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Tumor Mitochondria and the Bioenergetics of Cancer Cells

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Prologue

It is a good thing for the entire enterprise that mitochondria and chloroplasts have remained small, conservative, and stable, since these two organelles are, in a fundamental sense, the most important living things on earth. Between them they produce the oxygen and arrange for its use. In effect, they run the place.

My mitochondria comprise a very large proportion of me. I cannot do the calculation, but I suppose there is almost as much of them in sheer dry bulk as there is the rest of me. Looked at in this way, I could be taken for a very large, motile colony of respiring bacteria, operating a complex system of nuclei, microtubules, and neurons for the pleasure and sustenance of their families, and running, at the moment, a typewriter.

I am intimately involved, and obliged to do a great deal of essential work for my mitochondria. My nuclei code out the outer membranes of each, and a good many of the enzymes attached to the cristae must be synthesized by me. Each of them, by all accounts, makes only enough of its own materials to get along on, and the rest must come from me. And I am the one who has to do the worrying.

Now that I know about the situation, I can find all kinds of things to worry about. Viruses, for example. If my organelles are really symbiotic bacteria, colonizing me, what's to prevent them from catching a virus, or if they have such a thing as lysogeny, from conveying a phage to other organelles? Then there is the question of my estate. Do my mitochondria all die with me, or did my children get some of mine along with their mother's; this sort of thing should not worry me, I know, but it does.

From *The Lives of a Cell*
by LEWIS THOMAS

I. Introduction

The extent to which mitochondria play a role in the normal to neoplastic transformation process and in maintaining or promoting the transformed state is largely unknown. Despite the fact that mitochondria occupy

15–50% of the cytoplasmic volume of most animal cells and participate in more metabolic functions than any other organelle in the cell, it seems fair to state that cancer research, and consequently funding for it, has been directed away from mitochondrial studies in the past decade. The new student of cancer biology and biochemistry may ask 'Why?'.

Following WARBURG's hypothesis [WARBURG, 1930] that cancer cells may have an impaired respiratory capacity resulting in elevated rates of glycolysis, a study of the energy metabolism of cancer cells was the central focus of cancer research for many years [AISENBERG, 1961; WENNER, 1967, 1975; WEINHOUSE, 1972, 1976; FRIEDKIN, 1973]. In particular, mitochondria of cancer cells became a target of attack for cancer researchers shortly after KENNEDY and LEHNINGER [1948] demonstrated that these subcellular organelles are the principal site of oxidative metabolism and ATP synthesis in animal cells. However, a concentrated research effort on studies of mitochondria of tumor cells was short-lived. Once it appeared that a *capacity*[1] for a very 'high glycolysis' is neither an underlying factor in the normal to neoplastic transformation process, nor even an universal characteristic of tumors [AISENBERG and MORRIS, 1961, 1963; WEBER and MORRIS, 1963; SWEENEY *et al.*, 1963; POTTER, 1964], the interest in 'bioenergetics' of cancer cells and subsequent funding for such studies declined. In fact, as later sections of this review emphasize, it was never clearly established during this era to what extent highly glycolytic tumors have an *impaired respiratory* capacity. As will be emphasized in the following section and in section XII, all highly glycolytic tumors that have been studied in detail appear to be markedly deficient in mitochondria.

[1] Throughout this article the author will speak of certain tumor cells as having a *capacity* for *high glycolysis*. The word *capacity* is used to indicate that although tumor cells when grown (or placed) in glucose-containing media may produce large amounts of lactic acid relative to control cells, the tumor cells may not necessarily need to utilize glucose to grow. Thus, tumor cells may in certain cases grow equally well in a medium in which glucose is replaced with galactose or mitochondrial substrates, and under such conditions not produce elevated amounts of lactic acid. By *high glycolytic* tumors the author is referring to that class of tumors which have a growth rate equal to or exceeding that of Morris Hepatoma 7288C (section II). Among the experimental tumors that fit into this class are several other lines of Morris hepatomas, Ehrlich and other types of ascites tumors, Novikoff hepatoma, and the Walker 256 carcinosarcoma. Such rapidly growing tumors, in contrast to tumors of slower growth rate, have markedly elevated rates of lactic acid production (fig. 10). The author recognizes that even the most slowly growing hepatomas, when placed in appropriate media, have slightly elevated rates of lactic acid production [BURK *et al.*, 1967; WOODS *et al.*, 1968; WOODS and VLAHAKIS, 1973; LANOUE *et al.*, 1974].

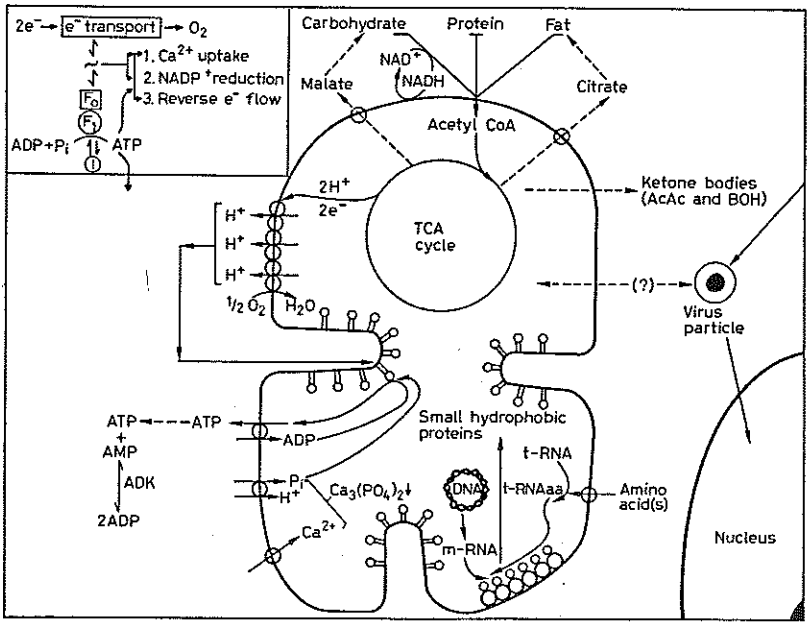

Fig. 1. Diagram illustrating the major functional activities of mitochondria (for purposes of illustration the outer membrane is not shown). *Oxidative phosphorylation:* Reducing equivalents derived from the TCA cycle during the catabolic phase of metabolism as well as reducing equivalents from certain flavoprotein-linked enzyme reactions (not shown) are utilized by the electron transport chain to reduce oxygen to water. During this process protons are translocated from the interior (or matrix) of the mitochondrion to the external side. The net result of this in→out proton translocation process is an electrochemical gradient of protons across the inner membrane. This gradient drives the entry of P_i (and aids the entry of ADP) into the mitochondrion. Once on the inside ADP and P_i are dehydrated to give ATP and water at the level of the oligomycin-sensitive ATPase complex (OS-ATPase), presumably at the expense of the major portion of the electrochemical gradient of protons. *Nucleotide buffering and metabolic regulation:* ATP that is not utilized within the mitochondrion is transported to the external side (intermembrane space) where it may come in contact with the enzyme adenylate kinase (ADK), located between the inner and outer membranes. Adenylate kinase, provided sufficient Mg^{++} is present, tends to buffer the adenine nucleotide pool of the cell. This enzyme is thought to play a key role in metabolic regulation, i.e., in adjustments of the ATP/AMP ratio depending upon the metabolic state. *Glycolysis:* During the catabolic phase of cell metabolism mitochondria, via shuttle systems (malate, α-glycerol phosphate, and perhaps others as well), are responsible for regeneration of NAD^+ for the substrate level phosphorylation step of the glycolytic pathway. *Ketogenesis:* During prolonged starvation, liver mitochondria produce the ketone bodies acetoacetate (AcAc) and β-hydroxybutyrate acid (BOH) which are transported out of the mitochondria and across the plasma membrane into the blood.

They eventually enter brain and muscle mitochondria where they are oxidized to provide energy for these tissues. *Gluconeogenesis:* During the anabolic phase of metabolism, amino acids from muscle proteins are converted to TCA cycle intermediates (muscle + liver reactions) and then via the TCA cycle to malate. Malate leaves the mitochondrion via a specific transport system and is converted via a series of reactions in the cytoplasm to glucose. *Lipogenesis:* During the anabolic phase of metabolism, the TCA cycle is regulated in such a way as to allow citrate to accumulate and then exit from the mitochondrion on a specific transport system. Once in the cytoplasm citrate is converted to acetyl CoA, the precursor for synthesis of fatty acids and cholesterol. *Protein synthesis:* Mitochondria contain a small DNA molecule ($\sim 10^7$ daltons) which codes for several small hydrophobic proteins essential for anchoring the OS-ATPase, some electron transport chain components, and perhaps transport systems to the inner membrane. These hydrophobic proteins are synthesized within mitochondria on mitochondrial ribosomes by a process which is sensitive to chloramphenicol but not cycloheximide. *Calcium uptake:* In the absence of a permeant anion, mitochondria can take up limited amounts of calcium (membrane loading) via a specific transport system, provided energy is supplied by either substrate oxidation or ATP hydrolysis. In the presence of a permeant anion calcium leaves the membrane and is taken up into the matrix (matrix loading). Provided P_i is the permeant anion, calcium is precipitated as calcium phosphate granules which also contain Mg^{++} and adenine nucleotides. Mitochondria can extrude calcium but the mechanism of this process remains unclear. *Virus formation:* Several reports in the literature indicate that mitochondria may be essential for virus production, in particular the production of certain RNA tumor viruses. *Inset:* The energy-conserving mechanism of mitochondria is shown in greater detail. During electron flow, energy is conserved in the form of a high energy state ($\sim$) which may represent an electrochemical gradient across the inner mitochondrial membrane (as indicated above), a high energy chemical intermediate, or an energized conformation of the inner membrane. This high energy state ($\sim$) can drive the synthesis of ATP, the uptake of Ca^{++}, or the reduction of $NADP^+$. Synthesis of ATP occurs at the level of the OS-ATPase molecule. This molecule consists of three functional units: F_1, a water-soluble component which catalyzes the hydrolysis or the synthesis of ATP; F_0, a proteolipid complex which presumably acts as a channel for protons in the inner membrane (it is this complex which is sensitive to oligomycin and dicyclohexylcarbodiimide [DCCD]), and 'I', a peptide inhibitor of ATP hydrolysis which has been suggested to be a regulatory peptide during ATP synthesis (presumably associated with F_1). ATP once synthesized can be utilized to drive cytoplasmic processes, or the three mitochondrial processes: Ca^{++} uptake, reduction of $NADP^+$, and reverse electron flow. While these latter processes are being driven, the ATPase inhibitor peptide is thought to be dissociated from the OS-ATPase complex.

To some extent the shift in research emphasis away from studies of tumor mitochondria and back to basic research on mitochondria of normal cells was healthy and fruitful. Indeed, it would seem that investigators in the field of bioenergetics learned how little they had actually known about the biochemistry of mitochondria of normal cells. Thus, subsequent studies between 1960 and 1970 revealed that the synthesis of ATP via oxidative phosphorylation is only one of the many important functions of mitochondria of animal cells. (See figure 1 for a diagramatic summary of the major functional activities of mitochondria.)

It is now known, as illustrated in figure 1, that mitochondria participate not only in electron transport coupled to adenosine triphosphate (ATP) synthesis [LARDY and FERGUSON, 1969; HATEFI and HANSTEIN, 1972; SENIOR, 1973; PENEFSKY, 1974; BALTCHEFFSKY and BALTCHEFFSKY, 1974; PEDERSEN, 1975; ERNSTER, 1977; SLATER, 1977; MITCHELL, 1977; RACKER, 1977; CHANCE, 1977; BOYER, 1977; HAROLD, 1977], but, in addition, they accumulate certain cations and anions [LEHNINGER *et al.*, 1967; LEHNINGER, 1970; CARAFOLI, 1974; SCARPA, 1977]; they contain a fully competent genetic apparatus which codes for several hydrophobic proteins [TZAGOLOFF *et al.*, 1973; SCHATZ and MANSON, 1974; KROON and SACCONE, 1974; SACCONE, 1976], and they are essential for both the anabolic and catabolic phases of metabolism [LOWENSTEIN, 1969]. Mitochondria participate in the regeneration of NAD^+ for glycolysis during the catabolic phase of metabolism, and supply the carbon skeleton for carbohydrate, cholesterol, and fatty acid synthesis during the anabolic phase of metabolism. In liver, mitochondria participate also in the synthesis of urea and ketone bodies. Significantly also, mitochondria can 'work in reverse'; that is, they can utilize ATP to either support the formation of NADH via the process of reverse electron flow; to support the formation of NADPH from $NADP^+$ and NADH (transhydrogenase); or to support 'membrane' or 'matrix' loading of calcium (fig. 1, insert). Finally, there are indications that viral replication and/or virus production in some animal cell lines may require mitochondrial participation (see references quoted under section XIV).

Today, students of bioenergetics view the biochemistry of animal cell mitochondria in a much broader context than did their predecessors in the 1950s. Extrapolating this broader biochemical view to cancer cells, it should be appreciated that mitochondria from such cells are now being studied not only with the purpose of establishing why some cancer cells have a *capacity* to exhibit 'high glycolysis', but also to establish to what extent mitochondria may play a role in the normal to neoplastic transition process, in maintaining

or promoting the transformed state, or in the clinical condition known as 'cachexia' (general physical wasting or malnutrition of the cancer patient). Also, the possibility that mitochondria may represent a target for chemotherapy is not without consideration.

What appears to be an unappreciated experimental fact about the bioenergetics of cancer cells *in vitro* and *in vivo* is that such cells (even those with a high glycolytic *capacity*) usually derive a significant fraction of their total ATP from mitochondrial oxidative phosphorylation (see sections VIII and XIII). In fact, some tumor cells *in vitro* have been shown to utilize very little glucose and, instead, rely almost entirely on fatty acids and/or amino acids (i.e., glutamine) as fuel sources (sections VIII and XIII). Thus, as will be emphasized in subsequent sections of this review, it now seems clear that a major source of energy essential for growth and cell division of tumors both *in vitro* and *in vivo* is derived from mitochondrial oxidative phosphorylation as well as from glycolysis.

With these thoughts in mind, it is the purpose of this review article to: (1) supply the reader with an overview of past and ongoing research on tumor mitochondria and their relationship to the overall bioenergetics of cancer cells, and (2) stimulate additional research and funding for this important but rather neglected area.

II. Tumor Lines Available for Mitochondrial Studies

As indicated in figure 2, over 50 experimental tumor lines are available for studies of mitochondrial properties. These tumor lines are usually carried in the rat, the mouse, or in tissue culture. In subsequent sections of this review, reference will be made to some of these tumor lines and the reader may wish to refer to figure 2 and its legend to obtain a rough estimate of the growth rate of a given tumor and its relative degree of differentiation.

Most investigators have chosen the rapidly growing or poorly differentiated tumor lines (lower 'box' in fig. 2) as a source of neoplastic tissue for mitochondrial studies. Such tumor lines include the Ehrlich ascites tumor (carried in the peritoneal cavity of mice), several other mouse or rat ascites tumors, the Walker 256 carcinosarcoma, tumor cells growing in tissue culture, and several Morris hepatomas (usually carried in the hind legs of rats). These tumor lines are deviated rather markedly from their tissues of origin as evidenced by histological, karyotype, biochemical, and morphological analyses. With respect to their bioenergetic properties, it will be emphasized in subsequent sections that this class of tumors is characterized by a *capacity* for 'high glycolysis' (i.e., high lactate production when glucose is present) and a marked reduction in mitochondrial content. The Morris hepatomas are probably the most thoroughly characterized of the 'rapidly growing' tumor class [Aisenberg and Morris, 1961, 1963; Weber and Morris, 1963;

Some available tumor lines.

Slowly growing or highly differentiated tumors

Morris hepatomas: 66, 21, 9618A, 47C, 28A, 7794B

Morris hepatomas: 20, 16, 9618B, 39A, 7787, 6, 8624, 9611B, 9098

Intermediate growth rate or well differentiated tumors

Morris hepatomas: 44, 42A, 9633, R3B, 9633F, 7794A, R-7, 38B, 38A, 8995, 9108, 5123C, 7793, 7316B, 7795, 7800, 9121F, 7316A, R1, 5123A, 9121
Reuber hepatoma: H35

Morris hepatomas: 5123D, 5123B, 5123tc

Rapidly growing or poorly differentiated tumors

Morris hepatomas: 8894, 9618A_2, 3924A, 7288C, H35tc_2. 7288ctc, H35tc_1, 3683F
Ascites tumors: Ehrlich, L1210, Novikoff hepatoma, AH130 hepatoma, AS-30D hepatoma
Walker 256 carcinosarcoma
Hepatocellular carcinoma (HC-252)
Tumor cells in tissue culture

Fig. 2. Tumor lines available for mitochondrial studies. Tumors growing in solid, ascites, or tissue culture form may be classified generally into three major categories: (1) slowly growing (highly differentiated), (2) intermediate growth rate (well differentiated), and (3) rapidly growing (poorly differentiated). The average transfer time is 1.2 months for the poorly differentiated class of solid tumors and 1.5–12.5 months for the well and highly differentiated classes. Tumor cells growing in ascites form have an average transfer

SWEENEY *et al.*, 1963; POTTER, 1964; MORRIS, 1965; NOWELL *et al.*, 1967; NOWELL and MORRIS, 1969; MORRIS and MERANZE, 1974; MORRIS, 1975].

Although rapidly growing, poorly differentiated tumors provide large amounts of experimental material in a short period of time, it is debatable whether they provide the best experimental system for ascertaining the role that mitochondria play in the normal to neoplastic transition. Rather, such tumors are most likely to yield information about the role of mitochondria in promoting or maintaining the malignant state.

At the other extreme are the slowly growing or highly differentiated tumors (upper 'box' in fig. 2). This class of tumors is composed exclusively of the Morris hepatomas. They have been shown to be deviated much less from their tissue of origin (liver parenchymal cell) than the rapidly growing, poorly differentiated tumors (see references cited above). With respect to their bioenergetic properties, it will be noted in subsequent sections that these tumors are characterized usually by a slightly elevated or near normal glycolytic capacity, and a normal or near normal mitochondrial content. The slowly growing, highly differentiated tumors probably represent the best class of experimental tumors for pinpointing changes in mitochondrial structure or function which occur either during or shortly after the normal to neoplastic transition.

In subsequent sections, reference occasionally will be made to mitochondrial studies carried out on regenerating or fetal liver. Regenerating liver is chosen as a source of rapidly proliferating, noncancerous tissue. It serves as an ideal control in hepatoma studies in those cases where the investigator wishes to establish whether a mitochondrial alteration is characteristic of the tumor or simply an alteration induced by rapid cell growth. Fetal liver is chosen to get some indication of whether a given alteration or property found in tumor mitochondria is characteristic also of the fetal state. Much work in recent years has shown that the more poorly differentiated a tumor becomes the more it tends to take on properties of fetal tissue, particularly with respect to its isozyme patterns [WEINHOUSE and ONO, 1972; WEINHOUSE *et al.*, 1972a; IBSEN, 1977] and its bioenergetic properties (see subsequent sections for references). Thus, a preponderance of fetal-like isozymes appears (with a decrease in the level of adult-like isozymes); the *respiratory capacity* of the tumor decreases, as evidenced by a marked reduction in mitochondria, and the *glycolytic capacity* of the tumor increases as evidenced by an enhanced level of glycolytic enzymes and increased lactic acid production.

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time of about a week. Significantly, most of the tumor lines summarized in this figure are known to be malignant, i.e., they metastasize and ultimately kill their hosts. The categorization of Morris hepatomas in this figure is based upon information from MORRIS and MERANZE [1974]. Morris hepatomas are obtained directly from Dr. HAROLD MORRIS (Howard University) or from some other investigator carrying these lines. Rats (usually the Buffalo strain rat) for transplantation purposes can be obtained from Simonson Laboratories, Gilroy, Calif. Ascites tumors are usually obtained from research laboratories known to be carrying such lines (i.e., via the 'grapevine'). Information about animal lines grown in tissue culture can be obtained from the American Type Culture Collection (Rockville, Md.). Finally, a number of mouse tumor lines (i.e., adenocarcinomas of the mammary gland, sarcomas and leukemic tumors) not included in the figure can be obtained from the Jackson Laboratory, Bar Harbor, Me.

Table I. Mitochondrial content of tumors relative to normal tissue

Tumor or rapidly growing tissue	Animal	Mitochondrial content % of normal
Hepatoma (dimethylazobenzene-induced)[a]	mouse	22–33
Hepatoma (Novikoff)[b]	rat	25
Hepatocellular carcinoma (HC-252)[c]	rat	25
Hepatoma 98/15[d]	mouse	35–50
Hepatoma C-57[e]	mouse	40
Hepatoma 3924A[f]	rat	40
Mammary adenocarcinoma[g]	mouse	50
Hepatoma 16[h]	rat	91
Hepatoma 7800[i]	rat	100
Fetal liver[f]	rat	50–60
Regenerating liver[j], [k] (2 days)	rat	85, 66

[a] SCHNEIDER [1946]. [b] HOWATSON and HAM [1955]. [c] CEDERBAUM *et al.* [1976]. [d] SCHNEIDER and HOGEBOOM [1950]. [e] FIALA [1953]. [f] SCHREIBER *et al.* [1970]. [g] MEHARD *et al.* [1971]. [h] LANOUE *et al.* [1974]. [i] PEDERSEN [1972]. [j] ALLARD [1952]. [k] GEAR [1970].

III. Content of Mitochondria in Tumor Cells

The content of mitochondria in a number of different tumor cells is tabulated in table I as *percent of normal.* Some of the values were determined from the amount of nitrogen or protein in the mitochondrial fraction after isolation. Other values were obtained on whole cells or tissues by enzyme assay (cytochrome oxidase or succinic dehydrogenase), from spectral measurements of cytochromes, or by counting mitochondria under the electron microscope.

It is interesting to note that, with the exception of Morris hepatomas 16 and 7800, all tumor lines examined have a marked deficiency of mitochondria ($\leq 50\%$ normal). Although most of the tumors listed are hepatomas, it is noteworthy that the content of mitochondria in a mouse mammary adenocarcinoma is reduced also by 50% when compared with normal mammary tissue. The near normal content of mitochondria in hepatomas 16 and 7800 is of particular interest since it is known that these tumors are relatively slow growing and have a normal or only a slightly elevated glycolytic *capacity* [AISENBERG and MORRIS, 1963; MORRIS, 1965; LANOUE *et al.*, 1974].

Similar to its reduction in rapidly growing tumors, the content of

mitochondria in fetal liver is reduced markedly also. For the 2 cases reported for regenerating liver, however, the content of mitochondria is only moderately reduced (15–34%). These findings would suggest that a reduction in the content of mitochondria in rapidly growing hepatomas is not entirely the result of rapid growth rate but, rather, to some extent a consequence of the normal to neoplastic transition.

