Hormonal Regulation of Capillary Fenestrae in the Rat Adrenal Cortex: Quantitative Studies using Objective Lens Staging Scanning Electron Microscopy

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HORMONAL REGULATION OF CAPILLARY FENESTRAE IN THE RAT ADRENAL CORTEX:
QUANTITATIVE STUDIES USING OBJECTIVE LENS STAGING SCANNING ELECTRON MICROSCOPY

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Abstract

High magnification studies of the fenestrated capillary endothelium in the zona fasciculata (ZF) of rat adrenal glands were performed using the objective lens stage of an analytical scanning electron microscope (SEM) equipped with a lanthanum hexaboride emitter (LaB_6). Resolution of surface substructure of the luminal membrane obtained with specimens decorated with gold/palladium (Au/Pd) was compared with that observed in others sputter coated with tantalum (Ta). High magnification (50,000x) of the fenestrated endothelium demonstrates that tantalum coating of the cryofractured adrenals improves the substructural detail compared to that seen in Au/Pd decorated specimens.

The procedures used in specimen preparation, metal deposition and secondary electron imaging (SEI) are described. Quality imaging achieved using the objective lens stage is a result of the elimination of the SE-III component derived from backscattered electrons.

Rat adrenals exhibited uniformly patent capillaries. High magnification micrographs of capillary walls were randomly recorded in two morphometric studies of the fenestral content of capillaries in the rat adrenal cortex.

Adrenocorticotropic hormone (ACTH), when administered to rats following dexamethasone (DEX) treatment, significantly reduced the fenestrae/μm^2 of endothelial surface and increased the mean size of fenestrae. After hypophysectomy, the number of fenestrae/μm^2 declined over 48 h; within 2 h after ACTH was given to rats hypophysectomized 48 hours earlier, the fenestrae/μm^2 had increased two-fold. These studies indicate that ACTH plays an important role in modulating fenestral content of the capillary endothelium in the adrenal cortex.

KEY WORDS: Adrenal cryofracture preparation, SE-I and SE-II contrasts, gold/palladium decoration, tantalum coating, fenestral morphometry, adrenocorticotropic hormone.

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Introduction

It has been demonstrated that high quality imaging of macromolecular membrane structures with the Scanning Electron Microscope (SEM) on metal coated biological tissue is possible by generating a type I high resolution contrast (SE-I) and recording it at high magnification [15, 17]. In order to obtain the topographic contrast of small features which are exclusively imaged by SE-I and II components, the SE-III component of the signal must be eliminated or greatly reduced [16]. Peters has demonstrated that the SE-III contribution to the SEI can be eliminated by shielding the objective lens pole-piece of a field emission SEM with an electron absorption device [14]. In the present study, the objective lens specimen stage in an analytical SEM using only the upper detector created a signal collection condition which effectively eliminated the SE-III component.

Surface information and contrast, directly dependent on the metal film thickness, structure, and decoration effects, are sufficiently improved in new analytical SEMs if they are equipped with high brightness electron guns and operated in the high resolution imaging mode [17].

Penning sputtering of 1-2 nm ultrathin films of chromium (Cr) or tantalum (Ta) produce a higher SE-I/SE-II signal (enriched SE-I signal) and creates the best topographic contrast of small features (<10 nm) such as fenestral substructure [13]. In the present study, conventional sputtering equipment, considerably less expensive than that employed by Peters, has been used to deposit discontinuous films of Au/Pd to decorate fenestrae and continuous Ta films to enhance the topographic contrast of fenestrae [6, 17].

Recent SEM studies of liver, kidney, ovary and adrenal tissues, employing perfusion fixation, have demonstrated that excellent preservation of the histological organization and the integrity of fine vascular components such as capillary walls is achieved when combined with cryofracture or similar techniques [1-4, 5, 11, 15, 21, 22]. The large numbers of capillary luminal surfaces exposed in radially cryofractured adrenals facilitate the random selection of

these vascular elements at low magnification for subsequent morphometric studies of their fenestral content at high magnification [3, 4, 5, 21].

The tropic hormone, ACTH, exerts two primary regulatory effects upon the adrenal cortex [7]. It stimulates the production of glucocorticoids by cells in the inner cortex. This steroidogenic response of adrenocortical cells is a rapid one, resulting in elevated plasma levels of glucocorticoids within 5-10 minutes after ACTH is administered [7]. Continuous secretion of low concentrations of ACTH by the anterior pituitary is required for the maintenance of normal functioning by these adrenocortical cells and chronic administration of ACTH stimulates growth of the adrenal cortex [7]. When the pituitary gland is surgically removed (hypophysectomy), a rapid atrophying of the adrenal cortex ensues. Although these effects of ACTH on the adrenal cortex are well known, there is little information about a possible role of ACTH in influencing the physiological activity and morphology of the endothelial cells which form the thin, fenestrated walls of the capillaries in the adrenal cortex.

Two preliminary morphometric studies of the effects of ACTH on the ultrastructure of these endothelial cells, as revealed by high resolution scanning electron microscopy, are described in the present paper. In the first study, changes in the fenestral content of the capillary endothelium in the rat adrenal 20 minutes after administering ACTH I.V. were determined using randomly recorded high magnification images of the luminal surfaces of the endothelium. The effects of ACTH on the size distribution and numbers of fenestrae/μm^2 were determined. In the second study, the effects of hypophysectomy and subsequent ACTH therapy upon the numbers of fenestrae/μm^2 and the topographic substructure of the endothelium were examined.

Materials and Methods

Hormone Treatment

Male Sprague-Dawley rats obtained from Charles River Breeding Laboratories, Wilmington, MA. weighing between 250 and 300 g, were used throughout these studies. In the first experiment, 12 rats received two dexamethasone (25 μg/100 g body wt.) I.V. injections, the first at 8 pm, the second at 8 am the next day. 2 h after the second injection, 6 of the rats received ACTH (100 ng/100 g body wt.); 6 rats, serving as controls, received 0.85% saline vehicle only. Flaxedil, a muscle relaxant, was given 15 minutes later (0.1 mg/100 g body wt.), followed by anesthetization with Nembutal, prior to fixation by 5 min vascular perfusion 20 min after the ACTH or saline injection. Seventeen hypophysectomized rats and three intact rats were used in the second experiment. The adrenal glands were fixed following the different treatments summarized in Table 1.

Tissue Preparation for Electron Microscopy

At designated times after hypophysectomy, each rat was anesthetized and its adrenals fixed by perfusion. The flushing solution and fixative were delivered by cannulation of the left ventricle of the heart with an 18-gauge syringe needle, using a Manostat peristaltic pump to maintain a constant flow rate of 85-90 ml/min. In the first experiment, the vasculature was flushed for 30 sec with heparinized phosphate buffered saline containing 4% sucrose, then perfused with the fixative containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. The adrenals were removed, defatted, and fixed in the same fixative overnight at 4°C. Each gland was razor-cut in half along its long axis, osmicated in 1% OsO_4 in 0.1 M cacodylate buffer at pH 7.4 for 45 minutes, and the adrenal halves were rinsed 3 times for 15 min in distilled H_2O prior to processing for SEM.

