is phenotypically very similar to the C^R7 cell line studied in this investigation. The selection procedure chosen for the Chang mutant was such that by design a cholesterol auxotroph was isolated. Chang and his co-workers found that their mutant was auxotrophic for both cholesterol and unsaturated fatty acid. It has a similar level of reductase activity to that found in C^R7, as well as a similar level of LDL receptor activity. Reductase and receptor activities are similarly unresponsive to exogenous lipoproteins. The incorporation of metabolites into cholesterol and lanosterol is similarly altered. Chin and Chang (1982) suggested that the requirement for unsaturated fatty acids in their mutant may be

secondary to the primary defect in its ability to express an increase in the activities of the early cholesterolgenic enzymes. As with the C^R7 Con-A resistant mutant, all of the defects in the Chang auxotroph reverted in concert. The possibility exists that the Chang auxotroph and the C^R7 cell line are very similar, and may have the same or similar defects.

One of the most striking features of the Con-A resistant phenotype is the characteristic temperature sensitivity these cells express. It has been suggested that the temperature-sensitivity of Con-A resistant mutants is a function of the temperature stability of individual proteins (Briles, 1982). Inadequately glycosylated glycoproteins may be unstable at higher growth temperatures. This investigation offers a possible alternative explanation for temperature-sensitivity in Con-A resistant mutants. Temperature-sensitivity of C^R7 is a result of membrane instability and membrane alterations. The C^R7 mutant has a demonstrated marked inability to appropriately regulate its lipid metabolism, and this regulatory deficiency can be lethal as evidenced by C^R7's inability to proliferate at 34°C on LPDS-M (Figures 15 & 16) and at 39°C; as seen in Figures 17, 18 & 19. The fluidity and function of mammalian cell membranes depends very much upon the stoichiometric relationship of membrane lipids. If a mammalian cell is unable to adaptively regulate its membrane lipid composition in response to changing temperatures, then the fluidity and function of the membrane may be lost or altered. It can be argued that the temperature-sensitive growth of the C^R7 cell line, which was apparent in cultures grown at 39°C on FCS-M, may be related to an extent to the fact that the lipid and lipoprotein components of fetal calf serum do not necessarily

provide the required amounts or ratios of lipids to maintain the growth of C^R7 at higher temperatures.

The non-permissive temperature for growth for C^R7 is 39°C (Figure 13). The data presented in Figures 21, 22, 25 & 26 establish that MVA-lactone (77 μM), dolichol (12 μM) or dolichyl-phosphate (12 μM) allow growth of C^R7 at 39°C, so long as cholesterol is present in the growth medium. Presumably, MVA-lactone would give rise, in vivo, to dolichol and dolichyl-phosphate which relieved temperature-sensitivity. Metabolites of MVA-lactone, other than dolichol and its phosphate esters, had no such effect (Figure 24).

Previous studies had demonstrated that the Con-A resistant mutant C^R7 is defective in oligosaccharide biosynthesis and that the specific defect in this biosynthesis was likely to be in the assembly of lipid-linked intermediates (Wright et al, 1979). This conjecture was strongly supported and further developed by the work of Krag et al (1977, 1979) in the Con-A resistant cell line B211. The lesion in B211 was demonstrated to be a defect in the biosynthesis of lipid-linked oligosaccharides but went further in identifying a specific deficiency in the transfer of glucose from dolichol-phosphoryl-glucose to lipid-linked oligosaccharide (see Figure 1). A consequence of this defect is the synthesis of improperly glycosylated glycoproteins (Krag et al, 1977, 1979).

It is pertinent to note that in vitro synthesis of lipid-oligosaccharide and incorporation of mannose into mannosyloligosaccharidyl-lipids was restored to normal levels in B211 membranes by dolichyl-phosphate, although the full-sized lipid-linked oligosaccharide intermediate was not synthesized (Krag, 1979). These

findings, in conjunction with data presented in this thesis, suggest that a massive exogenous amount of dolichol, or dolichyl-phosphate is able to enhance defective glycoprotein synthesis in C^R7 sufficiently to overcome temperature-sensitivity but insufficiently to reverse other phenotypic traits. Thus, dolichol and dolichyl-phosphate had no effect on the Con-A resistance of C^R7 (Figure 49).

While there is no doubt that C^R7 grew at 39°C in LPDS-M, to which cholesterol and mevalonate or dolichol or dolichyl-phosphate were added (Figures 22 & 26), the growth rate was lower than in FCS-M with added MVA-lactone alone or with added dolichol or dolichyl-phosphate (Figures 21 & 25). The addition of cholesterol to LPDS-M allowed growth of C^R7 at 34°C at the same rate as in FCS-M. Apparently, therefore, factors other than cholesterol and dolichol or dolichyl-phosphate must be needed to achieve optimal growth at 39°C in LPDS-M.

The cellular levels of dolichol found in C^R7 and WT under various conditions are shown in Table 11. The three fold increase seen in WT at 39°C is consistent with the idea that glycoproteins are important for thermal stability of cells. In C^R7, after 36 hours at the non-permissive temperature, an even greater increase in the dolichol level was found. C^R7 is therefore more than capable of increasing dolichol synthesis, at least as efficiently as the wild type, but for C^R7 this increase is insufficient to permit growth at 39°C. Apparently, as mentioned earlier, a relatively massive amount of dolichol and/or its phosphate are required to enhance defective glycoprotein synthesis sufficiently to allow growth at 39°C. Hence, it seems that C^R7 is defective, not in dolichol synthesis, but in its utilization for glycoprotein synthesis.

