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2

Functions of Subcellular Structures

2.1. BACTERIAL WALLS AND MEMBRANES

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STUDIES of the structure and biosynthesis of bacterial walls are intimately associated with the mechanism of action of several antibacterial agents.

2.1.1. *Bacterial Walls*

The cell envelope of gram-positive bacteria is composed of two separate and distinct structures: a thick (200–800 Å) external layer constituting the outer wall, and an alternating electron-dense electron-transparent layer representing the inner plasma membrane. Although isolated walls viewed under the electron microscope generally appear as empty and rather amorphous bags retaining the size and shape of the original cell, their complex multilayered structure has recently been revealed with the aid of improved sectioning and staining techniques. One or more outermost layers are interlinked in a specific and organized manner and contain the chemical determinants responsible for the immunological and phage fixation properties of the cells. These are bound to a deep rigid layer, about 100 Å thick, which accounts for about 50% of the weight of the isolated wall. The rigid layer of the gram-negative bacteria is much thinner (20–30 Å) and represents as little as 5 to 10% of the cell envelope by weight. It is sandwiched between the plasma membrane and an outer highly complex multiple-track layer of lipoproteins and lipopolysaccharides, i.e. the O-antigens or the bacterial endotoxins.

The rigid layer of the wall is found in all procaryotic cells (with the exception, however, of the halobacteria), where it functions as an insoluble, supporting structure allowing the bacteria to live under hypotonic environmental conditions. This layer is visualized as a network of glycan strands interlinked by means of peptide chains. It is thus a peptidoglycan polymer. (The terms mucopeptide, glycopeptide and murein used by some authors are synonymous with peptidoglycan.)

Although the wall peptidoglycan is an immense macromolecule completely surrounding the cell, both the glycan and the peptide moieties are small, water-soluble oligomers; rigidity and insolubility hence are properties of the intact polymer. A loss of integrity resulting from the breakdown of either the glycan or the peptide component brings about the solubilization of the entire polymer. During cell expansion and division, the safe enlargement of the net results from a strict coordination between the creation of additional receptor sites by hydrolysis and the insertion of newly synthesized building blocks into the gaps thus formed. Such

coordination in the cell machinery is a hallmark of well-balanced growth. Under normal conditions, and despite the fact that the wall is one of the most dynamic structures of the whole cell, the mechanical strength of its peptidoglycan network is never impaired.

2.1.2. *Structure of the Bacterial Wall Peptidoglycan*

In all bacterial peptidoglycans, the glycan chains consist of alternating β-1,4 linked 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine; GlNAc) and 2-acetamido-2-deoxy-3-O-(D-1-carboxyethyl)-D-glucose (N-acetylmuramic acid; MurNAc) pyranoside residues, i.e. a chitin-like structure with the exception that every other sugar is substituted by a 3-O-D-lactyl group (Fig. 1). The only possible variations encountered to date are the presence of O-acetyl groups on C6 of some of the N-acetylmuramic acid residues or the replacement of N-acetylmuramic acid by N-glycolylmuramic acid (in *Mycobacterium* sp. and related bacteria).

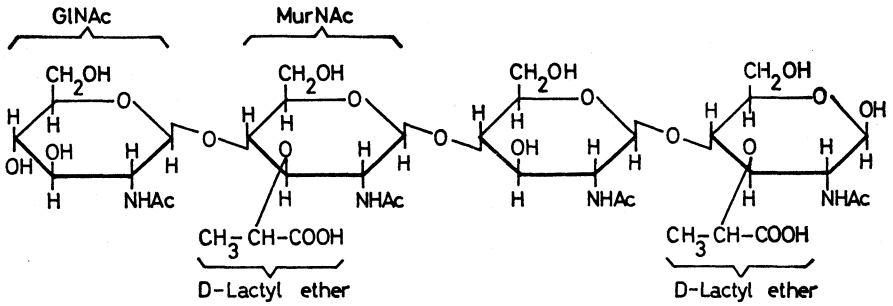

FIG. 1. A portion of a glycan strand. In the peptidoglycan network, the COOH of the D-lactyl groups are peptide-substituted.

The peptide moieties of the wall peptidoglycans are essentially composed of *tetrapeptide subunits*, which substitute through their N-termini the D-lactic acid groups of the glycans, and *peptide bridges*, which cross-link the tetrapeptide subunits so that adjacent glycan chains are paired (Figs. 2 and 3). The tightness of the net thus depends upon the length of the glycan strands, the frequency with which the glycan strands are peptide-substituted, and the frequency with which the peptide subunits are cross-linked. Depending upon the bacteria, the chain length of the glycan strands averages from 20 to 140 hexosamine residues, the percentage of peptide-substituted N-acetylmuramic acid residues in the glycan chains varies from 50 to 100, and the average size of the peptide moieties is between two and ten cross-linked peptide subunits. Evidently, many terminal groups are present in both the glycan and the peptide parts of the net. They reflect, at least in part, the dynamics of bacterial growth.

The consistency of structure seen in the glycan moiety is not reflected in the peptide substituents. The tetrapeptide subunits have the general sequence R_1-γ-D-glutamyl-R_3-D-alanine. The R_1 residue, that is, the one which substitutes the glycan chains, is usually L-alanine but can be L-serine or glycine. D-glutamic acid

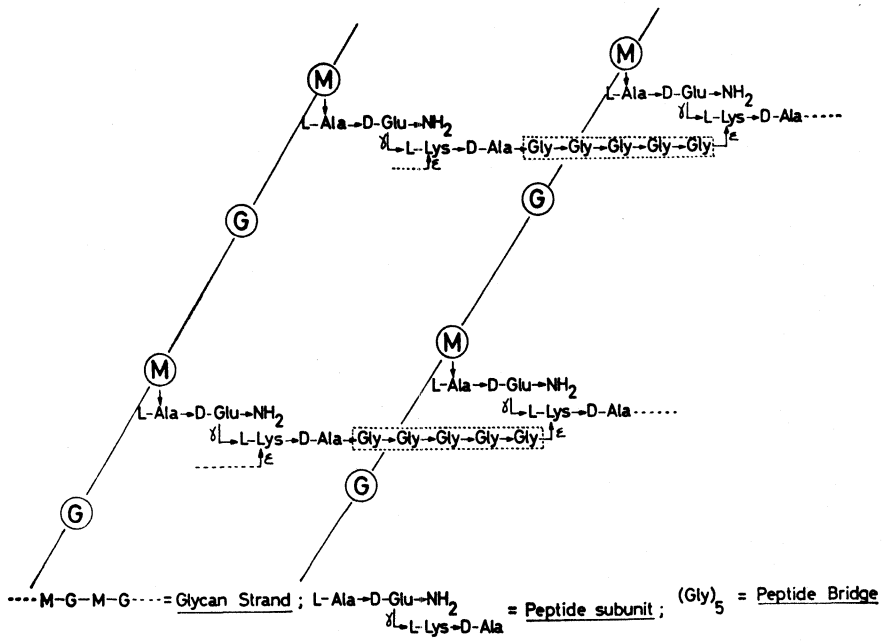

FIG. 2. Monolayer representation of a portion of the wall peptidoglycan in *Staphylococcus aureus*. M = MurNAc, G = GlNAc. The arrows indicate the CO → NH direction of the linkage. Usual α-peptide linkages are represented by horizontal arrows. The peptidoglycan in gram-negative bacteria most likely occurs as a two-dimensional monolayer network (20–30 Å thick). The peptidoglycan in gram-positive bacteria (about 100 Å thick) is probably a three-dimensional multilayered network. One may visualize that some of the peptide bridges are used to interconnect several superimposed peptidoglycan sheets.

is always linked through its γ-carboxyl group to the third amino acid in the sequence. The third residue is sometimes a neutral amino acid such as homoserine; most often, however, it is a diamino acid such as L-lysine (Fig. 2), L-ornithine, LL-diaminopimelic acid or *meso*-diaminopimelic acid (Fig. 3). L-lysine and L-ornithine are linked to D-glutamic acid through their α-amino group so that the

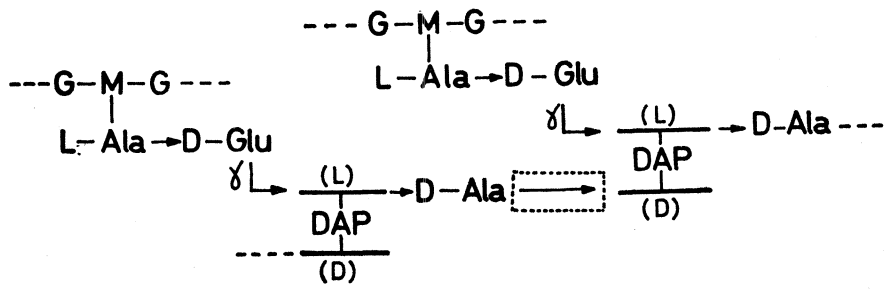

FIG. 3. The peptide subunit and bridging in *Escherichia coli*.

ε- and δ-amino groups, respectively, are free. Both the amino and carboxyl groups of diaminopimelic acid, linked respectively to D-glutamic acid and D-alanine, are located on the same asymmetrical carbon which, in the case of *meso*-diaminopimelic acid, is the L-carbon (Fig. 3). Also depending upon the bacterial species, the α-carboxyl group of glutamic acid can be either free or amidated (the second amino acid in the sequence then being a D-isoglutamine residue) (Fig. 2), or substituted by an additional amino acid such as glycine. Similarly, the carboxyl group of the diaminopimelic acid residue not engaged in peptide bonding is either free or amidated.

The cross-linking between the tetrapeptide subunits always involves the carboxyl group of the terminal D-alanine residue of one peptide subunit and, usually but not always, the free amino group of the diamino acid of another peptide subunit. The peptide bridges may consist of direct peptide bonds (Fig. 3) such as D-alanyl-(D)-*meso*-diaminopimelic acid linkages (gram-negative bacteria; some gram-positive *Bacillaceae*), i.e. a bond extending from the amino group located on the asymmetrical D-carbon of *meso*-diaminopimelic acid of one peptide subunit to the C-terminal D-alanine residue of another. Peptide bridging between peptide subunits may also be mediated via a single additional amino acid (a D-isoasparagine residue in many *Lactobacillaceae*) or via an intervening peptide chain such as a pentaglycine (in *Staphylococcus aureus*) (Fig. 2), an L-alanyl-L-alanyl-L-alanyl-L-threonine sequence (in *Micrococcus roseus*), or one or several peptides each having the same amino-acid sequence as the peptide subunit (as in *Micrococcus lysodeikticus*). Finally, in a few bacteria such as some plant pathogenic *Corynebacteria* and *Butyribacterium rettgeri*, the peptide bridges extend from the α-carboxyl group of D-glutamic acid of one peptide subunit again to the C-terminal D-alanine of another subunit. The latter type of cross-linking necessarily involves a diamino-acid residue. D-ornithine and D-lysine were found to be involved in this type of bridging.

2.1.3. *Biological Effects of Isolated Wall Peptidoglycan*

Peptidoglycans chemically isolated by means of hot formamide from the walls of streptococci, of *Gaffkya tetragena* and of some other gram-positive cocci exert general and/or dermal endotoxin-like effects such as a rise in temperature, a fall in the number of circulating granulocytes in the blood or the growth of hard nodular lesions in rabbit dermis. 5-hydroxytryptophan may be involved in the pyrogenic response [10] and the importance of a toxic peptidoglycan in virulence has been postulated [11].

2.1.4. *Biosynthesis of Bacterial Wall Peptidoglycan*

In spite of extremely wide variations in structural details, all the bacterial peptidoglycans are probably synthesized by mechanisms which are consistent throughout the bacterial world. A multiple-stage synthesis has been proposed which takes place first inside the cell, next on the plasma membrane and finally within the growing wall [2, 3, 4].

Stage 1. The two nucleotide precursors, uridine-5′-pyrophosphoryl-N-acetylglucosamine (UDP-GlNac) and uridine-5′-pyrophosphoryl-N-acetylmuramic acid-pentapeptide (UDP-MurNAc-R_1-γ-D-Glu-R_3-D-Ala-D-Ala) (Fig. 4), are synthesized by means of soluble enzymes. UDP-MurNAc originates from UDP-GlNAc. A three-carbon fragment is transferred from 2-phosphoenol pyruvate (the glycolytic intermediate) to UDP-GlNAc and the resulting UDP-GlNAc-pyruvate enol ether is then reduced to UDP-MurNAc. The first three amino acids of the pentapeptide substituent are then added sequentially in the presence of ATP and either Mg^{++} or Mn^{++}, each step being catalysed by a specific enzyme. The last D-alanyl-D-alanine sequence, however, is added *en bloc* to the incomplete precursor UDP-MurNAc-R_1-γ-D-Glu-R_3, thus giving rise to the complete precursor UDP-MurNAc-R_1-γ-D-Glu-R_3-D-Ala-D-Ala.

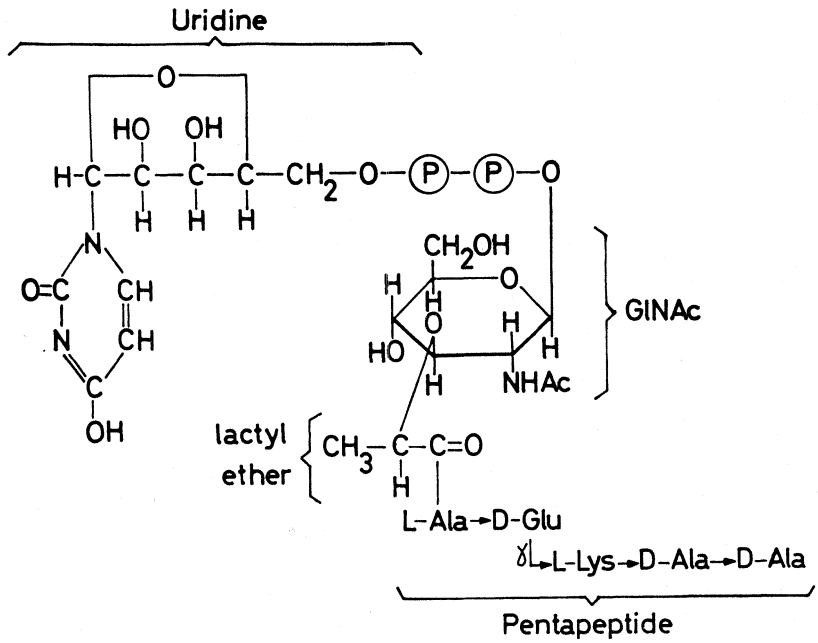

FIG. 4. The nucleotide precursors UDP-GlNAc and UDP-MurNAc-pentapeptide in *Staphylococcus aureus*. Note that D-glutamic acid is linked to the α-amino group of L-lysine through its γ-carboxyl group and that the α-carboxyl group of D-glutamic acid is free. The same precursors are found in *Escherichia coli* except that *meso*-diaminopimelic acid replaces L-lysine.

