

# LIPID SYNTHESIS, INTRACELLULAR TRANSPORT, AND STORAGE

## III. Electron Microscopic Radioautographic Study of the Rat Heart Perfused with Tritiated Oleic Acid

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### ABSTRACT

Rat hearts pulse-labeled by perfusion *in vitro* with 9,10-oleic acid- $^3\text{H}$  for 15 or 30 sec were shown to take up the fatty acid extensively. In hearts postperfused with unlabeled medium for 15 sec or more, 90% of the radioactivity was recovered in esterified lipids. The radioautographic reaction was localized initially over elements of the sarcoplasmic reticulum and mitochondria. After longer periods of postperfusion (2-20 min), there was concentration of silver grains over lipid droplets. In mitochondria and sarcoplasmic reticulum isolated from hearts postperfused for 1 min or more, most of the esterified lipid was in the form of triglyceride. The ratio of the specific activity of isolated sarcoplasmic reticulum triglyceride to mitochondrial triglyceride changed from a value of 3.2 to 1.3 during 5 min of postperfusion. Under conditions of hypothermia, considerable uptake of free fatty acid occurred. The radioactivity recovered in the heart was mostly in the form of free fatty acid, and the radioautographic reaction was seen over sarcoplasmic reticulum and mitochondria, but not over lipid droplets or myofibrils. The results are interpreted to show that intracellular transport of free fatty acid, which occurs also when esterification is repressed, proceeds through intracellular channels, i.e. the sarcoplasmic reticulum. Esterification of fatty acid into triglycerides occurs mostly in the sarcoplasmic reticulum, especially in the region of the dyad, in the vicinity of which lipid is stored in the form of droplets.

### INTRODUCTION

During the past decade, the ultrastructural organization of heart muscle (16, 18, 25, 34, 41) and its metabolic requirements (2) have been extensively investigated. As in skeletal muscle, the transverse tubules of the sarcolemma have been shown to be continuous with the sarcolemma (11, 18, 28, 34) and have been ascribed a prominent role in the conduction of electrical impulses

(11, 13). The rest of the sarcoplasmic reticulum has been compared to the endoplasmic reticulum in other cells (25) and has been said to participate in the general metabolic processes of the cell, including synthesis of energy-rich compounds (17).

In recent years, attention has been directed to the role of lipids as the respiratory fuel for continuous work of the contracting myocardium (2).

The isolated perfused rat heart continues to contract long after the available glycogen stores have been exhausted (31), even though no extraneous caloric source is supplied. Since no increase in protein catabolism (42) or amino acid utilization could be found, the endogenous lipids were considered to be the only utilizable energy store. Opinions differ, however, as to which type of endogenous lipid is used. Shipp et al. (33) concluded, from experiments with rat hearts pre-labeled in vivo, that phospholipids are used mainly; Olson and Hoeschen (20) in a similar type of experiment arrived at an opposite conclusion, namely that the endogenous triglyceride is utilized preferentially.

containing the unlabeled medium, the other the labeled medium. Recirculation perfusion was employed during pulse labeling, while the perfusion with nonradioactive medium was of the nonrecirculation type (40). The perfusion medium (15 ml) consisted of Krebs-Henseleit bicarbonate buffer pH 7.45, 5 mM glucose, 0.14 mM bovine serum albumin (fatty acid poor) (Pentex Inc., Kankakee, Ill.) and 0.42 mM 9,10-oleic acid-<sup>3</sup>H (specific activity 2.5 mc/mole, Radiochemical Centre, Amersham, England). The labeled fatty acid was complexed to dialyzed serum albumin (35), and its radiochemical purity was determined by gas liquid chromatography as described before (37). The distribution of radioactivity in the separated methyl ester fractions was as follows: 0.5% in palmitic acid, 1.5% in palmitoleic acid, 1%

TABLE I  
*Differential Centrifugation of Rat Heart Muscle Homogenate*

10% homogenate in sucrose 0.25 M centrifuged 3500 g min				
Supernatant A		Sediment resuspended in 1/2 original volume centrifuged 3500 g min		
Supernatant B + Supernatant A centrifuged 80,000 g min			Debris discarded	
Mitochondria resuspended, centrifuged 70,000 g min		Supernatant centrifuged 3 × 10 <sup>6</sup> g min		
Mitochondria	Supernatant dis- carded	Supernatant dis- carded	Sarcoplasmic Reticulum (SR)	

In the present study, advantage was taken of the fact that in a perfusion system in vitro, labeled free fatty acids are taken up by the rat heart, oxidized to carbon dioxide, and converted into complex lipids (19, 32, 35). Such an isolated system presented an opportunity for us to obtain a highly labeled preparation and thus to visualize some of the processes concerned with fatty acid uptake and its intracellular transport, using electron microscopic radioautography.

## MATERIALS AND METHODS

### *Heart Perfusion*

Male albino rats of the Hebrew University strain fed Purina laboratory chow and weighing 180-200 g were used; from some animals food was withheld for 24 hr. The hearts were perfused as described before (35) but with minor modifications. The perfusion apparatus consisted of two sets of chambers, one

in stearic acid, 97% in the oleic acid fraction. The nonradioactive perfusion medium was the same as above, but did not contain added fatty acid.

### *Subcellular Fractionation*

Mitochondria and elements of the sarcoplasmic reticulum (SR) were separated by differential centrifugation from heart homogenates prepared in 0.25 M sucrose according to the scheme shown in Table I. For the determination of purity of the subcellular fractions, the pellets were fixed in osmium tetroxide and sections of Epon-embedded material were examined under the electron microscope. The mitochondrial or SR pellets derived from hearts labeled by perfusion with oleic acid-<sup>3</sup>H were resuspended in water, and the lipids were extracted with 20 vol of chloroform:methanol (2:1 v/v).

### *Extraction and Fractionation of Lipids*

The lipids extracted in chloroform:methanol (2:1 v/v) were purified according to Folch et al. (10).

Radioactivity was determined in the chloroform and aqueous phases and in the interphase (37). The distribution of radioactivity in lipid classes was determined in fractions separated by thin-layer silica gel chromatography (37). Separation of triglycerides, free fatty acids, diglycerides, and phospholipids was carried out in petroleum ether (30–60°):diethyl-ether:glacial acetic acid, 80:20:1 (v/v). For the determination of specific activity of triglycerides, the fraction separated on a thin-layer HR silica gel plate (Desaga, Darmstadt, Germany) was scraped and transferred into a column, 0.5 cm in diameter, and the triglycerides were eluted with 20 ml of chloroform, aliquots of which were taken for glycerol (14) and radioactivity determination (37).

