Mitochondrial Structure: Two Types of Subunits on Negatively Stained Mitochondrial Membranes

Abstract. *Projecting subunits were seen on the cristae of all 11 types of mitochondria examined by electron microscopy. These consisted of a stem, 30 to 35 Å wide and 45 to 50 Å long, and a round head 75 to 80 Å in diameter. The outer membranes of rat liver mitochondria showed regular arrays of hollow cylindrical subunits 60 Å by 60 Å.*

Two types of periodic structures, associated with the membranes of mitochondria, have been observed in mitochondria negatively stained by a surface-spreading technique (*1, 2*). With this technique mitochondria from a variety of cell types can be examined, without previous isolation and with minimum manipulation. Isolated mitochondria can also be examined similarly.

A needle was pushed into the tissue to remove a few cells which were then spread on the surface of 1 percent potassium phosphotungstate solution (*p*H 6.0 to 7.0) by dipping the needle slowly into the solution (*3*). Surface tension ruptured the cell membranes and spread the cytoplasmic structures as a thin film on the surface. Specimen grids, coated with a carbon-strengthened Formvar film, were touched to the surface to pick up the film. (The phosphotungstate contained approximately .01 percent bovine serum albumin to give improved wetting of the carbon-Formvar film.) After excess phosphotungstate had been removed with filter paper, the grid was allowed to dry; it was then examined in the Siemens Elmiskop I electron microscope at magnifications of 14,000 to 40,000. Mitochondria of the following tissues were examined: normal rat liver, kidney, and hepatoma; normal mouse liver, kidney, and brain; leukemic mouse thymus and lymph nodes; two types of mouse plasma-cell tumors and normal bovine pancreas.

Mitochondria were isolated from rat liver and kidney by the procedure of Lardy and Wellman (*4*) although the medium suggested by these authors was replaced by that of Chance and Hagihara (*5*). Lysed mitochondria were prepared by exposing isolated mitochondria to very hypotonic conditions (1 m*M* phosphate buffer at *p*H 7.0) and then re-isolating them by centrifugation. The isolated mitochondria were negatively stained by dipping a needle into the mitochondrial suspension and then into the phosphotungstate solution. The film of spread mitochondria was picked up with a grid as described above. The distribution of mitochondria on the grid was adjusted by diluting the mitochondrial suspension with the appropriate medium.

Nonlysed mitochondria, from both preparations of cells spread on a surface and spread-isolated mitochondrial preparations, showed several different forms according to the degree of flattening and of penetration by the phosphotungstate. When not flattened, the cristae and outer membrane were readily recognizable because of their similarity to thin-sections, but fine inner structure was not visible. Many of the mitochondria, however, were flattened out and very regular structures were clearly visible attached to the surface of the inner membranes or cristae. Part of a mitochondrion from a mouse liver preparation is shown in Fig. 1*a*, and cristae of a mitochondrion from an isolated and lysed rat liver mitochondrial preparation in Fig. 1*b*. The surfaces of the cristae in both types of preparations are covered with close-packed projecting subunits. The units are shown at high magnification in Fig. 1*c*. They consist of a narrow stem 30 to 35 Å wide and 45 to 50 Å long and an approximately spherical head 75 to 80 Å in diameter. The center-to-center distance between subunits was 100 Å. Similar subunits were seen in all types of mitochondria examined. They were more clearly visible in isolated and lysed mitochondrial preparations apparently because of release of pieces of cristae from the mitochondrion and removal of substances of low molecular weight from around the subunits.

There seems little doubt that the subunit associated with inner membrane or cristae is identical with that (the elementary particle) described in a preliminary report by Fernández-Morán (*7*). Frozen sections of mitochondrial and electron transport particle preparations were negatively stained by Fernández-Morán. The three-dimensional shape of the cristae could be seen in the spread preparations; whereas some of the cristae appeared as thin, plate-like structures, many consisted of surprisingly long (up to 0.5 μ) filaments (Figs. 1*a* and 1*b*). Why the cristae present a more elongated appearance

