# THE NATURE AND ORIGIN OF THE MINIALBUMIN FOUND IN CADMIUM-POISONED ANIMALS

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In a recent report,[1] we described the isolation and characterization of low-molecular albumins in the urine of dogs and monkeys poisoned chronically with cadmium chloride, given by repeated intravenous injections. The present paper describes experiments designed to provide more information on the origin and mechanism of formation of the minialbumins. Such knowledge would be important in relation to the possible role of cadmium ions in the regulation of albumin metabolism, and would also help to resolve uncertainty with regard to the tissues most affected by the metal. The evidence that cadmium can be a nephrotoxic agent is well reviewed by Bonnell.[2]

Amino-aciduria[3] and glycosuria, hypercalciuria and other defects of both proximal and distal tubular function[4] appear to be indicative of a Fanconi type of renal tubular disorder. Abnormal renal function tests have been demonstrated in a number of cases[5] but, on the contrary, the kidneys of poisoned workmen who have died after years of persistent proteinuria may show no histological evidence of tubular necrosis.[6] Our own view that the proteinuria of cadmium intoxication is a specific entity[1,7] is not favoured by other investigators. Creeth *et al.*[8] consider the mixture of urinary proteins observed in cadmium poisoning to be that typical of any non-specific renal tubular defect. Our earlier publications have included data which do not appear to support this concept, and the present work provides, we believe, further evidence in support of the view that the low-molecular albumin, found in the urine of men and animals poisoned by cadmium, arises as a result of a characteristic biochemical lesion.

The pattern of urinary globulins may be associated with the Fanconi type of renal tubular syndrome, but it has transpired from our work that the urinary minialbumin probably comprises a mixture of polypeptides formed by fragmentation of the serum albumin molecule, mainly within the liver cells. The smallest fragments which have been recognized are all approximately similar in size (MW 5,000), and it is presumed they have widely diverse amino-acid composition. The over-all composition of the mixture, nevertheless, closely simulates that of the parent molecule. The peptides have a remarkable tendency to aggregate to form molecules whose molecular weight may be 10,000, 20,000 or even that of normal serum albumin (67,000). Urinary minialbumin, we regard, as the component MW 20,000 in this series which passes into the urine when reabsorption of the protein from the glomerular filtrate is impaired by cadmium ions.

For purposes of discussion, all mixtures of polypeptides, whose amino-acid composition, electrophoretic mobility, antigenic behaviour and stability in acid-ethanol are closely similar to these characteristics in normal serum albumin, are referred to, throughout, as minialbumins, regardless of whether found in urine, peritoneal dialysate, serum or tissues.

## EXPERIMENTAL OBSERVATIONS AND RESULTS

*Experimental animals and their maintenance.* Male vervet monkeys (*Cercopithecus aethiops*) were housed and fed, and specimens collected, as already described.[1] The rats used were normal adult female albino animals, locally bred and weighing 240 - 300 G. The animals were maintained on a normal diet and were not starved before the experiments. The rats were supported on a perforated porcelain disc inside a glass container and the urine could thus be easily collected.

The rabbits employed for the production of antisera were normal healthy males, locally bred, and each weighed approximately 2 kg. They were fed on a standard mixed diet.

*Poisoning of the animals.* Poisoning of monkeys was performed according to the method of Kench *et al.*[1] Cadmium was administered to the rats as a 1% w/v solution of cadmium chloride in 0·9% w/v sodium chloride, at a dosage level of 15 mg. cadmium/kg. body-weight. An equivalent volume of physiological saline was given to the control animals. The animals were lightly anaesthetized and the cadmium chloride or saline injected intraperitoneally over a few minutes.

*Peritoneal dialysis.* This procedure was performed on monkeys only. Indwelling teflon catheters were surgically inserted into the peritoneal cavity of monkeys, anaesthetized by intravenous injection of sodium pentothal solution (2 ml. of 2·5% aqueous solution). The dialysis fluid was sterile, non-pyrogenic Ringer's lactate, peritoneal dialysis solution (Dianeal), which was warmed before infusion. The solution contained $Na^+$, $Mg^{2+}$, $Ca^{2+}$, $Cl^-$, lactate and 15% glucose, with 1 mg./l. bisulphite. The fluid was run in and out of the peritoneal space of the animal, under gravity, for 30-min. periods.

*Collection and preparation of sera and tissue extracts.* Serum was employed throughout for preparation of albumin, as heparin interfered with the precipitation procedure. Blood was withdrawn from the saphenous vein of the lower hind leg of the monkeys and allowed to clot. Blood from the rats was obtained from the aorta and from the hepatic portal vein immediately after killing the animals.

Clear supernatant extracts of liver and kidney tissues were prepared as follows: the tissues were homogenized in M/15 phosphate buffer pH 7·0 and then centrifuged at 105,000 g. for 100 min.

## SPECIAL INVESTIGATIONS OF ALBUMINS

### *Separation of Albumin Fraction*

Albumin was prepared in many instances by the trichloracetic acid (TCA)-acetone method of Vallance-Owen *et al.*,[9] which is particularly suited to handling of voluminous specimens such as dialysates and urine. Trichloracetic acid and acetone were removed from the albumin solution by dialysis in Visking cellophane sacs against M/15 phosphate buffer pH 7·0. The dialysed solution of albumin was freeze-dried and redissolved in column buffer. The albumin obtained was found to be free of globulins by either cellulose acetate or immunoelectrophoretic techniques. One unfortunate complication we discovered was an interfering substance being extracted from the sacs by the acetone, or by ethanol if this was used as the solvent.

The contaminant from the Visking dialysis tubing was biuret positive, absorptive at 280 mμ, and had a molecular weight ranging widely around 5,000. It could be extracted from the sacs by boiling with dilute sodium carbonate solution or in the presence of bile salts, but this treatment irreversibly increased the porosity, so that, afterwards, lysozyme (MW 14,500) diffused freely through the membranes. Thus, both untreated and treated membranes were for

different reasons equally unsuitable for our purpose.

