Further Evidence to Suggest That Microbodies Do Not Exist as Individual Entities

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Male wild-type mice (Cs[a] strain) were treated with ethyl-α-*p*-chlorophenoxyisobutyrate (CPIB), a hypolipidemic drug which enhances hepatic catalase synthesis and induces rapid and significant increase in the number of microbody (peroxisome) profiles in liver cells. Numerous microbody profiles, several of them appearing in clusters and retaining membranous continuities, were observed in liver cells of CPIB-treated mice. They showed a significant variation in size and configuration, and the presence or absence of the nucleoid or core did not appear to bear any relation to the size or shape of microbody profiles. Nucleoids were encountered frequently in microbody profiles measuring as small as 0.1 μ in diameter. Numerous continuities between two or more anucleoid and/or nucleoid-containing microbody profiles of different sizes and shapes were seen. These findings are inconsistent with the concept that the smaller peroxisomes are the possible precursors or progenitors of their larger counterparts. Detailed examination of numerous electron micrographs revealed irregular dilatations and tortuosities of the endoplasmic reticulum (ER) containing electron-opaque peroxisomal material displaying the characteristic appearance of matrix and usually containing irregular cores. Transitions of rough ER to smooth ER in which microbody proteins accumulated were also apparent. Numerous continuities between several microbody profiles and continuities between microbody profiles and ER are interpreted as accumulations of peroxisomal proteins in dilated tortuous channels of ER. These observations strongly suggest that the microbody proteins constitute a common pool, circulating constantly in the dilated ER channels. The size, shape and number of microbody profiles appear to reflect the amount of peroxisomal proteins present in the pool. These observations clearly suggest that the microbodies do not exist as individual entities (Am J Pathol 70:421–438, 1973).

MICROBODIES (peroxisomes) constitute a distinct class of cytoplasmic organelles containing catalase and several oxidative enzymes such as urate, oxidase, D-amino acid oxidase, L-α-hydroxy acid oxidase and isocitrate dehydrogenase. In an attempt to investigate the synthesis and turnover of rat liver microbodies, Poole, Higashi and DeDuve [1] separated these organelles according to size by zonal sedi-

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mentation and showed that the specific radioactivity of catalase is independent of size of isolated particles and that there is a homogeneity of peroxisomal enzyme distribution in particles of different sizes. Based on these turnover studies, they suggested that microbodies either exist "as individuals, each with a life history independent of the others" or that the microbody proteins form a common pool from which they exchange material.[1] We presented persuasive morphologic evidence to suggest such an exchange and also demonstrated the origin of hepatic microbodies from the endoplasmic reticulum (ER), from our studies on acatalasemic mice treated with ethyl chlorophenoxyisobutyrate (CPIB).[2] Administration of CPIB to these genetically mutant acatalasemic mice resulted in a marked increase in microbody number in hepatic parenchymal cells.[3,4] Examination of large numbers of microbodies in the livers of CPIB-treated acatalasemic mice revealed numerous continuities between microbodies and the ER and interconnections between two or more adjacent microbodies.[2] Since these morphologic observations clearly indicated that the size, shape and number of microbodies largely reflect the quantity of microbody protein that is synthesized and accumulated in the localized dilatations of the ER channels, through which they possibly exchange material, the possibility that the microbodies "exist as individuals" [1] in the liver cells was considered unlikely.[2]

Because of the genetic abnormality of the animals used in our studies we felt that the "applicability of the proposed biologic significance of microbody-to-microbody connections" required elucidation in other species.[2] Recently, Novikoff and Novikoff,[5] in their elegant studies of peroxisomes in absorptive cells of mammalian small intestine, observed multiple peroxisome-ER attachments. They also suggested that these organelles may be "interpreted as localized dilatations of smooth ER retaining multiple membranous continuities." [5] We now present additional morphologic evidence for the microbody-ER relationship in hepatic parenchymal cells of wild-type normal mice (Cs^a strain) treated with CPIB. These observations clearly suggest that microbodies (peroxisomes) are the profiles of peroxisomal proteins accumulated in the dilated and tortuous ER channels which are continuous with one another.

Materials and Methods

Male wild-type Cs^a strain mice were used in these experiments. These animals were from a colony derived from mice originally obtained through the generous cooperation of Dr. R. N. Feinstein, Argonne National Laboratory, Argonne, Ill. All animals used in these experiments were 2 to 3 months old and weighed between 20 to 30 g. Ethyl-α-*p*-cholorophenoxyisobutyrate (CPIB, Clofibrate, Atromid-S®) was administered in ground Purina chow *ad lib* in a concentration of 0.25%.[6,7] One

to 2 animals were killed at various intervals following the commencement of CPIB treatment. Small pieces of liver tissue were fixed at 0 to 4 C for 1 to 2 hours in 2% osmium tetroxide buffered to pH 7.4 with S-collidine (2,4,6-trimethylpyridine; Vaughn, Inc, Memphis, Tenn). After fixation, the tissues were dehydrated in a graded series of alcohols and embedded in epoxy resins. Thin sections were stained with lead hydroxide [8] and were examined with an electron microscope.

Results

The livers from male Cs^a mice were examined after 1, 3, 5, 7, 14, 19 and 36 days of CPIB treatment. A significant increase in number of microbodies in liver cells of these animals was noted during the early stages of CPIB administration, reaching a steady state during the second week. Abundant peroxisomes were observed in the livers of mice killed 7 days after beginning CPIB treatment, and they persisted throughout the duration of CPIB feeding. In addition to microbody proliferation, a significant increase in smooth endoplasmic reticulum was also seen. Focal degranulation of rough endoplasmic reticulum was evident and was most marked in dilated ER segments.

Microbody Profiles: Size, Shape and Nucleoids

Numerous microbodies, several in closely-knit clusters, were present throughout the cytoplasm of hepatocytes (Figure 1). In general, microbodies varied a great deal in size and shape; the variation was most pronounced in microbodies constituting a cluster. Exact measurements of microbodies were not feasible because of their irregular configuration, continuities with one another and the presence of abundant continuities with dilated ER channels (Figures, 1–15, 20–25, 30–41). However, the approximate diameters of most microbodies varied from less than 0.1 μ to over 0.6 μ. The majority were elongated, oval or tortuous, and their shapes strongly indicate that these structures, in fact, represent accumulation of electron-dense peroxisomal proteins in the dilated ER channels (Figures 2–13, 15–22, 24, 25). Nucleoids or electron-dense cores were present in many peroxisome profiles of varying sizes (Figures 4, 5, 12, 13, 15, 16, 18, 20–22). Several small microbody profiles measuring 0.1 to 0.25 μ had irregular linear or twisted cores (Figure 4). Although nucleoids or cores were generally seen in larger microbody profiles, it was not uncommon to observe microbody profiles as large as 0.5 to 0.7 μ lacking nucleoids (Figures 20, 29). Anucleoid microbody profiles were recognizable by the characteristic appearance of finely granular or homogeneous electron-dense material (Figures 14, 20 and 39). Previous cytochemical studies on the livers of CPIB-treated animals published earlier from this laboratory [3,4] have clearly indicated that the nucleoid-

bearing as well as anucleoid microbody profiles contained cytochemically demonstrable catalase and are distinguishable from lysosomes.

