URINARY CALCULI IN GERMFREE RATS*

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PLATES 31 AND 32

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During our investigations on the morphology and metabolism of germfree animals it was observed that bladder stones occurred in germfree rats. The scope of the present investigation is to describe the frequency, morphology, and chemical composition of these urinary calculi.

As the formation of these urinary calculi might be related to changes in the composition of the urine, investigations of the electrolyte excretion were performed.

EXPERIMENTAL

Germfree Methods.—The germfree rats studied were reared according to the technique of Gustafsson (1, 2) and reared on the semisynthetic diet D7. The diet was sterilized by autoclaving for 20 minutes at 121°C. The composition of diet D7 is given in Table I. According to analysis of the diet by a commercial laboratory the following values on a dry weight basis of diet composition are given: protein 21.4 per cent; calcium 1.02 per cent; phosphorus 0.48 per cent, and calcium/phosphorus ratio 2.1.

The rats were killed in the course of our studies on other topics such as the mechanism of the cecum enlargement in the rats and the formation of γ-globulins. Some of the rats were ex-germfree; *i.e.*, they had been transferred from the germfree isolators to the animal rooms, given an enema with the intestinal contents from conventional rats, and thus obtained the flora of conventional rats. For metabolic studies animals were kept in metabolism cages where feces and urine were collected every 24 hours according to standard methods. A total of 323 animals of both sexes was autopsied during a 2 year study period.

Chemical Methods.—Urines were analyzed for sodium and potassium with a flame photometer, for calcium by the method of Kramer and Tisdall (3), for magnesium by the method of Simonsen, Westover, and Wertman (4), for phosphorus by the method of Taussky and Shorr (5), for citrate by the method of McArdle (6), and for creatinine by the method of Hare (7). pH in urine was determined by glass electrode with a radiometer pH meter, model 22 with scale expander.

For determination of the chemical composition of the urinary calculi the stones from separate animals were collected and powdered. The air-dried material was weighed and a

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fraction dissolved in 0.1 M sulfuric acid and aliquots of the solution used for citrate determination. A portion of the air-dried material was weighed in a platinum crucible, dryashed, and quantitatively analyzed for magnesium, calcium, and phosphorus. The nitrogen content of the stones was determined by the micro-Kjeldahl method.

TABLE I

Composition of Diet D7

Casein	22 per cent
Wheat starch	63 per cent
Arachis oil	10 per cent
Salt mixture HMW*	4 per cent
Vitamin mixtures	1 per cent
Vitamins added per 100 gm diet:	
Vitamin A	2100 I.U.
Vitamin D	400 I.U.
Vitamin E	50 mg
Vitamin K_1	1 mg
Thiamine	5 mg
Riboflavin	2 mg
Pyridoxine	2 mg
Calcium pantothenate	10 mg
Nicotinamide	20 mg
Choline	200 mg
Inositol	100 mg
p-Aminobenzoic acid	30 mg
Biotin	0.1 mg
Folic acid	2 mg
Vitamin B_{12}	0.002 mg
Ascorbic acid	100 mg

* According to Hubbell, Mendel, and Wakeman (15).

The organic acids present as calcium salts in the urinary calculi were separated by partition chromatography on columns of Celite (8). The following solvent systems were used.

Solvent system	Stationary phase	Moving phase
1	0.25 M sulfuric acid	*n*-Butanol:chloroform 35:65 v/v
2	0.25 M sulfuric acid	Ether

9.6 ml of the stationary phase was supported on 12 g of Celite. About 10 mg of the powdered stone material was dissolved in 0.4 ml of the stationary phase; 0.5 gm of Celite was added and the mixture was transferred to the top of the column. The effluent was collected with an automatic fraction collector and the organic acids were determined by titration with 0.02 M sodium hydroxide.