Borgford (1984) suggested that, if improper glycosylation in C^R7 is a result of deficient synthesis or utilization of a dolichol-cycle intermediate, it is reasonable to assume that the mutant will be more sensitive to inhibitors of the dolichol cycle reactions. This seems to be the case. An inhibitor of glycosylation which exerts its influence in the dolichol cycle is 2-deoxyglucose. 2-deoxyglucose is converted to the metabolite GDP-2-deoxyglucose, an analog of GDP-mannose. This metabolite is then thought to substitute for GDP-mannose in transfer reactions and sequester dolichol from the dolichol cycle. Bacitracin is another inhibitor of glycosylations generally believed to exert its effect by complexing with dolichol pyrophosphate and preventing reutilization of the lipid. The mutant cell line demonstrated approximately a two fold greater sensitivity to these drugs than the wild type (Borgford, 1984). This supports the contention that C^R7 is defective not in dolichol synthesis but in its utilization for glycoprotein synthesis.

The C^R7 mutant is glycosylation defective. It is not possible to say whether glycosylation directly or indirectly influences aspects of lipid metabolism, if at all. However, glycosylation could be important to lipid metabolism through a cascade of events since a variety of mammalian cell proteins are glycosylated. Perhaps glycosylation is not a passive process in mammalian cells but rather operates in a manner which may turn on or off certain aspects of metabolism.

There will obviously be certain constraints on the nature of a mutation affecting oligosaccharide biosynthesis. The foremost of these is that the mutation be non-lethal. Cells in which glycosylation is abolished altogether have never been isolated. Isolates must therefore

possess some glycosylating activity. It is uncertain whether independently selected Con-A resistant mutants have defects in independent, or different gene products. Selection of mutants with the same selective agent and by the same protocol likely isolates cells possessed by very similar if not identical defects. On the contrary, Lec R I mutants, for example, were independently selected for resistance to a number of different lectins and characterization of these mutants showed that they were all resistant by virtue of the same biosynthetic defect (Briles, 1982). Coincidently, the target oligosaccharide of each of the selective lectins was the same. It is interesting to note that independently isolated Con-A resistant mutants express strikingly similar and pleiotropic phenotypes, for example, C^R7 and B211. Therefore, it was suggested that C^R7 is defective in the transfer of lipid-linked glucose to lipid-linked oligosaccharide.

One important use of dolichol is its role in glycoprotein synthesis where it is a component of lipid-monosaccharides and lipid-oligosaccharides which are ultimately transfered to glycoproteins. C^R7 is glycosylation defective. Glycoproteins are believed to be involved in thermal stability of cells, so the glycosylation deficiencies of C^R7 might be involved in its temperature-sensitivity. The ability of C^R7, WT and Rc to incorporate mannose into lipid-monosaccharide, lipid-oligosaccharide and glycoprotein at 34°C and 39°C was measured.

Figures 41 and 42 show that C^R7 incorporated much less mannose into all three compounds at 34°C than did WT and a revertant cell line. This is consistent with a reduced ability to use dolichol. At 39°C, all three cell lines incorporated less mannose into lipid-oligosaccharide

and glycoprotein than at 34°C but the incorporation into lipid-monosaccharide was about the same. Again C^R7 incorporated much less than WT or Rc.

It is suggested that increased dolichol levels in wild type and the mutant at 39°C (Table 11) reflect the cells' attempt to overcome the apparently reduced ability to synthesize lipid-oligosaccharide and glycoprotein at this temperature (Figures 40 & 41). In the case of WT and revertant this strategy is successful since they are viable at 39°C. With C^R7, as shown in Figure 40, the synthesis of glycoproteins is very limited at 39°C despite the increased dolichol content, and may be too little to allow viability.

Stanley (1984) has speculated that the Lec 5 phenotype may result from a structural membrane abnormality causing multiple alterations in function of membrane-associated glycosylation enzymes. C^R7 has been shown to be abnormal in the biosynthesis of some membrane-lipids and that its cholesterol auxotrophy results from an inability to convert lanosterol to cholesterol (Table 22). This became especially true for C^R7 at 39°C, at which it is temperature-sensitive (Table 22; Figure 13). The possibility therefore exists that the presence of lanosterol, coupled with a lack of cholesterol, in the membranes of C^R7 causes abnormalities like those suggested by Stanley (1984).

Following this reasoning, it is suggested that temperature-sensitivity in C^R7 may result from the defective utilization of dolichol by membrane-bound glycosylases leading to the reduction, or absence, of glycoproteins involved in cellular thermostability. Large exogenous amounts of dolichol, dolichyl-phosphate or MVA-lactone (which is precursor to dolichol), permit sufficient synthesis of these critical

glycoproteins to allow growth at 39°C (Figures 21, 22, 25 & 26). Other metabolites of MVA-lactone would be expected to be ineffective, as was observed (Figure 24). In support of these ideas, it would be helpful to know whether C^R7 cells, grown at 39°C in the presence of dolichol, dolichyl-phosphate, or MVA-lactone, contain significantly more dolichol than cells grown in unsupplemented media. Such measurements were not done, but, perhaps deserve future consideration.

Similarly, the abnormal presence of lanosterol and its effects on membrane properties provides a plausible explanation for the Con-A response exhibited by the mutant. The presence of abnormal amounts of lanosterol and the consequences of this on membrane properties could explain either a decreased synthesis or availability of Con-A receptors resulting in the findings shown in Figure 52. This figure shows that the addition of lanosterol, or MVA-lactone, which is predominantly converted to lanosterol in C^R7 (Tables 21a & 21b), to the growth medium markedly enhanced Con-A resistance in C^R7. Wild type cells, with their normal complement of, and ability to synthesize, cholesterol were unaffected. The previously observed abnormalities in receptor-mediated binding and uptake of LDL (Borgford & Burton, 1982; Borgford et al, 1986) could be explained on the same basis, as the LDL receptor is known to be a glycoprotein (Schneider et al, 1982).

