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Proximal Tubule Function in Chronic Serum Sickness Glomerulonephritis of Rats (41990)

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Abstract. Fatal immune complex glomerulonephritis can be induced in rats by chronic intravenous administration of bovine serum albumin. There are three distinct stages, mild, moderate, and severe, in the development of renal immunopathology and pathophysiology in this model of chronic serum sickness. The work described here was undertaken to evaluate aspects of proximal tubule function in those different stages. Tissue water distribution, oxidative metabolism, and transport of representative organic anions and cations were measured in renal cortical slices. In mild chronic serum sickness all functions were normal except the transport of *p*-aminohippurate (PAH, organic anion), which was significantly decreased. This decrease appeared to be attributable to immunization with Freund's adjuvant. In the moderate stage of chronic serum sickness, proximal tubule functions and morphology appeared essentially normal. Only Na–K-ATPase activity was somewhat lower than in controls. However, proximal tubule dysfunction was a feature of severe chronic serum sickness. A significant inhibition of anion and cation transport was observed. Reduction in transport functions occurred together with impaired oxidative metabolism and severe reduction in Na–K-ATPase activity. Abnormalities of mitochondrial structure, a decrease in number of mitochondria, and a significant increase in intracellular H_2O content provided additional evidence of degenerative changes in proximal tubule cells during the severe stage of chronic serum sickness. It was concluded that decreased transport of organic ions by the basolateral membrane in proximal tubules of rats with severe chronic serum sickness resulted from a breakdown in the metabolic machinery of the tubule epithelium rather than a specific injury to organic ion transport systems.

Chronic intravenous injection of heterologous serum proteins produces immune complex glomerulonephritis in rats and rabbits (1, 2). Deposits of immunoglobulin, complement, and antigen accumulate within the mesangium and capillary wall of the glomerulus, leading to extensive damage. In a study relating immunopathology to pathophysiology, three discrete stages were identified in the natural history of chronic serum sickness glomerulonephritis of rats (3). At first, in mild chronic serum sickness, immune deposits were limited to the glomerular mesangium; the histology and function of the kidney were essentially normal. As the disease progressed, large amounts of immune complexes were deposited in the peripheral capillary wall of the glomerulus. In moderate chronic serum sickness those deposits were accompanied by proteinuria, although most other aspects of kidney function were within normal limits. It was only in the severe stage of chronic serum sickness, in which diffuse proliferative lesions developed in glomeruli, that a significant compromise of whole kidney function became apparent. Despite evidence of impairment of kidney function, including proteinuria, reduced glomerular filtration rate, and decreased sodium excretion, there was little evidence of proximal tubule insufficiency. Measurements of glucose excretion and fluid reabsorption did not suggest that abnormalities of proximal tubule function resulted from chronic serum sickness. However, throughout the study, emphasis was placed primarily on aspects of glomerular, rather than tubular, function. In other studies of experimental chronic serum sickness and in systemic lupus erythematosus, the human disease with which it is most often compared, it has been shown that tubules, as well as glomeruli, may suffer severe histological damage (4, 5).

The work described in this report was undertaken to assess in detail the relationship of function to pathology in proximal tubules of rats with chronic serum sickness nephritis. Specific organic anion and cation transport

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functions were measured in each of the stages of disease by means of the tissue slice technique of Cross and Taggart (6). Collapse of the proximal tubule lumen in the kidney tissue slice precludes measuring transport properties of the brush border membrane; the uptake of organic ions by tissue slices is presumed to reflect activities of the basolateral membrane only (7). Oxygen consumption of the slice, Na–K-ATPase activity, and tissue distribution of water were also analyzed, in order to detect possible alterations in cell metabolism that could affect organic ion transport by the basolateral membrane.

Methods. *Animals.* Female LEW rats weighing approximately 125 g, purchased from Charles River Breeding Laboratories (Wilmington, Mass.), were used in this study.

Immunizations to produce chronic serum sickness (CSS). To induce CSS, rats were given two sc injections of 3.0 mg bovine serum albumin (BSA) in Freund's adjuvant at 2-week intervals. Daily iv injections of 2.0 mg BSA in 0.4 ml isotonic saline were introduced gradually by a scheme described in detail elsewhere (2). Two groups of rats served as controls: (1) age-matched normal rats and (2) rats given sc injections of adjuvant alone. Titers of circulating antibodies to BSA were measured weekly by Ouchterlony immunodiffusion tests (2). Urinary protein excretion was determined by means of a biuret test.

