THE BIPHASIC NATURE OF RENAL CALCIFICATION

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Pathological calcification has been the subject of many investigations, both histological and chemical in nature, but the material studied in each case appears to have been limited to lesions which were sufficiently established to be recognized by microscopic examination. If the mature lesion is but an end stage in a series of complex chemical reactions, it is evident that such material is not likely to reveal the primary chemical events that determine the process. To elucidate these it is necessary to study the lesion in all stages, before and throughout the process of calcification. Such a study was seen to be possible when it was observed, in confirmation of previous reports (1–6), that the cortex of the rat kidney may become calcified after damage with uranium. The insult to the tissue in this case can be adequately quantitated and timed; under standard conditions the lesion evolves in a relatively predictable fashion, so that the early stages may with some confidence be regarded as antecedent to a subsequent calcification.

In the present work this opportunity was exploited by the combined application of histological and microanalytical procedures. The former served to orient the chemical findings in relation to well known anatomical changes; the latter demonstrated that the process of calcification was divisible into two components: a primary accumulation of calcium in association with an unidentified anion, and a secondary conversion of this complex into a precipitate of calcium phosphate.

Procedure

The animals used in this study were male rats of the Sherman strain, weighing 233 ± 22 gm. Normal values for the concentrations of calcium, inorganic phosphate phosphorus, and total phosphorus in the renal cortex were established by analyses of tissues from 16 rats; the variations in the values thus obtained were not correlated with the variation in weight within the limited range of weights that entered into the study. The experimental group of 33 rats was divided into 4 subgroups, containing 6, 15, 6, and 6 animals; these subgroups received 2, 10, 20, and 30 mg./kg. of $UO_2(NO_3)_2 \cdot 6H_2O$ respectively, given by the intraperitoneal injection of an aqueous solution (0.2 to 0.6 per cent). At intervals varying from 1 to 12 days after the injection rats from each subgroup were sacrificed for analyses of their kidneys. In each case portions of kidneys were fixed in 5 per cent neutral formalin and Zenker's solution for histological examination. Until the time of sacrifice all animals had free access to water and to fox chow pellets.

At the time of sacrifice each animal was etherized; the kidneys were removed quickly, dropped into a freezing mixture of acetone and dry ice, and taken into a cold room (0°C.) for dissection. After removal of the perirenal fat the kidneys were quartered by two symmetrical cuts at right angles; the medulla was excised from each piece with a small scalpel and the remaining pieces of cortex were weighed in two portions, one for the direct estimation of the total phosphorus concentration and the second for acid extraction. The calcium and acid-soluble phosphorus compounds were separated from the second portion by three extractions with a total of 25 to 50 volumes of ice-cold 5 per cent trichloracetic acid.

Calcium was determined by the method of Sobel and Sobel (7). Normal kidneys, and those with lesser grades of calcium accumulation, yielded extracts that were too dilute for direct application of this method. These extracts were concentrated by a preliminary evaporation to dryness in the centrifuge tubes; the walls of the tubes were washed down with 0.2 ml. of 1 N HCl; the sample was dried once more and finally taken up into 0.2 ml. of acetate buffer (0.1 M, pH 5.02).

Inorganic phosphorus was estimated by the Fiske-Subbarow method (8). The value thus obtained is slightly in excess of the probable true value because it includes any phosphorus that had been combined with creatine and possibly some that had been linked to adenosine; however, it can be estimated from the data of LePage (9) that these compounds in normal kidneys would contribute less than 0.1 mg./gm. to the estimated value. Providing that there is no marked increase of these high energy compounds with renal damage—an unlikely possibility—the inclusion of this small amount of ester phosphorus is of no consequence in relation to the considerable accumulation of inorganic phosphorus in calcified tissue. Total phosphorus was estimated by King's (10) modification of the Fiske-Subbarow procedure (8).

