STUDIES OF THE MITOCHONDRIAL ENERGY-TRANSFER SYSTEM OF BROWN ADIPOSE TISSUE

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ABSTRACT

An investigation of the mechanisms of norepinephrine action and heat production in brown adipose tissue from newborn rabbits has been carried out. Data obtained with the use of biochemical techniques has been correlated with morphological data from electron microscopy. Norepinephrine was found to stimulate the respiration of brown fat in vitro. Inhibitors of glycolysis abolish this effect, whereas inhibitors of oxidative phosphorylation do not, at least not to the same extent. Brown fat is readily permeable to added Krebs cycle intermediates. Substrate level phosphorylation, but no electron transport–coupled phosphorylation, could be demonstrated in isolated mitochondria. It is suggested that the rate of fatty acid oxidation is limited by the availability of phosphate acceptor systems which break down ATP formed at the substrate level and thus provide ADP for further substrate level phosphorylation. The theory of respiratory control by the action of reesterification of fatty acids is discussed in the light of these findings. Under the electron microscope, brown fat mitochondria are characterized by their large size, tightly packed cristae, and by the different types of granules in the matrix. No elementary particles are seen when the mitochondria are examined by the negative-staining technique. The absence of electron transport–coupled phosphorylation together with the apparent absence of elementary particles seems to be of particular significance.

INTRODUCTION

Brown fat is a highly specialized tissue whose main function is the production of heat (4, 41). Under conditions of little physical activity and high demand for thermogenesis, brown adipose tissue serves as a heating system for the entire body. Such conditions are found during arousal from hibernation, in newborn mammals, and during cold adaptation.

Generally, heat can be considered a byproduct of physiological and biochemical activities, and heat production is thus an expression of the thermodynamic inefficiency of energy-generating or energy-transferring systems. However, brown fat apparently contains a system whose sole purpose is the production of heat; the lack of other important biochemical activities in this tissue seems to preclude the possibility that it produces heat through an inefficient process with some other goal.

Norepinephrine (NE) is a regulator of physiological activity. Within adipose tissue, the regulatory mechanism is considered to involve the availability of free fatty acids and their reesterification (5, 41); the fatty acids are thought to act both as substrate and phosphate acceptor (4, 12).

The mechanism of NE regulation via the availability of free fatty acids seems to require some

modification in order to fit data obtained from brown fat. When added to this tissue, NE seems to cause a prompt and pronounced increase in respiration, both in vivo and in vitro. The mechanism which triggers this increase might be of a different biochemical nature from the quantitatively predominant reaction that transfers the energy of stored fat into heat.

The biochemist usually defines mitochondria as the intracellular organelles capable of carrying out respiration with oxidative phosphorylation coupled to that respiration. The accepted concept is that the generation of high-energy bonds fulfills the need of the cell to transform substrate bond energy into a form that can be utilized for energy-requiring processes.

It is believed that a complex morphological organization is necessary for this coupling of terminal electron transport to the formation of high energy bonds. (The membrane-free mitochondrial derivatives in some invertebrate sperm might be an exception to this hypothesis; see reference 2.) Of particular interest in this connection is the "elementary particle" of Fernández-Morán (14), which is considered by Racker et al. to be the site of the coupling factor bringing about ATP formation from the primary high energy bond formed during terminal electron transport (32). These particles can be made visible in the electron microscope by the so called negative-staining technique. It has also been claimed that the particles can be seen in sectioned mitochondria, but these claims are open to serious doubt.

Mitochondria from brown adipose tissue are structurally well defined (26) and contain a normal respiratory chain (17). However, the theoretical lack of necessity for oxidative phosphorylation in a tissue concerned only with thermogenesis poses a

TABLE I

Norepinephrine Stimulation of Oxygen Consumption by Brown Fat Fragments in vitro under Different Conditions

Substrate	Additions	μatoms O/g wet weight/hr −NE	SD	+NE	SD	No. of experiments
—	—	85	± 33	282	± 125	46
	2,4-Dinitrophenol 7.5×10^{-5} M	107	± 37	136	± 73	27
	Oligomycin 2.5 μg/ml	94	± 45	191	± 122	14
	2,4-Dinitrophenol + oligomycin	135	± 88	229	± 128	7
	Iodoacetate	70	± 35	84	± 53	6
	Sodium flouride	33	± 12	27	± 10	5
Pyruvate + malate, 15 mM + 15 mM	—	184	± 55	247	± 145	9
	2,4-Dinitrophenol	290	± 105	295	± 144	6
	Oligomycin	172	± 96	314	± 183	6
	Iodoacetate	184	± 103	195	± 89	5
α-Ketoglutarate, 15 mM	—	123	± 45	510	± 259	29
	2,4-Dinitrophenol	214	± 93	270	± 144	14
	Oligomycin	155	± 73	313	± 177	6
	2,4-Dinitrophenol + oligomycin	198	± 101	279	± 166	6
	Iodoacetate	184	± 75	184	± 68	5
α-Glycerophosphate 10 mM	—	254	± 62	436	± 192	10
	Iodoacetate	301	± 96	270	± 100	5

Tissue from one newborn rabbit (1–6 days old). The Warburg flasks contained 35 mg of tissue suspended in 2-ml solution of 119 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.7 mM $CaCl_2$.

When added, the following concentrations were used: pyruvate 15 mM, malate 15 mM, α-ketoglutarate 15 mM, glycerophosphate 10 mM, 2.4 dinitrophenol 7.5×10^{-5} M, iodoacetate 0.1 mM, sodium fluoride 10 mM, oligomycin 2.5 μg/ml and 1.5 μg norepinephrine-bitartrate/ml.

Gas phase O_2. Temperature 37°C. O_2 insufflation: 15 min. Thermoequilibration: 10 min. Time of experiment: 2 hr.

question: do brown fat mitochondria carry out oxidative phosphorylation during thermogenesis, or does the highly developed structure of these mitochondria have a purpose other than the generation of high-energy bonds?

The present paper examines brown fat on two levels: first, it investigates the metabolic pattern of the tissue under different hormonal conditions; and secondly, it investigates the biochemical and morphological properties of brown fat mitochondria. These combined investigations might not only shed light on the mechanisms of NE action and thermogenesis, but also help elucidate features of the well recognized problem of the relationship between mitochondrial structure and oxidative phosphorylation.

Preliminary reports of the findings have previously been presented (1, 19).

MATERIALS AND METHODS

Preparation of Brown Fat Fragments

Newborn rabbits, 12 hr–10 days old, were stunned by a blow to the head. Brown adipose tissue from the scapular and neck regions was removed (for the anatomy see references 12, 15, and 33); brown fat from the jugular side of the neck was discarded because of contamination with blood vessels. It has been noted that the replacement of brown fat with white fat begins in the scapular pads; thus, with rabbits 8–10 days old, fat was collected from the neck only. Each experiment was carried out with fat from one animal only.

After removal from the animal, the fat was placed in a Krebs-phosphate-Ringer solution at room temperature. The tissue was taken from this solution in portions, blotted on filter paper, separated from surrounding connective tissue, cut into small pieces (1–3 mg), and placed into preweighed Warburg vessels also containing Krebs-phosphate-Ringer buffer. 20–100 mg of tissue, depending on the amount of fat obtained and the number of variables to be tested, were added to each vessel; in a single experiment all vessels contained the same amount of tissue (within 10%).

Substrate and other substances were added to the vessels; then the vessels were attached to manometers and placed in a Warburg bath at 37°C. Next, the vessels were flushed with 100% oxygen for 15 min. After an additional 15-min period for temperature equilibration, the readings were begun.

An average of about 90 min elapsed from the time of sacrifice to the first reading. There were several indications that this was not an excessive time lapse: experiments in which the time lapse was significantly shorter yielded results entirely analogous to those of the other experiments. Also, in some experiments, readings were taken for as long as 3 hr (the average being 2 hr), during the whole of which time respiration was essentially constant. Finally, the tissue suspensions were exposed to substrate and other substances for only 25–30 min before readings were begun.

Preparation of Brown Fat Mitochondria

Mitochondria were prepared using the common sucrose method (13) with the following modification: the fat cake which forms on top of the supernatant during high-speed centrifugation was carefully separated from the mitochondrial pellet by pouring off the liquid supernatant and leaving the fat on the wall of the centrifuge tube. This permitted transfer of the mitochondria with 0.25 M sucrose and a pipette to another centrifuge tube for washing.

Other methods employed were the mannitol method (9) and preparation of mitochondria from tissue pretreated with proteinase (27). Different variables—such as the addition of albumin, EDTA, Tris buffer, ATP, and salt solutions, all at varying concentrations—were tested in conjunction with these three preparation methods.

