stimulated and carbamylcholine-stimulated acinar cells of the pancreas.

Cyclic GMP in the pancreas appeared to be localized predominantly in the apical plasmalemma membrane and the lumen of the acinar cells (Fig. 2a). After carbamylcholine stimulation, the cyclic GMP immunofluorescence was markedly increased in the apical plasmalemma membrane and in the lumen within 30 seconds (Fig. 2b), and in the lumen and zymogen granules in the apical part of the acinar cells after 10 minutes (Fig. 2c). However, the cells in the islets of Langerhans, which are involved in endocrine secretion of glucagon and insulin, did not show any significant change in cyclic GMP immunofluorescence (Fig. 2d). Concentrations of cyclic AMP in the resting and stimulated pancreatic acinar cells were not altered and cyclic AMP was diffusely localized along the apical portion of plasma membrane and cytoplasm (Fig. 2, e and f). No significant immunofluorescence was observed in pancreatic acinar cells after treatment of the sections with nonimmune serum and fluorescein-labeled goat antiserum to rabbit IgG (Fig. 2g), or with antiserum to cyclic GMP which had been incubated with 5 μM cyclic GMP.

Since this immunocytochemical procedure is performed on unfixed cryostat sections, it seems probable that the free cyclic nucleotide would be lost during the staining procedure and that only the nucleotide bound to cellular compartments would be visible. We have found that, even though most of the cyclic GMP in the medium is in a free form, most of the cyclic GMP in the tissue appears to be bound. Thus, it seems that most of the cyclic GMP present in the tissue is associated with the apical portion of the plasma membrane which gives rise to an intense cyclic GMP immunofluorescence in the lumen during exocytosis of zymogen granules.

Ong *et al.* (*6*) reported that cyclic GMP was present on the nuclear element and plasma membranes of a number of cells in liver, small intestinal brush border, thyroid, and testis. These authors suggested that this nucleotide may serve a regulatory function in nuclear directed events and membrane functions. Our observation of cyclic GMP in the lumen, the apical plasmalemma membrane, and the granules of pancreatic acinar cells suggests another role for this nucleotide in the secretion of enzymes.

The mechanism by which cyclic GMP is released is not known, but we postulate that it may be released by exocytosis as are the pancreatic enzymes. In support of this hypothesis is the finding that the zymogen granules contain a high concentration of cyclic GMP, and the release of the cyclic GMP parallels the release of the amylase after stimulation with carbamylcholine and other agents. Even though more than 90 percent of cyclic GMP in the medium is not associated with proteins, most of this nucleotide is apparently associated with macromolecules in the cells. In contrast, cyclic AMP is not released into the medium. It appears that cyclic GMP becomes dissociated during or after secretion into the medium. This dissociation may be brought about by differences in the ionic composition between the medium and the cytoplasm of the cells. The role of the cyclic GMP present in the pancreatic secretion is not clear. Whether it is involved in the activation of enzymes after they are released requires further investigation.

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Duchenne Dystrophy: Alteration in Muscle Plasma Membrane Structure

Abstract. *Freeze fracture studies of skeletal muscle from eight patients with Duchenne dystrophy showed nonuniform distribution and depletion of particles on both protoplasmic and extracellular faces of the muscle plasma membrane. The findings support the view that the muscle surface membrane is abnormal in this disorder and indicate that alterations are present in the internal molecular architecture of the membrane.*

Biochemical studies have suggested a muscle cell surface membrane abnormality in Duchenne muscular dystrophy (*1*). Moreover, electron microscopic studies have shown focal defects of the muscle cell surface membrane in nonnecrotic fibers (*2, 3*), and electron cytochemical studies have demonstrated peroxidase permeation through the defects (*3*). It has been suggested that this defect is an early and possibly basic one (*3*).

Table 1. Particle distribution per square micrometer on plasma membranes. Values are mean and standard deviation for each subject, except the values in italics, which are group means and standard errors of the means.

P face		E face	
Duchenne	Control	Duchenne	Control
112 ± 11	257 ± 6	44 ± 8	159 ± 7
123 ± 43	284 ± 11	89 ± 40	159 ± 7
129 ± 18	275 ± 20	74 ± 13	155 ± 11
93 ± 45	239 ± 43	50 ± 24	133 ± 12
123 ± 50	262 ± 18	31 ± 17	151 ± 10
134 ± 25		36 ± 20	
50 ± 15		23 ± 12	
113 ± 9		17 ± 8	
*110 ± 27**	*262 ± 18*	*45 ± 9**	*151 ± 4.3*

*$P < .001$ (Duchenne value versus corresponding control value).

