groups. An examination of time trends by age-specific groups led us to suspect that a cohort or generational effect might be wholly or at least partially responsible for the observed increase in prostatic cancer mortality rates among blacks. To this end, we have analyzed prostatic cancer mortality rates among men aged 45 to 84 years for separate birth cohorts of U.S. nonwhites extending from 1846 to 1925. While it would have been preferable to utilize black rates for the analyses, these are not available. However, since blacks make up the large majority of nonwhites, only a small effect could be attributed to other nonwhites.

The cohort data are shown graphically in Fig. 2. Rates for all ages peak in the birth cohort of 1896 to 1900. Cohorts born prior to that time show a consistent increase in rates for all age groups, and cohorts born after 1896 to 1900 show a fairly consistent decline. An analysis of variance (F statistic) was computed and confirmed that the cohort effect was indeed nonrandom ($P < .001$). The reason that the age-adjusted rates calculated from current data continue to increase is that the earlier cohorts, those born before 1900, comprise the older age groups which contribute most heavily to the summary rates. If trends among younger nonwhite cohorts continue, and as the effect of the earlier cohorts fades, it can be expected that overall mortality rates from prostatic cancer in blacks will begin to decline.

By means of a log-linear statistical model (*3*), the magnitude of the cohort effect can be assessed. The age-adjusted prostatic cancer rates among nonwhites with the cohort effect statistically removed are shown in Fig. 1 (dashed line). These modeled rates essentially parallel the observed nonwhite rates until 1955. At that time, the slope in the modeled rates decreases until 1965 and then shows a slight increase in 1970. A similar decrease was observed among whites 15 years earlier.

A frequently offered explanation for the secular increase in cross-sectional prostatic cancer mortality rates is that it can be accounted for by improvements in the medical care, screening, and accuracy of diagnosis among blacks over time. The nature of prostatic cancer makes it particularly sensitive to such changes, so that the increases might be artifacts of reporting systems. The proportion of nonwhite males dying from ill-defined and unknown causes declined from 5.7 percent in 1930 (*4*) to 3.3 percent in 1970 (*5*), and this might have had a minor influence on prostatic cancer rates. However, these reasons would not explain the fact that the increases observed for the 1896 to 1900 cohort are followed by decreases in subsequent cohorts, at least for men under the age of 70 for whom data are available.

We have carried out a similar analysis of mortality data for whites and no such generational pattern was seen, a finding consistent with the fact that cross-sectional rates for whites have shown very little change in the past five decades.

Research into the historical experience of the 1896 to 1900 and earlier nonwhite birth cohorts might provide clues to agents associated with prostate cancer. Given the distance in time, we acknowledge the potential difficulties of such a task.

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3. The log-linear model is:

$$\log (r_{ij(k)}) = \mu + \alpha_i + t_j + c_{(k)}$$

where the logarithm of the rate of prostatic cancer $r_{ij(k)}$ is modeled by the sum of a constant represented by μ, the effects of the ith age group represented by a_i, the effects of the jth time period represented by t_j, and the effects of the kth cohort represented by $c_{(k)}$. Note that the term $c_{(k)}$ may be considered as a time-age interaction since each cohort is uniquely defined by the pair of variables, time period, and age. The components of this model were estimated by the method of least squares, and the fit of the model to the observed data is extremely good. The proportion of the variation explained by the model is 99.3 percent. The estimates of the components of the model with the values of $c_{(k)}$ set to zero yield an assessment of the "cohort effect." The F-statistic (with 14 and 47 degrees of freedom) for testing the statistical significance of this reduced model is 20.38 [$F_{14,57}(.05) = 1.87$]; thus, the contribution due to differences among cohorts to the variation in nonwhite prostatic cancer rates is not likely to have occurred by chance. This reduced model produces estimates of age-adjusted rates for prostatic cancer in which the cohort effect has been removed statistically, as shown in Fig. 1.
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Calcareous Deposits in the Renal Sac of a Molgulid Tunicate

Abstract. *Weddellite (calcium oxalate dihydrate) and calcite (anhydrous calcium carbonate phase) are components of concretions in the renal sac of the ascidian tunicate* Molgula manhattensis. *The presence of weddellite along with urate in the concretions suggests a resemblance to human kidney stones, although, unlike the latter, the concretions in* Molgula *do not seem to be pathologic deposits.*

The role of the renal sac in the ascidian family Molgulidae (Tunicata, phylum Chordata) has remained a puzzle since the structure was first described more than a century ago (*1*). Usually thought to function as a kidney, chiefly because concretions in its lumen contain uric acid (*2–4*), the renal sac nevertheless possesses various features that are unusual for an excretory organ. For example, the bladder-like, sausage-shaped structure has no apertures or ducts at any time in the life cycle of *Molgula* (*4*). Its large lumen sequesters solid concretions easily visible to the naked eye and also—in at least five species of *Molgula*, including *M. manhattensis*—consistently harbors dense populations of fungus-like microorganisms (*4*).

