Report of the Workshop on the Feasibility of a Chronic Neurotoxicity Study of Aluminum Administered in the Drinking Water of Animals

Ottawa, Ontario, Canada
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Co-sponsored by
Health Canada
and the
United States Environmental Protection Agency

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Acknowledgements

This Workshop was jointly funded by Health Canada (Environmental Health Directorate, Health Protection Branch) and the U.S. Environmental Protection Agency (Office of Water, Office of Science and Technology). Dr. Shalini Gupta acted as the Scientific Coordinator of the Workshop. Thanks are extended to all participants who contributed their valuable time and expertise to the Workshop.

Summary -- Aluminum Workshop

Aluminum is known to be a neurotoxic element. As aluminum is used widely as a coagulant in water treatment, the issue of safe levels of aluminum in drinking water is of considerable interest to public health officials and regulatory agencies. Although acute exposure to high levels of aluminum is well tolerated, chronic exposure to aluminum has been shown to produce encephalopathy in patients undergoing renal dialysis, and aluminum has been implicated in the aetiology of amyotrophic lateral sclerosis and in the dementia associated with Parkinson's disease. A causal role for aluminum in the development of Alzheimer's disease has been suggested on the basis of neuropathological studies of the brains of patients and epidemiological studies of the association between Alzheimer's disease and exposure to aluminum in drinking water. Animal studies on the neurotoxicity of aluminum have also been positive. However, both the epidemiological studies and the animal studies have been criticized on methodological grounds, and it has not been possible, nationally or internationally, to establish health-related guidelines for aluminum in drinking water.

Health Canada, as part of its Drinking Water Safety Program convened an international Workshop to investigate the feasibility of a study of the chronic neurotoxicity of aluminum administered in the drinking water of animals which could be used to establish a health-based guideline for human exposure to aluminum in drinking water. Experts from Australia, the United Kingdom, Canada, Norway, and the United States participated in the Workshop. Participants were asked to contribute papers addressing the issues of establishing an animal study. The Workshop was co-sponsored by the U.S. Environmental Protection Agency, Office of Water, Office of Science and Technology.

A consensus for a study was obtained on all questions raised, except that it was not possible to agree on the use of a single species of animal. Arguments were made for the use of mice, rabbits, and transgenic mice carrying risk factors for Alzheimer's disease, if sufficient funding can be obtained. However, there was a consensus that if funding is restricted, then the species should be given priority in the order listed.

A summary of the presentations and discussion, the specific recommendations on the suggested study(ies) and a copy of the contributed papers are included in the attached report.

October 24, 1997

Résumé -- Atelier sur l'aluminium

On sait que l'aluminium est un élément neurotoxique. Puisqu'il est fréquemment utilisé en tant que coagulant dans le traitement de l'eau, la question de niveaux sécuritaires d'aluminium dans l'eau potable suscite beaucoup l'intérêt des autorités du domaine de la santé publique et des organismes réglementaires. Bien qu'une exposition aigue à des niveaux élevés d'aluminium soit bien tolérée, il a été démontré qu'une exposition chronique à l'aluminium pouvait entraîner une encéphalopathie chez les patients recevant une dialyse rénale, et l'aluminium a été impliqué dans l'étiologie de la sclérose latérale amyotrophique et dans la démence associée à la maladie de Parkinson. Il a également été suggéré que l'aluminium pouvait être une cause du développement de la maladie d'Alzheimer, suite à des études neuropathologiques sur des cerveaux de patients et à des études épidémiologiques de l'association entre la maladie d'Alzheimer et l'exposition à l'aluminium dans l'eau potable. Des études sur la neurotoxicité de l'aluminium chez les animaux ont également été positives. Toutefois, les études épidémiologiques et les études chez les animaux ont fait l'objet de critiques en ce qui concerne la méthodologie employée, et il n'a pas été possible, à un niveau national ou international, d'établir de recommandation en matière de santé pour l'aluminium dans l'eau potable.

Santé Canada, dans le cadre de son Programme sur la qualité de l'eau potable, a convoqué un atelier international afin d'évaluer la faisabilité d'une étude sur la neurotoxicité chronique de l'aluminium administré à des animaux dans leur eau de consommation qui pourrait être utilisée afin d'établir une recommandation en matière de santé pour l'exposition des êtres humains à l'aluminium dans l'eau potable. Des experts venant de l'Australie, du Royaume-Uni, du Canada, de la Norvège et des États-Unis ont participé à cet atelier. On a demandé aux participants de contribuer des documents scientifiques concernant la question d'établir une étude chez les animaux. La *Environmental Protection Agency* (Office of Water, Office of Science and Technology) des États-Unis a apporté une aide financière à cet atelier.

