Calcium: some aspects of subcellular accumulation and distribution in milk

by

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(ABSTRACT)

Distribution and bioavailability of ^{47}calcium in milk labeled by extrinsic and intrinsic methods was investigated. Milk from Sprague Dawley rats was labeled by both methods, and milk from a cow was labeled by the extrinsic method. Retention of ^{47}Ca from milks administered to young male Sprague Dawley rats was determined through whole body counting for 6 days after administration of milk. Percent of ^{47}Ca dose retained was 72% for extrinsically labeled cow milk, 62% for extrinsically labeled rat milk, and 55% for intrinsically labeled rat milk. Samples were fractionated by ultracentrifugation and by gel exclusion chromatography. ^{47}Calcium distributions in rat milk labeled intrinsically or extrinsically were similar. The majority of ^{47}Ca was found in a particulate, > 30,000 molecular weight fraction (about 60% for cow milk, about 90% rat milks). The amount of milk calcium retained by rats appeared to be related to the amount of noncasein micelle-associated calcium.

When administered by intraperitoneal injection into rats, ^{45}Ca specific activity of milk peaked in 60 to 90 minutes. Specific activity was highest in cytosol, and lower in Golgi apparatus and rough endoplasmic reticulum. Specific activities in subcellular fractions changed in parallel with specific activities of milk. Rapid turnover of Ca was observed in endoplasmic reticulum and Golgi apparatus; this was expected since secretory proteins and associated Ca are transported through these organelles for secretion.

In vitro ^{45}Ca accumulation was compared in Golgi apparatus and endoplasmic reticulum from liver and mammary gland of lactating Dunkin Hartley guinea pigs. In the presence of ATP, highest accumulation per unit total fraction protein was found in Golgi apparatus (mammary gland 28% of available ^{45}Ca, liver 11%) while 8% was accumulated by endoplasmic reticulum fractions. Calcium accumulation was not the result of binding, as preincubation of vesicles with calcium ionophore resulted in less than 10% of the accumulation found without ionophore. The ATPase inhibitor sodium orthovanadate, and the ATP analog AMP-PNP, reduced ^{45}Ca accumulation in all fractions. Protonophore caused a small reduction in ^{45}Ca accumulation in all cases. Citrate accumulation by fractions was not observed under conditions used for ^{45}Ca accumulation.

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LITERATURE REVIEW

Milk of cows has a calcium content of about 30 mM (Jenness, 1974). This high calcium content contributes to the nutritional value of milk. Approximately 65% of the calcium in cow milk can be removed by dialysis or will pass through an ultrafiltration membrane, this portion of milk calcium is referred to as diffusible (Mepham, 1983). The nondiffusible portion of milk calcium largely is associated with casein micelles. Milk of rats contains about 80 mM calcium, but only about 3% of this calcium is diffusible (Mepham, 1983). Protein content of rat milk, 84g/l, is much higher than protein content of cow milk, 34g/l (Jenness, 1974; Jenness and Sloan, 1974). In both rats and cows, caseins account for about 80% of the total protein content of milk. Calcium binds with avidity to caseins, and calcium-caseinate complexes account for most of the nondiffusible portion of milk calcium (Zittle et al., 1958). Jenness (1974) observed that there is a positive correlation between the amounts of casein and calcium in milk. Caseins assemble into aggregates called micelles in lumina of Golgi apparatus cisterna and in secretory vesicles within mammary epithelial cells (Farrell, 1988). Calcium is required for formation of casein micelles <u>in vitro</u>, and it is believed that calcium is required also for micelle formation <u>in vivo</u> (Waugh,

1971; Farrell, 1988). About 55% of the diffusible portion of the calcium in cow milk is complexed with citrate. Cow milk contains about 8 mM citrate. Rat milk is much lower in citrate (0.1 mM) than is cow milk, and little of the calcium in rat milk is in complex with citrate (Jenness and Sloan, 1974).

How cells can synthesize and secrete milk which has mM calcium concentrations is a question which can be addressed through study of the ability of intracellular organelles to sequester calcium. Calcium transport by fractions from cells of tissues such as bone, intestine, muscle, nerve, liver and mammary gland has been studied. Endoplasmic reticulum and Golgi apparatus from liver have been shown to accumulate calcium in the presence of ATP (Hodson, 1978; Joseph et al., 1984). Calcium was found to be accumulated, and not simply bound to membrane surfaces, by use of the calcium ionophore, A23187. Subcellular fractions either released calcium, or calcium accumulation was inhibited in the presence of ionophore (West, 1981; Chan and Junger, 1983; Prentki et al., 1984). In studies with subcellular fractions from mammary gland, it was found that Golgi apparatus has the capacity to accumulate calcium in an energy-dependent manner (Bamurucker and Keenan, 1975; Neville and Peaker, 1981; West, 1981; Virk et al., 1985). Only the first-named group found evidence for calcium

accumulation by endoplasmic reticulum from mammary gland. Calcium sequestration is of importance in function of milk secreting mammary epithelial cells. These cells must accumulate calcium from blood (3 mM calcium), transport it across the cell, and secrete a product with a [calcium] 10 times or more greater than that of blood. In the process of concentrating calcium, cytosolic calcium must be maintained at μM levels. At cytosolic calcium levels above the μM range, microtubule mediated processes such as exocytosis, and several enzymatic reactions would be affected adversely (Schliwa et al., 1981).

From intital studies with liver, mitochondria were believed to be responsible for hormonally induced changes in intracellular [calcium] (Prpiz et al., 1978). However, later it was found that hormonal stimulation of liver cells resulted in release of calcium from, presumably, endoplasmic reticulum, while mitochondria served to buffer cytosol from large changes in [calcium] (Prentki et al., 1983; Joseph et al., 1983; Shears and Kirk, 1984). Saponin-permeabilized hepatocytes were used in an attempt to distinguish mitochondrial from non-mitochondrial calcium accumulation. Mitochondrial calcium accumulation was blocked through use of the mitochondrial uncoupler 2,4-dinitrophenol and the mitochondrial ATPase inhibitor, oligomycin (Burgess et al., 1983). This study yielded

evidence that a non-mitochondrial compartment, presumably endoplasmic reticulum, primarily was responsible for calcium accumulation within hepatocytes. Calcium accumulation above a cytosolic concentration of 0.18 μM was found only in this non-mitochondrial compartment upon addition of ATP.

Chan and Junger (1983) found that plasma membrane vesicles from rat liver could accumulate calcium in the presence of ATP. Since mitochondrial poisons were without effect, it was concluded that contamination of plasma membranes by mitochondria was not responsible for these results. Other nucleotides (CTP, GTP, UTP and ADP) would not substitute for ATP in stimulation of calcium accumulation by plasma membrane vesicles. Vanadate inhibited both calcium accumulation and ATPase activity. Chan and Junger (1983) concluded that calcium accumulation into plasma membrane vesicles was dependent on Ca^{2+}, Mg^{2+}-ATPase activity.

Many workers have reported that calcium is accumulated into vesicles derived from endoplasmic reticulum (ie., microsomal vesicles) in the presence of ATP (Moore et al., 1975; Walz, 1982; Unger et al., 1984; Heilmann et al., 1984). Moore et al. (1975) reported that other nucleotides would not substitute for ATP in promoting calcium sequestration by microsomes from rat liver. Walz (1982) made a

similar observation with agranular (smooth) endoplasmic reticulum from liver. Calcium efflux from endoplasmic reticulum of rat insulinoma cells was stimulated specifically by ADP, and ATP-dependent calcium accumulation was inhibited by addition of hexokinase and glucose to incubation medium (Prentki et al., 1984). The non-hydrolyzable ATP analog adenyl-imidodiphosphate (AMP-PMP) and inhibitors of ATPase of sarcoplasmic reticulum (mersalyl and tetracaine) inhibited ATP-dependent calcium accumulation by endoplasmic reticulum from photoreceptor cells (Unger et al., 1984).

Isolated preparations enriched in elements of Golgi apparatus have been reported to accumulate calcium in the presence of ATP. Many of these studies have been with Golgi apparatus isolated from lactating mammary glands of various species. With mammary gland Golgi apparatus, nucleotide specificity for calcium accumulation was not as strict as with endoplasmic reticulum from other tissues (West, 1981). West (1981) found that ADP added to incubation mixtures in an amount equimolar with ATP inhibited calcium accumulation by 50%. Calcium-stimulated ATPase activity was found in Golgi apparatus preparations from cow (Baumrucker and Keenan, 1975) and mouse (Watters et al., 1984) mammary glands. Hodson (1978) obtained evidence for ATP-dependent accumulation of calcium by Golgi apparatus from rat liver.

While studies to date clearly implicate components of the endomembrane system in ATP-dependent calcium accumulation, several aspects of calcium transport remain to be clarified. Several investigators have used oxalate to trap calcium. Presumably oxalate enters vesicles and serves to bind and trap calcium transported into vesicles. In some studies oxalate was found to have a positive effect (Moore et al., 1975; West, 1981; Virk et al., 1985), while in others oxalate had little or no effect (Hodson, 1978; Prentki, 1984). Differences observed upon addition of oxalate cannot be explained by differences in tissue type or subcellular fraction, as clear differences have been observed within tissue and fraction type. Measurements of stoichiometry of calcium accumulation and ATP hydrolysis also have produced variable results. West (1981) found a ratio of 1 molecule of calcium accumulated to 50 molecules of ATP hydrolyzed, while Virk et al. (1985) obtained a ratio of 0.3 to 0.7:1 for Golgi apparatus from rat mammary gland. Protonophores have been found to reduce calcium uptake by Golgi apparatus from mammary gland (West, 1981; Virk, 1985). This may be due to an effect of protonophores on an electrogenic proton pump, driven by NADH and ATP, as was found in Golgi apparatus from rat liver (Barr et al. 1984).

INTRODUCTION I

To establish bioavialiability of Ca in milk, studies were performed using isotopically labeled milk. Before using this approach, it was necessary to determine if incubation of milk with isotopic calcium creates a labeled calcium distribution in milk equivalent to that of endogenous milk calcium. A comparison of intrinsically labeled milk with extrinsically labeled milk was made using rats. Milk was intrinsically labeled by intraperitoneal injection of ^{47}Ca into lactating rats. Extrinsically labeled milk was obtained by incubation of milk with ^{47}Ca. Cow milk was extrinsically labeled to evaluate differences in ^{47}Ca distribution in relation to differences in milk composition. Milk was fractionated by several techniques to determine the amount of ^{47}Ca which was associated with lipid globules, complexed with proteins, or which was soluble or in low molecular weight salts. Rats were intubated with ^{47}Ca-labeled milk to determine if retention was the same regardless of milk composition or method of labeling.

Objectives were to determine the distribution of radiolabel in milk labeled extrinsically versus intrinsically, compare bioavailability of calcium in these milks, and to determine if species variation in milk composition affected these parameters.

MATERIALS AND METHODS I

Milk collection and labeling

Pregnant Sprague Dawley rats were obtained from Dominion Laboratories, Dublin, VA, and housed at 21°C on a 12:12 light/dark schedule. Deionized water and standard laboratory rat chow were supplied ad libitum. Lactating rats, with litters adjusted to 14 pups at delivery, were milked four to six days postpartum. Approximately 12 hours before milking, all but two pups were removed from each dam. Just prior to milking, dams received pentobarbital, 60 mg/kg, and 5 IU oxytocin intraperitoneally (ip). Rats were milked with a vacuum aspiration device similar to that described by Gupta et al. (1970). Intrinsically labeled milk was obtained by injecting each of 10 rats ip with 10 μCi $^{47}CaCl_2$ in 0.2 ml sterile 0.9% saline, 10 to 15 hours before milking. Isotope stock contained 50 μCi/ml and specific activity was greater than 200 μCi/mg Ca. Forty milliliters of milk was obtained. Radiation was measured by three 40 second counts using an Ortec Model 402 MX whole body counter with window settings for ^{47}Ca of 220 (lower limit) and 760 (upper limit). The radioactivity of the intrinsically labeled milk was approximately 55,000 CPM/ml. Ten rats not injected with isotope provided a total of 23 ml of milk for extrinsic ^{47}Ca labeling. Milk from these rats and milk from a Holstein cow were

extrinsically labeled by adding ^{47}Ca to a final level of approximately 55,000 CPM/ml, equal to that obtained from intrinsic labeling. The volume of $^{47}CaCl_2$ solution added to these milks was under 1 µl/ml. Samples were then allowed to equilibrate at about 21°C for 1.5 hours.

Bioavailability studies: intubation and retention

Male Sprague Dawley rats, weighing approximately 175 g, were obtained from Charles River Laboratories, Wilmington, MA. Animals were housed under conditions described above. Rats were weighed and food was removed 24 hours prior to treatment. Milk was administered to unanesthetized rats via stomach tube. The dose was 1.5 ml of intrinsically labeled rat milk (n=12) or 1.5 ml of extrinsically labeled rat or cow milk (n=10). Three 40 second whole body counts were used to determine the quantity of radioisotope placed into rats, using instrumentation and settings described above. All animals received food and water ad libitum for the duration of this experiment.

^{47}Calcium retention was assessed by whole body counting on days 1, 2, 3, 4, and 6 after dosing. All measurements of radioactivity were corrected for background and decay. One milliliter of labeled milk was used as a standard, counted on day 0 and on all subsequent days, to calculate radioactive decay. Retention values were compared using

the Statistical Analysis Systems General Linear Model procedure (1985). This program allows for an unbalanced sample size. Treatment differences were compared using least square means from analysis of the model below:

$$Y_{ijkl} = \mu + T_i + S_j + (TS)_{ij} + C_{(ij)k} + D_l + (TD)_{il} + (SD)_{jl} + (TSD)_{ijl} + E_{ijkl}$$

where

Y_{ijk} is observed dependent variable

μ is mean of Y

T_i is fixed effect of ith isotope labeling treatment, i=1,2

S_j is jth source of milk j=1,2

$C_{(ij)k}$ is random effect of kth rat within ith treatment and jth source, k=1,10 or 1,12

D_l is fixed effect of lth day l= 1,7

$(TS)_{ij}$ is the interaction of treatment and source

$(TD)_{il}$ is the interaction of treatment and day

$(SD)_{jl}$ is the interaction of source and day

$(TSD)_{ijl}$ is the interaction among treatment, source, and day

E_{ijkl} is random residual

Treatment, milk source, and treatment by source were tested for significance at the 0.05 level with least square means. Animals were weighed at the time of the last body count.

Distribution of ^{47}Ca in milk

Milk samples were fractionated using centrifugal micro-concentrators. Duplicate 200 µl whole milk samples were centrifuged in Centricon 30 microconcentrators (Mr cutoff of 30,000) for 30 minutes at 5,000 X g and at 20°C. One milliliter of distilled, deionized water was added and centrifugation was continued for 15 minutes. Solution which passed through the Centricon 30 membrane was loaded into a Centricon 10 (Mr 10,000 cutoff) and centrifuged as above for 60 minutes. Radioactivity was determined on three ultrafiltrate fractions for each milk sample: > 30,000 D, 10-30,000 D, and < 10,000 D.

Fractionation of milk by ultracentrifugation was performed on duplicate samples, using 800 µl of intrinsically or extrinsically labeled rat milk or cow milk. Samples were centrifuged in a swinging bucket rotor at 100,000 X g for 12 hours at 4°C. Radioactivity was measured in three phases: floating lipid globules, supernatant, and pellet.

Skim milk was prepared by centrifugation of whole milk at 3,000 X g for 15 minutes at 2°C. Four hundred µl of rat or cow skim milk was fractionated by gel permeation chromatography using a PD-10 column (Sephadex G-25M, bed volume 9 ml) which was equilibrated and eluted with phosphate buffered saline, pH 7.4, at 21°C. Eluates were collected in 400 µl fractions and radioactivity was

determined using a LKB Model 1282 Compugamma Counter. The energy detection range of the instrument was 10 - 2,000 keV, covered by 256 logarithmically arranged energy level numbers. Window settings, representing these energy levels, were 180 (lower limit) and 236 (upper limit).

Sephadex G-150 columns were used to obtain greater resolution in fractionation. A second set of milk samples was prepared for this fractionation as a higher specific activity of the milk was required. One primparous Sprague Dawley rat was removed from her pups, injected with 35 μCi $^{47}CaCl_2$ (0.7 ml isotope stock) and 5.1 ml of milk were obtained. Extrinsically labeled rat and cow milk were prepared as before, to approximately equal ^{47}Ca levels as were present in intrinsically labeled rat milk, 400,000 CPM/ml. Skim milk samples of 300 to 800 μl were loaded onto a 2.5 X 50 cm column of Sephadex G-150, which was equilibrated and eluted with distilled, deionized water. Flow rate was 0.4 ml/minute at 21°C, and 2.3 ml fractions were collected for determination of radioactivity and absorbance at 280 nm.

Materials

Isotope, $^{47}CaCl_2$, was obtained from Amersham, Arlington Heights, IL. PD-10 gel filtration columns were obtained from Pharmacia, Piscataway, NJ. Centricon centrifugal

microconcentrators were obtained from Amicon Corp., Danvers, MA. All other reagents and chemicals were from Sigma, St. Louis, MO or from Fisher, Raleigh NC.

RESULTS AND DISCUSSION I

Calcium retention studies

Amounts of calcium retained by young rats were different for different sources of milk and for different methods used to introduce radiolabeled calcium into milk (Figure 1). Retention was greatest with extrinsically labeled cow milk, intermediate with extrinsically labeled rat milk and lowest with intrinsically labeled rat milk ($p<0.05$). Least squares mean retentions over the six days following administration were about 72%, 62% and 55% for cow milk, extrinsically labeled rat milk and intrinsically labeled rat milk, respectively. Retention was higher with cow milk than with either rat milk sample on each of the 6 days of the experiment. Retention of calcium from rat milk was higher with extrinsic rather than intrinsic labeling throughout the experiment, but differences were only significant for days 3 and 4 ($p<0.05$). There were no significant differences in rate of loss of ^{47}Ca from rats administered cow or rat milk over the time course of this experiment. With all milk samples, rate of loss of radioactivity from rats was about 2% per day. Differences in retention were not due to differences in beginning body weights of rats, which was about 205g for all treatment groups. At termination of the experiment on day 6, mean body weights in each treatment group was 249g.

Figure 1. ^{47}Calcium retention in rats after dosing with labeled milk.

Rats were intubated with 1.5 ml of milk containing approximately 82,500 CPM. Three 40 second whole body counts were used to determine the quantity of radioisotope placed into each rat (instrumentation described in text). This initial count was designated 100%. Retention was assessed on subsequent days by whole body counting, with counts retained represented as a percent of original dose. Statistical differences are discussed in the text.

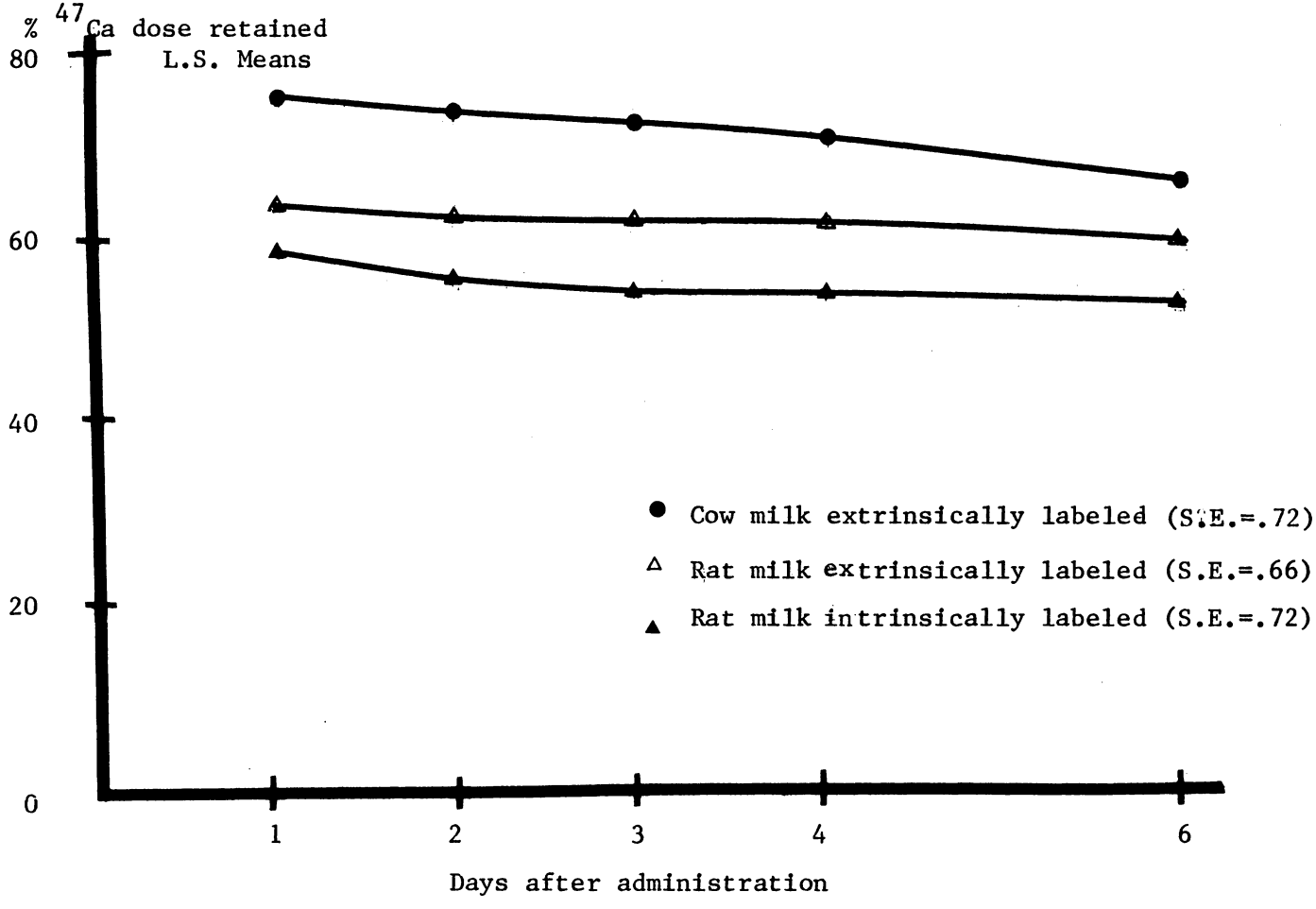

Figure 1. ^{47}Calcium retention in rats after dosing with labeled milk.

Fractionation of labeled milk

Rat milk has a total calcium content of 80 mM, and cow milk has 30 mM total calcium (Mepham, 1983). However, only about 3% of the calcium of rat milk can be removed by dialysis, but 34% of the calcium of cow milk can be removed by dialysis. From this information it can be calculated that rat milk has 2.5 mM diffusible calcium whereas cow milk has 10.4 mM diffusible calcium. It is believed that most of the non-dialyzable calcium of milk is in complex with inorganic phosphate and with phosphoproteins, primarily caseins (Jenness, 1974; Mepham, 1983). If only that portion of milk calcium which is not tightly complexed with casein micelles can be absorbed (Wilkinson, 1976), then higher retention of calcium would be expected from cow milk than from rat milk.

Milk from cows and from rats was fractionated to determine if there were differences in distribution of radiolabeled calcium in milk labeled by intrinsic or extrinsic methods. When fractionated by ultrafiltration, there was little difference in distribution of ^{47}Ca in fractions of extrinsically or intrinsically labeled rat milks (Table 1). About 94% of the ^{47}Ca in both samples was in the >30,000 mw fractions, and 5 to 6% was in the <10,000 mw fractions. With both extrinsically and intrinsically labeled rat milks, less than 1% of the isotope was in the

Table 1. Distribution of ^{47}Ca in milk samples.[a]

Fractionation Method	Fraction	Rat I	Rat E	Cow E
Ultrafiltration	MW [b] > 30,000	94.5	93.3	60.0
	10,000 - 30,000	0.3	0.8	0.8
	< 10,000	5.2	5.9	39.2
Ultracentrifugation[c]	Lipid globules	2.2	2.4	1.8
	Supernatant	9.0	8.3	42.2
	Particulate	88.8	89.3	56.3

[a] Percent of total, averages of duplicates, I = intrinsically labeled, E = extrinsically labeled.

[b] Whole milk samples (200 ul) were fractionated using centrifugal microconcentrators as described in Materials and Methods I. Microconcentrators were equipped with a selective filtration system for specific molecular weight retention.

[c] Whole milk samples (800 ul) were ultracentrifuged at 100,000 X g for 12 hours, 4°C.

10,000 to 30,000 mw fraction. With the extrinsically labeled cow milk sample, 60% of the isotope was in the >30,000 mw fraction, less than 1% in the 10,000 to 30,000 mw fraction, and 39% was recovered in the <10,000 mw fraction.

Fractionation of milk samples by ultracentrifugation gave results similar to those obtained by ultrafiltration and provided information on the amount of radiolabeled calcium which was associated with lipid globules (Table 1). In all milk samples, the amount of ^{47}Ca associated with lipid globules was about 2% of the total. The amount of ^{47}Ca in the supernatant produced by centrifugation correlated with amounts in <10,000 mw fraction obtained by ultrafiltration. The particulate fraction obtained by centrifugation had about the same amount of ^{47}Ca as did the >30,000 mw fraction from ultrafiltration.