Transitions from ER to Microbody Profiles

Several continuities between ER and microbodies were observed at various periods following CPIB treatment and were frequent during the early stages (Figures 12–22). The ER membranes close to the microbody profile (connecting stalk) were devoid of ribosomes (Figures 12, 13, 15–20). Transitions of rough ER to dilated smooth ER membranes in continuity with the microbody profiles are clearly apparent in Figures 15 and 16. Nucleoids or cores are visible in the microbody profiles which are continuous with the ER channels (Figures 4, 12, 13, 15, 16). Direct continuities between rough ER channels and microbody profiles, without intervening smooth ER segment, were very rarely encountered. Although dilated ER vesicles with few attached ribosomes were found attached to some of the microbody profiles, none of them contained electron-dense microbody proteins. From several microbody profiles examined it appears that the loss of ribosomes from ER membranes may be a prerequisite for the accumulation of peroxisome proteins.

Continuities Between Smooth ER and Microbody Profiles: Transport or Circulation of Microbody Proteins

Because of the presence of large numbers of microbodies in areas rich in tubular smooth ER, continuities between smooth ER and microbody membranes were conspicuous in livers examined 7 days after CPIB treatment (Figures 21, 22, 26–31). However, due to the three-dimensional nature of the ER-microbody relationships, it is not unusual to find distortions of such continuities in most electron micrographs. Examination of closely knit aggregates of microbody profiles showed several examples of two or more microbody profiles connected with each other by irregularly anastomosing channels of ER (Figures 25–27, 30). In Figures 27 and 30, widely separated, nearly spherical microbody profiles are connected with one another by long smooth ER channels.

Continuities Between Microbody Profiles

Several elongated and tortuous peroxisome profiles were continuous with each other (Figures 1–3, 5, 6, 23, 34–41). These continuities were abundant in the livers of Cs^a mice after the achievement of CPIB-steady state. The maximum increase in the number of microbody profiles and in catalase activity occurs between 7 to 12 days after starting CPIB

treatment. Continuities between two or more adjacent microbody profiles are illustrated in Figures 2, 3, 5, 35–41. The presence of more than one nucleoid in these profiles (Figures 35–37 and 40) makes it unlikely that we are dealing with a "single microbody" with an unusual shape. Figures 24, 34 and 36 illustrate what appear to be multiple direct continuities between several adjacent microbody profiles. They may, in fact, be considered as accumulations of peroxisomal proteins in dilated tortuous channels of ER. Several peroxisomal profiles showed pseudopod-like irregular extensions (Figures 29, 31–33), reminiscent of amoeboid movement. From these morphologic appearances, it is conceivable that the peroxisomal proteins circulate within the dilated channels of smooth ER.

Discussion

Ethyl-α-*p*-chlorophenoxyisobutyrate (CPIB) treatment induces a remarkable increase in hepatic microbody population in male rats [4,6,7,9] and Cs[a] mice, and in both sexes of acatalasemic mice.[3] Furthermore, it stimulates the rate of catalase synthesis.[10] The presence of multiple membranous continuities between adjacent microbodies, and between microbodies and ER channels, suggested that the peroxisomal proteins exist as a common circulating pool. Although additional morphologic observations were in progress, it was cautioned that, since the acatalasemic mice have a genetic abnormality involving catalase,[11] further studies may be required before extending these observations to normal animals.[2] The present studies, therefore, were undertaken to obtain morphologic evidence for microbody-ER relationships and to detect continuities between microbody profiles in normal Cs[a] mice treated with CPIB. Although it might be asserted that the mice used in these studies are not truly normal because they were given CPIB, it is clear that they are "normal" in the sense that they do not have genetic aberrations in catalase.

The present findings were similar to our previous observations of microbody-ER relationships in the livers of acatalasemic mice [2] and substantiate the earlier speculations about the origin of microbodies in rat liver by Novikoff and Shin.[12] Furthermore, these findings in CPIB-treated adult mice are in agreement with the studies of Essner [13] and of Tsukada, Mochizuki and Konishi [14] on the origin of peroxisomes in the fetal mouse and fetal rat livers, respectively. However, it is to be pointed out that Legg and Wood [15] and Fahimi [16] do not believe that continuities between microbodies and membranes of ER exist in adult rat liver.

Irregularities in size and shape of microbody profiles appear to reflect varying quantities of microbody protein that accumulate in ER channels

of differing sizes and shapes. Furthermore, we also believe that the increase in the *number* of microbody profiles in the livers of CPIB-treated animals is due to the increase in the "circulating pool" of peroxisomal proteins resulting from CPIB-stimulated synthesis. It is now clear that CPIB enhances the synthesis of catalase [10] and isocitrate dehydrogenase,[17] which are the two major components of peroxisomal proteins.[18] Furthermore, our studies on the CPIB-induced microbody proliferation in rats treated with allylisopropylacetamide to inhibit catalase synthesis [19] suggested that CPIB might induce the synthesis of yet unidentified proteins that make up between one-half and two-thirds of the total microbody (peroxisome) proteins.[18] It is to be pointed out, however, that Azarnoff and Svoboda [20] have observed a decrease in activities of D-amino acid oxidase and L-α-hydroxy acid oxidase in the rat liver homogenates following CPIB treatment. These two enzymes combined account for less than 5% of total peroxisomal proteins.[18] Because of significant increase in liver weight and total proteins of liver in CPIB-treated animals, an increase in the activity of these trace enzymes of peroxisomes may not be detectable, since biochemical determinations of enzyme activity were performed on liver homogenates and expressed as units per milligram protein.[20] Although it is essential to isolate microbodies in CPIB-treated animals and characterize the enzymatic composition, there appears no reason to suspect that microbody profiles appearing after CPIB treatment are abnormal. The presence of large quantities of peroxisomal proteins in the common pool is considered to be responsible for the filling of numerous ER channels which are discernible as microbody profiles.

Novikoff and Novikoff,[5] in detailed studies on peroxisomes of mammalian small intestine, presented additional observations on the ER-peroxisome relationships which are similar to our earlier findings on hepatic microbodies in acatalasemic mice [2] and to the observations presented in this report on normal mice treated with CPIB. They proposed the term "microperoxisome" to designate the smaller peroxisomal profiles described recently in a variety of cell types.[21-29] They also interpreted the numerous microbodies in CPIB-treated livers of acatalasemic mice observed in our studies as microperoxisomes and suggested that the larger peroxisomes in liver and kidney develop by enlargement of microperoxisomes.[5] Although the peroxisomal profiles identified recently in several cell types appear somewhat smaller than the hepatic and renal counterparts, the microbody profiles in normal and neoplastic Leydig cells of rats [26,29] contain elongated tubular inclusions which are identical to those observed in the larger peroxisomes of rat kidney.[30,31] Further-

more, the presence or absence of nucleoid in hepatic peroxisomes of CPIB-treated animals [2-4,6,7] does not appear to bear any direct relationship to the size of these microbody profiles. Therefore, judging from the multiplicity of microbody-ER continuities and from the marked variations in size and shape of these profiles in the livers of CPIB-treated acatalasemic and wild-type mice, it is difficult to differentiate the peroxisomes from the so-called "microperoxisomes" of Novikoff and Novikoff.

