NATURALLY OCCURRING PROTEIN DROPLETS IN THE PROXIMAL TUBULE OF THE RAT'S KIDNEY.

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INTRACELLULAR hyaline droplets in the proximal tubules of the kidney have been described after the injection of protein solutions in the toad (Gerard, 1935), salamander (Lambert, 1936) and in mammalian species (Smetana, 1947). Since then these inclusions have been analysed by different techniques and the general view is that they represent protein material re-absorbed from the glomerular filtrate (Oliver, 1948; Rather, 1948). The more recent studies in this field have been concerned with the structural and functional relationship between these inclusions and cell organelles by phase contrast microscopy (Zollinger, 1950; Rüttimann, 1951), histochemical procedures (Oliver, Moses, MacDowell and Lee, 1954) and cell fractionation by differential ultracentrifugation and enzymic determinations (Kretchmer and Dickerman, 1954). In all the published reports the droplets studied were seen after intraperitoneal injections of mostly low-molecular proteins, although occasional reference is made to the occurrence of droplets not specifically induced by protein injections.

During preliminary attempts to induce droplets by injection of egg albumen in rats we noted the presence of large numbers of naturally occurring droplets in mature male controls but not in mature females. This observation, together with the known difference of proteinuria in the male and female rat (Bell, 1933) and the effect of androgens on the spontaneous proteinuria of rats (Sellers, Goodman, Marmorston and Smith, 1950) led us to attempt to record the occurrence of naturally occurring droplets, to find whether there was a difference in the two sexes and, if so, to see whether this difference could be correlated with the degree of proteinuria.

MATERIAL AND METHODS.

Rats.

Male and female albino rats were kept on a diet consisting of "Research" rat cubes supplemented with white bread and greens twice a week.

Protein estimation in urine.

At 8 to 9 a.m. food was withheld and the rats were allowed free access to a 10 per cent glucose in 0·5 per cent saline solution. Four hr. later they were individually placed in urine-collecting glass cages for a period of 7 to 8 hr. The collecting cages consisted of inverted 5-l. glass jars whose bases had been cut off and in which a wide-mesh wire floor supported the animal 2 in. above the neck of the bottle. Urine was collected through a glass funnel into a covered rubber-topped specimen jar after passing through a narrow-mesh wire faeces trap. All glassware was siliconed. During the period of collection the animals were allowed

10–15 ml. of the glucose solution in two doses. Emptying of the bladder was stimulated at the beginning and end of the collecting period by forced inhalation of ether.

Protein was estimated by the Biuret method (Foster, Rick and Wolfson, 1952), and a solution of crystalline bovine albumen was used as a standard.

Protein excretion was expressed as μg./100 cm.^{2} body surface/hr. Meeh's formula was used for deriving body surface from body weight ($S = KW^{2/3}$ with $K = 11{\cdot}36$) as determined by Carman and Mitchell (1926).

Microscopical preparations.

The kidneys were removed under ether anaesthesia, care being taken to avoid undue congestion during removal. They were weighed, longitudinally bisected and placed immediately in Helly's fluid. The left kidney was used for paraffin sections stained by H.E., Heidenhain's haematoxylin, phosphotungstic acid haematoxylin (P.T.A.H.), Bensley's modification of Altmann's anilin acid fuchsin, 0·5 per cent phloxine in 20 per cent alcohol using alkali alcohol differentiator and Ehrlich's haemalum nuclear stain, and Weigert's method for fibrin. Phase contrast microscopy and supravital preparation with Janus Green B were used on fresh material in selected cases. Pyridine extraction followed by Baker's acid haematin or Sudan black was used on frozen and paraffin sections.

Urine collection from the bladder.

This was carried out after injection of 0·06 mg. nembutal/100 g. body wt. and 2 ml. of 3 per cent ethanol in water/100 g. body wt. given by gastric intubation. After 1 hr. of anaesthesia, urine was collected by a suprapubic incision through a 22 gauge needle attached to a syringe.

RESULTS.

Normal rats.

Proteinuria was determined in 10 male and 10 female rats every two weeks from the ages of 30 to 120 days. Batches of 10 males and 10 females were killed every two weeks. An increase in protein excretion was found in male rats from 30 days onwards. A maximum of 180 μg./100 cm.^{2} body surface/hr. was reached by 90 days, approximately $2\frac{1}{2}$–3 times that of female rats of the same age in whom protein excretion, apart from minor fluctuations, was relatively constant and did not show the marked elevation at puberty (Figure). This difference in protein content in the urine was reflected also in urine withdrawn by bladder puncture in 10 male and 10 female rats 4 months old. The value was 201 μg. $\pm$ S.D. 35/100 cm.^{2} body surface/hr. for the male group and 71 μg. $\pm$ S.D. 25/100 cm.^{2} body surface/hr. for the female group.

