FINE STRUCTURE OF CELLS

UNION INTERNATIONALE DES SCIENCES BIOLOGIQUES
INTERNATIONAL UNION OF BIOLOGICAL SCIENCES
SERIES B - NR 21

FINE STRUCTURE OF CELLS

SYMPOSIUM HELD
AT THE VIIITH CONGRESS OF CELL BIOLOGY
LEIDEN 1954
WITH FINANCIAL SUPPORT OF U.N.E.S.C.O.

P. NOORDHOFF LTD - PUBLISHERS - GRONINGEN - THE NETHERLANDS

I.U.B.S. PUBLICATIONS - 1 RUE VICTOR COUSIN - PARIS (5e) - 1955

CONTENTS

SECTION I. MITOCHONDRIA

SECTION II. NUCLEAR AND CHROMOSOME STRUCTURE

SECTION III. SUBMICROSCOPIC ORGANIZATION OF CYTOPLASM

SECTION I

Mitochondria

GEORGE H. HOGEBOOM

The isolation and biochemical properties of liver mitochondria

One of the most important aspects of the somewhat controversial field of cytochemistry is the role played by the mitochondrion in the metabolism of the cell. Beginning with Claude's pioneering search for systematic procedures for the isolation of cellular structures by means of differential centrifugation (1,2), mitochondria have received a great deal of attention from biochemists. The result has been the accumulation of extensive data bearing on the biochemical properties of this cell component. Since by far the greatest proportion of the information now available has come from studies of mitochondria isolated from mammalian liver, I shall limit my remarks to this phase of the research. Rather than attempt to cover all of the findings, which have been reviewed on numerous occasions (cf. (3—6)), I shall attempt to emphasize a number of unresolved problems that demand further exploration. In addition, I should like to describe a new technique of centrifugation that may be of help in solving some of these problems.

The need for a critical reappraisal of cytochemical methods and data has arisen from important recent advances in cytological techniques. Thus in the past, mitochondria have been identified as pleomorphic cytoplasmic structures, displaying certain distinctive staining properties but much too small to be otherwise characterized cytologically. They have often been mistaken for other cellular structures and to some biochemists, in fact, apparently have little, if any cytological significance. Their pleomorphism, together with difficulties in examining them in detail, have occasionally led to some doubts as to their status as specific cellular organelles. As other investigators attending the Congress will demonstrate, however, these uncertainties have now been eliminated by electron microscopy (7—9), to such an extent that the mitochondrion would appear to be as characteristic a cellular entity as is the cell nucleus.

The isolation of mitochondria is accomplished by the centrifugal

fractionation of broken cell suspensions (cf. (10, 11)). In this fractionation technique, the liver cells are first mechanically disrupted by means of a „homogenizer", consisting of a rotating cylindrical pestle that fits a glass test tube (12), and the contents of the cell are dispersed into a suitable medium, usually isotonic or hypertonic sucrose solution. The cell nuclei, together with residual intact liver cells, connective tissue, and about 10 to 15 per cent of the free mitochondria, are removed by low speed centrifugation. The mitochondria remaining in the supernatant are collected by centrifugation at higher speed in an angle rotor and are carried through two cycles of resuspension and resedimentation in order to provide as complete as possible a separation from the more slowly sedimenting microsomes. The mitochondrial fraction obtained by this procedure contains essentially no cell nuclei, approximately 80 per cent of the mitrochondria of the original homogenate, and a small, though appreciable proportion of the microsomes. Cytological studies have not as yet revealed the presence of other cytoplasmic elements in significant amounts.

