A BIOCHEMICAL STUDY OF THE MINIALBUMIN TO BE FOUND IN THE URINE OF MEN AND ANIMALS POISONED BY CADMIUM*

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The early observations of Friberg (1950)[1] stimulated much of the subsequent interest in cadmium poisoning. He noted a high incidence of proteinuria in workers in the alkaline battery industry under his care. Pedersen (findings published in Friberg's monograph, page 37) examined the protein and found it to have a molecular weight of 25 - 30,000. The protein has been found consistently since then in the urine of men and animals poisoned by cadmium, by inhalation or parenteral route.

Smith, Kench and Lane[2] carried out a series of investigations in Manchester on the incidence and degree of proteinuria in industrial workers exposed to cadmium, which have confirmed and extended the observations of Friberg. They determined the incidence and degree of proteinuria in relation to cadmium exposure and excretion. Urinary cadmium and excreted protein appeared to vary independently of one another in the series of 95 workmen exposed to the hazard, but the individual who worked in the most heavily contaminated atmosphere consistently excreted most cadmium and protein in his urine. The quantity of urinary protein was 1·0 - 3·2 G/l.[3]—which is much in excess of the average value of 133 mg./24 hr. found by Webb and his co-workers[4] in young, normal, adult males.

As regards the nature of the urinary protein, Friberg described it as having the electrophoretic mobility of α_2-β-globulins. The proteinuria could not unequivocally be demonstrated by the heat coagulation test, but complete precipitation was effected by addition of 10% w/v aqueous trichloracetic acid (TCA) (1 vol. TCA to 1 vol. urine).[3,5] Olhagen (p. 36 of Friberg's monograph) observed that the urinary protein was precipitated when the urine was fully saturated with ammonium sulphate, but not on 50% saturation. The sequence of events, as observed by the Manchester workers, was the initial appearance of α_2-globulins, soon followed by a β-globulin fraction. Later, protein fractions appeared, migrating as serum albumin and γ-globulins, until eventually the urinary protein pattern was similar to that of serum. Kekwick[6] observed that, even when the pattern was complex in this way, the urinary proteins all sedimented in the ultracentrifuge in the range 20 - 30,000.

These observations may be compared with those made on normal urine or on the urinary proteins of patients suffering from renal or other diseases. The small quantity of protein present in normal urine includes γ-globulins of low molecular weight, 10,600,[4] while the α_2, β- and other globulins excreted normally[4] or in conditions of renal tubular necrosis[7] have molecular weights similar to those found in cadmium poisoning. Creeth and his colleagues are, therefore, convinced that the proteinuria of cadmium poisoning comprises a mixture of globulins which is typical of any non-specific renal tubular defect or necrosis, as obtains in the Fanconi syndrome, in hepatolenticular degeneration or in galactosaemia. The proteinuria of cadmium poisoning is, in their view, neither specific nor unique, and it also appears that they regard the first urinary peak as probably an α_1-globulin. As regards the albumin found in normal urine, Webb *et al.*[4] were unable to distinguish between it and normal serum although, more recently, Merler *et al.*[8] have reported that normal human urinary albumin has, in fact, a smaller molecular weight, $S_{20,w}$ of 2·6 S as compared with 4·2 S for crystalline serum albumin. The mobility of serum albumin and of other serum proteins is also somewhat augmented in urine,[9] an effect ascribed to adsorbed urinary pigments. The albumin excreted in the urine of nephritic and of nephrotic patients,[3,10] as of a person poisoned with metallic mercury,[11] is indistinguishable from normal serum albumin in molecular size.

On the basis of the close similarity in amino acid structure and electrophoretic mobility of the urinary and serum albumins of workmen and of animals poisoned by cadmium, and of the low molecular weight of the urinary proteins, Kench and his colleagues[3,12] have postulated that the special feature of cadmium intoxication is the excretion of an albumin of low molecular weight. Cadmium-poisoned workpeople so far examined have excreted only low molecular albumin unless renal disease from other causes was coexistent. Cadmium-poisoned rabbits[12] excrete both albumins of low and of normal molecular weight in the urine. The metabolic turnover of the low molecular species appeared to be more rapid than that of normal albumin, and, on intravenous injection of the C^{14}-labelled urinary proteins into normal rabbits, much more activity was recovered in the urine as protein than when C^{14}-labelled serum proteins were similarly injected. The question of renal permeability to serum proteins of different molecular weights is very pertinent to this situation. Thus, Hardwicke[13] has demonstrated, both by immunological procedures and by use of gel filtration through Sephadex G 200, that renal clearances of serum proteins increase steadily with diminishing molecular weight.

The experimental evidence in support of the concept of a specific biochemical lesion, caused by cadmium ions, leading to the excretion of a low molecular albumin,[12] was only tentative in kind, since techniques for separating albumin molecules on the basis of difference in molecular size (*cf.* myoglobin and haemoglobin[14]) were not available to us. The present paper is an account of biochemical studies designed to separate and characterize, with greater exactitude than was previously possible, the urinary albumins of cadmium-poisoned animals, as a necessary prerequisite to a study of its tissue source and the mechanism of its formation.

Apart from its molecular weight—in its behaviour variously in trichloracetic acid-acetone, on immunoelectrophoresis and Ouchterlony plates and in its amino acid

*Much of this work was presented at the Annual Conference of the South African Society of Pathologists held in Cape Town, April 1965; most of it is embodied in a thesis submitted by A. C. Gain for the Ph.D. degree, University of Cape Town, December 1965. Full experimental details are available in the thesis and will be presented elsewhere.

composition (established earlier)—the low-molecular protein in the albumin fraction of the urine of cadmium workers was similar to normal human serum albumin. We, therefore, now refer to it as minialbumin.

No minialbumin could be detected in the urine of patients suffering from non-specific renal tubular damage, as for example, in an advanced case of hepatolenticular degeneration, Fanconi syndrome, acute tubular necrosis or nephrotic syndrome. Rabbits, dogs and monkeys have been chronically poisoned by intravenous injection of cadmium chloride, and, after some months, these animals, too, excrete minialbumins closely similar in all the respects already mentioned with their own normal serum albumins and the normal molecular albumin also found in the urine. If it is assumed that all the minialbumin formed in the cadmium-poisoned monkey is excreted in the urine, then the number of such diminutive molecules would correspond to approximately 26% of the number of normal serum albumin molecules being synthesized in the liver. In cadmium workers the corresponding figure is approximately 53%.