Alternatively, the HCl-ethanol method of Fernandez *et al.*[10] was employed, as it did not require contact of cellophane membranes with organic solvents. The method was found to be quite satisfactory. In this procedure, globulins are first precipitated by addition of an HCl-ethanol mixture, and the resulting supernatant fluid separated and treated with sodium acetate to precipitate the albumin. This could be collected by centrifugation and then dissolved in sodium chloride solution (0·2 M or more) for passage through cross-linked dextran gel Sephadex G 75. The pore size of this gel permitted good separation of the different types of albumin under investigation. Details of column dimensions, buffer systems and flow rates are given individually with the illustrations, as these factors varied according to the experiment. Invariably, eluates from the column were monitored at 262 m$\mu$ by a LKB Uvicord ultraviolet absorptiometer and recorder.

Protein preparations were preserved by lyophilization after removal of salt by dialysis in boiled cellophane sacs. Buffers for all albumin preparations contained 500 mg. sodium azide per litre as preservative. The pH of the albumin solution during the TCA procedure fell to approximately 2·5, whereas it did not fall below 4·5 in the HCl-ethanol method. Both techniques gave closely similar results as judged by quantities of normal and minialbumins present, and in the characteristics of the individual albumins.

Specimens of urine were dialysed for 24 hours against running water and then for 1 - 2 hrs. against distilled water before precipitation and fractionation of the proteins. Dialysates were treated likewise.

An investigation on the albumins present in peritoneal dialysates of the monkey F is presented in Fig. 1. Many

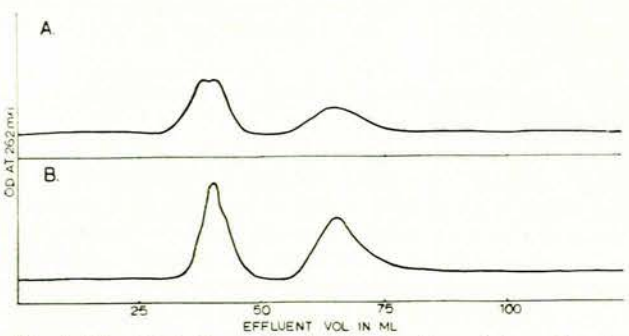


*Fig. 1.* Albumins in the peritoneal dialysates of a cadmium-poisoned monkey F, following bilateral nephrectomy. A = peritoneal dialysate 1 day after nephrectomy; B = peritoneal dialysate 6 days after nephrectomy and 10 hrs. after a pulse dose of 6 mg. cadmium given intravenously as cadmium chloride. Albumins prepared from the dialysate by the TCA-acetone method, and separated on Sephadex G 75 M/15 phosphate buffer, pH 7·0, containing 0·5 M sodium chloride. Column = 25 x 2 cm. Flow rate 20 ml./hr. Optical density (OD) recorded at 262 m$\mu$. Normal albumin is eluted first from the column and minialbumin later, reading from left to right on the optical density tracing.

experiments on this animal were described in our previous communication.[1] He had been chronically poisoned with cadmium for several months, and then, in two stages, underwent bilateral nephrectomy. The animal appeared to withstand the operation well and was sitting up in his cage on the 1st postoperative day. Following nephrectomy, peritoneal dialysis was performed on the 2nd, 4th and 6th postoperative days. A single dose of 6 mg. cadmium (as cadmium chloride) was administered intravenously at 10 p.m. on the 5th postoperative day, and he was dialysed 10 hours later. Unfortunately, the monkey died unexpectedly 2 hours later.

Minialbumin was found to be present in all peritoneal dialysates, and the quantity did not appear to fall during the 6 days following complete nephrectomy. Moreover, after the booster dose of cadmium on the 5th day, there was a marked rise in output of minialbumin in the peritoneal dialysate collected 10 hours later; the rise in concentration was from 110 to 180 mg./100 ml. dialysis fluid.

In subsequent experiments, we have been unable to detect minialbumin in the peritoneal dialysates of normal monkeys, but the appearance of minialbumins in sera and peritoneal dialysates following intravenous injection of cadmium chloride can regularly be demonstrated (Fig. 2).

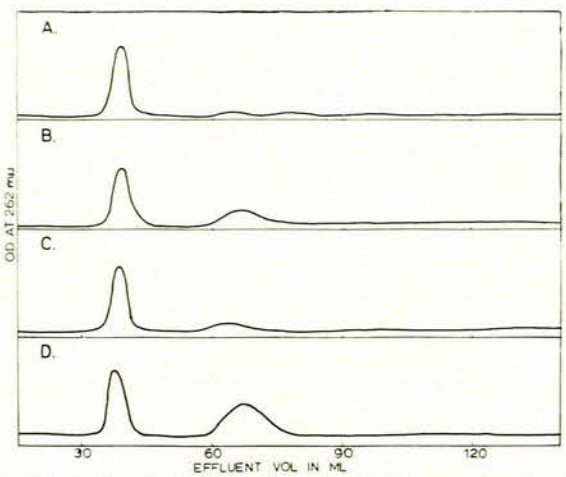


*Fig. 2.* Albumins in the sera and peritoneal dialysates of a chronically-poisoned monkey K, given pulse doses of cadmium. A and C = peritoneal dialysate and serum respectively 168 hrs. after previous cadmium injection; B and D = peritoneal dialysate and serum respectively 1½ hrs. after pulse dose of 6 mg. cadmium given intravenously as cadmium chloride. Albumins prepared and separated as in Fig. 1. Column = 40 x 1·8 cm. Flow rate 15 ml./hr. Optical density (OD) recorded at 262 m$\mu$. Some sac contaminant can be seen as a small peak in A following just behind minialbumin.

The rise in the peak of circulating minialbumin is especially marked 90 mins. following a pulse dose of 6 mg. cadmium, but in the chronically-poisoned monkey there is always some minialbumin present. There is a steady fall in the urinary excretion of minialbumins following a single booster dose of cadmium in monkeys chronically poisoned by cadmium (Fig. 3). The usual timing of events in chronic poisoning, as observed consistently in the 6 monkeys we have studied, is the appearance of minialbumins in the serum after intravenous injection of 25 - 30 mg. cadmium (10 - 12 mg. weekly for 2 - 3 weeks) and minialbuminuria becomes evident after 60 - 90 mg. cadmium and 6 - 7 weeks of poisoning, provided the poisoning regime is steadily maintained. Thus, passage of minialbumin into the urine usually begins 4 weeks after its first appearance in the serum. If the injections are stopped in these short-term intoxication studies, the gravity of the biochemical lesion falls away in a few days, as seen in Figs. 2 and 3.

