THE PRODUCTION OF CHRONIC RENAL DISEASE IN RATS BY A SINGLE INTRAVENOUS INJECTION OF AMINONUCLEOSIDE OF PUROMYCIN AND THE EFFECT OF LOW DOSAGE CONTINUOUS HYDROCORTISONE

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In a previous experiment (Lannigan, Kark and Pollak, 1962) it was shown that a single intravenous injection of an amino-nucleoside (6-dimethyl-aminopurine-3 amino-d-ribose) prepared from the antibiotic Puromycin (Lederle) produced proteinuria in rats which persisted to the end of the experiment at 143 days. The glomeruli during this period showed no evidence of basement membrane thickening or cellular proliferative changes on light microscopy. By electron microscopy various degrees of fusion of the foot processes were noted at an early stage and this persisted in some glomeruli up to 112 days.

Proteinuria was estimated by expressing urine from the bladder on to an Albustix (Ames Co.) test paper. No measurements were made of protein output.

The present communication describes further experiments in which following a single intravenous injection of aminonucleoside of Puromycin in rats, daily protein output was measured and the experiments were continued for 60 weeks. The effect of low dosage continuous hydrocortisone on a similarly treated group of rats was also studied for the same period.

METHODS

Four groups of 10 white rats were used with an average weight of 180 g. Group I received a single intravenous injection of 10 mg. aminonucleoside per 100 g. body weight as a 2 per cent solution in saline. Group II received a similar injection and 6 days later soluble hydrocortisone (Efcortelan-Glaxo) was added to the drinking water in a dose equivalent to 3 mg. per kg. body weight. A fresh solution was made daily. Group III received an intravenous injection of saline followed by Efcortelan in a dose similar to group II. Group IV received an intravenous injection of saline.

The rats were kept in metabolic cages several to a cage, Group I and Group II throughout the experiment and Groups III and IV for most of the time. Specimens of urine (24 hr.) were collected from Tuesday till Saturday of each week and on Monday a 48 hr. collection of urine was made. Protein was estimated by the Biuret method on pooled urine for each group. For comparison between groups the results are expressed as the average of the 6 readings per week divided by the number of animals in each group, *i.e.* expressed as the average protein output per rat per day during 1 week. Paper electrophoresis was carried out at intervals. On a few occasions urinary protein output was measured on individual rats and blood was taken from a tail vein for serum protein estimations.

One animal from each group was killed at 9, 23, 35 and 48 weeks and daily urine protein output was measured on these individual rats for a few days before. Blood was taken for serum protein and serum creatinine estimations. Serum creatinine was estimated after adsorption on Lloyd's reagent (Owen, Iggo, Scandrett and Stewart, 1954).

The organs were weighed and for light microscopy tissues were fixed in 10 per cent formol-saline and embedded in paraffin. Sections of kidney were stained with haematoxylin and eosin, Van Gieson, periodic acid-Schiff, Mallory's phosphotungstic acid haematoxylin,

Lendrum's acid picro-Mallory and the periodic acid methenamine silver method. Other tissues were stained by H. and E., Van Gieson and PAS. For electron microscopy, kidney tissue was fixed in buffered osmium tetroxide and embedded in methacrylate or Araldite (CIBA).

In one animal from each group blood pressure estimations were made at 48 weeks by direct cannulation of the femoral arteries under ether anaesthesia before being killed.

All animals were fed standard diets and were weighed at regular intervals.

The experiment was continued for 60 weeks.

RESULTS

All animals survived the experiment except 3 in group I which died at 2, 3 and 39 weeks. The first 2 were cannibalised and no tissues are available. The third was found moribund.

The animals gained weight as the experiment progressed but in Groups I, II and III the weight increase was slower than in the controls. (Group IV). In Group IV the weight increased more rapidly in the early months than in the other Groups and at the termination of the experiment the average weight was 370 g. in Group IV compared with 275, 252 and 263 g. in Group I, II and III respectively. The control group was, however, kept in larger cages for the first 20 weeks of the experiment and this may have had some effect on the growth rate.