#### *Preparation of Tissue for Electron Microscopy and Radioautography*

Aliquots of the perfused hearts were fixed in sodium cacodylate-buffered glutaraldehyde for 1 hr (30) and, following washing in five changes of buffered 0.25 M sucrose for 2–3 hr, were postfixed in two changes of 2% osmium-tetroxide fixative (4) for 45 min each. Some hearts were fixed directly by injection of 2% osmium-tetroxide fixative through the aortic cannula, and areas which blackened immediately were excised and the fixation was continued in two changes of fresh fixative, 45 min each. In all instances, dehydration was carried out at 0° in two changes (5 min each) of 70 and 95% ethanol and three changes each of pure Epon. Infiltration with complete Epon mixture was carried out at 4° overnight and continued for 2–3 hr at 37° (36). The loss of radioactivity during the entire procedure was monitored, and results of a representative experiment are shown in Table II. The radioactivity remaining in the tissue was determined following its homogenization in 50 vol of chloroform:methanol (2:1 v/v). The colored extract of the labeled lipids was treated with a few drops of 30% hydrogen peroxide (36).

Sections showing silver-to-gold interference colors were prepared with an LKB microtome with glass knives. Electron microscopic radioautography was carried out according to Caro and van Tubergen (3), with the Ilford L4 research emulsion. Following exposure of 1–8 wk, the radioautographs were developed in Microdol X for 5 min at 20°, and fixed in Kodak fixer (Eastman Kodak Co., Rochester, N.Y.) for 5 min. Some grids were developed for 1½ min in paraphenylenediamine (physical developer; see reference 3) and fixed in Kodak F24 fixer for 2 min. All sections were washed briefly and stained with lead citrate (27) by a method for precipitate-free staining (6). Grain counts were performed on electron micrographs taken at an instrument magnification of 3,000 and enlarged thereafter. 200–400

TABLE II  
*Fate of Radioactivity in Rat Heart Muscle During Specimen Preparation for Electron Microscopy*

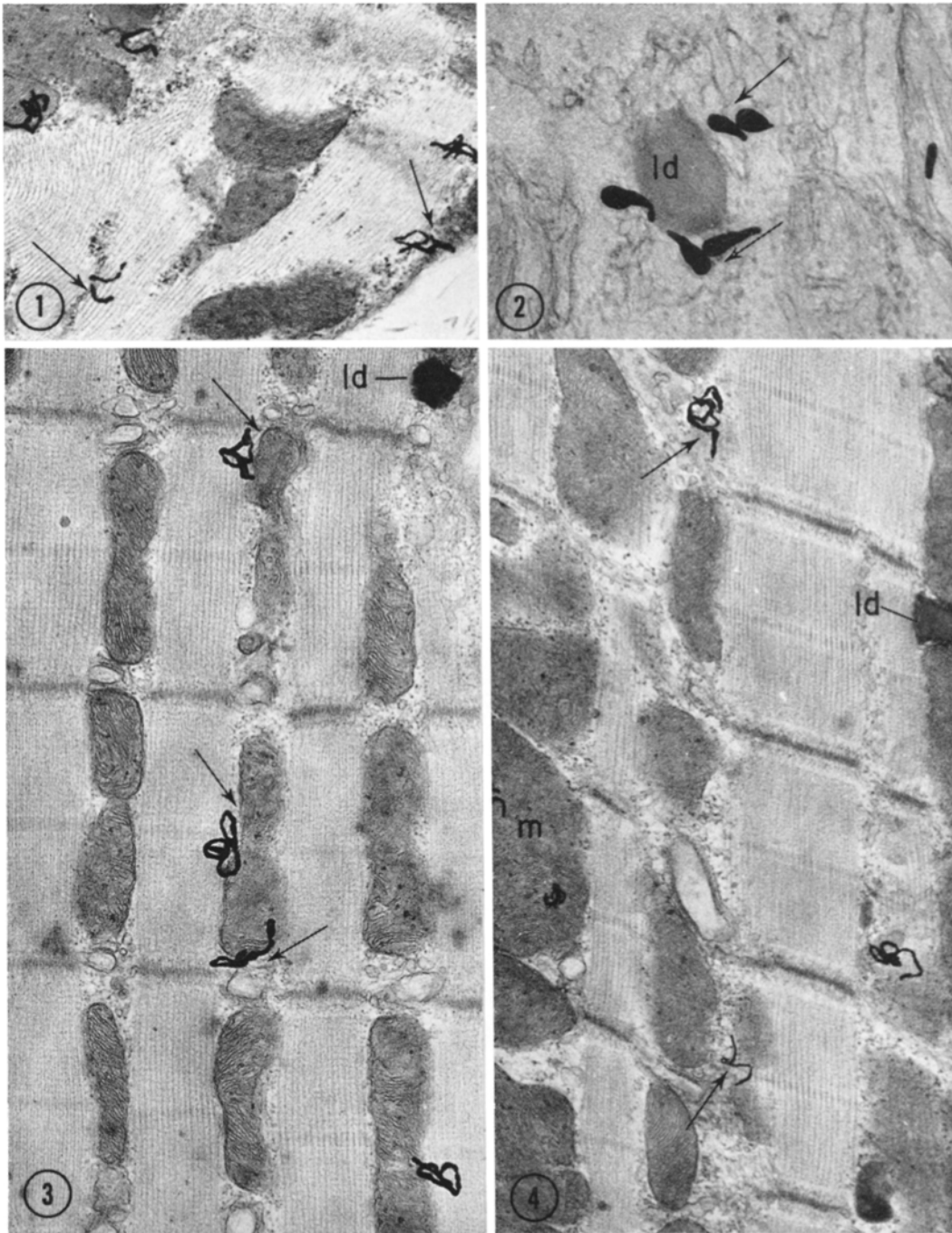
	Radioactivity recovered
	%*
Glutaraldehyde	3.4
Sucrose	1.3
Ethanol, 70%	0.5
Ethanol, 95%	0.6
Epon	3.0
Epon mixture	1.0
Tissue	78.2
Recovery	88.0

\* Aliquots of rat heart muscle perfused for 1 min with 9,10-oleic acid-<sup>3</sup>H were homogenized in chloroform:methanol (2:1 v/v), and the radioactivity recovered per unit weight was taken as 100%. Other aliquots were fixed in glutaraldehyde, washed in sucrose, postfixed in osmium tetroxide, and dehydrated as shown above. Only negligible amounts of radioactivity were found in osmium tetroxide from heart tissue prefixed in glutaraldehyde. The radioactivity found in glutaraldehyde and sucrose remained in the aqueous phase following extraction of the sample according to Folch et al.

total grain counts were obtained for each time interval studied. Those grains which were partially over mitochondria were included in the mitochondrial count if 2/3 or more of the grain was located over the mitochondrion (9); otherwise, the grain was counted as label over SR which adheres closely to the mitochondrion. Since in numerous electron micrographs examined the areas occupied by the myofibrils were mostly not labeled, only those grains which were not adjacent to the SR or mitochondria were included in the myofibril count.