The chemical activities of the renal sac have been surmised almost exclusively from the presence of uric acid in the organ. However, uric acid is not the sole precipitate in the renal sac lumen. If renal sac concretions of *M. manhattensis* are immersed in 5.25 percent sodium hypochlorite (NaClO; Clorox), much of the concretionary material, including uric acid, is quickly digested, leaving behind a residue that includes transparent dipyramidal crystals and, in some animals, a number of spheroid, opaque concretions (*5*). We have examined the composition of this NaClO-resistant, bioinorganic fraction isolated from *M. manhattensis* collected in San Francisco Bay, California.

We have seen tetragonal, dipyramidal crystals, with or without columnar faces (Fig. 1), in all sexually mature *M. manhattensis* from San Francisco Bay and have also found them in specimens of *M. manhattensis* from Woods Hole, Massachusetts, and Chesapeake Bay (Maryland and Virginia). These crystals lie "free" in the renal sac fluid as single or

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(rarely) twinned crystals or are found attached to or embedded within other concretionary material. The crystals usually are colorless, but occasionally are yellow or brown; their size is variable, but rarely exceeds 0.1 mm in diameter. They are morphologically similar to crystals of calcium oxalate dihydrate (weddellite) (*6*). Qualitative electron microprobe analysis shows calcium to be the major elemental constituent of the crystals; traces of sulfur and chlorine are also present. The infrared absorption spectrum of a sample of crystals pooled from 100 *M. manhattensis* adults matches that of weddellite (*6, 7*), with major absorption bands at 1324 and 1620 to 1650 cm^{-1} (*8*). Thus, we conclude that the dipyramidal crystals of *M. manhattensis* are weddellite.

Spheroid concretions resistant to NaClO oxidation were found in 5 of 72 animals collected from southern San Francisco Bay in February 1976, and in 22 of 95 animals collected from central San Francisco Bay in March 1976 and November 1976 to April 1977. These concretions are easily discerned, even before NaClO treatment, as opaque spheroids up to 0.7 mm in diameter (Fig. 2). Up to 11 such concretions have been found in a single animal. The spheroids are white, pale yellow, amber, or red-brown; their color tends to fade with extended immersion in NaClO.

The spheroids are readily soluble in 0.1*N* HCl. The infrared absorption spectrum of NaClO-treated spheroids matches that of calcite, with major absorption bands at 1445 to 1450 and 876 cm^{-1} and a minor band at 712 cm^{-1}. Qualitative microprobe analysis of NaClO-treated spheroids shows calcium to be the major elemental constituent. Magnesium is only a trace constituent, with sulfur and chlorine also present in lesser amounts. Thus, we conclude that the spheroids are calcite.

Both the weddellite crystals and calcite spheroids can be seen in live animals through the wall of the intact renal sac, before dissection and NaClO treatment, and are found in freshly collected animals. This indicates that the presence of these substances is not an artifact of the NaClO treatment or of maintenance of the animals in aquariums.

Other ascidian families possess concretion-filled blood cells ("nephrocytes") or (in the Corellidae and Ascidiidae) small, closed "renal vesicles," both of which have been considered analogous to the molgulid renal sac. Most evidence suggests that the concretions of nephrocytes and renal vesicles are composed of uric acid, other purines, or other organic substances (*2, 9*). Goodbody (*2*) has reported, however, that concretions isolated from the renal vesicles of *Ascidiella aspersa* contain chiefly calcite and that the renal vesicle concretions of *Phallusia mammillata* and *Ascidia nigra* contain, in addition to uric acid, small amounts of calcium. Thus, nonskeletal precipitation of calcium occurs in at least one ascidian family besides the Molgulidae.

Neither the functions nor the metabolic sources of the calcareous deposits in *Molgula* are yet known. The deposits cannot serve as a calcium reserve for skeletal construction, since *Molgula* has no mineralized structures in its body other than the renal sac concretions themselves. However, the deposits might provide calcium reserves for other purposes, such as ionic regulation or other metabolic processes involving calcium. Both ultrastructural and physiological data (*4, 10*) suggest that there is ionic regulation of renal sac fluid. Evidence to date (*10*) indicates that osmotic regulation of body fluid and renal sac fluid in the estuarine *M. manhattensis* occurs only at ambient water salinities of less than 16 parts per thousand.

