MEMBRANE CHOLESTEROL, TUMORIGENESIS, AND THE BIOCHEMICAL PHENOTYPE OF NEOPLASIA

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"If ye kin make a model, ye understand it; if ye kanna, ye dinna."
Attributed to Lord Kelvin

I. INTRODUCTION

A. A Proposal on the Role of Membrane Cholesterol Enrichment in Tumorigenesis

This review sets forth arguments for the proposal that abnormalities in the biosynthetic regulation of lipids — particularly that of cholesterol — constitute early events in tumorigenesis, and underlie many of the phenotypic hallmarks of malignant neoplasia. We envisage that deregulation in the biosynthesis of cholesterol as well as other lipids occurs very early after a primary carcinogenic event, and continues over time, such that tumor cell membranes become enriched with cholesterol. We focus on the role of cholesterol in biological membranes, especially with regard to the regulation of metabolism, and when cholesterol is present in excess in tumors, on the potential of the sterol-enriched membranes to sustain and even facilitate the state of neoplasia. Our discussion of this subject is selective and topically parametered. We have limited our hypotheses to those which appear to support the following proposition. If an initiating carcinogenic event (unspecified) induces an increase in the synthesis and accumulation of cellular cholesterol, particularly in membranes, this leads to the development of a host of altered metabolic patterns within the affected tissue. Such altered metabolic profiles often present themselves as significant indicators of the neoplastic phenotype.

At the outset it is important to understand the stage of neoplastic transformation at which our discussion unfolds. The development of a primary malignancy from an otherwise normally differentiated tissue manifests a long period of time between what is assumed to be the carcinogenic trigger or event and the diagnostic appearance of the tumor. This "latency" period, or early masking of the eventual neoplastic phenotype, was recognized many years ago, and gave rise to the well-known, two stage concept for the mechanism of carcinogenesis: initiation and promotion.[1] Consequently, time plays

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a crucial role in the eventual expression of the primary tumor cell phenotype and in our ability to recognize early events in the change from normal to altered metabolic patterns of neoplasia. Given this phenomenon of delayed expression, and realizing that the malignant cell traces its ancestry to a normal differentiated one, we are entitled to ask whether a "precancerous" state can be identified within the target tissue (i.e., at a point in time prior to the ability to ascertain the presence of a malignancy histopathologically). For example, we wish to know *when* a tumorigenic characteristic can be identified during the precancerous or latent period. Moreover, one would hope to establish, on a molecular level, the nature of such an early indicator of tumorigenesis and ascertain that it is a hallmark of the precancerous state for diverse tissues. Regardless of how these questions might be answered, it is clear that once the triggering carcinogenic event occurs the ultimate result probably will be cellular transformation to the neoplasm subsequent to promotion. Afterwards, one may identify the fully cancerous state according to one of its most general descriptions — the relatively autonomous growth and proliferation of the afflicted cell.

If a unifying concept for the process of tumorigenesis is to be considered, then any metabolic alteration that is proposed as a fundamental and early molecular event in neoplastic transformation must satisfy two principles. First, it must occur in a wide variety of tissue types soon after the introduction of the triggering carcinogenic event, because tumorigenesis is not restricted to only a few target tissues. Second, the fundamental metabolic alteration should be shown as capable of generating or being ultimately involved with all major biochemical properties that are characteristic of tumor cell metabolism. The arguments that we shall present in this review illustrate how the loss of normal control over cholesterol biosynthesis appears to fulfill both criteria.

The metabolic sequelae that we propose to occur subsequent to the primary tumorigenic lesion leaves untouched, and as controversial as ever, the target of this lesion, and thus, the subject of the ultimate causality of cancer. Whether the primary carcinogenic insult is taken to be at the genetic (information is added to or removed from chromosomal DNA) or the epigenetic level (the information content of the cellular genome remains unaltered but is expressed in an aberrant fashion), or both, is not treated in our discussion. In this regard, it is sufficient to point out that the documented metabolic alterations seen in neoplastic cells, including those that we propose later in this article, have never seriously been ascribed to mutant *structural* gene products as the *prima facie* cause of the neoplastic property.[2]

Nevertheless, a vast literature covering all areas of malignant transformation has been accumulating for decades. We believe, as do some others working in this field, that a central theme to which much of these data may be related involves the rather early (precancerous?) loss of regulation of sterol metabolism, and the attendant accumulation of cholesterol in cell membranes. We are beginning to perceive that this alteration in cholesterol synthesis regulation may turn out to be a potentially significant biological marker of neoplasia along with others that continue to receive attention. This suggestion has also been made by Siperstein.[3]*

Onto the increasing number of observations on the misregulation of cholesterol synthesis in tumors, and stemming from this would-be fundamental metabolic alteration, we have grafted a hypothesis with extensive implications for other aspects of tumor cell

* Several putative biological markers of neoplasia have already been described, and other candidates are periodically proposed. The appearance of human chorionic gonadotropin as a marker for choriocarcinoma, as well as for carcinomas of the lung, liver, breast, pancreas, ovary, etc., is one example.[4] Others include tumor specific antigens,[5] neoplastic cell surface glycoproteins,[6] plasminogen activators,[7] and a variety of serum and urine enzyme activities such as acid and alkaline phosphatases, 5′ nucleotidase, glycosyltransferases, assorted glycolytic enzymes, and asparagine synthetase.[8,9]

metabolism. The details of the hypothesis appear to support, and in turn, lead to many other known features that have been documented for a number of established malignant tumors. Some of these are increased glutamate and glutamine oxidation; enhanced aerobic glycolysis; altered amino acid levels in host body fluids; diminished urea cycle activity in hepatomas; increased polyamine synthesis and accumulation; alterations in the patterns of mitochondrial bioenergetics, including those concerned with reducing equivalent shuttles; and as a causative phenomenon from which these metabolic aberrations arise, alterations in the composition and function of various kinds of tumor cell membranes. With regard to cellular membranes, the relative differences in the lipid content (specifically, as represented by the cholesterol level or the cholesterol/phospholipid ratio) of tumor cell membranes compared with those of the tissue from which the tumor arose, may in fact govern many of the anomalous patterns of metabolism that have been noted in tumors. The cholesterol-modulating effects on cell membrane function are, understandably, important to contemplate. Indeed, cholesterol is a fundamental membrane constituent, and cellular membranes are the preeminent sites for "global" regulation of overall metabolism, and consequently, of phenotypic expression of cellular metabolic patterns.

We shall begin our discussion with some general information on the cholesterol molecule and its proposed role as a lipid component of biological membranes. More detailed and comprehensive reviews on this subject are available elsewhere.[10] Then, we will briefly highlight some of the features of regulated cholesterol synthesis in normal vs. neoplastic cells, and document evidence that proliferating cells display a special requirement for sterol synthesis. We will then present our views on how an aberrant regulation of cholesterol biosynthesis in tumors, occurring early in the temporal sequence of the tumorigenic process, can directly lead to other documented characteristics of tumor cell metabolism, particularly those aspects concerning cellular respiration. Finally, our discussion will lead back to the cancer-stricken host, where recent findings show that plasma membrane fragments, rich in cholesterol, are shed from the tumor into sera and pleural effusions of the afflicted organism. There are clear indications that these membrane fragments, shed from the tumor as microvesicles, contain tumor antigenic determinants. These are quite exciting data, since such cholesterol-rich tumor membrane vesicles could serve as an acellular target for cell-mediated destruction by the host, permitting the tumor cells, themselves, to escape at some critical time during tumor establishment *in situ*. The key to all of these patterns, both metabolic and cellular, may lie in the membrane lipid modifications of tumor cells produced soon after exposure to the carcinogenic insult.

II. CHOLESTEROL AS A STRUCTURAL COMPONENT OF BIOLOGICAL MEMBRANES

Cholesterol, an alicyclic lipid molecule, whose structure includes four fused rings, a single hydroxyl group at C-3, and an unsaturation at C-5, 6 is ubiquitously distributed throughout the eucaryotic phyla, and a cholesterol derivative is a required component of at least one primitive procaryotic organism, the methanotroph *Methylococcus capsulatus*.[11] The molecule is very insoluble in aqueous media (a maximum of 4.7 μM as the micelle) and has a critical micelle concentration of about 30 nM at 25°C.[12] Cholesterol is the principal sterol of vertebrate tissues, with special abundance in brain and nervous tissue where it is found in the myelin sheaths associated with phospholipids as micellar structures. Probably every membranous component or organelle of vertebrate cells contains cholesterol to at least some extent. The molecule may be enzymatically esterified at the C-3 hydroxyl position by transfer of an acyl

residue from acyl-CoA moieties (usually saturated) or from lecithins. These cholesteryl esters are transported within the plasma of the circulatory system as complexes with lipoprotein fractions, the low density lipoprotein (LDL) component being responsible for the bulk of this transport. Cholesterol serves as the parent precursor substrate for the biosynthesis of bile acids in the liver, of steroid hormones in the adrenal cortex, and of sex hormones generated by the testis and ovary.

Perhaps one of the most important roles assumed by cholesterol, yet the most difficult to appreciate in any but the most general terms, is that of its physical association with biological membranes.

Since the study of cell membrane structure and function began to accelerate over 40 years ago, it has been tacitly assumed that biological membranes, especially those of eucaryotic organisms, contain sterols as well as phospholipids. However, it was only after the cholesterol content of the erythrocyte membrane was reported by Ways and Hanahan[13] that it became generally accepted that a substantial cholesterol presence was not unique to myelin membranes (which contain nearly half the brain's cholesterol), but that other subcellular membranes involve considerable amounts of cholesterol as well.[14,15]

That cholesterol functions principally as a membrane structural component gained support from at least two prominent findings. One such study indicated that the development of insect pupae from larvae required added cholesterol, which, for the most part, remained unmetabolized during the pupation period.[16] Insects do not synthesize cholesterol *de novo,* yet nevertheless require it for growth and development. During pupation, the recovery of unmodified cholesterol implies that it fulfills a structural role as a component of cell membranes. Such a role would be visualized as controlling, to an apparently significant extent, the changing functions of cell membranes during larval morphogenesis, without the molecule's utilization as a metabolic substrate. With the added findings that protozoans as well as anaerobically grown yeast require sterols that are not metabolized by the cell,[17,18] it may now be generally accepted, but with caution, that the free cholesterol content of a eucaryotic cell, upon extraction and analysis, is almost totally derived from cellular membranes, where it serves in an architecturally vital manner.

Although there are numerous derivatives of cholesterol present in nature, most eucaryotic cell membranes do not tolerate significant modifications to the unique molecular structure of cholesterol. The C-3 hydroxyl group is an absolute requirement; cholestane, which has no C-3 substitution, is incapable of replacing cholesterol as a growth supporter of anaerobic yeast, indicating that the importance of the hydroxyl group may lie in its potential to hydrogen bond at the aqueous interface of the membrane. The cholesterol ring nucleus configuration has been shown by X-ray analysis to be all-*trans-anti*[19] which leads to a flat planar structure with a thickness, viewed edge-on, of a bit over two angstroms, taking into account the C-18 and C-19 β-face methyl substituents.[20] Nes et al.[21] have concluded that the growth supporting stereochemistry of cholesterol's C-17 isooctyl side chain requires that it extend in a direction away from the polar C-3 hydroxyl function. Thus, the overall molecular dimensions of cholesterol would appear to approximate a solid rectangle about 18 Å long $\times$ 5 Å high $\times$ 3 Å thick. This particular structure is uniquely selected for by higher vertebrate cell membranes to the exclusion of nearly all other sterol molecules.

It should, however, be pointed out that cholesterol is subject to structural modification in addition to its normal metabolic conversion to steroid hormones and bile acids, which are not, of course, architectural components of cell membranes, although they interact with them. For example, cholesterol is susceptible to considerable primary oxidation both in vivo or in vitro, which need not involve enzymic catalysis. Under relatively mild

autooxidizing conditions, a wide variety of oxidized derivatives of cholesterol are generated.[22,23] When the oxidized cholesterol derivative, cholesterol-α-oxide (cholestan-5α, 6α-epoxy-3β-diol), is administered to rats and mice subcutaneously, it is found to be locally carcinogenic.[24] A similar observation has been made for the cholesterol oxidation product, 6β-hydroxy-4-cholesten-3-one.[25] Neither oxidation product is a cell membrane constituent, yet their carcinogenic potential would require either that they themselves be metabolized, or exert influence over normal metabolic regulation in the cell. These findings support the fact that vertebrate cell membranes uniquely express a highly delimited specificity for the molecular configuration of cholesterol as a structural component. Parenthetically, the data also raise intriguing questions about the in vivo potential of a pharmacologically innocuous, indeed vital, membrane molecular component to become, somehow, transformed into a carcinogen.

According to data compiled by Jain,[26] the mole fraction of cholesterol almost never exceeds 0.5 for a diversity of biological membranes, but varies to a considerable degree below that value as a function of the particular cell membrane fraction examined (Table 1). Thus, whatever roles cholesterol fulfills as a component of cell membranes, it would appear to be dispensable *quantitatively* to some extent. Yet, its presence confers distinctive physico-chemical attributes upon the membrane, and therefore upon specific membrane-associated functions.[123] This, of course, is supported by many studies, beginning with the observation that cholesterol or other sterols are nearly absent from virtually every bacterium examined, without affecting bacterial survival or proliferative capacity.[27]*

III. EFFECTS OF CHOLESTEROL ON MEMBRANE STRUCTURE AND FUNCTION

A. On Bilayer Structure

The alteration of the physical characteristics of biomembranes containing differential amounts of cholesterol has been investigated by various techniques in several kinds of model and natural membrane systems.[10,38,39] Studies with sterol/phospholipid mixed liposomes via nitroxyl electron spin resonance methods,[40,41] indicate that sterols interdigitate between the pairs of phospholipid acyl chains in the bilayer. An increasing presence of cholesterol has been observed to effect an increasing order or structural rigidity in the lamellar core of the liposomal bilayer.[42] Cholesterol is known to form micellar complexes with the acyl chains of lecithins and sphingomyelins, thereby condensing or causing restriction of mobility of these hydrocarbon chains in the hydrophobic environment.[10,43] The rigidifying effect of cholesterol implies that such an ordering of phospholipid acyl chains is strongly temperature dependent, and therefore the phase transition temperature (T_c) of mixed polar lipid/sterol bilayer systems has been studied as a function of sterol content. The cholesterol enrichment of such systems causes decreased mobility of polar lipid acyl chains above their T_c, leading to more condensed and rigid domains in the bilayer.[10,44,45] Consistent with these observations, model system studies with cholesterol, phospholipid, and mixed cholesterol/lecithin compressed monolayers have indicated that the area occupied by the sterol/phospholipid complex is only 75% that of the sum of the individual molecular areas. On the

* Apart from *Methylococcus,* mentioned previously, certain strains of *Mycoplasma* require sterols as growth factors, especially sterols which, like cholesterol, can raise the viscosity of lipid bilayers. However, even these sterol-requiring *Mycoplasma* strains may be made to grow in the absence of significant amounts of cholesterol. It is then observed that saturation of the acyl chains of the membrane's polar lipids increases, rendering the membranes less fluid.[28-30]

Table 1
RELATIVE CHOLESTEROL CONTENT OF MAMMALIAN CELL MEMBRANES[26,31,32]

Isolated fraction	Source	Cholesterol/P-lipid (mole:mole)	Cholesterol[a] (mole % total lipid)
Plasma membrane			
Erythrocyte	Various	1.30	44.2
Myelin	Rat	1.18	40
Lymphocyte	Pig, calf, hamster	1.47	50
Hepatocyte	Rat	0.76	26
Muscle	Rat	0.24	8.1
Nuclear envelope			
Liver	Pig	0.10	3.4
Liver	Rat	0.23	7.8
Liver	Mouse	0.17	5.7
Lymphocyte	Pig, calf, hamster	0.25	8.5
Microsomal membrane			
Brain	Guinea pig	0.24	8.1
Liver	Guinea pig	0.15	5.1
Liver	Rat	0.16	5.4
Liver	Mouse	0.23	7.8
Mitochondria[b]			
Liver	Rat	0.11	3.7
Intestine	Rat	0.09	3.1
Muscle	Rat	0.15	5.1
Brain	Guinea pig	0.03	1.0
Liver	Guinea pig	0.03	1.0
Kidney	Guinea pig	0.015	0.05
Heart	Beef	0.03	1.0

[a] Due to both experimental and computational inconsistencies in the literature, especially insofar as the derivation of phospholipid content, the values given here were recalculated on the basis of an average mol wt of 750 for the glycerophosphatide class of lipids. Total lipid (including cholesterol, mol wt 386) was therefore assumed to have a mol wt of 1136. The reader should be aware that membrane phospholipid content employed here was *not* based on experimentally assessed lipid phosphorus, as some authors have reported. Consequently, the values are to be viewed as general approximations to aid in establishing comparisons between various membrane fractions. The primary literature should be consulted in calculating the values for this table.[33,34] The observant reader will also be aware that experimental methods for isolating specific cell membrane fractions are varied; some techniques result in considerable cross-contamination of specific membrane fractions, adding to the generalized nature of the data tabulated here.