Much more work needs to be done to establish why rapidly growing tumors almost without exception have a 50% or more reduction in mitochondria. One possibility seems to be that the normal to neoplastic transformation process results in a transition from a normal to a fetal-like mitochondrial biogenesis and/or degradation pattern, particularly in rapidly growing, highly glycolytic tumors.

Finally, it should be appreciated that many tumors other than those summarized in table I have been shown to have a low mitochondrial content [WENNER and WEINHOUSE, 1953; AISENBERG, 1961; WENNER, 1967; COLOWICK and NAGARAJAN, 1972][2].

In a subsequent section (section XII) the possible relationship of the low mitochondrial content of rapidly growing tumors to their *capacity* for high glycolysis is considered.

[2] It is important to emphasize that before 1959 many investigators were in general agreement that the content of mitochondria in the rapidly growing, highly glycolytic class of tumors was markedly reduced. In fact, enzymatic data on this point was presented as early as 1950 by POTTER *et al.* In 1959, however, CHANCE and HESS showed on the basis of spectral measurements that the cytochrome content of Ehrlich ascites cells is in the same range as that of muscle and yeast. Unfortunately, these studies were taken by some [AISENBERG, 1961] to suggest that the mitochondrial content of the rapidly growing, highly glycolytic class of tumor cells may be normal, a suggestion which may have distracted some investigators (in view of the anti-Warburg sentiment at the time) from more thoroughly investigating the metabolic significance of the marked reduction in mitochondria in rapidly growing tumors. Significantly, in preparing this review article, the author was able to find more than 20 literature references showing that the mitochondrial content of rapidly growing, highly glycolytic tumors is markedly reduced relative to their tissue of origin, but was unable to find a single literature reference showing that such tumors have a normal mitochondrial content when compared carefully with their tissue of origin. Therefore, although it may well be true that the endogenous or glucose-supported oxygen consumption rates of 'highly glycolytic' tumors are in the same range as those of a variety of normal tissues [WEINHOUSE, 1956, 1976], the reader should bear in mind that the *total respiratory capacity* of such tumors (in terms of mitochondrial content, i.e., in terms of the total DNP-stimulated or state III rates which may be exhibited by these mitochondria) may well be reduced markedly when comparisons are made directly with the *known tissue of origin*. This point seems to have been 'glossed over' in many writings about the respiratory properties of highly glycolytic tumor cells.

IV. Preparation of Tumor Mitochondria

It is essential to work with mitochondrial preparations which can be considered ultrastructurally and functionally intact (or as ultrastructurally and functionally intact as possible) in order to obtain convincing and reliable results in most studies involving tumor mitochondria. Mitochondria should normally show continuous inner and outer membranes, and a densely staining matrix space when examined under the electron microscope (i.e., after glutaraldehyde fixation, positive staining, and thin sectioning) to be considered *ultrastructurally intact*. It is likely that the method used for preparation is too harsh when freshly isolated mitochondria appear swollen, exhibit lightly staining matrices, and/or have some inner and outer membrane damage. Mitochondria should, in most cases, exhibit acceptor control of respiration to be considered *functionally intact*. That is, they should respire slowly in the presence of an oxidizable substrate (state IV rate), respire rapidly for a brief period of time when limiting amounts of the phosphate acceptor ADP are added (state III rate) and, finally, return to the slow state IV rate when the ADP is converted to ATP [CHANCE and WILLIAMS, 1955]. The ratio, state III rate/state IV rate is called the *acceptor control ratio*. Respectable values for rat liver mitochondria are ≥ 5 when succinate or β-hydroxybutyrate is substrate. [For a review summarizing exact details for the preparation and characterization of mitochondria from liver, Morris hepatomas, and ascites tumor cells, see PEDERSEN *et al.*, 1977.]

Unfortunately, there is no generally applicable procedure for the preparation of ultrastructurally and functionally intact tumor mitochondria [DEVLIN, 1967; SORDAHL and SCHWARTZ, 1971; PEDERSEN *et al.*, 1977; KASCHNITZ *et al.*, 1977]. Suffice it to say that, for every tumor to be studied for the first time with respect to its mitochondrial properties, preliminary experiments should be carried out to define the most optimal preparative conditions. Accordingly, the information on preparation media summarized in table II should provide the new investigator with two helpful suggestions. First, it should be possible to disrupt the cell membrane by homogenization with a Teflon pestle-glass homogenizer when the tumor tissue of choice appears soft. However, if the tumor tissue of choice appears hard (as is the case for many rapidly growing tumors), or if ascites cells are used, it may be necessary to pretreat the cells with a proteolytic enzyme such as Nagarse prior to the homogenization step. Secondly, for reasons which are not completely clear, some tumor homogenates appear to contain uncoupling agents and/or membrane-damaging agents (perhaps free fatty acids, lysolecithin, and/or divalent cations). Therefore, in addition to the usual isotonic sucrose or mannitol medium used to prepare mitochondria from normal tissue, it is advisable in the case of tumor mitochondrial isolation procedures to include also bovine serum albumin (BSA), preferably fatty acid-deficient or free, and EDTA or EGTA.

With respect to the latter suggestion, it is not the author's intention to imply that the presence of uncoupling agents or membrane-damaging agents in tumor homogenates is the only reason why 'intact' tumor mitochondria are frequently more difficult to prepare than 'intact' control mitochondria. As will be indicated below and in sections to follow, certain tumor mitochondria, because of membrane compositions which are different from control mitochondria, may be either more fragile or more stable than mitochondria from control tissues. Should they be more fragile, BSA and chelating agents may have a stabilizing effect on their structure. For example, WHITE and TEWARI [1973] have shown that, during

Table II. Methods of cell disruption and isolation media used for preparation of tumor mitochondria with acceptor control of respiration

Method of cell disruption	Tumors studied	Isolation media
Homogenization with glass beads[a]	Ehrlich ascites (mouse)	0.25 M mannitol, 0.001 M EDTA, and 0.017–0.05 M triethanolamine buffer, pH 7.4.
Homogenization with Dounce homogenizer[a]	Ehrlich ascites (mouse)	0.25 M sucrose containing 0.001 M EDTA, pH 4–5
Nagarse followed by homogenization with Teflon pestle-glass homogenizer[b, c]	Ehrlich ascites, L-1210 ascites, AS-30D hepatoma (mouse, rat)	0.22 M mannitol, 0.07 M sucrose, 0.002 M HEPES, 0.05% BSA, pH 7.4
Homogenization with Teflon pestle-glass homogenizer[d]	Mammary adenocarcinoma (mouse)	0.25 M sucrose, 0.001 M Tris-HC1, 0.001 M EDTA, 1% BSA, pH 7.2–7.4
Homogenization with Teflon pestle-glass homogenizer[e]	Hepatocellular carcinoma (HC-252) (rat)	0.25 M sucrose, 0.01 M Tris-HC1, 0.001 M EDTA, 1% BSA, pH 7.4
Homogenization with Teflon pestle-glass homogenizer[b, f]	Morris hepatomas 9618A, 16, 7800 and 3924A (rat)	0.22 M mannitol, 0.07 M sucrose, 0.002 M HEPES, 0.05% BSA, pH 7.4
Homogenization with Teflon pestle-glass homogenizer[g]	Yoshida hepatoma AH-130, Morris hepatoma 5123 (rat)	0.22 M mannitol, 0.07 M sucrose, 0.02 M Tris-HC1, 0.002 M Tris-EDTA, 0.2% BSA, pH 7.4
Nagarse followed by homogenization with Teflon pestle-glass homogenizer[h, i]	Morris hepatomas 3683, 7777, 3924A, 5123D, and 7800 (rat)	0.22 M mannitol, 0.07 M sucrose, 0.002 M HEPES, pH 7.4 (1 or 2% BSA depending on the step) 1% BSA, pH 7.4

[a] WU and SAUER [1967]. [b] PEDERSEN and MORRIS [1974]. [c] PEDERSEN *et al.* [1977]. [d] SORDAHL and SCHWARTZ [1971]. [e] CEDERBAUM *et al.* [1976]. [f] PEDERSEN *et al.* [1970]. [g] FEO *et al.* [1973]. [h] KASCHNITZ *et al.* [1976]. [i] KASCHNITZ *et al.* [1977].

isolation, Novikoff hepatoma mitochondria bind significant amounts of BSA (~2% of the total mitochondrial protein in the mitochondrial fraction is BSA), and that in a sucrose gradient the presence of BSA increases the lower banding density of the tumor mitochondria to a normal density.

As emphasized in the previous section, the content of mitochondria in most rapidly growing tumors is low. Consequently, the yields of mitochondria from such tumors are much lower than those obtained from control tissues. In order to maximize mitochondrial yields, it is advisable to wash the nuclear fraction several times [BUSTAMANTE *et al.*, 1977; PEDERSEN *et al.*, 1977].

Although mitochondria prepared by homogenization-differential centrifugation techniques may appear ultrastructurally and functionally intact, they almost assuredly contain some contamination by lysosomes and perhaps other subcellular fractions as well. If the investigator is interested in obtaining more homogenous mitochondria, the recent review by the author and his colleagues should be consulted [PEDERSEN *et al.*, 1977]. Very briefly, however, it should be noted that mitochondria can be further purified in one of two ways to remove much of the microsomal contamination. First, it may be possible to remove much of the contamination from the mitochondrial fraction with retention of acceptor control of respiration. This is accomplished by treating the freshly isolated mitochondrial fraction with a very low concentration of digitonin (i.e., a concentration much lower than that necessary to remove the outer membrane) followed by sedimentation [LOWENSTEIN *et al.*, 1970; SCHNAITMAN and GREENAWALT, 1968; PEDERSEN *et al.*, 1977]. Secondly, the mitochondrial fraction can be subjected to centrifugation in sucrose gradients [PEDERSEN *et al.*, 1977]. Although this technique is effective in removing much of the microsomal contamination, it is not as effective as the digitonin method in removing lysosomes. Moreover, liver mitochondria may lose most of their acceptor control of respiration after sedimentation in sucrose gradients. Significantly, however, some slowly growing tumors retain acceptor control of respiration after sedimentation in sucrose gradients, i.e., hepatomas 5123, 7800, and perhaps 16 [PEDERSEN *et al.*, 1977]. For reasons which are unclear but which are most likely related to differences in membrane and/or matrix composition, mitochondria from such hepatomas may be more resistant to the hydrostatic pressure occurring during sedimentation in sucrose gradients than are mitochondria from control liver [WATTIAUX *et al.*, 1971; WATTIAUX, 1974].

In summary, it can be stated that mitochondria which exhibit acceptor control of respiration can be prepared from most tumors studied to date by applying standard homogenization-differential centrifugation techniques. In some cases, it is desirable to utilize the proteolytic enzyme Nagarse prior to the homogenization step to break down the cell membrane. Unlike the preparation of mitochondria from control tissues, however, the isolation medium for tumor mitochondria usually requires EDTA and/or BSA (for reasons which are not completely clear) in addition to the usual isotonic sucrose or sucrose-mannitol components. Also, yields of mitochondria from many tumor tissues are lower than yields from control tissues because of the lower content of mitochondria in many tumors. Yields can be maximized by washing the nuclear fraction several times. Finally, tumor mitochondria, similar to control mitochondria, can be further purified by mild digitonin treatment, or by sedimentation in sucrose gradients in order to remove contamination by other subcellular fractions.

V. Morphology, Ultrastructure, and Configurational Changes

Several investigators have examined tumor mitochondria *in situ* in the electron microscope [DALTON, 1964; HRUBAN *et al.*, 1965, 1966, 1973; HACKENBROCK *et al.*, 1971; GALEOTTI *et al.*, 1974; WHITE *et al.*, 1974] (see fig. 3A–D for the author's pictorial summary of these studies). In the early study of HRUBAN *et al.* [1965], mitochondria from the following hepatomas

were examined *in situ:* Novikoff (10–21 days), Morris 3683 (12–20 days), low catalase (19–42 days), high catalase (18–42 days), Reuber H35 (28–71 days), and Morris 5123 (30–69 days). These investigators noted that mitochondria of each hepatoma have a characteristic size and shape. In the rapidly growing tumors, mitochondria are usually smaller with few cristae. In the slow-growing tumors, mitochondria tend to be larger, with characteristics closely resembling those of normal hepatocytes or with cristae densely packed in central regions. These observations are generally supported by other *in situ* studies [DALTON, 1964; WHITE *et al.*, 1974]. DALTON [1964] notes that intermediate or slowly growing Morris hepatomas such as 5123B, 7794B, and 7787 have mitochondria which are similar to hepatic mitochondria, whereas mitochondria from the rapidly growing 3683 hepatoma are small and sometimes exhibit longitudinal cristae. In addition, WHITE *et al.* [1974] show that *in situ* mitochondria of the Novikoff hepatoma have a deficiency of cristae. There are exceptions to these generalizations [DALTON, 1964; HRUBAN *et al.*, 1973]; for example, mitochondria from the slowly growing tumor 7787, rather than exhibiting the appearance of normal hepatoma mitochondria, have few cristae which extend into the central matrix [DALTON, 1964].

The observations noted above, that tumor mitochondria have a characteristic size and shape, are supported by sedimentation and electron microscopic studies carried out by CORNBLEET *et al.* [1974] and by MARTIN *et al.* [1974]. By these techniques, it was concluded that a significant proportion of the mitochondria isolated from Morris hepatomas 16 and 21 are respectively smaller and larger in size than those from normal liver. In addition, results from enzyme distribution studies as well as sedimentation studies indicate that more mitochondria of Morris hepatoma R3B are larger in size and/or more dense, whereas those of Morris hepatoma 7794A are smaller in size and/or less dense than host (or normal) liver mitochondria.

Mitochondria *in situ* in Ehrlich ascites tumor cells [HACKENBROCK *et al.*, 1971] and in Morris hepatoma 3924A [GALEOTTI *et al.*, 1974], both rapidly growing tumors, have been shown to undergo the orthodox → condensed configurational changes reported earlier by HACKENBROCK [1966] for isolated mouse liver mitochondria (fig. 3C). Such changes are induced in isolated mouse liver mitochondria by the addition of ADP (to the respiring mitochondria) to initiate oxidative phosphorylation. In Ehrlich ascites tumor cells, orthodox → condensed configurational changes are induced by 2-deoxyglucose (DOG), an inhibitor of glycolysis which allows oxidative phosphorylation to proceed via ADP produced at the level of the hexokinase step:

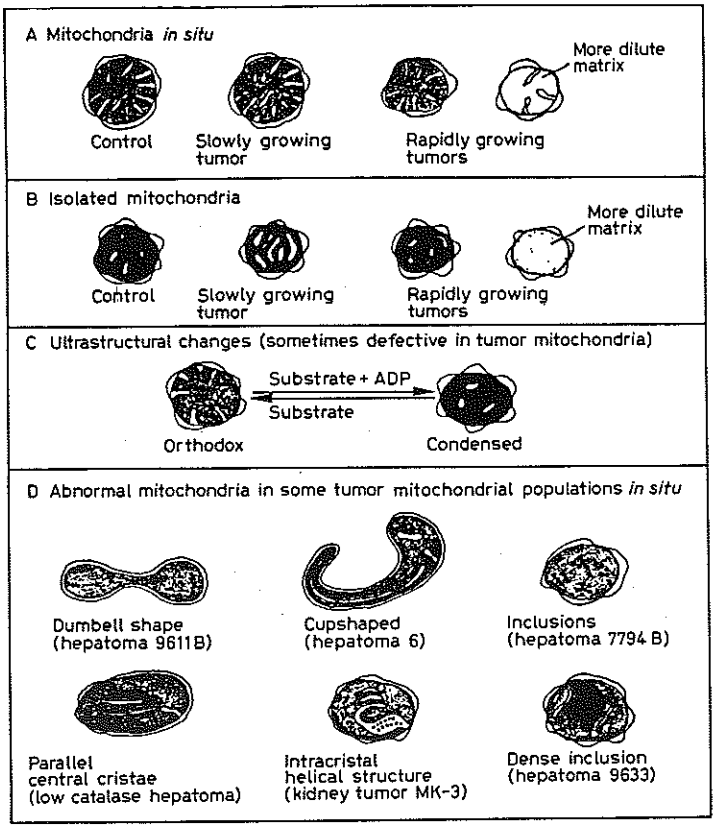

Fig. 3. A Mitochondria *in situ*. Mitochondria are usually in what HACKENBROCK coined the 'orthodox configuration' [HACKENBROCK *et al.*, 1971; GALEOTTI *et al.*, 1974]. The matrix stains lightly and the cristae can be seen to be associated with the inner membrane. Mitochondria from slowly growing tumors and from some rapidly growing tumors are similar in appearance to control (normal) mitochondria. However, mitochondria from other rapidly growing tumors have few cristae and even more lightly staining matrices. *B* Isolated mitochondria. Control mitochondria as isolated are usually characterized by very densely staining matrices and are said to be in a 'condensed configuration' [HACKENBROCK, 1966]. Freshly isolated mitochondria from slowly growing tumors also have densely staining matrices whereas mitochondria from rapidly growing tumors, depending on the tumor and/or method of isolation, may have either densely staining or lightly staining matrices. *C* Ultrastructural changes. (1) *In situ:* Mitochondria from the two rapidly growing tumors, Ehrlich ascites and Morris hepatoma 3924A have been shown to undergo orthodox to condensed configurational changes [HACKENBROCK *et al.*, 1971; GALEOTTI *et al.*, 1974]. (2) *In vitro:* Coupled mitochondria from other rapidly growing tumors have been shown to undergo either no configurational change or to undergo irreversible changes

DOG + ATP ⇌ DOG-6-P + ADP. In Morris hepatoma 3924A cells, orthodox → condensed configurational changes are induced by increasing the temperature from 1 to 38 °C.

In situ studies have revealed also that some mitochondria within the mitochondrial population of certain tumors are abnormal [HRUBAN *et al.*, 1965, 1966, 1973, unpublished observations]. Either they exhibit unusual shapes (dumbbell or cup) or they contain unusual cristae or inclusions (fig. 3D).

Finally, several studies have been carried out to examine the ultrastructure of freshly isolated tumor mitochondria and to ascertain whether such mitochondria undergo configurational changes characteristic of normal mitochondria (i.e., whether they can undergo *orthodox-condensed* configurational cycles as detected by electron microscopy, or *swelling-contraction* cycles as detected by optical density measurements) (fig. 3B). Mitochondria from Morris hepatoma 9618A, Morris hepatoma 7800, and a mammary adenocarcinoma (slow, intermediate, and rapid growth rate tumors, respectively) exhibit normal or near normal morphology [PEDERSEN *et al.*, 1970; MEHARD *et al.*, 1971]. On the other hand, mitochondria from the rapidly growing hepatomas 3924A and Novikoff have dilute-appearing (lightly staining) matrices with few cristae [PEDERSEN *et al.*, 1970; WHITE and TEWARI, 1973]. Significantly, mitochondria from hepatoma 3924A were prepared in the same BSA-containing medium as mitochondria from hepatomas 9618A and 7800.

Most studies carried out to examine the ability of tumor mitochondria to undergo configurational changes or to swell have been confined to rapidly growing tumors. SORDAHL *et al.* [1969] examined the ability of mitochondria from two different mouse mammary adenocarcinomas and a rat hepatoma to undergo orthodox to condensed configurational changes. Despite the fact that these mitochondria, when prepared in BSA-containing media, exhibit acceptor control of respiration, they fail to undergo complete orthodox → condensed cyclic transitions. Moreover, MARTIN *et al.* [1974] showed that

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(i.e., the condensed configuration can be converted to the orthodox but the reverse process does not occur [SORDAHL *et al.*, 1969]). *D* Abnormal mitochondria in some tumor mitochondrial populations. HRUBAN *et al.* [1965, 1972, 1973, unpublished observations] examined most of the Morris tumors *in situ* in the electron microscope. As indicated some populations of tumor mitochondria contain mitochondria of abnormal shape or mitochondria containing unusal cristae, helical-like structures, or inclusions. The author is grateful to Dr. ZDENEK HRUBAN of the University of Chicago for supplying the electron micrographs from which these drawings were made.

mitochondria from Morris hepatoma 21 have the expected orthodox and condensed conformations during state IV and state III respiration, respectively. However, when changes in fluorescence of the probe 8-anilino-1-naphthalene sulfonic acid were studied, the addition of uncoupler to the energized tumor mitochondria (Morris hepatomas 21 or 16) failed to result in an increase in fluorescence as observed for control mitochondria. The authors interpret these results, together with results from detergent-induced swelling studies, to reflect organizational differences at the membrane level for Morris hepatomas 21 and 16.

Both ARCOS [1971] and FEO and co-workers [FEO, 1967; FEO *et al.*, 1970, 1973a–c; FEO and MATLI, 1970] have emphasized that many tumor mitochondria have abnormal swelling properties relative to control mitochondria. Thus, in contrast to normal liver mitochondria, mitochondria from the AH130 Yoshida ascites hepatoma (which exhibit high acceptor control ratios) appear spontaneously swollen [FEO and MATLI, 1970]. Little additional swelling can be induced by swelling agents. Also, in contrast to normal liver mitochondria, contraction can be induced by the addition of Mg^{++} without ATP. Mg^{++} is evidently passively accumulated by the tumor mitochondria and a fraction of the total is irreversibly bound to the mitochondrial inner membrane. Additional studies by FEO *et al.* [1970, 1973a–c] show that rat liver and AH130 hepatoma mitochondria respond differently to the damaging action of the proteolytic enzyme trypsin, suggesting differences in membrane structure and composition.

Work reviewed in this section suggests that many tumor mitochondria (especially those from rapidly growing tumors) differ from normal mitochondria in their morphology, ultrastructure, and ability to undergo configurational changes. Such studies are consistent with the view that many tumor mitochondria differ from normal mitochondria in their membrane and/or matrix compositions. Support for this view is summarized in subsequent sections of this review.

