Original article

Identification of the 16°C compartment of the endoplasmic reticulum in rat liver and cultured hamster kidney cells

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In many systems transfer between the endoplasmic reticulum and the Golgi apparatus is blocked at temperatures below 16°C. In virus-infected cells in culture, a special membrane compartment is seen to accumulate. Our studies with rat liver show a similar response to temperature both *in situ* with slices and *in vitro* with isolated transitional endoplasmic reticulum fractions. With isolated transitional endoplasmic reticulum fractions, when incubated in the presence of nucleoside triphosphate and a cytosol fraction, temperature dependent formation of vesicles occurred with a Q_{10} of ≈ 2 but was apparent only at temperatures greater than 12°C. A similar response was seen *in situ* at 12°C and 16°C where fusion of transition vesicles with *cis* Golgi apparatus, but not their formation, was blocked and transition vesicles accumulated in large numbers. At 18°C and below and especially at 8°C and 12°C, the cells responded by accumulating smooth tubular transitional membranes near the cis Golgi apparatus face. With cells and tissue slices at 20°C neither transition vesicles nor the smooth tubular elements accumulated. Those transition vesicles which formed at 37°C were of a greater diameter than those formed at 4°C both *in situ* and *in vitro*. The findings show parallel responses between the temperature dependency of transition vesicle formation *in vitro* and *in situ* and suggest that a subpopulation of the transitional endoplasmic reticulum may be morphologically and functionally homologous to the 16°C compartment observed in virally-infected cell lines grown at low temperatures.

transition vesicle — endoplasmic reticulum — temperature — rat liver

INTRODUCTION

Reduced temperature has been shown to affect post-translational processing and secretion in a number of cell systems. Tartakoff [19] demonstrated that at 10°C secretory proteins of the exocrine pancreas accumulated in pre-Golgi apparatus transition vesicles, while at temperatures of 22°C or greater, progress through the Golgi apparatus and into condensing vacuoles occurred. Secretion of IgM and the appearance of HLA-1 on the cell surface decreased gradually in lymphoid cells as the temperature was reduced from 37°C to 20°C but no intracellular accumulation was observed [3]. Histamine secretion from mast cells also decreased markedly at temperatures below 18°C [10].

With hepatic cells, secretory proteins were blocked in a pre-Golgi apparatus compartment at 18°C, but at 20°C these proteins were exocytosed normally [5]. This was similar to rat pancreatic cells where, at 16°C, most of the labeled secretory proteins remained in the endoplasmic reticulum while at 20°C the medial Golgi region was reached [18].

These and other data, especially with the intracellular transport and surface expression of protein in virus-infected cells [4, 6, 8, 12, 17, 20, 21] has led to the concept of a 16°C compartment. This pre-Golgi apparatus compartment would differ from the bulk of the endoplasmic reticulum and would presumably be located in the secretory pathway between the transitional endoplasmic reticulum and the Golgi apparatus. Despite the considerable evidence for the existence of such a compartment based on the interruption of processing and transport of secretory and membrane proteins, the data on the morphological identification of such an intermediate compartment are more limited and somewhat conflicting.

This study was undertaken to utilize quantitative morphometric methods to identify that endomembrane compartment of rat liver which may accumulate at temperatures below 18°C and in cells in culture for comparison. Studies were then conducted with isolated transitional endoplasmic reticulum fractions from rat liver to determine if a similar mechanism of control by temperature of transition vesicle formation could be duplicated in the cell-free system.

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* Correspondence and reprints.
This article is dedicated to the memory of Professor Pierre Favard.

MATERIALS AND METHODS

Preparation of liver slices

Male Holtzman rats were sacrificed and livers removed. Unless stated otherwise, slices of liver averaging about 1.5×0.3×0.3 mm were cut by hand with a razor blade as described [14]. Slices were incubated in phosphate-buffered saline at the temperatures indicated for 30 or 60 min and fixed directly with 1% osmium tetroxide.

Cell culture

BHK (baby hamster kidney) cells were grown in MEM (Eagles Minimum Essential Medium) supplemented with 7% fetal calf serum, 10% triptose phosphate, 100 U/ml penicillin and 100 μg/ml streptomycin [22].

Temperature incubations

All temperature experiments were carried out in constant temperature incubators (±0.5°C). The incubators were housed in a refrigerated room (4°C) to ensure that the room temperature never exceeded that of the chambers. Equilibration of culture media, membrane preparation and liver slices to the temperature of the chambers occurred rapidly in a matter of a few minutes. Fixations were at the same temperature as the incubations.

Electron microscopy

Cells or isolated fractions were fixed after temperature incubation in 2.0% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2

followed by post fixation in osmium tetroxide in the same buffer. Dehydration was through an acetone series with embedment in Epon [11]. Thin sections were observed and photographed using a Philips EM 200 electron microscope.

Isolation of transitional endoplasmic reticulum

Endoplasmic reticulum fractions were isolated as described [13]. Livers were homogenized in 2 vol of a medium containing 37.5 mM Tris maleate, pH 6.5, 0.5 M sucrose, 5 mM MgCl₂ and 1% dextran for 45 sec with a Polytron 20 ST operated at 6,000 rpm. The homogenates were centrifuged for 15 min at 6,000 g to remove nuclei, plasma membrane fragments and Golgi apparatus, and the supernatant diluted 1:5 with homogenization medium was centrifuged at 10,000 g to remove mitochondria. The supernatant containing the endoplasmic reticulum was layered onto a discontinuous sucrose gradient consisting of 2.0, 1.5 and 1.3 M sucrose layers. After centrifugation at 85,000 g for 90 min, the membranes from the 1.3 M sucrose/sample interface were withdrawn using a Pasteur pipette and pelleted by centrifugation for 20 min at 70,000 g as the starting fraction for temperature incubation in a reconstituted system capable of *in vitro* formation of transition vesicles (see below).