Proteinuria.—Increased proteinuria was evident in Groups I and II within 3 days and quickly reached a maximum at the 15th day after injection, the highest daily reading being 760 mg. per rat in Group II. At the tenth day oedema was noted in some rats and this disappeared by 3 weeks. A rapid decline in proteinuria occurred and by the 6th week the protein output stabilised at a level approximately 4 times the control group. A second rise in protein output occurred in Group I at the 29th week and after a delay of 8 weeks a similar but lesser rise occurred in Group II. With the exception of a few weeks at the beginning of the experiment and again at the 47th week the mean protein output in Group I exceeded that of Group II. Fig. 1 shows the variations in protein output for the duration of the experiment. The abrupt alteration in the output of all groups at 20 weeks resulted from the introduction of new metabolic cages which permitted a more efficient collection of urine. The results for the first 20 weeks are therefore too low. Because 3 animals in Group I died the number of animals in Group I became less than those in the other groups and at the end of the experiment there were 3 rats in Group I and 6 in the other groups. Protein output of individual rats was measured for 2 or 3 days from the 32nd to 34th weeks in Groups I and II. The results are shown in the Table. Because of the exigencies of the

TABLE—*Urinary Protein Output in Individual Rats in Groups I and II at 32–34 Weeks and at the End of the Experiment*

	32–34 weeks			60 weeks		
	mg. protein/day	Mean	S.D.	mg. protein/day	Mean	S.D.
Group I	72, 85, 190, 195, 271, 688	250·2	227·2	100, 230, 310	213·3	106·0
Group II	6, 12, 12, 30, 30, 48, 70, 130	42·3	41·3	55, 80, 135, 150, 240, 530	198·3	174·8
	$t = 2·57$ (12 d.f.) $P < 0·05$			$t = 0·13$ (7 d.f.) not significant		

experiment and the expedient of using pooled urine it was not possible to isolate components of variance attributable to individual animals in the day to day variation in protein output. For this reason only an insensitive statistical test of significance could be applied. There is, however, a significant difference at 32 weeks but not at 60 weeks.

By paper electrophoresis, at the 15th day, the bulk of the protein was albumin but many other proteins were also present (Fig. 2). These have not been studied in any detail. Albumin, however, remained the most prominent protein throughout the experiment.

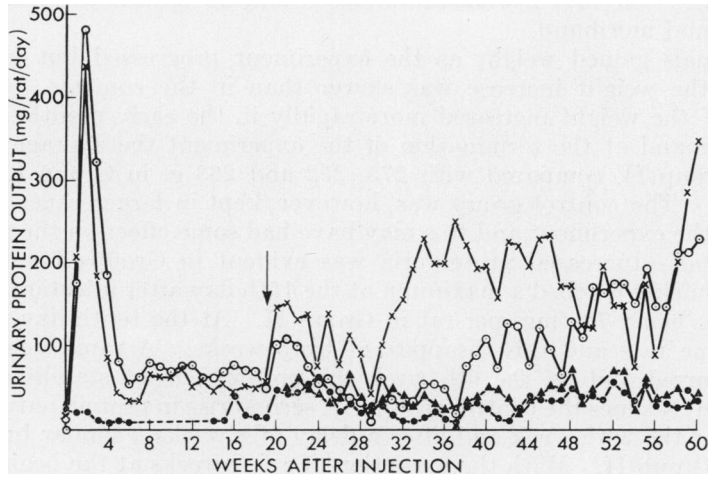


FIG. 1.—Daily urinary protein output. Each point represents the mean of 6 daily readings per rat on pooled urine for each group. The arrow indicates where new metabolic cages were introduced.

×—× Aminonucleoside. Group I.
○—○ Aminonucleoside + Efcortelan. Group II.
●—● Efcortelan. Group III.
▲—▲ Controls. Group IV.