Analytical Techniques

Oxidative phosphorylation, ATPase, and P_i-ATP exchange were measured as previously described

TABLE II

Oxidation of α-Ketoglutarate by Brown Fat Tissue in vitro

Tissue mg wet weight/ Vessel	Additions	Amount consumed in 2 hr/vessel	
		O_2	α-Keto-glutarate
		μmoles	*μmoles*
49.7		3.45	—
50.3	NE	12.2	—
53.0	Malonate	2.91	—
50.0	α-Ketoglutarate	5.0	0.45
49.1	Malonate + NE	2.21	—
50.7	α-Ketoglutarate + NE	8.7	1.09
53.2	Malonate + α-keto-glutarate	6.65	1.89
50.6	Malonate + α-keto-glutarate + NE	15.1	1.17

Conditions the same as in experiment of Table I except: concentration of α-ketoglutarate 5 mM, malonate 3 mM, weight of tissue about 50 mg/ vessel.

TABLE III

Oxidation of Pyruvate by Brown Fat Tissue in vitro

Tissue mg wet weight/Vessel	Time of incubation	Additions	Amount consumed/Vessel	
			O_2	Pyruvate
	min		*μmoles*	*μmoles*
51.6	30	—	1.6	—
51.2	"	NE	2.6	—
52.7	"	Pyruvate + malate	4.3	3.14
51.6	"	NE + pyruvate + malate	5.4	3.14
50.0	120	—	6.1	
51.5	"	NE	17.8	
52.3	"	Pyruvate + malate	16.6	8.50
50.0	"	NE + pyruvate + malate	28.3	8.50

Conditions the same as in experiment of Table I except concentration of pyruvate 10 mM and malate 10 mM.

(20, 40). ATP, pyruvate, and α-ketoglutarate were determined with standard enzymatic technique (6), and energy-linked transhydrogenase was determined as described by Danielson et al. (11). Succinate-linked NADH reduction was measured according to Löw and Vallin (21).

Electron Microscopic Techniques

Morphological examinations were carried out on tissue from the same animals used for biochemical studies. Small (2-mm-sided) cubes were excised from the scapular brown fat, fixed in 3% glutaraldehyde in a cacodylate buffer (39), washed in the buffer, postfixed in 1% osmium tetroxide in a phosphate buffer (24), dehydrated in ethanol, and embedded in Epon (22). The embeddings were sectioned with an LKB Ultrotome microtome, and the sections stained with uranyl acetate according to Brody (7) and with lead hydroxide according to Reynolds (34).

Some of the mitochondrial suspensions prepared for biochemical studies were also used for studying brown fat mitochondria with the negative-staining technique. Equal volumes of 2% sodium phosphotungstate (pH 7) and mitochondria in sucrose (about 5 mg protein/ml) were mixed, and the mixture sprayed onto grids covered with carbon-coated collodium films. In attempts to make the elementary particles (14) or inner membrane subunits (29) visible, the mitochondria were treated in various ways before they were mixed with phosphotungstate: distilled water was used in order to disrupt the mitochondria osmotically and liquid nitrogen in order to freeze-thaw them; mitochondrial fragments, which consist mainly of free cristae, were obtained by sonification (Raytheon Sonic Oscillator Model DF 101, at 16 kc for 30 sec); and the mitochondria were aged for 2 hr at 0°C.

RESULTS

Biochemical Studies on Incubated Tissue Fragments

The experiments summarized in Table I demonstrate that norepinephrine (NE) stimulates the

TABLE IV

ATP Content of Brown Fat Incubated under Different Conditions

Additions	−NE	+NE
	958	591
2,4-Dinitrophenol	512	533
Oligomycin	615	554
2,4-Dinitrophenol + oligomycin	415	342
Iodoacetate	319	308
Iodoacetate + 2,4-dinitrophenol	326	266
α-Ketoglutarate	268	282
Pyruvate + malate	388	332
α-Glycerophosphate	324	309

Nonincubated tissue contains:
Orthophosphate = 5.54 μmoles/g wet weight
ATP = 220 mμmoles/g wet weight
Total nucleotide (260 mμ absorption) 4.79 μmoles/g.

Values expressed as mμmoles per gram of wet weight. For experimental conditions, see Table I. The effects of the additions on respiration correspond to those demonstrated in Table I.

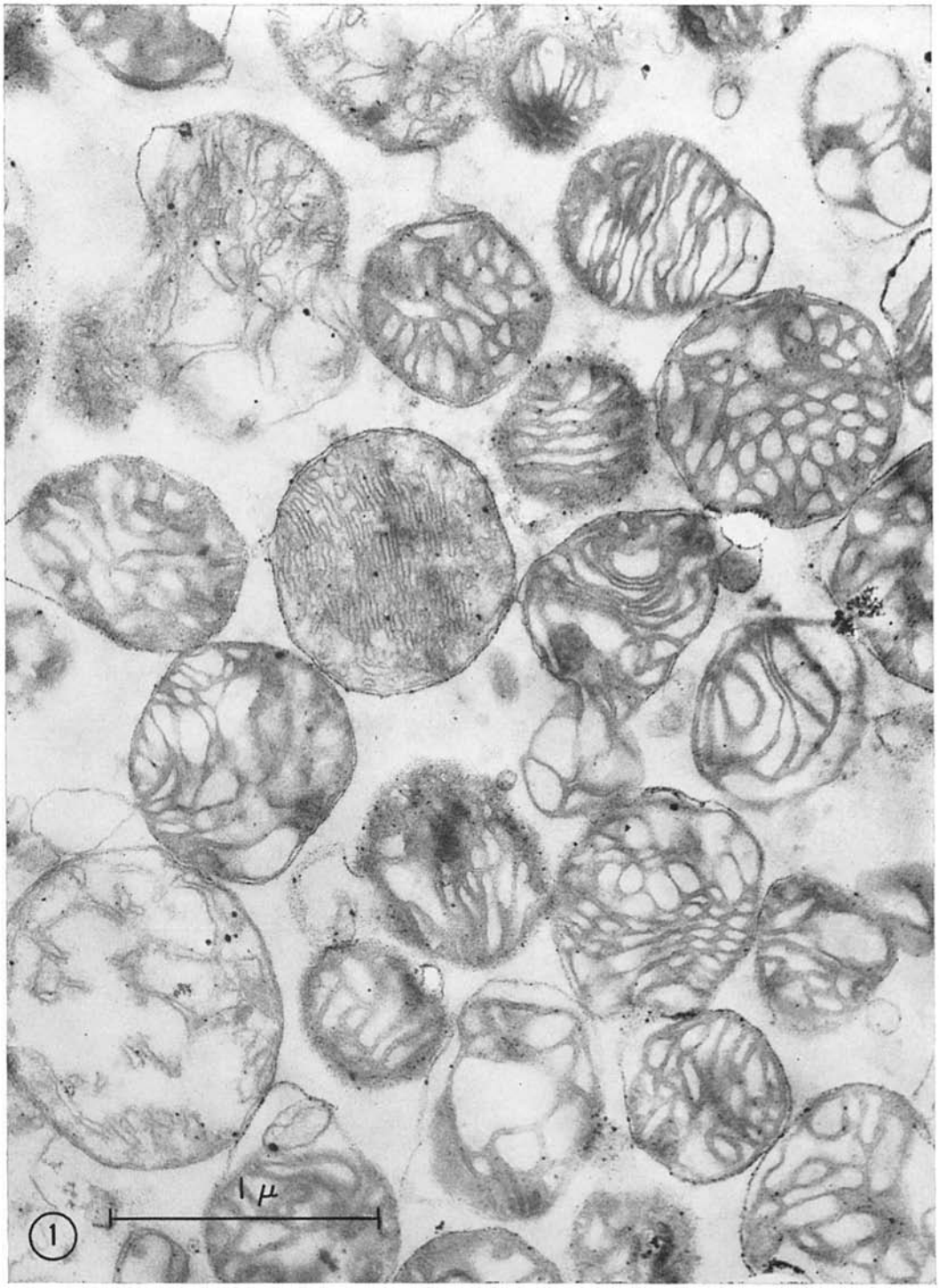

FIGURE 1 Section through a pellet of mitochondria prepared from rabbit brown fat. Occasional lipid droplets can be found in the pellet, although most fields like this one are free from major contaminants. × 40,000.

in vitro respiration of brown fat fragments. This confirms the results of other investigators (4, 12, 17).

It is also evident from Table I that glycolytic inhibitors such as monoiodoacetate and fluoride abolish the NE effect. Dinitrophenol and oligomycin, inhibitors of oxidative phosphorylation, have a somewhat less pronounced reductive effect on the NE stimulation.