Reconstitution of transition vesicle formation in the cell-free system

Part-rough, part-smooth transition elements of endoplasmic reticulum were isolated as described above and incubated in the presence of nucleoside triphosphate and cytosol at either 4°C or 37°C. In addition, the complete incubation systems contained an ATP regenerating system of 30 mM HEPES-KOH, 30 mM KCl, 2.5 mM Mg acetate, 80 μM ATP, 300 μM UTP, 2 mM creatine phosphate and 10 IU/ml creatine phosphokinase (final pH 7.0) [15, 16].
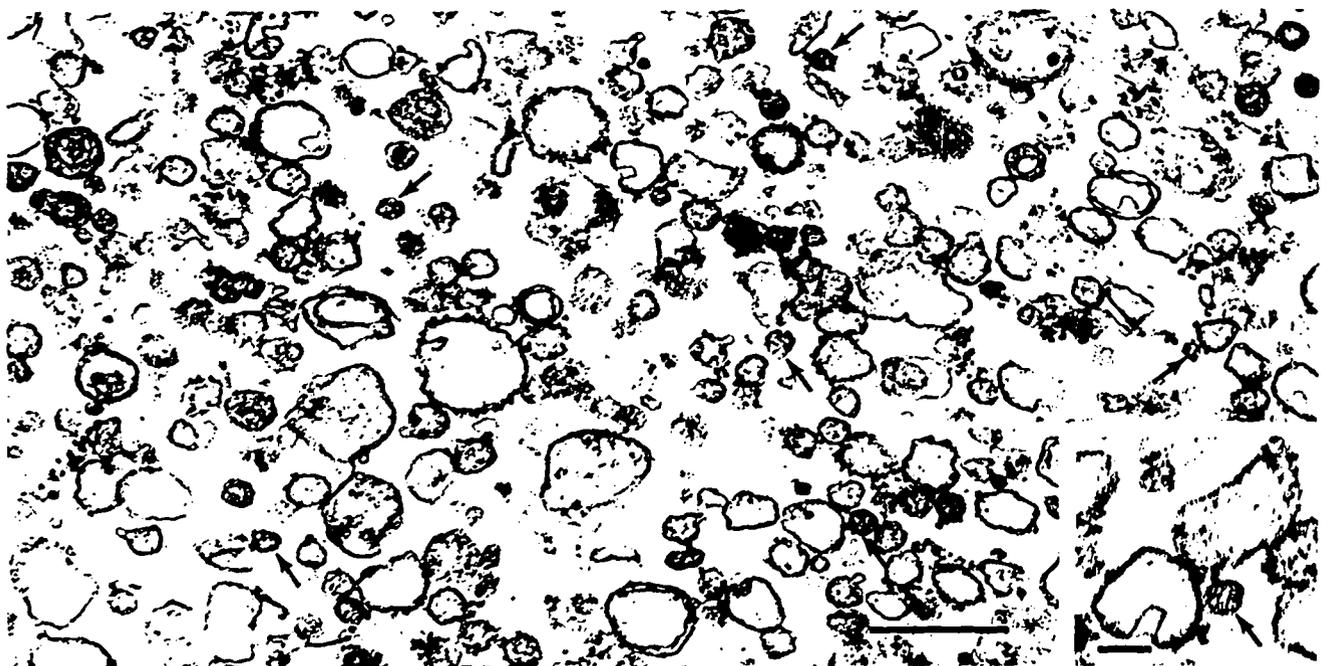

FIGURE 1. – Portion of an isolated membrane preparation consisting of part rough-part smooth endoplasmic reticulum treated for 60 min at 37°C with 60 μM ATP plus an ATP regenerating system and a concentrated cytoplasmic protein extract as described [13]. Numerous blebs or blebbing profiles are present in the preparations *(arrows)*. Bar = 0.5 μm. Inset bar = 0.1 μm.

RESULTS

Part-rough, part-smooth fractions of the transition endoplasmic reticulum fraction from rat liver when resuspended in ATP, ATP regenerating system and cytosol, were observed to contain ≈60 nm bebs and vesicles reminiscent of transition vesicles produced in situ (Fig. 1; see Nowack et al. [15] for a comparison). These vesicles appeared to vary in number as a result of incubation at different temperatures.

To quantitate the numbers of vesicles present, the preparations were pelleted onto nitrocellulose filters, prepared for electron microscopy, and the pellets were photographed through their entire cross sectional area. All vesicles were counted in 3 representative $40 \mu m^2$ areas. Only circular profiles with solid interiors representing filled vesicles rather than cross sections of hollow tubules, and in the approximate size range to be transition vesicles were scored as transition vesicles. Many were still connected to the transitional endoplasmic reticulum membranes by a narrow stalk (Fig. 1, inset).

The numbers of putative transition vesicles present in the resuspended membranes was approximately 1.5 membrane profiles per 100 membrane profiles at 4°C (Fig. 2). With increasing temperature, the number of additional vesicles followed a hyperbolic function of increase to approximatively double at 37°C with Q_{10} of increase of

about 2 over the temperature range 12°C to 37°C. By extrapolation, the temperature dependent formation of 60 nm vesicles below 16°C was insignificant.

The effect of temperature on transition vesicle production in situ was examined with liver slices as well. The morphology of the Golgi apparatus of the slices at 8°C (Figs. 3A and 4A) was representative of that observed both at 4°C and at 12°C. Golgi apparatus with characteristic stacked cisternae were present at all temperatures so that Golgi apparatus zones could be readily located. Over the temperature range 4°C to 12°C, transition vesicles were sparse. However, transition elements were well developed and numerous tubular smooth elements of the endoplasmic reticulum were evident within the Golgi zone adjacent to the cis Golgi apparatus face. These tubular smooth-surfaced membranes were more evident after 60 min of incubation (Fig. 4A) than after 30 min of incubation (Fig. 3A).