*Antigenic Behaviour of the Albumin Preparations*

The antigenic behaviour of the various minialbumins was compared with that of normal serum albumin by the technique of Ouchterlony[11] and by immunoelectrophoresis.

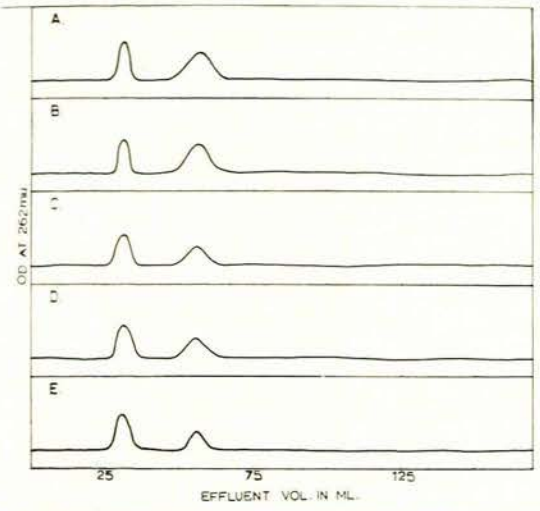


*Fig. 3.* Urinary albumins of the cadmium-poisoned monkey G, following a pulse dose of 6 mg. cadmium given intravenously. A - E = 1 - 5 days respectively after the dose. Albumins prepared and separated as in Fig. 1. Column 25 x 2·5 cm. Flow rate = 18 ml./hr. OD recorded at 262 mμ.

For these purposes, antisera to normal monkey albumin, to normal monkey serum and to normal rat serum was produced. This was done according to the method of Mandy *et al.*[12] Normal healthy rabbits each weighing approximately 2 kg. and fed on a balanced diet were employed. A 1% solution of the appropriate antigen in physiological saline was sterilized by passage through millipore filters (porosity 0·5 - 0·22 μ) and injected intravenously 2 or 3 times weekly. The animals were test bled from the ear after 5 - 6 weeks and after a 5-day resting period. Subsequently, bleeding and inoculations were carried out every 2 - 3 weeks.

Immunoelectrophoresis[13] was performed in 1% agarose gel (Seravac Laboratories, Cape Town) prepared in 0·05 M tris (hydroxymethyl) amino methane (tris)-HCl buffer pH 8·4, in a perspex tank, over a period of 25 - 30 minutes with potential difference of 300 V. Phenol red, which runs slightly ahead of albumin, was employed as a marker. The antigen-antibody precipitin reaction was allowed to proceed overnight in a moist chamber at the ambient temperature. After dialysing out excess antiserum, the precipitin lines were fixed with an ethanol-acetic acid-water mixture, stained with 1% aqueous nigrosin solution, and finally cleared with 5% w/v acetic acid. The dried plate could then be photographed.

Ouchterlony plates were prepared with 1% agarose gel in 0·05 M tris-HCl buffer pH 8·4. Diffusion and precipitation was allowed to proceed overnight. The fixing and staining procedures thereafter were as described above for immunoelectrophoresis.

All buffer solutions used in these studies of antigenicity contained 500 mg. sodium azide/litre, as preservative. We have collected together a number of immunoelectrophoretic patterns in Fig. 4 which illustrates the close similarity in electrophoretic mobility and antigenic behaviour between serum albumin of normal monkeys and minialbumins from sera, peritoneal dialysates and urine of chronically-poisoned animals. Figs. 4 and 5 are representative of consistent observations on 6 poisoned monkeys (F, K, G, O, B, M).

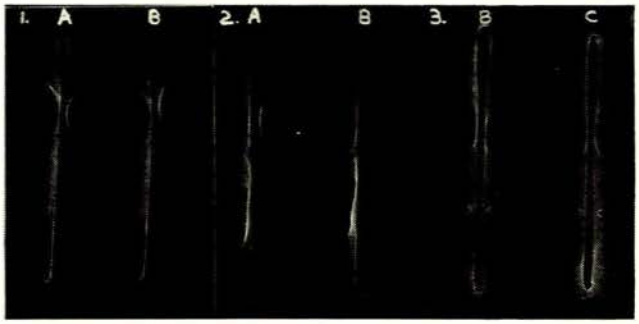


*Fig. 4.* Immunoelectrophoresis of albumins prepared from normal and cadmium-poisoned monkeys. 1A, 1B, 2A, 2B—left hand side of well = whole normal monkey serum. 1A right, 3B left = serum normal albumin from poisoned monkey. 1B, 3B right = serum minialbumin from poisoned monkey. 2A and 2B right = normal and minialbumin respectively from peritoneal dialysate of poisoned monkey. 3C left and right = urinary normal and minialbumin from poisoned monkey. Antiserum in central well: Plates 1 and 2—rabbit anti-whole normal monkey serum; plate 3—rabbit anti-normal serum albumin from normal monkey.

As seen in plates 1 and 2, the rabbit antiserum had good titres against a wide variety of serum globulins, but no trace of globulin contaminant can be seen in the albumin preparations. Minialbumins from serum or peritoneal dialysate of poisoned animals moved slightly faster than the corresponding normal albumin. With a specific antiserum prepared against normal monkey serum albumin (plates 3B, 3C), serum and urinary normal albumins and minialbumins are indistinguishable from one another on the plates.

