increased the rate of oxygen consumption slightly during the first hour. This was followed by a gradual decrease in rate until after 11 hours, then there was complete inhibition.

8. Toxins from *Streptococcus pyogenes* markedly inhibited the rate of oxygen consumption although there was no hemolysis.

Received October 27, 1949. P.S.E.B.M., 1949, v72.

Studies on Proteinuria in the Rat. (17515)

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Most investigations of protein excretion in the urine have assumed that protein in quantity is an abnormal constituent of the urine. It has furthermore been commonly assumed that mammalian kidneys extract from plasma a protein-free fluid in each species. The existence of a normal proteinuria in the Slonaker strain of white rats has been described qualitatively by Addis.(1,2) Protein excretion has been studied by Smetana(3) by injecting dye-labelled foreign protein into the blood of several mammals and observed deposition of dyed particles within the lining cells of the proximal convoluted tubules. Oliver(4,5) used similar technics both *in vivo* and in perfused isolated kidneys, with similar results. Dock(6) injected Evans blue intraperitoneally in rats and subsequently observed dye deposition in the renal tubules. The present studies again demonstrate a normal proteinuria, both albuminuria and globulinuria, under a variety of environmental conditions, in the albino rat. The passage of plasma protein through the rat kidney is studied by labelling the native plasma protein with Evans blue, and examining, in fixed tissues, the disposition of the Evans blue-protein complex.

Procedures. A. *Protein determinations.* Urine was collected from rats, weighing from 180 to 250 g each, while in individual metal metabolism cages. During periods of urine collection, none of which was longer than 24 hours, no food was present in the cage. The animals studied included 14 male animals of the Wistar strain, on "Rockland Rat Diet", and located in a constant temperature room at 27° C; 12 animals, 8 males and 4 females, of the Sprague-Dawley-Holtzman strain, at ordinary room temperature, that had been on a diet of fortified powdered milk for a month; and 8 male rats of the Sprague-Dawley-Holtzman strain that had been subjected to a constant air temperature of 4° C for 3 months.

a) The urine was collected individually, centrifuged clear, and tested for protein content by heating and adding acetic acid. The precipitate so obtained was spun down washed and tested by the biuret test, xanthoproteic test, charring, and Millon test.

b) Qualitative protein fractionations of 72-hour urine specimens from 9 normal rats were performed. Urine was saturated with ammonium sulfate and a precipitate was interpreted as albumin. Globulin was precipitated by half saturation with ammonium sulfate. In both instances the precipitates were tested with protein reagents as above.

c) Quantitative protein measurements were made on 24-hour pooled urine from 5 normal male rats. Total protein was determined by precipitating with trichloracetic acid, centrifuging, dialyzing against distilled water at 5° C till chloride-free, and drying to con-

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stant weight at 95° C. Its globulin was determined by precipitating with equal parts of 4 M potassium acid phosphate solution (by the method of Butler),(7) centrifuging, and redissolving in physiological saline, then dialyzing against distilled water until chloride- and phosphate-free. At completion of the dialysis the globulin had reprecipitated. Albumin was determined by precipitating from the supernatant urine, after the globulin was precipitated, with an equal part of 20% trichloracetic acid, centrifuging, resuspending the precipitate in water, and dialyzing until chloride- and phosphate-free.

B. *Evans blue (T-1824) determinations.* Evans blue forms a complex with plasma proteins. This property is made use of in the present experiments to label plasma proteins in their passage through the kidneys. Rawson(8) ascertained that, at pH 7.4, in solutions of human or canine plasma albumin, up to 14 molecules of Evans blue are linked to one molecule of plasma albumin. The greatest stability of the dye-protein complex is attained up to ratios of 8 molecules of Evans blue per molecule of plasma albumin. At such concentrations, the dye is wholly and preferentially bound by the albumin fraction, and in increasing proportions as the concentration of the dye increases. If the dye concentration is increased sufficiently, the dye may also be bound by the globulin fraction, but preferentially by the alpha-globulin. That, in the equilibrium system between the protein-dye complex and the free dye and plasma protein, the concentration of free dye is infinitesimal, is indicated by the observation of Rawson(8) that ultracentrifugation of a solution of dye-protein complex centrifuges down a blue-stained layer and leaves an unstained supernatant. Trypan blue is also bound by plasma protein; this complex is more unstable than the Evans blue-protein complex. When the trypan blue-protein complex is dialyzed in a cellophane bag the trypan blue is separated from the protein and stains the cellophane. Evans blue, however, remains bound to protein under such circumstances, and leaves the dialyzing membrane colorless. The Evans blue molecule has 4 sulphonic-acid groups, and has a molecular weight of 960. The attachment of several such dye molecules to a molecule of plasma protein modifies the charge and the weight of the colloidal particle, and the addition of Evans blue to a solution of albumin has been found to lower appreciably the isoelectric point of the protein.(8)