The identity and number of organic acids in each peak isolated with column chromatography were established by gas-liquid chromatography using an Argon pye chromatograph with an ionization chamber as detector. The titrated fractions from a peak were combined, evaporated, and dissolved in 0.1 M hydrochloric acid. The free organic acids were extracted with ether. The solvent was evaporated and the organic acids were converted into methyl

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esters by the addition of a slight excess of ethereal diazomethane. The methyl esters were analyzed with gas chromatography on columns of silicone oil and polydiethyleneglycol adipate supported on Celite 545.

Infrared absorption spectra were obtained with a Perkin-Elmer model 221 recording spectrophotometer equipped with sodium chloride optics. Disks were prepared by mixing 0.5 to 1.0 mg of finely ground stone material with 300 mg of potassium bromide and compressing under vacuum for 3 minutes at 15 tons per cm^2.

RESULTS

A. Frequency and Appearance of Urinary Calculi in Germfree Animals.—As is evident from Table II, 35 of 70 autopsied germfree males had bladder stones whereas 113 males reared in the animal rooms on exactly the same autoclaved

TABLE II

Frequency of Vesical Stones in Germfree and Conventional Animals Autopsied during a 2 Year Period

	Germfree		Conventional	
	Males	Females	Males	Females
Total No. of animals	70	46	113	94
No. of animals with stones	35*	1	0	0
Per cent of total	50	2	0	0

* 4 animals died of uremia.

diet did not present a single case of bladder stone. The frequency was thus 50 per cent in the germfree and zero in the conventional rats. Stones blocked the urethra in 4 cases, and the animals died in hydronephrosis and uremia.

The occurrence of bladder stones in the females studied during the same time as the males was much lower; only 1 of the 46 germfree rats had bladder stones, which is a frequency of 2 per cent. As was the case with the males, none of the 94 conventional females reared during the same period had any bladder stone.

The stones seemed to be present in all age groups. The youngest animal with urinary stones in this series was thus 42 days and the oldest 393 days old. The calculi were usually present in a rather large number; in some cases several hundred have been encountered. In most cases the number of the stones was between 3 and 10 (Fig. 1 B).

The stones were studied in some ex-germfree rats by consecutive x-ray examinations. It was then found that the calculi disappeared during the ex-germfree period by disintegration, which was also obvious when the animals were sacrificed (Fig. 1 C).

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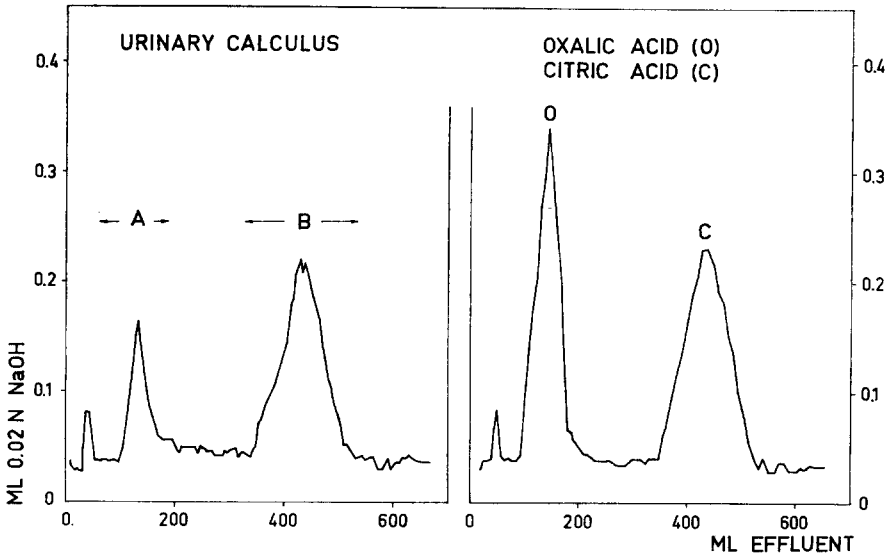
The bladder stones usually have a smooth surface, white or grayish white in appearance. The organic matter forms a stroma which becomes evident when minute stones in the folds of the bladder are sectioned *in situ* (Fig. 2). The figure shows that the stones are composed of concentric layers. This becomes also evident when the calculi disintegrate during the ex-germfree period (Fig. 1 C).