There seems to be little question that the mitochondrion plays a prominent role in cell respiration. In addition to the terminal respiratory enzyme system, cytochrome oxidase (13, 14), most of the reactions within or related to the Krebs citric acid cycle are concentrated in the fraction. Some investigators have claimed, in fact, that the Krebs cycle reactions are *entirely* confined to mitochondria (cf. 15). As demonstrated in Fig. 1, however, the situation is not as clearly defined as these claims would indicate. Here an attempt has been made to summarize our present knowledge concerning the role of mitochondria in the Krebs cycle and related oxidative and phosphorylative reactions. Wherever the reaction is accompanied by a closed star, the data have been sufficiently clear-cut to indicate that the enzyme is localized exclusively in mitochondria. This situation holds only for cytochrome oxidase; adenylate kinase (16), which catalyzes an equilibrium between adenosine tri-, di-, and monophostphates; glutamic dehydrogenase (17), converting glutamate to α-ketoglutarate; and succinic dehydrogenase (18), which oxidizes succinate to form fumarate. The open stars denote reactions either that are carried out at a reasonably rapid pace by the mitochondrial fraction but cannot be readily studied in homogenates or that are concentrated in mitochondria but also occur in significant amounts in other cell fractions. One of the most interesting is the synthesis of ATP (or adenosinetriphosphate) from adenosinediphosphate and inorganic phosphate. This important reaction proceeds only when

energy is supplied through the aerobic oxidation of such metabolites as succinate, glutamate, or α-ketoglutarate (16). Although the rate of synthesis of ATP by mitochondria is very rapid, it is not possible, for technical reasons, to determine whether or not the enzyme system

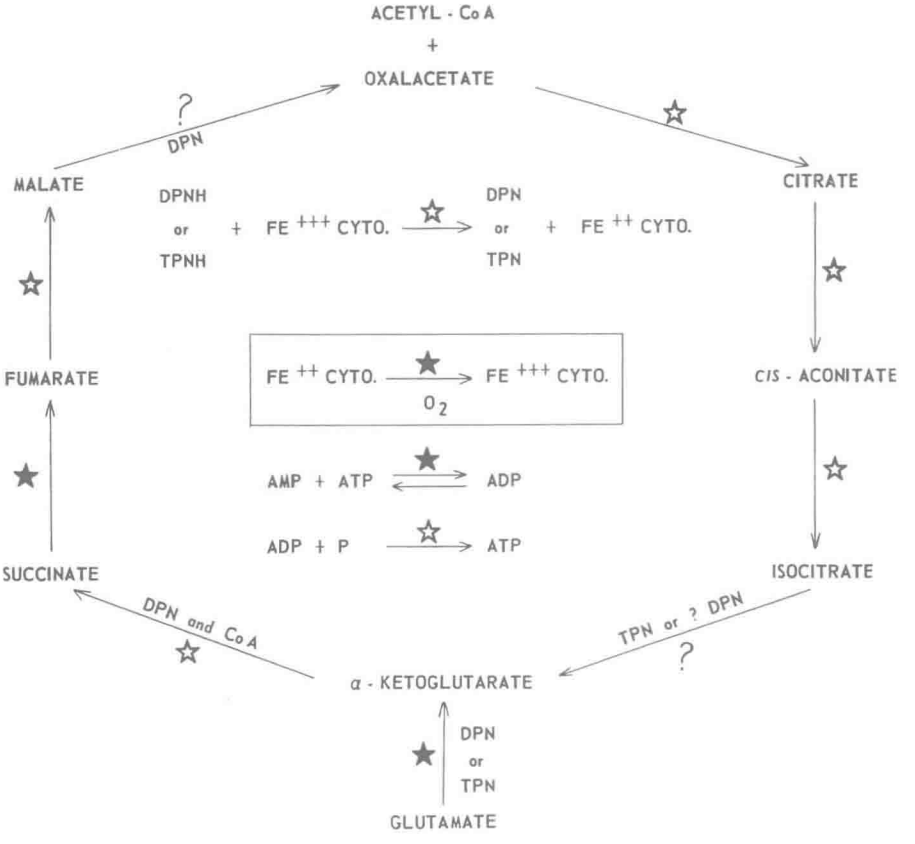

Figure 1. The role of mitochondria in the Krebs cycle and related oxidative and phosphorylative reactions. For an explanation of the symbols, see the text.

responsible for the reaction is present in other cell fractions. We therefore cannot conclude with certainty that the system is an exclusive property of mitochondria.