Un consensus a été obtenu pour toutes les questions soulevées, à l'exception du choix d'une seule espèce d'animaux a utiliser. Les avantages d'utiliser des souris, des lapins et des souris transgéniques ayant des facteurs de risques pour la maladie d'Alzheimer ont été discutéss si des fonds suffisants peuvent être amassés. Cependant, un consensus a été établi sur le fait que si les fonds disponibles étaient limités, alors l'ordre de priorité de l'animal à utiliser est tel qu'indiqué ci-dessus.

Un résumé des présentations et des discussions, les recommandations spécifiques pour l'étude (ou les études) suggérée(s) et une copie des documents scientifiques qui ont été contribués sont inclus dans le rapport ci-joint.

Le 24 octobre 1997

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Introduction

Aluminum is known to be a neurotoxic element. As aluminum is used widely as a coagulant in water treatment, the issue of safe levels of aluminum in drinking water is of considerable interest to public health officials and regulatory agencies. Although acute exposure to high levels of aluminum is well tolerated, chronic exposure to aluminum has been shown to produce encephalopathy in patients undergoing renal dialysis, and aluminum has been implicated in the aetiology of amyotrophic lateral sclerosis and in the dementia associated with Parkinson's disease. A causal role for aluminum in the development of Alzheimer's disease has been suggested on the basis of neuropathological studies of the brains of patients and epidemiological studies of the association between Alzheimer's disease and exposure to aluminum in drinking water. Animal studies on the neurotoxicity of aluminum have also been positive. However, both the epidemiological studies and the animal studies have been criticized on methodological grounds, and it has not been possible, nationally or internationally, to establish health-related guidelines for aluminum in drinking water.

Health Canada, as part of it's Drinking Water Safety Program, convened an international Workshop to investigate the feasibility of a study of the chronic neurotoxicity of aluminum administered in the drinking water of animals; such a study could be used to establish a health-based guideline for human exposure to aluminum in drinking water. The Workshop was held in Ottawa on 3–4 September 1997, with support from Health Canada and the U.S. Environmental Protection Agency.

Experts from Australia, the United Kingdom, Canada, Norway, and the United States participated in the Workshop (see Appendix 13). Participants were asked to contribute papers addressing one or more of the following issues:

1. What problems are involved in the assessment of the human health effects of aluminum in drinking water?

2. How important are aluminum speciation and substances that affect speciation, such as silica, fluoride, and pH?

3. Is an animal study crucial in human risk assessment (i.e. is an animal study an appropriate next step)?

4. Which is the best animal model, and what are the appropriate end-points to be measured (e.g. neurotoxicity, behavioural changes, aluminum transfer to the serum and brain)?

5. In an animal study with ^{26}Al, how much aluminum is absorbed? Where does it accumulate? What are the pathways for accumulation? What are the effects of age on accumulation?

6. What methods should be used to avoid contamination from aluminum, which is ubiquitous in nature, during an animal study?

The contributed papers were circulated prior to the meeting and are included in this report (see Appendices 1 to 12).

Summary of Presentations

On the first day of the Workshop, each contributed paper was presented by its main author and discussed by the participants.

Dr. Barry Thomas (Health Canada), who chaired the Workshop, began by welcoming the participants on behalf of Health Canada. He then described the procedures by which the quality of drinking water is regulated in Canada. The responsibility rests with the provinces, but not all provinces have laws governing water quality. Some rely on municipal authorities. At the federal level, Health Canada publishes guidelines on drinking water quality, covering more than 80 elements. Agreement on the guidelines is achieved by a Federal–Provincial Subcommittee on Drinking Water, which makes recommendations to the Federal–Provincial Committee on Environmental and Occupational Health. Aluminum is not included in the guidelines, and attempts to reach agreement, based on aesthetic or health-related considerations, have so far failed. In particular, a proposal for an upper limit of 100 µg/L was rejected in 1996 because of inadequate scientific evidence. If a suitable animal study can be mounted, then this would help to resolve the impasse. However, Health Canada is not in a position to fund such a study in its entirety, so joint funding would be necessary.

Dr. Amal Mahfouz (U.S. Environmental Protection Agency, or EPA) also welcomed the participants. The EPA was pleased to collaborate with Health Canada in convening the Workshop because of new laws — passed in 1996 in the United States — that emphasize that sensitive populations, especially children, should be considered in formulating regulations. A list of elements that are to be given priority will be published shortly, and aluminum will be included. Aesthetic-based guidelines of 50–100 µg/L already exist in the United States, but there is no clear message from existing data to support the establishment of health-based regulations. Too many confounding factors are involved, and a protocol is needed to address these. The protocol should be aimed at risks for children, the general public, and the elderly.

