1959

Mitochondria and cellular secretion, with special reference to the adrenal cortex

BOSTON UNIVERSITY
GRADUATE SCHOOL

Thesis

MITOCHONDRIA AND CELLULAR SECRETION, WITH SPECIAL REFERENCE TO THE ADRENAL CORTEX

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(A.B., Boston University, 1955)

Submitted in partial
fulfillment of the requirements
for the degree of
Master of Arts
1959

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ACKNOWLEDGMENT

This thesis was prepared under the supervision of Dr. Donald I. Patt, member of the faculty of Boston University. The author is deeply indebted to him for his continued guidance and encouragement throughout all phases of her work.

The author also wishes to express her appreciation to the personnel of the Boston University Research Laboratories for the assistance given her in the collection of data for this thesis.

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ABSTRACT

Mitochondria are the sites in which the products of digestion are oxidized to provide the energy for cellular activities, such as contraction and secretion.

In the adrenal cortex, the mitochondrial membrane is the site of hydroxylating enzymes essential in the synthesis of cortisone and other corticoids from cholesterol.

Pritchard's silver is the recommended stain for use in quantitative studies of mitochondria in the adrenal cortex.

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MITOCHONDRIA AND SECRETORY ACTIVITY- WITH SPECIAL REFERENCE TO THE ADRENAL CORTEX

INTRODUCTION

The purpose of this thesis is to review the evidence for the relationship of mitochondria to secretion and to consider the role of mitochondria in the formation of adreno cortical steroids.

Part One will consist of three sections: (1) an introduction to the background of research on mitochondria and secretion, with consideration of some correlations between structure and function; (2) a summary of some recent experimental work correlating changes in mitochondria with cellular injury and with secretory cycles in various glands, and especially in the adrenal cortex; (3) a discussion of a possible mechanism for the participation of mitochondrial enzymes in the synthesis and secretion of adrenocortical steroids.

Part Two will consist of the author's study of staining techniques for mitochondria in tissue secretions of the adrenal cortex of the rat, carried out under the direction of Dr. Donald I. Patt, Professor of Biology, Boston University.

The outline for Part I is based on Lindberg & Ernster's "Chemistry and Physiology of Mitochondria and Microsomes."

PART ONE

I. BACKGROUND

A. HISTORICAL

The high points of research on mitochondria before the discovery by Bensley and Hoerr[11], in 1934, of the isolation of mitochondria by washing and centrifugation have been outlined by E. V. Cowdry.[11]

Mitochondria were discovered by Altmann[11] in 1890, some thirty years after Gerlach had popularized the use of staining techniques in cytological research. Altmann, using an acid fuchsin anilin oil staining mixture noted granules, rods, and filaments that superficially resembled bacteria in many different kinds of cells. He called them "bioblasts" and believed them to be elementary vital units existing in all cells. Though they are no longer associated with the mechanism of heredity, they are important in cellular metabolism and in secretion. Their role in the process of secretion remains to be elucidated and forms the subject of this paper.

The second major step in the characterization of mitochondria was taken by Michaelis[11] in 1900, and by Laguesse,[11] in the same year, who described the staining of mitochondria with Janus green B (diethylsafranineazodimethylaniline hydrochloride) in fresh living cells. This technique became

widely used as a tool for the identification of mitochondria, but it was not until 1953 that the mechanism of Janus green staining was described.

Showacre,[50] and Lazarow and Cooperstein[31, 32] demonstrated that Janus green B is first reduced to diethyl safranine and to leuco safranine by the lactic dehydrogenase and glucose dehydrogenase systems of the cell, and then reoxidized by the cytochrome c - cytochrome oxidase system which is concentrated in the mitochondria. Complete anaerobic conditions and cyanide poisoning abolish the selective staining of the mitochondria, by inhibiting the cytochrome oxidase system. Thus their importance in cellular metabolism was demonstrated. In 1899, Benda introduced the term "mitochondria" (meaning "thread" and "granule," Gr.) to describe the cell organelles discovered by Altmann. Other workers used the term "chondriosomes."[6]

As early as 1909, the relation of mitochondria to cellular secretion was suggested by Regaud[11] in his <u>eclectosome theory of mitochondrial function</u>, in which he stated that the mitochondria of animal cells act like plastids or "electosomes," choosing and selecting substances from the cytoplasm and condensing them on the surface or the interior, and changing them into diverse products.

This idea was further developed by E. V. Cowdry[12], into

the Surface Film Theory of the Function of Mitochondria, which appeared in 1926. Evidence for the mitochondrial origin of plastids in plant cells had been presented by Alvarado, Maximov, Meves, and others, while in animal cells mitochondria had been shown to accumulate more than 100 substances from the cytoplasm of different cells.[11] Cowdry's theory of the role of mitochondria in accumulation and secretion stated that: (a) the molecules of certain solutes orient at the mitochondrial-cytoplasmic interface; (b) the molecules of the approximated substances are incorporated into the mitochondria; (c) the substances are then concentrated in the mitochondria (indicated by the increased intensity of staining reaction, or the increased amount of starch or fat and the enlargement of the mitochondria); (d) the incorporated substances react with the mitochondrial material to produce new substances; (e) each step in the process is accompanied by a mitochondrial change demonstrated by increased resistance to the solvent action of acetic acid and by changes in size, shape, and staining properties.

Supporting evidence for the theory was: (a) the occurrence of the mitochondrial film in all living, active cells; (b) measurements of acinous cells of the pancreas which showed that the combined surface area of the mitochondria was greater than the surface area of the nuclear or cytoplasmic membrane; (c) migration and localization of mito-

chondria in different cells and in different physiological states; (d) differential absorption action in various cells due to differentiation in the proteolipids of the mitochondria to produce different physical properties of the film; (e) the uniformity of the mitochondrial diameter in cells of the same type, while varying in length, and from type to type. (f) The dependence of the activity of the mitochondria on the cytoplasmic composition; (g) the sensitivity to injury of mitochondria in secreting cells. Thus, though mitochondria were clearly linked to secretory processes in cells as early as 1926, their exact function was not known, but believed to be "multiple, differing, perhaps, even in successive stages of cytomorphosis and between cells of different categorization."

In 1926, Robertson[11] made the suggestion that the molecules at the mitochondrial surface might orient so that the reactive carboxyl and amino-acid groups would point outward to the surrounding cytoplasm due to the lipoid properties of the mitochondria.

Then, in 1932, Guilliermond[11] confirmed the earlier reports that the plastids of plants derive from mitochondria. But Regaud's "electosome theory" of the relation of mitochondria to secretion in animal cells remained unproven.

Research continued, however, and in 1933, Horning[11] demonstrated a correspondence between the distribution and

orientation of mitochondria and intense enzymatic activity in protozoan and metazoan cells.

Then an important new technique was developed by Bensley and Gersh,[11] who applied Altmann's staining methods to frozen-dried tissues. Thus by the avoidance of chemical fixatives they opened the way for histochemical research on mitochondria.

Prior to 1934, therefore, the main lines of research had been established into the nature and function of mitochondria. Extensive work had shown that: (a) mitochondria were basic cell components, found in all plant and animal cells, and were as essential to the cytoplasm as chromatin was to the nucleus; (b) they provided a very extensive surface of interaction with the cytoplasm; (c) they were mostly lipo-protein in composition; (d) they were centers of constructive metabolism of products essential to life. See Fig. 1.

B. MORPHOLOGICAL AND METHODOLOGICAL

1. ISOLATION AND IDENTIFICATION

The turning point in research on mitochondria occurred in 1934, when as previously stated, Bensley and Hoerr[11] isolated mitochondria from tissue homogenates. By 1953 the isolation procedure had been standardized as follows:

> The tissue is removed from the animal as quickly as possible, cooled in ice-cold sucrose solution and cut into small pieces. The pieces of tissue are then homogenized. The homogenate may be filtered through gauze to remove any coarse particles. Unbroken cells, nuclei and cell debris are removed by low-speed centrifugation. The supernatant is then spun at a high rate

and the mitochondria are obtained as a pellet. For the isolation of microsome material, the clear or almost clear supernatant is spun again at still higher speeds.

The most important result of the use of this technique was the discovery of the multiplicity and heterogeneity of cytoplasmic particles.

First, Claude[8] discovered the microsome layer in 1940, which contained sub-microscopic particles of about 50-150 mu. This layer was found to consist of fragments of the "endoplasmic reticulum" characterized as a system of membranes and small, dense particles by George E. Palade[40, 41] of the Rockefeller Institute for Medical Research.

Then Laird, Nygaard, Ris, and Barton[30] separated a densely packed lower layer of mitochondria from a loosely packed fluffy layer which contained particles similar in shape, osmotic behavior and succinoxidase activity to mitochondria, but intermediate between mitochondria and microsomes in their ribonucleic acid (RNA) to protein ratio and phospholipid content, and showing twice the alkaline phosphatase activity of the mitochondria or the microsomes. Electron microscope studies reviewed by Novikoff[38] have shown the fluffy layer to be a mixture of mitochondria and microsomes, but de Duve[15] believes there may be elements called "lysosomes," containing the acid phosphatases and hydrolases, which may be separate particles or precursors of mitochondria.

To sidestep this problem and avoid the arbitrary separa-

tion of particles, Kuff and Hogeboom,[26] together with Dalton,[13] devised a new technique based on the "size distribution analysis of the cytoplasmic particles as determined from the rate of sedimentation of their specific biochemical constituents."

> Livers of fed rats were homogenized and the homogenate cleared of nuclei, whole cells, and connective tissue by preliminary low-speed centrifugation. The resultant suspension, the "cytoplasmic extract," was centrifuged at varying speeds (depending on the size of the particle to be analyzed), for a given length of time. A 50% sucrose solution was then mechanically injected at a slow and constant rate through a hypodermic needle inserted into the tube a few millimeters from the bottom. The fluid column was thus floated upward and passed into a sampling device which permitted the collection of timed samples of fluid levels from the column. The volume of the sample was determined by weight, and the samples analyzed for biochemical composition.