[b] The outer mitochondrial membrane contains substantially more cholesterol than the inner membrane. These differences are not specified in the table, although some authors whose work was consulted have attempted to determine experimentally the sterol content of each membrane independently.[35,37]

other hand, this phenomenon appears to depend on the species of phospholipid and the conditions employed in the monolayer studies.[46-48]

By extension of the data from a variety of studies with model lipid monolayer/bilayer systems,[49-53] it is fairly certain that in natural biomembranes cholesterol is inserted anisotropically into each half of the bilayer. In this orientation, the polar hydroxyl group at C-3 abuts the aqueous interface on either side of the bilayer and is localized within reasonably close lateral distance to the ionic membrane surface charges. The long axis of the cholesterol molecule is thus perpendicular to the plane of the membrane, and parallel with the acyl chains of conjugated membrane lipids (Figure 1). As supported by the lipid

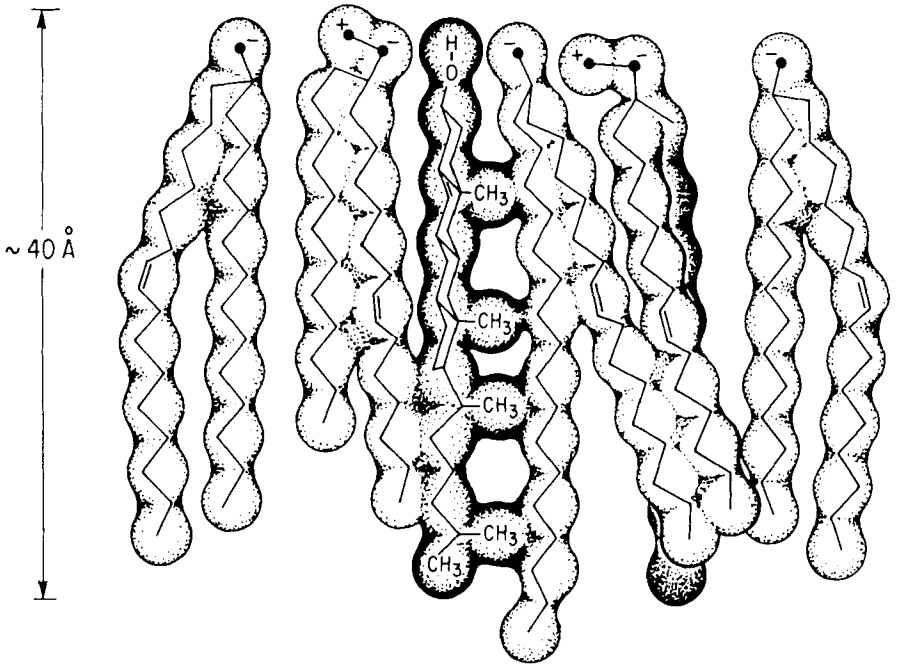


FIGURE 1. The proposed disposition of cholesterol in one bilayer leaflet of a membrane, illustrating the condensing or clustering of neighboring polar lipid moieties. Also indicated is the coulombic attraction between polar lipid head groups, as well as the possiblity of their H-bonding with the sterol 3-OH group at the aqueous interface.

bilayer model system studies, the presence of abundant cholesterol in certain biomembranes (e.g., erythrocytes, myelin) seems to generate a pseudocrystalline or ordered structure in the polymethylene acyl chain core region of the natural bilayer.[53a] Thus, the hydrocarbon chains of phospholipids in each of the bilayer leaflets assume extended and rigidified orientations when the cholesterol content is high. In biomembranes with a low cholesterol content (e.g., retinal rod outer segment), a more disordered and fluid bilayer core exists.

B. On Membrane Permeability, Mediated Transport, and Other Membrane Functions

Not surprisingly, membrane-associated functional phenomena have displayed alterations commensurate with sterol compositional modifications. The reduction of diffusional permeability that varies directly with increasing cholesterol content has been observed with liposomes constructed of egg lecithins.[54,55] Considerable experimental data confirm this cholesterol-mediated inhibition of solute release from within liposomes.[56–61] In one recent study,[62] the absence of the α-face axial C-14 methyl group on cholesterol (as distinct from lanosterol, which possesses this substituent) dramatically lowers the permeability of the bilayer to glucose diffusion. These results emphasize the structural specificity possessed by cholesterol with regard to its effects on the physical properties of the membrane, and therefore, on membrane function.

Similarly, biologically correlative systems have indicated that when *Mycoplasmas* were grown in the presence of varying amounts of cholesterol, the cholesterol enrichment of their membranes reduced diffusional permeability to glycerol and erythritol. This result was interpreted as being due, presumably, to the laterally condensing effect of cholesterol on the membrane lipids, and its ability to raise the membrane viscosity.[63,64]

Clearly, changes in membrane viscosity would be expected to alter the function of both membrane lipids and proteins. Thus, alterations in cholesterol levels of both model lipid bilayers as well as natural biomembranes produce a variety of permeability changes correlative with membrane viscosity alterations.

The cholesterol enrichment of lecithin liposomes has been shown to decrease permeability to glycerol,[56] as well as to chloride and sodium ions.[58,59] Manipulation of the cholesterol content of mixed phosphatidylcholine bilayer films endows them with either anion or cation permselectivity.[61] In the case of valinomycin-mediated Rb^+ transport across model bilayer membranes, cholesterol incorporation was observed to lower the rate constants for both the association and translocation of the valinomycin-Rb^+ complex.[60] Similarly, cholesterol enrichment of erythrocytes inhibited K^+ and Na^+ influx by 70 to 76%.[65] Other data have indicated that the Na/K-ATPase and adenylate cyclase enzymes of rat kidney fibroblast plasma membranes exhibited a reduction in activity as a function of cholesterol content, with the adenylate cyclase activity falling in direct proportion to the incremental cholesterol enrichment of the cell membrane via liposomal loading.[66]

It is also interesting that in addition to, and possibly because of, these alterations in membrane permeability to ions, cholesterol level modification of cell membranes has been observed to produce electrophysiological changes as well.[67,68] Anticipating the discussion further along in this review, it might be noted in this regard that Morris hepatoma 7777 cells, whose membranes are cholesterol rich, manifest plasma membrane resting potentials that are only 50% those displayed by normal rat hepatocytes. Highly reduced membrane resting potentials are also observed in transplantable murine corneal fibrosarcoma cells compared to normal mouse corneal fibroblasts *in situ*.[69] Cone, in fact, has proposed a correlation between the lowered transmembrane potential and the proliferative capacity of tumors.[70] However, no correlation has yet been made to include differential membrane sterol content as one of the underlying causes of altered membrane potentials in tumor cells.

Finally, lateral mobilities of membrane affiliated (i.e., intrinsic) proteins, including receptor loci, are also altered by changes in membrane sterol content. Subsequent to exogenous cholesterol enrichment, concanavalin A- and phytohemagglutinin-treated erythrocytes displayed a decreased susceptibility to agglutination.[71] Such a loss of agglutination susceptibility was interpreted as being due, at least in part, to the reduced lateral mobility of lectin receptors in the cholesterol-enriched membranes, thereby decreasing the propensity for "lectin patch" formation. The recent technique of micro surface domain fluorescence recovery after laser photobleaching was used to investigate the lateral mobility of a membrane-affiliated amphipathic apolipoprotein, Apo C-III, bound to phosphatidylcholine bilayers with and without added cholesterol. Those bilayers containing cholesterol manifested an Apo C-III diffusion rate that was retarded by a factor of more than ten compared with bilayers devoid of cholesterol.[72]

Physical studies have indicated that the variation in the measured effective membrane viscosity as a function of cholesterol enrichment or depletion is apparently symmetric; fluidity increases as the membrane cholesterol level decreases at temperatures above the normal T_c for the particular membrane.[73,74] However, one must be cautious to avoid overinterpreting generalized membrane viscosity results that are derived from probe techniques, such as polarization of fluorescence,[73,75] because of potentially "selective" membrane domains reported on by such methods.[76] Reevaluation of such methodologies has led to the inferential existence of a patchwork mosaic for the arrangement and distribution of membrane constituents. Such mosaic domains of lipid, for example, would exhibit very different properties than might be attributed to homogeneous lipid distributions. In fact, the concept of a mosaic patchwork structure with regard to the membrane's lipid components becomes the basis of an attractive model of biological

membranes developed by Jain and White,[77] which they refer to as the "plate model" of membrane structure. Detailed experimental support for mosaic-like lipid domains, some of which can be visualized as less fluid than others because of their enrichment with cholesterol, has been provided by recent studies on fluorescent lifetime heterogeneities observed in mixed phase phospholipid model systems compared with isolated cell plasma membranes.[76]

Considering, therefore, evolving concepts of biological membrane structure and function, it is becoming apparent that the most widely quoted structural model, the fluid mosaic model advanced by Singer and Nicolson,[78] is currently under modification. Perhaps this is because the fluid mosiac model provides only a general framework for establishing the cause of lateral diffusion of membrane components (lipids as well as intrinsic proteins) in the plane of the membrane. One weakness of the fluid mosaic model as originally expounded, has been its inability to explain the vast lipid heterogeneity or polymorphism observed in biological membranes, of which the differential cholesterol content of various cellular membranes is one major example. Lipid polymorphism is currently being assessed with a view towards regional *nonbilayer* structure of biological membranes. In a recent revew, Cullis and de Kruijff[79] conceive of a "metamorphic mosaic" for biological membrane lipids, extending the structural role of lipids beyond that of the mosaic plate model proposed by Jain and White.[77]

C. Delimited Membrane Cholesterol Content for Optimal Function

The direct correlation of biomembrane function with its sterol content is somewhat more subtle than the data on the diffusion permeability or the viscosity studies would indicate at first sight. For example, sterol-deficient mammalian cell mutants have been recently developed, and their catalytic membrane transport properties studied. Saito and Silbert[80] reported that one such mutant cell line displayed an absolute requirement for cholesterol addition to the culture medium to sustain cell growth. Plasma membranes isolated from these sterol auxotrophs were shown to be cholesterol-poor (actually desmosterol-poor; desmosterol, a proximal cholesterol precursor, is the final sterol product in these cells). With regard to membrane function, these sterol-deficient mutants, compared with wild type cells, displayed a dramatically lower V_{max} for the facilitated transport of both 3-O-methyl glucose and thymidine. It was further demonstrated that wild type transport kinetics could be restored by incubating the mutant cell with cholesterol/phospholipid (sterol-enriched) liposomes.[81] Consequently, in this system normal kinetic characteristics of substrate transport across the plasma membrane not only require a reasonable magnitude of membrane cholesterol to be present, but appear to be reversible by manipulating the magnitude of this membrane cholesterol.

Other studies by these investigators support the importance of sterol-regulated membrane properties. It was observed that in order to maintain normal sugar transport activities in cells depleted of their endogenous membrane sterol content, the sterol-poor cells displayed metabolic compensatory changes that led to increased saturation of plasma membrane phospholipid acyl chains.[82] This kind of compensatory lipid metabolism has also been observed with sterol-requiring *Mycoplasma* variants.[28-30]

Evidence also shows, for example, that cholesterol or the cholesterol/phospholipid ratio of many lipid-containing viruses that infect and transform vertebrate cells is significantly higher than that of uninfected host cell plasma membranes.[83] This has been reported for RNA tumor viruses,[84] Semliki Forest virus,[85] and Sindbis virus,[86] among others.[83] Furthermore, Sindbis virus particle membranes were shown to possess a reduced fluidity compared with the host cell plasma membrane.[87] It is currently accepted that for viruses that bud through the plasma membrane, it is the host cell's membrane lipid composition that determines the lipid composition of the viral coat.[83,88]

A likely connection between the cholesterol content of viral and host cell membranes

mentioned above, and altered substrate transport kinetics in the virally infected host cell, is worth drawing here. A number of laboratories have shown that the rate of facilitative substrate transport across the cell membranes of virally transformed cells in culture is dramatically altered. Hatanaka et al.,[89] Isselbacher,[90] and others (Hatanaka,[91] Parnes and Isselbacher[92]) have obtained marked *increases* in the rates of amino acid, glucose, mannose, galactose, and glucosamine transport into virally transformed tumor cells. The glucose analog, 2-deoxyglucose, was carried with an apparent V_{max} of 83.5 (nmol/min/mg) in virally transformed murine sarcoma cells, compared to one of 10.2 for control cells.[93] Unfortunately, very few attempts to correlate enhanced transport activity with lipid compositional alterations that arise subsequent to viral infection have been performed on the same experimental systems. But, since lipid-containing viruses obtain their membrane lipid components from the host cell plasma membrane through which they bud,[88] the possibility has been raised that the budding process occurs on membrane domains of transformed cells that are relatively enriched with cholesterol, perhaps in mosaic-like "patches".[94] The contention that the process of virally directed tumorigenesis and aberrant membrane functions can be correlated with membrane cholesterol levels is at least supported by the few studies that have addressed the question. For example, Bailey et al.[93a] reported elevations in cholesterol synthesis of up to fivefold with SV-40 transformed human fibroblasts relative to control cells. This increased sterol synthesis corresponded with higher than normal levels of HMG CoA reductase (the rate-limiting enzyme in the sterol synthesis pathway) in these virus transformants.

Considered together, data on membrane cholesterol content, facilitative (as opposed to simple diffusive) substrate transport, and, on top of this, the complicating factor of viral infectivity and release associated with the propagation of many experimental tumors, introduce additional puzzling elements that bear investigation. Data such as these lead to intriguing questions about the spatial disposition and content of cholesterol in membranes of virally transformed (and malignant) cells. Do virally infected and transformed cells display distinctive and altered plasma membrane cholesterol levels or domains relative to normal cells? If so, are these differences consistently higher or lower?

Under normal circumstances a particular biomembrane of a given cell type contains a rather narrowly specified cholesterol content commensurate with the functions performed by that membrane (Table 1). The inner mitochondrial membrane, for example, is cholesterol poor relative to the plasma membrane, and the different activities of both membranes may be said to be reasonably correlative, in this sense, with their cholesterol levels. Available information on the functional properties of both model system and biomembranes with respect to their lipid composition seems to indicate that either too much or too little membrane cholesterol may perturb a functional homeostasis insofar as the bioregulatory properties controlled by membranes are concerned.

IV. CELLULAR RESPONSES TO DIFFERENTIAL MEMBRANE CHOLESTEROL CONTENT

All cells in an organism respond to their "history" of prior environmental stimuli by means of the regulatory functions mediated, first, by the activities of the plasma membrane. Intracellular responses to this environmental history then proceed to establish the metabolic phenotype of the cell, which, in turn, defines its growth characteristics. With biologically relevant experimental systems, it has become clear that membrane cholesterol exerts its effects on membrane structure in a manner that is reflected by the patterns of cell metabolism, growth, and division.

Should an externally derived stimulus elicit, among other changes, a qualitative and quantitative redistribution of cholesterol deposition into cellular membranes, then

according to the evidence from studies with natural biomembranes and lipid mono- and bilayer model systems discussed above, the ensuing metabolic profile of the cell will manifest patterns that may be causally traced back to the altered cholesterol (or to the cholesterol/phospholipid) content of its membranes. Such functional alterations may be attributable to the effects exerted by the cholesterol molecule on the physical properties of the membrane bilayer, as discussed previously. The data of Silbert's group[80] constitute merely one elegant example of how metabolically pertinent membrane functions may be manipulated by, in this case, the plasma membrane cholesterol content of viable cells.

A. Sterol Availability as a Prerequisite for Cell Proliferation

The dependence of cell growth on the presence of membrane cholesterol arises from two facts. First, inasmuch as cholesterol is a primary lipid constituent of the plasma and most intracellular membranes of eucaryotic cells, the additional membrane material that enables cell growth and division depends on the ready availability of an adequate supply of the sterol. Second, as mentioned above, the increasing body of evidence indicates that some discrete level or content threshold of cholesterol appears to be an absolute requirement for eucaryotic membrane functional integrity. Since diverse cellular membranes display equally diverse functions, we can expect that the cholesterol levels of particular cell membranes would be as varied as the functions they govern. Consequently, it also may be expected that a significant variation in the normal cholesterol level for a particular membrane would indeed elicit changes in a number of membrane characteristics, including fluidity, ion permeability, substrate transport, and activities or affinities of membrane-bound enzymes and receptors. And it may be via these changes in membrane cholesterol levels that ensuing functional alterations in membrane-associated activity can contribute to the regulation of cell growth, division and even proliferation.

In this regard, data which correlate membrane cholesterol levels with characteristics of tumor metabolism and the dynamics of cell growth and proliferation are appearing with increasing frequency. Such studies at the cellular level, however, usually do not focus upon the functional distinctions between discrete subcellular membrane fractions and cell membranes *in toto*. Much of the work concentrates on the plasma membrane fraction, inasmuch as the plasma membrane is both the cell's first sensor to and interface with the external milieu.

All cell cultures, regardless of cell type, have been observed to synthesize cholesterol *de novo* when they are grown in sterol-depleted culture medium. Extensive work, particularly by Chen, Heiniger, and Kandutsch, has established the imperative nature of cholesterol's presence for cell proliferation in culture, or for that matter in whole animal studies, either via supplementation of the experimental system, or via *de novo* synthesis. In studies with mice, constantly proliferating cells appeared to synthesize cholesterol at relatively high rates, whereas nonproliferating cell populations did not.[95] The addition to the diet of specific inhibitors of cholesterol synthesis was observed to suppress the growth of young mice. Nevertheless, whole animal studies have been declared to be ambiguous, since the regulation of sterol synthesis at the molecular level is difficult to establish with such systems. In tissue culture, however, a variety of cholesterol synthesis inhibitors have been shown to have explicit effects on some of the molecular events associated with cell growth dynamics. Kandutsch and Chen[96] observed that the specific inhibition of sterol synthesis in L-cells grown in vitro resulted in a progressive decline in the rate of DNA synthesis, reaching zero after about 3.5 days. Concomitantly, the membrane sterol concentration of the cells dropped to approximately half that of control, and the cells ultimately died. Addition of either mevalonate or cholesterol to the culture medium

reversed these effects. In a manner reflecting the transport results obtained by Silbert's group with the sterol auxotrophic variants,[80] Rb^+ transport kinetics were altered in the sterol-depleted L-cells, and endocytosis activity ceased.[97,98]

Previously, Chen et al.[99] had demonstrated that *de novo* cholesterol biosynthesis in fact *precedes* that of DNA by some 22 hr in phytohemagglutinin-stimulated mouse lymphocyte cultures, a model system for the study of growth control in cells. Moreover, it was observed that specific inhibitors of cholesterol synthesis added to the cultures abolished DNA synthesis. This repression of blastogenesis appeared to occur only when the inhibitors were added early enough to prevent sterol synthesis from achieving its peak, as measured by both thymidine and acetate incorporation.