Kielley and Kielley, in a study of oxidative phosphorylation (16), made several interesting observations that have a bearing on the properties of mitochondria. Thus it was found that the synthesis of ATP is rendered inoperative by any procedure resulting in structural damage to mitochondria. The disappearance of oxidative phosphorylation is

accompanied by the appearance of a very active adenosinetriphosphatase, which removes the terminal phosphate group from ATP to form ADP (16). Dinitrophenol (cf. 19)) and ageing (16), or preincubating in the absence of substrate, have the same effect as does structural damage to the particles. Although these findings are by no means fully understood, it seems safe to conclude that the synthesis of ATP is dependent on the structural integrity of mitochondria. This is also true to a varying extent for other reactions, including fatty acid oxidation and the oxidation of succinate (20).

With respect to the other reactions shown in Fig. 1, the enzymes converting α-ketoglutarate to succinate and malate to oxalacetate cannot be satisfactorily assayed as single step reactions in homogenates. That mitochondria carry out the synthesis of citrate from acetyl coenzyme A and oxalacetate is based on indirect but nonetheless rather convincing evidence. Thus if fluoroacetate is injected into female rats, a procedure resulting in the accumulation in the livers of relatively large amounts of citric acid, and the livers are then fractionated, about 70 per cent of the citric acid is recovered in the mitochondrial fraction (6). The cytochrome reductases, which catalyze the reduction of cytochrome c in the presence of the reduced pyridine nucleotides (21, 22), and fumarase, (23) catalyzing the hydrolysis of fumarate to malate, are found in both the mitochondrial and the microsomal fractions. Aconitase, which catalyzes an equilibrium among citrate, aconitate, and isocitrate, is distributed between the mitochondrial and soluble fractions (24). Isocitric dehydrogenase, converting isocitrate to oxalosuccinate, which in turn decomposes, either spontaneously or enzymatically to α-ketoglutarate, is largely recovered in the soluble fraction of the liver cell (22), only about 12 per cent of the total activity being present in the mitochondrial fraction.

As mentioned earlier, several of the more complicated reactions carried out by mitochondria, such as oxidative phosphorylation, are dependent for their function on the structural integrity of the particles. It is of interest to note that the reverse situation holds for a number of other reactions. Thus the acid phosphatase (25), glumatic dehydrogenase (17), ribonuclease (26), and desoxyribonuclease (26) activities of the mitochondrial fraction are appreciably enhanced by damage to the particles. If the mitochondria are actually disrupted by mechanical means, all of these enzymes, as well as fumarase (23) and adenylate kinase (16), are apparently extracted into true solution. The most plausible explanation for these findings is that the mitochondrial mem-

brane forms a barrier between enzyme and substrate. Additional support for the view that the mitochondrial membrane is a relatively impermeable structure arises from the finding that most of the citric acid in the livers of rats poisoned with fluoroacetate is recovered in the mitochondrial fraction. Furthermore, damage to the mitochondrial membranes by exposure to a hypotonic medium results in rapid and complete extraction of the citric acid (6). Apparently, a relatively large amount of water-soluble protein of unknown function is also contained by the mitochondrial membrane. Thus if the particles are mechanically disrupted and the preparation then centrifuged at 150,000 × gravity, 50 to 60 per cent of the total nitrogen remains in the supernatant (20, 27). Examination of this supernatant in the optical ultracentrifuge has revealed that most of the soluble nitrogen is in the form of proteins with sedimentation constants ranging between 4 and 12 Svedberg units (27), the most prominent component behaving in many respects like a type of globulin (6).

As noted previously, the procedure of disruption of mitochondria results in the release of several enzymes into solution. A number of other enzymes, however, including cytochrome oxidase (20, 28), DPN-cytochrome reductase (20, 28), and adenosinetriphosphatase (29), remain firmly attached to sedimentable fragments of mitochondria. It is of interest that cytochrome c (28), a soluble protein of relatively low molecular weight, falls into the latter class and seems to be in close structural proximity to the succinic dehydrogenase and cytochrome oxidase systems.