The observations presented here and in our previous studies [2] clearly indicate that the size and shape of these profiles depend on the amount of microbody constituents trapped in the tortuous ER channels of varying sizes and shapes. This conclusion is consistent with biochemical data of Poole and co-workers [1] and supports the theory that the peroxisomal proteins constitute a common pool. In our studies on acatalasemic mice treated with CPIB, we suggested that catalase is redistributed through the continuities between adjacent microbodies and via ER channels. Catalase and other peroxisomal enzymes appear to possess certain physicochemical affinities and "exist in dynamic equilibrium" in a common circulating pool.[2] We indicated that the peroxisomal proteins accumulated in the ER channels can be isolated as distinct particles because "on homogenization the membranes rupture and appear to enclose varying quantities of microbody protein material" which "can account for the differences in size of the isolated particles" [2] and for the homogeneity of enzyme distribution in particles of different sizes.[1] Because of multiple ER-peroxisome continuities demonstrated in the liver and in the absorptive cells of small intestine,[15] it is reasonable to assume that the *microbodies*, as seen in the electron micrographs, are simply the profiles of accumulated electron-opaque peroxisomal proteins, which constantly circulate in the ER channels *in vivo*. The distinction of microperoxisomes from the larger peroxisomes in livers is merely academic, in view of the homogeneity of peroxisomal enzymes in isolated particles of different sizes.[1] These morphologic studies, together with the turnover data on peroxisomal catalase of Poole and co-workers,[1] clearly substantiate the view that the microbodies do not exist as individuals. Furthermore, the suggestion that microbodies are not unit organelles, but merely represent circulating peroxisomal enzymes which are electron opaque, is consistent with their proposed functional role. A readily circulating enzyme system in the ER channels is more effective than individual particles for rapid detoxification of hydrogen peroxide and may also serve as shuttle systems for the transfer of electrons from one part of the cell to another.

Additional support for the concept that microbodies do not exist as individuals capable of growth and maturation, comes from our studies on the reversal of CPIB-induced microbody proliferation. When CPIB treatment is discontinued, the catalase activity returns to pretreatment levels within 7 days and there is simultaneous reduction in the number of microbody profiles. The reduction in the number of microbody profiles was not associated with any significant increase in lysosomes or autophagy.[32] The imperceptible disappearance of microbodies after withdrawal of CPIB from the diet is believed to be the result of reduction in the circulating peroxisomal protein pool brought about by the cessation of the enhanced synthesis.

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[*Illustrations follow*]

Legends for Figures

Fig 1—Mouse liver; CPIB, 14 days. Numerous microbody (*mb*) profiles are seen in clusters during this CPIB-induced steady state. The *short arrows* indicate continuities in electron-opaque peroxisomal material. Increase in smooth endoplasmic reticulum (*ER*) is evident (× 14,600).

Figs 2 and 3—Direct continuities between two or more adjacent microbody profiles were frequently observed in CPIB-treated mouse liver after the establishment of CPIB steady state. Two microbody profiles each with a distinct nucleoid are continuous with each other. Note the obvious difference in the electron opacity (△) of microbody proteins in the connecting ER channel. The presence of nucleoids in both microbody profiles and differences in electron density rule out the possibility that these profiles are indeed "one microbody" with irregular shape. *Arrow* indicates microbody-ER continuity (× 63,000).

Fig 4—The presence or absence of nucleoid (*n*) has no relation to the size or shape of microbody profiles. Nucleoids are seen in the smaller, as well as the larger microbody profiles. The smaller peroxisome profile is also continuous with ER *(short arrow)* (× 69,000).

Figs 5 and 6—Two microbody profiles with nucleoids are continuous *(arrows)* with a common ER channel in **5,** whereas in **6,** the profiles are connected with a desmosome. The third microbody profile in **6** is continuous with ER *(arrow)* (× 51,000).

Figs 7–11—Mouse liver; CPIB, 3 days. These elongated and tortuous microbody profiles strongly suggest accumulation of peroxisomal proteins in dilated ER channels. The *short arrows* in **7** indicate an ER channel connecting the microbody profiles above and below. Incomplete filling of the ER channels by peroxisomal proteins is depicted by the *arrow heads* in **7, 10, 11.** Nucleoids *(n)* or ill-defined cores are also visible, even in partially filled ER channels (× 51,000).

Fig 12—Mouse liver; CPIB, 1 day. An irregularly elongated microbody profile and a nearly spherical microbody profile reveal multiple continuities *(arrow heads)* with rough endoplasmic reticulum *(ER).* Note the absence of ribosomes on the ER membranes adjacent to peroxisome densities. Nucleoids *(n)* are present in the larger as well as in the smaller microbody profiles (× 49,900).

Figs 13 and 14—Mouse liver; CPIB, 5 days. The microbody profiles are continuous *(arrow heads)* with the ER membranes. *n* = nucleoid (**13,** × 65,000; **14,** × 46,000).

Fig 15—Rough endoplasmic reticulum *(ER)* channels show dilatation and absence of ribosomes *(arrow heads)* and contain electron-opaque microbody proteins in the dilated portions (× 87,000).

Fig 16—Two microbody profiles are connected with each other (△) and the lower one of these is continuous *(arrow head)* with the endoplasmic reticulum *(ER)*. The third microbody profile is also continuous *(arrow head)* with the ER. Ribosomes are present on ER membranes, but are characteristically absent in proximity of microbody profile (× 58,000).

Figs 17–20—Continuities *(arrow heads)* between rough endoplasmic reticulum *(ER)* and microbody profiles were frequently encountered during the early stages of CPIB treatment. The dilated segments of ER close to the microbody profile are devoid of ribosomes (× 58,500).

Fig 21—Smooth endoplasmic reticulum *(ER)* channels of varying length are connecting *(arrows)* widely separated microbody profiles. The ER membranes which are continuous with microbody profiles mb_1 and mb_2 have anastomosed with one another *(between arrows)* (× 58,000).

Fig 22—Numerous continuities between microbody profiles and smooth ER channels *(arrows)* are easily recognizable in the livers after chronic CPIB treatment. The microbody profiles mb_1 and mb_2 clearly suggest accumulation of peroxisomal proteins in dilated ER channels (× 48,000).

Fig 23—Nucleoid containing microbody profile and a larger anucleoid microbody profile are continuous with each other (× 51,000).

Figs 24 and 25—Several microbody profiles constituting a cluster are continuous with one another *(arrow heads and arrows)*. The four microbody profiles in **24** are considered as microbody proteins filling a dilated and a tortuous ER channel (× 64,500).

Fig 26—Two microbody profiles containing nucleoids are connected *(arrows)* with a common tubular smooth endoplasmic reticulum *(ER)* channel. The *arrow head* indicates continuity of one of the microbody profiles with the rough ER (× 29,000).

Fig 27—Four microbodies are connected with one another *(arrow heads)* by long channels of smooth endoplasmic reticulum *(ER)* (× 60,000).