A series of 8 male rats were given intracardiac injection of 0.5 ml of 5% Evans blue solution. The blood volume of the rat is reported as 4 ml per 100 g body weight,(9) of which approximately one half is plasma. Plasma protein concentrations are 5 to 7% (10) with about a 5 to 2 ratio of albumin to globulin.(10) It is readily calculated that, in the dose administered, all the dye combined with the plasma proteins. Starting 3 hours after administration of the Evans blue, urine was collected for 24 hours, observed qualitatively for color, and tested for protein with heat and acetic acid. The animals were then decapitated and autopsied. Kidneys were formalin-fixed in two series, unstained and stained with hematoxylin and eosin respectively, so as to facilitate study of both the course of the dye and of any possible renal damage that might have resulted from the injected Evans blue. In order to observe *in vitro* formation of the dye-protein complex in protein-containing urine, one drop of 5% Evans blue solution was added to the 24-hour urine sample of each of 4 untreated normal rats, and within two minutes this urine was tested for protein, as above.

Next, a second series of 13 male rats received intracardiac injections of 0.4 ml of 5% Evans blue. These rats were all decapitated, 7 rats at 10 minutes after injection of Evans blue; and 6 rats, 90 minutes after injection. Kidney slices from each of these rats were hardened in formalin solution for

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the preparation of frozen sections.

Results. A. *Urinary proteins.* Two male rats maintained in the constant temperature room at 27°C gave negative tests for urinary protein. The other 28 rats, including animals of the 2 strains studied, and animals maintained at the several above-mentioned temperatures and diets, showed proteinuria. The amounts of protein, as observed grossly by the relative degrees of turbidity or flocculation on treatment of the urine with heat and acetic acid, varied in all groups, except in the group acclimatized to cold, from a moderate turbidity to a heavy flocculent precipitate. Urine samples collected from the animals kept in the cold room all showed heavy flocculent precipitates. All urine specimens fractionated by ammonium-sulfate precipitation contained both albumin and globulin. The urinary proteins were determined as 6.6 mg of protein in an average urine quantity of 8 ml per 210 g rat per 24 hour period. As separately determined, 3.0 mg of globulin, and 3.3 mg of albumin were excreted per rat in this period. No attempt was made to estimate the variation of proteinuria in an individual, but there was an appreciable variation in the bulk precipitate in the same rat in several 24-hour qualitative protein determinations.

B. *Evans blue studies.* Several rats, not included in the numbers reported above, died within a few minutes of injection with Evans blue, presumably from cardiac or pulmonary injury. All the 24-hour urine samples of the first group of 8 rats injected with Evans blue were blue-stained. The urine of all these animals was positive for protein as it had been in each animal when tested before the dye injection. The protein precipitate in all instances showed blue, and, when allowed to settle, was found to have withdrawn the dye from solution, the supernatant retaining no visible tinge. Normal rat urines to which a drop of Evans blue was added *in vitro* had their proteins precipitated with heat and acetic acid; precipitates so obtained were blue, and the supernatant showed no blue stain. All injected animals were decapitated. On macroscopic examination, in 21 animals injected with Evans blue, the tissues were, to a greater or lesser degree, blue stained. The liver contained the most deeply dyed tissue. Kidney sections showed heavily stained cortical papillae which were sharply demarcated from the lightly tinted medullary tissue. Selective staining of the cortex was most intense in the animals killed after 24 hours, and least apparent in the specimens taken ten minutes after injection of the Evans blue. Microscopic studies of the kidney tissues of Evans blue-treated animals revealed several findings (see Table). All fixed hematoxylin and eosin stained sections taken from animals even 24 hours after Evans blue injection showed no morphological renal damage.

(a) In frozen kidney sections cut from animals killed 10 minutes after injection, blue-staining granules were observed, all superficially distributed in the cytoplasm (that is, toward the tubular lumen) of proximal tubular cells of many nephra. Several sections contained proximal tubular cells with a diffuse blue stain toward their luminal surface, as well as a similarly distributed, granularly disposed stain. The glomerular and capsular spaces were uniformly and distinctly clear of visible material, blue or otherwise. Blue-stained casts were found in the proximal tubules and in the collecting tubules of all specimens.

(b) In frozen sections of kidneys removed from animals killed 90 minutes after Evans blue injection, blue-staining granules were again observed in the cytoplasm of proximal tubular cells. Now, however, no diffuse distribution of the blue dye was apparent. Blue-staining granules in the cytoplasm were distributed superficially as well as more deeply throughout the cells, although the stained particles seldom approached the basement membrane, and were more concentrated toward the lumen. Cells in this group of sections contained appreciably more dye than in the ten-minute group. Also, the dyed particles were not merely in the shape of irregular granules of varying size as in the 10-minute sections, but frequently had a filamentous and often branching appearance, such as to suggest the possibility that the dye had become adherent to mitochondria.