The protein concentration rose from 250 mg. per 100 ml. to 4·60 g. per 100 ml. at the 15th day in Groups I and II. It fell with the protein output but did not fall below 800 mg. per 100 ml. urine. For most of the experiment the concentration fluctuated between 1 and 3 g. per 100 ml. In Groups III and IV the concentration rarely rose above 300 mg. per 100 ml. This confirms the findings in the previous experiment where the Albustix method was used.

Serum proteins.—At the 15th day the total serum protein level was not reduced but albumin was markedly reduced. Recovery took place with the drop in urinary protein excretion and for the remainder of the experiment the total serum proteins remained near normal. In a few cases in Groups I and II the serum albumin was lowered and this occurred where proteinuria was high.

Serum creatinine.—By the method used the highest serum creatinine in Group IV was 0·36 mg. per cent. This was not exceeded in Groups II and III but in Group I two animals at 39 and 48 weeks had serum creatinine levels of 2·08 and 0·8 mg. per cent respectively.

Blood pressure.—In one animal of Group I and II the blood pressure readings from the femoral artery were within the normal range at 48 weeks. (Mean blood pressure 95–98 mm. of mercury).

Pathology

Macroscopic appearances.—Ascites was not found at necropsy in any animal.

Heart.—Heart/body weight ratio in Group IV did not exceed 0·0049. At the end of the experiment 2 animals in Group I, 3 in Group II and 1 in Group III exceeded this value. In Groups I and II these were cases in which proteinuria was severe.

Adrenals.—The adrenals were difficult to weigh accurately but no naked eye differences were noted in the animals receiving steroids.

Kidneys.—In 4 cases in Group I and 1 case in Group II the kidneys showed fine granularity of the surface. The kidney/body weight ratio did not differ in the various groups.

Other organs showed no special features.

Light microscopic appearances.—Nothing relevant was found in organs other than the kidneys.

Group I.—At 9 weeks the kidneys were virtually normal apart from occasional hyaline droplets in glomerular epithelial cells and occasional tubules distended with casts. The glomerular basement membrane appeared normal. At 35 weeks similar appearances were noted but in addition there was a thickening of the axial zones in some glomeruli with an increase in material giving staining reactions similar to glomerular basement membrane (Fig. 3). In animals killed at later stages more advanced degrees of damage were noted (Fig. 4). The degree of involvement varied from glomerulus to glomerulus and some glomeruli appeared normal. Glomerular adhesions were occasionally found and lesions resembling fibrous crescents were noted (Fig. 5), the earliest crescents being found at 39 weeks. No cellular increase was noted in the tuft. The exact nature of the sclerosing process is difficult to elucidate but there appears to be an increase of material in the axial zones similar to basement membrane together with areas of capillary collapse. The basement membrane of open loops did not appear to be thickened but fibrillation of the membrane was evident in a few glomeruli.

An additional type of lesion was noted in 5 cases. This consisted of small areas of eosinophilic material within the tuft and often near the peripheral loops (Fig. 6). This material was PAS positive, usually stained red with the acid picro-Mallory and orange with the phosphotungstic acid haematoxylin stain. It was frequently deposited in already damaged glomeruli and in some of the fibrous crescents a similar material was found. These lesions are similar to the so-called exudative lesions found in diabetes mellitus and other chronic renal diseases. In the animal dying at 39 weeks capillary thrombi were also found in some capillary loops.

Variable numbers of casts were found in tubules and the lumina of the affected tubules were frequently distended (Fig. 7). Hyaline droplets were increased in number in some proximal tubules.

Occasionally small focal areas of lymphocytic infiltration were noted in the interstitial tissues of the cortex. Interstitial fibrosis was not however prominent.

Hypertensive changes were noted in the vessels in one animal at 39 weeks.

Group II.—The lesions were similar in nature to those of Group I. Sclerotic changes were noted from 35 weeks but the lesions were not as severe as in Group I except in 2 cases. Two animals showed only minimal glomerular changes at the termination of the experiment. "Exudative" lesions were found in 2 cases.