A histological examination of the kidneys revealed small calculi in the collecting ducts (Fig. 3). The epithelium of the urinary system including that of



TEXT-FIG. 1. Left curve, chromatographic separation of organic acids in 5.0 mg of urinary calculus. Right curve, chromatographic separation of oxalic (O) and citric acid (C). Solvent system 1. Column, 12 gm of Celite.

the bladder had a normal appearance (Fig. 2), and there were no signs of hyperkeratosis or other signs of vitamin A deficiency.

B. Chemical Composition of the Urinary Calculi.—Standard methods for qualitative analysis of urinary calculi revealed that the stones were composed of calcium salts of organic compounds. The results of the quantitative analysis of the ash showed that the inorganic part of the stones is made up almost entirely of calcium. The presence of magnesium and phosphorus could not be detected by the methods used. Nitrogen analysis gave values of 0.5 to 1 per cent.

The organic acids present in the urinary calculi were separated by column chromatography, and a typical elution curve is shown in the left part of Text-fig. 1. The acidic products are recovered in two peaks, the main part in the

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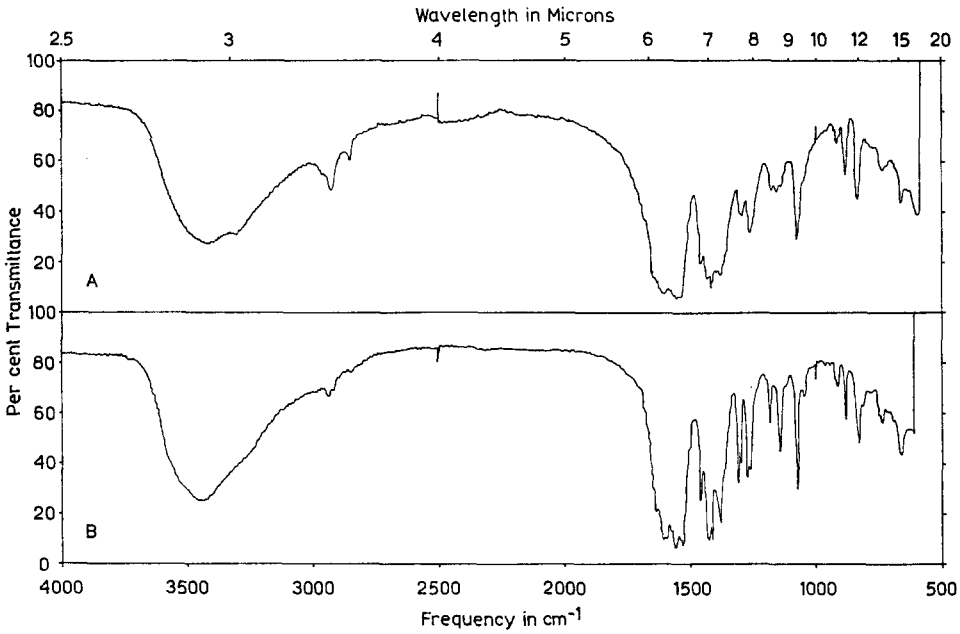
fractions 350 to 500 ml of effluent (Text-fig.1 B) and the residue in the fractions 100 to 180 ml of effluent (Fig. 1 A). As seen from a reference chromatogram (Text-fig. 1, right part) the fractions *A* and *B* are eluted at the same rate as oxalic and citric acid respectively. Peak *A* was rechromatographed with phase

TABLE III

Analysis of Calculi

Rat No.	Calculus		
	Weight	Calcium	Citrate (as citric acid)
	mg	*per cent*	*per cent*
115 - 5	43.4	21.8	47
136 - 8	49.3	23.2	51
137 - 6	84.6	25.4	39
168 - 1	12.4	20.7	58
176 - 1	26.0	21.2	50
192 - 2	80.2	21.9	53
192 - 4	23.7	21.8	56
212 - 1	79.8	24.7	25
Calcium citrate, 4 H_2O		21.07	67.4



TEXT-FIG. 2. Infrared absorption spectrum of a urinary calculus (A) and of calcium citrate, 4 H_2O (B).

