well, our studies extend previous work in that freeze-fracturing has provided a visual representation of the extent of exocytosis by stimulated cells. Finally, under conditions known to inhibit the secretion of catecholamines, namely the absence of calcium (*5*), we have shown that freeze-fracture profiles of exocytosis sites are infrequent.

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12. Supported by PHS research grant HL-15691, NSF grant GB-12117X, the John A. Hartford Foundation, and the Council for Tobacco Research. U.S. is an established investigator of the American Heart Association. H.W. is a visiting scientist from the University of Innsbruck, Austria, on a Max Kade fellowship.

30 October 1972

Insulin Release by Emiocytosis: Demonstration with Freeze-Etching Technique

Abstract. *The technique of freeze-etching for electron microscopy applied to isolated islets of Langerhans has permitted a successful evaluation of emiocytotic events on the cell surface. The frequency of these events in stimulated cells suggests that emiocytosis represents a significant mechanism for insulin release.*

It is well established that specific membrane-bound cytoplasmic granules of the pancreatic B cell represent the main storage form of insulin within the gland (*1*), but doubts remain about the structural basis of insulin release. Lacy and Hartroft (*2*) suggested that beta granules were released by emiocytosis (or exocytosis), a process which implies the extrusion of the granule core through an opening created by the coalescence of the granule-limiting membrane with the cell membrane. Although unequivocal evidence of such a process is available in the literature (*3, 4*) (see also Fig. 1E), conventional electron microscopy has so far failed to demonstrate a clear correlation between the frequency of emiocytotic events and the measured insulin release. Thus, the view of Lacy and Hartroft was challenged by several authors, who advocated alternative routes of insulin release (*3, 5*).

To investigate the importance of emiocytosis in stimulated insulin release, we made use of the freeze-etching technique (*6*), which yields surface views of large areas of cell membranes and thus facilitates the analysis of events, such as emiocytosis, occurring at the plasma membrane.

Batches of islets were isolated from the pancreases of fed rats by collagenase digestion (*7*). They were incubated for 15 minutes to 1 hour at 37°C in a bicarbonate-buffered medium containing albumin (0.5 percent, weight to volume) equilibrated against an atmosphere of 95 percent O_2 and 5 percent CO_2. Control islets were incubated in the absence of glucose or in the absence of calcium, two conditions in which no sustained release of insulin occurs (*8*). Glucose alone (3.0 mg/ml), or a combination of glucose (3.0 mg/ml), theophylline (1.4 m*M*), and K^+ (20 milliequivalents per liter) were incorporated into the incubation medium of the "stimulated islets." Elevated concentrations of K^+ and theophylline have been shown to potentiate the insulin-releasing action of glucose in this system (*8, 9*). At the end of incubation, the islets were centrifuged into a pellet, fixed in 2 percent glutaraldehyde in phosphate buffer (0.1*M*, *p*H 7.2), and then soaked for 1 to 2 hours in a 20 percent solution of glycerol, also buffered with phosphate. The pellets were frozen by immersion in Freon (*10*), cooled to −150°C with liquid nitrogen, then freeze-etched according to the method of Moor and Mühlethaler (*11*) in a Balzers freeze-etching device. The fracturing temperature was −100°C and the etching time 1 minute. Platinum-carbon replicas were washed in sodium hypochlorite to remove adherent organic material, then in distilled water, and recovered on 200-mesh copper grids. The replicas were examined in a Philips EM 300 electron microscope. We have examined the central part of the islets, which consists mostly of B cells in the rat (*12*).

The freeze-etching procedure results either in a fracture through the cell cytoplasm, revealing the intracellular organelles, or in a split occurring within the cell membranes. The splitting of the plasma membranes (*13*) exposes two complementary fracture faces, the A-face oriented toward the extracellular compartment and the B-face oriented toward the cytoplasm (*14*).