Gel exclusion chromatography was used to extend fractionation studies. When fractionated by Sephadex G-25 columns, most radioactivity in rat milk was collected in the void volume, irrespective of labeling method (Figure 2). Radioactive calcium added to cow milk eluted in two fractions, one corresponding to the void volume and the second in the included volume (Figure 2). Separation in Sephadex G-150 columns confirmed these results. Most radioactive calcium in cow milk eluted in two peaks (Figure

Figure 2. Gel filtration of ^{47}Ca-labeled milk on PD-10 columns.

Skim milk samples (400 uI) were fractionated on PD-10 (Sephadex G-25M) columns. Bed volume was 9 ml. Samples were eluted with phosphate buffered saline, pH 7.4, at 21°C. Fraction volume was 400 ul.

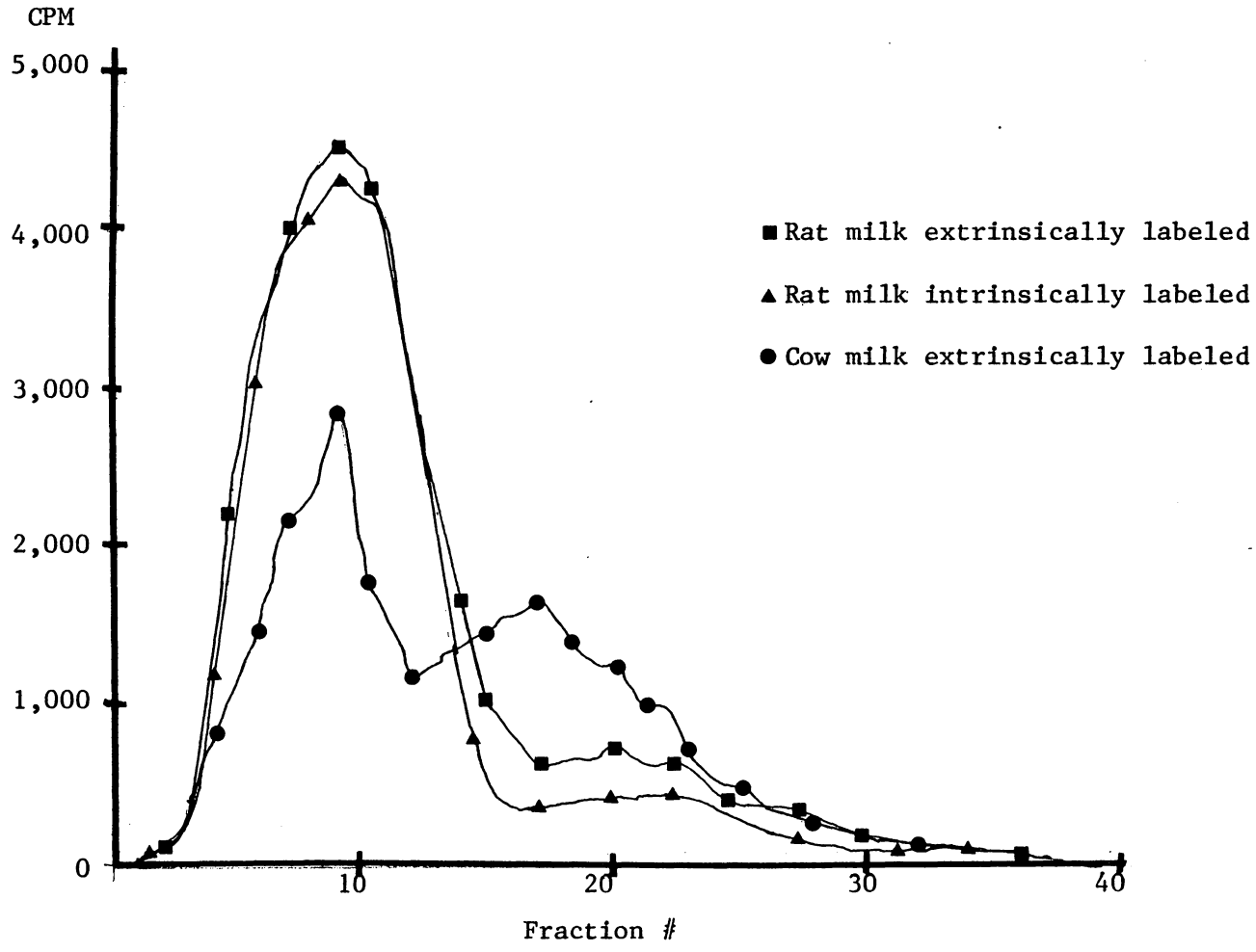

Figure 2. Gel filtration of ^{47}Ca-labeled milk on PD-10 columns.

3). The first peak corresponded with the peak of A_{280} absorbing material in the void volume and most probably contained calcium which was associated with casein micelles. The second peak from cow milk contained little A_{280} absorbing material and most probably contained small calcium salts. In contrast to the results with cow milk, most of the ^{47}Ca in extrinsically and intrinsically labeled rat milk eluted with the major protein peak in the void volume of the column (Figure 4).

Based on results from retention and fractionation studies, it appeared that the amount of milk calcium retained by rats may have been related to the amount of calcium which was not associated with casein micelles. Rat milk has a higher calcium content than cow milk, but most of the rat milk calcium was associated with casein micelles. Cow milk has less total protein and less total calcium than rat milk, but in cow milk about 40% of the calcium was not associated with casein micelles. These differences may account for the different initial calcium retentions of rats dosed with cow or rat milks. However, the amount of calcium initially retained was greater than the amount of non-casein micelle associated calcium in these milks in all cases. This may mean that calcium associated with casein micelles is absorbed more slowly than calcium from salts. From these results it appears

Figure 3. Gel filtration of ^{47}Ca-labeled cow milk on Sephadex G-150 columns.

Skim milk (800 ul extrinsically labeled) was fractionated on a 2.5 X 50 cm Sephadex G-150 column. Distilled-deionized water was used for elution of sample, and 2.3 ml fractions were collected at 0.4 ml/minute, 21°C. Elution profiles of radioactivity are given in panel A and protein in panel B.

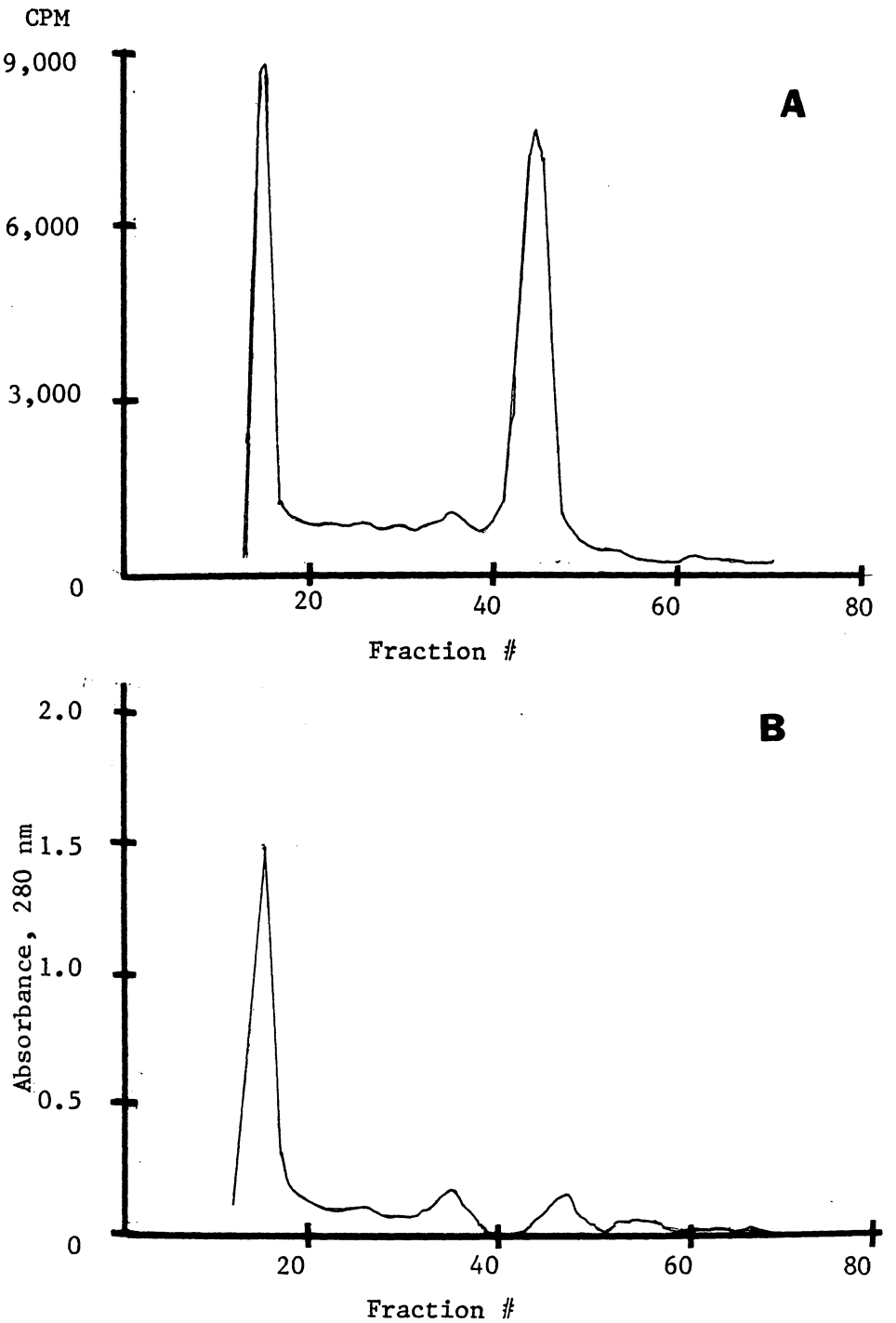

Figure 3. Gel filtration of ^{47}Ca-labeled cow milk on Sephadex G-150 columns.

Figure4. Gel filtration of ^{47}Ca-labeled rat milk on Sephadex G-150 columns.

Skim milk (800 ul of extrinsically labeled, 300 ul of intrinsically labeled) was fractionated as described in Figure 3. Elution profiles of radioactivity and protein for intrinsically labeled rat milk are shown in panels A and B respectively, and extrinsically labeled rat milk panels C and D, respectively.

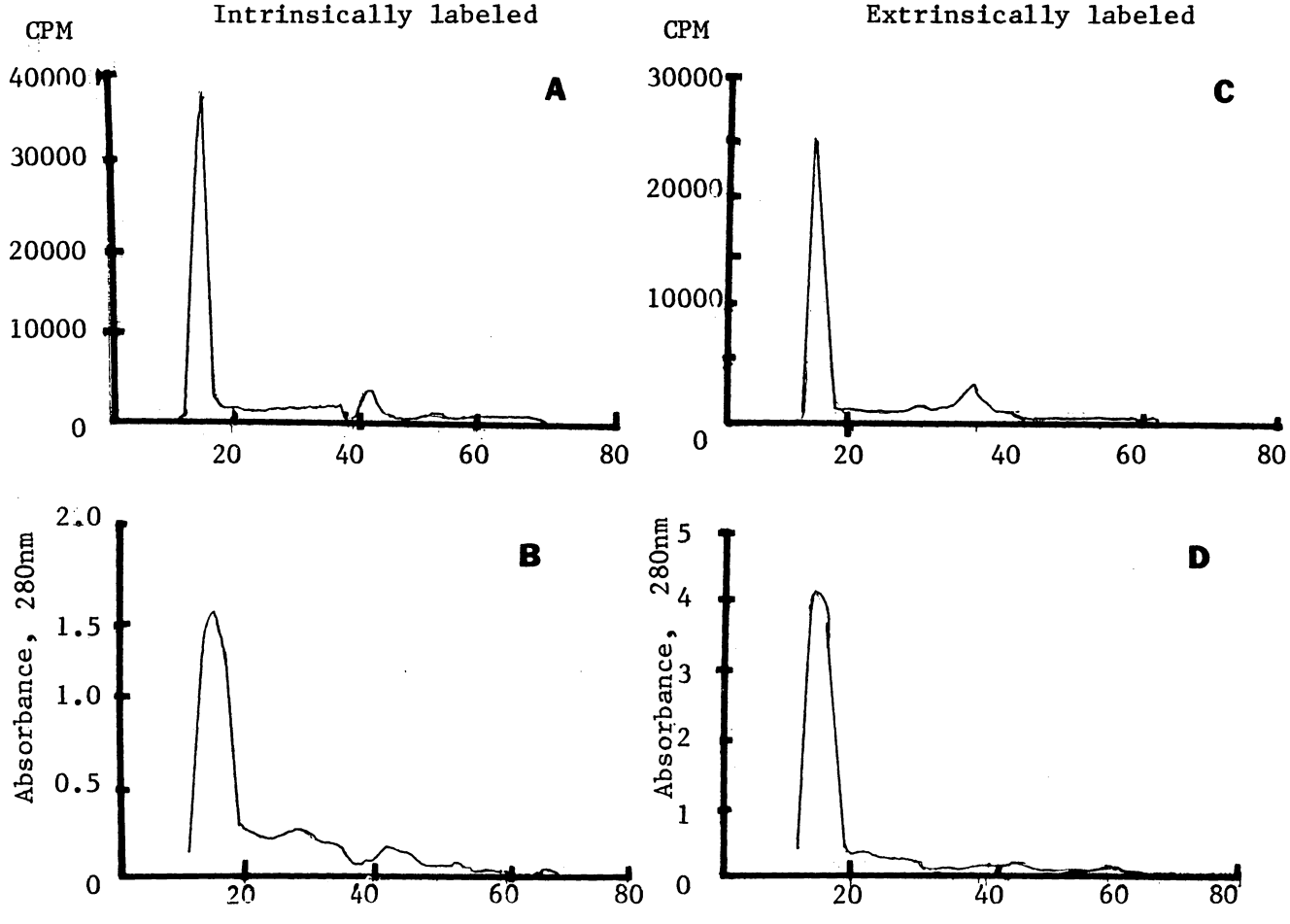

Figure 4. Gel filtration of ^{47}Ca-labeled rat milk on Sephadex G-150 columns.

that radiolabeled calcium added to cow milk equilibrated with the intrinsic calcium of this milk. Given this, then the retention data show that the total amount of calcium absorbed by rats to which milk was administered was greater with rat milk than with cow milk. Once absorbed, there appeared to be no differences in retention with source of calcium, as rates of loss per day were the same irrespective of milk source.

In interpreting these results, it must be remembered that distribution of calcium in milk fractions as measured in the laboratory may not reflect distribution in milk fractions during the digestive process. In the physiological conditions under which milk calcium is absorbed, milk is acidified in the stomach, neutralized in the small intestine, and exposed to digestive proteases in both the stomach and intestine. My findings do indicate that species differences in distribution of calcium in milk may affect bioavailability of milk calcium.

INTRODUCTION II

To gain a complete understanding of milk formation, a question which must be addressed is how cells synthesize and secrete milk with mM Ca content and how subcellular organelles participate in this process. In vivo ^{45}Ca administration was attempted first to determine relative distribution of Ca in the mammary epithelium. Mitochondrial fractions were not examined in this study. Mitochondria have been shown to have the ability to accumulate Ca, but mitochondria do not appear to play an important role in transport of Ca for secretion into milk (Prentki et al., 1984; Burgess et al., 1983; Neville & Watters, 1983). Ultrastructural localization of Ca, by electron microscopic examination of tissue treated to cause Ca precipitates, or by electron probe X-ray analysis of tissue, was not atttempted, since these techniques have not been successful in providing information on localization of calcium in organelles such as such as endoplasmic reticulum and Golgi apparatus (Wooding & Morgan, 1978; Cameron et al., 1986; Somlyo et al., 1985).

Before a comparison of calcium accumulating ability of various organelles can be made, differences in organelle composition must be considered. Calcium accumulation is a process controlled by membranes, and therefore comparison between organelles should be made on a per unit of membrane

basis. The percent of protein and phospholipid of the organelle present in membrane and contents was determined for this reason. Calcium accumulation was examined in rough endoplasmic reticulum and Golgi apparatus isolated from liver and mammary tissue of lactating animals. Liver and mammary tissues were compared since literature contains reports of accumulation of calcium by liver endoplasmic reticulum (Moore et al., 1975). Guinea pigs were used for studies of calcium accumulation by subcellular fractions because rats provide relatively less mammary tissue and all mammary glands are not equivalent in rat (ie.,pectoral and inguinal glands differ from each other). Age and sex were found to be factors which effected energy-dependent calcium sequestration by endoplasmic reticulum from rat liver (Moore et al., 1975). In addition, the liver of a lactating animal may accumulate calcium at a higher rate than the liver of a non-lactating animal. For these reasons, liver and mammary gland always were obtained from animals of the same physiological status.

Various parameters, including time of incubation, protein concentration, and calcium concentration, were optimized for <u>in vitro</u> calcium accumulation experiments. Preparations of endoplasmic reticulum and Golgi apparatus from liver and mammary gland were compared for ^{45}Ca accumulation with and without exogenous ATP. The role of

ATP in calcium accumulation was assessed by addition of a nonhydrolyzable ATP analog (AMP-PNP) or a Ca^{2+}-ATPase inhibitor (vanadate) to vesicle preparations (Chan & Junger, 1983). True vesicle accumulation was determined using the calcium ionophore, A23187 (Reed & Lardy, 1973). As citrate has been called the "harbinger of lactogenesis" (Peaker & Linzell, 1975) and citrate uptake has been found in Golgi apparatus from mammary tissue (Zulak & Keenan, 1983) the possibility of calcium and citrate cotransport was investigated using ^{14}C-labeled citrate. The possibility of the requirement of a proton gradient for calcium accumulation was evaluated using carbonyl cyanide m-chlorophenyl hydrzone (CCCPH) (Harold, 1970).

The overall objective of this work was to compare calcium accumulation activity of two subcellular fractions, endoplasmic reticulum and Golgi apparatus, isolated from two different tissue types, liver and mammary gland. A quantitative assessment of the calcium accumulating capacity of these fractions was made on a total protein basis, as well as on a membrane protein or phospholipid basis. The affects of a calcium ionophore, a protonophore, ATP, an ATP analog and an ATPase inhibitor on calcium accumulation were determined. These comparisons were made to determine if these organelles share common means of Ca accumulation, without tissue-specific differences.

MATERIALS AND METHODS II

In vivo calcium distribution

The uptake and distribution of radiolabeled calcium (^{45}Ca) in mammary glands was examined using Sprague Dawley rats (Dominion Research Laboratories, Dublin, VA). Rats were housed in the Virginia Polytechnic Institute & State University (V.P.I. & S.U.) Laboratory Animal Resources vivarium, in rooms maintained at 21°C, with 12:12 light/-dark schedule. Purina Rat Chow and tap water were supplied ad libitum.

Within two days of parturition, litter size was adjusted to nine. Dams were separated from pups and were given one intraperitoneal (ip) injection of 20 μCi of $^{45}CaCl_2$(28 mCi/mg) in 0.3 ml of 0.9 % sodium chloride between the 9th and 11th days of lactation. Animals were killed by cervical dislocation at 3, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, and 360 minutes postinjection. Two minutes prior to sacrifice, animals were given oxytocin ip (5 IU), anesthetized with ether, and were milked. Inguinal mammary glands were dissected and fractionated to prepare Golgi apparatus (Keenan et al., 1972a) rough endoplasmic reticulum (Adelman et al., 1973) and cytosol (12 hour supernatant from centrifugation of homogenate at 100,000 X g, 2°C). Serial blood samples were taken via the tail vein from two rats (15 to 180 minutes after injection of ^{45}Ca)

to determine when ^{45}Ca peaked in the circulation. Protein concentrations of milk, homogenate, and subcellular fractions were determined according to Lowry et al. (1951) using bovine serum albumin as standard. Radioactivity of fractions was measured by liquid scintillation counting, using a LKB 1217 Wallac Rackbeta scintillation counter. The energy detection range of the instrument was 1-2,800 keV covered by 256 logarithmically arranged energy level numbers. Window settings, representing these energy level numbers, were 25 (lower limit) and 165 (upper limit). Ecoscint scintillation fluid was used. Samples were counted for five minutes.

Fraction isolation and enzymatic evaluation

Dunkin-Hartley guinea pigs (Hazelton Research Laboratories, Danver, PA) were raised and bred at the V.P.I. & S.U. Laboratory Animal Resources vivarium under conditions stated above, except that standard guinea pig chow was fed *ad libitum*. Guinea pigs at 7 to 14 days of lactation were asphyxiated with carbon dioxide. Mammary glands and liver were excised and chilled on ice in 0.25 M sucrose. Tissues were weighed and homogenized for isolation of Golgi apparatus (Keenan et al., 1972a) or rough endoplasmic reticulum (Sunshine et al., 1971). Subcellular fractions were evaluated for purity using enzyme markers for plasma

membrane and mitochondria, two other fractions known to transport calcium. Phosphodiesterase I, E.C. 3.1.4.1 (plasma membrane marker) and succinate-2-(p-nitrophenyl)-5--phenyltetrazolium reductase, E.C. 1.3.99.1 (mitochondrial marker) were measured spectrophotometrically by methods of Brown et al. (1976) and Pennington (1961), respectively. To determine enrichment of Golgi apparatus, lactose synthase, E.C. 2.4.1.22, and galactosyl transferase, E.C. 2.4.1.67, were measured using glucose as acceptor for fractions from mammary tissue and N-acetylglucosamine as acceptor for fractions from liver (Morre', 1971). Nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome c reductase, E.C. 1.6.2.4, an indicator of endoplasmic reticulum enrichment, was determined according to Mackler (1967). Spectrophotometric measurements were made with Model 250 or 260 Gilford spectrophotometers.

Morphological analysis

Membrane pellets suspended in 0.25 M sucrose were fixed in 2% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4, for one hour on ice. Samples were centrifuged at 250,000 X g for 30 minutes at 4°C. Pellets were washed with 0.1 M cacodylate buffer, resuspended with a pipet in 1 % liquid agar, and centrifuged at 1,000 X g at 20°C for 5 minutes. Agar pellets were cut into pieces of about 2 X 2 mm, and

postfixed in 1 % osmium tetroxide for four hours, except where noted. Sample were rinsed with buffer and dehydrated with serial changes of 25, 50, 70, 90, 95, and 100% ethanol, repeating the last step three times. Infiltration with Spurr's low viscosity resin was achieved using 50:50, 75:25, and 100:0 resin to ethanol ratios sequentially. Samples were maintained in each concentration of resin for four hours, with constant rotation (variable speed rotary mixer, Pelco Electron Microscopy Supplies, Irvine, CA). Specimens were hardened in Beem capsules at 60°C for 48 hours. Sections (80-100 nm in thickness) were cut with an American Optical Ultracut microtome, triple stained with potassium permanganate, uranyl acetate, and lead citrate (Soloff, 1973) and examined in a JEOL 100C transmission electron microscope.

Separation of vesicular contents from membranes

Sodium carbonate treatment of subcellular fractions was used to separate membranes from vesicular contents (Fujiki et al., 1982). Protein (Lowry et al., 1951) and phospholipid (phospholipid extraction, Folch et al., 1957; inorganic phosphate determination, Rouser et al., 1966) were measured before and after sodium carbonate treatment . Vesicular contents were concentrated using centrifugal microconcentrators (Centricon 10, Mr 10,000 cutoff) at

5,000 X g, 4°C, until retenate volume was approximately 200 µl. Membrane pellets and concentrated vesicular contents were electrophoresed in a sodium dodecyl sulfate polyacrylamide gel system (PAGE) according to Laemmli (1970). In addition, morphology of membrane pellets was assessed by electron microscopic examination of fixed and thin-sectioned material as described above. Samples were postfixed in osmium for one hour and sections were stained with lead citrate and uranyl acetate.

Protein synthesis and secretion

In vitro protein synthesis and secretion from liver and mammary gland slices were compared using radiolabeled amino acid incorporation in a pulse chase experiment. After cervical dislocation, liver and mammary glands were obtained from two lactating guinea pigs. Tissue slices of about 1 mm thickness were prepared with a Stadie-Riggs hand microtome and placed in Krebs-Ringer bicarbonate buffer, pH 7.4, on ice. Slices were weighed, placed in flasks (about 1 g of slices/flask) and incubated for 20 minutes at 37°C in 3 ml of Krebs-Ringer buffer containing 30 µCi L-[4,5-3H] leucine (specific activity of 164 Ci/mmol) with a 95% air : 5% CO_2 atmosphere. After rinsing with Krebs-Ringer buffer, slices were immersed in 3 ml of Medium 199 supplemented with 10 mM unlabeled leucine (chase medium), the flasks

were flushed with 95% air : 5% CO_2; and incubated for three hours at 37°C. Aliquots of media, 500 µl, were taken at 30 minutes, 1, 2, and 3 hours after immersion of slices in chase medium. Secreted proteins were precipitated by adding 5 ml of 10% trichloroacetic acid (TCA) and holding at 0°C for 20 minutes, followed by centrifugation at 4,000 X g for 30 minutes at 0°C. Supernatants were removed and pellets were solubilized overnight at room temperature with 500 µl NCS tissue solubilizer. Radioactivity of samples was determined by liquid scintillation counting using 5 ml of Ecoscint per vial. The window settings on the LKB 1217 scintillation counter were 16 (lower limit) and 112 (upper limit). Samples were counted for five minutes.