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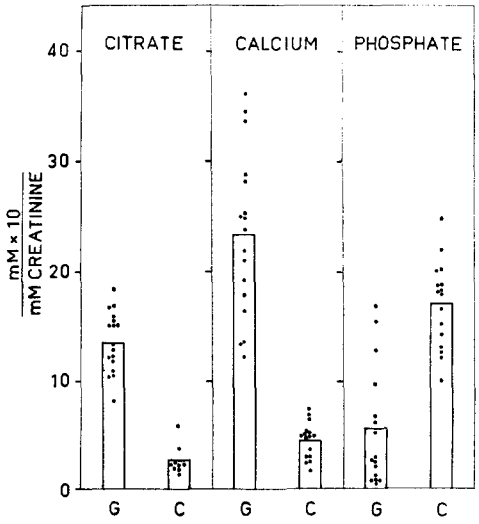
system 2 together with a tracer dose of oxalic acid-1, 2-C^{14}. Titration revealed one peak with a position identical with that of the labeled oxalic acid. The nature of the organic acids present in the urinary calculi was further investigated by chromatographic analysis. Peaks *A* and *B* each consist of only one organic acid with a chromatographic behaviour of oxalic and citric acid respectively.

Qualitative analysis thus showed that the calculi are composed of the calcium salts of citric and oxalic acid. Quantitative analysis of citrate in the calculi showed that the percentage of citrate varied between different animals (Table III). This was also apparent from chromatographic analysis of the organic



TEXT-FIG. 3. Daily urinary excretion of citrate, calcium, and phosphate in germfree (G) and conventional (C) rats. Each point represents the mean value of 3 days' urinary excretion in one animal. The bars represent the mean values for the different groups.

acids, which showed a varying oxalic acid/citric acid ratio. In this part of the study one stone was found which contained citric acid as the only organic acid. Text-fig. 2 gives the infrared spectrum of a potassium bromide disk preparation of this stone. The spectrum is almost similar to that of pure calcium citrate, 4 H_2O.

C. Differences in the Urinary Electrolyte Excretion of Germfree, Ex-Germfree, and Conventional Rats.—Urine of male germfree rats was compared with that of conventional male animals of corresponding weight and age. Because of the possibility of water evaporation from the urine, determination of actual urinary electrolyte concentration was not performed. The daily urinary excretion was determined and the excretion values expressed on the basis of millimol (mM) electrolyte × 10/mM creatinine. It was found that the daily urinary

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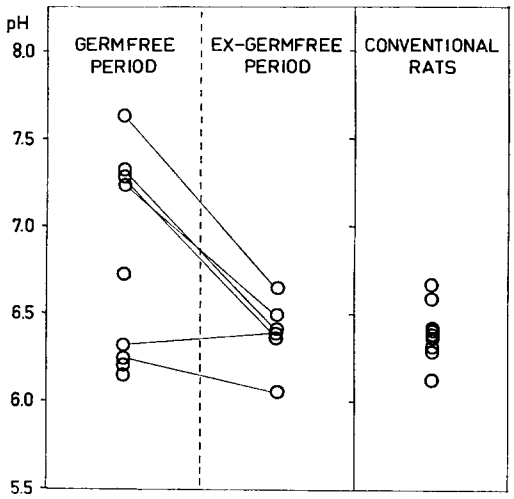
excretion of creatinine only varied slightly between animals from the different groups and still less from day to day in the same animal.