With slices incubated at 16°C (Fig. 4B) or 18°C (Fig. 3B and 4C), the most obvious morphological alteration from the appearance at 12°C was an increase in the number of transition vesicles. Especially with 60 min of incubation, the vesicles accumulated in clusters, usually near the cis Golgi apparatus face between the cis Golgi apparatus cisternae and the transitional endoplasmic reticulum. The tubular transitional membranes were still present at both 16°C and 18°C but were less evident than at 8°C or 12°C.

At 20°C, the appearance of the Golgi apparatus zone was changed markedly from that at 16°C or 18°C (Fig. 3C and 4D). The number of transition vesicles was reduced compared to 18°C and those vesicles present were no longer present in clusters. Additionally, the tubular smooth membrane elements present throughout the Golgi apparatus zone at temperatures of 18°C and below were much less in evidence. With further increases in temperature, the numbers of transition vesicles appeared to be increased slightly but the tubular elements of the endoplasmic reticulum did not reappear within the Golgi apparatus zone.

Overall, the numbers of transition vesicles present at the cis Golgi apparatus face showed an increase with increasing temperature to an optimum at 16°C (30 min) or 18°C (60 min) with an abrupt decline in transition vesicle numbers between 18°C and 20°C (Fig. 5). As with the isolated fractions (Fig. 2), the numbers of transition vesicles did not appear to increase until temperatures exceeded 12°C. Neither did they accumulate in large numbers at temperatures above 18°C presumably through release of the temperature block at temperatures below 18°C that allowed for their formation but retarded their fusion with the Golgi apparatus.

The distribution of diameters of transition vesicles present in situ at 4°C differed from those present in vitro with isolated transition elements at 4°C (Fig. 6). Those formed in vitro exhibited a larger diameter of 72 μm compared to 55 μm in situ. Comparing vesicles formed in situ at 37°C with those formed at 4°C (Fig. 6), the diameters also were shifted to larger values in the distribution profile and the average diameter was increased to 65 μm. A similar increase in vesicle diameter was seen with the transition vesicle formed in vitro from 73 μm at 4°C to 88 μm at 37°C.

Since most previous investigations of temperature effects on transitional endoplasmic reticulum have been with cultured cells, studies were carried out with BHK cells under identical conditions of temperature incubations used for liver slices. At 16°C (Fig. 7A), the general appearance
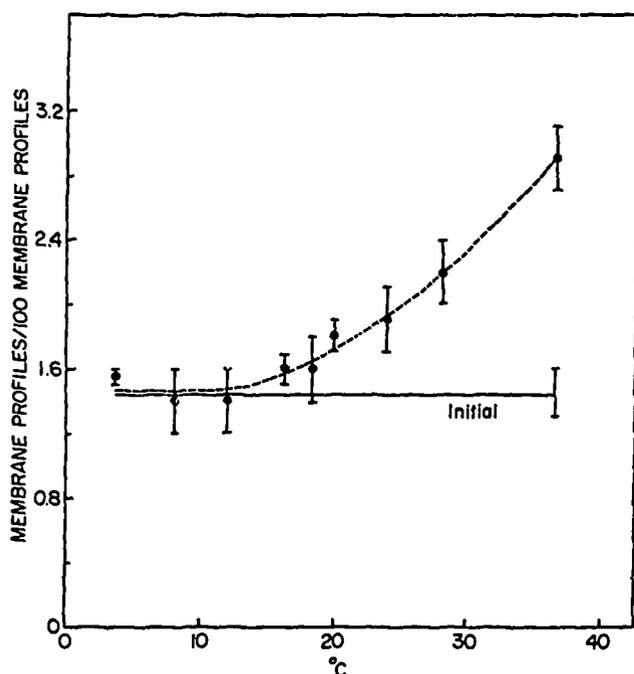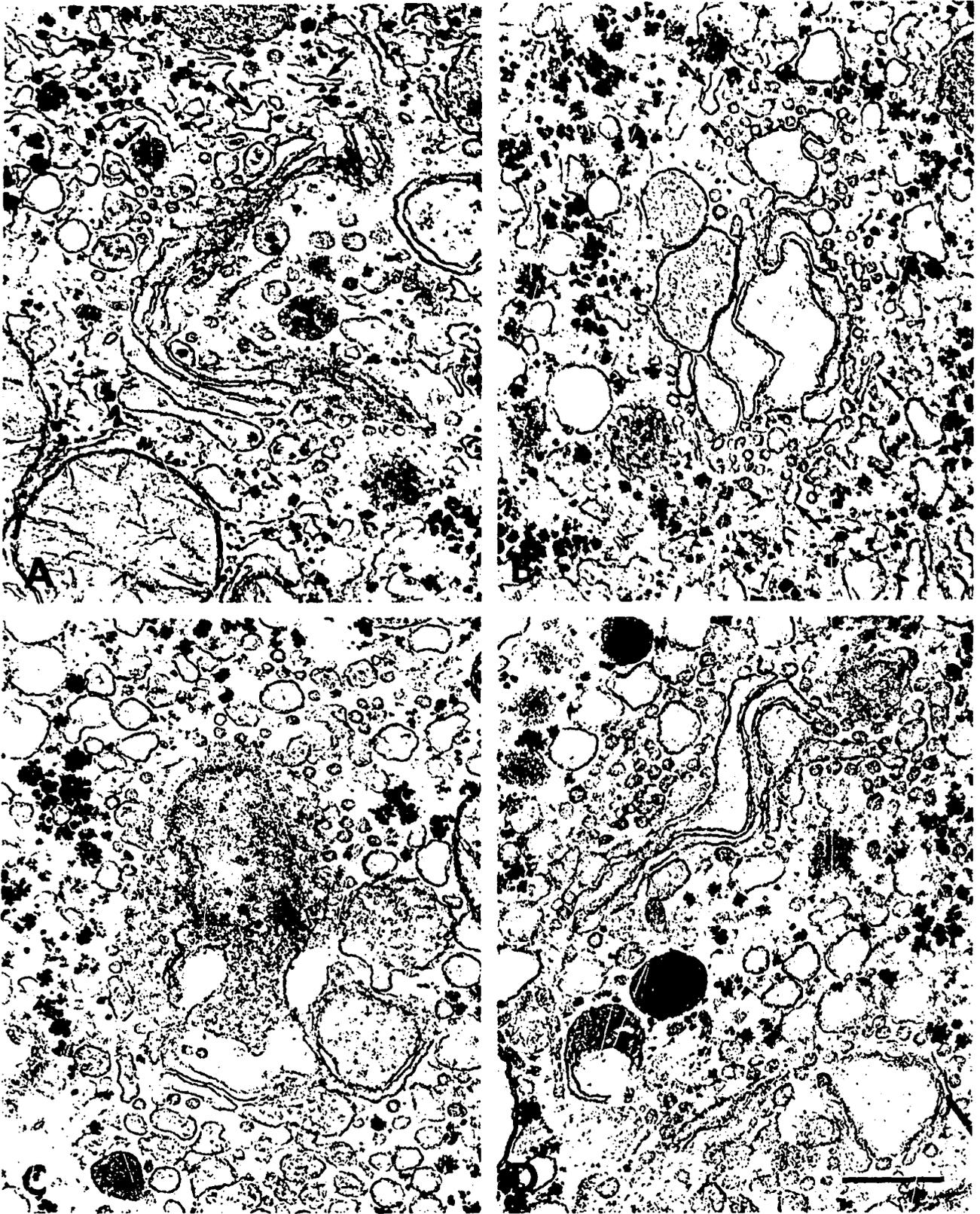