In the second experiment, the vascular system was first cleared by perfusing for 30 sec with lactate Ringer's solution containing 2% polyvinyl pyrrolidone (PVP), and heparin (1000 units/liter). Because this flushing solution, together with the replacement of sucrose with PVP in the fixative, resulted in improved preservation of microtubules in adrenal specimens prepared for TEM studies, these changes were made in the fixation procedures. The fixative was composed of 2% glutaraldehyde, 0.8% paraformaldehyde, 1% PVP, 0.05 M cacodylate buffer at pH 7.4, and heparin. After a 5 minute perfusion with fixative, the adrenals were excised, trimmed of fat, cut in half and left in the fixative overnight at 4°C. The specimen vials were randomly numbered to eliminate bias in the quantitative SEM study. Adrenal tissues were then rinsed with several changes of 2% PVP in 0.05 M cacodylate buffer. The PVP was removed from the samples by repeated rinsing in ice-cold 0.05 M cacodylate buffer over a 4 h period. The adrenal halves were then osmicated in 1% OsO_4 in the

Table 1
Hypophysectomy and ACTH Therapy

Group	Number of Rats	Duration of Hypophysectomy	Duration of ACTH Therapy Prior to Perfusion Fixation
A	3	none (intact controls)	---
B	1	8 h	none (saline vehicle only)
C	4	24 h	none (saline vehicle only)
D	4	48 h	none (saline vehicle only)
E	3	48 h	1 h ACTH
F	5	48 h	2 h ACTH

same buffer for 2 h, followed by three 15 min rinses in the buffer.

Adrenal halves from the DEX/ACTH study were dehydrated in a continuous gradient apparatus bringing the tissue from 0 to 100% ethanol, using a constant flow rate of 2 ml/min for 3 h at $4^{o}C$. Each adrenal half was individually wrapped in a packet of parafilm under ice-cold ethanol, frozen in Freon-22 slush ($-160^{o}C$) and placed in LN_2 [1, 8]. Individual packets were placed on the copper base of a LN_2 trough fitted to a modified Smith-Farquhar tissue chopper (Ivan Sorvall, Norwalk, CT). The length of the stroke of the blade was adjusted so that it would strike only the top of the adrenal packet during the cryofracturing. The resulting adrenal quarters were thawed in ice-cold absolute ethanol, loaded into a Polaron critical point dryer (CPD) and dried from CO_2 using the exchange method [12].

Osmicated specimens from the hypophysectomy/ACTH experiment were rinsed several times in distilled H_2O before being dehydrated and cryofractured [1, 8]. The quartered adrenals were thawed in an absolute ethanol-filled boat within the gradient apparatus and the fluid continuously exchanged with Freon 113 (TF) for 90 min at 3 ml/min. The Freon-113 filled boat was loaded into the CPD and dried from CO_2 by the exchange method [12]. Specimens in both studies were mounted cryofracture face-up (shiny surface) with silver paste (SPI Supplies) on aluminum stubs.

Metal Deposition

Metals were deposited onto mounted adrenals by low voltage sputtering with either an Emscope SC-500 or a Polaron E-5400 sputter coater unit. Gold-palladium targets, (Polaron 50 mm dia., 95:5; Emscope, 60 mm dia., 60:40) were used to deposit discontinuous films. Since measurements made with quartz film thickness monitors (FTM) on both units are subject to large errors (6x or greater), especially in those involving thin films of less than 10 nm, it is stressed that the film thicknesses given in this paper were estimated based on FTM readings and sputter conditions [6, 13]. This measurement system, with standardized voltage, current, pressure and target distance conditions provides a comparison from one deposition to another but does not provide a measure of absolute film thickness. Sputter chambers and target metals were etched with atmospheric gases for 5 minutes at a plasma current of 50 mA to remove contaminants. Specimens and filter paper were loaded onto the water cooled stage ($18^{o}C$) containing a centrally located quartz crystal and the chamber was evacuated to 5.3 Pa ($4x10^{-2}$ torr.). Target distance was 25 mm for the transverse magnetic shroud design of the Polaron cathode, and 15 mm for the high efficiency Emscope cathode. Chambers were purged with high purity Argon (Ar) at 26.6 Pa ($2x10^{-1}$ torr.) for 15 minutes, then re-evacuated to 5.3 Pa. Cathode voltages of 70 or 480 VDC were applied in the Emscope and Polaron coaters, respectively, to maintain a plasma current of 5 mA. FTM readings of 1 nm were usually obtained after applying these cathode voltages for 30-45 seconds. Chambers were then filled to atmosphere with Ar. Whatman filter paper placed at specimen height prior to deposition, under the conditions stated above, produced a very light gray interference color in both sputter units. Repetitive filter paper tests were made under conditions identical to those used to deposit metal on adrenals and the deposition colors on the filter papers were compared.

Tantalum foil (Alfa Products (00330) Danvers, MA.) was fitted into the shrouded Polaron cathode and a new glass chamber was used for Ta sputter-coating. The system was etched with atmospheric gas at 50 mA for 15 minutes prior to venting and loading of specimens and filter paper. Once the chamber had been evacuated to 4 Pa ($3x10^{-2}$ torr.) and purged with Ar at 26.6 Pa for 15 minutes, a cathode voltage of 535 VDC was applied and a 15 mA plasma current was maintained for 14 minutes. Upon termination of deposition, Ar was vented into the chamber and filled to atmospheric pressure. A light gray deposition appeared on the filter paper.

Microscopy and Quantitative Methodology

Specimens were placed in the upper stage of either an ISI SS-60 or an ISI DS-130 analytical SEM. Both instruments were operated at 25-30 kV with a tungsten source or at 20 kV with a LaB_6 source. A final aperture of 50 μm and the smallest possible spot size were used in both instruments in order to obtain a beam with the smallest diameter at these accelerating voltages. The SEM was calibrated using two magnification standards, a 1000-mesh gold cross grid and 1 μm latex spheres, observed at 1000 x and 10,000 x, respectively.

The zona fasciculata (ZF) was located on the cryofractured surface of each adrenal quarter at low magnification. Ten capillaries in the hypophysectomy/ACTH experiment, and four capillaries in the DEX/ACTH experiment, were randomly selected in each sample and photographed at a magnification of 50,000x on a CRT with 2500 lines using Polaroid type 55 film and an 80 second exposure. Negatives were enlarged to 100,000x in a Beseler MX-45 enlarger with a point light source. Micrographs (20.32 cm x 25.40 cm) were used for counting of fenestrae and measurement of their diameters. A 10 cm x 10 cm square, corresponding to 1 $μm^2$, was placed in the center of each micrograph and both the diameter of each fenestra and the total number of fenestrae within this area were determined in the DEX/ACTH study. The diameter of each fenestra was measured along the x and y axes to obtain the average of these values. The porosity of the endothelium was expressed as the ratio of the total area composed of fenestrae to the total area in $μm^2$. Since micrographs of four capillaries/adrenal, and six adrenals/treatment were utilized in the first study, data from 24 $μm^2$ of luminal surface area/treatment were compared using the student's t test. In the second study, ten capillaries at the cryofractured surfaces of each adrenal were randomly selected and photographed. The number of fenestrae/$μm^2$ was

determined for a total area of 10 μm^2/animal; a statistical analysis was made to determine if the fenestrae/μm^2 differed significantly between intact, hypophysectomized and ACTH treated, hypophysectomized rats.