VI. Membrane Proteins

Previous sections of this review have emphasized that many investigators believe that there may be alterations in membrane composition and/or structure in many tumor mitochondria. Work summarized in subsequent sections of this review tends to reinforce this view. It is significant, then, that in the few studies where inner membrane proteins of control and tumor

Table III. Differences in protein subunit composition of the inner mitochondrial membrane of control and hepatoma tissue

	Number of subunits				
		differences	present *in control*	present *in hepatoma*	
Hepatoma(s) Studied	total in control[e]	control vs. hepatoma	but low or absent in hepatoma	but low or absent in control	Comment
7800[a]	23	6	3 (82, 58, 46)[d]	1 (75, 50)[d]	the 58,000-dalton protein has a mobility near that of the B subunit of F_1-ATPase
3924A, 9098, H35, H35-tc2[b]	12–15	2	1 (55–60)[d]	1 (35–50)[d]	the 55,000–60,000-dalton protein appears to have a mobility corresponding to the B subunit of F_1-ATPase
3924A, 20[c]	Not given	2–3	Not given	Not given	the 2–3 differences noted are in mitochondrially synthesized protein

[a] PEDERSEN *et al.* [1971].
[b] CHANG *et al.* [1971].
[c] IRWIN and MALKIN [1976].
[d] Number in parentheses represents approximate subunit molecular weight $\times 10^{-3}$.
[e] It should be noted that the total number of proteins in control membranes are those detected by staining with Coomassie Brilliant Blue and/or Amido Schwartz. Proteins present in the membrane in very low concentration, such as transport proteins, would go undetected by this method. Also, both the studies of PEDERSEN *et al.* [1971] and CHANG *et al.* [1971] were carried out with inner membrane + outer membrane proteins. However, since the outer membrane constitutes only about 4–6% of the mitochondria from liver, whereas the inner membrane constitutes 19–25%, it seems unlikely that any of the major protein bands detected are outer membrane proteins.

mitochondria have been examined by electrophoresis in sodium dodecyl sulfate (SDS), several notable differences have been observed. Thus, as indicated in table III, CHANG *et al.* [1971] concluded, after examining delipidated inner membranes of 5 different hepatomas, that two major differences exist in the subunit (polypeptide) compositions of control and tumor membranes. First, a major protein band (estimated by the author to

have a mobility near or equal to that of the B subunit [57,000 daltons] of the F_1-ATPase [CATTERALL and PEDERSEN, 1971; CATTERALL *et al.*, 1973] is deficient or absent in all hepatoma lines examined. Secondly, there appears to be a smaller protein band ($\sim$ 50,000 daltons) present in the tumor membranes that is absent from control membranes.

Studies carried out by PEDERSEN *et al.* [1971] on hepatoma 7800 show greater resolution of inner membrane proteins than do the studies of CHANG *et al.* [1971], in that 23 total subunit protein bands rather than 12–15 are observed upon SDS gel electrophoresis. A total of 6 membrane subunit differences is observed between control and 7800 membranes. Three of these involve subunit proteins which are present in control membranes but low or absent in the tumor membranes, whereas two others involve subunit proteins which are present in the tumor but low or absent in the control. As in the studies of CHANG *et al.* [1971], a protein band of about 58,000 daltons is missing from the inner membrane of hepatoma mitochondria. However, protein bands corresponding to the A and B subunits of the mitochondrial F_1-ATPase appear to be present in 7800 membranes. The mobility of the missing 58,000-dalton protein appears to fall between the mobility of the A and B subunits.

KOLAROV *et al.* [1973] compared submitochondrial particles of liver, Ehrlich ascites carcinoma, and Zajdela hepatoma with respect to their SDS gel electrophoretic profiles. Although they note that there are significant (unspecified) differences between the polypeptide profiles of tumor and liver submitochondrial particles, they indicate that submitochondrial particles from the two tumors have essentially identical polypeptide patterns.

SENIOR *et al.* [1975] examined the inner membrane profiles of mitochondria isolated from rat mammary gland and from mammary tumor R3230AC. They note several differences in the SDS gel electrophoretic profiles between normal and tumor mitochondria. In particular, one of these differences is a polypeptide in the 50,000 molecular weight range which the authors suggest may reflect a change in the amount or nature of the F_1 component of mitochondrial ATPase.

Finally, IRWIN and MALKIN [1976] labeled the proteins synthesized by control liver and hepatoma mitochondria with ^{35}S-methionine. Interestingly, they find 2–3 differences in labeled subunit proteins between control mitochondria and mitochondria from hepatomas 3924A and 20. Mitochondria are known to code for and synthesize several hydrophobic proteins which constitute parts of the oligomycin-sensitive ATPase complex (OS–F_1), cytochrome oxidase, and the cytochrome b-c_1 complex [TZAGOLOFF *et al.*,

1973; SCHATZ and MANSON, 1974; KROON and SACCONE, 1974; SACCONE, 1976]. Therefore, an extension of IRWIN and MALKIN's [1976] work would be of great interest in order to ascertain to what extent hydrophobic protein production by mitochondria has been altered by the normal to neoplastic transition.

The author is unaware of any studies in which the subunit composition of outer membrane, intermembrane space, and matrix proteins have been carefully analyzed in control and tumor mitochondria by gel electrophoresis in SDS. Suffice it to say, however, that some tumor mitochondria are deficient in certain outer compartment enzymes such as monoamine oxidase and adenylate kinase. Also, several tumors examined to date have been shown to be markedly deficient in the matrix enzyme glutamate dehydrogenase (section X).

VII. Lipid Composition

The limited number of studies in which the lipid content and/or composition of tumor mitochondria have been examined tend to support the general thesis that mitochondria from tumor cells have altered membrane compositions [RUGGIERI and FALLANI 1968a,b; BERGELSON *et al.*, 1970; FEO *et al.*, 1973a–c, 1975; PANI *et al.*, 1973a, b; MORTON *et al.*, 1976; HOSTETLER *et al.*, 1976; REITZ *et al.*, 1977]. FEO *et al.* [1975] find that the cholesterol to phospholipid ratio is higher in mitochondria from hepatomas AH130, 3924A, and 5123 than in mitochondria from control liver. The inner mitochondrial membrane becomes more enriched in cholesterol than does the outer membrane. The amount of additional cholesterol found in tumor mitochondria is quite striking (i.e., about fourfold higher in hepatomas; compare ~ 20 μg/mg protein in hepatoma 3924A mitochondria to ~ 5 μg/mg protein in liver mitochondria), and does not seem to be accounted for by increased contamination by other membrane-containing fractions. The phospholipid content of tumor mitochondria either remains constant (AH 130 hepatoma) or decreases (5123 hepatoma) [PANI *et al.*, 1973a].

Additional data of FEO *et al.* [1975] indicate that an increase in the cholesterol to phospholipid ratio of hepatoma mitochondria seems to be associated (at least in part) with the decreased ability of these organelles to undergo P_i-induced swelling. Thus, cholesterol to phospholipid ratios are significantly correlated to the extent of large amplitude and small amplitude swelling. This is also the case with regard to both extent and initial rate of the

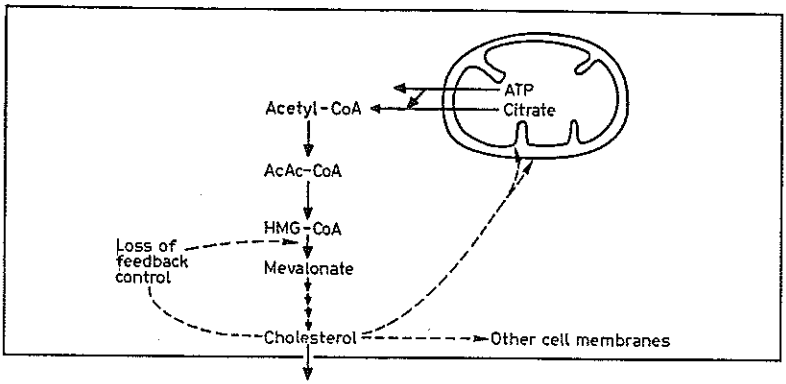


Fig. 4. Relationship of cholesterol biosynthesis to mitochondrial metabolism in tumor cells. Similar to normal cells, tumor cells require mitochondrial ATP and citrate for the biosynthesis of cholesterol. In contrast to normal cells, and for reasons that are not clear, the *in vivo* feedback mechanism for regulation of cholesterol biosynthesis is lost in all tumor cells examined to date. Mitochondria from such tumor cells contain an enhanced level of cholesterol in both their inner and outer membranes which may be a consequence of the loss of feedback regulation of cholesterol synthesis. AcAc-CoA = Acetoacetyl-CoA; HMG-CoA = β-hydroxy-β-methylglutaryl-CoA.

absorbance decrease which accompanies valinomycin-induced K^+ uptake by mitochondria. However, the decreased capacity of tumor mitochondria to swell does not appear to be related solely to alerations in cholesterol content. Cholesterol-enriched liver mitochondria undergo only a partial reduction in P_i-induced or valinomycin-K^+-induced swelling. It seems likely, therefore, that altered membrane-dependent properties of tumor mitochondria are related to alterations in both membrane lipid and protein composition (see previous section).

FEO *et al.* [1973a–c] suggest that the increased content of cholesterol in tumor mitochondria may be related to the loss of feedback inhibition of cholesterol synthesis reported by SIPERSTEIN *et al.* [1966] (fig. 4). This is an interesting suggestion and certainly should be followed up with more experimentation, especially in view of the fact that loss of cholesterol feedback control now seems to be a more characteristic phenotype of hepatoma cells than does the *capacity* for a high rate of glycolysis.

Thus, the defective feedback inhibition of cholesterol biosynthesis by dietary cholesterol has now been reported for all hepatomas tested *in vivo*, including mouse tumors, 19 rat hepatomas (both minimal deviation and poorly differentiated), several aflatoxin-induced trout hepatomas and two human hepatomas [see WALLACH, 1975a, for a general discussion of this subject].

As indicated above, the phospholipid content of tumor mitochondria from AH130 is unchanged, whereas that of Morris hepatoma 5123 is lower than that of control liver mitochondria. In contrast, the total phospholipid content of mitochondria from Morris hepatoma 7777, a very rapidly growing hepatoma, is increased by 50–75% when compared to control liver [HOSTETLER *et al.*, 1976; REITZ *et al.*, 1977]. The amount and chemical composition of individual mitochondrial phospholipids in hepatoma 7777 show some striking differences from phospholipids isolated from control tissue. For example, MORTON *et al.* [1976] report that cardiolipin is reduced, while diacylglycerolphosphorylserine and monoacylglycerolphosphorylethanolamine are elevated. In addition, the level of polyunsaturated fatty acids in the mitochondrial phospholipids is lowered in hepatoma 7777, whereas there is an increase in the level of monounsaturated fatty acids. There is a relatively high level of oleic acid in position 2 of hepatoma phospholipids. RUGGIERI and FALLINI [1968a,b] also report that hepatomas show a decrease in polyunsaturated acyl chains.

In following up these studies, REITZ *et al.* [1977] show that in hepatoma 7777 the acyl coenzyme A: 1-acyl glycerophosphorylcholine acyl transferases, which aid in controlling the fatty acid composition of phospholipids, are markedly increased in activity toward both polyenoic as well as monoenoic fatty acids. These authors therefore suggest that the polyenoic acids are not available for use in the resynthesis of the phosphatidylcholines in the tumor.

The phospholipid patterns of tumor mitochondrial membranes, in analogy to normal controls, appear to be distinctly different from the phospholipid patterns of other cellular membranes (although there is one report [BERGELSON *et al.*, 1970] which suggests that the phospholipid patterns of hepatoma 27, mouse hepatoma 22, and Zajdela hepatoma are the same as other cell membranes).

In summary, it appears that those tumor mitochondrial membranes examined to date have elevated levels of cholesterol, a normal, reduced, or increased total phospholipid content and significant changes in the content of individual phospholipids with a decrease in polyunsaturated acyl chains, and an increase in monounsaturated acyl chains. The phospholipid distribution pattern is different from other cellular membranes for most cases studied.

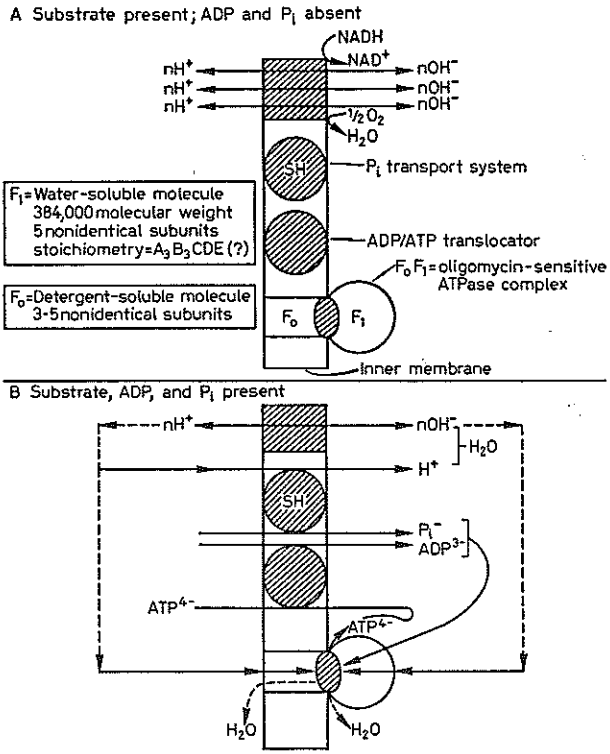


Fig. 5. Current view of the interrelationships among electron transport, ion transport, and ATP synthesis in mitochondria. *A* Substrate present: ADP and P_i absent. During electron transport an electrochemical gradient of protons is established across the inner mitochondrial membrane and comes to equilibrium with the electron transport process (for details of the electron transport chain see figure 7). Backflow of protons through the ATPase system and the P_i transport system does not occur in the absence of ADP and P_i. The rate of respiration under these conditions is referred to as the *state IV* rate. When NADH is substrate, protons are translocated from the matrix to the cytoplasmic surface at three regions along the electron transport chain. These three regions correspond to the known regions along the electron transport chain where sufficient energy is conserved to drive the synthesis of ATP. The H^+/O site ratio and therefore the H^+/ATP ratio may be as high as 4 [REYNAFARJE *et al.*, 1976] giving a total of 12 H^+/O for electron flow from NADH to molecular oxygen. The molecular details involved in the establishment of the electrochemical gradient of protons across the mitochondrial inner membrane are for the most part unknown. *B* Substrate, ADP, and P_i present. The electrochemical gradient of protons is used to drive the influx of P_i^- on a specific H^+/P_i^- symport system, and the uptake of ADP^{3-} on a specific ADP^{3-}/ATP^{4-} antiport system. The driving force for this process is the formation of H_2O as the electrochemical gradient of protons is collapsed. Charge neutrality is maintained with four net charges moving into the matrix (P_i^- and

VIII. Respiration, Oxidative Phosphorylation, and Acceptor Control[3]

Since WARBURG [1930] first suggested that the high glycolytic rate of many tumor cells is a result of impaired respiration, many investigators have focused their attention on the respiratory properties of tumor cells and, more specifically, on the respiratory properties of mitochondria therefrom [see AISENBERG, 1961 for a review of work before that date; see WALLACH, 1975b

[3] Prior to reading this section, it may be helpful to the new investigator to examine figures 5 and 7 and the legends to these figures in order to obtain an overview of the electron transport chain as well as current thoughts on how electron transport may be coupled to ATP synthesis. In brief, it should be appreciated that ATP synthesis takes place at the level of the oligomycin-sensitive ATPase complex (F_0F_1 or H^+-translocating ATPase), and that the primary driving force for ATP synthesis is thought to be an electrochemical gradient of protons established across the mitochondrial inner membrane by the electron transport chain [MITCHELL, 1966, 1977]. The electron transport chain is now known to be a system which translocates protons from the matrix to the cytoplasmic surface of the mitochondria. Although the exact nature of the coupling of this gradient to ATP synthesis at the level of the oligomycin-sensitive ATPase complex remains unknown (e.g., are chemical intermediates or conformational changes involved?), it is known that rendering the inner membrane 'leaky' to protons results in low acceptor control ratios and a reduced efficiency (or total inhibition) of ATP synthesis. Therefore, the rates of electron transport and the relative magnitudes of the acceptor control ratio and the P/O ratio are now thought to be dependent directly on the capacity of the inner mitochondrial membrane to maintain an electrochemical gradient of protons.

ADP^{3-}) and four net charges moving out of the matrix (ATP^{4-}). The electrochemical gradient of protons is thought to drive also the synthesis of ATP at the level of the oligomycin-sensitive ATPase (OS-ATPase, F_0F_1 complex, or H^+-translocating ATPase). Again the driving force for the process is the formation of water. As many as 3 protons/ATP may be involved at the level of the ATPase step (BRAND and LEHNINGER, 1977]. Although the molecular components of the oligomycin-sensitive ATPase system of liver and heart are known [for reviews see SENIOR, 1973; PENEFSKY, 1974; PEDERSEN, 1975], the molecular details associated with coupling the electrochemical gradient of protons to the synthesis of ATP remain unknown.

The rate of respiration when ADP, P_i, and oxidizable substrate are present is referred to as the *state III rate*. The state III rate divided by the state IV rate is called the *acceptor control ratio*. It should be clear from the diagram that both oxidative phosphorylation and the acceptor control properties of intact mitochondria are directly related to the capacity of the inner mitochondrial membrane to maintain an electrochemical gradient of protons. Proton leaks presumably result in low acceptor control ratios and a reduced efficiency of ATP synthesis.

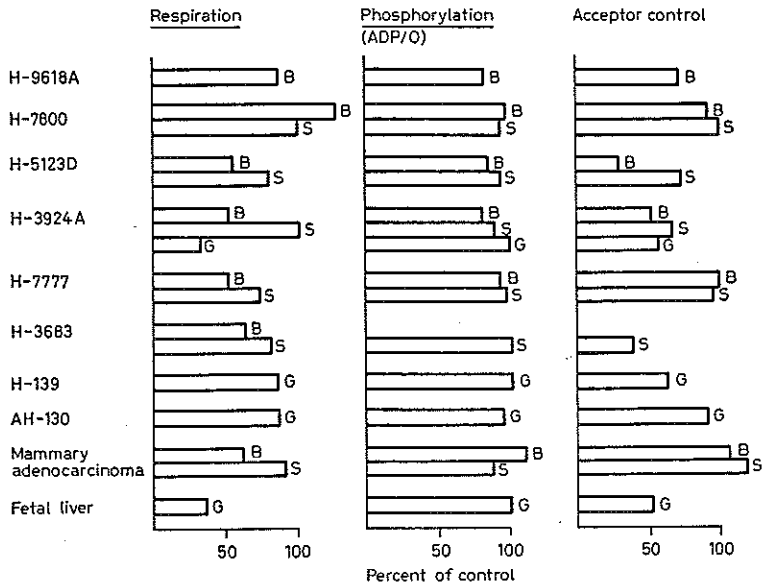
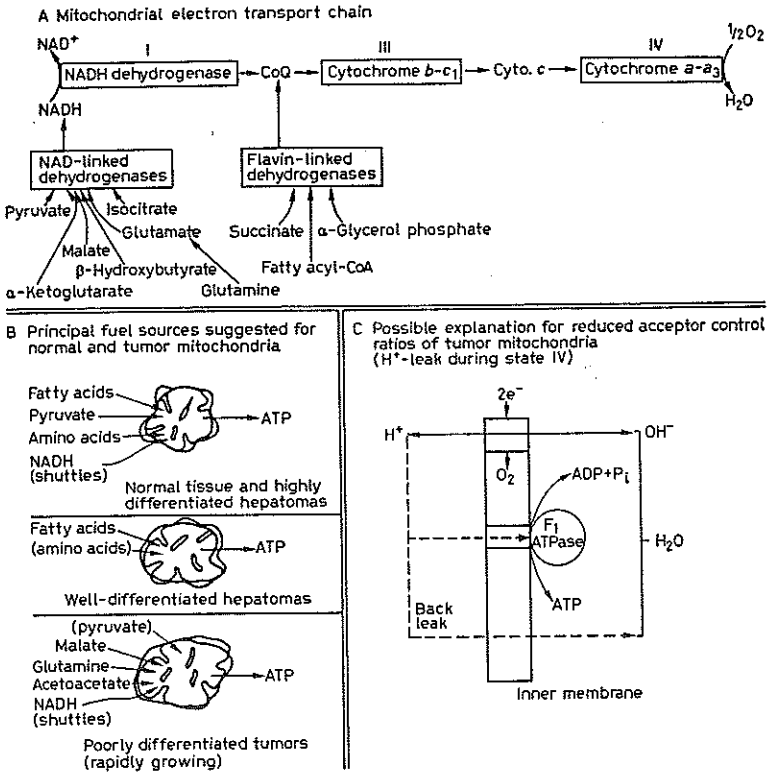


Fig. 6. Respiration, oxidative phosphorylation, and acceptor control of respiration of tumor mitochondria relative to control tissue. Values are based on the data of PEDERSEN *et al.* [1970] for hepatoma 9618A, KASCHNITZ *et al.* [1976] for hepatomas 7800, 5123D, 3924A, 7777 and 3683, SORDAHL *et al.* [1969] for hepatoma 139, FEO *et al.* [1975] for hepatoma AH 130 and fetal liver, and MEHARD *et al.* [1971] for mammary adenocarcinoma. Values from KASCHNITZ *et al.* [1976] are based on averages of the ranges given; maximal acceptor control ratios for normal liver were taken as 10 (although in some cases acceptor control ratios exceeded this value). The figure indicates that although some tumor mitochondria have reduced rates of respiration and lower acceptor control ratios than controls, all tumor mitochondria have a normal *capacity* to carry out oxidative phosphorylation. B = β-Hydroxybutyrate, S = succinate, or G = glutamate as respiratory substrate.

Fig. 7. A Mitochondrial electron transport chain showing sites of entry of substrate electrons. Principal sites of entry of substrate electrons are at the level of the NADH dehydrogenase complex and coenzyme Q. Prosthetic groups of the NADH dehydrogenase complex (complex I) directly involved in electron flow are FMN and iron sulfur moieties. Prosthetic groups of the cytochrome bc_1 complex (complex III) are heme-like moieties and iron sulfur moieties. Prosthetic groups of the cytochrome aa_3 complex (complex IV) are heme moieties and perhaps copper in nonheme form. Hepatoma mitochondria are frequently deficient in one or more iron sulfur centers (table IV). *B* Principal fuel sources suggested for normal and tumor mitochondria. Mitochondria of normal tissues and highly differentiated Morris hepatomas usually oxidize either fatty acids, pyruvate or amino acids (or combinations of these substrates), as well as NADH via 'shuttle' systems. Some slowly growing, well differentiated Morris hepatomas preferentially oxidize fatty



acids, and perhaps some amino acids. Glucose (and therefore pyruvate) is not a major fuel source for some well differentiated, slowly growing Morris hepatomas. This is because such tumors have low hexokinase activities and, unlike liver, do not respond to glucose or insulin to induce glucokinase. Rapidly growing tumors have been reported to oxidize a variety of energy sources depending on the source of the tumor. NADH (via 'shuttles'), some pyruvate, malate, glutamine or the ketone body, acetoacetate, have been shown to be oxidized by mitochondria from certain rapidly growing tumors. Unlike slowly growing hepatomas, rapidly growing hepatomas do not readily oxidize fatty acids (see text for references). *C* One possible explanation for reduced acceptor control ratios of tumor mitochondria. In general, it can be stated that tumor mitochondria have ADP/O (P/O) ratios similar to control mitochondria (fig. 6) provided serum albumin and sometimes EDTA are present in the isolation and assay medium. Nevertheless, the same tumor mitochondria frequently exhibit lower acceptor control ratios. It is suggested that unlike control mitochondria, the inner mitochondrial membrane of tumor mitochondria may be leaky to protons during state IV respiration. This may in some cases tend to favor endogenous ATP hydrolysis rather than ATP synthesis.

for a brief review of more recent work]. Studies on isolated mitochondria do not support the view of a generalized impairment of the mitochondrial electron transport chain. Certainly some tumor mitochondria may respire poorly on a given oxidizable substrate, but the same mitochondria may respire normally with another substrate (fig. 6, 7B). Also, some tumor mitochondria have a marked deficiency in one or more electron transport chain components [WALLACH, 1975b; LANOUE *et al.*,1974; MYERS and BOSMANN, 1974], whereas other tumor mitochondria have a normal content of electron transport chain components [SCHREIBER *et al.*, 1970; PEDERSEN *et al.*, 1970; WHITE and TEWARI, 1973].