Dr. Deborah Rice (Health Canada) noted in her presentation that the submitted papers were of two types: those concerned with the role of aluminum in the aetiology of Alzheimer's disease, and those addressing the broader question of the general neurotoxicity of aluminum. In her view, the proposed animal study should not focus on a single disease orientation, and a "cradle to grave" approach is warranted. There is clear evidence that aluminum is neurotoxic, and we know from the studies of other neurotoxic metals, such as lead, that it is important to consider the developmental period in their assessment. We need to study cognitive effects in general, including learning and memory. It is clear from the literature on the effects of lead on rodents that the effective doses for such sensitive end-points are much lower than those for end-points of the general screening type. Neuropathological examinations should also be more general than those used in relation to Alzheimer's disease. Rabbits and transgenic mice are useful for the latter, but rodents should probably be used for a more general approach. In the discussion of Dr. Rice's paper, the problem of the

blood–brain barrier was raised, especially in the use of rats, but it was felt that this would not be a major concern. Attention was drawn to recent clinical evidence of aluminum toxicity in infants and children and to the finding that the doses that produced dialysis encephalopathy were lower in children than in adults.

Dr. John Savory (University of Virginia) presented arguments for the use of old rabbits in the proposed study. Rabbits are genetically closer to primates than rodents, and old rabbits have been shown to be sensitive to the effects of aluminum given intracisternally, with the production of neurofilamentous aggregates in the regions of the brain involved in Alzheimer's disease. Early features seen in Alzheimer's disease, such as oxidative stress and apoptosis, are also seen. Dr. Savory also argued for the use of aluminum maltolate, as inorganic salts of aluminum precipitate at neutral pH, whereas aluminum maltolate does not hydrolyse over a pH range of 2–9. Behavioural end-points have not been systematically studied in the rabbit model, but they could be developed. In response to questions concerning oral administration, Dr. Savory replied that experiments on young rabbits using exposure for several months by the oral route produced only minimal changes in the brain, and the effects of oral administration in older rabbits have not been studied. The possibility of differential absorption in patients with Alzheimer's disease was also discussed.

Dr. Mari Golub (University of California, Davis) presented the results of neurotoxicity studies of aluminum lactate in the diet of mice, using a screening battery of end-points developed and standardized for other neurotoxicants. These experiments have shown that aluminum affects the same standard end-points, now adopted by the EPA. Based on her experience and that of other laboratories, Dr. Golub proposed a study of the developmental and general neurotoxicity of aluminum in drinking water using these guidelines and conducted under Good Laboratory Practice conditions. In designing such studies, careful consideration must be given to the diet of the animals. Most commercial grain-based foods for rodents contain high levels of aluminum, so a purified or semi-purified food should be used. The diet should contain no more than the National Research Council recommended amounts of trace metals, as these have been shown to influence the uptake of aluminum. Consideration must also be given to the fact that rodents drink while they eat and that aluminum leads to enhanced food motivation. Topics discussed following Dr. Golub's presentation included possible additions to the EPA standard end-points, the distribution of pathological changes in the nervous system of rodents, and the possible effect of purified diets on the longevity of the animals.

Dr. Judie Walton (Australian Institute for Biomedical Research) prefaced her presentation by describing the cellular changes associated with aging. Changes in the cells on the surface of bone, in the kidney glomeruli, and in the lining of the gastrointestinal tract could affect the absorption and excretion of aluminum. This is in keeping with the results of preliminary studies in rats that showed that, after oral dosing with aluminum, plasma aluminum levels peaked later in older than in younger animals. Dr. Walton went on to discuss the factors that should be taken into account when choosing an animal model for lifetime exposure, such as length of life, ease of maintenance, amenability to

testing, eating and drinking behaviour, and the availability of inbred strains susceptible to the formation of plaques and tangles in the brain. Mice that have been genetically altered to produce amyloid might be used. Dr. Walton also described her experience with the use of ^{26}Al in kinetic studies and the need for such studies prior to studies of neurotoxicity. Following Dr. Walton's presentation, there was further discussion of the ages at which animals should be studied and the possibility of using tissue cultures. Dr. Walton commented that tissue cultures might suffice for the study of simple effects, but normal brain cells cannot be maintained in culture.