The over-all data support the view that cadmium ions may provide a valuable tool in the study of the relationships between chemical structure, antigenic behaviour, and metabolism of serum albumin and perhaps of other proteins in man and animals.

EXPERIMENTAL OBSERVATIONS AND RESULTS

Experimental Animals and their Maintenance

Male vervet monkeys (*Cercopithecus aethiops*), which had been captured in the wild state, were the experimental subjects. Young full-grown individuals of steady weight (about 5 kg.) and, therefore, in nitrogen balance, were chosen. They were kept in stainless steel metabolic cages with floors of ½ inch wire mesh which allowed urine to pass through but held back most of the stools and food debris. The cages were placed over stainless steel trays which were covered with mosquito netting to filter the urine free from residual contaminants.

The animals were fed daily between 11 a.m. and 3 p.m. on carrots, cabbage, sweet potatoes, oranges and dried maize. They do not drink water and none was provided.

Poisoning of the Animals

The technique employed was similar to that developed by Kench *et al.*[12] for cadmium intoxication of rabbits. Blood was withdrawn from the saphenous vein in the lower hind leg using an all-glass 10 ml. Luer-Lock syringe with off-centre attachment and 1½ inch 21-gauge needles. The needle, previously heparinized, was left in the vein and the blood flow halted by closing the Luer-Lock stopcock. The blood was then displaced from the syringe into a beaker containing 0·5 ml. heparin, with which it was well mixed. Cadmium chloride ($CdCl_2.2\frac{1}{2}H_2O$) solution, containing 2 mg. Cd^{++} per ml. and sodium chloride added to a final concentration of 0·55% w/v Cl, was then stirred gently into the heparinized blood—a manoeuvre which allows the acidic cadmium chloride solution to be fully buffered by the blood without the occurrence of precipitation. The mixture was then sucked up into the same syringe, which was reattached to the needle in the vein, and the injection given over a period of about 2 minutes. The needle was finally removed and stasis achieved by placing a piece of cottonwool plus Hibertane ointment over the wound, which was secured with Elastoplast.

Injections were made twice weekly at an approximate dosage of 2 mg. cadmium per kilogram body-weight.

During the first 2 - 3 months period of intoxication, 15 - 18 mg. of cadmium were injected weekly. When severe poisoning had occurred with gross proteinuria, dyspnoea and agitation of the animal, the dose was lowered to 6 - 8 mg. of cadmium weekly.

Quantitation of Proteins in Serum and Urine of Poisoned Animals

The protein concentrations of specimens of serum and urine were measured daily during the intoxicating period by a modified biuret procedure,[15] using a known pure bovine albumin preparation, standardized by the micro-Kjeldahl procedure.[16] Serum albumin-globulin fractionation was performed using 23% w/v Na_2SO_4 solution.[17]

Urinary proteins rose steadily in amount in the cadmium-poisoned monkey during the process of poisoning, until an average of nearly 200 mg. was being excreted daily. The concentrations of serum proteins—albumin 3·5 - 4·0 G/100 ml. and globulins 2·0 - 3·0 G/100 ml.—remained unaltered during high-dosage administration of cadmium chloride as during the state of chronic intoxication.

Special Investigations of Urinary Proteins

1. *Separation of Albumin Fraction*

Urinary albumins have, in the course of this study, been separated from globulins present in the urine by a number of procedures.

(*i*) Zone electrophoresis on ethylated cellulose according to the method of Porath and Flodin,[18] as adapted by Campbell and Stone.[19]

(*ii*) Separation on columns of the cationic ion-exchange resin, carboxy methyl cellulose (CMC), using salt and pH gradients.[20]

(*iii*) The trichloracetic acid procedure described by Vallance-Owen *et al.*[21] based on the solubility of serum albumin in acid-alcohol mixtures. This technique has been critically appraised by Michael[22] who could detect no changes in physico-chemical or antigenic properties of albumin as a result of the fractionation procedures. This has also been our experience.

All three procedures provided homogeneous albumin fractions when electrophoresed in a Durrum-type tank, the protein migrating as a single compact band. However, the excellent recoveries and the relative simplicity and reproducibility of the trichloracetic acid method were important advantages for our particular work, and after preliminary trials with all 3 procedures, it was thereafter employed for all subsequent experiments on serum, urine or peritoneal dialysate.

In brief, the preparation was performed as follows: The urinary proteins were first precipitated en masse with a 10% w/v aqueous solution of trichloracetic acid (TCA) which was added in a volume equal to that of the urine, which had already been concentrated 3 - 4 times by dialysis and pervaporation alternately.* The precipitated proteins were then centrifuged at 2,000 r.p.m. for 10 minutes at 0°C in a MSE major refrigerated centrifuge with a swing-out head and the supernatant solution discarded. The precipitate was then washed with 5% w/v aqueous TCA. To the washed precipitate, 1% TCA in 96% aqueous ethanol was added and the albumin thereby extracted. After extraction and centrifugation, the

*The urinary specimens were first dialysed against running tap-water overnight, and, later, against cold distilled water. All dialysis tubing was boiled for at least 30 minutes in distilled water before use, to decrease the pore size, a necessary precaution to prevent escape of any low-molecular albumins.

supernatant fluid (SNF) was removed and dialysed in visking sacs at 0°C for 24 hours in a refrigerator, against several changes of distilled water to remove the alcohol and excess TCA. The protein at first precipitated during dialysis but later redissolved. It was found that if the earlier treatment of the total protein precipitate was with 1% TCA in 96% aqueous acetone instead of in ethanol, albumin remained in solution throughout the whole period of removal of the TCA-acetone solvent. In this manner a pure aqueous solution of albumin was obtained, as judged by a single symmetrical peak on paper electrophoresis (Fig. 1).

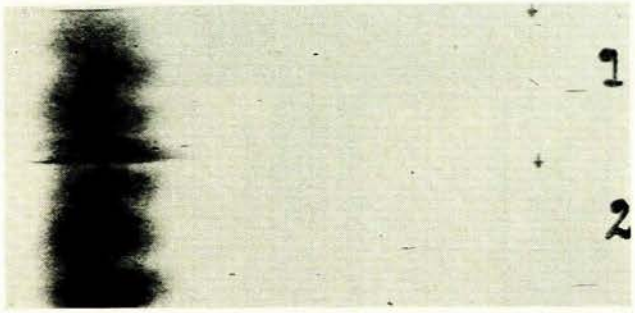

Fig. 1. Homogeneity of albumin fraction prepared by the TCA method. 1 = Urinary albumin of cadmium-poisoned monkey F, 2 = Serum albumin of normal monkey.