Why is C^R7 Con-A resistant and WT not? Con-A binds oligosaccharide chains with a high mannose content (Brown & Hunt, 1978). Furthermore, in order for Con-A to exert its complete cytotoxic effects it must not only bind the appropriate receptor but also must be internalized (Brown & Hunt, 1978). It has been shown that in hamster cells there is a pathway for converting mannose to fucose and that this pathway is of

greater importance in Con-A resistant cells (as is C^R7) than in wild type or revertant Con-A sensitive cells (Wright et al, 1978, 1979). Various glycosidase forms were previously examined in hamster cells (Blaschuk et al, 1980a) and significant differences in the activities of these glycoprotein enzymes were found between Con-A resistant C^R7 cells and Con-A sensitive cells (Blaschuk et al, 1980b). The synthesis of a class of glycoproteins (high in fucose not mannose) (Wright et al, 1979) coupled with aberrant activities of glycoprotein enzymes (Blaschuk et al, 1980b) would undoubtedly have wide ranging effects on numerous biological properties of the lectin resistant cell including modifications to surface membrane components. The preceding is summarized in Figure 8. Therefore C^R7 is Con-A resistant due to an aberrant surface membrane with glycoproteins high in fucose content; low in mannose. Wild type and revertant cell lines are Con-A sensitive because they possess "normal" membranes properties with surface glycoproteins high in mannose residues.

If lanosterol or MVA-lactone (largely converted to lanosterol in C^R7, see Tables 21a & 21b) entered C^R7's membrane, the result could be further modification of enzyme activities. (The aberrant composition of C^R7's membranes hypothesized to contain increased lanosterol content at the expense of cholesterol has not yet been unequivocably demonstrated. However, this suggestion is clearly consistent with data already acquired.)

On the other hand, as a result of higher lanosterol content in the mutant, topological changes in the membrane might occur. Such changes could mask or make unavailable receptors in C^R7. These receptors could contain high mannose or those already unresponsive to the action of

Con-A binding, more specifically, those surface components high in fucose.

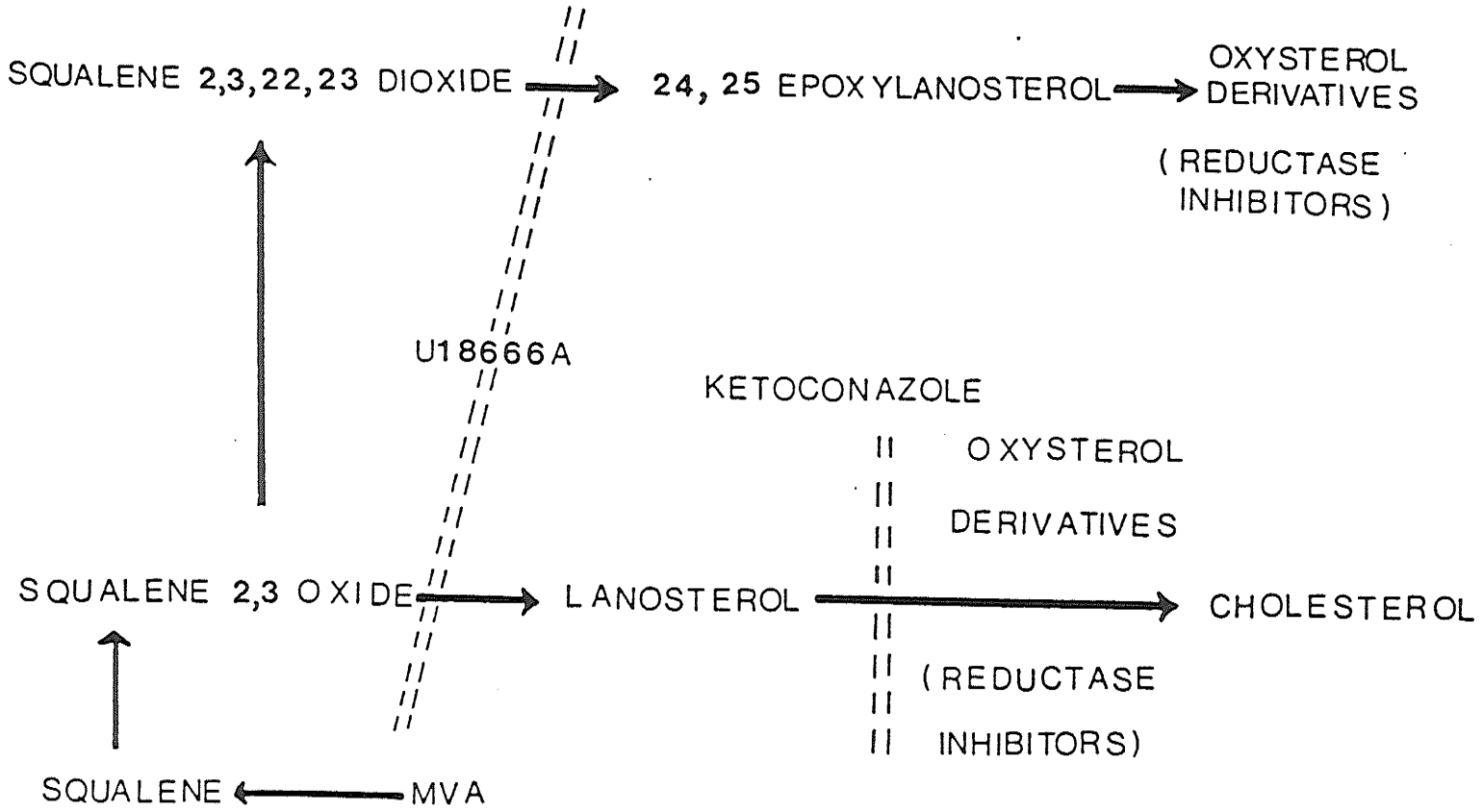
In WT cells with their "normal" membrane and surface components, the addition of lanosterol has no effect on Con-A binding. Presumably, lanosterol is unable to successfully compete with cholesterol for binding sites in the membrane and thereby modify (or, at least in this case) a fully competent membrane. Therefore, WT cells, in the presence of lanosterol or MVA-lactone, in the growth medium remain Con-A sensitive (Figure 52). In WT cells, lanosterol or MVA-lactone is successfully converted to cholesterol, and is not blocked at lanosterol, (see Tables 21a & 21b).