The tubular lesions were similar to those in Group I but in addition three animals showed focal calcium deposits in collecting tubules.

Hypertensive vascular changes were noted in 1 animal at the end of the experiment.

In both these groups the severity of the lesion at the time of death corresponded roughly with the degree of proteinuria.

Group III.—No glomerular lesions were noted. Calcification of the collecting tubules was not observed.

Group IV.—In one case occasional hyaline droplets were observed in glomerular epithelial cells. In one other animal a polymorph infiltration was noted in the peri-pelvic tissues but not in the renal substance.

Electron microscopy

Only preliminary studies have been carried out. In the early cases there is increased folding and thickening of the basement membrane at the axial zones but the peripheral basement membrane is not thickened. In the Group I animal killed at 9 weeks the foot process layer is almost intact. In later cases focal loss of the foot process layer is observed (Fig. 8) and in the animal dying spontaneously at 39 weeks there is extensive fusion of the foot process layer. Vacuolation of epithelial cytoplasm which is prominent during the severe initial phase of proteinuria returned after 9 months in cases with heavy proteinuria.

The exudative lesions showed deposition of osmiophilic material in various positions. Sometimes it was closely applied to the outer layer of the basement membrane and in others it seemed to involve the epithelial cells, a broad band of osmiophilic material passing through the cytoplasm (Fig. 9). In one glomerulus similar material filled the capillary lumina. This was thought to be thrombus and on light microscopy stained blue with PTAH stain.

DISCUSSION

This experiment confirms the previous finding that, after a single intravenous injection of aminonucleoside, proteinuria continues. Serum protein and urinary protein studies have not been investigated in sufficient detail to permit an accurate correlation. At the peak of the initial severe proteinuria there is a marked re-

EXPLANATION OF PLATES

FIG. 2.—Paper electrophoresis of urine and serum in rats at 15 days and 39 weeks. (Group I).

FIG. 3.—Irregular thickening in the axial zones. Group I. 36 weeks. P.A. silver ×320.

FIG. 4.—More severe deposition of silver positive material in the axial zones. P.A. silver ×320.