2. *Separation of Urinary Albumins of Differing Molecular Weights*

This was achieved by gel filtration[23] using the cross-linked dextran gel, Sephadex G 75, which allowed ready separation of molecules of the sizes of the albumins with which we were concerned. The aqueous solution of albumin, as prepared above, was applied carefully to the top of a column (40 x 1·8 cm.) of the gel, and allowed to run into the gel. On complete entry, a phosphate-sodium chloride buffer (M/15 phosphate buffer pH 7·0 and 0·5 M NaCl) was pumped through the column at a rate of 15 ml. per hour. The eluate from the column was monitored at 262 mμ. by a LKB Uvicord ultraviolet absorptiometer with a recorder, and then collected in 5 ml. fractions. The solution of the urinary albumins from a cadmium-poisoned monkey (F), produced a trace with two distinct peaks, indicating that there were two proteins of differing molecular weights in the albumin preparation (Fig. 2). The same twin peaks were given on Sephadex chromatography

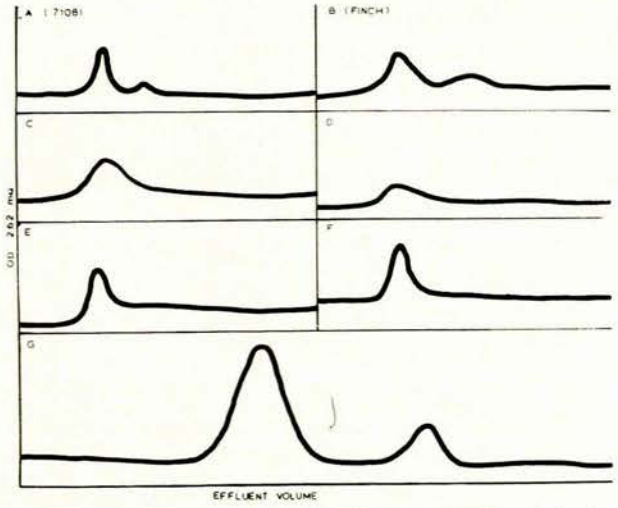

Fig. 2. Separation of urinary albumins of normal and of low molecular weight on dextran gel (Sephadex G 75). A = Cadmium worker 7108, B = Cadmium worker Finch, C = Acute renal failure, D = Hepatolenticular degeneration, E = De Toni-Fanconi syndrome, F = Chronic glomerulonephritis, G = Cadmium-poisoned monkey F.

whether the albumin fraction had been prepared on CM cellulose, by zone electrophoresis on ethylated cellulose, or by the preferred TCA method.

If, before running on Sephadex, the urinary albumin solution was enriched with a solution of normal monkey serum albumin, then the graphic recording of the Uvicord-monitored column effluent had a similar pattern to the urinary albumins alone; but the protein peak first to emerge, i.e. the protein with the greater molecular weight, was augmented, with a greater area under the curve. This pointed to an albumin in the urine with a molecular weight similar to that of normal serum albumin, 66,000, as well as an albumin with a lower molecular weight.

A number of other similar separations are shown in Fig. 2. Finch and 7108 refer to lyophilized urinary albumin from cadmium-poisoned workmen, prepared in Manchester in 1957. Until they were analysed this year, the specimens had stood at room temperature in small sealed bottles. Some denaturation had evidently taken place, since some of the protein remained insoluble in TCA-acetone. The albumin which dissolved was separable into two components, corresponding in size to normal serum albumin and to minialbumin. The presence of normal serum albumin was an unexpected finding, since the sedimentation coefficients of other specimens from the same individuals in May 1958 (kindly determined by Dr. R. A. Kekwick) were respectively 1·96 and 1·99, values which correspond to a molecular weight of approximately 20,000.

Albumin prepared from the urine of patients suffering from various renal disturbances have been examined as follows: acute nephritis (1 case), acute renal failure (4), De Toni-Fanconi syndrome (1) and hepatolenticular degeneration (1). In each instance, molecular separation on Sephadex G 75 showed only one distinct peak appearing in the region and at the effluent volume associated with albumin of a normal molecular weight. No albumin of lower molecular weight was detectable.

3. *Characteristics of the Urinary Minialbumin of Cadmium Poisoning*

(*i*) *Sedimentation and diffusion coefficients and molecular weight.* These parameters of minialbumin were measured in the UCT/CSIR Virus Research Unit, either by Dr. A. Polson himself or under his supervision. The sedimentation coefficient was determined using a Beckman Spinco analytical ultracentrifuge, model E, in which the concentration gradient curve is directly observed throughout the run by suitable optical equipment. The ultracentrifugal patterns of the urinary minialbumin of the cadmium-poisoned monkey (F) are presented in Fig. 3, alongside those for the serum albumins of a normal monkey and a normal rabbit.

The sedimentation constant of the minialbumin $S_{20,w}$ was calculated to be $2{\cdot}2 \times 10^{-13}$ cm./sec./dyne (2·2 S).

The diffusion constant of the minialbumin was measured by the method of Cohen and Bruins[24] as modified by Lamm and Polson.[25] The minialbumin was prepared in 0·9% w/v sodium chloride solution at a concentration of 0·6 G/100 ml. From the experimental observations, the calculated diffusion constant $D = 10{\cdot}61 \times 10^{-7}$ sq. cm./sec.

The molecular weight of the minialbumin was calculated from the sedimentation and diffusion coefficients according to the equation of Svedberg[26] and assuming that the partial specific volume of minialbumin is the same as that of normal serum albumin, their amino acid compositions being so similar.

The molecular weight of minialbumin, thus derived, = 20,060.

