LEVELS OF ORGANISATION IN CELL FUNCTIONS

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IT is the most extraordinary phenomenon of nature that in the enzymatic events controlling life processes there is present a great deal of direction, order and co-ordination all of which show themselves by way of a harmonious and healthy existence. This order sets in right from cell division and multiplication. Thus, from the very beginning in the life of the fertilizing egg, the development of each tissue is co-ordinated with that of all others and this harmonious integration is apparently maintained by elaborate systems of physiological control throughout the life-span of the organism. The living form is thus an intricate and highly developed mosaic of varying and individually specific components.

For a fuller understanding of the basic chemical phenomena underlying this organisation, it has become incumbent to study isolated systems, away from the whole organism. In this process however there must be a clear recognition of the organisational features lost.[1] For, it is axiomatic in biology that the organism is more than the sum of the component tissue. An excised heart can be made to live outside the animal organism for several hours. Muscular contractions and relaxations can be observed with isolated muscle fibres for quite some time. Yet these components contribute more to the life processes that constitute the complicated morphological forms of complete animals.

Notwithstanding the arrangement and integration of parts at each level of complexity, a considerable amount of knowledge has accumulated through independent developments in the fields of enzyme chemistry and cytology. During the last two decades, chemical events in the body have been examined by a variety of means at the cellular and even sub-cellular levels. One of the first attempts in this direction has involved histological studies with tissue preparations. Even greater progress has been made on the dynamics of cellular processes through respiratory studies with tissue slices, homogenates and particulate fractions. This bold approach has paid excellent dividends and literally hundreds of different enzymatic mechanisms have been uncovered. An ever-increasing number of the finer reaction mechanisms that contribute to the sum total of metabolic processes are now explicable in terms of simple chemical equations. Such studies have for example enabled *in vitro* reconstruction of the entire process of glycolysis by adding together some twenty different enzymes that include phosphatases, phosphorylases, kinases, enolases, isomerases, mutases and dehydrogenases. The synthesis of starch, glycogen, sucrose, etc., could now be accomplished by relatively simple model reconstructions. From studies on such isolated systems one could go into integrated pictures of larger areas of metabolism and physiologic behaviour.

Sub-Cellular Fractions

This concerted attack on the enzymic armoury of the plant and animal kingdom has revealed an unique architectural pattern or 'chemical geography' within the living cell itself. In other words, there is a beautiful though complex structural pattern in the cell which is equalled only by a similar complex chemical organisation. In fact, it could be argued that cellular architecture has evolved itself in such a way as to permit an orderly sequence of metabolic processes or spatial disposition of enzymatic units which excludes interference among the many biochemical reactions that occur simultaneously.[2,3] Thus, specific cellular structures have apparently specific chemical properties. Systematic procedures in cell fractionation techniques have been devised for the isolation of cytoplasmic elements by means of differential centrifugation in isotonic media of broken cell suspensions.[4,5] It is now well recognized that there are only limited numbers of particle types which are sharply distinguishable from one another by specific enzymatic properties and chemical characteristics.[2] Thus a heavy nuclear fraction with relatively less activity for most enzymes is followed by a grayish mitochondrial residue with a whole hodgepodge—but NOT a random hodgepodge—of enzymes and there is left a supernatant which could be further resolved at high speed into sub-microscopic microsomes and a soluble non-sedimentable fraction. The characterisation of the enzymatic and other properties of these sub-cellular fractions are now the subject of intensive study in different laboratories. The nuclei contain all the desoxy-ribose nucleic acid (DNA) of the cell and are responsible for the transmission of species characteristics. They are nearly devoid of oxidizing enzymes. Most of these and especially those concerned with the oxidation of the Krebs cycle intermediates re-

side in the mitochondria. On the other hand, the entire group of enzymes concerned with glycolysis are in the soluble phase. Thus, the breakdown of glucose into carbon dioxide would imply the participation sequentially of the enzymes in the supernatant and those in the mitochondria. It seems that at least with diphosphopyridino nucleotide (DPN) the enzyme system responsible for the synthesis of this coenzyme resides in the nucleus.[6] This would indicate a biochemical interaction between the nuclei and the cytoplasmic contents. Doubtless there are other similar such relationships.

Mitochondria

Numerous recent reports have concerned themselves with the properties of the mitochondrial bodies. They are found in all types of animal cells as well as in other forms of plant and microbial life. They have however been studied more extensively in the animal cells.[3] To the cytoenzymologists, they are always the characteristic landmarks in the intracellular landscape and represent the physical housing for a complex of enzymes, a kind of 'chemical microkitchen'. Among these enzymes are those responsible for the oxidation of the Krebs intermediates, fatty acids and certain amino acids.[7,8] While it is possible for any one enzymatic process to be isolated for purposes of study from the other processes catalysed by this complex, none of the enzymes can be separated from the others in the complex except by rather drastic means. Enzymes show varying tendencies to be detached from the mitochondrial bodies.[9] A whole spectrum of split products intermediate between the mitochondrion and soluble protein can be covered by the application of various devices for fragmenting and disintegrating mitochondria. In such manipulations probing into the mitochondrion, there is again considerable loss of organisational features.

