SOME OBSERVATIONS ON THE PROTEINURIA OF RABBITS POISONED WITH CADMIUM

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The experimental observations described here were made in an attempt to elucidate certain known biochemical effects of cadmium in man and animals.

Workmen may be exposed to cadmium-oxide fume in the process of manufacture of copper-cadmium alloys for electrical cable, whilst cadmium-oxide dust is a hazard in the alkaline battery industry. In either case, continuous exposure may lead to chronic cadmium poisoning associated with the development of emphysema and the excretion in the urine of a characteristic protein of low molecular weight.[1-5] From the many reported cases of death from chronic cadmium poisoning, it is clear that the salient point of attack of cadmium-oxide fume is the lung parenchyma, leading ultimately to severe emphysema.[6,7] Characteristically, there is no clinical history of a chronic cough nor histological evidence of chronic bronchitis. The presence of severe emphysema without chronic bronchitis seems to be an important feature of chronic cadmium-fume poisoning.

Although cadmium has been found widely distributed in the body, especially in the soft tissues (hepatic and renal) clear-cut evidence of tissue damage in any organ other than the lungs has not been adduced. Amino aciduria[8] occurs almost invariably in man, but, although severe renal tubular lesions arise in rats,[9] the evidence that progressive renal damage[10] occurs in man is only suggestive.

From the biochemical viewpoint, the urinary protein associated with cadmium poisoning is of special interest, in that, although it has been shown to comprise several components electrophoretically, the molecular weight was consistently in the range 20 - 30,000.[1,11]

In our series of cadmium workers the quantity of urinary protein was 1·0 - 3·2 G./l.[12] — much in excess of the average normal value of 133 mg./24 hr. found by Webb and his co-workers.[13]

Physico-chemical studies[14] have shown the presence in normal urine of γ_2-globulins of low molecular weight, 10,600, but failed to distinguish between the urinary albumin fraction and normal serum albumin. The urinary albumin of nephritic and nephrotic patients[15,12] and persons poisoned with metallic mercury[16] is likewise normal serum albumin. The proteinuria of cadmium poisoning is distinguished by the presence of an albumin, closely similar in amino-acid composition to normal serum albumin (M.W. 69,000), but having a molecular weight of 20 - 30,000.[12]

It was conceivable that such a protein could arise through an inhibitive action of cadmium on a late stage in the biosynthesis of serum albumin, and our present experiments were designed to explore this hypothesis. The rabbit, chronically poisoned with cadmium, does excrete a urinary protein of low molecular weight, but this was accompanied by normal serum albumin, presumably as a consequence of glomerular dysfunction. In contrast to human subjects, glycosuria and amino aciduria, owing to interaction of cadmium ions with the renal tubular cells, was never observed. A notable incidental feature of severe intoxication was the remarkable viscosity of the blood, reminiscent of that associated with some cases of human hyperglobulinaemia.[17]

Species differences were not unexpected, but the additional complexities encountered in the poisoned rabbit, and the insuperable technical problem, to us, of separating the serum and urinary albumins, rendered an unambiguous study quite impossible. However, in as much as such closely-related proteins may become amenable to separation on 'sephadex' (cross-linked dextran) columns,[18] and as cadmium, of whose biochemical behaviour virtually nothing is known, promises to be a powerful tool in the study of protein metabolism, this work, though incomplete, appeared worthy of record.

MATERIALS, METHODS AND RESULTS

Experimental Animals

Young adult male rabbits reared from known healthy stock and weighing 2 - 2·5 kg. were the subjects of study. Where possible, litter mates were used. The animals were maintained on the MRC diet[19] plus fresh cabbage, and kept in all-perspex cages to avoid metallic contamination. The cages were specially designed to allow separate collection of urine and faeces.

In all, observations were made on 38 animals during the course of the investigation.

Cadmium Sulphate

Radiocadmium was obtained from Harwell in the form of thin foil which had been irradiated for 4 weeks and then allowed to decay for 1 week before despatch to exhaust the short-lived component of Cd,[115] leaving predominantly the isotope which decays to indium by emission with a half-life of 43 days. The decay was followed for several months and was such as to exclude the presence of other radioactive materials.

The foil was dissolved in concentrated sulphuric acid slightly in excess of the theoretical requirement, and when all metal was dissolved, the excess sulphuric acid was evaporated under reduced pressure. The cadmium sulphate remaining was dissolved in distilled water to give a stock solution, containing 10 mg. Cd/ml., from which the standard for radioactivity measurements was prepared. The radioactive counts were determined in a liquid counter (type M6).