The freeze fracture technique provides the microscopist with the opportunity to visualize and quantitate fine details of membrane ultrastructure. The work of Branton and colleagues has demonstrated that the fracture preferentially passes along the midline of the membrane through the hydrophobic phospholipid interior. Both fracture faces are covered with particles, and it is now accepted that these particles are proteins and structural components of the membrane (*4*). Utilizing this technique we report widespread alterations in the internal molecular architecture of the muscle plasma membrane in Duchenne muscular dystrophy.

Quadriceps muscle plasma mem-

branes from eight patients with Duchenne dystrophy were studied (5). Progressive proximal muscle weakness and enlarged calves were present since early childhood. All had markedly elevated creatinine phosphokinase in excess of 1000 international units per liter and muscle biopsies consistent with this disorder. Four out of eight had evidence of X-linked recessive inheritance. The age at the time of biopsy was between 4 and 10 years.

For control data, normal quadriceps human muscle was obtained from five individuals (one adult male volunteer without neuromuscular disease, one nonweak female control, and three male children, ages 3, 5, and 11, undergoing orthopedic operations. Histochemical studies of the biopsies were normal).

At low magnifications, both the protoplasmic (P) and extracellular (E) faces of normal muscle plasma membrane contain openings that correspond to caveoli (Fig. 1, A and B). The P face characteristically shows square arrays (6), large particles, and a population of smaller particles. All particles appear distributed in a uniform fashion (Fig. 1A) with a mean density of 244 ± 44 [standard deviation (S.D.)] per square micrometer (Fig. 2). The group mean value was 262 ± 18 [standard error (S.E.)] (Table 1). The E face particles were less numerous (Fig. 1B), with a mean density of 127 ± 27 (S.D.) per square micrometer (Fig. 2), and corresponding pits of the square arrays were present. The group mean value was 151 ± 4.3 (S.E.) (Table 1). No significant difference in particle density was noted between children and adults.

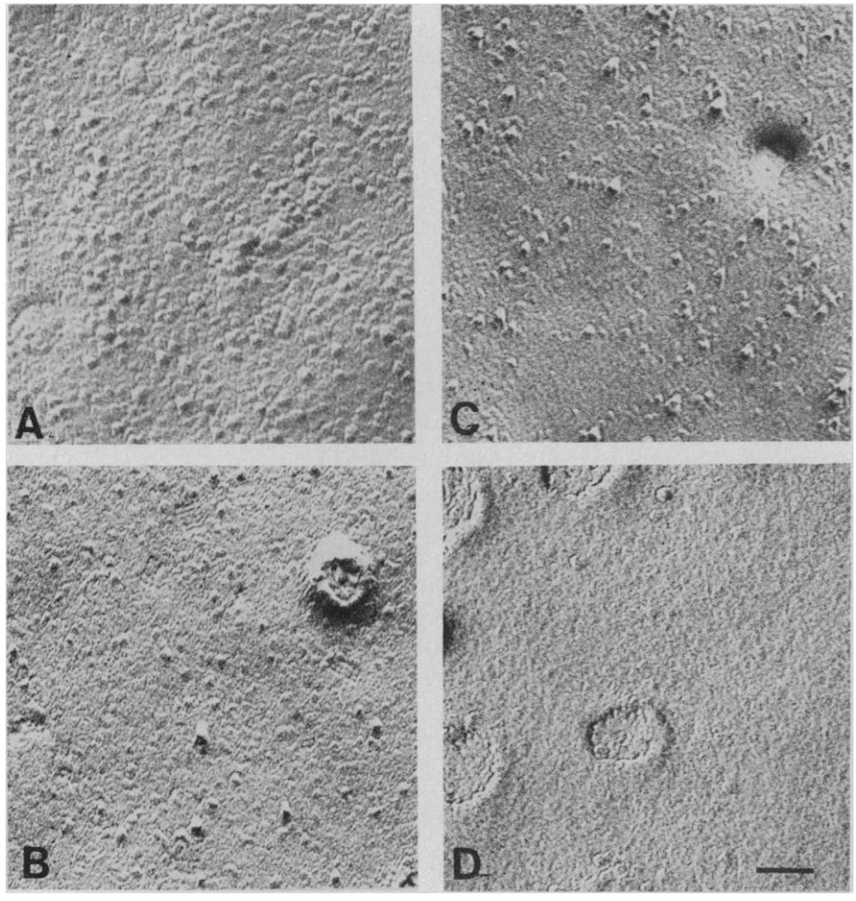

Fig. 1. High magnification micrographs illustrating the difference in intramembranous particle density between normal and Duchenne sarcolemma. (A) Normal P face; (B) normal E face; (C) Duchenne P face; (D) Duchenne E face (all ×78,000). Marker equals 0.1 μm.

