induced by reduction of renal arterial pressure. Although there was also generally a decrease in renal venous renin activity when mannitol was given to dogs in whom the arterial pressure had not been lowered, the change for the entire group was not statistically significant. The present study extends these findings and demonstrates a consistent and statistically significant decrease in renin secretion in normotensive animals.

In contrast to mannitol diuresis, alone, the superimposition of complete ureteral occlusion produced a marked rise in renin secretion. These data constitute evidence against the theory that renin secretion is increased as a result of increased intratubular macula densa sodium concentration, since early distal sodium concentration is, if anything, further decreased during ureteral occlusion (8) from the already low values of free-flow mannitol diuresis (6, 7). In contrast, as described in the introduction, the changes in renin secretion observed during both free-flow diuresis and stop-flow are consistent with (although, of course, do not prove) the hypothesis that renin secretion is inversely related to the total load (rather than concentration) of sodium entering the distal tubule per unit time. A possible mechanism by which changes in sodium load might be detected by the macula densa cells has been described previously (1).

These data also provide additional evidence that neither a decrease in renal blood flow nor in arterial blood pressure is a prerequisite for increased renin secretion; indeed, the increased secretion was associated with an increased blood pressure (probably secondary to increased plasma angiotensin) and renal plasma flow. The changes in renal blood flow are similar to those reported by several other investigators for similar conditions (see Circulation Res., Supp. I to Vols. 14 and 15, 1964).

Summary. Renin secretion was decreased during mannitol diuresis, alone, but was increased by the superimposition of ureteral occlusion. The data are consistent with the "sodium load" hypothesis for control of renin secretion but not with the "sodium concentration" hypothesis.

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Antinuclear Antibodies—Attempts at Ultrastructural Localization* (33056)

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Considerable interest has evolved over the last 10 years concerning the *raison d'être* as well as the pathogenic significance of antinuclear antibodies among human disease states. The elution from isolated lupus glomeruli of antibodies showing distinct anti-DNA or anti-

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TABLE I. Profile of Reactivities of Antinuclear Antibodies among Six SLE Sera Chosen for Ultrastructural Localization Study.

Patient	Immunofluorescent titer with mouse kidney sections	Nucleoprotein latex titer	Reaction with SM antigen		Reaction with soluble nucleoprotein	
			Precip.[a]	CF[b]	Precip.[a]	CF[b]
1. L.O'S.	256	256	0	0	+	0
2. D.Un.	256	128	0	64	+	4
3. L.Ph.	128	256	0	8	+	0
4. M.Wa.	128	16	0	8	0	0
5. M.Kr.	32	0	+	0	0	16
6. L.Pe.	8	0	+	0	0	0

[a] Precipitin in agarose gel.
[b] Complement fixation reaction.

nucleoprotein reactivity (1–3) has produced strong evidence that these γ-globulins may participate as part of antigen–antibody complexes localizing within glomerular lesions. Recently, by use of human antibodies to DNA and immunofluorescent procedures, it has been shown that DNA itself is present within some glomerular lesions as the possible antigenic contribution to pathogenic complexes (4). Moreover, eluted γ-globulin from lupus glomeruli was shown by these same workers to contain marked concentrations of antibody activity for various nuclear antigens.

The present study was primarily directed at an electron microscopic localization within the nucleus of high titered antinuclear antibodies using sera from patients with active disseminated lupus erythematosus (SLE). Ferritin-labeled γ-globulin fractions from sera with strong reactivity for nuclear antigens were employed.

Materials and Methods. Sera from 38 patients with disseminated lupus erythematosus were collected and stored in frozen aliquots at −20°C until fractionation and antibody assay procedures were undertaken. Of the 38 patients' sera studied, only 6 showed consistently broad enough antinuclear reactivity to be deemed worthy of attempts at antinuclear localization. A profile of the multiplicity of reactivities for various nuclear antigens is shown in Table I. Agglutinating antibodies for latex particles coated with nucleoprotein (5,6), precipitins for nucleoprotein (7) and the nuclear antigen previously designated as SM antigen by Tan and Kunkel (8), immunofluorescent antinuclear reactivity against nuclei seen in 6-μ sections of mouse kidney (8), and complement fixation for nucleoprotein, SM antigen and DNA (8–10) were studied.