Determination of the stage of CSS. Determination of the stage of CSS represented by each animal was made according to criteria described in detail in an earlier publication (3). Rats were 3 months old when daily iv injections to produce CSS were begun. For 1 month after the start of daily iv injections of BSA, all rats maintained normal urinary protein excretion (<10 mg/24 hr) and exhibited only a mesangial distribution of immune reactants. Those animals had mild CSS. Proteinuria developed in the second month of daily iv injections when the rats were approximately 4 months old. Animals considered to have moderate CSS were those exhibiting proteinuria (>50 mg/24 hr) for 2 to 3 weeks, which were, therefore, between 4 and 5 months old. Immune deposits in the kidneys of rats with moderate CSS were located predominantly in the glomerular capillary wall. Continuation of daily immunization produced severe CSS in the third month of BSA injections. Rats with severe CSS had persistent proteinuria (>50 mg/24 hr) for more than 6 weeks. In addition, severe CSS was characterized by the formation of ascites fluid and extensive accumulation of immune deposits in the glomerular capillary wall. There was evidence of capillary loop necrosis. Those severe manifestations of renal disease (fluid retention and glomerular necrosis) were not observed in moderate CSS. Because of the prolonged immunization required to produce severe CSS, rats in that stage of disease were 5 to 6 months old.

Immunofluorescence tests. Kidney tissue samples for direct immunofluorescence tests were obtained and frozen when kidney slices were prepared for function tests. Details of the procedures used for immunofluorescence tests in our laboratory have been published elsewhere (4, 8). FITC-conjugated antisera to BSA, rat immunoglobulins, and rat complement were purchased from Cappel Laboratories, Cochranville, Pennsylvania.

Histopathology. To evaluate tubule morphology in detail, several rats ($n \geq 3$) in each of the three stages of CSS were killed especially for study by light and electron microscopy. To achieve optimal fixation and preservation of the morphology of proximal tubules, the left kidney was fixed *in situ* by perfusion with 1% glutaraldehyde in modified Tyrode's buffer (9). Some pieces were embedded in paraffin, cut to 4 μm thickness, stained with hematoxylin–eosin, and examined with the light microscope. Other pieces of each kidney were postfixed in 1% osmium tetroxide, embedded in Epon–Araldite, and prepared, by staining with lead hydroxide and uranyl acetate, for examination with the electron microscope (Siemens 101).

Slice uptake. Fresh kidneys were obtained from rats in different stages of CSS, age-matched normal rats, and rats immunized with adjuvant alone. The animals were killed by cervical dislocation. The renal artery was perfused with ice-cold chilling solution (130 m*M* NaCl, 20 m*M* KCl, and 1.5 m*M* $CaCl_2$) to remove blood. Slices of renal cortex (0.2–0.4 mm thick, weighing 50–100 mg) were cut by hand and stored in isotonic chilling solution prior to incubation.

The degree to which renal cortical tissue could accumulate various organic ions was assessed according to the method of Cross

and Taggart (6). Briefly, slices were incubated for 60 min at 25°C in 3 ml of a modified Cross–Taggart incubation medium (140 m*M* NaCl, 10 m*M* KCl, 1.5 m*M* $CaCl_2$, 5 m*M* Na phosphate; pH 7.4) containing the organic ion under study. Tetraethylammonium (TEA) and *p*-aminohippurate (PAH) were selected as a representative organic cation and anion, respectively. The initial concentration of PAH was 7.5×10^{-5} *M* and of TEA, 1.0×10^{-5} *M*. Trace amounts of the respective ^{14}C-labeled organic ion (New England Nuclear, Boston, Mass.) were also added. In separate experiments to estimate the extracellular fluid space, trace amounts of ^{14}C-labeled inulin (Amersham, Arlington Heights, Ill.) were also added to the medium. The incubation was performed in a Dubnoff metabolic shaker (100 cycles/min) with a 100% oxygen atmosphere. After 60 min incubation, tissues were removed from the medium, blotted on filter paper, and weighed. Slices used for organic ion uptake studies were digested in 1 *N* NaOH for at least 20 min at 60°C; an aliquot of the medium and tissue digest (neutralized with HCl) was analyzed for [^{14}C]PAH or -TEA. Slices used for determination of tissue water distribution were dried at 95°C for 24 hr by which time they had reached a constant weight. The tissue water content was determined from the difference in weight before and after drying. Dried tissue samples were weighed and extracted with 0.1 *N* HNO_3 for 48 hr. Aliquots of the medium and the nitric acid extracts were analyzed for [^{14}C]inulin. The ^{14}C activity was measured by means of a Beckman 350 scintillation spectrometer, as described elsewhere (10). The uptake data are expressed as the ratio of the tissue concentration of compound (moles/g wet tissue) divided by that of the medium (moles/ml medium), i.e., slice to medium (S/M) ratio. The S/M inulin ratio was used as a measure of the extracellular fluid space of the slice.