FIGURE 2. — Temperature dependency of the presence of vesicular membrane profiles of the isolated membrane preparations illustrated in Figure 1 with morphologies and of a size range similar to those of transition vesicles in situ. Results are averaged from 2 experiments ± mean average deviations. Preparations were sedimented as uniform, thin pellets onto nitrocellulose filters and sections were cut perpendicular to the nitrocellulose. Preparations were photographed at random near the top, in the middle, and near the bottom of the pellet sampling the entire pellet cross section.

FIGURE 3. – Rat liver slices incubated for 30 min at different temperatures in phosphate-buffered saline. **A**, 8°C. The appearance at 8°C is representative of the appearance of the Golgi apparatus in the temperatures of 4°C and as well 12°C. Transition vesicles *(small arrows)* are sparse and numerous smooth membrane elements *(large arrows)* penetrate into the Golgi apparatus zone. **B**, 18°C. Transition elements *(large arrows)* are still abundant but transition vesicles *(small arrows)* are increased markedly. **C**, 20°C. Both transition elements present in the Golgi apparatus zone and free transition vesicles are now reduced. **D**, 26°C. Transition vesicles are increased from 20°C but transitional endoplasmic reticulum elements of the Golgi apparatus zone remain reduced from those seen at 18°C and below. Bar = 0.5 μm.
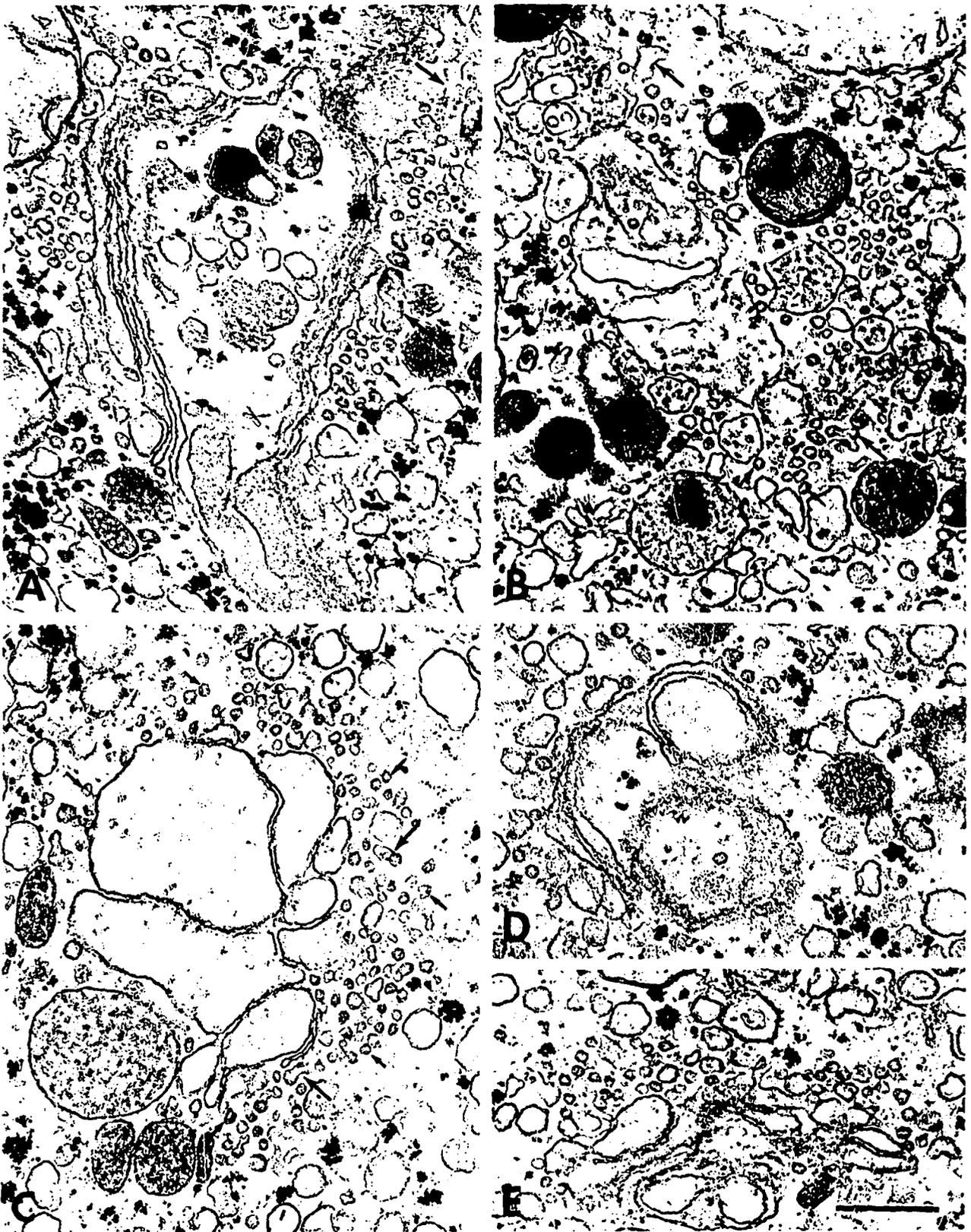
FIGURE 4. – Rat liver slices incubated for 60 min at different temperatures in phosphate-buffered saline. **A**, 8°C. Golgi apparatus morphology at this temperature is representative of the appearance of the Golgi apparatus at temperatures of 4°C and 12°C as well. Tubular smooth elements of the transitional endoplasmic reticulum *(large arrows)* are even more abundant than at 30 min. **B**, 16°C. Some tubular transitional endoplasmic reticulum elements are still evident *(large arrows)* but the transition vesicles *(small arrows)* are increased in number. **C**, 18°C. Results are the same as for 16°C except that the tubular transition endoplasmic reticulum elements *(large