In the light of recent work, other phenotypic traits of C^R7 may be explained by the presence of anomalous amounts of lanosterol in its membranes. Treatment of rat intestinal epithelial cells with ketoconazole, a lanosterol demethylation and cytochrome P450 inhibitor, caused inhibition of HMG-CoA reductase. This effect was caused by the generation of polar sterols, inhibitory to HMG-CoA reductase, from methyl sterols, for example, lanosterol, which accumulated in the presence of ketoconazole (Gupta et al, 1986). Furthermore, a failure to convert lanosterol to cholesterol, which already exists in the mutant, or created in WT by the presence of ketoconazole, could result in the production of oxysterols via an alternative pathway. The existence of an alternate pathway for formation of oxysterols which branchs off from the normal pathway at the level of squalene 2, 3 oxide via squalene oxidocyclase (Panini et al, 1984) is illustrated in Figure 63.

HMG-CoA reductase activity in C^R7 was not upshifted in response to removal of lipoprotein cholesterol from the growth medium (Borgford &

Figure 63: Pathways for oxysterol formation (from Panini et al, 1984; Rudney & Sexton, 1986).

U18666A and Ketoconazole are inhibitors of the steps indicated.


SQUALENE 2,3,22,23 DIOXIDE
24, 25 EPOXYLANOSTEROL
OXYSTEROL DERIVATIVES
(REDUCTASE INHIBITORS)
U18666A
KETOCONAZOLE
OXYSTEROL DERIVATIVES
SQUALENE 2,3 OXIDE
LANOSTEROL
CHOLESTEROL
(REDUCTASE INHIBITORS)
SQUALENE
MVA

Burton, 1982; Borgford et al, 1986; Table 14). This could be explained by the presence of inhibitory polar sterols generated from the abnormally large amount of lanosterol present in C^R7. Furthermore C^R7 is also defective in unsaturated fatty acid synthesis (Borgford et al, 1986; Figure 39). If lanosterol accumulation results from a P450-like cytochrome defect in C^R7, defective desaturase activity would be expected since it involves a similar cytochrome-linked process (Oshino & Sato, 1971).

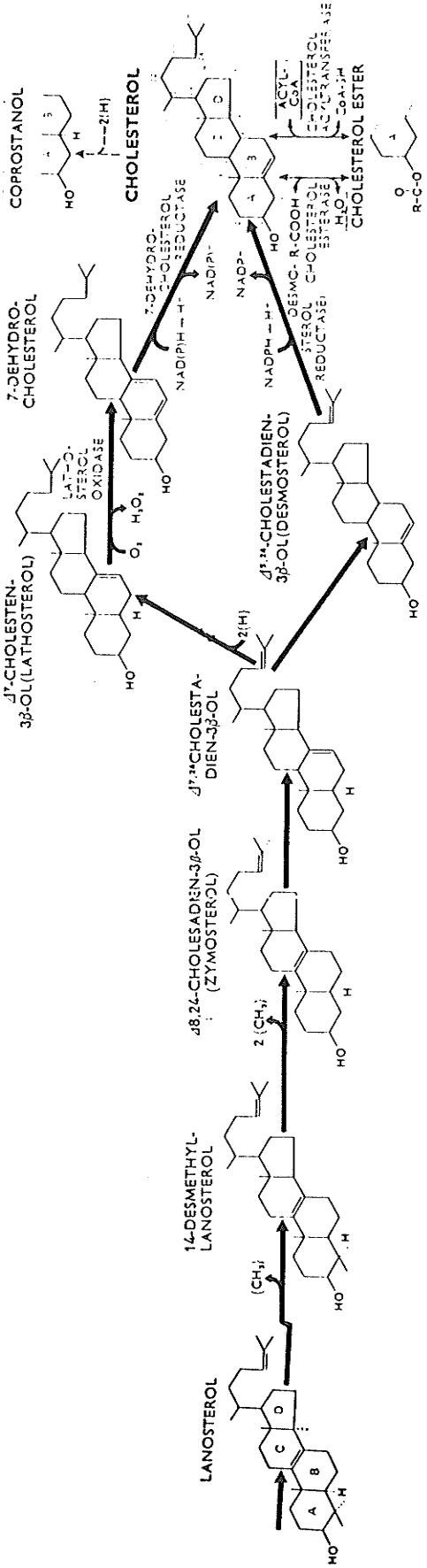
The hypothesis that the phenotypic abnormalities of C^R7 can be explained by a defect in P450-like cytochrome mediated processes leading to accumulation of lanosterol in cellular membranes, is somewhat speculative. It is, however, potentially amenable to experimental investigation.

Clearly, this investigation has demonstrated that the Con-A resistant variant, C^R7 is a cholesterol auxotroph because it fails to adequately convert lanosterol to cholesterol (Figure 16, Table 22). This failure results in an accumulation of lanosterol, and a dearth of cholesterol. This failure is at the root of the abnormal biochemistry of C^R7. The phenotypic abnormalities of C^R7 are therefore envisioned as secondary consequences of the accumulation of lanosterol.

What then is the defect(s) in the variant, C^R7? Why can't C^R7 successfully convert lanosterol to cholesterol? This investigation suggests a possible answer.

Ketoconazole, which is a substituted imidazole (Borelli et al, 1979) blocks the conversion of lanosterol to ergosterol in yeast by inhibiting the cytochrome P-450 dependent step of C-14 demethylation (Van den Bossche et al, 1980). It was also shown to block the

Figure 64: The pathway for the conversion of lanosterol to cholesterol.


LANOSTEROL
14-DESMETHYL-LANOSTEROL
Δ8,24-CHOLESTADIEN-3β-OL (ZYMOSTEROL)
Δ7,24-CHOLESTADIEN-3β-OL
Δ7-CHOLESTEN-3β-OL (LATHOSTEROL)
7-DEHYDRO-CHOLESTEROL
Δ5,24-CHOLESTADIEN-3β-OL (DESMOSTEROL)
CHOLESTEROL
COPROSTANOL
CHOLESTEROL ESTER
LATH-O-STEROL OXIDASE
7-DEHYDRO-CHOLESTEROL REDUCTASE
DESMO-STEROL REDUCTASE
CHOLESTEROL ESTERASE
ACYL-CoA CHOLESTEROL ACYLTRANSFERASE

conversion of lanosterol to cholesterol by preventing the demethylation of lanosterol at C-14 in human subjects with advanced prostate cancer (Kraemer & Pont, 1986).

