Contamination of Distilled Water, HCl, and NH₄OH
with Amino Acids, Proteins, and Bacteria

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Contamination of distilled water with free and bound amino acids and with microbes has been overlooked in most laboratories. Unless special precautions are taken, distilled water will be contaminated with airborne microbes, probably pseudomonads, which multiply in water of conductivity quality. Amino acids and proteins that may be found in the water therefore presumably are derived from these airborne organisms. Chemical reagents such as hydrochloric acid and ammonium hydroxide solutions, which have water as an integral part, also have been found to be contaminated with amino acids. This paper deals with the extent of such contamination and describes means to provide water and hydrochloric acid (6 mol/liter) that would be suitable for determination of amino acids in concentrations of 10⁻⁹ mol/liter or less in materials of biological interest as well as rocks, lunar soil, meteorites, etc.

Additional Keyphrases: analysis on the ultramicro scale
- purity of reagents
- preparation of uncontaminated water

Compositional studies on proteins whose availability because of difficulty of preparation may be limited to the 10⁻²⁵ µg range, or the determination of free amino acids in biological fluids of limited availability—such as anterior fluid of a single human eye—demands that all procedures be adequately controlled to rule out accidental extraneous contamination. Similarly, a search for amino acids in meteorites, geologically ancient rocks, abiotic synthetic reaction mixtures, lunar materials, etc., demand the most rigorous attention to proper controls. This need for great care first became apparent when free amino acids were being sought in acid extracts of meteorites (7), and the search for meaningful controls led to the discovery of a wide spectrum of amino acids in finger-prints (2), an observation soon confirmed elsewhere (3, 4). Thus, simply by handling, amino acids can easily be transferred in appreciable amounts to material of interest.

Subsequently, free and bound amino acids originating from micro-organisms or solubilized products of them were found in distilled water. The organisms were apparently airborne and entered the outlet of stills or de-ionizing systems, where they multiplied. After sterilization, unless special precautions were taken to protect the water outlet, organisms could again be found, usually within a few days. The organisms most frequently encountered belonged to the family Pseudomonadaceae. It is likely—as discussed by Leifson (5-7) and Favero et al. (8), and as indicated below—that microbial contamination of distilled water is ubiquitous.

As a corollary we were led to search for amino acids in commercial analytical reagents of which water is an integral part, such as hydrochloric acid and ammonium hydroxide. Micro-organisms in the water used in the manufacture of these reagents could be a source of contamination. We wished to confirm previous reports of amino acids in hydrochloric acid (3, 9, 10) and to determine whether hydrochloric acids from different sources varied much in the extent of their contamination.

Amino acid contamination of water and hydrochloric acid would affect results of protein structural studies where accurate quantitative data are sought, particularly when ultramicro methods are being used. Contamination with high-molecular-weight protein or peptides would also be of interest to immunologists, because organisms in the water, though nonviable after autoclaving, would still be potential sources of unrecognized antigenic material.

We report here the extent of microbial and amino acid contamination likely to be encountered in distilled water, hydrochloric acid, and ammonium hydroxide solutions. Means to diminish such contamination are also described. Some of these problems have been briefly discussed in connection with the search for amino acids in lunar samples from the Apollo lunar mission (11).

Materials and Methods

Water. Originally, our distilled water was obtained from a system comprised of steam still, storage

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tanks, and distributing lines. Water and organisms from this system were studied.

Water was also collected from other laboratories in sterile containers, which were then shipped for study.

Finally, after many ways of preparation were considered, water of adequate purity was obtained from a Model 1-R recycling system with 0.2 μm (av pore diameter) post-filtration (Continental Water Corp., El Paso, Tex. 79926).

These aspects of purity and preparation are dealt with fully below.

Reagents. Analytical-grade hydrochloric acid and ammonium hydroxide were used.