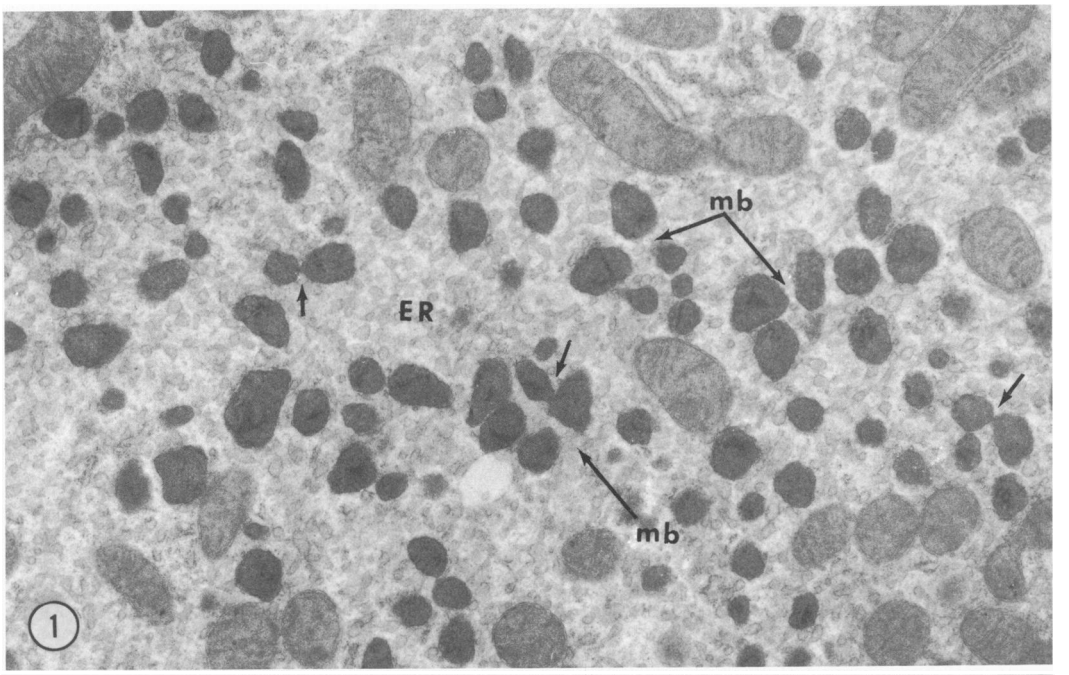
Figs 28–31—Accumulations of electron-opaque microbody proteins in dilated portions of smooth endoplasmic reticulum *(ER)* channels are evident. *Arrows* indicate junctions of microbody profiles with the ER channels and anastomoses between ER channels (× 63,000).

Figs 32 and 33—The irregular appearance of several microbody profiles, some of them containing a nucleoid *(n)*, strongly suggest that microbody proteins circulate through the vesicles of endoplasmic reticulum *(arrows)*. Variance in electron opacity *(arrows)* is also suggestive of transport of peroxisomal proteins. See also Figures 28, 29 and 31 (**32,** × 59,000; **33,** × 69,000).

Fig 34—Four microbody profiles are continuous with one another *(arrows)*. These are considered as dilated endoplasmic reticulum channels filled with electron-opaque microbody proteins. Nucleoids are also visible (× 72,000).

Figs 35–38—Additional examples of accumulation of varying quantities of peroxisomal proteins in dilated endoplasmic reticulum *(ER)* channels, presenting as multiple continuities *(arrows)* between adjacent microbody profiles. This is obvious in **36** and **38,** in which ER channels are visible. Nucleoids *(n)* are present in one or more microbody profiles that are continuous with each other (**35–37,** × 59,000; **38,** × 48,000).

Figs 39–41—These microbody profiles connected with each other by endoplasmic reticulum *(ER)* reflect flow of microbody proteins into vesicles of ER. The connecting ER channels show differences in the degree of electron density. Nucleoids are visible in two microbody profiles that are connected with each other in **39** (**39,** × 57,000; **40,** × 65,000; **41,** × 57,000).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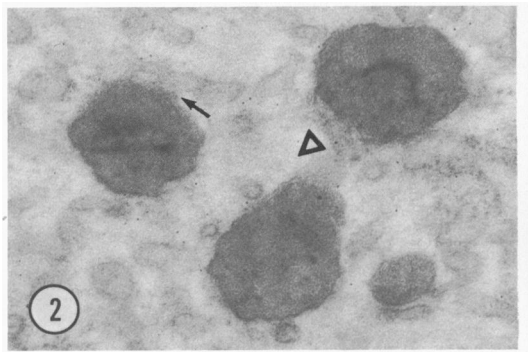
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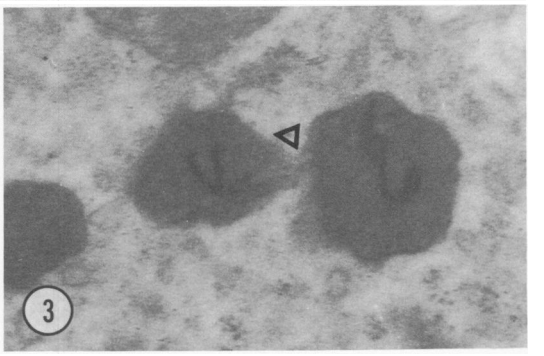
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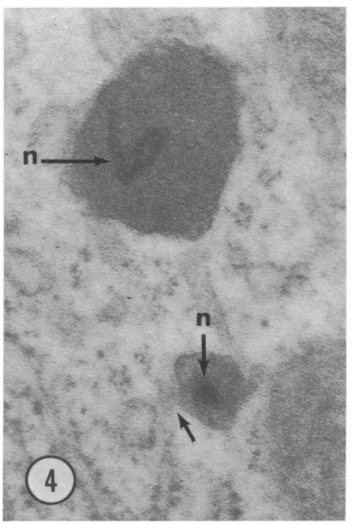
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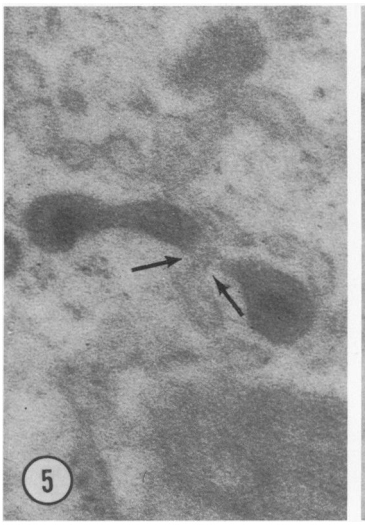
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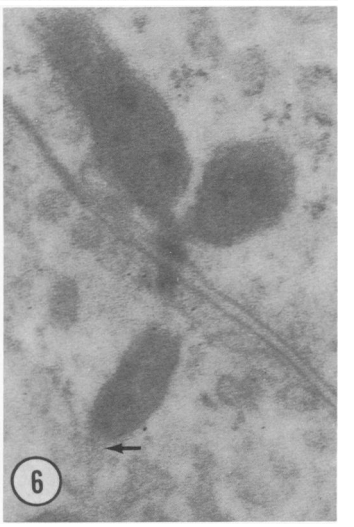
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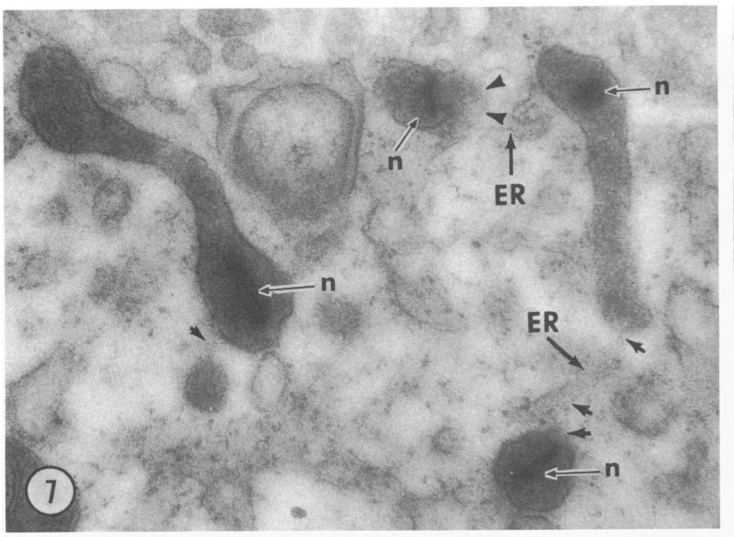