TABLE V

Oxidative Phosphorylation of Brown Fat Mitochondria

	O_2	P	P/O
α-Ketoglutarate	95.0	21.9	0.23
α-Ketoglutarate + malonate	59.2	41.0	0.69
Glutamate	15.4	6.1	0.40
Succinate	61.5	5.35	0.087
Pyruvate	0	—	—
Pyruvate + malate	81.7	17.2	0.21
Malate	1.1	—	—
Isocitrate	1.0	—	—
Butyrate	0	—	—
α-Glycerophosphate	44.6	11.2	0.25
β-OH Butyrate	5.1	—	—

Four newborn rabbits/experiment. Data expressed as μatoms/g mitochondrial protein/min. Time of incubation 30 min. Gas phase: air. Temperature: 37°C. Concentration of substrates: 10 mM, malonate: 3 mM. About 6 mg mitochondrial protein per flask were suspended in 2 ml of a medium containing: 50 mM KCl, 10 mM $MgSO_4$, 1 mM ATP, 10 mM P_i^{32}, 25 mM Tris buffer (pH 7.5), 25 mM glucose, and 28 units hexokinase.

The addition of α-ketoglutarate, pyruvate + malate, or α-glycerophosphate stimulates the oxygen uptake of the tissue, and the hormone effect persists in the presence of these substrates, although the relative increase caused by NE in the latter two cases might be diminished.

The highest rates of respiration recorded in these investigations were obtained when brown fat fragments were incubated in the presence of α-ketoglutarate plus NE. As Table II demonstrates, α-ketoglutarate is oxidized to only a very slight extent under these circumstances; this compound obviously enhances the NE effect on respiration in a nonstoichiometric manner.

A similar experiment with pyruvate-malate as substrate is demonstrated in Table III. It is seen that the added substrate is rapidly oxidized, but that the rate of pyruvate oxidation is not influenced by NE.

Three other things should be noted about Table I. First, in the presence of pyruvate-malate, dinitrophenol stimulates respiration much more significantly than when the tissue is utilizing only endogenous substrate. Secondly, several substrate combinations other than those shown in the table—

TABLE VI

Conditions Tested without Significant Effect on the Efficiency of Oxidative Phosphorylation of Mitochondria Isolated from Brown Fat

Mitochondria isolated from (Standard procedure):
- Rabbits, of different ages.*
- Rabbits, newborn, chilled in ice.
- Rabbits, newborn, cooled in air.
- Rabbits, newborn, fasted for 2 days (loss of fat and response to norepinephrine).
- Rats, 3 hr old (without fat).
- Rats, newborn, treated with reserpin.
- Rats, newborn, treated with actinomycin D.

Variables tested for the isolation of mitochondria from brown fat of newborn rabbits:

Mediums tested:

0.44 M sucrose	0.25 M sucrose + AMP + ADP + ATP
0.25 M sucrose	0.25 M sucrose + Mg
0.25 M sucrose + albumin	0.25 M sucrose + Mn
0.25 M sucrose + AMP	0.25 M sucrose + EDTA
0.25 M sucrose + ADP	0.25 M sucrose + proteinase
0.25 M sucrose + ATP	Buffers of different pH

* Newborn = 6 hr—5 days, young = 5–20 days.

e.g., α-ketoglutarate plus α-glycerophosphate—have been tested without noteworthy results. And finally, results similar to those shown in Table I have also been obtained by the use of brown fat from newborn rats, the only difference being that the oxygen consumption per gram wet weight is approximately twice as high as in rabbits; this difference might be explained on the basis of the very low lipid content in brown adipose tissue from newborn rats.

Table IV summarizes data on the amount of ATP in brown fat incubated under different conditions. As can be seen, the level is rather low. Maximum values are obtained when the tissue is incubated without substrate or inhibitors; it is most striking that increased respiration in the presence of NE or substrate is accompanied by a decrease in the ATP level.

When fragments of brown fat from newborn rabbits are incubated in an oxygenated Krebs-Ringer solution, a very slight release of free fatty acids into the medium is observed. This release can be significantly increased by the addition of NE. Our findings confirmed those of Dawkins and Hull (12): a release of 1.1 μmoles of free fatty acid/g/hr is stimulated to 3 μmoles upon addition of NE.

Biochemical Studies on Isolated Mitochondria

Brown adipose tissue is rich in mitochondria with well developed cristae (26). When isolated by the common sucrose method (13), these mitochondria appear well preserved in electron micrographs (Fig. 1). In spite of the structural integrity, the mitochondria, isolated by different procedures, do not exhibit any significant capacity for oxidative phosphorylation, which amplifies earlier findings (23).

The respiration and phosphorylation carried out by isolated brown fat mitochondria in the presence of different substrates are shown in Table V. As is evident, attempts to obtain phosphorylation in these isolated mitochondria have, so far, been without success, except when glutamate or α-ketoglutarate plus malonate is the substrate.

Some of the variables employed before and during the isolation procedure are listed in Table VI. Special attention has been paid to the possibility that free fatty acids liberated during the isolation procedure may act as an uncoupler of oxidative phosphorylation. This problem has also been particularly recognized in the case of brain tissue, and methods suitable for the preparation of phosphorylating brain mitochondria (16) have been explored to no avail. Nor has an improvement in P/O ratio been obtained by albumin addition. Nor, furthermore, does brown fat from newborn rats, which contains comparatively little lipid, yield mitochondria with a higher phosphorylating capacity.

Finally, if brown fat and liver tissue are mixed and homogenized together, and if mitochondria are isolated from this homogenate, the resulting P/O ratio is found to be almost exactly the value which would be calculated by assuming that the

TABLE VII

Some Enzymic Capacities of Mitochondria Isolated from Brown Fat

Activities expressed as μmoles/g protein/minute of mitochondria from rabbit brown fat tissue.

ATPase (Mg^{++}-stimulated)	68
ATPase (Mg^{++}-stimulated) in the presence of oligomycin	5
ATPase (dinitrophenol-stimulated)	37
ATPase (dinitrophenol-stimulated) in the presence of oligomycin	3
ATPase (Mg^{++} + dinitrophenol)	115
ATPase (Mg^{++} + dinitrophenol) in the presence of oligomycin	3
$P_i \rightleftharpoons$ ATP exchange	Lacking
ATPase of submitochondrial particles	150
ATPase of submitochondrial particles + oligomycin	11
Adenylate kinase	434
Energy-dependent transhydrogenase (reduction of NADP from NADH)*	Lacking
Succinate-linked reduction of NAD*	Lacking

* Tested on submitochondrial particles as well as on intact mitochondria.

liver fraction of the mitochondrial suspension has a P/O ratio of 3 and that the fat fraction has a P/O ratio of 0.2.

In a personal communication, Hohorst and Stratmann have described a method for the isolation of mitochondria from the brown fat of newborn guinea pigs. The respiration of this preparation can be influenced by ADP and inhibited by oligomycin; the oligomycin inhibition can be released by dinitrophenol. We have been able to confirm these results with guinea pigs, but not with rabbits or rats.

Table VII demonstrates the results of testing brown fat mitochondria for the presence of certain enzymic reactions which are accepted to be partial reactions of the mitochondrial energy-generating system. It will be noted that the small but significant ATPase activity is sensitive to oligomycin; this is the only indication, so far discovered, of any phosphorylation coupled to electron transport or of the formation of any high-energy intermediate in mitochondria from brown fat. Attempts to increase the P/O ratio with low concentrations of oligomycin (18) have been unsuccessful. The experiment in Table VIII demonstrates the insensitivity of the phosphorylation of brown fat mitochondria to dinitrophenol, and, further, the presence and absence of respiratory control when α-ketoglutarate or pyruvate + malate, respectively, are used as substrates.

These results clearly suggest that the slight phosphorylation obtained when brown fat mitochondria are incubated with Krebs cycle substrates originates from the substrate-level phosphorylation accompanying the transformation of α-ketoglutarate to succinate. Similar results have been obtained with brown fat mitochondria from cold-adapted rats by Smith et al. (42).

Electron Microscopic Studies of Sections

In the electron microscope, brown fat cells are easily recognized by their large mitochondria and by an almost complete lack of endoplasmic reticulum. These features are readily seen in brown fat from newborn rabbits; furthermore, other features not previously reported have been found to characterize the cells of this tissue.

In the examined animals, the nucleus is located peripherally; and sometimes it is separated from the plasma membrane by only a very narrow zone of cytoplasm. The nucleus is usually deformed in its outline by several large fat droplets. The nucleolus is prominent.

A Golgi apparatus has been found close to the nucleus; it consists of a few inflated vacuoles. In the same region of the cell a centriole has been observed, and surrounding it are several microtubules, seen in cross-section in Fig. 2. Thus, this region can be compared to the cell center of other cells, though it is located far from the geometric center.

The cell membrane exhibits several small invaginations and numerous micropinocytotic vesicles which might account for the high permeability of this tissue to added substrates.