Stage 2. This stage comprises the assembly of the two nucleotide precursors into disaccharide pentapeptide units, β-1,4-GlNAc-MurNAc-pentapeptide, and their transport via an intermediate carrier to a final acceptor, i.e. the growing wall peptidoglycan (Fig. 5) Several particulate enzymes and a C_{55} polyisoprenoid alcohol phosphate, most probably located on the cytoplasmic membrane, are involved in a complex cyclic reaction that may be visualized as a sequence of three transfer reactions. First, the MurNAc (pentapeptide)-monophosphate residue

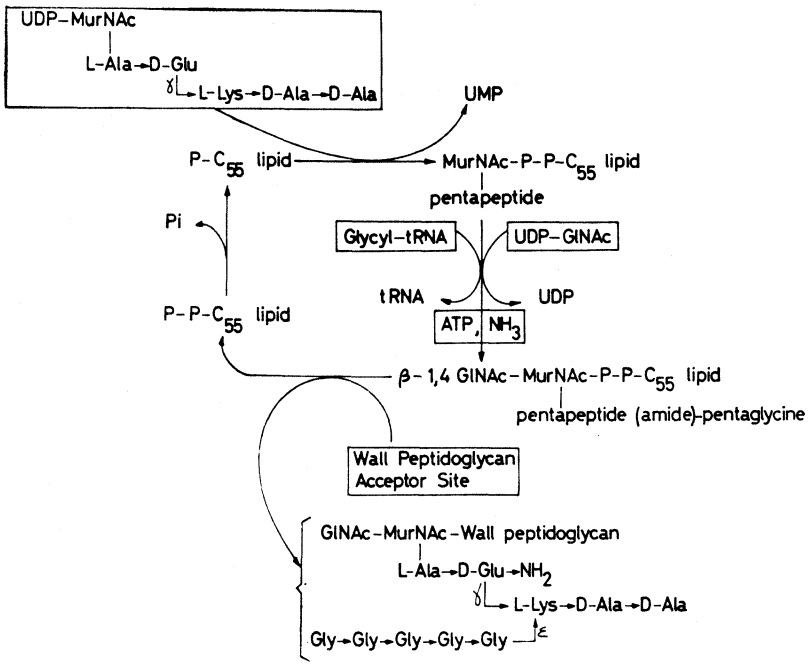

FIG. 5. The lipid cycle in *Staphylococcus aureus* with formation of uncross-linked peptidoglycan strands. In *Escherichia coli* the same lipid cycle occurs with the exception that D-glutamic acid is not amidated and that no additional amino acids are incorporated into the pentapeptide moiety.

is transferred from UDP-MurNAc pentapeptide to the P-C_{55} lipid, resulting in the formation of UMP and in the attachment of MurNAc-pentapeptide to the lipid by means of a pyrophosphate bridge (MurNAc (pentapeptide)-P-P-C_{55}). Secondly, GlNAc is transferred by transglycosylation from UDP-GlNAc with the liberation of UDP and the formation of β-1,4-GlNAc-MurNAc (pentapeptide)-P-P-C_{55} lipid. Thirdly, the disaccharide pentapeptide is transferred to the wall receptor sites (i.e. non-reducing N-acetylglucosamine termini). Concomitant with this, the liberated C_{55} lipid pyrophosphate is dephosphorylated with formation of inorganic phosphate, following which the P-C_{55} carrier can then begin a new cycle. This cyclic reaction is evidently the mechanism by which the disaccharide peptide units are transported through the plasma membrane from the intracellular sites of synthesis to the extracellular sites of incorporation.

Stage 3. In many cases, the pentapeptide moiety cannot be utilized to extend the wall peptidoglycan network without prior chemical modification. Depending upon the bacteria, these modifications may consist of the amidation of some carboxyl groups (in the presence of ATP and NH_3) (Fig. 5), the substitution of the α-carboxyl group of D-glutamic acid by one additional amino acid, or the incorporation of those amino-acid residues which in the completed peptidoglycan will function as "specialized" peptide cross-linking bridges. In *S. aureus*, for example,

pentaglycine is added to the α-amino group of L-lysine (the third amino acid in the pentapeptide sequence) while the disaccharide pentapeptide is bound to the lipid carrier (Fig. 5). The glycyl donor for the incorporation is glycyl-tRNA. Ribosomes, however, are not involved in this process, which thus differs from a "normal" protein synthesis. Again, such a mechanism is not ubiquitous. An amino-acyl-tRNA is not always required for the bridge incorporation in the pentapeptide subunit. The initial acceptor for the tRNA-dependent incorporation likewise may not be the lipid intermediate but the soluble nucleotide precursor itself.

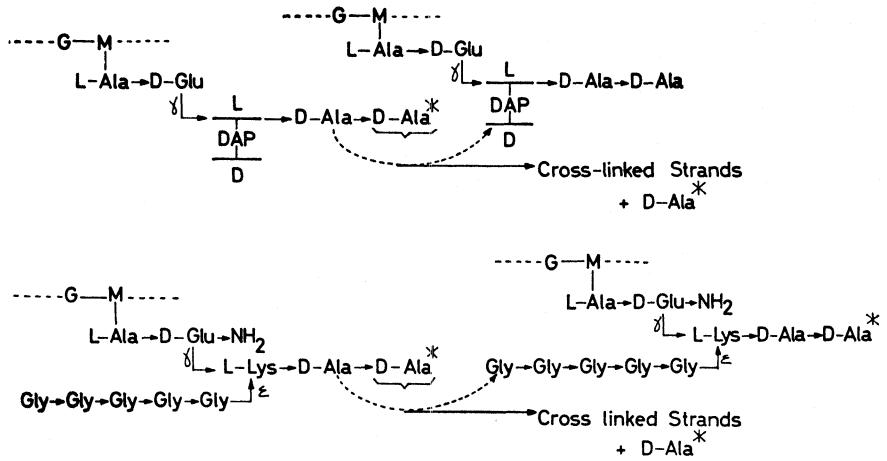

FIG. 6. The transpeptidation reaction with formation of cross-linked peptidoglycan strands and the transformation of pantapeptide units into tetrapeptides. Top: in *Escherichia coli* (cf. Fig. 3). Bottom: in *Staphylococcus aureus* (cf. Fig. 2).

Stage 4. The insertion of newly synthesized and, where necessary, suitably modified disaccharide peptide units into the growing peptidoglycan must be followed by the closure of the peptide bridges if the process is to yield a rigid, insoluble two- or three-dimensional network. This last reaction occurs outside the plasma membrane, that is at a site where ATP is not available. It has been proposed that the bridge closure between two peptide subunits results from a transpeptidation (i.e. a reaction not requiring any exogenous energy source) in which the bond energy of the terminal D-alanyl-D-alanine dipeptide of one peptide unit is utilized to transfer the carboxyl group of the penultimate D-alanine residue to the amino group acceptor of a second peptide unit, with concomitant release of the terminal D-alanine (Fig. 6). The nature of the amino-acceptor varies, of course, with the bacteria. The end product, however, is always an insoluble network of glycan chains substituted by cross-linked tetrapeptide subunits (R_1-γ-D-Glu-R_3-D-Ala), and the C-terminal D-alanine residues of these tetrapeptides are always involved in the bridging. A direct demonstration of this transpeptidation reaction has been achieved in one case, that of *Escherichia coli.* The hypothesis that this reaction is in fact a general bacterial mechanism rests upon indirect but convincing evidence.

2.1.5. *Antibiotics that interfere with the Biosynthesis of Bacterial Wall Peptidoglycan*

In the presence of certain antibiotics, the processes involved in bacterial growth continue to take place with the exception that the cells can no longer synthesize a normal rigid wall peptidoglycan. As a result, the cells are unable to withstand the high internal pressure and die of osmotic disruption.

D-cycloserine is a competitive inhibitor of both the alanine racemase which catalyses the reversible interconversion of the two optical antipodes (L-alanine → D-alanine) and of the D-alanyl-D-alanine synthetase. Consequently, D-cycloserine blocs the synthesis of the muramyl nucleotide precursor (Fig. 4) at an early stage and induces the accumulation of the incomplete precursor UDP-MurNAc-X_1-γ-D-Glu-X_3 which lacks the essential terminal D-alanyl-D-alanine dipeptide. It is believed that the molecular basis for the D-cycloserine inhibition lies in the structural analogy between the antibiotic and D-alanine (Fig. 7) [2].

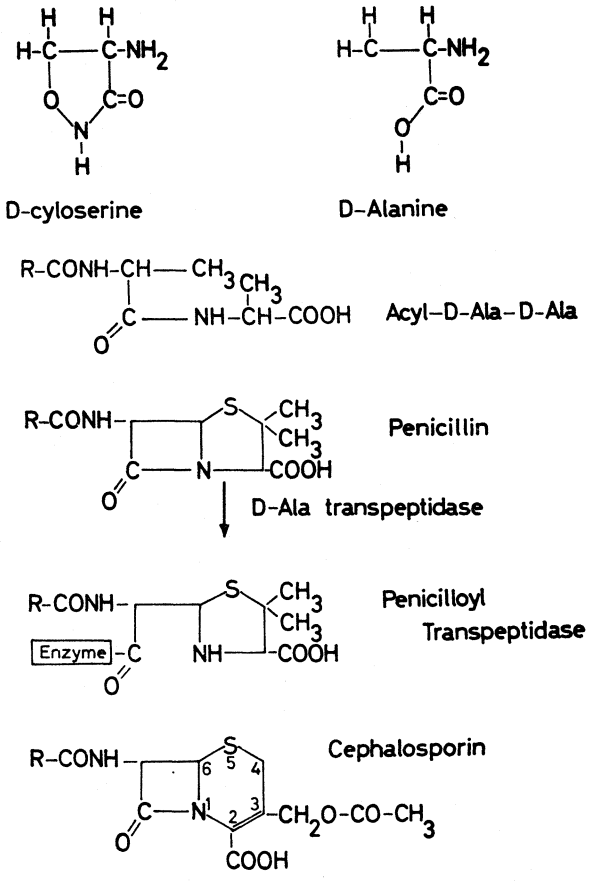

FIG. 7. Molecular basis for antibiotic actions of D-cycloserine and penicillin.

O-carbamyl-D-serine competitively inhibits the alanine racemase but does not inhibit the D-alanyl-D-alanine synthetase. In the presence of O-carbamyl-D-serine, there is accumulation of the same incomplete nucleotide precursor as in the previous case.

Vancomycin is known to combine readily with the isolated complete precursor UDP-MurNAc-X_1-γ-D-Glu-X_3-D-Ala-D-Ala whose specific combining site is the C-terminal D-alanyl-D-alanine sequence [5]. The attachment of vancomycin to the nucleotide precursor does not prevent transphosphorylation to the P-C_{55} lipid carrier (and the formation of UMP. See stage 2 of the biosynthesis, Fig. 5). However, the vancomycin-nucleotide precursor-lipid complex no longer constitutes a substrate for the transglycosylation enzymes and hence cannot be used for further syntheses. The antibiotic's chemical structure is largely unknown. It contains phenols, chlorophenols, aspartic acid, N-methylleucine and glucose, forming a complex with the isolated UDP-MurNAc-pentapeptide precursor when equimolar proportions of peptide and antibiotic (calculated on the basis of one residue each of glucose, aspartic acid and N-methylleucine) are present.

Ristocetin exerts its effects by a mechanism similar to that of vancomycin. Its chemistry is likewise not known. Like vancomycin, it is an amphoteric substance containing amino and phenolic groups and sugar residues (glucose, mannose, arabinose and rhamnose).

Bacitracin, a polypeptide antibiotic, interferes like vancomycin and ristocetin with the lipid cycle (Fig. 5), preventing the nucleotide precursors from being used to form linear uncross-linked peptidoglycan strands. Bacitracin's mechanism of action, however, is entirely different [6]. It specifically inhibits the enzymatic dephosphorylation of the pyrophosphate C_{55} polyisoprenoid alcohol which appears as the end product of the cycle reaction. As a result, the P-C_{55} lipid carrier fails to be regenerated and is thus prevented from re-entering the cycle. The molecular basis of this inhibition has not been elucidated.

Penicillins and *Cephalosporins* are specific inhibitors of the peptide bridge closure reaction (Fig. 6), so that an uncross-linked wall peptidoglycan deprived of mechanical strength is formed [2, 7, 8]. It has been proposed that penicillin G [(6-phenylacetamido) penicillanic acid, i.e. essentially an acetylated cyclic dipeptide of L-cysteine and D-valine] as well as other penicillins and cephalosporins function as analogues of the D-alanyl-D-alanine dipeptide sequence at the C terminus of the pentapeptide units (Fig. 7) [2]. The D-alanine transpeptidase that is presumed to be capable of recognizing the acyl-D-alanyl-D-alanine configuration would accept penicillin as an analogue. This would irreversibly inhibit the enzyme via acylation through the highly reactive CO–N bond in the lactam ring with the formation of a penicilloyl-transpeptidase complex.

Novobiocin, an antibiotic consisting of a substituted phenol, a substituted coumarine and noviose (the carbamate ester of a derivative of L-rhamnose), is known to induce the accumulation of uridine nucleotide precursors in bacteria when used at dose levels near the growth inhibitory concentration. However, novo-

biocin also inhibits protein and nucleic acid synthesis. It may well be that the inhibition of peptidoglycan synthesis and other effects exerted by this antibiotic result from another as yet undiscovered primary metabolic lesion [9].

2.1.6. *Conclusion*

The primary structure and biosynthesis of the bacterial wall peptidoglycans have been elucidated and are now understood at a molecular level. These studies have provided evidence for the exact mechanism of action of several highly selective antibacterial compounds. A striking feature is the importance of the D-alanyl-D-alanine sequence as a target for a number of these antibiotics. D-cycloserine and O-carbamyl-D-serine inhibit the synthesis of the dipeptide, with the result that the wall peptidoglycan nucleotide precursor remains incomplete. Vancomycin and ristocetin attach themselves to the complete nucleotide precursor due to the presence of the D-alanyl-D-alanine sequence and make it unavailable for further syntheses by membrane-bound enzymes. Finally, in the presence of penicillin and related antibiotics, the D-alanyl transpeptidase would recognize the acyl-D-alanyl-D-alanine configuration in the antibiotic and, by accepting it as an analogue, would be irreversibly denatured.

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2.2. MITOCHONDRIA

2.2.1. *Structure and Functions*

by ROSSI, C. S. (Padova) and CARAFOLI, E. (Modena)

2.2.1.1. *Introduction: Electron Microscopy*

MITOCHONDRIA have long been known as the sites of cellular respiration; the complex of enzymes responsible for the Krebs cycle, for the operation of the respiratory chain and for the coupling of respiration to phosphorylation, are associated with them. The transformation of the energy of the foodstuff molecules into that of ATP has been regarded for a long time as the only *raison d'être* of the mitochondria. However, in the recent years it has become obvious that mitochondria can use oxidative energy to carry out a number of energy-dependent operations different from the synthesis of ATP. For example, they can accumulate certain cations and anions against concentration gradients, and catalyse the energy-dependent reduction of NADP by $NADH_2$. Very recently, it has been shown that mitochondria are equipped with a complete genetic apparatus. They possess a type of DNA different from that of the cell nucleus, and the various RNA's required for protein synthesis.

A large part of the biochemical machinery of the mitochondria is housed in a highly complex membrane organization which is essential for the proper functioning of the mitochondria; as a consequence, the classical enzymological approach of solubilizing and purifying the various components to study the reactions in soluble form has met with formidable difficulties.

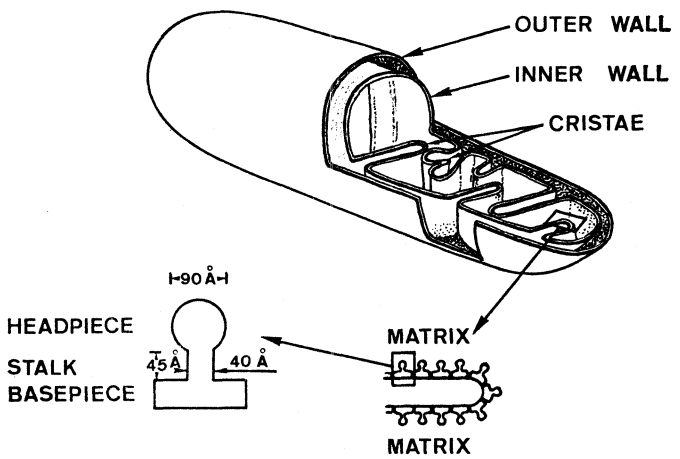

FIG. 1. Schematic representation of the mitochondrion, showing a typical double-membrane structure, consisting of an outer and an inner membrane. At the matrix side of the inner membrane and of the cristae the elementary particles of 90 Å diameter are illustrated.

The basic structural elements of all mitochondria are: (1) an outer membrane, (2) an inner membrane, (3) a number of infoldings of the inner membrane which are called "cristae", (4) two compartments, one between the outer and inner membrane, one within the inner membrane; the content of the latter is called matrix (Fig. 1). While these basic components are present in mitochondria from all the cell types studied so far, there are large variations in their organization. In Figure 2 are shown mitochondria isolated from different tissues, after negative staining with ammonium molybdate. Their shape and structural organization are different; those isolated from liver, kidney and adrenal cortex are spherical, while those isolated from brain and muscle are elongated. As a rule mitochondria in the intact tissues are always elongated. When isolated, they may or may not swell to become spherical. The swelling probably depends on whether or not the cristae form complete septa. If this is the case, the swelling is prevented, and the elongated shape is maintained (see, for example, Fig. 3 (A)). It is seen in Figure 2 that the cristae organization varies greatly in different mitochondria; in those from liver, which have been most extensively studied, the cristae are relatively few and poorly developed; the matrix space is comparatively very abundant. In kidney-cortex mitochondria the cristae are much more numerous and form rather closely packed configurations. In those from skeletal muscle or heart the cristae are extraordinarily numerous and very densely packed; the matrix space is thus very poorly represented; frequently the cristae form complete septa. The cristae form complete septa also in the mitochondria from neurons; however, they are far less numerous than in kidney or muscle mitochondria. In the mitochondria from the adrenal cortex the cristae are frequently swollen (Fig. 2 (C)), so that the inner sac of the mitochondrion assumes a sponge-like appearance. In some types of mitochondria, the cristae may assume very peculiar configurations. For example, in blow-fly muscle mitochondria they are disposed like sheets and so densely packed that scarcely any matrix space is visible. Each cristal sheet has many holes, placed at regular intervals so that the holes of many cristal sheets are aligned: this arrangement very likely facilitates the diffusion of soluble components in the mitochondrion.