Acute and Chronic Cadmium Poisoning

Since most heavy metals and their compounds (cadmium is not exceptional in this regard) are poorly absorbed from the gastro-intestinal tract and the inhalation route is difficult to control, intravenous injection offered the best means of controlled intoxication.

Early attempts to produce systemic effects by injection of cadmium compounds into the marginal ear veins were un-

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Dr. R. A. Kekwick, of the Lister Institute of Preventive Medicine, London, kindly made ultracentrifugal studies of the protein, and the remainder of this section is almost a literal transcription of his personal communications to us.

The samples of the dried specimens were dissolved in a phosphate-sodium chloride buffer, pH 8·0, total ionic strength 0·35, and dialysed overnight in cellophane tubes against excess buffer. During dialysis, approximately 50% of the preparation passed through the cellophane tubing into the surrounding buffer, but this gave no precipitate with salicyl sulphonic acid, whereas the contents of the tube gave a heavy precipitate. The ultracentrifuge picture for the urinary protein preparations relates to the non-diffusion residue; the diffusible material was not further examined. The protein was diluted to a concentration of 1·0 G./100 ml. and centrifuged at a speed of 54,000 rev./min. which gives about 250,000 Gravities, and the interval between exposures was 10 minutes. Serum proteins from rabbit R.E.1 were similarly prepared and examined in a concentration of 1·5 G./100 ml., concentrations in all specimens being determined with a dipping refractometer.

The ultracentrifuge photographic records are shown in Fig. 1. The urinary proteins from both rabbits appear as 2 components in the ultracentrifuge (Fig. 1. c, d). In each, protein(s) of low sedimentation coefficient are found in the range of the urinary protein of workmen poisoned with cadmium, of which Fig. 1. b is an example, with $S_{20,w}$ 1·99, corresponding to a molecular weight 20 - 30,000.* In R.E.1 (Fig. 1. c) the faster sedimenting component was reasonably well defined, and Dr. Kekwick was able to calculate its sedimentation coefficient, the value corrected to water at 20°C., $S_{20,w}$, at 1% protein being 4·07. This is very similar to 4·04, the coefficient calculated for human nephritic urinary protein (Fig. 1. a) and for human serum albumin (4·4 S). Although there appeared to be two components present also in R.E.3 (Fig. 1. d), both were polydisperse and it was not possible to calculate a sedimentation coefficient for either of them. In general, however, the urinary proteins of both rabbits appeared to be sedimenting at closely similar rates, and the difference in the behaviour of the urinary proteins in the ultracentrifuge was probably just a reflection of the relative proportions of serum albumin and of low-molecular protein present.

Serum from rabbit R.E.1 (Fig. 1. e) gave a normal type of picture and there was no evidence of the presence of any slowly sedimenting proteins such as occurred in the urines. However, the concentrations of such proteins which might reasonably be anticipated may well be below the limit of detection by the ultracentrifuge.

(b) Metabolism

When proteinuria was plentiful and continuous in the chronically poisoned rabbits (R.E.1 and R.E.3) each was given a single intravenous injection of a mixture of the C^{14}-labelled amino acids, glycine, and lysine. The amino acids were both generally labelled, as supplied by the Radio Chemical Centre, Amersham, and were administered in 0·9% sodium chloride solution in a dosage of 100 μC (3·65 mg.) glycine and 40 μC (0·97 mg.) lysine per kg. body weight. Thereafter, venous blood and urine were collected at short intervals during the first 24 hours; urine by urethral catheter, but later pooled 24-hourly specimens were examined. The radioactivity of the glycine prepared from the mixed proteins of serum and of urine was determined in the many samples.

Following dialysis and lyophilization, the proteins of serum and urine were redissolved in water and then thoroughly mixed with 10% w/v trichloracetic acid (TCA). The precipitated proteins were centrifuged and repeatedly resuspended in 5% w/v TCA, and finally washed with acetone to remove free isotopically-labelled amino acids and dried over phosphorus pentoxide.

The protein (10 mg. or less per batch) was hydrolysed by heating in sealed pyrex tubes with 6 N. hydrochloric acid (1 ml. per mg. protein) at 100°C. for 24 hours. The hydrolysate was evaporated to dryness under reduced pressure at 37°C. or below. Where the hydrolysate contained an appreciable quantity of humin, as judged by its dark colour, it was redissolved in water, filtered, and evaporated as before.