Na–K-ATPase activity. The Na–K-ATPase activity was determined in a crude homogenate of renal cortex, as described elsewhere (11). Briefly, tissue was homogenized in an ice-cold solution containing 0.25 *M* sucrose, 30 m*M* histidine, and 0.2% sodium azide (pH 7.4). The amount of inorganic phosphate liberated in the presence (total ATPase) and absence (Mg-ATPase) of KCl was determined. The difference was attributed to Na–K-ATPase. Total ATPase activity was measured for 10 min at 37°C in an incubation medium containing 5 m*M* ATP, 5 m*M* $MgCl_2$, 100 m*M* NaCl, 20 m*M* KCl, 0.5 m*M* EDTA, and 20 m*M* Tris. The incubation medium for the determination of Mg-ATPase activity was identical, except for the omission of KCl and the inclusion of ouabain (0.1 m*M*). The concentrations of inorganic phosphate and tissue protein were analyzed by the method of Fiske and SubbaRow (12) and Bradford (13), respectively.

Oxygen consumption. The O_2 consumption of renal cortical slices was assessed by measuring the change in O_2 saturation of 5 ml of well-stirred incubation medium containing approximately 50–100 mg of tissue (14). This was accomplished using an oxygen monitor with Clark-type oxygen electrodes (Model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio). Electrode drift in the absence of tissue was measured and subtracted from the change in saturation observed with tissue present. The change in O_2 saturation was converted to microliters of O_2 consumed per milligram of tissue per hour, taking the solubility of O_2 in normal Ringers to be 0.0375 μl O_2/ml/Torr at 25°C (International Critical Tables, 1928).

Statistics. A minimum of seven rats was used for each determination. The data were analyzed statistically using Student's *t* test, either paired or unpaired, depending on experimental design.

Results. Because the natural course of chronic serum sickness, from initial immunization to the terminal stage, covers a large fraction of the normal life span of a rat, all physiological parameters were evaluated as a function of age. The range of values ($\bar{x} \pm 1$ SE) obtained from normal rats is indicated in each figure (shaded area).

Control groups. Although O_2 consumption and Na–K-ATPase activity did not change with age in normal, untreated female LEW rats (Fig. 1), PAH transport was observed to decrease significantly (3.7 ± 0.2 vs 2.7 ± 0.3, $P < 0.01$) from 3 to 9 months (Fig. 2). In contrast, the uptake of TEA increased (14.7 ± 0.8 vs 17.5 ± 0.7, $P < 0.02$) over the same period (Fig. 2). Rats immunized with adjuvant alone did not differ from normal in any parameters except PAH transport and Na–K-ATPase activity (Figs. 1 and 2). Two weeks