By calling attention to such published data, it is not the author's intention to imply that the Warburg theory is incorrect, but only to indicate that a generalized defect at the level of the mitochondrial electron transport chain does not exist in tumor cells. Indeed, as emphasized earlier (see footnote 1 and section III), there is strong evidence that the respiration *capacity* of highly glycolytic tumors is impaired, as suggested originally by WARBURG [1930]. This impaired respiratory *capacity* seems to be due, at least in part, to a low mitochondrial content rather than to an inhibition of the electron transport chain *per se*.

Perhaps the most intriguing aspect of the respiratory properties of tumor cells lies in the oxidizable substances they utilize for fuels (fig. 7B). Some well differentiated, slowly growing hepatomas contain little or no hexokinase and, unlike liver and highly differentiated hepatomas, they do not respond to dietary glucose or insulin to induce the enzyme glucokinase [SHATTON *et al.*, 1969; WEINHOUSE, 1972]. Consequently, such tumors (at least *in vitro*) do not readily utilize glucose as an energy source (and therefore, pyruvate or NADH via 'shuttles' at the level of mitochondria) but, rather, they rely upon fatty acids as a fuel source [BLOCK-FRANKENTHAL *et al.*, 1965; WEINHOUSE, 1972; WEINHOUSE *et al.*, 1973]. Significantly, then, some well differentiated tumors probably rely almost entirely upon mitochondrial ATP (rather than glycolytic ATP) as a direct energy source for growth, a fact that might have important chemotherapeutic significance.

Mitochondria from poorly differentiated, rapidly growing tumors, on the other hand, evidently do not utilize fatty acids as a major fuel source [BLOCK-FRANKENTHAL *et al.*, 1965; WEINHOUSE, 1972; WEINHOUSE *et al.*, 1973; CEDERBAUM and RUBIN, 1976]; in fact, the fatty acid activation enzyme, acyl thiokinase, is reduced in activity by at least 85% in such tumors [WEINhouse *et al.*, 1973]. These tumors appear to utilize NADH via 'shuttles', small amounts of pyruvate derived from glucose and/or one or more

substrates which are not oxidized normally in large amounts by control mitochondria. Along these same lines, GREENHOUSE and LEHNINGER [1977] report that, in some ascites cells, as much as 33% of the cells' ATP may be obtained at the level of mitochondria by oxidation of reducing equivalents from glycolytically produced NADH via the malate-aspartate shuttle.

HANSFORD and LEHNINGER [1973] report that mitochondria isolated from L-1210 ascites tumor cells oxidize malate by converting it to pyruvate via a malic enzyme characteristic of the tumor mitochondria. KOVAČEVIĆ [1971] and KOVAČEVIĆ and MORRIS [1972] report that mitochondria from rapidly growing hepatomas and Ehrlich ascites tumor cells oxidize glutamine, evidently as a result of a P_i-dependent glutaminase characteristic of certain tumor mitochondria which convert glutamine to glutamate [KOVAČEVIĆ, 1974]. WAGLE *et al.* [1963] report that the rapidly growing Morris hepatomas 3924A and 3683 have a marked increase in the oxidation of isoleucine and valine. Finally, FENSELAU and WALLIS [1973] and FENSELAU *et al.* [1975] report that rapidly growing hepatomas can oxidize the ketone body acetoacetate. Some hepatoma mitochondria, unlike liver mitochondria, contain the enzyme succinyl CoA:acetoacetate coenzyme A transferase, which initiates ketone utilization by converting acetoacetate to the activated CoA derivative which, in turn, can be cleaved thiolytically to yield 2 equivalents of the important metabolic intermediate acetyl CoA [FENSELAU *et al.*, 1976]. It would therefore appear that, as tumor cells become more malignant, their capacity to utilize fatty acids as a fuel source decreases; they may favor ketone body utilization over ketone body production (in some hepatomas); and they can oxidize frequently in addition to NADH (via 'shuttles'), and some pyruvate, ketone bodies, and other metabolic substrates not oxidized normally by control tissues.

Along these lines, it should be mentioned also that some normal and transformed animal cells grown in tissue culture need not derive a large fraction of their total energy for growth from high aerobic glycolysis supported by glucose. Rather, they can grow equally well on galactose, which results in normal rates of glycolysis (low lactic acid production) or on glutamine [EAGLE *et al.*, 1958; ZIELKE *et al.*, 1976; BUSTAMANTE and PEDERSEN, 1977]. When the latter substrates are used, the major source of energy for growth is derived most likely from mitochondrial ATP rather than from glycolytic ATP.

Another rather intriguing feature of some tumor mitochondria is their high levels of endogenous respiratory substrate(s). In particular, the endogenous respiration rate of mitochondria from hepatoma AH130 can be stimu-

lated much more by ADP than can the endogenous respiration of liver mitochondria [FEO *et al.*, 1973a–c].

The *oxidative phosphorylation capacity* of tumor mitochondria appears to be normal, provided the mitochondria are isolated in BSA-containing media or in media containing both BSA and EDTA (fig. 6).

Some tumor mitochondria, perhaps because of the fragility of their inner membranes, are especially difficult to isolate (even in BSA-EDTA-containing media) in a form which carries out oxidative phosphorylation. For example, PEDERSEN and MORRIS [1974] and MORTON *et al.* [1976] have failed to observe significantly high phosphorylation with mitochondria from hepatoma 7777. However, KASCHNITZ *et al.* [1976, 1977] report that when the hepatoma 7777 membrane is disrupted with the proteolytic enzyme Nagarse rather than with a Teflon pestle (glass homogenizer), the resultant tumor mitochondria isolated in a BSA-containing medium have ADP/O ratios similar to those of control mitochondria. It remains to be established whether tumor mitochondria *in situ*, which do not have the protective company of BSA and EDTA, carry out oxidative phosphorylation *as efficiently* as normal cells.

Although mitochondria isolated from tumor cells have a normal capacity for oxidative phosphorylation, the *acceptor control ratios* (rate of state III resp./rate of state IV resp.) of such mitochondria are frequently less than those exhibited by control mitochondria (fig. 6). The reasons for this are unclear, but, as indicated in figure 7C, the inner mitochondrial membranes of tumors, unlike control mitochondria, may be more leaky to protons (and perhaps other small ions) in state IV than in state III. Such a leak would favor a higher rate of state IV respiration in the tumor mitochondria and perhaps, in some cases, favor endogenous ATP hydrolysis rather than ATP synthesis.

In summary, it seems clear that there is not a generalized impairment of the respiratory chain in tumor mitochondria, although some tumor mitochondria do have low contents of electron transport chain components or enzymes which feed substrate electrons into the electron transport chain. However, on a per cell or per tissue basis, the *maximal respiratory capacity* of many tumor cells relative to that of tissues of origin is most likely decreased (i.e., in rapidly growing, highly glycolytic tumors) because of the low content of mitochondria. Also, tumor mitochondria in general do not preferentially oxidize a given type of substrate. The view perhaps still held by some investigators – that tumor cells *in general* preferentially derive their energy source from catabolism of glucose – is not true. The fuel source for tumor cells, and ultimately the available substrate fuel for mitochondria, depends on the source of the tumor and its degree of differentiation. Finally, isolated tumor mitochondria in general have a normal capacity to carry out

oxidative phosphorylation provided BSA and (sometimes) EDTA are included in the isolation medium (and usually in the assay medium as well). In contrast, the acceptor control ratios of isolated tumor mitochondria are frequently lower than the acceptor control ratios of mitochondria from control tissues.

IX. Uncoupler-Stimulated ATPase Activity

Work carried out in 8 different laboratories has shown that under certain assay conditions well coupled mitochondria isolated from 14 different tumor lines, unlike normal mitochondria, have a reduced capacity to catalyze the hydrolysis of ATP in the presence of uncoupling agents such as DNP [EMMELOT *et al.*, 1959; DEVLIN and PRUSS, 1962; PEDERSEN *et al.*, 1971; KOLAROV *et al.*, 1973; THORNE and BYGRAVE, 1973a, b; LANOUE *et al.*, 1974; PEDERSEN and MORRIS, 1974; SENIOR *et al.*, 1975; KASCHNITZ *et al.*, 1976]. The tumors studied include slow, rapid, and intermediate growth rate Morris hepatomas (3683, 3924A, 7777, 7800, 7794A, 16, and 9618A), a primary rat hepatoma, By252 hepatoma, Zajdela hepatoma, a rat hyperplastoma, mammary tumor R3230AC, and *some* strains of L1210 and Ehrlich ascites tumors. Oxidative phosphorylation catalyzed by mitochondria from these tumors is inhibited in an apparently normal fashion by DNP. However, the capacity of DNP to *maximally* enhance ATPase activity seems to be markedly impaired unless ATP is added before introduction of the uncoupling agent. Mitochondria from hepatoma 7800 have been examined most closely with respect to their capacity to elicit DNP-stimulated ATPase activity, and it seems clear in this case that the tumor mitochondria must see ATP before DNP to elicit maximal ATPase activity (fig. 8A, B) [KASCHNITZ *et al.*, 1976]. This is not the case for normal liver mitochondria, host liver mitochondria, or for mitochondria from regenerating liver tissue [PEDERSEN and MORRIS, 1974].

The important observation involving the relative order of addition of uncoupler and ATP in distinguishing between the ATPase activities of normal liver and certain hepatoma mitochondria was not appreciated until the recent studies of KASCHNITZ *et al.* [1976]. As they report in table VI of a recent paper, hepatoma 7800 mitochondria (prepared by a proteolytic procedure involving Nagarse) have an ATPase activity of about 400 nmole/min/mg when ATP is added before DNP (50 μM) and 226 nmole/min/mg when ATP is added after DNP.

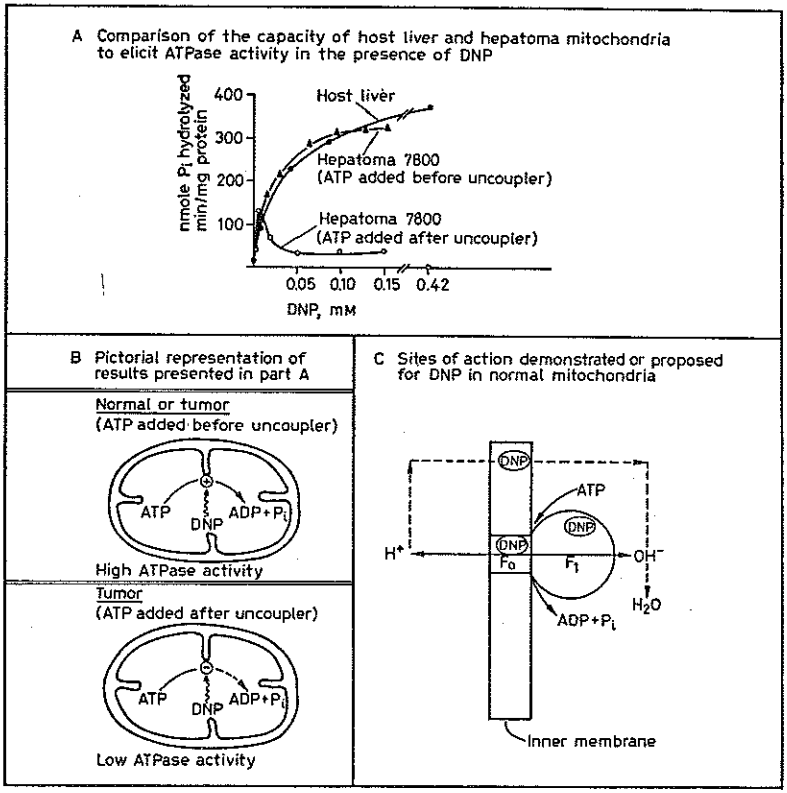


Fig. 8. A Comparison of capacity of host liver and hepatoma mitochondria to elicit ATPase activity in the presence of DNP. The figure shows that hepatoma 7800 mitochondria have normal DNP-stimulated ATPase activity provided ATP is added before DNP. If ATP is added after DNP the tumor mitochondria exhibit a normal response up to about 20 μM DNP, a concentration in the range sufficient to inhibit oxidative phosphorylation. Higher concentrations of uncoupler, which readily stimulate ATPase activity of host liver mitochondria, have little effect on the ATPase activity in hepatoma 7800 mitochondria. Host liver mitochondria exhibit a reproducibly high DNP-stimulated ATPase activity regardless of the order of addition of DNP and ATP. Data are those from the author's laboratory. A P_i-release assay was used [PEDERSEN and MORRIS, 1974]. Acceptor control ratios of liver and hepatoma mitochondria with succinate as substrate were 7.5 and 7, respectively. The range of uncoupler over which hepatoma mitochondria exhibit a normal response (when ATP is added after DNP) varies from 15 to 50 μM DNP; the ATPase activities range from about 75 to 200 nmol P_i/min/mg. *B* Pictorial representation of results presented in part A. The diagrams emphasize that under conditions used by the author and several other investigators (see text for references) to isolate and assay hepatoma mitochondria, DNP (depending upon its concentration) may have either a positive

More recent work (fig. 8A) carried out in the author's laboratory – using mitochondria prepared by the standard Teflon pestle (homogenization) differential centrifugation method [SCHNAITMAN and GREENAWALT, 1968] – has confirmed the results of KASCHNITZ *et al.* [1976] and further emphasizes the necessity for mitochondria from hepatoma 7800 to see ATP before DNP in order to elicit normal ATPase activity. In the author's hands, the differential effect in ATPase activity between host liver and hepatoma 7800 mitochondria (when ATP is added after uncoupler) is particularly striking at very high uncoupler concentrations, i.e., concentrations in excess of that necessary to inhibit oxidative phosphorylation. In fact, if mitochondria from hepatoma 7800 do not see ATP prior to DNP, the effect of DNP (relative to its effect on host liver mitochondria) is to inhibit rather than stimulate ATP hydrolysis (fig. 8A). The 'order of addition' effect needs to be examined carefully in many other tumors in order to obtain some indication of its general importance.

Significantly, the results presented in figure 8A also emphasize the importance of varying uncoupler concentrations and comparing host liver and tumor from the same animal in distinguishing the ATPase activities of certain tumor and control mitochondria. Thus, if a very low uncoupler concentration (15–25 μM) is chosen, similar ATPase activities will be observed in control and hepatoma 7800 mitochondria (regardless of the order of addition of uncoupler), but if a higher uncoupler concentration is chosen, marked differences will be observed provided ATP is added after uncoupler. To date, the ATPase activities of well-coupled mitochondria from Morris hepatomas 9618A, 16, and 7800, and defined strains of Ehrlich ascites and L1210 ascites tumor cells have been examined as a

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or negative effect on ATPase activity. *C* Sites of action demonstrated or proposed for DNP in normal mitochondria. PULLMAN *et al.* [1960] showed that a concentration of DNP greater than necessary to inhibit oxidative phosphorylation stimulates ATP hydrolysis catalyzed by the F_1-component of the OS-ATPase complex. MITCHELL [1968] suggested that uncouplers like DNP, because of their lipophilic, weak acid nature, may simply interact with the lipid phase of the membrane in such a way that protons arising on the outside of the mitochondrion can be conducted back to the inside. Support for this hypothesis is derived from the effect of uncouplers on lipid bilayers [HOPFER *et al.*, 1968]. More recently HATEFI [1975] has demonstrated that uncouplers bind rather specifically to a protein of about 30,000 molecular weight, which is a part of what HATEFI calls complex V (a complex capable of catalyzing oligomycin and uncoupler-sensitive ATP-P_i exchange).

It is not known in what way (s) DNP interacts differently in certain tumor mitochondria than in control mitochondria when added before ATP. Whether the inability of some tumor mitochondria to hydrolyze ATP under certain conditions (i.e., when very high uncoupler concentrations are present) provides tumor cells with a selective advantage over their normal neighbors also remains unknown.

function of uncoupler concentration (ATP added after uncoupler). In all cases the *ATPase activity* vs. *uncoupler concentration* profile of the tumor mitochondria differs markedly from that of control mitochondria [PEDERSEN and MORRIS, 1974].

The reason why the ATPase of many tumor mitochondria responds differently to uncoupling agents is not understood. Several investigators have emphasized that uncoupling agents like DNP, in addition to binding to an uncoupler binding site (most likely the protein described by HATEFI [1975] and/or membrane phospholipids), also bind directly to the F_1-component of the oligomycin-sensitive ATPase complex [STOCKDALE and SELWYN, 1971; CANTLEY and HAMMES, 1973; SENIOR and TOMETSKO, 1975]. Thus, one possible explanation for differences in uncoupler-stimulated ATPase activities of control and certain tumor mitochondria is that the tumor mitochondria may have a structural alteration in a DNP binding site on F_1 [SENIOR and TOMETSKO, 1975].

The physiological significance of a low capacity of well coupled tumor mitochondria to elicit ATPase activity (ATP added after uncoupler), especially at very high uncoupler concentration, is not understood either. Certainly, it is not related to the capacity of some tumor cells to elicit a high glycolytic rate since low uncoupler-stimulated ATPase activities are observed in mitochondria isolated from both 'low glycolytic' and 'high glycolytic' tumors. Perhaps there is no physiological significance to the observation at all, and it may be related to the inability of various investigators, including the author, to isolate *completely* intact tumor mitochondria. However, until it is proven otherwise, the possibility remains open that a defective ATPase in tumor mitochondria may provide certain tumor cells with a selective advantage over their normal neighbors. Thus, at very late stages in tumor growth, glycolytically produced ATP in viable cells might be preserved in the presence of very high concentrations of uncoupling agents arising exogenously from necrotic cells. Under such conditions, mitochondrially produced ATP would be completely or partially blocked since uncoupling agents have a normal capacity to inhibit oxidative phosphorylation in tumor mitochondria. Such a suggestion is not entirely without precedence since it is well established that the isolation media for the preparation of well coupled mitochondria from many tumors require bovine albumin and/or EDTA (section IV).

Finally, it should be noted that the reduced capacity of tumor mitochondria to elicit maximal uncoupler-stimulated ATPase activity under certain conditions is evidently not restricted to proton-conducting uncoupling agents like DNP. Thus, THORNE and BYGRAVE [1973a, b] show that both the DNP and Ca^{++}-stimulated ATPase activities of Ehrlich ascites mitochondria are reduced by at least 40%. These findings are discussed in more detail in section XI.

X. Other Enzymatic Activities

There are a number of reports that certain enzymatic activities are either reduced or elevated in mitochondria isolated from tumor cells (table IV). Some of these alterations may simply be consequences of the normal to neoplastic transition and may play no role in maintaining or promoting the malignant state. Thus, as noted in table IV, the activities of monoamine oxidase and α-glycerol Pi dehydrogenase are reduced in mitochondria isolated from some tumors, but elevated in mitochondria isolated from other tumors. Other alterations in enzyme activity at the mitochondrial level, particularly those found in rapidly growing tumor cells, may favor or help promote the capacity of such tumors to catalyze high rates of glycolysis. Thus, the Walker 256 tumor contains very low mitochondrial amounts of cytochromes; Morris hepatoma 7777 has decreased levels of mitochondrial iron sulfur centers 6, 5, and 9; and all ascites cells examined to date contain a large fraction of the total cell hexokinase in the outer mitochondrial membrane (table IV). The latter enzyme, when bound to the outer mitochondrial membrane of Ehrlich ascites cells, has been implicated in the Crabtree effect, i.e., the inhibition of respiration by glucose addition [KOOBS, 1972]. More recently, mitochondrially bound hexokinase has been implicated also in the high glycolytic capacity of a hepatoma line growing in tissue culture (section XII) [BUSTAMANTE and PEDERSEN, 1977]. Finally, other enzymatic alterations in tumor mitochondria seem to reflect an expression of the normal to neoplastic transition process to provide the tumor cell with an 'energetic advantage' over its normal neighbors. Thus, as indicated in table IV and as emphasized previously in this review, rapidly growing tumors, unlike control tissues, may contain enzymes such as Pi-dependent glutaminase, succinyl CoA: acetoacetate coenzyme A transferase, or malic enzyme, which promote oxidation of substrate (glutamine, acetoacetate, or malate) not normally oxidized at high rates by control mitochondria. The additional findings (table IV) that some tumor mitochondria have enhanced rates of ADP transport and low uncoupler-stimulated ATPase activities suggest that such mitochondria may be programmed to utilize every means available to maximize their own ATP production, or at least to preserve total cell ATP by restricting its useless hydrolysis induced by exogenous or endogenous uncoupling agents.

There are some enzyme or protein alterations in tumor mitochondria which are difficult to rationalize at this time. For example, the activity of β-hydroxybutyrate dehydrogenase which catalyzes the reversible conversion of β-hydroxybutyrate to acetoacetate is reduced in 19 different hepatomas (table IV). Yet, some hepatoma mitochondria have an enhanced capacity to oxidize acetoacetate [FENSELAU and WALLIS, 1973]. Additionally, conconavalin A-binding sites are reduced in hepatoma mitochondria (table IV). Yet, many tumor mitochondria have a more efficient Ca^{++} uptake and retention capacity (section XI), processes thought to be mediated by glycoprotein transport system(s) [PRESTIPINO *et al.*, 1974]. Also, in regard to this point, BOSMANN *et al.* [1974] report that the neoplastic transformation of chick embryo fibroblasts by Rous sarcoma virus induces an increase in the biosynthesis of mitochondrial glycoprotein. Finally, the activity of adenylate kinase (which catalyzes the reversible reaction ATP + AMP $\rightleftharpoons$ 2 ADP) has been reported to be near normal in mitochondria isolated from the slowly growing Morris hepatoma 9618A, but reduced by more than 40% in mitochondria isolated from the rapidly growing Morris hepatoma 3924A [PEDERSEN *et al.*, 1970]. Yet, there are only slight differences in the relative levels of ATP, ADP, and AMP in these two hepatomas [CRISS, 1973].