The photographs in Fig. 1, A and B, show extended areas of plasma membranes in replicas of unstimulated and stimulated islet cells, respectively. In both situations, a number of depressions of various sizes are present on the exposed A-faces. The topographical relationship between these depressions and secretory granules are evidenced in replicas where the cleavage plane changes from following the cell membrane to pass through the cytoplasm. Since a continuity of the plasma membrane with the inner surface of the granule membrane was observed at the level of the largest depressions (1500 to 2000 Å in diameter; Fig. 1, C and D), we believe that the latter features represent emiocytotic stomata. It seems also that the membranes of several granules might fuse together within the cell, their content being discharged together through the same aperture (Fig. 1D). The small depressions, or pits (300 to 500 Å in diameter), observed together with the stomata on membrane faces probably correspond to the openings of microvesicles, either pinocytotic or exocytotic in nature (Fig. 1F).

Although the number and distribution of these features varied to some extent from one cell to another and within different areas of the same cell, pits and stomata were markedly increased in number in stimulated B

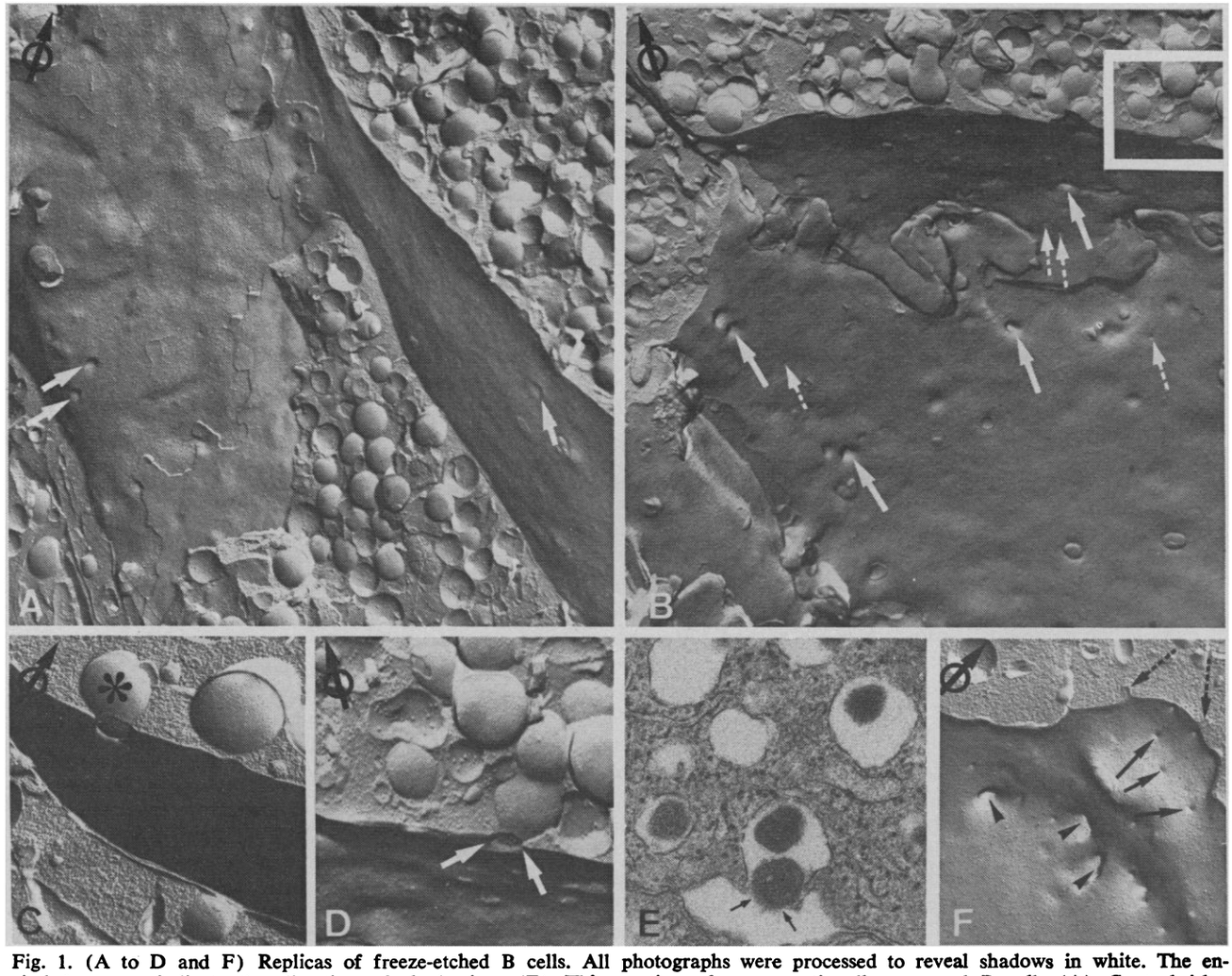

Fig. 1. (A to D and F) Replicas of freeze-etched B cells. All photographs were processed to reveal shadows in white. The encircled arrows indicate the direction of shadowing. (E) Thin section of a conventionally prepared B cell. (A) Control islet. The exposed A-face of the cell membrane exhibits a few depressions or emiocytotic stomata, indicated by white arrows. The fracture plane through the cytoplasm reveals numerous globular profiles, most of them representing secretory granules (× 16,000). (B to F) Stimulated islets. (B) An increased number of depressions or emiocytotic stomata (solid arrows) appear on the A-face of the cell membrane. The dashed arrows point to pits. The relationship of a stoma to the opening of a secretory granule is seen in the area outlined by the framed square (see high magnification in D) (× 17,000). (C) The asterisk indicates a secretory granule connected to the extracellular space by a stoma in the plasma membrane (× 25,000). (D) Two secretory granules open through the same stoma, indicated by the converging arrows (× 33,000). (E) Two granule cores are exposed to the extracellular space through the same stoma, indicated by the converging arrows; compare with D (× 33,000). (F) Plasmalemmal pits are illustrated facing forward (solid arrows) as well as in cross fracture (dashed arrows). The arrowheads indicate emiocytotic stomata (× 25,000).