Oxidative Phosphorylation

Of special significance in this connection is the fact that the mitochondrion is also the seat of all oxidative phosphorylation.[8,10] In the oxidation of the substrates of the citric acid cycle by the mitochondrial enzymes, the need is felt for addition of inorganic phosphate, adenosine-5-phosphoric acid (AMP) or, more strictly, adenosine-diphosphate (ADP) and Mg^{++}.[7,11,12] Otherwise, oxidation slows down after a while. In the unfractionated tissue homogenate representing the entire cellular material, no such additions are observed to be necessary. This will therefore denote a lower degree of organisation in the mitochondria as compared to the cell. The fact is, oxidations in the cell are coupled with simultaneous initiation of various endergonic reactions. This problem of energy transmission is fundamental to our understanding of the chemistry of the cell. If the two processes can occur in close juxtaposition at the respective enzyme surfaces the possibility of the dissipation of energy produced in the reaction as heat is reduced enormously. The oxidative processes thus enable the cell to do osmotic work, mechanical work such as contraction, ciliary movement and chemical synthesis. Unexpended energy of oxidation is stored as adenosine triphosphate (ATP) and phosphocreatine.

In a purified mitochondrial system, an oxidative process does not proceed unless there is an acceptor of the high energy bond ($\sim$) which could be formed as follows[7]:

$$\text{Substrate} + \text{oxidase} \xrightarrow{-2e} \text{Oxidized substrate} \sim \text{reduced oxidase}.$$

2. Oxidized substrate $\sim$ reduced oxidase + phosphate $\rightarrow$ Phosphate $\sim$ reduced oxidase + oxidized substrate.
3. Phosphate $\sim$ reduced oxidase + ADP $\longrightarrow$ reduced oxidase + ATP.

According to this concept, phosphate is not necessary for the primary reaction. However, in its absence, the primary complex is stabilised and the over-all reaction velocity becomes limited by the spontaneous rate of breakdown of this complex. Like AMP or ADP, various energy-requiring systems could be equally well coupled with the oxidation. Thus, glucose and hexokinase (not present in mitochondria) or creatine and creatine phosphopherase or thiamine and carboxylase can be added. They are referred to as trapping systems for the $\sim PO_4$. Alternately, adenosine triphosphatase (ATPase) can be added. The respiration of mitochondrial preparations is enhanced by additions of nuclei or supernatant preparations because of their contributions of ATPase or of endergonic systems.[11] ATPase functions by breaking down the $\sim PO_4$. Other uncoupling agents that could act at this step and thus promote oxidation without esterificaion of inorganic PO_4 are dinitrophenol, atabrine, certain antibiotics, thyroxine, barbiturates, and so on. There is a suggestion that there may be fundamental differences in the mechanism by which various agents uncouple oxidative phosphorylation in mitochondria.[13]

Thus, in addition to the organized chain of carrier enzymes which transfer electrons from substrate to oxygen, the mitochondria possess the auxiliary enzyme system which couple phosphorylation of ADP to the exergonic electron transport process. The number of phosphate molecules esterified per mole of substrate oxidized in a single two-electron step, usually expressed as the P/O quotient (disappearance of inorganic phosphate : Oxygen uptake) is approximately three[12,13] and corresponds to a thermodynamic efficiency of some 60-70%.[10] Two of these coupled phosphorylation mechanisms are located between reduced DPN (DPNH) and cytochrome C and the third between cytochrome C and oxygen.[14,15]

Mitochondrial Structure and Function

The most important property of the mitochondrial bodies resides in their efficient use of oxidisable substrates. The morphological and physical properties of mitochondria have attracted special attention because they are closely interlinked with mitochondrial functions. The mitochondria in living cells appear under an electron microscope like beaded rodlets enveloped by a membrane having an inner and an outer zone.[3,9] The presence of this membrane and its importance have been repeatedly brought out from various biochemical studies. When isolated freshly in hypertonic sucrose, a large number of these particles retain their original rod-like structure although a good per cent. of spherical forms are also present. The transformation to spherical shapes is complete on further dilution of the suspending medium.[9] The morphological alteration from elongated to spherical shape at isotonic concentrations however is not accompanied by any major changes in the biochemical properties of the mitochondria. Preparations with isotonic sucrose are widely used for various studies and represent the choicest material for study of oxidative phosphorylation. Actively metabolising mitochondria control the transport of solute and water across their membranes[16] and are known to concentrate to a small but significant extent certain ions, Krebs intermediates, etc.[17,18] This selective power of mitochondria which is energy-dependent is efficiently geared to the systems participating in electron transport and phosphorylation and points to the active and semipermeable nature of the surrounding membrane.