The rest of the cytoplasm contains mitochondria, fat droplets, ribosomes, and glycogen particles. The fat droplets do not seem to have the distinct limiting membrane suggested earlier (10). The electron opacity and the inner structure of the fat droplets vary from one embedding to another; thus, these features seem to be influenced by small variations in the preparation technique. The ribosomes appear in rosettes of five or more. Most of the animals used were fasted overnight before sacrifice, and hence the amount of glycogen in

TABLE VIII

Experiment Demonstrating Substrate Level Phosphorylation in Mitochondria from Brown Fat

Substrate	System	μatom O/mg/30 min	μmole P/mg/30 min	P/O
Pyruvate + malate	Complete	2.26	0.496	0.220
	+ DNP	1.77	0.476	0.269
	− ADP-hexokinase	2.12	—	—
α-Ketoglutarate + malonate	Complete	1.21	1.34	1.11
	+ DNP	1.77	1.68	0.952
	− ADP-hexokinase	0.67	—	—

Condition the same as for experiment in Table V.

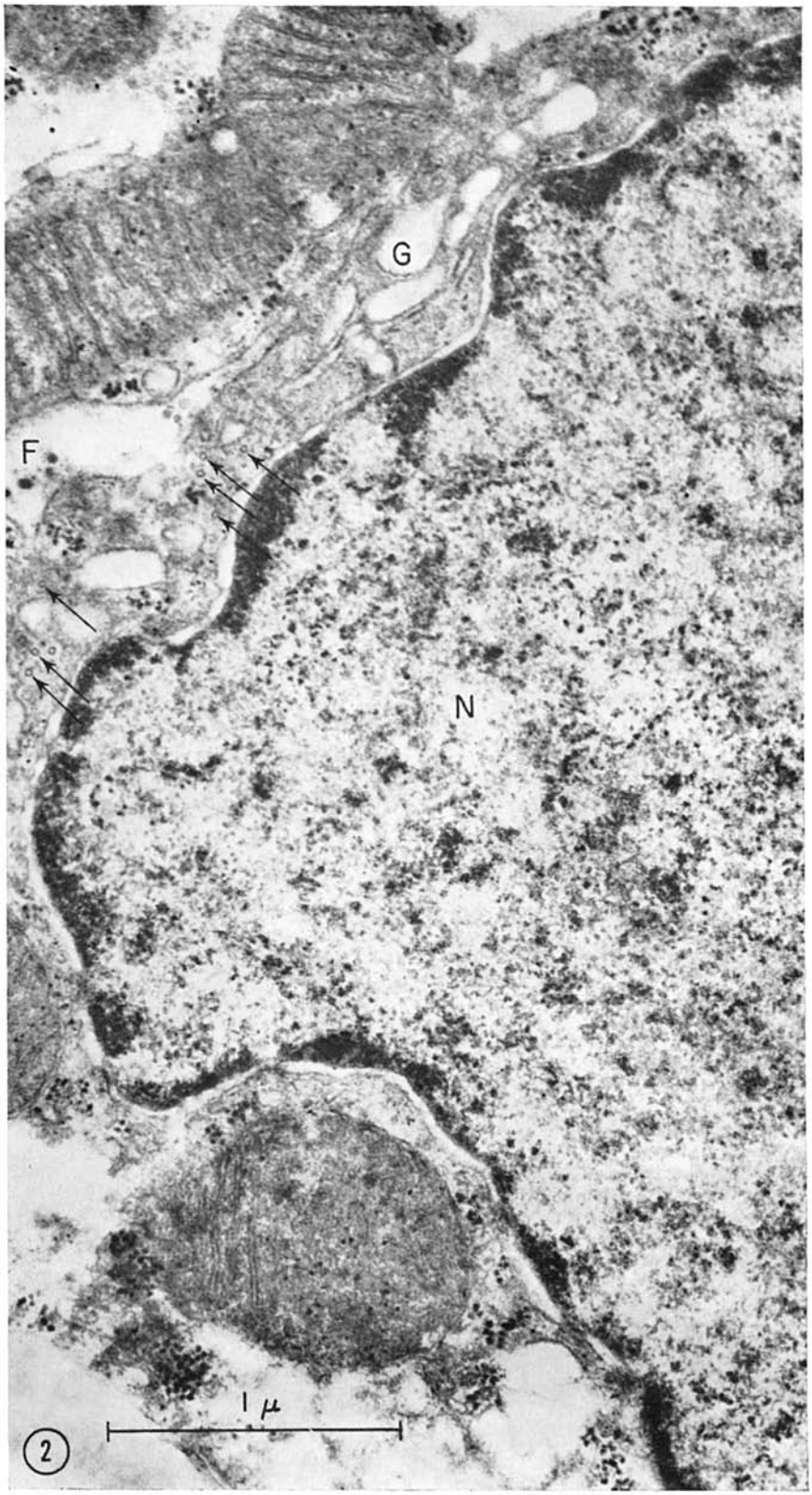

FIGURE 2 Section through the brown fat tissue, showing the peripheral location of the nucleus (*N*). The border between two fat cells are in the upper left part. Transected collagen fibers (*F*) in the intercellular space. Between the nucleus and cell surface there is a small Golgi apparatus (*G*). Several microtubules (arrows) are found in this region. Note the similarity in "staining" properties between the ribosomes and certain granules in the mitochondria (lower left corner). × 44,000.

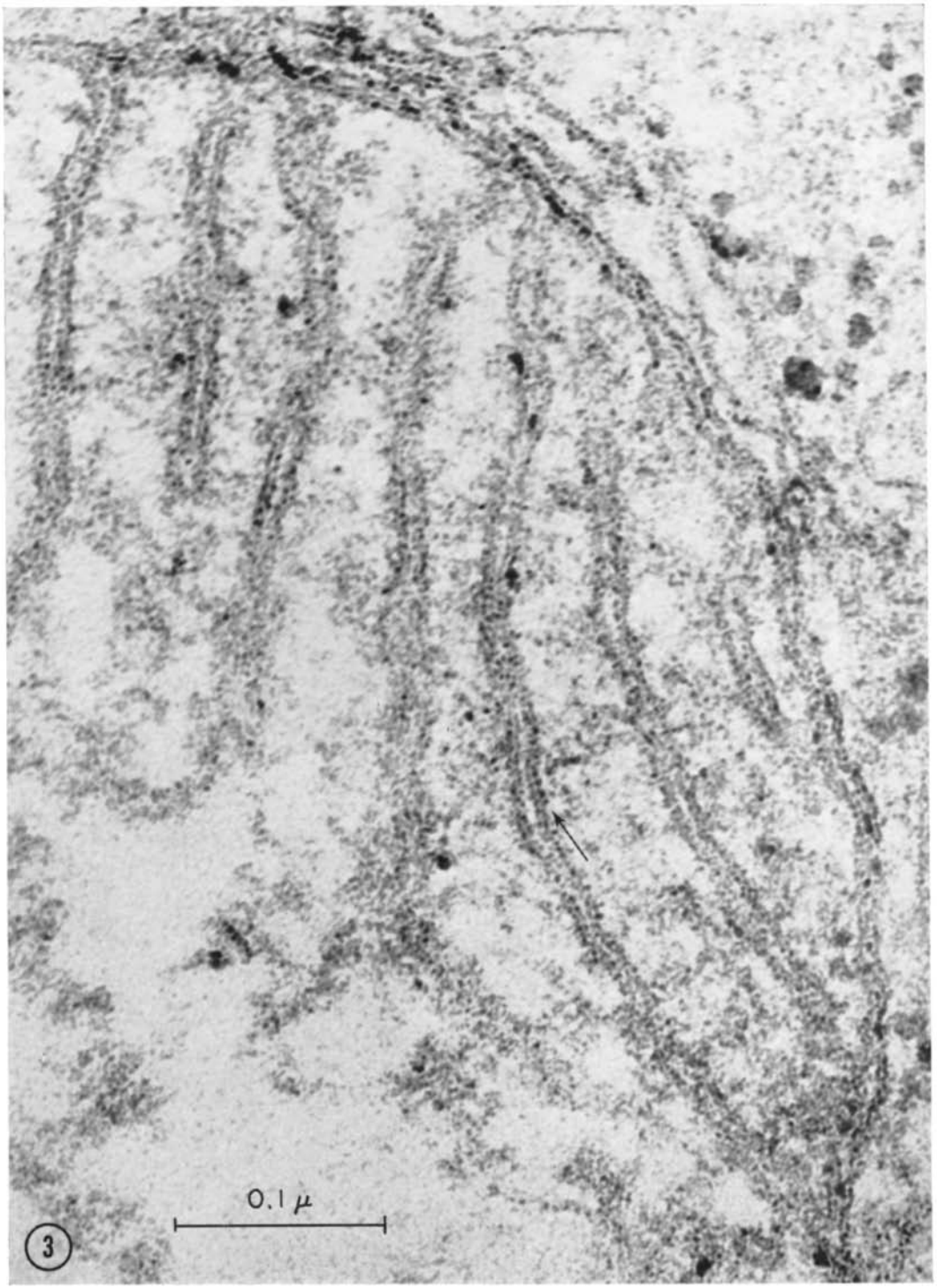

FIGURE 3 At higher magnifications, the membranes of the mitochondria appear double and with indications of a "globular substructure" (see text). × 310,000.

