The sequence -Asn-Phe-Phe-Trp-Lys- was confirmed in an acetylated, permethylated tryptic digest of SRIF by direct mass spectrometry (*10*).

The linear tetradecapeptide was synthesized by solid-phase methodology and purified by gel filtration in presence of 2-mercaptoethanol (*10*). After purification, the synthetic peptide had the biological activity of the native SRIF (Tables 1 and 2); at concentrations $\geqslant 1$ n*M*, native or synthetic SRIF inhibits the secretion of growth hormone from monolayer cultures of dispersed cells of rat adenohypophysis. In one experiment, native SRIF, at a concentration of 20 n*M*, inhibited significantly the spontaneous secretion of growth hormone by enzymatically dispersed cells derived from the pituitary gland of a patient with confirmed active acromegaly (*13*). The biological results with native and synthetic SRIF do not help to resolve the question of the reduced or oxidized state of the Cys residues in the peptide when it is recognized by the pituitary receptors; both the isolation procedure and the conditions that are used in the bioassays might convert one form into the other.

With the exception of primates, no simple adequate laboratory animal model seems to exist, which would exactly duplicate in vivo what is known of the physiological regulation of the secretion of growth hormone in humans. However, we found that the crude hypothalamic extract was able to inhibit (Table 2) the elevation in the plasma of growth hormone, as determined by radioimmunoassay, induced in rats by intravenous injection of sodium pentobarbital (*14*). This effect is specific for the hypothalamic extract as it is not duplicated by similar extracts of sheep cerebellum. Synthetic SRIF inhibits the secretion of growth hormone in similarly prepared assay rats (Table 2).

Native or synthetic SRIF has no effect on the basal secretion of LH or FSH (follicle stimulating hormone) in vitro at concentrations at which it inhibits maximally the secretion of somatotropin.

The peptide SRIF has been isolated and characterized with the use of an in vitro assay method of considerable reliability (*6*); the effects observed in vivo proceed from a method the rationale of which is less clearly established. Thus a physiological role for SRIF remains to be demonstrated. Should SRIF be active in humans (*15*), its possible clinical significance, particularly in the treatment of acromegaly and in the management of juvenile diabetes, has not escaped our attention.

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12. Abbreviations for amino acids are Ala, alanine; Asn, asparagine; Cys, cysteine; Gly, glycine; Lys, lysine, Phe, phenylalanine; Ser, serine; Thr, threonine; Trp, tryptophan; Asp, aspartic acid.
13. Radioimmunoassay for human growth hormone, by courtesy of Drs. S. Yen and T. Siler, University of California School of Medicine, San Diego. Fragments of a human acromegalic pituitary were obtained at time of surgical hypophysectomy, courtesy of Drs. Levin, Wilson, and Aubert, University of California School of Medicine, San Francisco.
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15. We propose to name the peptide described here *somatostatin*, from somato(tropin), a pituitary factor affecting statural growth, and stat(in), from the Latin "to halt, to arrest" (as in hemostat and bacteriostatic). This is in keeping with the efforts of several international nomenclature committees (with which the final decision should remain) aiming at creating trivial names for biologically active polypeptides rather than maintaining the use of acronyms.
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Exocytosis in the Adrenal Medulla Demonstrated by Freeze-Etching

Abstract. *Replicas of fractured chromaffin cells are indicative of a range of activities thought to characterize exocytosis, including attachment of secretory vesicles to the plasma membrane, fusion, extrusion of contents, and membrane retrieval. Exocytosis sites are abundant on stimulated cells but are infrequent when calcium is omitted from the system.*