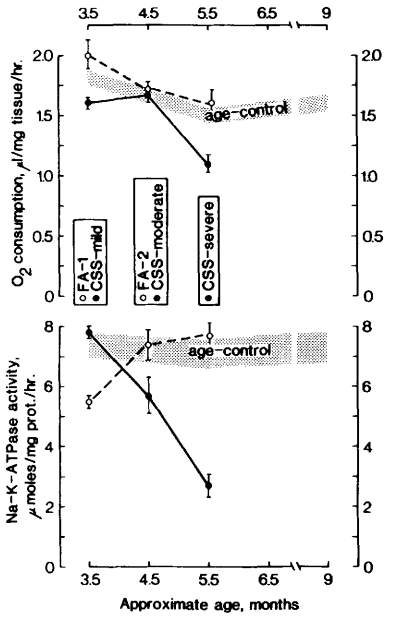

FIG. 1. O_2 consumption (upper graph) and Na-K-ATPase activity (lower graph) in mild, moderate, and severe stages of chronic serum sickness (CSS). In rats immunized with Freund's adjuvant (FA) alone, both parameters were measured at times corresponding to mild, moderate, and severe CSS. In normal, unimmunized rats, O_2 consumption and Na-K-ATPase activity were measured at different ages, covering a range corresponding to the natural history of CSS. Mean values (±1 SE) for these age-matched controls are indicated by the shaded area on the graph. A minimum of seven animals was used to obtain each mean value.

after the second adjuvant administration both functions were found to be significantly decreased (3.7 ± 0.2 vs 2.5 ± 0.3, $P < 0.01$, for S/M PAH, 7.4 ± 0.4 vs 5.5 ± 0.2, $P < 0.001$ for Na-K-ATPase). A return to normal activity was noted 1 month later.

The average ratio of total kidney weight to total body weight was 0.8–1.0% in all control groups. The average tissue water content was 75.6 + 0.3% ($n = 8$). No changes in those values were observed with advancing age or adjuvant administration.

The morphology of glomeruli and proximal tubules was completely normal in all control groups.

Mild CSS. In rats with mild CSS, TEA transport, O_2 consumption, and Na-K-ATPase activity were normal (Figs. 1 and 2). However, PAH transport (Fig. 2) was reduced (2.4 ± 0.3 vs 3.7 ± 0.2, $P < 0.01$) to the degree observed in animals immunized with adjuvant alone (see above). Minor changes in glomeruli, typical of the initial stages of immune complex glomerulonephritis were noted (2). The morphology of proximal tubules in kidneys from rats with mild CSS was essentially normal (Fig. 3).

Moderate CSS. In the first few weeks after the onset of proteinuria, tubule transport functions and O_2 consumption were not different from normal. A slight reduction in Na-K-ATPase activity (5.7 ± 0.6 vs 7.1 ± 0.5, $P < 0.05$) was detected (Fig. 1). Granular immune deposits were observed in the glomerular capillary wall as well as the mesangium. Proliferative lesions were noted in glomeruli. The morphology of proximal tubules in rats with moderate CSS was not qualitatively different from that observed in the mild stage of disease. Microvilli and

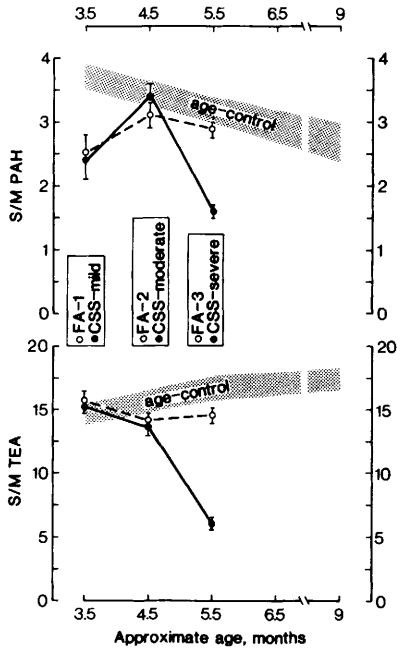

FIG. 2. PAH uptake (upper graph) and TEA uptake (lower graph) in mild, moderate, and severe stages of chronic serum sickness (CSS). Uptake is expressed as the slice to medium ratio (S/M) of organic ion concentration (see Methods). In rats immunized with Freund's adjuvant (FA) alone, uptake of PAH and TEA was measured at different ages, covering a range corresponding to the natural history of CSS. Mean values (±1 SE) of PAH and TEA uptake in those age-matched controls are indicated by the shaded area on the graph. A minimum of seven animals was used to obtain each mean value.