Results

Histology of the Adrenal Cortex

Procedures used in this study to prepare cryofractured specimens from rat adrenals fixed by perfusion are similar to those described for the rat ovary, except that the adrenals were cut in half in order to facilitate postfixation in osmium tetroxide [1]. Cryofracturing of adrenal halves yielded large radial surfaces which extended from the outer capsule through the entire width of the cortex to the medulla (Fig. 1). To assure that micrographs were only taken of capillaries within the zona fasciculata, specimens were always positioned so that the area to be photographed was at least 150 μm from the capsule of the adrenal gland. The sinusoids within this zone were then photographed at high magnification for morphometric analysis. Numerous luminal surfaces of capillaries, fractured longitudinally, were found on the cryofractured face of each adrenal quarter (Fig. 2). The most prominent feature of these endothelial surfaces were the large areas containing numerous small fenestrae (Fig. 3). Microvilli were seen projecting from the adrenocortical cells into the perivascular space surrounding each capillary. The distortion-free appearance of this perivascular compartment and these fragile cellular projections reflects the quality of tissue preservation achieved by perfusion-fixation and cryofracturing [11].

Electron Sources and Signal Strength

Specimen imaging at high magnification was achieved using the objective lens stage in both the SS-60 and DS-130 SEMs and high magnification contrast was obtained by collecting only the SE-I and SE-II type signals using a detector located above the lens [10, 17]. Both instruments were operated with a standard tungsten (W) source before being outfitted with an ion getter pumped LaB_6 source. Contours of small features such as 30 nm fenestrae were greatly improved by the increased intensity of the beam and the decreased chromatic aberration of the electron probe emitted from the LaB_6 source. When this source was used, probe diameters were reduced from 3.5 to 2.5 nm in the SS-60, and from 3.0 to 2.0 nm in the DS-130 SEM, thus improving the resolving power of both instruments.

Small diaphragmed fenestrae (30-50 nm), decorated with a 3 nm discontinuous film of Au/Pd and observed on the SS-60 SEM operated at 30 kV with a W-source generated a weak high magnification signal and produced an image with poor topographic and substructural detail (Fig. 4). When identical metal decorated specimens displaying fenestrae of the same dimension were observed with a DS-130 SEM operated with a W-source at 25 kV, signal strength and topographic resolution were only slightly improved

(Fig. 5). Although operation of the SS-60 SEM at 20 kV with a LaB_6 source significantly improved signal strength, topographic contrast of small features less than 30 nm remained weak (Fig. 6).

Topographic Contrast and Metal Deposition

Although low voltage sputtering of 3 nm discontinuous Au/Pd films resulted in SE images of fenestrae with pronounced contours, substructural detail of the endothelial cell surfaces was lacking due to the high background noise contributed by the SE-II signal component (Figs. 4-6). These micrographs were soft focus printed
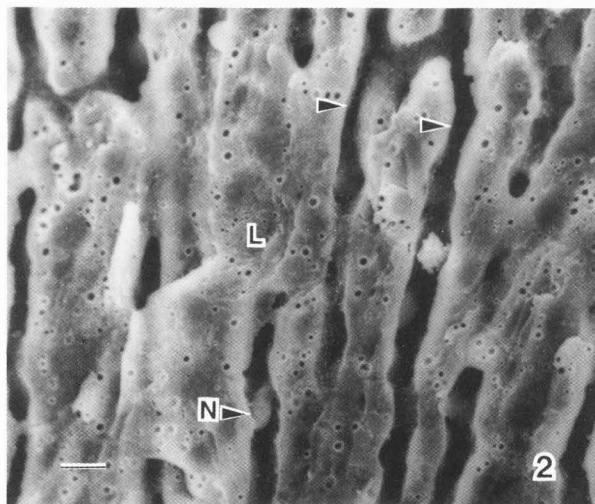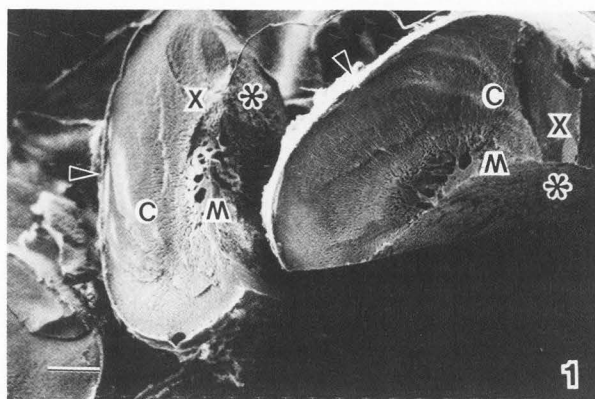

Fig. 1: Micrograph of two fractured adrenal quarters reveal both razor cut (*) and cryofractured (X) faces. Capsular (arrows) cortical (C) and medullary regions (M) are located within the smooth fracture face. Bar = 300 μm.

Fig. 2: Capillaries (arrows) traversing the zona fasiculata are randomly selected for fenestral analysis in micrographs at high magnification. Note the holes (L) which correspond to the lipid droplet content of steroidogenic cells. An endothelial cell nuclear (N) bulge is observed on the luminal surface. Bar = 10 μm.
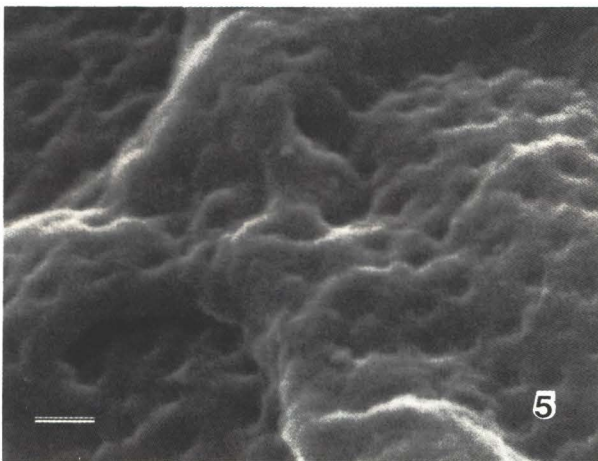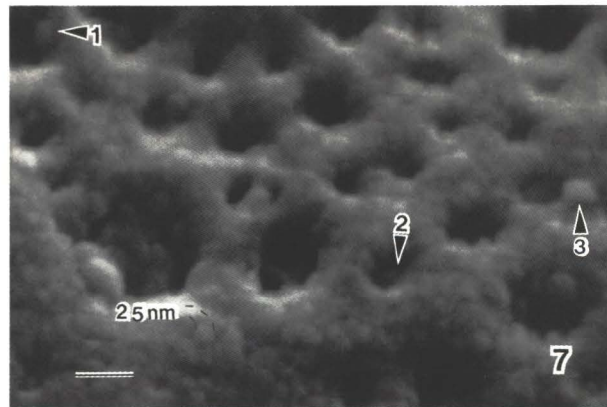
Fig. 4: High magnification micrograph of fenestrated endothelium taken on a SS-60 SEM operated with a W-source. Weak signal strength creates a SE image with poor S/N, but contrast contours the fenestral rim (arrows). From a 3 nm Au/Pd decorated adrenal gland. Bar = 100 nm.

Fig. 5: High magnification micrograph of a 3 nm Au/Pd decorated endothelial surface taken on a DS-130 SEM operated with a W-source. Comparable S/N with Fig. 4. Bar = 100 nm.

Fig. 6: A LaB$_6$ source on the SS-60 SEM improves the SE image of small fenestrae (F) decorated by a 3 nm Au/Pd deposition. Due to discontinuities in the film (arrows) a high background noise nearly obscures the central particle (P). Bar = 100 nm.