In vitro calcium uptake

Subcellular fractions from guinea pigs and incubation buffer were held on ice until the time of mixing. After addition of membranes, tubes were vortexed, the mixture was sampled and filtered for a zero time point, and tubes were then immersed in a 37°C shaking water bath. The incubation buffer contained 2 mM adenosine 5'-triphosphate, 5 mM phosphocreatine, 5 mM sodium azide, 6 mM magnesium chloride, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), pH 7.0, 100 mM potassium chloride, 5 mM potassium oxalate, 250 mM sucrose, 3 U/ml creatine kinase,

0.5 mg protein/ml, and 20 uM calcium except where indicated. After incubation (time denoted in figures) reaction mixtures were filtered through a vacuum filtration manifold at 27 inches of mercury, using 0.45 μm pore, 25 mm diameter Millipore HAWP filters, and rinsed with 8 ml buffer containing 2 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA), 25 mM HEPES, pH 7.0, 100 mM potassium chloride, and 0.25 M sucrose. Total incubation volume was 2 ml, consisting of 400 μl of sample and 1600 μl of reaction buffer. The volume of reaction mixture for filtration was 800 μl. Fractions were incubated without adenosine triphosphate (ATP) and creatine kinase to evaluate ATP-independent calcium uptake. Quantification of ^{45}Ca uptake was by liquid scintillation counting of air dried filters, as described earlier. Nine ml of Ecoscint scintillation fluid was used per filter.

Calcium ionophore incubations

The influence of calcium ionophore A23187 on calcium uptake was evaluated. Vesicles were incubated as described above, with ATP and ^{45}Ca for 10 minutes at 37°C. ^{45}Calcium uptake was measured after 10 minutes. Half of the remaining reaction mixture was transferred to a separate tube containing A23187 (final concentration 2 μM). Calcium uptake was assessed at 15, 20, 30 minutes for control and

ionophore reaction mixtures (5, 10, and 20 minutes after A23187 addition). Instead of adding A23187 after ten minutes of ^{45}Ca accumulation, subsequent experiments assessed ^{45}Ca accumulation after preincubation of fractions with 2 μM A23187 for 20 minutes on ice.

Preincubation of vesicles

To insure complete equilibration of subcellular fractions with compounds to be tested before the addition of ^{45}Ca, subsequent experiments included a preincubation period. Protonophore CCCPH (160 μM) was preincubated with subcellular fraction for 20 minutes, on ice, to determine the role of proton gradient maintenance in calcium uptake. Final CCCPH concentration upon addition of incubation buffer was 32 μM. The effect of vanadate, a Ca-dependent ATPase inhibitor, was determined by preincubation of vesicles with 0.5 M vanadate which, on addition of incubation buffer, was diluted to 0.1 M. The ATP analog AMP-PNP was preincubated with fractions, 20 minutes on ice, at a final concentration of 2 mM. Vesicles were hypotonically lysed on ice (diluted 1:50 in distilled, deionized water) centrifuged (250,000 X g, 2°C, for 30 minutes) and pellets were resuspended in 0.25 M sucrose.

Increasing calcium concentration

The effect of increasing Ca concentration on vesicle aggregation was evaluated using a Varian Cary 219 spectrophotometer, Varian Industries, Pal Alto, CA. The reference cuvette contained 0.1 mg vesicle protein/ml in incubation buffer containing 60 μM $CaCl_2$ and no ATP. This concentration of Ca was used as a baseline as ^{45}Ca uptake at this concentration was nearly linear. The sample cuvette contained the same amount of vesicle protein to which $CaCl_2$ was added to final concentrations of 120, 240, 360, or 660 μM. Change in absorbance upon calcium addition was monitored from 700 to 300 nm. Absorbance values changed in the wavelenght range of 338 to 447 nm as calcium was added, so further scans were recorded in this range using a slit width of 2.0, and an absorbance range of 0.2. In addition, membrane fractions fixed as previously described in 2% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.4) containing 20 or 240 μM Ca were examined by electron microscopy.

Statistical analysis

Statistical analysis of data was done using the Statistical Analysis Systems General Linear Model program (1985). This program allows for unbalanced designs. Treatment differences of subcellular fraction and tissue source were compared by pairwise comparison of least square

means from analysis of the model below:

$Y_{ijk} = u + S_i + T_j + (ST)_{ij} + E_{ijk}$

where

Y_{ijk} is observed dependent variable

u is mean of Y

S_i is fixed effect of ith subcellular fraction, i=1,3

T_j is fixed effect of jth tissue, j=1,2

$(ST)_{ij}$ is the interaction of subcellular fraction i and tissue j

E_{ijk} is the random residual

Materials

Chemicals and reagents were obtained from Sigma Chemical Company, St. Louis, MO, or Fisher, Raleigh NC, except as noted. Creatine phosphate and creatine kinase were from Boehringer Mannheim, Indianapolis, IN. AG 1X-2 resin was obtained from Bio-Rad Laboratories, Richmond, CA. Sodium orthovanadate was supplied by Aldrich Chemical Company, Milwaukee, WI. Polyacrylamide gel electrophoresis supplies were obtained from Bethesda Research Laboratories, Gaithersburg, MD; Centricon centrifugal microconcentrators from Amicon Corp., Danvers, MA; electron microscopy supplies from Polysciences, Warrington, PA; NCS tissue solubilizer from Amersham, Arlington Heights, IL; Ecoscint scintillation fluid from National Diagnostics, Manville,

NJ. Radioisotopes were purchased as follows: $^{45}CaCl_2$, 28 mCi/mg, from ICN Biomedical, Irvine, CA; uridine diphospho--D-[U-^{14}C] galactose, 202 mCi/mmol, and L-[4,5-^{3}H] leucine, 164 Ci/mmol, from Amersham, Arlington Heights, IL; and [1,5-^{14}C] citric acid, 57 mCi/mmol, from New England Nuclear, Boston, MA. Filters, HAWP 2500, for uptake experiments were purchased from Millipore, Bedford, MA. Vacuum manifold FH225 was manufactured by Hoefer Scientific Instruments, San Francisco, CA.

RESULTS AND DISCUSSION II

Calcium distribution in vivo

When administered by intraperitoneal injection, radioactive calcium reached highest levels in blood 20 minutes post injection (not shown). Specific activity (CPM/mg protein) of calcium in milk peaked between 60 and 90 minutes post injection (Figure 5). Compared with milk, specific activities of all subcellular fractions examined were much lower, except during the first 30 minutes after injection. Of the subcellular fractions examined, specific activities were highest in cytosol, lowest in endoplasmic reticulum and intermediate in Golgi apparatus. The time course of increase and decrease in specific activity of calcium in cytosol and Golgi apparatus paralleled that for milk. The peak specific activity in endoplasmic reticulum occurred at 45 minutes post injection and declined rapidly thereafter. Specific activity of the mammary tissue homogenate reached nearly maximum values within 20 minutes after injection, and specific activities in homogenates changed little thereafter. These results with homogenates may have been due to the presence of entrained blood, since maximum specific activity in blood was also found at 20 minutes, and mammary glands were not perfused to remove blood. At no time point in the experiment were specific activities in Golgi apparatus or endoplasmic reticulum

Figure 5. ^{45}Calcium distribution in milk and fractions from mammary gland.

Twenty microcuries of ^{45}Ca in 0.3 ml of 0.9% sodium chloride was injected i.p. into Sprague Dawley rats on the 10th (x) day of lactation. Just prior to sacrifice, rats were anesthetized with ether, given oxytocin (20 U, i.p.) and milked. Rats were killed by cervical dislocation. Fractions were prepared from mammary gland tissue and radioactivity and protein concentration determined.

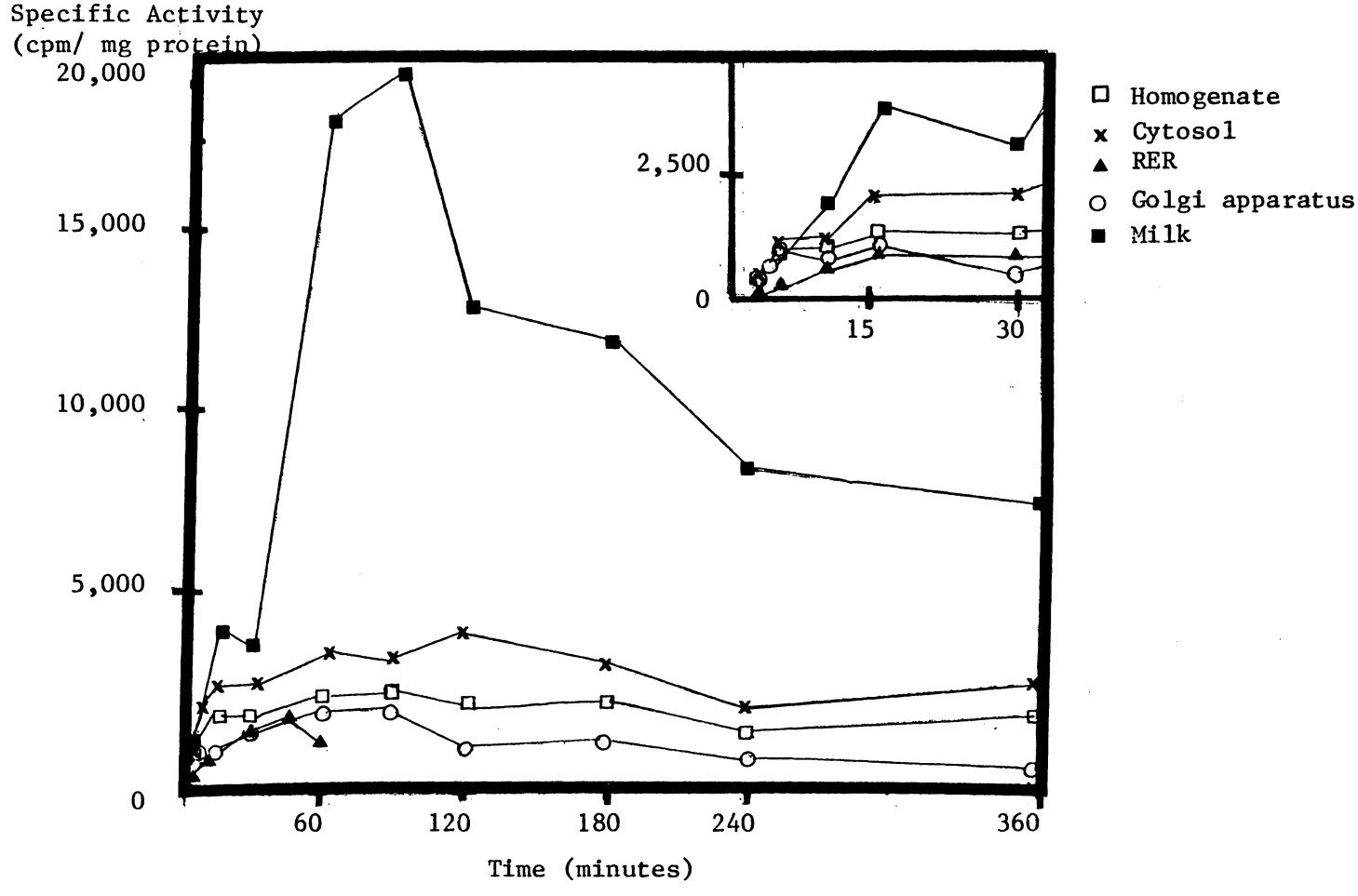

Figure 5. ^{45}Calcium distribution in milk and fractions from mammary gland.

higher than those of the homogenate. Rapid turnover of calcium in each of these compartments would be predicted, since secretory proteins and associated calcium are transported through these organelles for secretion into alveolar lumina.

Overall, results of this study were not inconsistent with the view that the calcium which is secreted into milk may be accumulated within lumina of Golgi apparatus and endoplasmic reticulum (West, 1981; Baumrucker and Keenan, 1975). However, if these endomembrane compartments are primary sites of accumulation of calcium for secretion into milk, then data from the present study can be interpreted as suggestive of rapid transport of calcium out of these compartments, possibly into secretory vesicles for exocytosis (Peaker, 1978). Whole body dilution of the radioisotope led to low specific activities in the fractions examined and made interpretation of data difficult. This dilution effect, coupled with the large animal to animal variations observed, indicated that the _in vivo_ approach would not provide the resolution necessary to make a quantitative assessment of the contribution of endoplasmic reticulum and Golgi apparatus to accumulation of milk calcium. For these reasons, the _in vivo_ approach was abandoned in favor of studies of the ability of fractions to accumulate calcium _in vitro_.

Uptake studies with subcellular fractions

Conditions for the calcium uptake assay were established using Golgi apparatus from mammary gland, a subcellular fraction known to accumulate calcium (West, 1981; Virk et al., 1985; Baumrucker & Keenan, 1975). Incubation mixtures contained no ATP or 4 mM ATP, 1 mM oxalate, 10 mM histidine hydrochloride, pH 7.0, 20 μM $^{45}CaCl_2$, 10 mM magnesium chloride, 100 mM potassium chloride, and 0.14 mg Golgi apparatus protein in a final volume of 2 ml. Time points over 35 minutes incubation at 37°C were chosen for assay. ATP increased the amount of calcium accumulated by Golgi apparatus (Figure 6). In the absence of ATP, calcium was accumulated for the first 5 minutes of incubation, but after this time there was a nearly linear net loss of calcium from the Golgi apparatus vesicles through the remainder of the 35 minute incubation. In the presence of ATP, calcium was accumulated rapidly during the first 5 minutes of incubation. Accumulation continued at a lower, but nearly linear rate, in the presence of ATP between 5 and 35 minutes of incubation. For this experiment, Golgi apparatus isolated from one animal was used so as to avoid animal to animal variation. For subsequent experiments, 10 minute incubation times were chosen as the standard assay because available data indicates that Golgi apparatus vesicle integrity is maintained over this time period, with

Figure 6. ^{45}Calcium accumulation by mammary gland Golgi apparatus.

Subcellular fractions from one guinea pig and incubation buffer were held on ice until the time of mixing. After addition of membranes, tubes were vortexed, mixture sampled and filtered for a zero time point and tubes immersed in a 37°C shaking water bath. Incubation buffer contained 4 mM ATP, 1 mM potassium oxalate, 10 mM histidine hydrochloride, pH 7.0. Reaction volume was 2 ml containing 0.14 ml of Golgi apparatus protein. Reactions were stopped by filtering through 0.45 um pore filters, and rinsed with incubation buffer, without ATP, but with 2 mM EGTA.

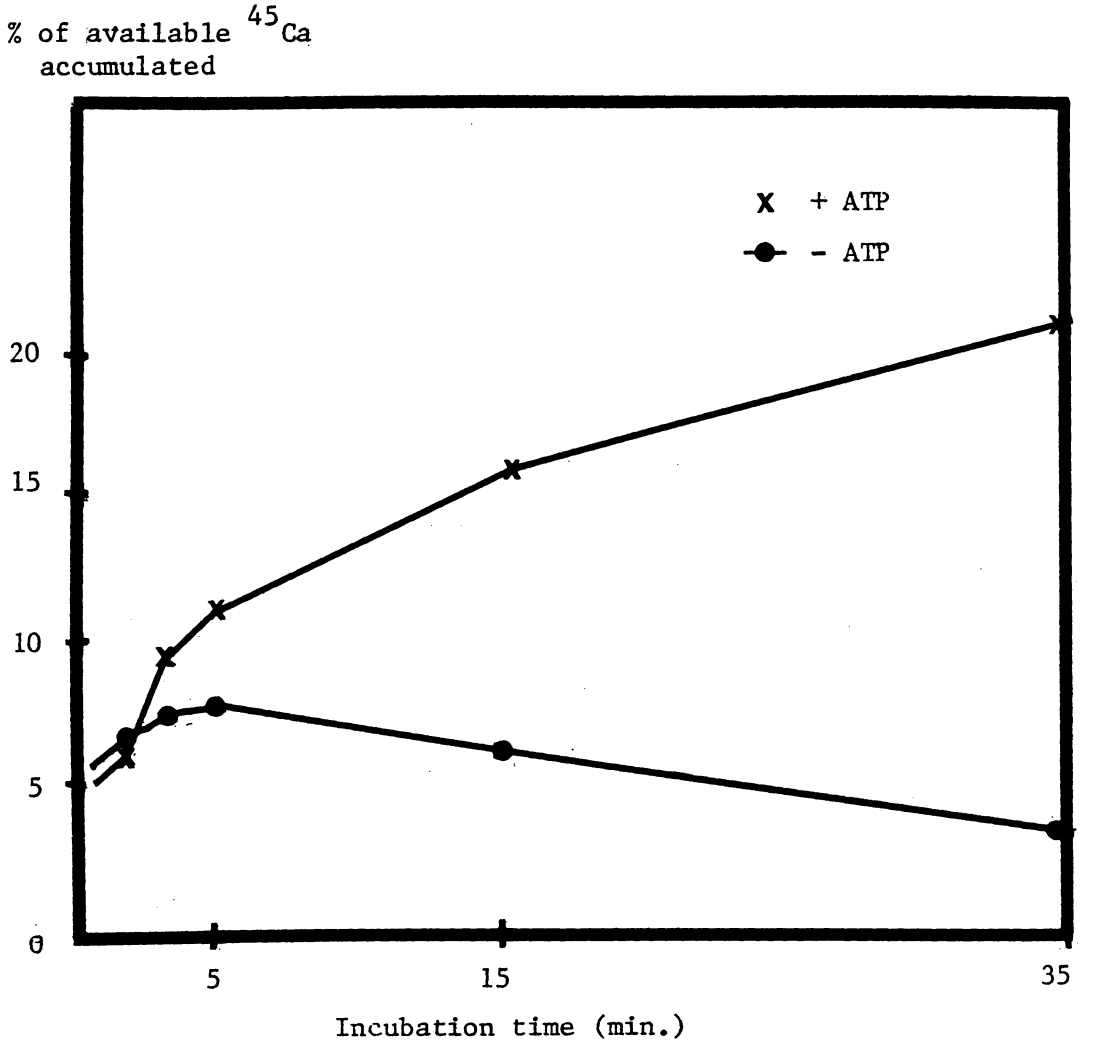

Figure 6. ^{45}Calcium accumulation by mammary gland Golgi apparatus.

or without ATP in the incubation mixture (Hodson, 1978).

The linear range of calcium accumulation with respect to amount of vesicle protein in the incubation mixture was determined for Golgi apparatus and endoplasmic reticulum from liver and from mammary gland. In these experiments an ATP-creatine kinase regenerating system was included in the incubation mixture, and the amount of vesicle protein ranged from 50 μg to 1 mg/ml (Figure 7). For isolation of these fractions, tissues from three animals were pooled. In the case of liver Golgi apparatus, only two protein levels were assayed, due to a low yield of this fraction in this instance. With endoplasmic reticulum and Golgi apparatus from mammary gland, and endoplasmic reticulum from liver, there was a nearly linear increase in calcium accumulation between about 0.25 and 0.75 mg of vesicle protein/ml. For subsequent experiments, a level of 0.5mg vesicle protein/ml was chosen, since this was within the nearly linear range for mammary gland Golgi apparatus and liver endoplasmic reticulum, and yielded easily quantifiable amounts of calcium accumulation with all fractions.

To determine the calcium concentration which yielded maximum accumulation, assays were performed over a range of 20 to 240 μM $CaCl_2$ (Figure 8). As the amount of calcium in the incubation mixture increased, the percent of available

Figure 7. ^{45}Calcium accumulation with varying protein concentrations.

Pooled tissue homogenates were prepared from 3 guinea pigs prior to subcellular fractionation. Incubations were for 10 minutes, at 37°C, using the incubation buffer described in Materials and Methods II, with the exception that 60 uM $CaCl_2$ was used.

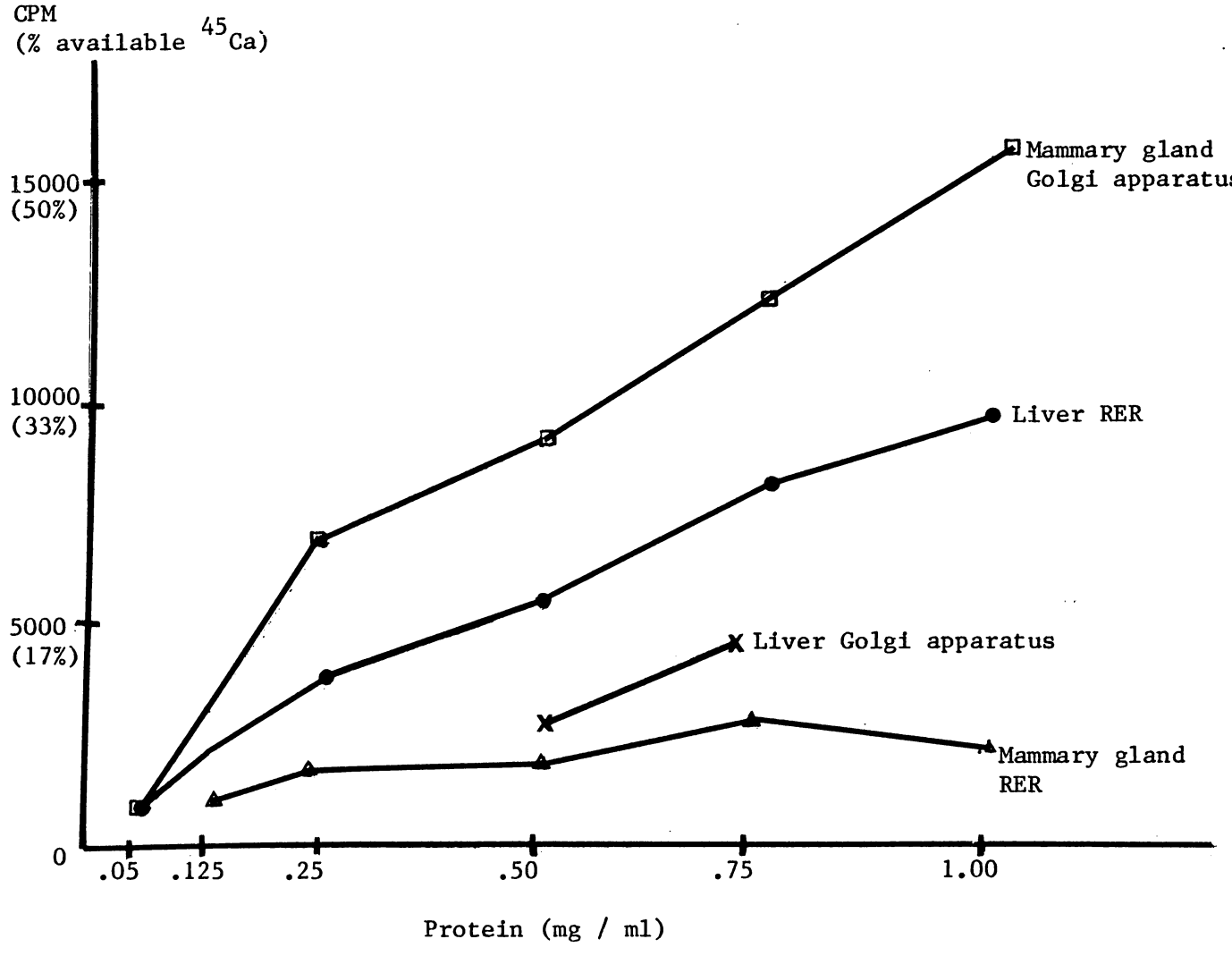

Figure 7. ^{45}Calcium accumulation with varying protein concentrations.

Figure 8. ^{45}Calcium accumulation with varying calcium concentrations.

Pooled tissue homogenates were prepared from 3 guinea pigs for 20 to 60 uM $CaCl_2$ incubations, prior to subcellular fractionation. A second set of pooled homogenates from an equal number of animals was used for 60 to 240 uM $CaCl_2$. Incubations were for 10 minutes, at 37°C, using the incubation buffer described in Materials and Methods II.