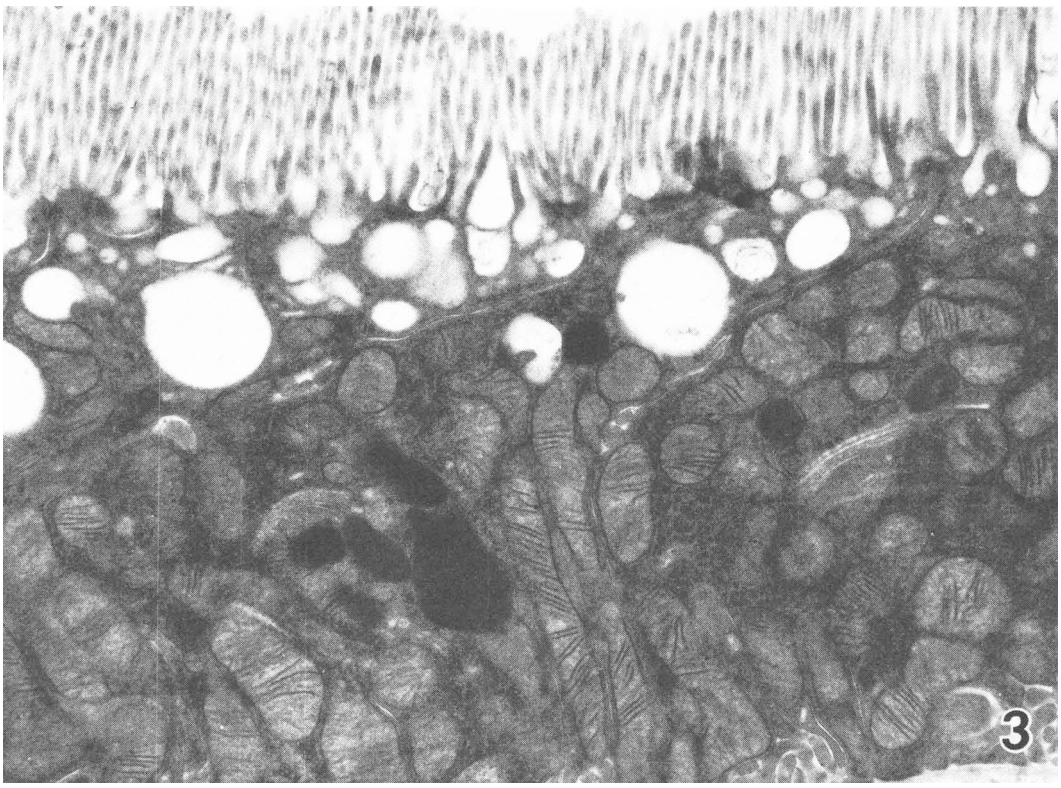

FIG. 3. An electron micrograph of part of a proximal tubule cell from a rat with mild chronic serum sickness. Microvilli and mitochondria appear normal. (×23,500)

basolateral infoldings were essentially normal in appearance. Vacuoles, absorption droplets, and lysosomes were somewhat increased compared to normal as has been reported previously (15).

Severe CSS. In the final stage of CSS, significant severe impairment of both TEA (6.0 ± 0.2 vs 17.5 ± 0.7, $P < 0.001$) and PAH (1.6 ± 0.1 vs 3.2 ± 0.2, $P < 0.001$) transport functions was measured (Fig. 2). In addition, O_2 consumption was reduced (1.1 ± 0.10 vs 1.5 ± 0.06, $P < 0.01$) and Na–K-ATPase activity was far below normal (Fig. 1).

Although the ratio of kidney weight to body weight remained normal (0.8–1.0%) in mild and moderate CSS, a significant increase in that ratio was detected in severe CSS (1.3 ± 0.07, $P < 0.001$) providing confirmatory evidence of the kidney enlargement described previously to be a feature of severe CSS in LEW rats (3). The increased ratio could be attributed to an increased H_2O content of the kidney tissue in severe CSS compared to age-matched controls (80.8 ± 0.3 vs 77.7 ± 0.34%, $P < 0.001$). An increase in intracellular H_2O content appeared to account for the total increase in tissue H_2O content, as extracellular water, estimated by the S/M inulin ratio, was not different in severe CSS (0.36 ± 0.01) and age-matched controls (0.37 ± 0.01).