Even though uric acid and calcium oxalate are typical components of human kidney stones (*11, 12*) and calcium carbonate is both an occasional component of kidney stones and a major constituent of human gallstones (*12*), it is unlikely that the renal sac concretions of *M. manhattensis* are pathologic deposits. Uric acid and weddellite have been found in all *M. manhattensis* adults collected from San Francisco Bay over a period of 3 years: a disease so universally present within a population is difficult to imagine. We have not proved that the calcite spheroids are benign deposits in *M. manhattensis*; even so, those individuals possessing calcite concretions do seem to be healthy animals in all outward respects. Field-collected *M. manhattensis* individuals can be considered "infected" only

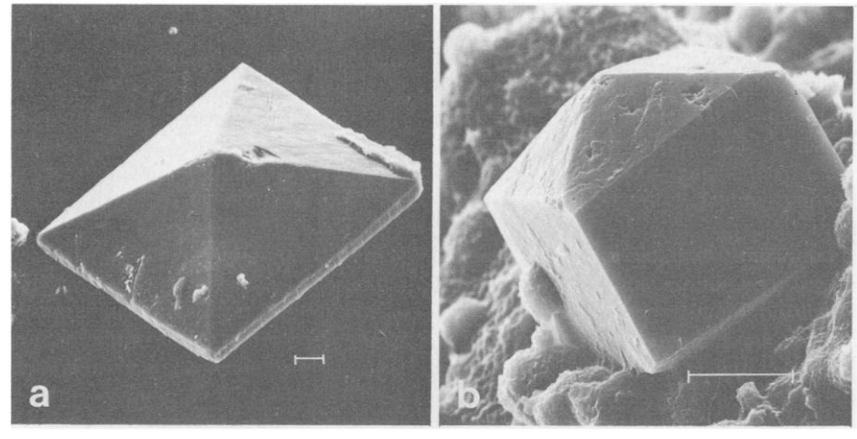

Fig. 1. Scanning electron micrographs of weddellite crystals isolated from the renal sac lumen of *Molgula manhattensis*. (a) Dipyramidal crystal. (b) Dipyramidal crystal with columnar face, on the surface of the concretion. Scale bars, 10 μm.

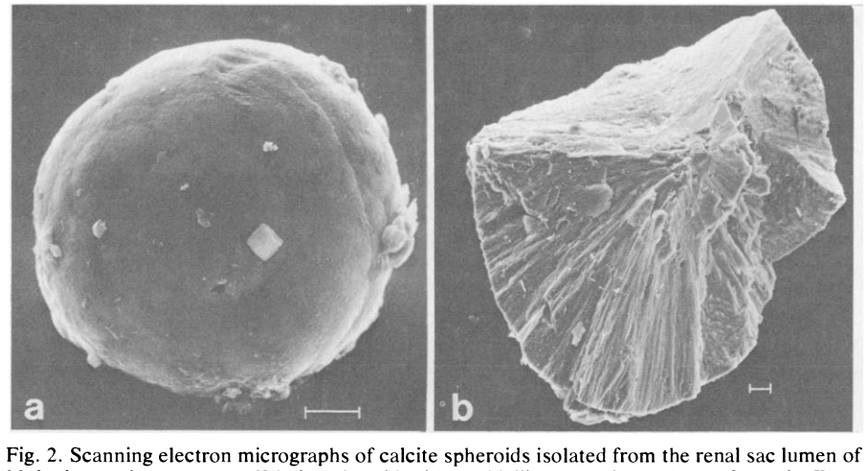

Fig. 2. Scanning electron micrographs of calcite spheroids isolated from the renal sac lumen of *Molgula manhattensis*. (a) Whole spheroid with weddellite crystals on the surface. (b) Fractured spheroid, showing internal structure. Scale bars, 10 μm.

in the sense that they all contain fungus-like symbionts in the renal sac (*4*); aquarium culture should show whether these microorganisms are metabolically associated with any of the solid deposits in the renal sac. The production of oxalate by many fungi (*13*) raises the possibility that the weddellite crystals in *Molgula* are a metabolic product of the renal sac symbionts, rather than of *Molgula* itself.

Although the role of the renal sac remains unclear, our results do indicate that in *M. manhattensis* the chemical activities of the sac are not limited to the production and accumulation of uric acid. The contents and metabolic activities of the renal sac are thus unexpectedly complex and suggest the need for further scrutiny of the organ's role in the biology of the Molgulidae.