In addition, because of previous work by others (11–13) indicating presence of 7S γM within some sera from patients with SLE, sucrose density gradient separations (14) of 0.3 ml of serum diluted 1:1 with 0.15 *M* NaCl were performed. After 16 hours of ultracentrifugation at 35,000 rpm in a 10–40% sucrose gradient, 20–25 fractions were collected and assayed for ANF[1] agglutinating antibodies using the latex nucleoprotein reagent and immunofluorescent staining of mouse kidney sections. Fractions were also examined in immune diffusion against anti-γM antisera to check for presence of low molecular weight γM.

Methods used to prepare ferritin conjugates of SLE gamma globulins. Sera were separated using starch block electrophoresis (15). The cathodal fractions containing high titers of antinuclear antibodies were concentrated with negative pressure and collodion membranes. These γ-globulin fractions or 7S γ-globulins isolated by DEAE-cellulose chromatography (16) were used for ferritin conjugation. The procedure of Singer and Schlick (17,18) was employed for conjugation of ferritin to antibody globulin, using γ-globulin at 15–25 mg/ml (starch block)

[1] ANF=Antinuclear factor.

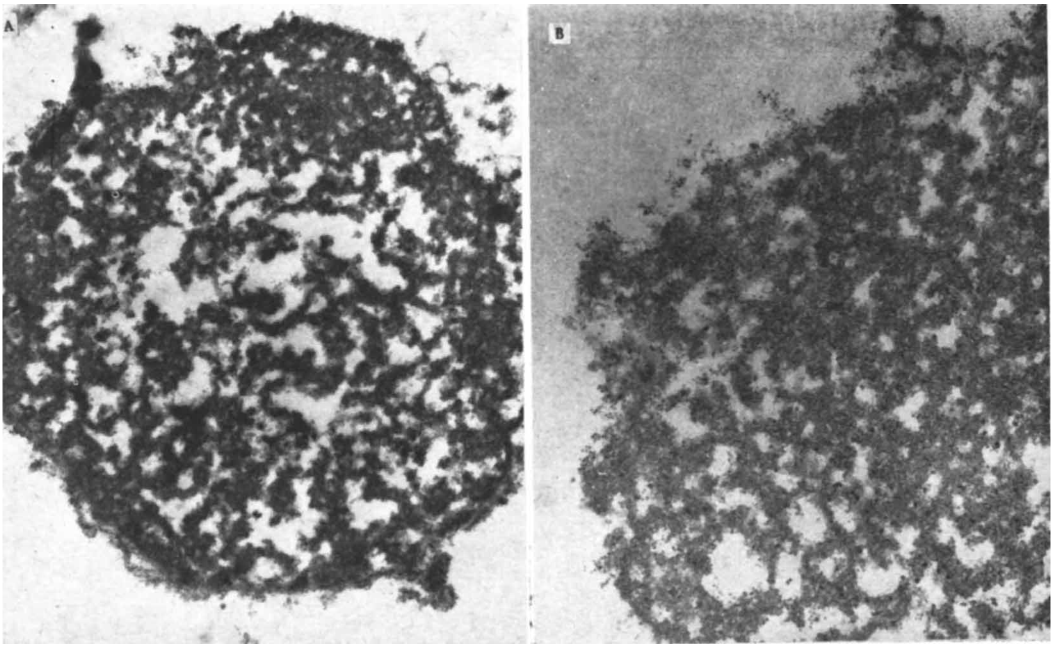

FIG. 1. A. Represents calf thymus nucleus treated with ferritin-conjugated γ-globulin from serum L.Ph. (Table I) containing high titers of antinuclear antibody. Ferritin is noted densely distributed around outer nuclear membrane as well as the interstices of the nuclear structures. × 37,525. B. Indicates electron micrograph of control experiment in which ferritin alone was incubated with isolated calf thymus nuclei. In this instance, despite thorough washing, ferritin is noted adherent to the outer nuclear membrane and interstices. × 110,000.