arrows)* are fewer and the transition vesicles *(small arrows)* are even more abundant. **D**, 20°C. As with the 30 min incubation, at this temperature both the tubular transition element adjacent to the Golgi apparatus and the numerous transition vesicles are reduced. **E**, 26°C. Transition vesicles again are increased from 20°C but as with the 30 min incubation, tubular transition elements of the Golgi apparatus zone remain reduced from those seen at 16°C and below. Bar = 0.5 µm.
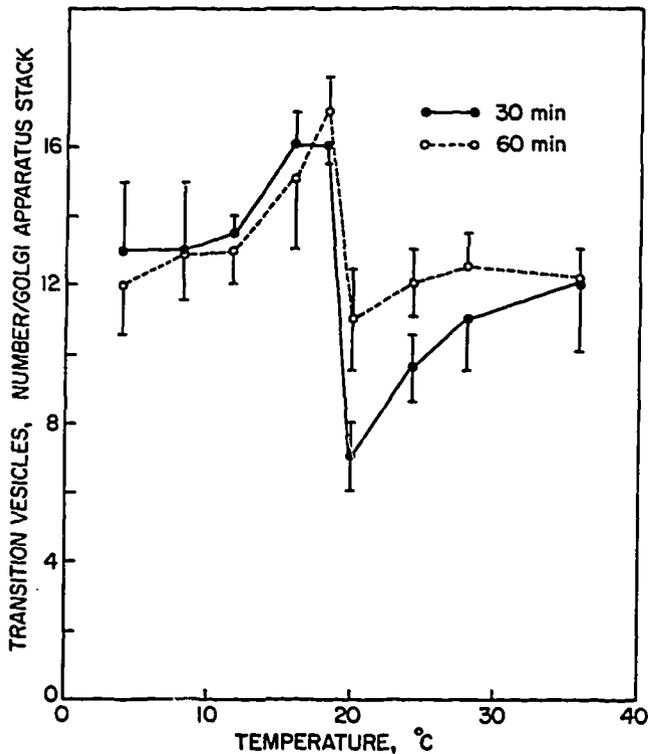
FIGURE 5. – Quantitation of the number of transition vesicles per Golgi apparatus stack as a function of temperature for liver slices incubated in phosphate-buffered saline for 30 or 60 min. Golgi apparatus were photographed at random. Results are averages from duplicate experiments ± mean average deviations with counts averaged from 12 Golgi apparatus at each temperature.

The use of inhibitors to interrupt the flow of membranes between the endoplasmic reticulum and the Golgi apparatus might be expected to have effects similar to low temperatures. Therefore, BHK cells were treated with substances to block membrane flow. Potassium cyanide blocks secretion, presumably by reducing the cellular energy charge, but did not result in an accumulation of transition vesicles (Fig. 9B) compared with control preparations (Fig. 9A) with 15, 30 or 60 min of incubation. In contrast, 1 mM $CoCl_2$ conditions known to block vesicular flow in other systems and axonal transport (see *Discussion*), did produce morphological changes very similar to those observed with the low temperature block (Fig. 9C). Numbers of transition vesicles were approximately doubled relative to the control preparations and the peripheral cytoplasm contained numerous tubular transition elements of the endoplasmic reticulum similar in appearance to those observed with incubation at 16°C (Fig. 7A). These morphological changes were observed with incubation times with 1 mM $CoCl_2$ as short as 15 min and the alterations after 60 min (not illustrated) of incubation were similar to those after 30 min of incubation (Fig. 6, Fig. 9C).