In light of these results, a recent review by Chen et al.[100] proposed that since sterol synthesis or acquisition seems to be imperative for normal cell proliferation in culture, excessive *de novo* sterol synthesis may be capable of pushing cell populations into anomalous proliferative activity. This argument establishes, then, a specific molecular correlation with a major phenotypic characteristic of malignant cell growth: the deregulated dynamics of cell division and the cellular cholesterol level.

The proposition of Chen et al.[100] receives peripheral support from the studies of Sinensky[101] who obtained a variant Chinese hamster ovary cell line resistant to killing by the cholesterol synthesis inhibitor 25-hydroxycholesterol in cholesterol-depleted culture medium. Sinensky's variant is defective in the negative feedback regulation of sterol synthesis by supplements of cholesterol itself, 25-hydroxycholesterol, and sterol-rich lipoproteins.[102] These cells continue to synthesize sterol in the presence of inhibitors of cholesterol synthesis, and in delipidated growth medium they display cell generation times in the vicinity of 3 to 4 days. When cholesterol is added as a supplement to the culture medium (~ 5 $\mu g/m\ell$) the variant cells continue to synthesize sterol, thereby elevating their cellular cholesterol content. The result of such elevated sterol content was shown to be the two- to fivefold reduction in cell generation time; that is, increased rates of cellular proliferation.[103]

If aspects of sterol biosynthesis are, in fact, directly involved with cellular proliferation, then there might be an expected influence of one or more specific intermediates in the cholesterol synthesis pathway on DNA synthesis, because the replication of DNA is clearly the central feature associated with proliferating cells. Recently, Siperstein's laboratory[103a] has reported that the activity of the rate-limiting enzyme in the sterol synthesis pathway, HMG CoA reductase, increases at or just prior to the rise in DNA synthesis during the S-phase of the normal BHK-21 cell cycle. Two observations from this study imply that the product of the HMG CoA reductase reaction, mevalonate (or a mevalonate-derived metabolite), effectively and specifically regulates DNA synthesis, and therefore, the proliferation of these cells in culture. First, the reductase inhibitor compactin[103b,103c] completely blocked *de novo* DNA synthesis. Second, the compactin-induced inhibition of S-phase DNA synthesis could be completely reversed by the addition of mevalonate. A most intriguing observation was the fact that large mevalonate additions to compactin-inhibited cultures promoted DNA synthesis to levels higher than that of the control cell population.

B. Rate of Synthesis and Membrane Cholesterol Levels in Tumor Cells

Animals that bear tumors of diverse origin manifest marked hyperlipidemia,[104] and many exhibit dramatic increases in circulating plasma cholesterol.[106,106a] Yet, any generalization as to the cholesterol "load" in tumor-burdened hosts must be entertained with caution, and it is imperative that the circulating plasma cholesterol levels be distinguished from the tumor membrane cholesterol content. While this distinction would appear to be simple in principle, data on circulating plasma cholesterol levels in

experimental animals and in humans carrying tumors are disappointingly inconsistent. To dramatize this inconsistency and the unreliability of employing circulating sterol levels as an indicator of tumorigenesis, consider the recently reported, large scale clinical study of 3000 individuals who were screened for 13 years in an attempt to correlate serum cholesterol with the propensity to develop cancer.[106b] The results showed that individuals who developed cancer within 1 year had a *higher* initial serum cholesterol level, while those whose cancer appeared between 1 and 6 years displayed lower circulating cholesterol profiles. On the other hand, the lipid composition of tumor cell membranes consistently manifests an enrichment in lipids over those from normal cells, with higher cholesterol levels often being the most apparently enriched lipid component.[104,107,108a]

The plasma membrane fraction of hepatic tumors indirectly indicates general lipid enrichment by displaying lower isopycnic densities (~1.12 to 1.16 g/cc) in sucrose gradients, compared with equivalent membrane fractions from normal liver (~1.16 to 1.18 g/cc).[108] Where rates of cholesterol synthesis have been examined in such tumors, they have been found to be substantially increased. This phenomenon holds for human leukemias,[109] guinea pig leukemia L2,[110,111] murine leukemias,[112,113] and rat hepatomas.[106,114] The rate of sterol synthesis from acetate is dramatically increased in Morris hepatomas 9121 and 7787, relative to that observed in host liver preparations.[115] Kandutsch and Hancock[116] similarly noted an enhancement in the rate of sterol synthesis in spontaneous murine hepatomas. The phenomenon has obtained further support from Snyder[117] and from Wood[118] whose laboratories have observed increased total cholesterol levels and cholesterol/phospholipid ratios in both slow and rapidly proliferating Morris hepatomas, compared to normal liver. These and other like data are reviewed and referenced by Chen et al.[100]

Additional results of a similar nature include cholesterol/phospholipid analyses on plasma membrane isolates from normal as well as SV-40 transformed hamster lymphocytes. Employing N_2 cavitation disruption techniques, thereby providing cleaner plasma membrane fractions in higher yield, Schmidt-Ullrich et al.[119] documented a 54% increase in the cholesterol/phospholipid ratio of SV-40 transformed cell membranes. Further studies[120] establish the cholesterol enrichment of at least the plasma membranes of virally transformed cells in culture, and call to mind altered transport kinetics observed with such infected cells, as reviewed by Hatanaka[91] and Parnes and Isselbacher.[92]

Therefore, certain generalizations regarding the cellular presence of cholesterol, especially in tumors, may be drawn from these accumulated and diverse data. They may be summarized as follows:

1. Cholesterol is an obligatory, not an ancillary component of the membranes of all viable mammalian cells.
2. The relative cholesterol content of cell membranes varies with the functional role served by the particular membrane and becomes, to some degree, a distinguishing hallmark of the particular membrane type.
3. The relative cholesterol content of a specific membrane type cannot be significantly changed without affecting at least some of the metabolic functions served by that membrane.
4. Variation in the relative cholesterol content of cell membranes alters the phenotypic characteristics of cells insofar as maintenance of viability and the capacity to proliferate.
5. Cellular sterol synthesis appears to precede DNA synthesis in cell populations stimulated to divide in vitro.
6. Tumor cells appear to synthesize sterols (as well as fatty acyl moieties[121,122]) more

rapidly than do their normal cell counterparts, and their membranes often indicate an enrichment in cholesterol, either in absolute terms or relative to membrane phospholipid levels.

Recognition of the relationships between cellular sterol synthesis, membrane sterol content and function, and the dynamics of cellular growth and proliferation emphasize two comprehensive questions that are worthy of detailed scrutiny, especially with regard to neoplastic development. First, what are the mechanisms by which *de novo* sterol synthesis is known to be regulated? Second, if aberrant regulation of sterol synthesis arises in a population of cells, what are the metabolic ramifications of such deregulation, and what are its consequences for cellular growth and division?

V. REGULATION OF CHOLESTEROL BIOSYNTHESIS

A. Control Mechanisms in Normal Cells

The two sources of cholesterol available to the animal, and therefore to the tumor it bears, are via the diet and via *de novo* synthesis in the cell. In higher vertebrates, cholesterol synthesis is performed chiefly by the liver, with lesser contributions provided by the adrenal cortex, skin, intestine, and some other tissues. The *de novo* biosynthesis of cholesterol, and of fatty acid moieties that comprise the acyl chains of membrane polar lipids, begins with acetyl CoA. Every carbon of the cholesterol molecule is derived from the acetate residue. Of the more than 30 or so discrete, enzyme-catalyzed steps in the cholesterol pathway, the first half of these involve soluble cytoplasmic enzymes. There is one notable exception. The third reaction in the biosynthetic pathway is the conversion of (S)-hydroxymethylglutaryl-CoA (HMG CoA) to (R)-mevalonic acid, and takes place on the endoplasmic reticulum catalyzed by the membrane-bound enzyme HMG CoA reductase (mevalonate:NADP oxidoreductase, EC 1.1.1.34). Later reactions in the pathway, from squalene to cholesterol, are catalyzed by a complex sequence of endoplasmic reticulum-associated enzymes, principally because of the aqueous insolubility of pathway intermediates starting with the polyisoprenoid squalene.[123,124]

In mammals (e.g., mouse, rat, man) the rate of *de novo* synthesis in the liver is under strict regulation by the amount of dietary cholesterol absorbed by the intestine.[114,125,126] The predominant source of circulating serum cholesterol appears to arise as a result of cholesterol synthesis in the liver, and this tissue exhibits the most dramatic sensitivity to dietary negative feedback control over cholesterol synthesis. But tight regulation is also observed in every cell capable of sterol synthesis.[127-130] It therefore must be borne in mind that *every* nucleated somatic cell, in addition to the liver hepatocyte, possesses the genomic information required for the regulated *de novo* synthesis of cholesterol, regardless of whether or not the particular tissue expresses it. Thus, in the guinea pig, Swann, Wiley, and Siperstein reported that, in fact, every tissue manifesting significant cholesterol synthesis appears to be susceptible to feedback regulation by diet manipulation.[131] And in this respect it is interesting that guinea pig serum contains almost exclusively low density lipoproteins.[132]

It is well established that the reaction catalyzed by HMG CoA reductase is the principal rate-limiting step in cholesterol biosynthesis for all tissues that have been examined (see Rodwell et al.[133]). Pure cholesterol, oxygenated derivatives of cholesterol, lipoproteins, and intracellular cytosolic proteins (e.g., the "sterol carrier protein" of Scallen et al.[134]), all have been proposed to act, in a manner not fully understood, as modulators of this enzyme's activity.[96,135-138] Some of the proposed mechanisms of regulation of reductase activity invoke changes that could occur in its immediate environment in the membrane; this microenvironment is conceived as being directly

perturbed by one or more of these agents.[139] Short-term regulation of enzyme activity, and consequently of cholesterol synthesis, may also occur in vivo upon ATP-mediated phosphorylation and dephosphorylation of HMG CoA reductase.[140-142]

In addition to such fine-tuned control of reductase activity, longer-term regulation of cholesterol synthesis occurs according to a more involved set of principles. The activity of HMG CoA reductase is known to undergo a diurnal rhythm, peaking during the dark cycle (see Rodwell et al.[133] for details). This diurnal rhythm in the tissue activity of reductase correlates with a fluctuation both in enzyme synthesis and cholesterol synthesis.[143] Protein synthesis inhibitors eliminate this diurnal fluctuation in reductase activity,[144] which, under normal circumstances, may be as much as tenfold in the rodent.[145] The other side of this issue, the possible cyclic fluctuation in proteolytic degradation of the reductase as a contributor to the diurnal enzyme activity, is unclear. However, the in vivo half-life estimate of the liver enzyme is believed to be fairly short, perhaps 3 hr.[146]

Over and above the rhythmic cycle in reductase activity during a 24-hr period, cholesterol synthesis in the liver, which correlates directly with the activity of the enzyme, responds dramatically to dietary cholesterol feeding. Within 4 hr of feeding a diet containing 5% cholesterol to rats, the activity of the reductase begins to fall precipitously.[133] Dietschy and Brown[147] have demonstrated that cholesterol synthesis and reductase activity in the liver can be varied by more than 100-fold with cholesterol manipulation of the rat's diet. This responsiveness to dietary addition of cholesterol is also preserved in cultured fibroblast systems[148] and to some extent probably in all other cells that normally manifest cholesterol synthesis in vivo.[131,135a]

B. Loss of Regulation of Synthesis as an Early Event in Tumorigenesis

In tumor cells, however, there appears to be quite a different situation. The expected dietary feedback control over *de novo* cholesterol synthesis (and, in certain cases, fatty acid synthesis as well[299]) has been shown to be lost in many of the experimental tumors that have been investigated. The phenomenon has been demonstrated in hepatomas, precancerous liver, and leukemias.[3,32,100,149] This loss of lipid synthesis regulation, particularly with respect to cholesterol, appears to be a far more consistent characteristic of experimental hepatomas than does the propensity for enhanced glycolysis.[150] In fact, the normal degree of feedback regulation of cholesterol biosynthesis is reported as absent, in every hepatoma so far investigated (more than twenty), including those of rodents, fish, and man.[32,150,151]

On the other hand, tissue culture studies on the feedback control of cholesterol synthesis have afforded mixed results, such that certain culture systems retain while others lose this aspect of regulation over cholesterogenesis. Bailey et al.[93a] observed that although SV-40 transformed human fibroblasts displayed a dramatic deregulation in the rate of cholesterol synthesis, these tumor cells retained effective feedback control over HMG CoA reductase activity when serum lipoproteins (e.g., LDL) were added to the system. The authors witnessed an essentially similar binding of ^{125}I-labeled LDL to both tumor and normal fibroblasts, and concluded that a lesion in some control locus other than the membrane LDL receptor feedback loop[135] is responsible for enhanced cholesterol synthesis in the virus-derived tumors.

In vivo, this loss of feedback control over cholesterol synthesis has been demonstrated to occur within *2 weeks* after dietary treatment of rats with hepatic carcinogens.[149] Sabine noted that the loss of cholesterol synthesis regulation appeared months prior to any physical manifestation of the presence of the tumor. Yet, noncarcinogenic hepatotoxins were found to have no effect on the dietary feedback control of cholesterol biosynthesis. In addition to phenomena involving sterol synthesis, Perkins and Kummerow[152]

observed significant increases in the levels of phosphatidylethanolamine and phosphatidylinositol of rat liver plasma membranes subsequent to only 2 weeks of feeding the animal the potent hepatocarcinogen N-2-fluorenylacetamide. Concomitantly, the cholesterol content of these liver plasma membranes was found to be 1.3 times that of control liver plasma membranes. Such data imply that aberrations in the regulation of general lipid synthesis in the liver may be considered among the primary events in the normal to neoplastic cell transformation sequence in vivo.[32] The liver, as the principal site of cholesterol synthesis, normally maintains a sensitive metabolic homeostasis, which together with growth control, is discernably upset in rats after only 2 weeks of dietary supplementation with the carcinogen diethylnitrosamine.[153]

This interplay, in vivo, between the dietary manipulation of lipid intake and tumorigenesis highlights some rather interesting parallels, especially with regard to tumor promotion.[154] Rats fed a high fat diet along with hepatocarcinogens manifested a greater primary tumor incidence than did rats fed the carcinogens mixed with a normal rat chow diet.[155] Further, cholesterol was found to be a potent dietary cocarcinogen for the induction of colon cancer by dimethylhydrazine in rats.[156] Even when not exposed to carcinogen feeding, mice maintained on high fat diets develop more spontaneous primary gastrointestinal[157] and mammary[158] tumors than controls. And in humans there appears to be a substantial correlation between breast cancer mortality rates and total dietary fat intake.[159]

On the one hand, certain data suggest that the enhanced rate of sterol synthesis in particular tumors might possibly reflect an increase in total lipid synthesis overall, since cholesterol/free fatty acid ratios appear to remain relatively constant in lymphoid tumor tissues,[160] even when the absolute lipid content differs from normal. Yet, in studies with other tumors, it is the membrane sterol enrichment that stands out. For example, Hilf et al.[161] found the free cholesterol content of a large number of human breast carcinomas to be twofold greater, and the cholesterol ester content more than threefold greater than normal breast tissue.

In the absence of tight control over cholesterol synthesis that has been observed to occur so shortly after dietary introduction of hepatocarcinogens and so long prior to the histological manifestation of the primary tumor mass, the accumulation of cholesterol in precancerous cellular membranes over time could encourage alterations in the normal patterns of metabolism. As was already indicated, isolated membrane fractions derived from a variety of tumors display altered cholesterol compositions.[107,162-165] It is therefore clear that the early loss of control over cholesterol synthesis in a variety of tumors is a fairly common phenotypic hallmark of the precancerous state. All indications point to the high probability of the accumulation of membrane cholesterol during maturation of the tumor may assume critical importance to the process of tumorigenesis and to diverse metabolic characteristics of neoplastic cells.[166]

VI. MOLECULAR RAMIFICATIONS AT THE METABOLIC LEVEL

A. Tumor Cell Mitochondria

With regard to questions on the altered cell metabolism of tumors, the role of specific intracellular membranes is clearly defined. Mitochondrial membranes of tumor cells appear to be especially pertinent in this context. Not only do these organelles constitute, functionally, the unique bioenergetic locus of aerobic eucaryotic cells, but mitochondria participate directly in a large number of other vital metabolic processes that rely on regulated metabolite flux between the cytoplasmic milieu and the mitochondrial matrix. The inner mitochondrial membrane, due to its highly infolded structure, possesses a great surface area and constitutes the largest component of the total mass of cell membranes,

specifically in liver and heart tissues. Because the inner mitochondrial membrane behaves as one of the most metabolically important permselective barriers, alterations in its lipid composition may be assumed to perturb a host of cellular metabolic patterns. As reviewed by Pedersen[150] and others, tumor mitochondria have been found to possess considerably more cholesterol than do normal mitochondria in experimental systems studied to date. The proposals for altered metabolic patterns in tumors, which we shall elaborate upon shortly, are predicated on this phenomenon.