and 4 mg/ml (DEAE isolation). Control specimens included: (i) γ-globulins from goat antihuman ceruloplasmin antiserum, (ii) rabbit antihuman albumin antiserum, (iii) isolated γ-globulins from individual and pooled normal human sera, and (iv) ferritin alone. All conjugates were dialyzed against 0.25 *M* sucrose, 0.003 *M* $CaCl_2$ for 8 hours. Some indication of the relative degree of conjugation of the ferritin was sought using Ouchterlony analysis with rabbit antisera to human γ-globulin and to horse spleen ferritin. In most instances, a relatively low efficiency of conjugation was achieved. This was estimated by fusion of the precipitins of ferritin and γ-glomulin indicating ferritin-γ-globulin complexes possessing both determinants on the same molecule. Considerable enrichment of the proportion of ferritin-conjugated γ-globulins was achieved in several experiments utilizing the method of Amstey (19) which employs DEAE elution of conjugates with salt gradients designed to secure maximum ferritin–γ-globulin complex, as measured by immune diffusion experiments.

Substrates for ferritin-conjugated antibody staining were preparations of calf thymus nuclei prepared by the Allfrey procedure (20) and shown previously (21) to consist of at least 97–98% nuclear constituents by electron microscopic monitoring. Conjugated globulins were incubated with 0.3–0.5 ml of concentrated suspension of calf thymus nuclei for 15 min at room temperature, 20 min at 37°C, and then 30 min at room temperature with frequent gentle agitation. Five ml of 0.25 *M* sucrose, 0.003 *M* $CaCl_2$ was then added, and after centrifugation at 2000 rpm for 3 min (International centrifuge), supernatant was decanted and pellet was resuspended and immediately fixed and embedded for ultrastructural studies, as previously outlined (22,23).

Results. Table I indicates the general pattern of antinuclear reactivity of the sera chosen for study. It can be seen that the