The 2·4 dinitrophenyl (DNP) derivatives of the amino acids were then prepared and the 2·4 dinitrophenyl glycine isolated as described by Neuberger, Perrone and Slack.[21] The final solution in peroxide-free ether was determined colorimetrically at wavelength 460 mμ in a 'unicam' spectrophotometer SP 500. The solution was evaporated to dryness *in vacuo* and the radioactivity measured on solid samples of 'infinite thinness' according to the procedure of Henriques, Henriques and Neuberger.[22]

The changes in specific activity with time of DNP glycine in the mixed proteins of serum and of urine in rabbit R.E.1 are shown in Table III and graphically in Fig. 2, in which logarithm of specific activity is plotted against time. The data we were able to collect for rabbit R.E.3 are much more fragmentary, but the activity-time curves for serum and urinary proteins run a similar course to those presented here for R.E.1.

TABLE III. RADIOACTIVITY OF TOTAL PROTEINS IN SERUM AND URINE OF CADMIUM-POISONED RABBIT R.E.1

Time following injection of C^{14}-glycine and lysine (days)	*Serum*	*Urine*	*Time following injection of C^{14}-glycine and lysine (days)*	*Serum*	*Urine*
0·0 a. 1·58 hr.	755	129	5·0	588	720
b. 3·58 hr.	1,079	1,412	7·0	519	574
c. 6·50 hr.	1,160	2,288	8·0	—	364
d. 11·00 hr.	1,242	5,120	10·0	—	325
1·0	1072	—	11·0	395	297
2·0	905	1,143	18·0	244	113
3·0	780	882	25·0	143	79

The values given in columns 2 and 3 are measurements of the radioactivity of DNP-glycine prepared as described in the text and expressed as counts/min./mg. at 'infinite thinness'.

Finally, an attempt was made to ascertain the fate of the protein appearing in the urine of cadmium-poisoned animals when introduced by intravenous injection into the circulation of normal and poisoned animals.

The experiment was arranged as follows:

Rabbit injected	*Nature of injected preparation**
Normal (RF10)	30 mg. urinary proteins.
Normal (RF11)	80 mg. urinary proteins.
Normal (RF12)	100 mg. serum proteins.
Poisoned (28 × 2 mg. Cd RF8)	10 mg. urinary proteins.
Poisoned (26 × 2 mg. Cd RF7)	100 mg. serum proteins.

* All proteins were C^{14}-labelled from Cd.-poisoned animals.

Urine from the experimental animals was collected for 48 hours following the injection, pooled, and the total radioactivity of any protein found therein measured as DNP-glycine. In both the normal and in poisoned animals (RF12 and 7) less than 1% of the injected C^{14}-labelled serum proteins was recovered in the urine. In contrast, in each of the animals (RF 10, 11 and 8) that received radioactive urinary proteins, more than 50% of the injected radioactivity was recovered as protein in the urine.

DISCUSSION

By repeated small injections of cadmium sulphate, rabbits were brought to a condition of chronic intoxication which has provided the background for the biochemical lesions we hope to clarify. Through the intravenous route, cadmium was widely distributed throughout the body tissues, but mainly in the liver and kidneys, and these organs alone developed significant histological changes. Lung parenchyma was apparently unharmed although moderate concentrations of cadmium were found there. This suggests

* A perfectly spherical molecule with this sedimentation coefficient would have a molecular weight of 13—20,000. (A. Polson, 1962 — personal communication.)

that the lung does not specially concentrate the metal, as do, for example, the basal nuclei of the brain with copper in hepato-lenticular degeneration, nor is lung tissue particularly sensitive to the action of cadmium. The damage to the lungs that occurs in workmen inhaling cadmium-oxide dust or fumes may be ascribed to the local action of cadmium on the epithelial cells of the lung alveoli as a result of its high concentration there, probably in ionic form. Although there was some evidence of renal tubular degeneration, manifestations of this, such as amino aciduria and glycosuria, were absent.

Perhaps the most significant observation of our experiments is the urinary excretion in the poisoned rabbit of a protein with a low molecular weight, similar to that observed in cadmium workers. It will be a matter of interest to examine how general is this effect of cadmium on protein metabolism in other animal species. The much increased blood viscosity in the rabbit, which may implicate the proteins of the serum, enhances the probability that cadmium may effect unusual disturbances in serum-protein metabolism.

A complication with the rabbit as an experimental subject, not encountered in man, was the passage of normal serum albumin into the urine. This could not be separated from the accompanying low-molecular protein. Although the labelled urinary proteins could not, therefore, be clearly identified, the evidence from clearance studies indicates that, in contrast to normal serum albumin, the low-molecular protein passes rapidly out of the circulation through the renal glomeruli, irrespective of whether the latter are normal or have been exposed to the action of cadmium.