Table IV. Enzyme activities or proteins deficient or elevated in some tumor mitochondria (enzymes associated with nucleic acid metabolism are considered in section XIV)

Enzyme or protein	Localization	Tumor(s)
Deficient		
Monoamine oxidase-(benzylamine)[a]	outer membrane	Morris hepatomas 9618A, 7800, and 3924A
Monoamine oxidase(^{14}C-tryptamine or ^{14}C-serotonin)[b, c]	outer membrane	Novikoff hepatoma, Melanoma cells, SV-40-3T3 cells
Rotenone-insensitive NADH-cytochrome *c* reductase[b, c]	outer membrane	Novikoff hepatoma; Melanoma cells
Adenylate kinase[a–c]	intermembrane space	Morris hepatomas 7800 and 3924A; Novikoff hepatoma, Melanoma cells
Glutamate dehydrogenase[d, e]	matrix	Morris hepatomas 16, 7800, 3924A, and 7777
Ornithine transcarbamylase[d]	matrix	Morris hepatomas 9618A, 7800, and 3924A
Superoxide dismutase[f]	soluble	Morris hepatoma 3924A and Ehrlich ascites cells
Cytochromes *a*, *b*, *c* + c_1, and a^3[g]	inner membrane	Walker 256
Cytochrome oxidase[h]	inner membrane	Mammary tumors (CfZ and C3H)
Iron sulfur centers[e]	inner membrane	Morris hepatomas 16 and 7777
NADH cytochrome *c* reductase[b]	inner membrane	Novikoff hepatoma; Melanoma cells
Succinic dehydrogenase[b, h]	inner membrane	Novikoff hepatoma; Mammary tumors (CfZ and C3H)
α-Glycerol P_i dehydrogenase or oxidase[h–j]	inner membrane	Morris hepatoma 7800, Leukemic spleen (L1210), Leukemic B82T tumor, Mammary tumors (CfZ and C3H)
β-Hydroxybutyrate dehydrogenase[k, l]	inner membrane	18 different Morris hepatomas and Novikoff hepatoma (assayed in the tumor homogenate and not directly in mitochondrial fractions), Hepatocellular carcinoma HC-252
Uncoupler-stimulated ATPase	inner membrane	(14 different tumors, see section IX)
Conconavalin A binding sites[m]	membrane	Morris hepatomas 7800 and 7777
Elevated		
Monoamine oxidase (^{14}C-tryptamine)[h]	outer membrane	Mammary tumors (CfZ and C3H)
P_i-dependent glutaminase[n]	matrix	Ehrlich ascites cells

Table IV (continued)

Enzyme or protein	Localization	Tumor(s)
Succinyl CoA-acetoacetate coenzyme A transferase[o]	matrix	Morris hepatoma 7288ctc
α-Glycerol P$_i$ dehydrogenase[l]	inner membrane	Novikoff hepatoma
ADP/ATP transport system[p, q]	inner membrane	Mammary tumor (R3230 AC) and unspecified Morris hepatoma
Malic enzyme[r]	unknown	L1210 ascites cells
Hexokinase[s–w]	outer membrane	Many rapidly growing tumors

[a] PEDERSEN *et al.* [1970]. [b] WHITE *et al.* [1974]. [c] WHITE *et al.* [1975]. [d] PEDERSEN and GAMBLE [unpublished data]. [e] LANOUE *et al.* [1974]. [f] DIONISI *et al.* [1975]. [g] GALEOTTI *et al.* [1971]. [h] WHITE and NANDI [1976]. [i] HUNT *et al.* [1970]. [j] SACKTOR [1964]. [k] WEINHOUSE [1966]. [l] CEDERBAUM and RUBIN [1976]. [m] GLEW *et al.* [1973]. [n] KOVACEVIC [1974]. [o] FENSELAU *et al.* [1976]. [p] SENIOR *et al.* [1975]. [q] SUL *et al.* [1976]. [r] HANSFORD and LEHNINGER [1973]. [s] ROSE and WARMS [1967]. [t] MCCOMB and YUSHOK [1959]. [u] WU and RACKER [1959a]. [v] BUSTAMANTE and PEDERSEN [1977]. [w] ACS *et al.* [1955].

Other enzyme alterations at the mitochondrial level are involved in nucleic acid metabolism and will be considered in section XIV.

In summary, it can be stated that the activity of some enzymes in freshly isolated tumor mitochondria are decreased, while others are unchanged or elevated. Some of the alterations may provide the tumor cell with a selective energetic advantage over its normal neighbors, others may promote the expression of glycolytic reactions, and some may simply be consequences of the normal to neoplastic transformation and play no role in promoting or maintaining the malignant state.

XI. Transport Systems

A. Cation

Freshly isolated mitochondria from animal cells take up Ca^{++}, and under certain conditions of assay they also release Ca^{++}. Ca^{++} uptake by mitochondria has been studied in great detail during the last 15 years, and much has been learned about its mechanism [for reviews, see LEHNINGER *et al.*, 1967; LEHNINGER, 1970; CARAFOLI, 1974; SCARPA, 1977; for an overview, see figure 9 and the accompanying legend]. Suffice it to say here that Ca^{++} transport is mediated by a specific, ruthenium red-sensitive carrier (presumably a glycoprotein), and that net uptake requires energy which can be supplied by substrate oxidation or by ATP hydrolysis (fig. 9A). The physiological significance of Ca^{++} transport by mitochondria is largely unknown. It has been suggested by BYGRAVE [1967] and by LEHNINGER [1971]

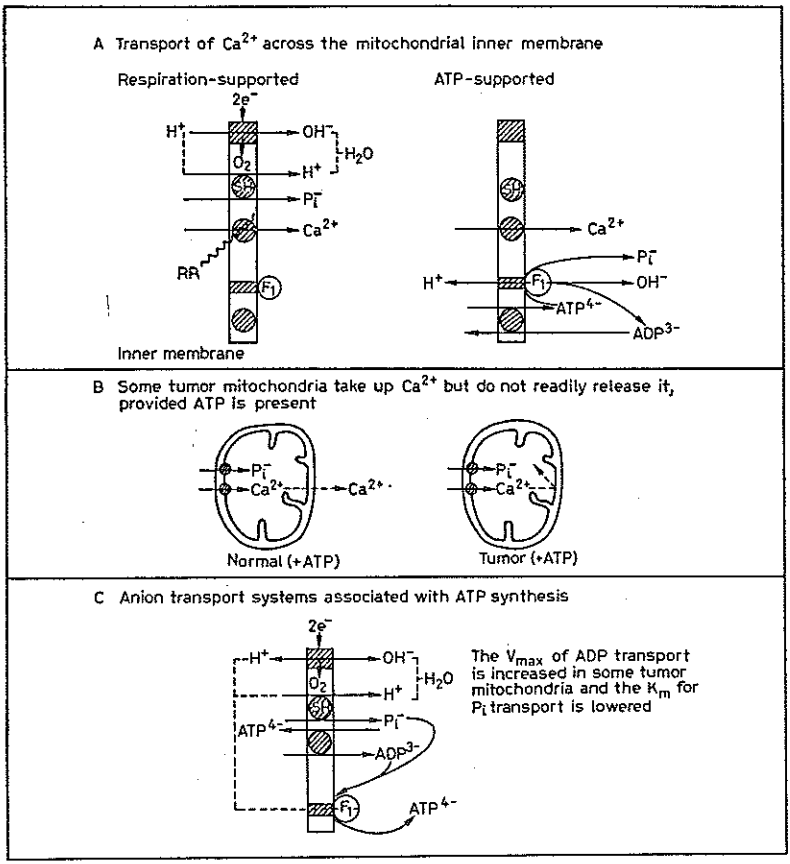

Fig. 9. A Transport of Ca^{++} across the mitochondrial inner membrane. In order for Ca^{++} to be transported into the mitochondrial matrix, energy must be supplied by substrate oxidation (*substrate-supported uptake*) or by ATP hydrolysis (*ATP-supported uptake*). In the presence of either energy source an electrochemical gradient of protons is established across the mitochondrial inner membrane ($^+\!\rightarrow -$) and Ca^{++} is thought to be pulled in by this gradient on a specific, ruthenium red-sensitive (RR), 'uniport' system. When oxidizable substrate is the energy source, the electron transport chain establishes the electrochemical gradient, whereas when ATP is the energy source the oligomycin-sensitive ATPase complex (OS-ATPase) establishes the gradient. In the former case, it is thought that unless an anion enters with the Ca^{++} (*matrix loading*) that Ca^{++} remains bound to the inner membrane (*membrane loading*). In the example shown the anion P_i enters the matrix on a specific, mercurial-sensitive, P_i/H^+ 'symport' system (or P_i/OH^- 'antiporter'). At high assay concentrations of Ca^{++} and P_i, and when ATP (or ADP) and Mg^{++} are present, CaP_i is precipitated in the matrix in the form of amorphous deposits

that Ca^{++} uptake by mitochondria may be necessary to assure a low cytoplasmic Ca^{++} concentration ($\sim$ 1 μM). The basis for this suggestion is derived from the known facts that animal cells are bathed in a sea of Ca^{++} (blood $Ca^{++} \cong 2$ mM) and contain in their cytoplasmic compartment enzymes which are activated by Mg^{++} and inhibited by Ca^{++} [BYGRAVE, 1967; LEHNINGER, 1971].

The mechanism by which mitochondria of normal animal cells release Ca^{++} has received less attention; in fact, it is not at all clear whether release of Ca^{++} by mitochondria is a physiologically significant event. Under certain conditions of assay, i.e., high Ca^{++} ($\geqslant$ 1 mM) or high P_i ($\geqslant$ 1 mM), mitochondria will release Ca^{++} shortly after accumulation. However, release of the accumulated Ca^{++} most likely results because the mitochondria become uncoupled by the Ca^{++}, swell, and perhaps in some cases rupture.

Tumor mitochondria which have been studied to date take up Ca^{++} more efficiently than normal mitochondria and release it less readily. REYNAFARJE and LEHNINGER [1973] show that mitochondria from L1210 ascites cells transport Ca^{++} at a much higher rate than normal liver mitochondria, and that the apparent affinity for Ca^{++} uptake is 3- to 4-fold higher in L1210 mitochondria. In addition, THORNE and BYGRAVE [1973a, b, 1974a], working with Ehrlich ascites cells, and CARPENTIERI and SORDAHL [1975], working with murine lymphoblasts in culture, show that tumor mitochondria readily accumulate Ca^{++}, but, unlike mitochondria from normal cells, fail to release the Ca^{++} as readily (i.e., such mitochondria probably fail to uncouple, swell, and rupture as readily).

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which also contain Mg^{++} and adenine nucleotide (massive loading). *B* Ca^{++} uptake and release by tumor mitochondria. Tumor mitochondria studied in detail to date seem to be more efficient in accumulating and retaining Ca^{++} than normal mitochondria. The retention process of tumor mitochondria requires ATP (at least in the case of Ehrlich ascites mitochondria). Evidently, there is an impairment of the normal release mechanism for Ca^{++} in tumor mitochondria. The molecular details of the release mechanism for Ca^{++} in normal cells is not understood. *C* Anion transport systems associated with ATP synthesis. For synthesis of ATP, both ADP and P_i enter mitochondria on separate transport systems. ADP^{3-} exchanges for ATP^{4-} on one transport system and P_i moves in symport with H^+ (or in antiport with OH^-) on another transport system. The energy necessary for both transport and ATP synthesis is thought to be derived to a large extent from the electrochemical gradient of protons across the mitochondrial inner membrane. In some tumor cells the various systems associated with ATP synthesis seem to be more efficient than in normal cells. Thus, the V_{max} of ADP uptake is increased, the K_m for P_i uptake is lowered, and when high uncoupler concentrations are present, the capacity for ATP hydrolysis at the level of F_1 is diminished.

THORNE and BYGRAVE [1973a, b] note that in Ehrlich ascites tumor mitochondria the presence of ATP is essential to prevent release of Ca^{++}. Normal liver mitochondria release Ca^{++} shortly after uptake even in the presence of ATP. The requirement for ATP in retarding release of Ca^{++} in tumor mitochondria is not understood in molecular terms, although ATP may preserve the integrity of the mitochondrial inner membrane better in tumor cells than in normal cells. It is of interest, however, that the ATP effect is sensitive to oligomycin, and that the Ehrlich ascites mitochondria are shown to have a 40–50% reduction in both Ca^{++} and DNP-stimulated ATPase activity. Thus, it does not seem too presumptuous to state that the ATP effect in retarding Ca^{++} release by Ehrlich ascites mitochondria may in some way involve the oligomycin-sensitive ATPase complex (OS-F_1).

Perhaps relevant also to the ATP effect in retarding the release of Ca^{++} are the studies of PEDERSEN and MORRIS [1974] on the low glycolytic Morris hepatoma 7800. Mitochondria isolated from this hepatoma accumulate significantly more Ca^{++} in the presence of ATP ('massive loading conditions') than do liver mitochondria. As indicated earlier in this review, mitochondria from Morris hepatoma 7800 are highly deficient in DNP-stimulated ATPase activity unless the mitochondria see ATP before the uncoupler.

Additional studies carried out by THORNE and BYGRAVE [1974a, b] show that Ca^{++} does not readily stimulate respiration in Ehrlich ascites mitochondria as it does in liver mitochondria. It is assumed by these workers that Ca^{++} does not uncouple ATP synthesis from respiration under such conditions, although direct measurements of oxidative phosphorylation have not been reported. Although Ehrlich ascites mitochondria may not be uncoupled in the typical manner by Ca^{++}, it should be pointed out that ATP synthesis is most likely prevented in Ehrlich ascites mitochondria by Ca^{++} as in normal mitochondria. Thus, as THORNE and BYGRAVE [1974c] show, Ca^{++} inhibits the ATP/ADP translocation step in Ehrlich ascites mitochondria which would prevent net uptake of ADP and therefore ATP synthesis.

THORNE and BYGRAVE [1974a] have extended their studies to other tumor mitochondria. Similar to Ehrlich mitochondria, they find that low concentrations of Ca^{++} fail to stimulate respiration in AH130 hepatoma mitochondria and in mitochondria from the slowly growing, low glycolytic Morris hepatomas 5123C and 9618A. Respiration of mitochondria from regenerating liver responds in the normal manner to Ca^{++} addition by eliciting a marked stimulation. Unfortunately, other studies carried out previously on Ehrlich mitochondria (see above discussion) were not carried out on the hepatoma mitochondria. Thus, whether or not 5123C, 9618A and

AH 130 hepatoma mitochondria accumulate Ca^{++} more efficiently and release it less readily than normal liver mitochondria, and whether or not ADP/ATP translocation and/or ATP synthesis are inhibited by Ca^{++}, was not established.

Studies by EBOLI *et al.* [1974] further support the view of THORNE and BYGRAVE [1973a, b, 1974a–c] that tumor mitochondria are less sensitive to the deleterious actions of Ca^{++}. Thus, in mitochondria isolated from both Ehrlich ascites cells and from Morris hepatoma 3924A, respiration supported by glutamate-malate is not inhibited by Ca^{++} (40–120 μM). This is in contrast to results obtained with control liver mitochondria where respiration supported by glutamate-malate is markedly inhibited by Ca^{++}.

The possible relationship of a more efficient Ca^{++} uptake and retention mechanism in tumor mitochondria to the neoplastic state is unclear. If, in fact, it is true that mitochondria serve a role in normal cells to maintain a low intracellular Ca^{++} concentration [BYGRAVE, 1967; LEHNINGER, 1971], then to maintain the same intracellular Ca^{++} concentration in rapidly growing, highly glycolytic tumor cells the mitochondria will have to either work 'harder' or more efficiently. This is because the content of mitochondria in such cells is usually (if not always) reduced by 50% or more (section III). The problem tumor mitochondria face in this regard is compounded if one assumes that other Ca^{++} uptake systems may be less efficient or defective in certain tumor cells. Thus, the studies of CITTADINI *et al.* [1973] show that when succinate is substrate, mitochondria account for all the calcium accumulated by Ehrlich ascites cells.

Finally, it should be noted that mitochondria are thought to have transport systems for cations other than Ca^{++}, notably Na^+ (Na^+/H^+ antiporter), K^+ (K^+/H^+ antiporter), ornithine (uniporter), and perhaps Mg^{++}. However, these transport systems are not well understood even in normal mitochondria, and therefore have not been examined as yet in tumor mitochondria.

B. Anion

As indicated in figures 1, 5 and 9, mitochondrial anion transport systems play an important role in cell metabolism by directing metabolic traffic back and forth across the mitochondrial inner membrane in a specific and controlled manner. Thus, anion transport systems operate during the catabolic phase of cell metabolism to promote breakdown of carbohydrate, fat, and protein and, ultimately, to promote electron transport and ATP synthesis. In this regard mitochondria contain anion transport systems essential for the operation of 'shuttles' involved in the regeneration of NAD^+ for glycolysis; they contain transport systems which allow for the entry of oxidizable substrates such as pyruvate and some dicarboxylic acids and, most importantly perhaps, they contain transport systems which allow for the entry of ADP and P_i, the substrates for ATP synthesis.

Anion transport systems in mitochondria play an equally important

role during the anabolic phase of cell metabolism. Thus, both malate and citrate leave the mitochondria on specific transport systems to allow gluconeogenesis, fatty acid synthesis, and cholesterol synthesis to take place in the extramitochondrial phase of the cell. Additionally, ATP leaves the mitochondria on a specific transport system (in exchange for ADP) to provide the source of energy in the cytoplasm to drive anabolic processes.

Despite the essential role of mitochondrial anion transport systems in cell metabolism, little effort has been expended in examining the properties of these transport systems in tumor mitochondria. Moreover, many of the studies that have been done involve swelling assays rather than initial rate assays. The swelling assay (swelling of mitochondria in the ammonium salt of a given anion [CHAPPELL, 1968]) provides evidence for the existence or nonexistence of a given transport system in tumor mitochondria, and a rough 'guesstimate' of the activity level of the transport system relative to that of control mitochondria. However, to obtain K_m and V_{max} information, initial rate studies must be done.

The initial rate of ADP transport into mitochondria has been examined in a mammary tumor (R3230AC) and in a Morris hepatoma of unspecified origin [SENIOR *et al.* 1975; SUL *et al.*, 1976]. In both cases ADP transport into mitochondria was found to proceed with a higher than normal V_{max} and a lower than normal K_m. P_i entry into mitochondria from Ehrlich ascites cells was found to proceed with a lower K_m than in liver mitochondria [THORNE and BYGRAVE, 1975]. ATP transport out of mitochondria from Zajdela hepatoma [KOLAROV *et al.*, 1973] proceeds at a lower rate and with a higher K_m than in liver mitochondria.

Swelling studies in ammonium salts suggest that the following anion transport systems are present at normal or near normal activity levels in certain tumor mitochondria:

P_i – Normal in mammary adenocarcinoma [MEHARD *et al.*, 1971]; in mammary tumor (R3230AC) [SENIOR *et al.*, 1975]; in Morris hepatomas 16, 7800, and 7777 [LANOUE *et al.*, 1974], and in hepatocellular carcinoma HC252 [CEDERBAUM and RUBIN, 1976].

Succinate, malate and *citrate* – Normal in mammary tumor (R3230AC) [SENIOR *et al.*, 1975].

Glutamate – Normal in Morris hepatoma 7800 [LANOUE *et al.*, 1974].

Swelling studies in ammonium salts suggest that the following anion transport systems are present at lower than normal activity levels in certain tumor mitochondria:

Malate – Low in Morris hepatomas 16, 7800, 7777 [LANOUE *et al.*, 1974] and in hepatocellular carcinoma HC252 [CEDERBAUM and RUBIN, 1976].

Glutamate – Absent in Morris hepatomas 16 and 7777 [LANOUE *et al.*,

1974] and low in Morris hepatomas MK-3, 7316A, 5123 tc, 7777 and Ehrlich ascites cells [KOVAČEVIĆ and MORRIS, 1972].

It is rather obvious that much more work needs to be carried out on the anion transport systems of tumor mitochondria to better define their kinetic properties (i.e., their K_ms and V_{max}s). In particular, it would be of interest to know whether the kinetic properties of transport systems vary with the degree of malignancy of the tumor. Thus, in rapidly growing tumors where the content of mitochondria is markedly reduced, are the mitochondrial anion transport systems more efficient in promoting the entry of ADP, P_i and oxidizable substrates than in slowly growing tumors where the content of mitochondria is near normal?

XII. High Glycolysis of Rapidly Growing Tumors

A. Increased Lactic Acid Production – Are Mitochondria Limiting for Oxidation of NADH or Pyruvate?

The question as to why rapidly growing tumors have a propensity to convert the normal glycolytic end product pyruvate to lactic acid in the cytoplasm rather than transporting the pyruvate into the mitochondria for oxidation has puzzled biochemists for many years. (See fig. 10 for an overview of the lactic acid-producing capacities of normal tissues and tumors.) As LANOUE *et al.* [1974] point out, 'On a purely logical basis, the production of excess lactate could be due to: (a) ineffective mechanisms for reoxidation of NADH generated during glycolysis by the mitochondrial electron transport chain, or (b) glycolytic rates exceeding the oxidative capacity of the mitochondria for pyruvate' (this possibility was suggested by WEINHOUSE [1956]. These two possibilities are considered below in light of both old and more recent data summarized in the literature.