Fig. 7: A high resolution SE-I signal is generated from a continuous coat of Ta on the fenestrated endothelium. Topographic features on the membrane are blanketed with 5 nm of Ta, yet particle size may be determined (diameter ~2x metal coating thickness). Note particles in several orientations (1,2,3). Bar = 100 nm.
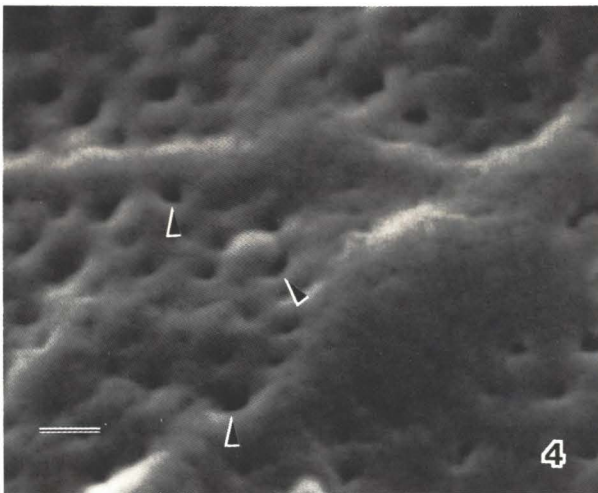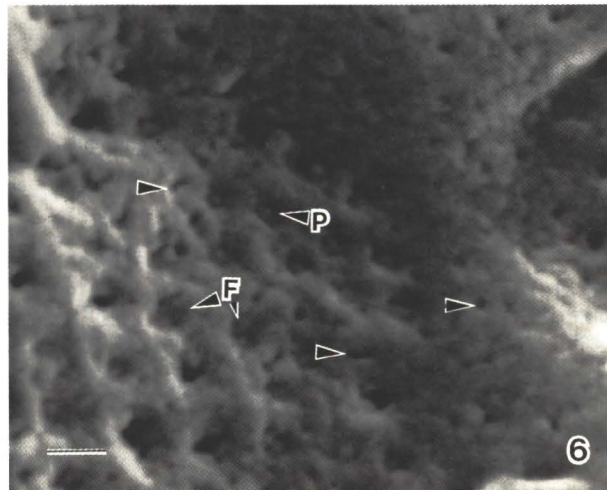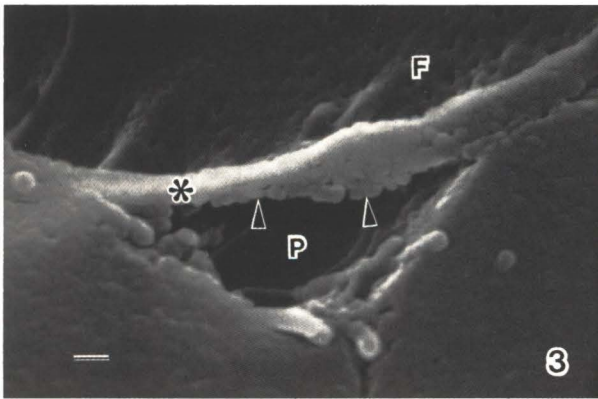
Fig. 3: A perivascular space (P) is formed by the abluminal wall (arrows) of a thin endothelial cell cytoplasmic expanse (*). Note the fenestrae (F) clustered on the luminal surface of the endothelium. Bar = 200 nm.

to eliminate high noise contrast [18].

Improvement in both topographic contrast of fenestrae and imaging of fine cell surface details was achieved by sputtering continuous 5 nm Ta films on the specimens, apparently due to a partial enrichment of the SE-I signal component. Discontinuities in the metal film, such as those created by deposition of 3 nm Au/Pd, known to generate background noise, are avoided by Ta coating. Excellent images with high signal to noise ratios (S/N) of the metal coated endothelial surface were routinely obtained at high magnification for quantitative studies. Subfenestral particles could be distinguished in several orientations and enhanced topography was evident. Soft focusing of SE-I micrographs enhanced detail recognition (Fig. 7). Membranous particles 10-20 nm in diameter were clearly coated with, but not obscured by the thin Ta film. Although imaging of surface structures 20-30 nm in diameter was achieved it is pointed out that these dimensions include the actual particle size plus twice the thickness of the metal film.

Endothelial Morphology and Fenestral Quantitation

Forty-eight capillaries from the DEX/ACTH study and two hundred capillaries from the HYPOX/ACTH study were photographed at high magnification. Upon examination at intermediate magnifications (15,000x), the capillary walls were found to be composed of numerous flattened cytoplasmic expanses filled with fenestrae. These areas correspond to the sieve plates which have been described in SEM studies of adrenal morphology [11]. Most of these fenestrae are covered by a thin membranous diaphragm, at the center of which is seen a granular particle. Bulges encountered at irregular intervals along the capillary walls correspond to the endothelial soma and their nuclear content. When more than 50% of a randomly selected area of the luminal surface of a capillary was occupied by a nuclear bulge, the area was not used for the quantitative study of fenestral content and another capillary surface was randomly selected. The distribution of clusters of fenestrae typical of the capillary endothelium within the zona fasciculata is shown in Fig. 8. The flat cytoplasmic sheets of two adjoining endothelial cells occasionally were observed at their junction, which appeared as an interdigitating and overlapping union (Fig. 9). Short microvillar-like structures and spherical bodies were observed projecting from the luminal surface of the endothelium (Fig. 10).

ACTH, when administered to rats previously given dexamethasone, significantly reduced the fenestral content of capillaries within the zona fasciculata from 33.08 ± 5.07 fenestrae/μm^2 in rats receiving saline vehicle to 15.58 ± 1.25 fenestrae/μm^2 in those given the hormone (p < 0.02). While a total of 374 fenestrae were counted in 24 μm^2 of luminal surface area of capillaries of adrenals from the six rats receiving ACTH, 794 fenestrae were found in the same surface area in adrenals of rats adminis-

tered vehicle only (Table 2). This reduction in fenestral content, however, did not result in a significant change in capillary porosity, increasing from 4.09% in DEX controls to 4.16% in ACTH treated rats. The diameters of fenestrae ranged from 20 to 150 nm. In rats receiving vehicle only, 87% of the fenestrae had diameters of 30-45 nm, whereas only 38% of the fenestrae in the adrenals of rats receiving ACTH fell within this size range. In contrast, 55% of the fenestrae in the latter group possessed diameters of 50-80 nm, compared to only 10.6% of those in adrenals of rats given saline vehicle (Fig. 11).

In the second study, the numbers of fenestrae/μm^2 of capillary luminal surface within the zona fasciculata of the adrenal declined steadily following hypophysectomy. Compared to the fenestral content of these capillaries in intact rats, their numbers decreased by 23%, 34%, and 69% by 8, 24, and 48 h after hypophysectomy, respectively. The large arrays of fenestrae typical of adrenocortical endothelium in intact rats were seen infrequently in rats hypophysectomized 48 h earlier. Only 12.45 ± 0.47 fenestrae/μm^2 were found at this time compared to 39.95 ± 0.74 fenestrae/μm^2 in the adrenals of intact rats (p < 0.001; Table 2). Cytoplasmic protrusions from the luminal surface of endothelial cells became more numerous with time after hypophysectomy. At 48 h, the luminal surfaces of the endothelium appeared corrugated (Fig. 12).