#### RESULTS

Rat hearts perfused in vitro for periods up to 30 min showed a satisfactorily preserved ultrastructure. During the experimental period the heart rate was regular, 200–240 beats per min, and the flow ranged from 6 to 8 ml per min. The following experiments were performed: pulse labeling for 15 sec and fixation without postperfusion washout; pulse labeling for 15 sec and postperfusion for ¼–5 min; pulse labeling for 30 sec and postperfusion from 15 sec to 20 min; continuous labeling up to 20 min followed by 30 sec of postperfusion wash-



FIGURES 1-4 Radioautographs of sections of rat heart muscle, pulse labeled by perfusion with oleic acid- $^3\text{H}$  for 15 sec and fixed by perfusion with osmium tetroxide. The radioautographic reaction is seen over the sarcoplasmic reticulum (arrows) and mitochondria (*m*). Lipid droplets (*ld*) are not labeled. The silver grains in Fig. 2 are much reduced in size by development in a "physical developer," and their localization to the elements of the SR is seen (arrows). Fig. 1,  $\times 18,500$ . Fig. 2,  $\times 60,000$ . Fig. 3,  $\times 20,000$ . Fig. 4,  $\times 20,000$ .

out; continuous labeling for 1–2 min at 4° without washout. In all the experiments, the hearts were perfused with oleic acid-<sup>3</sup>H and the chromatographic identification and radioautographic localization of the labeled lipid was carried out.

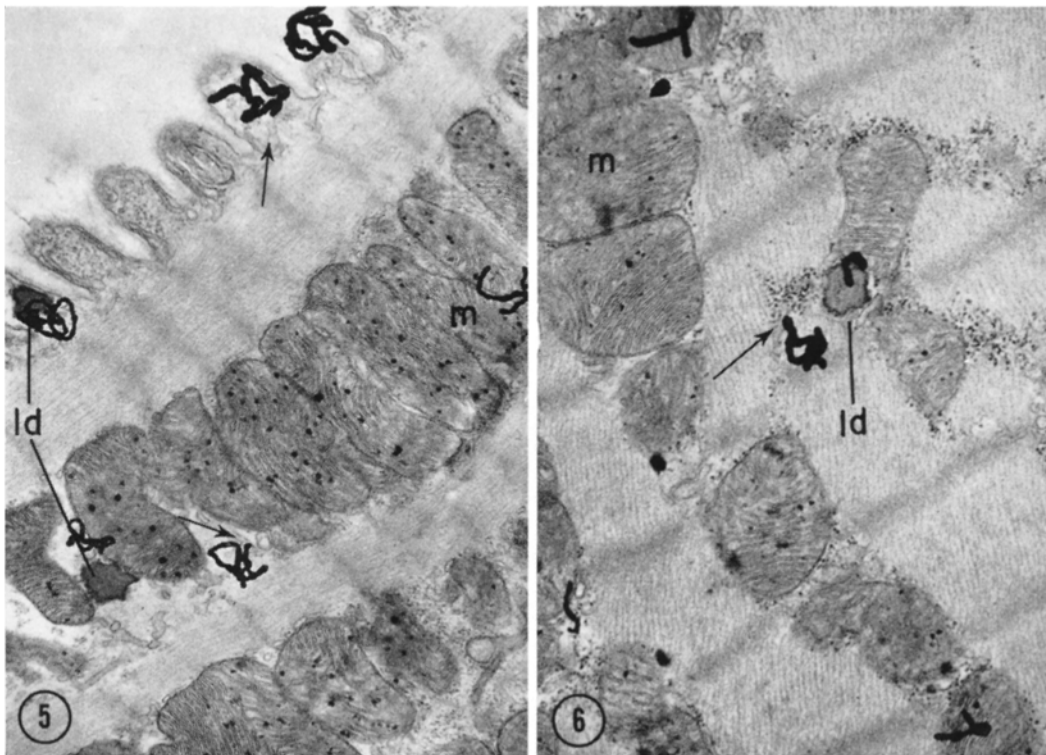
### Pulse Labeling

The hearts pulse labeled for 15 or 30 sec either were fixed immediately (Figs. 1–4) or were washed by perfusion with nonradioactive medium for 15 sec (Figs. 5 and 6). Pulse labeling for 15 or 30 sec resulted in a rapid uptake of the fatty acid by the muscle cells, and when postperfusion wash-out was continued for 15 sec or more, about 90% of the label was in esterified form (Table III). The radioautographic reaction was seen mostly over elements of the sarcoplasmic reticulum (SR) and mitochondria. Some grains were associated with subsarcolemmal vesicles, whereas others were seen in different regions of the longitudinal SR and occasionally close to the transverse tubule, in the region of the dyad (Figs. 1–6). After 15 sec of

labeling, the lipid droplets were mostly not labeled (Figs. 2–4; Table IV), while in hearts labeled for 30 sec a higher per cent of labeled droplets was found (Figs. 5, 6; Table V). Fig. 2 demonstrates the advantage of the development with physical developer. Owing to the reduction of the grain size, it is possible to determine that the grains are localized still over the SR, in the vicinity of the lipid droplet. In these and numerous other electron micrographs, which were used for grain counting, only a small per cent of the grains was found over the myofibrils (Tables IV and V).

### Pulse Labeling and Postperfusion

When the hearts were pulse labeled for 15 or 30 sec and then perfused with nonradioactive medium, there was a rapid fall in the labeled free fatty acid, some of which had been washed out from the vascular tree, while most was esterified into neutral lipids (Table III). The fall in heart radioactivity which occurred between 2 and 20 min of postperfusion with nonlabeled medium



FIGURES 5 and 6 Radioautographs of sections of heart muscle pulse labeled for 30 sec and washed by perfusion with nonlabeled medium for 15 sec. Silver grains are seen over subsarcolemmal cisternae, elements of the SR (arrows), mitochondria (*m*), and lipid droplets (*ld*). Fig. 5,  $\times 19,000$ . Fig. 6,  $\times 19,000$ .