In general, four distinct particle types were indicated: (a) glycogen inclusions; (b) mitochondria; (c) and (d) not distinct, but possibly lysosomes and microsomes, as both contained large amounts of pentose nucleic acid (PNA).

The succinic dehydrogenase activity, generally thought to be exclusively located in the mitochondria, was determined for extracts of mitochondria spun at 3148 to 14,290 r.p.m. The size distribution of the particles centered around radii of 1.22 and 1.27 u, which was in agreement with earlier measurements of rat liver mitochondria. See Fig. 2.

Succinic dehydrogenase activity, however, may be obtained with mitochondrial fragments, and a preferable enzymic criterion for the identification of mitochondria is the

capacity for oxidative phosphorylation.

Although Janus green B has been used to characterize mitochondria in the intact cell, the staining of isolated structures with the dye does not necessarily prove them to be mitochondria, as Lazarow and Cooperstein[31,32] demonstrated that the mitochondrial, microsomal and supernatant fractions of liver cell homogenates were all capable of reducing the dye under appropriate conditions.

2. SIZE AND STRUCTURE

Early observations by Cowdry[10] with the light microscope, stressed the uniformity of the mitochondrial diameter in a given cell type. Palade, [39,40] however, using the electron microscope, found variation in every cell type. Liver mitochondria, for example, varied in diameter from 0.35 to 0.74 u.

The differences in the mitochondrial diameters are characteristic for the various cell types, ranging from slender (0.2-0.3 u dia.) in endothelia, epithelia of pancreatic ducts, pancreatic centro-acinar cells, pancreatic islet cells, and acinar cells of the salivary gland, to thicker (0.5 u dia.) in epithelium of the proximal convoluted tubules of the nephron (0.4-0.6 u dia.) in pancreatic acinar cells, (0.5-0.7 u dia.) and in heart muscle, (0.4-1.0 u dia.). In an electron microscope study of isolated mitochondria obtained from lymphosarcoma of the rat, Claude and Fullam[8] found an average mitochondrial diameter of 0.6 to 1.3 u.

In histological demonstrations, Cowdry[10] found mitochondrial diameters to vary from 0.2u to 7.0u as the extreme limits. The mitochondria may be granular, rod-like, or filamentous in appearance.

In tissue culture, with phase contrast optics, Lewis and Lewis[34] observed them to be in constant movement, with pear-shaped and dumbbell-shaped forms appearing.

In electron micrographs of sectioned material mitochondria are round or oval, sharply outlined and of different density than the cytoplasm.

> The form of the mitochondria undergoes profound alteration when the cell is subject to external or internal stimuli. When the cell is absorbing or secreting, or is subjected to hypotonic conditions, the mitochondria swell, become rounded, nd may assume a volume many times the resting value. In a hypertonic medium on the other hand, they shrink and the rods are readily converted to angular formations.

The electron microscope has also been a most useful tool in the elucidation of the ultrastructure of the mitochondria.[3, 43]

In a fundamental study of methods of fixation of tissues for electron microscope study, Palade[44] found that the diameter of the mitochondria varied with the pH of the fixative and was smallest at neutrality. He recommended a fixative of 1% osmium tetroxide (OsO_4), buffered at pH 7.3-7.5 with acetate-veronal buffer as giving best results. Since then potassium dichromate (chrome-osmium), $KMnO_4$, and formalin have also been developed as fixatives, but OsO_4 is still preferred.

Dalton and Felix[13] evaluated the criteria for the acceptance of electron microscope data and the identification of artifacts of fixation, and confirmed the findings of Palade that each mitochondrion has (a) a limiting membrane; (b) a mitochondrial matrix that appears structureless at present levels of resolution; (c) a system of internal ridges (cristae mitochondriales) that protrude from the inside surface of the membrane toward the interior, some perpendicular to the long axis of the mitochondrion and occurring in series and parallel at more or less regular intervals. Dalton and Felix[13] also established the presence of a double limiting membrane and double internal membranes, continuous with, and apparently formed by an inward folding of the inner of the two limiting membranes.

Having numerous internal membranes increases the surface area at which mitochondrial enzymes and substrate reaction may take place. They may also serve to maintain the spatial organization of the mitochondrial enzyme systems.

3. NUMBER

A method for counting mitochondria in tissue sections is described in detail by Thurlow,[54] who demonstrated a constant number of mitochondria per unit volume of cytoplasm in normal nerve cells. This constant differed for nerve cells of different types, and was thought to be significant indication of functional differentiation of the nerve cell.

The method for counting isolated mitochondria is described by Allard, Mathieu, de Lamirande, and Cantero.[2]

Lindberg and Ernster[36] in a brief review of some important quantitative studies on mitochondria state that:

> The number of mitochondria varies from tissue to tissue, and is relatively high in highly active cells. The number of mitochondria may also vary in the same tissue under different physiological conditions....An increase in mitochondria has also been observed in vigorously secreting tissues such as secreting tumours. Junqueira[25] (1951) has found a direct correlation between the mitochondrial count and the secretory activity in the salivary glands of the mouse.

Further research may uphold this observation with respect to the adrenal cortex as well. One such study is discussed on page 25 of this work.

4. INTRACELLULAR DISTRIBUTION

Mitochondria are known to swell during the accumulation of heparin in mast cells and zymogen in excretory cells of the pancreas, and become droplets of secretion. The mitochondria are originally oriented basally in the secretory cells, and migrate to the apex of the cell during the accumulation process where they are finally excreted by an unknown mechanism.[36]

As revealed with cytological techniques, the mitochondria are small, granular, and evenly distributed throughout the cytoplasm of the adrenal cortex.*

C. PHYSIOLOGICAL AND BIOCHEMICAL

1. CHEMICAL COMPOSITION

Lindberg and Ernster[36] have summarized the main points concerning the chemical composition of mitochondria;

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* See Part II ff.

Chemically, mitochondria consist mainly of lipoprotein, in addition they contain small amounts of RNA and of low-molecular components. Proteins, lipids, and nucleic acid together account for 92% of the dry weight of the mitochondria.

Protein	65-70% dry wt.
Lipid (mainly Phosphatides)	25-30% dry wt.
Nucleic Acid	
RNA concentrated in core	
DNA some in membrane.	

The chemical distinction between mitochondria and microsomes in animal tissues has been based primarily on the higher ribonucleic acid (RNA) content of the latter, the Palade granules consisting of approximately equal parts of protein and ribonucleic acid (RNA). In plant material, however, only a slight difference in nucleic acid content between microsomes and mitochondria has been found.

2. GENERALIZED FUNCTIONS

A brief summary of the generalized functions of mitochondria is here given, prior to consideration of the evidence linking mitochondria to secretory processes in cells. Mitochondria are of importance in cellular metabolism as they contain the enzymes for catalyzing the Krebs cycle, coupling oxidation and phosphorylation, and forming adenosine triphosphate. In their role as energy suppliers, they have been termed "the powerhouse of the cell."[50]

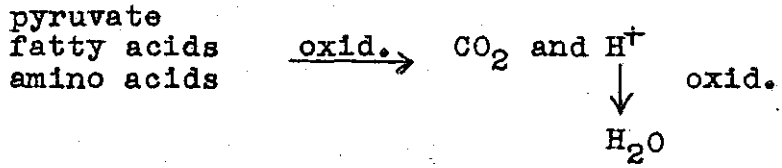
Lindberg and Ernster[36] have written the most comprehensive review of the chemistry and physiology of mitochondria. Junqueira and Hirsch,[25] in a study of secretion in

the pancreas and salivary glands, have summarized the relevant data which has been adapted and reproduced in Figs. 2, 3, and 4.

The general chemical reactions localized in the mitochondria may be classified under three major headings: (a) oxidations; (b) electron transport; (c) oxidative phosphorylation.

a) OXIDATIONS

The oxidations which take place in the mitochondria are exergonic reactions and provide the energy needed by the cell to carry on the life processes. Fatty acids, amino acids, and pyruvate are oxidized to carbon dioxide and water. The pyruvate substrate obtains from the extra-mitochondrial oxidation of carbohydrates, principally glucose.



The amino acids and fatty acids derive from the breakdown of proteins and fats. Siekevitz[30] states that:

> Inside the mitochondria these compounds undergo a complex series of reactions to form intermediate products, all of which are interrelated by a chain of enzymes called the citric acid cycle. The oxidations remove carbon atoms one at a time from the intermediate products of carbohydrate and protein breakdown, and two at a time from the products of the breakdown of fats. The carbon dioxide produced by the oxidations is ultimately exhaled. Many, but not all, of the enzymes which preside at these steps have been localized in the mitochondria.

b) ELECTRON TRANSPORT

Hydrogen ions are oxidized stepwise by a series of mitochondrial enzymes, known as the electron (or hydrogen) transport chain. When the electrons and hydrogen ions reach oxygen they combine with it to form water.

Some pathways have been identified in mitochondrial preparations, and in reconstructed soluble enzyme preparations, and are illustrated in Fig. 4. Other electron carriers are known, but their intramitochondrial paths have not been determined.

The three main factors in the known system are the pyridine nucleotide coenzymes, the flavoprotein reductases, and the cytochrome-cytochrome oxidase system.

c) OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation is the transformation of energy liberated by oxidation into the chemical energy of the ATP pyrophosphate bond. Conversion of ADP to ATP requires pyruvic acid, oxygen, and phosphoric acid - $CH_3CO\ COOH$ & 5 O & 15 ADP & 15 $H_3PO_4 \rightarrow 3CO_2$ & 17 H_2O & 15 ATP (Green[21]). ATP is thought to provide energy for different cell activities, such as the synthesis of proteins and other compounds, contraction, the production of light and electricity, osmotic work, and cell secretion.