Although the lipid composition of diverse tumor mitochondrial membranes has not been exhaustively investigated, those studies that have been performed indicate that the lipid content of these organelles is substantially altered from what is found in normal mitochondria.[164,165,167-169] For example, Feo et al.[164] found that mitochondria from serially transplanted hepatomas AH 130, 3924A, and 5123 possessed elevated cholesterol/phospholipid ratios compared to normal liver mitochondria. In hepatoma AH 130, the cholesterol content of the inner membrane was consistently about fourfold higher. Relative to normal mitochondria, the phospholipid content of rat hepatoma 5123 decreased, remained about the same in hepatoma AH 130, and was dramatically increased in mitochondria from hepatoma 7777.[169] In general, it would appear that those hepatoma mitochondrial membranes that have been examined exhibited both a higher total cholesterol content as well as a variation in the total amount and kind of phospholipid.

Tumor mitochondrial membrane enzyme activity has been shown to undergo readily observable changes as a function of the cholesterol content of their membranes. With mitochondria isolated from Morris hepatoma 7777, Morton et al.[163] correlated changes in energy-linked reactions with observed alterations in membrane lipid composition. Kaschnitz et al.,[170] studying mitochondria from the same rat tumor line, 7777, documented shifts in enzymatic activity which correlated with tumor growth rate. It is noteworthy that hepatoma 7777 tissue has been shown independently to possess a generally enriched cell membrane cholesterol composition[163] and, furthermore, to display enhanced rates of cholesterol biosynthesis relative to normal liver.[106]

The role played by variable cholesterol content in perturbing mitochondrial membrane function is worth considering, and has been investigated by Graham and Green,[171] who exogenously enriched isolated normal rat liver mitochondria with cholesterol in vitro via coincubation with cholesterol-rich serum lipoproteins. They reported a correlation between the amount of cholesterol incorporated into the mitochondrial inner membrane and an increase in succinate dehydrogenase activity. Parenthetically, it is interesting that independent work subsequently has demonstrated that tumor mitochondrial succinate dehydrogenase activity varies directly with the degree of tumor malignancy.[172]

As was mentioned previously (Table 1), the inner mitochondrial membrane normally is cholesterol poor. Yet recent preliminary reports indicate that upon moderate cholesterol enrichment in vitro, the rate of electron transfer from NADH to cytochrome c is stimulated.[173] It has also been observed that, compared with control preparations, liver mitochondria from rats maintained on a diet enriched with cholesterol exhibited increases of two- to threefold in cholesterol concentration, as well as increases in the time for which a given load of exogenous Ca^{2+} is retained in the matrix.[174] These, and other data demonstrate that experimental manipulation of the cholesterol content of mitochondrial membranes can modulate lateral, diffusion-limited steps that are conceived to occur between the bioenergetically functional redox components of the inner membrane, and by extension, the activity of the ATP utilizing components as well.[175]

With regard to this last observation, other mitochondrial studies that are indirectly

indicative of inner membrane cholesterol enrichment in tumors have illustrated that with mitochondria from Morris hepatoma 5123C an Arrhenius plot for ADP translocation kinetics displayed no breaks between 0 to 30°, whereas with host mitochondria, a distinct phase transition was observed at about 6°.[176] Paralleling these observations, Senior et al. reported that ADP uptake rates increased in mitochondria isolated from tumors.[177] Alteration of adenine nucleotide exchange transport kinetics by mitochondrial membrane lipid modification, together with other similar data, has led to the proposal that oxidative phosphorylation is regulated, in part, by the immediate lipid environment of the adenine nucleotide carrier and other anion translocators of the inner membrane (see Vignais for review).[178] It was previously pointed out by Feo et al.[164] that significant correlations exist between mitochondrial membrane cholesterol, decreased hypoosmotic swelling amplitudes that accompany anion transport (Graham and Green[171]), and energy-linked conformational changes. Most significantly, Feo et al.[164] reported that different rat hepatoma lines exhibited elevated cholesterol levels ranging between 3.9 to 6.2 times that of normal liver mitochondria.

B. Further Parallels between Tumor and Cholesterol-Enriched Normal Mitochondrial Function

This laboratory has uncovered some interesting findings regarding the apparently direct effects of mitochondrial membrane cholesterol levels on some general parameters of oxidative phosphorylation. We have been able to enrich normal rat liver mitochondria with cholesterol in vitro in a rapid and controlled fashion, by incubating the organelles with cholesterol-coated Sephadex® beads[179] (Figure 2). These cholesterol-enriched normal mitochondria carried out oxidative phosphorylation efficiently, as indicated by ADP/O ratios that remained essentially unchanged from controls. Recently, Parks and McLean-Bowen reported similarly undisturbed basic, general functions with mitochondria derived from several sterol mutants of yeast that possessed variable sterol compositions.[175] Further studies with the cholesterol-enriched rat liver mitochondria, however, indicated that select activities differed from normal, and in fact, mirrored those changes that had been observed with mitochondria from tumor tissue. The respiratory (acceptor) control ratios with succinate, for example, were consistently lower, by as much as 36%, compared to control mitochondrial preparations. Latent ATPase activity increased two- to threefold as a function of the extent of cholesterol enrichment, and at low concentrations of dinitrophenol (20 μM), the uncoupler-stimulated ATPase activity appeared to be significantly enhanced by high levels of membrane cholesterol (Table 2).

Meanwhile, many other laboratories have noted a variability, usually a substantial reduction, in the respiratory (acceptor) control ratios of mitochondria from different hepatomas.[150,164,170,180-182] In addition, the work of Kaschnitz et al.,[170] referred to above, reported that the latent ATPase activity of mitochondria from a variety of hepatomas was between 2 to 3 times that of normal mitochondria. It would appear, then, that our exogenous cholesterol enrichment of normal mitochondrial membranes gave rise to some of the same functional properties noted with hepatoma mitochondria.

The data of Table 2 are further supported by some more recent observations from our laboratory with Morris hepatomas 3924A and 16 (Table 3). Tumor 16 is a slow growing, "minimally deviated" hepatoma, displaying many superficial characteristics that are similar to normal or host liver, except for its uncontested malignancy. Tumor 3924A is a very rapidly proliferating, highly malignant tumor which does not resemble liver in any histologically obvious way. The data of Table 3 illustrate two experimental facts. First, both tumor mitochondrial preparations demonstrate a dramatic reduction in the respiratory (acceptor) control ratios with succinate or glutamate as substrate. Second, we found that mitochondria from tumor 3924A contained about 6.8 times the amount of

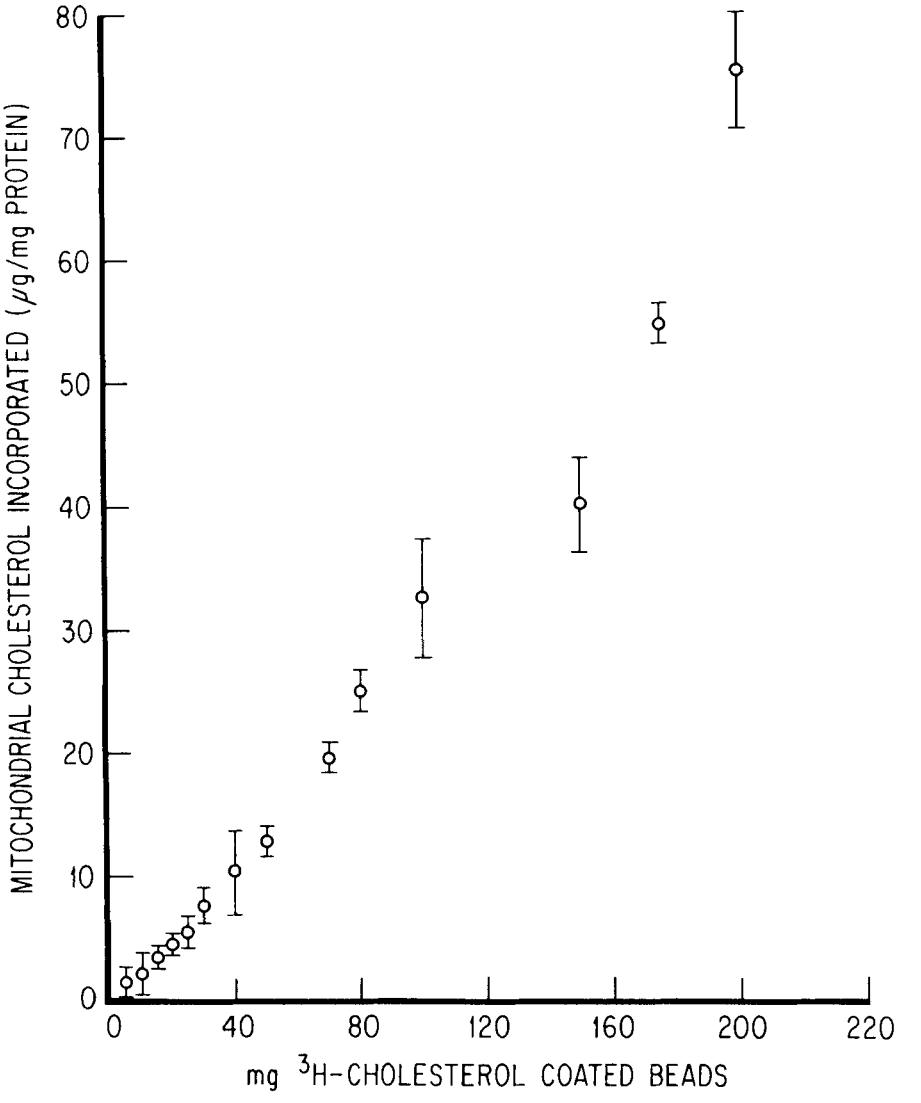


FIGURE 2. Cholesterol enrichment of isolated mitochondria. Mitochondrial suspensions (30 to 40 mg protein/ mℓ) were added to 25 mℓ erlenmeyer flasks containing 0 to 200 mg of benzene-washed "nude" Sephadex® G-10 beads or [^{3}H]-cholesterol-coated beads, and incubated (0°, 15 min) with very gentle shaking in a reciprocal shaker. The beads were separated from the suspension by low speed centrifugation over a 60% buffered sucrose cushion. Aliquots of the resulting cholesterol-enriched mitochondria were assayed for cholesterol incorporation by scintillation counting and checked via a colorimetric assay method.[298] The vertical bars represent the standard deviation of the mean for assays in triplicate.

membrane cholesterol compared with control ACI rat liver mitochondria, while the mitochondria from tumor 16 had 3.5 times the total cholesterol of its control liver (Buffalo rat) preparation.

The question that arises immediately is whether the fundamental similarities observed with hepatoma mitochondria and with normal cholesterol-enriched mitochondria are fortuitous, or whether the observed phenomena indicate a basic, functional, membrane-related aberration caused by excessive mitochondrial membrane cholesterol. At the

Table 2
EFFECTS OF INCORPORATED CHOLESTEROL ON RESPIRATORY ACTIVITY IN RAT LIVER MITOCHONDRIA

Parameter	Basal level[a]	Enrichment level I[b]	Enrichment level II[c]
Cholesterol content nmol/mg protein	26.2 ± 0.3 (5)	189.6 ± 17.8 (4)	336.3 ± 9.3 (4)
ADP:O ratio	1.81 ± 0.13 (16)	1.80 ± 0.09 (16)	1.71 ± 0.14 (16)
Respiratory control ratio	4.10 ± 0.18 (16)	3.72 ± 0.14 (16)	2.61 ± 0.13 (16)
ATPase activity[d] nmol P_i/min/mg protein	3.96 ± 0.22 (4)	5.63 ± 0.27 (4)	9.38 ± 0.43 (4)
ATPase activity in presence of 20 μM 2,4-DNP[e]	42.7 ± 2.4 (3)	73.1 ± 3.2 (4)	125.2 ± 5.7 (3)

Note: Data are expressed ± standard deviation. Numbers in parentheses indicate number of individual mitochondrial preparations. Each preparation was assayed at least twice for each experimental condition.

[a] Mitochondria from Long-Evans rat liver were incubated with 250 mg benzene-washed dextran beads (Sephadex® G-10);
[b] 250 mg cholesterol-coated dextran beads;
[c] 500 mg cholesterol-coated dextran beads, as described by Coleman et al.[179]
[d] Data are for intact mitochondrial ATPase in the absence of added Mg^{2+}. Comparison of ATPase ± Mg^{2+} allowed estimation of damaged mitochondria (<8%). Latent ATPase determined after 24 min.
[e] ATPase activity determined after 8 min with 20 μM 2.4-dinitrophenol. Other conditions as previously described.[179]

Table 3
RESPIRATION-LINKED ACTIVITIES OF HEPATOMA 16 AND 3924A MITOCHONDRIA: CORRELATIONS WITH MEMBRANE CHOLESTEROL CONTENT

Mitochondrial source	Substrate	ADP:O ratio	RCR[a]	Total cholesterol[b] (μg/mg protein)
Control (Buffalo) liver	Succinate	1.7	7.1(4)	7.6 ± 1.2
Control (ACI) liver	Succinate	1.8	7.0(3)	5.9 ± 0.3
Host (Buffalo) liver	Succinate	1.8	7.2(2)	6.5 ± 2.2
	Glutamate	2.7	7.5(2)	
Host (ACI) liver	Succinate	1.8	6.7(3)	5.4 ± 1.6
	Glutamate	2.9	6.6(3)	
Tumor 16 (Buffalo)	Succinate	1.6	3.3(3)	26.8 ± 9.7
	Glutamate	2.3	2.7(3)	
Tumor 3924A (ACI)	Succinate	1.8	3.6(4)	40.4 ± 10.1
	Glutamate	2.6	4.7(3)	

[a] RCR, respiratory (acceptor) control ratio. Values in parentheses indicate individual number of mitochondrial preparations, each of which was assayed in triplicate.
[b] Cholesterol was assayed colorimetrically by a procedure modified from Webster.[298] Data are reported ± standard deviation; N = 5 (Tumor 3924A) and N = 4 (Tumor 16), assayed in triplicate. Conditions for mitochondrial isolation included a BSA-medium containing 220 mM mannitol, 70 mM sucrose, and 2 mM N-2-hydroxyethyl-piperazine-N'-ethane sulfonate, pH 7.4. Other conditions or procedures were according to Coleman et al.[179]

present time, we feel that conclusions drawn from the existing data must be offered with strong caveats until more robust experiments can confirm, clarify, and extend these findings. Such experiments have been ongoing in the laboratory of P. S. Coleman, and it is of interest to point out that recent data from these studies not only corroborate but extend the above findings to aspects discussed below. These results will be communicated elsewhere shortly.

VII. CORRELATIONS AT THE MITOCHONDRIAL/CYTOPLASMIC INTERFACE: A PROPOSAL ON THE INTERMEDIARY METABOLISM OF TUMORS

A. Supplying Acetate for Enhanced Sterol Synthesis in Tumors

The documented early loss of control over cholesterol synthesis, the subsequent enrichment of mitochondrial and other cellular membranes with cholesterol, and the altered features of various plasma membrane and mitochondrial functions all may be interrelated phenomena in experimental hepatomas as well as in other tumors. One manifestation of these changes, which has far reaching implications for recognizing the establishment of a neoplastic metabolic phenotype, could involve an altered pattern of mitochondrial anion exchange transport between cytosol and matrix.

Since all of the skeletal carbons of cholesterol are derived from acetyl CoA, as are the fatty acid moieties of polar lipids, the decontrol of sterol biosynthesis in tumors would require elevated turnover of cytoplasmic acetyl CoA as synthetic precursor.[300] Acetyl CoA is metabolically generated via numerous biochemical conversions (oxidative decarboxylation of pyruvate, β-oxidation of long chain fatty acids, or oxidative deamination of some amino acids, mainly via the pyruvate junction) which occur almost exclusively within mitochondria, not in the cytosol.[183] Since acetyl CoA cannot passively diffuse across the inner mitochondrial membrane, it reaches the cytoplasm disguised as citrate. Thus, mitochondrial acetyl CoA must first be converted to citrate, upon which it may either serve as fuel for cellular bioenergetics via the tricarboxylic acid (TCA) cycle, a catabolic pattern; or in support of anabolism, it may be specifically transported out to the cytosol on the inner membrane's tricarboxylate carrier, in exchange for incoming malate. Once citrate appears in the cytoplasm, it is cleaved by the enzyme ATP-citrate lyase (EC 4.1.3.8) to yield acetyl CoA and oxaloacetate,[184,185] the latter being reduced to malate which reenters the mitochondrion in exchange for another mole of exiting citrate. In this compartmentalized fashion, mitochondrial acetyl CoA becomes cytoplasmic acetyl CoA, where it supplies the precursor acetate fragments necessary for sterol and fatty acid synthesis.[185,186]

Coincident with and independent of cleavage to acetyl CoA, cytoplasmic citrate also acts as a positive modulator of the activity of acetyl CoA carboxylase, the rate-limiting enzyme in the fatty acid synthesis pathway.[186] Furthermore, cytoplasmic citrate exerts some control over glycolysis by allosterically inhibiting the enzyme phosphofructokinase.[187] Within the mitochondrial matrix, citrate has been reported to reduce pyruvate dehydrogenase activity.[188]

By altering the distribution of required substrates, such as citrate, between mitochondria and cytoplasm, in concert with cytoplasmic utilization of citrate carbons to support enhanced sterol biosynthesis, a shift in the patterns of respiration would ensue.[189–191] Such metabolite rerouting would draw upon an altered participation of both mitochondrial and cytosolic compartments compared with the ordinary modes of respiratory substrate flux through the TCA cycle. In other words, if citrate is considered to traditionally serve as a catabolic TCA cycle intermediate in normal cells, one can conceive of tumor mitochondria supplying increasing amounts of citrate from matrix to

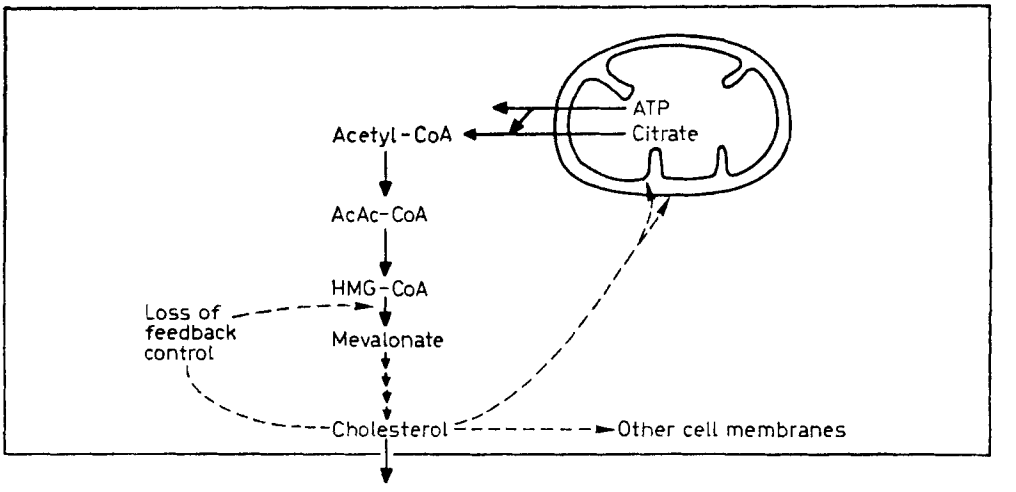


FIGURE 3. How mitochondrial acetyl CoA is supplied to the cytosol for cholesterol synthesis. (Adapted from Pedersen, P. L., *Progress in Experimental Tumor Research*, Vol. 22, Wallach, D. F. H., Ed., S. Karger, Basel, 1978, 210. With permission.)

cytoplasm, thereby providing the metabolic source for long term deregulation of sterol synthesis and the accumulation of sterol in tumor cell membranes (Figure 3).