OBSERVATIONS

In the control series of 16 untreated rats, analyses of the renal cortices yielded the following mean values and standard deviations: inorganic phosphate phosphorus, 0.56 ± 0.061 mg./gm. wet tissue; total phosphorus, 3.24 ± 0.18 mg./gm.; calcium 0.18 ± 0.082 mg./gm. Statistical analysis of the data revealed that the variance of each item reflected actual differences between the animals, while the differences between kidneys of the same animal were not significantly greater than those between replicate analyses of the same tissue sample. Since the variations of calcium and phosphate concentrations were uncorrelated, it is probable that at least part of the calcium in normal kidneys is associated with some anion other than phosphate. Moreover, since the concentration of calcium in normal kidneys exceeds the concentration of this element in blood, it is evident that there is an extravascular accumulation of calcium under normal circumstances.

The effects of uranium are shown in Table I, distributed according to dose and the time between injection and sacrifice. In this table the values that differ significantly ($p < 0.01$) from those of the control group are indicated by bold face type. It can be seen that marked accumulations of both calcium and phosphorus had developed within a few days after injection, the speed and magnitude of the accumulations generally being related to the size of the dose. It was of interest to find, however, that the animals in the highest dosage group (30 mg./kg.) had accumulated less than the corresponding animals in lower

dosage groups; since histological examination showed the extent of necrosis to be maximal in the highest dosage group this observation suggests that necrosis alone does not determine the accumulation. Moreover, it is clear that much of the inorganic phosphate phosphorus in damaged tissue must have been contributed by the circulating blood since its quantity in calcified tissue often far exceeded the total amount of phosphorus normally present; simple degradation

TABLE I

Dose of $UO_2(NO_3)_2 \cdot 6H_2O$		Days after injection								
		1	2	3	4	5	6	8	9	12
mg./kg. body weight										
2	Ca	0.10	0.14	**1.27**		0.16		0.10		0.40
	P	0.70	0.52	0.42		0.49		0.41		0.38
10	Ca	0.09	**2.39**	**3.16**		**6.48**		**2.87**	**2.87**	
	P	**0.74**	**0.58**	0.42		**1.37**		**2.31**	**1.85**	
	Ca		0.41	**0.94**	**0.44**					
	P		0.50	**0.89**	0.59					
	Ca		**0.49**	**1.27**	**12.53**					
	P		0.60	**0.79**	**7.41**					
	Ca		**0.68**	**0.85**	**1.74**					
	P		0.65	0.65	0.70					
20	Ca	**0.51**	**0.51**	**5.06**	**0.77**	**5.23**	**8.82**			
	P	**0.80**	0.69	**1.51**	0.58	**3.31**	**5.18**			
30	Ca	**2.60**	**1.17**	**1.43**	**1.78**	**2.84**	**2.66**			
	P	**0.83**	0.70	**1.24**	**1.32**	**2.73**	**1.98**			

Milligrams of calcium (upper figure) and inorganic phosphate (lower figure) per gram of the renal cortex following a single injection of uranium nitrate. Values which differ significantly ($p < 0.01$) from the controls are indicated by bold face type.

of organic phosphates, therefore, could not have caused the accumulation of inorganic phosphate.

The relative accumulations of calcium and phosphate give an indication of the composition of the pathological deposit. Calcium, if precipitated with phosphate as $CaHPO_4$, would exhibit a Ca/P ratio by weight of 1.29; the more basic hydroxyapatite, $Ca_5(PO_4)_3OH$, would yield a higher ratio, 2.16. Somewhat more basic precipitates might be formed by the sorption of $Ca(OH)_2$ onto the crystal lattice of hydroxyapatite, but it is unlikely that the Ca/P ratio would exceed 2.59, which corresponds to $Ca_2(PO_4)OH$ (11). These considera-

tions are of interest in relation to the graphic analysis of the data, presented in the figure. On this plot each point indicates the concentrations of calcium and of inorganic phosphate phosphorus in a single specimen of tissue. The size of the circles suggests the analytical uncertainty of the measurements and the appendages to the symbols show whether or not the animal had been