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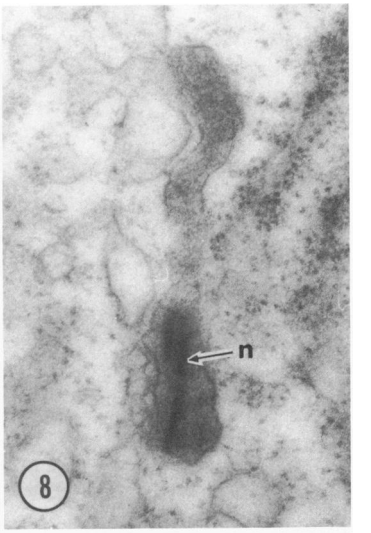

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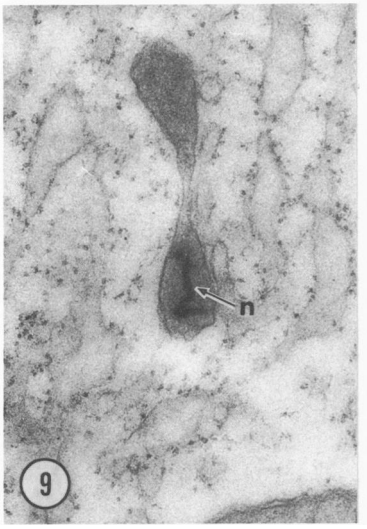

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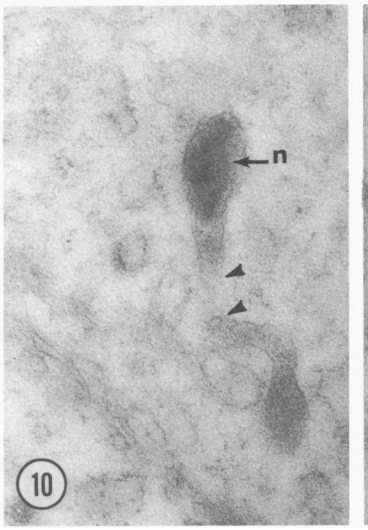

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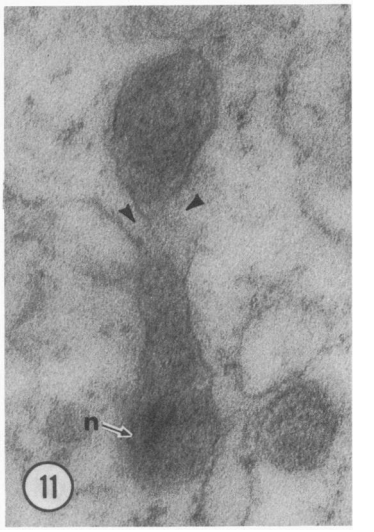

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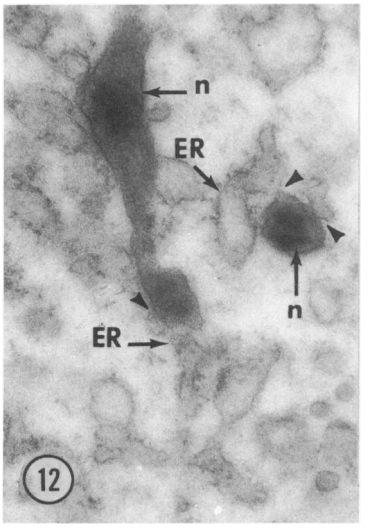

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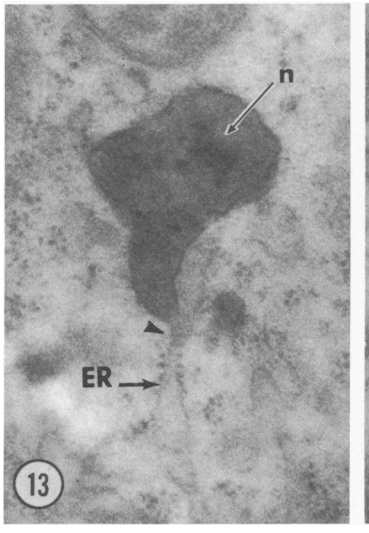

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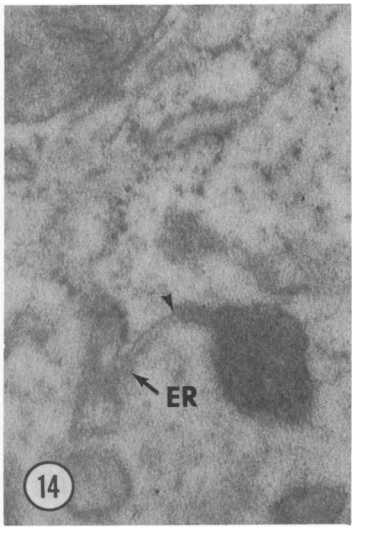