cells (Fig. 1B), as compared to resting B cells (Fig. 1A). Preliminary results indicate that the number of emiocytotic stomata increased significantly ($P < .005$) from 18.8 ± 3.5 per 100 μm^2 in the control islets to 32.7 ± 4.1 per 100 μm^2 in islets stimulated by glucose alone and to 39.0 ± 7.5 per 100 μm^2 in islets stimulated by the combination of glucose, elevated K^+, and theophylline.

The findings reported here suggest that the conflicting opinions about emiocytosis probably result from the sampling limitations of conventional electron microscopy. Indeed, direct evidence for emiocytotic release in a thin section is obtained only if its plane goes through an emiocytotic aperture. Since such apertures may have a small diameter, the probability of seeing in a single section a granule undergoing emiocytosis is small. This could account for the past failure to demonstrate a close relationship between emiocytosis and measured insulin release. On the contrary, emiocytotic events are easily counted in freeze-etched replicas since these yield large areas of membrane for examination.

We have been able to demonstrate by the freeze-etching technique that emiocytotic events in B cells are increased by conditions which increase insulin release. The evidence given does not, however, rule out the possibility of concomitant release by other mechanisms.

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15. Supported in part by grants 3.299.70, 3.553.71, and 3.384.70 SR from Fonds National Suisse de la Recherche Scientifique, and by a grant-in-aid from Hoechst-Pharmaceuticals, Frankfurt-Hoechst, Germany. F.M.-L. is on leave of absence from the Department of Pathology, University of Brussels, Brussels, Belgium. We thank J. G. Schofield and A. Perrelet for suggestions; and M. Sidler-Ansermet and M. Bernard for technical assistance.

17 August 1972

Freeze-Fractured Acholeplasma laidlawii Membranes: Nature of Particles Observed

Abstract. *Freeze-fracture of* Acholeplasma laidlawii *membranes from cells incubated in the presence of puromycin or omission of amino acids reveals a decrease in the number of particles between 50 and 100 angstroms in the hydrophobic fracture plane, which strongly suggests that these particles are protein. Additional evidence indicates that they may be involved in substrate transport.*

The freeze-fracture technique in conjunction with electron microscopy has been a unique tool for studying membrane structure. Although there has been considerable controversy (*1*) as to where the primary fracture plane in biological membranes occurs, it has been convincingly demonstrated that the major fracture plane in membranes is down the center of the hydrophobic portion of the lipid bilayer (*2*).