Dr. Donald McLachlan (University of Toronto) began his presentation by stressing the public health importance of the relationship between aluminum in drinking water and Alzheimer's disease. Based on the results of epidemiological studies in Ontario, it has been estimated that the consumption of drinking water containing aluminum at concentrations in excess of 100 µg/L accounts for 16 000 cases of Alzheimer's disease per year. However, despite strong evidence from epidemiological and other studies implicating aluminum, an animal study is desirable to clinch the issue. There are strong arguments for using genetically susceptible animals, and Dr. McLachlan proposed the use of three sets of mice: wild type, transgenic mice with presenilin-1, and transgenic mice with both presenilin-1 and the human amyloid mutation. These should be tested at four aluminum concentrations — 0, 250, 500, and 1000 µg/L — and sacrificed at 8 and 16 months. Discussion of Dr. McLachlan's paper included the availability of genetically altered mice, the areas of the brain to be examined, and the possible use of radiochemistry to overcome problems of background noise. Dr. Golub commented that inbred strains might not be typical for behavioural testing and that C57 mice would be preferable, as their response is known.

Dr. Robert Yokel (University of Kentucky) reviewed the data on the absorption of aluminum and the factors that influence it. Studies suggest that about 1% of orally administered aluminum is absorbed. The doses used in animal studies of the absorption of natural aluminum have been 1000 times greater than the daily human dose of aluminum in water, but the use of ^{26}Al allows much smaller doses to be studied. It has been shown that food inhibits the absorption of aluminum, but the effect varies with the fibre content of the food and the consequent faecal recycling that takes place. Factors that increase the absorption of aluminum are the solubility of the aluminum compound used, lower gastric pH, carboxylic acid concentration, and the presence of uraemia. Silicon, fluoride, and calcium probably decrease absorption. Most of the absorbed aluminum is stored in the skeleton and the liver, the concentration in the brain being much lower. Aluminum concentration in the tissues has been reported to increase with age, with a longer half-life of elimination. However, there is evidence from isotope studies that aluminum may be permanently retained in the brain. A pharmacokinetic study should be run concurrently with any neurotoxicity study, preferably using ^{26}Al in the aluminum species representative of human drinking water. Dr. Yokel's presentation was followed by a general discussion of the availability of aluminum compounds, the effects of water treatment and food additives, and the cost of isotope studies.

Dr. Kenneth Bailey (United Kingdom Water Research Centre) suggested that animal studies leave much to be desired and that it may be better to rely on the data from observational studies in humans (e.g. the studies of dialysis patients). If an animal study is undertaken, then detailed consideration of the type of diet, the aluminum compound to be used, and the end-points to be measured is required. Drinking water is presumably the appropriate route of administration, but a pilot study using gavage should be used to establish the dose range.

Dr. Trond Flaten (Norwegian University of Science and Technology) began his presentation by emphasizing that the possibility that aluminum causes Alzheimer's disease is the main concern. It has not been possible, from the end-points used in epidemiological and occupational studies, to derive a guideline for aluminum in drinking water. More sensitive end-points are required, including early cognitive impairment and other neurological effects. Because of the problems of the speciation of aluminum in drinking water and the many substances that affect it, more than one species of aluminum must be used in a study in animals, at least in the pilot stage. Drinking water is a "soup" of organic ligands. The rabbit would be a suitable animal to study because of its susceptibility to aluminum and the fact that it demonstrates neurofibrillary pathology. If a general neurotoxicity study is mounted, then biomedical sub-studies should be included in the design. Dr. Flaten's presentation stimulated further discussion of the question of which aluminum compound to use and the need to focus the study on the problem of Alzheimer's disease.

Dr. William Forbes (University of Ottawa) emphasized the multifactorial nature of the problem of measuring the neurotoxicity of aluminum in drinking water, owing to the many species of aluminum present and the effect of pH and other elements, such as silica, fluoride, and possibly iron and calcium. Dr. Forbes illustrated this problem by presenting a multifactorial analysis of the data from an epidemiological study in Ontario. Another important feature of the analysis was the presence of a J-shaped relationship between the risk of dementia and the concentration of aluminum. This must be taken into account in designing animal studies, which generally assume a linear dose–response relationship. The complexity of the problem of aluminum speciation in drinking water is such that, at least in the short term, any guideline must be based on the total amount of aluminum present. In the ensuing discussion, the wisdom of this approach was questioned, as much of the aluminum that passes the filter is particulate.

Dr. Michael Gardner (United Kingdom Water Research Centre) presented a detailed analysis of the problem of aluminum speciation in drinking water and its implication for the design of toxicity studies. The dissolved fraction of aluminum in water is often an insignificant proportion of the total aluminum present, and this proportion varies considerably with pH. Within the dissolved phase, aluminum takes several forms: free aluminum ions, ions complexed by inorganic ligands, and ions complexed by organic ligands. The states of equilibrium between these species can be calculated if the equilibrium constants are known, but they are not known for the organic complexes. The rates at which the theoretical equilibria are reached vary between species, and there may be loss of dissolved aluminum by precipitation during storage. The aging of precipitated solids is also important,

as precipitated minerals less readily redissolve with time. Dr. Gardner concluded that the aluminum used in toxicity tests should be in a form similar to that present in drinking water, which should be determined from a range of waters using standardized techniques. The speciation in the water used should be monitored throughout the study. Dr. Gardner's presentation stimulated a lengthy debate about the type of aluminum to be used in the study, in particular whether it is more important to "mimic" the mixture of forms actually present in drinking water or to use a single compound, the bioavailability of which could be more easily standardized.