At each stage of the electron (hydrogen) transport chain a definite amount of energy is liberated:

Three molecules of ADP are phosphorylated during the passage of a pair of electrons from DPNH to molecular oxygen, (P:O ratio equals three), corresponding to a thermodynamic efficiency of 60-70%.

See Fig. 5.

Guanosine diphosphate is the co-enzyme in the transformation of succinyl-co-enzyme A to succinic acid, responsible for the formation of ATP. The other three phosphorylating enzymes are unknown.

Attempts have been made to identify these enzymes. Claude[8] demonstrated that isolated mitochondria swell in hypotonic sucrose. Tapley[51,52,53] and others have shown that this swelling can be inhibited in intact mitochondria by the addition of certain "uncoupling agents," which uncouple oxidation and phosphorylation, such as 2, 4-dinitrophenol, dicumarol, and pentachlorophenol. Enzyme systems isolated from disrupted mitochondria have been tested with these substances.

Gamble and Lehninger[18] disrupted rat liver mitochondria by chemical and mechanical means, and analyzed some of the mitochondrial fragments with a light-scattering photometer. These fragments showed changes in shape and aggregation after the addition of compounds which cause swelling in intact mitochondria, and might represent the enzyme chains responsible for the swelling in intact mitochondria.

Aboad and Alexander[1] isolated a multienzyme complex from

rat brain mitochondria disrupted by treatment with a detergent, Triton. The enzyme units carried on oxidative phosphorylation with much the same P:O ratio, substrates, and co-factor requirements as intact brain mitochondria.

Plaut[46] isolated an enzyme from pig and rat liver and beef heart which catalyzed an exchange reaction between the terminal phosphate of adenosine triphosphate (ATP) and inorganic phosphate (P^{32}), and is specific for adenine nucleotides. The reaction requires Mn^{++} and is stimulated by adenosine diphosphate (ADP). As an exchange of P^{32} with the terminal P of adenosine triphosphate (ATP) can occur in intact mitochondria in the absence of substrate oxidation, the enzyme has properties like those of intact mitochondria.

Thyroxine and its analogs, however, do not uncouple oxidative phosphorylation in the isolated enzyme complexes, though they produce pronounced swelling of liver and kidney mitochondria, and slight swelling of diaphragm and heart. Tapley and Cooper[9,52,53] suggest therefore, that these substances may produce their effect in intact mitochondria by altering the structure in some way (involving the movement of water or intracellular electrolytes or the permeability of the membrane), rather than by direct interaction with the enzymes.

Isolated rat mitochondria from various organs were suspended in a sucrose solution. The substance to be tested was added, and the volume changes in the mitochondria were

observed with a spectrophotometer.

In general, these findings are tending to confirm the statement by Gamble and Lehninger[18] that:

> Mitochondria may contain relatively durable enzyme units, loosely coupled together, possibly in a uniformly distributed manner. There may be a significant difference in the organization of phosphorylating and non-phosphorylating enzyme groups in the mitochondria studied.

3. SPECIAL FUNCTIONS

In addition to their generalized functions as producers of energy in cell metabolism, mitochondria contain enzymes which aid in the formation of substances to be accumulated or secreted, including the adrenocortical steroids.

This is one of the "special functions" of mitochondria as stated by Lindberg and Ernster[36] in their review of the physiology of cytoplasmic particles.

> The reactions associated with the metabolism of fatty acids, the synthesis of nitrogenous end products and condensation for purposes of detoxification of aromatic substances with activated radicals, and the formation of substances to be accumulated or secreted are special functions based on the fundamental pattern.

In discussing the role of mitochondria in relation to cell secretion, Junqueira and Hirsch[25] distinguish three types of materials passing out of a cell:

> a) Secretions: Products of more or less complex complex molecules that are built up, accumulated in the cytoplasm, and later extruded to the outside of the cell. The best known examples are hormones, digestive enzymes, mucus, and poisons.
> b) Recrements: Inorganic substances such as water and ions flow into cytoplasm during ingestion and are eliminated as such (from "Rekrete" according to Frey-Wyssling 1945).
> c) Excretions: This third group is of waste material derived from the breakdown of cellular components.

Utilizing these distinctions they define secretion as

> ...a chain of processes in which the following three main steps may be observed: (1) Ingestion, comprising the penetration of raw material into the cell such as amino acids, sugars, fatty acids, water, ions, etc. (2) Synthesis, in which more or less complex molecules are built up, concentrated, and stored in granules, vacuoles, crystals, etc. (3) Extrusion, the expulsion of these secretions from the cell.

The mitochondrion as a whole may effect the ingestion of raw materials through "active transport" across the mitochondrial membrane. Rosenberg[49] defines active transport as "the transport from a lower to a higher chemical potential." More generally, he states that:

> Active transport is the movement of substance which is influenced by other forces in addition to chemical (or analagous) potential gradient of this substance.

This is sometimes referred to as the "secretory activity" of mitochondria. Lehninger[33] states that:

> ...the apparent ability of these bodies to effect accumulation or secretion of some substances, such as water and certain electrolytes, presumably against gradients of chemical potential. These energy-requiring secretory properties (italics mine) of mitochondria are apparently geared to the process of oxidative phosphorylation. Although other enzymes and enzyme systems have been found in mitochondria, electron transport with its coupled phosphorylations and the secretory activity (italics mine) appear to represent the most fundamental functions of mitochondria.

According to Davies,[4]

> It is now known that the cell is not the simplest unit which is able to maintain active transport. Mitochondria are able to both secrete and accumulate a variety of inorganic and organic cations and anions.

Bartley and Davies[4] showed that well-oxygenated actively

metabolizing kidney cortex can actively transport H^+, Na^+, K^+, orthophosphate, adenosine polyphosphates, pyruvate, fumarate, oxaloacetate, α- keto-glutarate, and citrate.

Sheep kidney cortex was homogenized and a mitochondrial precipitate obtained by centrifugation. The particles were then incubated aerobically at room temperature with adenosine triphosphate, magnesium phosphate and substrates. After incubation the substances were generally found and were concentrated in the mitochondria.

The "secretory activity" referred to involving recrements and substances to be oxidized, is better described as active transport, however, reserving the term "secretion" for the entire process of ingestion, synthesis of complex compounds and extrusion as defined above.

The processes of active transport and secretion require energy as compared with the process of metabolism which supplies energy.

The mitochondria, as the main energy producer of the cell appear to supply the energy for the processes of cell secretion.

In a comparative study of the cytological and biochemical phenomena related to cell secretion in normally functioning and non-secreting salivary gland, Junqueira and Hirsch[25] concluded that "the role of mitochondria in the processes of cell secretion appears to be mainly that of energy suppliers."

Materials and Method: Normal or excretory duct ligated submaxillary glands of mice and rats. Results (in ligated glands): (1) disappearance of secretory granules; (2) decrease in cell and gland size;

(3) decreased activity of digestive enzymes; (4) no secretion; (5) no degenerative changes for one year; (6) some glycolysis (in normal and ligated glands); (7) decreased QO_2 consumption and succinic dehydrogenase activity; (8) decrease in amount of ATP, ADP, and PC.

The decreased oxygen consumption and succinic dehydrogenase activity in the ligated glands suggest that the mitochondria are the main source of energy in cell secretion. The decrease in the energy rich phosphorus compounds of the cell suggest that they are the immediate sources of energy for cell secretion.

Price, Fonnesu, and Davies[46] have presented evidence that "high-energy" phosphate esters may also supply the energy for the movement of water and ions in respiring rat liver mitochondria. Tapley[51], however, feels that there is as yet no convincing evidence that water per se is actively transported by mitochondria. Further discussion of this question is another problem, however, and will not be discussed here.

II. MITOCHONDRIAL CHANGES

A. A. REACTIONS TO INJURY

Changes in mitochondria in various glands as reactions to cellular injury have been extensively studied, and form a basis of comparison for studies correlating mitochondrial changes with secretory cycles. The mitochondria are sensitive to internal and external stimuli. In a hypertonic medium they shrink, the rod-shaped forms becoming angular while in a

hypotonic medium, or when the cell is absorbing or secreting they swell to many times their normal size.

Cowdry[12] summed up the chief types of reactions as follows: (a) granulative degeneration of filamentous mitochondria; (b) "chondriolysis," the passing into solution and disappearance of granular mitochondria; (c) the enlargement of granular mitochondria to form droplets which disappear or change to fatty elements (fatty degeneration); (d) agglutination of granules to form clumps, then large droplets, which change to lipoids.

Ciacco[24] assorted grades of changes of mitochondria due to poisoning and infectious diseases:

(1) Preplastorrhexis - slight change of form;
(2) Plastorrhexis - change like the breaking up of the cell nucleus (fragmentation);
(3) Plastopycnosis - change like pycnosis of the nucleus (shrinkage);
(4) Plastovacuolisation - vacuole formation;
(5) Plastolysis - declination of staining and disappearance of mitochondria like in chromatolysis and karyolysis.

Emmel[17] studied mitochondrial changes and pH changes in the rat's kidney following interruption and restoration of the renal circulation. Ligation of the renal artery was followed by an immediate rise in tissue acidity and mitochondria were observed to fragment. Reorganization of the mitochondria in the normal elongated forms found in the renal cortex followed restoration of the circulation and the return of the pH to normal values.

Kyu, Matsuoka, and Ishiwatari[27] have noted the special

sensitivity of mitochondria in the zona fasciculata of the adrenal to experimentally induced shock.