Fig. 5 is a photograph of two Ouchterlony plates, in which rabbit anti-whole normal monkey serum was placed in the central well, and various albumin preparations in the peripheral wells numbered 1 - 6. The plates show clearly continuous fusion of all the precipitin bands around

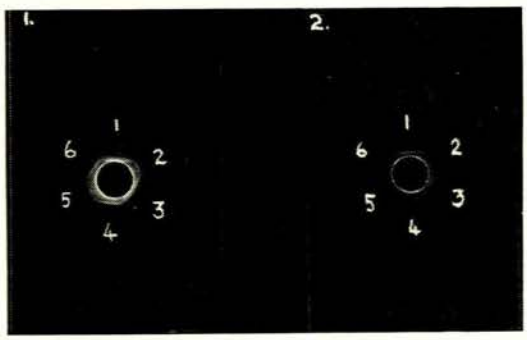


*Fig. 5.* Ouchterlony plates showing the antigenic behaviour of albumins from serum, peritoneal dialysates or urine of cadmium-poisoned monkeys. Plate 1: 1 and 3 = serum normal albumin from poisoned monkey, 2 = serum minialbumin from poisoned monkey, 4 and 6 = urinary normal albumin from poisoned monkey, 5 = urinary minialbumin from poisoned monkey. Plate 2: 1 and 3 = normal albumin from peritoneal dialysate of poisoned monkey, 2 and 4 = minialbumin from peritoneal dialysate of poisoned monkey, 5 = serum normal albumin of poisoned monkey, 6 = serum normal albumin of normal monkey. Antiserum used: rabbit anti-whole normal monkey serum.

the central well, without intersection or formation of spurs (the minialbumin in well 2 of plate 2 was too dilute to give a precipitin reaction). In brief, all albumins—whether normal or miniature in size, from serum of normal monkeys or from sera, peritoneal dialysates or urine of the 6 cadmium-poisoned monkeys we have studied—gave the reaction of identity in this test and were antigenically indistinguishable against this antiserum.

*Amino-Acid Composition*

The amino-acid composition of each electrophoretically-pure albumin was determined. The proteins were hydrolysed according to the method of Moore and Stein,[14] in 6 N HCl in sealed, oxygen-free, evacuated pyrex tubes, for 22 hours at 110°C. The resulting mixture of amino acids was dried by lyophilization, and analysed in a Beckman Spinco amino-acid analyser, Model 120 B, according to the method of Spackman *et al.*[15] Data on serum normal albumin and minialbumin of the cadmium-poisoned monkey M are presented in Table I and in the form of histograms in Fig. 6. With the exception of the lower proportions of lysine and ½-cystine in minialbumin, there is a close similarity between the amino-acid composition of the two proteins. We are unable to make a firm statement as to whether the smaller differences, seen in threonine, serine, glycine, valine and isoleucine, are significant or within experimental error. Similar findings have already been reported by us for monkey F, and we have so far found low values of lysine and ½-cystine in the minialbumins of all 3 monkeys we have investigated, but, in other respects, the amino-acid compositions of normal serum albumin and minialbumins are remarkably alike.

TABLE I. AMINO-ACID COMPOSITION OF THE ALBUMINS OF THE CHRONICALLY-POISONED MONKEY M

| *Aminoacid* | *Serum normal albumin* | | *Serum minialbumin* | |
|---|---|---|---|---|
| | *G residues /100 G* | *Residues* | *G residues /100 G* | *Residues* |
| Lysine | 11·98 | 63 | 10·40 | 54 |
| Histidine | 3·24 | 16 | 3·18 | 16 |
| Ammonia | 1·03 | — | 2·98 | — |
| Argenine | 4·70 | 20 | 4·37 | 19 |
| Aspartic acid | 9·25 | 54 | 9·66 | 56 |
| Threonine | 3·26 | 22 | 3·93 | 26 |
| Serine | 2·90 | 22 | 3·57 | 27 |
| Glutamic acid | 16·48 | 86 | 15·95 | 83 |
| Proline | 3·45 | 24 | 3·44 | 24 |
| Glycine | 1·13 | 13 | 1·95 | 23 |
| Alanine | 7·46 | 70 | 7·22 | 68 |
| ½-cystine | 4·76 | 31 | 3·13 | 21 |
| Valine | 6·85 | 46 | 6·22 | 42 |
| Methionine | 1·71 | 9 | 1·52 | 8 |
| Iso-leucine | 0·40 | 2 | 1·07 | 6 |
| Leucine | 10·90 | 65 | 10·35 | 61 |
| Tyrosine | 4·34 | 18 | 4·56 | 19 |
| Phenylalanine | 6·67 | 30 | 6·42 | 29 |
| Totals | 100·51 | 591 | 99·88 | 582 |

The number of residues has been calculated on the basis of a molecular weight of 67,000 for albumin in both cases. This albumin was prepared by the method of Fernandez *et al.* Albumin prepared by the TCA method of Vallance-Owen *et al.* gave similar results.

*Sedimentation and Diffusion Coefficients and Molecular Weight of the Minialbumins*

These parameters were kindly determined on a number of our preparations by Dr. A. Polson, as described previously. His report on one preparation of serum minialbumin from the monkey M is as follows: 'The synthetic boundary cell was used, and a 0·6% solution of protein in 0·9% w/v NaCl was run in the Beckman Spinco analytical ultracentrifuge, Model E. The bulk of the material (approximately 60%) had a sedimentation constant less than 1 Svedberg unit, but, on account of its inhomogeneity, no accurate sedimentation constant could be calculated. The rest of the protein travelled as a peak for which the sedimentation constant was calculated as $S_{20,w}=4{\cdot}75$. Within the limits of error of the experiment, this coefficient corresponds to that of normal serum albumin.'

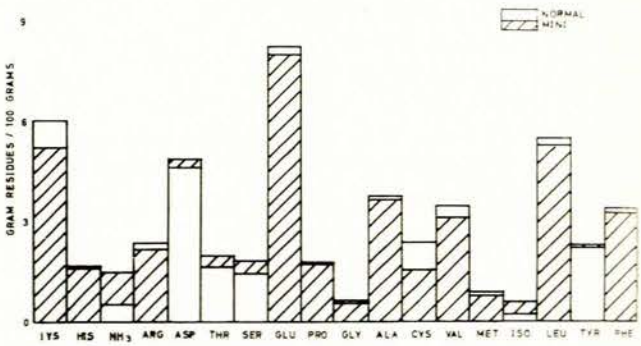


*Fig. 6.* Amino-acid composition of normal albumin and minialbumin obtained from the serum of the cadmium-poisoned monkey M. In the histogram the amino acids are expressed as gram residues per 100 G albumin, rather than as the number of residues per molecule of albumin, because of the heterogeneity of the small albumin.