B. Replacement of Glucose by Glutamate as Principal Respiratory Substrate

In order that increasing amounts of mitochondrial citrate be provided to the cytoplasm as a molecular ancestor to sterol accumulation in tumors, the tricarboxylate (citrate) carrier of mitochondria might be expected to exhibit properties specific to this task of more effective citrate export. Indeed, Halperin et al.[191] reported the K_m of the citrate transporter of hepatoma 7777 to be much lower ($<75\ \mu M$) than that of normal host liver mitochondria ($250\ \mu M$). It will be recalled that mitochondria from hepatoma 7777 are cholesterol rich, and that this tumor displays enhanced rates of sterol synthesis.[106,163] Whether the tricarboxylate carrier activity of other tumor mitochondria behaves similarly awaits further experimental clarification.

If one postulates the diversion of citrate carbons toward lipid biosynthesis, then the question arises as to the identity of an alternative and primary respiratory fuel for oxidative phosphorylation in tumors. One of the cell's principal molecular fuels is glucose, which, subsequent to partial (anaerobic) catabolism, is converted to citrate within the mitochondrion. When increasing amounts of citrate leave the mitochondria for use in deregulated sterol synthesis, then in effect, glucose carbons will have been removed from further catabolic utilization by the TCA cycle. As a result of such metabolic rerouting, the question becomes one of substrate availability for two opposing and competitive metabolic pathways: one catabolic and energy yielding, the other anabolic and energy requiring.[300]

McGee and Spector[192] found that in Ehrlich ascites tumors, glucose carbons (as ^{14}C) were incorporated as the major skeletal elements of lipids and their immediate precursors, with little ^{14}C recoverable as $^{14}CO_2$. This would support the metabolic pattern just proposed. Yet, Pedersen's review[150] emphasizes that tumor cells still derive the majority (60 to 95 %) of their ATP from repiration-coupled phosphorylation. Consequently, if glucose-derived catabolites are excluded from directly supporting tumor cell bioenergetics, a substitute respiratory substrate is necessary to fuel tumor cell

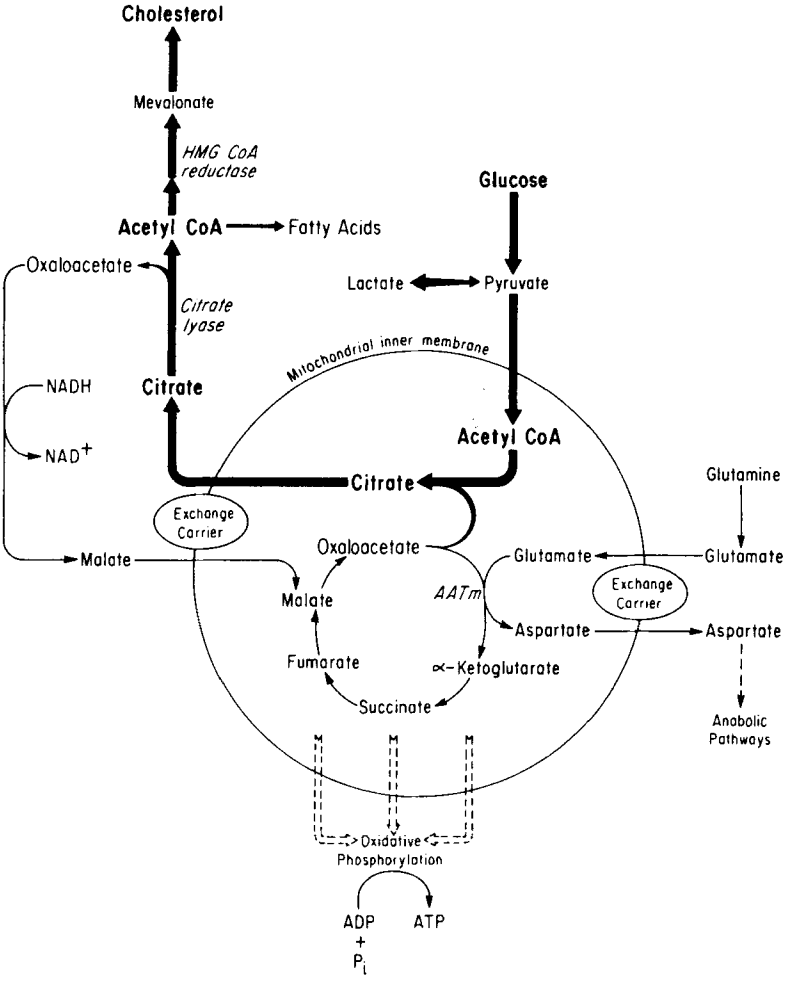


FIGURE 4. Rerouting of glucose carbons and concomitant changes in respiratory metabolic patterns subsequent to the early deregulation of cholesterol synthesis in tumors. Enhanced cholesterol synthesis alters the balance of substrates supporting respiration and bioenergetics. Mitochondrial citrate is diverted from participation in the TCA cycle and this pattern obviates the utilization of glucose as a respiratory substrate precursor. Tumor respiration is supported effectively by glutamate (see also Reitzer et al.).[296] An abbreviated TCA cycle is maintained by mitochondrial aspartate aminotransferase activity (AAT_m). Further details are discussed in the text.

oxidative phosphorylation. Furthermore, in consideration of the altered metabolite flux postulated above, the substrate substituting for glucose should not only (1) support oxidative phosphorylation effectively, but in addition (2) should not be metabolically converted in the mitochondrion by way of an acetyl CoA intermediate.

We propose that glutamate replaces glucose as the fundamental respiratory precursor in tumors, as shown in Figure 4. The immediate consequence of this substitution of glutamate as respiratory fuel is an abbreviated, yet efficient, TCA cycle that bypasses the involvement of acetyl CoA and citrate as fuels for cellular bioenergetics.

The loss of regulation over cholesterol biosynthesis and the accumulation of sterol in tumor cell membranes over time, raises questions about the distal ramifications of this

altered metabolic pattern at the mitochondrial/cytoplasmic interface. These questions will be addressed below in terms of some of the more established hallmarks of neoplastic development.

VIII. CONSEQUENCES FOR RESPIRATION AND ALTERNATIVE METABOLIC FLOW IN TUMORS

A. The Truncated TCA Cycle

Figure 4 illustrates the diversion of glucose carbons toward sterol biosynthesis in tumors via mitochondrial citrate as the intermediate metabolite. This metabolic pattern, which is central to our hypotheses, emphasizes an increased reliance on glutamate as the primary bioenergetic fuel in tumors. Overall, such a pattern is conceived as arising very early subsequent to some unspecified tumor "promotion" event. This altered metabolic flux becomes increasingly expressed during the time- and tissue-dependent deregulation of cholesterol synthesis, and leads to membrane sterol enrichment, which may be one of the most significant characteristics in the eventual establishment of the neoplastic phenotype. One metabolic consequence of Figure 4 is an abbreviated or truncated TCA cycle, resulting from the increased utilization of glutamate as respiratory fuel. This would imply, first, that the oxidative decarboxylation of citrate up to the generation of α-ketoglutarate would be diminished. A second feature of the pattern would imply that of the total citrate formed within the mitochondrion, a greater portion than normal would be preferentially exported to the cytoplasm for use in lipid (particularly cholesterol) synthesis.

Some form of abridged TCA cycle in tumor mitochondrial reactions was suggested by the work of Busch and colleagues more than 20 years ago. It was observed that neither ^{14}C-labeled pyruvate nor acetate equilibriated with the TCA cycle in Walker 256 rat sarcoma in vivo,[193,194] whereas labeled glutamate and succinate did.[195,196] Supporting these observations, 6C3HED murine lymphoma cells have been shown to prefer either glutamate or glutamine as respiratory fuels, compared with glucose; i.e, more than 80% of the glucose underwent catabolic conversion to lactate, and its carbons were not retrieved as CO_2 in this tumor.[197] The same phenomenon has been demonstrated recently for HeLa cells.[296]

The alleged restructuring of metabolic patterns in tumors at the mitochondrial/cytoplasmic interface shown in Figure 4 provides only limited implications for other characteristics observed in tumors. Therefore, Figure 5 extends these proposals as we conceive them to occur in tumors exhibiting significant capacities for aerobic glycolysis. One obvious outcome of this extended pattern is that glucose, although actively catabolized in tumors, is not burned to any major extent via the aerobically linked TCA cycle. The decreased reliance on glucose for respiration is usually reflected by the observed increase in cytoplasmic lactate production noted with many malignant cell systems. Pedersen has emphasized and documented the dramatic reduction in the number of mitochondria per cell in rapidly proliferating tumors.[150] A reduced cellular complement of mitochondria signifies a potentially lower capacity for the oxidative decarboxylation of pyruvate in the tumor cell such that cytosolic lactate dehydrogenase is free to compete more effectively for glycolytically-derived pyruvate. Thus, Figure 5 postulates that a smaller-than-usual percentage of glucose-generated pyruvate enters the tumor mitochondrion, most of it undergoing conversion to lactate in the cytoplasm.

Yet, despite the underutilization of glucose carbons in aerobic catabolism, tumor mitochondrial respiratory activity would continue, supported principally by glutamate oxidation. The truncated TCA cycle illustrated in Figures 4 and 5 is maintained by intramitochondrial transamination of oxaloacetate with incoming glutamate to yield the

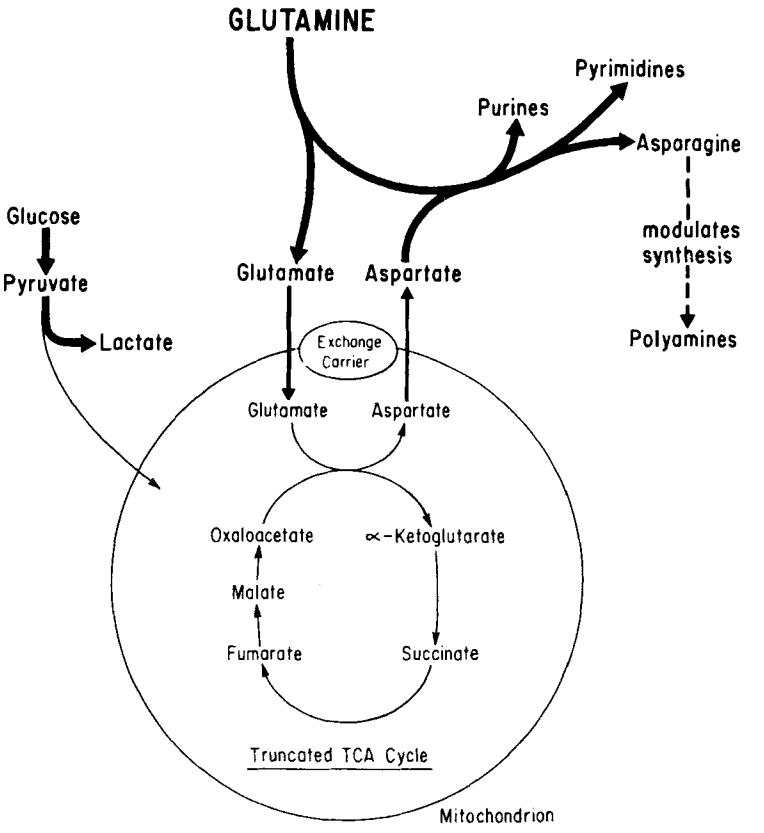


FIGURE 5. Metabolic implications of tumor respiration supported by glutamate. Glutamine is the principal amido donor and is required for the synthesis of purines, pyrimidines, and asparagine. In each of these syntheses, glutamate is the byproduct and supports tumor cell respiration, according to Figure 4. Aspartate, resulting from glutamate oxidation in the mitochondrion, is exported in exchange for incoming glutamate, and is then employed in the pathways of purine, pyrimidine, and asparagine synthesis. Channeling the amido groups into these synthetic routes lowers the availability of free ammonia and, thereby, urea cycle activity in tumors. See text for detailed discussion. (With permission from Lavietes, B. B. and Coleman, P. S., *J. Theor. Biol.*, 85, 523, 1980. Copyright by Academic Press Inc. (London) Ltd.)

TCA cycle intermediate α-ketoglutarate. Glutamate is derived, predominantly, via the cellular synthesis or uptake of glutamine, and is imported into mitochondria in exchange for the export of one of the transamination products, aspartate.

Figure 5 proceeds to make some general and far-reaching statements that derive from the substitution of glutamate for glucose as fuel for tumor cell respiration.[297] For example, increases in the synthesis of asparagine, polyamines, purines, and pyrimidines can be expected from the utilization of mitochondrially exported aspartate, subsequent to glutamate transamination in the matrix. These metabolic phenomena have been documented in a variety of cancers, and some supportive comments about the proposed patterns are appropriate. Some of the more apparent consequences of the pattern shown in Figure 5 and their experimental support, are given below.

First, the abridged or truncated TCA cycle predicts an asymmetry in the activities of various TCA cycle enzymes relative to that expected for the operation of the full or

traditional cycle. Such asymmetry has been reported. Cederbaum and Rubin[172] characterized these enzyme activities in a series of rat hepatomas by measuring $^{14}CO_2$ produced upon oxidative decarboxylation of selected ^{14}C-labeled TCA cycle substrates. They recovered elevated amounts of $^{14}CO_2$ from α-ketoglutarate, succinate, and malate, but not from acetate and citrate.

With regard to the mitochondrial source and supply of α-ketoglutarate in liver tissue, it is generally recognized that this TCA cycle intermediate could be derived in the matrix from glutamate by oxidative deamination rather than via specific amidotransfer, as proposed in Figures 4 and 5. However, the extent of such deamination has been observed to be insignificant in Ehrlich ascites tumor mitochondria.[198] Furthermore, in a study of human hepatoma enzyme imbalances, the activity of glutamate dehydrogenase, which is responsible for the deamination of glutamate to α-ketoglutarate, was found to systematically *decrease* as the histopathologically defined malignancy of these tumors became more pronounced.[199] Classically, the glutamate dehydrogenase reaction in liver mitochondria is believed to comprise the main source of free ammonia required for urea synthesis. Thus, it might be predicted, on the basis of the above data, that urea cycle activity would be simultaneously diminished in rapidly growing, highly malignant hepatomas. In fact, this prediction is supported by experimental observations with rat hepatomas, where urea cycle maneuvers appear to be severely limited relative to normal liver.[200,201]

Although not yet studied, cholesterol enrichment of tumor mitochondrial membranes may play a causative role in the shift away from oxidative deamination of glutamate in hepatocellular carcinomas. The implied relationship may be drawn as follows. Tomkins et al.[202] showed that steroidal molecules behaved as potent inhibitors of the isolated and purified enzyme glutamate dehydrogenase. Recently, Pons et al.[203] reexamined this phenomenon in a study on the catalytic activity of the isolated enzyme that embraced many steroid derivatives. Interestingly, those steroids possessing the greatest inhibitory effect on the enzyme have molecular structures that most closely resemble the parent steroid, cholesterol. It is important to establish, then, whether the excess mitochondrial membrane cholesterol encourages the metabolic pattern of Figure 5 in hepatoma mitochondria by causing a rerouting from oxidative deamination to transamination of incoming glutamate.

La Noue et al.[204] have studied the glutamate-aspartate exchange carrier of mitochondria. They showed that aspartate efflux (and thus, concomitant glutamate import) in isolated heart mitochondria was greater than that in normal liver organelles. The authors suggested that this may be due to the absence of glutamate dehydrogenase activity in the heart, a tissue that exhibits no urea cycle. The experimental observation that hepatomas prefer to metabolize glutamate via the activity of aspartate aminotransferase rather then via glutamate dehydrogenase,[198-201] suggests that glutamate-aspartate exchange transport may tend to be more active in these tumors than in normal hepatocyte mitochondria, perhaps because of their enrichment with cholesterol; but this has not yet been investigated.