> Materials and Method: Shock induced in rabbits and guinea pigs by one or several doses of antigen (horse serum). Shock induced after one injection causes mitochondria to diminish in size, become irregular in shape and fragment. The changes become more marked after each sensitizing injection. After five such injections the shock dose was administered three weeks after sensitization was complete. Results: Plastorrhexis and plastopycnosis develops increasingly with each injection before shock is induced. There is decreased stainability and irregularity of distribution of the mitochondria. In the zona fasciculata repeated sensitization produces extensive plastopycnosis, disappearance of mitochondrial staining and the nuclei become fuchsinophile.

B. RELATION TO SECRETORY CYCLES

1. In Various Glands

The exact function of mitochondria in relation to secretory processes has not been firmly established.

Zollinger[36] demonstrated that kidney mitochondria have the capacity to accumulate different substances and are transformed into droplets of secretion:

> If ovalbumin, haemoglobin, gelatin, or sucrose is injected intra-venously or intra-peritoneally into an animal, the test substance is taken up by mitochondria. This process is readily followed under phase contrast optics . . . Ovalbumin, haemoglobin, and vital stains accompanied by plasma proteins first appear as small scattered droplets in the mitochondrial core. They then coalesce and undergo a chemical change, as, for example, accumulated ovalbumin could be stained between 24 and 40 hours after injection, but not earlier or later, with special fibrin stains. The accumulated material finally occupies the entire mitochondria body, whereupon the mitochondrial membrane degenerates and the mitochondrion becomes a drop of secretion.

Investigations on other glands, however, have not always demonstrated the transformation of mitochondria into secretion granules, though an inverse relation between the number of granules and the number of mitochondria has been shown.

Hirschowitz[23] demonstrated this relation between the number of mitochondria and the number of secretory granules, believed to contain pepsinogen, the precursor of the enzyme pepsin, in the gastric chief cells of the rabbit.

> Materials and Method: A single dose of pilocarpine injected into a fasting rabbit initiated a complete secretory cycle in gastric chief cells. Results: After one hour the granules, abundant in the resting phase started to disappear except at the base of the cell, which becomes smaller. The mitochondria are scattered throughout the cell. After three hours the number of granules decreases, the number of mitochondria increases, and they become more slender and wavy. The pepsin content of the gastric juice now increases at the expense of the mucosal pepsinogen content. After six hours replacement signs appear. The cells enlarge, the mitochondria enlarge and organize at the base of the cell, and the granules begin to appear at the free border of the cell. After sixteen hours the cycle is complete. The pepsinogen level increases to the fasting level and restocking of cells with granules occurs.

A light and electron microscope study of the albumin-secreting cells of the hen oviduct by Hendler, Dalton, and Glenner[22] did not show either the mitochondria or the Golgi apparatus to be directly involved in the synthesis of an amorphous precipitate believed to be newly synthesized egg-white proteins.

Cunningham, et al, and Potter and Ward,[36] however, observed an increase in the number of mitochondria in immature blood cells in the phase of active protein synthesis.

Mitochondria may supply the energy for the "activation" of amino acids as the first step in protein synthesis, by the combination of the amino acids with high-energy phosphate groups. The linkage of the amino acids and the establishment of the complex structure of the protein molecule seem to be functions of the microsome or endoplasmic reticulum, however. Zamecnik et al.[55] summarizes the evidence for this theory of protein synthesis.

2. In The Adrenal Cortex

Mitochondria vary in form in the adrenal cortex of different animal species. Hoerr[24] found rods and elongated mitochondria in the cells of the zona glomerulosa of the guinea pig. The mitochondria of the zona fasciculata were larger and almost wholly granular. Occasionally a single cell of the fasciculata was found to contain all rod-like forms. Bennett[5] observed elongated mitochondria throughout the cortex of the cat. Payne[44] has noted that in fowl only spherical mitochondria are normally present. Miller[37] observed that while in intact untreated rats spherical mitochondria predominate, the zona glomerulosa and zona fasciculata also showed rod and filamentous forms with Baker's acid hematin method.

In an investigation of the relation of mitochondria to

secretory activity in the cells of the adrenal cortex of the cat, Bennett[5] found that "mitochondria and Golgi apparatus are present in the cell early in its life cycle and persist till the death of the cell, apparently long after secretion is completed." He found "no cytological evidence that these structures participate directly in the formation of the secretion of the gland."

Deane and Greep,[14] however, correlated changes in mitochondria with steroid secretion in the adrenal cortex of the rat after hypophysectomy. They found that mitochondria remain crowded granules of an unchanged small size in the zona glomerulosa. In the zona fasciculata they become smaller and lose their characteristic irregularity. In degenerating cells they may be fused and not discrete bodies. The ketosteroids gradually disappear from the zona fasciculata after hypophysectomy, but persist in the zona glomerulosa. The secretory activity of the zona fasciculata diminishes to almost zero.

Miller,[37] following this work, applied quantitative techniques in the fascicular zone of the rat's adrenal. He found an increase in the total number of mitochondria after a single injection of ACTH, thought to coincide with the synthesis or restoration of cortical hormone or a precursor of the hormone.

> Materials and Method: Normal and hypophysectomized rats of the Vanderbilt strain between 35 and 80 days old used. Standard hypophysectomy. The number of eosiniphils estimated in a Fuchs-Rosenthal counting chamber. Adrenals fixed in 10% formol-calcium after decapitation or anaesthesia with sodium amytal or after perfusion with formol-Calcium through the aorta.

Tissues mordanted according to Baker's method in acid-hematin test for phospholipid. Embedded in gelatin and sectioned at 2u and stained with Haidenhain's iron hematoxylin. Control and experimental animals anaesthetized with nembutal and blood samples drawn. Experimental animals then injected with ACTH or epinephrine and killed at intervals after the injection. Total number of mitochondria and the number of elongated mitochondria were counted in sections of cells in the outer third of the zona fasciculata. Cells which had a nucleus were chosen to keep the volume of cytoplasm constant except where experimental conditions altered the size of the cell. Results: In all tests the increase in the total number of mitochondria was preceded by a transient increase in the number of elongated mitochondria. This phenomenon occurred only in the zona fasciculata.

A decrease in the number of eosinophils was the criterion for the release of cortical hormone into the peripheral blood stream. There was no temporal relation between the proliferation of mitochondria and the discharge of cortical hormone.*

The proliferation of mitochondria may be brought about by the division of elongated mitochondria to form spherical mitochondria.

Payne[44] found elongated mitochondria in the adrenal cortical cells of fowl and believes that they may represent the origination of new mitochondria by division.

Materials and Method: Adrenals fixed in a 1:2:1 mixture of 2% osmic acid, 3% potassium dischromate, and 1% chromic acid. Then stained with acid-fuchsin

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* Hartroft and Eisenstein[16,20] have furthered earlier findings which indicate that the zona fasciculata produces glucocorticoids, particularly corticosterone, while the zona glomerulosa is functionally specific for aldosterone.

> and methyl green. Observations: The cortical cells of fowl lie in separate groups or masses irregularly scattered in the gland. In general the peripheral cells are smaller and less active. In young fowl there are fewer secretory droplets. In the interior of the gland the cortical cells are in varying stages of secretory activity: (1) filled with droplets; (2) having discharged secretions; (3) recuperative state; (4) degenerative state. They may return to the functional condition from the degenerative state. The mitochondria are all normally spherical. Central cell mitochondria are larger, but their size may vary in the same cell. Destaining the mitochondria reveals 1 to 10 dark red granules standing out in contrast to the non-granular mitochondrial matrix.

The presence of elongated mitochondria with a slight separation of two granules indicates division rather than fusion activity. There is no evidence of division or fusion in mitochondria with more than two granules.

There is some evidence to indicate that cortical hormone or a precursor of the hormone is being synthesized during periods of mitochondrial proliferation. Miller[38] suggests that mitochondrial proliferation may begin as soon as 1/2 hour after injection with ACTH even though the increase in elongated mitochondria is not statistically significant until 4 hours after injection. The total number of mitochondria shows a significant increase from 7 to 10 hours after injection with ACTH in intact animals. Rats which had been hypophysectomized for five days, when injected with ACTH, showed the same sequence of events, but they were delayed by three hours.

III. MITOCHONDRIA AND ADRENOCORTICAL STEROID SECRETION

Hayano, Saba, Dorfman, and Hechter[21] have reviewed the hydroxylation of steroids, with special reference to the adrenals.

Enzymes which play an essential role in the biogenesis of adrenal steroid hormones have been isolated in the mitochondria of the adrenal cortex. These enzymes catalyze hydroxylations (the introduction of hydroxyl groups into the steroid nucleus) and are known as hydroxylases. The 11-B hydroxylating system has been localized primarily in the mitochondrial fraction. The system involved in the primary degradation of the cholesterol side chain has also been localized in the mitochondria. The 17-and 21-hydroxylase systems were localized in the "soluble fraction," while the 3-B hydrogenase which converts pregnenolone to progesterone was localized in the "microsomal fraction."

Fig. 5 is an adaptation of a diagrammatic summary of the reactions in the sequence of corticosteroid biogenesis from cholesterol (and acetate), indicating enzyme localization in the adrenal cortex based on the method of tissue homogenation, and isolation and identification of components.

Intact mitochondria were found to hydroxylate progesterone at C 11-B more than disrupted mitochondria. Oxidative phosphorylation is no longer considered a direct factor in the reaction, but only stimulatory.

Fig. 6 is a schematic representation of the localization

of enzymes on a mitochondrial surface. The possible spatial relations have been suggested to correlate *in vivo* and *in vitro* data on the biosynthetic sequence.

How the transportation of steroids within the cell is effected, considering their relative insolubility, is problematical . The existence of transporting compounds has been ppstulated, but without any definitive evidence that they exist. It would seem just as reasonable to relegate this function to the mitochondria and portions of the endoplasmic reticulum, inasmuch as both are in a constant state of motility within the actively metabolizing cellnand both represent sites of enzyme localization.