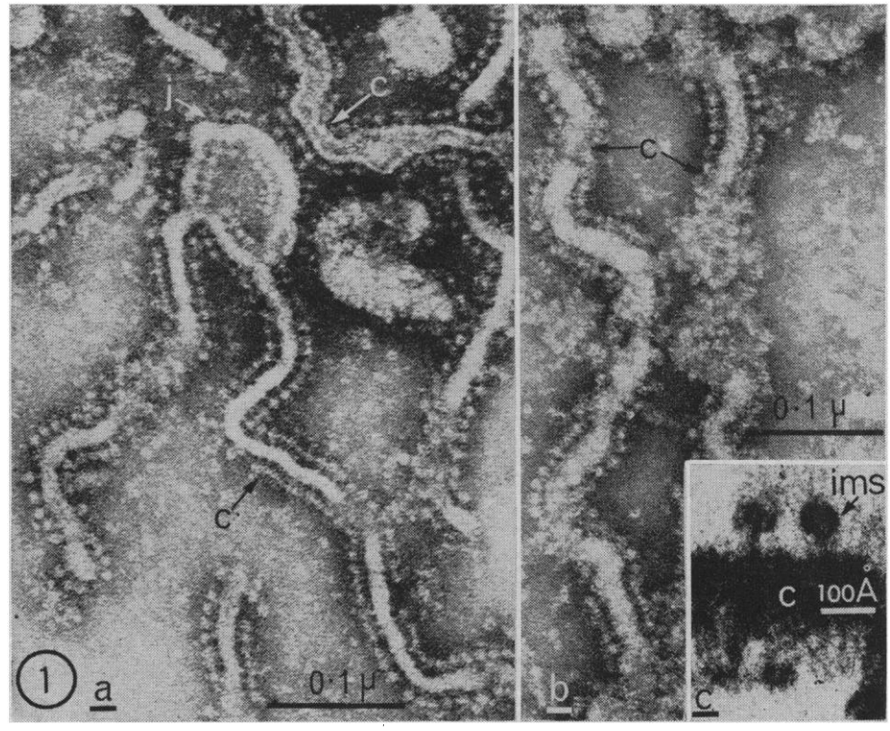

Fig. 1. The subunit associated with the inner membranes or cristae. (*a*) Part of a mitochondrion from a negatively stained preparation of mouse liver. A few of the cristae (*c*) are shown. The cristae consist of long filaments which branch at some points (*j*). The surfaces of the cristae are covered with projecting subunits (× 192,000). (*b*) Negatively stained cristae (*c*) prepared by spreading isolated lysed rat liver mitochondria. The subunits on the cristae appear similar to those of Fig. 1*a* (× 192,000). (*c*) Higher magnification—a few subunits from the same preparation as Fig. 1*b*. The spherical heads are 75 to 80 Å diameter and the stems 30 to 35 Å wide and 45 to 50 Å long. The center-to-center spacing is 100 Å. Reversed print (× 770,000).

in negatively stained preparations than in thin section preparations is not now clear.

Another, different type of substructure has been seen on the outer membrane of rat liver mitochondria, but not on the other types of mitochondria examined. This structure (Fig. 2) consists of hollow cylinders, 60 Å wide and 60 Å long, with a central hole 20 Å in diameter. The center-to-center distance was 80 Å. The axis of each cylinder is perpendicular to the plane of the membrane. It has not yet been established whether the cylinders are incorporated into the structure of the membrane or project from its surface. This structure was visible on projecting portions of the outer membrane (Fig. 2*a*) or in narrower portions of the outer membrane. It joined together two larger pieces of membrane (shown at high magnification in Fig. 2*b*). The structure occurred most frequently in lysed rat liver mitochondrial preparations but also in preparations of rat liver cells spread on a surface.

Observations of the outer membrane subunit do not at present indicate whether the subunit is located on the whole, or only on a small part, of the outer membrane. The former is possible since the subunit may only be in favorable orientation for negative staining in the projecting or constricted portions of the outer membrane. In the latter case, these portions of the outer membrane with subunits may have a specialized function as suggested, for example, by the special association of endoplasmic reticulum with mitochondria liver cells (*8*). Recently a periodic structure associated with the outer membrane of mitochondria in thin sections of cat retinal-rod cells has been reported (*9*). However, the center-to-center separation of this unit is larger (160 Å) than that reported here.