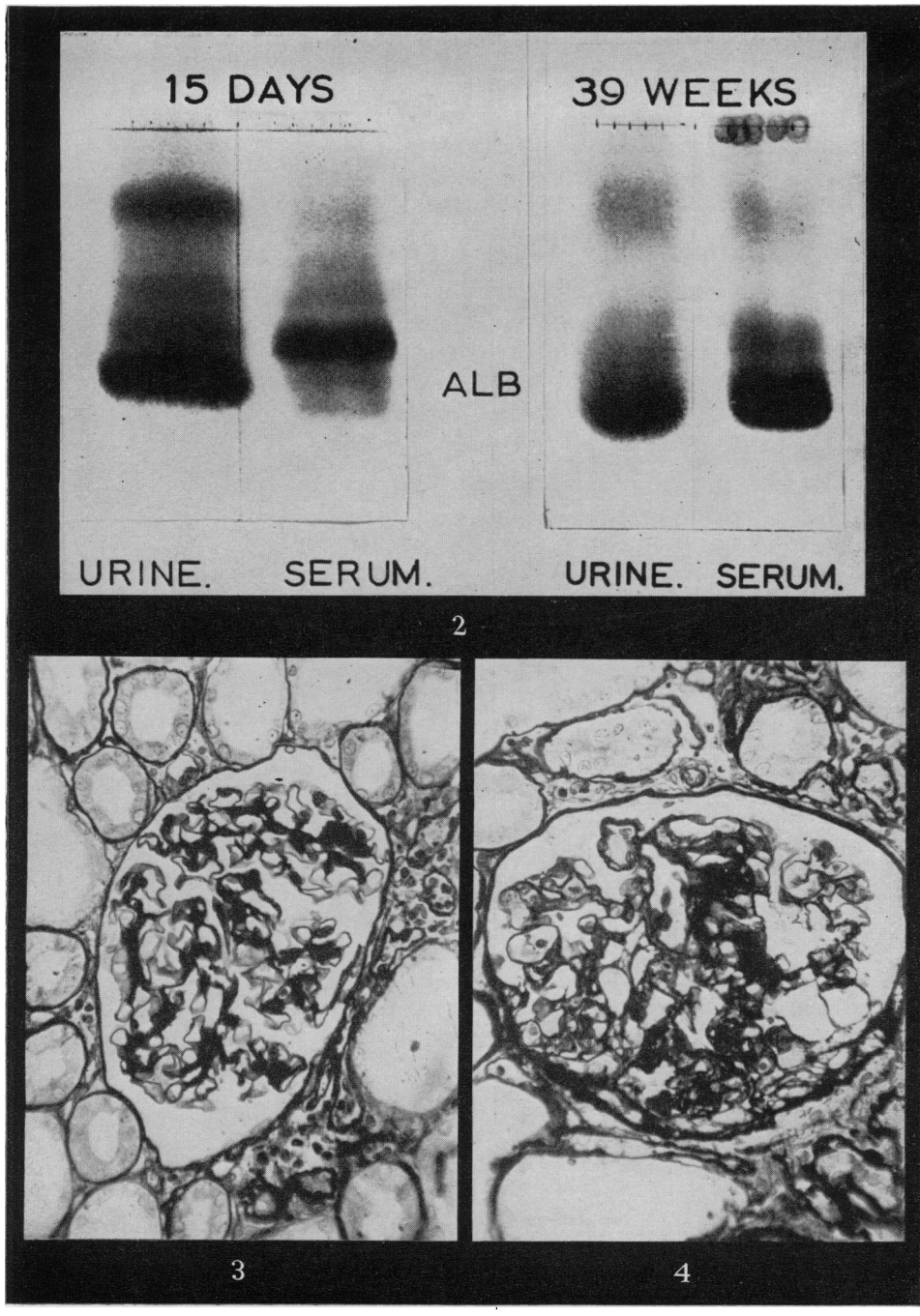
FIG. 5.—Fibrous adhesion and "crescent". Group II. 60 weeks. Picro-Mallory ×360.

FIG. 6.—"Exudative" lesions and glomerular adhesions. Group I. 39 weeks. Acid Picro-Mallory ×360.

FIG. 7.—Numerous casts, some in dilated tubules. Group II. 60 weeks. Picro-Mallory ×280.

FIG. 8.—Increased deposition of basement membrane like material in the axial zone (A), and partial fusion of the foot process layer. C. indicates capillary lumen. Group I. 48 weeks. Osmic-methacrylate ×13,500.