This same preparation run previously in higher salt concentration appeared to consist only of molecules of weight less than 10,000. Serum minialbumin prepared from monkey K (Fig. 2) and kindly examined in the ultracentrifuge by Dr. T. H. Mead, contained mainly protein of MW 10,000, but greater and smaller components were also present.

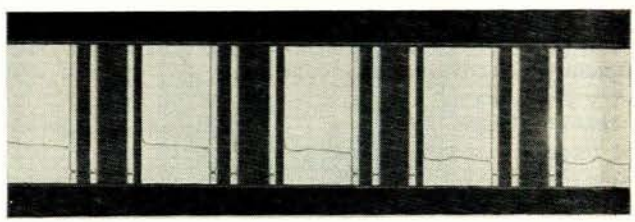

*Fig. 7.* Ultracentrifugal pattern of serum minialbumin prepared from the cadmium-poisoned monkey M. Observations were made using a Beckman Spinco analytical ultracentrifuge, Model E. The protein was examined as a 0·6% solution in 0·9% w/v aqueous sodium chloride in a synthetic boundary cell at a centrifuge speed of 56,100 r.p.m. Photographs were taken of the peak at intervals of 32 minutes.

These observations are in line with those previously reported for the urinary albumins of cadmium workers. In some mysterious manner, the minialbumins had apparently grown in molecular size during storage. The effect was almost certainly due to the fact that the preparations were ultracentrifuged in a lower salt concentration (0·9% NaCl) in Cape Town as compared with that used by Dr. R. A. Kekwick in London (phosphate-sodium chloride buffer total ionic strength 0·35).

*Minialbumin Formation in Rats Acutely Poisoned with Cadmium*

A series of rats were killed at varying intervals of time after a single intraperitoneal injection of cadmium, and the albumins in the serum separated on dextran gel, Sephadex G 75. A good response was elicited 90 mins. after the injection (Fig. 8). Concurrently with the appearance of minialbumin in the serum, a pre-albumin fraction arose in the electrophoretogram. The sera were applied to cellulose acetate strips and electrophoresis carried out for 20 mins. in a perspex tank, in veronal buffer pH 8·6, ionic strength 0·75, at a potential difference of 120 V and current of 3·5 - 4·5 ma. The strip was fixed and stained in a fixative-dye solution containing Ponceau S, trichloracetic and sulphosalicylic acids. The plates were cleared and dried and scanned in the 500 - 520 mμ range in the analytrol. The records are shown in Fig. 9. A distinct pre-albumin fraction is evident at 90 mins., but it has fallen markedly 180 mins. after the injection. It is proposed to study this change in more detail in further experiments.

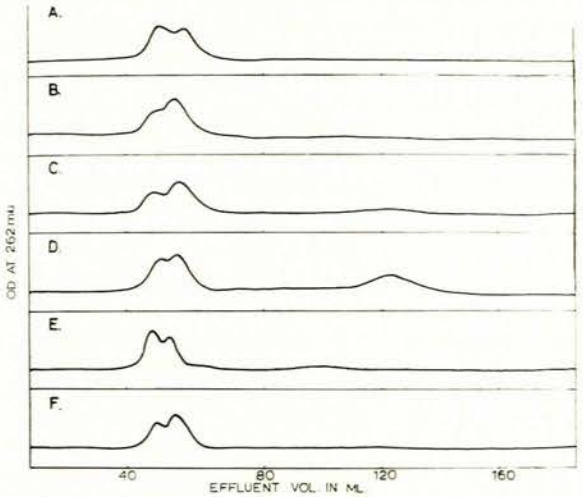


*Fig. 8.* Albumins prepared from sera of rats acutely poisoned with cadmium. A = control—equal volume of saline injected and animal killed after 60 mins. B - F = 15 mg. cadmium/kg. body-weight injected intraperitoneally, and the animals killed at 0, 40, 90, 180 and 1,440 mins. respectively. Albumins prepared by HCl-ethanol method and separated on Sephadex G 75 in M/15 phosphate buffer, pH 7·0 containing 0·2 M NaCl. Column 42 x 1·8 cm. Flow rate 12·5 ml./hr. OD recorded at 262 mμ.

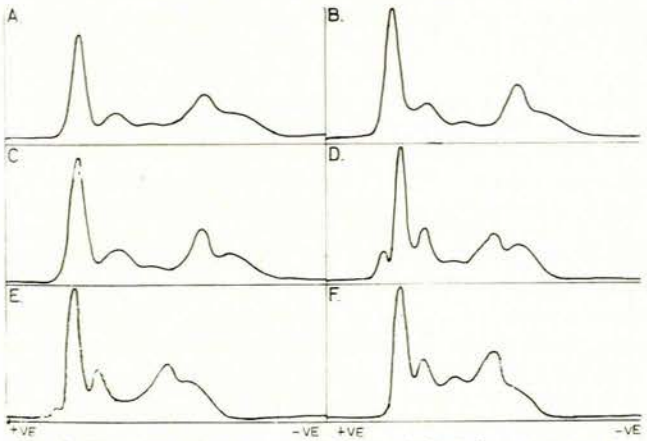


*Fig. 9.* Electrophoretic studies on the sera obtained from rats acutely poisoned with cadmium. A = control animal—saline—killed at 60 mins. B - F = sera from animals killed at 0, 40, 90, 180 and 1,440 mins. respectively after intraperitoneal injection of 15 mg. cadmium/kg. body-weight.

Fig. 10 presents observations on the albumins present in the liver of normal rats, and in animals during acute cadmium poisoning. Two features of especial interest are the presence of minialbumin in normal rat liver, and the greatly increased concentration of minialbumin in the tissue 90 and 180 mins. after a single intraperitoneal injection of cadmium. It is also noteworthy that there is a decline in the numbers of normal albumin molecules present after 2 hours of intoxication.