Discussion and Recommendations

On the second day of the Workshop, the following issues arising from the papers were debated:

1. Is an animal study needed, or should reliance be placed on further epidemiological studies?

2. Should the focus of such a study be directed to the relationship between aluminum and dementia, or should it encompass the general neurotoxicity of aluminum, including effects on neural development?

3. What is the most appropriate species of animal to study: mice, rats, rabbits, transgenic mice?

4. Which aluminum compound should be used: inorganic (sulphate, chloride); organic (citrate, lactate, maltolate)?

5. How should the aluminum be administered: drinking water, gavage, intracisternally?

6. What range of doses should be used, and at how many levels?

7. At what ages should the animals be exposed: young, old, lifetime?

8. Which end-points should be studied: behaviour, histology, tissue levels?

9. Should other constituents of drinking water be incorporated: silicate, fluoride, pH?

10. How should aluminum in the diet and the environment be controlled?

11. Should kinetic studies be incorporated, perhaps using ^{26}Al?

A consensus was obtained on all these questions, except that it was not possible to agree on the use of a single species of animal. Arguments were made for the use of mice, rabbits, and transgenic mice carrying risk factors for Alzheimer's disease, if sufficient funding can be obtained. However, there was a consensus that if funding is restricted, then the species should be given priority in the order listed.

The following recommendations were made:

- A study of the neurotoxicity of aluminum in the drinking water of animals would provide agencies with useful information for developing guidelines for human exposure.

- The study should focus on the consequences of life span exposure on neurotoxicity, assessed in young and aged adults. Exposure should begin *in utero* (at implantation) and continue throughout life.

- The appropriate species of animal for the study are (in order of priority) mouse and rabbit. The transgenic mouse carrying risk factors for Alzheimer's disease and premature aging should also be considered.

- Aluminum should be administered in drinking water after a pilot study to determine the maximum tolerated dose. Subsequently, a control dose, the maximum tolerated dose, and two intermediate doses should be used. Based on epidemiological studies, one dose level to be used might be that equivalent to 200 ppb in human drinking water.

- Although the use of an inorganic compound such as aluminum sulphate would be desirable, solubility considerations, stability in solution, and ability to dissociate in the stomach of the animals will probably require the use of an organic compound, such as aluminum maltolate. The solution should be made with deionized water and then filtered through a 0.22-µm filter. To simulate patterns of human consumption, some of the water should also be given in the absence of food.

- Purified diet (National Research Council recommended nutrient composition) with less than 7 mg Al/kg food should be given to mothers (beginning 2 weeks before mating) and their offspring.

- The end-points employed by the US EPA neurotoxicity testing guidelines should be used for behavioural testing, with the addition of delayed alternation and multiple fixed interval–fixed ratio performance. Behavioural tests should be conducted beginning at age 50 days and 18 months (in mice). Behavioural tests should be those that are not confounded by repeated experimentation. Brain histopathology examination of at least three coronal sections of fore-, mid-, and hind-brain should be taken, using appropriate stains, such as H&E, Bielschowsky, and immunohistological staining for amyloid and phosphorylated tau. Biochemical assessment of aluminum, beta amyloid, and a cholinergic marker should be carried out. Blood specimens should be obtained for biochemical and haematological studies.

- Sufficient dams should be treated to provide 20 animals per sex per group (1 male and 1 female per litter) for functional neurotoxicity testing in young and aged adults. The pathology/chemistry assessment at the end of the study should be performed on those animals undergoing behavioural assessment. An additional 5 animals per sex per group (1 male and 1 female per litter) should be used for additional pathology/chemistry assessment at the same age at which the first behavioural testing is concluded.

- Kinetic studies using ^{26}A should be included to determine if aluminum from drinking water accumulates in the brain.

- Proper control of contamination with aluminum is essential and should be demonstrated. The study should be conducted under Good Laboratory Practice conditions.

- A lifetime exposure study of rabbits is recommended to take advantage of their sensitivity to aluminum-induced encephalopathy and the related neuropathological features seen in this species. The protocol suggested for the mouse neurotoxicity studies could be modified for this species, which has a life expectancy of approximately 8 years. Modified behavioural testing using the classically conditioned defensive eye blink reflex should be considered.