(*ii*) *Amino acid composition.* The amino acid composition of normal monkey serum albumin has been compared with the values for the urinary albumins of the cadmium-poisoned monkey (F). The albumins were prepared in the usual manner by TCA-acetone fractionation, and 1 ml. of a solution of each containing 2 - 3 mg. protein was hydrolysed in 6 N HCl in a sealed pyrex tube according to the procedure of Hirs *et al.*[27] Hydrolysis was carried out for 22 hours or for 70 hours. The hydrolysate was subsequently analysed by the ion-exchange chromatographic method of Moore, Spackman and

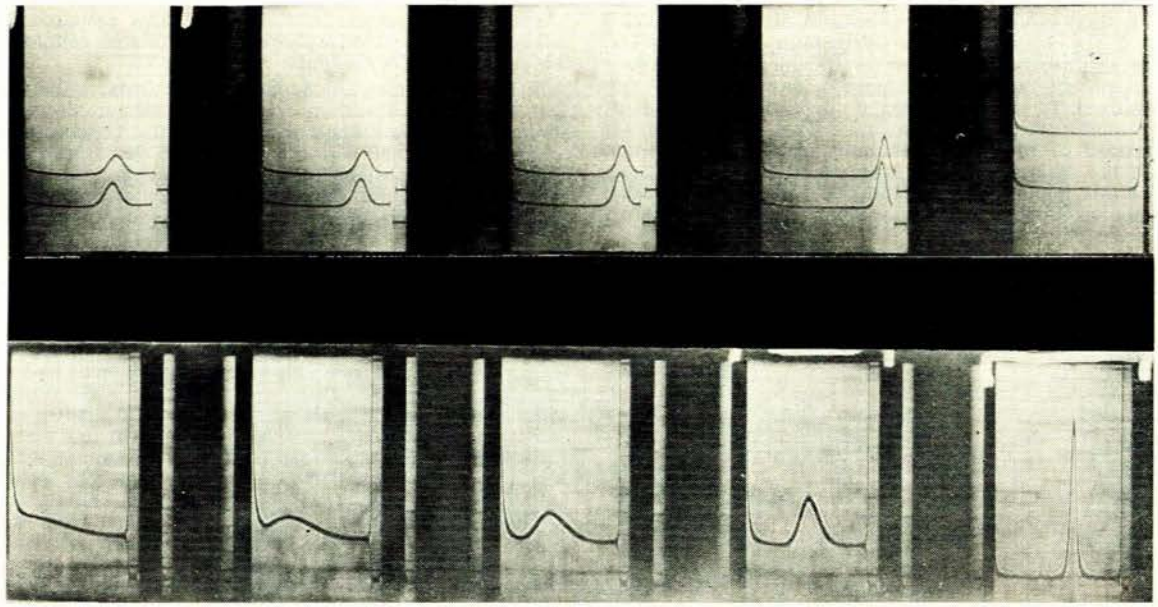

Fig. 3. Ultracentrifugal patterns of albumins. *Above.* Normal monkey serum albumin, normal rabbit serum albumin. *Below.* Urinary minialbumin of cadmium-poisoned monkey F.

Observations were made using a Beckman Spinco analytical ultracentrifuge, model E. Minialbumin was examined as a 0·66% w/v solution in 0·5% w/v aqueous sodium chloride in a synthetic boundary cell at a centrifuge speed of 56,100 r.p.m. The temperature was 24·7°C and photographs were taken of the peak at intervals of 32 minutes. Photographs of the larger serum albumins of rabbit and of monkey were taken at 16-minute intervals.

Stein,[28] a known quantity of nor-leucine being added to each protein preparation before hydrolysis in order to check the recoveries in the over-all analytical procedure. From the percentage amino acid composition of the albumins (G each amino acid/100 G protein), the numbers of the individual amino acid residues in each albumin expressed per gram molecular weight (66,000) of normal serum albumin have been computed and presented as histograms in Fig. 4.

With the possible exception of lysine and of cysteine, which apparently represent a relatively lower proportion of the minialbumin molecule, the proportions of amino acids in the urinary albumins of the cadmium-poisoned monkey were not significantly different from one another or from normal monkey serum albumin.

In other experiments, likewise, no significant differences were observed with regard to the quantities of bound hexose and sialic acid associated with the amino acids in the various albumin molecules.

(*iii*) *Metabolic turnover studies of the albumins in the cadmium-poisoned monkey.* Two aspects were investigated, the metabolic turnover of minialbumin in the poisoned animal, and the fate of minialbumin injected intravenously into a normal monkey.

In the first experiment, 1 mC L-lysine-C^{14} (generally labelled) was injected intravenously into the cadmium-poisoned monkey F, as a single pulse-dose. Blood and urinary specimens were collected; thereafter, 5 carefully-timed collections were made during the first 24 hours and less frequently in the following days, for a period of 3 weeks. In all, 26 blood specimens and 27 samples of urine were obtained from the animal.

The radioactivity of serum albumin was measured on dried protein prepared as follows: The albumin was prepared by the TCA-acetone method and then precipitated from aqueous solution with 10% TCA. The precipitate was washed twice in a centrifuge tube with 5% w/v TCA and then with 95% v/v ethanol followed by a mixture of ethanol, ether and chloroform (2:2:1 v/v/v) to dehydrate the protein adequately. The precipitate was then suspended in pure diethyl ether and plated onto a small filter paper disc (Whatman 541) in a modified Buchner funnel, and the protein thoroughly air-dried. The filter paper discs were then transferred to small stainless steel or plastic planchettes (1·5 cm. diameter), in which the protein could be weighed to 5 decimal places. The radioactivity was measured in an end-window Geiger-Müller counter at infinite thickness.

The urinary albumins were counted in solution in the Packard Tri-carb Scintillating Counter, Model 314 EX, standardized initially and daily with a Tri-carb standard containing C^{14} in toluene. Each vial was individually assayed at maximum efficiency, with recounting of protein preparations from day to day to check for fluctuation in the readings. The aqueous solutions of urinary normal and minialbumins, as collected from the column of dextran gel (Sephadex G 75), were concentrated by pervaporation in visking tubing. The protein concentration of each urinary albumin sample was determined by both the biuret and the micro-Kjeldahl technique, and carefully measured aliquots taken for scintillation counting. Quantities of protein were taken such that optimal counts were obtained throughout the assay.

The results of the experiment are presented in Fig. 5. The rates of logarithmic decay, as portrayed by the linear decline of specific activity of the individual albumins, indicate a greater rate of turnover of minialbumin as compared with the albumins of normal size in serum and urine. The curves are functions of the loss of C^{14} label—particularly of C^{14} lysine—from the albumin molecules by degradation and by dilution of radioactive molecules by newly synthesized unlabelled albumin. Urinary albumin appears to have a slightly greater rate of turnover than has the albumin in the serum. The lower peak value of specific activity of minialbumin, as compared with the larger albumins, may be accounted for in part by the fact that the relative proportion of lysine is less, and the possible maximum radioactivity by incorporation of C^{14} lysine could be diminished accordingly.