DISCUSSION

Using part-rough, part-smooth transitional element fractions isolated from rat liver, Nowack *et al.* [15] demonstrated the temperature dependent transfer of mem-
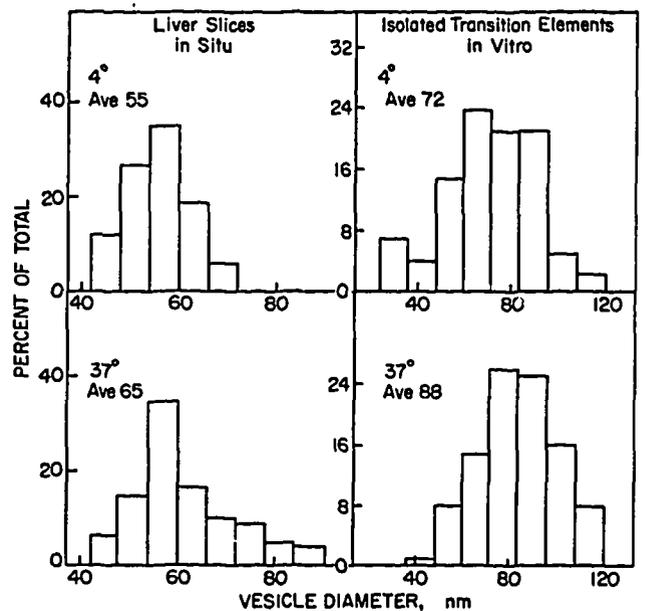
of the Golgi apparatus zone of the BHK cells was similar to that for liver. Transition vesicles present in the cytoplasm adjacent to the *cis* Golgi apparatus face were numerous and accompanied by abundant tubular transitional elements in the peripheral cytoplasm. With cells incubated at 20°C, both the tubular transitional elements of the endoplasmic reticulum and the numbers of transition vesicles were reduced markedly (Fig. 7B). At higher temperatures, *e.g.* 37°C, the appearance was similar to that at 20°C except that the numbers of transition vesicles may have increased slightly. Cells incubated at temperatures below 16°C were similar in appearance to those incubated at 16°C except for small variations in numbers of transition vesicles (Fig. 8). Results with 30 min of incubation (Fig. 7) and 60 min of incubation (not illustrated) were comparable.

Quantitation of vesicle numbers of transition vesicles associated with Golgi apparatus stacks of BHK cells gave results (Fig. 8) similar to those observed for liver slices (Fig. 5). The most characteristic feature of these data was the precipitous drop in number of transition vesicles between 18°C and 20°C. Thereafter, with increasing temperature, the number of vesicles present increased slightly. As with liver cells, the average diameter of the transition vesicles in BHK cells at 4°C was $55 \pm 4 \mu m$ but increased to $66 \pm 5 \mu m$ at 37°C.

FIGURE 6. – Distribution of transition vesicle diameters at 4°C *(upper)* and 37°C *(lower)* comparing liver slices *(left)* and isolated transition elements *in vitro (right)*. Transition vesicles *in situ* in liver slices have a mean average diameter of 10 nm greater than those at 37°C. Transition vesicles formed *in vitro*, while of overall larger diameter, show a comparable shift to larger diameters at the higher temperature.
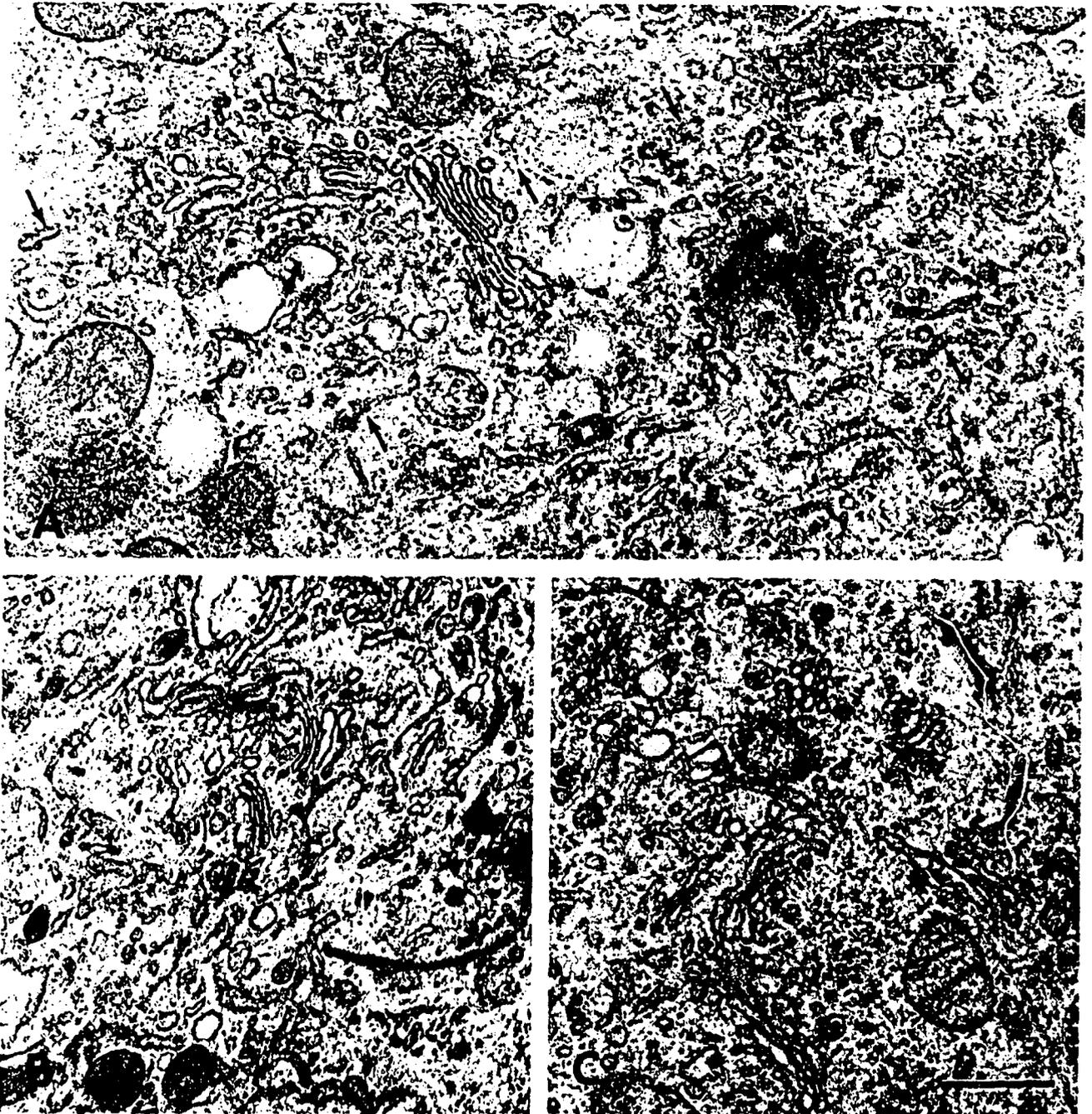
FIGURE 7. — Portion of the Golgi apparatus of BHK cells grown in MEM supplemented with fetal calf serum for 30 min at the temperatures indicated. A, 16°C. Numerous tubular smooth elements of the endoplasmic reticulum *(large arrows)* surround the Golgi apparatus stacks. Transition vesicles *(small arrows)* also are numerous. B, 20°C. Both the tubular smooth elements of the endoplasmic reticulum and the numbers of transition vesicles are reduced. C, 37°C. The appearance at 37°C is similar to that at 20°C except that transition vesicles appear to be more numerous. Bar = 0.5 µm.