It is now established that the total area of the cristae and the respiratory activity are closely related; for example, the rate of respiration of liver mitochondria, which possess a limited number of cristae, is lower than that of heart mitochondria and far lower than that of blow-fly muscle mitochondria, which are extremely rich in cristae. The total area of the inner membrane and cristae of rat-liver mitochondria is about 40 square metres per gram of protein, while that of muscle mitochondria is 10 times greater. The reason for this close correlation is that the respiratory chains are localized in the inner membrane and in the cristae (see p. 156).

Until recently, little interest had been focused on the matrix, which was usually considered a diluted aqueous solution of different proteins, without any structural organization. Calculations by Hackenbrock have however shown that the protein concentration in the matrix is far too high for a true solution: some of it must therefore be organized in some sort of structural arrangement. Electron microscopy on frozen-etched material has shown an organized reticular network in continuity with the inner membrane. All mitochondria studied so far contain some "dense granules" in the matrix; long regarded as mineral deposits, they have recently been

shown to contain no appreciable amounts of Ca^{++} or Mg^{++}. Their composition and function are still largely a matter of speculation.

Recently Hackenbrock has described sites of contact of the inner and the outer membrane of freshly isolated mitochondria (Fig. 6). There are 4–5 contact sites per average rat-liver mitochondrial profile, and there are 115 of these sites in an average liver mitochondrion; their nature and significance is unknown.

When observed in the electron microscope after positive staining, both sides of the inner and outer membrane, and of the cristae, appear smooth. When, however, unfixed mitochondria are observed in the electron microscope after negative staining (e.g. with phosphotungstate or ammonium molybdate) the matrix side of the inner membrane and of the cristae are covered with globular particles of about 90 Å diameter (Fig. 4) which are connected to the membrane by a stalk. These "elementary particles" [6] might be "tripartite units" [7]: knob, stalk and basepiece in the inner membrane. The reality of these structures has been contested because they are not regularly seen in positively stained mitochondria.

2.2.1.2. *Ultrastructural Changes During Metabolic Activity*

Usually, the isolated liver mitochondria have a spherical shape: both the inner and the outer membrane appear fully extended, the cristae are narrow, and the matrix is rather clear (Fig. 5). This appearance, called "orthodox" is typical of mitochondria during the absence of phosphorylation (state 4 in the terminology of Chance and Williams). Upon initiation of phosphorylation by addition of ADP, a dramatic change occurs (Fig. 6): the inner membrane and the cristae undergo extensive shrinkage, the matrix space becomes considerably reduced and the content of the matrix compartment appears much denser. The outer membrane remains extended; the compartment enclosed between the two membranes, which is very limited in the orthodox mitochondria, is therefore considerably increased. This "condensed configuration" has been related by Hackenbrock to the mechanism of energy coupling. The structural organization of the mitochondria varies with the metabolic activity, and the conformational changes of the inner membrane might be a means of energy conservation.

Heart mitochondria can exist in three different configurations: non-energized, energized and energized-twisted [7]. The energized configuration is induced by either respiratory substrate or ATP; inorganic phosphate and other permeable anions induce the transition to the energized-twisted configuration. Inhibitors of respiration or of ATP hydrolysis prevent the formation of the energized or of the energized-twisted configuration. Uncouplers decompose the energized state.

2.2.1.3. *Topography of the Mitochondrial Enzymes*

When mitochondria are disrupted into small vesicles or fragments with detergents, or by sonic oscillation, some enzymes and enzyme systems remain associated with the particles, while some others are recovered in the soluble phase. The cytochromes and the flavoproteins of the respiratory chain are exclusively recovered in the membrane fractions and seem to be firmly bound to the membrane. The ability to couple oxidation to phosphorylation is usually lost upon fragmentation; however, if the submitochondrial particles are prepared very carefully, they can

retain at least a part of it. Most of the enzymes of the Krebs cycle (Fig. 19, p. 173) are recovered in the soluble phase; they seem to be part of the matrix and to operate in close functional link with the cristae and the respiratory carriers. Complex methods based on density-gradient centrifugation have permitted the separation, in different laboratories, of the inner and outer membranes of mitochondria, mainly by the use of specific markers, like monoaminoxidase, which is located exclusively in the outer membrane [18]; however, the two membranes differ also in other important aspects: all of the cholesterol is in the inner membrane, and all of the cardiolipin in the outer membrane. The outer membrane does not contain the 90 Å particles. The inner membrane contains also the ion transport mechanism (see p. 166).

Contrary to the majority of authors, Green and his group maintain that their method of subfractionation yields an outer membrane fraction which possesses *all* of the tricarboxylic (TCA) Krebs-cycle activities of the mitochondrion.

Attempts are made to localize certain enzymatic activities in various parts of the inner membrane. The 90 Å knobs have been isolated; they contain one of the coupling factors (F_1 in Racker's terminology). It is possible to obtain from mitochondria sealed vesicles which are either "correctly" oriented or turned "inside out" (Fig. 7): using the 90 Å knobs as markers for the matrix side, it has been possible to conclude that cytochrome c is located at, or bound from, the outer side of the inner membrane.

2.2.1.4. *The Respiratory Chain*

Figures 8 and 9 show the sequential arrangement of the respiratory carriers. These carriers have prosthetic groups that can be reversibly oxidized and reduced by gaining or loosing electrons and have redox potential increasing from $-0{\cdot}35$ to $+0{\cdot}81$ V. The net drop of about 1·1 V corresponds to a total drop in free energy of about 52,000 cals. (Fig. 9). The arrangement of the respiratory carriers permits a stepwise liberation of this energy, which can then easily be trapped and conserved.

Research on the respiratory chain began more than 30 years ago with the pioneering work of Warburg and Keilin. However, despite intensive studies, some points are still controversial, for instance the role and position of coenzyme Q. Some authors maintain that all of coenzyme Q is present in the main respiratory chain; others believe that this coenzyme constitutes a common pool which distributes reducing equivalents to different respiratory chains. Cytochrome b has also been questioned as an obligatory component of the main respiratory chain; the same applies to non-heme iron, which may step in only when energy coupling occurs. All the components of the respiratory chain, with the exception of cyto-

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FIG. 2. (A) A mitochondrion isolated from rat liver, and negatively stained with isotonic ammonium molybdate. The negative stain penetrates the outer membrane, and within the cristal infoldings (dark areas), which appear to be poorly developed. Very few dark areas are visible within the profile of the inner membrane. Note the spherical appearance of the organelle. (B) A mitochondrion isolated from rat kidney cortex and treated as in (A). The organelle is spherical, and has a cristate system much more developed than in liver mitochondria. The matrix space is clearly visible. ×80,000. (C) A mitochondrion isolated from beef adrenal cortex and treated as in (A). Also

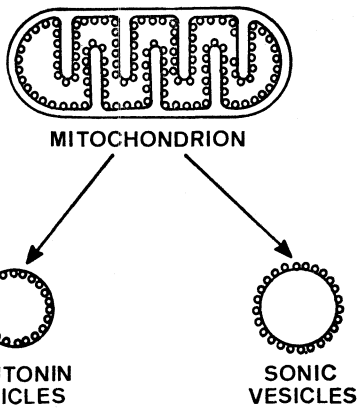

FIG. 7. Orientation of the elementary particles in submitochondrial fragments obtained by sonic oscillation and by digitonin treatment.

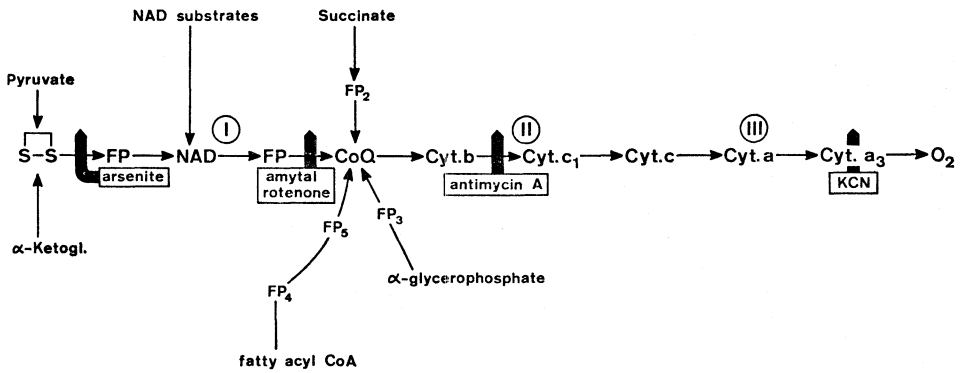

FIG. 8. Schematic representation of electron transport in the respiratory chain. The sites of energy conservation are indicated by the roman numerals.

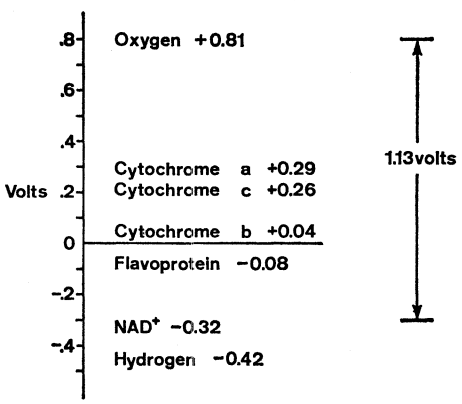

FIG. 9. Oxidation-reduction potentials of the components of the respiratory chain. The values are given for pH 7, and 25°C.

COMPLEXES OF ELECTRON CARRIERS OF MITOCHONDRIAL RESPIRATORY CHAIN

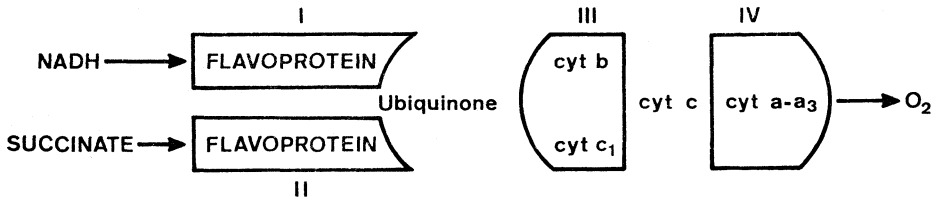

FIG. 10. Schematic representation of the organization of the respiratory carriers in the four complexes, described by Green and associates.

chrome c and coenzyme Q, are very tightly bound to the inner mitochondrial membrane; this fact is likely to affect their properties after isolation: indeed, the behaviour of some components of the respiratory chain in the isolated state is different than in intact mitochondria. For this reason, the complete reconstitution *in vitro* of the respiratory chain from isolated and purified components is an extraordinarily difficult endeavour. Green *et al.* have tried to isolate not single respiratory carriers, but "respiratory complexes" representing a section of the chain. So far they have isolated four complexes: NADH-CoQ reductase, succinate-CoQ reductase, reduced CoQ-cytochrome c reductase and cytochrome c oxidase (Fig. 10). The complexes contain, in addition to two or more carriers, other structural components of the mitochondrial membranes, so that the carriers are presumably maintained in the proper environment and configuration. The entire

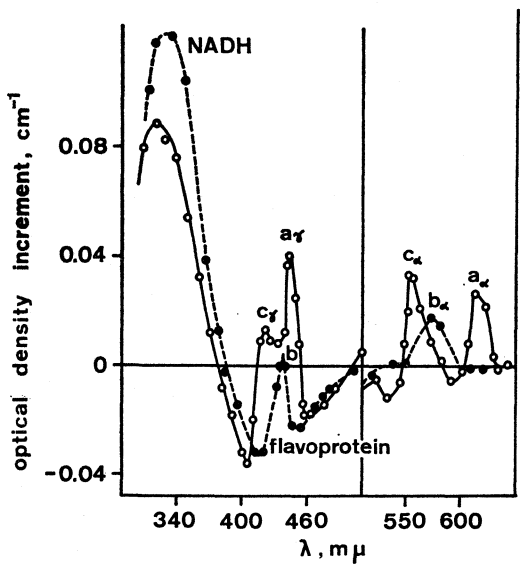

FIG. 11. Difference spectra of the respiratory carriers of rat-liver mitochondria. The solid line illustrates the optical density under aerobic conditions, and the dashed line under anaerobic conditions.

respiratory chain can thus be reconstituted upon mixing the complexes stoichiometrically.

Racker and his group have obtained normal electron transfer from succinate to oxygen by combining several soluble mitochondrial components, Mg^{++}, and phospholipids. However, they found it essential to add a "sedimentable" preparation of cytochrome b, which in the electron microscope appeared to consist of vesicles; this particulate preparation could conceivably provide the structural material on which the carriers must be properly organized before a "normal" electron flow can be obtained.

Chance and his colleagues, by using extremely sensitive double-beam spectrophotometry, could eliminate both the turbidity and the light scattering of the particles and succeeded in measuring difference spectra (reduced minus oxidized) of the individual carriers in the intact mitochondria (Fig. 11). From these difference spectra, and from the molecular absorbancy index of the various electron carriers, one can calculate their concentration in the mitochondria. Most of the carriers are found in single molar ratios to each other, but the molar concentration of CoQ is far greater than that of the other components of the respiratory chain; as mentioned, it may serve as a common donor of reducing equivalents.

2.2.1.5. *Oxidative Phosphorylation*

A large drop in free energy occurs at three steps of the respiratory chain. At these three steps, oxidative energy is converted into the bond energy of ATP; this process is called respiratory chain phosphorylation. The association between the phosphorylation of ADP and the operation of the respiratory chain was established by Lehninger almost 15 years ago. It was known at that time that no phosphorylation occurs during the oxidation of NADH by crude-tissue homogenates, despite the fact that NADH was known as the electron donor to the respiratory chain. Lehninger demonstrated that NADH is oxidized by non-phosphorylating microsomal systems, and not by intact mitochondria. However, by briefly exposing intact mitochondria to hypotonic conditions, he was able to increase their permeability, thus inducing penetration and vigorous oxidation of externally added NADH. Under these conditions, the oxidation was accompanied by phosphorylation; later studies proved that P/O ratios approaching 3 could be obtained when NADH was used as the electron donor:

$$NADH + H^+ + 3\,Pi + 3\,ADP + 1/2\,O_2 \rightleftharpoons NAD^+ + 4\,H_2O + 3\,ATP\,.$$

The location of the phosphorylation steps within the respiratory chain was established in a series of studies carried out with different approaches. An essential step was the demonstration provided by Lehninger that one of the three phosphorilations takes place between cytochrome c and oxygen. The use of artificial electron acceptors such as ferricyanide has permitted to approximate the location of the other two phosphorylations: one occurs in the NAD–FAD* region, the other in the cytochrome b–cytochrome c region (Fig. 8). The approach used by Chance and his colleagues was based on the phenomenon of respiratory control, that is, on the inhibition of respiration which takes place when the phosphate acceptor, ADP, is

* FAD = flavinadeninedinucleotide.

absent from the medium. In the absence of ADP (state 4 of Chance; see p. 162) all of the respiratory carriers are essentially in the reduced state. Upon addition of ADP, the respiration increases (the respiratory control is released) and the carriers become more oxidized. Chance and Williams added limited amounts of ADP, and were able to identify three "cross-over points" in the respiratory chain where the exhaustion of ADP by phosphorylation caused the carrier on the oxygen side of the cross-over point to become more oxidized, and that on the substrate side more reduced. They are localized between NADH and FAD, between cytochromes b and c, and between cytochrome a and oxygen; only at these three sites the drop in free energy is large enough for the synthesis of one molecule of ATP (Fig. 9).

The "gross" location of the phosphorylation at these three sites is still valid today; however, attempts have been made to locate the sites more precisely and some adjustments have become necessary. Good evidence, for example, indicates that the first phosphorylation step lies on the oxygen side of FAD. Some authors believe that the total number of phosphorylations is higher than three: in particular, two sites could be operative between cytochrome c and oxygen. The complete picture described so far belongs to liver and mammalian mitochondria in general. Mitochondria from other organisms may have different patterns of biochemical organization.