Two h after ACTH was administered to rats hypophysectomized 48 h earlier, the fenestral content had increased to 26.89 ± 1.21 fenestrae/μm^2 (Table 2). At this time, the flat areas exhibiting ordered arrays of fenestrae had inceased in size (Fig. 13). Fewer surface irregularities, often appearing as folds in the capillary wall, and reduced numbers of cytoplasmic projections into capillary lumina were observed 2 h after administering ACTH.

Discussion

Fixation by vascular perfusion when combined with various slicing or cryofracture methods, has been successfully applied in recent SEM studies of fenestrated capillary endothelia in several organ systems. These preparative techniques result in excellent preservation of fine structural details and provide large surface areas, which contain numerous profiles of capillary lumina in such highly vascularized organs as the liver, kidney, and adrenal [3, 4, 5, 11, 15, 21, 22]. Individual capillaries can be randomly selected at low magnification and then photographed at high magnification for morphometric analysis. Quantitative SEM studies employing these techniques were used to determine the diameters of sinusoids and fenestrae in order to assess the possible occurrence of lobular gradients in the rat liver [21, 22]. The nondiaphragmed fenestrae found in the liver sinusoids measure about 100 nm in diameter and provide an open connection to the space of Disse. Perfusion fixation of rat adrenals

Table 2

Morphometric Analysis of Fenestral Content of Capillaries in the
Zona Fasciculata of Rat Adrenals

A. Expt 1	Treatment	Total # of Fenestrae	Fenestrae/μm^2	Porosity
	(1) DEX/SALINE (6)	794	33.08 ± 5.07	4.09%
	(2) DEX/ACTH (6)	374	15.58 ± 1.25^1	4.16%

B. Expt 2	Treatment	Fenestrae/μm^2
	(1) Control (3)	39.95 ± 0.74
	(2) 8 h hypox (1)	30.7
	(3) 24 h hypox (4)	26.33 ± 2.59^1
	(4) 48 h hypox (4)	12.45 ± 0.48^2
	(5) 48 h hypox; 1 h ACTH (3)	13.63 ± 0.94
	(6) 48 h hypox; 2 h ACTH (5)	26.89 ± 1.21^3

1. p < 0.02
2. Control vs 48 h hypophysectomy: p < 0.001
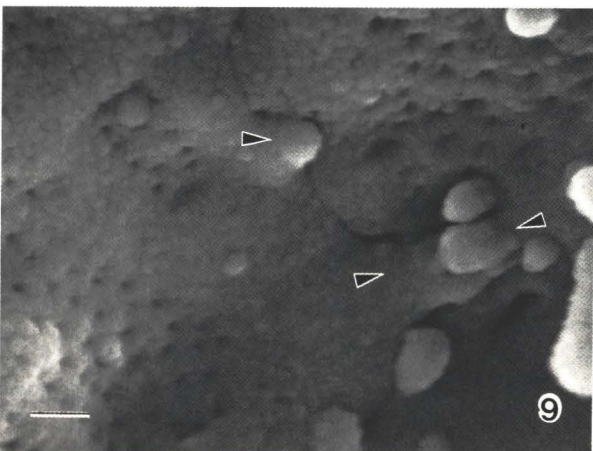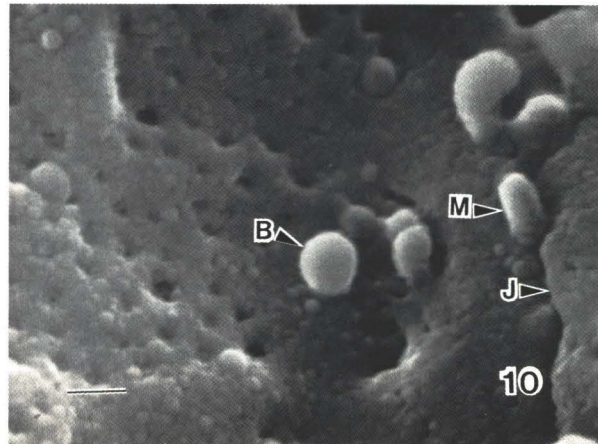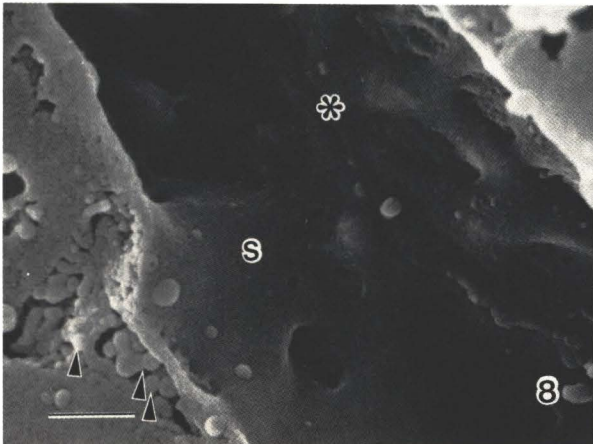3. 48 h hypox vs 48 h hypox + 2 h ACTH: p < 0.01

Fig. 8: The luminal surface (S) of two endo-thelial cell cytoplasmic expanses display a junc-tional (*) region. The perivascular space con-tained spherical structures (arrows) of unknown composition. Bar = 1 µm.

Fig. 9: A high resolution SE-I micrograph of an endothelial cell junction showing an overlapping of finger-like cytoplasmic projections (arrows). Bar = 200 nm.

Fig. 10: Spherical and elongated cytoplasmic protrusions or blebs (B) and a short micro-villus-like (M) profile on the luminal endothe-lial surface are observed near an endothelial cell junction (J). Bar = 200 nm.