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Reversed-Phase, High-Pressure Liquid Chromatography of Peptides and Proteins with Ion-Pairing Reagents

Abstract. *Reversed-phase, high-pressure liquid chromatography has been successfully applied to the analysis of peptides and proteins by the addition of hydrophilic (for example, phosphoric acid) or hydrophobic (for example, hexanesulfonic acid) ion-pairing reagents, or both, to the mobile phase. Examples described included proteins such as insulin, glucagon, and 1-24 ACTH pentaacetate (ACTH is adrenocorticotrophic hormone).*

The isolation of peptides and proteins, frequently present in only trace amounts in biological systems, has enlarged our understanding of the molecular biology of many complex biochemical and endocrine pathways. When high-pressure liquid chromatography (HPLC) (*1*) was introduced as an analytical tool, it was anticipated that this technique would allow the rapid but selective separation of natural polypeptides (*2*). The early applications of HPLC to the analysis of underivatized peptides and proteins were not entirely successful, however, with the use of either liquid-solid or liquid-liquid reversed-phase systems. Poor resolution was frequently observed to be associated with peak broadening and long retention times (*3, 4*). These features are not altogether unexpected when one considers the complex ionic equilibria that these amphoteric compounds can undergo. In a recent publication (*5*) we offered a solution to the problems of poor resolution and reproducibility by suggesting that hydrophilic ion-pairing reagents may be used with reversed-phase systems. We show here that these reagents do in fact make it possible to analyze and purify a wide range of underivatized peptides and proteins by HPLC.

Ion-pair partition chromatography or paired-ion chromatography has recently been applied to a variety of substances (*6–9*). The use of ion-pairing reagents such as the PIC reagents [tetrabutyl ammonium phosphate and heptanesulfonic acid (Waters Associates)] has resulted in increased retention times for highly polar molecules by the formation of hydrophobic ion-pair complexes (*10*). The hydrophilic ion-pairing reagents such as H_3PO_4 (*5*), perchlorates (*7, 8*), methylsulfonates (*7*), and picrates (*6*) have greatly extended the scope and potential of reversed-phase, ion-pair partition chromatography. When used alone or in combination with hydrophobic ion-pairing reagents, hydrophilic ion-pairing reagents result in marked alterations in retention times, allowing the resolution of complex mixtures (*5–7*). On a reversed-phase chromatographic support, increased polarity due to the formation of

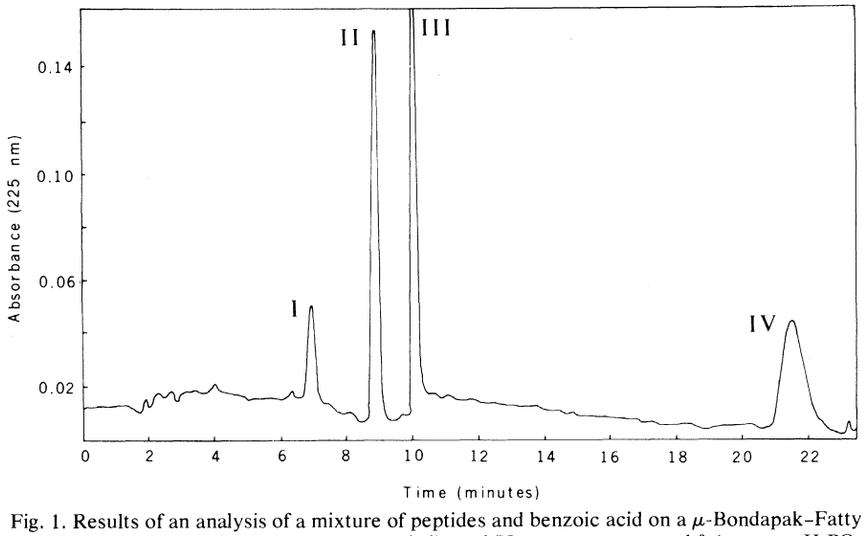

Fig. 1. Results of an analysis of a mixture of peptides and benzoic acid on a μ-Bondapak–Fatty acid analysis column with 25 percent acetonitrile and 75 percent water, and 0.1 percent H_3PO_4 as the mobile phase. Peak I corresponds to Met-Arg-Phe-Ala, peak II to Leu-Trp-Met-Arg, peak III to benzoic acid, and peak IV to Leu-Trp-Met-Arg-Phe. One nanogram of each component, made up in the mobile phase, was loaded in a volume of 25 μl; the flow rate was 1.5 ml/min, the pressure 144 atm, and the temperature 22°C. All the HPLC was carried out with a Waters Associates system as detailed in (*5*). The μ-Bondapak–Fatty acid analysis and μ-Bondapak-C_{18} columns (10-μm particles, 30 cm long and 4 mm in inside diameter) were purchased prepacked from Waters Associates. All other apparatus, materials, and methods were as outlined in (*5*).

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