2.2.1.5.1. Inhibitors

When ADP is missing from the medium, respiration is limited. Respiration respiration in the absence of ADP: appropriately, they are called uncouplers. When they are present, energy is not conserved, but is dissipated as heat. The classical can only proceed if phosphorylation occurs; respiration is "coupled" to phosphorylation, and the coupling is mandatory. However, certain compounds can release example is 2,4-dinitrophenol. Many other compounds of pharmacological interest act as uncouplers: hormones like thyroxine, the antivitamin K, dicumarol, and long chain fatty acids. Certain polypeptidic antibiotics like gramicidin and valinomycin also display uncoupling activity, but they cannot be considered as "true" uncouplers, since their action depends on the transport of monovalent cations between the medium and the mitochondria. Similarly, Ca^{++} only uncouples when it is actively transported into the mitochondria from the extramitochondrial spaces. The molecular mechanism of action of uncouplers is still a matter of debate. Uncouplers powerfully stimulate the hydrolysis of ATP, as shown below.

A second group of widely used inhibitors is represented by (a) the barbiturate amytal and the fish poison rotenone, which inhibit at the level of the first coupling site, (b) the antibiotic antimycin A, which blocks the respiratory chain in the region of cytochrome b, and (c) cyanide, which interacts with cytochrome oxidase and blocks the chain at its end.

A third group of compounds include the antibiotics oligomycin and aurovertin which inhibit phosphorylation. Due to the mandatory coupling between respiration and phosphorylation, they block also the respiration when it is coupled to phosphorylation, but not when it is uncoupled. Very likely, they combine irreversibly with some intermediate in the coupling sequence. The removal of the oligomycin (or aurovertin) block in phosphorylating mitochondria by uncouplers would indicate that uncouplers react at a site located between the respiratory chain

and the point of attack of oligomycin and aurovertin. Accordingly, these two antibiotics inhibit the ATP-ase induced by uncouplers (see also p. 167).

A fourth class of compounds, identified more recently, inhibit one of the "permeases" which are thought to be present in the mitochondrial membrane. The inner membrane of most mitochondria is poorly permeable to a number of essential substrates and metabolites; the penetration of these substances is mediated by a series of carriers, so far only hypothetical, which should be specific for different molecules. Thus a carrier is postulated for ATP and ADP, one for inorganic phosphate, one for succinate and other TCA cycle intermediates. The glycoside atractyloside inhibits the transport of ADP–ATP, and in so doing logically limits not only the synthesis of ATP, but also coupled respiration and, in the reverse direction, ATP-ase. Butylmalonate inhibits the penetration of succinate, limiting both coupled and uncoupled respiration. The SH-reagent mersalyl inhibits the transfer of inorganic phosphate; this causes inhibition of phosphorylation due to lack of inorganic phosphate at the coupling site, and in turn inhibition of coupled respiration. Respiration in the presence of uncouplers is on the other hand unaffected. Some inhibitors have been shown to have a "mixed" mechanism of action; for instance progesterone acts as a true uncoupler at the third phosphorylation site and as an inhibitory uncoupler at the first site.

The inhibitors mentioned above have been most useful in elucidating a number of problems and have been instrumental in developing new areas of research. The intelligent use of gramicidin and valinomycin in the laboratories of Chappell and of Pressmann has been very fruitful in research on the transport of K^+ and other monovalent cations in mitochondria.

2.2.1.5.2. Mechanism

Despite 15 years of efforts, the mechanism of energy conservation in the respiratory chain is still unsolved. Three main theories are discussed by experts.

The *chemical* theory (proposed by Slater in 1953, modified by Lehninger, Chance and Williams, Boyer, Green and others) follows the general pattern of the substrate-level phosphorylation observed during glycolysis. It is postulated that

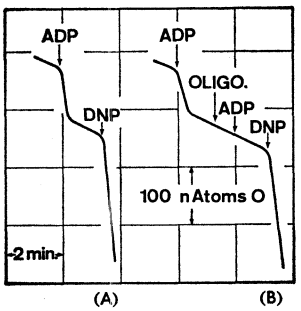

FIG. 12. Control of respiratory rate. Oxygen electrode. (A) The tracing illustrates the stimulation of the rate of oxygen consumption brought about by ADP; the extra-oxygen taken up is stoichiometric with the amount of ADP added (and phosphorylated). After exhaustion of ADP the rate of respiration returns to its original level. (B) The tracing illustrates the effect of oligomycin and of DNP. The details are described in the text.

a high-energy intermediate is formed between the carrier that undergoes oxidation and an energy carrier, termed I [4]:

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2 .$$

The intermediate $A \sim I$ then reacts with a second energy carrier, termed X, to set free the carrier A and to form the energy intermediate $X \sim I$:

$$A \sim I + X \rightleftharpoons A + X \sim I .$$

Pi (inorganic phosphate) reacts with $X \sim I$; I is liberated and a third high-energy intermediate $X \sim P$ is formed:

$$X \sim I + Pi \rightleftharpoons X \sim P + I .$$

$X \sim P$ reacts with ADP to form ATP, and X is set free.

A simple experiment (Fig. 12) embodies the most significant experimental findings in oxidative phosphorylation, and may be fitted into this rather complex formulation.

In the absence of ADP mitochondria respire very slowly. When ADP is added, there is a burst in respiration (state 3 of Chance and Williams)*, which subsides when all the ADP has been phosphorylated. At this point, the addition of DNP again increases the respiration, which remains in the activated state indefinitely. In the tracing on the right side, oligomycin is added before ADP; the ADP-induced stimulation of respiration is abolished, but addition of DNP still increases the respiration. According to the chemical theory, ADP releases the respiration since it sets into motion the entire chemical machinery of the coupling system, setting free X, which can accept I, releasing it from the combination with the carrier. DNP would hydrolyse $X \sim I$ (or $A \sim I$), thus dissipating the energy of the intermediate before it can react with Pi to form $X \sim P$. The reaction of DNP with a non-phosphorylated, rather than with a phosphorylated intermediate, has been suggested, since no Pi is necessary for DNP to induce the uncoupling effect. It is clear that this would result in an indefinite stimulation of respiration and also in the hydrolysis of any preformed, or added, ATP, since the reactions of the coupling sequence are reversible. Oligomycin prevents the reaction of Pi with $X \sim I$; clearly, this would inhibit phosphorylating respiration and also ATP-ase. DNP acts at a point in the energy coupling sequence closer to the respiratory chain than oligomycin. This nice interpretation raises the serious difficulty that it needs high-energy intermediates, none of which so far has been isolated.

* Chance and Williams have defined five metabolic states for isolated mitochondria. *State 1*, in which respiration is slow and limited by lack of phosphate acceptor (ADP). In state 1, also, the level of the respiratory substrates is low, while that of inorganic phosphate is adequate. *State 2*, in which respiration is limited only by lack of respiratory substrate. *State 3*, in which respiration is fast, since respiratory substrate, inorganic phosphate, and phosphate acceptor (ADP) are present in adequate amounts. The rate-limiting component in state 3 is the respiratory chain. *State 4*, in which the respiration is slow: respiratory substrate and inorganic phosphate are adequate, but the phosphate acceptor is lacking. *State 5*, in which the rate of respiration is zero, due to lack of oxygen. More recently, a *State 6* was identified, a state of inhibited respiration during uptake of divalent cations in the absence of permeant anions.

This uncomfortable situation led Mitchell [16] to the formulation, in 1961, of the so-called *chemiosmotic hypothesis*, which in its original formulation did not need any high-energy intermediates. It postulates that the basic process energized by electron transport is the separation of H^+ and OH^- across the mitochondrial membrane, which is assumed to be impermeable to those species. Electron transport

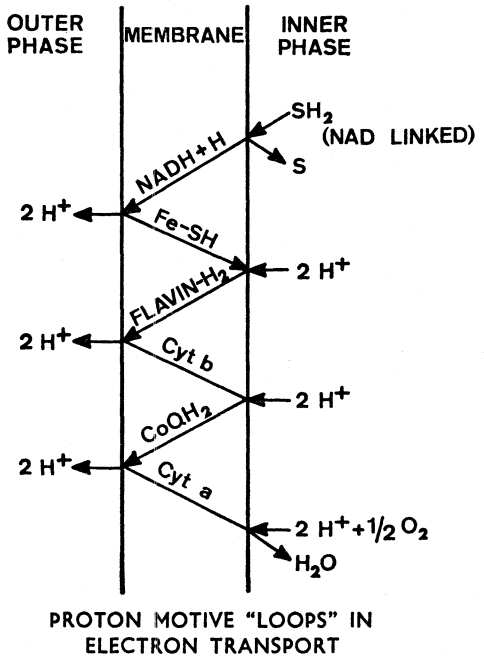

FIG. 13. Schematic illustration of the asymmetric orientation of the respiratory carriers in the mitochondrial coupling membrane. This specific orientation ("loops") drives the separation of charges across the mitochondrial membrane, and thus creates a pH gradient.

produces H^+ on one side of the inner mitochondrial membrane, and consumes H^+ from the opposite side of it, since the molecules of the electron carriers are supposed to be oriented asymmetrically across the inner membrane (Fig. 13). The carriers are arranged in a folded manner to form three "loops". The H^+ gradient thus established across the inner membrane is the driving force for the formation of ATP from ADP and Pi, since it reverses the action of an ATP-ase vectorially located in the inner membrane:

$$ADP + Pi \rightleftharpoons ATP + H^+ \text{ (inside)} + OH^- \text{ (outside)}.$$

Mitchell postulates that this reaction is pulled to the right by the fact that the H^+ produced by the ATP-ase at the inner side of the membrane is removed by the excess OH^- of the intramitochondrial compartment, while OH^- generated in the external phase is similarly removed by the H^+ generated by electron transport. Actually it is not even necessary to postulate the building of a large pH gradient.

Since the inner membrane is supposed to be highly impermeable to ions, the translocation of H^+ across it would generate an electrochemical potential. Energy can thus be preserved in the form of a proton gradient, and/or in the form of a membrane potential. Mitchell provided experimental evidence for the production of protons during oxidation of substrates, and also for the translocation of protons across the membrane during the hydrolysis of ATP. However, the significance of the proton movements in these experiments has been questioned and found to be a phenomenon more complicated than it was originally suggested.

The chemiosmotic theory requires that uncouplers increase the permeability of the coupling membrane to protons, thus collapsing the pH or electrochemical potential, and indeed several recent reports on mitochondria, or on artificial membranes, are in line with the concept.

The chemiosmotic theory requires that a proton gradient artificially created across the membrane by addition of acid or alkali to the external medium should lead to the synthesis of ATP. This last prediction has been verified [10] by a sudden

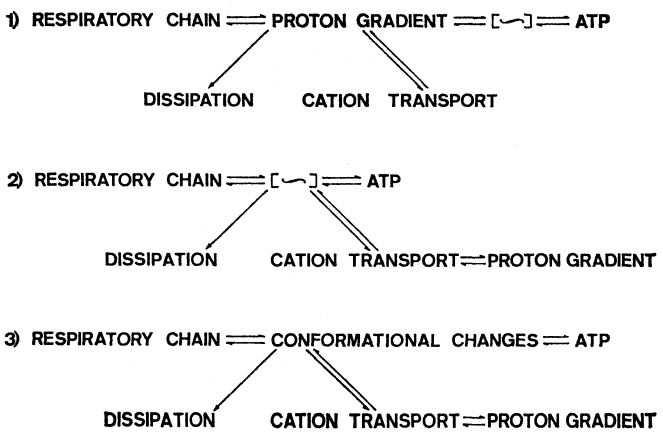

FIG. 14. The three leading theories for the mechanism of energy conservation in mitochondrial systems.

transition from an acid to an alkaline environment in isolated chloroplasts; most important, the ATP production was sensitive to uncouplers.

Energy coupling may be brought about by *changes in the conformation of the inner mitochondrial membrane*, as suggested by Boyer some years ago. Interest in it has been revamped by the previously mentioned studies of Hackenbrock, and by the analogous studies carried out by the group of Green. The implication of these studies was that the ultrastructural modifications reflected the conservation of oxidative energy into conformational energy, which could then be transduced into the chemical energy of ATP.

More recently, the problem has been attacked by Chance and his associates [1], using the fluorescent probes successfully employed in the study of the conformational changes of proteins. These dyes do not fluoresce in water, but become fluorescent in hydrophobic environments. They can be bound to mitochondria, or to

mitochondrial membrane fragments. Upon initiation of energy coupling, changes in the fluorescence of the bound dye are immediately observed and interpreted as reflecting a conformational change of the membrane. The time sequence of energy coupling and fluorescence changes have been found to be in line with the idea that the change in conformation preceeds the energy coupling and not vice versa.

These studies are still in their infancy; however, they have opened an extremely interesting and new avenue of investigation.

Figure 14 summarizes the mechanisms of energy conservation, as postulated by the three major theories.

2.2.1.6. *Protein Synthesis and Nucleic Acids*

Mitochondria contain a complete genetic apparatus, and are active in protein synthesis. Knowledge on this particular aspect of mitochondrial function has developed only recently, and is now increasing at a very rapid rate. For more than 15 years it was known that mitochondria could incorporate labelled amino acids into protein. However, in most of the early studies, the rates of incorporation were extremely low as compared to those obtained with the fragments of the endoplasmic reticulum. Moreover, in these studies bacterial and ribosomal contamination were a serious problem. Very recently, improvement of the methods has yielded rates that compare favourably with those of the endoplasmic reticulum: it has also been possible to exclude—at least for the case of liver—any contribution by bacterial or ribosomal contamination.

Protein synthesis in mitochondria is dependent on the supply of ATP; either oxidative phosphorylation, or a steady supply of ATP must be provided. From a pharmacological standpoint, it is interesting that the incorporation of amino acids is affected by thyroid hormone *in vivo*. The labelled amino acids are incorporated into an insoluble protein fraction present in the membrane and none of the soluble mitochondrial enzymes studied so far become labelled to any appreciable extent. The process of protein synthesis in mitochondria, as monitored by the incorporation of amino acids, displays some peculiar characteristics: it is inhibited by a variety of other amino acids, possibly due to competitive effects among different amino acids for a common transport mechanism. Also peculiar is the sensitivity to chloramphenicol, and the insensitivity to cycloheximide, which is typical of bacterial systems, and not of microsomal systems. Then, there is the observation that actinomycin-D (a known inhibitor of the nuclear DNA-dependent RNA polymerase), inhibits protein synthesis in mitochondria after treatments have been applied which affect the permeability of the membrane, thus permitting penetration of the antibiotic. This last observation indicates synthesis of messenger RNA in mitochondria via a specific DNA-dependent RNA polymerase. Protein synthesis in mitochondria is thus apparently dependent on the continuous synthesis of RNA; this is possibly due to a peculiar lability of mitochondrial messenger RNA.

During the past few years ribosomes have been identified in a variety of mitochondria, although their exact dimensions have not yet been established. Ribosomal-type RNA isolated from mitochondria have sedimentation properties different from those of extramitochondrial ribosomal RNA. As for the other components of the machinery for protein synthesis, activating enzymes for a variety

of amino acids and several species of sRNA have recently also been found in mitochondria.

It is now established beyond any doubt that all mitochondria contain a DNA which has different size, melting point, and base composition from the DNA found in the nucleus of the same cell. Electron-microscopy studies have shown that it is circular. Submitochondrial fragments appear enriched in DNA with respect to the intact mitochondria they are derived from. This observation suggests that mitochondrial DNA is associated with the membrane, in keeping with the finding previously mentioned that the membrane, and not the "soluble" phase, is the most important site of mitochondrial protein synthesis.

The existence of a complete genetic apparatus, and of active processes of protein synthesis in mitochondria is of course of high interest to the problem of mitochondrial biogenesis. At the moment, two alternatives are considered. Mitochondria could be produced by division of pre-existing mitochondria, or via a process of *de novo* synthesis from single precursors. Experiments on *Neurospora crassa* support the first alternative, whereas experiments by Linnane and his associates on yeast cells favour the second [24]. Mitochondria are not assembled from simple, soluble building blocks, but seem to arise from pre-existing membrane precursors. These "promitochondria" have recently been the subject of many active investigations.

Final consideration: as mentioned, only a membrane protein resembling structural protein becomes labelled during incubation of mitochondria with radioactive amino acids. Very likely, then, most of the mitochondrial proteins are genetically coded by nuclear DNA and synthesized elsewhere in the cell.