Interpretation of the findings is rendered difficult by a number of unknown factors such as the rates of renal clearance of the urinary albumins, of metabolic interconversions, if any, between minialbumin and the other albumins, and the relative importance to the individual proteins of recycled C^{14} lysine or other labelled amino acid. The available evidence, though tentative, points to a somewhat greater turnover rate for minialbumin and a corresponding shorter biological half-life, when judged by the radioactivity of the excreted protein.

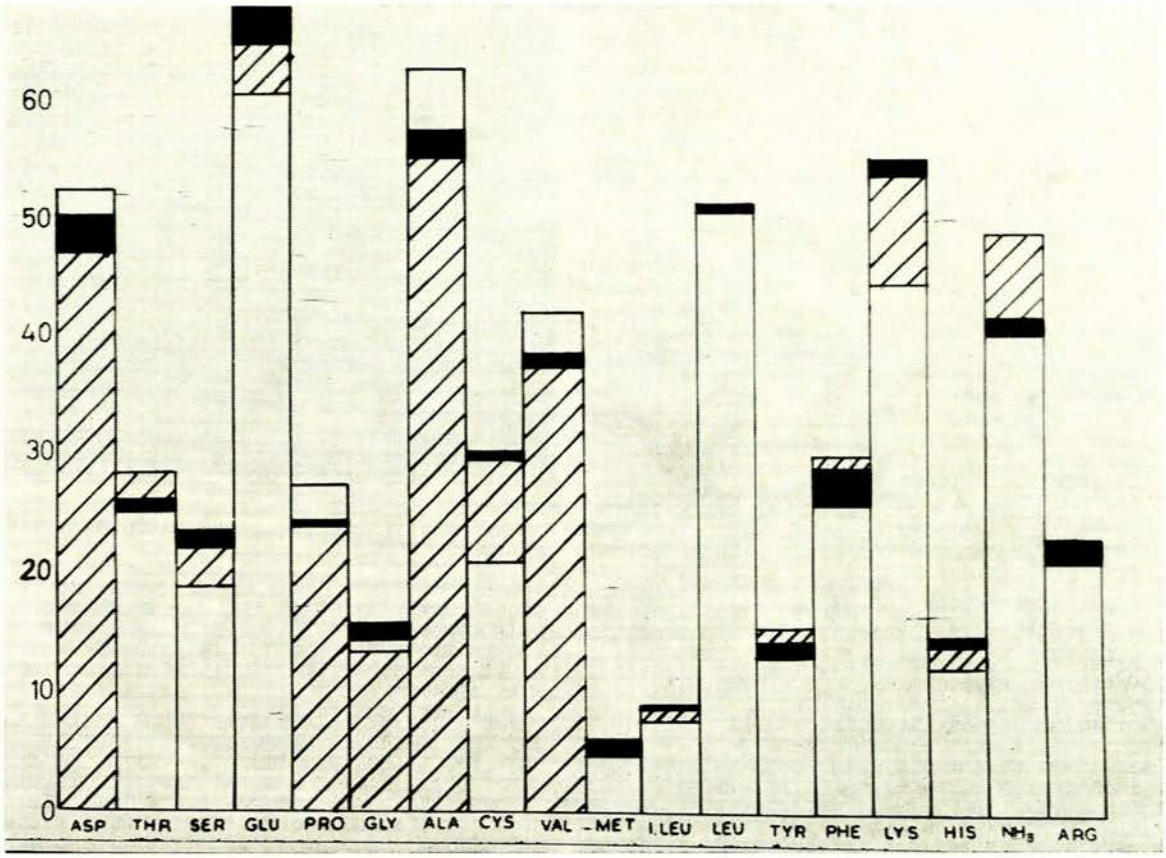

Fig. 4. Amino acid composition of monkey serum and urinary albumins. The ordinate represents numbers of amino acid residues per gram molecular weight of normal serum albumin (66,000).

Normal serum albumin ■ Urinary normal albumin □ Urinary minialbumin.

The metabolic fate of infused C^{14} minialbumin was studied in order to gain further insight into the metabolic interrelationships of the albumins. A total of 79·7 mg. C^{14}-labelled minialbumin was available from the previous experiment and this was dissolved in 4 ml. sterile isotonic saline, and injected intravenously into a normal monkey (R). The monkey showed no signs of anaphylactic shock or other reaction after the injection. Blood and urinary specimens were collected following the injection, fractionated in the usual manner, and the specific radioactivity of the albumins measured. No minialbumin could be detected in the serum of the animal at any time,

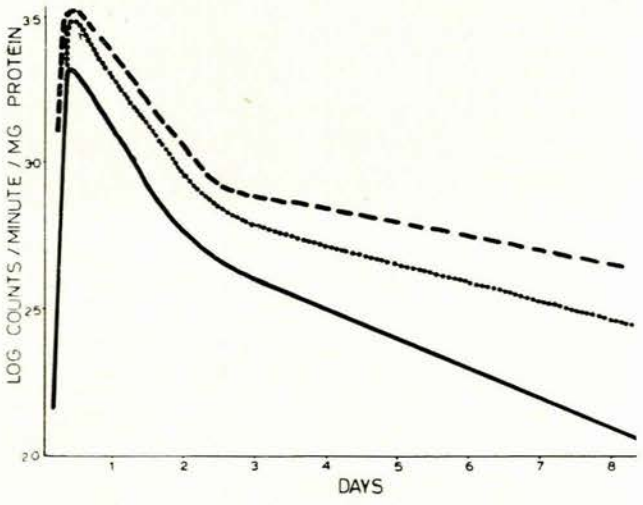

Fig. 5. Metabolic turnover of serum albumin and urinary albumins of cadmium-poisoned monkey F. Specific radioactivity of serum albumin (— — —); of urinary normal albumin (. . . . .); and of urinary minialbumin (———).