Text-fig. 3 gives the daily urinary excretion of citrate, calcium, and phosphate in germfree and conventional rats. The citrate and calcium excretion in germfree rats is 4.8 and 5.0 times higher than in conventional rats. The differences between the two animal groups are statistically highly significant. On the contrary the mean daily phosphate excretion in germfree rats is only a third of the mean value observed for conventional rats. There were some germfree rats with higher values of phosphate than the minimum values in the conven-



TEXT-FIG. 4. Comparison of urinary pH in germfree, ex-germfree, and conventional rats. Each point represents the mean value of 3 days' urinary excretion in one animal. The ex-germfree period is between the 3 to 5 days after contamination with intestinal contents from conventional rats. The lines connect values in the same animal.

tional group. The difference between germfree and conventional rats in phosphate excretion is, however, also statistically significant (t value 10.05, $f = 31$).

As is evident from Text-fig. 4 there were great individual variations in the urinary pH of germfree rats. Some animals had values in the range of conventional rats, *i.e.* pH between 6.1 and 6.7, but other germfree animals had urinary pH above 7.0. After contamination with a suspension of feces from conventional rats the elevated urinary pH decreased to values observed for conventional rats.

The urinary electrolyte excretion was studied in germfree rats which had been transferred to normal environment and acquired the flora of conventional rats. As shown in Text-fig. 5 the calcium excretion decreased and the phosphate excretion increased to quantities similar to those in conventional animals.

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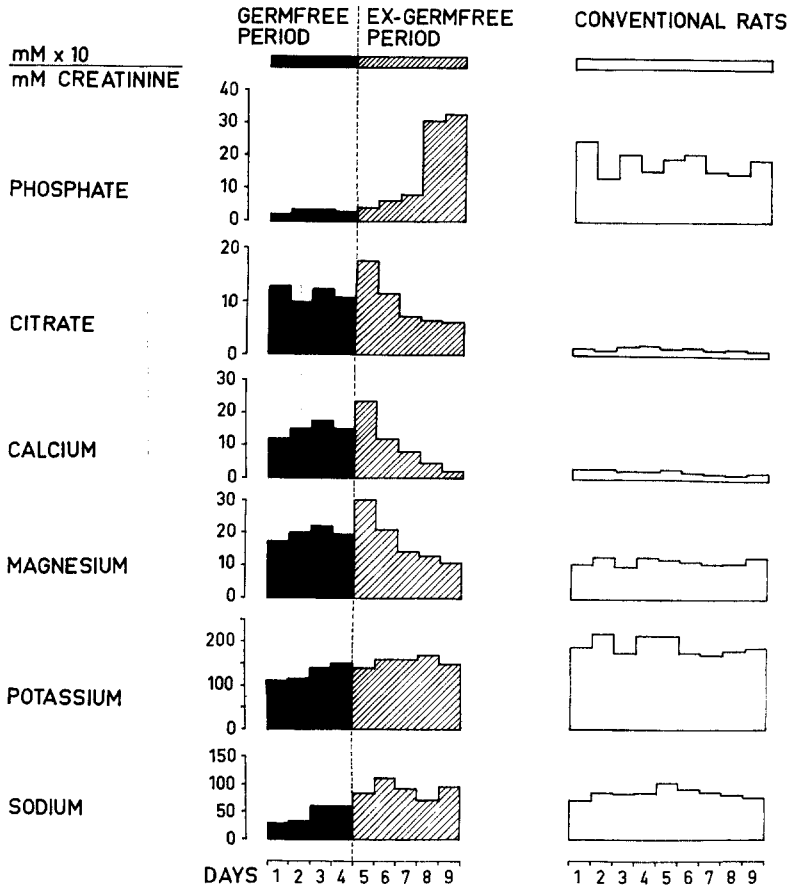
The ratio of calcium to phosphate was thus reversed. The citrate excretion decreased more slowly than the calcium excretion and on the 5th day after



TEXT-FIG. 5. Mean daily electrolyte excretions of 3 conventional and 3 germfree rats, which on the 5th day were contaminated with fecal suspensions from conventional rats.

infection it was still higher than in conventional rats. Only slight changes in the magnesium, potassium, and sodium were observed.