B. Shifts into Anabolic Patterns

Upon the influx of glutamate into mitochondria as fuel for tumor cell bioenergetics via transamination, aspartate becomes a principal byproduct and is stoichiometrically exported from the mitochondrion. The fate of the exiting aspartate leads directly to a variety of synthetic patterns of metabolism that are of considerable importance to proliferating cells.

In tumor cell systems, a number of aspartate-related observations have been documented by many investigators. The fundamental observation has been the increase

in aspartate or aspartate-derived amino acid levels in host body fluids (serum, urine, tissue culture medium).[205-208] Figure 5 considers these variegated observations by illustrating that enhanced mitochondrial export of aspartate in exchange for incoming glutamate can encourage increases in the synthesis of molecular moieties known to be primary indicators of proliferating cells, such as purines, pyrimidines, and asparagine. In the biosynthesis of each of these metabolites, an amide transfer from the basic donor molecule, glutamine, occurs early in the synthetic pathway. The glutamate which results is then brought into the mitochondrion in exchange for aspartate, the latter of which is a required metabolite at later stages of the same synthetic pathways. Some of the relationships between this metabolic flow and the metabolic phenotype of tumors are discussed below. First, however, one aspect of such metabolic interplay should be stressed.

The appearance of elevated cytoplasmic aspartate in tumors, as proposed in Figures 4 and 5, is visualized as a direct result of increased tumor reliance on glutamate as respiratory fuel. Thus, emphasis on aspartate-consuming reactions may be conceived as deriving from events that concern tumor cell respiration. To the best of our knowledge, the relationships between these metabolic characteristics to one another and to respiration and bioenergetics in tumors have not been previously described.[297]

1. Elevations in Purine and Pyrimidine Synthesis

Consistent with the characteristics of proliferating cell populations is the increase in nucleic acid precursor metabolites. Therefore, elevated purine and pyrimidine synthesis is expected in growing tumors. The synthesis of these moieties requires the direct participation of both aspartate and glutamine. The glutamate that results from glutamine amidotransfer during *de novo* nucleic acid synthesis becomes available for the support of mitochondrial respiration and the formation of aspartate, as shown in Figure 5.

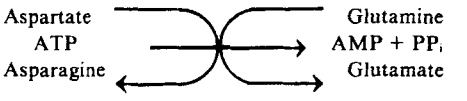
Elevated rates of glutamine and aspartate utilization in purine and pyrimidine anabolism are congruent with the observed lack of urea cycle activity in hepatomas alluded to previously. Rat ascites AS-30D cells were shown to be deficient in the mitochondrial enzyme ammonia-dependent carbamoyl phosphate synthetase, as a consequence of which they form no urea.[201] The concluding logic of this phenomenon is that with increased rates of purine and pyrimidine synthesis in proliferating tumor cell systems, which involves cytoplasmic glutamine-dependent carbamoyl phosphate synthetase among other enzymes, it is to be expected that such anabolic, amide-consuming reactions would leave little free ammonia to be converted to urea. Thus, the metabolic flow, illustrated in Figure 5, may be seen to serve two purposes for tumor cell maintenance. First, it allows for the support of respiration by making glutamate continually available as respiratory fuel, thereby generating aspartate for anabolic removal. Second, the proposed pattern encourages the biosynthesis of critical metabolites required by replicating cells, and obviates, to a substantial extent, a purely wasteful spillover of aspartate into the cytoplasmic milieu.

2. Other Routes for the Utilization of Aspartate: Elevated Asparagine Synthesis

In many tumors, cytoplasmic asparagine formation may be another major route for the utilization of mitochondrially generated aspartate. A clearcut experimental example exists. The 6C3HED murine lymphoma may be obtained as two subline variants, one of which cannot make asparagine because it lacks the enzyme asparagine synthetase.[209] This subline relies on the extracellular milieu for the asparagine it requires for protein synthesis. For this reason, the bacterial enzyme L-asparaginase, when added externally to neoplastic asparagine auxotrophs, serves to kill the cells by depriving them of this

necessary amino acid. By lacking asparagine synthetase, this subline expells large quantities of aspartate into its surroundings when cultured.[205] A revertant subline of this 6C3HED lymphoma, however, possesses wild type levels of asparagine synthetase activity, and has been shown to release to its surroundings equivalently large amounts of asparagine.[205] Furthermore, high levels of asparagine synthetase activity itself have been noted in the serum of tumor-bearing hosts, suggesting that the enzyme is not only present at rather high intracellular levels, but that it leaks from and may qualify as a diagnostic aid for the presence of many tumors.[9] Horowitz et al.[20] have documented considerable increases in asparagine synthetase activities within a variety of tumor cell types compared with controls, and our laboratory has observed similarly enhanced asparagine production in Morris hepatomas 3924A and 16 relative to host liver.[211]

Mammalian asparagine synthetase is a glutamine-dependent enzyme.[212,213]



As mentioned previously, significant increases in aspartate levels have been observed in host extracellular fluids of both experimental tumor-bearing animals and in human patients.[205-208] But it is important to note that such studies usually do not distinguish asparagine from aspartate, since the amino acid analysis procedures employed cause the hydrolysis of asparagine to aspartate.

An interesting corollary to enhanced asparagine synthesis in tumors, which arises from the utilization of glutamine and aspartate according to Figure 5, is the observed increase in "serum glutaminase activity" of many tumor-burdened animals.[214] The asparagine synthetase reaction may be visualized, in one respect, as a glutaminase reaction. Indeed, the relationships between glutamine-requiring amidotransferases and glutaminases are difficult to distinguish with experimental cellular systems. For example, isolated asparagine synthetase preparations from RADA1 tumors exhibit considerable glutaminase activity, even higher than that manifest by glutamine-dependent carbamoyl phosphate synthetase.[213] In view of this fact, we suspect that elevated asparagine synthetase together with glutamine-dependent carbamoyl phosphate synthetase activities of proliferating tumors may be responsible for this increase in so-called glutaminase activity. Chakrabarty and Shrivastava[215] have indicated that successful chemotherapy in tumor-bearing mice diminishes "serum glutaminase activity", and it will be recalled that Cooney et al.[9] demonstrated an increase in the levels of serum asparagine synthetase activity as tumor growth progressed. Consequently, as effective chemotherapy promotes tumor regression, and if serum asparagine synthetase activity is commensurately reduced, one could expect a proportional decline in "serum glutaminase activity", as has, in fact, been reported.[215]

3. *Accumulation of Polyamines*

The observation that polyamine species, such as putrescine, spermine, and spermidine, are synthesized and accumulate to a greater degree in tumors has recently received attention as a characteristic of cells that are unable to regulate their growth capacity.[216-220] In tumor-burdened animals, altered urinary polyamine levels have been suggested as still another potentially useful clinical hallmark for determining tumor cell growth kinetics and the effectiveness of chemotherapy.[221] The increased asparagine levels, which assume importance as an anabolic sink for aspartate produced during tumor cell respiration, also may be implicated as an indirect regulator of polyamine

synthesis. Experimental data that argue in favor of such a role for cellular asparagine have been obtained.

Increased activity of the first enzyme, ornithine decarboxylase, in the polyamine synthesis pathway via putrescine has been shown to correlate with the extent of neoplastic transformation.[222,223] Furthermore, Chen and Canellakis[224] demonstrated that asparagine was a required metabolite for the expression of ornithine decarboxylase in cultured N18 neuroblastoma cells. Two observations from the work of these authors are striking and supportive of the altered metabolic patterns outlined in Figure 5. First, half-maximal stimulation of ornithine decarboxylase activity was observed upon supplying the cell cultures with 5 m*M* asparagine, a concentration well within physiological levels. Second, an equal concentration of glutamine appeared to be only half as effective as the asparagine.

From the standpoint of assessing the regulation of polyamine synthesis via indirect enzyme-linked cooperativity, the data of Prouty[225] constitute additional support. It was observed that ornithine decarboxylase activity in HeLa cells was stimulated by glutamine to about the same extent as that reported by Chen and Canellakis with neuroblastoma cultures. Nevertheless, Prouty found no direct effect of glutamine on the activity of the purified enzyme in vitro. According to the pattern of metabolite flow given in Figure 5, glutamine would be expected to be a less effective stimulant of ornithine decarboxylase than an equivalent asparagine concentration, because asparagine is clearly not the only product of cellular glutamine metabolism. The data discussed above[224,225] support this view (see also Canellakis et al.[226]).

What remains to be raised, however, is a specific postulate on how asparagine could indirectly regulate the enhanced synthesis and accumulation of polyamines in tumors. Figure 6 proposes two mechanisms by which elevated cellular asparagine levels lead to this widely observed phenomenon. As already described, asparagine may act to stimulate the first enzyme in the path toward polyamines, ornithine decarboxylase. But just as important is the finding that catabolic (decarboxylated) derivatives of asparagine, which turn out to be metabolites that are also common to mitochondrial bioenergetics, are *inhibitors* of putrescine oxidation by their effects on the enzyme diamine oxidase.[227] Therefore, polyamines can accumulate in tumor cells according to a flow of metabolites that may be under the distal direction of cellular asparagine levels, as shown in Figure 6.

It will be appreciated, by referring to Figures 4 and 6, that the cytoplasmic levels of the asparagine catabolites oxaloacetate and pyruvate would be raised as a result of elevated glycolysis in combination with the export of mitochondrial citrate and its subsequent cleavage. Figure 6 thus draws attention to the possibility that the metabolic "funnel" which leads to the increased generation of polyamines in tumors, may arise, ultimately, because of enhanced glutamate-aspartate exchange transport during the mitochondrial respiratory maneuvers of tumor cells.

C. Glutamine Dependence and Intracellular pH in Tumors

The anabolic patterns illustrated in Figures 4 and 5 rely heavily on the presence of a sufficient cellular level of the amido donor glutamine. As proposed, glutamine amidotransfer also creates the glutamate necessary to support tumor cell mitochondrial bioenergetics. The seemingly extravagant use of glutamine for the operation of these metabolic routes may explain the generally observed avidity for glutamine displayed by tumors. Many laboratories have demonstrated that glutamine levels may be either growth limiting, or that the presence of glutamine is imperative for the survival of human and animal neoplasms in tissue culture systems.[228-231] It has also been shown that when supplied to tissue culture medium, glutamine stimulates DNA synthesis in B65 rat neuroblastoma cells.[232]

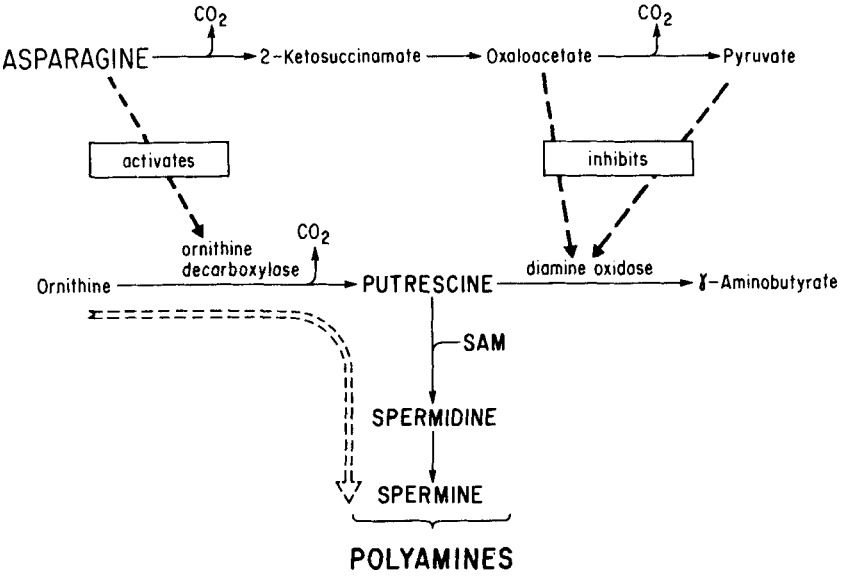


FIGURE 6. Elevated levels of asparagine in tumors can effect the accumulation of polyamines. Asparagine stimulates ornithine decarboxylase activity, while asparagine catabolites inhibit putrescine oxidation via diamine oxidase. In this manner, the build-up of putrescine can enhance the production of spermidine and spermine according to the metabolic flow indicated. (With permission from Lavietes, B. B. and Coleman, P. S., *J. Theor. Biol.*, 85, 523, 1980. Copyright by Academic Press Inc. (London) Ltd.)

Early studies on the nutritional requirements of neoplastic and normal tissues in vitro indicated what appeared to be the augmented need of tumors for glutamine. For example, with cocultures of Ehrlich ascites cells and normal tissue slices, Shrivastava and Quastel[233] observed that the tumor took up and readily metabolized the glutamine released from the nonproliferating normal tissue. This cross-feeding emphasized the ability of the tumor to prey parasitically on its surroundings, especially with respect to the requirement for sufficient glutamine to sustain its proliferation. Further, the metabolic distribution of intracellular ^{14}C, upon injection of ^{14}C-glutamine into Yoshida sarcoma-bearing rats, has been traced into some of the primary metabolites shown in Figures 4 and 5: glutamate, aspartate, and succinate.[234] Glutamine metabolic turnover also has been directly correlated with the degree of malignancy of a graded series of rat hepatomas,[214,235] and parallels the dependence of the tumor on glutamine for the support of cellular respiration.[236,237]

In a broad-based overview of tumor cell glutamine dependence, Roberts and Simonsen[238] reported that plasma glutamine levels are found to be lower than normal in patients afflicted with the following neoplasms: chronic leukemias, breast cancer, Hodgkin's disease, lymphosarcoma, and reticulum cell sarcoma. Such generalized observations are consistent with augmented glutamine dependence in neoplastic tissues, and are consonant with the patterns of metabolism illustrated in Figures 4 and 5.

In addition, a decrease in the intracellular pH of tumors, arising from the abundance of glycolytically generated lactate, has often been presented as a precipitating force for neoplastic transformation.[239,240] However, low pH, in itself, is an insufficient stimulus for tumorigenesis, and leaves a host of unanswered questions on the factor(s) that act as its trigger. In normal kidney mitochondria, for example, a low environmental pH stimulates glutamate oxidation,[241] and normal kidney tissue function is sustained by glutamine

metabolism in vivo even under conditions of tissue acidosis.[242] Similarly, the burning of galactose instead of glucose, encourages some tumor cells to generate relatively modest amounts of lactate and maintain normal intracellular pH while sustaining their growth rate.[150,243] Furthermore, cultured human diploid fibroblasts can grow in the complete absence of glucose by metabolizing glutamine.[244] And glutamine has been shown to act as a growth-limiting respiratory metabolite for murine L-cells in culture.[245,246]

It is interesting, in view of the proposed respiratory dependence on glutamate of Figures 4 and 5, that glutamate has been found to substitute for glutamine in L-cells as well as in human diploid cell cultures.[247,248] Low pH growth conditions have been observed to stimulate glutamate uptake by Ehrlich ascites cells,[249] presumably by protonating the γ-carboxyl group so that the molecule may be brought inside the cell by neutral amino acid carriers of the plasma membrane.[250]

The phenomenon of reduced cellular pH together with that of glutamine(glutamate)-supported tumor cell growth permits us to argue that once glutamate oxidation is initiated in tumors, it can become a self-perpetuating phenomenon. This develops as follows:

1. The glucose molecule, having been replaced by glutamate as tumor respiratory substrate, is released from its role as precursor for further catabolism and made available for both lactate and sterol synthesis.
2. As lactate production is enhanced, intracellular acidity rises, and stimulated cellular glutamate uptake and utilization can reinforce this aspect of the glucose-independent respiratory pattern.

Finally, our interpretation of the basis for the observed increase in serum glutaminase in tumor-burdened hosts[214,215] (see Section VIII.B.2) is also pertinent to the apparent avidity for glutamine expressed by such systems. The so-called serum glutaminase activity, in fact, may be a phenotypic indicator of accelerated glutamine utilization in vivo, compared with more direct and explicit assays for glutamine-dependent reactions that are applicable to in vitro systems.