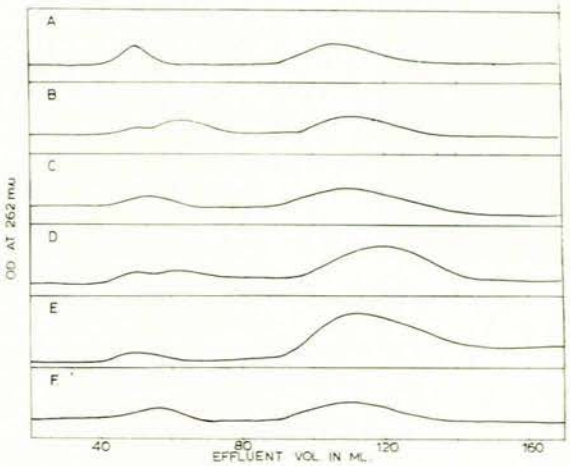


*Fig. 10.* Albumins prepared from the liver extracts of rats acutely poisoned with cadmium. A = control—saline—killed at 60 mins. B - F = extracts from animals killed at 0, 40, 90, 180 and 1,440 mins. respectively after intraperitoneal injection of 15 mg. cadmium/kg. body-weight. Albumins prepared and separated as in Fig. 8.

Similar extracts of the kidneys of poisoned rats were prepared and the changes in albumin fractions are shown in Fig. 11. A gel column of smaller capacity was employed

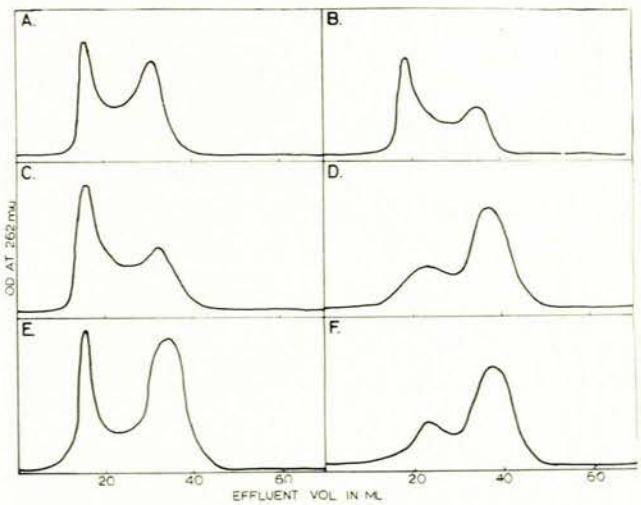


*Fig. 11.* Albumins prepared from kidney extracts of acutely poisoned rats. A = control—saline—killed at 60 mins. B - F = extracts from animals killed at 0, 40, 90, 180 and 1,440 mins. respectively after intraperitoneal injection of 15 mg. cadmium/kg. body-weight. Albumin prepared and separated as in Fig. 8. Column 16 x 1·6 cm. Flow rate 18 ml./hr. OD recorded at 262 mμ.

in this experiment, and the albumins overlap. There appears to be a considerable quantity of minialbumin before administration of cadmium. Following poisoning, still more minialbumin was found in the renal tissues and the effect appeared to outlast that in the liver. Whether all the minialbumin to be found in the kidney comes from the liver is an extremely important question apropos the origin of the urinary minialbumin of men and animals poisoned with cadmium. We hope to be able soon to publish decisive evidence on this problem.


*Fig. 12.* Antigenic behaviour of albumins found in the serum, liver and kidney of cadmium-poisoned rats. 1=serum normal albumin from acutely poisoned rat, 2=serum minialbumin from acutely poisoned rat, 3=liver normal albumin from acutely poisoned rat, 4=liver minialbumin from acutely poisoned rat, 5=kidney normal albumin from acutely poisoned rat, 6=kidney minialbumin from acutely poisoned rat. Antiserum—rabbit anti-whole normal rat serum.

The albumins in these tissue extracts behaved antigenically as do the albumins we have already found in sera, peritoneal dialysates and urine (Fig. 12).

*Possible Direct Chemical Action of Cadmium Ions on Normal Albumin Present in Blood or Serum*

It was important to establish whether minialbumin can be formed by the direct chemical action of cadmium ions on normal serum albumin present in blood or serum. To test this possibility, 2 mg. Cd (as $CdCl_2$) was added drop by drop with continuous stirring to 6 ml. venous blood of a normal monkey, containing 0·5 ml. heparin as anticoagulant. The mixture was allowed to stand for 2 hours at room temperature, after which the red blood cells were removed by centrifugation and the albumins prepared from the plasma by the TCA-acetone method and passed through a Sephadex G 75 column (Fig. 13A).

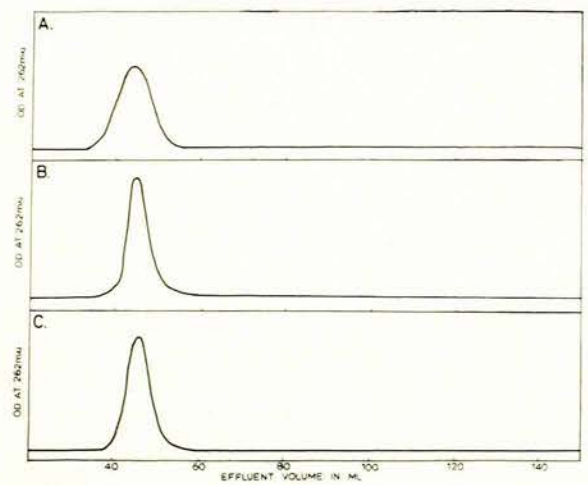


*Fig. 13.* Incubation of cadmium chloride with normal monkey serum or blood. A = albumins prepared from normal monkey blood after incubation with $CdCl_2$. B = albumins of normal monkey serum incubated with physiological saline (control). C = albumins of normal monkey serum after incubation with $CdCl_2$. In each experiment albumin in M/15 phosphate buffer, pH 7·0 and 0·5 M NaCl was passed through a Sephadex G 75 column 40 x 1·8 cm. Flow rate 15 ml./hr. OD was recorded at 262 $m_\mu$.