Since the first few days of transmission from germfree to ex-germfree status must be considered to be a period of unbalance, the urinary excretion of calcium and phosphate was followed for a longer period to exclude temporary changes caused for instance by a reduced food intake. Text-fig. 6 shows the variation in the ratio urinary calcium/urinary phosphate during 40 days following the transfer of the germfree rats to conventional environment. The

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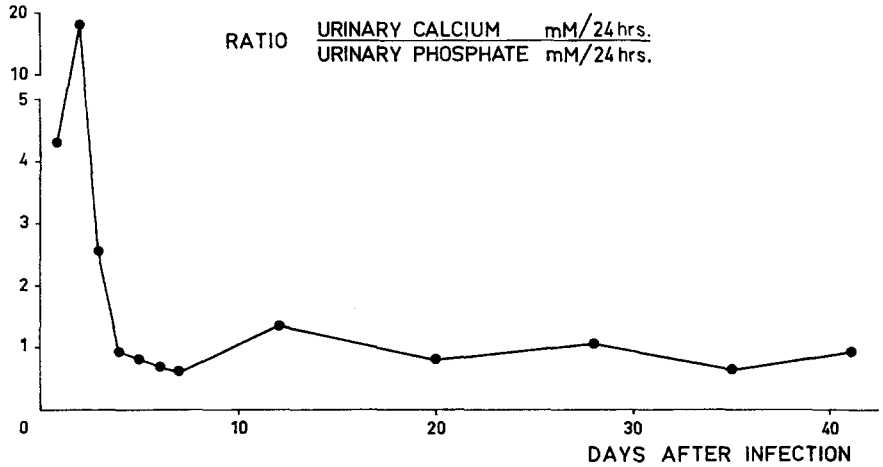
normal ratio obtained after 4 days of ex-germfree living is lasting during the following experimental period.



TEXT-FIG. 6. Mean ratio of urinary calcium and phosphate in three ex-germfree rats.

DISCUSSION

Our data have demonstrated a high incidence of urinary calculi in germfree rats reared on a semisynthetic diet with a composition within the limits of present standards. The facts that not a single conventional animal on the same diet had urinary calculi and that the stone formation tendency disappeared when the germfree animals were contaminated with the intestinal flora from the conventional controls strongly suggest that the absence of the microbial flora of the intestinal tract is the determining factor in the calculus formation. The mineral pattern of the urine with high calcium, high citrate concentration combined with high pH in germfree rats can readily explain the formation of calcium citrate calculi. It is too early to discuss whether the increased urinary excretion of calcium is a phenomenon secondary to an increase in the intestinal absorption related to the absence of the microbial flora or to other factors controlling mineral metabolism.

Verkoren's (9) discovery in rats of bladder stones containing calcium citrate has been verified by several authors. Thus Schneider and Steenbock (10) showed that a diet low in phosphate caused urinary calculi in rats. The stone frequency was increased if salts increasing the pH of the urine were added to the diet (11). Sager and Spargo (12) found that on a low phosphate diet the frequency of calcium citrate stones was increased when the protein content of the diet was decreased. The influence of low phosphate and low protein diet has recently been studied by van Reen *et al.* (13, 14). In the studies by Sager and

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Spargo and van Reen *et al.* it was found that the urinary concentration of calcium and citrate was high and phosphate low in the groups of animals with high incidence of stones. A similar excretion pattern of electrolytes was found in our germfree animals. During the ex-germfree period, however, a profound change in the urinary composition took place, acquiring the pattern of conventional animals with low calcium and citrate and high phosphate. It is interesting that during the ex-germfree period some stones disintegrated. Although there are certain similarities between the results of our studies in germfree rats and those of van Reen *et al.* one can hardly suppose a common etiologic factor inherent in the diet, as our conventional animals did not present a single case of stone and had a mineral pattern in the urine different from the animals in the studies cited in which all animals were conventional.