(Table III) was not accounted for by the initial washing out of intravascular label and could be interpreted as utilization of the labeled lipid by the heart. Following postperfusion of 2 and 5 min, there was some change in the distribution of the radioautographic reaction over the muscle cell. While grains were still present over the elements of the SR (Figs. 7, 8, 11), there was more label over the mitochondria (Fig. 8) and lipid droplets (Figs. 7 and 8) than at the earlier time interval studied. Concentration of silver grains over lipid droplets was even more prominent after 20 min of postperfusion, and at that time label was present also over mitochondria and SR (Figs. 9, 10, 12; Table IV). Figs. 11 and 12 show again the easier localization of silver grains, following physical development, to elements of the SR, mitochondria, and lipid droplets, 2 and 20 min after postperfusion.

#### Continuous Labeling

Continuous perfusion with labeled oleic acid resulted in a fatty acid uptake progressive with

time (Table III). The radioactivity present in the heart after 30 sec of postperfusion washing was more than 90% esterified, mainly in the form of triglyceride. The radioautographic reaction, present at first mostly over SR and mitochondria, became concentrated over lipid droplets; representative examples are shown in Figs. 13-15, and the results of grain counts are summarized in Table V.

#### Labeling at 4°

In order to visualize the transport of free fatty acid from the circulation into the heart muscle cell, we made an attempt to inhibit esterification, by perfusing the heart under conditions of hypothermia. As seen in Table III, following 1 or 2 min of perfusion at 4° a considerable uptake of fatty acid occurred, and analysis of labeled lipids recovered from the heart has shown that 80% of the label was in unesterified fatty acid. The radioautographic reaction was seen over the longitudinal elements of the SR and over subsarcolemmal vesicles and mitochondria, but the myofibrils and

TABLE III  
Incorporation of 9,10-Oleic Acid-<sup>3</sup>H into Esterified Lipids by the Perfused Rat Heart

Perfusion with			Distribution of radioactivity		
Oleic acid- <sup>3</sup> H	Nonlabeled medium	Oleic acid- <sup>3</sup> H uptake $\mu$ moles per g wet wt	Fatty acid	Tri- and diglycerides	Phospholipids
min	min		%	%	%
Pulse labeling					
1/4	0	28	43.0	35.5	21.5
1/4	1	28	10.0	72.0	18.0
1/4	5	21	7.0	78.5	14.5
1/2	1/4	56	12.1	67.4	20.5
1/2	1/4	53	11.1	64.9	24.0
1/2	2	30	12.9	70.6	16.5
1/2	5	20	11.5	73.0	15.5
1/2	10	12	6.0	77.4	16.6
1/2	20	8	6.7	63.7	29.6
Continuous labeling					
1	1/2	92	7.0	74.7	18.3
1	1/2	79	6.8	78.7	14.5
2	1/2	149	6.1	81.0	12.9
5	1/2	265	5.6	80.7	13.7
20	1/2	337	3.3	84.4	12.3
Continuous labeling at 4°					
1	0	80	81.3	11.7	7.0
2	0	112	76.3	14.6	9.1

In all experiments, except the "continuous labeling" group, the hearts were derived from rats fasted for 24 hr.

TABLE IV  
Distribution of Grains Over Cytoplasmic Structures in Muscle Cells of Rat Hearts Perfused Continuously with 9,10-Oleic Acid-<sup>3</sup>H

Cytoplasmic structures	Perfusion time						
	<i>min</i> ¼	<i>min</i> 1*	<i>min</i> 1	<i>min</i> 2	<i>min</i> 5	<i>min</i> 10	<i>min</i> 20
	Distribution of grain counts						
	%	%	%	%	%	%	%
Mitochondria	30.8	43.2	39.3	38.1	36.3	34.2	35.4
Sarcoplasmic reticulum‡	65.6	54.4	43.3	39.0	39.0	30.4	27.4
Lipid droplets	1.2	1.2	13.5	18.4	23.7	33.4	35.2
Myofibrils	2.4	1.2	3.9	4.5	1.0	2.0	2.0
Total counts	325	330	230	262	198	209	278

\* Perfused at 4°C.

‡ Includes the transverse tubular system.

All hearts, except those labeled for ¼ min and for 1 min at 4°, were washed by perfusion for 30 sec.

TABLE V  
Distribution of Grains Over Cytoplasmic Structures in Muscle Cells of Perfused Rat Hearts, Pulse Labeled for 30 Sec with 9,10-Oleic Acid-<sup>3</sup>H and Postperfused with Nonradioactive Medium

Cytoplasmic structures	Postperfusion with nonradioactive medium			
	<i>min</i> ¼	<i>min</i> 2	<i>min</i> 5	<i>min</i> 20
	Distribution of grain counts			
	%	%	%	%
Mitochondria	27.3	35.7	34.3	29.0
Sarcoplasmic reticulum*	56.1	33.9	34.3	28.5
Lipid droplets	13.9	25.1	28.0	42.0
Myofibrils	2.7	5.3	3.4	1.0
Total count	209	212	347	200