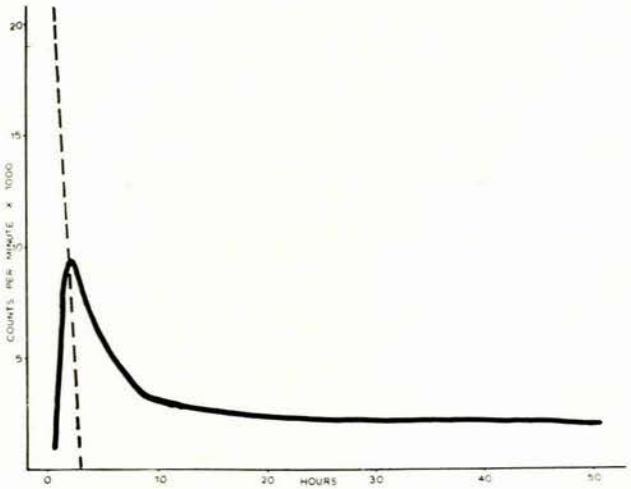

Fig. 6. Fate of C^{14}-minialbumin injected intravenously into a normal monkey R. Specific radioactivity of normal serum albumin (———) and of urinary minialbumin (— — —).

but serum albumin became rapidly labelled with a peak of radioactivity at 2 hours (Fig. 6). There were no counts incorporated into the serum globulin fraction. Of the injected C^{14}-minialbumin, 40 mg. appeared in the first urinary specimen which could be collected, 3 hours after the injection. This was the only specimen of urine which contained radioactive protein. Slight radioactivity was detected in the urine at 23 hours, but no protein was detectable, and presumably these were labelled products of albumin catabolism.

The apparent rapid transfer of C^{14} from minialbumin to normal serum albumin suggests some precursor relationship between the smaller and the larger albumin molecules. Such an important conclusion must be supported by much more experimental evidence, and a number of alternative mechanisms excluded before acceptance of this hypothesis. One obvious explanation could be that C^{14} minialbumin was adsorbed to normal serum albumin, but this seemed less likely as a result of the following experiment:

2·5 mg. C^{14}-minialbumin was added to a solution containing 7·5 mg. unlabelled normal monkey serum albumin. The individual solutions were counted before mixing, and the mixture was allowed to stand for 5 hours in a waterbath at 37°C. The two albumins were then separated on Sephadex G 75, collected, concentrated in boiled dialysis sacs, and again counted. The experimental data are presented below:

	Counts before mixing	*Counts after separation from mixture*
C^{14}-minialbumin	8,432	>8,452
Normal monkey serum albumin	1,723	1,637

Background count was 1,717 c.p.m.

This simple experiment does not exclude the possibility that, *in vivo*, a firmer type of complex might exist between minialbumin and normal serum albumin, which might account for the radioactivity in serum albumin, as one of the metabolic fates of the infused C^{14}-minialbumin in addition to the large proportion (50%) rapidly excreted unchanged into the urine.

(*iv*) *Antigenic behaviour of minialbumin.* The antigenic properties of minialbumin have been examined in two ways, by immunoelectrophoresis[29] and on Ouchterlony[30] plates. The gel for both techniques was 1% agarose (purchased from Seravac Laboratories S.A. Ltd., Cape Town) in 0·05 M tris (hydroxy methyl) amino methane-HCl buffer, pH 8·4.

Electrophoresis was performed in perspex tanks, with a PD of 300 V for approximately 20 minutes, progress being judged by the movement of a phenol-red marker. After removal of the plates from the electrophoresis tank, antiserum was placed in the longitudinal slot separating the two runs. Antigen-antibody reactions leading to lines of precipitation within the gel, were allowed to proceed overnight, the plates being housed in petri-dishes kept moist with damp filter paper. The immunoelectrophoretic patterns were then photographed by contact printing before or, more usually, after staining with 0·02% aqueous nigrosine.

Fig. 7 shows the immunoelectrophoretic precipitation patterns of the urinary mini- and normal albumins of the cadmium workman, Finch, separated as already described. The antiserum in the middle well was Coombs anti-human serum (Burroughs Wellcome). In each instance there is only one precipitin band to be seen, bands being closely paired with regard to their positions in the gel. No globulin contaminant was revealed on immunoelectrophoresis, although the antiserum had high titres against a wide range of serum globulins.

The antigenic behaviour of various albumins were tested by the two-dimensional gel diffusion procedure first described by Ouchterlony in 1948. The antibody was placed in the central well of the agarose gel deposited on the glass slides, while the antigens to be examined were put into the 6 circumferential wells, as seen in Fig. 8. The proteins diffuse through the gel and, on meeting, antibody and specific antigen form precipitin bands. The reaction was usually complete after 24 hours, but with antigens or antibodies of weak titre, 48 hours were needed for development of the precipitates. Precipitin bands

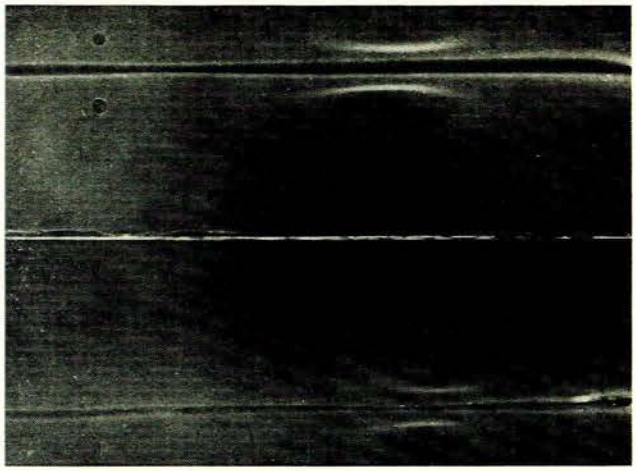

Fig. 7. Immunoelectrophoretic pattern of urinary albumins of cadmium-poisoned workman Finch. *Above.* Urinary normal albumin. *Below.* Urinary minialbumin.

were visible to the naked eye as white lines at varying distances from the central well. The plates were sometimes photographed unstained by contact printing, but more usually after the precipitin bands had been stained. The plates in Fig. 8 are almost self-explanatory, but the important features are as follows:

Plate I. Coombs anti-human serum in the central well has given the reaction of identity between urinary normal albumin and minialbumin of the cadmium worker (Finch), alternating in wells 2 - 5, and at increasing concentrations of antigen. The precipitin bands fuse without intersection or formation of spurs. In contrast, normal monkey albumin and normal bovine albumin give no clear-cut precipitin bands.

Plate II. There is reaction of identity between the urinary albumin of a cadmium worker (7108) and normal human serum albumin (wells 1 and 2).