11

13

12
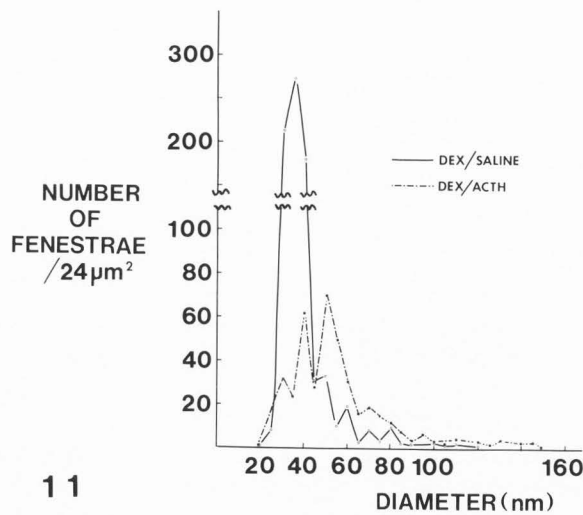
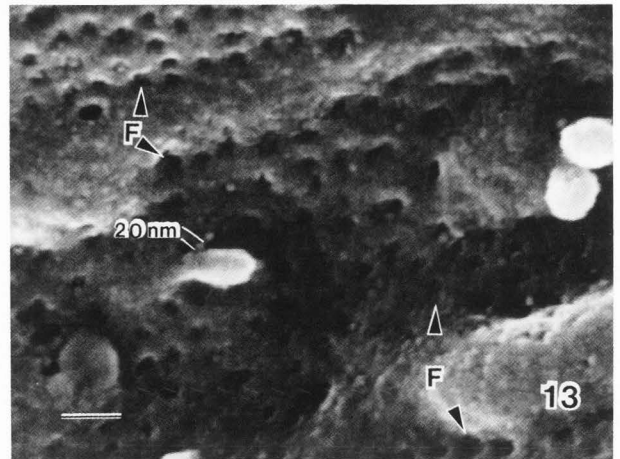Fig. 11 Distribution of fenestrae as a function of their diameters in the DEX and DEX/ACTH treated rats. ACTH treatment reduced the numbers of 30-40 nm fenestrae and increased the numbers of fenestrae over 100 nm in diameter.
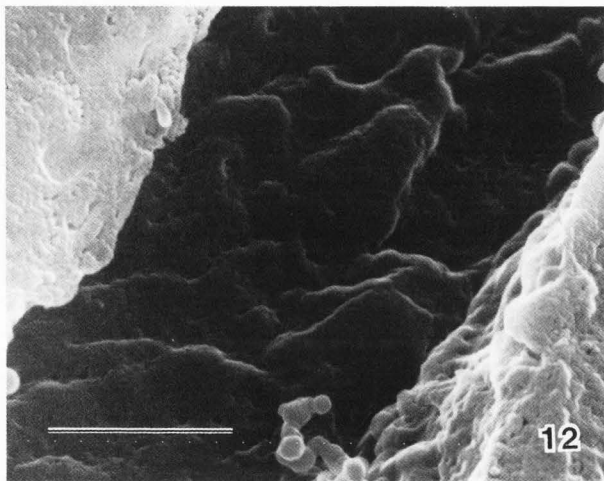
Fig. 12 A capillary from the zona fasciculata of a 48-h hypophysectomi zed rat lacks the characteristic patches of ordered fenestrae. The luminal surface of the endothelium often appeared corrugated. Bar = 1 μm.

Fig. 13 Micrograph of a typical large fenestrated (F) area of capillary observed 2 h after ACTH was administered to rats hypophysectomized 48 h earlier. Bar = 200 nm.

achieved a similar degree of preservation of perivascular spaces and endothelium which contain generally smaller (30-150 nm range) diaphragmed fenestrae [11, 19]. In this paper, a detailed description of vascular perfusion methods and fixatives used to preserve the fine structure of vascular and parenchymal cell types in the rat adrenal is provided.

Delicate exchange of fluids used to dehydrate adrenal specimens and their subsequent cryofracture under LN_2 minimizes the risk of high surface tension conditions. Cryofracture methods, known to expose large surface areas with minimal damage to the fracture faces of rat ovary, were used here to facilitate accurate location of the zona fasciculata in rat adrenals [1]. Using the exchange method with either ethanol or Freon-113, critical point drying was regulated both in terms of temperature and flow rates; such careful regulation has been shown to minimize the introduction of artifacts during the drying of delicate biological samples [12].

Low voltage sputter coating with discontinuous Au/Pd films yield low contrast images of fenestral and other substructural features of the luminal surfaces, lacking topographic information about the shape, height and spacing of superficial granular elements (Fig. 6). Small fenestrae with diameters of 30-45 nm could be measured with confidence because their outlines possessed sharp contours, but metal decoration created a high background noise which suppressed topographic contrast of surface fine structure. Local scattering conditions on Au/Pd decorated fenestrae produced micro-roughness contrast (SE-II component) due to discontinuities in the film [17]. Weak grainy spots resembled fenestral particulate substructure but little could be discerned as to topographic orientation since metal accumulated at the top of particles and not along their sides.

In order to improve the substructural surface information of fenestrae for greater accuracy in quantitative measurements, adrenal sam-

ples were coated with fine grained Ta films rather than decorated with discontinuous Au/Pd films. Early morphometric studies of capillaries in the liver and kidney assessed the fenestral content from specimens which had been decorated with Au/Pd [5, 21, 22]. The resultant images obtained from these specimens were comparable with similarly decorated adrenal specimens; however, soft focusing greatly reduced the background noise caused by micro-roughness in micrographs of the adrenal [18].

Advances in metal coating of biological specimens with Ta has greatly improved the S/N ratio at high magnification. In a recent study of the fenestrated endothelium in kidney, Peters has demonstrated that after a 5 nm deposition of Ta the endothelial surface was composed of closely packed particles [17]. Since membrane features shadowed one another during deposition, their bases received less metal than their tops, such that their tips enlarged and formed a continuous particulate surface. In the present study, images enriched in type I contrasts routinely provided excellent topographic contrast of the endothelial fenestrae and revealed a distinct granular substructure in the form of 20 nm particles closely apposed to one another (Figs. 7 & 13). The compact and continuous coat of Ta, although relatively thick (5 nm), contoured substructure thereby providing relief contrast, and prevented micro-roughness background noise, thus increasing the SE-I/SE-II ratio. Detail recognition was further enhanced by soft focusing SE-I micrographs during enlargement.

Although Ta deposition used in the present study for metal coating endothelial fenestrae was not as precisely controlled as that obtained by Penning sputtering [13], comparable fine structural details were revealed. The present study has demonstrated that metal deposition with conventional sputter coating equipment, when used under conditions of clean vacuum, can yield continuous fine grained films of Ta useful for high resolution SEM.

Advanced cathode configurations for conventional sputter coaters may provide the higher plasma energies necessary to dislodge tightly bound target atoms. The time required for plasma discharge of Ta in a conventional sputter system must be reduced to minimize the thermal load upon the specimen [6]. Ideally, film thickness should be limited to 1-2 nm, to reduce the excitation area from which secondary electrons are emitted and enhance the imaging of surface fine structural detail [13, 17].

Analytical SEMs used in this study were equipped with an objective lens specimen stage and a detector placed above the specimen, resulting in the elimination of the SE-III signal component [10, 17]. It is clear that a high brightness LaB_6 gun, which creates a small probe size and has a beam intensity great enough to excite those luminal surfaces of capillaries recessed beneath the cryofractured surface, is adequate for high magnification signal recording. Images obtained from these instruments when equipped with a tungsten gun created a low S/N ratio and lacked sharp fenestral contours.

Recent quantitative studies of fenestrae in kidney and liver, which utilized conventional SEMs, collected images which, when photographed, contained a low S/N ratio and lacked substructural detail [5, 21, 22]. The recently developed technique for producing continuous ultrathin metal films of Cr and Ta, which enrich the SE-I signal, when combined with imaging with an analytical SEM that eliminates the SE-III signal, presently offers the best approach for high resolution imaging of endothelial fenestrae in bulk samples for morphometric SEM studies.

The present investigation has demonstrated that ACTH, within 20 min after being administered to rats pretreated with dexamethasone, descreases the fenestrae/μm^2 and causes a shift in their size distribution from predominantly 30-45 nm to 50-80 nm in diameter. These changes occur without any significant change in the porosity of the capillary endothelium within the zona fasciculata of the rat adrenal gland. While 87% of the fenestrae in the adrenal cortex of controls fell within the 30-45 nm size range, only 38% of the fenestrae were of this size 20 min after ACTH treatment. Although fenestrae with diameters from 20-150 nm were found, approximately 95% of the total numbers observed in both groups of rats had diameters of 20-80 nm. Based on measurements in freeze-etch specimens, Ishimura et al. [9] have reported that the fenestrae in mouse adrenal capillaries vary in diameter from 50-400 nm, with those smaller than 100 nm comprising 81% of the total number.