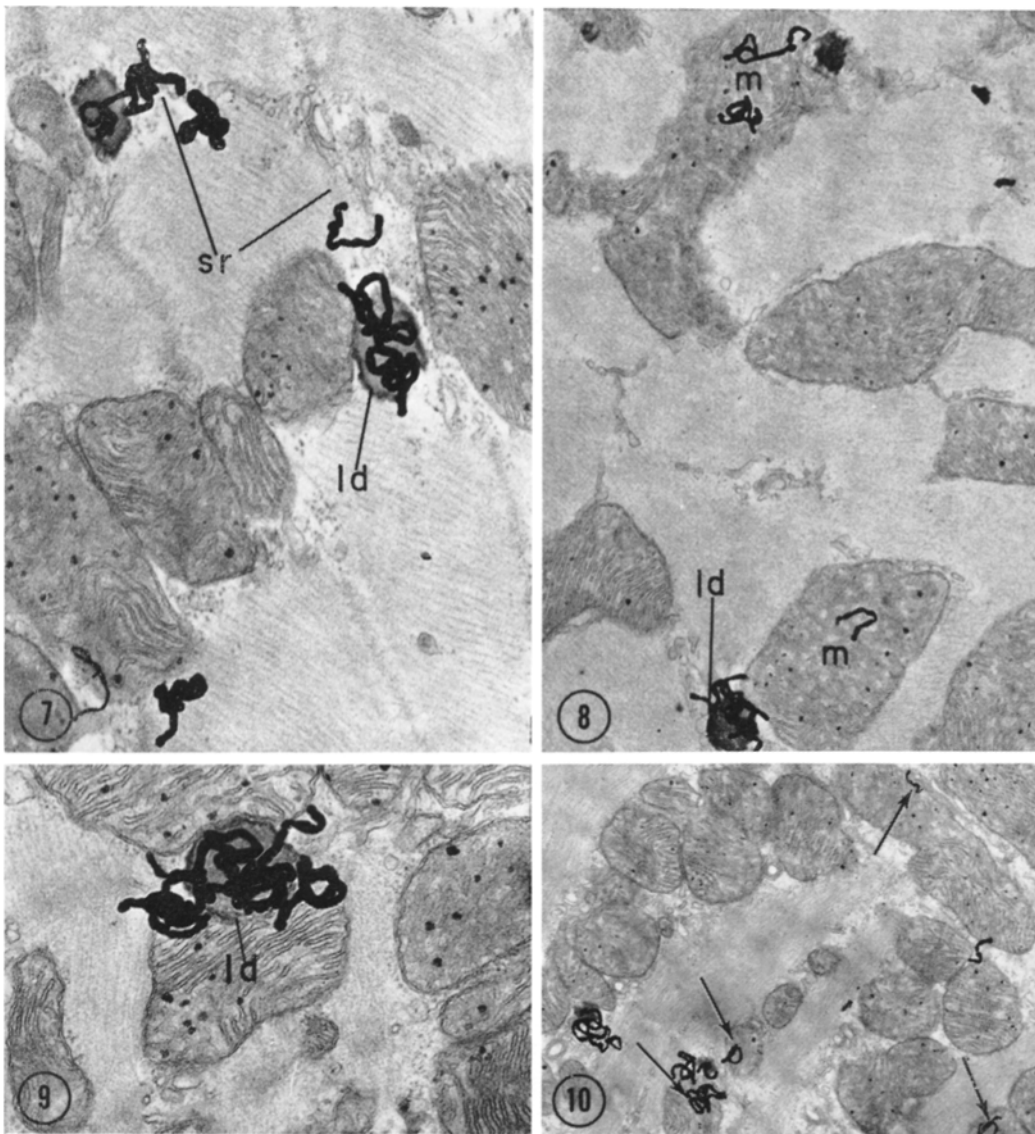
\* Includes the transverse tubular system.

lipid droplets were not labeled (Figs. 16-20; Table IV).

#### Specific Activity of Mitochondrial and SR Glycerides

In order to correlate the radioautographic reaction found over the mitochondria and the SR (as shown in previous figures) with a more closely identified lipid class, we prepared pure fractions of mitochondria and SR from hearts labeled for 15 sec and washed by nonrecirculation perfusion for 1 and 5 min. In both the SR and mitochondrial fractions, most of the radioactivity was found in

glycerides (Table VI). Radioautographs of sections obtained from pelleted mitochondria and SR, isolated from hearts pulse labeled for 15 sec and postperfused for 1 min, are shown in Figs. 21 and 22. The radioautographic reaction present over the mitochondria, though scanty, shows that the labeled triglycerides are of mitochondrial origin. The specific activity of the triglyceride in these isolated subcellular fractions was determined. The ratio of the SR to mitochondrial triglyceride specific activity changes from a value of 3.2 at 15 sec to 1.3 after 5 min of washout (Fig. 23).



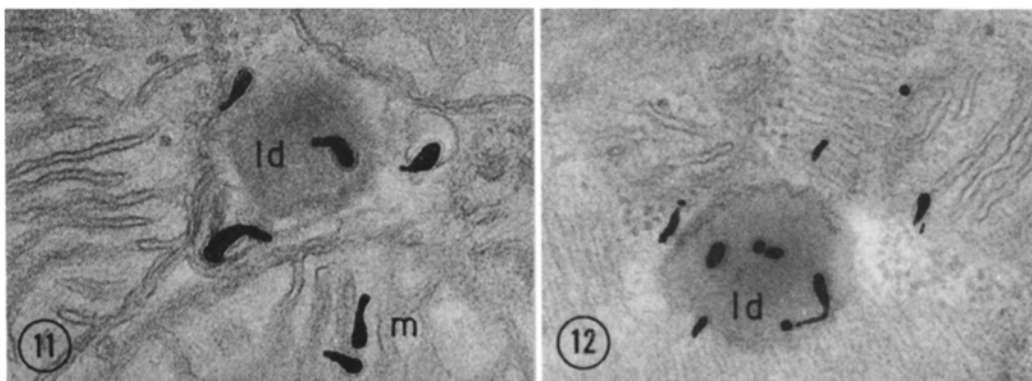
FIGURES 7-10 Radioautographs of sections of heart muscle pulse labeled for 30 sec and postperfused with nonradioactive medium for 2 and 5 min (Figs. 7, 8) and 20 min (Figs. 9, 10). There is concentration of label over lipid droplets (*ld*), and grains are seen over mitochondria (*m*, arrows) and SR. Fig. 7,  $\times 26,000$ . Fig. 8,  $\times 18,500$ . Fig. 9,  $\times 30,000$ . Fig. 10,  $\times 12,000$ .

#### DISCUSSION

In the present study, good preservation of the ultrastructure of the perfused rat heart was observed. Perfusion under conditions of hypothermia caused some distention of the transverse tubules, but in other preparations the expansion of the extracellular space mentioned by Orth and

Morgan (21) was not so apparent, probably owing to the inclusion of albumin in the perfusate. In view of the ultrastructural integrity of the heart muscle cell, the study of the passage of the labeled fatty acid from the vascular system, its site of entry into the muscle cell, its intracellular distribution, and metabolic fate was attempted.