2.2.1.7. *Ion Transport Activities*

As discussed before, the energy conserved by the mitochondrial machinery in the molecule of ATP, or as a different form of biological energy, can be finally utilized for endoergonic processes: muscular contraction, osmotic work, etc.

The osmotic work which will be considered in this chapter is essentially the work of translocating solutes across the mitochondrial membrane. This activity of the mitochondrion is not a passive process. The characteristic feature of this kind of transport is the movement of specific molecules or ions against a concentration or electrochemical potential. Thus, this "active translocation" requires the expenditure of metabolic energy together with a highly specialized mechanism to couple the energy-producing enzymatic reactions with the vectorial reactions responsible for the active transport.

The most intensely investigated ion translocating activity is the energy-linked movement of monovalent (K^+) and divalent (Ca^{++}, Sr^{++}, Mn^{++}) metal ions across the mitochondrial membrane [2, 3, 14]. The molecular mechanisms operating in the two types of translocation (monovalent and divalent cations) differ significantly in their basic features.

The major features of the active translocation of divalent cations can be briefly summarized as follows: (1) the uptake requires the operation of the respiratory chain and the conservation of energy, and is sensitive to respiratory inhibitors (rotenone, antimycin-A, etc.); (2) the uptake is prevented by uncoupling agents like DNP, thyroxine, etc.; (3) ion translocation can be carried out by mitochondria

in the absence of electron flow, provided an appropriate supply of energy coming from the hydrolysis of ATP is available; (4) oligomycin, the powerful inhibitor of oxidative phosphorylation, does not affect the rate and the extent of ion transport coupled to electron flow. On the other hand, the same antibiotic completely prevents the active process when supported by ATP hydrolysis. The response of the translocation to oligomycin indicated that the uptake depends on a high-energy intermediate (or state) generated at a site between the electron carriers and the oligomycin sensitive site (Fig. 15).

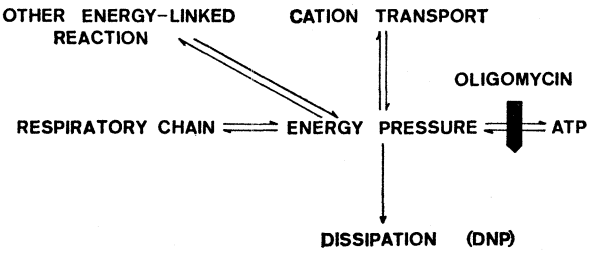

FIG. 15. Cation transport and other energy-linked functions of mitochondria. Effect of oligomycin and dinitrophenol.

The basic properties described above are essentially independent on the concentration of metal ion which is presented to the mitochondria. When enough divalent cation is present in the medium, the organelles can accumulate up to 1·5–2·0 μM of divalent metal per mg protein, in the so-called "massive loading" process. For the massive accumulation of Ca^{++} the presence of inorganic phosphate in the medium is essential; Pi follows Ca^{++} across the membrane to form insoluble salts inside. When loaded to their full capacity, mitochondria observed in the electron microscope contain large electron-dense granules which have the same composition of calcium hydroxyapatite, the major component of bone salt [23].

The massive accumulation of divalent cations leads to profound and irreversible alterations of mitochondrial structure and function: swelling, loss of respiratory control and of the capacity to phosphorylate ADP. Such damages do not occur under the so-called "limited loading" conditions, in which mitochondria are incubated in an appropriate medium in the presence of very small concentrations of Ca^{++} (0·1–0·2 μM). The addition of small amounts of calcium to mitochondria (respiring in state 4) evokes a rapid stimulation of respiration. This stimulation is transient, and the respiratory rate returns to its original resting state as soon as the external concentration of Ca^{++} falls to 1·0–1·5 μM. Thus, during the respiratory jump 95–98% of the added Ca^{++} is removed from the suspending medium and translocated within the mitochondrial structure (see Fig. 16). Measurements of the Ca^{++} accumulated and of the oxygen taken up during activated respiration have revealed that about two calcium ions are translocated as a pair of electrons traverses each energy-conserving site of the respiratory chain (Fig. 16). When phosphate is added to the medium, it passively follows Ca^{++}. The Ca^{++} accumulated during the activated respiration is actively maintained in mitochondria during the subsequent resting respiration.

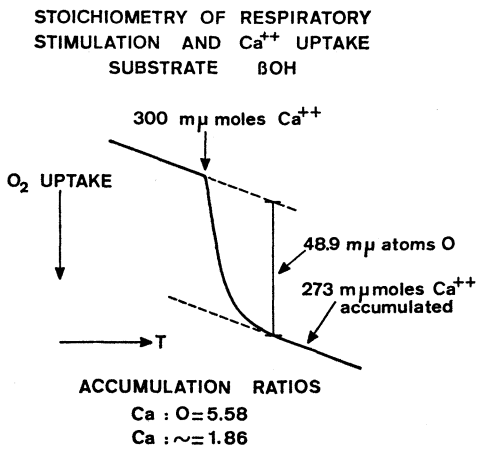

FIG. 16. Stimulation of respiration by Ca^{++}. The addition of 300 nmoles of Ca^{++} to rat-liver mitochondria in the presence of β-hydroxybutyrate as the respiratory substrate produces a sharp increase in the rate of oxygen uptake (Ca^{++} jump). The oxidation rate abruptly returns to the initial level when a stoichiometric amount of Ca^{++} has disappeared from the suspending medium. The experimental details are described in the text.

During the Ca-dependent activation of respiration the external medium becomes acidic due to an ejection of H^+. Under limited loading conditions and in the absence of phosphate or any other permeant anion the ion ratio of H^+ ejected to Ca^{++} taken up is about 1 : 0.

Under limited loading conditions, the capacity of mitochondria for oxidative phosphorylation, and the respiratory control, are completely retained: mitochondria can accumulate Ca^{++} first, and then phosphorylate ADP, and vice versa. Most important is the observation that, when ADP and Ca^{++} are presented to the mitochondria simultaneously, Ca^{++} is translocated first, followed by the phosphorylation of ADP. Thus, it can be concluded that oxidative phosphorylation and ion transport are alternative processes.

Freshly isolated intact mitochondria, when exposed to appropriate media, accumulate K^+, and other monovalent cations, during the oxidation of substrate. This active process is markedly enhanced by the antibiotic valinomycin and occurs in exchange with intramitochondrial H^+, with an ion ratio 1 : 1. The stoichiometry between K^+ translocation and energy-conserving sites may vary according to the experimental conditions, and may be as high as 8–9 (for further details see [5bis, 9, 15, 17, 20]).

The molecular mechanism of the translocation of divalent cations remains obscure. According to the chemical theory of energy-coupling, the discharge of a high-energy chemical intermediate is the cause of the translocation of Ca^{++} across the mitochondrial membrane [20]: Ca^{++} interacts with a protonated form of $X \sim I$ (this reaction is DNP-sensitive and oligomycin-insensitive) in the presence of H_2O; the high-energy intermediate is discharged and simultaneously 2 $CaOH^-$ move into the mitochondrial matrix. The final result is reported in the following equation:

$$2\,Ca^{++}(out) + H_2O\,(X \sim I) + H_2O \rightarrow 2\,CaOH^-(in) + H_2\,(X \sim I) + 2\,H^+(out).$$

The key point in this mechanism is that H^+ ejection is the result, not the cause, of the translocation of the cation. Thus, the above formulation provides a mechanism to account for H^+ production (or, which is the same, for the charge separation across the mitochondrial membrane) and also for the $H^+ : Ca^{++}$ stoichiometric ratio. But, again, the handicap of this mechanism lies in the fact that the high-energy intermediates have never been isolated.

The chemiosmotic hypothesis of Mitchell has also been applied to the mechanism of mitochondrial ion transport: the electrochemical gradient across the mitochondrial coupling membrane is visualized to drive oxidative phosphorylation, *ion transport*, and other energy-linked functions without the intervention of any high-energy intermediate. The protons ejected are visualized to drive Ca^{++} into mitochondria by an exchange-diffusion mechanism. Thus, according to this hypothesis, the ejection of H^+ is the cause, and not the effect, of divalent cations transport.

Energy-linked movements of Ca^{++} have been demonstrated in mitochondria *in vivo* [3]. Mitochondria could thus play a role in the regulation of the Ca^{++} concentration in the cytosol, with consequences of obvious importance for the physiology of the cell.

2.2.2. *Oxidation of Fatty Acids in Mitochondrial Systems*

by ROSSI, C. R. and ALEXANDRE, A. (Padova)

2.2.2.1. *Fatty Acyl-CoA Derivatives*

FATTY ACIDS are oxidized only in the form of fatty acyl-CoA derivatives, and mitochondria from mammalian tissues contain the full equipment of enzymes necessary for the synthesis and the degradation of fatty acyl-CoA. The enzymes involved in the oxidative process are located in the mitochondrial matrix, and the inner mitochondrial membrane sequesters the oxidative process from the rest of these organelles. On the contrary, the fatty acids activating enzymes (thiokinase) seem to be present in different compartments of the mitochondrion and widely distributed among the subcellular fractions. The significance of this may lie in the fact that the conditions required for fatty acyl-CoA oxidation differ from those required for other CoA—SH dependent pathways.

The synthesis of acyl-CoA is accomplished by two different enzymatic reactions: in the first an ATP-specific thiokinase is involved:

$$\text{R—COOH} + \text{ATP} + \text{CoASH} \rightleftharpoons \text{R.CO.SCoA} + \text{AMP} + \text{PP}. \qquad (1)$$

A second mechanism [22] requires GTP as the energy donor:

$$\text{R—COOH} + \text{GTP} + \text{CoASH} \rightleftharpoons \text{R.CO.SCoA} + \text{GDP} + \text{P}. \qquad (2)$$

Reaction (1) is catalysed by three different acyl-thiokinases, specifically depending on the length of the fatty acyl chain, and although catalysed by a single enzyme, actually proceeds in the two-step sequence of reactions (3) and (4):

$$\text{R—COOH} + \text{ATP} \rightleftharpoons \text{R.CO.AMP} + \text{PP}; \qquad (3)$$

$$\text{R.CO.AMP} + \text{CoASH} \rightleftharpoons \text{R.CO.CoA} + \text{AMP}. \qquad (4)$$

In the first of these reactions a mixed anhydride of the carboxyl group of fatty acid and the phosphate group of AMP is formed.

Fatty acyl-CoA oxidation is accomplished by a basic sequence of reactions which, by repetition, breaks the fatty acyl chain in 2-carbon fragments. The first step of oxidation involves the formation of the α-β-unsaturated fatty acyl-CoA:

$$R—CH_2—CH_2—COSCoA + FAD \rightleftharpoons R—CH{=}CH—COSCoA + FADH_2. \quad (5)$$

The three acyl dehydrogenases which are involved in this reaction are named according to the length of the carbon chain of the compound reacting most rapidly. All these enzymes contain FAD. An electron-transferring flavoprotein (ETF) is interposed between the dehydrogenase and some components of the respiratory chain, e.g. non-heme iron, coenzyme Q or cytochrome b.

This oxidative step is followed by hydration of the unsaturated compound to the corresponding L-β-hydroxyacyl-CoA derivative:

$$R—CH{=}CH—COSCoA \rightleftharpoons R—CHOH—CH_2—COSCoA. \quad (6)$$

The enoyl hydrase is responsible for this reaction.

A second dehydrogenation then takes place with the formation of the β-keto-acyl-CoA derivative. This reaction is catalysed by a NAD-dependent dehydrogenase:

$$R—CHOH—CH_2—COSCoA + NAD^+ \rightleftharpoons R—CO—CH_2—COSCoA + {+}NADH + H^+. \quad (7)$$

Finally, the β-ketoacyl reacts with CoASH according to the following reaction catalysed by thiolases:

$$R—CO—CH_2—COSCoA + CoASH \rightleftharpoons R—COSCoA + CH_3—COSCoA. \quad (8)$$

The several thiolases which have been described differ according to their chain-length specificity. The enzymes are all thiol enzymes (RSH) and the inter-

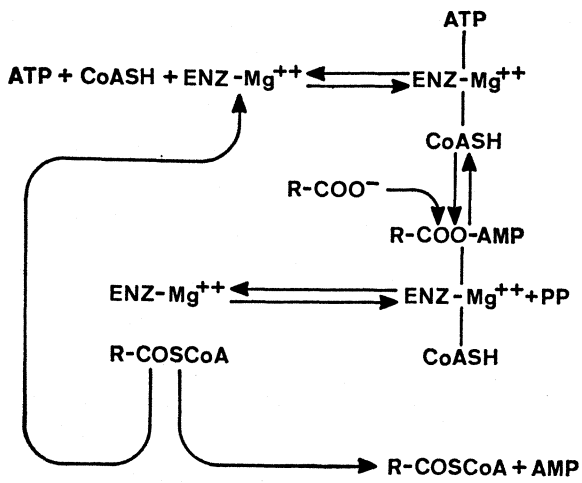

FIG. 17. Proposed mechanism for ATP-energized fatty acid thiokinase.

action of them with the β-ketoacyl derivatives leads to the formation of a β-keto-acyl-enzyme intermediate and of acetyl-SCoA, according to the following equation:

$$R—CO—CH_2—COSCoA + RSH \rightleftharpoons R—CO—SR + CH_3—COSCoA . \quad (9)$$

In turn the β-ketoacyl-enzyme intermediate interacts with a CoASH molecule, to give the free enzyme and a fatty acid derivative of CoASH which is shorter by two carbon atoms:

$$R—CO—SR + CoASH \rightleftharpoons RSH + R—COSCoA . \quad (10)$$

The overall schematic representation of these reactions is shown in Figure 17. Acetyl-CoA mixes with the acetyl-CoA derived from other metabolic pathways, such as the oxidative decarboxylation of pyruvate or from amino-acid metabolism. However, it seems that acetyl-CoA derived from fatty acid oxidation is not used in fatty acid synthesis since fatty acyl derivatives of CoASH may act as "feed-back" regulators of fatty acid synthesis.

In recent years the inner mitochondrial membrane has been commonly recognized as the barrier separating the locus of fatty acyl-CoA oxidation from other biochemical events of the cell. This barrier appears to be impermeable to CoASH and to CoASH derivatives. As a result the fatty acyl-CoA externally added to intact mitochondria are not oxidized, unless carnitine is present in the system. The existence of carnitine acyl transferase:

$$R—COSCoA + OH—R' \rightleftharpoons R—COO—R' + CoASH \quad (11)$$

provided the basis for the present concept that fatty acyl-CoA must first be converted into carnitine esters in order to be translocated inside of the inner mitochondrial membrane (see scheme of the Fig. 18). In the inner mitochondrial compartment, by reversing the reaction (11), the acyl are handed back to "internal"

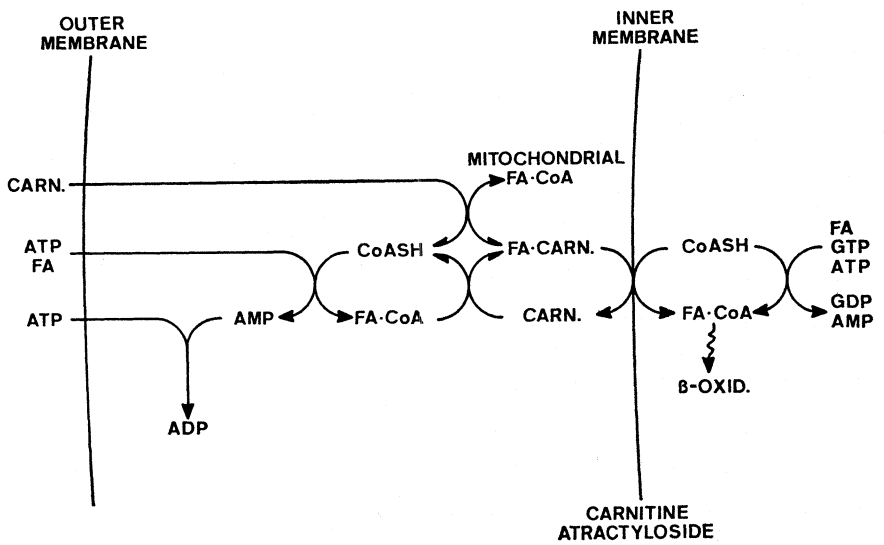

FIG. 18. Organization of fatty acid activation in mitochondrial systems.