Intracellular droplets mainly in the upper two-thirds of the proximal tubules were found in male rats from 60 days onwards, the earliest arranged in small groups of small droplets in occasional cells. These increased in number and size at 75 days and were found in most of the cells in the proximal tubule at 90 days. The droplets were phloxinophilic, stained with P.T.A.H., Weigert's fibrin stain, Heidenhain's haematoxylin and supravitally with Janus Green B and were prominent under the phase contrast microscope. Mitochondria did not appear to be notably deficient in cells containing droplets in the single sections studied (see Plate). After pyridine extraction the droplets were not stained by acid haematin or Sudan black, but these reactions were positive before pyridine extraction. In contrast, only occasional female rats, up to 5 in a group of 20 at 120 and 180 days showed the presence of occasional droplets in a few cells of the proximal tubules.

Thus at puberty in male rats there is a well marked increase in the degree of proteinuria and, at the same time, abundant intracellular droplets are noted in

the proximal tubules of the nephron. Females show neither a rise in proteinuria nor intracellular droplets.

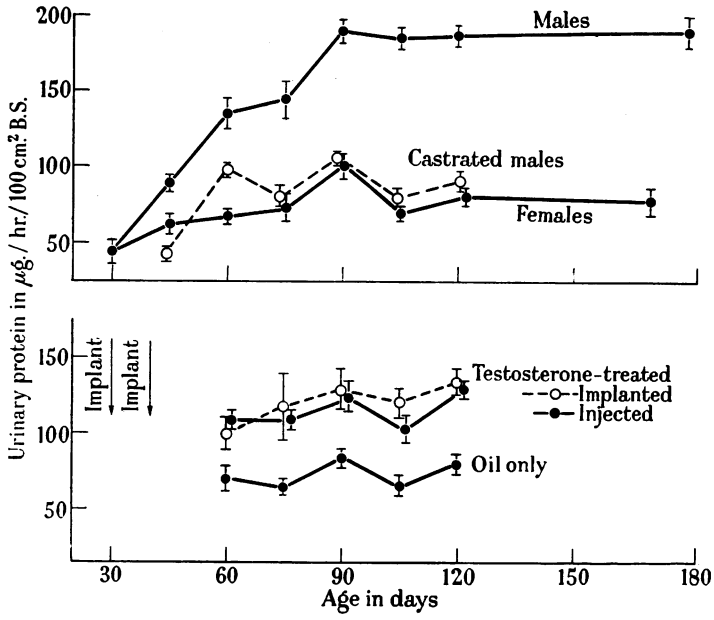


FIGURE.—Proteinuria in male and female rats.
Upper graph—males and castrated males.
Lower graph—testosterone-treated females.

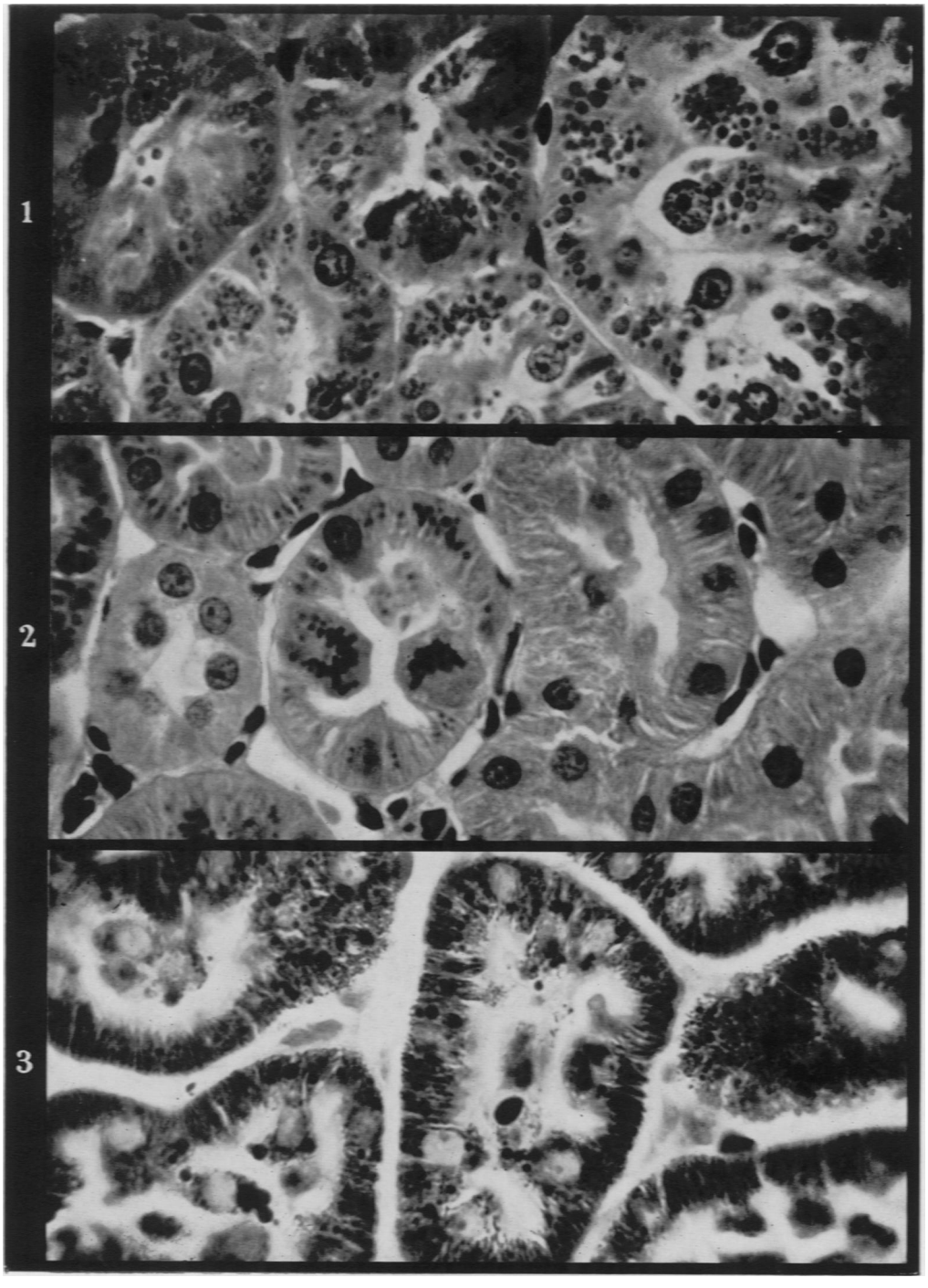
Castrated male rats.