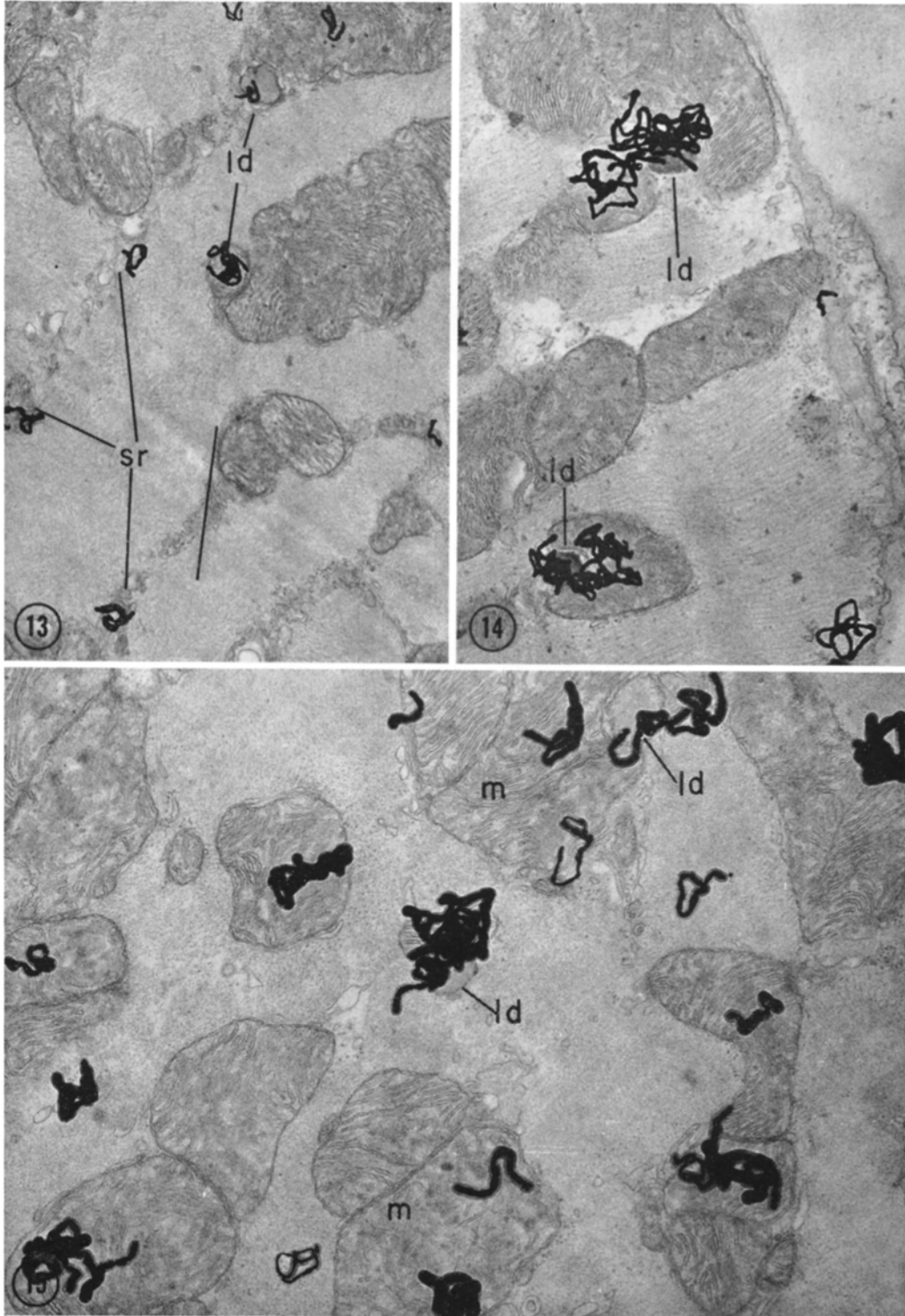
FIGURES 11 and 12 Radioautographs developed in "physical developer." Both sections are from hearts labeled for 30 sec and postperfused for either 2 min (Fig. 11) or 20 min (Fig. 12). The silver grains in the vicinity of the lipid droplet in Fig. 11 are seen over profiles of SR and over a mitochondrion (*m*); in Fig. 12 there is concentration of label over the lipid droplets (*ld*). Fig. 11,  $\times 55,000$ . Fig. 12,  $\times 51,000$ .

TABLE VI  
Distribution of Radioactivity in Sarcoplasmic Reticulum (SR) and Mitochondria  
in Rat Hearts Pulse-Labeled with 9,10-Oleic Acid- $^3\text{H}$  for 15 Sec and  
Washed with Nonlabeled Medium

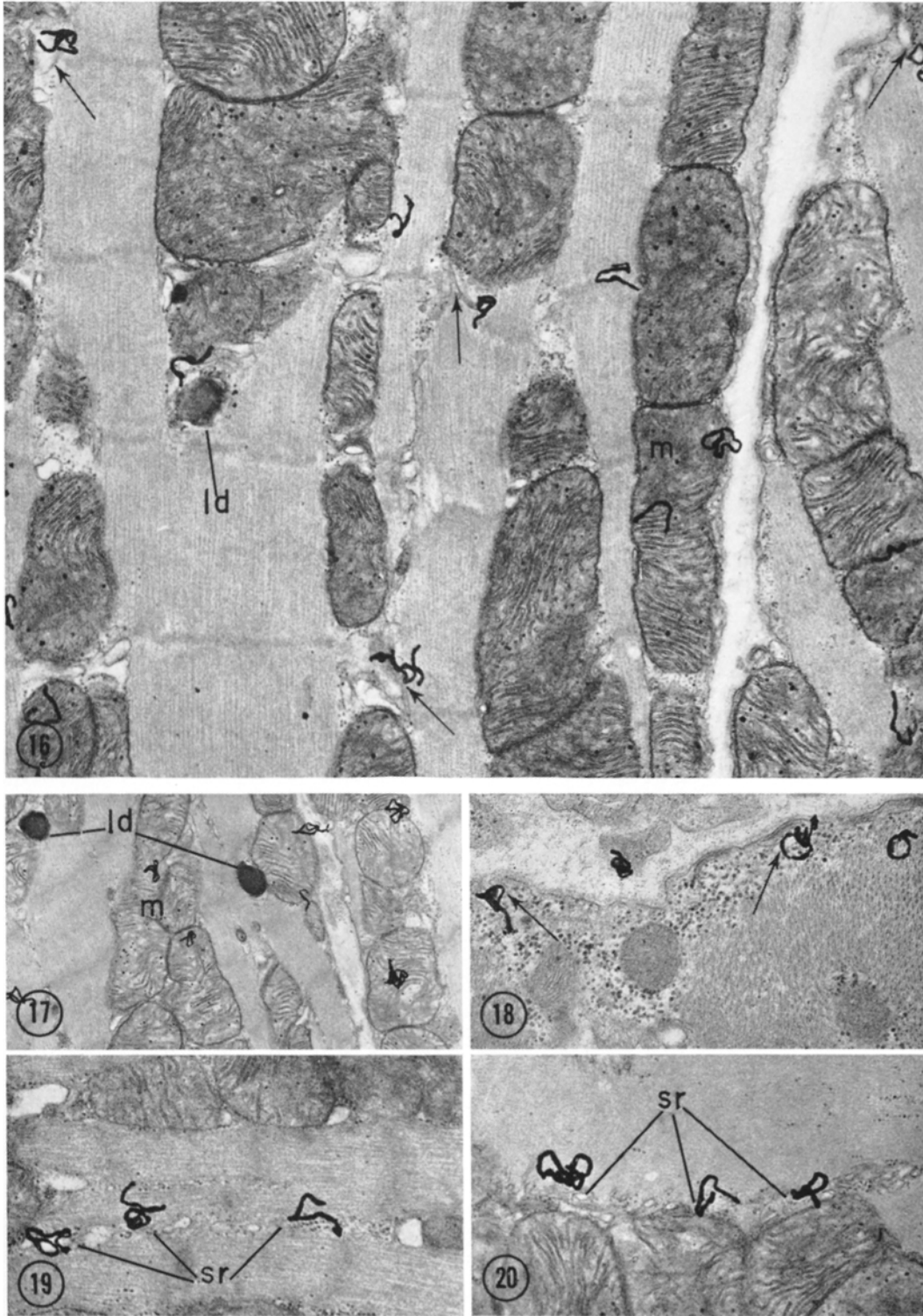
Postperfusion	Fraction	Distribution of radioactivity		
		Fatty acid	Tri- and diglycerides	Phospholipids
<i>min</i>		%	%	%
1	SR	12.3	58.2	29.5
	Mitochondria	15.5	74.1	10.4
5	SR	10.8	52.8	36.4
	Mitochondria	11.5	77.7	10.8