However it has become apparent during studies on oxidative phosphorylation that the mitochondrial membrane has a limited ability to carry out these functions and to maintain the integrity of mitochondrial structure and its activities. Kielley and Kielley[19] found that the synthesis of ATP was rendered inoperative by any procedure resulting in structural damage to mitochondria. A number of factors are known to cause a swelling of these particles due to imbibition of water. Exposure to hypotonic conditions, ageing, presence of traces of heavy metal ions or certain anions, other environmental alterations and physiological states result in an acceleration of swelling of mitochondria which may finally lead to their disintegration into smaller particles.[16,26] Mitochondria in intact cells have also been shown to be sensitive to such changes.[21] Among the biochemical alterations observed are the loss of several intramitochondrial components such as nucleotides and other co-factors, certain ions involved in oxidative phosphorylation and small quantities of soluble proteins.[26] Inhibition of certain enzymes and an activation of others which are latent in fresh mitochondria, especially of ATPase,[19,27-30] and an extreme lability of oxidative phosphorylation system in homogenates and mitochondria[19,21,26] have been consistently reported.

Several attempts at prevention of the swelling of the mitochondria as well as restoration of oxidative phosphorylation, by returning the component(s) presumed to be lost once the swelling had taken place, have met only with partial success.[17,18,31] Protection by adenine nucleotides and Mg^{++}[5,26,32,33] had been observed. Similarly addition of DPN has reportedly some effect.[25] Nearly complete restoration on addition of trichloroacetic acid extracts of fresh mitochondria has also been observed to occur.[32]

It seems evident that the swelling of mitochondria precedes rather than follows the uncoupling phenomenon. Little is known about the events which lead to the swelling of these particles under unfavourable conditions nor about the mechanisms by which phosphorylation is restored on addition of mitochondrial extracts or components. The concept that a supply of ATP is essential at all times for maintenance of mitochondrial integrity is probably not satisfactory.[34] It has been suggested that the swelling of mitochondria is probably an enzymic process rather than a disappearance of any protecting mechanisms.[35] Thus lysolecithinase from snake venom could act on mitochondrial phospholipids (in which it is rich) to give rise to lysolecithin which attracts water molecules with subsequent distortion, swelling, and even rupture of the membrane. However, the pre-

sence of lysolecithinase in mitochondria remains to be demonstrated. Again it has been observed that the swelling is more rapid under aerobic conditions and there is the possibility that oxygen reacts directly with a certain key group probably sulphydryl.[34] Thus, in contrast to the slow and relatively limited swelling produced by other methods, sulphydryl binding agents produce a rapid and more pronounced swelling, suggesting the participation of free sulphydryl groups in the maintenance of mitochondrial structure and/or permeability.[36]

Particulate but no longer mitochondrial systems capable of catalysing phosphorylation during the oxidation of selected substrates have recently been obtained by Lehninger *et al.*[37] by digitonin extraction of rat liver mitochondria and by Green *et al.*[38] by fragmentation of beef heart mitochondria and fractional separation in presence of ethanol and phosphate. Although therefore it seems possible to obtain functional and structural sub-units of the parent mitochondrion, the question as to whether these particles capable of both oxidation and phosphorylation are vesicular or solid cannot be definitely answered at the present time.[39] The fact that they do bind K^+ like the intact mitochondria[40] would suggest a re-evaluation of the role of the mitochondrial membrane.

Despite recent achievements on the isolation of sub-cellular electron transport particles it would seem necessary to concede to the importance of mitochondrial integrity for the maintenance of oxidative phosphorylation and therefore of normal cellular metabolism. An expendable structural feature of an enzyme housing in an *in vitro* system may not be so in the complex milieu of the living cell. In a consideration of protein structure to function, Steinberg and Mihalyi[4] point out that "non-essential" features of enzymes and other biologically essential molecules may arise from their role in orienting the active protein to other structural features and enzymes in the cell. They may also determine its thermodynamic efficiency in the cell under different physiological conditions.

Vitamin B_{12} and Oxidative Metabolism

Subtle changes in the geometrical organization of the multi-enzyme arrays and the matrix in which these functionally interdependent units are housed are conceivable under different biological conditions and hence intracellular control of oxidative phosphorylation may occur at any one of several sites in the coupling processes susceptible to interference. Indeed, it is now recognized that uncoupling of oxidative phosphorylation could arise out of diverse known and unknown modalities.[13]

A reference may now be made to certain suggestions implicating vitamin B_{12}, albeit indirectly, in the control of mitochondrial morphology. The observations have arisen from studies on protection afforded by this vitamin against experimentally induced thyrotoxicosis or liver injury.