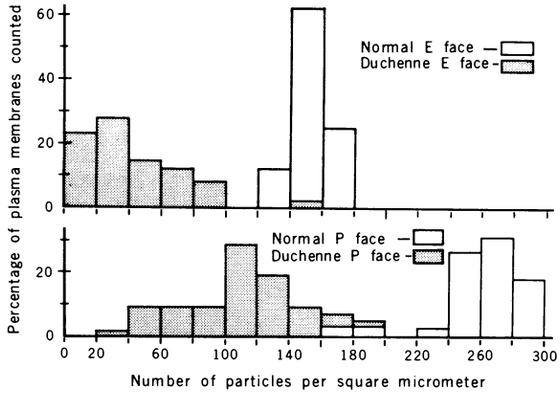

Fig. 2. Distribution of particle density per square micrometer in Duchenne versus normal plasma membrane.

In contrast, the plasma membrane from Duchenne muscle showed nonuniform distribution of particles, and many contained extensive areas in which only a few particles were seen (Fig. 1, C and D). This finding was noted in both P and E faces and in some instances resembled the cleaving behavior of pure lipid bilayers (7). Square arrays were rarely seen in the P faces (Fig. 1C) of the plasma membrane studied. The mean particle density in the Duchenne P face was 127 ± 27 (S.D.) per square micrometer (Fig. 2), and the group mean value was 110 ± 27 (S.E.) (Table 1). The mean particle density in the Duchenne E face was 47 ± 38 (S.D.) (Fig. 2). The group mean value was 45 ± 9 (S.E.) (Table 1).

Depletion and altered distribution of intramembranous particles may be an early sign of membrane abnormality in Duchenne dystrophy preceding the formation of focal defects at the cell surface membrane.

Whether the diminution of particle density in the muscle plasma membrane was present in utero or occurred during the course of the disease is uncertain. The presence of a 14 percent overlap in particle counts between the Duchenne and normal P faces (see Fig. 2) supports the first possibility. However, further studies on very early cases and possibly in carriers are required to clarify this question.

It is also uncertain whether the observed changes are conditioned by a muscle defect, an abnormality in innervation, or both. Preliminary data do suggest, however, that the mechanism that alters membrane structure in Duchenne dystrophy may be different than that observed in denervation, since freeze fracture studies on two patients with peripheral neuropathy with normal serum enzymes and decreased nerve conduction velocities have shown an increase in the number of plasma

membrane intramembranous particles. Whether this is related to the stage of denervation or reinnervation is unknown (*8*).

Significant progress has been made in correlating function with intramembranous particle populations in skeletal muscle sarcoplasmic reticulum (*9*). It is anticipated that function will eventually be linked to the intramembranous particle populations seen in the muscle plasma membrane so that alterations in distribution and number of particles will be correlated with changes in specific plasma membrane functions in Duchenne dystrophy.

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5. The specimens were removed at rest length, attached to a stick or U-shaped muscle clamp, and fixed immediately in 3 percent glutaraldehyde in 0.1*M* phosphate buffer, *p*H 7.4. Fascicles were dissected out and gradually infiltrated in glycerol in water up to a concentration of 30 percent. Freezing was carried out in Freon 22 and fracture and replication were performed in a Denton DF E5 freeze-etch unit and a Balzer BAF 301 freeze-etch apparatus. Tissue was digested in a commercial bleaching solution (Clorox). The detached replicas were washed twice in distilled water and finally picked up on uncoated 300-mesh grids. The material was examined and micrographed in an AEI EM-6B electron microscope operated at 60 kv. Particle counts per square micrometer were carried out on random areas of the protoplasmic (P) face and extracellular (E) face of the plasma membranes on micrographs enlarged to ×60,000. Plasma membranes from a minimum of eight fibers (1 μm^2 each) were counted in each biopsy. Particles were only counted when they were clearly above the surface of the replica. Particle size was not emphasized in this study. The randomness of the sample was ensured by photographing any area of the fracture that was recognized as plasma membrane. Individual square micrometers counted were chosen by blind selection.
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Endothelial Damage and Thrombocyte Adhesion in Pigeon Atherosclerosis