APPENDIX 1: Chairman's Introduction
Dr. B. Thomas

Welcome to Ottawa. We have scientists here from Australia, the United Kingdom, Norway, and the United States of America. I want at the outset to acknowledge the considerable financial contribution to this Workshop that has been provided by the United States Environmental Protection Agency (EPA). Without their help, I am sure that this event would not have occurred. Dr. Amal Mahfouz is representing the U.S. EPA.

I want to give you some history that will show you how we came to decide that a meeting of experts was needed on the issue of aluminum in drinking water.

Drinking water quality in Canada is largely the responsibility of provincial and territorial governments and local municipalities. However, the federal government, through Health Canada, publishes a set of national guidelines called the *Guidelines for Canadian Drinking Water Quality*, which are used by all jurisdictions as the basis for ensuring the safety of drinking water. Health Canada conducts the risk assessments, does some national surveys of water quality, and conducts research. The final value for the guideline includes risk management, which is largely provided by the provinces. The decision on the guideline is made by the Federal–Provincial Subcommittee on Drinking Water, whose recommendation is sent for final approval to a more senior committee, called the Federal–Provincial Committee on Environmental and Occupational Health.

Currently, aluminum is not listed in the Canadian guidelines. Some years ago, Health Canada did a risk assessment of aluminum and recommended an aesthetic value similar to the existing World Health Organization (WHO) guideline. However, the Federal–Provincial Subcommittee rejected the recommendation on two grounds. First, the aesthetic value had no scientific basis — the only citation was the WHO guideline, which also lacked a significant body of science in support of the guideline. Representatives from the Prairie provinces pointed out that they had aluminum concentrations in their finished water as high as 1000 µg/L with no aesthetic problems. These water supplies have a high pH, which keeps the aluminum in solution. The second problem was that the risk assessment did identify a body of literature describing aluminum as a neurotoxin, even though the conclusion was that there was insufficient evidence to set a health-based guideline. The provinces asked Health Canada to see if they could develop a guideline that addressed the health concern. In 1996, a guideline was developed that used a series of animal studies to set a tolerable daily intake (TDI) and a guideline. The review did note that all of the animal studies were weak but that several of them gave similar TDIs, even though the toxic end-points were different. The weakness of these studies led the

Federal–Provincial Subcommittee to again reject the proposed guideline of 100 µg/L. It was reasoned that the high cost of implementing such a guideline would have to be based on strong science to make it justifiable. There was also fear that some small towns would merely discontinue coagulation and filtration rather than face the cost of introducing a more sophisticated system. This would obviously increase health risks rather than reduce them. The Subcommittee has agreed to the development of a "free" or dissolved aluminum guideline based on achievable treatment technology, which will not have the force of a health-based guideline but will show the need to keep aluminum residuals as low as practicable. The proposed guideline is now out for public comment until 31 December 1997, and copies of the proposal are available for anyone who is interested. The WHO has also taken a similar decision based on the Canadian experience. The numerical value is under discussion, but it will probably be in the 100–200 µg/L range.

There have been many workshops and reviews on aluminum toxicity in drinking water, with much emphasis being placed on the question of whether it affects the incidence of Alzheimer's disease (AD). Our position has always been that aluminum is a neurotoxin; if it gets into the central nervous system, it may have much wider effects than just AD. Nevertheless, all these meetings and reviews have always come to much the same conclusion — inadequate data to reach any conclusions, either positive or negative. We agree that both the animal toxicity data and the human epidemiological data are weak and prevent us from reaching any firm conclusions on a causative effect or a TDI. This meeting therefore is not to again review all the existing data, but to see if progress can be made by conducting an appropriately designed study. We have decided that it is unlikely that a human epidemiological study could provide the answers we need at this time, so this will not be the main focus. Obviously, a better epidemiological study with well-characterized exposure measurements and disease status would be useful, but it would still leave many unanswered questions.

We are therefore asking this group of international experts to give advice on what type of study would be the most likely to give us useful information on the potential for aluminum in drinking water to cause neurotoxicity. We have invited chemists who are experts on aluminum speciation because of the growing body of evidence that this may influence the bioavailability of aluminum. It is not intended that the discussion delve into the details of exactly how the study would be conducted, such as designing the actual protocol. Protocol design would come later if a study is seen as feasible and useful.

If some consensus and recommendations come out of our deliberations, the next challenge will be to see how such a study can be conducted. Like many governments, we are undergoing major budget cuts that preclude even a modest Health Canada study. Our present thinking is that a joint

effort may be required, but first let us see if there is any agreement on what would be a useful study. Some basic questions that will need to be addressed include the following:

1. Animal model — species?

2. Duration of the study — subchronic or chronic?

3. Dosage form — speciation of aluminum in the drinking water; aluminum content and form in the food?

4. End-point(s) — neurotoxic indicators?

APPENDIX 2: Aluminum in Drinking Water

Dr. A. Mahfouz

The 1996 *Food Quality Protection Act* and the 1996 *Safe Drinking Water Act* require the regulation of contaminants in food and water at levels that are protective of children. Other potentially sensitive populations, such as the elderly and immunologically impaired, are also of special concern because of their enhanced susceptibilities to some of these contaminants.