When small quantities of an iodo-protein are included in the diet of experimental animals or when small doses of thyroxine are given to them parenterally, they show increased requirement for several of the food factors—among them, chiefly, B vitamins. This is a recognized procedure for induction of deficiency in certain of the B vitamins, the others being given in excess. Of especial significance is the increased requirement for vitamin B_{12}. In thyrotoxic rats, there is usually a rapid weight loss and when this comes to about 10% the animals rapidly die off.[42] If excess B_{12} is present in the diet, there is protection against weight loss and mortality.[43]

Again, a single injection of the steatotic poison carbon tetrachloride can cause fatty liver in the rat. This degeneration is also protected against by prior administration of vitamin B_{12}.[44]

Uncoupling of oxidative phosphorylation has been reported in experimentally induced hyperthyroidism[42,45,46] as well as in steatotic liver injury.[47] Mitochondrial preparations from such livers show a good parallel in their behaviour and characteristics with normal mitochondria subjected to hypotonic conditions. They have lowered pyridine[45,48] and adenine[49] nucleotide levels, and exhibit decreased oxygen consumption[50,51] and lowered P/O ratios.[45,47] No effect of CCl_4 could be seen on mitochondrial integrity in *in vitro* studies.[47] Though *in vitro* uncoupling of oxidative phosphorylation by thyroxine has been reported,[52–55] this is not observed to take place in mitochondria held in isotonic sucrose.[49] Thus, thyroxine is effective *in vitro* only when the hormone is pre-incubated with mitochondria[56] or when the mitochondria are subject to hypotonic conditions[57] in its presence so as to facilitate entry of the hormone. Thyroxine acts by binding Mg^{++} once it enters the mitochondria[58,59] and thyroxine-induced uncoupling could be reversed by Mg^{++}. Thyroxine also fails to uncouple oxidative phosphorylation[60] in digitonin preparations[37] of mitochondria. These results are therefore suggestive of the fact that any *in*

vitro effect of thyroxine is not due to direct interference with the enzymes of oxidative phosphorylation and that its effect as well as that of CCl_4 *in vivo* could arise from an impairment of mitochondrial integrity due to osmotic damage. This phenomenon obviously underlies the observed uncoupling of phosphorylation from respiration.

In recent work from this laboratory,[61,62,44,63] it has been demonstrated that, in experimentally induced nutritional stress such as thyroprotein feeding or CCl_4 poisoning, there is depletion of tissue vitamin B_{12} levels and an impairment of several metabolic processes. A disturbance in glutathione metabolism and of osmotic damage to mitochondria precede all other derangements. A rapid reduction in liver stores of glutathione in the CCl_4-poisoned rat has also been reported by Patwardhan and coworkers.[64] Tapley and Cooper[36,65] observed that the action of thyroxine *in vitro* is probably due to its primary action on mitochondrial structure. Dianzani has made similar observations in steatotic rats.[47,48] Since oxidative phosphorylation is the only major pathway linking energy-requiring with energy-yielding processes, it is obvious that the uncoupling produced by mitochondrial swelling is reflected as metabolic derangements.

It has been suggested that the nucleotides are bound to sites in the mitochondria which are probably also the ones at which oxidative phosphorylation takes place. It would appear that the immobilisation of co-factors like AMP, ADP, DPN, Mg^{++}, etc., from their site of activity makes them unavailable for participation in oxidative phosphorylation. It is also possible that the nucleotides are bound to proteins enzymatically active and confer stability on these enzymes and that unbinding and subsequent loss of these from mitochondria makes the proteins more labile and enzymatically inactive.

The morphological and metabolic derangements of hyperthyroidism and fatty infiltration are all protected against by vitamin B_{12} *in vivo*.[61,44,63] An interesting feature relates to changes in pyridinonucleotides (PN). A marked reduction in PN and a decrease in PN/PNH (oxidised to reduced pyridinonucleotides) in CCl_4 toxicity suggests a shift of fatty acid metabolism towards synthesis. PN is important because it is concerned in oxidation of fatty acids and in at least 3 steps in the citric acid cycle (isocitric, α-ketoglutaric and malic dehydrogenations) whose normal functioning is necessary for fatty acid oxidation. The influence of vitamin B_{12} on correction of CCl_4 damage is explicable in terms of its favourable effect on PN and PN/PNH.[66]

Since mitochondria are apparently protected against changes attendant on swelling by AMP or ATP,[25,26] DPN[25] and sulphydryl,[34,36] the protection observed with vitamin B_{12} might arise from its known role in nucleotide and sulphydryl metabolism. Obviously the diverse effects observed with this vitamin, in extremely small amounts, on the physiology of the animal organism must arise from a very basic function it has in cellular integrity and economy.

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