Abstract. *Scanning electron microscopy studies of spontaneously occurring atherosclerosis in pigeons reveal dramatic alterations in endothelial integrity. An irregular endothelium at the intimal cushion region of 5-week-old birds gives rise to extensive areas of pitted endothelium and subendothelial exposure. Thrombocytes, thrombocyte aggregates, and leukocytes are associated with the developing lesion.*

Ultrastructural changes in spontaneously occurring atherosclerosis have been described by several authors (*1*). Particular attention in these studies has been given to pathologic alterations in the arterial walls that climax in mature lesions composed of smooth muscle cells and connective tissue elements (*2*). Although the general sequence of events in spontaneous atherosclerosis has been elucidated, little specific information is available on the initiation of this disease. Recent observations using animal models, including chickens, dogs, swine, rabbits, and nonhuman primates (*3*) have been of value in providing more specific information on atherogenesis, and results obtained with these animal models suggest that both smooth muscle cell proliferation and lipid accumulation are manifestations secondary to continued endothelial injury (*4*). This hypothesis, however, is based upon experimentally induced atherosclerosis, since both the extent of arterial disease and the localization of spontaneous lesions vary in most species.

Arterial lesions in the White Carneau pigeon, unlike other animals, develop spontaneously and are highly predictable with respect to arterial localization (*5*). Furthermore, the similarity of pigeon intimal cushions to those found in humans (*5, 6*), and the fact that the time course for lesion progression is well defined in this species (*7*), make the White Carneau a desirable model for identification of early events in spontaneous atherosclerosis. We report a scanning electron microscope study of aortas from the atherosclerosis-susceptible White Carneau pigeon. Our studies, focusing upon endothelial integrity and the role of circulating elements at the site of spontaneous lesion development, reveal significant endothelial damage, with thrombocytes and leukocytes adherent to both the damaged endothelial cells and exposed subendothelial fibrils.

Twenty White Carneau pigeons ranging in age from 5 weeks to 3 years were anesthetized by intravenous administration of pentobarbital (32 mg) and anticoagulated with 250 units of heparin prior to perfusion under pressure (110 mm-Hg) with 2.5 percent glutaraldehyde buffered to *p*H 7.2 with 0.1*M* phosphate. Subsequent to perfusion the excised aortas were fixed further by immersion overnight in fresh fixative before being dehydrated through graded alcohols and dried from CO_2 by the critical point method. Preparation of aortas by this technique permitted direct observation of aortic endothelium from below the celiac bifurcation, where spontaneous lesions develop, to areas 15 mm cephalad to the celiac origin (Fig. 1a). Included in such preparations are the vestigial ligament, openings of the intercostals, the intimal cushion region, and areas sufficiently far from the lesion to serve as control tissues.

Endothelium from the nonlesion control regions, as shown in Fig. 1b, was intact. The well-delineated endothelial cells had distinct marginal folds and multiple surface projections. This was true for birds of all ages and for birds with varying degrees of intimal cushion proliferation. Irregular patches positioned over the aortic surface near the intimal cushion region were occasionally found in birds as young as 5 weeks. When observed at high magnification, the endothelium in these irregular areas was disrupted and had extensive ruffling of luminal plasma membranes (Fig. 1c). In addition to the membrane ruffles, areas of endothelial desquamation were found in the 5-week-old birds. This damage, localized at orifices of the intercostals and in the area of the vestigial ligament, was most pronounced at the celiac bifurcation, where an extensively pitted and cratered endothelium resulted in large areas of subendothelial exposure (Fig. 1d). Blood cells adherent to the damaged surfaces frequently formed a semiconfluent cytoplasmic sheet. These observations were consistently found in pigeons of all ages, but were most conspicuous in older birds with identifiable lesions. Thrombocytes in the atheromatous area were attached to subendothelial fibrils and to altered endothelial cells by the extension of multiple cytoplasmic fingers (Fig. 1, e and f). Thrombocyte aggregates were also associated with necrotic endothelial cells (Fig. 1g). In addi-