FIG. 9.—Exudative lesion. Layer of osmiophilic material within epithelial cytoplasm between capillary basement membranes. Group I. 39 weeks. Osmic-methacrylate ×13,500.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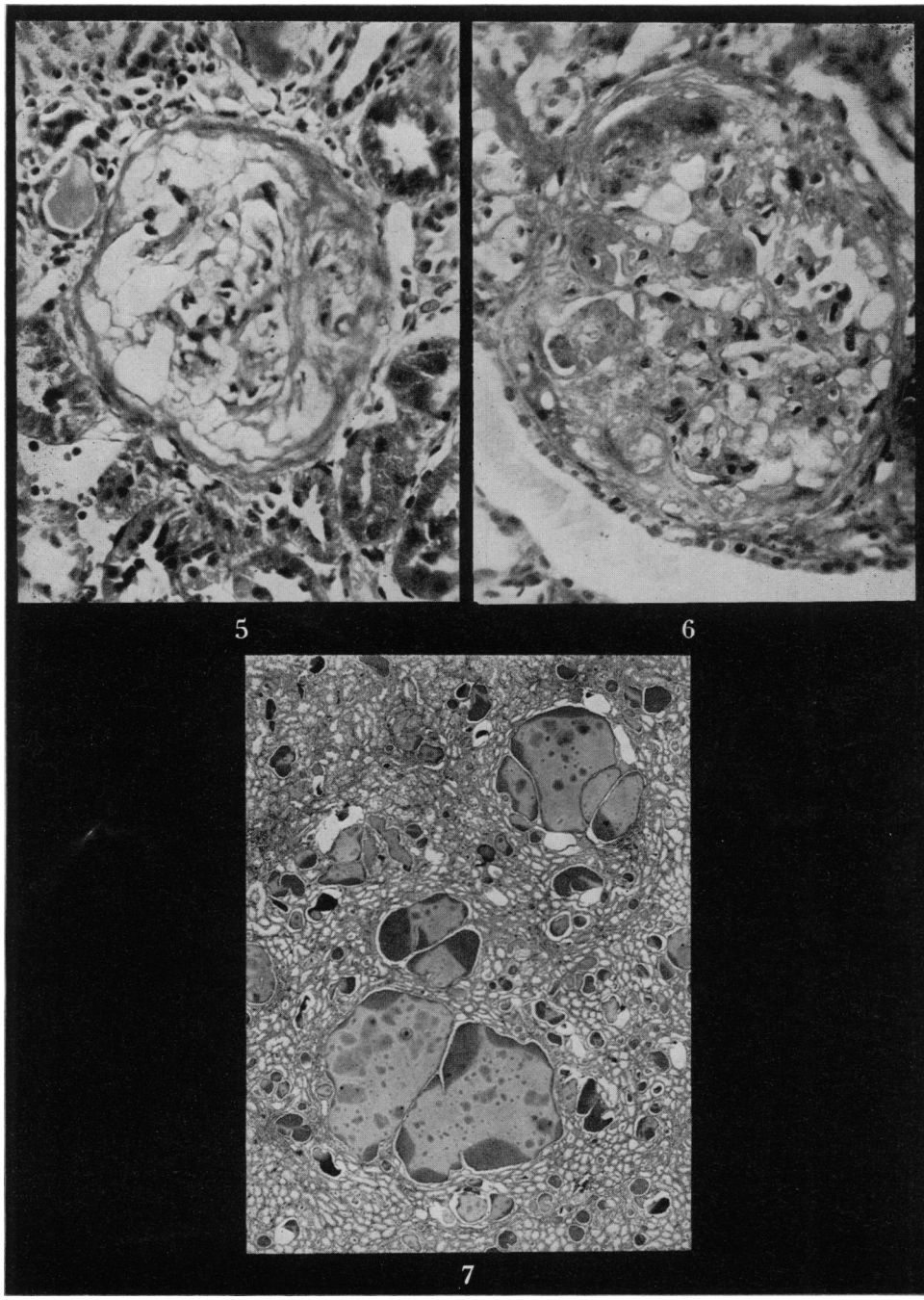