There is now abundant evidence that secretion from exocrine, endocrine, and nervous tissue occurs by a process termed exocytosis [for reviews see (*1*)]. In exocytosis, it appears that the secretory vesicle fuses with the plasma membrane, creating a stoma which allows passage of the vesicle contents to the extracellular space. Biochemical studies of the adrenal medulla provide strong support for the concept, having demonstrated that the total soluble contents of chromaffin cells are secreted without concomitant release of cytoplasmic constituents (*2*). As well, profiles indicative of exocytosis have been shown by electron microscopy of thin sections (*3, 4*). However, difficulties are encountered in providing morphological evidence for exocytosis, probably owing to the infrequency with which the point of fusion of vesicle and plasma membrane coincides with the plane of a thin section. As a consequence, studies of thin sections cannot provide a visual representation of the extent of exocytosis in the adrenal medulla, a surface phenomenon resulting in the massive release of catecholamines. In the study reported here, we overcame this difficulty by applying freeze-fracture techniques to expose large areas of the plasma membrane of chromaffin cells; we used both stimulated and unstimulated adrenal glands.

Golden hamsters (weighing approxi-

mately 150 g) were anesthetized with intraperitoneal injections of chloral hydrate (300 mg per kilogram of body weight). The chest was opened, heparin (1000 units in 1 ml) was injected into the left ventricle, and a cannula was placed into the descending portion of the aortic arch. A cut was made in the right atrium to drain blood and to monitor the perfusion. Tyrode's solution (137 m*M* NaCl, 2.7 m*M* KCl, 1.8 m*M* $CaCl_2$, 0.32 m*M* NaH_2PO_4, 0.001 m*M* $MgCl_2$, 5.56 m*M* glucose, 11.6 m*M* $NaHCO_3$, *p*H 7.2), aerated with a mixture of O_2 and CO_2 (95 : 5) and maintained at 37°C, was perfused for 6 to 10 minutes at 6 ml/min. In some experiments, calcium [an ion required for the release of catecholamines (*5*)] was omitted from the Tyrode's solution. As soon as the effluent from the right atrium was free of blood, the perfusion solution was changed to glutaraldehyde (2.5 percent in 0.05*M* cacodylate buffer, *p*H 7.4, containing 0.17*M* sucrose), or a stimulatory agent was introduced into the Tyrode's line. The stimulatory agent (carbachol, 2.8 mg/ml; angiotensin II, 10 μg/ml; or bradykinin, 10 μg/ml) was perfused for 1 minute with Tyrode's solution and then for up to 6 minutes with the glutaraldehyde solution. In all experiments, tissues were fixed by perfusion of the glutaraldehyde solution for 25 minutes. The adrenal glands were then removed, and fixation was continued in vitro overnight. Subsequently, the medulla was dissected from the cortex and either put into 30 percent glycerol for freeze-fracturing (*6*) or put into cacodylate buffer wash and prepared for thin sectioning (*7*).