The U.S. Environmental Protection Agency is required under the 1996 *Safe Drinking Water Act* to develop the first list of drinking water priority contaminants and to publish its final list in February 1998. The Office of Water has been working on this list during the last year and intends to publish a Federal Register Notice with the proposed list in the next few weeks for public comments. Aluminum (Al) is one of the chemicals included on this list. Based on the anticipated public comments on the Federal Register Notice, contaminants on this list will be prioritized for regulation. Criteria are developed for this selection, including criteria for both toxicity and exposure. Data gaps may exist that need to be filled in order to characterize the hazard and dose–response for some of these contaminants.

Al is one of the contaminants that is currently regulated based on its aesthetic properties in drinking water at a range of 50–200 µg/L. There is rising public concern about the health effects of Al and a substantial body of literature pointing in this direction. The issue of developing a Health Advisory value based on health instead of aesthetics has been raised. However, the available toxicity studies in animals are usually performed at concentrations that exceed the level of concern with respect to aesthetic properties. Also, the epidemiological studies do not clearly indicate levels of concern that exceed the aesthetic range. While both the present animal data and epidemiological data point out the clear association between Al and neurotoxic effects and Alzheimer's-like response, a clear dose–response is not well defined owing to the interference of other confounding factors from water or food, or both.

It is well known that Al toxicity is influenced by many factors (e.g. pH, organic matter, temperature, other metals, etc.) and varies with the source water used for drinking water, diet of exposed populations, metabolism, and genetics. It is important to eliminate as much as possible the impact of these confounding factors in developing a protocol for a new study on Al.

It is therefore crucial to take into consideration all these issues in designing a protocol for a study that can characterize Al risk to exposed populations, especially children and other sensitive populations.

This is a challenging task, and it may not be possible to address all of these issues in one study. A systematic approach may be needed to design a series of studies on developmental effects using at least two animal species (including the rabbit, with its known susceptibility to Al), a chronic study that may include a prenatal exposure, and an epidemiological study that would include exposed children and the elderly population.

The animal studies need to improvise for a no-observed-adverse-effect level (NOAEL) and a lowest-observed-adverse-effect level (LOAEL). The available literature would be very helpful in selecting these levels. Harnessing confounding factors in these studies is also a challenge, and these factors need to be well defined in the design of the protocol of any new study.

Finally, multimedia exposure to Al is a fact, and the issue of relative source contribution will be an issue that needs to be improvised for in this series of investigations.

APPENDIX 3: Aluminum Neurotoxicity

Dr. D. Rice

The issue driving public (and government?) concern in relation to aluminum (Al)-induced neurotoxicity is the possible link between Al intake and the development of Alzheimer's disease (AD). However, Al also produces severe neurotoxicity in humans as a consequence of dialysis or administration of fluid parenterally. In addition, there is a reasonable-sized animal literature demonstrating behavioural and neurochemical changes following perinatal exposure. When considering a standard for Al in drinking water, it must be recognized that the mandate is to protect against even subtle adverse consequences of lifetime exposure, including prenatal through old age. This has important implications for choice of experimental model, developmental period and duration of exposure, and neurotoxic end-points examined.

It may be instructive while exploring these issues with regard to Al to keep in mind the development of our understanding of the health consequences of ubiquitous exposure to lead, another neurotoxic metal made bioavailable by anthropogenic activity. It was recognized in antiquity that workers engaged in lead mining or smelting developed severe peripheral neuropathy, as well as changes in affect and emotional behaviour. More than a century ago, it was realized that children exposed to high levels of lead developed encephalopathy, a sequela of which was mental retardation in some cases. Over the last 20 years, intense research in children and animal models has characterized the types of cognitive impairment produced by body burdens of lead once regarded as "normal." Recent analyses have estimated the cost to the (U.S.) economy for every 1 µg/dL increase in blood lead levels in children in the billions of U.S. dollars per year, mostly as a result of decreased earning power (lost wages). There are at least three important principles to be extracted from our experience with lead: (1) neurotoxic effects may be different in adults and children, (2) effects may be different at high and low exposure levels, and (3) even very subtle deficits can have an enormous cost to society. Currently, attention is focused on the possibility that lifetime or developmental exposure to lead (or other neurotoxic agents) accelerates the aging process or contributes to diseases of old age.