(c) Kidney sections of animals killed 24 hours after injection received no post mortem

TABLE I.
Distribution of Evans Blue in Kidney Sections Taken 10 Min., 90 Min., and 24 Hr After Dye Injection.

Structures observed	10 min.	90 min.	24 hr
Blue-stained casts	+	+	+
Diffuse stain of superficial surface of tubular cells	+	—	—
Granular stain of tubular cells superficial	+	+	+
Luminal half of cells	—	+	+
Throughout cytoplasm	—	—	+
Stained granules in tubular cells showing various filamentous and rod-shaped forms	+	+	Indeterminate
Blue-stained arterial and arteriolar elastic membranes	+	+	+

stain, but were passed through paraffin to make permanent sections. These tissues showed the densest aggregations of intensely blue-stained particles, so numerous as to make any distinction between massed particles and single filaments very dubious. In these sections the dye was fairly evenly distributed throughout the cell, from luminal border to basement membrane (see Fig.). Again, in these sections, blue-stained casts were observed both in the proximal tubules and in the collecting tubules. Of much interest is the finding that all kidney sections contained areas of unstained proximal tubules which were estimated to be at a comparable level along the tubules as were intensely stained regions of other tubules in the same section. Hence it would seem probable that the dye-protein complex was filtered through the glomeruli in high dilution, and partly reabsorbed in the proximal tubules, possibly through the intermediation of mitochondria. Sections of the liver showed blue-dyed granules in the Kupfer cells and also showed blue-stained droplets of varying sizes in the cytoplasm of the liver parenchymal cells. Adrenal parenchymal cells and cardiac muscle fibers were unstained by Evans blue.

Discussion. Proteinuria occurs almost uniformly in normal laboratory rats. About half of this proteinuria is globulinuria. Proteinuria is a much less common finding in humans, but it does occasionally occur in the absence of demonstrable renal lesions.(11,12) An interesting parallel between rat and human proteinuria is the prevalence of tubular casts in the normal rat kidneys and the prevalence of tubular casts in the kidneys of humans known to have had gross proteinuria, whether frankly pathological or not.

The source if the urinary proteins, and the method of their passage into the urine, are two points of interest.

a. Source of the protein. The renal glomerulus has long been considered by many renal physiologists to be impermeable to plasma protein molecules. Such an attribute would make the capillaries of the glomerular tuft quite unique, as complete impermeability to protein cannot be ascribed to any other capillary bed. Thus, the human cerebrospinal fluid has a modicum of protein (20 to 65 mg %) with up to 6 mg of this as globulin. Warren(13) found the extracellular fluid of the human leg to contain 0.9% of protein. It is possible, of course, that the renal tubules secrete the urinary protein; but there has been no evidence adduced to this effect. That the protein in rat's urine is plasma protein is indicated by the passage of Evans blue into the rat's urine, as Evans blue is firmly and

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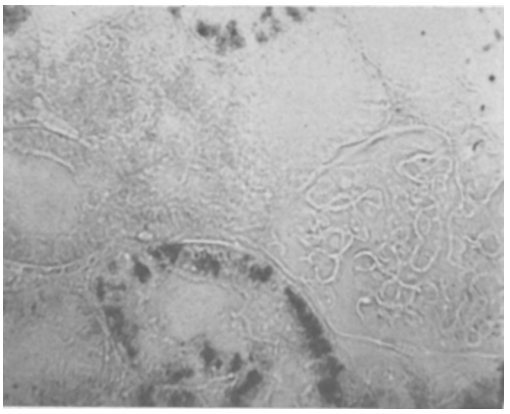

FIG. 1.
Kidney section of rat killed 24 hr after Evans blue injection (× 400). Glomerulus on right is unstained; some tubules are stained, others not.

preferentially bound to plasma protein. The data on Evans blue excretion must, however, be interpreted with reservations, as the protein-Evans blue complex represents an appreciable modification of the native plasma proteins, both as to molecular size and charge. Evans blue is a strongly acid substance and, as such, modifies the isoelectric point of the protein and might possibly modify the passage of the protein molecule across a membrane. There is also the possibility of a subtle effect of the Evans blue upon the kidney itself. However, no renal histopathology is noted after Evans blue injection.