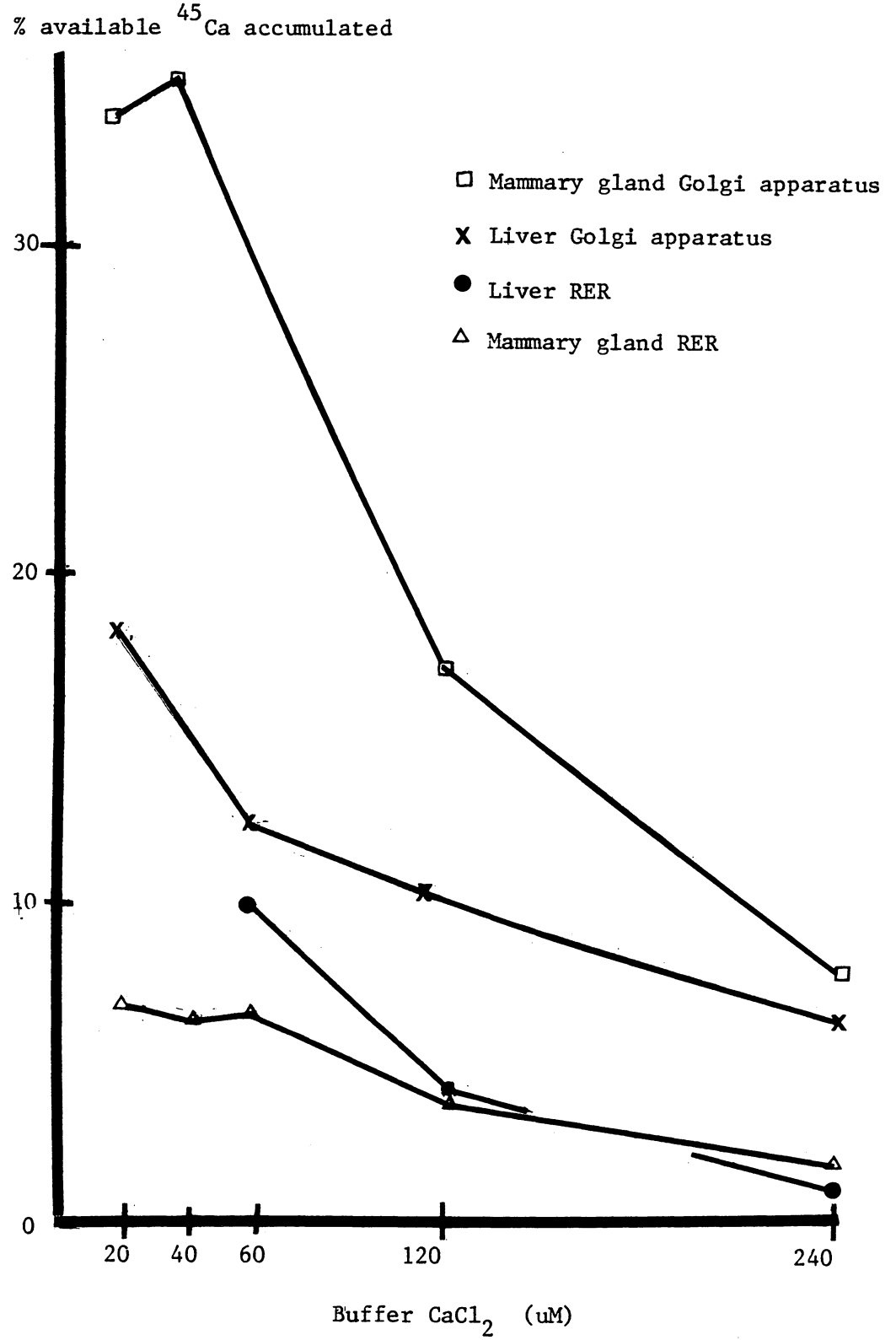

Figure 8. ^{45}Calcium accumulation with varying calcium concentrations.

calcium accumulated by vesicles decreased, but the absolute amount of calcium accumulated increased. At concentrations above 240 μM, ^{45}Ca appeared to be trapped by membranes within 20 seconds of incubation, and this ^{45}Ca could not be released by washing vesicles with EGTA-containing buffer. The presence or absence of ATP in incubation mixtures appeared to have no effect on the amount of ^{45}Ca trapped at calcium concentrations above 240 μM (results not shown).

In an attempt to determine if vesicle aggregation was responsible for apparent calcium entrapment at higher calcium concentrations, fractions were fixed as described in Methods for subsequent electron microscopic examination. Results presented here are representative of those obtained with four preparations of each fraction. At least two grids with 10 or more sections from each preparation were examined. With Golgi apparatus from mammary gland (Figure 9) and liver (Figure 10), membranes appeared to be more closely packed together or aggregated when fixed in buffer containing the higher calcium concentration. In addition, fixation at 240 μM calcium resulted in a darker staining of membranes with heavy metals used during postfixation (compare A with B in Figures 9 and 10). Increased calcium concentration in fixation solutions appeared to cause dissociation of ribosomes from endoplasmic reticulum from both mammary gland (Figure 11) and liver (Figure 12). This

Figure 9. Mammary gland Golgi apparatus in 20 uM and 240 uM $CaCl_2$.

Transmission electron micrograph (TEM) of isolated mammary gland Golgi apparatus fixed in 0.1 M cacodylate buffer, pH 7.4, with either 20 uM $CaCl_2$ or (B) 240 uM $CaCl_2$. Fractions were post-fixed and stained as described in the text. C marks cisterna, t tubule, m casein micelle. Magnification = 44,000 (A) 70,000 (B). Dark staining vesicles are from trans side of Golgi apparatus.

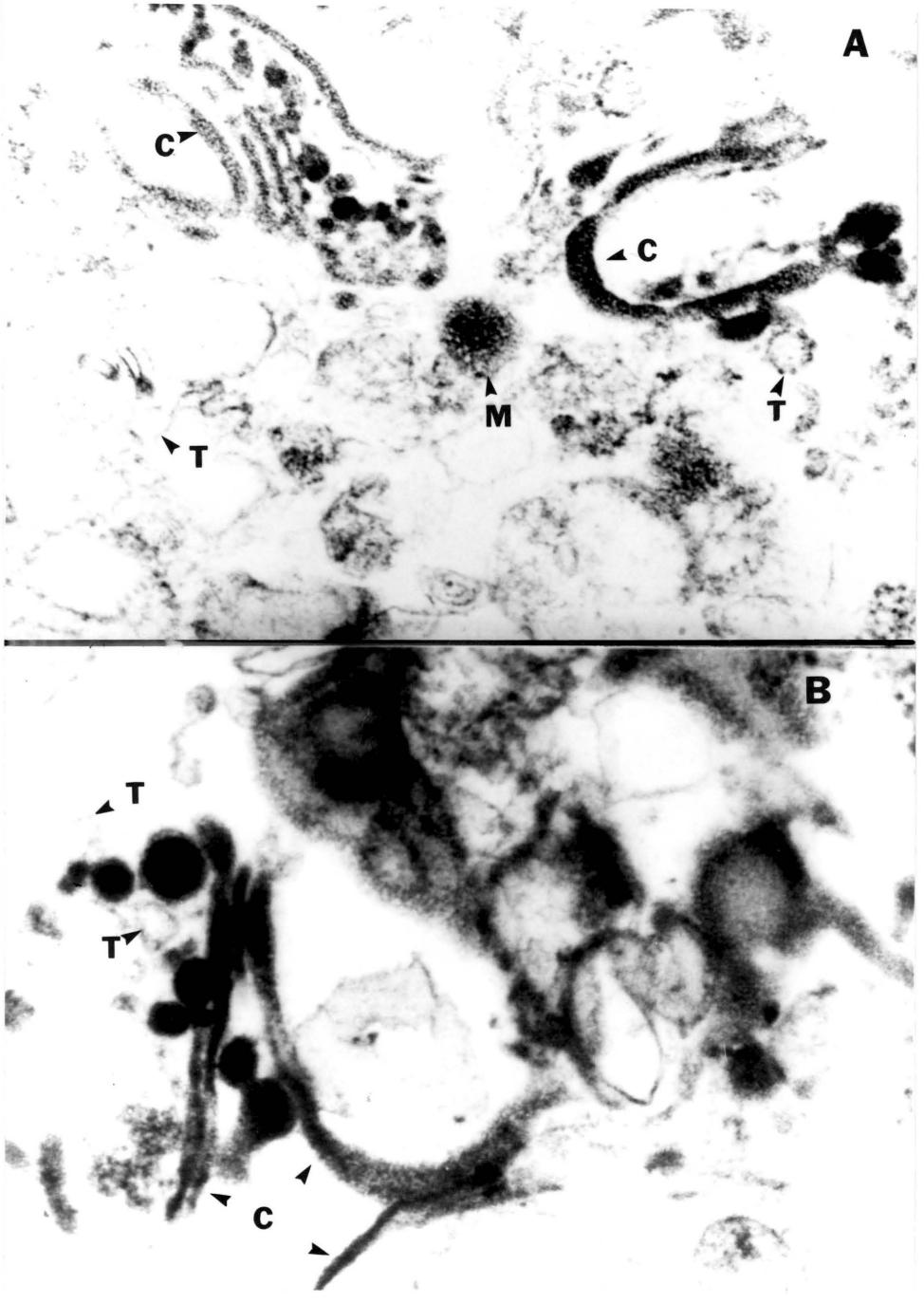

Figure 9. Mammary gland Golgi apparatus in 20 uM and 240 uM calcium chloride.

Figure 10. Liver Golgi apparatus in 20 uM and 240 uM calcium chloride.

Transmission electron micrographs of isolated liver Golgi apparatus prepared as described in Figure 9. (A) 20 uM $CaCl_2$, (B) 240 uM $CaCl_2$. C marks cisterna, T tubules, G glycogen granules. Magnification= 46,000 X. Darker staining vesicles originate from trans side of Golgi apparatus stacks.

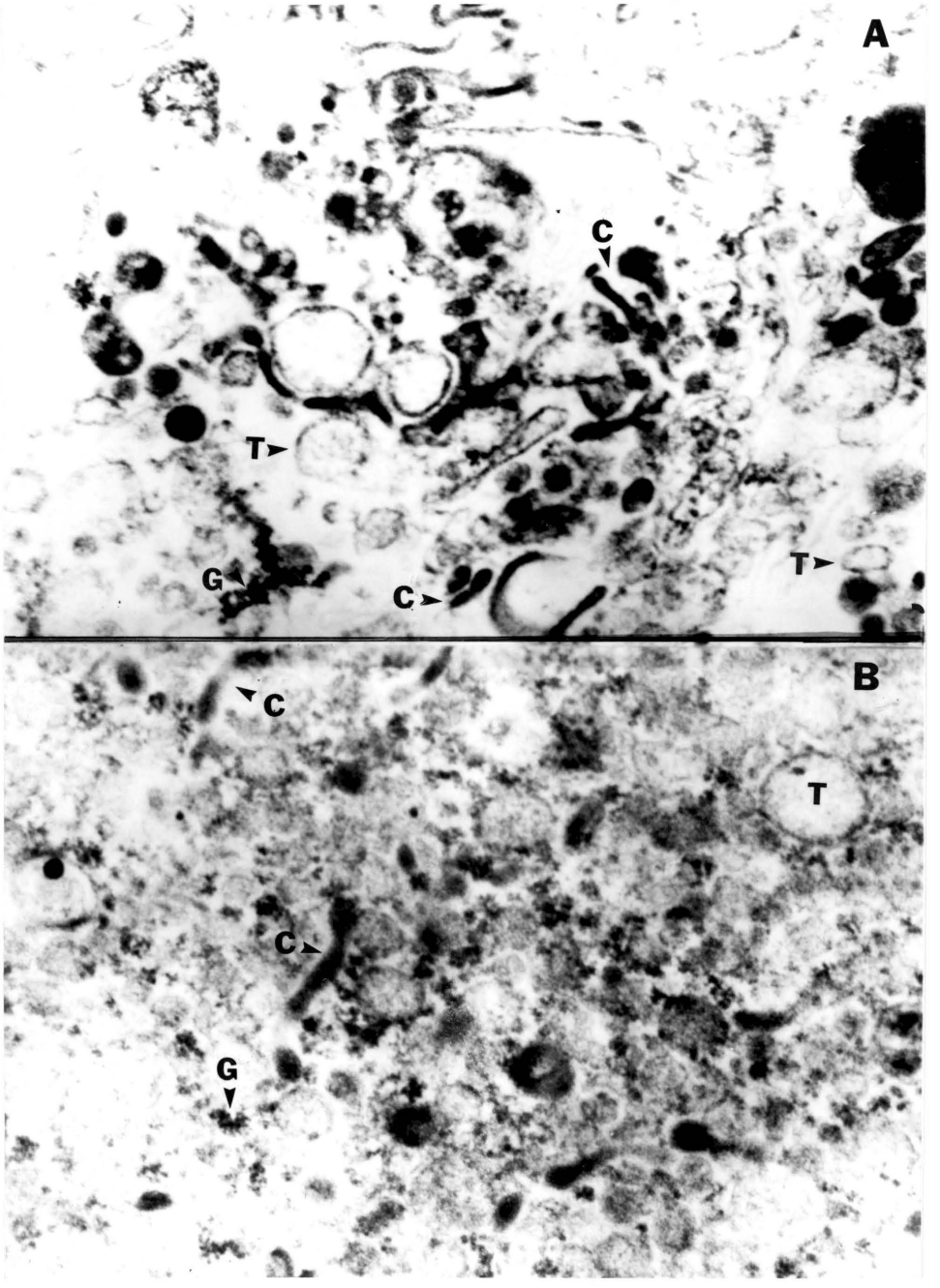

Figure 10. Liver Golgi apparatus in 20 uM and 240 uM calcium chloride.

Figure 11. Mammary gland RER in 20 uM and 240 uM $CaCl_2$.

Transmission electron micrographs of isolated mammary gland RER, prepared as described in Figure 9. (A) 20uM $CaCl_2$, (B) 240 uM $CaCl_2$. Arrowheads mark ribosomes, L marks lumen of microsomes. Magnification = 48,000 X.

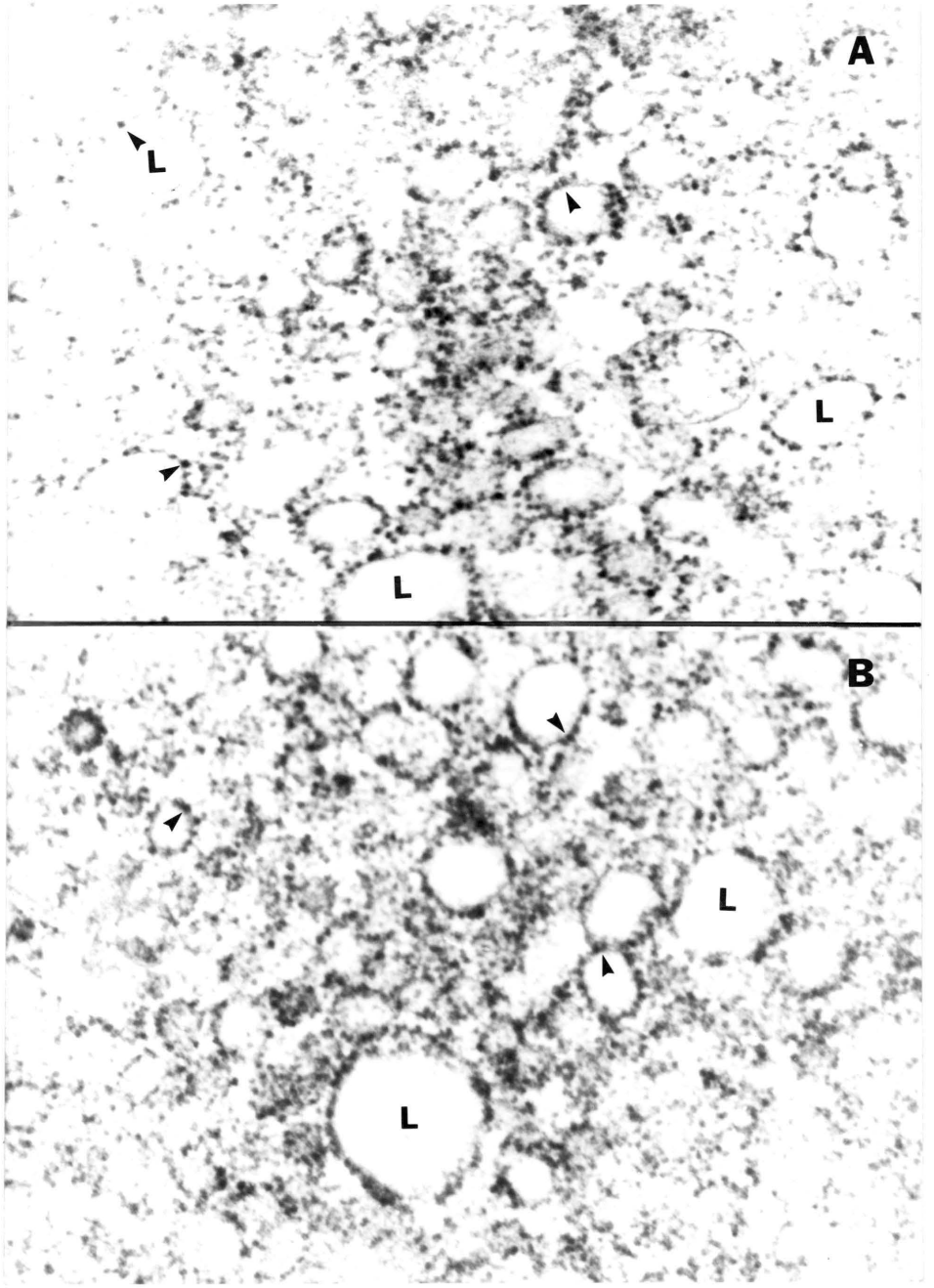

Figure 11. Mammary gland RER in 20 uM and 240 uM $CaCl_2$.

Figure 12. Liver RER in 20 uM and 240 uM $CaCl_2$.

Transmission electron micrographs of isolated liver RER prepared as described in Figure 9. (A) 20 uM $CaCl_2$, (B) 240 uM $CaCl_2$. Arrowheads mark ribosomes, L lumen of microsomes, G glycogen. Most ribosomes appeared to be dissociated from membranes at the higher $CaCl_2$ concentration. Magnification =44,000 X.

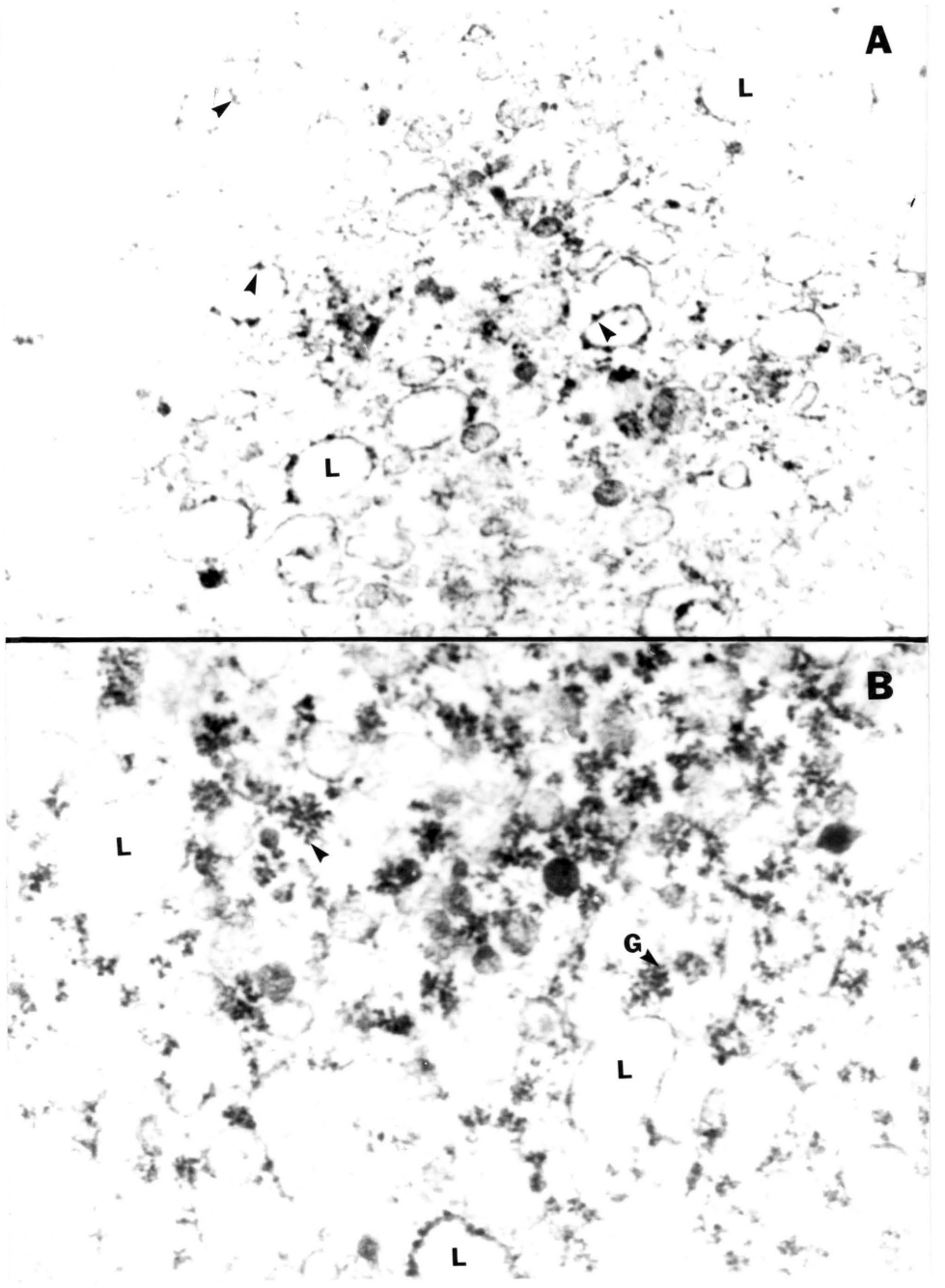

Figure 12. Liver RER in 20 uM and 240 uM $CaCl_2$.

effect on dissociation of ribosomes was greater with preparations from liver than from mammary gland. Observation of this effect in endoplasmic reticulum preparations from liver was complicated by the presence of glycogen in the fraction. However, the same amount of glycogen should have been present irrespective of the amount of calcium in the fixation buffer, since the same volume of vesicles, from the same isolation, were taken for fixation under the two conditions. Thus, the increased number of particles not associated with membranes and of the same size as membrane-bound ribosomes observed upon fixation in 240 μM calcium most probably are ribosomes. In contrast to Golgi apparatus preparations, increased [calcium] did not lead to pronounced aggregation of endoplasmic reticulum vesicles, although there was increased aggregation of endoplasmic reticulum vesicles at higher [calcium]. As with Golgi apparatus, endoplasmic reticulum was more densely stained in the presence of 240 μM calcium than in the presence of 20 μM calcium (compare A with B in Figures 11 and 12).

In an attempt to obtain an assessment of the extent of vesicle aggregation induced by calcium, a spectrophotometric approach was evaluated. Absorbance by a suspension containing 0.1 mg vesicle protein/ml (20% of that used experimentally) and from 60 to 660 μM calcium was measured by scanning from a wavelength of 447 nm to 338 nm.

Throughout this wavelength range, there was increased absorbance with increasing calcium concentrations with all fractions (Figure 13). However, absorbance did not increase inversly as the 4th power of wavelength, as would have been expected if the result were due to increased scattering properties of the suspension. Results may have been due to aggregation and settling of vesicles during scanning. For this reason, absorbance was estimated at a fixed wavelength of 360 nm, at a constant time after addition of calcium (Figure 14). At this wavelength, absorbance increased to nearly the same extent with all fractions except endoplasmic reticulum from mammary gland with increasing [calcium]. Endoplasmic reticulum from mammary gland apparently aggregated to a greater extent than did endoplasmic reticulum from liver and Golgi apparatus from either organ. The amount of light scattered is related to the refractive index of a solution, the number of particles in a set volume, and the size or molecular weight of the particles (Campbell and Dwek, 1984). Of these parameters, that which would have changed under the experimental conditions described, was particle size because of aggregation. Absorbance did not change with increasing calcium content in buffer without vesicles. Based on these facts and observations, the increase in apparent absorbance observed when [calcium] was increased

Figure 13. Spectral profiles of fractions with increasing calcium concentration.

Effect of increasing Ca concentration was monitored by recording the change in absorbance over the wavelength range of 338 to 447 nm. Reference cell cuvette contained 0.1 mg/ml vesicle protein in incubation buffer with 60 uM $CaCl_2$ (no ATP). Sample cell cuvette contents were identical with the exception that Ca concentration was sequentially increased by addition of $CaCl_2$ from a stock solution. Absorbance readings were taken after each addition of $CaCl_2$. Fractions were liver Golgi apparatus (A) and RER (B), and mammary gland Golgi apparatus (C) and RER (D).