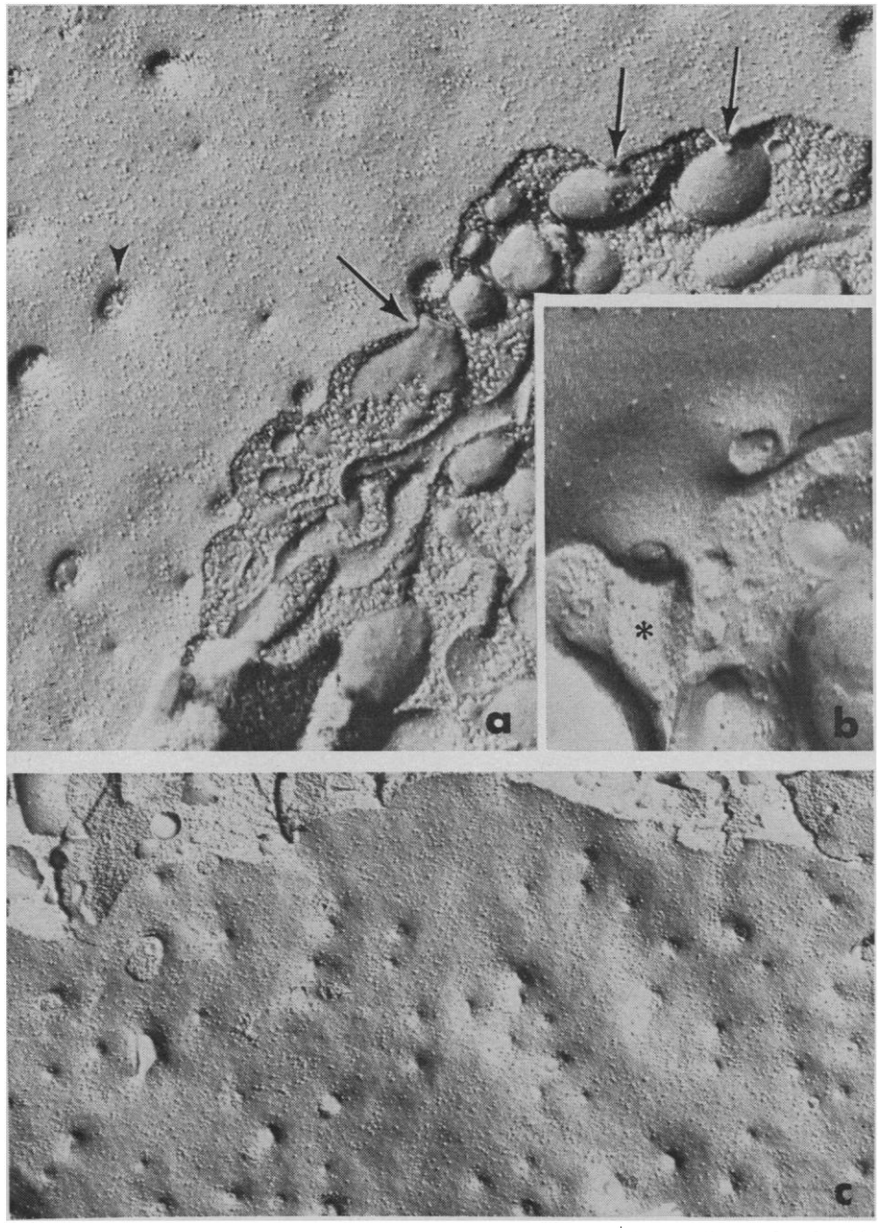

Fig. 1. (a) A chromaffin cell of the golden hamster adrenal medulla perfused with angiotensin II (10 μg/ml). The fracture exposes the cytoplasmic contents and a large extent of the extracellular aspect of the inner leaflet (IFF) of the plasma membrane. One of the most striking features of this leaflet of the plasma membrane is the large number of indentations, which take the form of wide pits and small depressed plaques. In three places (arrows) the fracture coincides with a point of attachment between a vesicle and the plasma membrane. These points may correspond to the small depressions noted above. The rough material (arrowhead) in wider pits may be the contents of the vesicles in the process of extrusion (see also Fig. 2d) (× 55,000). (b) Replica of the cytoplasmic aspect of the outer leaflet (OFF) of the plasma membrane of a chromaffin cell. This aspect reveals necks of vesicles fused with the plasma membrane (•) and from which vesicles have been detached during fracturing. The gland was perfused with carbachol (2.8 mg/ml) (× 85,000). (c) Replica of the extracellular aspect of the inner leaflet (IFF) from a chromaffin cell of a gland perfused with bradykinin (10 μg/ml). Note the abundance of exocytosis sites (× 40,000).

Chromaffin cells are readily identified by the large numbers of secretory vesicles (catecholamine-storing vesicles, chromaffin "granules") exposed by fractures through the cytoplasm (Fig. 1a) (*8*). The vesicles are about 2000 Å in diameter and their membranes are studded with particles 85 to 100 Å in diameter. Fractures within the plane of the plasma membrane reveal either the extracellular aspect of the inner leaflet (inner fracture face, IFF) or the cytoplasmic aspect of the outer leaflet (outer fracture face, OFF) (*9*), demonstrated by Fig. 1, a and b, respectively.