Heavy granular to ribbon-like deposits of immunoglobulins and complement were detected in the glomerular capillary wall; focal weak granular deposits of immunoglobulins were occasionally observed along the tubular basement membrane but were not an outstanding feature of tubule immunopathology in these rats. Proliferative glomerulonephritis, characteristic of severe immune complex-mediated injury, was present. Glomeruli were enlarged with focal areas of necrosis. Proximal tubule cells were flattened, with some reduction in the height, but not in the number of microvilli (Fig. 4). There was focal reduction

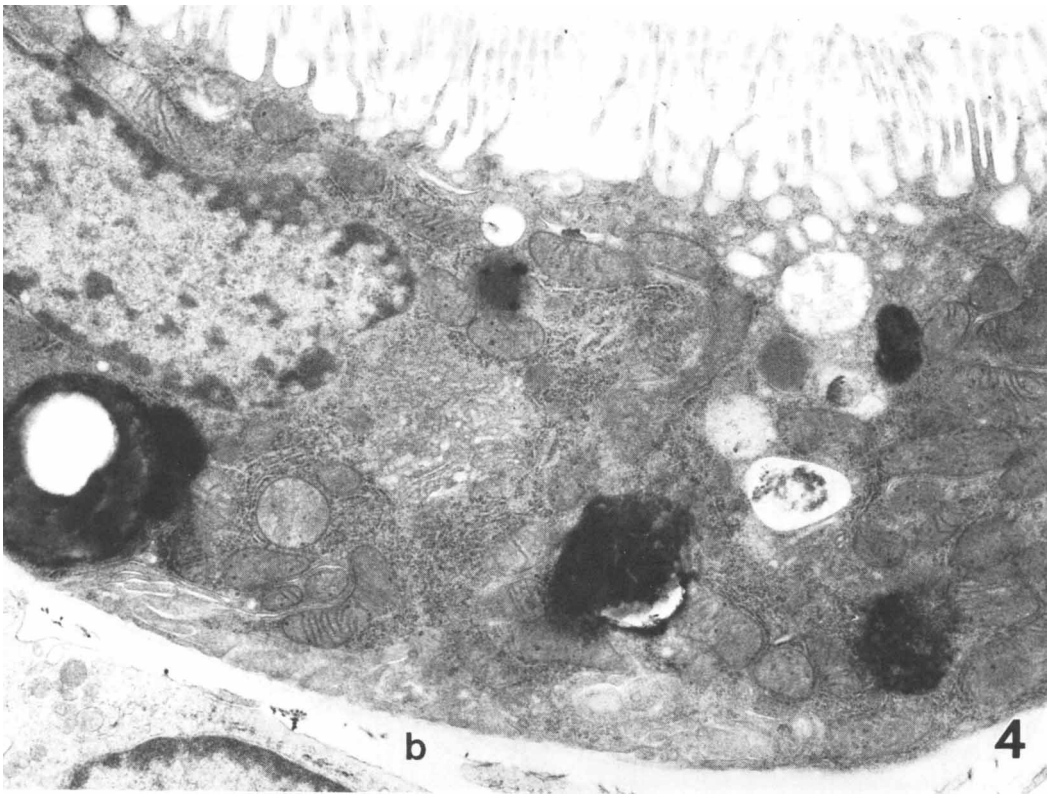

FIG. 4. An electron micrograph of part of a proximal tubule cell from a rat with severe chronic serum sickness. The number of mitochondria is decreased. Some mitochondria are swollen and show a decreased number of cristae. Several cytophagosomes containing lipids are present. The tubular basement membrane is indicated by "b". (×23,500)

in the number of basal infoldings and some decrease in the length of basolateral membrane ramifications. The most striking abnormality was a reduction in the number of mitochondria (Figs. 4 and 5). Widespread degenerative changes in the structure of mitochondria included swelling and loss of cristae (Fig. 5).

Discussion. Secretion of organic ions by epithelial cells into the proximal tubule fluid occurs by active transport. Although different carrier proteins appear to be responsible for the transport of organic anions and cations, general depression of tubule cell metabolism will lead to impaired transport of all organic ions. In severe chronic serum sickness, a great reduction was observed in the uptake of both organic anions and cations. That impairment in active transport probably resulted from the breakdown of proximal tubule cell metabolism rather than specific alterations in membrane transport systems.