SUMMARY

In a colony of germfree rats 50 per cent of the males had urinary calculi composed of calcium citrate and calcium oxalate. Genetically closely related conventional animals on the same sterilized diet did not present a single case of stone formation. The tendency to calculus formation disappeared when germfree animals were contaminated with the intestinal flora from conventional rats.

The calculus formation can readily be explained by the high calcium, high citrate, and high pH of the urine. This pattern was changed to that of conventional rats when the germfree rats were infected with intestinal microorganisms.

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EXPLANATION OF PLATES

PLATE 31

FIG. 1. Autopsy specimens of vesical calculi from a germfree rat (B) and from an ex-germfree rat (C), which was contaminated with intestinal bacteria during 90 days. × 5.

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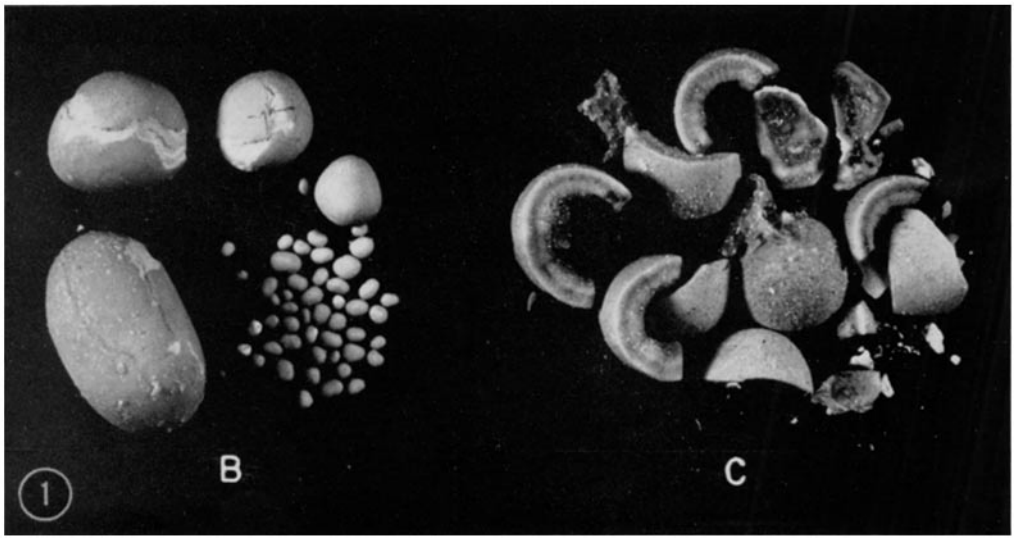

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PLATE 32

FIG. 2. Vesical calculus *in situ* from a germfree rat. × 100

FIG. 3. Urinary calculi in the collecting ducts in the outer zone of the medulla of kidney from a germfree rat. The lumen of the kidney pelvis is to the right.