brane from transitional endoplasmic reticulum donor that was prepared from liver slices labeled with [^{14}C]leucine to unlabeled Golgi apparatus acceptor. Transfer was 8-fold greater at 37°C than at 4°C commensurate with a Q_{10} of 2 for the overall transfer process. Little or no transfer was observed at 4°C compared to 37°C even in the presence of the complete incubation system containing ATP, ATP regenerating system and cytosol.

In the present study, the transition element fractions from rat liver exhibit temperature dependent formation of vesicles similar to that observed *in situ*. The formation of ≈ 50–70 µm transition vesicle-like structures over and above those initially present began at temperatures of 18°C and above. The origin of at least a major fraction of these vesicles was from the transition endoplasmic reticulum vesicles present in the fraction. Many of the vesicles re-
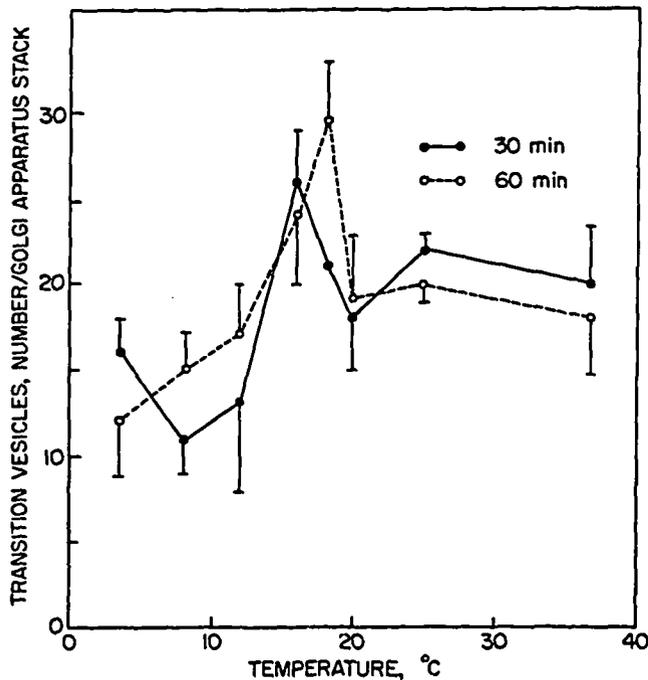
FIGURE 8. – Quantitation of the numbers of transition vesicles per Golgi apparatus stack as a function of temperature for BHK cells grown in MEM supplemented with fetal calf serum for either 30 min or 60 min at the temperatures indicated. Results are averaged from two experiments ± mean average deviations and represent counts averaged from approximately 30 Golgi apparatus stacks taken from 6 different cells for each temperature.

mained attached to larger endoplasmic reticulum vesicles by a narrow stalk (Fig. 1). The attachment of the vesicles during tissue homogenization is ruled out by the absolute dependency of their presence upon ATP [13]. Specificity is also shown by the absence of such vesicles from incubated preparations consisting only of conventional rough endoplasmic reticulum collected from gradient fractions of density greater than those yielding the responsive transitional membrane elements [15].

In situ, transitional endoplasmic reticulum of liver slices incubated at different temperature conditions behaved similarly. Over the range 4°C to 12°C, there was little evidence of transition vesicle formation. However, between 16°C and 18°C not only were transition vesicles formed but they accumulated as shown by others, presumably due to an inability of the vesicles to fuse with the cis Golgi apparatus at temperatures of 18°C or below [3, 5, 10, 18, 19]. Quite abruptly, between 18°C and 20°C, the number of transition vesicles declined as the temperature block that prevented vesicle fusion with the Golgi apparatus was relieved [3, 5, 18, 19].