Cleaning of glassware. The following procedure is that of Hamilton and Nagy (11), a modification of that reported by Hare et al. (12). All glassware was scrubbed with soap and water, rinsed, soaked for 30 min in sodium bicarbonate solution (50 g/liter), rinsed again, and dried at 160 °C. The glassware was then wrapped in aluminum foil (0.0254 mm thickness) and baked for at least 4 h at 625 °C in a muffle furnace (Model 7075; Hotpack Corp., Philadelphia, Pa. 19135). This procedure was designed to provide a glass with all negatively charged active sites combined with sodium and a surface that was devoid of organic contamination. Controls were run, in which amino acids were added to flasks in 10,000-fold the minimum concentration detectable by the ion-exchange chromatographic methods used. After the baking process, no amino acids could be detected. In a much more rigorous test, 4.5 ml of beef heart infusion neopeptone medium with defibrinated sheep blood was inoculated with Group A streptococci and incubated for 16 h, then transferred to a 50-ml flask, evaporated, and baked for 3 h at 625 °C. Some charred carbon then remained in the flask, but extraction and analysis revealed that the residue contained a total of about 2.4 μg of amino acids. With such heavy protein contamination it was assumed that probably a 12- or 16-h baking would have resulted in complete decontamination.

Because the aluminum foil is also decontaminated by the baking process, the glassware could be expected to be free of contamination for a reasonable time, but we did not determine an actual shelf life.

Amino acid analysis. To determine amino acids in water or reagents, we evaporated 200 to 1000 ml to dryness in a rotary evaporator. Half of the residue was then analyzed for free amino acids, as was the other half after acid hydrolysis. Analyses for amino acids were done by a single-column ion-exchange chromatographic procedure (13). Samples were hydrolyzed in hydrochloric acid (6 mol/liter) in sealed borosilicate-glass ampoules at 110 °C for 22 h. Excess acid was then evaporated and the residue was transferred with 0.1 mol/liter hydrochloric acid to the ion-exchange column.

Bacteriological procedures. Preliminary culturing was usually done by streaking 0.1 and 0.2 ml of water on tryptose-blood-agar plates and incubating at 37 °C for 48 h. If the water was known to be heavily contaminated, the sample was diluted with water and an equivalent of 10 or 30 μl of the original solution was streaked. To obtain quantitative data, we used the dilution pour-plate method on nutrient agar, with and without NaCl solution (10 g/liter). Plates were incubated at 20 and 37 °C for 3 to 4 days. From the number of colonies counted, we calculated the number of organisms per milliliter of the original water. Standard microbiological techniques of culturing for anaerobic organisms were also used. These cultures were read after 48-h incubation. Other standard bacteriological methods were also applied.

Samples of water were collected from various sources with aseptic precautions. When possible, counts were made immediately (at zero time) and then after 24- and 48-h storage at room temperature.

Results

Hydrochloric acid. Two-hundred milliliters of analytical-grade concentrated hydrochloric acid (rel. density, 1.17) was evaporated in a flash evaporator. The entire residue was transferred with water to the analytical ion-exchange column. Since there was no difference between residues that were analyzed at once, and residues that were subjected to 6 mol/liter hydrochloric acid by hydrolysis, this latter step was not used in the routine analysis of concentrated hydrochloric acid. All the hydrochloric acids examined contained aspartic acid, threonine, serine, glutamic acid, glycine, alanine, isoleucine, and leucine. Total amino acids (in μg/liter of the original liquid) were as follows: Du Pont (East Chicago, Ill.) 2.5; Du Pont (Graselli, N. J.) 4.5; J. T. Baker, 3.1; Baker and Adamson 6.4; Mallinckrodt 73. The Mallinckrodt sample contained ornithine, indicating the possibility of hand contamination or the complete degradation of arginine; arginine was not present in the sample. A sample of J. T. Baker "Ultrex"-grade acid contained some ornithine, lysine, and histidine, but no arginine. Its total amino acid contamination was 18.0 μg/liter.

If the concentrated acid was diluted with an equal volume of water and slowly distilled (50 ml/h) through a 2.5 × 60 cm column packed with Rachig rings, the total amino acid contamination decreased significantly. If, as a preliminary, the column had been thoroughly flushed by distilling (and discarding) about 1200–1500 ml of acid, a single passage through the column still decreased total contamination to less than 1 μg/liter. Having established the extent of contamination of reagent acid and the means to decrease contamination to less than 1 μg/liter, we could then determine the nature and extent of amino acid contamination in ammonium hydroxide and water.