A most interesting structural feature revealed in these preparations is the presence of particles between 50 and 100 Å in the hydrophobic region of the fracture plane. The precise chemical nature and function of these globular intercalations, which interrupt the continuity of the membrane's lipid bilayer, has been the subject of conjecture. That these interruptions are more abundant in membranes with high metabolic activity, such as chloroplasts, bacteria, and mitochondria, and are absent in the more metabolically inert biological membranes such as myelin and in synthetic liposomes (*1*) suggests that they may play a dynamic role in membrane function.

Digestion of red blood cell membranes (*1*, *3*) by the broad spectrum proteolytic enzyme, Pronase, for 10 hours results in almost total loss of these particles in fracture faces, which suggests that they are protein. Similar digestion of *Acholeplasma laidlawii* membranes has presented inconsistent results; that is, a moderate reduction in the number of particles was observed in some experiments and little or no reduction in others. Thus, the nature of these particles in *A. laidlawii* in regard to Pronase digestion is uncertain. In addition, since Pronase has been reported to exhibit esterase activity (*4*) the possibility that particles are lipid cannot be ruled out.

In this report we present evidence, obtained by a different approach, suggesting that these particles in *A. laidlawii* are protein in nature and that at least some of them may be involved in substrate transport across the cell membrane.

Acholeplasma laidlawii cells were grown in lipid-poor medium supplemented with oleic acid (30 mg/liter) (*5*). At 16 hours of growth, puromycin (25 μg/ml) was added to a portion of the growing cells, and [^{14}C]glutamine and [^{3}H]palmitic acid were added to monitor protein and lipid synthesis. Controls consisted of portions of the growing cells containing radioactive glutamine and palmitic acid to which no antibiotic was added. Samples were taken at 2-hour intervals, and the membranes were prepared (*5*) and freeze-fractured in a Balzers freeze-etching device.

Isotope counting was accomplished by using a Packard Tri-Carb liquid scintillation spectrometer. Isopycnic density gradient analyses were done as described by Kahane and Razin (*6*). Incorporation of ^{14}C-labeled thymidine, uridine, and glucosamine was monitored on separate portions of the same batch of cells used for the experiments described above. Active transport of the nonmetabolizable sugar [^{14}C]2-deoxyglucose was followed by methods described by Zupnik and Tourtellotte (*7*), by using cells treated as described above with the omission of radioactive glutamine and palmitic acid. The rates were determined as counts per minute per milligram of membrane lipid.

For experiments demonstrating lipid synthesis in a medium devoid of amino acids, cells were washed three times with the following buffer (buffer K): 0.1*M* tris(hydroxymethyl)aminomethane (tris), 0.2*M* KCl, 0.01*M* $MgCl_2$,

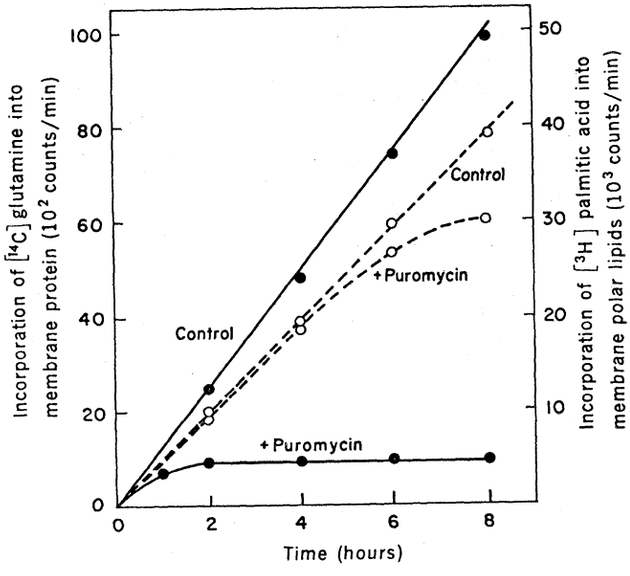

Fig. 1. Effect of puromycin on membrane lipid and protein biosynthesis in *Acholeplasma laidlawii*. (Open circles) Polar lipid synthesis; (filled circles) protein synthesis. The controls contained no puromycin.