It should be pointed out that the properties of the mitochondrial membrane are of great importance in a consideration of the validity of enzyme assays. Thus in studies of the distribution of enzymes among cell fractions, it would now appear to be imperative to determine routinely by disruption of the mitochondria whether optimum conditions for the assay of enzymes have been obtained.

One of the most difficult problems that we have to face, in arriving at definitive conclusions concerning the biochemical properties of mitochondria, is emphasized by the quantitative differences in the distribution of enzymes between the mitochondrial fraction and other cell fractions. As indicated earlier, the experimental data generally fall into one of three categories: first, an apparently exclusive localization of an enzyme in the mitochondria (e.g., cytochrome oxidase); second, the distribution of an enzyme mainly between the mitochondrial and soluble fractions (e.g., aconitase, acid phosphatase, and catalase (30));

and third, the distribution of an enzyme mainly between the mitochondrial and microsomal fractions (e.g., the cytochrome reductases and uricase (31)). To those of us who would like to believe that the structure of the cell is a reflection of its chemistry, this is a somewhat distressing situation, since the exclusive localization of an enzyme in a given cell structure would appear to occur only in special instances. It is evident from the results summarized in Fig. 1, for example, that a rather vivid imagination and a good deal of wishful thinking are required if one is to reach the conclusion that the citric acid cycle is confined solely to mitochondria. What, then, is the true situation? At present, there are two possibilities, the first and teleologically less satisfying being that the mitochondrion is not the only cell structure taking part in this integrated series of reactions. The second possibility, which I should like to discuss now, is that our method of isolation of mitochondria may be producing artifacts that confuse the picture.

It is conceivable, of course, that a completely false picture of the intracellular distribution of a soluble enzyme may arise as a result of the adsorption of the enzyme on particulate material. In several recent investigations, in which direct experimental tests have been made for the occurrence of artifacts of this type (26, 32), it has not been possible to incriminate mitochondria to any serious degree. Microsomes, on the other hand, are capable of adsorbing significant amounts of such enzymes as ribonuclease (26) and fumarase (23). It therefore seems unlikely that adsorption artifacts could account for many of the biochemical properties that are exhibited by the mitochondrial fraction.

It is also possible that some damage to mitochondrial membranes occurs during cell disruption and subsequent fractionation, with the result that appreciable amounts of soluble or even particulate material could escape into the medium. A striking example of the ease with which a soluble enzyme can be extracted from mitochondria is found in the work of Berthet and Duve (25) on acid phosphatase. In our own laboratory [1], we have recently made some studies of liver homogenates prepared in isotonic sucrose solution containing 6 to 12 per cent dextran, a polymer of glucose having an average molecular weight of 70,000. In this medium, as in hypertonic sucrose, the free mitochondria retain their normal rod-like shape. On dilution of the homogenates prepared at the lower dextran concentration, many of the mitochondria gradually assume the spherical shape seen in homogenates prepared in isotonic sucrose without

[1]) Hogeboom, G. H., E. L. Kuff, and M. J. Striebich, unpublished experiments.

dextran. On numerous occasions, we have observed that a single elongated mitochondrion can give rise to more than one spherical granule. This observation indicates that a discontinuity in the mitochondrial membrane has occurred and immediately leads us to the question as to what proportion of the mitochondria isolated by standard procedures are similarly damaged. It is apparent that such a phenomenon could result in the release both of soluble enzymes that would appear in the soluble fraction of the cell and of particulate enzymes that would appear in the microsomal fraction. Furthermore, the mitochondrial fraction might contain larger fragments differing biochemically from intact mitochondria. Since this type of redistribution in all probability does occur to some extent but cannot be readily detected experimentally, we have taken the stand that the concentration of enzymes in the mitochondrial fraction is of cytochemical significance (3, 5, 6), despite variations in distribution among other cell fractions. It is obvious, however, that a better alternative would be the discovery of a more suitable medium for the isolation of mitochondria.