Ammonium hydroxide. An open 1-pint bottle of concentrated ammonium hydroxide solution (analytical grade) was placed in a 5-gallon polyethylene jar with lid. The bottom of the jar was filled to a depth
of 5 cm with sulfuric acid solution (2 mol/liter), and the whole was maintained at 60 °C. After four days, ammonium fumes were negligible, and the fluid contents of the bottle was then evaporated to dryness, transferred with 6 mol/liter HCl to a borosilicate ampoule, which was sealed and kept at 110 °C for 22 h. After hydrolysis, excess acid was removed and the whole was placed in the amino acid analyzer. J. T. Baker analytical reagent had no amino acid contamination, whereas Mallinckrodt analytical reagent contained aspartic acid 79.9, threonine 1.2, serine 2.1, glycine 52.6, and alanine 8.9 μg/liter. Only one bottle from each manufacturer was analyzed. Other halogen acids were not examined.

Water. The extent of contamination of water from our original distilled-water system and from three other laboratories is shown in Table 1. In all samples kept at room temperature, microbial contamination increased rapidly. A remarkable feature of the organisms was their ability to grow in water of conductivity quality, without even a faint turbidity in the most heavily contaminated samples. Because our criteria of purity did not include scanning for absorption in the ultraviolet or measurements of surface potential, we did not systematically look for the type of contamination described by Bangham and Hill (14). However, we found that the absorbance, measured at 280 and 215 nm, did not correlate with the extent of bacterial contamination. The most thoroughly investigated organisms were those from our own original water system. From colonies of these organisms, nonchromogenic, nonfermentative, gram-negative, nonflagellated rods were obtained. The organisms were cytochrome oxidase positive, urease negative, H₂S negative, indole negative, methyl red negative, Voges-Proskauer negative, and citrate positive. Some organisms, but not all, reduced nitrate and hydrolyzed gelatin. The organisms grew well in nutrient agar at 20 °C. However, the total colony counts were 90% of those grown at 37 °C, indicating that these bacteria were mesophiles (organisms that grow best at temperatures between 20 and 55 °C). In the presence of sodium chloride (10 g/liter) at 20 °C the number of colonies was 38% of the number that grew in the absence of salt, and at 37 °C it was 80%. This suggested that if Caulobacter (6) were present, they did not constitute the major population of the organisms encountered.

The principal organisms were tentatively identified as belonging to the genus Pseudomonas. Spore formers, anaerobes, gram-positive bacteria, or cocci of any kind were not isolated. Organisms isolated from the other sources (Table 1) also formed colorless colonies and were gram-negative nonflagellated rods, presumed to belong to the Pseudomonadaceae also.

Bacterial counts varied with the day of the week, the time of the day, and the rate of consumption of distilled water. In general, contamination was greater after weekend inactivity and at the beginning of the day. High consumption of water tended to flush the system out and diminish the count. After acid hydrolysis of organisms from Laboratory No. 1 and Laboratory No. 3, the amino acid patterns were qualitatively essentially the same. A typical pattern is shown in Figure 1.

The data shown were obtained from the hydrolysis of organisms in 14 ml of water containing 10⁸ organisms per milliliter, as determined by dilution plate count. A hydrolysate equivalent to 2 × 10⁸ organisms would be about the least we could detect with our present chromatographic instrumentation.

Water put through a 0.2-μm (av pore size) Millipore filter to remove whole organisms still contained

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Table 1. Number of Micro-organisms in De-ionized or Distilled Water

<table>
<thead>
<tr>
<th>Time of culturing after collecting water sample, h</th>
<th>Colonies/ml</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
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<tr>
<td>48</td>
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| Laboratory 1                                      |             |
| De-ionized water (18 × 10⁴ Ω/cm)                  | 0.5 × 10⁴   |
| Distilled water (storage tank)                    | 1.5 × 10⁴   |

| Laboratory 2                                      |             |
| Triple dist. (directly from still)                |             |
| Triple dist. (stored in bottle)                   |             |
| Laboratory 3                                      |             |
| Distilled (closed reservoir)                       |             |
| (after 3 weeks in sterile container)              |             |
| Laboratory 4                                      |             |
| Distilled (closed reservoir)                       |             |
| (after 3 weeks in sterile container)              |             |
| Blank (Millipore filter, 0.22-μm filter)                  |             |

* Delayed in transit 4 days.