The inhibition of organic ion uptake, detected only in the most severe stage of glomerulonephritis, occurred together with a large reduction in the supply of energy available for all cell functions, as estimated by O_2 consumption. Reduction in the number and degeneration of the structure of mitochondria, also observed only in rats with severe chronic serum sickness, provided additional evidence of serious damage to the machinery providing energy for all metabolic processes, including active transport. Although inhibition of the active transport of organic ions in severe chronic serum sickness appeared to be closely linked to deterioration of the energy supply, it remains possible that alteration of certain membrane components, specifically related to organic ion transport, may have contrib-

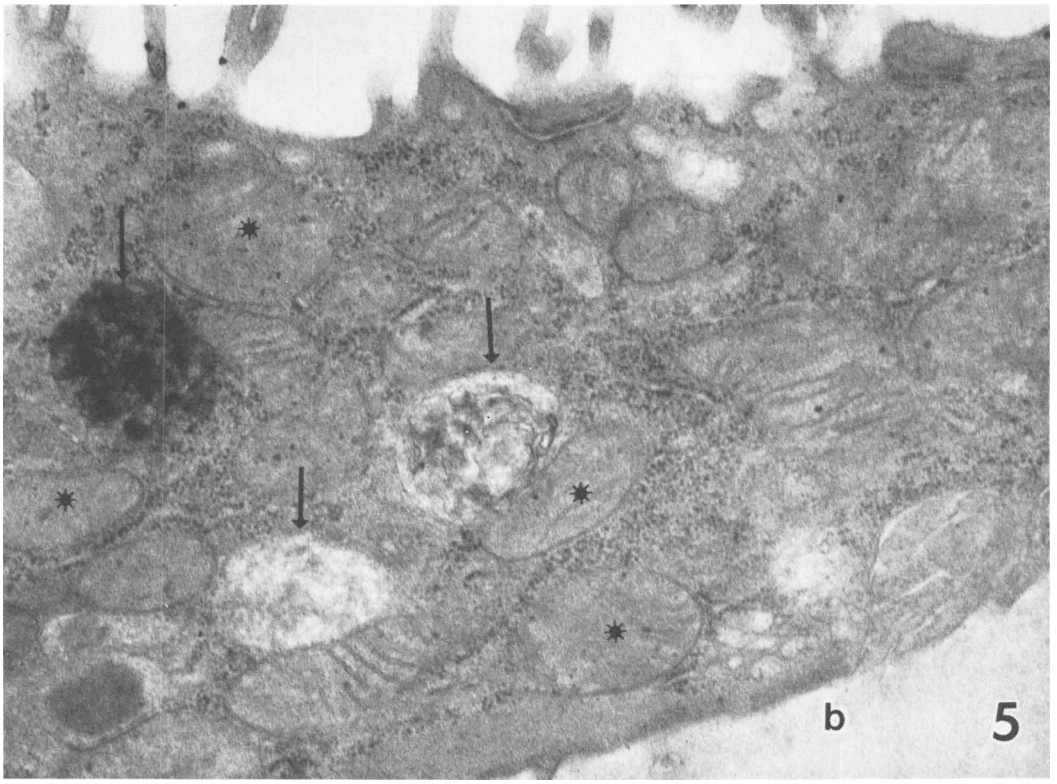

FIG. 5. A higher magnification electron micrograph of part of proximal tubule cell from the same rat with chronic serum sickness shown in Fig. 2. Some mitochondria, indicated by asterisks, exhibit extensive loss of cristae. Arrows indicate vacuoles containing myelinic figures, probably resulting from degeneration of mitochondria. (×38,000)

uted, at least in part, to the functional impairment that has been measured.

Any possible cause and effect relationship between reduction in the available energy supply and structural changes in mitochondria cannot be evaluated from experiments reported here. Measurements of function are usually thought to be more sensitive than observations of morphology for detection of tissue injury. However, except for a very modest reduction of Na–K-ATPase activity in moderate chronic serum sickness, no significant compromise of either tubule transport activity or overall cell metabolism was observed to precede damage to the ultrastructure of tubule cells. Careful investigation of this topic in the future will require quantitative morphometric analysis together with functional measurements of isolated mitochondria in each of the different stages of serum sickness. For example, it will be important to evaluate separately each segment of the proximal tubule with respect to mitochondrial morphology, distribution, and number.