With respect to the first possibility, it is known that normal animal cells have one or more 'shuttle' systems involving both cytoplasmic and mitochondrial reactions for regeneration of NAD^+ from the NADH produced during glycolysis (fig. 11). Shuttle systems that seem to be most important are the *α-glycerol-phosphate shuttle*, the *malate-aspartate shuttle*, and, perhaps in some tissues, the *fatty acid shuttle*. Evidence for a fourth shuttle, the *β-hydroxybutyrate-acetoacetate shuttle* [BOXER and DEVLIN, 1961] has not been obtained at the enzymatic level (only one rather than two forms of β-hydroxybutyrate dehydrogenase is present in mitochondria). To explain the high rates of lactate production in rapidly growing tumor cells, 'defective' or 'rate-limiting' shuttle mechanisms were postulated in the late 1950s and

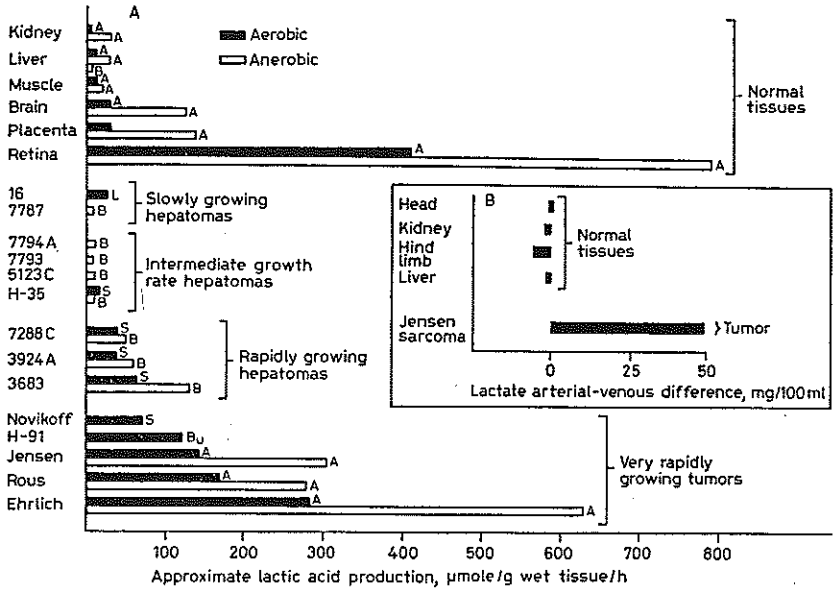

Fig. 10. Lactic acid production of normal tissues and tumors (*in vitro* and *in vivo*). *A* Values were taken or calculated from data included in the following monographs or papers: AISENBERG [1961], SWEENEY *et al.* [1963], BURK *et al.* [1967], LANOUE *et al.* [1974] and BUSTAMANTE and PEDERSEN [1977]. The figure is presented in this fashion to provide the reader with a general overview of the relative lactic acid-producing capacities of various tumors and not to provide absolute numbers for lactic acid production. Values should be considered as very approximate because averages of ranges have been used, and because in some cases it was necessary to convert from µl gas/mg dry weight/h to µmol lactate/g wet tissue/h. This was done by multiplying by 0.15 to give µmol lactate/g wet tissue/min [WEINHOUSE 1976] and then by 60 min/h. The conversion assumes that 200 mg dry weight tissue = 1 g wet weight tissue. Various authors report different experimental conditions. Therefore, the reader should consult the original reference for experimental details. A = Aisenberg; B = Burk; Bu = Bustamante; L = LaNoue and S = Sweeney. *B* Values were taken from WARBURG [1926], and are expressed as arterial-venous differences (i.e., the difference in the concentration of lactic acid in the blood flowing into and out of the tumor). Similar *in vivo* observations have been made for the Rous sarcoma growing in Plymouth Rock chicken wings, for a human forearm tumor [CORI and CORI, 1925], and for several rat tumors [GULLINO *et al.*, 1967].

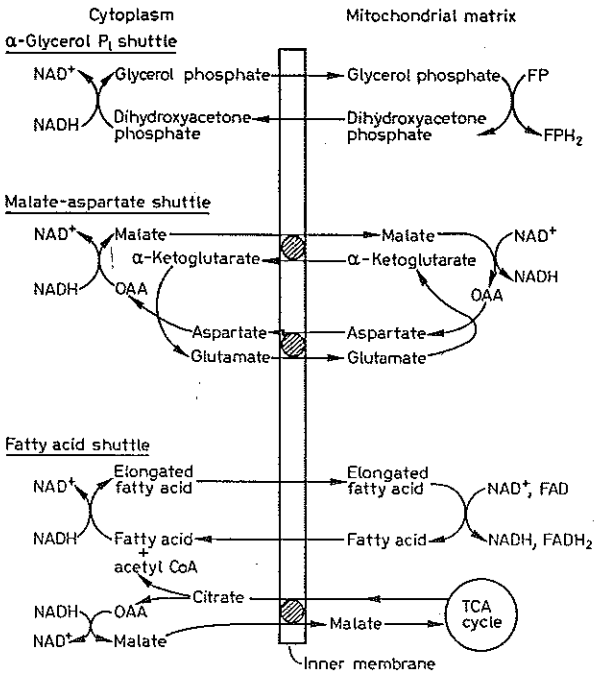

Fig. 11. 'Shuttles' involved in regeneration of cytoplasmic NAD$^+$. Regeneration of NAD$^+$ by 'shuttle' systems is necessary for glycolytic reactions to proceed normally in the cytoplasm. If 'shuttle' mechanisms are impaired, pyruvate conversion to lactate in the cytoplasm will be favored since the lactic dehydrogenase reaction provides an alternate route for regeneration of NAD$^+$. Three shuttle systems are shown: the *α-glycerol-phosphate shuttle,* which is not thought to require transport systems; the *malate-aspartate shuttle,* which is mediated by two anion carriers (malate/α-ketoglutarate carrier and the aspartate/glutamate carrier), and the *fatty acid shuttle.* Details of the fatty acid shuttle have not been worked out. (Provided acetyl CoA can readily leave the mitochondria, a citrate/malate carrier would not be required. Otherwise, the shuttle may function as suggested by the diagram in which case citrate would leave the mitochondria via a carrier-mediated mechanism in exchange with malate). Although some tumor cells are markedly deficient in one type of shuttle mechanism, they appear to have normal activity with respect to another type of shuttle mechanism. It would seem therefore, that high lactate production by certain tumor cells utilizing glucose does not result because 'shuttle' mechanisms become rate-limiting for oxidation of NADH.

early 1960s, the basic idea being that if NADH could not be converted to NAD^+ via shuttles rapidly enough to keep pace with glycolysis, then the NADH → NAD^+ conversion essential for glycolysis to proceed would necessarily be mediated by lactic dehydrogenase in the cytoplasm (i.e., pyruvate + NADH ⇌ lactate + NAD^+).

The 'defective' or 'rate limiting' shuttle theory to explain enhanced lactate production in certain tumors reached its high point in the early 1960s as a result of the studies carried out by DELBRÜCK *et al.* [1959], BOXER and SHONK [1960], SACKTOR and DICK [1960], and BOXER and DEVLIN [1961]. In particular, the extensive study of BOXER and SHONK [1960] showed that α-glycerol-phosphate dehydrogenase activity is very low or absent in 31 different animal tumors, whereas the lactic dehydrogenase levels are normal or elevated.

More recent studies carried out by DIONISI *et al.* [1970] show that the α-glycerol-phosphate dehydrogenase activity is about 20 times higher in the hyperdiploid Ehrlich cells (wild type) than in the hyperdiploid Lettré-Ehrlich cells (mutant). However, both cell types are shown to have not only high rates of lactate production, but the wild type is shown to produce α-glycerol-phosphate upon glucose addition. These investigators conclude that 'the high rate of aerobic glycolysis in ascites cells cannot be dependent as suggested by other authors, on the lack of a system for the transfer of glycolytic NADH to the respiratory chain'.

Further studies by DIONISI *et al.* [1974] and by EBOLI *et al.* [1976a] show that tumor cells of the mutant Ehrlich strain contain an active malate-aspartate shuttle, whereas the wild type strain does not. It is suggested that the wild type strain may use, in addition to the α-glycerol-phosphate shuttle, the fatty acid shuttle [CEDERBAUM *et al.* 1973]. In any case, the studies of DIONISI *et al.* [1970, 1974] and EBOLI *et al.* [1976a] emphasize that tumor cells probably do not rely on a single type of shuttle for regeneration of NAD^+, and that in both strains of Ehrlich cells 'defective' or 'rate-limiting' shuttles are not the cause of high lactate production.

The conclusions of DIONISI *et al.* [1970, 1974] and EBOLI *et al.* [1976a] are supported by the recent studies of GREENHOUSE and LEHNINGER [1976, 1977] on the malate-aspartate shuttle in 6 different tumor lines: L1210 ascites cells, AS-30D ascites hepatoma cells, Novikoff hepatoma, Krebs II carcinoma, and two strains of Ehrlich ascites cells. In experiments where malate-aspartate shuttle activity was measured together with oxygen uptake, it was concluded that the malate-aspartate shuttle is sufficiently active to reoxidize all the cytosolic NADH equivalent to the pyruvate formed by glycolysis in each of the 6 tumor strains examined. GREENHOUSE and LEH-

NINGER [1976] also indicate that their data disprove the earlier contention of KOVAČEVIĆ [1971], that the malate-aspartate shuttle cannot function at a sufficiently high rate because of the low concentration of aspartate in tumor cells.

LANOUE *et al.* [1974] also examined the malate-aspartate shuttle in hepatomas (Morris hepatomas 7777, 7800, 7794A, and 16) and compared this activity to that of control liver. Although all hepatomas exhibited a diminished capacity for transporting reducing equivalents from cytosol to mitochondria, the extent of the effect was found to vary between tumors and not to be a function of growth rate. Since the glycolytic capacity of Morris hepatomas, as measured by lactate production, is a function of growth rate [WEBER and MORRIS, 1963; SWEENEY *et al.*, 1963] it therefore can be inferred that diminished malate-aspartate shuttle activity does not correlate with lactic acid production activity in Morris hepatomas. Thus, the results of LANOUE *et al.* [1974] on both low and high glycolytic, transplantable hepatomas seem to be consistent with the observations on ascites tumors of DIONISI *et al.* [1974], EBOLI *et al.* [1976a], and GREENHOUSE and LEHNINGER [1976, 1977].

Other recent studies are consistent also with a normal capacity of tumor cells to oxidize NADH to NAD^+ via 'shuttle' mechanisms. Thus, CEDERBAUM and RUBIN [1976] report that although the fatty acid shuttle is low in hepatocellular carcinoma HC-252, the α-glycerol phosphate and malate-aspartate shuttles are only slightly lower than those of host liver. Additionally, PAPA *et al.* [1973] and EBOLI *et al.* [1976b] present evidence that the normal and mutant strains of Ehrlich ascites cells contain mitochondrial transport systems for α-ketoglutarate and glutamate which are essential for the function of the malate-aspartate shuttle.

Since 'shuttle' mechanisms evidently operate in 'highly glycolytic' tumors at a rate sufficient to reoxidize all of the NADH produced during glycolysis, the author is led to conclude 'by deduction' that high lactate production in such tumors most likely results because the mitochondria (when considered *in toto*) have a limited capacity to oxidize the pyruvate formed[4]. Although this possibility has not received the experimental scrutiny that the 'ineffective shuttle hypothesis' has, it does not seem unreasonable. In fact, it seems likely that in rapidly growing tumor cells the pyruvate

[4] The author is cognizant of the fact that the type of deductive reasoning used in this section may not be completely valid. For example, as BUSH [1962] has emphasized, high net lactic acid production by some tumor cells utilizing glucose may result at least in part because the tumor, unlike the control tissue used for comparison, fails to readily reutilize lactate.

oxidation capacity may be reduced in the presence of glucose simply because the *reduced number of mitochondria* (section III) are not as effective in competing for the available ADP, P_i (or both) as is the *enhanced level of glycolytic enzymes* (section below). Thus, the maximal state III rate of respiration is probably rarely attained in 'highly glycolytic' tumors utilizing glucose. Certainly this appears to be the case for hepatoma cells (H-91 subline of AS-30D) in tissue culture. When grown on glucose these cells are highly glycolytic as evidenced by high lactate production. However, respiration is not operating maximally (i.e., at state III rates) under these conditions because DNP addition can be shown to result in a marked stimulation [BUSTAMANTE and PEDERSEN, 1977].

In summary, it would appear that high lactate production is observed in rapidly growing tumor cells, not because shuttles for regenerating NAD^+ are limiting but, rather, because the pyruvate oxidation capacity of such cells is limiting. The pyruvate oxidation capacity of such cells may be limiting because of suboptimal levels of ADP, P_i (or both) at the site of oxidative phosphorylation (preventing the attainment of maximal state III rates) rather than because the respiratory chain or pyruvate transport system of individual mitochondria is limiting[5].

B. Glucose Utilization –
Relationship to Mitochondrially Bound Hexokinase[6]

From the above discussion, it would appear that during glycolysis in rapidly growing tumor cells, ADP, P_i (or both) become limiting in mito-

[5] The conclusions arrived at in this section seem to be consistent with the view expressed earlier by WEINHOUSE and collaborators [1968]. They state: 'These findings provide evidence for the suggestion offered many years ago by JOHNSON and by LYNEN that the Pasteur effect may reflect competition for ADP and P_i at transphosphorylation sites of glycolysis and respiration. They suggest also that the high aerobic glycolysis which is, in general characteristic of highly dedifferentiated tumors may be, in part, a resultant of their *low respiratory activity* and *high levels of glycolytic transphosphorylating enzymes*.'

[6] As indicated in table IV of this review, a number of investigators have reported that a large fraction of the total cell hexokinase activity of highly glycolytic tumors is associated with the mitochondrial fraction. As of this writing, it has not been clearly established whether this particulate hexokinase is associated with the mitochondria *per se* or with some particulate fraction which is tightly associated with the mitochondria of tumor cells. Although in this section the author, similar to other investigators (see table IV for references), treats the subject as though the hexokinase activity found in the mitochondrial fraction is localized in the outer mitochondrial compartment, he believes that further experimentation is essential to rigorously identify the particulate fraction which binds much of the hexokinase activity of highly glycolytic tumor cells.

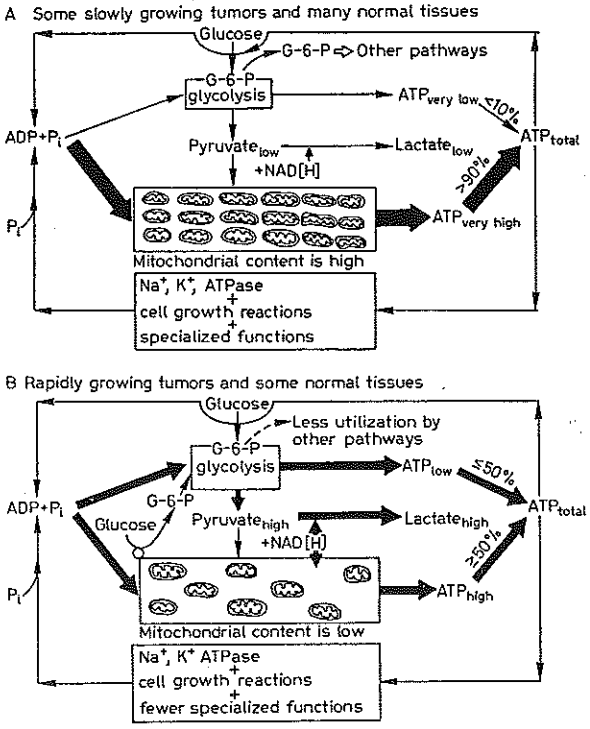

Fig. 12. A ATP synthesis-ATP hydrolysis cycle in some slowly growing tumors and many normal cells. The diagram emphasizes that the ratio of mitochondrial oxidative phosphorylation systems to glycolytic systems is high in some slowly growing tumors and many normal tissues; that mitochondria are more effective competitors for the available ADP and P_i of the cell than glycolysis; that the pyruvate formed is oxidized preferentially by the mitochondria rather than being converted to lactate; that greater than 90% of cell ATP is provided by mitochondrial oxidative phosphorylation and less than 10% by glycolysis; that ATP formed is coupled to energy-dependent reactions (such as hexokinase, Na^+, K^+ ATPase, cell growth reactions, and reactions involved in specialized cell functions) in order to regenerate ADP (or AMP) and P_i. *B* ATP synthesis-ATP hydrolysis cycle in some rapidly growing tumors and some normal tissues. The ATP synthesis-ATP hydrolysis cycle of rapidly growing, very highly glycolytic tissues (i.e., Ehrlich and other ascites cells) is considered in order to emphasize the contrast with tissues considered in *A*. The diagram emphasizes that, relative to the tissues discussed in *A*, the ratio of mitochondrial oxidative phosphorylation systems to glycolytic systems is low; that glycolysis is much more effective in competing for the available ADP and P_i; that the pyruvate formed (or at least a large fraction of it) is preferentially converted to lactate rather than oxidized by mitochondria; that hexokinase is bound to the mitochondrial fraction; that mitochondrial oxidative phosphorylation and glycolysis contribute about equally to the ATP supply of the cell, and that ATP is coupled to fewer specialized cell functions and therefore must rely more on the Na^+, K^+ ATPase and cell growth reactions for regeneration of ADP (or AMP) and P_i.

chondria to the extent that maximal state III rates cannot be obtained (fig. 12). Moreover, the resultant respiratory limitation appears to be due at least in part to the high level of glycolytic phosphorylation enzymes relative to the reduced level of oxidative phosphorylation systems when considered on a *whole cell basis.* Significantly, for high lactic acid formation to occur this view does not require necessarily that the rapidly growing tumor cells utilize more glucose more rapidly than do normal cells. The mere fact that the mitochondria are so dramatically reduced in many rapidly growing tumor cells is likely to make them poorer competitors (on a whole cell basis) for the available ADP, P_i (or both) than the glycolytic system, even when a normal flux of glucose catabolism occurs.

Experimentally, it has been observed that the net amount of glucose utilized by some rapidly growing tumor cells is much greater than that of control cells [CORI and CORI, 1925; WARBURG *et al.*, 1926; WOODS *et al.*, 1968], whereas, the net amount of glucose utilized by other rapidly growing tumor cells appears to be normal or near normal [ASHMORE *et al.* 1958; BUSH, 1962]. It might be inferred, therefore, that in those cases where enhanced glucose utilization does occur, that this will amplify the 'already high' lactic acid-producing capacity of the tumor both by amplifying the superior competitive capacity of the glycolytic system for ADP, P_i (or both), and by producing excess pyruvate.

Whether or not rapidly growing tumor cells elicit an enhanced glucose utilization rate may depend on the environment in which they find themselves, i.e., low or high oxygen tension, low or high glucose, etc. Certainly, different solid tumors have differing vascular systems. Those with poor vascular systems may have many cells which see an environment of low hormonal and/or oxygen concentration [see BUSH, 1962 for a discussion]. In such cases, high glucose utilization may have to take place to keep pace with the energy demands of the cell. Also, different tumor cells may have different affinities for glucose either at the level of their transport systems or at the level of hexokinase. Thus, depending on the glucose environment to which they are exposed (i.e., low or high glucose) such cells may utilize normal amounts of glucose or enhanced levels of glucose when compared to control tissues.

Regardless of whether rapidly growing tumors are found under a given set of experimental conditions to exhibit a normal or an enhanced glucose utilization rate, one thing seems clear: In general, such tumors have the *capacity* to utilize more glucose than do slowly growing tumors and many normal tissues (fig. 13). Glycolytic enzymes are both elevated [WU and RACKER, 1959a, b; WEBER, 1972] and appear in large part as fetal rather than adult-like isozymes [WEINHOUSE and ONO, 1972; WEINHOUSE *et al.*,

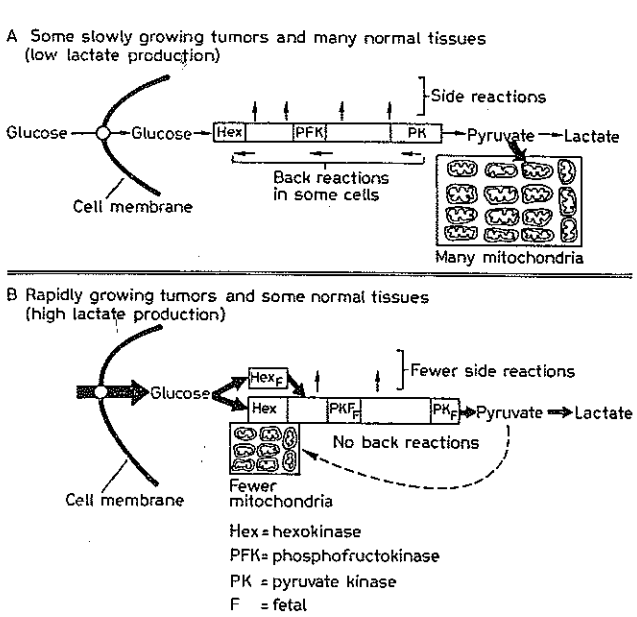

Fig. 13. Molecular differences between low (A) and highly glycolytic tissues (B) which support a more unifying concept for the high glycolytic capacity of rapidly growing tumors. As emphasized in the figure, the basic elements of the 'unifying concept' for high lactate production in rapidly growing tumor cells are twofold: (1) The rapidly growing tumor cell has been programmed genetically to more efficiently channel glucose to pyruvate. This is effected in a variety of ways which may differ slightly from one cell type to the other. The major genetically directed alterations, all of which are usually expressed in rapidly growing tumors, are the following: (a) elimination or reduction of some side reactions and back reactions which oppose net pyruvate formation; (b) an increase in the level of some glycolytic enzymes; (c) the synthesis of a greater proportion of fetal-like glycolytic isozymes with different kinetic and regulatory properties (perhaps with a greater affinity for ADP and P_i), and (d) the binding of a significant portion of the cell hexokinase to the outer mitochondrial membrane or some other cell membrane (perhaps to more efficiently convert glucose to G-6-P or to prevent product inhibition by G-6-P). (2) The rapidly growing tumor cell has been programmed genetically also to synthesize fewer mitochondria (usually a 50% or more reduction in most highly glycolytic tumors; see section II). On the basis of '1' and '2' above, a more unifying concept for the high glycolytic capacity of rapidly growing tumors can be stated very simply as follows: The net result of two genetic programs (one at the glycolytic level and one at the mitochondrial level) is that the *ratio* of glycolytic systems (operating more efficiently in a single metabolic direction) to mitochondrial systems is *much higher* in high than in low glycolytic tissues. This enhanced ratio may lead to increased lactate production both by enhancing the competitive capacity of the glycolytic system for the available ADP, P_i (or both) in the cell (fig. 11) and by allowing for the enhanced phosphorylation and catabolism of glucose.

1972a, b; IBSEN, 1977]. In some rapidly growing tumors (particularly in hepatomas), enzymes involved in the synthesis of glucose are markedly reduced or absent [WEBER and MORRIS, 1963; SWEENEY *et al.*, 1963]. Moreover, enzymes (other than those involved in the glucose-6-phosphate shunt) which 'bleed off' intermediates from the glycolytic pathway, are frequently reduced in activity (i.e., α-glycerol phosphate dehydrogenase). Finally, hexokinase, the enzyme involved in introducing glucose into the glycolytic pathway is not only elevated and fetal-like in many rapidly growing tumors, but a large fraction of the total cell activity (40–60%) is frequently bound to the mitochondrial fraction[6] [HOCHSTEIN, 1957; MCCOMB and YOUSHOK, 1959; WU and RACKER, 1959a; GEIGER, 1963; ROSE and WARMS, 1967; BUSTAMANTE and PEDERSEN, 1977]. This membrane-bound type of association may allow for a more direct (or more efficient) type of phosphorylation of glucose by the ATP produced during oxidative phosphorylation (fig. 12–14)[7,8].