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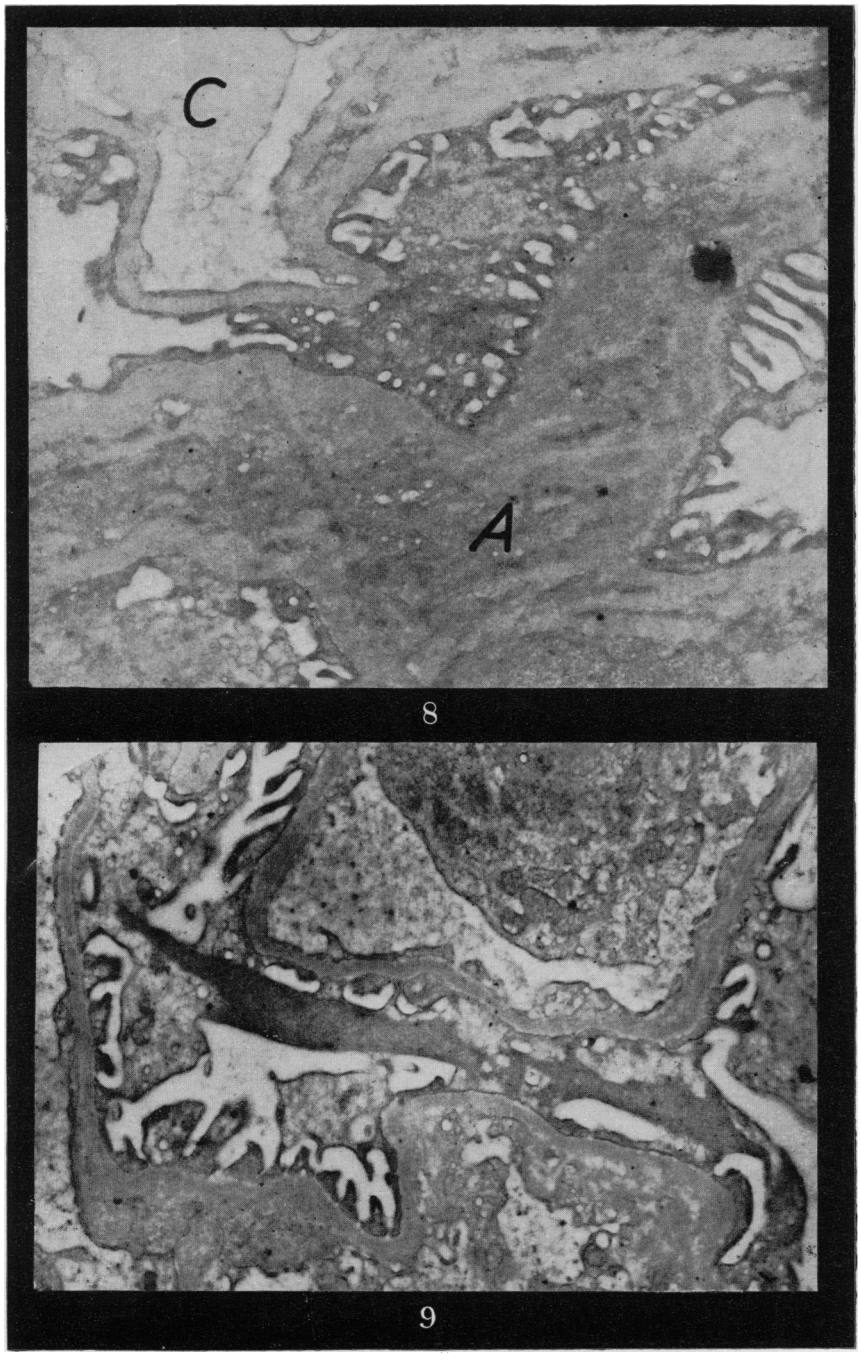

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duction in serum albumin and an increase in what is probably a large molecular weight glycoprotein. The rapid fall in proteinuria may in part be due to albumin depletion but the albumin in the serum regains normal values at about 6 weeks which suggests that there is a partial recovery of the renal lesion with a drop in the protein leak through the glomerulus.

In the previous experiment electron microscopic studies showed the persistence of foot process fusion in some glomeruli up to 112 days after injection. As even in the early stages not all glomeruli were involved it is not possible to be certain of the degree of recovery if any since quantitive studies by electron microscopy are not possible. By light microscopy the numbers of glomerular hyaline droplets decreased and this may be interpreted as an index of recovery in some glomeruli. In the daily subcutaneous injection technique Vernier, Papermaster and Good (1959) were able to show partial restoration of the foot process pattern after 4 weeks since by this method every glomerulus is involved after about 2 weeks.

Mild sclerosing lesions were noted at 35 weeks and these increased in severity although there was no constant time sequence. The development of these chronic lesions was associated with a second rise in proteinuria and the degree of glomerular damage correlated roughly with the degree of proteinuria.