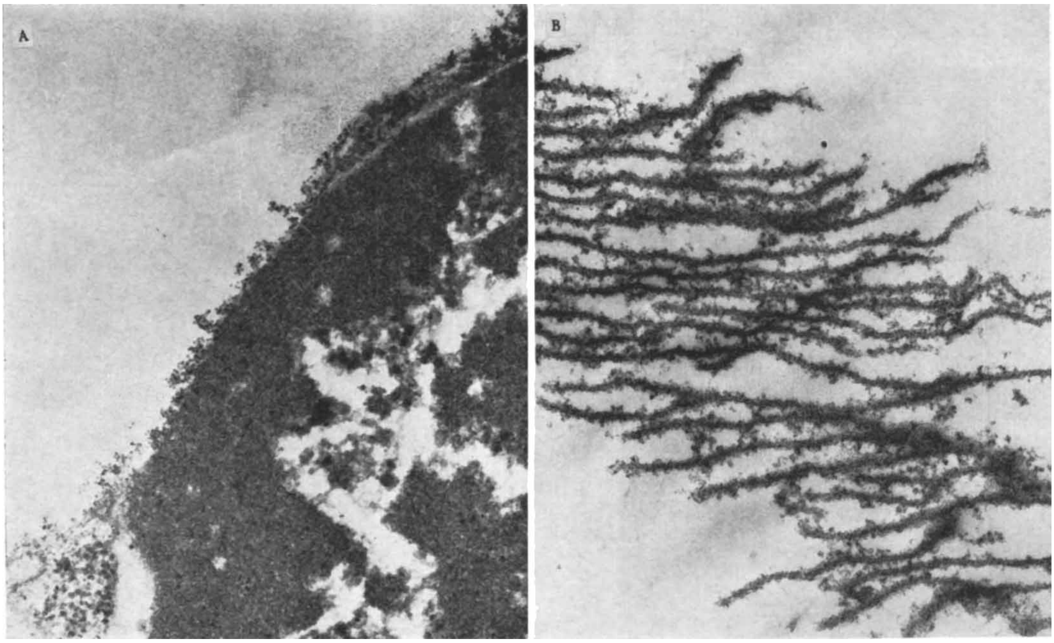

FIG. 2. A. Shows calf thymus nuclear surface with adherent ferritin; this preparation was run as a control in which calf thymus nuclei were stained with γ-globulin from rabbit anticeruloplasmin antiserum conjugated with ferritin. The photograph indicates adherent nuclear ferritin "staining" in this control experiment. × 60,600. B. Shows lamellae of calf thymus nuclear membranes exposed to ferritin conjugated SLE γ-globulin containing high titers of antinuclear antibody. Unfortunately, such intense nuclear membrane ferritin localization was also seen when ferritin alone or ferritin conjugated to normal human γG γ-globulin was used (× 60,000). These experiments indicated that ultrastructural distribution of ferritin in such experiments could not be directly correlated with antinuclear antibody.

latex–nucleoprotein agglutination reaction reflected concomitant strongly positive tests with mouse kidney immunofluorescent staining, and in many instances parallel positive precipitins against nucleoprotein or SM antigens. No sera were found in this study which showed precipitins in agarose gels with DNA.

Sucrose gradient separations revealed that in three of the most active sera (O'S., Ph., and Wa) the agglutination of nucleoprotein-coated latex particles was entirely in the 19S region. However, immunofluorescence of mouse kidney sections showed predominant 7S γ-globulin distribution (titers of 1:28–1:256) from sucrose gradients. No low molecular weight γM was detected in any of the sera used for ultrastructural study.

Ferritin-conjugated globulins derived from fractions of lupus sera possessing high titers of antinuclear antibody activity showed diffuse staining of isolated calf thymus nuclei. In many instances the most intense staining, as judged by density of ferritin label, appeared on the nuclear membranes. These reactions can be seen in Figs. 1 and 2. However, controls using ferritin-conjugated globulins from normal human sera, untreated rabbit antisera, and ferritin alone showed essentially similar ferritin affinity for isolated calf thymus nuclei or nuclear membranes. These positive control reactions indicated that antinuclear antibody localization could not be attributed to the specific antibodies labeled from SLE globulins. Such nonspecific staining of isolated nuclei by ferritin alone or completely unrelated (antihuman ceruloplasmin or albumin) ferritin-conjugated γ-globulins indicated that components of nuclei—possibly extremely basic proteins such as histone—reacted with or adhered to ferritin or ferritin-conjugated molecules. It was felt that this technique could not be applied to studies of antinuclear antibodies. Further washing of ferritin-conjugates mixed with isolated nuclei did not diminish ferritin affinity as judged by subsequent observations under the electron microscope.

Discussion. Important structural definition of various human antibodies to autologous antigens might be gained if ferritin-labeled autoantibodies could be used in the same fashion as fluorescent-conjugated γ-globulins. Unfortunately, using preparations previously shown to consist of relatively pure calf thymus nuclei (21), antibody specificity of ferritin-conjugated γ-globulins derived from antibody fractions possessing a broad spectrum of antinuclear antibodies could not be documented. Both isolated calf thymus nuclei and nuclear membranes showed intense uptake of ferritin-conjugated control preparations as well as of unconjugated ferritin alone. This difficulty may be related to charge affinities inadvertently introduced into the reactants since basic proteins such as nuclear histones might be expected to react in nonspecific fashion with ferritin or ferritin-conjugated γ-globulins. The importance of strict controls in this regard is underscored by this study.

Efficiency of ferritin conjugation was low as indicated by the use of concomitant anti-γ-globulin and antiferritin antisera giving lines of identity in Ouchterlony analysis with conjugates. This was improved using the recently described method of Amstey (19) but indicates that better high-yield procedures for labeling antibodies for electron microscopic localization are sorely needed.

These findings are presented as evidence that antibody localization, at least with isolated calf thymus nuclei, cannot be reliably approached using currently accepted methods of ferritin conjugation and staining.

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