Apart from normal albumin and minialbumin in the TCA-acetone preparation, no protein could be detected from

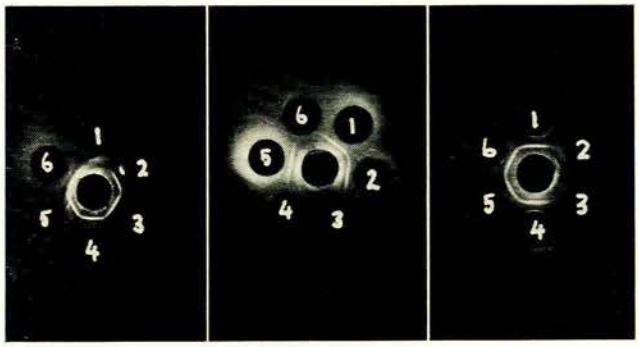

Fig. 8

Plate I *Plate II* *Plate III*

Fig. 8. Antigenic behaviour on Ouchterlony plates of various albumin preparations.

Plate I. Coombs anti-human serum in central well. 1 = Serum albumin normal monkey, 2 = Urinary albumin cadmium worker Finch, 3 = Urinary minialbumin cadmium worker Finch, 4 = Urinary normal albumin Finch, 5 = Urinary minialbumin Finch, 6 = Normal bovine serum albumin.

Plate II. Coombs anti-human serum in central well. 1 = Normal human serum albumin, 2 = Urinary normal albumin cadmium worker 7108, 3 = Pre-albumin peak, 4 = Pre-albumin peak, 5 = Urinary minialbumin cadmium worker 7108, 6 = Post-minialbumin peak.

Plate III. Rabbit anti-normal monkey albumin in central well. 1 = Normal monkey serum albumin, 2 = Urinary normal albumin cadmium-poisoned monkey F, 3 = Urinary minialbumin monkey F, 4 = Urinary normal albumin monkey F (concentrated 10 x), 5 = Urinary minialbumin monkey F (concentrated 10 x), 6 = Urinary normal albumin monkey F (concentrated 10 x).

the urine of the cadmium worker 7108 (material forming minor peaks in the eluate from the dextran gel column, Sephadex G 75, were placed in wells 3, 4 and 6).

Plate III. The central well contains rabbit anti-normal monkey serum albumin, prepared by 6 weekly intramuscular injections into an adult rabbit, of 5 - 10 mg. monkey albumin in 1 ml. water, emulsified with 1 ml. oil adjuvant.

Wells 1, 4, 5 and 6 show reaction of identity with one another: the preparations in wells 2 and 3 were too dilute to give clear lines of precipitation.

The Origin of Minialbumin

A detailed report on investigations relating to the origin of minialbumin will be published shortly. One aspect only of this problem will be considered in the present paper, namely, whether any fragmentation of serum albumin can occur during the initial mixing of cadmium chloride with whole blood.

Whole normal monkey blood (6 ml.) was mixed with 6 mg. Cd (as $CdCl_2$) in the usual manner as for intravenous injection. Thereafter, serum albumin was prepared by the TCA-acetone procedure and fractionated by gel filtration. No minialbumin could be detected, only the single peak of normal serum albumin being present (Fig. 9), and we can safely conclude that minialbumin is produced either in the circulating blood or at some tissue site in the body.

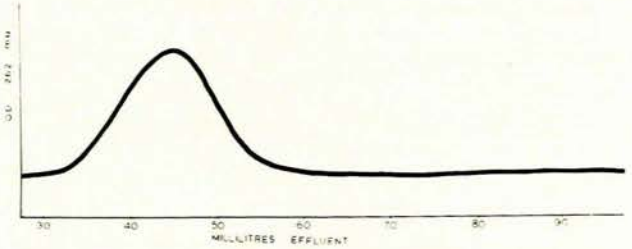

Fig. 9. Gel filtration of serum albumin prepared from whole monkey blood treated with cadmium chloride—6 ml. whole normal monkey blood mixed with 6 mg. Cd (as $CdCl_2$) as for IV injection. Serum albumin prepared and fractionated in the normal way.

DISCUSSION

Workmen exposed to cadmium oxide dust or fume suffer clinically from an incapacitating emphysema,[31] the main cause of the morbidity and mortality of men poisoned by cadmium in industry. Proteinuria is commonly found in such affected persons, but the incidence and quantity is not closely associated with the severity of the lung lesion. Cadmium administered intravenously to rabbits,[32] dogs and monkeys gives rise to proteinuria without causing lung damage. On the other hand, much evidence pointing to disturbances in renal function or to histological changes in the kidney has been accumulated from patients and experimental animals. Thus Bonnell and his associates[32] have described renal lesions consisting essentially of tubular atrophy and interstitial fibrosis in chronically poisoned rats, although Kench *et al.*[12] observed only minimal changes at postmortem examination of experimental rabbits. In an addendum to this paper Dr. Timme reports definite degenerative changes in the proximal tubules of the cadmium-poisoned monkey F, with occasional frank necrosis. Aminoaciduria is a frequent finding in poisoned workmen, progressing from the appearance alone of the hydroxy amino acids, serine and threonine, in mildest intoxication to a generalized aminoaciduria in severe poisoning.[33] Glycosuria may accompany the proteinuria and aminoaciduria. A fine intralobular cirrhosis of the liver was observed in poisoned rabbits; and occasional areas of focal necrosis, but without evidence of cirrhosis, were noted in the monkey liver.

As regards the proteinuria itself, the present study has demonstrated unequivocally the presence of a minialbumin as well as normal albumin in the urine of the cadmium-poisoned monkey. Poisoned monkeys, dogs and rabbits, excrete less minialbumin relatively to normal serum albumin when compared with man. The number of minialbumin molecules formed in the cadmium-poisoned monkey is equal approximately to 26% of the number of albumin molecules of normal molecular weight being synthesized in the liver, whereas the corresponding figure for poisoned workmen is 53%.

The minialbumin appears to have a somewhat greater turnover rate than have serum and urinary albumins of normal molecular weight in the cadmium-poisoned monkey. In high doses about 50% of minialbumin passed through the kidney of a normal monkey, but the quantity injected in 2 minutes (80 mg.) corresponds roughly to the quantity presented to the kidneys of severely poisoned monkeys in 24 hours. Of the 40 mg. retained, much of the C^{14} of minialbumin appeared in the albumin fraction in a form inseparable by gel filtration. The relatively large metabolic load of minialbumin which can be retained by the normal monkey kidney suggests that a certain degree of renal damage must occur before the protein can pass into the urine. Perhaps the onset of the proteinuria is delayed by the presence, in normal renal cortical tissue, of the cadmium-binding protein metallothionein,[34] but, at present, we have no useful information on this question. We have been unable to detect minialbumin in the urine of a small series of patients suffering from non-specific renal tubular defects, but this aspect of the problem also deserves further investigation.