Data to test the alternative hypothesis have been collected by Hechter and his associates from mitochondria which were isolated from the adrenal cortex by centrifugation in sucrose at 8,000 G for 20 minutes and incubated 2 hours in cofactor-phosphate medium. The major product, which was identified by infrared analysis proved to be progesterone. No pregnenolone or 17-, 21-, or 11-hydroxylated compounds (derivatives of progesterone) were found.

It was concluded that 17- and 21-hydroxylases were not associated with the mitochondria, but rather that, in theses experiments at least, some part of 3B-ase

activity was firmly associated with the mitochondria. This can be accounted for by postulating the adsorption of microsomes on the surface of the mitochondria to form a "mitochondrial complex" at specific sites.

Further studies led to the conclusion that 17α- and 21-hydroxylase were also adsorbed on the mitochondrial surface.

> Materials and Methods: Mitochondria from 100 g of adrenal cortex was suspended in 400 ml. cofactor buffer medium containing 0.025M sucrose, incubated with 50 ug of cholesterol -4-C^{14} for 2 hours at 38°C, (O_2). Results: Progesterone, 11B-hydroxyprogesterone, corticosterone, cortisol obtained. Contamination was avoided by spinning down the mitochondria in batches and by washing.

This evidence supports the theory that the extra-mitochondrial enzymes are mobile and become adsorbed on specific mitochondrial sites in orderly fashion, so that an integrated pattern is developed in which steroids are transferred successively from one enzyme to another, and the entire sequence of cholesterol to corticoids occurs on a single surface. The various end products leave the surface of the mitochondrial complex and diffuse out into the cytoplasmic medium and then out of the cell.

PART TWO

THE STAINING OF MITOCHONDRIA IN TISSUE SECTIONS IN THE ADRENAL CORTEX OF THE RAT

1. Statement of Aims

A method for staining mitochondria was sought which would permit quantitative studies to determine their number per unit volume of cytoplasm in adrenocortical tissue in the rat. The criteria for selecting the method were: (a) differentiation sufficient to allow discrimination and counting of individual mitochondria (under high power or after being photographed and enlarged); (b) suitability for mass processing of tissue sections; (c) reliability of results with standardization of method; (d) ease in correlating results with cytological studies of lipid changes.[7]

The importance of studying mitochondrial changes in the adrenal gland is indicated by Part One of this thesis. If mitochondria are important in the secretory activity of adrenal cells, application of quantitative techniques to adrenal tissue might be most illuminating. An inverse relation between the number of secretory granules and the number of mitochondria may indicate the transformation of mitochondria into secretory granules. This could then be followed in detail with phase contrast optics, as demonstrated by Zollinger in the kidney.* An increase in the number of mitochondria prior to an increase in the amount of secreted substance may

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* See page 22

indicate that mitochondria play an active role in the synthesis of that substance.

2. Fixation

All tissues were fixed in Regaud's (80 cc. 3% potassium dichromate and 20 cc. 40% formaldehyde solution added at time of using) after initial attempts with Helly's (100 cc. distilled water, 5 gms. mercuric chloride, 2.5 gms. potassium dichromate, - to 20 cc. add 1 cc. neutral formalin before use) and Bensley's formol-bichromate-sublimate (neutral formalin 10 cc., water 90 cc., potassium dichromate 2.5 gms., corrosive sublimate 5 gms.) showed mitochondria less well, with running together of fat granules, and precipitate in tissue.[35]

3. Staining Techniques

A. Bensley's acid fuchsin-methyl green[35] - Fix in Regaud's, and refrigerate for two days, changing fluid daily. Mordant in 3% potassium dichromate four days, changing after two days. Wash overnight in running water. Dehydrate in graded alcohols, clear, and infiltrate in autechnicon. Imbed in paraffin, melting point 57°C. Sectioned 5u. Deparaffinize in xylene 10 minutes. Hydrate in absolute alcohol 2 minutes, 95% alcohol 1.5 minutes, 70% alcohol 1.5 minutes, 50% alcohol 1.5 minutes, distilled water 1.5 minutes. Pretreat tissue for staining: 1% aqueous potassium permanganate 1 minute. Rinse in distilled water, then 5% oxalic acid 1 minute. Wash in distilled water 1 minute, 2.5% aqueous potassium dichromate 1/2 minute. Rinse in distilled water. Stain in Altmann's acid fuchsin (vigorously shake 1 cc. aniline with

20 cc. distilled water a few minutes; shake again from time to time in 24-hour period; filter; add 4 gms. acid fuchsin; shake from time to time in 24-hour period before use; prepare 20 cc. only as it will deteriorate) that has been heated to 60°C. Place a few drops on a slide and allow to cool 5 minutes. Wash in distilled water 1 minute. Drop 1% methyl green in aqueous solution 5 seconds. Dehydrate in absolute alcohol 2 minutes. Clear in 50/50 absolute alcohol and xylene 2 minutes, pure xylene 5 minutes. Mount in permount.

Potassium permanganate extracts the mordantizing elements of the fixative; oxalic acid extracts potassium permanganate. Treatment with 2.5% potassium dichromate improves staining with acid fuchsin, and helps prevent too much counterstaining.

Results - Mitochondria are crimson, nuclei are green. Differentiation is not sufficiently good to observe individual mitochondria in most cells. Methyl green is both the differentiator and counterstain and time is most critical. Lipids are well preserved with mitochondria densely packed in cytoplasm around lipid droplets. Advantage is excellent preservation of lipids. Disadvantage is the critical nature of differentiation necessitating individual treatment of slides with only fair results.

B. Acid fuchsin - sodium carbonate - Following a suggestion by Baker,[6] 1951, I tried differentiating with 0.1% sodium carbonate. Follow A through staining with acid fuchsin. Differentiate under microscope. Wash in distilled

water. Dehydrate as in A.

Results - Mitochondria are crimson. Excellent differentiation was obtained in some cells in some sections but the technique could not be standardized. Advantages are that a colorless differentiator and use of a weak solution allows leisurely differentiation. Disadvantages are individual treatment of slides and lack of uniform results; but the technique is worth investigating further where mass processing is not required, as careful differentiation can produce good results.

C. Heidenhain's hematoxylin[35] - Follow A through treatment with oxalic acid. Mordant in 5% ferric ammonium sulfate 18 hours. Rinse in distilled water 1 minute. Stain in Heidenhain's hematoxylin 24 hours. (Hematoxylin 0.5 gms.; distilled water 100 cc.; Lithium carbonate-saturate 3 drops and allow to ripen). Differentiate under microscope in 2% ferric ammonium sulfate. Wash in distilled water 1 hour. Dehydrate in graded alcohols (50% - 1 minute, 70% - 1 minute, 95% - 1 minute, absolute alcohol - 2 minutes, 50/50 - 2 minutes, xylene - 5 minutes). Mount in permount.

Results - Mitochondria are black; cytoplasm is yellowish to brown. Differentiation is not very good. Lipids are not well preserved. This method is not suitable for mass processing of tissues. Differentiation can quite easily pass too far. Results were not uniform.

D. Harman's Fast Green FCF[19] - Follow A through treatment with oxalic. Stain in fast green FCF (4% solution w/v in aniline water, 10% v/v) heated to 62° C. Put a few drops of stain on a slide and let cool for 6 minutes. Rinse rapidly in distilled water. Immerse in saturated aqueous picric acid 10 minutes. Rinse in distilled water. Immerse in 1% phosphomolybdic acid 1 minute. Wash in distilled water 1 minute. Counterstain with 1% safranin in 50% ethanol, 1 second. Dehydrate and clear as in C.

Results - Mitochondria are green; cytoplasm is red. Lipids are well preserved. Differentiation is fair but not adequate for counts of individual mitochondria. Much difficulty has been encountered with the counterstain which tends to overstain. If diluted to 0.1%, ethanol bleaches during dehydration.

E. Lacy's Silver[28] - Follow A through treatment with oxalic. Place slide in 1.5% silver nitrate 15 minutes (not critical). Remove slide and wipe around section - leave a thin film of silver nitrate on section. Place slide section downwards over a solid watch-glass filled to the top with Aoyama reducer (Hydroquinone 1 g., 40% formaldehyde 15 cc., distilled water 85 cc., anhydrous sodium sulfite 0.15 g.) diluted with 5 parts water for 1 minute. Remove slide. Do not let reducer in watch-glass overflow. Wash in distilled water 15 minutes. Dehydrate and mount as in C. Carry out reduction away from strong light.

Results - Mitochondria are black on a colorless back-

ground. This method does not work with mercuric chloride in the fixative. If over-impregnated, sections can be gold-toned and differentiated. Most sections showed diffusion effects; three staining areas in the tissue were recognizable under both low and high power: (a) Central area - silver was deposited only in cells, no intracellular deposit was seen. Mitochondria were dark brown to black on a colorless background; evenly sized and distributed in the cells, clearly distinguishable individually, and could be counted. (b) Intermediate area - silver was deposited inter- and intracellularly. In the cells, granules are uneven in size and tend to outline the cell and lipoid inclusions, and may be artifacts. The overall appearance is fuzzy. (c) Peripheral area - yellow to colorless with large irregular sized clumps of black-purple silver deposit. Individual cells were not clearly distinguishable. Disadvantages are the need for individual processing, and diffusion effects. The silver nitrate is carried over in the section and provides most of the silver that is deposited. The mechanism of action of silver nitrate on mitochondria is suggested by Lacy. Both mitochondria and lecithin react to silver nitrate solution in the same way. The method probably depends on the phospholipid content of the mitochondria. Acid hematin tests show mitochondria contain a phospholipid, and potassium dichromate fixes phospholipid (which is also retained in gelatin-imbedded sections).