The relationship that the hexokinase may have to the enhanced glucose utilization capacity of rapidly growing tumors needs further study. Of all the glycolytic enzymes, it would seem to the author that hexokinase may be the most important in amplifying the lactic acid-producing capacities of the rapidly growing class of tumors. Thus, it is hexokinase which allows for entry of glucose into the glycolytic sequence. Should this enzyme in rapidly growing tumor cells be present at low or normal levels, should it be product-inhibited by glucose-6-phosphate, or should it be found exclusively in the cytoplasm fraction away from the mitochondrial ATP-producing system, tumors so affected may not have as enhanced a glucose utilization capacity as they do. It seems very important then that in many rapidly growing tumor cells (if not all) hexokinase is present at much higher levels than in control

[7] With respect to the possible role that mitochondrially bound hexokinase may play in the high glycolytic capacity of certain tumors the following closing passage from the 1968 paper of WOODS *et al.* seems noteworthy: 'The "glucolytic potential" as measured in terms of the anerobic glucolytic capacity correlates well with growth in melanomas, tissue culture fibrosarcomas, and hepatomas. The key factor may be the critical balance of glucose-6-phosphate formation in growth-controlling regions of the cell (e.g., mitochondria and/or ribosomal regions of the cytoplasm).'

[8] WEINHOUSE has in some of his writings emphasized the possible importance of hexokinase in contributing to the 'high glycolysis' of certain tumor cells. For example, he stated in 1972: 'The high hexokinase and pyruvate kinase levels of the poorly differentiated tumors readily explain their high glycolytic activity on the basis of competition for ADP between the glycolytic and respiratory systems, coupled with rapid regeneration of ADP via hexokinase.'

tissues and a significant fraction of it is bound to the mitochondrial fraction (fig. 12–14)[9].

The important role that mitochondrially bound hexokinase may play in rapidly growing tumor cells has been emphasized by KOOBS and his colleagues [KOOBS and MCKEE, 1966; MCKEE *et al.*, 1966; KOOBS 1972]. These workers present evidence to indicate that mitochondrially bound hexokinase may be involved in limiting (or inhibiting) respiration when Ehrlich ascites tumor cells are utilizing glucose (Crabtree effect). The continuous phosphorylation of glucose by ATP (involving mitochondrially bound hexokinase) may reduce the phosphate level available for oxidative phosphorylation and therefore prevent maximal state III respiration rates from being obtained. The net reaction scheme would be as follows:

$$\text{Glucose} + \text{ATP} \xrightarrow[\text{(mitochondrially bound)}]{\text{hexokinase}} \text{G-6-P} + \text{ADP},$$

$$\text{ADP} + \text{P}_i \xrightarrow[\text{phosphorylation}]{\text{oxidative}} \text{ATP} + \text{HOH}$$

$$\text{Net: Glucose} + \text{P}_i \longrightarrow \text{G-6-P} + \text{HOH}$$

From this scheme, it can be seen that mitochondrially bound hexokinase may contribute together with other phosphorylating glycolytic enzymes such as pyruvate kinase [WU and RACKER, 1959a, b; WEINHOUSE, 1970; MELI and BYGRAVE, 1972; GONSALVEZ *et al.*, 1974, 1975] to the superior competitive capacity of the glycolytic system for the available ADP and P_i in the cell.

The important role of hexokinase in contributing to the high lactic acid-producing capacity of rapidly growing tumors is illustrated by two other types of experiments. First, the addition of yeast hexokinase to homogenates of slowly growing, hexokinase-deficient tumor cells (Morris hepatoma 5123), which produce low amounts of lactic acid, results in an enhancement of lactic acid production to the level normally exhibited by rapidly growing tumors [WEINHOUSE, 1966]; secondly, recent experiments in the author's laboratory show that hepatoma cells in culture (H-91 subline as AS-30D ascites hepatoma) will grow as well in galactose as in glucose-containing media, but when grown in galactose (in contrast to when grown in glucose-containing media) they produce only moderately elevated lactic acid levels

[9] ACS *et al.* [1955] recognized that mitochondrially bound hexokinase may be important in tumor glycolysis.

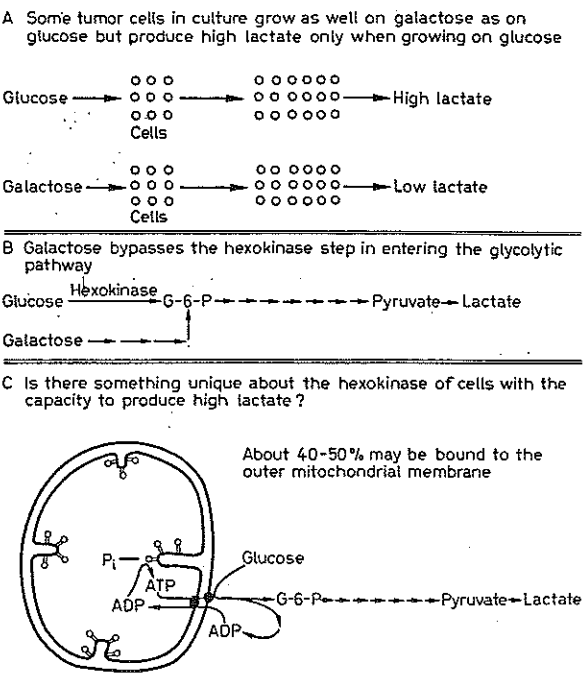

Fig. 14. The important role of hexokinase in contributing to the high glycolytic capacity of rapidly growing tumor cells. *A* Some tumor cells in culture grow equally well on galactose as on glucose as a carbohydrate source, but only when grown on glucose do they produce very high levels of lactic acid (see text for references). *B* The major difference in catabolic pathways for glucose and galactose catabolism lies at the level of hexokinase. Glucose must be phosphorylated via the hexokinase reaction but galactose phosphorylation and its eventual conversion to G-6-P bypasses the hexokinase step. *C* Much of the cell hexokinase in many rapidly growing tumors is bound to the mitochondrial fraction of the cell where it can be phosphorylated directly by the ATP produced during oxidative phosphorylation.

when compared with control liver [BUSTAMANTE and PEDERSEN, 1977]. The major difference between pathways for galactose and glucose catabolism is that galactose, in its conversion to glucose-6-phosphate, bypasses the hexokinase step (fig. 14). With respect to the latter study, it is also important to note that in hepatoma cells, the level of hexokinase is elevated markedly in relation to control tissue; 40–50% of the activity is bound to the mitochondrial fraction, and it is not readily product-inhibited by glucose-6-phosphate [BUSTAMANTE and PEDERSEN, 1977]. Moreover, the mitochondrial content of H-91 cells is reduced by about 50% when compared to control liver.

From the discussion presented in both parts *A* and *B* of this section, the reader hopefully will appreciate that not one, but several factors acting synergistically appear to be involved in the high lactic acid-producing capacity of rapidly growing tumors. The author has made an attempt to simplify these complexities by emphasizing the following points about the bioenergetics of rapidly growing tumors: (1) Shuttle systems for reoxidation of NAD^+ do not appear to be limiting and, therefore, are most likely *not* the cause of high lactic acid production. (2) The capacity of the mitochondrial population to oxidize pyruvate as rapidly as it is formed appears to be the major limiting factor. (3) The limited capacity of the mitochondrial population to oxidize pyruvate may be generally related to the inability of mitochondria within the population to achieve maximal state III rates for reasons unrelated to 'a defective respiratory chain' or a 'defective pyruvate transport system' (although some tumor mitochondria may have defects at these levels). (4) The reason maximal state III respiration rates may not be obtained may be because ADP, P_i (or both, depending on the tumor) are limiting at the site of oxidative phosphorylation in mitochondria. (5) The reason ADP, P_i (or both) may be limiting at the site of oxidative phosphorylation in mitochondria may well be due to the fact that the reduced level of mitochondria within rapidly growing tumor cells (50% or more reduction [section III]) makes the mitochondria poorer competitors for the available ADP and P_i than the glycolytic system, which is markedly enhanced. (6) Enhanced glucose utilization may or may not take place in rapidly growing tumor cells, depending on the hormonal, oxygen, and glucose concentrations surrounding the cells. Moreover, enhanced glucose utilization does not appear to be necessary for all rapidly growing tumor cells to exhibit a greater lactic acid production than control cells. However, all rapidly growing tumor cells probably have the *capacity* to utilize much more glucose than their tissue of origin. Moreover, when enhanced glucose utilization does occur, it most likely amplifies the 'already high' lactic acid-producing capacity of the tumor, both by amplifying the superior competitive capacity of glycolysis for ADP and P_i, and by producing more pyruvate. (7) When enhanced glucose utilization occurs, the major enzyme responsible in many tumor cells may be hexokinase, which to a large extent (40–60%) is bound to the mitochondrial fraction. The action of this enzyme, which is present at high levels in rapidly growing tumors, is to channel more glucose-6-phosphate into glycolysis, and at the same time it may limit P_i levels at the site of oxidative phosphorylation. The net result is to enhance the 'already high' lactic acid-producing capacity of the rapidly growing tumor cell. (8) In those cases where rapidly

growing tumor cells reutilize lactic acid less efficiently than the tissue of origin, this will contribute to the high *net* lactic acid production of the tumor relative to the tissue of origin.

Perhaps more than anything else the first seven points tend to emphasize that a fine balance may exist in both normal and cancer cells between mitochondrial oxidative phosphorylation and glycolysis for the available ADP, P_i (or both) (fig. 12). The possibility that ADP, P_i (or both) may be rate-limiting for glycolysis and respiration in rapidly growing tumor cells has been considered by numerous investigators [CHANCE and HESS, 1959a, b; WU and RACKER, 1959a, b; KOOBS and MCKEE, 1966; WENNER, 1967, 1975; MAZUMDER and WENNER, 1977; LEE *et al.*, 1967; LO *et al.*, 1968; COE and LEE, 1969; KOOBS, 1972; GONSALVES *et al.*, 1974, 1975]. In many normal cells and in slowly growing tumor cells, the balance between mitochondrial oxidative phosphorylation and glycolysis for the available ADP, P_i (or both) seems to be maintained in such a way that mitochondria can accommodate most of the pyruvate formed from glucose. In rapidly growing tumors and in some normal cells, however, this balance appears to be in favor of glycolysis for a number of reasons alluded to above, and lactate formation rather than pyruvate oxidation is favored. Evidence that a balance does exist in both normal and tumor cells (between glycolysis and oxidative phosphorylation for the available ADP, P_i [or both]) is provided by experiments that show that the glycolysis of both cell types is enhanced when cells are transferred from an aerobic environment (where the mitochondria are functioning) to an anerobic environment (where the mitochondria are not functioning) [WEINHOUSE, 1972].

C. Other Factors Involved in the High Glycolysis of Rapidly Growing Tumors

The views presented above to account for the high lactic acid-producing capacity of rapidly growing tumor cells, although complex in some respects, may be oversimplified in other respects. For example, other reactions in the cell which utilize or consume ATP may have to be considered as well. Figure 12 represents an attempt by the author to put the important relationships among glycolysis, oxidative phosphorylation, and other cell reactions into focus as they relate to ATP synthesis and ATP hydrolysis. It will be appreciated that ATP synthetic and ATP-utilizing reactions in actively metabolizing animal cells are most likely in a 'dynamic steady state'. Glycolysis and mitochondria require ADP and P_i to synthesize ATP which is in turn utilized to drive biosynthetic reactions, to maintain the Na^+, K^+ balance of the cell via the Na^+, K^+ ATPase, to phosphorylate glucose via hexokinase, and to drive many other ATP-dependent reactions such as the efflux of

Ca^{++} from the cell or the influx of Ca^{++} into mitochondria or microsomes (not shown in fig. 12). ADP and P_i (and in some cases AMP) are formed from the utilization of ATP, and mitochondria and glycolysis are again required to synthesize ATP. Although the concentrations of ATP, ADP, and P_i in the cell may not change much during active metabolism, there is much ATP synthesis and ATP utilization taking place.

As RACKER and his colleagues [RACKER, 1972; SCHOLNICK *et al.*, 1973; SUOLINNA *et al.*, 1974, 1975] emphasize, the activity of the Na^+, K^+ ATPase and other cell ATPases may be important in helping sustain glycolysis. Thus, it might be predicted that when cellular ATPases are inhibited glycolysis and oxidative phosphorylation will be inhibited, and when cellular ATPases are stimulated glycolysis and oxidative phosphorylation will be stimulated. The Na^+, K^+ ATPase and other cell ATPases must be considered, therefore, in any complete picture which emphasizes the interaction of ADP and P_i with glycolysis and mitochondria. In support of this view, VAN ROSSUM *et al.* [1971], SCHOLNICK *et al.* [1973], and GALEOTTI *et al.* [1977] have shown that in certain rapidly growing tumor cells, i.e., Ehrlich ascites and Morris hepatomas 3924A, the addition of ouabain (an inhibitor of the Na^+, K^+ ATPase) suppresses lactate production. In other studies, RACKER and his colleagues [SUOLINNA *et al.*, 1974, 1975] have shown that in addition to ouabain, flavonoids such as quercetin also suppress lactate production of certain tumors. Quercetin is an inhibitor of several membrane-bound ATPases [LANG and RACKER, 1974; SUOLINNA *et al.*, 1975], but also of mitochondrially bound hexokinase [GRAZIANI, 1977][10].

Although it seems clear from these studies that cell ATPases help sustain glycolysis in tumor cells, what is not clear is whether such ATPases help sustain glycolysis more in tumor cells than in normal cells. As shown in figure 12, cell ATPases must supply the necessary ADP and P_i for glycolysis and oxidative phosphorylation in normal cells as well as tumor cells. RACKER [1977], however, tends toward the view that one or more ATPases of tumor cells may be sufficiently activated to cause high glycolysis. Thus, he states in a recent monograph [RACKER, 1977], 'I propose that the high aerobic glycolysis of tumor cells is caused by activation of an ATPase which may be in the mitochondria, in the plasma membrane, or even in a virus'. The word 'ATPase' is used in a very general sense and could be any activated cellular reaction which leads to the hydrolysis of ATP to yield ADP and P_i. For example, the hexokinase reaction, which is elevated in highly glycolytic tumors and partially responsible for high lactate formation (section XII.B), can be considered a 'partial ATPase' since it yields ADP as a product. In any case, before RACKER's interesting (but very general) view can be evaluated critically, additional experiments will have to be carried out in which the 'ATPase activities' of tumor cells are compared carefully with *known* tissues of origin (e.g., hepatoma vs. liver).

XIII. ATP Production by Tumor Cells – Relative Contributions by Glycolysis and Mitochondria

Glycolysis *in vitro* accounts for a much smaller fraction of the total ATP produced in most tumor cells than does mitochondrial oxidative

[10] GRAZIANI *et al.* [1977] have also shown that Quercetin inhibits lactic acid production in Ehrlich ascites tumor cells.

phosphorylation. As mentioned earlier in this review, slowly growing and intermediate growth rate Morris hepatomas, when placed in glucose-containing media, produce normal or moderately elevated levels of lactic acid relative to control liver (sections I and II; fig. 10). Moreover, some of these tumors are markedly deficient in hexokinase and, rather than utilizing glucose, they may preferentially utilize fatty acids as a major fuel source. Fatty acids, of course, are oxidized directly at the level of the mitochondria. It seems reasonable to suggest, therefore, that *in vitro* the slow and intermediate growth rate classes of Morris hepatomas most likely rely on mitochondrial oxidative phosphorylation, either totally or greater than 90%, for their source of ATP (fig. 12).

As might be predicted, rapidly growing, highly glycolytic tumors *in vitro* rely more on glycolysis for ATP than do the slow and intermediate growth rate hepatomas. However, even in the rapidly growing class of tumors it would seem that mitochondrial oxidative phosphorylation still accounts for the major source of ATP in most cases. This was emphasized more than 15 years ago by AISENBERG [1961] when he wrote, 'Even the ascites cell which displays the most extreme metabolism of the type we associate with tumor cells (i.e., high aerobic and anerobic glycolysis) derives only about half of its energy from glycolysis (fig. 12), whereas for most solid tumors only 15–40% of the total aerobic energy available comes from glycolysis. Furthermore, the prominent Crabtree effect (inhibition of respiration during glucose utilization) of the tumor slice indicates that, if glycolytic substrate is not available, the tumor slice is able to extend its respiratory rate to produce oxidatively the energy that would ordinarily be produced by glycolysis.' Indeed, it would seem that the latter suggestion of AISENBERG [1961] has been confirmed in other laboratories. For example, studies of tumor cells in culture show that glucose can be replaced with other carbohydrates (i.e., fructose or galactose) without altering the growth properties of the cell [EAGLE *et al.*, 1958; BUSTAMANTE and PEDERSEN, 1977]. Significantly, cells grown on these alternative carbohydrate sources, which bypass the hexokinase step (section XII. B; fig. 14) produce much less lactic acid than cells grown in glucose. It would appear, therefore, that when cells in culture grow on glucose they may obtain, at the most, 50% of their ATP from glycolysis (assuming they can glycolyze as rapidly as the Ehrlich cell), whereas when they are grown on other carbohydrate sources they probably derive less than 10–20% of their ATP from glycolysis *per se* and as much as 90% from mitochondrial oxidative phosphorylation. Moreover, it would appear that for cells in culture a very 'high glycolysis' (therefore, a very high

lactic acid production, e.g., in the range of the Ehrlich cell) is not essential for malignant growth.

In assessing the relative contributions of mitochondrial oxidative phosphorylation and glycolysis in supplying the energy needs of cancer cells, it is more important to the cancer problem to know what is taking place *in vivo* than *in vitro.* Unfortunately, obtaining *in vivo* information about the bioenergetics of cancer cells is difficult, and less definitive information is available. However, the early *in vivo* experiments of CORI and CORI [1925] on the Rous sarcoma, and of WARBURG *et al.* [1926] on the Jensen sarcoma remain impressive even today. Both studies show that tumors *in vivo* utilize much glucose and produce large amounts of lactic acid. In fact, the amounts of lactic acid formed by these tumors relative to the amount of lactic acid formed by normal tissues seems to be greater *in vivo* than *in vitro* (fig. 10). It is possible that tumors *in vivo* are not as 'oxygenated' as tumor cells *in vitro* [URBACH, 1956; URBACH and NOELL, 1957] or that some other factors limits respiration. In any case, the tumor cells *in vivo* may rely somewhat more on glycolytic reactions for their source of ATP than tumor cells *in vitro.*

The possibility that oxygen deprivation may promote high rates of glycolysis for some tumor cells *in vivo* does not seem to be the case for Morris hepatoma 5123, fibrosarcoma 4956, or Walker 256 carcinosarcoma. GULLINO *et al.* [1967] were unable to find any indication that these neoplastic tissues could supplement an *in vivo* oxygen deficiency by an increase in glycolysis.

In summary, it seems clear that all tumor cells *in vitro* do not obtain the same proportion of their ATP from mitochondrial and glycolytic reactions. The slowly growing and intermediate growth rate hepatomas most likely obtain the bulk of their ATP from mitochondrial reactions, whereas the rapidly growing tumors obtain significant amounts from both sources with the greater proportion of ATP still being derived from the mitochondria. The situation *in vivo* remains unclear at this time. The available published data suggest that glycolytic reactions may be more important in supplying the source of ATP for tumor cells *in vivo* than *in vitro,* but the 'degree of this glycolytic importance' relative to mitochondrial oxidative phosphorylation is not at all clear.

XIV. Genetic Apparatus, Viral and Chemical Carcinogens

One of the major approaches to cancer research under way in this and other countries today involves examining the interaction of viral and chemical

carcinogens with the genetic apparatus of the nuclei of animal cells. However, the possibility that carcinogen interactions with *mitochondrial DNA* as well as with nuclear DNA may be necessary to effect transformation and/or help maintain the transformed state has not been given serious consideration. With this thought in mind, the purpose of this section is threefold: (1) to summarize briefly what is known about the genetic apparatus and associated protein synthetic machinery of mitochondria of normal cells; (2) to summarize what molecular alterations have been found at the level of these systems in tumor cells, and (3) to emphasize that very suggestive evidence exists in the literature implicating either a direct or indirect association of carcinogens with mitochondria.

Mitochondrial DNA is a circular duplex molecule with a perimeter of about 5 μm and a molecular weight of about 10^7 daltons [for a review, see NASS, 1969]. It comprises less than 2% of the total cell DNA. Two to six molecules of DNA are thought to be present in each mitochondrion and to be associated, at least in part, with the inner mitochondrial membrane. Genetic and other types of studies have shown that mitochondrial DNA codes for mitochondrial ribosomal RNA, mitochondrial t-RNAs, and several small hydrophobic proteins ($\leq$ 30,000 daltons) which comprise parts of the cytochrome oxidase molecule (complex IV), parts of the cytochrome b-c_1 complex (complex III), and parts of the F_0 portion of the oligomycin-sensitive ATPase complex ($F_0 F_1$) [for reviews or monographs, see TZAGOLOFF *et al.*, 1973; SCHATZ and MANSON, 1974; KROON and SACCONE, 1974; SACCONE, 1976]. Mitochondrial protein synthesis takes place on mitochondrial ribosomes which are distinct in polypeptide composition from cytoplasmic ribosomes [LEISTER and DAWID, 1974].

The reader should refer to figure 15 for an overview of the mitochondrial genome. The genetic map shown, that of the yeast *Saccharomyces cerevisiae*, was kindly provided by Drs. ANNA MARIA COLSON and ANDRÉ GOFFEAU of the University of Louvain in Belgium, and B. DUJON and P. SLONIMSKI of Centre de Biologie Moléculaire, Gif-sur-Yvette, France. For details, see SLONIMSKI and TZAGOLOFF [1976] SACCONE and KROON [1976], and BÜCHER *et al.* [1976]. Locations on the map are shown for genes which code for mitochondrial proteins and mitochondrial RNA. Open regions are shown also indicating that there may be some unidentified gene products of mitochondrial DNA.

In tumor cells several alterations have been noted at the level of the mitochondrial genome and its associated protein synthetic machinery. These include changes in: (a) the amount and 'forms' of DNA; (b) the activities of DNA and RNA polymerase; (c) the rate of turnover of DNA; (d) the activity of poly A polymerase; (e) the degree of methylation of t-RNA species, and (f) the rate of protein synthesis and the type of polypeptides synthesized. In general, it can be stated that these molecular changes are most pronounced in the rapidly growing class of tumors, and that they are poorly understood in terms of their relationship to the neoplastic state.