Interestingly, not only was there an effect of temperature on transition vesicle numbers but on the distribution of diameters as well. At 37°C in situ, the distribution of transition vesicle diameters was shifted in the direction of larger diameters such that their overall average diameter was increased from 55 to 65 μm. For these measurements, the transition vesicles were identified not only from their characteristic appearance but from their spatial location within the cytoplasm. In situ measurements from structures other than transition vesicles would have been largely eliminated. When a
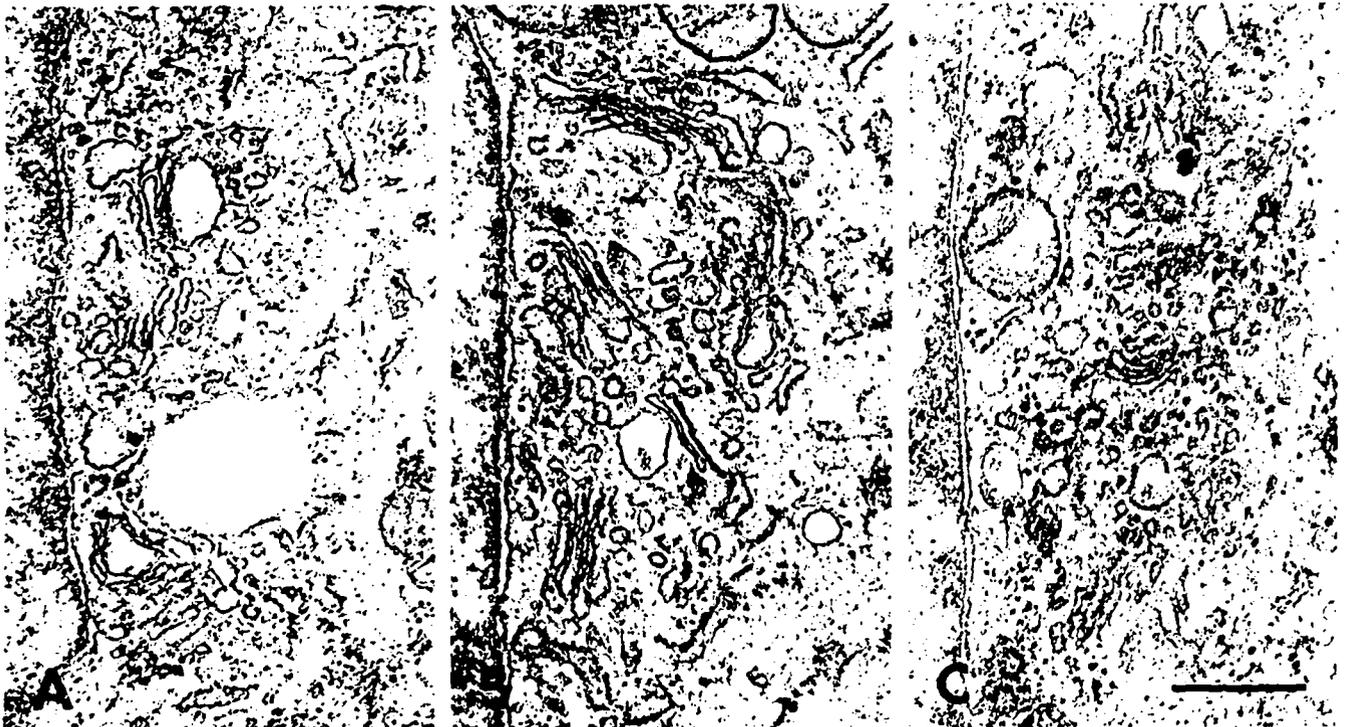

FIGURE 9. – Effect of CoCl$_2$ and KCN on transitional endoplasmic reticulum of BHK cells. A, Control cells incubated in the absence of inhibitor. B, Incubation for 30 min in the presence of KCN. The appearance is similar to that of control cells. C, Incubation with 1 mM CoCl$_2$ for 30 min. Numerous tubular transition elements and transition vesicles are present in the Golgi apparatus zone similar to those seen at 16°C. Bar = 0.5 μm.

similar analysis was carried out with those vesicles scored as transition vesicles in the *in vitro* incubated fractions, the average diameters were greater both at 4°C and at 37°C. However, even *in vitro*, the temperature effect on vesicle diameter, although small, was still evident. The average vesicle diameter at 4°C was 72 μm while the average diameter at 37°C was 88 μm.

Thus the response of transitional elements of the endoplasmic reticulum from liver to temperature *in vitro* paralleled that observed with liver slices *in situ*. Temperature-dependent formation of transition vesicles occurs primarily at temperatures above 12°C, becoming significant at approximately 20°C. The vesicle diameter was greater at 37°C than at 4°C both *in situ* and *in vitro* with the isolated fractions.

A 16°C compartment intermediate between the endoplasmic reticulum and the Golgi apparatus has been reported for cultured cells infected with temperature sensitive virus strains [8, 17, 20, 21]. In order to attempt to define a 16°C compartment for liver, parallel experiments were carried out with BHK cells incubated at different temperatures in normal culture medium. As with liver cells, both at 16°C and at 18°C, transition vesicles accumulated. However, at temperatures of 18°C and below, there was also an accumulation of smooth tubular elements of the endoplasmic reticulum in the peripheral cytoplasm surrounding the Golgi apparatus most often in near proximity to the *cis* face. We suggest this structure to be morphologically and structurally homologous to the 16°C compartment of virus-infected cells [21]. The smooth tubular endoplasmic reticulum elements seen with liver slices at temperatures of 18°C and below and especially at 8°C and 12°C also appear to be structurally homologous to the 16°C compartment seen in cultured cells. While the formation of a 16°C compartment by the isolated membrane fractions is not immediately obvious, preliminary indications are that the stalked vesicles or that some of the membrane blebs still attached to endoplasmic reticulum fragments seen in isolated preparations exposed to ATP and cytosol but not incubated at temperatures of 16°C and above may be the equivalent structures.

Both cyanide and cobalt have been reported to inhibit the transfer step between endoplasmic reticulum and Golgi apparatus [9]. Cyanide presumably blocks through its ability to deplete the energy charge of the cell whereas how cobalt inhibits is not known. With BHK cells, cyanide did not result in vesicle accumulations. At least in the cell-free [16] and perforated [1, 2] cell systems, vesicle formation and transfer shows an absolute energy dependence. Thus, an accumulation of vesicles under conditions of energy depletion would not be expected.

In contrast to cyanide, the cobalt treatment resulted in increased numbers of transition vesicles with the BHK cells resembling the response to 16°C. Similar results could not be shown with liver due to the extreme cytotoxicity of liver slices to the same cobalt concentrations and treatment conditions that resulted in transition vesicle accumulations with the cultured cells.

Cobalt ions were selected initially for comparison to low temperature because of their reported ability at 1 mM to inhibit fast axonal transport [7]. Previous findings suggested that cobalt was acting at a rate-limiting step in the initiation of axonal transport perhaps at a site where calcium or zinc might be involved in the initial coupling of protein to the transport.

ACKNOWLEDGEMENTS

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