CoASH and thus reforming the true immediate substrate for the oxidation. According to this hypothesis, acyl carnitine, which is not a substrate for the enzymes of the oxidative process, is an admirable substrate for fatty acid degradation in intact mitochondrial systems. In addition, mitochondria depleted of their "endogenous" energy donors (ATP, GTP) are unable to oxidize added fatty acids unless ATP and carnitine are both present in the system. This observation clearly proves that acyl-CoA is formed outside of the inner mitochondrial membrane—i.e. outside of the oxidation compartment—and is transported to the inner compartment via the carnitine-linked transport mechanism. In agreement with these results an ATP-dependent thiokinase has been identified in the outer mitochondrial membrane, and an acyl-carnitine transferase has been found in the inner mitochondrial membrane.

Furthermore, a second type of ATP-dependent acyl thiokinase is located in the inner compartment of the mitochondrion (mitochondrial matrix). This enzyme uses endogenous CoASH and ATP formed through the oxidative phosphorylation machinery.

Finally, a third acyl thiokinase is located in the inner mitochondrial compartment and depends on GTP as the energy donor.

2.2.2.2. *The Krebs Cycle* (Fig. 19)

Pyruvate oxidation together with fatty acid oxidation, is the main source of acetyl-CoA, whose main metabolic fate is the breakdown to water and carbon dioxide, through the sequence of reactions known as tricarboxylic acid (TCA) cycle, or Krebs cycle (Krebs): this cycle represents the main contributor of reducing equivalents to the mitochondrial respiratory chain. All of the enzymes of the Krebs citric acid cycle are mitochondrial. According to the majority of authors, they are located in the matrix.

Condensation of acetyl-CoA with oxaloacetate to produce citrate is the first reaction of the TCA cycle. This reaction is catalysed by the condensing enzyme (or citrate synthetase) which has been obtained in crystalline form:

$$\text{acetyl-CoA} + \text{oxaloacetate} + H_2O \rightarrow \text{citrate} + \text{CoA} + H^+ .$$

The reaction goes to completion as written, and is inhibited by physiological concentrations of ATP and of palmityl-CoA.

Citrate is then reversibly isomerized to isocitrate, via a successive dehydration to cis-aconitate and rehydration to isocitrate. The overall reaction is catalysed by aconitase, and Fe^{++} and a sulphydryl group are required components. At equilibrium, 90% citrate, 4% cis-aconitate and 6% isocitrate are present.

Isocitrate dehydrogenase catalyses the synthesis of oxoglutarate from isocitrate through an oxidation-decarboxylation process. Two isocitrate dehydrogenases are present in the cell. The overall reaction is identical for both enzymes :

$$\text{isocitrate} + \text{NAD (NADP)} \rightleftharpoons \text{oxoglutarate} + H^+ + \text{NADH (NADPH)} + CO_2 .$$

While the NAD-linked dehydrogenase is only of mitochondrial origin, the NADP-linked one is both mitochondrial and cytoplasmic. The NAD-linked enzyme, which appears to be responsible for most of the mitochondrial oxoglutarate oxidation,

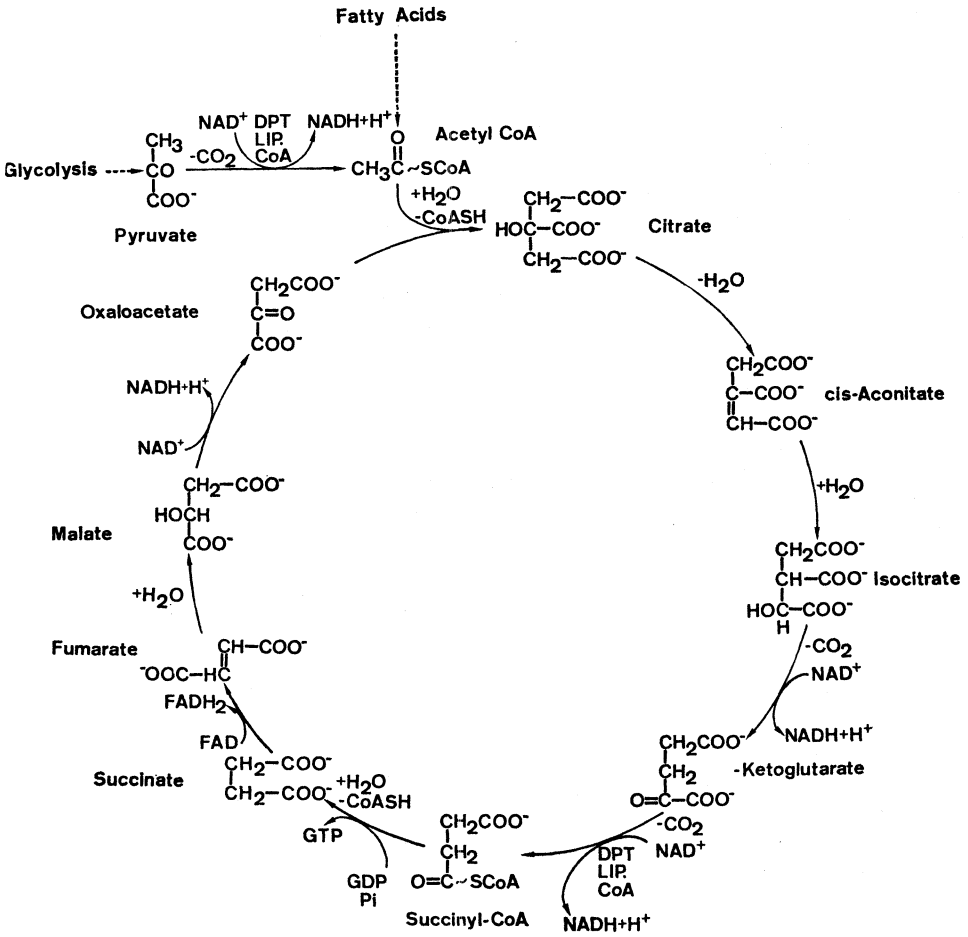

FIG. 19

exhibits an allosteric control, ADP and NAD^+ being positive modifiers, ATP and NADH negative modifiers. The enzyme requires Mg^{++} or Mn^{++}.

Oxoglutarate undergoes oxidative decarboxylation to succinyl-CoA, via multi-enzyme reaction similar to the reaction pattern of pyruvate. The multi-enzyme complex (mw about 2×10^6) is an octamer of an elementary unit containing each of the three contributing enzyme proteins: oxoglutarate decarboxylase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. The overall reaction involves thiamine pyrophosphate, lipoic acid, CoASH and NAD^+; succinyl-CoA is the end product:

$$\text{oxoglutarate} + \text{ThPP} + \text{lipoic acid} + \text{CoASH} + NAD^+ \rightleftharpoons \text{succinyl-CoA}.$$

Oxoglutarate is first transferred to enzyme-bound thiamine pyrophosphate (ThPP) with evolution of CO_2 and formation of ThPP-bound α-hydroxy-γ-carboxypropyl-ThPP (active succinic semialdehyde). The semialdehyde radical is then transferred

to oxidized lipoic acid with resulting oxidation at the aldehyde to acetyl radical and reduction of lipoamide to the sulfhydryl compound; succinyllipoamide is generated and ThPP is recycled. The succinyl radical is then transferred to the sulfhydryl group of CoASH yielding reduced lipoamide and the end product succinyl-CoA. Reoxidation of reduced lipoamide is catalysed by the complex-bound flavoprotein dihydrolipoyl dehydrogenase, hydrogen acceptor being NAD^+.

Succinyl-CoA is then split into succinate and free CoASH via a phosphorolytic cleavage in the presence of GDP:

$$\text{succinyl-CoA} + \text{P} + \text{GDP} \rightleftharpoons \text{succinate} + \text{GTP} + \text{CoASH}.$$

This is a typical example of substrate-level phosphorylation. The GTP generated in this reaction undergoes different fates: (a) transphosphorylation with ADP to give ATP and GDP; (b) transphosphorylation with AMP to give ADP and GDP; (c) direct activation of fatty acids; (d) phosphoenol-pyruvate synthesis from oxaloacetate.

An iron containing flavoprotein, succinate dehydrogenase, catalyses oxidation of succinate to fumarate:

$$\text{succinate} + \text{fp} \rightleftharpoons \text{fumarate} + \text{reduced fp}.$$

Hydrogen atoms are transferred to enzyme-bound FAD, and hence to ubiquinone and the respiratory chain. Succinate dehydrogenase is competitively inhibited by the homologue of succinate, malonate. Only the *trans*-isomer fumarate is generated.

In the next step, fumarase catalyses the hydration of fumarate to L-malate:

$$\text{fumarate} + \text{H}_2\text{O} \rightleftharpoons \text{L-malate}.$$

Fumarase appears to be a tetramer of four identical units. L-malate is finally oxidized to oxaloacetate by the NAD-linked malate dehydrogenase:

$$\text{L-malate} + \text{NAD}^+ \rightleftharpoons \text{oxaloacetate} + \text{NADH} + \text{H}^+.$$

In this reaction oxaloacetate, the partner of acetyl-CoA for condensing enzyme, is finally regenerated.

Hydrogens for substrate oxidation in the Krebs citric acid cycle are transferred to oxygen via the carriers in the respiratory chain; during this process ATP is formed.

The operation of the Krebs citric acid cycle is first of all regulated by the availability of ADP to the respiratory chain. When little or no ADP is present, the flow of reducing equivalents down the respiratory chain is limited. As a result, a marked fall in the flux of substrates along the cycle is observed. In mammalian mitochondria ADP could control the rate of the citric acid cycle also by virtue of its activating effect on isocitric dehydrogenase activity.

The concentration of ATP also modulates the rate of citric acid cycle. As pointed out before, physiological levels of ATP inhibit the activity of condensing enzyme, thus slowing down the rate of acetyl-CoA degradation.

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2.3. DRUGS AND LYSOSOMES

by WATTIAUX, R. (Namur)

2.3.1. *General Properties of Lysosomes*

IN this first section we shall give a brief account of the general properties of lysosomes. The reader interested in a more extensive coverage of the subject is referred to earlier reviews [2, 3, 6].

2.3.1.1. *Definition*

Lysosomes are described as cytoplasmic particles containing numerous acid hydrolases of various specificities. Under normal conditions, the membrane of the granules is impermeable to the substrates of these enzymes; however, a variety of chemical or physical agents are able to alter the lysosomal membrane and thus provide the enzymes with free access to the external substrates (Fig. 1). Lysosomes are found in many animal tissues, and increasing evidence is recently being uncovered that they are also present in plants.

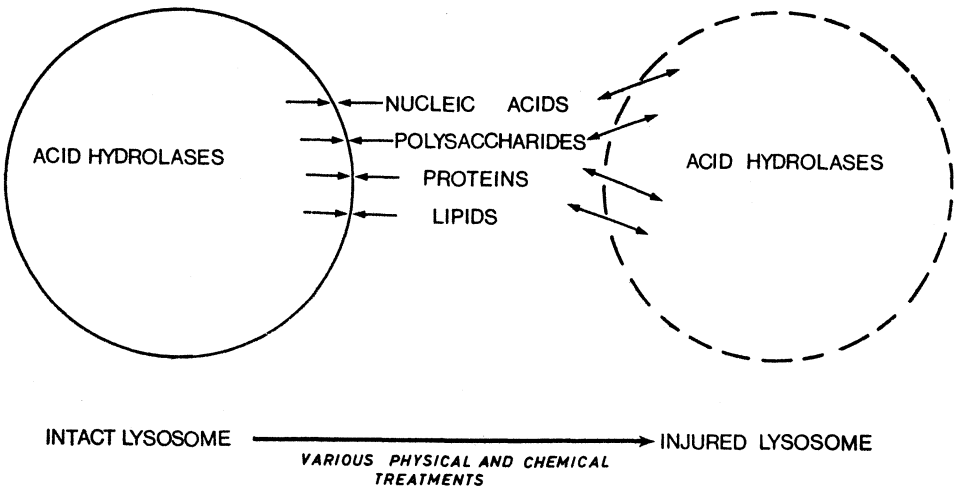

FIG. 1. Schematic representation of the properties of lysosomes.

2.3.1.2. *Formation*

Relatively little is known concerning the mode of formation of these particles; this problem is discussed extensively in a review article [6]. We may summarize by saying that the acid hydrolases appear to be synthesized in the rough endoplasmic reticulum and thence conveyed to the Golgi apparatus through the smooth endoplasmic reticulum; there, they are released within vesicles which may be regarded as the *primary lysosomes*. As we shall see later, these granules represent only a fraction of the lysosomal system.

2.3.1.3. *Functions*

Lysosomes take part in the intracellular digestive processes involving exogenous materials taken up by the cell (heterophagic function) as well as endogenous components (autophagic function).

The heterophagic function may be schematized as follows. The extracellular components such as macromolecules, viruses, etc., which will reach the lysosomes

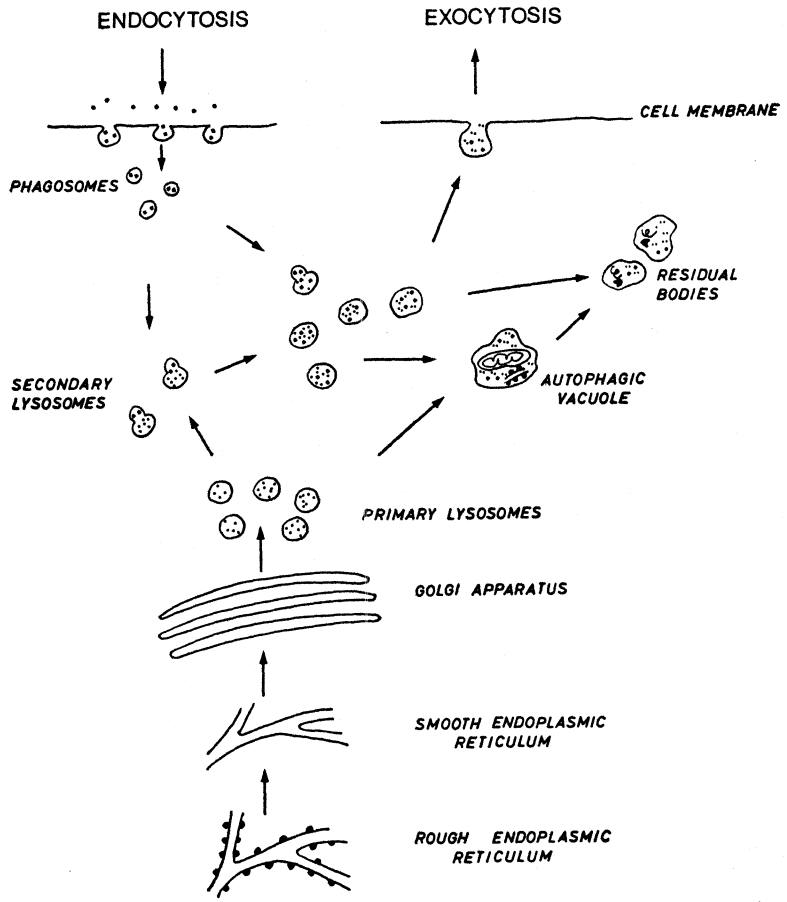

FIG. 2. Schematic representation of the heterophagic and autophagic functions of lysosomes.

are engulfed by endocytosis. The vesicle (*phagosome*) resulting from this process fuses either with a primary lysosome or with a *secondary lysosome*, i.e. a granule that has already been the site of hydrolytic phenomena. In this way, the newly enclosed component is exposed to the lytic action of hydrolases and can be more or less degraded. The degradation products either diffuse into the cytoplasm, are eliminated from the cell by exocytosis, or accumulate in the lysosomal system. The exocytosis of the lysosome contents may still allow the enzymes to act on the extracellular substances (Fig. 2).

The autophagic function is performed in autophagic vacuoles consisting of a cytoplasmic area surrounded by a membrane, where mitochondria, fragments of endoplasmic reticulum, etc., are degraded. Considerable experimental data indicate that lysosomal enzymes take part in this focal autolysis. It is not clearly understood how the hydrolases reach the vacuoles, but in some cases fusion may occur between a lysosome and a vacuole containing the cellular component to be digested.

These digestion phenomena take place in an enclosed area of vacuoles involved in numerous fusion and division processes.

Besides this intracellular digestion apparatus, there is another set of vacuoles into which secretion proteins are transported. The two systems exhibit a high degree of similarity. As Palade and coworkers have shown in their studies of the exocrine cells of the pancreas, proteins of secretion (digestive enzymes) are also synthesized in the rough endoplasmic reticulum, and are conveyed by the smooth reticulum to the Golgi apparatus where they are condensed and released within secretion granules (zymogen granules). Enzymes are finally extruded into the extracellular fluid where they perform their digestive function.