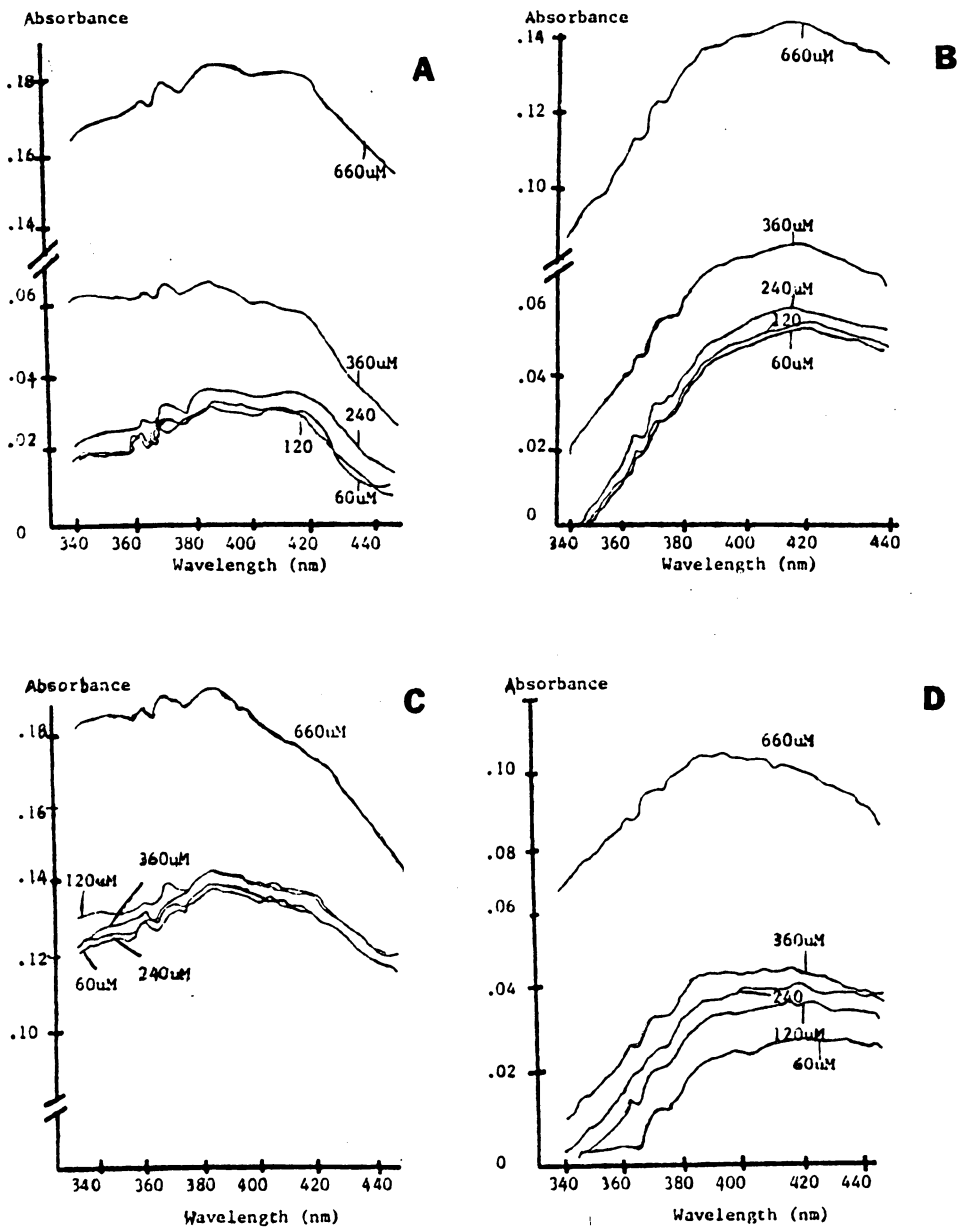

Figure 13. Spectral profiles of fractions with increasing calcium concentration.

Figure 14. Ca concentration-dependent change in absorbance of fractions.

Data from Figure 13 was compared by plotting the change in absorbance at 360nm of 0.1 mg vesicle protein as calcium chloride concentration was increased from 60 uM (baseline) through 660 uM. Experimental conditions were described in Figure 13.

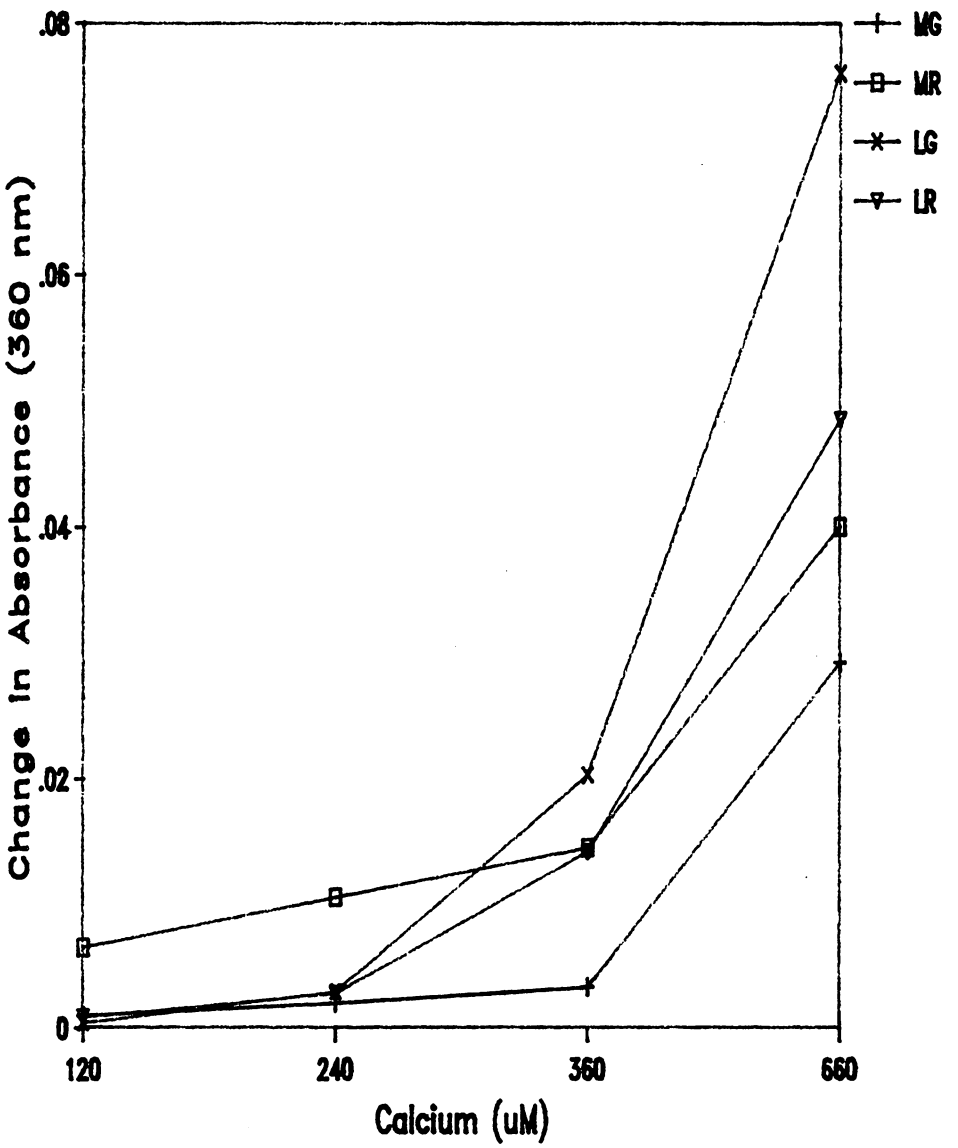

Figure 14. Calcium concentration-dependent change in absorbance of fractions.

in vesicle suspensions must have been due to calcium-induced aggregation of vesicles.

Enzymatic evaluation of fractions

To assess the degree of purification achieved by separation from homogenates, activities of enzymes which are markers for various cell fractions were measured in Golgi apparatus and endoplasmic reticulum preparations from liver and mammary gland. Enrichment of marker enzymes was determined by calculating the ratio of specific activity (units of enzymatic activity/mg protein/hr) of the fraction to the specific activity of the homogenate (Table 2). Galactosyl transferase, a Golgi apparatus marker, was enriched 13.9-fold and 15.5-fold in Golgi apparatus isolated from guinea pig liver and mammary gland, respectively. Values obtained for Golgi apparatus from mammary gland are in agreement for those with rat (13-fold) (Keenan et al., 1970) and cow (16-fold) (Keenan et al., 1972b). Specific activity in Golgi apparatus from guinea pig liver was nearly identical to that reported for mouse liver by Croze and Morre' (1984). However, the enrichment noted by Croze and Morre' (64-fold) was much greater than that reported here (13.9-fold), possibly due to the lower specific activity of the mouse liver homogenate (0.002 μmol galactose incorporated/mg protein/hr). This may

Table 2. Enzymatic evaluation of subcellular fractions[a].

	Galactosyl Transferase n=3	NADPH Cyt. c Reductase n=3	Succinate INT Reductase n=5	Phosphodiesterase n=5
Liver homogenate	0.01 ± .003	0.19 ± .010	0.164 ± .0425	0.23 ± .044
Liver Golgi apparatus	0.11 ± .056 (13.9)	0.68 ± .099 (3.6)	0.084 ± .0384 (0.51)	0.62 ± .284 (2.7)
Liver RER	0.01 ± .004 (0.7)	1.82 ± .367 (9.6)	0.037 ± .0200 (0.23)	0.24 ± .087 (1.0)
Mammary homogenate	0.04 ± .005	0.15 ± .028	0.060 ± .0151	0.78 ± .202
Mammary Golgi apparatus	0.65 ± .083 (15.5)	0.16 ± .021 (1.1)	0.030 ± .0084 (0.51)	2.02 ± .984 (4.1)
Mammary RER	0.01 ± .001 (0.1)	0.36 ± .045 (2.4)	0.029 ± .0061 (0.52)	0.32 ± .107 (0.4)

[a]Units are umoles/mg protein/hr ± S.D. Assay methods are described in Materials and Methods II. ( ) is specific activity of fraction ÷ specific activity of homogenate, indicating relative enrichment based on homogenate.

reflect a species difference or a physiological difference in abundance of Golgi apparatus in liver of lactating animals.

NADPH cytochrome c reductase was enriched 9.6- and 2.4-fold in endoplasmic reticulum from guinea pig mammary gland and liver, respectively (Table 2). Literature gives values of 2- to 4-fold enrichments for endoplasmic reticulum from both liver and mammary gland (Croze and Morre', 1984; Keenan et al., 1972a; 1972b). Endoplasmic reticulum isolation usually involves an overnight centrifugation step, while the procedure used to obtain data reported here involved centrifugation for 4 hours. Howell et al. (1978) obtained evidence that NADPH cytochrome c reductase activity declined when Golgi apparatus subfractions were held a 0 to 4°C, while this activity did not decline appreciably in homogenates held under identical conditions. Thus, the higher enrichment currently reported may have been due to a lesser loss of enzymatic activity than observed during longer preparation times.

Based on activities of the mitochondrial marker succinate INT reductase, none of the fractions used in this study appeared to have any appreciable contamination with mitochondria (Table 2). Enrichment of succinate INT reductase was 0.5-fold or less in all fractions. In comparison, mitochondrial preparations from cow (Huang and

Keenan, 1971) and guinea pig (I. H. Mather, personal communication) mammary glands have been found to be enriched about 9-fold in this enzymatic activity.

Phosphodiesterase I, a plasma membrane marker, was not enriched relative to homogenates in endoplasmic reticulum from mammary gland or liver (Table 2). Enrichments of about 3- to 4-fold were found in Golgi apparatus preparations from each organ. These results were expected, based on the known presence of plasma membrane enzymes in Golgi apparatus (Farquhar et al., 1974; Morre' et al., 1979). The plasma membrane marker 5'-nucleotidase has been found to be enriched about 4-fold in Golgi apparatus from cow mammary gland (Keenan et al., 1972b). Golgi apparatus preparations from mouse liver were enriched 3-fold in the plasma membrane marker p-nitrophenyl phosphatase (Croze and Morre', 1984). Plasma membranes from rat liver (Fleischer and Kervina, 1974; Brown et al., 1976) and mammary gland (Huggins et al., 1980) typically are enriched 25- to 35-fold in plasma membrane marker enzymes.

Morphological examination of preparations which had been fixed and sectioned for electron microscopy showed that fractions were contaminated to a limited extent with other organelles. Most elements observed in micrographs of Golgi apparatus fractions from mammary gland (Figure 15A) and liver (Figure 15B) could be identified as originating

Figure 15. Golgi apparatus fractions from mammary gland and liver.

Transmission electron micrographs of isolated fractions. Mammary gland (A), 66,000 X. Liver (B), 47,000 X. Cisternae are marked C, tubules T. Fractions from both tissues appear similar, with little indication of contamination by other subcellular fractions. Trans elements stain more darkly than cis. Samples were prepared for electron microscopy as described in Materials and Methods II, without Ca present in fixation buffer.

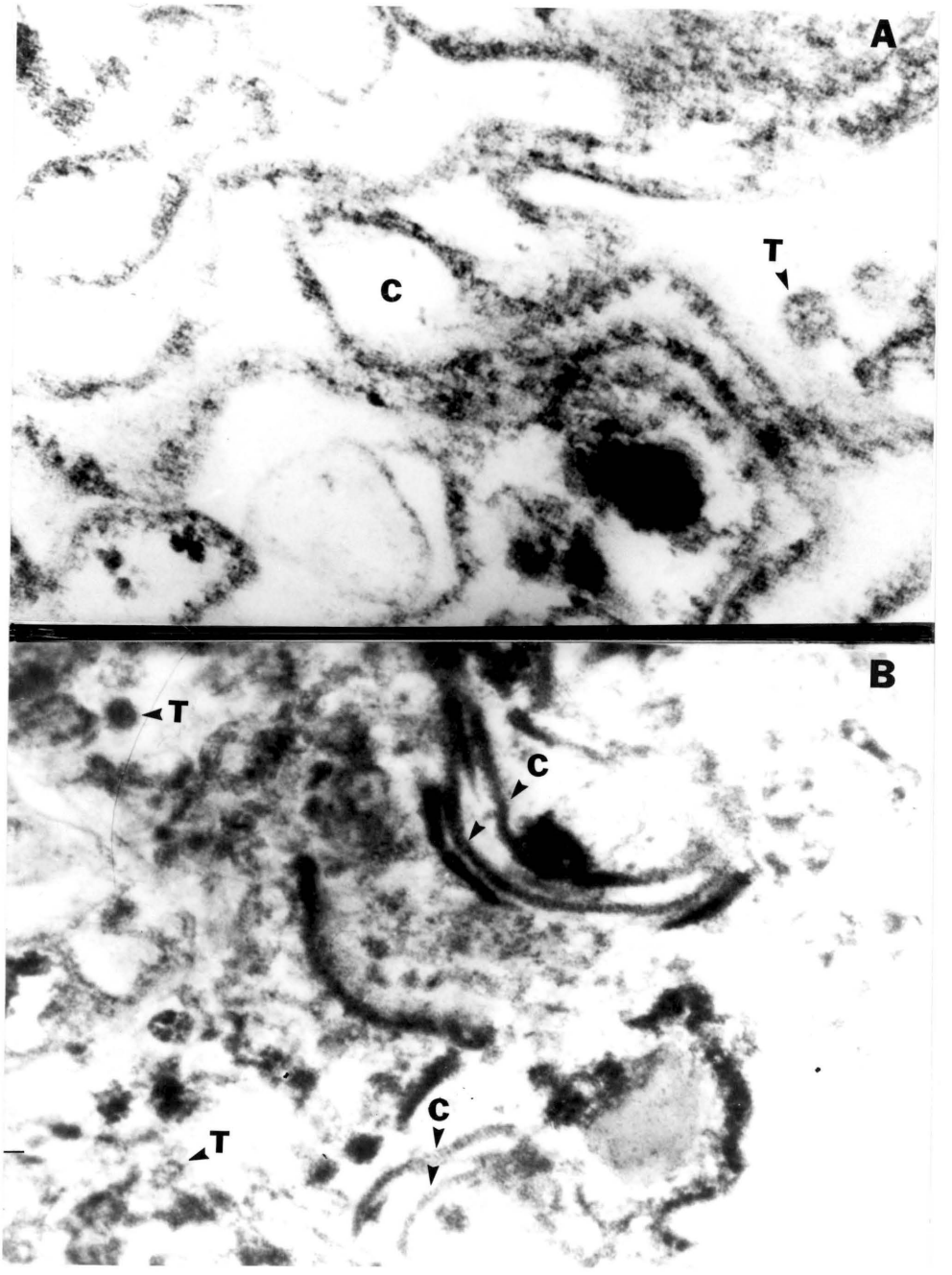

Figure 15. Golgi apparatus fractions from mammary gland and liver.

from Golgi apparatus, with reference to established morphological features of Golgi apparatus (Morre' et al., 1970). Cisterna and tubules and vesicles from the peripheral tubular network of dictyosomes were abundant in these preparations. Differential osmication of cis and trans elements of Golgi apparatus dictyosomes, typically observed in situ (Fawcett, 1981), was evident in the isolated material. The trans elements stain much darker than cis elements. Mitochondria, vesicles with ribosomes, and plasma membranes joined by junctional complexes were not observed. Endoplasmic reticulum fractions isolated from mammary gland (Figure 16A) and liver (Figure 16B) consisted almost entirely of vesicles with surface associated ribosomes. Some nonmembranous, particulate material was present in liver endoplasmic reticulum which was heterogenous in size. This was assumed to be contaminating glycogen (Morre' et al., 1970). Mitochondria and membranes recognizable as of Golgi apparatus or plasma membrane origin were not observed in endoplasmic reticulum preparations.

Protein and phospholipid distribution in fractions

Incubation in alkaline sodium bicarbonate solution at 0 to 4°C has been shown to be effective in removal of content proteins from liver endoplasmic reticulum and Golgi

Figure 16. Rough endoplasmic reticulum fractions from mammary gland and liver.

Transmission electron micrographs of isolated fractions. Mammary gland (A). Liver (B). L marks lumen of microsomes, arrowheads mark ribosomes. Most microsomes appear oval or circular. All membranes are associated with ribosomes. No tissue difference is evident, nor is contamination by other subcellular fractions. Magnification = 50,000 X.

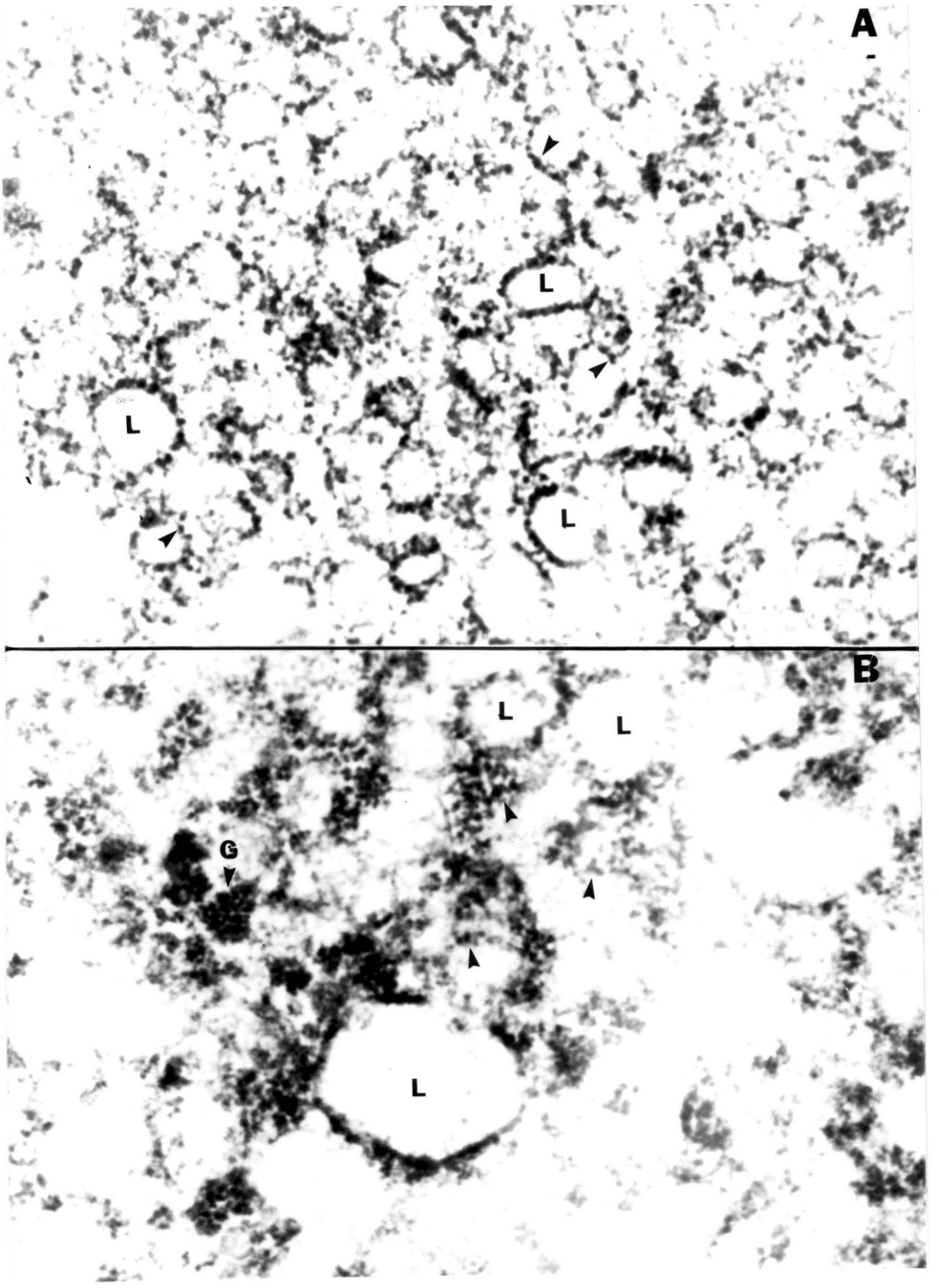

Figure 16. Rough endoplasmic reticulum fractions from mammary gland and liver.

apparatus (Fujiki et al., 1982; Howell and Palade, 1982). This method was applied to fractions used in this study, and the amount of protein and phospholipid retained in the membrane fraction, recovered by centrifugation of the suspension, was determined (Table 3). Only 32% and 36% of the protein in endoplasmic reticulum and Golgi apparatus, respectively, from mammary gland was recovered in the membrane fraction after sodium carbonate treatment. Since most of the starting phsopholipid (91% and 88% for endoplasmic reticulum and Golgi apparatus, respectively) was recovered in the membrane fraction, it was apparent that membrane degradation was not responsible for the large amount of protein released. Thus, it appears that about 65% of the total protein in fractions from mammary gland may be content proteins in the lumina of vesicles. With fractions from liver, 80% of the endoplasmic reticulum protein and 60% of the Golgi apparatus protein was recovered in the membrane fraction. Nearly all of the starting phospholipid of liver fractions was recovered with membranes after sodium carbonate treatment. Results on amount of protein and phospholipid released from fractions from liver are in agreement with results of others (Fujiki et al., 1982; Howell and Palade, 1982). Morphological examination of membranes recovered from mammary gland fractions after exposure to alkaline sodium carbonate

Table 3. Percent of protein and phospholipid found in membranes*

Tissue	Fraction	% of Total in Membrane Fraction[a]
	Protein Distribution	
Mammary gland	RER	36% ± 0.6
	Golgi apparatus	32% ± 4.6
Liver	RER	80% ± 1.7
	Golgi apparatus	60% ± 5.9
	Phospholipid Distribution	
Mammary gland	RER	91% ± 7.0
	Golgi apparatus	88% ± 10.6
Liver	RER	99% ± 1.0
	Golgi apparatus	98% ± 1.7

*Membranes were separated by treatment of fractions with 0.1 M sodium carbonate according to Fujiki et al., 1982.

[a]Both contents and membranes were assayed giving "total" values. Analysis of fractions before sodium carbonate treatment allowed assessment of recoveries, which were >90%. n = 4 preparations.

showed that this treatment was effective in disrupting vesicle integrity with concommitant loss of vesicle contents. Elements recovered after treatment of Golgi apparatus appeared as distened and elongated vesicles, with electronlucent interiors (Figure 17A). Elements recovered from endoplasmic reticulum were primarily sheet-like, and there were few or no symmetrical vesicles present (Figure 17B). Morphology of mammary gland fractions after this treatment was similar to that observed with liver fractions (Fujiki et al., 1982; Howell and Palade, 1982).

Protein synthesis and secretion

Slices of tissue from mammary gland released ^{3}H-leucine labeled proteins into incubation medium at a greater rate than did slices from liver (Figure 18). After 30 minutes of incubation in chase medium, mammary tissue slices had released 6.5 times more TCA-precipitable radioactivity than liver slices and 18 times more by 2 hours. In contrast to mammary tissue slices, in which there was a decrease in TCA-precipitable radioactivity in media between 2 and 3 hours, radioactivity in medium in which liver slices were incubated increased slightly. Decrease in radioactivity observed with mammary gland reflected the chase effect of incorporation of unlabeled leucine into proteins. Since secretory protein synthesis

Figure 17. Membranes from mammary gland after sodium carbonate treatment.