F. Pritchard's Silver[47] - Follow A through oxalic acid. Place slide, agitating gently, in silver bath 20 seconds (to 5 cc. of 10.2% silver nitrate add ammonium hydroxide drop by drop until the precipitate which forms is dissolved. Add 5 cc. of 3.1% NaOH and just dissolve the resulting precipitate with a few drops of ammonium hydroxide. Make the solution up to 50 cc. with distilled water. Dilute with an equal volume of distilled water with the addition of 2 drops of 8% ammonium hydroxide per 50 cc. of mixture.) Drain excess solution. Quickly plunge the slide with agitation into 0.1% commercial formalin in distilled water. Reduction takes place in 10-15 seconds.

Use fresh formalin for each section. Wash in distilled water and examine under the microscope. The mitochondria are specifically blackened and show distinctly. (If nuclei and other structures stain yellowish or brownish, remove with 2% aqueous potassium ferricyanide, differentiate under microscope - this procedure did not prove necessary, however).

Results - Mitochondria were black; cytoplasm colorless. Mitochondria were evenly sized and distributed through cell. No intercellular deposition of silver was seen except slightly around edges of section in some cases. Mitochondria can be counted in an individual cell by focusing up and down. With slight adjustments in the amount of formalin used, the method can probably be adapted to mass processing of sections. The results were very reliable and could be reproduced in section

after section. After a while it is possible to tell if reduction is complete by the gross appearance of the slide. The lipids do not show clearly as in the acid fuchsin and fast green FCF methods, but the superiority of the mitochondrial demonstration makes it most suitable for quantitative studies. A control should be run to verify whether or not the blackened, spherical granules are really mitochondria rather than artifacts.

Control - Frozen sections fixed and stored for one year in 10% neutral buffered formalin were rinsed in distilled water and stained as above and mounted in Kaiser's glycerin jelly. A second group were pretreated with 1 part glacial acetic acid and 3 parts absolute alcohol from 1 to 5 minutes to remove mitochondria and then stained as above. A third group were pretreated with 2.5% potassium dichromate and then stained and mounted as above. Results were ambiguous. Silver was deposited in irregular sized clumps on almost all slides. Nothing resembling mitochondria was seen. As the most important variable in silver staining is fixation and condition of the tissue, no conclusions can be drawn from this series.

4. The Theory of Silver Staining as Discussed by Zon[56] - When ammonium hydroxide is added to silver nitrate, first silver hydroxide is precipitated and then, as more base is added, the silver hydroxide redissolves to form a compound $Ag(NH_3)_2NO_3$. The state of equilibrium in a solution of silver

diammino-nitrate is as follows: The salt ionizes completely into a complex cation $Ag(NH_3)_2$ and the nitrate anion. The complex cation dissociates slightly giving silver ions and by hydrolysis ammonium and hydroxyl ions. The excess of ammonium hydroxide above two moles for each one of silver is necessary to depress the number of silver ions so that silver hydroxide will not be precipitated.

Silver deposition upon the stained elements plays a part in silver staining processes. The rate of reduction of silver within and on histological structures is an important factor. Some factors controlling the rate of reduction are 1) the absorption of silver hydroxide and ammonia, 2) the affinity of silver for proteins, and 3) the protective power of the gel structures. In absorption the anion is not taken up. Ammonium hydroxide and silver hydroxide are absorbed. The ratio of base, amine and silver plays an important role in controlling the rate of reduction. The conditions are so complex, however, that optimum ratios for deposition must be determined by experiment. The amount of silver absorbed depends upon the concentration in the surrounding medium. The union follows the Freundlich absorption isotherm. Absorption of silver occurs only because a decrease in free energy takes place. As the concentration increases, the absorbent becomes saturated and successive increments of absorbate cause less free energy change and are held less avidly. The rate of reduction, therefore, of absorbed silver compounds increases rapidly as the concentration of

the silver solution increases.

The third factor upon which difference in staining properties of various structures depends is that they have gel structures which allow precipitation of metallic silver to occur readily. In silver stained sections the colors assumed by tissue elements are exactly those which may be seen in silver solutions of different particle size. Silver stained structures contain colloidal particles of silver. Since the gel structure can affect the rate of crystal growth, and the degree of dispersion of a solution is dependent upon the ratio of the rate of nucleus formation to the rate of crystal growth, the results indicate that each structure has a different protective power.

Bielchowsky-type stain - The tissues take up ammonium hydroxide and silver hydroxide in various ratios. Each histologic structure has a different affinity for silver and different protective power. In the reducing solution absorption of formaldehyde takes place and reduction of silver hydroxide occurs rapidly or slowly depending on the above factors. Those structures in which reduction of silver hydroxide proceeds fastest will acquire a deposit of silver from the solution carried over mechanically from the silver-bath. This mechanically carried silver will tend to diffuse out, and if reduction is too slow will be unavailable for deposition. Fixatives change the protective power of the

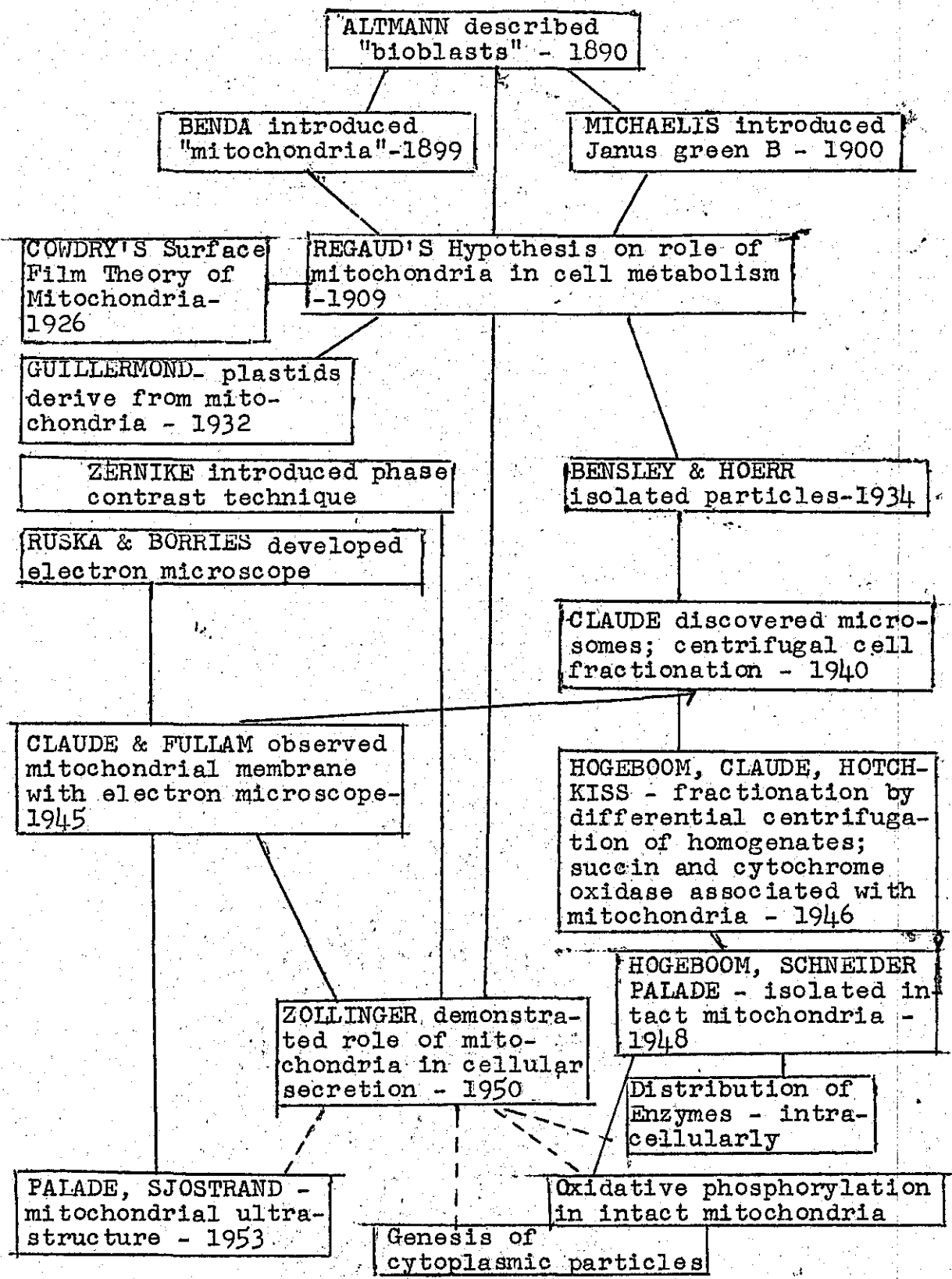
gels and their ability to absorb silver and are therefore the most important variable in silver staining.

Ladman and Mitchell[29], using the Bodian Protargol method and the electron microscope showed particles of silver, 0.4u in one dimension within the mitochondria of rat retina. They also found that in paraffin sections silver stain has greater sharpness and clarity than the anilin-acid fuchsin-methyl green method. It would be interesting to apply their methods to the staining of adrenal tissues.

5. Summary

Several techniques for the selective processing and staining of mitochondria were studied for the purpose of ascertaining one best suited for use in further investigations of changes in mitochondria during adrenocortical regeneration in the rat. The methods of Pritchard, 1952, and Lacy, 1954, were found most satisfactory.