The secretion of the digestive enzymes presupposes a fusion of the secretion granule membrane with the plasma membrane; in the same manner, the fusion of the lysosome membrane with the membrane of the phagosome, which is in turn a part of the plasma membrane, is a prerequisite for the heterophagic function of the lysosomes. This suggests that striking structural analogies exist between the plasma membrane, the secretion granule membrane and the lysosome membrane. As a matter of fact, there is a definite possibility that lysosomes may fuse with secretion granules. We think it important to stress the close relationship between the two systems because a compound that is able to affect lysosomes could theoretically also affect the secretion process. However, it has not yet been possible to show that an endocytosed substance has reached the endoplasmic reticulum system, which suggests that fusion between the phagosome membrane and the endoplasmic reticulum membrane is an impossibility. At some point between the production of acid hydrolases in the endoplasmic reticulum and their appearance in the lysosomes, a change of membrane structure must occur; the Golgi apparatus is the most plausible site for this transformation to take place. A detailed analysis of this problem has been made [5, 6].

The cell utilizes the heterophagic and autophagic functions of lysosomes in numerous cellular processes. The granules take an active part in cell defence against bacteria, viruses and toxic macromolecules. Autophagic function is useful in phenomena leading to the disappearance of intracellular structures during organ regression, metamorphosis and related processes; it most likely plays a more general rôle in the catabolic phase of the turn-over of cellular components. These functions associated with autonomous cell life can be integrated in a variety of physiological events such as immunity reactions, developmental processes or the liberation of products endowed with special biological properties [6].

2.3.1.4. *Morphology*

Lysosomes are characterized by their polymorphism and are not readily distinguished on the basis of a particular size or structure. The morphological identification of lysosomes is therefore based chiefly on cytochemistry, particularly cytochemical localization of the acid hydrolases.

2.3.2. *Drugs and Lysosomes*

De Duve [4] proposed classifying drugs which act on lysosomes into three groups. The first category would include substances able to affect the rate of flow of matter through the vacuolar apparatus; these agents regulate endocytosis, phagosome–lysosome interaction and autophagic vacuole formation. In the second group are the drugs which modify reactions inside the lysosome. The third category includes substances which affect the stability of the lysosome membrane. Obviously, certain drugs can be classed in more than one group; for example, a lysosomal-membrane modifier may affect lysosome–phagosome interaction, act as an inducer of pinocytosis or inhibit some lysosomal enzymes. The reader will find various examples in de Duve's publication. Here, we shall consider methods of investigating *in vitro* and *in vivo* drug effects on lysosomes.

2.3.2.1. In vitro *effects*

In general, a compound may affect lysosomes *in vitro*: (1) by changing the permeability of their membranes, or (2) by inhibiting or activating their enzymes.

(a) *Effects on the lysosomal membrane.* The following is a convenient experimental method for testing the stability of the lysosomal membrane *in vitro.*

As pointed out previously, under normal conditions the lysosome membrane is impervious to hydrolase substrates. This may be evidenced *in vitro* by incubating a preparation of granules in isotonic sucrose for a relatively short time. Let us take as an example the granule preparation isolated from rat-liver tissue (Fig. 3). If acid hydrolase activity is measured on granules incubated with a substrate no longer than 10 min at pH 5, at 37°C and in 0·25 M sucrose, a very low activity is observed. This is defined as the *free activity* and is characteristic of experimental conditions where the lysosomal membrane is well preserved. If the granules are subjected to a treatment which can alter the membrane, enzymatic activity increases considerably. In some cases this treatment will allow the hydrolases to have free access to their external substrates, with the result that the enzymes are completely "unmasked" and exhibit *total activity*. An increase in free activity is often accompanied by a degree of solubilization of the enzymes.

It follows that a substance's action on the lysosomal membrane *in vitro* can be evaluated by testing its effects on the free (and soluble) activity of one or several lysosomal hydrolases. It is also possible to determine whether a compound inhibits or enhances the effectiveness of a treatment modifying free acid hydrolase activity.

By way of example, we shall show (Fig. 3) the effects on rat-liver lysosomes of two liposoluble substances of biological interest: cholesterol and progesterone. A mitochondrial fraction of rat liver containing 70% of the tissue lysosomes was used in these experiments. Following incubation at pH 5 in 0·25 M sucrose, a gradual increase in free acid phosphatase activity is observed, indicating a progressive alteration of the lysosomal membrane (Fig. 3). If prior to incubation the granules are exposed to progesterone, there is a more rapid increase in free acid-hydrolase activity; in contrast, after contact with cholesterol, free activity increases more slowly (Fig. 3). The total activity of the acid phosphatase is not affected by the drugs. One may conclude that progesterone labilizes and cholesterol stabilizes the rat-liver lysosomal membrane *in vitro.*

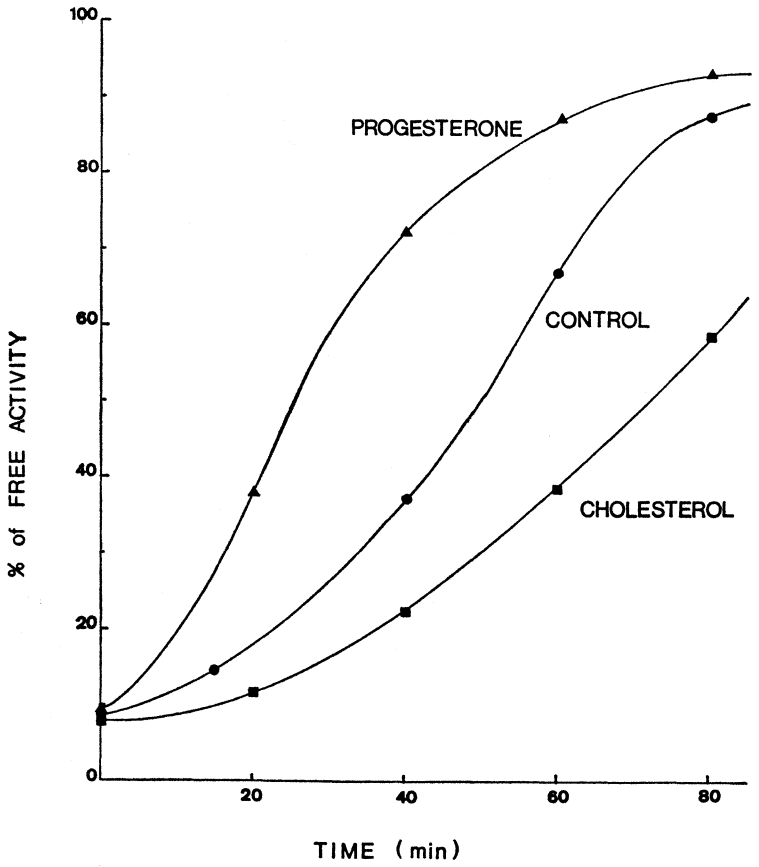

FIG. 3. Effects of progesterone and cholesterol on the release of acid phosphatase. A mitochondrial fraction of rat liver was incubated for various periods of time at 37°C in isotonic sucrose, pH 5; the free activity of acid phosphatase was measured at the time indicated. Progesterone and cholesterol were dissolved in dioxane and added at a concentration of 0·125 mg/ml; dioxane alone was added to the control. (After de Duve, Wattiaux and Wibo, 1961.)

TABLE 1. CLASSIFICATION OF SOME DRUGS ACCORDING TO THEIR EFFECTS ON THE LYSOSOMAL MEMBRANE

Labilizers	Stabilizers
Progesterone	Cholesterol
Testosterone	Cortisone
Diethylstilboestrol	Prednisolone
Vitamin A	Chloroquine and related compounds
Vitamin E	Antihistamines
Polyene antibiotics	Phenothiazines

Another experimental procedure consists of determining the enzyme activity which is released in a soluble form after treatment of the granules with drugs. Table 1 lists a number of drugs that have been shown to be stabilizers or labilizers of the lysosomal membrane *in vitro*. This table is not exhaustive, additional information may be found in references [1, 7, 11].

Such a technique may be applied to mitochondria and peroxisomes since some enzymes are also "masked" in these organelles. Thus it is possible to compare the effects of a substance on the membrane of these subcellular structures and on the lysosome membrane.

It may be asked whether the study of a substance's effects on the lysosomal membrane is of therapeutic interest. The discovery that lysosomes play a role in inflammatory processes is in itself sufficient to justify such an investigation. Indeed some symptoms of inflammation probably result from an increase in fragility of the lysosomal membrane and the release of the granular content, leading to cellular and extracellular injuries. Hence a compound able to stabilize the lysosomal membrane may possess anti-inflammatory properties. Many well-known anti-inflammatory drugs do indeed stabilize the lysosomal membrane [11].

(b) *Effects on lysosomal enzymes.* The functions of lysosomes depend on their enzymes. As illustrated by certain metabolic diseases, a genetically-determined deficiency in one lysosomal hydrolase may lead to severe cellular disturbances. Therefore, a drug able to inhibit or activate one or several lysosomal enzymes may exert profound effects on the behaviour of the lysosomal system. Drugs that act specifically on acid hydrolases and become concentrated after administration *in vivo* in the lysosomal system are particularly interesting. When a substance of pharmacological interest appears to affect some acid hydrolases *in vitro*, it is useful to investigate whether a significant percentage of the compound is recovered in the lysosomes after administration to the animal. Conversely, if a substance appears capable of accumulating in the lysosomes *in vivo*, its *in vitro* effects on acid hydrolases should be tested, using a concentration comparable if possible to that found in the lysosomal apparatus.

A characteristic example is the case of some bisazodyes. Lloyd *et al.* [8] have shown that trypan blue is a powerful inhibitor of acid hydrolases and that when injected in the rat the dye concentrates in the lysosomes of phagocytic tissues. They reason, therefore, that the dye could conceivably inhibit acid hydrolases *in vivo*. According to these authors, there is a relationship between this phenomenon and the teratogenic and carcinogenic effects of the dye.

2.3.2.2. In vivo *effects*

The effects of a drug on the lysosomal system *in vivo* can be detected by various changes affecting the components of the system. To demonstrate these changes, it is helpful to compare the properties of lysosomes in the normal and treated animal. In Table 2 we have summarized the experimental methods available for this purpose. To illustrate, let us consider the technique applied to the study of the *in vivo* effects of a non-ionic detergent, Triton WR1 339, on rat-liver lysosomes [9, 10].

TABLE 2. EXPERIMENTAL METHODS FOR STUDYING THE PROPERTIES OF LYSOSOMES

Lysosomal properties	Methods for detecting changes in lysosomal properties
Acid-hydrolase activity	Measurement of total enzymatic activity on homogenates
Size	Differential centrifugation Morphological examination
Density	Centrifugation in density gradients
Chemical composition	Chemical analysis of purified preparation
Membrane permeability	Determination of free acid-hydrolase activity
Morphological appearance	Morphological examination including cytochemical methods

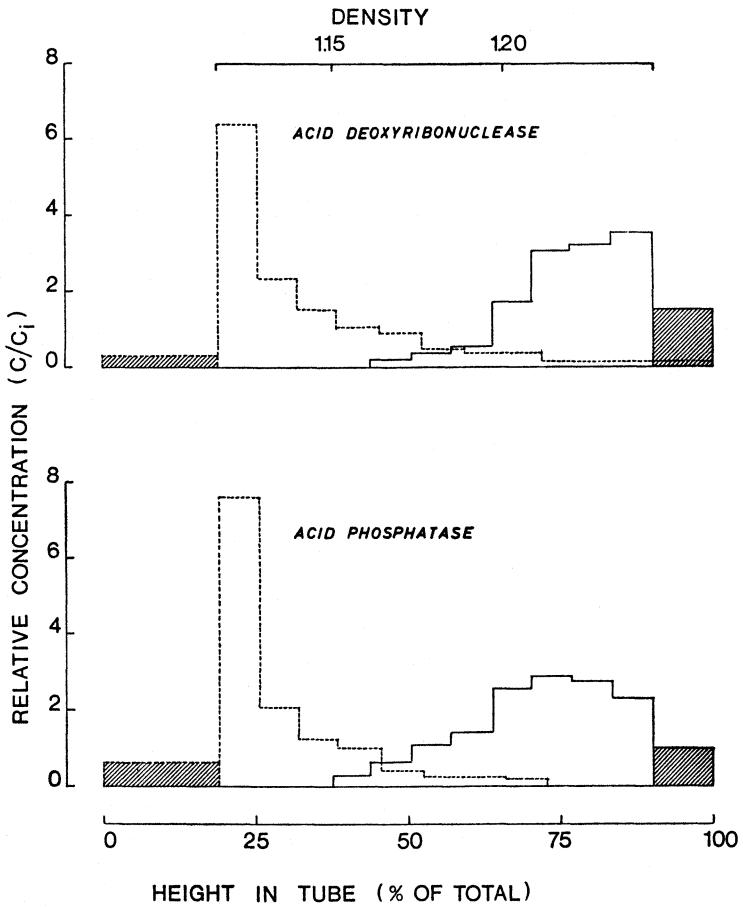

FIG. 4. Effects of Triton WR1 339 injection on the equilibrium density of rat-liver lysosomes in a sucrose gradient as ascertained by the distribution of acid phosphatase and acid deoxyribonuclease. Solid line: control animal. Broken line: Triton WR1 339 treated animal. (After Wattiaux, Wibo and Baudhuin, 1963; and Wattiaux, 1966.)

Triton WR1 339 is a polyethylene glycol derivative of polymerized *p-tert*-octylphenol. It causes a considerable increase in lipaemia and in the rate of cholesterol synthesis by the liver; it is also an antituberculotic. When injected in the rat, the compound produces changes in lysosomal properties. These alterations can be identified by means of some of the techniques listed in Table 2. Let us examine, for instance, how the lysosomes are distributed after isopycnic centrifugation in a sucrose gradient (Fig. 4). With granules from the liver of a normal rat, the lysosomes equilibrate at a density greater than 1·20 as ascertained by the acid phosphatase and acid DN-ase distribution; however, with granules from the liver of a rat injected with Triton WR1 339, lysosomes are found in a lower density region of the gradient (1·10–1·12). The density of the rat-liver lysosomes is thus markedly affected by treatment of the animal with the detergent. Morphological examination in the electron microscope shows that lysosomes are also greatly enlarged following Triton WR1 339 administration. Granular stability *in vitro* is modified as shown by a higher resistance to pH 5 incubation but a lower resistance to hypotonicity. Following the injection of radioactive Triton WR1 339, centrifugation experiments show that the detergent accumulates in the lysosomes. We may therefore surmise that there is a correlation between the storage of this low-density substance in the granules and their changes in size and density.

These results also point to a possible link between the presence of the detergent in the lysosomal system and its effects on tuberculous infection. Triton WR1 339 is not bacteriostatic; it is effective only inside the infected cell and acts by preventing bacillus proliferation. A plausible hypothesis is that the compound enhances the defensive power of the lysosomes against tuberculous infection by sensitizing mycobacteria to the effects of acid hydrolases. In addition, the intracellular distribution of the detergent suggests that practically all the Triton WR1 339 found inside the cell is located in the lysosomes. This provides convincing evidence that certain drugs can be selectively introduced into the lysosomal system and can thus selectively affect reactions inside the lysosomes.

2.3.3. *Conclusion*

The enzymatic processes which take place in the lysosomes are relatively simple; the organization of these subcellular structures likewise appears to be simpler than that of other cytoplasmic structures such as the mitochondria or endoplasmic reticulum. Drug effects on these organelles thus seem relatively easy to detect. However, we wish to stress the importance of using a sufficient number of experimental techniques in a pharmacological investigation of these granules. The study of a single drug effect on the lysosomal system is usually inadequate; for instance, a compound may not change the free activity of acid hydrolase but produce marked alterations of lysosomes *in vivo*. The case of Triton WR1 339 is significant in this respect; the detergent has no striking effect on lysosomes *in vitro* but affects the organelles inside the cell.

We have attempted in this chapter to emphasize certain experimental approaches which in our opinion are capable of yielding additional information on the effects of drugs on lysosomes. The examples selected were all taken from studies on rat liver. It is preferable, at least at the beginning of a pharmacological study, to

make use of this particular biological system since normal rat-liver lysosomes have been studied extensively by well-established techniques. However, this does not mean that one should be confined to the system; if the investigator finds that *in vivo* a drug acts more specifically on a given type of cell, he should by all means instigate studies using the lysosomes of these cells.