* Delayed in transit 2 days.

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amino acids, after acid hydrolysis, indicating that soluble proteins or peptides were present. Presumably these were solubilized products of microbiological origin.

Discussion

The import of these findings with respect to biochemistry, immunology, and organic geochemistry bears emphasis. Leifson (5–7) in an extensive study on organisms in distilled water, found that water samples from several laboratories in Chicago; from California; Vancouver, B. C.; Washington, D. C.; England; Japan; and New Zealand were all contaminated. The organisms investigated were in general gram-negative flagellated rods belonging to the family Pseudomonadaceae, except for Caulobacter vibrioides (Caulobacterae). He also proposed names for five other new species, belonging to four genera, cultures of which have been deposited with the American Type Culture Collection. Evidently, the organisms encountered in the present work are not the same as those described by Leifson, but do belong to the genus Pseudomonas. Our bacteriological findings also confirm those of Favero et al. (8), more particularly in respect to the rapid proliferation, extent of contamination, and complete absence of turbidity of contaminated water.

Results of our efforts to prepare uncontaminated water by distillation were disappointing. Sterile water could be produced by a still that could be steamed out before collection (e.g., the Loughborough Still; Bellco Glass, Inc., Vineland, N. J. 08360). In water from this still we found small amounts of free amino acids (before acid hydrolysis), but the bulk of the amino acids were found after hydrolysis; hence we presumed that large peptides, proteins, or whole organisms were carried over by entrainment. As stated under Results, when the feed water to the still was filtered (Millipore), bound amino acids were decreased but were not eliminated. Permanganate oxidation was also deemed unsafe because of permanganate carryover (11), and destructive oxidation by gaseous oxygen needed approaches (15–17) that were more sophisticated than those attempted here. We investigated two commercial deionizing systems and one laboratory-built de-ionizer. They were abandoned, partly because of excessive color from the resin, partly because of incomplete removal of material containing amino acids, and partly because no way was provided to prevent microbial contamination. Several sources of bottled sterile water for injection (USP quality, pH 7.5, from pharmaceutical companies) were examined, but contamination with amino acids varied considerably amongst the sources, and even from bottle to bottle from the same sources. These too were abandoned.

The method of preparing water of adequate purity was the Continental Water System (see Materials and Methods). This system provided approximately 1200 liters of high-grade water before filters had to be replaced. The resistivity was greater than 15 × 10⁶ Ω/cm; the total amino acid contamination (after acid hydrolysis) was less than 1 μg/liter; there was no microbial contamination; and the water was nontoxic to cultured mammalian cells and to various strains of Group A streptococci.

The final outlet from this system was a silicone rubber tube, which hung down, thereby minimizing ingress of airborne organisms. Occasionally, the output tube became contaminated, as evidenced by the appearance of 10–20 colonies per ml. If this occurred, the output tubing was decontaminated by soaking it for an hour in undiluted sodium hypochlorite (“Chlorox”) solution.

We have had about 2.5 years of experience with this system, and the quality of the water has been consistent and adequate for all needs, including the search for amino acids in lunar materials (18). It is apparent that, given water and commercial hydrochloric acid both of low contamination, a good quality of 6 mol/liter hydrochloric acid may be obtained simply by mixing. However, we have found all commercial sources vary appreciably from lot to lot. Therefore, we favor distillation at least once. If low iron content is desirable, two or more distillations are recommended.

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References


Fig. 1. Strip-chart recording of amino acid analysis pattern of acid-hydrolyzed Pseudomonas sp.

1. taurine; 2. cysteic acid; 3. aspartic acid; 4. threonine; 5. serine; 6. proline; 7. glutamic acid; 8. glycine; 9. alanine; 10. valine; 11. isoleucine (with unknown peak at trailing edge); 12. leucine; 13. tyrosine; 14. phenylalanine; 15. ethanolamine; 16. ammonia; 17. methionine; 18. ornithine; 19. lysine; 20. histidine; and 21. arginine. Peak for valine = 469 ng. Abscissa, time in minutes.

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