Transmission electron micrographs of isolated fractions after treatment with 0.1 M sodium carbonate (Fujiki et al., 1982). Golgi apparatus membranes (A) retained elongated vesicle conformation, while RER (B) became arranges in sheets, with few circular tubules remaining. Magnification = 40,000 X. Prepared for electron microscopy as described in Materials and Methods II., without Ca present in fixation buffer.

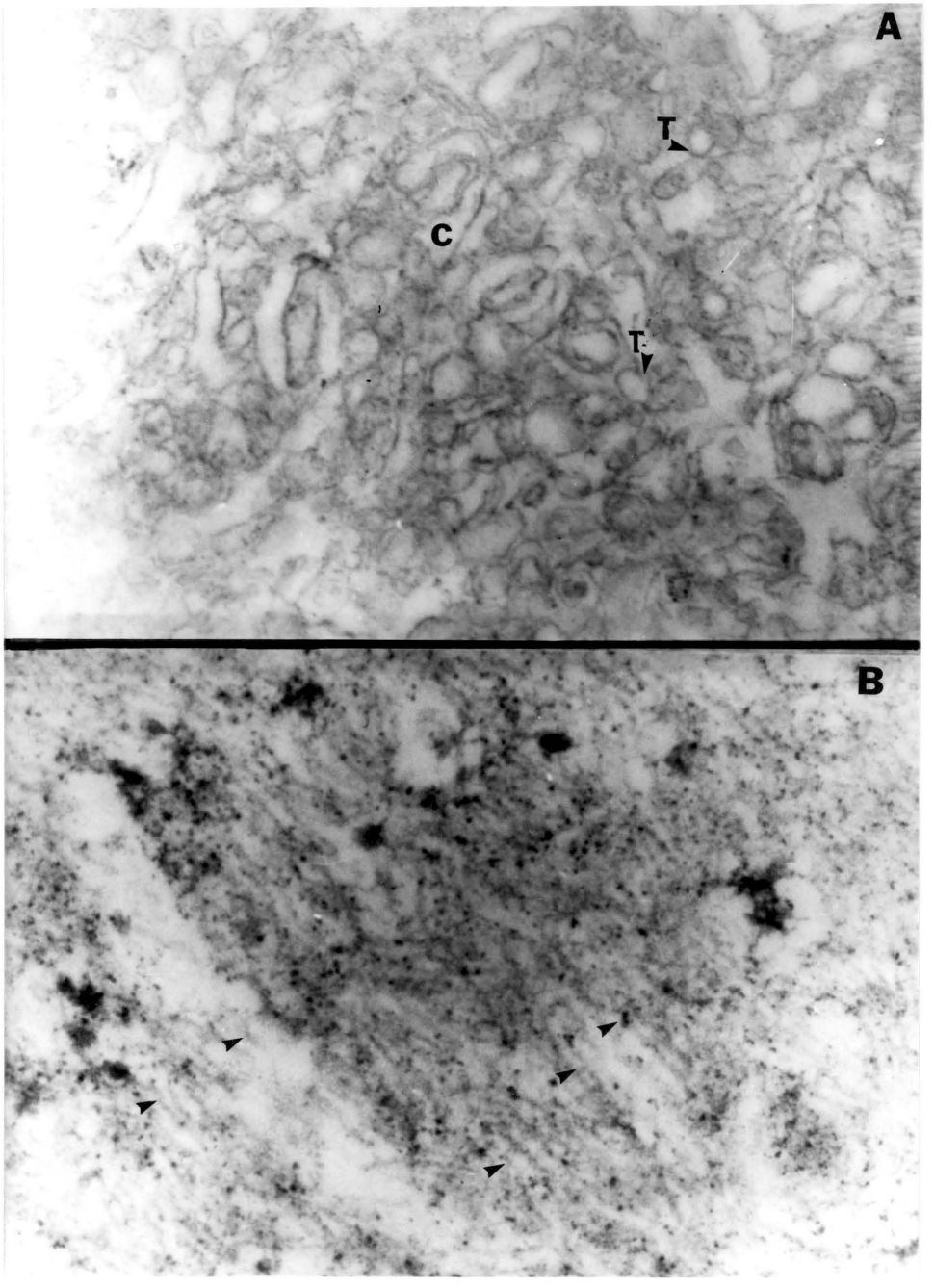

Figure 17. Membranes from mammary gland after sodium carbonate treatment.

Figure 18. ^{3}H-leuicine in TCA-precipitated media.

Mammary gland and liver slices were incubated for 20 minutes, in Krebs-Ringer buffer (KRB), pH 7.4, containing 30 microcuries ^{3}H-leucine, at 37°C. Slices were then rinsed with KRB and immersed in Medium 199 (supplemented to obtain 10 mM leucine, pH 7.4) and incubated for 3 hours at 37°C. Aliquots of media were removed at designated time points, from which secreted proteins were TCA-precipitated, centrifuged, and solubilized. Radioactivity was determined by liquid scintillation counting.

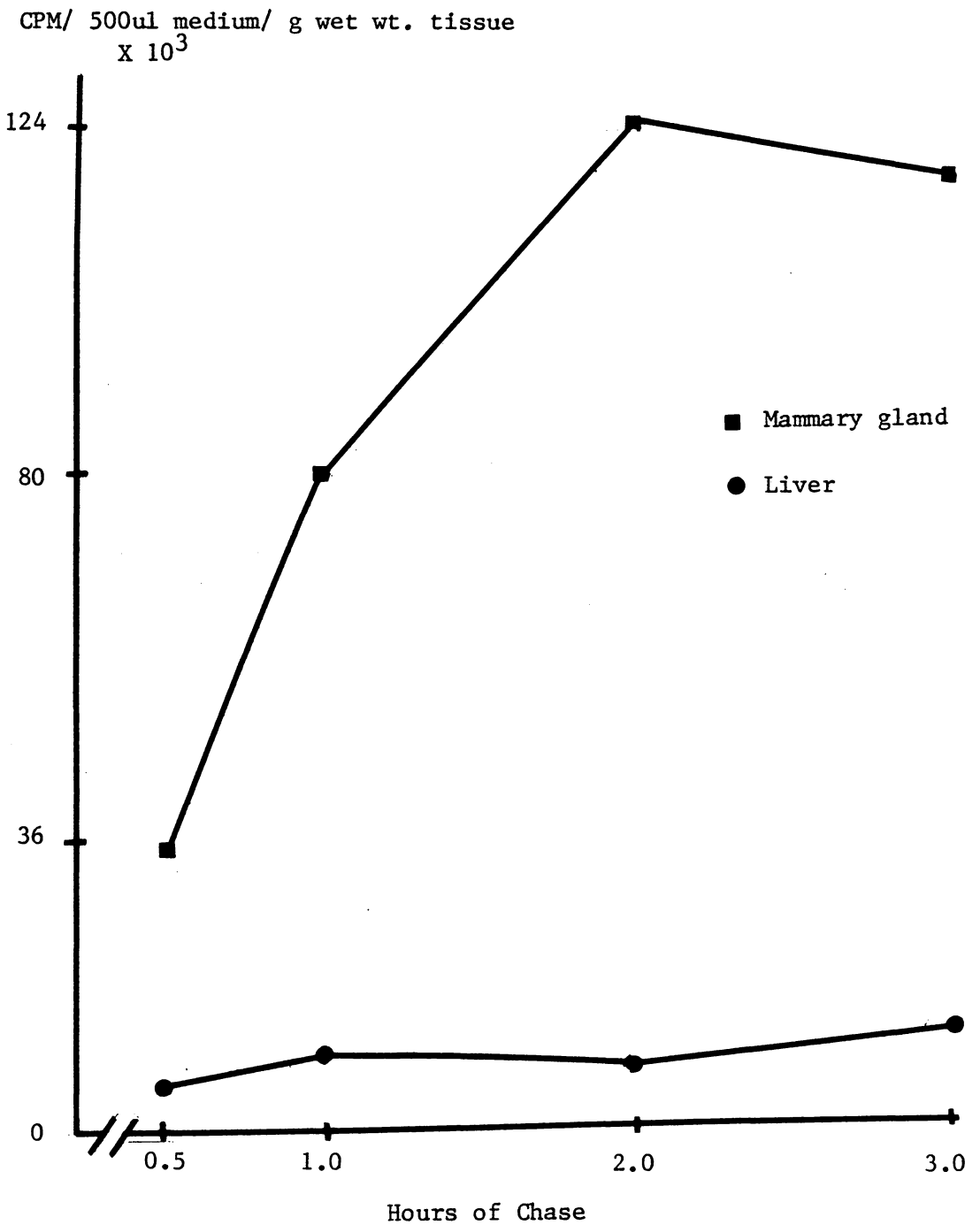

Figure 18. ^{3}H-leucine in TCA-precipitated media.

appeared to occur more slowly in liver, it would be expected that the chase effect would appear later, due to slower depletion of the pool of labeled amino acid. These data are consistent with the differences observed in amounts of fraction protein released by treatment of mammary gland and liver fractions with sodium carbonate. The higher rate of protein release found with mammary gland slices may be correlated with a higher amount of content (secretory) versus membrane protein in comparison with liver.

Calcium accumulation by subcellular fractions

In the presence of ATP, Golgi apparatus from mammary gland accumulated 28% of the ^{45}Ca in incubation buffer in 10 minutes at 37°C, and Golgi apparatus from liver accumulated 11% of the ^{45}Ca (Figure 19). Endoplasmic reticulum accumulated 8% of the ^{45}Ca in incubation medium regardless of tissue source. These incubations were with 0.5 mg fraction protein/ml. As the amount of total protein which was membrane-associated was not constant for all fractions, these values do not reflect uptake on a standardized unit of membrane protein. Uptake values were adjusted for the different percentages of membrane protein in each fraction.

Figure 19. Total ^{45}Ca accumulation in the presence of ATP. ^{45}Ca accumulation was measured as described in Materials and Methods II. Results give accumulation as a percent of total label available for uptake (least square means, ∓ S.E.). Abbreviations are as follows: mammary gland homogenate, MH; liver homogenate, LH; mammary gland Golgi apparatus, MG; liver Golgi apparatus, LG; mammary gland RER, MR; liver RER, LR; both Golgi apparatus and RER fraction results pooled from mammary gland, M; the same pooling of fraction results from liver, L; pooling results by fraction, homogenate H, Golgi apparatus GA, RER the same. Statistical analysis of data indicated differences as follows; fractions with a common superscript letter have unequal ^{45}Ca accumulation. a, b $p<0.001$, c $p<0.005$, d,e $p<0.0001$.

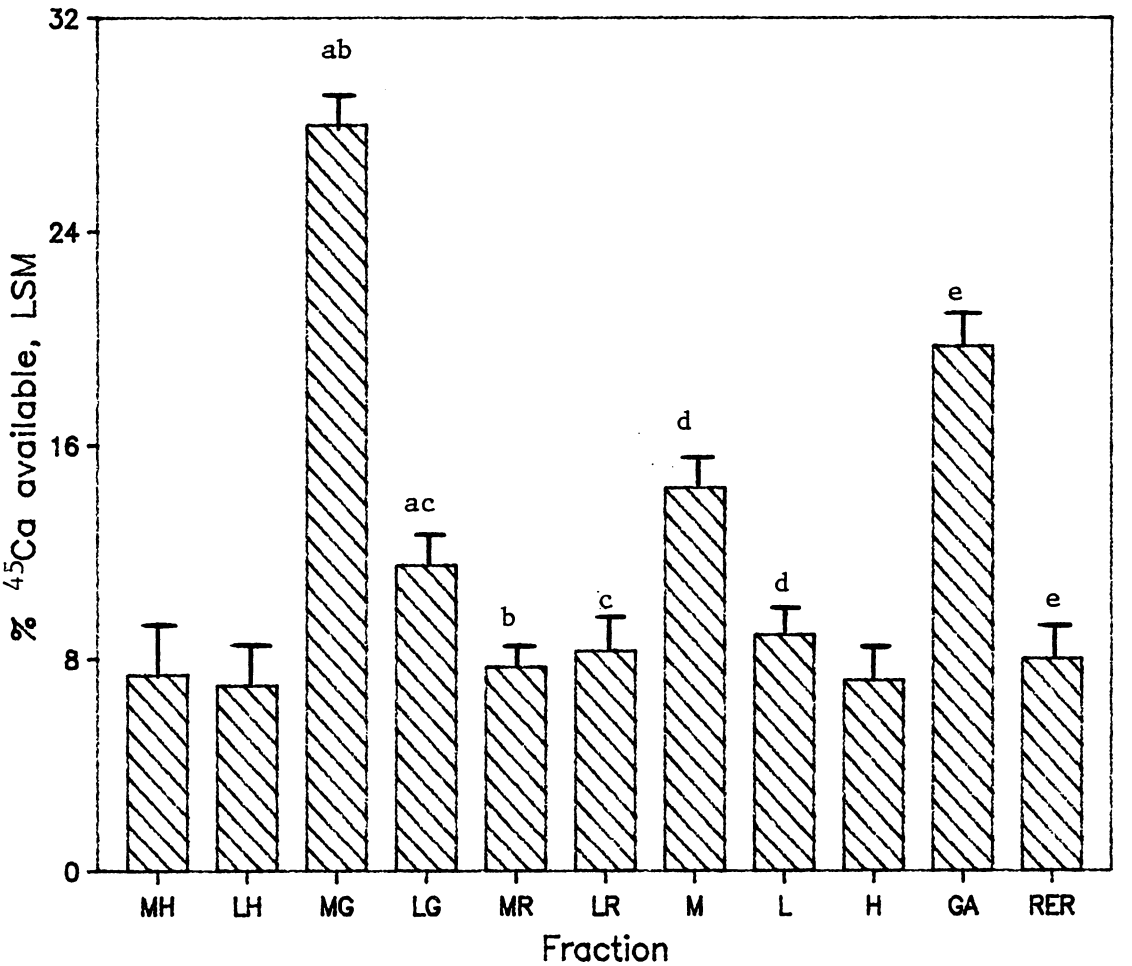

Figure 19. Total ^{45}Ca accumulation in the presence of ATP.

Golgi apparatus from each tissue accumulated higher amounts of calcium per unit membrane protein than did endoplasmic reticulum. Relative accumulations, based on one mg of membrane protein, were 88% and 18% for Golgi apparatus, and 22% and 10% for endoplasmic reticulum, from mammary gland and liver, respectively (Table 4). Of all fractions examined, Golgi apparatus from mammary gland was most active in calcium accumulation.

Calcium accumulation per unit of membrane was also assessed based on phospholipid content (Table 5). Sodium carbonate treatment followed by phospholipid assay allowed quantification of the amount of membrane phospholipid per mg total fraction protein. Dividing percent accumulated ^{45}Ca by the respective mg phospholipid/mg protein value gave accumulation per unit of membrane phospholipid (Table 4). Expressed on this basis, Golgi apparatus fractions from both tissues gave higher values for calcium accumulation than did endoplasmic reticulum from either tissue.

Using values for enrichment of marker enzymes in each fraction, it was possible to calculate the relative amounts of cell protein represented in these two fractions. These values could then be used to assess the relative contributions of Golgi apparatus and endoplasmic reticulum to calcium accumulation. For example, in mammary gland homogenate galactosyl transferase activity was 0.04 μmol/mg

Table 4. ^{45}Calcium accumulation on a membrane protein and phospholipid basis.

Fraction	Relative ^{45}Ca accumulated		
	mg total[a] protein	mg membrane[b] protein	mg membrane[c] phospholipid
Mammary gland Golgi apparatus	28% (3.5)	88% (3.5)	44% (6.3)
Mammary gland RER	8%	27%	7%
Liver Golgi apparatus	11% (1.4)	18% (1.8)	22% (1.7)
Liver RER	8%	10%	13%

[a]Ten minute accumulation of available ^{45}Ca was measured using 0.5 mg total subcellular fraction protein/ml as described in Materials and Methods II. Parenthetical numbers indicate ratio of accumulation by Golgi apparatus divided by accumulation by RER for each tissue.

[b]Results in the first column were adjusted based on the percent membrane protein of the corresponding subcellular fraction (Table 3). Resultant accumulation by RER and Golgi apparatus from one tissue type were then added and relative % of total Ca accumulation calculated for each fraction.

[c]Results in the first column were adjusted based on mg phospholipid (PL), mg protein (Table 5), followed by adjustment by the % of PL which was membrane (Table 3). The relative % of total Ca accumulation was calculated as described in b.

Table 5. Phospholipid concentrations of subcellular fractions *

Tissue	Fraction n=4 preps	ug PL/mg Protein ± S.D.
Mammary gland	Homogenate	340 ± 55
	RER	1,110 ± 65
	Golgi apparatus	570 ± 31
Liver	Homogenate	190 ± 37
	RER	610 ± 17
	Golgi apparatus	490 ± 31

* Phospholipids of subcellular fractions were extracted (Folch et al., 1957) and inorganic phosphate determined by spectrophotometric assay (Rouser et al., 1966). Phospholipid was calculated based on 4% inorganic phosphate composition of phospholipid. Protein determination was made on a separate aliquot of sample.

protein/hr, while Golgi apparatus isolated from this homogenate has a specific activity of 0.65. After correction for amount of protein which was membrane protein, it was calculated that 6% of mammary gland homogenate protein was in Golgi apparatus. Following the same calculation, liver Golgi apparatus accounted for 7% of homogenate protein, and liver and mammary gland endoplasmic reticulum were 10% and 42% of homogenate protein, respectively. Using these values, the relative contribution of each fraction to calcium accumulation was compared (Table 6). In liver, endoplasmic reticulum and Golgi apparatus were nearly equal in overall calcium accumulation activity. In mammary gland, endoplasmic reticulum would account for 65% and Golgi apparatus for 35% of the "total" calcium accumulation activity of these two fractions. Using literature values for surface areas of endoplasmic reticulum and Golgi apparatus in mammary epithelial cells (Verley and Hollman, 1971), it was calculated that these fractions were nearly equal in overall calcium accumulation activity (Table 6). Literature values for surface area of these fractions in hepatocytes gave much lower values for abundance of Golgi apparatus than that determined by enzymatic assay (Bollender et al.,1978). Based on these literature values, Golgi apparatus contributes much less than endoplasmic reticulum to calcium accumulation (Table

Table 6. Relative calcium accumulation by Golgi apparatus and RER.

Fraction	Accumulation[1] (% available)	Accumulation corr.[2] for relative % of cell	Accumulation corr. based on S.A. of membrane
Mammary gland Golgi apparatus	28% ± 1.1	35%	49%[3]
Mammary gland RER	8% ± 1.2	65%	51%[3]
Liver Golgi Apparatus	11% ± 1.1	48%	8%[4]
Liver RER	8% ± 1.1	52%	92%[4]

[1] Measured directly by filtration assay as described in Materials and Methods II, on equal total protein basis.

[2] % of total cell protein represented by each fraction was determined by using enrichment values for marker enzymes as described in text. Calcium accumulation (first column) was then multiplied by this number, and the relative contribution of each fraction of one tissue type was then determined.

[3] Calculations based on membrane surface area values from Verley & Hollmann, 1971.

[4] Calculations based on membrane surface area values from Bollender et al., 1978.

6). However, this incongruity may be due to sex or physiological differences, since male rats were used in the study of Bollender and colleagues.

ATP and calcium accumulation

Mammary gland Golgi apparatus was more active than any other fraction studied in ability to accumulate calcium on a unit membrane protein basis. Mammary gland Golgi apparatus was also different from the other fractions in calcium accumulation activity in the absence of exogenous ATP (Table 7). Earlier workers observed a slight calcium accumulation in the absence of ATP by Golgi apparatus from mouse and cow mammary gland (Neville et al., 1981; Baumrucker & Keenan, 1975). Neither group discussed this observation, although Neville et al. presented data suggestive of calcium being accumulated into membranous vesicles, and not just binding to membranes in the absence of ATP. In contrast, West (1981) reported that calcium was not accumulated by Golgi apparatus from rat mammary gland in the absence of ATP. Golgi apparatus and endoplasmic reticulum from rat liver did not accumulate calcium in the absence of ATP (Hodson, 1978; Moore et al., 1975). The near zero values obtained in the present study for calcium accumulation without ATP by fractions other than mammary gland Golgi apparatus are in agreement with results

Table 7. ATP affect on ^{45}calcium accumulation.[1]

Least Square Means ± S.E.

Fraction[2]	ATP-dependent[3] accumulation	ATP-independent[4] accumulation	% of total accumulation[5] that is ATP-independent
Mammary gland Golgi apparatus	22.0±1.98^{ab}	5.4±0.45^{ab}	21.1±3.19
Mammary gland RER	7.1±1.98^{a}	1.8±.45^{a}	25.4±3.19
Liver Golgi apparatus	10.5±1.98^{b}	1.8±0.45^{b}	15.9±3.19
Liver RER	4.9±1.98	0.9±0.45	16.8±3.19
Combined Res.[6] Mammary Gland	14.6±1.40^{c}	3.6±0.32^{c}	23.3±2.26^{c}
Combined Res. Liver	7.7±1.40^{c}	1.4±0.32^{c}	16.3±2.26^{c}
Combined Res. Golgi apparatus	16.3±1.40^{d}	3.6±0.32^{d}	18.5±2.26
Combined Res. RER	6.0±1.40^{d}	1.4±0.32^{d}	21.1±2.26

[1]Comparisons were made by column. Accumulation was unequal in fractions with a common superscript letter, $p<0.05$. Data is % of available ^{45}Ca.

[2]n=6 preparations.

[3]ATP-dependent accumulation was that accumulation which occurred in the presence of 2 mM ATP minus accumulation that occurred in the absence of ATP.

[4]ATP-independent accumulation was that accumulation which occurred in the absence of ATP in the incubation buffer.

[5]Calculated : ATP-independent ^{45}Ca accumulation divided by total accumulation for that fraction in the presence of ATP.

[6]Abbreviation for results.

of Hodson and Moore et al.

One possible explanation for ATP-independent accumulation of calcium by Golgi apparatus from mammary gland may be that the pore properties of this fraction are different from those of the other fractions. White et al. (1984) found that both charged and uncharged solutes of molecular weight below about 300 penetrated membranes of Golgi apparatus from rat mammary gland. Differences in pore properties alone may not explain the differences in Golgi apparatus from mammary gland and liver. The enhanced calcium accumulation activity of Golgi apparatus from mammary gland may be due to the presence of mammary gland specific secretory proteins, such as caseins and α-lactalbumin, which are known to bind calcium with high affinity (Boulet et al., 1971; Bryant and Andrews, 1984). Greater calcium accumulation found with Golgi apparatus versus endoplasmic reticulum from mammary gland may be due to differences in extent of phosphorylation of milk proteins within these two endomembrane compartments. Caseins are phosphorylated by kinases which are concentrated in Golgi apparatus, and thus caseins within Golgi apparatus should have higher affinities for calcium than those in endoplasmic reticulum (Bingham & Farrell, 1974). The addition of acid-precipitated caseins to incubation buffer, at concentrations equal to the amount of vesicular protein

used, reduced calcium uptake by 55% for fractions from mammary gland and 85% for fractions from liver (current work). This result can be explained as being due to calcium binding to extravesicular caseins, thus making Ca unavailable for uptake. As expected, calcium uptake by mammary gland vesicles, which contain caseins, was less affected by extravesicular caseins than were liver vesicles.

The ranking order of fractions studied, with respect to calcium accumulating activity, was the same for total uptake, ATP-dependent uptake and ATP-independent uptake (Table 7). Combining data for endoplasmic reticulum and Golgi apparatus according to tissue source revealed a significant tissue difference ($p < 0.0001$ to $p < 0.003$), with fractions from mammary gland being more active in calcium accumulation than those from liver. Combining data by subcellular fraction regardless of tissue source, Golgi apparatus was found to have more activity in calcium accumulation than endoplasmic reticulum ($p < 0.0001$). Comparison of the percent of total ^{45}Ca uptake that is ATP-independent for each fraction did not follow the above pattern. The only difference found in these data was that of tissue source, with mammary gland fractions accumulating a higher percentage of calcium by ATP-independent means than those from liver (23% and 16%, respectively).

Cotransport of calcium and citrate

When 70 μM citrate was included in buffer used for calcium uptake studies, the amount of ^{14}C-citrate which was accumulated was less than 1% of the total citrate in the buffer for all fractions, with or without ATP. All fractions accumulated calcium to the same extent in the presence and absence of citrate. Thus, under these experimental conditions, calcium accumulation did not appear to be coupled to citrate accumulation. Zulak and Keenan (1983) found that citrate was accumulated by endoplasmic reticulum and Golgi apparatus from bovine mammary gland. They found citrate accumulation to be inhibited by 1 to 5 mM ATP, and that this inhibition was overcome by inclusion of an amount of calcium equimolar with the amount of ATP. For current work, buffers contained 2 mM ATP and 20 μM calcium, and these differences may explain why citrate accumulation was not observed.