The metabolic mechanics that have been discussed with respect to the importance of glutamine, glutamate, aspartate, and asparagine as principal catabolic and anabolic intermediates in a wide variety of neoplastic systems are collated in Table 4. The basic metabolic syndrome that links every one of these characteristics to each other is their collective derivation from the proposed need for tumor cells to support respiration and associated bioenergetics with glutamate in lieu of glucose. Thus, it is fair to point out that though these phenomena have been individually documented by numerous laboratories, their relationships to the early deregulation and enhanced synthesis of cholesterol and to the altered patterns of respiration that ensue during tumorigenesis appear to have been overlooked.[296,297]

IX. ALTERED REDUCING EQUIVALENT SHUTTLES AND THE GLUTAMATE-ASPARTATE EXCHANGE TRANSLOCASE IN TUMOR MITOCHONDRIA

A. The Tricarboxylate-Pyruvate Shuttle in Tumor Cells

Among the consequences of the impermeability of the inner mitochondrial membrane to the simple diffusion of virtually all metabolites, is the presence of at least nine substrate-specific translocases integral to the membrane structure. The glutamate-aspartate exchange transport carrier, heavily stressed in our proposals, is one of these. The resulting compartmentalization of cytoplasmic and matrix metabolite pools applies

Table 4
CORRELATIONS BETWEEN THE METABOLIC PHENOTYPE OF TUMORS AND THE METABOLIC PATTERNS OF FIGURES 4 AND 5

Metabolic phenotype	Experimental system	Ref.
Diminished use of glucose for respiration; high lactate production	Murine 6C3HED lymphoma	197
	Walker 256 carcinoma	193, 194
	Ehrlich ascites (2 sublines)	190
	Murine L1210 leukemia	190
	Murine Krebs II carcinoma	190
	AS-30D hepatoma	190
	Human HeLa cells	296
Enhanced glutamate or glutamine oxidation	Murine 6C3HED lymphoma	197
	Rat hepatomas	236
	Human HeLa cells	296
Elevated tumor "glutaminase" activity	Various rat hepatomas	214, 235
Elevated host serum "glutaminase" activity	Ehrlich ascites host mice	215
Decreased plasma glutamine levels in tumor-bearing hosts	Human breast carcinoma; Hodgkin's disease; reticulum cell sarcoma; leukemia	238
Glutamine is growth-limiting or required for cell survival	ERLD murine leukemia	231
	Murine sarcoma 180	229
	Human leukemia	228
	Murine 6C3HED lymphoma	228
	Murine MOPC-31C plasmacytoma	230
Tumors feed on glutamine produced and exported by normal cells in coculture	Ehrlich ascites; Walker 256 carcinoma	233
Elevated asparagine synthetase activity	Murine leukemias and lymphomas resistant to L-asparaginase, including L5178; 6C3HED; P388; ERLD; RADA1; P1798; EL4; Meth A; EARAD 1; Balb-♂ RL2; K36; E♂ G2	9, 205, 209, 210
	Novikoff hepatoma	9, 263
	Rat hepatomas	262
	B16 melanoma	9
	Ehrlich ascites	9
	Lewis lung carcinoma	9
Increased extracellular levels of aspartate and aspartate-derived amino acids	Murine 6C3HED-R lymphoma	205
	Walker 256 carcinoma host serum	206
	Human cervical carcinoma host serum	207
	Human leukemia host urine	208
Modulation of polyamine synthesis by asparagine	N18 neuroblastoma (tissue culture; expression of ornithine decarboxylase activity)	224, 226
	Virus-transformed BHK (tissue culture; inhibition of putrescine oxidation via diamine oxidase)	227
Elevated aspartate aminotransferase activity and depressed glutamate dehydrogenase activity	Human hepatomas	199
	Ehrlich ascites tumor mitochondria	198

Note: These are some metabolic characteristics of tumors relating glutamine/glutamate oxidation to the patterns illustrated in Figures 4 and 5.

as well to energy-yielding reducing equivalents, in the form of NADH, for which no specific membrane translocase is known. Thus, cytoplasmic NADH, arising from the glycolytic production of pyruvate, is unable to directly penetrate the matrix space for oxidation by the electron transfer chain of the inner mitochondrial membrane. To satisfy the requirement of regenerating oxidized nicotinamide adenine dinucleotide, indirect routes have evolved that rely on the coordinate coupling of anionic substrate translocations together with specific enzymes of matrix and cytoplasmic compartments. Such cyclic routes are described as reducing equivalent shuttles. The principal one, the malate-aspartate shuttle, is believed to be the means by which extramitochondrial NADH is oxidized for reuse during, for example, continued operation of glycolysis in aerobic tissues (Figure 7A).[251]

With the proposal that glutamate assumes prominence as the major respiratory substrate in tumors, altered shuttle systems may develop in order to regenerate NAD^+ for continued, and, in many instances, enhanced glycolytic activity. There has been considerable discussion recently on the significance of various substrate shuttles as they relate to tumor cell metabolism.[172,198,252-255]

In many rapidly growing malignant tumors, most cytoplasmic NADH, produced via glycolysis, is reoxidized during the conversion of pyruvate to lactate via lactate dehydrogenase (Figure 4). Many laboratories have confirmed that perhaps only 20% of the glucose taken up by these tumors is oxidized in the mitochondria to CO_2.[190,197,256,257] Several investigators have attributed this residual 10 to 20% of the glucose-derived CO_2 explicitly to the activity of the malate-aspartate shuttle.[256,258] For example, Greenhouse and Lehninger support this conclusion, not by means of direct shuttle activity measurements, but by observation that amino-oxyacetate, an inhibitor of cytoplasmic aspartate aminotransferase (ATT_c), obviates the reoxidation of this residual cytoplasmic NADH. Their logic can be appreciated by reference to Figure 7A. What is overlooked in this analysis, however, is the symmetry of the malate-aspartate shuttle mechanism, especially with regard to the presence of amidotransferring isozymes in both the matrix and the cytosol; AAT_m and AAT_c. The inhibitor amino-oxyacetate has been found to be effective with *both* intra- and extramitochondrial amidotransferases.[257] Furthermore, amino-oxyacetate has been shown to block urea synthesis,[259] which indicates the inhibitor's ability to penetrate the mitochondrial inner membrane. It is clear, therefore, that by inhibiting both cytoplasmic and mitochondrial isozymes of AAT with amino-oxyacetate, one would virtually guarantee the nearly complete inability of the cell to reoxidize the residual NADH remaining in the cytosol subsequent to lactate dehydrogenase activity.

The proper functioning of the traditional malate-aspartate shuttle (Figure 7A) demands the exit of both aspartate and α-ketoglutarate in stoichiometric exchange for the entry of glutamate and malate. If, as we propose, mitochondrial α-ketoglutarate, arising via the AAT_m reaction, is to be employed primarily as an energy-yielding TCA cycle intermediate in tumor mitochondria, then its cycling through the shuttle mechanism would appear to be a wasteful diversion depriving the cell of over 60% of the ATP otherwise made if the α-ketoglutarate were to proceed only through the remainder of the truncated TCA cycle (Figure 4). Specifically, operation of the malate-aspartate shuttle generates 1 mol of NADH intramitochondrially. This results in the ultimate production of only 3 mol of ATP via oxidative phosphorylation, such limited ATP production being due to the diversionary maneuvers of α-ketoglutarate away from its role as TCA intermediate during shuttle operation. Alternatively, the linear processing of α-ketoglutarate via the remainder of the TCA cycle theoretically provides for 8 mol of ATP by means of mitochondrial electron transfer-linked bioenergetics (*plus* one substrate-level phosphorylation).

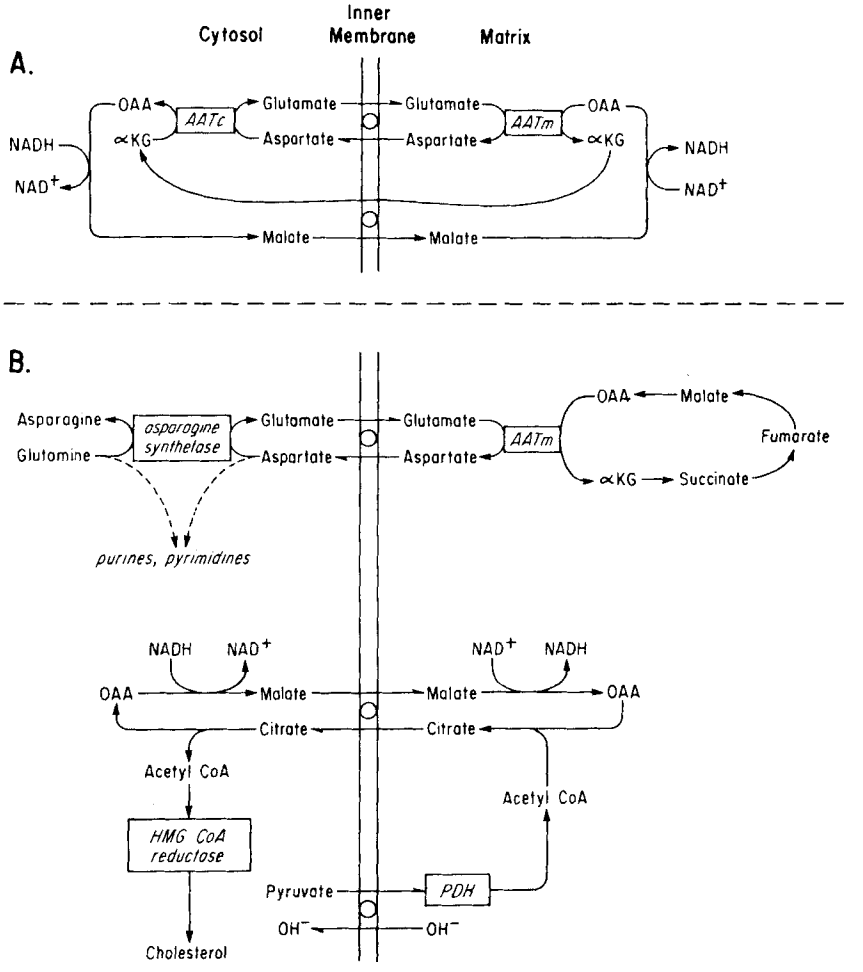


FIGURE 7. (A) The mammalian malate-aspartate shuttle. The participation of both cytoplasmic and mitochondrial aspartate aminotransferase isozymes is obligatory. Redrawn from Lehninger.[251] (B) The citrate-pyruvate shuttle and the glutamate-aspartate exchange translocase are coordinated in this alternative reducing equivalent pathway. Operation of the citrate-pyruvate shuttle also provides for the supply of cytoplasmic acetyl CoA needed for long-term accumulation of cholesterol in tumor membranes, as shown in Figure 3. The glutamate-aspartate exchange translocase utilizes AAT_m, similar to that shown in Figure 7A, but converts the exported aspartate, in part, to asparagine via the elevated asparagine synthetase levels in tumors. This shuttle pathway is open-ended compared with the traditional malate-aspartate shuttle. (With permission from Lavietes, B. B. and Coleman, P. S., *J. Theor. Biol.*, 85, 523, 1980. Copyright by Academic Press Inc. (London) Ltd.)

Whatever logic resides in the above analysis, it gathers support from findings on obligatory aerobic energy production and the depletion of the cellular complement of mitochondria in many tumors.[150] On the other hand, the tumor as well as the normal cell still requires a mechanism for moving cytoplasmic reducing equivalents derived from glycolysis into the mitochondrion. An alternative shuttle mechanism that fulfills this requirement and is consistent with both the empirical data of Greenhouse and Lehninger[190,256] and Chiaretti et al.,[258] as well as with the metabolic flow proposed in Figures 4 and 5, is given in Figure 7B.

The reducing equivalent shuttle we stress as an alternative to the malate-aspartate mechanism is that which operates via the tricarboxylate-malate exchange carrier. This is the translocase which, when investigated by Halperin et al.,[191] was reported to exhibit a greater than threefold reduction in its apparent K_m for citrate in hepatoma 7777 mitochondria. In order for reducing equivalent transfer to function according to this scheme, the pyruvate-OH^- translocase must be coordinately operative. The citrate-malate exchange scheme not only satisfies the need for a reducing equivalent shuttle, but is simultaneously capable of providing for the cytoplasmic acetyl CoA requisite to sterol synthesis.

It also becomes evident that the activity of the cytoplasmic enzyme ATP-citrate lyase on the exiting citrate may aid to accelerate glycolysis by releasing the ATP- and citrate-induced inhibition of the key rate-limiting glycolytic enzyme, phosphofructokinase.[260] This event could not only insure cytosolic pyruvate levels sufficient to saturate lactate dehydrogenase, but also allow the remaining pyruvate to serve simultaneously as substrate for mitochondrial pyruvate dehydrogenase, thus supplying the acetyl CoA necessary for citrate (and ultimately cholesterol) synthesis.

With respect to the effectiveness of such an alternative reducing equivalent shuttle in tumors, it is of interest that Greenhouse and Lehninger[256] reported that 1,2,3-benzene tricarboxylate, which specifically blocks the mitochondrial citrate-malate translocase, is also a more efficient inhibitor of residual cytoplasmic NADH oxidation than are reagents such as n-butylmalonate, which inhibit dicarboxylate exchange transport. One should note, therefore, that the exchange carriers responsible for the malate-aspartate shuttle are dicarboxylate translocases.

At the same time, the active operation of the glutamate-aspartate exchange process, while not involved in the proposed reducing equivalent shuttle mechanism, is viewed as necessary for metabolite flow during the anabolic patterns according to Figure 5. It can also be seen that the malate-α-ketoglutarate transporter is not applicable to this shuttle system. The α-ketoglutarate formed in the matrix via transamination by AAT_m continues to fuel the truncated TCA cycle. The glutamate-aspartate exchange process that we illustrate here differs from the malate-aspartate shuttle with respect to the fate of the exported aspartate. In Figure 7B it is clear that cytoplasmic aspartate reacts preferentially with asparagine synthetase rather than with AAT_c, thereby satisfying the need for a continuing supply of intermediates required for the anabolic shifts characteristic of proliferating cells (Figure 5).

The proposal on the preferred reaction of aspartate via asparagine synthetase over AAT_c receives support from data on the properties of these enzymes when purified. While the activity of isolated pig heart AAT_c is inhibited under reduced pH conditions, that of glutamine-dependent asparagine synthetase is insensitive to pH fluctuations between 6.5 and 8.5.[213] The suggestion, then, is that in tumors, where high lactate production tends to reduce the cytosolic pH, asparagine rather than oxaloacetate would be the preferred product of aspartate reactivity. In addition, asparagine synthetase manifests an apparent K_m with aspartate between 0.3 and 0.9 m*M*,[213,262,263] whereas the K_m values for AAT_c (aspartate) from pig heart[264] and human heart[265] are 4.4 m*M* and 1.1 m*M*, respectively. If the apparent K_m of tumor cell AAT_c for aspartate is assumed to be similar to that of normal tissues, and if AAT_c levels are equivalent in tumors and normal cells, then asparagine synthetase would compete more effectively for the aspartate exiting from tumor mitochondria. Clearly, the patterns proposed in Figure 7B would apply only to those tumors possessing significant asparagine synthetase activity. This is usually signified by tumor resistance to L-asparaginase treatment, a characteristic displayed by the majority of solid tumors investigated.[9]

Finally, it was already mentioned (Section VII.B) that most of the glycolytically

derived acetyl CoA in Ehrlich ascites tumors was found to be directed towards lipid synthesis.[192] The concomitant activity of the tricarboxylate-pyruvate shuttle and the glutamate-aspartate exchange transport systems, therefore, may be tightly correlated. Thus, we find that McGee and Spector also observed that when Ehrlich cells were supplied with amino acids (e.g., glutamine, asparagine) in addition to glucose, lipid biosynthesis was stimulated.[192]

B. Overview: Altered Metabolic Patterns at the Mitochondrial/Cytoplasmic Interface in Tumors

In view of the preceding proposals on tumor metabolic flow, we conceive of the emerging outline as involving, basically, an imbalance or shift in the normal metabolic patterns expected of *all* aerobic cells, normal or neoplastic. No new metabolites peculiar to the tumorous condition have been invoked in the proposed schemes. Yet, the metabolic logic involved might be accused of overemphasizing events at the mitochondrial/cytoplasmic interface, to the exclusion of more distal, and arguably no less important, metabolic phenomena (occurring, say, in the nucleus). This would, we feel, be too limiting an appraisal of the arguments presented. Obviously, the deregulation of cholesterol synthesis, which occurs very early in tumorigenesis, together with tumor membrane cholesterol enrichment over relatively long time periods cannot be thought of as events whose *only* cellular target is the inner mitochondrial membrane and its associated functions.[266] On the other hand, we feel certain that the problem of the cytoplasmic availability of acetyl CoA, which is required for enhanced cholesterol synthesis of long duration, still leads inextricably to the metabolic events that take place within the matrix of these organelles.[267,300] At the present time, then, it remains to be shown whether or not the full metabolic patterns proposed in Figures 4, 5, 6, and 7B contain a firm basis in experimental fact.[296,297]

X. SHEDDING OF CHOLESTEROL-ENRICHED TUMOR CELL SURFACES: A MANEUVER FOR CONTINUED TUMOR GROWTH IN THE HOST

Sufficient documentation has been presented in many of the preceding sections to support the proposition that altered membrane cholesterol levels during tumorigenesis can lead to changes in metabolic patterns mediated by membranes, particularly but not exclusively, those of tumor mitochondria. It is even known that malignant growth in rats appears to affect the cholesterol content of *normal* host animal tissues.[268] Thus, for the purposes of the following discussion, it seems unprofitable to continue to provide, by weight of numbers, an even longer list of experimental documentation that supports the correlation between aberrant cholesterol biosynthesis, membrane cholesterol accumulation, and tumor cell growth.

The reader will appreciate immediately that the phenomenon of tumor cell growth, with its affiliated metabolic characteristics, constitutes in itself merely one facet of the diversity of life. Recognizing this, we might expect the existence of cancer to stimulate no more profound a response than great scientific curiosity. After all, from its own standpoint the cancer cell is not a pathological pariah; just different. It is a fact that under appropriate nutritional and environmental conditions, tumor cells are immortal. Clearly, then, it is the animal harboring the growing tumor whose destiny is less than bright. Consequently, trying to understand the performance of biomembranes in tumors as a function of their cholesterol content may be accomplished by experiments which are designed from a vantage point other than that of specific enzyme analyses and perturbations in the metabolic pathways they regulate. An alternative approach

recognizes that cancer research may proceed with considerable vigor at the level of the whole animal and its struggle to defend itself from tumor invasiveness and death. In this regard, we shall now consider, briefly, certain factors that allow the propagation of the tumor in the host to flourish, usually without effective constraint.