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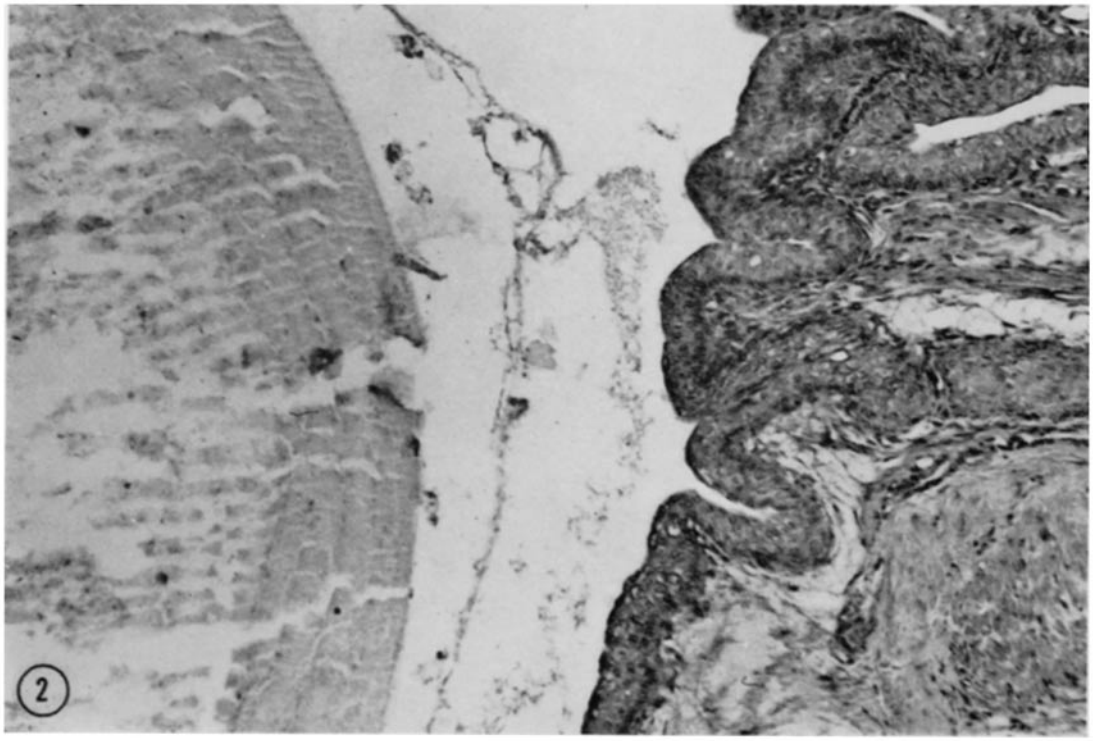

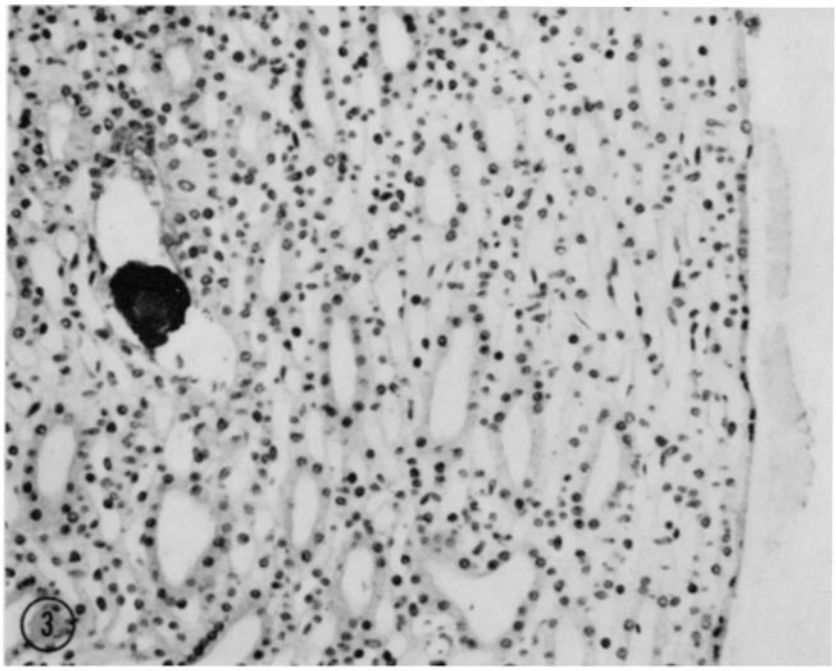

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