Another feature of the low-molecular protein is shown by the amino-acid investigations (Table III, Fig. 2). The rate of incorporation of glycine into the mixture of urinary

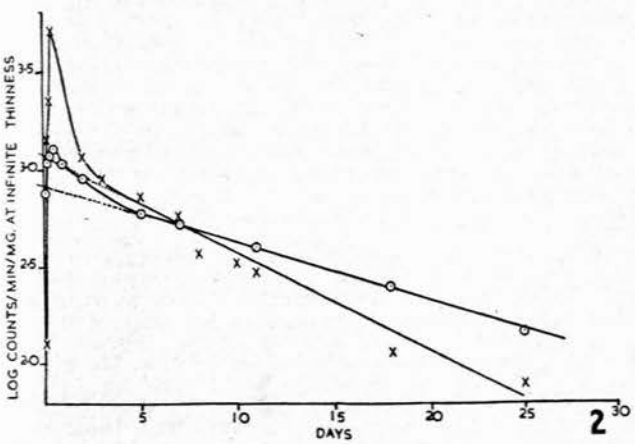

Fig. 2. Changes in the specific activity of DNP glycine contained in the serum and in the urinary proteins of the cadmium-poisoned rabbit R.E.1. The radioactivity of the separated DNP glycine was measured on solid samples of 'infinite thinness'.
o——o = serum proteins.
x——x = urinary proteins.

proteins — rich in low-molecular protein — is considerably faster than into serum proteins, which, from ultracentrifugal examination and isotope clearance, must contain traces only of the low-molecular species. In the succeeding period of exponential decline, the specific activity of serum proteins measured in terms of C^{14}-glycine fell much more gradually than did that of the urinary proteins.

If we assume that the body-pool of low-molecular protein remains very small, owing to rapid renal excretion, the data are consistent with a more rapid fractional increase in specific activity of the low-molecular protein as compared with normal serum proteins, to be followed by an equally rapid fall-off in activity as the specific activity of free glycine, continually incorporated into new protein molecules, falls owing to the removal of the radioactive amino-acid molecules along various metabolic pathways.

This implies that the low-molecular protein has a shorter half-life or turnover time, which would be anticipated if this was an intermediate in normal or aberrant protein biosynthesis, perhaps comparable with Bence-Jones protein.

Owing to the fact that the low-molecular weight protein could not be separated from large molecules in urine or in serum by ultracentrifugal or other means, it was not possible to determine the concentration of the low-molecular component in either medium. It is possible that this problem may be resolved in the future by use of cross-linked dextrans (sephadex) or similar material employable as a molecular sieve. When this is achieved, it will be possible to compute the specific activity of the individual albumins in man — both of low and of normal molecular weight — the size of body pool of each, and the rates of synthesis, catabolism, and urinary loss in grams per day.

At present it is possible to arrive at certain conclusions regarding these parameters only if we make certain broad assumptions:

1. That the average serum-albumin concentration of the cadmium workmen[12] was 4·5 G./100 ml., and the volume of distribution of this protein 4,000 ml.

Then the total exchangeable body-pool = 4·5 × 40 = 180 G.

2. If we accept the frequently reported value of 0·05 as the correct fractional daily rate of catabolism for serum albumin, then:

Rate of catabolism of serum albumin per day = 180 × 0·05 G. = 9 G./day.

Gitlin and his colleagues[23] have given the amount of albumin synthesized per day by nephrotic children as 6·3 - 8 G./day, which values they regard as within the upper limits of the normal range.

3. The workmen may justifiably be considered as being in the steady rate in respect of protein metabolism, so that the expression: rate of protein synthesis = rate of catabolism + urinary loss, will generally apply. They excreted daily on the average approximately 1·6 G. of low-molecular albumin.[12]

If, on the basis of our rabbit experiment, we assume that very little of this aberrant protein is catabolized in the short time that it is in the body, then this excreted protein is equivalent in quantity to the daily rate of synthesis.

Thus the proportion of aberrant albumin in the cadmium workmen $= \frac{1{\cdot}6}{9{\cdot}0} \times 100 = 20\%$.

Further experimental studies have been designed to test the truth of these assumptions and to localize with more precision the biochemical lesions of cadmium poisoning.

SUMMARY

1. Rabbits were chronically poisoned by repeated intravenous injections of cadmium sulphate. Cadmium was

found distributed in all organs — particularly in liver and in kidneys, the only ones to show histological abnormality.

2. The animals excreted in the urine a mixture of normal serum albumin and low-molecular proteins; the low-molecular proteins as found in human cadmium poisoning.

3. Changes in the blood were found, consistent with the presence of abnormal serum globulins.

4. Radio-isotopic studies with glycine and lysine are described which suggest that the low-molecular protein is metabolized more rapidly than normal serum proteins, and is eliminated promptly through the renal glomeruli.

5. Quantitation of the disorder of protein metabolism present in cadmium poisoning is discussed.

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