Affect of calcium ionophore A23187

In an attempt to establish that calcium was transported into vesicles and not just bound to membranes, the calcium ionophore A23187 was added to incubation mixtures 10 minutes after initiation of calcium accumulation. In the presence of ionophore, there was a loss of accumulated calcium from endoplasmic reticulum and Golgi apparatus in

the first 5 minutes after ionophore addition (Figure 20). After 5 minutes, the amount of calcium in vesicles exposed to ionophore remained relatively constant. West (1981) reported retention of only 25% of radioactive calcium just minutes after addition of A23187 to a crude membrane fraction from mouse mammary gland. The degree of loss of accumulated calcium reported by West was similar to the amount of loss observed with fractions from liver (Figure 20A and 20B). With fractions from mammary gland, Golgi apparatus retained a higher percentage of calcium than did endoplasmic reticulum in the presence of A23187 (Figure 20C and 20D). This may be reflective of the sequestration of calcium with phosphorylated proteins in mammary gland Golgi apparatus. When fractions were preincubated with A23187 before addition of ATP and calcium, calcium accumulation was reduced to values which were less than 10% of the amount accumulated in the absence of ionophore in all fractions (Table 8). These observations were similar to those of West (1981) for calcium accumulation by Golgi apparatus from rat mammary gland in the presence and absence of A23187. Pooling data for subcellular fractions from each tissue type revealed that Golgi apparatus retained more calcium than endoplasmic reticulum in the presence of A23187 ($p < 0.03$) (Table 8). Whether this is due to differences in membrane composition

Figure 20. ^{45}Ca accumulation after ^{2+}Ca ionophore addition.

Vesicle protein was incubated with ^{45}Ca for 10 minutes under standard conditions described in Materials and Methods II. At 10 minutes, one aliquot was filtered to determine ^{45}Ca accumulation. The remaining incubation sample mixture was divided in half. Incubation was continued as before for one half, while the second half had ^{2+}Ca ionophore (A23187, 2 uM) added. Accumulation of both sets were followed by filtration of aliquots at 5, 10, and 15 minutes after ionophore addition. Panels are results with liver Golgi apparatus (A), liver RER (B), mammary Golgi apparatus (C), and mammary RER (D).

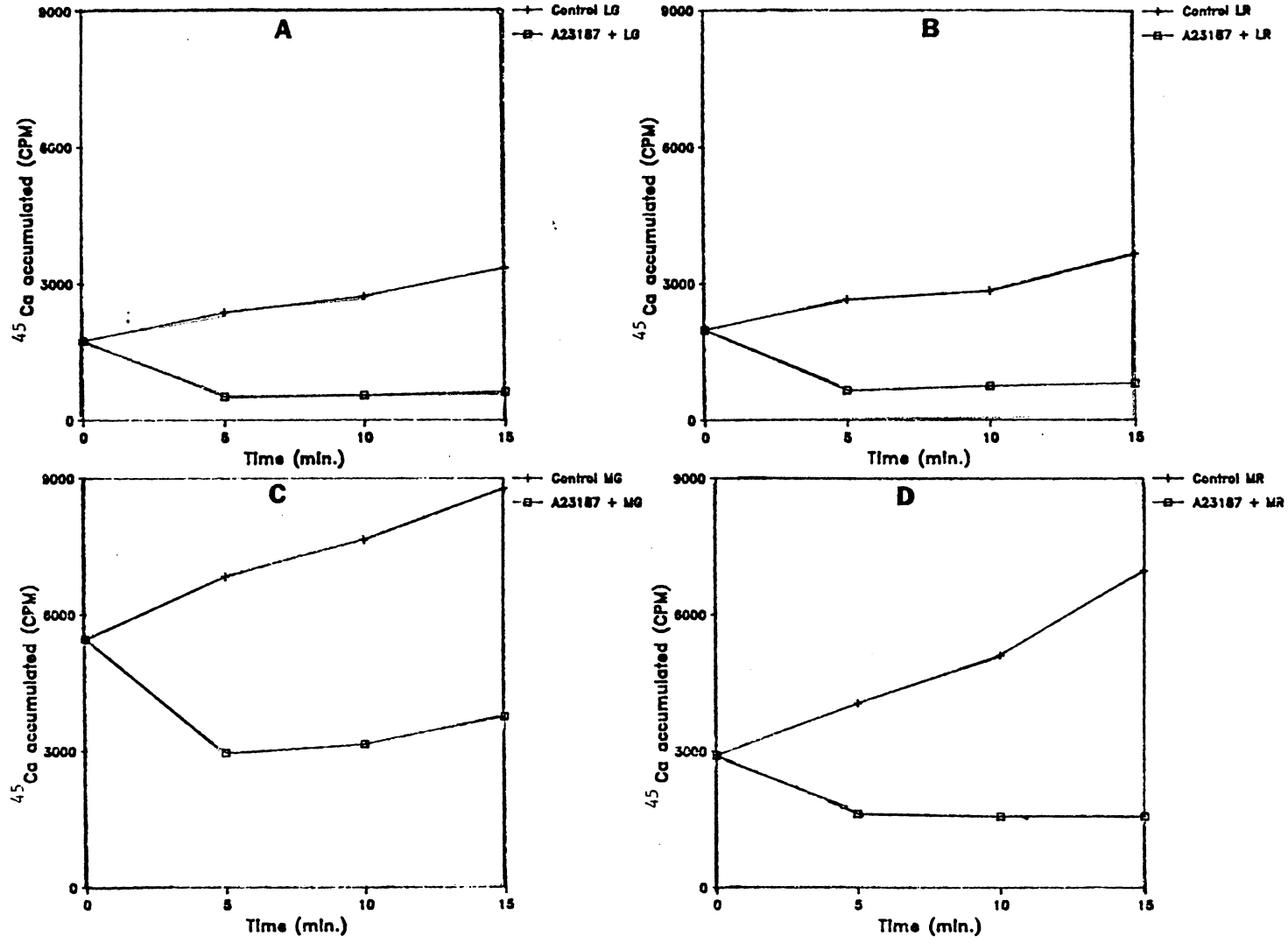

Figure 20. ^{45}Ca accumulation after ^{2+}Ca ionophore addition.

Table 8. Affect of Ca^{2+} ionophore on ^{45}Ca accumulation*

Fraction	% of control ^{45}Ca L.S. Means ± S.E.
Mammary gland homogenate	7.8 ± 1.83
Liver homogenate	7.8 ± 1.83
Mammary gland Golgi apparatus	9.7 ± 1.83
Liver Golgi apparatus	11.2 ± 1.83
Mammary gland RER	4.2 ± 2.58
Liver RER	4.9 ± 2.00
Combined homogenate results	7.8 ± 1.29
Combined Golgi results	10.4 ± 1.29 [a]
Combined RER results	4.5 ± 1.63 [a]

*Subcellular fractions were preincubated with 2uM A23187 for 20 minutes on ice, followed by measurement of ^{45}Ca accumulation under conditions described in Materials and Methods II. Fractions for controls were held on ice for an equal time, but ionophore was not added before ^{45}Ca accumulation was measured.

[a] Ca^{2+} ionophore effect was different in combined Golgi apparatus versus combined RER results, $p<0.03$.

or vesicular protein content is not clear.

Effect of hypotonic lysis

In an attempt to determine the affect of lumenal content on calcium accumulation, vesicles were subjected to hypotonic lysis to remove content at least partially. After hypotonic exposure subcellular fractions were returned to isoosmotic medium, allowing reformation of vesicles prior to assay of ^{45}Ca accumulation. Mammary gland Golgi apparatus vesicles were found to accumulate 30% of the amount of calcium accumulated by untreated vesicles under standard incubation conditions (Figure 21). Hypotonically treated liver Golgi apparatus and mammary gland endoplasmic reticulum vesicles accumulated about 25% of control amounts of calcium, and those from liver endoplasmic reticulum accumulated about 15% of the amount of calcium accumulated by non-hypotonically lysed vesicles.

Effects of hypotonic lysis and return to isoosmotic medium on vesicles was evaluated by electron microscopic examination of treated fractions. Hypotonically lysed vesicles appeared to be virtually the same as untreated preparations in morphology. Vesicles and tubules appeared to be intact, completely surrounded by membrane, indicating that vesicles did reform after being returned to isoosmotic medium. Interiors of treated vesicles did appear more

Figure 21. Effect of hypotonic lysis on ^{45}Ca accumulation.

Isolated subcellular fractions were hypotonically lysed on ice with distilled, deionized water, centrifuged, and pellets resuspended in 0.25 M sucrose. $^{45}Calcium$ accumulation was measured for fractions before and after lytic treatment. Prelysis accumulation was considered the control. Fraction designations are the same as given in Figure 19. Lytic effect was unequal in fractions with a common superscript letter. a $p<0.009$, b $p<0.05$, c $p<0.007$.

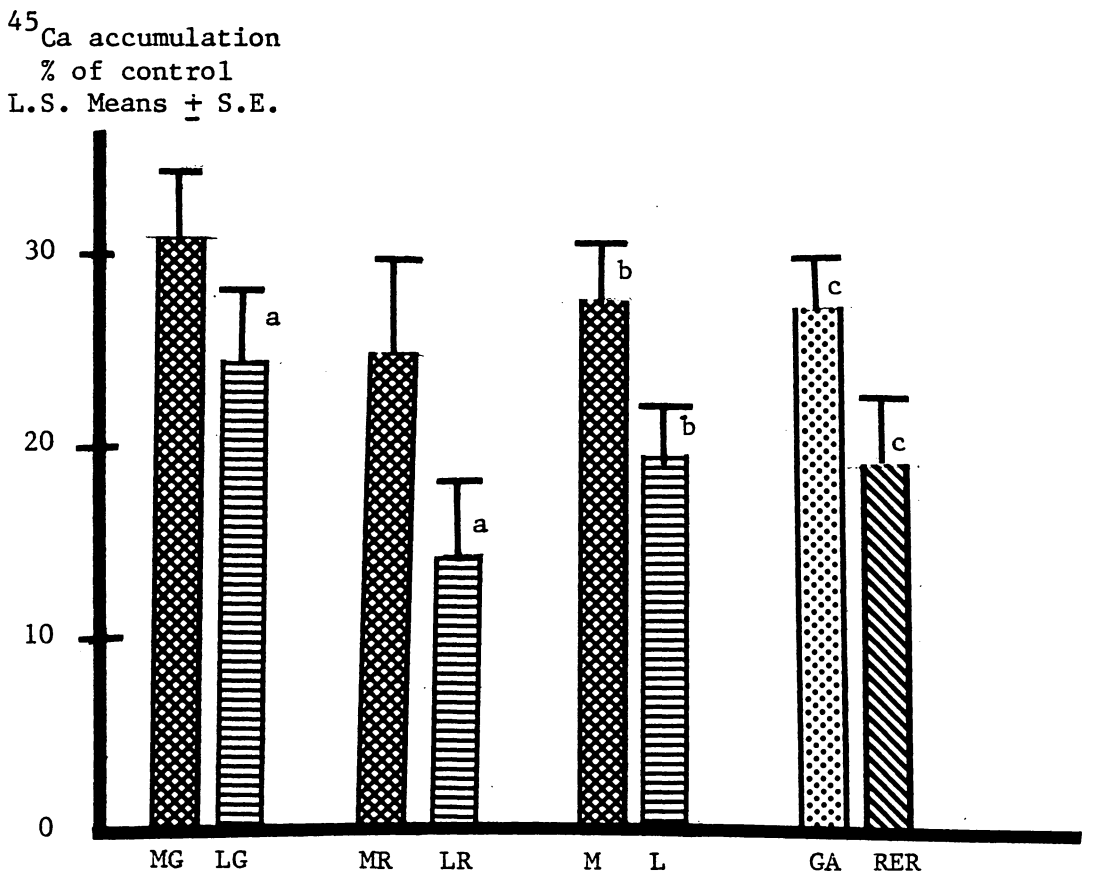

Figure 21. Effect of hypotonic lysis on ^{45}Ca accumulation.

transparent or electron-lucent than did vesicles in control preparations, suggesting that content was removed at least partially by hypotonic lysis (results not shown).

Original vesicle preparations and membranous and soluble fractions recovered after hypotonic lysis were examined by SDS-polyacrylamide gel electrophoresis. Different polypeptide patterns were observed in comparison of membranous with soluble fractions from each vesicle preparation (Figure 22). A polypeptide of about 55 kDa was found predominantly in soluble fractions of endoplasmic reticulum from both tissues. A protein of about 68 kDa, presumably albumin, was present in higher relative abundance in soluble fractions of each liver fraction. The soluble fraction from mammary gland Golgi apparatus contained relatively larger amounts of polypeptides of about 67 kDa and 155 kDa than did the membrane fraction. A 118 kDa protein in rat liver endoplasmic reticulum and a 70 kDa protein in rat mammary gland Golgi apparatus have been associated with calcium accumulation (Heilmann et al., 1984; West and Clegg, 1982). Electrophoretic patterns provided no evidence for loss of proteins of these molecular weights during hypotonic lysis.

One possible explanation for the lessened calcium accumulation by hypotonically treated vesicles is that vesicle integrity may have been lost to some extent. If

Figure 22. SDS-PAGE of fraction proteins, before and after hypotonic lysis.

Fractions were hypotonically lysed as described in the text. Total protein before subcellular fractions were hypotonically lysed is designated Tot. Pellet protein obtained after lysis is designated Pel., and soluble content protein is Con. Subcellular Fraction designations are the same as in Figure 19. MW standards are myosin, 200 KD; B-galactosidase, 116 KD; phosphorylase B, 92 KD; bovine serum albumin, 68 KD; and ovalbumin, 43 KD. Gel was 8 % acrylamide, 1.5 mm thick, 70 ug of sample protein per lane, stained with 0.2 % Coomassie Blue.

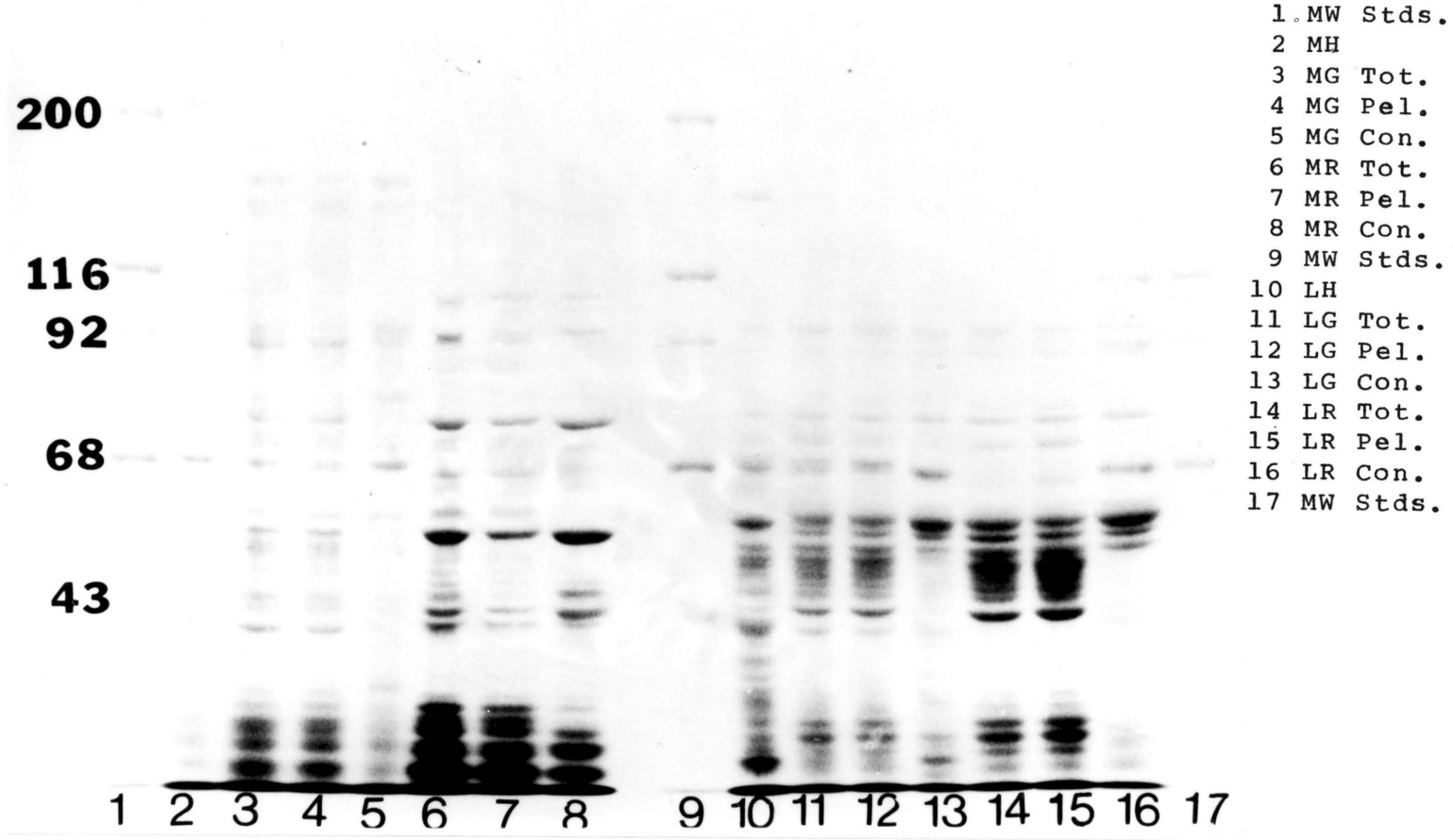

Figure 22. SDS-PAGE of fraction proteins, before and after hypotonic lysis.

such were the case, calcium may have been transported into vesicles, but an uncontrolled efflux also would have occurred. At least some of the calcium which remained in hypotonically treated vesicles may have been trapped by content proteins which had not been removed by lysis. Another possible explanation for diminution in calcium accumulation is the possible loss of enzymatic activity needed for calcium uptake, or the loss of calcium transporters during lysis and resealing.

Affect of protonophore CCCPH

The potential importance of maintanance of a proton gradient for calcium accumulation was evaluated by preincubation of vesicles with 160 μM CCCPH. With all fractions there was a small (about 15 to 22%) reduction in calcium accumulation by fractions after preincubation with protonophore (Table 9). Reduction with liver Golgi apparatus was greater than with the other fractions, but none of the differences between fractions were statistically significant at $p < 0.05$. Virk et al. (1985) reported a 26% reduction in calcium transport and a reduction in pH of 0.33 units when Golgi apparatus from rat mammary gland was incubated with CCCPH. From these results, it does not appear that maintenance of a proton gradient is essential for calcium accumulation by Golgi

Table 9. Affect of protonophore CCCPH on ^{45}calcium accumulation.*

Fraction	% of control ^{45}Ca L.S. Mean ∓ S.E.
Mammary gland Golgi apparatus	86.4 ∓ 3.57[a]
Liver Golgi apparatus	77.4 ∓ 3.57[a]
Mammary gland RER	84.9 ∓ 3.57
Liver RER	85.1 ∓ 3.57

*Subcellular fractions were preincubated with 160uM CCCPH for 20 minutes on ice. Final CCCPH concentration was 32uM upon addition of incubation buffer. Fractions for controls were held on ice an equal time, but CCCPH was not added before ^{45}Ca accumulation was measured.

[a]Fractions with a common letter superscript differ in CCCPH's effect on ^{45}Ca accumulation., $p < 0.09$.

apparatus or endoplasmic reticulum.

Affect of inhibition of ATPase

Vanadate, an ATPase inhibitor, was added to incubation mixtures to determine if ATPase activity was necessary for ATP-dependent calcium accumulation. In the presence of vanadate, calcium accumulation was reduced by 60 to 75% of control values in all vesicle preparations (data not shown). Virk et al. (1985) found that vanadate inhibits Ca^{2+}-ATPase activity and greatly diminishes calcium accumulation in Golgi apparatus from rat mammary gland. Similarly, vanadate inhibits ATPase activity and calcium accumulation by plasma membrane vesicles from rat liver (Chan and Junger, 1983). Vanadate also has been shown to inhibit the Ca^{2+}-ATPase of sarcoplasmic reticulum (Ortiz et al., 1984).

Dependence of calcium accumulation on ATP hydrolysis was addressed further by incubation of fractions with the nonhydrolyzable ATP analog AMP-PNP. When fractions were preincubated with 2 mM AMP-PNP prior to addition of calcium and ATP, calcium accumulation was reduced to 40 to 70% of values obtained with fractions incubated in parallel without AMP-PNP. Based on these results, and those obtained with vanadate, it can be suggested that ATPase activity is in part necessary for calcium accumulation by

endoplasmic reticulum and Golgi apparatus from both tissues.

SUMMARY AND CONCLUSIONS

From the results reported here, it is apparent that both Golgi apparatus and endoplasmic reticulum from mammary gland and liver have the ability to accumulate calcium *in vitro*. In the presence of ATP, Golgi apparatus accumulates more calcium than endoplasmic reticulum on a per mg of fraction protein basis. However, when results were calculated on the basis of the total cellular protein represented in endoplasmic reticulum and Golgi apparatus, or on the total surface area of these endomembrane compartments, endoplasmic reticulum appeared to be equal to or greater than Golgi apparatus in calcium accumulation capacity. Fractions from mammary gland accumulate more calcium than do those fractions from liver. A significant component of calcium accumulation into Golgi apparatus and endoplasmic reticulum from each tissue appeared to be ATP-independent. Both ATP and ATPase activity were necessary for maximum calcium accumulation by all fractions examined. Much literature in this area advances the concept of a strict coupling of calcium transport to ATP hydrolysis, however the evidence for this is not conclusive. There is large variation in reported ratios of moles of inorganic phosphate released from ATP to moles of calcium accumulated. In contrast to results of many other groups, Kuhn and White (1977) found no hydrolysis of ATP by

Golgi apparatus from rat mammary gland. Calcium accumulation by Golgi apparatus and endoplasmic reticulum from mammary gland and liver did not appear to be dependent on maintenance of a proton gradient, but accumulation may have been pH sensitive. From results obtained in this study, I suggest that differences in calcium sequestration activity of proteins in lumenal contents of fractions could account for tissue differences in calcium accumulation and retention by fractions.

For future research, the question of whether calcium transport is linked to transport of other molecules, such as citrate or ATP, would be an interesting area for exploration. Elucidating the nature of the ATP requirement for maximum accumulation of calcium would be particularly challenging. Calcium and ATP may be cotransported in the form of a complex, ATP hydrolysis may be necessary to provide energy for a component of calcium transport, or ATP transport and utilization for phosphorylation of lumenal proteins may be necessary for sequestration of transported calcium.

Based on the distribution of radiolabeled calcium in milk and the retention of this radiolabeled calcium by rats dosed with milk, several tentative conclusions can be drawn. First, a higher percentage of the calcium in cow milk was retained by the rat in comparison with rat milk,

yet rat milk contains more calcium and more protein than cow milk. Results from this study support the idea that non-casein associated calcium is more readily absorbed than is calcium associated with caseins (Schachter et al., 1960). Also, results from the current study indicated that, by various methods of fractionation, radiolabeled calcium added to rat milk after collection could not be distinguished from radiolabeled calcium incorporated into milk prior to its secretion from mammary epithelial cells. This equivalent ^{45}Ca distribution suggests that exogenously supplied calcium can equilibrate with endogenous calcium, and that exogenous addition of ^{45}Ca may be a vaild approach to take in studies of absorption and retention of milk calcium.

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Colocalization of α-Lactalbumin and a Major Casein in Secretory Vesicles of Rat Mammary Epithelial Cells

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Summary

Whether both casein and noncasein (serum or whey) proteins of milk are contained within the same secretory vesicles of milk secreting mammary epithelial cells was explored. Antibodies to a major casein and to α-lactalbumin of rat milk were localized in thin sections with colloidal gold-conjugated second antibodies. Antibodies to the casein component bound to an antigen present within lumina of Golgi apparatus cisternae and within secretory vesicles. This antigen was also recognized in structures within secretory vesicles and within alveolar lumina which were ultrastructurally identified as casein micelles. Antigens recognized by antibodies to α-lactalbumin also were present in Golgi apparatus cisternae and within secretory vesicles. Both anti-casein and anti-α-lactalbumin antibodies recognized antigens within the same secretory vesicles. These observations show that one major noncasein protein of rat's milk is present in casein-containing secretory vesicles.

Keywords: Mammary epithelium; Golgi apparatus; Secretory vesicles; Exocytosis; Immunogold localization.

1. Introduction

Secretory vesicles in mammary epithelial cells appear to be the major vehicles for exocytosis of the proteins, lactose, ions and water which constitute the nonlipid or serum phase of milk (reviews KEENAN and DYLEWSKI 1985, MATHER and KEENAN 1983). However, the question as to whether secretory vesicles are homogeneous or heterogeneous with respect to their protein content has not been resolved. Proteins in milk of many species can be subdivided into two groups, the caseins and the noncasein serum or whey proteins. Caseins are operationally defined as phosphoproteins which assemble into micelles and which can be precipitated from milk at pH values between about 4 and 5. Several different polypeptides in the casein fraction of rat milk have been characterized (HIROSE *et al.* 1981, VISSER *et al.* 1981), and at least three of these are primary gene products (MAKI *et al.* 1985, ROSEN *et al.* 1975). α-Lactalbumin, a protein essential for synthesis of lactose (review EBNER and SCHANBACHER 1974), is a major serum protein of rat milk (JENNESS 1979). This protein, which is a primary gene product, exists in rat milk in several forms differing in extent of glycosylation (PRASAD and EBNER 1980, PRASAD *et al.* 1979).