These findings, as well as the discrepancies in the chemical data, have led us to wonder whether the mitochondrial fraction is biochemically heterogeneous. From a cytological standpoint, the remarkably characteristic fine structure of mitochondria, as revealed by the studies with the electron microscope of Palade (7, 8), Sjöstrand and Rhodin (9), and others, makes it difficult to believe that qualitative biochemical differences should exist among the mitochondria of a single tissue or, indeed, even among the mitochondria of different tissues. It is possible, however, that quantitative biochemical variations might occur, perhaps, for example, as a reflection of variations in the maturity of individual mitochondria.

Suffice it to say that the results of several investigations (33—35) have indicated that the mitochondrial fraction, isolated from mammalian liver by present methods, is biochemically inhomogeneous and may, in fact, include particles that differ qualitatively in their properties. The significance of these experiments is somewhat difficult to assess, mainly because in no instance can one conclude definitely that the techniques themselves could not have produced the heterogeneity observed. In general, the methods have consisted of the centrifugal fractionation of preparations of isolated mitochondria, either by the collection of particles that sediment completely at increasing centrifugal forces in the angle centrifuge or by the collection of particles that move under high centrifugal force to their isodensity level within a fluid column in which a

density gradient has been set up. A third procedure has been the centrifigution at high speed in a horizontal rotor of a very concentrated suspension of mitochondria and the subsequent biochemical study of particles obtained from various levels of the firmly packed sediment.

As indicated by my previous remarks, in addition to the presence of some microsomes, it is possible that the preparations contained appreciable quantitites of mitochondrial fragments that differ biochemically from intact mitochondria. Furthermore, although the idea of separating particles according to their respective densities (34, 36) is an ingenious one, the resultant necessity for subjecting mitochondria to a medium of high and variable osmotic pressure is a distinct disadvange. Whether this treatment can produce artifacts remains to be seen.

Ideally, an investigation, based on rates of sedimentation, of the biochemical heterogeneity of a suspension of cellular particles should satisfy the following experimental requirements; the particles should be morphologically intact, centrifugation should be carried out under convection-free conditions, and a study should be made of the sedimentation boundaries formed by partially sedimented particles. In practice, the latter requirement can be met by centrifuging in a preparative type of rotor, sampling the fluid column at successive levels, and carrying out specific biochemical analyses on the samples obtained. The second requirement, namely, the elimination of convection to such an extent that monodisperse particles will form a sharp and stable sedimentation boundary and give sedimentation constants in agreement with known values, is difficult to fulfill. It is this phase of the problem that has occupied much of our time during the last year. If you will allow me now to digress from the subject of mitochondria, I should like to describe in some detail a new technique of analytical centrifugation that we hope will be of help to us in future cytochemical investigations.

First of all, it has long been recognized that the resolving power of angle rotors in separating particles of different sedimentation rates is low because of a relatively short path of sedimentation and because of convection resulting from side wall interference with sedimentation. Actually, convection-free conditions can at present be obtained only in the optical ultracentrifuge, where undisturbed sedimentation occurs in the sector-shaped cell described by Svedberg and his collaborators (37). Although refractive index patterns of sedimentation boundaries obtained in the optical centrifuge have yielded important data relating both to homogeneity and molecular weight, a lack of analytical specificity, a rather low sensitivity, and a requirement for relatively pure preparations

renders this instrument unsuitable for the problem at hand. Attempts have therefore been made to devise a procedure permitting the use of specific analytical methods in a study of the sedimentation behaviour of particles in a crude state and in great dilution (38, 39),