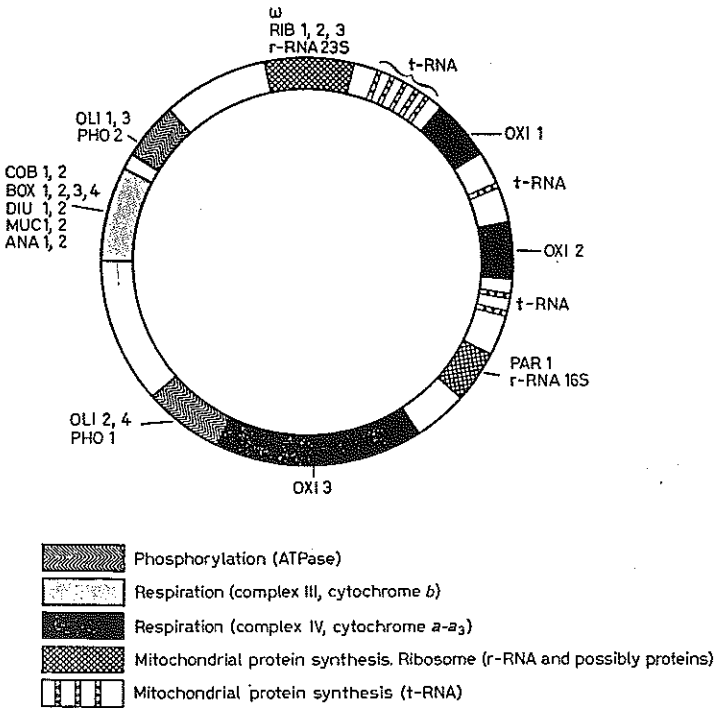

Fig. 15. Schematic localization of genes in the mitochondrial DNA of the yeast *Saccharomyces cerevisiae*. The map was kindly provided by Drs. ANNE MARIE COLSON and ANDRÉ GOFFEAU of the University of Louvain, Belgium, and constructed on the basis of the following references [SLONIMSKI and TZAGOLOFF, 1976; SACCONE and KROON, 1976; BÜCHER, *et al.*, 1976]. Abbreviations are as follows: ANA 1,2: antimycin resistance; BOX 1,2,3,4: cytochrome aa_3 and *b* deficiency; COB 1,2: cytochrome *b* deficiency; DIU 1,2: diuron resistance; MUC 1,2: mucidin resistance; OLI 1,2,3,4: oligomycin resistance; OXI 1,2,3: cytochrome oxidase deficiency; PAR 1: paromomycin resistance; PHO 1,2: oxidative phosphorylation deficiency; RIB 1: ribosome 1 = chloramphenicol resistance; RIB 2: ribosome 2 = spiramycin resistance; RIB 3: ribosome 3 = erythromycin resistance; ω = polarity factor. The length of the different segments is only indicative.

NEUBERT [1966] showed that the content of mitochondrial DNA in the rapidly growing hepatoma 3924 A is high, but in the slowly growing hepatoma 7787 it exceeds the liver value by only a factor of two. In reviewing the content of DNA in mitochondria from a number of tissues, NASS [1969] notes that the DNA content of rapidly growing tumors (2.5–4.7 μg/mg protein) is much greater than that of adult normal tissues (0.24–0.8 μg/mg protein) and

rapidly growing cells (0.9–1.8 μg/mg protein). More recently, WHITE *et al.* [1975] have shown that the content of mitochondrial DNA in the Novikoff solid hepatoma and in Novikoff cells in monolayer culture is four-to fivefold greater than the DNA content of liver mitochondria.

CLAYTON and VINOGRAD [1967, 1969], SMITH and VINOGRAD [1973], and WHITE *et al.* [1975] have shown that tumor mitochondria contain, in addition to the normal 5-μm circular duplex form of DNA, more complex forms called *catenanes* and *circular* dimers. Catenanes are those DNA forms in which two or more monomer-length circles are topologically bonded as the links in a chain. Circular dimers are DNA molecules which are twice the monomer size (i.e., ~ 10 μm) and which consist of two monomer genomes in tandem arrangement. Significantly, the circular dimers are most characteristic of cancer cells, and they may constitute as much as 50% of the total mitochondrial DNA of some tumors. Circular dimers have been observed in mouse L-cells, SV-40 transformed BALB/c3T3 cells, leukocytes of patients with granulocytic leukemia, and in 14 human solid tumors. Although they are rarely observed in noncancerous tissues, they are found in normal human and beef thyroid tissues.

Despite the differences observed in relative amounts and forms of mitochondrial DNA in normal vs. tumor cells, the base composition of the mitochondrial genome is evidently unaltered in the two cell types [WHITE *et al.*, 1975]. Thus, the increased amounts of DNA and the increased number of complex forms observed in mitochondria of rapidly growing tumors is probably not related to alterations in base composition but, rather, to an impaired or anomalous mechanism of DNA replication, or to an altered mitochondrial biogenesis pattern.

NEUBERT *et al.* [1968] and BASS [1977] have examined this problem in some detail and find that the mitochondria of rapidly growing hepatomas have increased activities of DNA and RNA polymerase and an increased rate of protein synthesis. However, the turnover, of DNA (measured using ^{3}H-thymidine) and protein (measured using ^{35}S-methionine) of such tumors is markedly reduced. In fact, in the rapidly growing hepatoma 3924A the mitochondrial DNA is found to have an average turnover half-life of greater than 80 days (compared to a value of 7–15 days for normal liver). If it can be assumed that the half-life (of turnover) of mitochondrial components is related to both synthesis and degradation, it would appear that the mitochondria of rapidly growing tumor cells may be degraded less readily than the mitochondria of normal cells. However, before making such assumptions, the reliability of methods used to measure the turnover half-lives of mito-

chondrial components must be considered also. Thus, CHANG *et al.* [1968] note that ^{3}H-cytidine is incorporated into mitochondrial DNA much more rapidly than ^{3}H-thymidine. These workers emphasize the need for caution in utilizing only one labeled precursor as an index of DNA synthesis.

Alterations have been noted also in other mitochondrial components associated with protein synthesis in mitochondria. JACOB *et al.* [1974] report that poly A polymerase, the enzyme responsible for catalyzing the synthesis of the poly A portion of messenger RNA, is markedly deficient in activity in the rapidly growing hepatomas 3924A and 7777. When isolated, however, the enzyme is highly active and is capable of adding 600 nucleotides to a poly A primer [ROSE *et al.*, 1975]. It is suggested that the low activity of the mitochondrial enzyme may reflect the nonavailability of the primer-binding sites or the occupation of available binding sites with an inefficient primer. Mitochondrial 4S RNA species (t-RNAs) are altered in tumor mitochondria both with respect to their degree of methylation of bases and the amount of ribothymidine they contain. The degree of methylation and the amount of ribothymidine are decreased in the 4S RNA species isolated from the rapidly growing hepatomas 5123D and 7777 [RANDERRATH *et al.*, 1974; CHIA *et al.*, 1976]. Although the significance of these defects is not understood, RANDERRATH *et al.* [1974] do not believe they reflect a reduced ability of the t-RNAs to interact with mitochondrial ribosomes.

Finally, the most important question about the mitochondrial genome and its associated protein synthetic machinery in tumor cells has been answered only partially. That is, does the mitochondrial DNA of tumor cells code for any unusual proteins which are not coded for by the mitochondria of normal cells? The preliminary experiments of IRWIN and MALKIN [1976] using ^{35}S-methionine suggest that two to three proteins synthesized by mitochondria of hepatoma 20 and 3924A may be different in molecular weight from proteins synthesized by normal liver mitochondria. These findings, together with those of BASS [1977], which show a two- to threefold increase in mitochondrial protein synthesis in hepatomas 7800 and 5123D relative to control liver mitochondria, suggest that the mitochondrial DNA of tumor cells may code for some proteins which are not coded for by the mitochondrial DNA of normal cells.

With these thoughts in mind, it seems important to identify the gene products of the mitochondrial DNA of cancer cells. Identification of these gene products may help establish whether or not viral carcinogens interact directly with the mitochondrial genome. Certainly the evidence summarized in table V is highly suggestive of a close relationship between mitochondria

Table V. Evidence suggesting either a direct or indirect association of viruses with mitochondria of animal cells

System	Observation	References
Chick leukaemia myeloid cells	the mitochondrial DNA of cells transformed by avian myeloblastosis virus were shown to contain an abnormally high proportion of catenated dimers or oligomers.	RIOU and LACOUR [1971]
Rous sarcoma	RSV was detected in the mitochondria both by electron microscopy and by infecting chick embryo fibroblast (CEF) cultures *in vitro* with the purified mitochondrial fraction, and by determining the number of transformed cells three days after infection	MACH and KARA [1971], KARA *et al.* [1971]
Chick-embryo fibroblasts	chloramphenicol, an inhibitor of mitochondrial protein synthesis was shown to suppress RSV production with little effect on focus formation	RICHERT and HARE [1972]
Chick livers	reticuloendotheliosis virus (REV) was shown to induce morphological, physical, and enzymatic alterations in chick target organ mitochondria; mitochondria isolated from REV-infected livers are effective in transmitting reticuloendotheliosis when inoculated intraperitoneally into day-old chicks; purified mitochondria from infected livers contain up to 16 particles having the size and shape of unenveloped (naked) C-type RNA tumor virus	BALCAVAGE *et al.* [1972]
Hela cells	infecting cells with herpes simplex virus causes a 1.86-fold increase in the amount of radioactivity incorporated into supercoiled mitochondrial DNA compared with mock-infected control samples	RADSAK and FREISE [1972]
Leukemic patients	virus-like particles were observed in the mitochondria of 6 patients with acute myeloblastic leukemia but not in 28 normal bone marrows	SCHUMACKER *et al.* [1973]
Chick-embryo fibroblasts	when the fibroblasts were infected by T5, a temperature-sensitive mutant of Schmidt-Ruppin RSV, the level of catenated dimeric and oligomeric mitochondrial DNA was shown to be temperature-dependent and to correlate with the phenotypic manifestation of transformation	NASS [1973]
Chick-embryo fibroblasts	RSV-transformed cells pretreated with camptothecin were shown to have elevated mitochondrial RNA, DNA, and protein synthesis; the elevation of these activities was shown to be directly related to cell transformation and not merely to cell viral infection; also, RSV-transformed cells were shown to synthesize	BOSMANN *et al.* [1974]

Table V (continued)

System	Observation	References
	greater amounts of glycoprotein autonomously than normal uninfected control cells	
Reptilian cell lines	intramitochondrial virions (IMV) of identical morphology were observed by thin-section electron microscopy in the reptilian lines VSW, VH-2, and GL-1	LUNGER and CLARK [1973, *1974*]
BALB 3T3 cells	using a non-cytotoxic dose of ethidium bromide, a differential inhibitor of mitochondrial DNA and RNA synthesis, some early event(s), required after infection of cells with murine sarcoma virus, were shown not to occur	ROA and BOSE [1974, 1975]
Mouse fibroblasts and human amnion cell line	electron-dense particles 80–100 nm in diameter were observed in electron micrographs of mitochondria of transformed mouse fibroblasts and in a human amnion cell line infected with supernatants of a human mammary carcinoma cell culture	KEYDAR *et al.* [1975]
Baby hamster kidney cells	virus-like particles were shown to be associated with the mitochondria in an ethidium bromide-resistant strain of RSV transformed cells	SOSLAU [1976]

and viruses in some animal cell lines. Significantly, 6 different studies (representing 6 different animal cell lines) show that intramitochondrial virions can be observed in the electron microscope. Other studies show that viral transformation induces elevated rates of mitochondrial DNA, RNA, and protein synthesis [BOSMAN *et al.*, 1974; RADSAK and FREISE, 1972], or that 'specific' inhibitors of mitochondrial protein or nucleic acid synthesis suppress viral integration, replication, or production [ROA and BOSE, 1974; VARMUS *et al.*, 1974a, b; RAMAREDDY *et al.*, 1975; BUSTAMANTE and PEDERSEN, 1976]. Of course, results of studies involving inhibitors are subject to interpretations which may not necessitate direct viral interaction with mitochondria. Thus, an inhibitor like ethidium bromide may inhibit viral-related processes by interacting directly with the viral nucleic acid in the cytoplasm rather than interacting specifically with the mitochondrial genome [VARMUS *et al.*, 1974a, b]. Moreover, an inhibitor like chloramphenicol may interact with other components of the cell rather than interacting specifically with the mitochondrial ribosome [BUSTAMANTE, 1977]. Nevertheless, it would seem to the author that the evidence summarized in table V is sufficiently suggestive of a close interaction between some tumor viruses and mitochondria to

warrant further experimental work. This view is reinforced by the recent study of ERSHOV *et al.* [1975], which shows that infectious RNA of Venezuelan equine virus can replicate within yeast mitochondria and be integrated into ribonucleoprotein complexes.

Along the same lines, it seems important to establish how chemical carcinogens interact with mitochondria, and to establish the relationship(s) of mitochondrial-chemical carcinogen interactions to the normal to neoplastic transition. GRAFFI showed as early as 1940 that carcinogenic hydrocarbons are accumulated by the mitochondria of animal cells. In more recent years, the interactions of chemical carcinogens with mitochondria have been studied *in vitro*, and the effect of carcinogen feeding (to rats) on the functional properties of freshly isolated mitochondria has been studied as well.

Aflatoxin B exerts several types of effects on freshly isolated rat liver mitochondria. These include a partial inhibition of respiration, a partial inhibition of protein synthesis, a partial inhibition of RNA synthesis, an induction of mitochondrial swelling, and an enhancement of uncoupler-stimulated ATPase activity [DOHERTY and CAMPBELL, 1973; BELT and CAMPBELL, 1973, 1975; BABABUNMI and BASSIR, 1976]. Moreover, dimethylnitrosoamine inhibits state III respiration of freshly isolated mouse liver mitochondria when glutamate is substrate [FRIEDMAN and HIGGINS, 1976]. Finally, HADLER and colleagues [HADLER, 1974; HADLER and DANIEL, 1972, 1973; HADLER and DEMITRION, 1975a, b; HADLER *et al.*, 1971a, b] have shown that certain carcinogen derivatives, proximate carcinogens, or ultimate carcinogens also interact directly with isolated mitochondria. 5-Hydroxy-1,2-napthalenedicarboxylic anhydride, which is derived from the carcinogenic polynuclear hydrocarbon dibenz[a,h]anthracene, inhibits mitochondrial oxidative phosphorylation in a fashion similar to rutamycin. Several proximate carcinogens (*N*-hydroxy derivatives of aromatic-substituted isomeric *N*-acetylaminofluorene), and the model ultimate carcinogen *N*-acetoxy-*N*-acetyl-2-aminofluorene induce ATP-dependent volume changes in isolated rat liver mitochondria in the presence of the antibiotic showdomycin. Parent carcinogens do not induce these changes (which appear to be related to the degree of carcinogenicity of the proximate or ultimate carcinogen).

Experiments in which rats are supplied with chemical carcinogens via the diet or injection also are suggestive of direct or indirect interactions of chemical carcinogens with the mitochondria of animal cells. FIALA and FIALA [1959] show that although 3′-Me-DAB-(3′-methyl-4-dimethylaminoazobenzene) and 3,4-benzpyrene feeding to the rat does not result in respiratory inhibition at the level of the mitochondria, such feeding does cause a marked lowering of cell respiration due to a reduction in the content of mitochondria per unit of DNA. Moreover, ARCOS and co-workers [ARCOS, 1971; ARCOS and ARCOS, 1958; ARCOS *et al.*, 1969a, b; BRYANT *et al.*, 1976] show that feeding rats 3′-Me-DAB has a pronounced effect on the swelling properties of freshly isolated mitochondria at 4 weeks of feeding. At this time, in contrast to early and later feeding times, the mitochondria exhibit little or no swelling in the presence of a variety of swelling-inducing agents. Also, the acceptor control ratio drops to a minimum at about this time, and the mitochondria are most resistant to the vicinal-dithiol uncoupling agent diacetyl. Mitochondria isolated from 3′-Me-DAB-induced hepatomas show low values of swelling comparable to those of liver mitochondria from rats fed 3′-Me-DAB for 4 weeks. Significantly, the 4-week time period corresponds to the onset of irreversibility of tumor induction under the conditions of ad-

ministration. Finally, WUNDERLICH *et al.* [1970, 1971/1972] show that rat liver mitochondrial DNA is methylated to a greater extent (3- to 7-fold) than nuclear DNA after administration of single doses of *N*-methyl-*N*-nitrosourea or dimethylnitrosoamine to the rat.

The interaction of chemical carcinogens with mitochondria has led HADLER [1974] to propose the following rather intriguing hypothesis of how chemical carcinogens might effect the normal to neoplastic transition by interacting at the mitochondrial level: 'We do hypothesize that when the mitochondrial machinery for oxidative phosphorylation is disturbed in a subtle fashion by an agent which is not present in adequate concentration to kill the cell, the mitochondrial membrane is altered. It then becomes possible for genetic material to leak through the mitochondrial membrane (in perhaps a single mitochondrion) and act like an exogenous oncogenic virus which has penetrated the cellular membrane. The mitochondrial genetic material may thus eventually alter the genome of the cell. In this way a cancerous cell may be generated.'

In summary, it would seem that the normal to neoplastic transition brings about numerous alterations at the level of the mitochondrial genetic apparatus and its associated protein synthetic machinery. Which of these effects (if any) is essential for neoplasia, and which are consequences of the neoplastic state is not known. Moreover, there is much evidence in the literature indicating that some viral carcinogens and many chemical carcinogens (or their derivatives) interact directly with the mitochondria of animal cells. Finally, some chemical carcinogens, when administered to experimental animals, not only enter the mitochondria but interact covalently with the mitochondrial genome.

XV. Summary, Conclusions, and Future Directions

Much has been learned about tumor mitochondria and the bioenergetics of cancer cells in the past 15 years. First, mitochondria of tumor cells frequently differ both structurally and functionally from mitochondria isolated from control tissues. In most cases (but not all) the degree of structural or functional change correlates with the growth rate or degree of differentiation of the tumor, the most pronounced changes from normal being observed in mitochondria from the fastest growing tumors. Structurally, mitochondria from tumor cells have a characteristic size and shape, and frequently differ from normal mitochondria in membrane lipid and polypeptide compositions. Functionally, tumor mitochondria frequently differ from normal mitochondria in the following ways: type(s) of substance(s) oxidized, requirement for BSA for normal energy coupling, magnitude of the acceptor control ratio, ATP requirement (before uncoupler) for normal uncoupler-stimulated ATPase activity, capacity to accumulate and retain Ca^{++}, rates of electron and anion transport, amount and forms of DNA, and rates of protein synthesis and organelle turnover.

Secondly, the more rapidly growing (or poorly differentiated) a tumor becomes the more its energy metabolism approaches the fetal-like state. The mitochondrial content is reduced by 50% or more and the glycolytic enzymes become elevated and fetal-like. The ratio of glycolytic systems to mitochondrial systems in rapidly growing cancer cells greatly exceeds that of normal cells. Moreover, a large fraction of the cell hexokinase is bound to the outer mitochondrial membrane, allowing direct and more efficient interaction between mitochondrial oxidative phosphorylation and glycolysis. The net result may be to endow the rapidly growing cancer cell with the capacity to produce much more lactic acid than its normal counterpart. This is in sharp contrast to the slowly growing (well or highly differentiated) cancer cell which produces only slightly elevated amounts of lactic acid. Such tumor cells appear to resemble closely their tissues of origin with respect to rates of glycolysis and mitochondrial content.

Thirdly, in all classes of tumors, the mitochondria are 'in charge' of supplying the major portion of the total cell ATP. In slow or intermediate growth rate tumors, the mitochondria account for 80–95% of the ATP produced by the cell, whereas in most rapidly growing tumors this figure is reduced only slightly to about 60–85%. Only in extreme cases (i.e., tumor cells growing in ascites or tissue culture form) is as much as 50% of the total cell ATP derived from glycolytic reactions.

Finally, carcinogenic agents which transform animal cells interact with the mitochondria as well as with the nucleus. Some transformed cell lines contain virus-like particles within the mitochondria. Other transformed cell lines appear to support viral replication within their mitochondria. Moreover, some chemical carcinogens have rather profound effects on the structural and functional properties of isolated mitochondria, whereas other chemical carcinogens, when administered to experimental animals, are found to penetrate the mitochondria and interact covalently with the mitochondrial genome.

Although we have learned much about the properties of tumor mitochondria and their overall relationship to the bioenergetics of cancer cells, we have failed to answer those questions which are most basic and fundamental to the cancer problem.

First, we have not established whether mitochondrial function (i.e., at the level of ATP synthesis, at the level of protein synthesis, or at some other level) is essential to the normal to neoplastic transformation process. This question can be answered best perhaps by attempting to transform cells in culture in the presence and absence of specific inhibitors of mitochondrial

function. It seems relevant to establish whether the cell-transforming agent (i.e., chemical or viral carcinogen) interacts directly with the mitochondria or indirectly with some mitochondrial product.

Secondly, we have not established to what extent mitochondrial function is necessary to maintain or promote the transformed state. Recent experiments have shown that when tumor cells in culture are deprived of oxygen they die [see GREGG, 1972, for a discussion]. However, whether the oxygen-requiring process involved is mitochondrial or extramitochondrial is not known. Moreover, although it seems clear that mitochondrial-hexokinase-glycolytic interactions are responsible, at least in part, for the high lactic acid production of very malignant cancer cells, we have not established whether acid production provides the cancer cell *in vivo* with a selective advantage over its normal neighbors. For example, does a low intracellular pH facilitate ATP synthesis via a Mitchellian type of mechanism, or does a low extracellular pH facilitate the transport of oxidizable substrates (within the blood) across the plasma membrane. Or, conversely, does a low extracellular pH suppress cell-cell interactions, or promote the activation of extracellular proteases essential for 'paving the way' for tumor growth.

Thirdly, we have not established to what extent mitochondria are involved in cachexia or malnutrition of the cancer patient. Certainly, the energy metabolism of the cell is highly dependent on the nutritional state of the animal since many respiratory enzymes require members of the vitamin B complex as part of their cofactor structure. RIVLIN and his colleagues [for a review, see RIVLIN *et al.*, 1974] are presently approaching this important question. Results obtained thus far document the resistance of tumor tissue to dietary vitamin deficiency, and suggest several mechanisms which enable the tumor to maintain a high concentration of vitamins despite dietary deficiency.

Finally, we have failed to supply the cancer chemotherapist with a reliable drug which will selectively inhibit the energy metabolism of the cancer cell. A first approach to this problem might be to define the fuel sources which are utilized preferentially by the major forms of human cancer. For those forms of cancer which rely predominantly on glucose as a fuel source, attempts to selectively suppress glucose utilization might be attempted by focusing on inhibitors of glucose or P_i transport into the cell, or by focusing on inhibitors of hexokinase (with regard to the latter point of attack it should be kept in mind that many very malignant cells have elaborated fetal-like forms of hexokinase, and that a large fraction of the total cell activity is bound to the mitochondrial fraction). For those forms of cancer

which utilize primarily fatty acids or amino acids as a fuel source, analogs of these substances may prove to selectively inhibit the energy metabolism of the tumor cell in question.

In conclusion, we can state that a study of the bioenergetics of cancer cells is a field in its infancy with the major questions relevant to the cancer problem remaining to be answered. It is no longer a field where the central issue at hand is to establish whether OTTO WARBURG was right or wrong.

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