Since the early work of BARGMANN *et al.* (1961), and WELLINGS *et al.* (1961), it has been recognized that some secretory vesicles contain structures identical in appearance to the casein micelles of milk. In micrographs of milk secreting cells, vesicles which contain a fibrillar of granular material are commonly observed; these vesicles may or may not contain casein micelles (see, for example, DYLEWSKI and KEENAN 1983, FRANKE *et al.* 1976). While it has been assumed that this fibrillar/granular material may be premicellar caseins, this has yet to be demonstrated (FARRELL 1978). Caseins and milk serum proteins are present in isolated fractions enriched in secretory vesicles (SASAKI *et al.* 1978). However, vesicles in these isolated fractions are heterogenous with respect to presence of casein micelles, and it cannot be determined that both caseins and serum proteins coexist within individual vesicles. TURKINGTON (1969) demonstrated that α-lactalbumin

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and caseins are synthesized within the same epithelial cells, but it remains to be established that proteins from these two groups exit the cell via the same secretory vesicles. In this paper we show, by ultrastructural localization of a major casein and α-lactalbumin, that these two proteins occupy the same secretory vesicles.

2. Material and Methods

2.1. Antigen Preparation

Milk was obtained from primiparous SPRAGUE-DAWLEY rats under pentobarbital anesthesia (60 mg/kg) by vacuum aspiration (GUPTA *et al.* 1970). Just prior to milking, animals were injected intraperitoneally with 40 I.U. oxytocin in 0.2 ml sterile saline. Lipid globules were removed by centrifugal flotation and casein and noncasein (whey) protein fractions were obtained from the skim milk by acid precipitation (VISSER *et al.* 1981). α-Lactalbumin was purified according to MCKENZIE and LARSON (1978). Caseins were separated in polyacrylamide gels containing sodium dodecyl sulfate (LAEMMLI 1970). The position of a major casein, designated casein C 4 (HIROSE *et al.* 1981), was determined by staining strips sliced from edges of gels with coomassie blue. Casein C 4 was eluted from unstained portions of gels and used for antibody production.

2.2. Antibody Preparation

Antibodies were produced in New Zealand rabbits following the immunization schedule described (DEENEY *et al.* 1985). Prior to use, antibodies were purified by immunoaffinity chromatography of IgG fractions (DEENEY *et al.* 1985). Specificity of antibodies was determined by transferring electrophoretically separated caseins and whey proteins onto nitrocellulose (TOWBIN *et al.* 1979), incubating with specific antibodies, and then with ^{125}I-protein A (Amersham Corp., Arlington Heights, IL/U.S.A.) Autoradiography was at −70 °C using Kodak XAR-5 film.

2.3. Immunocytochemistry

Samples of mammary tissue from primiparous rats were fixed, embedded in Lowacryl K4M and sectioned as described (DEENEY *et al.* 1985). Sections were collected on 100 mesh, formvar-coated nickel grids. Antibodies were localized on sections with goat-antirabbit IgG or with protein A to which colloidal gold particles were conjugated (DEENEY *et al.* 1985). For double labelling, sections were incubated sequentially with anticasein, 25 nm gold conjugated-antirabbit IgG, unconjugated protein A, anti-α-lactalbumin, and finally with 50 nm gold-protein A. Sections were extensively washed between each incubation (DEENEY *et al.* 1985). Sections were stained with 2% uranyl acetate in 80% ethanol-20% methanol followed with lead acetate (MILLONIG 1961). For controls, sections were incubated as above, but without primary antibody. Colloidal gold particles were from Janssen Pharmaceutica, Beerse, Belgium and from Polysciences, Warrington, PA/U.S.A.

Sections were examined and photographed with a Zeiss EM10 electron microscope operated at 60 kV.

3. Results

Rabbit antibodies to rat casein C 4 reacted strongly with this component in immunoblots of rat caseins (Fig. 1). In some preparations, weak reactivity was observed also with a minor component of the casein fraction. By analogy with the known origin of certain minor caseins by proteolysis of major caseins in cow's milk (EIGEL *et al.* 1979), this component in rat casein may be a degradation product of casein C 4. Antibodies against α-lactalbumin also reacted strongly with rat α-lactalbumin blotted onto nitrocellulose from SDS polyacrylamide gels. These antibodies reacted with both components of the α-lactalbumin doublet (Fig. 1). This doublet is due to the presence of both glycosylated and nonglycosylated forms of α-lactalbumin in rat's milk (MCKENZIE and LARSON 1978, PRASAD and EBNER 1980).

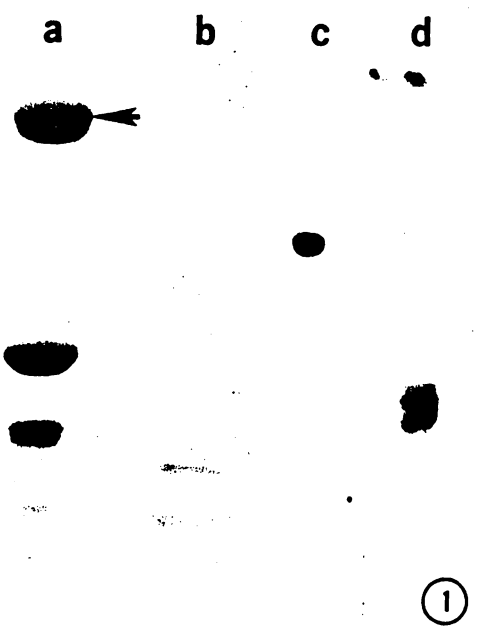
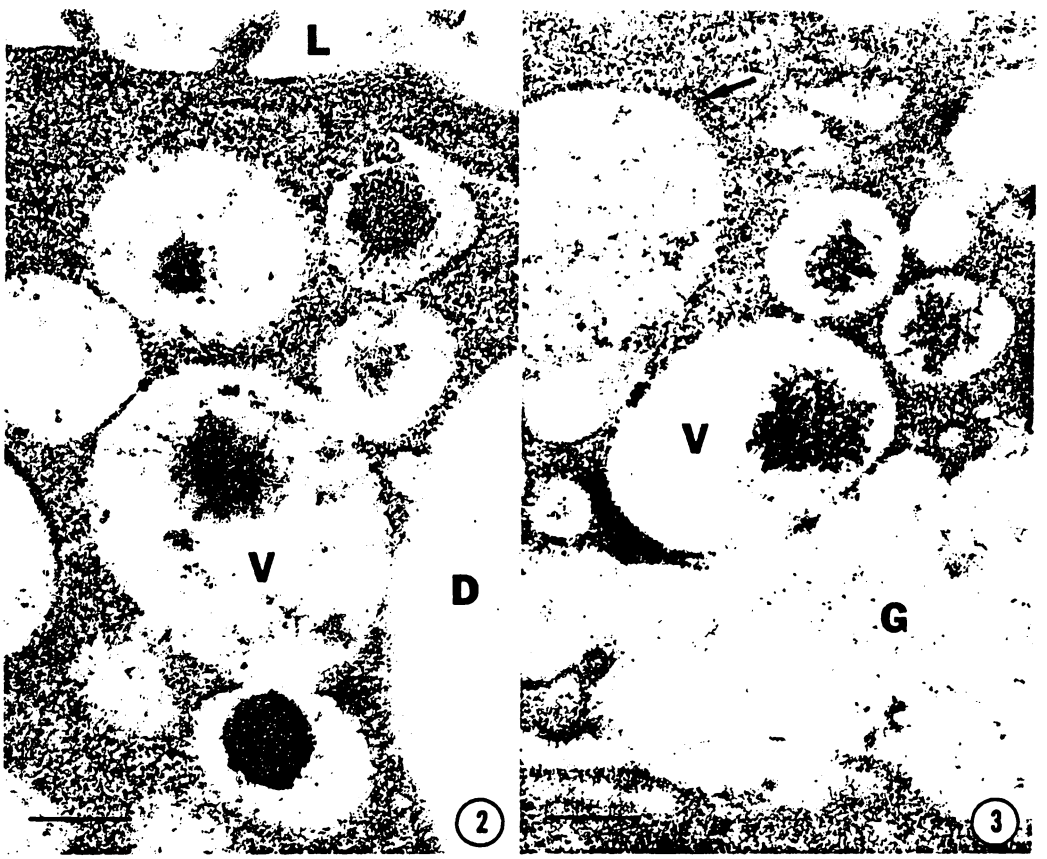


Fig. 1. Polyacrylamide gel electrophoretic pattern of caseins (lane *a*) and purified α-lactalbumin (lane *b*) from rat milk. Gel was stained with coomassie blue. Casein component C 4 (arrow) was eluted from preparative gels and used as antigen. Lane *c*, autoradiogram of a nitrocellulose blot of a casein fraction which had been incubated with anti-casein C 4 and then with ^{125}I-protein A. Lane *d*, autoradiogram of a nitrocellulose blot of α-lactalbumin incubated with anti-lactalbumin and then with ^{125}I-protein A

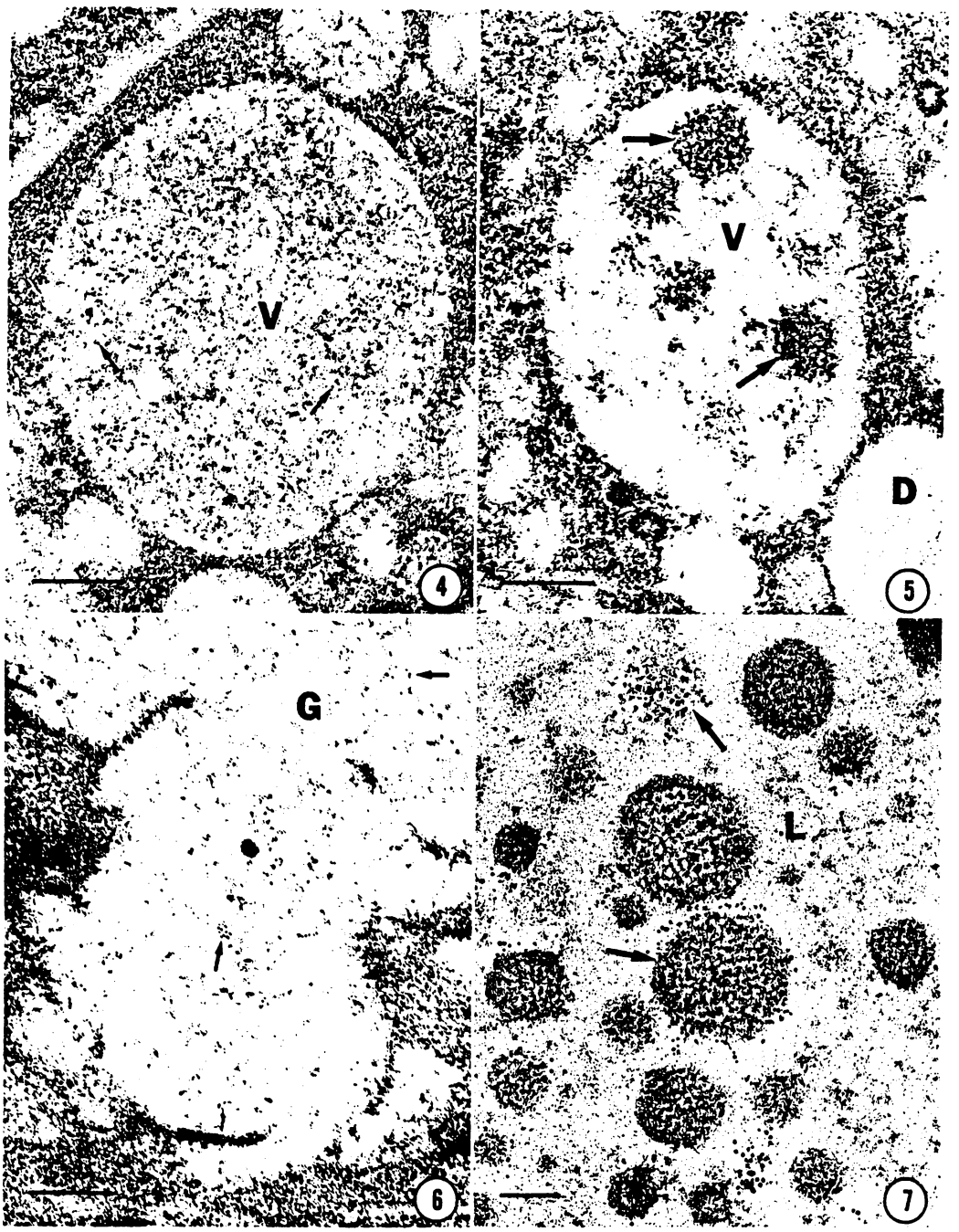
Without prior application of primary antibody (Figs. 2 and 3) there was little nonspecific binding of gold particles conjugated to IgG or to protein A. Antibodies to casein C 4 were localized over filamentous/granular materials in secretory vesicles which lacked recog-



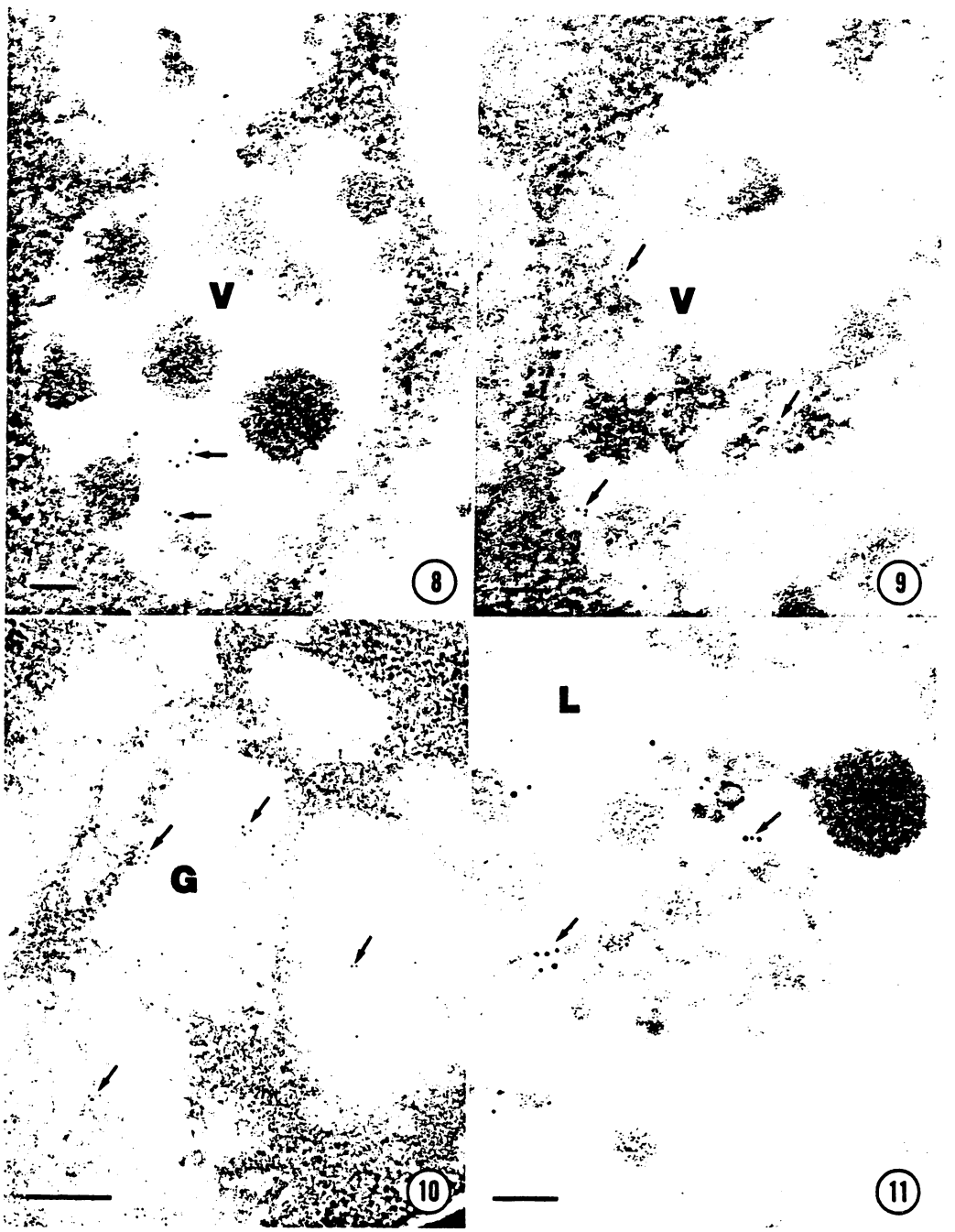
Figs. 2 and 3. Controls; sections were incubated without either primary antibody, then were placed onto drops of a dilution of 5 nm colloidal gold conjugated goat antirabbit antibody followed by protein A-15 nm gold complex. Survey fields of apical regions of epithelial cells showing relatively little nonspecific binding of gold particles. Alveolar lumen (*L*), secretory vesicles (*V*), cytoplasmic lipid droplet (*D*), Golgi apparatus (*G*) and 5 nm gold particle (arrow). Fig. 2, × 66,000; Fig. 3, × 59,000. Scale bars = 0.25 μm

nizable casein micelles (Fig. 4) and over both micelles and filamentous materials in secretory vesicles with casein micelles (Fig. 5). Immunogold conjugates were also observed over the entire area occupied by Golgi apparatus when sections were first incubated with anti-casein C4 (Fig. 6). Casein micelles in alveolar lumina were also heavily labelled with gold particles (Fig. 7). Within alveolar lumina, anticasein antibody was also present over areas which lacked casein micelles (Fig. 7). This is consistent with the observation that a proportion of the total caseins of milk are present in nonmicellar form (review, FARRELL and THOMPSON 1974).

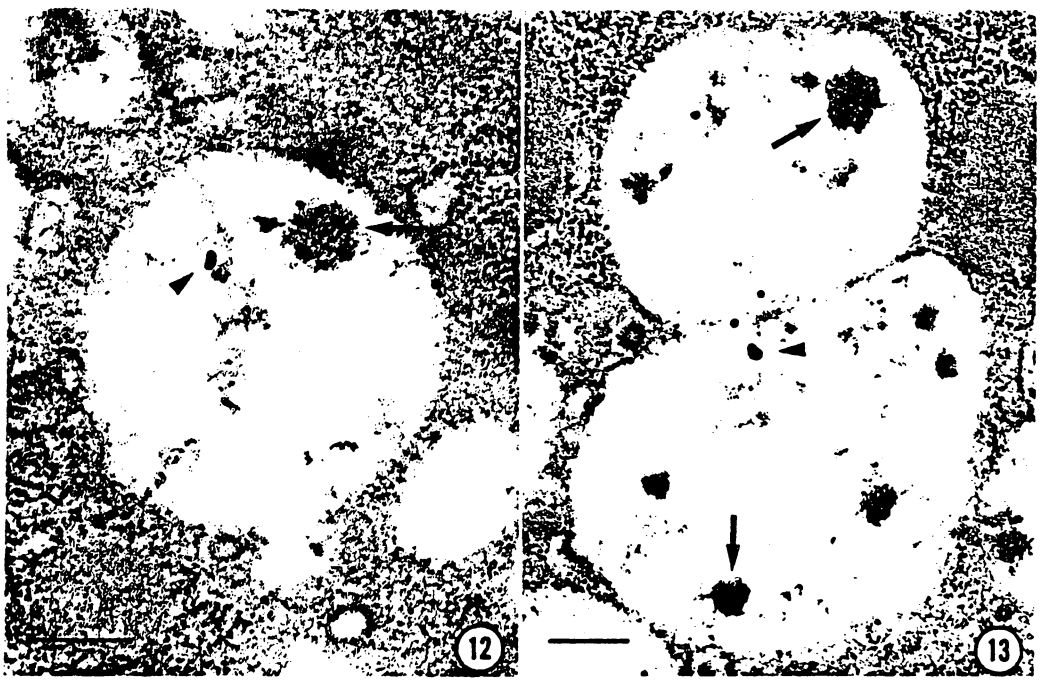
Antibody to α-lactalbumin recognized an antigen present in secretory vesicles characterized by the presence of casein micelles and in secretory vesicles which contained filamentous materials (Figs. 8 and 9). Anti-α-lactalbumin-gold conjugates were observed over areas within secretory vesicles which were not occupied by casein micelles or filamentous materials. These antibodies also were observed within lumina of Golgi apparatus cisternae (Fig. 10) and in areas of alveolar lumina (Fig. 11) which lacked morphologically distinct materials. When sections were incubated with anti-casein-25 nm gold-conjugated second antibody and then with anti-α-lactalbumin-50 nm gold-conjugated



Figs. 4 to 7. Sections incubated with anticasein antibody followed by second antibody-5 nm gold complex. Fig. 4, secretory vesicle (*V*) with gold particles over precondensed caseins. × 66,000, scale bar = 0.25 μm. Fig. 5, Secretory vesicle (*V*) with gold particles over casein micelles (arrows); cytoplasmic lipid droplet (*D*). × 66,000, scale bar = 0.25 μm. Fig. 6, Portion of Golgi apparatus dictyosome (*G*) with immunogold conjugates (arrows) present throughout cisternal lumina. × 58,000, scale bar = 0.25 μm. Fig. 7, Casein micelles (arrows) in alveolar lumen (*L*) labelled with gold particles. × 109,000, scale bar = 0.1 μm



Figs. 8 to 11. Sections incubated with anti-α-lactalbumin followed by second antibody—5 nm gold complex. Fig. 8, Secretory vesicle (*V*) with gold particles (arrows) over electron-translucent regions. × 98,000, scale bar = 0.1 μm. Fig. 9, Gold particles (arrows) over regions not occupied by condensed caseins in secretory vesicles (*V*). × 98,000, scale bar = 0.1 μm. Fig. 10, Golgi apparatus dictyosome (*G*) labelled with immunogold conjugates (arrows). × 61,000, scale bar = 0.25 μm. Fig. 11, Gold particles (arrows) present over electron-translucent regions of alveolar lumen (*L*). × 117,000, scale bar = 0.1 μm



Figs. 12 and 13. Sections double labellel with anti-casein-25 nm gold-conjugated second antibody and with anti-α-lactalbumin-protein A-50 nm gold. Secretory vesicles with 25 nm gold particles over casein micelles (arrow) and 50 nm gold particles (arrowheads) over electron-translucent regions. Fig. 12, × 66,000; Fig. 13, × 51,000, scale bars = 0.25 μm

protein A, both antibodies were localized over the same secretory vesicles (Figs. 12 and 13). Anti-casein antibody was present over micellar and filamentous materials while, within the same secretory vesicles anti-α-lactalbumin was present over electron lucent areas.

4. Discussion

Since micellar structures observed within secretory vesicles *in situ* are identical in ultrastructure to casein micelles of milk, it has been assumed that these intravesicular micelles are casein micelles (*e.g.*, DYLEWSKI and KEENAN 1983, FRANKE *et al.* 1976). That the micelles contained within secretory vesicles were recognized by antibodies to casein C4 confirms that caseins are constituents of these micelles. In species where this has been studied, all primary casein gene products are present within milk casein micelles (FARRELL 1978). While our immunocytochemical results were obtained with antibody to one major rat milk casein, we presume by analogy with other species that all primary casein gene products are present in micelles contained within secretory vesicles in rat mammary epithelial cells. Specific staining of granular/fibrillar materials with anti-casein, within lumina of Golgi apparatus cisternae and within secretory vesicles, confirms the casein nature of these materials and supports the contention that this material may be premicellar caseins (FARRELL 1978). All secretory vesicles observed in sections incubated with anti-casein and gold-conjugated second antibody contained gold particles, irrespective of whether or not casein micelles were present. This suggests that most or all secretory vesicles contain caseins.

α-Lactalbumin interacts with a galactosyltransferase responsible for the synthesis of lactose; this protein is necessary for synthesis of lactose at meaningful rates (EBNER and SCHANBACHER 1974). As would be anticipated from the Golgi apparatus localization of the galactosyltransferase (KEENAN *et al.* 1970), antibodies to α-lactalbumin revealed the presence of this protein in lumina of Golgi apparatus cisternae. These antibodies also showed α-lactalbumin to be present in secretory vesicles which contained micelles or fibrillar material

recognized by an antibody to casein C4. Identical observations were made with polyclonal antibodies prepared as described in Methods and with monoclonal antibodies to α-lactalbumin and casein component C4 (not shown) (KAETZEL and RAY 1984). As with anticasein antibody, all secretory vesicles observed in sections incubated with anti-α-lactalbumin-gold conjugate were marked by gold particles. These observations cannot exclude the possibility that primary casein and whey protein transcription products other than those recognized by the described antibodies are present in separate populations of secretory vesicles. However, these observations show that a major whey protein of rat milk is present in casein-containing secretory vesicles and suggest that a single population of secretory vesicles, qualitatively homogeneous with respect to secretory protein components, may exist in rat mammary epithelial cells.

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