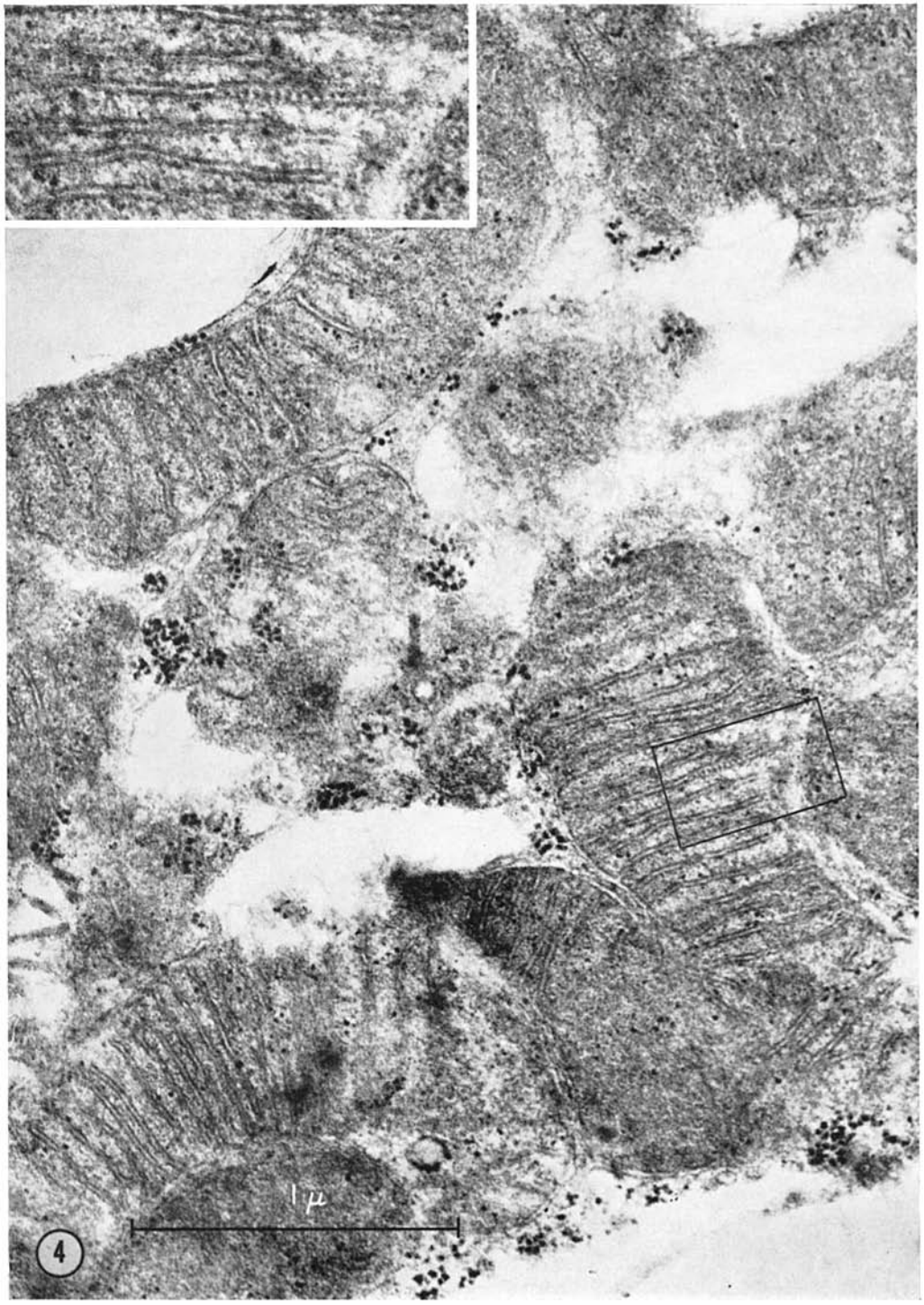

FIGURE 4 Mitochondria from the brown fat cells characteristically have straight and densely packed cristae. The mitochondrial matrix contains small dense particles, and there is another kind of particle in the cristae of a few mitochondria as shown at a higher magnification in the inset. × 47,000. Inset, × 90,000.

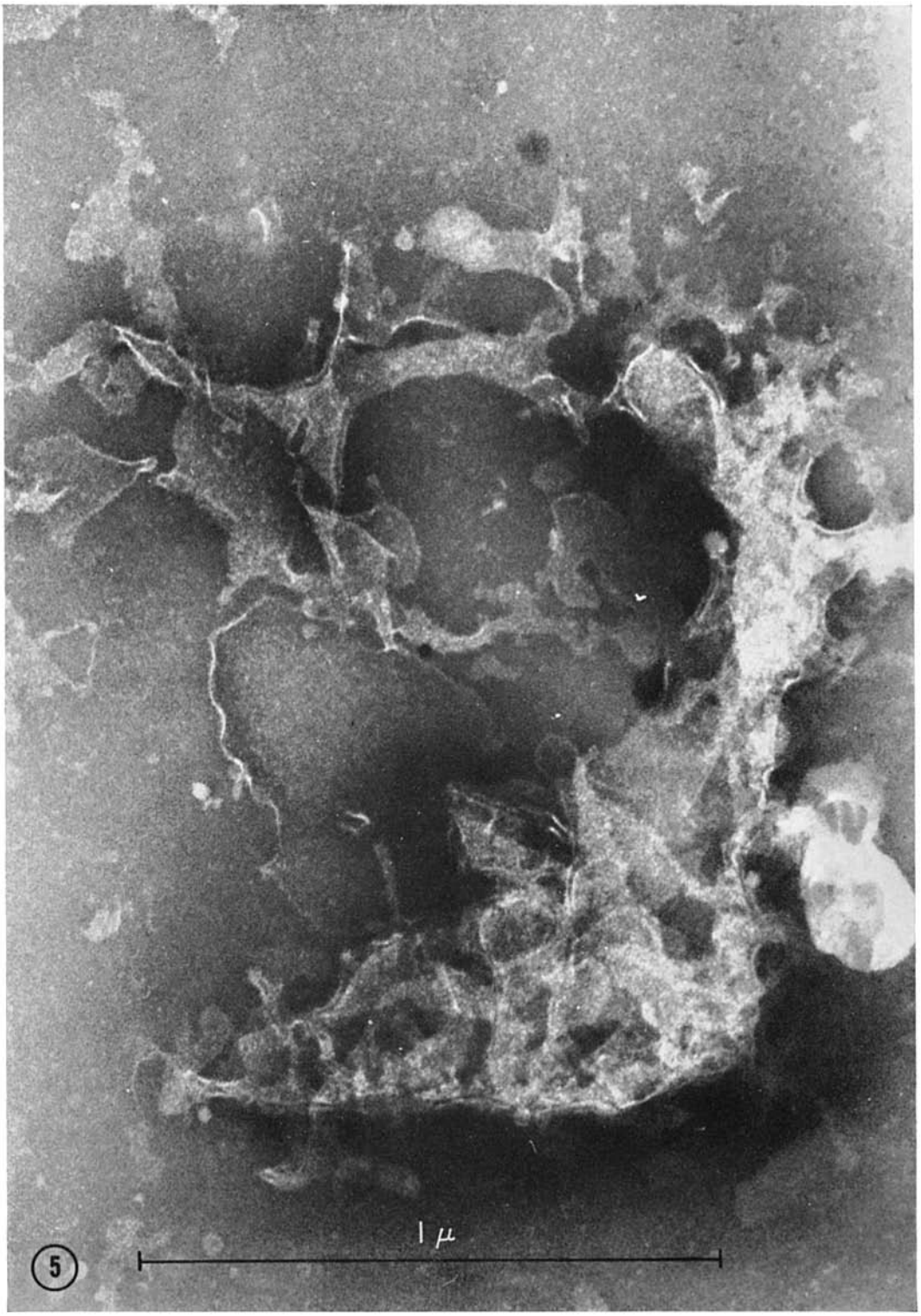

FIGURE 5 A brown fat mitochondrion in a negative-staining preparation. The discoidal shape of the cristae is reasonably well retained. The membranes appear smooth. × 89,000.