This investigation has shown that when provided with desmosterol or 7-dehydrocholesterol, the mutant is viable in a cholesterol-deficient medium (Figure 55). This is an important observation as desmosterol and 7-dehydrocholesterol are located at a key branch point in the pathway in the conversion of lanosterol to cholesterol (see Figure 64). The finding that these compounds allow C^R7 to survive in the absence of cholesterol suggests that the processes for the conversion of these two compounds to cholesterol are satisfactory in C^R7.

Studies with ketoconazole allowed the demonstration that, due to ketoconazole-induced lanosterol accumulation, wild type cells behaviour was similar to that of the mutant. In fact, wild type cells in the presence of ketoconazole mimicked C^R7 in that, at 39°C, WT exhibited temperature-sensitive growth (Figure 57). This was at a concentration of ketoconazole found not to be inhibitory for growth of wild type at 34°C (Figure 56). Furthermore, as the amount of ketoconazole present in the growth medium was increased, the inability of WT to grow at 39°C became more evident (Figure 60). Presumably with increased ketoconazole, the demethylation of lanosterol at C-14 decreased proportionally, such that cholesterol availability was reduced.

Regarding C^R7, the presence of ketoconazole reduced the mutant's ability to proliferate both at 34°C and at 39°C (Figures 58 & 59). It is hypothesized that the lesion present in C^R7, that is, its inability to demethylate lanosterol effectively is present at 34°C. However, when the mutant is challenged, for example, at 39°C with increased

temperature, in combination with its altered membrane composition, it is unable to make the appropriate compensatory response. It is only that the lesion now becomes critical.

Similarly, with ketoconazole present wild type cells exhibited a resistance to the cytotoxic effects of Con-A (Figures 61 & 62) while the mutant became even more resistant. Unlike the case with added lanosterol (recall this had no effect on WT), the presence of ketoconazole blocks the demethylation of lanosterol to cholesterol, and a defective membrane results. Unlike the case, with added lanosterol, where some process is required in which the fully competent membrane must be disrupted so that lanosterol can replace cholesterol in the membrane, this is not necessary with added ketoconazole. Presumably the drug is able to exert its effect actively on a metabolic process well established in the cellular machinery, the consequences of which are readily detected.

Why can't C^R7 demethylate lanosterol adequately? Lanosterol must be demethylated at both C-14 and C-4 during its conversion to cholesterol. Demethylation at C-14 is cytochrome P-450 and NADPH-dependent whereas demethylation at C-4 is cytochrome b_5 and NADH dependent. Earlier, work in lanosterol metabolism established that C-14α demethylation occurs prior to any demethylation at the C-4 position (Gautschi & Bloch, 1958; Berry & Chang, 1982).

Therefore, a determination of the cytochrome P-450 content of wild type and C^R7 cells at 34°C and 39°C was attempted. Although the amount detectable was small, the presence of cytochrome P-450 was demonstrated in wild type cells grown at either 34°C or 39°C. No cytochrome P-450 was demonstrated in C^R7 cells grown at either 34°C or 39°C, despite the

fact that the amount of cells used for the determination was the same for both wild type and mutant. By virtue of this fact, C^R7 mutant cells contain at least a decreased amount of cytochrome P-450 relative to the wild type cells. Obviously a more detailed investigation is warranted. However, from an economical and practical perspective, such an investigation may not be cost and time effective.

Nevertheless, due to the decreased P-450 content of the mutant (at least, relative to wild type), C^R7 is probably unable to demethylate lanosterol at C-14, or, at least at a rate less than the wild type. This rate is efficient enough to allow the WT to make cholesterol and other appropriate responses relative to thermostability. This is obviously true as WT cells proliferate at 39°C, the mutant does not (Figure 13). Similarly, the mutant doesn't do well on LPDS-M, wild type cells respond adequately (Figure 16, 17, 18 & 19).

Since demethylation at C-14 precedes and is thereby necessary in order for C-4 demethylation of lanosterol to occur, it may be said, although speculatively, that C-4 demethylation would not take place in C^R7.