An analysis of kidney function in rats with chronic serum sickness, based on micropuncture studies, has led to the proposal that transitions from one stage to another do not occur gradually, but rather as discrete, sudden events (3). Observations of proximal tubule function, made in these tissue slice experiments, are consistent with that hypothesis. Large differences were observed between moderate and severe serum sickness in PAH and TEA transport, O_2 consumption, Na–K-ATPase activity, and tissue H_2O content. The small standard error of the mean values for each of those parameters attests to the homogeneity of the groups, and suggests that individuals are only infrequently encountered whose kidney function is intermediate between the moderate and severe stages of

serum sickness. The conclusion, that deterioration of kidney function in experimental immune complex glomerulonephritis may occur abruptly rather than gradually, has important implications for our understanding of similar disease processes in man.

In a study of kidney function using micropuncture techniques, depressed PAH clearance in severe CSS was attributed to decreased renal plasma flow (3). Although no direct measurements of renal blood flow had been made, the large decrease in glomerular filtration rate that characterizes severe CSS was consistent with the conclusion that renal blood flow was significantly compromised. Evidence provided by the experiments reported here suggests that depressed proximal tubule cell metabolism may have also contributed to the observed reduction of PAH clearance in severe CSS. Renal blood flow depression, inferred from PAH clearances, may have been overestimated. To clarify that point, direct measurements, with a flow probe on the renal artery, are needed.

Given our present appreciation of kidney function in the various stages of chronic serum sickness, it is only possible to speculate about the factors responsible for the deterioration of tubule function that characterizes the severe stage of disease. Exposure of kidney slices to uremic serum *in vitro* has been described to produce an inhibition of PAH uptake that is not associated with depression of either TEA transport or oxidative metabolism (17). From experiments with isolated tubules it has been postulated that small molecules are present in uremic serum, probably arylacids, which could compete with PAH uptake (18). As an elevated plasma creatinine concentration distinguishes severe from moderate chronic sickness (3), it is possible that a similar mechanism, with more profound effects *in vivo* than those observed *in vitro,* is responsible for the deterioration in tubule cell function observed in severe serum sickness. Alternatively, other unidentified factors in uremic serum, with antimetabolic or cytotoxic activity, could lead to tubule cell degeneration resulting in reduced respiratory capacity with associated defects in transport function.

The present study confirms an earlier observation that PAH uptake decreases with age in normal rats (16). It has also revealed that TEA uptake tends to increase with age, adding another element to the differences distinguishing anion and cation transport processes.

Immunization with adjuvant alone was followed by a moderate, transient inhibition of the transport of PAH, but not TEA. This inhibitory effect of adjuvant treatment is the most probable explanation for a reduction in PAH transport observed in the mild stage of serum sickness. Although immunization with adjuvant alone also had a modest effect on Na–K-ATPase activity, the inhibition was not accompanied by reduced O_2 consumption, a parameter that is mainly influenced by enzyme-mediated transport of Na and K. Decreased PAH transport in rats with mild serum sickness was not associated with a lower Na–K-ATPase activity, indicating that the adjuvant may have a direct, specific effect on organic anion transport. No morphological abnormalities of the cell membrane were noted that could be correlated with this phenomenon.

In conclusion, severe impairment of proximal tubule function may develop in chronic serum sickness, a disease in which glomeruli, and not tubules, are the major target of immunological damage. Tubule dysfunction in chronic serum sickness appears to be secondary to deterioration of the metabolic machinery of cells of the proximal tubule epithelium. That deterioration occurs only in the most severe stage of glomerulonephritis and may result from systemic effects of the glomerular dysfunction that distinguishes severe chronic serum sickness from earlier stages of the disease. Similar impairment of proximal tubule function may contribute to renal insufficiency that may develop in patients with systemic lupus erythematosus, a human immune complex disease that has many features in common with experimental chronic serum sickness. The possible contribution of tubule dysfunction to the overall renal insufficiency that may accompany severe glomerulonephritis has not been carefully analyzed. Furthermore, although degeneration of tubule structure and deterioration of tubule function may frequently be associated with progressive glomerular disease in man, the mechanisms responsible for, and the natural history of, that association are poorly understood. Using the rat model of CSS it

should be possible to examine those issues more critically than has previously been possible. From the studies reported here, for example, it is clear that proximal tubule transport functions, energy metabolism, and morphology may remain completely normal in the face of glomerular disease of substantial severity, represented by moderate CSS. Clarification of the nature of tubule dysfunction that may be secondary to glomerular damage will also provide a background for the evaluation of those aspects of tubule dysfunction that may result from a specific injury to tubules, such as that occurring in Heymann nephritis (15).

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