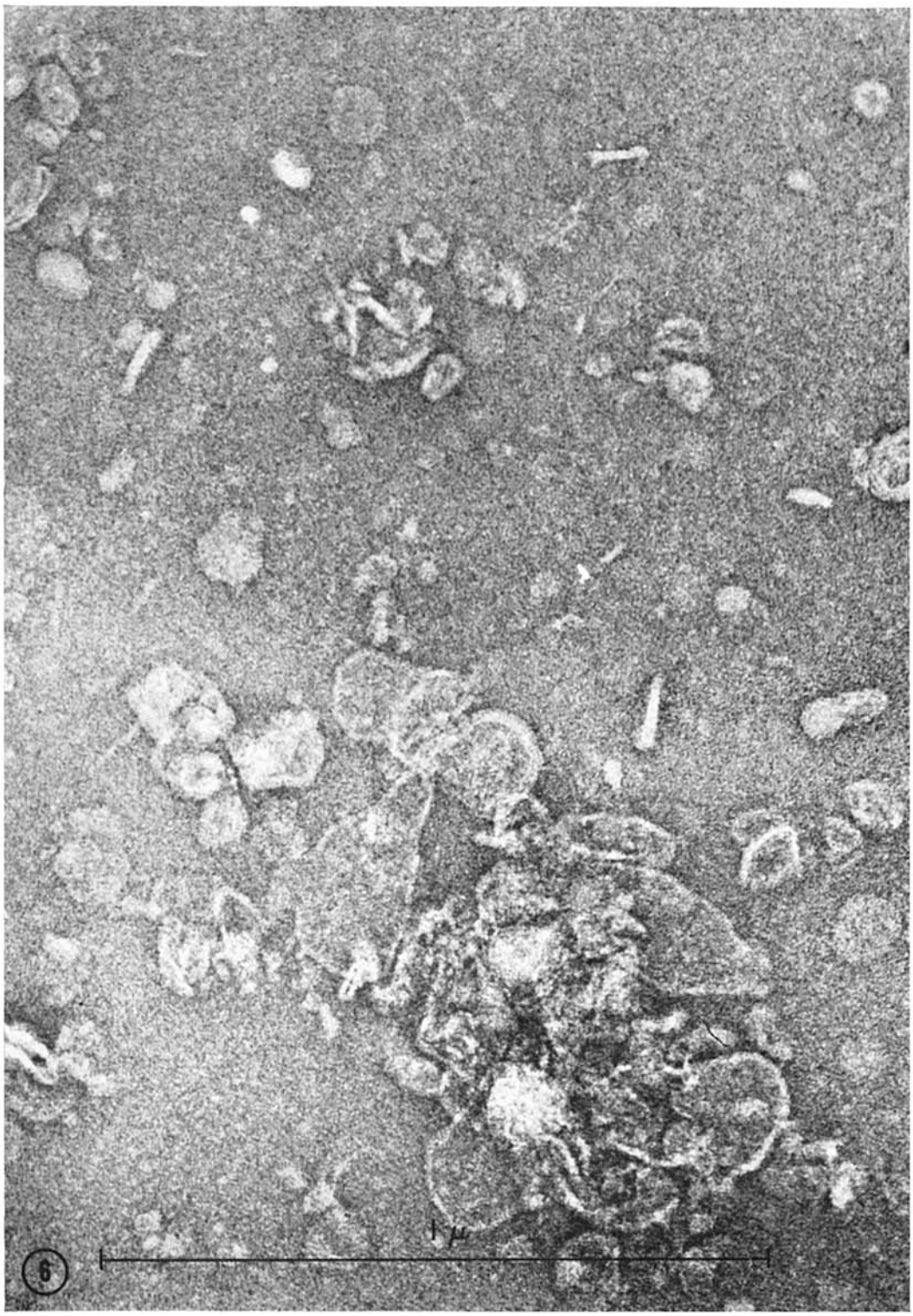

FIGURE 6 A negative-staining preparation of sonicated mitochondria shows submitochondrial particles; that is, a fraction of predominantly isolated crista-fragments. These fragments, like the membranes of negatively stained whole mitochondria, appear smooth. × 105,000.

their brown adipose tissue was low; however, an occasional glycogen cluster could be seen.

Rabbit brown fat mitochondria usually have closely packed cristae and a dense matrix. The cristae extend across the entire width of the mitochondria, and their open connection to the inner stratum of the outer layer of the mitochondria is often evident. After the double-section staining, the individual membranes are triple-layered and sometimes exhibit a "globular subunit structure," thus having the appearance of a rope ladder (Fig. 3).

Several kinds of granules are seen in the mitochondria. Relatively large (approximately 500-A) granules with an uneven contour are considered to be equivalent to the dense matrix granules described by Rhodin (35). Other granules of a more uniform size (130-A) possess the same staining properties as ribosomes in the cytoplasm (Figs. 2 and 4); these are located in the matrix close to the cristae and are believed to be mitochondrial ribosomes as described by André and Marinozzi (3).

A third type of granule is smaller than the others (about 80 A) and has a lower electron opacity; this type is shown in Fig. 4. These small granules

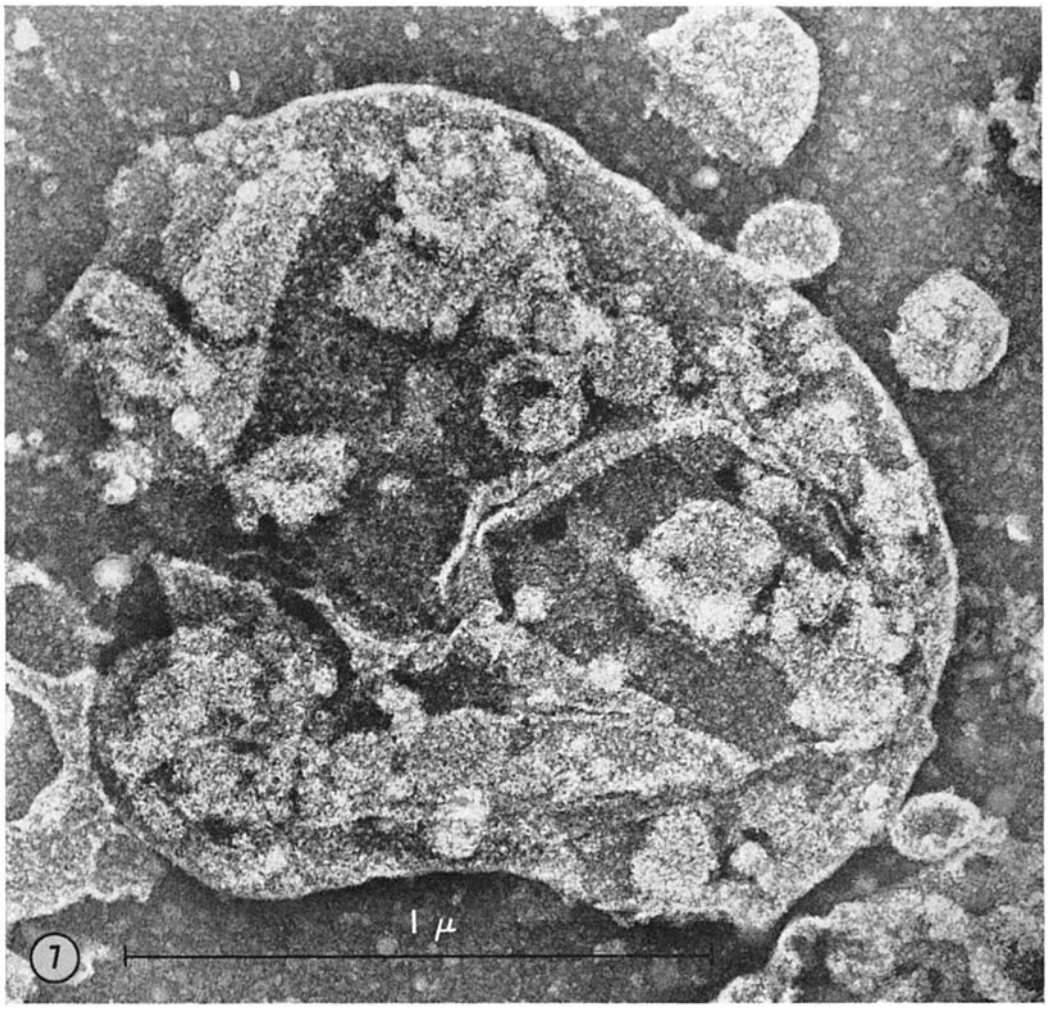

FIGURE 7 A negative-staining preparation of a frozen-thawed brown fat mitochondrion. There are no visible elementary particles, but the membranes have a coarser structure than in freshly prepared mitochondria. × 80,000.

characteristically appear as a row of regularly spaced dots and seem to be a component or modification of the inner stratum of the cristae. The nature of these granules is not understood. They resemble similar particles illustrated by Pease (30) and Picheral (31).

Electron Microscopical Studies of Negatively Stained Mitochondria

Rabbit brown fat mitochondria were also examined as whole mounts in negative-staining preparations (Fig. 5). The mitochondria are readily recognized and show a large number of cristae seen edge-on or flattened towards the supporting film. The membranes appear smooth and thus seem to lack the characteristic stalked elementary particles.