b. Course of the protein from blood to urine. Evans blue appears earliest in the cytoplasm along the luminal borders of proximal tubule cells, and in intratubular casts, which are presumably protein in nature. Subsequently the dye pervades the cells and so approaches their basement membranes. This suggests that the tubules are absorbing the Evans blue, and, perhaps the protein with it, from the tubular lumen. If Evans blue did not pass into the tubular lumen by transfer across the tubular cells, the dye must have gained ingress by passing across the glomerular-capsular membrane, probably to be concentrated in the tubules into visible casts. Morphologically, the membrane appears to be non-secretory endothelium and would probably not disrupt the Evans blue-plasma protein complex. Also, the tubular casts were stained blue, so that the Evans blue-plasma protein complex probably remains intact at least until it reaches the lumen of the tubule. Whether the tubular cells selectively absorb the Evans blue, leaving the protein free in the tubule, cannot be ascertained on the basis of the present experimental evidence. However, should unmodified plasma protein cross the glomerular-capsular membrane in the same manner as the Evans blue-protein complex, it would seem likely, by analogy with other blood-borne substances, as glucose and sodium, which are believed to cross the membrane freely, that the protein would be at least partially reabsorbed by the tubules. And similarly, it would then seem likely that the Evans blue is absorbed in the tubules in association with plasma protein.

The absence of Evans blue stain from the parenchyma of the adrenal and the muscle cells of the myocardium is in sharp contrast to the staining of kidney tubules and liver parenchyma. The latter two tissues are believed involved in plasma protein transfer, and it is therefore unlikely that the stain of these tissues is a non-specific or a general protoplasmic reaction. That the liver and kidney react diversely with the protein complex is indicated by the different dispositions of dye within the parenchymal cells of the two organs. The Evans blue-stained particulate material within the tubular cells could be interpreted as either an aggregation of the Evans blue-protein complex, or as a staining of existing cellular structures by the Evans blue. In this connection, the similarity in form and size of the intracellular aggregates of dye to that described for mitochondria is suggestive. The total absence of Evans blue from some proximal tubules, and the heavy staining of other proximal tubules, even 24 hours after injection of the Evans blue, indicates variation in activity among the nephra themselves.

Summary. Proteinuria was found to be a usual occurrence in albino rats, with an average excretion during fasting of 3.0 mg of globulin and 3.3 mg of albumin in 24 hours. Marked variation in the daily urinary protein excretion was observed. Evidence is obtained indicating glomerular filtration and tubular

reabsorption of an Evans blue-plasma protein complex, and, by inference, of normal plasma protein. Such tubular reabsorption possibly involves the mitochondria of the tubular cells.

Dr. E. F. Adolph gave invaluable suggestions, criticisms, and encouragement. Dr. James G. Wilson and Dr. Victor M. Emmel helped with the histological interpretations; their assistance is gratefully acknowledged.

Received November 1, 1949. P.S.E.B.M., 1949, v72.

Nutritional Value of Plant Materials. II. Prevention of Acute Uremia of the Newborn Rat by Vitamin B_{12}.* (17516)

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A very high incidence of mortality during the first 24-72 hours of the life of young rats born to mothers maintained on rations containing a commercial soybean protein preparation and DL-methionine as the only source of amino acids has recently been reported from this laboratory.(1) In about 50% of the litters cast, from one to all of the young develop a characteristic syndrome terminating in early death. At birth and for the first 24-36 hours these young, on visual inspection, appear to be in every respect normal, indistinguishable from those which survive and develop normally. They are strong and nursed by their mothers until the onset of the crisis. (The support of excellent lactation has been an outstanding feature of these rations). A rapid onset of dyspnea accompanied by cyanosis, weakness and emaciation is typical of the syndrome observed. Milk is always present in the stomachs of the young and very frequently one or two loops of the small intestine contain dark material which is visible through the abdominal wall. At this stage the blood urea as determined by the method of Archibald(2) reaches values of about 150-250 mg per 100 ml as compared to about 35 mg at birth and about 50-70 mg in normal appearing rats of the same age.(3) Death of these rats usually occurs within 4-6 hours after the first symptoms can be observed, although a few individuals have survived as long as 12 hours. Without inference concerning the nature of the biochemical or pathological lesion or the ultimate cause of death the syndrome is conveniently referred to as "acute uremia of the newborn."

Other investigators have reported early deaths of young rats(4-6) and mice(7) that had milk in their stomachs and they may have been dealing with the same syndrome. Zucker and Zucker(8) observed elevated concentrations of NPN and urea in the blood of rats of post weaning age which were fed rations devoid of animal protein. McGinnis *et al.*(9) refer to observation of an increased NPN concentration in the blood of chickens maintained on rations containing plant ingredients plus vitamin free casein. In this laboratory "acute uremia of the newborn" has never been observed on rations containing either alcohol extracted casein,(1) or a com-

* Paper No. 2514 Scientific Journal Series, Minnesota Agricultural Experiment Station.

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