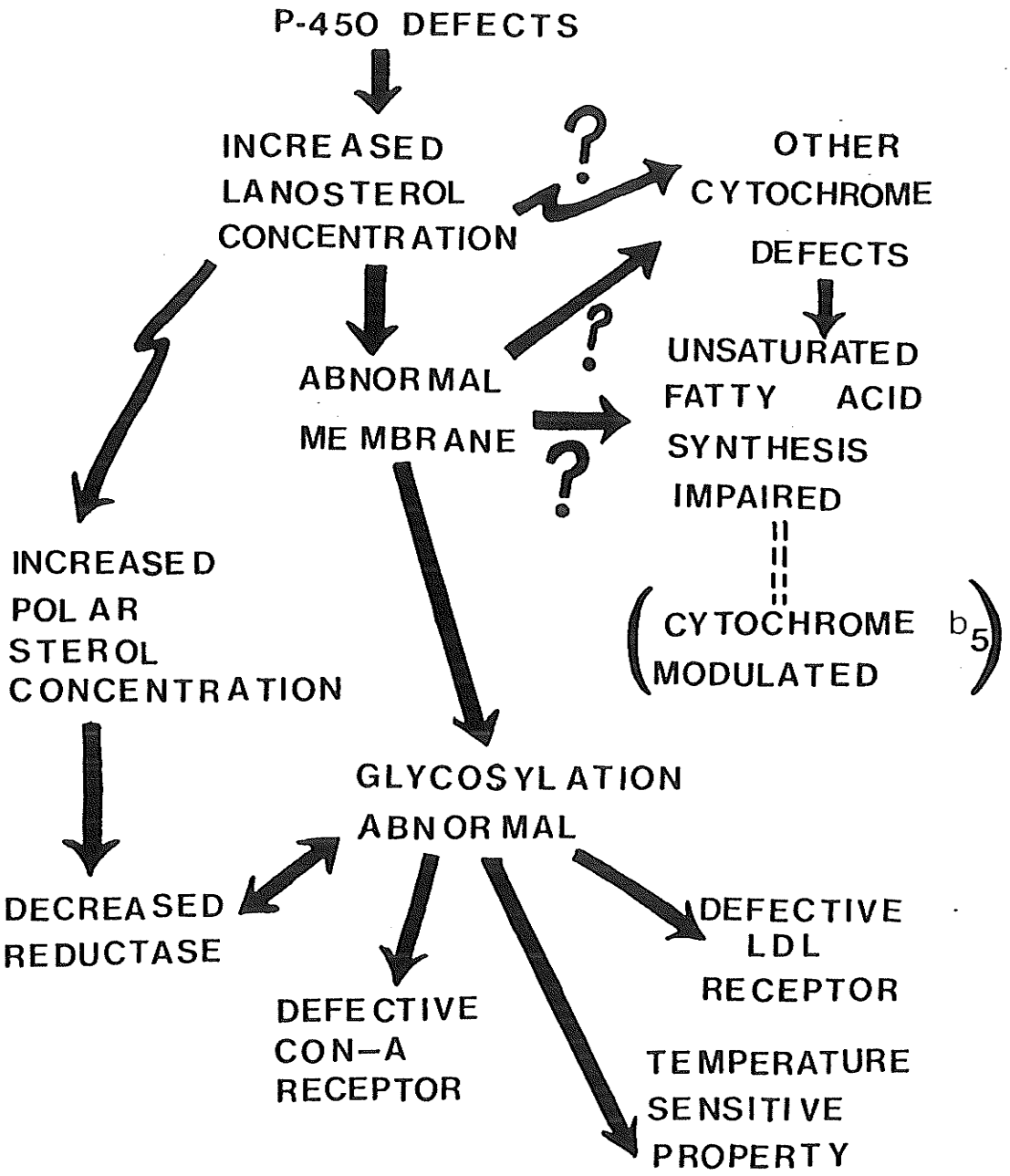
Demethylation at C-4 is cytochrome b_5 and NADH-dependent. Cytochrome b_5 content of these cells was not evaluated. However, C^R7 is defective in the synthesis of unsaturated fatty acids. This process is cytochrome b_5-dependent.

The possibility that cytochrome b_5 works in concert with cytochrome P-450 (although not well resolved), has been suggested (Estabrook, 1978). Therefore, a decreased content of cytochrome P-450 might be paralleled by a similarly decreased activity of cytochrome b_5.

Other cholesterol auxotrophs have been studied and shown to be defective in C-4 demethylation, more specifically in the 4α methyl sterol oxidase system (Chang & Berry, 1982). C-14 demethylation was, in this instance, apparently intact since it occured at identical rates in wild-type and mutant 215 (Berry & Chang, 1982).

The preceding ideas are summarized in Figure 65.

Figure 65: A diagrammatic explanation of the suggested defect in the Con-A resistant variant, C^R7.


P-450 DEFECTS
INCREASED LANOSTEROL CONCENTRATION
?
OTHER CYTOCHROME DEFECTS
UNSATURATED FATTY ACID SYNTHESIS IMPAIRED
(CYTOCHROME b_5 MODULATED)
?
?
ABNORMAL ME MBRANE
INCREASED POL AR STEROL CONCENTRATION
GLYCOSYL ATION ABNOR MAL
DECREASED REDUCTASE
DEFECTIVE CON-A RECEPTOR
DEFECTIVE LDL RECEPTOR
TEMPERATURE SENSITIVE PROPERTY

C^R7 - Return to Normal Biochemistry and the Effect of Exogenous Mevalonate

Mevalonate is a critical intermediate in cell metabolism (Quesney-Huneeus et al, 1979; Brown & Goldstein, 1980; Fairbanks et al, 1984; Siperstein, 1984). Compounds such as cholesterol, steroid hormones, and bile acids (steroidal isoprenoids) and dolichol, ubiquinone and isopentenyladenosine in certain tRNAs (non-steroidal isoprenoids) are products of mevalonate metabolism. In addition, a number of proteins are covalently modified by mevalonate or its derivatives (Schmidt et al, 1984). Mammalian cells in culture require the products of mevalonate metabolism for survival and growth (Brown & Goldstein, 1984; Goldstein et al, 1979). Mevalonate can be obtained either by de novo synthesis from HMG-CoA by the action of HMG-CoA reductase or by uptake from the extracellular milieu.

As this investigation progressed, it became obvious that a special relationship existed between mevalonate and the restoration to normalcy of the aberrant behaviour of the Con-A resistant variant, C^R7.

Mevalonate addition restored growth of C^R7 at the non-permissive temperature of 39°C to normal (Figure 21). Mevalonate addition to growth medium also had a profound effect on Con-A resistance of C^R7 at 34°C (Figure 52). Con-A resistance of C^R7 was increased. Mevalonate may have a similar effect on C^R7's response to Con-A at 39°C (Figure 53). This is difficult to assess with any certainty since at 39°C, no adequate control with C^R7 can be done (C^R7 will not grow at 39°C in the absence of mevalonate).

Furthermore, mevalonate addition restored C^R7's ability to incorporate mannose into lipid-monosaccharide, lipid-oligosaccharide, and glycoprotein to that exhibited by the wild type cells at 39°C (Figure

42). In the absence of mevalonate, all these processes were found to be defective in the variant at 39°C (Figures 40 & 41).