Fig. 1 Historical Survey of Research Trends
(Adapted from Lindberg & Ernster, Chemistry & Physiology of Mitochondria & Microsomes)



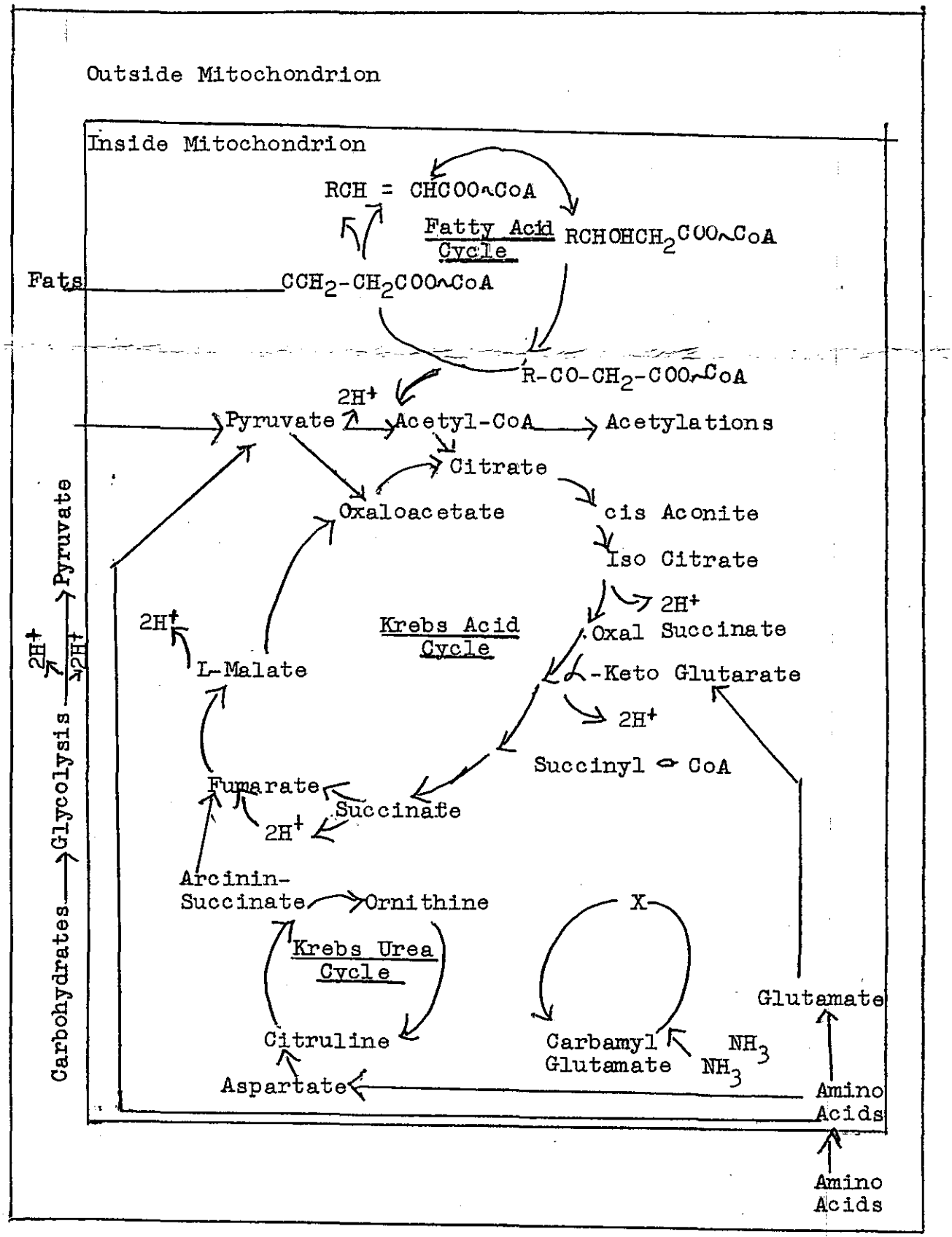


Fig. 2 Some oxidative pathways for the oxidation of lipids, amino acids, and carbohydrates in mitochondria. (Redrawn from Junqueira & Hirsch, Cell Secretion: A Study of Pancreas & Salivary Glands)

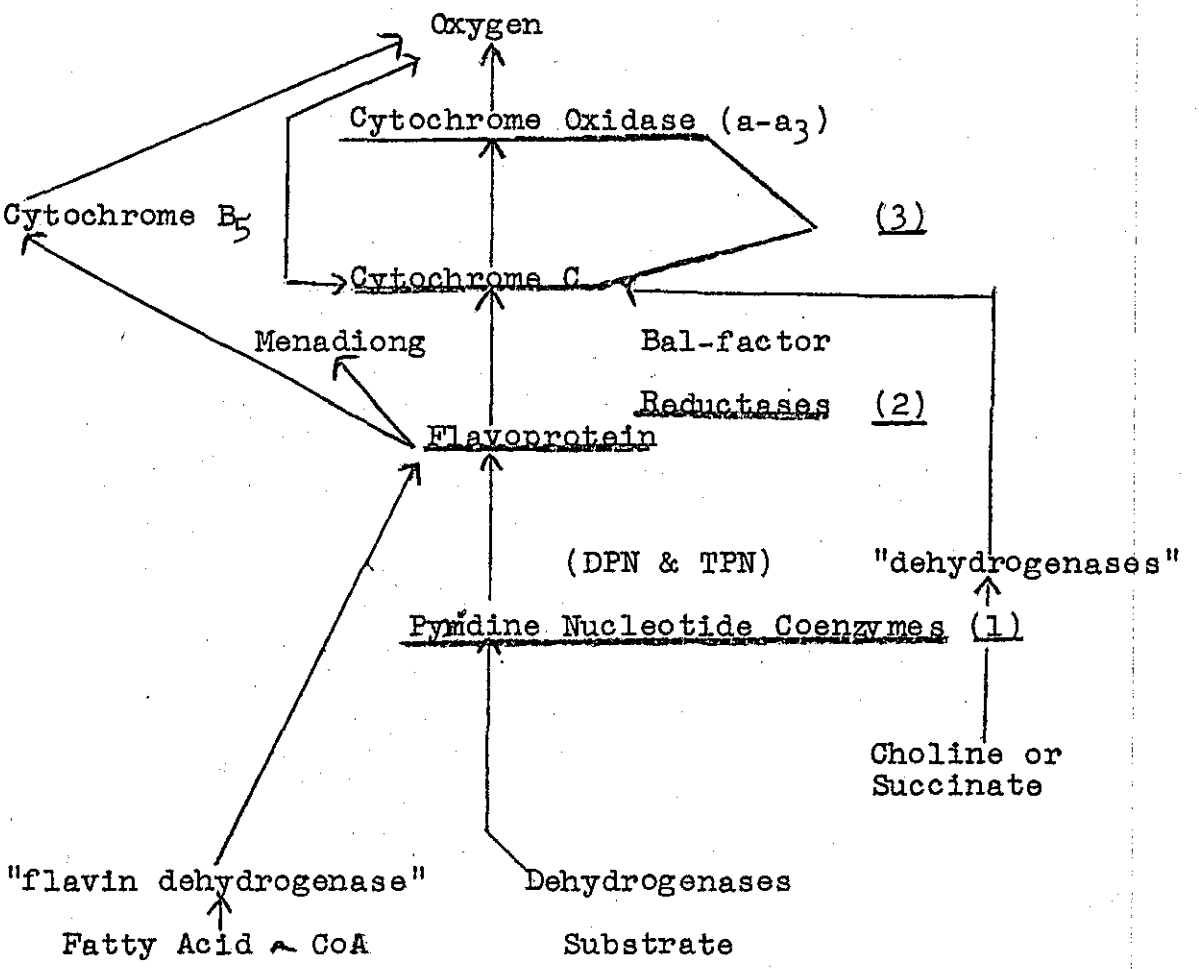


Fig. 3 Some pathways of electron transport in mitochondria. (Redrawn from Junqueira & Hirsch, Cell Secretion: A Study of Pancreas & Salivary Glands)