In a series of sampling experiments carried out several years ago, Kahler and Lloyd (40) compared the sedimentation behaviour of polystyrene latex particles in a horizontal, swinging-tube rotor and in a standard angle rotor. It was evident from the observed sedimentation boundaries that the resolving power of the swinging-tube rotor was far superior to that of the angle rotor. The results of these experiments made it apparent that a horizontal, preparative rotor of greater capacity and capable of attaining a much higher centrifugal force would be of value in investigating the sedimentation behaviour both of soluble proteins and of complex, macromolecular, cellular particles. Dr. E. G. Pickles, utilizing an ingenious principle for the attainment of high rotational speeds, designed two types of rotors to aid in solving this problem. The larger has a capacity of 30 ml. in each of three tubes and can be operated at a speed of 25,000 r.p.m., yielding a centrifugal force of 90,000 × gravity at the bottom of the tubes. The smaller, or SW-39 rotor, with which many of you are now undoubtedly familiar, is capable of swinging three tubes, each containing slightly over 5 ml., at 40,000 r.p.m., yielding a maximum centrifugal force of about 170,000 × gravity. The duralumin buckets, which are attached to the rotors by removable pins, reach a horizontal position during acceleration; at this point they come into contact with the body of the rotor and are no longer dependent on the pins for support. A vacuum seal is provided by threaded caps and plastic gaskets fitting the tops of the buckets.

In preliminary experiments with colored proteins, e.g. hemoglobin and hemocyanin, in the SW-39 rotor, it was found that sharp and stable sedimentation boundaries were formed, provided that deceleration between about 5000 r.p.m. and rest was very gradual (39), the time required being 20 to 30 minutes. Several methods for sampling the contents of the centrifuge tube were explored, the following technique (39) finally having been adopted: after centrifugation, the bucket was gently disengaged from the rotor and the plastic tube carefully removed and placed in a hole drilled in a block of lucite. The tube was then pierced near its base with a hypodermic needle and a concentrated solution of sucrose injected at a steady flow rate of about 0.1 ml. per minute. By this means, the fluid column was slowly forced upward without noticeable disturbance of the sedimentation boundary and through the perforated

base of a plastic cylinder. Samples were removed with a silicone-coated capillary pipette, weighed, and analyzed.

In order to provide a satisfactory background to future sampling experiments with preparations of unknown composition, it was desirable to work initially with a number of purified materials that could be characterized in the optical centrifuge. Sharp sedimentation boundaries were formed in all instances. As demonstrated in Table I, the sedimentation constants for a number of materials were in excellent agreement with values obtained with the optical centrifuge. This was true, furthermore, over a very wide range of sedimentation rates, — from 4 to over 2000 Svedberg units. It may also be noted that a number of sampling experiments were carried out with very dilute solutions. In these instances, the stabilizing effect of a slight sucrose concentration gradient

TABLE I

DETERMINATION OF SEDIMENTATION CONSTANTS BY A SAMPLING TECHNIQUE

COMPOUND	$s_{20,w} \times 10^{13}$ (OPTICAL ULTRACENTRIFUGE)	$s_{20,w} \times 10^{13}$ (SAMPLING EXPERIMENTS)	CONCENTRATION (PERCENT)
BOVINE SERUM ALBUMIN	4.16	4.13 4.47	0.87 0.087
HUMAN IMMUNE SERUM GLOBULIN	6.17	6.34	1.65, 0.83
BOVINE FIBRINOGEN	6.28	6.27	1.0
RAT LIVER CATALASE	10.9	11.0	1.0, 0.051
DESOXYRIBOSE NUCLEIC ACID	12 – 14	12.8	0.02
HEMOCYANIN	22.7	23.1 22.7	0.85, 0.68 0.091
POLYSTYRENE LATEX	2270	2130	0.008

led to sedimentation boundaries comparable in sharpness to those obtained with more concentrated solutions (39).

The next step in the investigation was to determine whether the technique of centrifugation and sampling was suitable for the study of enzymes in a crude state and in high dilution. Two enzymes that have been obtained in crystalline form and characterized in the optical centrifuge, namely, yeast alcohol dehydrogenase and jack bean urease, were chosen for study. The preparations used in the sampling experiments consisted of crude extracts of baker's yeast and of jack bean meal (41)