Medullary tissue perfused with the complete Tyrode's solution, alone or with stimulatory agents (*10*), shows areas of the plasma membrane which we interpret to be points of exocytosis. The abundance of such sites is illustrated in Fig. 1c. We suggest that the variation in size of the pits and indentations may represent the various stages of exocytosis. The small plaques are sites of attachment of the vesicles with the plasmalemma (Fig. 1a, arrows), while the wider, deeper pits may represent fusion; some of the wide pits contain plugs, which may depict vesicle contents in extrusion (Fig. 1a, arrowhead, and Fig. 2d).

The cytoplasmic aspect of the outer leaflet (OFF) provides the complementary view (Fig. 1b). Here, the necks

of fused vesicles are evident both where part of a vesicle is still attached and where the vesicle has been avulsed. When Fig. 1b is compared with Fig. 1, a and c, it appears that fewer particles adhere to the outer fracture face than to the inner.

Figure 1c is a survey at low magnification of a replica obtained from an adrenal gland perfused with bradykinin, and indicates the vast numbers of exocytosis sites encountered in stimulated medullas. Corresponding activity was observed in material from adrenals stimulated with angiotensin II and carbachol. In sharp contrast, Fig. 2a shows the appearance of the cytoplasmic aspect of the outer leaflet (OFF) of the plasma membrane from an adrenal gland perfused with calcium-free Tyrode's solution. Previous biochemical studies have shown that little or no secretion of catecholamines occurs in the absence of calcium [for a review see (*5*)]. Although occasional exocytosis sites could be found, material treated in this way was remarkable for the extensive sheets of smooth fracture faces covered by randomly distributed globular particles. Although intramembranous particles have been correlated with physiologic activity (*11*), their abundance and organization do not appear in these experiments to be affected by calcium. Also, as shown in Fig. 2a, the secretory vesicles within the cytoplasm of the cell appear normal.

Details of fused vesicles are shown at higher magnification in Fig. 2, b, d, and e. Figure 2c appears to be a coated pit, possibly involved in the retrieval of the membrane of a secretory vesicle (*4*). Figure 2b shows the outer fracture face of a fused vesicle. Figure 2d illustrates a pit in the inner fracture face. The appearance here is of a plug of material, possibly the vesicle contents during extrusion, the plane of fracture having traversed the neck of the vesicle (compare Fig. 1a, arrowhead). Figure 2e shows a fused secretory vesicle with small vesicles arising from it. This may represent a mechanism of membrane retrieval and has been illustrated in thin sections (*4*). A nerve terminal close to the chromaffin cell is replicated to the left of the field.

In summary, results of our freeze-fracture investigations complement previous studies of thin sections (*3*, *4*), evidence having been found of attachment of vesicles to the plasma membrane, fusion, extrusion of contents, and retrieval of the vesicle membrane. As