DPN - dipyridine nucleotides
TPN - tripyridine nucleotides

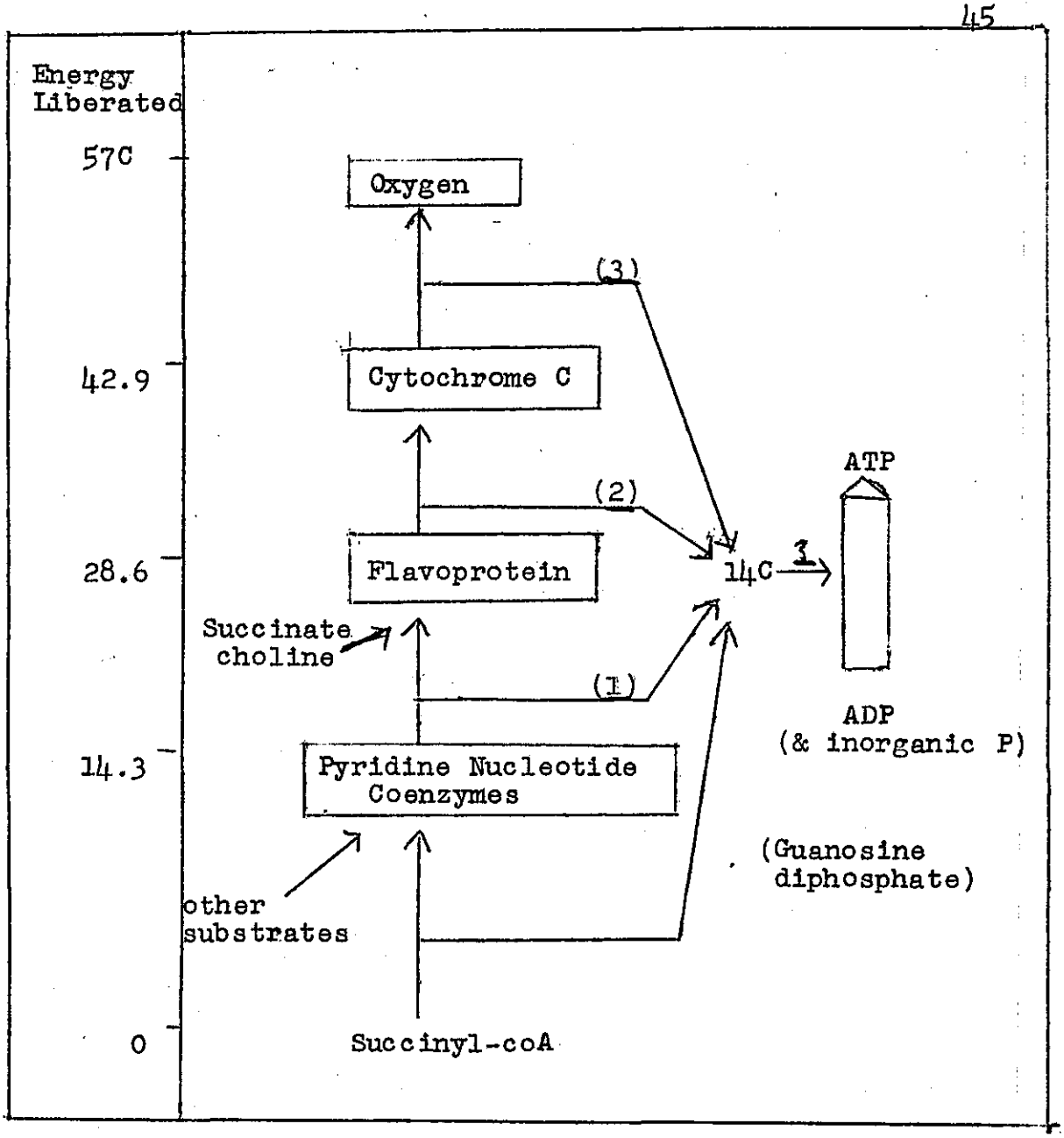


Fig. 4 Energy release and oxidative phosphorylation. (Redrawn from Junqueira & Hirsh, Cell Secretion: A Study of Pancreas & Salivary Glands.)

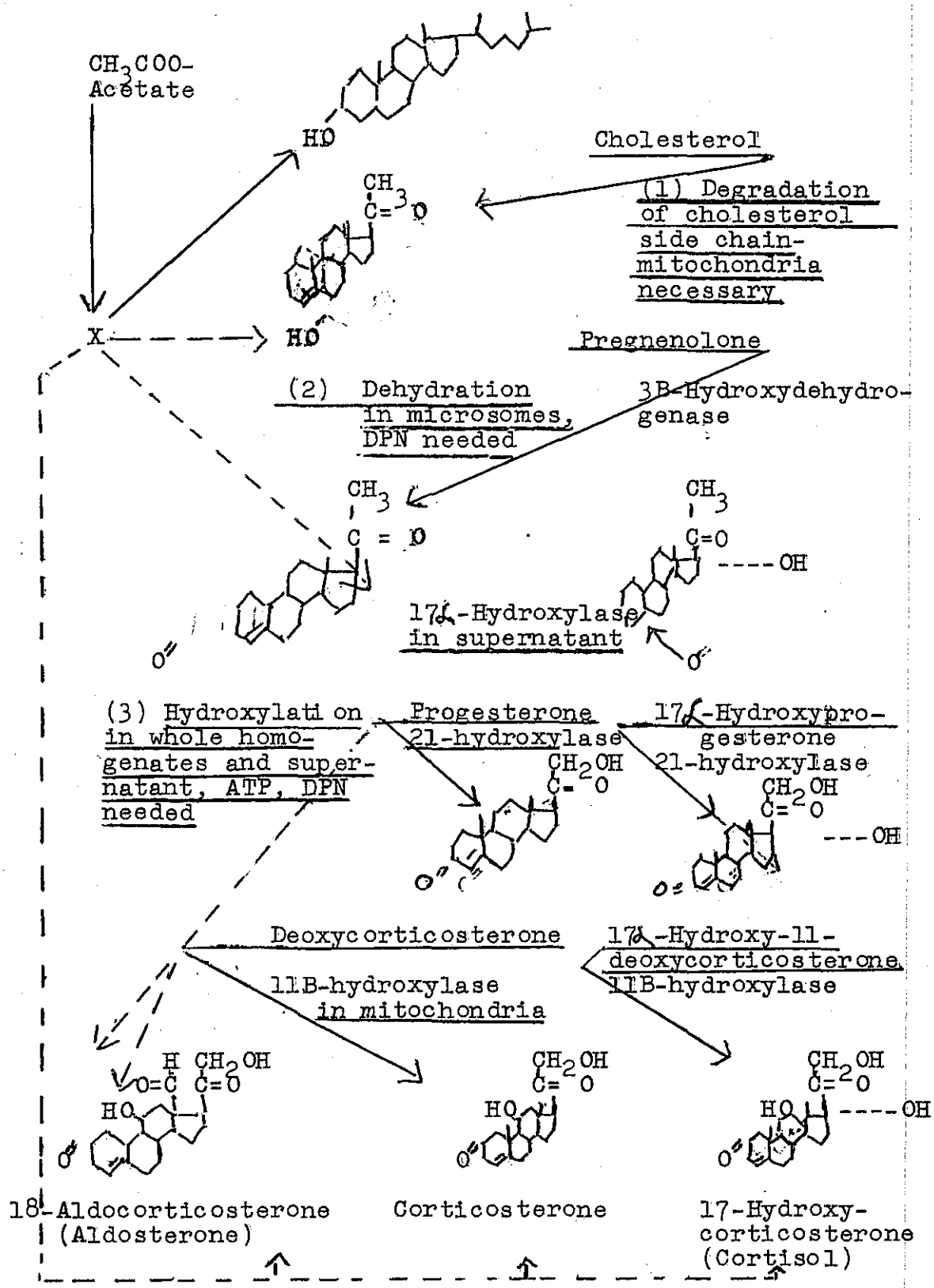
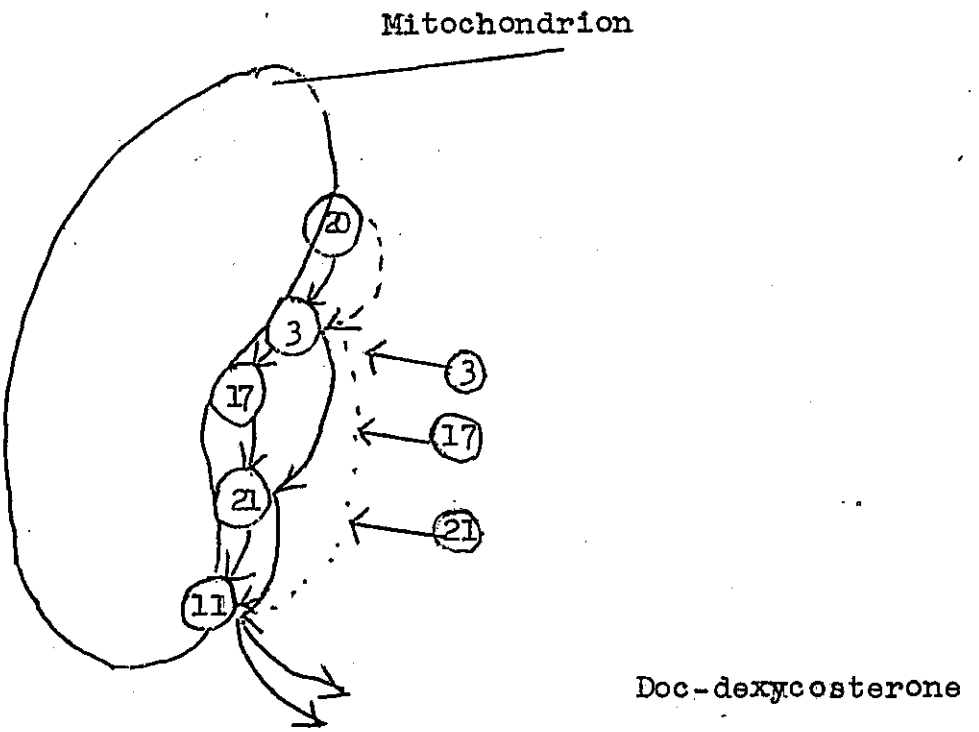


Fig. 5 Pathway of Corticosteroid Biogenesis
(Redrawn from Hayano et al, "Some Aspectsof the Biogenesis of Adrenal Steroid Hormones")



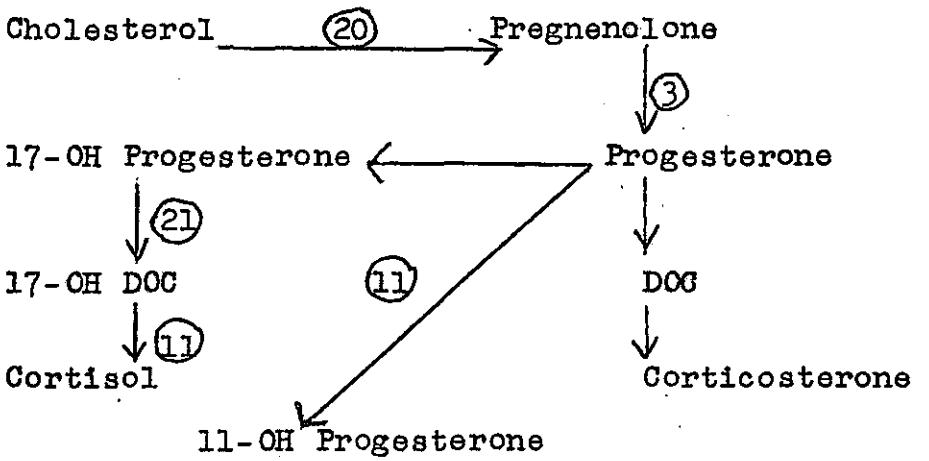


Fig. 6 A schematic representation of localization of enzymes on a mitochondrial surface. The enzyme system for the conversion of cholesterol to pregnenolone as well as 11-hydroxylase may be intramitochondrial and not on the surface. (Redrawn from Hayans et al. "Some Aspects of the Biogenesis of Adrenal Steroid Hormones").

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