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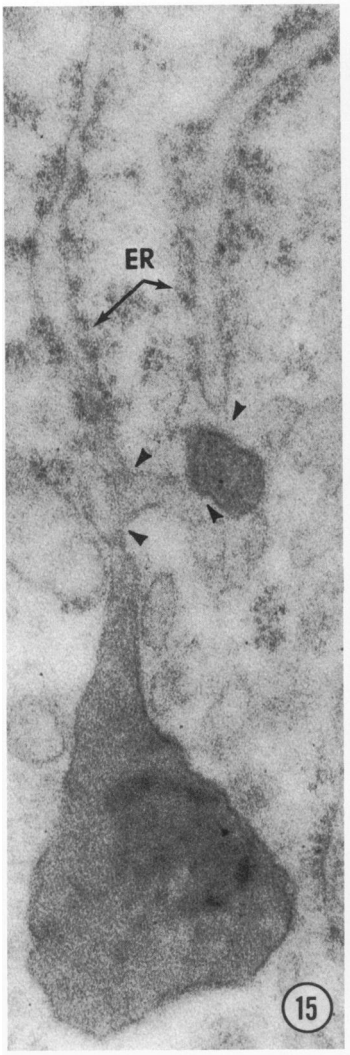
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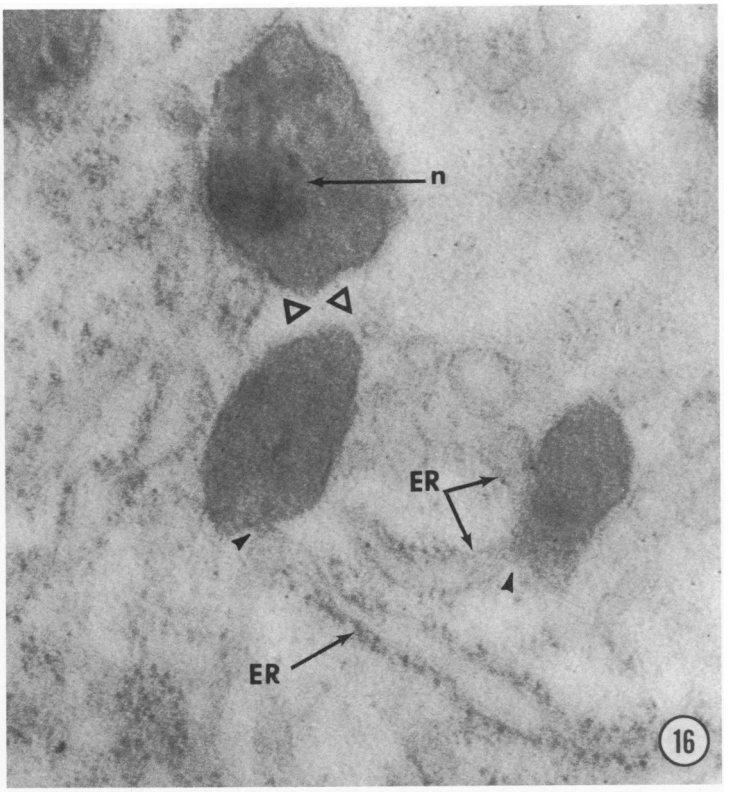
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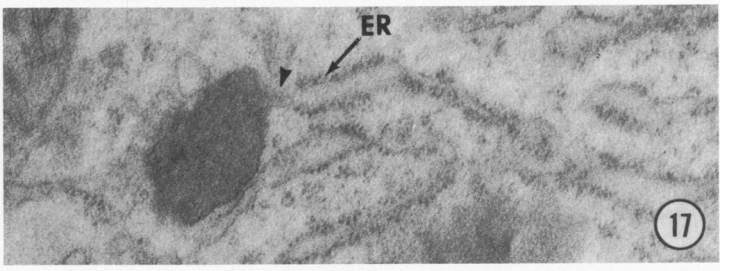
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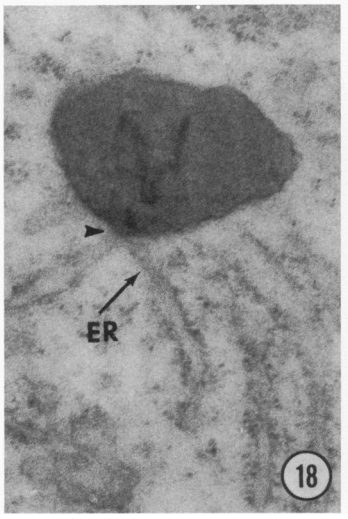
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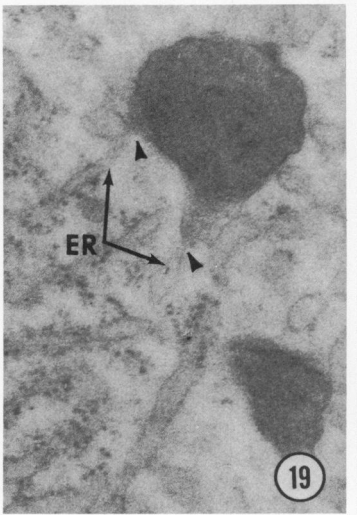
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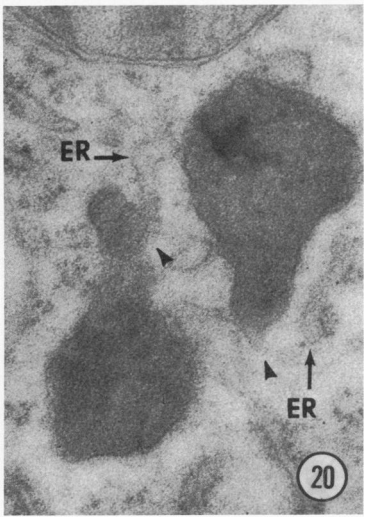
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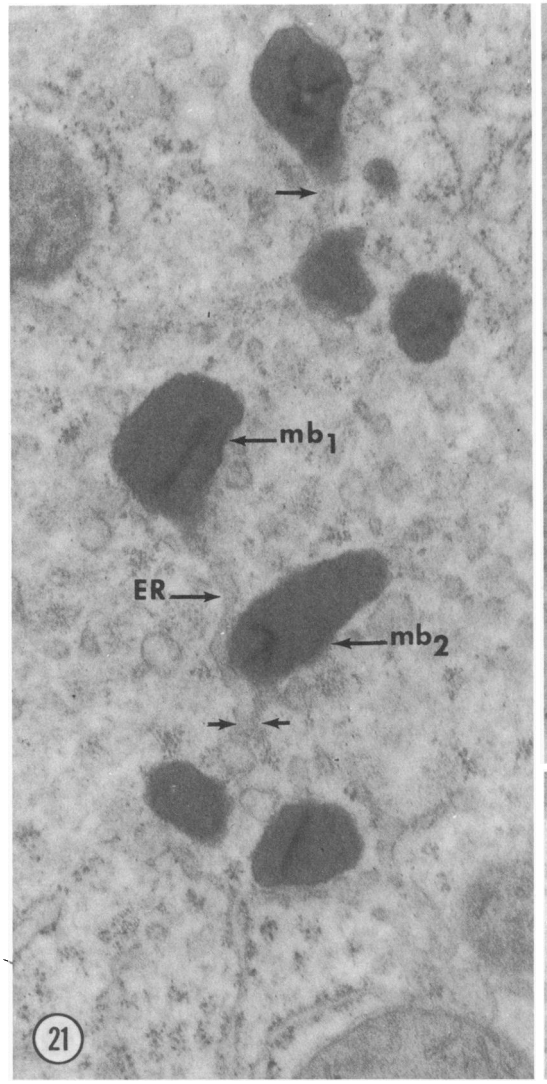

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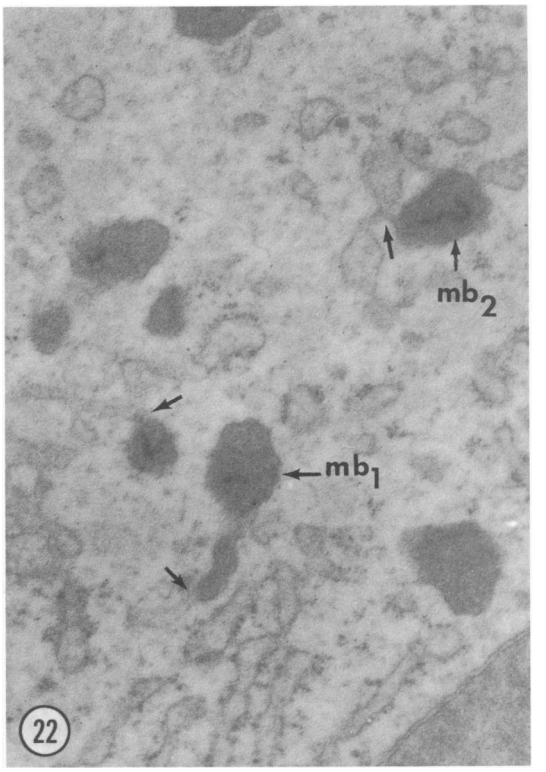