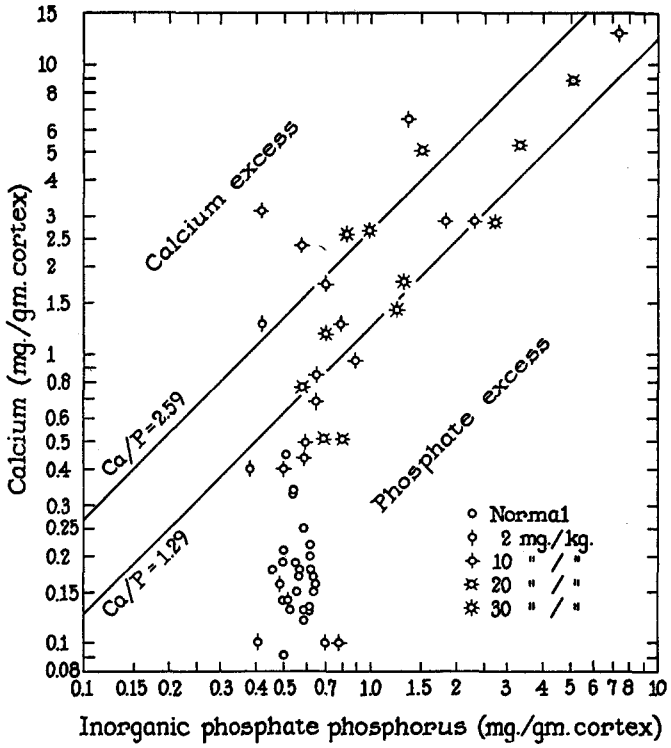

FIG. 1. Concentrations of calcium and inorganic phosphorus in mg./gm. of renal cortex in single specimens from control rats and rats injected with uranium nitrate. Plotted logarithmically, constant Ca/P ratios appear as parallel straight lines. The two lines indicate the lower and upper limits of Ca/P ratios for calcium phosphate precipitates. Points below both lines indicate a Ca/P ratio with a relative excess of phosphorus, whereas points above both lines indicate a Ca/P ratio with relative excess of calcium.

dosed with uranium. Since the axes of the graph are logarithmic, constant ratios of Ca/P appear as parallel straight lines, and thus allow a convenient subdivision of the graph into three areas, separated by the lines of Ca/P ratios that are maximal and minimal for calcium phosphate precipitates. Any point that lies below both of these lines is in a region of phosphate excess and implies that at least part of the phosphate must be associated with some cation other than calcium; similarly, any point above the lines implies the existence of an anion other than phosphate to balance the surplus calcium.

It can be seen in the figure that the findings in the normal kidney are located in a region of phosphate excess, and that the calcium normally has the greater percentage variability. With early or relatively mild lesions there is an increase in calcium without significant increase in phosphate; it is possible that this early stage of precalcification is no more than an exaggeration of the physiological accumulation of calcium, but the introduction of a foreign accumulating anion in damaged tissue cannot be excluded at present. In either case, it appears that an accumulation of calcium to or beyond the zone of calcium phosphate equivalence leads to a conversion of the deposit into a precipitate of calcium phosphate. This is evidenced by the fact that with increasing accumulations of calcium the points appear in the zone of calcium phosphate precipitates.

Because of the role that chondroitin sulfate appears to play in the calcification of bone (12), consideration was given to the possibility that this anion might be responsible for the primary accumulation of calcium in the damaged kidney. This possibility was excluded, however, by measurement of the capacity of homogenates to produce a metachromatic color shift of toluidine blue: even if the total metachromatic capacity had been due to chondroitin sulfate, the concentration of this substance in damaged kidney could not have exceeded *3* mg./gm. tissue. This corresponds to an equivalent concentration of about *6* μ eq./gm. (assuming an equivalent weight of 500), and is far less than the 50 or more μ eq. of calcium per gram that might appear in excess of phosphate. The unimportance of chondroitin sulfate in this process was confirmed by histochemical studies in which the toluidine blue stain was applied to tissue sections (12); such metachromasia as was detectable was slight, variable, and uncorrelated with the accumulation of calcium.

SUMMARY

Renal calcification, induced in rats by an injection of uranium, is accomplished in two stages: a primary accumulation of calcium in association with anions other than phosphate and a secondary conversion of this calcium complex into a precipitate of calcium phosphate. Except for the exclusion of chondroitin sulfate, the nature of the primary anions remains undefined. The accumulation of calcium in the kidney was converted into a precipitate of minimum solubility, and thus the evidence of its primary causation was obliterated. This may well hold true of calcification at other situations.

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