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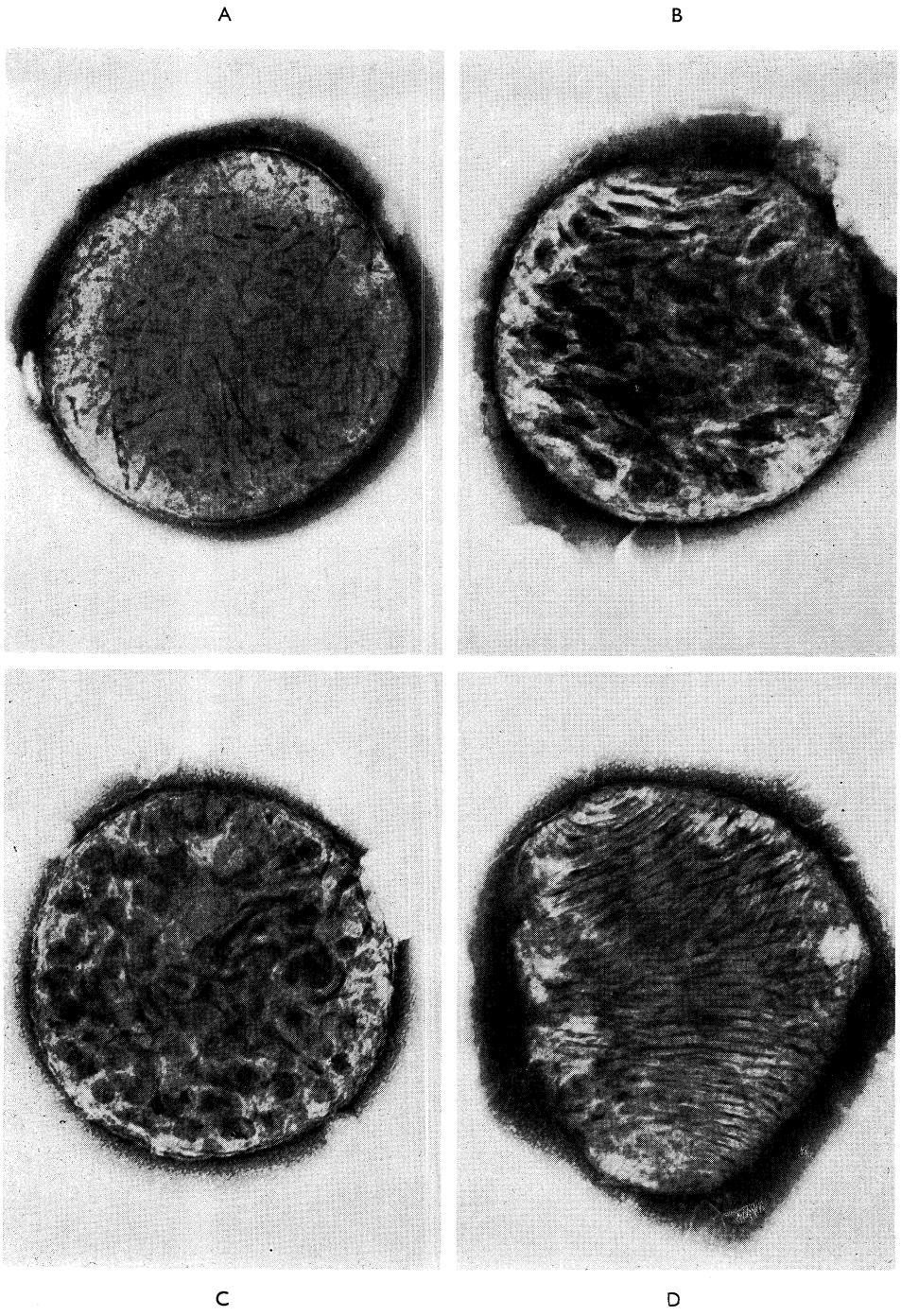
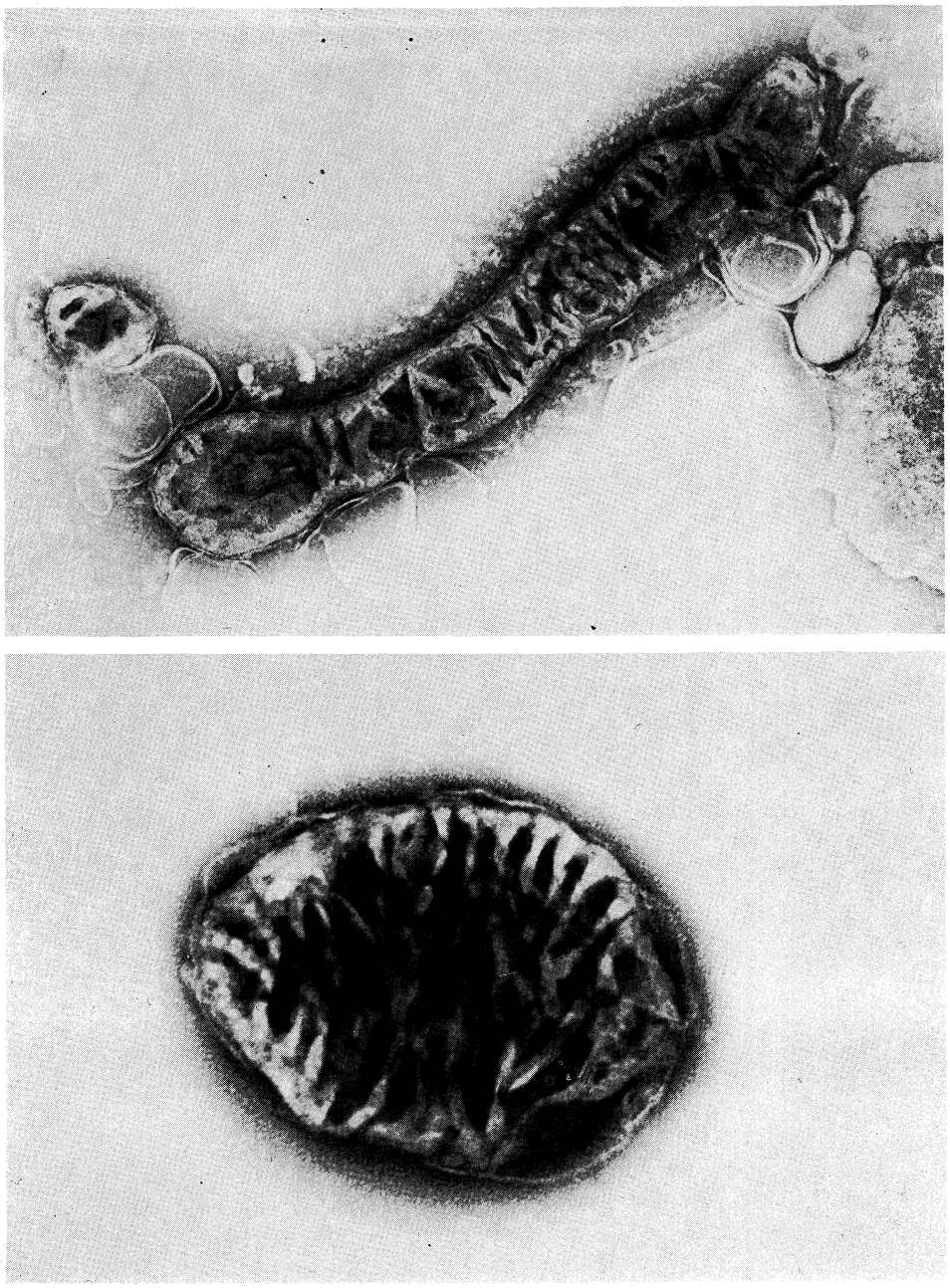


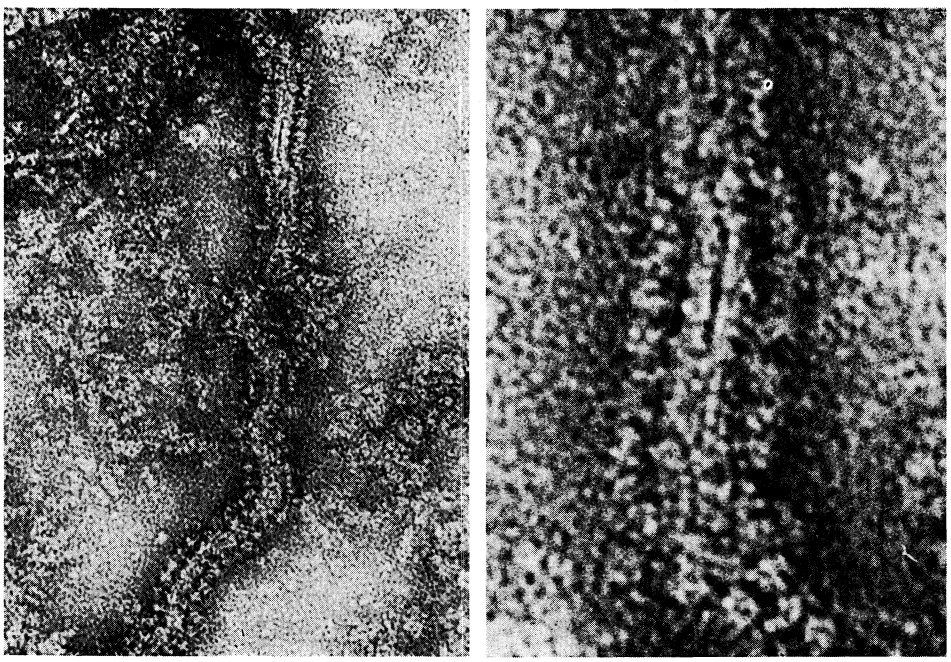
this organelle has a spherical shape, and a highly developed cristate system. However, many of the cristae are vesicular; the area enclosed within the inner membrane has a sponge-like appearance. ×80,000. (D) A mitochondrion isolated from rat heart, and treated as in (A). The shape of the organelle is not spherical, and the cristae are extraordinarily numerous. Most of them form complete septa and run parallel. The matrix space is very reduced. ×80,000. (Courtesy of Dr. U. Muscatello.)

A



B

FIG. 3. (A) A mitochondrion isolated from rabbit brain. Even after isolation and negative staining with isotonic ammonium molybdate, the mitochondrion has maintained a striking elongated appearance. The cristae are not very numerous, but many of them form complete septa. The matrix space is clearly recognizable. ×62,000. (B) A mitochondrion isolated from the masseter muscle of the rabbit, and treated as in Figure 1. Also this mitochondrion has a non-spherical shape. The cristae are numerous, but the matrix space is more abundant than in rat heart. In this mitochondrion the tri-dimensional organization is particularly evident. ×120,000. (Courtesy of Dr. U. Muscatello.)



A B

FIG. 4. (A) Rat-heart mitochondria, disrupted with sonic oscillation and negatively stained with isotonic ammonium molybdate. A detached crista is seen. Along its profiles, an array of protruding particles is evident. The particles are connected to the crista by a stalk. ×220,000. (B) A higher magnification detail of Figure 7. The particles measure about 90 Å in diameter; in some of them a substructure (subunits?) can be recognized. ×720,000. (Courtesy of Dr. U. Muscatello.)

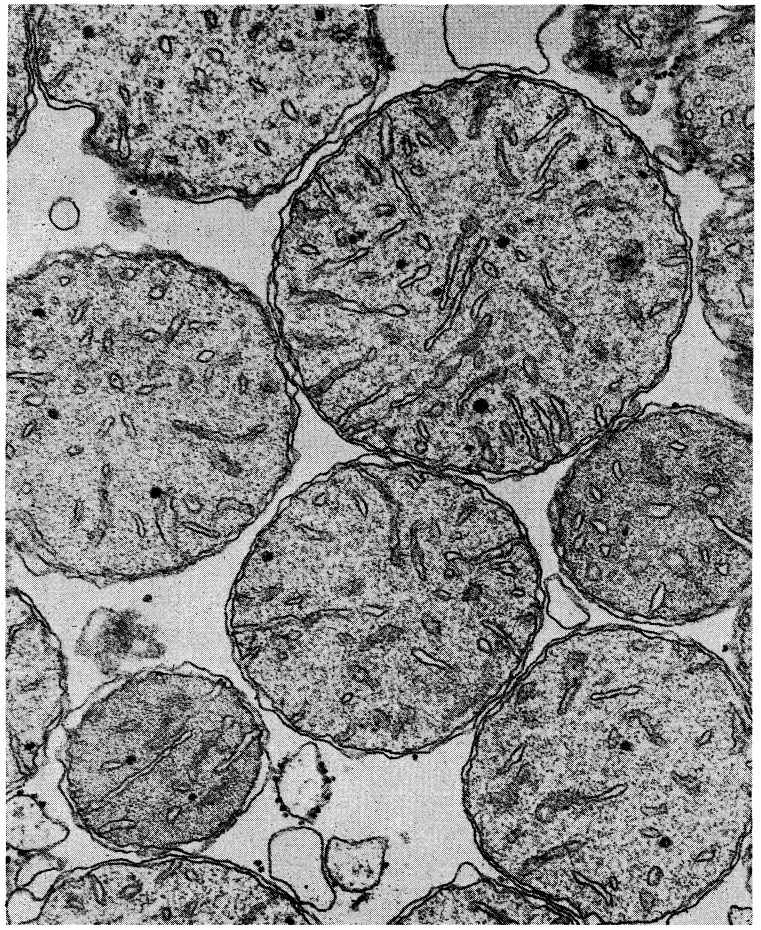


FIG. 5. The "orthodox" conformation of a rat-liver mitochondrion during resting (or state 4) respiration. Fixation in osmium tetroxide, staining in uranyl acetate and lead hydroxide. Both the outer and the inner membrane are extended, the cristae are flattened and the matrix appears as a moderately dense, granular material. Some dense granules are visible. ×110,000. (Courtesy of Dr. C. R. Hackenbrock.)

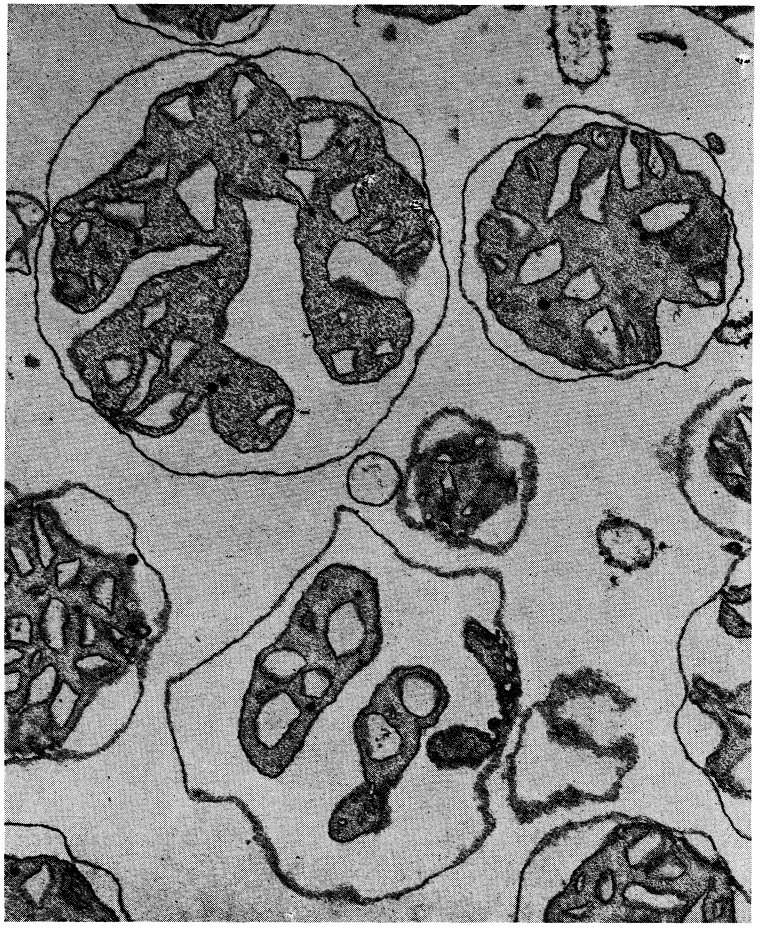


FIG. 6. The "condensed" conformation of a rat-liver mitochondrion during phosphorylating (or state 3) respiration. The outer membrane is extended, but the inner is randomly folded. The matrix volume is greatly diminished. The dense granules are still visible. Note the sites of contact between the outer and inner membrane. ×110,000. (Courtesy of Dr. C. R. Hackenbrock.)