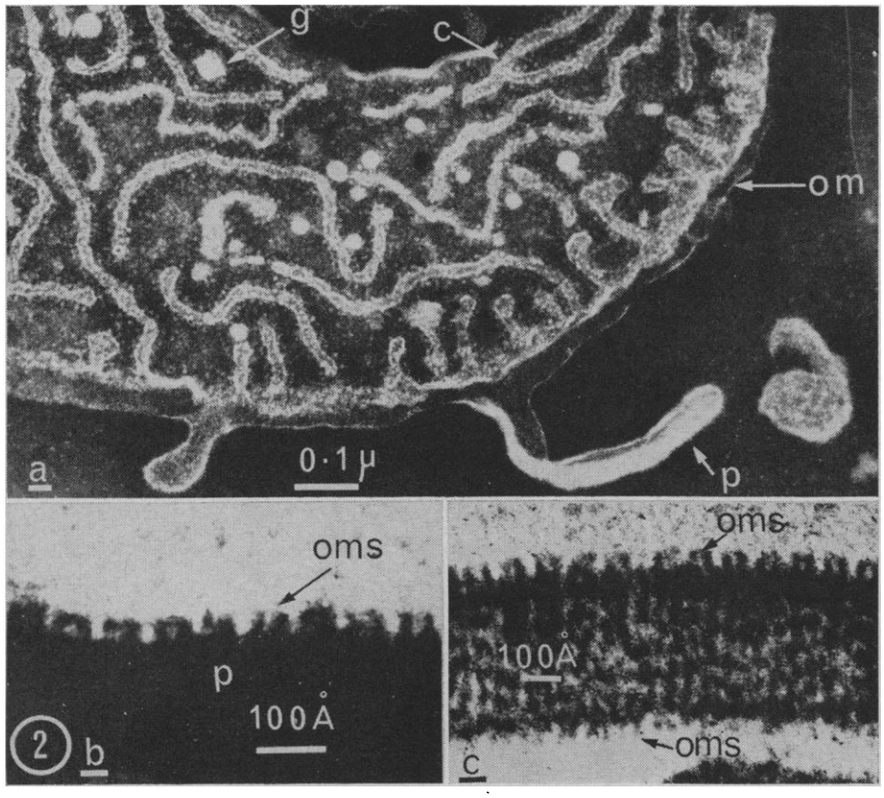

Fig. 2. The outer membrane subunit of rat liver. (*a*) Low magnification of a negatively stained mitochondrion showing the cristae (*c*) and the mitochondrial granules (*g*). The outer membrane (*om*) shows a projection (*p*) which has very regular periodic structures on its surface (× 87,000). (*b*) High magnification of the outer membrane subunit (*oms*) shown at *p* in Fig. 2*a*. The subunit consists of hollow cylinders 60 Å tall, and 60 Å wide, with a center-to-center spacing of 80 Å. Reversed print (× 945,000). (*c*) Portion of the outer membrane of a negatively stained spread preparation of isolated and lysed rat liver mitochondria. The region shown formed a narrow portion between two large pieces of the outer membrane. The outer membrane subunit (*oms*) is present on the surface of this portion of the membrane but is not visible elsewhere. Reversed print (× 547,000).

The small size and regularity of the subunits herein described suggest that they represent units of one or more enzyme molecules. Several workers have obtained direct evidence that the respiratory enzymes are attached to the membranes of mitochondria. The localization of the dehydrogenase-cytochrome system for oxidation of succinate and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$), in a membrane fraction of mitochondria, has been demonstrated by Siekevitz and Watson (*10*) and also by Ball and Barrnett (*11*). Green (*6*) fragmented mitochondria by ultrasonification to give small membrane fragments (electron transport particles) in which the cytochrome and dehydrogenase activities were mainly preserved. Some dehydrogenases have also been localized to the cristae of mitochondria by techniques of electron microscope cytochemistry. Barrnett and Palade (*12*) used potassium tellurite to localize succinic dehydrogenase to the cristae of mitochondria, and nitro-blue tetrazolium and tetranitro-blue tetrazolium have been used to localize succinate and $NADPH_2$ dehydrogenases (*13, 14*). Estabrook and Holowinsky (*15*) have considered various packing arrangements for cytochromes and dehydrogenases on the inner membranes of mitochondria. Their results indicate that a large part of the inner surface would be covered by these enzymes. Hence, it appears probable that the subunit associated with the inner membranes represents one or more components of the electron transport system.

Recently Green, Fernández-Morán, and co-workers (*6, 7*) purified a mitochondrial fraction containing particles of the same size as the subunit of the cristae and found that the electron transport systems were concentrated in this fraction. However, a difficulty arises over the small size of the units described in relation to the number of components believed to make up the electron transport chain, and the size of its individual components. The cytochromes (molecular weight 70,000) probably are comparable in size to hemoglobin (molecular weight 64,000), that is, 55 by 55 by 70 Å (*15*) although cytochrome *c* would have smaller dimensions. The flavoproteins (molecular weight 100,000 to 200,000) are probably larger than the cytochromes. It would appear to be impossible for more than a few such

molecules to be packed into the subunit associated with the inner membrane or cristae. However, the possibility exists that the subunits are not all identical and that several combinations of a molecule of flavoprotein with a few molecules of cytochromes can occur.