To examine the action on albumin in serum, 3 ml. of normal monkey serum was added to each of 2 containers, which were then treated as follows:

1. Control, 0·01 ml. physiological saline added.
2. 2 mg. Cd (as $CdCl_2$) added in physiological saline.

This is equivalent to the dosage of cadmium employed to cause acute poisoning in monkeys. The solutions were well mixed, passed through millipore filters into flamed sterile tubes, and then incubated at 37°C in a water bath, with gentle agitation, for 48 hours. Albumin was prepared from the two sera by the TCA-acetone procedure and fractionated on Sephadex G 75 gel (Figs. 13 B and C). No minialbumin was detected in any of the experiments. We can safely conclude that it does not arise by a direct action of cadmium on normal serum albumin.

## DISCUSSION

We have been concerned, in this and in earlier studies of the biochemical effects of cadmium, to elucidate the mechanisms which lead, in cadmium-poisoned men and animals, to the appearance of a low-molecular albumin in their urine. Other workers[5,8] have reported evidence supporting the view that this protein originates as a result of non-specific injury to the renal tubular epithelium cells. The experimental observations presented here appear to indicate, rather, that the urinary minialbumin arises, probably to a major extent, from a primary action of cadmium on the liver cells. The excretion of the protein in the urine occurs only after a period of weeks following the appearance of minialbumins in the circulating blood. The concentration of these compounds fell consistently with the onset of urinary excretion of minialbumin. On the other hand, the level of circulating minialbumins as seen in peritoneal dialysates, was maintained relatively unchanged for 6 days following bilateral nephrectomy, quite out of keeping with the observed high rate of metabolic turnover of minialbumin.[1]

Maintenance of the circulating blood level of minialbumin in the nephrectomized monkey implies a major source other than renal tissues. We have not, however, excluded the possibility that kidney tissue may be able to produce minialbumins from serum albumin on addition of cadmium ions, and experiments are presently being performed on this aspect of the problem. The presence of such minialbumin in the liver of untreated rats implies that small quantities must be present normally in the circulation, being filtered by the renal glomeruli and reabsorbed by the tubular cells. Whether cadmium ions play a role in the regulation of the production and concentration of these intermediates in normal animals remains to be seen. The effect of cadmium is certainly on intracellular enzymic systems—we have never been able to detect the formation of low-molecular products from albumin, from the direct action of cadmium on serum or blood.

As regards the nature of urinary minialbumin itself, its amino-acid composition is closely similar to that of serum albumin of molecular weight 67,000 and to the low-molecular albumins found in the serum of cadmium-poisoned animals. In the three analyses so far performed, however, the minialbumins show a deficit of lysine and ½-cystine, which, again, makes it possible to link together the circulating and excretory forms of the protein.

Both urinary and serum minialbumins show reaction of identity with normal serum albumin in Ouchterlony plates. Although the peaks of normal and low-molecular albumins were invariably well separated from one another in the eluates from columns of dextran gel, we were concerned lest some normal albumin might be present and responsible for this antigenic behaviour of the minialbumin fraction, although not for its amino-acid composition. This possibility was dismissed when it was found that two separate preparations of low-molecular albumins from serum, when centrifuged inadvertently in high salt concentration, contained no component with molecular weight greater than 10,000. On the other hand, we became aware of the fact that unless the proteins are in a salt concentration, 0·2 M or greater, there is a probability that some aggregation of minialbumin will take place and such aggregates will be found in the normal albumin fraction. Ready aggregation of minialbumin molecules under physiological conditions of pH and salt concentration gave the impression at first —until the critical influence of salt concentration on molecular size was appreciated—that we were dealing with a protean molecule. Separated on inert columns of dextran gels in sodium chloride solutions of 0·2 M or more, the minialbumin migrates as an entity of molecules of weight 10,000, sometimes with a lesser quantity of smaller molecules of MW 5,000. Lowering the sodium chloride concentration to 0·9% leads to aggregation, and a range of molecules up to the size of normal serum albumin is produced. In salt-free media, as employed for Ouchterlony immunological plates, minialbumin preparations diffuse and react as fully-constituted serum albumin molecules, and give the reaction of identity with it.

It is imperative in handling this protein, therefore, that the concentration of salt should be lowered to a safe level before the solution is dialysed, since the lower molecular species otherwise may rapidly escape. Boiled tubing of reduced porosity must be used. On the other hand, 0·9% sodium chloride or lesser concentration will facilitate rapid aggregation of minialbumin molecules, and it may be thought that normal albumin is a contaminant in the preparation. This certainly explains the anomalous behaviour of the urinary albumin from two cadmium-poisoned workmen, discussed in our earlier paper.[1] Dr. Kekwick determined the sedimentation coefficients of these albumins as 1·96 and 1·99, which correspond to a molecular weight of approximately 20,000.

Since the single chain of albumin includes only one tryptophan residue, and peptides of very different composition are obtainable on proteolytic degradation,[16] the possibility of a repeating unit appears to be excluded. The closeness of fit of the primary amino-acid composition of all the albumins is therefore inexplicable in terms of a disorder in RNA-coded synthesis of albumin. One can, however, readily envisage how such products could arise during catabolism of normal serum albumin. Disintegration of the molecular chains into large polypeptide fragments, of approximately equal size, may occur after release from the liver microsomes of the fully-formed albumin molecules, and before they pass from the cell into the blood stream. A complete mixture of such polypeptides would have an amino-acid composition identical with its parent molecule. Alternatively, such products could arise during catabolism of circulating serum albumin returning to the liver, since the liver plays an important role in albumin breakdown. Activation of proteinases concerned with this type of fragmentation, or inhibition of enzymes active in further breakdown would raise the concentration of mixtures, such as the minialbumins we now describe. The deficit of lysine and cystine in minialbumins might be due to attack by cadmium ions on one or more of the numerous disulphide bridges, which occur throughout the length of the molecular chain of serum albumin, with subsequent loss of one or more peptides, rich in lysine and cystine. Administration of thiol compounds such as cystine or dithiopropanol has been observed to be protective against the vascular injuries caused by cadmium.[17] It is also possible that a small peptide, rich in lysine and cystine, may have been lost at some point in the fractionation procedure. We were unable to detect any direct effects on serum albumin prepared, following incubation of cadmium with either whole blood or serum.