At the non-permissive temperature, the mutant had a marked inability to synthesize DNA, RNA and protein as measured by incorporation studies (Figures 43, 44 & 45). However, the addition of mevalonate to the growth medium relieved the defect affecting C^R7's inability to synthesize DNA, RNA, and protein (Figures 43, 44 & 45).

Truly, mevalonate plays a pivotal role regarding C^R7's behaviour at 39°C, the non-permissive temperature. Mevalonate may also play an equally crucial role regarding C^R7's behaviour at 34°C. This investigation was, however, concerned principally with the behaviour of C^R7 at 39°C. As a result, extensive studies of the influences of mevalonate on C^R7's behaviour at 34°C were not done. Such studies would be useful in order to more confidently assess the relationship between mevalonate and the behaviour of C^R7.

It is possible that the lesion of defective lanosterol demethylation present at 34°C, becomes critical at 39°C, at which temperature the demand for cholesterol (Figures 37) and glycoproteins (Figures 40 & 41) may be greater.

C^R7 synthesizes much more lanosterol, and much less cholesterol, than does WT at 34°C and this is magnified at 39°C (Tables 21a and 21b). It is a reasonable hypothesis that the presence of lanosterol, coupled with a relative deficiency of cholesterol, in cellular membranes of C^R7 might cause abnormally low activity of membrane-associated enzymes. Decreased activity of glycosyl transferases involved in glycoprotein synthesis, caused in this way, could result in altered membrane

glycoproteins and account for observations such as increased Con-A resistance. At 39°C, the demand for both cholesterol and membrane glycoproteins involved in cellular thermostability may be greater than at 34°C. Thus C^R7's deficiencies in glycoprotein synthesis may be lethal at 39°C. As well, the even greater disparity between lanosterol and cholesterol synthesis, which is apparent at 39°C (Table 22), would be expected to intensify the decrease in activity of membrane-associated enzymes. Uptake of a relatively massive amount of mevalonate at 39°C could allow enough synthesis of intermediates involved in glycoprotein synthesis, for example, dolichol, to ensure production of those glycoproteins critical to cellular survival at 39°C.

In other words, according to this hypothesis, the inability to convert lanosterol to cholesterol causes lanosterol accumulation resulting in low glycosyl transferase activity. This, in turn, leads to aberrant glycoprotein synthesis, thus causing temperature-sensitivity and other membrane changes, for example, changes in Con-A resistance. Adding MVA-lactone allows growth at 39°C perhaps by allowing sufficient dolichol synthesis to "push" the synthesis of critical glycoproteins to adequate levels. In this connection, it should be recalled that dolichol, or its phosphate, allowed growth of C^R7 at 39°C (Figure 25) and that C^R7 markedly increased its dolichol content at 39°C (Table 11). But, because mevalonate is converted primarily to lanosterol and not to cholesterol, it will not allow adequate growth of C^R7 on LPDS-M at any temperature (Tables 21a & 21b, Figures 22 & 23).

Another factor that may be pertinent, is the possibility that a temperature-sensitive mevalonate transport system may exist in C^R7. Such a system could be viewed as a compensatory response, by C^R7, at

39°C when the demand for mevalonate metabolites may become critical as discussed above. A mevalonate transport system has been described in met-18b-2 CHO cells by Faust and Kreiger.

Faust and Kreiger (1987) characterized mevalonate uptake and metabolism in a LDL-receptor negative CHO cell variant designated met-18b-2. These cells internalized mevalonate at rates 10-40 times greater than the progenitor cells from which they were derived. Regulation of HMG-CoA reductase activity and cholesterol esterification was dramatically more sensitive to mevalonate in met-18b-2 cells than in progenitor cells (Faust & Kreiger, 1987).

An interesting relationship perhaps exists between the levels of reductase activity and mevalonate availability. The reductase levels in the mutant C^R7 cells do not correlate with the observed rate of sterol synthesis (Table 14; Figures 37). The discrepancy probably lies with the reductase being defective but could be related to an inappropriate utilization of de novo synthesized mevalonate. The levels of reductase activity in the mutant cultured in the presence of mevalonate were not determined. Such an investigation might yield valuable information.

Increased uptake of labeled mevalonate in met-18b-2 cells was temperature-dependent and highly specific (Faust & Kreiger, 1987). It was suggested that met-18b-2 cells express a mevalonate transport activity not normally expressed in CHO cells, and this activity might be due to a specific mevalonate transporter that is differentially expressed in specialized tissues (Faust & Kreiger, 1987). A similar mevalonate transporter may exist in C^R7. Further investigation is needed to prove or disprove this suggestion.

HMG-CoA reductase serves at least two obligatory functions in the cell cycle. HMG-CoA reductase provides the mevalonate that is needed for the synthesis of cholesterol which, in turn, is required for the production of cell membranes and hence, cell growth (Quesney-Huneeus et al, 1983). Quite independent of its function as a cholesterol precursor, mevalonate has been demonstrated to play an essential role in the initiation of DNA replication (Quesney-Huneeus et al, 1983). Isopentenyladenine, or a related isoprene purine may mediate this effect of mevalonate in the cell cycle (Quesney-Huneeus et al, 1983). The preceding might be related to the demonstrated ability of added mevalonate to restore normal DNA synthesis (and by virtue of this, normal RNA and protein synthesis) to C^R7 at 39°C on FCS-M (Figures 43, 44 & 45). Perhaps, isopentenyladenine (or a related isoprene purine) would be effective in replacing mevalonate in this process. The effect on C^R7 at 39°C on LPDS-M is also unknown. However, all these processes are readily testable and may prove interesting.

A relatively recently discovered class of mevalonate metabolites is a set of proteins which are radiolabeled when the cells are exposed to radiolabeled mevalonate (Schmidt et al, 1984; Sinensky & Logel, 1985). Faust and Krieger (1987) compared the incorporation of labeled mevalonate into macromolecules in met-18b-2 and ldl A-7 cells (LDL receptor positive revertants). The mutants, met-18b-2 were LDL receptor negative. This was done by subjecting lysates of labeled cells to SDS-PAGE and autoradiography. No labeling was detected in extracts of ldl A-7 cells with as much as 162 μg of protein whereas significant labeling could be detected in extracts of met-18b-2 containing as little as 3.2 μg of protein.