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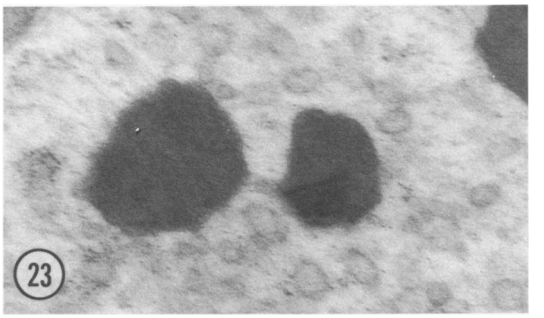

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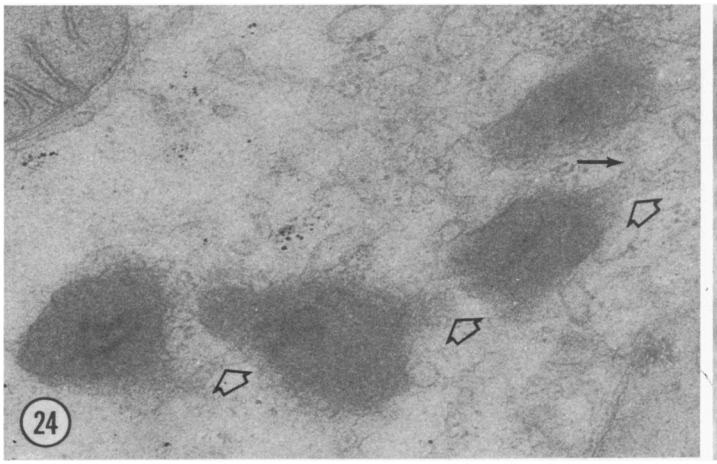

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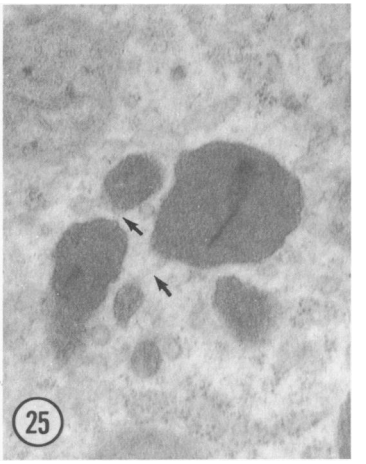