In order to be able to study these various steps with the help of radioautography, it was necessary to devise experimental conditions under which most of the radioautographic reaction at a given time interval would be due to one lipid class, namely, either free fatty acid or esterified lipid. Since, following perfusion of the heart under hypothermia, most of the labeled lipid was in the form of free fatty acid, the localization of the radioautographic reaction in these experiments demonstrated that the penetration and intracellular transport of the free fatty acid occur even when esterification is repressed. The lack of label concentration over lipid droplets (Figs. 16, 17) under these conditions, as compared to 1-min perfusion at  $37^\circ$  (Table IV), agrees well with the radiochemical data and may serve as additional

evidence that the lipid seen in the form of droplets is in esterified form. The lack of grains over the myofibrils following perfusion seems to indicate that the fatty acid is channeled intracellularly through selective pathways and not by random diffusion. The path of the free fatty acid can be tentatively reconstructed in the following manner: after having crossed the sarcolemmal membrane at the cell surface or at the level of the transverse tubule, the fatty acid reaches the lateral cisternae of the longitudinal reticulum through which it spreads further into the interior of the cell. Under conditions of hypothermia, the free fatty acid reaches the mitochondrion, which in vitro is known to bind free fatty acid at  $0^\circ$  (26). Whether this sequence of events is strictly applicable to conditions at  $37^\circ$  is difficult to state. 15 sec after



FIGURES 13-15 Radioautographs of sections of heart labeled for 1 min (Fig. 13), 5 min (Fig. 14), and 20 min (Fig. 15). Concentration of the silver grains over lipid droplets (*ld*) and mitochondria (*m*) is seen at the longer intervals of perfusion. Fig. 13,  $\times 15,500$ . Fig. 14,  $\times 20,000$ . Fig. 15,  $\times 27,000$ .



FIGURES 16-20 Radioautographs of sections of heart muscle labeled for 1 min at 4°. The radioautographic grains are seen over the elements of the SR (arrows) and mitochondria (*m*). Myofibrils and lipid droplets (*ld*) (Figs. 16, 17) are not labeled. Figs. 18-20 show localization of the silver grains over the elements of the SR including subsarcolemmal cisternae (arrows). Fig. 16,  $\times 20,000$ . Fig. 17,  $\times 12,000$ . Fig. 18,  $\times 20,000$ . Fig. 19,  $\times 20,000$ . Fig. 20,  $\times 20,000$ .

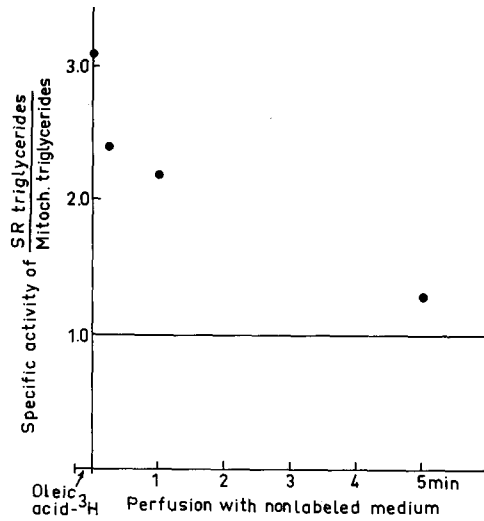
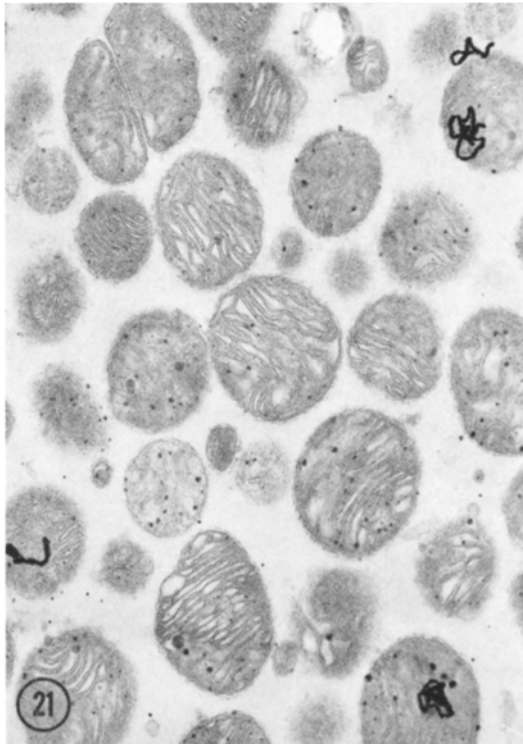


FIGURE 23 Ratio of specific activity of SR triglycerides to mitochondrial triglycerides at various time intervals of postperfusion with nonlabeled medium. All hearts were pulse labeled with 9,10-oleic acid- $^3\text{H}$  for 15 sec; each point represents one experiment.

perfusion with oleic acid- $^3\text{H}$  at  $37^\circ$ , the distribution of the grains over the SR and mitochondria was similar to that found in hearts perfused at  $4^\circ$ . However, while in the latter the radioautographic reaction was due to unesterified fatty acid (more than 80%), in the hearts perfused for 15 sec at  $37^\circ$ , more than 50% of the radioautographic reaction was due to esterified lipid. Thus, the question remains whether, after having crossed the sarcolemmal membrane, the fatty acid, as such, is transported intracellularly or is esterified immediately and reaches the interior of the cell as triglyceride. Since label over myofibrils was encountered rarely after perfusion at  $37^\circ$ , the preferential channeling through the SR seems to be valid also for the more physiological conditions.