A similar experiment was conducted with WT and C^R7 cells. Although not as dramatic as the above finding, more labeled mevalonate was found associated with C^R7 cell extract proteins especially at 39°C, and compared with wild type cells (Figure 47). The significance of this incorporation and of these labeled proteins, if any, is unknown.

All the unusual properties found in met-18b-2 cells described here were apparently due to their abnormally high levels of mevalonate uptake and subsequent conversion to many different cellular products. Two possible mechanisms for the rapid and saturable mevalonate uptake by met-18b-2 cells are possible. These are an increase in the intracellular rate of metabolism of either mevalonate or a mevalonate metabolite which could indirectly cause increased mevalonate uptake or the expression of an otherwise unexpressed transport activity which could directly increase mevalonate uptake (Faust & Kreiger, 1987). The latter was thought to be the most probable. The preceding might play a role in C^R7.

SUMMARY

SUMMARY

This investigation has addressed the issues of cell growth, lipid metabolism and temperature-sensitivity in a concanavalin-A resistant CHO cell variant, C^R7.

The growth parameters of C^R7 were well defined. Furthermore, this investigation once more underscored a previously noted close and specific interrelationship between the competent synthesis of glycoprotein and the normal expression of lipid metabolism.

The C^R7 cell line was previously shown to be defective in the glycosylation of proteins and is now known to be concomitantly deficient in the synthesis of cholesterol and fatty acids, and in particular unsaturated fatty acids. This is particularly true when C^R7 is presented with an increased demand for these factors, that is, increased temperature, or LPDS-M (cholesterol deficiency).

This investigation supported the finding that C^R7 is unable to adaptively regulate the levels of HMG-CoA reductase at 34°C. This finding was extended to the non-permissive temperature.

The revertant cell (Rc) demonstrated a complete reversion of the Con-A resistant phenotype supporting the conjecture that all the altered expression results from a single genetic lesion.

A discrepancy appeared between the *in vitro* activity of HMG-CoA reductase and the *in vivo* synthesis of sterols in the mutant at 39°C on FCS-M. This was previously observed at 34°C on FCS-M (Borgford, 1984). This discrepancy may indicate that HMG-CoA reductase is not the rate limiting enzyme in the synthesis of sterols. At least, this may be true in C^R7. Alternatively, the discrepancy may be caused by an inappropriate localization or orientation of the reductase protein.

This may be the result of a defective glycoprotein "tag" (Borgford, 1984). Defects in intracellular trafficking have been correlated with several of the glycosylation-defective phenotypes. For example, Lec 5 CHO mutants do not compartmentalize lysosomal enzymes appropriately into lysosomes (Krag & Robbins, 1982). C^R7 is probably Lec 5. A closer examination of HMG-CoA reductase and its regulation may be warranted. A subcellular localization of HMG-CoA reductase activity would be an intriguing undertaking and might provide useful information.

This investigation examined temperature-sensitivity in C^R7 and was able to delineate many aspects of this altered property. Furthermore, a possible explanation of this phenomenon and other aberrant properties of C^R7 was suggested.

C^R7 is probably temperature-sensitive and defective in other areas due to a cytochrome P-450 like defect. This defect results in an abnormally high level of lanosterol and a dearth of cholesterol in C^R7's membranes. Results from this study, although somewhat suggestive and preliminary, indicated that C^R7 is lanosterol demethylation defective. Regardless of this fact, this investigation, at a minimum, localized the defect in C^R7 to be between lanosterol and zymosterol (Desmosterol and 7-dehydrocholesterol were able to allow C^R7 to grow normally on LPDS-M at 34°C).

Two basic phenomena were elucidated in this study. They are the role of lanosterol in C^R7's abnormal behaviour and the role of mevalonate with the restoration of CR7's aberrant behaviour to normalcy.

Although this investigation revealed many interesting findings and more than adequately characterized C^R7's behaviour to date, a number of interesting questions remain. An examination of the actual levels of

lanosterol in these cells' membranes would yield valuable information which would either refute or support the findings of this thesis. The biochemistry of lanosterol in mammalian cell membranes is little, if at all, understood. Indeed, lanosterol may not have a role in "normal" membranes. Unfortunately such an investigation may prove to be cost and time ineffective, in light of the fact that mammalian cells in culture are not the ideal source of tissue for such a study. Such an investigation may also be complicated by the fact that lanosterol may be undetectable in "normal" membranes (as found in WT and Rc).

A detailed examination of cytochrome P-450 and of cytochrome b_5 content would be appropriate. However, such a undertaking should be approached with caution as it may prove to be problematic. It may prove to be a cost and time ineffective undertaking.

The best way to examine the reaction sequence of lanosterol to cholesterol in these cells would be with the use of radiochemically labeled substrates and isolated enzymes. Such radioactively labeled compounds are not readily available at this time.

Mevalonate has been shown to play a key role regarding C^R7's behaviour (previously discussed). Further studies of this relationship might reveal valuable information regarding regulatory aspects of C^R7, as well as WT and Rc cell lines.

The lesion of defective lanosterol demethylation (and its consequences) can account for the abnormalities seen in the CHO cell variant, C^R7 (see Figure 65). This lesion is present at 34°C but becomes critical in C^R7 at 39°C. This occurs probably because of the additional constraints placed on C^R7 by the stress of the elevated growth temperature.

This investigation represents further work in an ongoing study of lipid metabolism in the concanavalin-A resistant variant, C^R7. This present study examined individual aspects of lipid metabolism, and, specifically, established the relevance of lipid metabolism to temperature-sensitivity.

Through the efforts of this investigation, not only to elucidate but to understand the complex nature of C^R7, a number of intriguing possibilities for future study were discovered. It is hoped that these efforts might serve as the impetus for further scientific investigation into the biochemistry and cell biology of these CHO cell lines.

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