It is possible that elementary particles do exist on the cristae, but that it is unusually difficult to make them visible. Techniques recommended for the purpose of making these particles visible have, therefore, been employed (see Materials and Methods); none of these techniques resulted in preparations showing elementary particles. Fig. 6 shows the appearance of a preparation of isolated cristae-fragments after sonification of the mitochondria. In the freeze-thawing preparation, the surface of the membranes appears coarser than in the other preparations (Fig. 7), which may, or may not, reflect an induced higher porosity of the membrane.

The above findings indicate either a lack of elementary particles in rabbit brown fat mitochondria or an unusual difficulty in making these particles visible.

DISCUSSION

It is generally accepted that the physiological role of brown fat is nonshivering heat production, that free fatty acids are the fuel for this thermogenesis, and that the oxygen consumption of this tissue is controlled by NE (for reviews see 4, 12, 17, and 41). The data presented here are in accordance with these concepts.

The data in Tables I and III support the concept that endogenous fat is the substrate for the increase in respiration brought about by NE: consumption of exogenous substrate by brown adipose tissue is not increased upon the addition of the hormone, whereas oxygen consumption is. It emerges from Table II that the effect of added α-ketoglutarate on the in vitro respiration of brown fat is of a catalytic nature; thus, the presence of exogenous α-ketoglutarate enhances the oxidation of endogenous substrate, presumably fat.

A prerequisite for the cellular oxidation of triglycerides is their breakdown into free fatty acids through a lipase reaction. Among other factors involved, the availability of CoA and of energy for the activation of the free acids is mandatory for the initiation of the metabolic pathways leading to carbon dioxide and water. Since fatty acid oxidation occurs in the mitochondria, either this combustion must be subject to respiratory control (and thus rate-limited by the availability of a phosphate acceptor such as ADP) or else the coupling between respiratory and phosphorylating systems must be loosened to permit a free flow of electrons.

Table I demonstrates that the addition of iodoacetate or sodium fluoride to the incubation mixture completely abolishes the effect of NE on the respiration of brown fat fragments; and this suggests that glycolysis contributes at least one component necessary for the mitochondrial oxidation of free fatty acids. The fact that iodoacetate inhibits glycolysis at the level of phosphoglyceraldehyde dehydrogenase and the inability to reverse the effect of fluoride by adding α-glycerophosphate to the medium suggest that glycolytic ATP rather than glycerophosphate formed from dihydroxyacetonephosphate is necessary for the NE stimulation. Thus, oxidative phosphorylation does not seem able to supply the cell with enough ATP to compensate for the energy consumed in the activation of fatty acids; this might indicate that the respiratory chain in brown fat mitochondria is not coupled to a phosphorylating system during thermogenesis.

The inhibitory effect of dinitrophenol on the NE stimulation of respiration and the lack of effect of the uncoupler on the basal respiration of the tissue might suggest that the action of the hormone is exerted via an ATP-requiring mechanism that is not part of the thermogenesis as such. That the mechanism of NE action may involve unsuspected factors is hinted at by the striking similarity to be found in the manner in which the inhibitors employed here affect the NE control of respiration, on the one hand (Table I), and the lipase activity of adipose tissue, on the other (25).

It is a well established fact that catecholamines stimulate a lipase in adipose tissue which splits endogenous triglycerides (28, 36, 37). Cahill et al.

(8) and Ball and Jungas (5) advanced the hypothesis that the respiration of white fat was regulated by the availability of free fatty acids as a "phosphate-trapping agent" and that the fatty acids thus serve both as substrate and partner for reesterification of α-glycerophosphate formed in glycolysis. According to this mechanism, lipase would serve as the regulatory tool of NE for the liberation of free fatty acid as well as for respiration. The theory is sound and deals with accepted concepts of respiratory control.

Recently, Ball (4) and Dawkins and Hull (12) adapted this reesterification theory for the mechanism of NE-stimulated nonshivering heat production of brown fat and stressed the thermogenetic properties of this "overall ATPase." We cannot see any objections to the application of this theory for epididymal (white) fat, but, however attractive it might be, some modifications seem to be required to fit the conditions of rabbit brown fat. We have confirmed the data of Dawkins and Hull concerning lipase activity in rabbit brown fat and its stimulation by NE. We also find that 1 g of tissue in vitro under the stimulation of NE consumes about 300 μatoms of oxygen per hour, which is in agreement with their results and with those of Joel (17). Under these conditions, the lipase activity is about 14.5 μmoles of free fatty acids liberated per gram and hour. This value is calculated on the basis of glycerol release and with the presumption that all glycerol emanates from the lipase activity and that no glycerol is consumed. Under conditions of total respiratory control (P/O ratio 3), 14.5 μmoles of phosphate acceptor would permit $14.5/3 = 4.8$ μatoms of oxygen to be taken up. Even if this value should be doubled if fatty acid activation involves pyrophosphate formation rather than orthophosphate, it is still definitely too low to account for the uptake of 300 μatoms of oxygen. As pointed out, this calculation is based upon the two assumptions that glycerol is an inert substance in the system and that no other reaction competes with the reesterification of α-glycerophosphate. This is obviously not the case. Treble and Ball (43) find a significant glycerolkinase activity in brown fat of rat, while Dawkins et al. (12) deny its presence in brown fat from newborn rabbits. Whichever is the case, the high capacity of mitochondria of this tissue to oxidize glycerophosphate, as demonstrated in Table V, must limit the availability of this phosphate ester for reesterification of activated free fatty acids. This is also supported by our failure to demonstrate any incorporation of ^{14}C from labeled glycerol phosphate into neutral fat in tissue fragments. The disagreement implies that we have either to look for another kind of "overall ATPase" to serve as a phosphate acceptor system or else postulate that the mitochondria of brown fat have a very low phosphorylative efficiency. The first alternative is difficult to exclude as it might involve reactions of unknown character. But as long as no other NE-sensitive "overall ATPase" can be demonstrated, it might be justified for us to base a working hypothesis concerning the mechanism of nonshivering heat production on the well established NE-stimulated lipase.

The second alternative involving a low mitochondrial phosphorylating efficiency finds support in the findings by Lyskovsky et al. (23) and Smith et al. (42) and the data of Table IV. It emerges that the level of ATP in brown fat is rather low and that conditions that stimulate the respiration of the tissue in vitro, e.g. the addition of NE, decrease the level of ATP. If the decrease is caused by the increased consumption of ATP for the activation of free fatty acids, released through the effect of the hormone, then it is striking that the increased electron transport through the mitochondrial respiratory chain cannot make up the loss of ATP by electron transport–coupled phosphorylation.

In this connection, the lack of any demonstrable "elementary particles" in rabbit brown fat mitochondria is of great interest. Racker and coworkers (32) have hypothesized that these mitochondrial particles contain the factor which couples phosphorylation to oxidation. If it can be confirmed that these structures either are not present on the mitochondrial membranes in brown fat or else behave differently from those in mitochondria from other tissues, and if all the data indicating that brown fat mitochondria cannot carry out oxidative phosphorylation can be trusted—then Racker's hypothesis would be strongly supported by these coincident peculiarities of brown fat mitochondria. This Racker factor is, however, considered to be demonstrable as an oligomycin-sensitive ATPase, which, according to our findings, is present in these mitochondria. Also, a comparison of the results obtained by negative staining with those obtained by examinations of sections is not completely unequivocable: the globular subunit pattern of the mitochondrial cristae, i.e., their appearance as rows of small granules, would seem to indicate that elementary particles are

present; but such indications have not been borne out by the negative-staining method.

Although it is questionable to base theories on negative findings, it is suggestive to visualize the mitochondria from brown fat as being unable to carry out phosphorylation coupled to electron transport. The only energy-conserving reaction so far demonstrated in these mitochondria is the substrate-level phosphorylation connected with the transformation of α-ketoglutarate to succinate. This would imply the production of only one high-energy phosphate bond per Krebs cycle revolution, giving a P/O ratio of 0.2 for the combustion of pyruvate and a P/O ratio of 0.25 for the combustion of any of the Krebs cycle intermediates. In other words, the influence of one molecule of "energy-trapper" on the oxygen consumption of this tissue would be about 15 times that of a similar molecule in tightly coupled mitochondria with a P/O ratio of 3.

Returning to the calculations of the liberated fatty acids, 14.5 μmoles would permit a respiration of 14.5/0.2 = 72.3 μatoms of oxygen consumed. Although this is still a rather low value compared to the 300 μatoms of oxygen per gram of tissue and hour, it offers a basis for the formulation of a theory for the mechanism of thermogenesis in brown fat. The founding of such a theory on that concept that substrate-level phosphorylation is the rate-limiting step in mitochondrial oxidation of fatty acids finds experimental support in the reinduction by α-ketoglutarate of NE-stimulation in a malonate-inhibited system. Even if this were the case, the mode of action of malonate is poorly understood. Also, the finding by van den Bergh (44) that the oxidation of α-ketoglutarate stimulates the activation of fatty acids in rat liver mitochondria supports such a theory, which basically is founded on the discovery by Rossy and Gibson (38) of a GTP-dependent acyl-CoA synthetase.