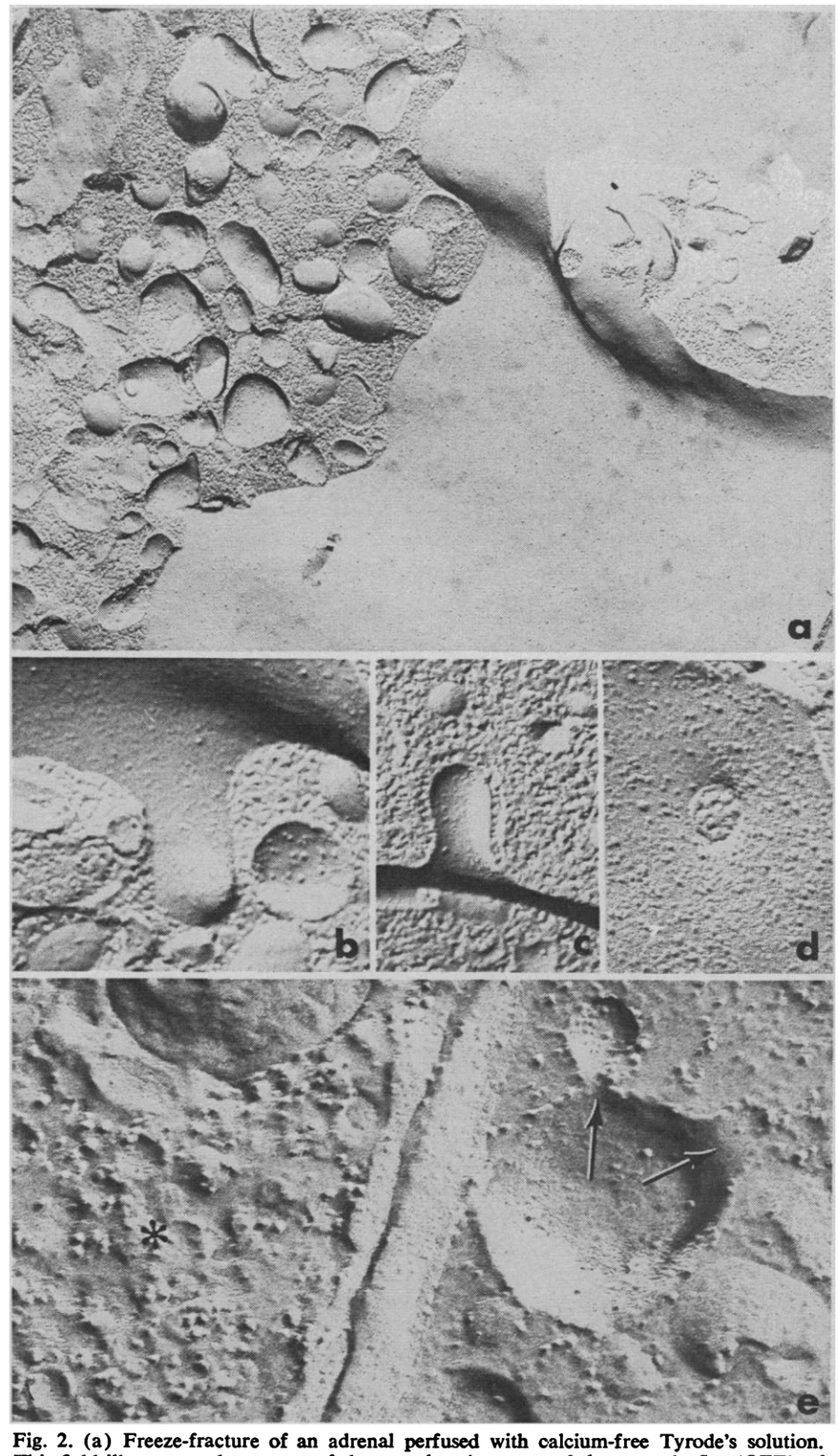

Fig. 2. (a) Freeze-fracture of an adrenal perfused with calcium-free Tyrode's solution. This field illustrates a large area of the cytoplasmic aspect of the outer leaflet (OFF) of the plasma membrane with the normal array of randomly scattered particles, but without signs of exocytosis (× 30,000). (b) The outer fracture face of a chromaffin cell. The plane of fracture appears to follow the contours of the vesicle, exposing a dome continuous with the cytoplasmic aspect of the outer leaflet of the plasma membrane (× 80,000). (c) Replica of the extracellular aspect of the inner leaflet (IFF) of a vesicle. The regular coating around the periphery suggests that this may be a coated pit (× 80,000). (d) Detail similar to that seen in Fig. 1a, which may show the contents of the vesicle where the fracture has traversed the neck of a fused vesicle (× 80,000). (e) Replica of a vesicle after fusion with the plasma membrane. Note the small vesicles (arrows) arising from the original vesicle membrane (see text). Part of a nerve ending is replicated to the left of the field (•) (× 130,000).

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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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