The mean diameter of fenestrae in the population ranging from 30-45 nm in the present study was 35.2 nm. Based on these measurements in SEM specimens, the fenestrae appear to be much smaller than those reported in TEM studies [20]. In a recent TEM study by Bearer and Orci [2], involving quick freezing and deep etching of capillary endothelia from a variety of tissues, the average diameter of fenestrae was 60 nm. We have determined the mean diameter of fenestrae to be 57.7 nm in thin sections of endothelial cells cut tangentially; the TEM specimens used for these measurements were from adrenals contralateral to those used for the SEM studies reported here. This difference between the size of fenestrae observed in SEM and TEM specimens is attributable to the greater shrinkage associated with specimen preparation for SEM and the reduction in fenestral dimensions resulting from metal coating of these specimens, i.e., the diameter is reduced by an amount equal to twice the thickness of the metal coat.

The rapid nature of the ACTH induced changes in fenestral numbers and size distribution in the first study described above suggests that they reflect physiological changes in endothelial cells which are coordinated with the increased steroidogenesis by adrenocortical cells.

The observation that hypophysectomy results in a steady loss of fenestrae from the capillary endothelium within the zona fasciculata of rat

adrenals indicates that the trophic action of ACTH may influence the maintenance and growth of the endothelial elements as well as the endocrine cells of this gland. Although the rapid atrophying of adrenocortical tissue as a consequence of hypophysectomy is well known, no detailed ultrastructural studies have been made of the effects of removing the pituitary on the endothelial cells in the adrenal cortex.

The movement of ions, small molecules and macromolecules across microvascular endothelia may occur by one or more of the following mechanisms: (1) diffusion through the plasma membrane and cytoplasm of endothelial cells; (2) permeation through endothelial cell junctions; (3) by endocytosis, exocytosis or transcytosis; and (4) through fenestrae [15, 20, 21]. Since the most prominent feature of the capillaries in the adrenal cortex of the rat appears to be the large numbers of fenestrae, each covered with a thin, membranous diaphragm, it is proposed that these labile structures may participate in the steroidogenic response of the adrenal cortex to ACTH, perhaps by allowing increased transcapillary exchange of LDL and steroid products of adrenocortical cells [11, 19]. Both the steady loss of fenestrae from capillaries within the zona fasciculata following hypophysectomy and their rapid reappearance with ACTH therapy suggest that this trophic hormone plays an important role in the maintenance of the differentiated state of endothelial cells in the adrenocortical microvascular system.

Acknowledgements

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Discussion with Reviewers

Reviewer I: Why have the authors used diameters for measuring pore size when the pores are of irregular shape? Why have they averaged the length and width measurements?

Authors: The size of fenestrae was determined by measuring their diameters because a preliminary study comparing this method with one involving measurement of their circumferences manually indicated that the former method was more accurate and reproducible than the latter. The rationale for determining fenestral diameters by averaging two measurements made perpendicular to one another is based on the circular nature of these pores consistently observed in thin sections with TEM when the plane of the section is parallel to the endothelial wall.

Reviewer I: Why have the authors used pores from surfaces which are tipped with respect to the specimen surface and hence are not an accurate measure?

Authors: In order to assure the randomness of sampling for the morphometric study of the size and distribution of fenestrae, individual capillaries were selected at low magnification, then brought to high magnification to photograph. Following this procedure, we did obtain micrographs in which the luminal surface of the endothelium was tilted with respect to the specimen plane. While we realized that this would result in some error in our measurements, we made the assumption that any error introduced by this procedure would be equally probable in samples from our control and treated rats. Since we were primarily interested in determining whether differences in fenestral dimensions occurred in response to either ACTH or hypophysectomy compared to controls in the two experiments, rather than in their absolute dimensions, we accepted this limitation in these preliminary studies.

Reviewer I: How did the authors make their random photographs? Did they know which group of animals they were photographing?

Authors: See Materials and Methods. The individual (R.A.) processing and photographing the SEM samples had no knowledge of the treatment given each adrenal sample, since the tissues were fixed by perfusion, placed in randomly numbered vials prior to their mailing to the SEM Facility at the Yerkes Regional Primate Research Center in Atlanta.

Reviewer I: How did the authors assure that the number of measurements made on each animal was statistically significant considering the amount of variation, the small number of animals, the small area used, and the small number of pores?

Authors: The data were analyzed using the VAX-VMS 780 computer and the statistical program, Minitab. Significance between the mean fenestrae/μm^2 of the different treatment groups was determined using the Minitab TWOSAMPLE T test, which does not assume equal variances for the groups compared.

Reviewer I: Could the authors provide more information on the time course of action of ACTH? Would one expect to see dramatic changes in 1-2 h?

Authors: ACTH is known to increase the synthesis and secretion of steroids by the adrenal cortex within 15 minutes. We have found no literature describing the effects of ACTH on the fine structure of the capillary endothelium within the zona fasciculata of rat adrenals after the time intervals employed in our two studies.

Reviewer I: Did the overall areas of the cytoplasm containing fenestrae change as well?

Authors: The present paper describes only the observations made in a SEM study of the luminal surfaces of endothelial cells. A TEM study of the fine structural changes in the endothelial cells from the adrenals contralateral to those used for the SEM studies described here is in progress.

K.-R. Peters: How do capillary surface structures compare on the razor-cut and cryofractured faces?

Authors: Although we have been primarily concerned with the cryofractured surfaces, razor-cut surfaces have been cursorily examined. Capillary surface structures, including the clusters of small fenestrae, appear well preserved on both of these surfaces; however, the part of the endothelial wall closest to the razor-cut surface frequently appears distorted, presumably as a result of mechanical injury. While the intercellular junctions in the endothelium and the perivascular spaces were undistorted on cryofractured faces, these spaces were frequently distended on the razor-cut surfaces.

K.-R. Peters: How did you distinguish between fenestral and stomatal diaphragms? What diameter do these diaphragms have in comparison to

the size of coated pits (i.e., in Fig. 5)? Fe-
nestrated capillaries have an approximate 1:6
ratio of stomatal and fenestral diaphragms. Is
the proportion of these diaphragms changed by
ACTH administration or hypophysectomy?

Authors: Fenestral and stomatal diaphragms can-
not be distinguished from one another in our SEM
preparations. Initial counts of the two types
of diaphragms in thin sections indicate that the
latter make up less than 10% of diaphragms in
the capillary endothelium of the rat adrenal
cortex. It has been shown in TEM studies that
these diaphragms can be distinguished based on
differences in their surface charges[23,24]. Fe-
nestral diaphragms will bind cationized ferritin
(CF) but not lectins whereas stomatal diaphragms
of transendothelial channels and vesicles bind
lectins but not CF. Since we were unable to
make any distinction between the different types
of diaphragms in our SEM morphometric study, we
realize that this would be a source of error in
our counts of fenestrae; however, based on TEM
observations not described in this report, we
believe that this error is small. Of particular
interest is the question of whether the relative
numbers of different types of diaphragms change
as a result of hypophysectomy or ACTH treatment.
This is being examined in a TEM study parallel-
ing the SEM study reported here. (See figure
14).

Coated pits have a reported size of 150-200
nm and have a characteristic surface structure
reflecting the clathrin coat whereas the pores
in Fig. 5 range between 30-50 nm. The endothe-
lium of the mouse adrenal cortex contains nu-
merous fenestrae from 50-100 nm and relatively
few in the 100-400 nm range[9].