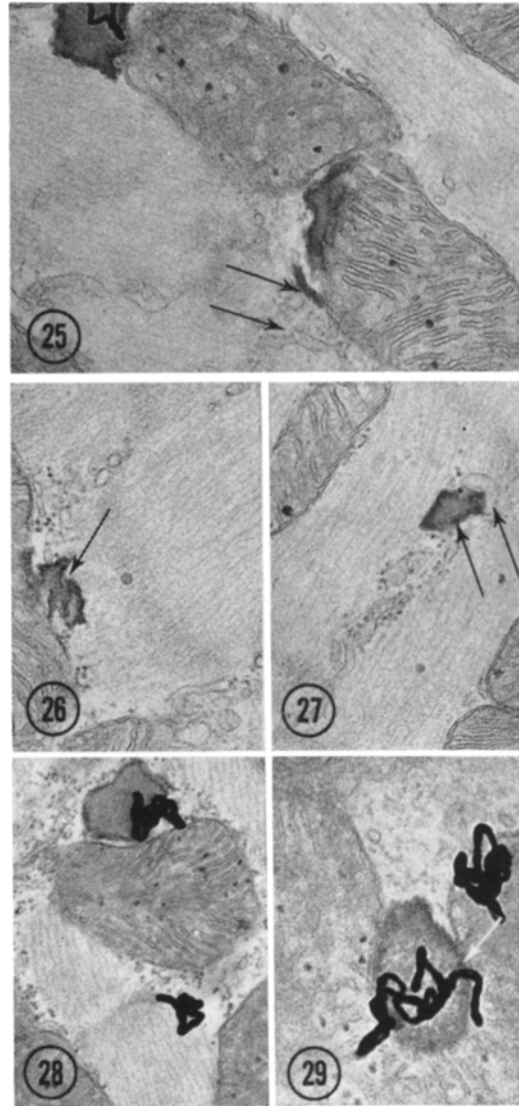
The next question investigated was the site of fatty acid esterification. The localization of the silver grains over the SR and mitochondria after 30 sec of labeling and 15 sec of postperfusion,

FIGURES 21 and 22 Radioautograph of pellets of isolated mitochondria (Fig 21) and sarcoplasmic reticulum (Fig 22) derived from hearts pulse labeled for 15 sec and postperfused for 1 min. The mitochondrial fraction is fairly homogeneous; the small dense particles in the SR fraction are probably glycogen. Fig. 21,  $\times 18,000$ . Fig. 22,  $\times 23,000$ .



FIGURE 24 Section of heart from a rat fasted for 24 hr. The lipid droplets are situated at periodic intervals, in close proximity to mitochondria. Note that all the droplets (arrows) are in the region of the Z line, an area in which most of the dyads are situated.  $\times 11,000$ .

when 90% of the lipid is in esterified form, pointed to both organelles as possible esterifying sites. The enzymes active in triglyceride synthesis have been localized to the endoplasmic reticulum (38). The SR of heart muscle is considered analogous to the endoplasmic reticulum of other cells, and glyceride synthesis occurs in isolated membranes of the SR of rat heart (unpublished results) under conditions similar to those reported previously for liver microsomes (38). It seems pertinent to mention that the SR of heart muscle is heterogeneous both structurally and functionally (8, 28, 29). The lateral sacs and the subsarcolemmal cisternae are considered to be part of a system of dyads and are distinguished from the rest of the longitudinal reticulum by the presence of ATPase activity (aldehyde resistant) (8, 28, 29). These elements have been shown to be the sites of  $Ca^{++}$  concen-



FIGURES 25-29 The mode of formation of lipid droplets is suggested in Figs. 25-27. The shape of the smallest lipid deposit resembles the configuration of the adjacent section through a cisterna of SR (arrows). With increase in size, the lipid aggregates acquire the form of a droplet (Figs. 25, 28, 29). The droplets are found also close to mitochondria, and sometimes fusion with the mitochondrial outer membrane is seen (white arrow, Figs. 25, 29). Fig. 25,  $\times 28,000$ . Fig. 26,  $\times 28,000$ . Fig. 27,  $\times 28,000$ . Fig. 28,  $\times 20,000$ . Fig. 29,  $\times 32,000$ .

tration (5, 12). These two findings are considered as suggestive of the location of the  $\text{Ca}^{++}$  pump (7, 12). The localization of lipid droplets in the region of the dyad, both intrafibrillar (Fig. 24) and subsarcolemmal (Fig. 5), noted in the present study, was mentioned by Barnett and Hagstrom (1) and is also evident in the electron micrographs of Maunsbach and Wirsén (16). It seems likely that lipid deposition would occur in the proximity of the site of glyceride formation, and thus the lateral sacs or the intermediate cisternae of the SR (24) seem to be the most likely site of triglyceride formation. The origin of the mitochondrial triglyceride has not been elucidated so far, but the two possibilities are either in situ synthesis from free fatty acid or translocation of preformed triglyceride. The finding that the specific activity of labeled triglyceride is higher in the isolated fraction of SR than in the isolated mitochondria at the early time intervals after perfusion, together with the finding that there is a progressive equilibration of the specific activities with time (Fig. 23), points to the SR as the main site of triglyceride formation.

Some controversy has emerged recently as to whether intracellular triglyceride can serve as a reservoir, which is utilized by the muscle cell during acute caloric deprivation. Masoro (15) has pointed out that since muscle triglyceride tends to rise during 72 hr of fasting, the cell does not utilize triglyceride as a source of energy. In the present experiments, the rapid fall in the labeled triglyceride between 2 and 20 min of postperfusion with nonlabeled medium (Table III) demon-

strated utilization of stored lipid, a finding in accordance with that of Olson and Hoeschen (20).

The rise in the concentration of label over the lipid droplets, relative to other sites of esterified lipid, with longer times of perfusion indicates that, as in the liver (36, 39), in muscle the triglycerides do not form a single metabolic pool and that the lipid found in droplet form has a turnover rate slower than that of the triglyceride in the intracellular organelles. The mode of formation of the droplet is suggested in Figs. 25-29. Since in outline, the smallest lipid deposits resemble the sarcoplasmic reticulum profiles, it seems likely that intracisternal deposition of lipid occurs initially and that the acquisition of droplet form is a later development. The lipid droplets, though often distinct from the mitochondrial membrane, are also seen in very close proximity to the outer mitochondrial membrane, which may be obscured by the lipid (Figs. 28, 29) (22, 23). This structural configuration as well as the finding of esterase activity in the mitochondrial membrane (1) might indicate that heart mitochondria can draw on stored esterified lipid for their energy requirements.

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