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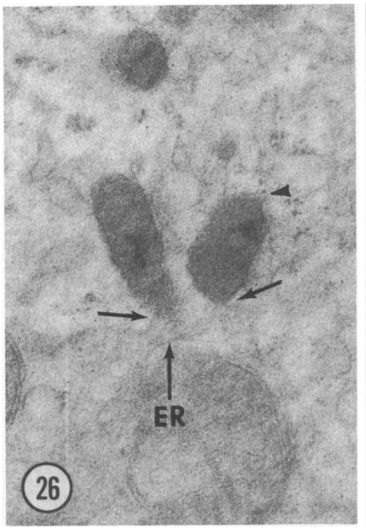
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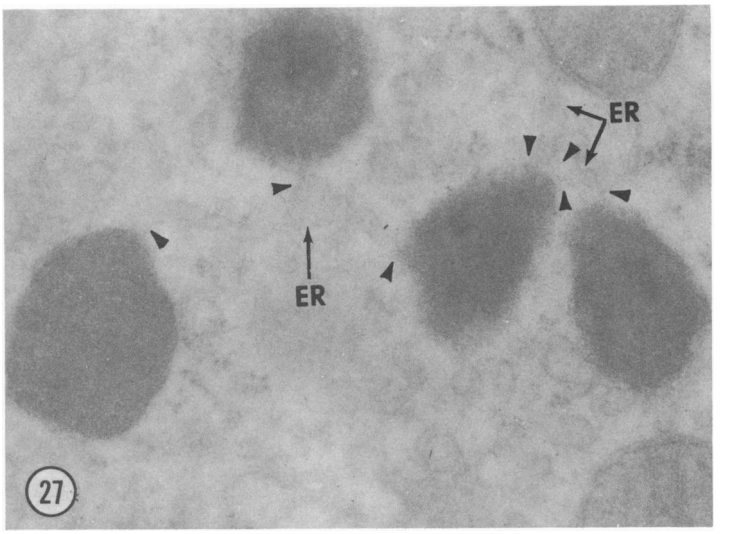
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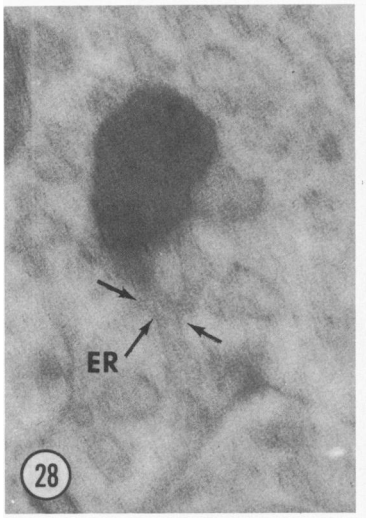
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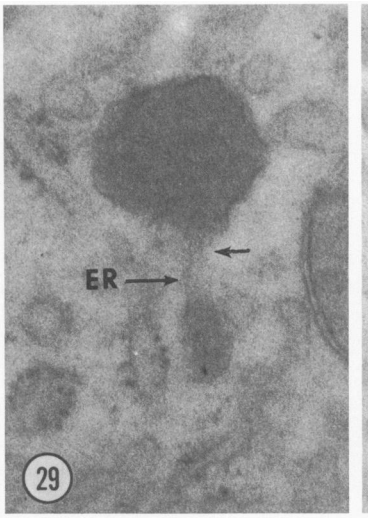
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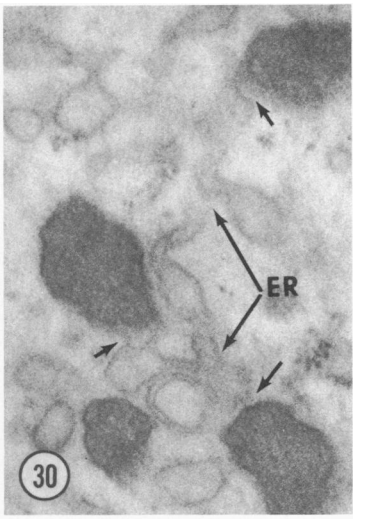
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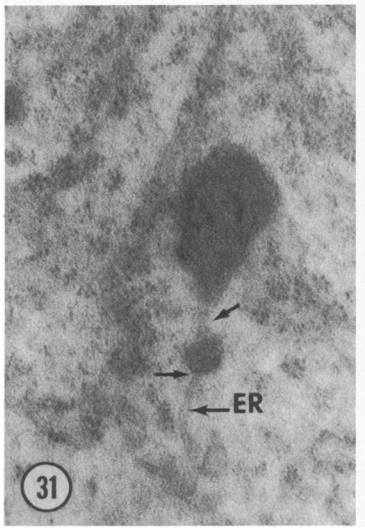
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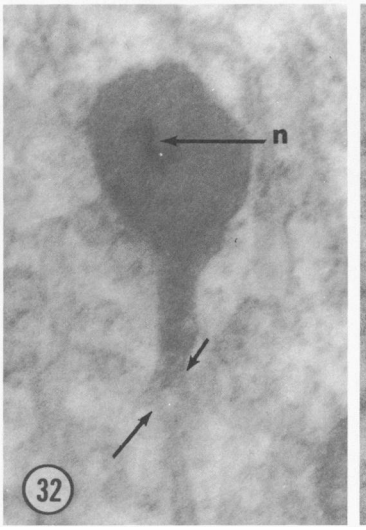
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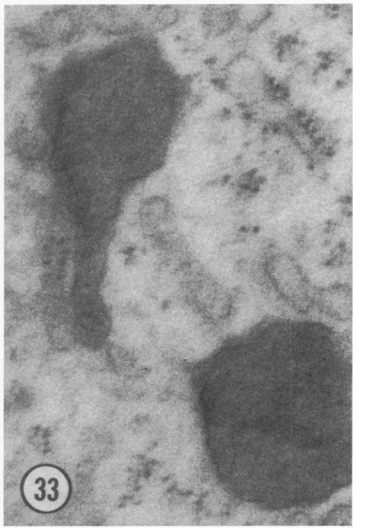
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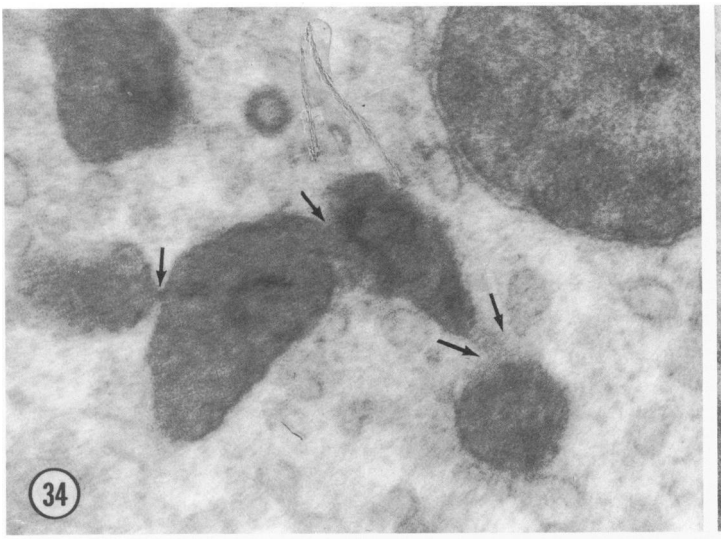
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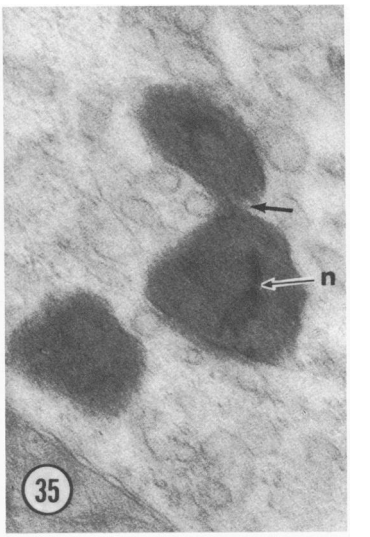
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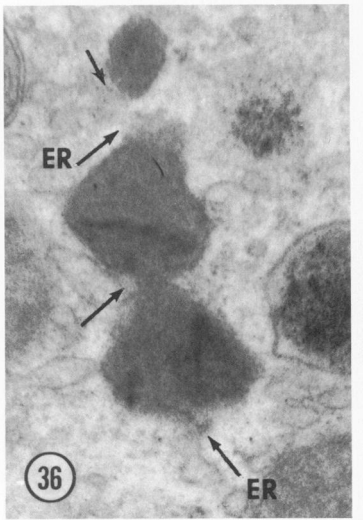
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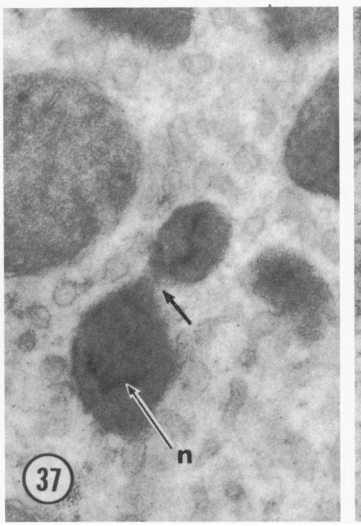
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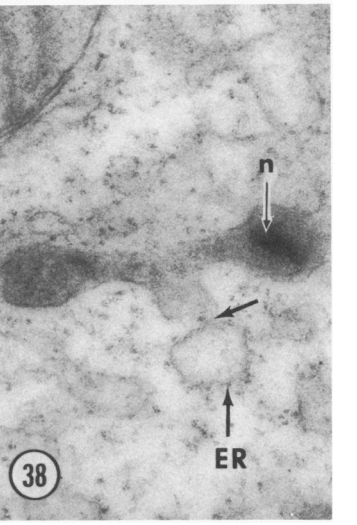
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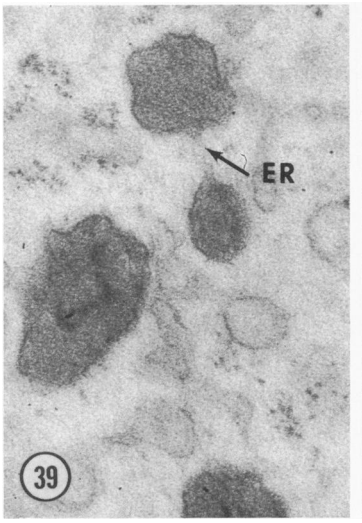
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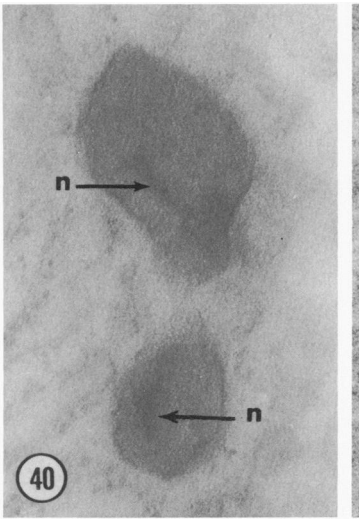
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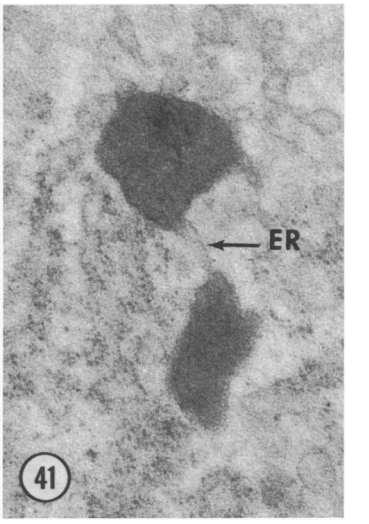
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