K.-R. Peters: How did you account for parallac-
tic distortions when measuring diaphragm diame-
ters from two perpendicularly oriented diame-
ters? Would such distortions not reduce your
measurements? Fenestrae are located at the bot-
tom of conical invaginations. In Fig. 13, on
surfaces perpendicular to the beam, openings are
very uniform and approximately double in size
when compared to openings imaged on tilted sur-
faces (Fig. 4). In grazing views, may the lumi-
nal rim of the invagination obscure the dia-
phragms of the fenestrae, reducing your diameter
measurements?

Authors: There is no doubt that some inaccuracy
results in determining the size of fenestrae by
measuring two perpendicular diameters, since our
random sampling procedure resulted in some
images of tilted luminal surfaces of endothelial
cells. Although we are aware that computerized
image analysis instruments make more accurate
measurements of surface areas by perimeter
tracing than are possible by our simple diameter
measurements, such a device was not available
for our studies. TEM studies have demonstrated
that diaphragmed fenestrae are round or octa-
gonal structures[20]. In the present SEM study
micrographs of tilted luminal surfaces yielded
reduced Y-axis measurements; however, X-axis

measurements of fenestral diameters should be
accurate. If the slope of a lumen was very
steep, it is possible that the luminal rim of
the invagination could mask the fenestral dia-
phragm and reduce the diameter measurement.

K.-R. Peters: In Fig. 7 most of the fenestral
diaphragms measure 60-70 nm in diameter
(measured parallel to the long axis of the
micrograph). This is in excellent agreement
with other published TEM and SEM data. However,
some diaphragms (dark holes) reveal only 20-30
nm large area. Since the fenestral diaphragm
complex is composed of the diaphragm, a central
knob and radial filaments, is it possible that
the smaller openings are the remainder of dis-
rupted diaphragms, partially buried under a
thick blanket of metal?

Author: Although a thick blanket of Ta has
coated the bases and rims of the fenestrae in
Figure 7, we do not feel that the 20-30 nm pores
in central-right portion of the micrograph are
the remainder of disrupted diaphragms. These
pores are roughly spherical when surface tilt is
taken into account and their central knobs are
visible. Two dark holes which are observed in
the upper left of the micrograph are interpreted
as part of a larger 80 nm fenestra.

K.-R. Peters: In Fig. 5 the diaphragmed open-
ings appear very uniform in size when surface
tilt is taken into account. However, in Fig. 4
the openings vary considerably. Could this dif-
ference be caused by varying thicknesses of
metal deposition rather than different imaging
procedures? Considering a uniform size of fe-
nestral diaphragms, absolute metal thickness in
Fig. 5 may be 3-5 nm, but in Fig. 4 it may be as
large as 10-20 nm.

Authors: Since 87% of the fenestrae in the dex-
amethasone-saline treated rats in the first
study fell within the 30-45 nm size range, it is
unlikely that this size fenestra can be ex-
plained in terms of small openings in disrupted
diaphragms, particularly since very few fenes-
trae exhibited dark holes in their diaphragms.
The variability in metal coat thickness could
explain the differences in fenestral sizes seen
in Figs. 4 and 5, in view of the limitations of
our sputter equipment and FTM readings in repro-
ducibly obtaining metal coats of the same thick-
ness. However, it should be pointed out that
SEM images in Figs. 4 and 5 were obtained from
adrenal specimens that had been treated dif-
ferently and the differences in fenestral diam-
eters may also be accountable in terms of these
treatments. The imaging procedures were essen-
tially the same for both figures since the
specimens were staged in the objective lens of
the SEM's used in this study.

E. Wisse: The histogram in Fig. 11 shows a
number of discrete peaks at approximately 10 nm
intervals. Is this an indication of a "quan-
tisized" size distribution, or do you consider
it to be "statistical noise" or as a third

possibility, might it be the "granularity", caused by using small integer numbers of scale units for the measurements?

Authors: The distribution of fenestrae shown in Fig. 11 represents the aggregate numbers, based on diameter measurements, that fall within each 5 nm interval over the range from 20 to 150 nm. The large peak associated with dexamethasone-saline treatment encompasses those fenestrae with diameters from 30-45 nm and includes 87% of the total number of fenestrae measured after this treatment. We believe that the fenestrae falling within this size range represent a single class of the diaphragmed pores found in the capillary endothelium within the rat adrenal cortex. The several smaller peaks depicting the distribution of larger fenestrae after ACTH treatment, however, may be artefactual and be a result of the small sample size and lumping together of fenestrae in 5 nm intervals.

E. Wisse: It is our experience that after perfusion fixation of liver tissue for SEM, such factors as leaving the tissue in glutaraldehyde for a long time, insufficient or exaggerated osmium fixation, rinsing in distilled water, insufficient dehydration, long exposure to dehydrating fluid, remoisturization after CPD or sputtering, may all lead to an increased granular and swollen appearance of the endothelium. Do you consider the 20 nm particles seen on the membrane surfaces, to be a preparation artefact?

Authors: The visualization of 20 nm particles on the luminal surface of the adrenocortical capillaries was only possible after the deposition of tantalum. Specimens coated with Ta displayed topographic contrast of particles smaller than 30 nm and consistently revealed the central knob in the fenestral diaphragms. Cryo-fractured endothelial surfaces of adrenals decorated with Au/Pd lacked this particulate substructure and the central particles of the diaphragms were observed less frequently (compare Figs. 6 and 7). The SEM images of the particulate nature of the endothelial surfaces in the adrenal cortex described here is similar to that recently reported in an SEM study of similarly coated fenestrated endothelial surfaces in the rat kidney[17].

E. Wisse: What is the effect of the addition of 4% sucrose or 2% PVP to washing or fixing fluids?

Authors: The 4% sucrose or 2% PVP have been used in our fixative and rinsing fluids because these two additives improve the quality of preservation of the smooth endoplasmic reticulum and of microtubules, respectively, in our TEM specimens. Since one adrenal from each rat is used for SEM, and the other, for TEM, we have adopted a fixation procedure that yields the best preservation of those ultrastructural features of adrenocortical cells of particular interest to us in our TEM studies.
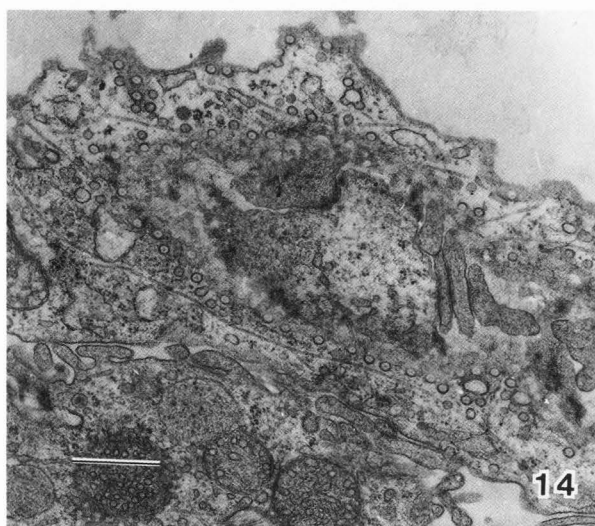

Figure 14: A TEM micrograph of a tangentially sectioned endothelial wall depicts large numbers of spherical 40-55 nm fenestrae which contain characteristic central knobs. Bar = 0.5 µm.

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