Twenty male rats were castrated at 30 days of age and 20 litter mates kept as controls. Urinary protein excretion was measured every 15 days and the rats were killed and kidneys removed and examined in the usual way at 105 and 120 days. The amount of protein in the urine was significantly lower than that of normal male rats and was approximately equal to that found in females. The kidney weights were 1·45 g. ± S.D. 0·2 as compared with 1·89 g. ± S.D. 0·10 in the litter mate controls, body weights being similar in the two groups. The proximal tubule cells appeared smaller in the castrated groups. Moderate numbers of inclusions were found in only 4 castrated male rats. All the controls contained abundant droplets.

Proteinuria in castrated rats is significantly reduced and the incidence of hyaline inclusions is much reduced.

EXPLANATION OF PLATE.

1.—Section of kidney of adult male rat; phloxine and Meyer's haemalum: shows abundant cytoplasmic inclusions (× 800).
2.—Section of kidney of 75 day-old male rat; phloxine and Meyer's haemalum: shows inclusions in mitosing tubule cells (× 800).
3.—Section of kidney of adult male rat; Bensley's modification of Altmann's aniline acid fuchsin: shows abundant mitochondria and inclusions in proximal tubules (× 800).



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Female rats treated with testosterone propionate.

Twelve female rats were injected subcutaneously with 5 mg. testosterone propionate in oil every second day from 50 days of age. Twelve control rats were given an equal volume of sterile almond oil. In a further 12 rats 25 mg. pellets of testosterone propionate (Organon) were implanted at 35 and 75 days. Proteinuria was measured in the usual way and the animals were killed and examined at 120 days of age. The protein excretion of animals receiving testosterone propionate was significantly higher than that of the controls (approximately 120 μg./100 cm.^{2} body surface/hr. compared with 60 μg./100 cm.^{2}/hr.). In six of the testosterone-treated female rats the level was greater than the average. Aerobic and anaerobic cultures were made of the kidney. No organisms were recovered and no microscopical evidence of renal inflammation was found.

The kidneys of the testosterone-treated rats weighed 1·84 g. $\pm$ S.D. 0·16 for the implanted, 1·85 g. $\pm$ S.D. 0·14 for the injected, compared with 1·30 g. $\pm$ S.D. 0·17 for the controls. No increase in the number of droplets was found in the experimental as compared with the control animals.

After treatment with testosterone propionate the protein excretion of female rats is increased to levels about mid-way between those of normal females and normal males, but the incidence of inclusions is not raised.

DISCUSSION.

The intrinsic difficulty in determining the relationship of these inclusions to protein re-absorption is the fact that we cannot obtain knowledge of the protein load of the glomerular filtrate. From this information and the amount of protein in the urine, an estimate of re-absorbed protein could be derived. An increased protein load at puberty in the male rat or a change in metabolism of the proximal tubule cells are both valid as interpretations of the present findings and similar arguments may be applied to the differences following castration.

The naturally occurring inclusions appear to conform exactly in morphology and staining reactions to those described after injections of foreign protein (Oliver *et al.*, 1954), but we were not able to demonstrate an inverse relationship between numbers of mitochondria and numbers of inclusions in any particular cell or group of cells.

The serial studies on cellular behaviour following the injection of low molecular proteins are subject to experimental error, as it seems possible that induced droplets may be confused with those occurring naturally. The conclusions may be rendered more valid if attention is directed to the evolution of naturally occurring inclusions in the species used and to the age and sex of the animals.

SUMMARY.

Abundant naturally occurring protein inclusions were found in the proximal tubules of male rats 60 days and older but not in any substantial numbers in female rats. These inclusions have been correlated with the level of proteinuria.

The effect of castration in male rats was to reduce the incidence of inclusions and to lower the level of proteinuria.

The effect of testosterone propionate in females was to raise the level of proteinuria. There was no increase in the content of inclusions.

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