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BIBLIOGRAPHY

1. Afzelius, B. A. 1966. The fine structure of brown fat mitochondria. Proceedings Sixth International Congress on Electron Microscopy, Kyoto. R. Uyeda, editor. Maruzen Co. Ltd, Tokyo. **2**:359.
2. André, J. 1962. Contribution à la connaissance du chondriome. Étude de ses modifications ultrastructurales pendant la spermiogénèse. *J. Ultrastruct. Res.* 3(suppl.):1.
3. André, J., and V. Marinozzi. 1965. Présence, dans les mitochondries, de particules ressemblant aux ribosomes. *J. Microscop., Paris.* **4**:615.
4. Ball, E. G. 1965. Some energy relationships in adipose tissue. *Ann. N. Y. Acad. Sci.* **131**:225.
5. Ball, E. G., and R. L. Jungas. 1961. On the action of hormones which accelerate the rate of oxygen consumption and fatty acid release in rat adipose tissue in vitro. *Proc. Natl. Acad. Sci. U. S.* **47**:932.
6. Bergmeyer, H. U. 1962. Methoden der enzymatischen Analyse. Verlag Chemie, Weinheim.
7. Brody, I. 1959. The keratinization of epidermal cells of normal guinea pig skin as revealed by electron microscopy. *J. Ultrastruct. Res.* **2**:482.
8. Cahill, G. F., Jr., B. LeBoeuf, and A. E. Renold. 1959. Studies on rat adipose tissue in vitro. III. Synthesis of glycogen and glyceride-glycerol. *J. Biol. Chem.* **234**: 2540.
9. Chance, B., and B. Hagihara. 1963. Direct spectroscopic measurements of interaction of components of the respiratory chain with ATP ADP, phosphate, and uncoupling agents. Proceedings of the 5th International Congress of Biochemistry, Moscow, 1961. N. M. Sissakian, editor. Pergamon Press, Oxford. **5**:3.
10. Cotte, G., F. Tasso, D. Picard, and A. M. Haon. 1964. La cellule de tissu brun au cours de la realimentation après le jeune. Proceedings of the 3rd European Regional Conference for Electron Microscopy, Prague, 1964. M. Titlbach, editor. Publishing House of the Czechoslovakian Academy of Science, Prague. 573.
11. Danielson, L., and L. Ernster. 1963. Energy-dependent reduction of TPN by DPNH. *In* Energy-linked Functions of Mitochondria. B. Chance, editor. Academic Press Inc., New York. 157–180.
12. Dawkins, M. J. R., and D. Hull. 1964. Brown adipose tissue and the response of new-born rabbits to cold. *J. Physiol. (London).* **172**:216.
13. Ernster, L., and H. Löw. 1955. Reconstruction of oxidative phosphorylation in aged mitochondrial systems. *Exptl. Cell Res. Suppl.* **3**:133.
14. Fernández-Morán, H., T. Oda, P. V. Blair, and D. E. Green. 1964. A macromolecular repeating unit of mitochondrial structure and function. *J. Cell Biol.* **22**:63.
15. Hull, D. D., and M. M. Segall. 1965. Sympathetic nervous control of brown adipose tissue and heat production in the new-born rabbit. *J. Physiol. (London).* **181**:458.
16. Jöbsis, F. F. 1963. A study of preparative pro-

cedures for brain mitochondria. *Biochim. Biophys. Acta.* **74**:60.

17. JOEL, C. D. 1965. The physiological role of brown adipose tissue. *In* Handbook of Physiology, Section 5, Adipose Tissue. A. E. Renold and G. F. Cahill, editors. American Physiological Society, Washington, D. C. 59.
18. LEE, C. P., and L. ERNSTER. 1965. Restoration of oxidative phosphorylation in "non-phosphorylating" submitochondrial particles by oligomycin. *Biochem. Biophys. Res. Comm.* **18**:523.
19. LINDBERG, O., J. DE PIERRE, E. RYLANDER, and R. SYDBOM. 1966. Studies of the mitochondrial energy-transfer system of brown fat tissue. *Commun. Third Meeting Fed. Europ. Biochem. Soc.*, Warsaw. 139.
20. LÖW, H., P. SIEKEVITZ, L. ERNSTER, and O. LINDBERG. 1958. Studies on the relation of the adenosinetriphosphate-inorganic phosphate exchange reaction of mitochondria to oxidative phosphorylation. *Biochim. Biophys. Acta.* **29**:392.
21. LÖW, H., and I. VALLIN. 1963. Succinate-linked diphosphopyridine nucleotide reduction in submitochondrial particles. *Biochim. Biophys. Acta.* **69**:361.
22. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
23. LYSKOVSKY, S., W. WANG, T. KOIKE, and M. K. DIMICK. 1959. The oxygen uptake and phosphorus uptake by particulate suspensions (mitochondrial) from liver, brown and white fatty tissues. *Federation Proc.* **18**:2, 272.
24. MILLONIG, G. 1962. Further observations on a phosphate buffer for osmium solutions. Proceedings of the 5th International Congress for Electron Microscopy, Philadelphia. S. S. Breese, editor. Academic Press Inc., New York. **2**:P-8.
25. MOSINGER, B. 1965. Action of lipomobilizing hormones on adipose tissue. *In* Handbook of Physiology, Section 5, Adipose Tissue. A. E. Renold and G. F. Cahill, editors. American Physiological Society, Washington D. C. 601–612.
26. NAPOLITANO, L. 1965. The fine structure of adipose tissue. *In* Handbook of Physiology, Section 5, Adipose Tissue. A. E. Renold and G. F. Cahill, editors. American Physiological Society, Washington D. C. 109.
27. OHNISHI, T., and B. HAGIHARA. 1964. Preparation of yeast mitochondria by an enzymatic procedure. *J. Biochem. (Tokyo).* **56**:484.
28. OKUDA, H., I. YANAGI, and S. FUJII. 1966. The mechanism of *in vitro* stimulation of lipolysis by adrenalin. *J. Biochem. (Tokyo).* **59**:438.
29. PARSONS, D. F. 1965. Recent advances correlating structure and function in mitochondria. *Intern. Rev. Exptl. Pathol.* **4**:1.
30. PEASE, D. C. 1962. Demonstration of a highly ordered pattern upon a mitochondrial surface. *J. Cell Biol.* **15**:385.
31. PICHERAL, B. 1966. Sur l'ultrastructure des mitochondries des cellules du tissu glandulaire du testicule de "Pleurodeles Waltlii" michah. (Amphibien urodèle). *Comp. rend. Acad. Sci. (Paris).* **262**:1769.
32. RACKER, E., B. CHANCE, and D. F. PARSONS. 1964. Correlation of structure and function of submitochondrial units in oxidative phosphorylation. *Federation Proc.* **23**:431.
33. RASMUSSEN, A. T. 1923/24. The so-called hibernating gland. *J. Morphol.* **38**:147.
34. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
35. RHODIN, J. 1954. Correlation of ultrastructural organization and function in normal and experimentally changed proximal convoluted tubule cells of the mouse kidney. Thesis, Karolinska Institute, Stockholm.
36. RIZACK, M. A. 1961. An epinephrine-sensitive lipolytic activity in adipose tissue. *J. Biol. Chem.* **236**:657.
37. RIZACK, M. A. 1964. Activation of an epinephrine-sensitive lipolytic activity from adipose tissue by adenosine 3′,5′-phosphate. *J. Biol. Chem.* **239**:392.
38. ROSSI, C. R., and D. M. GIBSON. 1964. Activation of fatty acids by guanosine triphosphate-specific thiokinase from liver mitochondria. *J. Biol. Chem.* **239**:1694.
39. SABATINI, D. D., F. MILLER and R. J. BARRNETT. 1964. Aldehyde fixation for morphological and enzyme histochemical studies with the electron microscope. *J. Histochem. Cytochem.* **12**:57.
40. SIEKEVITZ, P., H. LÖW, L. ERNSTER, and O. LINDBERG. 1958. On a possible mechanism of the adenosinetriphosphatase of liver mitochondria. *Biochim. Biophys. Acta.* **29**:378.
41. SMITH, R. E., and R. J. HOCK. 1963. Brown fat: thermogenic effector of arousal in hibernators. *Science.* **140**:199.
42. SMITH, R. E., J. C. ROBERTS, and K. J. HITTELMAN. 1966. Non-phosphorylating respiration of mitochondria from brown adipose tissue of rats. *Science.* **154**:653.
43. TREBLE, D. H., and E. G. BALL. 1963. The occurrence of glycerokinase in rat brown adipose tissue. *Federation Proc.* **22**:357.
44. VAN DEN BERGH, S. G. 1965. Two systems for the activation of fatty acids in rat-liver mitochondria. *Biochim. Biophys. Acta.* **98**:442.