The other characteristics of minialbumin are of great interest. Its molecular weight (20,000) is rather more than one-quarter of that of normal serum albumin, but we have found that, with the possible exception of its content of cysteine and of lysine, its amino acid composition is indistinguishable from that of normal serum albumin. Press and Porter,[35] in their studies on the degradation of human serum albumin with α-chymotrypsin, have separated 4 serologically active components, ranging in molecular weight from 7,000 to 23,400. The smallest component did not precipitate antibody but partially inhibited precipitation. Its amino acid composition differed greatly from that of the whole molecule, containing fewer residues of arginine and proline and entirely lacking serine and tyrosine. The minialbumin of cadmium poisoning appears to be quite a different entity from any fragment prepared by Press and Porter, and it precipitated antibody in a manner indistinguishable from that of normal serum albumin. The close similarity in antigenicity and amino acid composition between minialbumin and normal serum albumin suggests that the smaller molecule could arise as a result of the inhibitive action of cadmium ions at some stage in the synthesis or breakdown of normal albumin. For such a mechanism to operate implies the presence of a segment,

identical with minialbumin, within the long polypeptide chain which constitutes the normal serum albumin molecule. The close similarity in amino acid composition between minialbumin and the serum albumin molecule as a whole would, in this context, mean that the remainder of the albumin molecule should have a similar amino acid composition, although not necessarily the same primary arrangement of amino acid residues. It is possible that the small albumin encountered in cadmium poisoning may be formed entirely independently of normal serum albumin metabolism. If this were true, it would resolve the apparent anomalies between the amino acid composition and antigenic behaviour of the peptides prepared from serum albumin by tryptic digestion[35] and the corresponding characteristics of the minialbumin of cadmium poisoning.

Whatever the true mechanism, the evidence has already convinced us that cadmium ions will provide an important tool in the investigation of the synthesis of albumin and perhaps of other body proteins, and of the relationship between the size and structure of albumins and their antigenic behaviour.

SUMMARY AND CONCLUSIONS

1. Lyophilized preparations of urinary proteins of cadmium workers have been fractionated by the trichloracetic acid-acetone method and the albumin fraction subsequently separated into normal and low-molecular species by filtration through a column of dextran gel.

2. The low molecular protein migrated electrophoretically on paper as does serum albumin, and in TCA-acetone, on immunoelectrophoresis, in Ouchterlony plates, and in its amino acid composition was indistinguishable from human serum albumin. Only in its smaller molecular weight (approximately 25,000) did it differ from serum albumin and hence it has been named minialbumin.

3. No minialbumin could be detected in the urine of a small series of patients suffering from non-specific renal tubular damage, as for example, an advanced case of hepatolenticular degeneration, Fanconi syndrome, acute tubular necrosis or nephrotic syndrome.

4. Dogs and monkeys have been chronically poisoned by repeated intravenous injections of cadmium chloride, and after some months these animals, too, were found to excrete a minialbumin closely similar in all the respects already mentioned, with their own serum albumins and the normal molecular albumin found with them in the urine.

5. Diffusion-constant determinations and ultracentrifugation for the sedimentation constant proved the molecular weight of monkey minialbumin to be approximately 20,000.

6. Whole monkey blood when mixed with cadmium chloride in the usual manner, as for intravenous injection, did not give rise to any detectable minialbumin, only the single peak of normal serum albumin being present.

7. Monkey minialbumin had a somewhat higher metabolic turnover rate than its normal serum counterpart as shown by incorporation of C^{14}-lysine and radioactive decay of the serum and urinary albumin molecules.

8. The minialbumins of the various species differed antigenically from one another, as did their normal prototypes: Coombs anti-human serum did not give a precipitin reaction with monkey albumins, and a rabbit anti-monkey albumin preparation likewise was inactive against human albumin. No cross-reactions have been observed.

9. When 80 mg. C^{14}-minialbumin was injected intravenously into a normal monkey, about 50% of the protein appeared in the urine within 3 hours and the remainder was retained in the body. Much of the C^{14} was detected in the serum albumin fraction and was inseparable on gel filtration.

10. This appears to be the first recognized example of a simple chemical agent capable of bringing about a reproducible aberration *in vivo* in the molecular size of a protein. The possible biochemical mechanisms by which cadmium ions might lead to the *in vivo* formation of minialbumin have been discussed, and the hope expressed that cadmium may prove to be an important tool in the study of structure, metabolism and immunological behaviour of proteins.

We wish to express our gratitude to the following colleagues whose cooperation made this work possible: Dr. G. M. Potgieter for valuable advice and guidance on immunological techniques; Prof. A. Kipps for making available to us the facilities of the UCT/CSIR Virus Research Unit; Dr. A. Polson for determinations of the molecular weight of our proteins; Dr. M. C. Berman for stimulating discussion; Dr. A. H. Timme, Department of Pathology, for the histological studies and report; and Drs. W. P. U. Jackson, F. Harris and R. McDonald who kindly helped in our search for patients. We wish to thank Dr. Frances Ames in particular for her interest and for making it possible for us to investigate her patient suffering from hepatolenticular degeneration.

ADDENDUM

A Report on the Histology of the Cadmium-poisoned Monkey F

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A systematic investigation has been undertaken of the histological changes observed in the tissues of cadmium-intoxicated animals. A detailed report on liver and kidney tissues from biopsies and at postmortem examination is included in A. C. Gain's Ph.D. thesis. In the kidney biopsy definite degenerative changes were evident in the proximal tubules, with occasional frank necrosis, while attempts at regeneration on the part of the epithelial cells were minimal. The liver biopsy showed no evidence of cirrhosis. Occasional areas of focal necrosis were present. At postmortem examination, both the liver and kidney showed evidence of damage which could be attributed to the cadmium intoxication. In the kidney the changes were unmistakable, but those in the liver were less impressive and their significance much less certain. The fact that the postmortem specimen of liver showed more severe changes than are seen in the biopsy may be due to the metabolic disturbances following the nephrectomies (which were performed following the experiments described in the foregoing paper).

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