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Hypoglycemia: A Potent Stimulus to Secretion of Growth Hormone

Abstract. *In normal subjects, hypoglycemia produces an abrupt and sustained rise in levels of human growth hormone in plasma. This effect is independent of insulin, glucagon, or epinephrine. Prolonged fasting is accompanied by a rise in the hormone level in plasma. Measurement of this hormone after induced hypoglycemia is a specific test for pituitary somatotropic function.*

The physiologic role of endogenous growth hormone in vertebrate carbohydrate metabolism has been deduced from indirect studies (*1*). Measurement of this hormone in plasma by bioassay and immunoassay techniques has been impeded by interfering substances in plasma and by lack of a sensitive method. With a new immunoassay method employing I^{131}-labeled human growth hormone (*2*), which is capable of detecting 0.25 mμg/ml of human growth hormone in unextracted plasma, acute physiologic changes in hormone levels have been demonstrated for the first time.

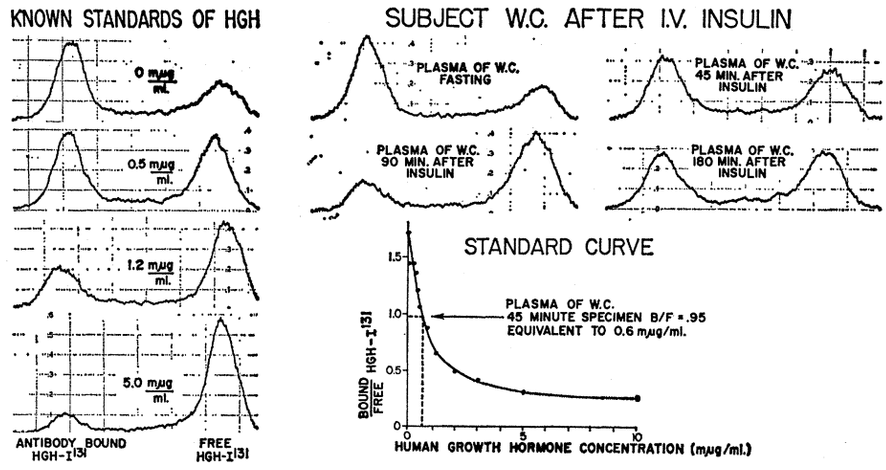

Fig. 1. Antibody-bound and free I^{131}-labeled human growth hormone (HGH-I^{131}) are separated by hydrodynamic flow chromatography on strips of filter paper and the strips are scanned for radioactivity. Shown are representative scans from known standard solutions (left) and from plasma samples of a normal subject (W.C.) before, during, and after insulin-induced hypoglycemia (upper right). All plasma samples were assayed at 1:10 dilution. Hormone values given in text and in subsequent figures are for undiluted plasma.

We measured endogenous human growth hormone in plasma by adapting, with modifications, methods previously employed for the immunoassay of plasma insulin (*3*). This method exploits the ability of purified and of endogenous growth hormone in plasma to inhibit competitively the binding of I^{131}-labeled growth hormone to growth hormone antibodies. Thus at fixed concentrations of antibody and labeled hormone, the ratio (B:F) of labeled hormone bound to the antibody (B) to unbound labeled hormone (F) decreases progressively with increasing concentration of unlabeled hormone. The concentration of endogenous hormone in a plasma sample is determined by comparison of the B:F ratio obtained from a solution containing the plasma with the B:F ratios obtained from standard solutions containing known concentrations of hormone (Fig. 1). No hormone was detected in plasma from several non-primate species or from totally hypophysectomized human subjects. Purified hormone, added to plasma, was recovered quantitatively.

In six normal subjects who were fasting, hypoglycemia, induced by insulin (0.1 unit/kg body weight, intravenously), was followed by an increase of at least 500 percent in plasma hormone concentration (Figs. 1 and 2) over fasting levels (0 to 3 mμg/ml) to values usually found in random plasma samples from acromegalic subjects (> 10.0 mμg/ml). Levels of hormone increased shortly after the onset of hypoglycemia, reached a peak about 30 minutes later, and persisted above fasting levels for several hours after the blood glucose level had returned to nor-

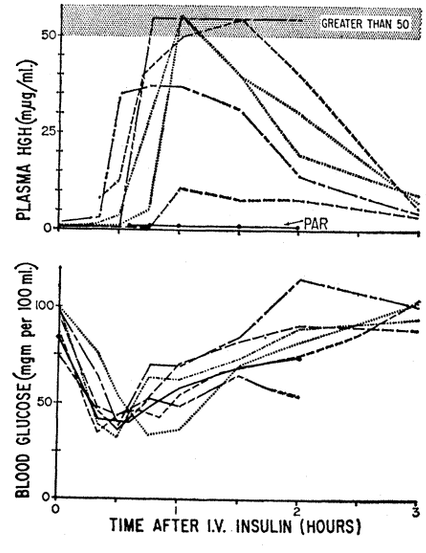

Fig. 2. Plasma human growth hormone (HGH) and blood glucose concentrations after insulin administered intravenously. (PAR, a hypophysectomized patient; other patients, normal.)