It appears remarkable that the peptides in the mixture are able to reassociate to form one or more intact albumins of normal molecular weight (67,000), giving a reaction of identity on Ouchterlony plates with normal serum albumin. This phenomenon implies that the antigenic sites have been preserved intact on individual peptides, whose disposition in the albumin molecule is of secondary importance. We have not so far, owing to dearth of material, been able to test whether these albumins are antigenically complete as compared with normal serum albumin. Since definite quantities of minialbumin are normally present in the liver, it is possible that some reassembly could actually occur *in vivo* without the need for a specific RNA code. This is a heretical concept, perhaps, but such a process could contribute to the micro-heterogeneity of serum albumin, as is well documented by Foster and his colleagues.[18,19] In this event, cadmium may prove to play a vital role in the physiological control of albumin metabolism.

We hope that our further investigations will shed light on these various possibilities, and on the more intimate relationships between cadmium ions and the intermediary metabolism of albumin.

## SUMMARY AND CONCLUSIONS

A low-molecular albumin (MW 10,000) has been demonstrated in the sera and in the peritoneal dialysates of monkeys chronically poisoned with cadmium, and, in one monkey, this minialbumin was found in the peritoneal dialysis fluid, 6 days after bilateral nephrectomy.

The protein was immunologically indistinguishable from albumin of normal size by precipitin techniques.

In all 6 monkeys studied, a minialbumin was detected, in the blood, 3 - 5 weeks before its appearance in the urine. In addition, in some preparations a component of MW approximately 5,000 could be seen. Previous work has shown that the urinary minialbumin has a molecular weight of approximately 20,000. In earlier experiments, described here, the quantity of this smallest component was exaggerated falsely by the presence of chromogenic material extracted from cellophane sacs by acetone. The excretion of urinary minialbumin fell away rapidly in 4 or 5 days following intravenous pulse doses of cadmium—in these experiments, the biochemical action of cadmium was relatively shortlived.

The serum minialbumins showed the reaction of identity with full-sized albumin by immunoelectrophoresis and Ouchterlony techniques. Amino-acid analysis of serum minialbumin prepared separately from 3 monkeys provided evidence of close agreement in composition between one another and

normal serum albumin, except that in each case the values for lysine and ½-cystine were lower in the smaller variant.

The minialbumins were clearly separated from molecules of normal size on Sephadex G 75 as long as the concentration of sodium chloride was 0·2 M or more, without the appearance of proteins of intermediate size. At lesser salt concentrations, as for example in 0·9% sodium chloride solution, aggregation occurs with the development of a grossly inhomogeneous mixture containing approximately 40% of albumin of MW 70,000 and 60% of smaller fragments down to peptides of MW 5,000.

Minialbumin which, from its elution volume from Sephadex G 75 columns appears to have a MW of 10,000, has been demonstrated to be present in the liver and kidney of normal rats. In acute cadmium poisoning, the proportion of minialbumin in the rat liver rose rapidly to a maximum at 90 minutes, when it then appeared in the circulating blood. The changes in the kidney were similar in trend but more prolonged. Simultaneously with minialbumin, a pre-albumin fraction made its appearance on electrophoresis in cellulose acetate strips.

Minialbumins are not formed by direct contact of cadmium ions with normal albumin in serum or blood; they must arise as the result of the action of the metal on some intracellular enzymic process.

The significance of these findings in relation to the origin of urinary minialbumin and the biochemical actions of cadmium are discussed.

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REFERENCES

1. Kench, J. E., Gain, A. C. and Sutherland, E. M. (1965): S. Afr. Med. J., **39**, 1191.
2. Bonnell, J. A. (1965): Ann. Occup. Hyg., **8**, 45.
3. Clarkson, T. W. and Kench, J. E. (1956): Biochem. J., **62**, 361.
4. Kazantzis, G. (1965): Ann. Occup. Hyg., **8**, 59.
5. Bonnell, J. A., Ross, J. H and King, E. (1960): Brit. J. Industr. Med., **17**, 69.
6. Potts, C. L. (1965): Ann. Occup. Hyg., **8**, 55.
7. Kench, J. E., Wells, A. R. and Smith, J. C. (1962): S. Afr. Med. J., **36**, 390.
8. Creeth, J. M., Kekwick, R. A., Flynn, F. V., Harris, H. and Robson, E. B. (1963): Clin. chim. Acta, **8**, 406.
9. Vallance-Owen, J., Dennes, E. and Campbell, P. N. (1958): Lancet, **2**, 336.
10. Fernandez, A., Sobel, C. and Goldenberg, H. (1966): Clin. chim. Acta, **12**, 194.
11. Ouchterlony, O. (1962): Progr. Allergy, **6**, 30.
12. Mandy W. J., Rivers, M. M. and Nisonoff, A. (1961): J. Biol. Chem., **236**, 3221.
13. Grabar, P. and Williams, C. A. jnr. (1955): Biochim. biophys. Acta (Amst.), **17**, 67.
14. Moore, S. and Stein, W. H. *in* Colowick and Kaplan, eds. (1963): *Methods in Enzymology*, vol. 6, p. 819. New York: Academic Press.
15. Spackman, D. H., Stein, W. H. and Moore, S. (1958): Analyt. Chem., **30**, 1190.
16. Press, E. M. and Porter, R. R. (1962): Biochem. J., **83**, 172.
17. Gunn, S. A., Gould, T. C. and Anderson, W. A. D. (1966): Proc. Soc. Exp. Biol. (N.Y.), **122**, 1036.
18. Foster, J. F., Soganin, M., Petersen, H. A. and Leonard, W. J. jnr. (1965): J. Biol. Chem., **240**, 2495.
19. Petersen, H. A. and Foster, J. F. (1965): *Ibid.*, **240**, 2503.