A. Exfoliation of Microvesicles by Tumors

Grohsman and Nowotny[269] demonstrated that the growth of the TA-3Ha ascites tumor in C57BL/10Sn or ICR mice was enhanced by the presence of the cell-free ascites fluid in normal syngeneic recipients. In the absence of the tumor, cell-free ascites fluid alone did not induce tumor growth. Upon fractionation of cell-free ascites fluid with ammonium sulfate, an active tumor growth-enhancing fraction was isolated and examined by electron microscopy. This active fraction was observed to consist of homogeneous membrane vesicles. Biochemical characterization of this fraction (P3) indicated that it was plasma membrane derived.[270] P3 was free of serum proteins and free immunoglobins, possessed activities for the plasma membrane marker enzymes 5′nucleotidase and α-glycerophosphatase, and revealed the presence of fatty acids (via gas-liquid chromatography) in almost exact ratios as those in plasma membranes. P3 had immunogenic properties when injected into tumor-free adult mice. After exposure in vitro to P3-containing ascites fluid, spleen cells lost the ability to adoptively transfer resistance. P3-containing ascites fluid was cytotoxic to normal thymocytes in vitro in the presence of complement. The authors suggested that in vivo, tumor cells are surrounded by membrane fragments derived either by shedding or as a result of cell death. They proposed that these fragments could form a target for the immune responses of the host, thus effectively blocking destruction of the tumor cells.

Membrane vesicles have also been isolated from YAC lymphoma cells.[271] YAC is a Moloney virus-induced lymphoma in A-strain mice. Electron microscopy showed virus-like particles associated with the vesicles. The vesicle preparation was used to both immunize against viable lymphoma cells and to induce new tumors, though oncogenicity was abolished by UV irradiation. In further studies, addition of the extracellular membrane preparation prevented in vitro association of YAC cells and nonactivated peritoneal exudate macrophages.[272] YAC cells and macrophages, by themselves, normally form stable cell contacts in vitro.

Van Blitterswijk et al.[273] found membrane vesicles in a particulate fraction of cell-free ascites fluid from GRSL tumor cells (a spontaneous leukemia in GR/A mice carrying surface MTV-induced antigen, MLr). Van Blitterswijk et al.[274] then measured the microviscosity of GRSL plasma membranes using the fluorescence polarization technique with 1,6 diphenyl-1,3,5-hexatriene (DPH) as a probe. Plasma membranes possessed a lower microviscosity (3.28 Poise) than did extracellular vesicle membranes (5.83 Poise). The measured cholesterol/phospholipid molar ratio was 1.19 for extracellular membranes and 0.37 for plasma membranes. The authors postulated that the membrane material is shed from the cell surface in a manner analogous to the budding of viruses, by selection of the most rigid parts of the cell membrane (Section III.C). In a subsequent paper,[275] the vesicular membranes were reported to be enriched with MLr antigen, 5′nucleotidase and sialic acid relative to GRSL plasma membranes. Electron microscopy of vesicles derived from GRSL cells showed them to be smooth surfaced, with an average diameter of 0.25 μm; some contained what appeared to be budding virus particles.

At the present time, the shedding of plasma membrane-derived microvesicles, principally by tumor cells, is a phenomenon observed with increasing frequency. The functional role played by these vesicles, if any, is currently unknown, or is at best conjectural. Furthermore, the intracellular events that induce plasma membrane vesicle

shedding or exfoliation are unknown. Yet, the appearance of cell surface projections, or blebs, is a widely observed phenomenon in dividing cells in vivo and in cultured cells, though the function(s) of these blebs is also unknown (see review by Bluemink and deLaat).[276]

An increased incidence of surface projections has been found in cells after transformation by a variety of transforming agents. Porter et al.[277] studied surface morphology of BALB/3T3 cells using scanning electron microscopy. Both transformants of spontaneous origin and those transformed by the action of SV-40 virus, murine sarcoma virus, or polyoma virus displayed an increased number of microvilli and spherical blebs after transformation. Similar observations were made by Malick and Langenbach[278] in nontumorigenic C3H/10T½ CL8 cells examined by scanning electron microscopy after transformation by the potent carcinogens 7,12-dimethylbenz(a)anthracene (DMBA) or 3-methylcholanthrene (MCA). MCA-transformed cells had numerous microvilli and ruffles, while DMBA-transformed cells displayed a pattern of increased blebs and cytoplasmic strands. Increased blebbing after transformation was also seen in hamster embryo cells transformed by X-irradiation.[279] In addition, Saxholme and Reith[280] found a positive correlation between the oncogenic potential of DMBA-transformed embryo fibroblasts (C3H/10T½) and the surface concentration of long microvilli (oncogenic potential is the ability of the cells to grow in immunosuppressed mice). Nontransformed cells had a smooth surface, while transformed cells had both short and long microvilli and numerous blebs.

Surface projections in rapidly dividing cells have been postulated to serve several functions. Porter et al.[277] suggested that they may be involved in meeting the nutritional requirements of the actively growing cells by providing an increased area for glucose transport. They have also been suggested to serve as areas for deposition of "excess" membrane which can be used during cell division or cell spreading.[281] Further, the shedding of surface proteins might occur by exocytosis of plasma membrane microprojections in lymphocytes.[282]

In view of the extensive literature reporting that rapidly dividing and transformed cells display an array of surface projections, it is not unreasonable to propose that some of these may be exocytosed into the surrounding medium to become the source of the extracellular membranous vesicles observed in the murine tumors and in the human leukemia discussed above.

We therefore wish to draw attention to the possible relationships that exist between the cholesterol enrichment of tumor cell plasma membranes and the phenomenon of tumor cell surface membrane exfoliation.

B. The Role of Cholesterol in Exfoliation of the Tumor Cell Surface

The array of cell surface projections observed in proliferating normal and transformed cell populations bears witness to the fact that alterations in the composition and qualitative disposition of membrane lipids can quite expectedly lead to visible changes in the morphology of cell surfaces. The propagation of animal viruses via budding of the virus particles from the infected cell surface is, presently, the clearest example of a sequential pattern by means of which the plasma membrane of transformed cells makes its appearance in the extracellular surroundings (Section III.C). Onto these observations, the proposal that such budding and exfoliation occurs at plasma membrane loci that are more rigid and cholesterol enriched[274] bears careful examination. Currently, however, there is a paucity of experimental information correlating the contribution of membrane cholesterol to the release of membrane material.

In one of the few published reports bearing directly on alterations in cell surface topology and cholesterol content, the depletion of membrane sterol was shown to depress

the endocytosis of horseradish peroxidase in cultured L-cells.[98] In these studies, the L-cells were first exposed to several oxygenated derivatives of cholesterol that were known to potently inhibit *de novo* cholesterol biosynthesis (Section IV.A). Such treatment resulted in the dramatic reduction in endocytic activity, with horseradish peroxidase as the cell surface label. Concomitant assays for cellular ATP levels in treated and control cultures displayed no differences. Apparently, the mechanism by which sterol synthesis is blocked after addition of oxygenated sterol derivatives had no affect on the cellular mechanisms for ATP generation. Thus, the conclusion derived from this work indicates the requirement for sufficient membrane cholesterol, via biosynthesis, and its direct participation in topologically visible membrane alteration.

Whether we are concerned with endo- or exocytosis, a certain degree of plasma membrane plasticity and deformability is a functional prerequisite. As indicated earlier (Section III.A,B) the differential presence of cholesterol has been demonstrated to modulate membrane structure, and consequently to intervene in the physical associations within membranes that determine such plasticity. Cooper et al.[283] both enriched and depleted erythrocyte membranes by exposing the cells to cholesterol-lecithin dispersions of varying cholesterol/phospholipid ratios. Enrichment of membranes with cholesterol was observed to increase the surface area of the cell membrane and yielded a 0.22% surface area increment for each 1.0% addition in cholesterol content. Whereas cholesterol-depleted erythrocytes displayed a spherical shape, the cholesterol-enriched cells appeared broad and flat with an irregular contour due to the folding of the cell periphery. It follows that an early deregulation of cholesterol synthesis in tumors would result, over a period of time (specific to the particular tumor), in a continuous accumulation and incorporation of cholesterol into cellular membranes, with a concomitant increase in membrane surface area. Our view, then, is that membranous material deposited in the form of surface villi or blebs could conceivably be shed into extracellular fluids. Support for this view may be found in studies that concentrate on plasma membrane surface area changes and their potential biological effects.

Recently, Luke and Kaplan[284] proposed that geometric changes in membrane shape can be predicted mathematically on the basis of increases in membrane surface area. In their model, the deposition of additional membrane molecular components increases the surface area of a hypothetical spherical bilayer. They calculate that as the surface area increases beyond a certain minimum, the free energy of curvature tends to rise exponentially (Figure 8A). If the relative free energy of curvature is mathematically restricted to a minimal value during the surface area increase, their computations indicate that a dramatic geometric transformation of the expanding spherical bilayer occurs. The topological transformation that results is the evolution of a dumbbell-shaped structure, with the two halves of the dumbbell connected by a narrowing neck-like region (Figure 8B, contour E). Luke and Kaplan[284] state, ". . . the neck is so narrow at E that it is natural to assume that the membrane fuses and that two separate vesicles are formed."

In the case of tumor cell plasma membranes, a continuously increasing surface area that arises from the steady accumulation of cholesterol, subsequent to the early decontrol of its synthesis, may cause an outpocketing of the membrane bilayer which eventually becomes pinched off, as the neck region separating it from the main body of the cell continues to narrow. The concept of outpocketing or bleb formation requires that the deposition of additional cholesterol over time should result in a relatively greater enrichment of the cytoplasmic side of the membrane bilayer leaflet compared with the extracellular leaflet face, in order to encourage the exfoliation process.

C. Tumor Surface Exfoliation and Host Immunosurveillance

The shedding of tumor cell surface fragments as microvesicles into the host circulation

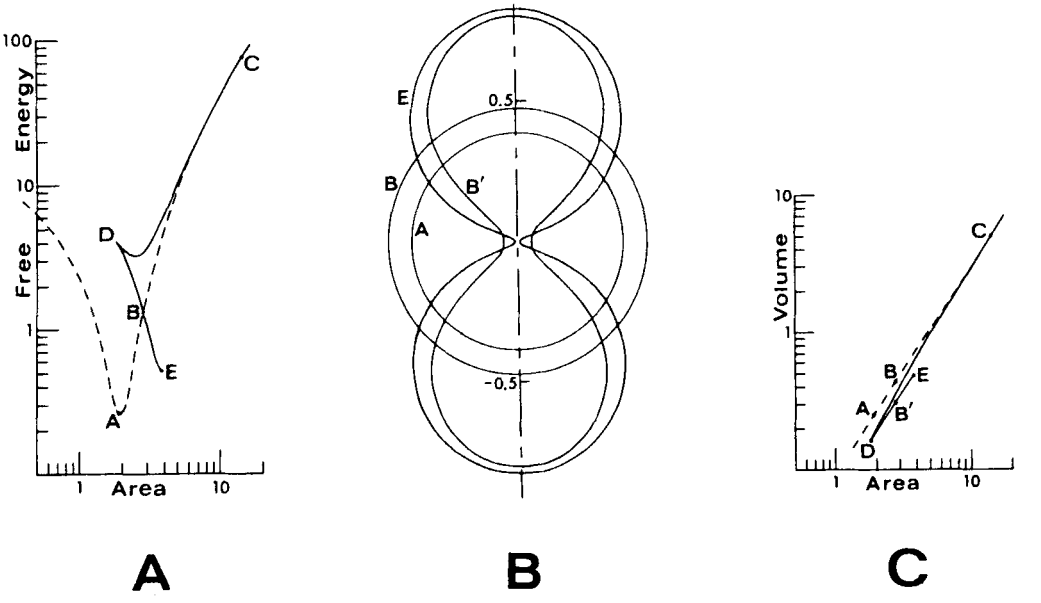


FIGURE 8. (A) As the surface area of a bilayer sphere enlarges, the free energy of curvature increases exponentially (curve A). At point B a singularity appears, and at point E the free energy declines rapidly even though the total surface area has increased somewhat. (B) Mathematically constraining the free energy of curvature to a minimum, the enlarged surface area of sphere B undergoes geometric transformation to B′ and finally to E. The contour shown by E is a dumbbell-shaped structure that can arise from increased cholesterol deposition into the original bilayer sphere, thereby enlarging its surface area. Continued cholesterol deposition would allow the neck region of contour E to fuse, and two vesicles would result. (C) The rise in the inclusion volume is less extreme upon such geometric transformation from sphere to dumbbell during surface area enlargement, and is finite at point E, where two smaller vesicles result. (Reproduced from Luke, J. C. and Kaplan, J. I. *Biophys. J.*, 25, 107, 1979. With permission of the Biophysical Society.)

or body fluids now directs our focus to the response of the tumor-burdened host toward these newly derived vesicular particles. With incontrovertible evidence that tumor surfaces are antigenic to the host,[285-287] albeit sometimes rather weakly, the role of tumor surface shedding to the propagation of the tumor in vivo is of major concern. The release of tumor-specific antigens into the host circulation has been widely observed,[5,6] and in many neoplastic cell systems these antigenic determinants have been shown to be membrane affiliated. For example, in rat sarcomas as well as hepatomas, soluble antigens may be released from tumor cell surfaces only upon limited papain digestion.[288-290] Furthermore, purified plasma membrane fractions from rat hepatomas displayed a significant enrichment in tumor-specific antigenic determinants relative to whole cell homogenates.[291,292] Most of the attention to soluble tumor antigens shed from cell surfaces has been directed at their role as "blocking factors" in host sera. These blocking factors have been reported to consist of antigens,[293] antibodies,[285] and antigen-antibody complexes.[294] Such factors may be perceived as capable of diverting host cellular cytotoxicity, both in vivo and in vitro, away from the tumor cell itself.

Evidence for the appearance of vesicles of tumor cell plasma membrane origin in the cell-free ascites fluid of several murine tumors, and the serum and pleural effusions of leukemia patients has been described.[269-275] These vesicular cell exfoliants were not only found to be enriched, relative to parent cell membranes, in plasma membrane marker enzyme activities and in certain tumor-specific antigenic determinants, but as well,

enriched in cholesterol.[295] All indications lead to the conjecture that these exfoliated vesicles readily interfere with the cellular immunological responses of the host to the tumor.[269,270,272]

The final correlation that we wish to raise, then, without extensive discussion, involves the documented enrichment of tumor cell membranes with cholesterol, and the promotion, thereby, of tumor cell surface shedding. It becomes distinctly feasible to conceive of these exfoliated cell surface vesicles as acellular targets for cell-mediated interaction with the host's immunosurveillance mechanisms. Exfoliated, cholesterol-rich microvesicles would thus serve in the role of passive "decoys", allowing the tumor cells themselves to escape immune-directed destruction during critical periods in the establishment of the malignancy. Once again, such postulates await further clarification, and experiments designed to probe these questions deserve encouragement.

XI. SYNOPSIS

All animal cell membranes contain, to varying degrees, cholesterol or an immediate molecular precursor. The membrane cholesterol appears to serve almost entirely as a mediator of diverse membrane functional activities. Cholesterol acts by modifying the physical state of the biomembrane insofar as contributing to relative membrane rigidity and fluidity, thereby exerting influence upon reaction-specific protein moieties embedded within the membrane bilayer. Cholesterol synthesis *de novo* seems to be an absolute requirement for cell division, presumably because the proliferating cell creates, during cell growth, additional membrane structures in preparation for distribution to its cellular progeny. The *de novo* synthesis of cholesterol proceeds under the constraints of various types of metabolic regulation. In many of the tumor systems so far examined, including those afflicting humans, the synthesis of cholesterol is deregulated. Evidence of the loss of control over tumor cell cholesterol biosynthesis is made manifest very early after exposure to a range of carcinogenic triggers or events. In several instances, the demonstration of decontrol of cholesterol synthesis in an appropriately treated animal is observed many months prior to the appearance of a malignant growth in the target tissue. Thus, over extended periods of time subsequent to the carcinogenic event, cholesterol accumulation will occur within the membranes of the precancerous cell. Upon unequivocal expression of the tumorous state, neoplastic cells display enhanced rates of sterol biosynthesis together with cellular membranes that are significantly enriched with cholesterol.

To foster such enhanced deposition of membrane cholesterol in tumors, a pattern of altered intermediary metabolism is proposed that centrally involves the metabolic acrobatics transpiring within tumor cell mitochondria and at the mitochondrial/cytoplasmic interface. These altered metabolic patterns show definitive correlations with a host of metabolic characteristics that have been documented for tumors. Among the most important metabolic shifts proposed is the substitution of glutamate for glucose as the fundamental respiratory catabolite precursor. A large and diverse tabulation of the phenotypic hallmarks of tumor cell metabolism may be explained by a sequence of events that begins with the biosynthetic deregulation and accumulation of cholesterol in tumor membranes, coupled with the increased reliance on glutamate to support tumor cell respiration and bioenergetics. Furthermore, cholesterol enrichment of tumor cell membranes is proposed to potentiate the exfoliation of the tumor cell plasma membrane in the form of antigenically competent, cholesterol-rich microvesicles. Such vesicle shedding within the tumor-burdened host is conceived as enhancing tumor proliferation in vivo, by allowing the tumor cell a camouflaged pathway for escaping the host immunoserveillance process.

XII. EPILOGUE

In one's enthusiasm for the grand simplicity of theory, it is easy to disregard the process by which theories are conceived. What has been presented in the preceding pages is a theory based upon a broad landscape of diverse experimental findings mostly concerning some limited aspects of membranes and tumorigenesis. We hold no illusions about being able to decipher every morsel of experimental data relevant to the subject. Rather than attempt to construct a unifying whole, we have had to be content with selective and (to us) explainable pieces of the refractory puzzle that is cancer. Therefore, we accept the probability that overnight some of the ideas we have put forth will turn out wrong, if not naive. What is to be hoped for, nevertheless, is that a few of the proposals contained here will stimulate the kinds of experimental research that, before too long, will lead us to a more confident understanding of how cells grow.

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