Chronic glomerular lesions of a similar type were noted by Wilson, Hackel, Horwood, Nash and Heymann (1958) after a repeated course of subcutaneous injections of aminonucleoside and Borowsky, Kessner, Hartroft, Recant and Koch (1961) showed similar findings after a course of subcutaneous injections or a prolonged course of orally administered aminonucleoside, the chronic lesions developing several months after withdrawal of the aminonucleoside.

The mechanism of production of these lesions is not clear but they bear a close similarity to certain types of renal disease in man, where following an acute nephrotic episode chronic progressive renal lesions develop.

An immunological mechanism appears unlikely in the initial phase since there is an almost immediate effect with intravenous aminonucleoside. The development of chronic renal lesions may result from the proteinuria *per se* or possibly by a secondary immune mechanism. There appears to be no cellular proliferative changes in the glomerular tuft itself. Some of the crescents and fibrous adhesions appear to be the result of an organising exudate rather than capsular proliferation. Tubular obstruction does not appear to play a significant part.

Hypertension

Hypertensive vascular changes were noted in 2 animals in which glomerular lesions were severe and heart/body weight ratio was increased in several others. However, in 2 cases in which moderate glomerular lesions were found the mean blood pressure was normal. This suggests that while hypertension may develop in association with the glomerular lesions, the sclerotic changes are not initially produced by hypertension. Wilson *et al.*, (1958) also noted the occurrence of hypertension.

The effect of steroids

The main effect of low dosage continuous steroids in this experiment is the delay in the onset of the secondary rise in proteinuria and this is probably due to

the slower development of the sclerosing glomerular lesions. Efcortelan was selected because of its solubility in water and the dosage employed was calculated on a weight basis slightly higher than the amount used in the treatment of the nephrotic syndrome in human patients (Blainey, Brewer, Hardwicke and Soothill, 1960). The amount absorbed was not known and the amount taken per rat in the drinking water depended on the fluid intake which was variable throughout the experiment. It is possible that a higher dose or a different steroid would have been more effective.

Fiegelson, Drake and Recant (1957) found that cortisone did not prevent the development of the aminonucleoside induced nephrotic syndrome and had no effect on the acute phase. Selye (1957), however, noted that the renal lesions in the acute phase were reduced in severity when cortisone was administered. Borowsky *et al.* (1961), gave a 3 months course of prednisone (0·4 mg. per rat daily by subcutaneous injection) immediately after a course of orally administered aminonucleoside and found no effect. They measured the effect by the development of chronic renal lesions up to 9 months. It is possible that some effect would have been obtained if the administration of steroids had been continued since in the present experiment the effect of steroids was not noted until about 6 months had elapsed.

The effect of steroids on the lesions in the present experiment bears some resemblance to the effect of certain varieties of the nephrotic syndrome where deterioration can be delayed by continuous administration of small doses.

Although in some respects the course of this experimental disease resembles that of certain types of human disease, the comparison should not perhaps be taken too closely. It does, however, provide a good experimental model for the study of the mechanism of production of chronic glomerular sclerosis and of the effect of therapeutic agents.

SUMMARY

Rats were given a single intravenous injection of 10 mg. aminonucleoside of Puromycin per 100 g. body weight and daily protein output was measured for 60 weeks. Significant proteinuria occurred within 3 days and reached a maximum at 15 days. A rapid fall in protein output followed and by 6 weeks levelled to approximately four times the control group. A second rise in protein output occurred after 29 weeks and continued to the end of the experiment.

A similar group of rats after injection with aminonucleoside received continuous low dosage hydrocortisone hemi-succinate in the drinking water. No effect on proteinuria was noted in the initial stages but the secondary rise in protein output was delayed for 2 months.

Chronic sclerosing glomerular lesions developed in both groups after 6 months and this was not associated with cellular proliferative changes. The severity of glomerular damage was roughly proportional to the degree of proteinuria at the time of death. Electron microscopy showed deposition of basement membrane-like material in the axial zones. The lesions in the steroid treated group were qualitatively similar but quantitatively less than in the untreated group.

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