Issues

1. Are we only concerned with AD? This presents a significant problem, since there is really no satisfactory animal model. It would be better to concentrate efforts on further epidemiological studies. However, to restrict the focus to AD is not a sufficient response to the issue.

2. What period and fraction of the life span are we concerned about? Developmental only, longitudinal, old age? There are legitimate arguments for each of these assessments.

3. What species? If we are only concerned about neurofibrillary tangles, rabbit or a transgenic mouse model is appropriate. If our concerns are broader (as I believe they should be), then a rodent model should be used, as there are few (or no) methods developed for sophisticated cognitive assessment in rabbits.

4. What end-points? If tangles are our only concern, neuropathology/neurochemistry is probably sufficient. However, if we are interested in possible cognitive/learning/memory deficits, then these must be studied explicitly. To my knowledge, end-points looked at to date in animal models are of the "screening" variety, with no careful assessment of cognitive function. How serious are we about characterizing Al neurotoxicity? Again, to draw a lesson from lead, it was "common knowledge" that the rat was not sensitive to developmental lead-induced behavioural impairment until careful assessment using sophisticated behavioural techniques by Dr. Deborah Cory-Slechta demonstrated clear reproducible impairment at blood lead levels relevant to environmentally exposed children. As the behavioural methodology moved from "screening" procedures or simple learning paradigms to detailed assessment of cognitive function, the body burden at which effects were detected in rodents decreased by orders of magnitude. If we are convinced that Al is neurotoxic, then the research strategy should employ methods suitable for hazard characterization rather than hazard identification.

Recommendations

1. Developmental study with continuing exposure throughout life using a protocol approximating the U.S. Environmental Protection Agency developmental testing guidelines.

2. Extend end-points beyond hazard identification paradigms to include targeted neurochemistry, based on our knowledge of the end-points affected by Al, as well as sophisticated assessment of learning and memory functions.

APPENDIX 4: Animal Models for Studies of Neurotoxicity Induced by the Presence of Aluminum in Drinking Water

Dr. J. Savory & Dr. J Rao

Introduction

Epidemiological studies have indicated that aluminum (Al) present in drinking water may be a risk factor for neurodegenerative disorders in humans. The first report from Martyn *et al*. (1989) indicated a significant increase in the risk of Alzheimer's disease (AD) in districts where mean water Al concentration was greater than 111 µg/L. After this initial study, a number of reports appeared with supportive data also indicating that Al in water was a risk factor (Flaten, 1990; Forbes *et al.*, 1991, 1994; Neri and Hewitt, 1991; Forbes and McAiney, 1992; McLachlan *et al*., 1996; Martyn *et al.*, 1997). Two reports from Britain and Europe (Wood *et al*., 1988; Wettstein *et al.*, 1991) failed to detect the risk relation between Al in water and AD. A more recent study from Martyn *et al.* (1997) indicated that Al in water at a concentration below 0.2 mg/L was not a risk factor for AD. Al salts are added as coagulants in water treatment plants, and the Al concentration in such treated water is between 1 and 5 mg/L. The World Health Organization (WHO) has recommended that Al concentrations in drinking water not exceed 0.2 mg/L, while the U.S. Environmental Protection Agency (EPA) in 1985 set 0.05 mg/L as a limit. The European Economic Community (EEC) has established a recommended guideline of 0.05 mg/L and a maximum permissible level of 0.2 mg/L. Further studies showed that fluoride and silicon (Si) concentrations in water may have a protective role against Al toxicity. However, Martyn *et al.* (1997) found no supportive data for a protective role of Si. Al speciation plays an important role in free Al concentration in water. At neutral pH, free Al is very low, being in the micromolar range. The major problem of the above epidemiological studies is the methodology of ascertaining dementia and extrapolating Al concentrations as risk factors. However, the role of higher concentrations of Al as a risk factor for AD cannot be ignored. To arrive at a definite conclusion about the relative risk concentration of Al in drinking water and its relationship to AD will require an evaluation of Al neurotoxicity in experimental animals.

AD is a complex neurodegenerative disorder for which no single pathogenetic mechanism has yet been established, but neuronal cell loss is an important feature of the disease. There is no suitable animal model with which AD neuropathological symptoms can be formulated. Recently, we have developed Al maltolate-treated aged rabbit as an animal model for AD. The hallmark neuropathological features of AD are the formation of neurofibrillary aggregates termed neurofibrillary tangles and neuritic plaques, and recent reports indicate the presence of oxidative stress and apoptosis. These pathological lesions are predominantly observed in cortex temporalis, subiculum, pyramidial neurons in hippocampus, and other regions of the AD brain (Nucleus lateralis dorsalis thalami; Nucleus nerve oculomotorii; Nucleus ruber). In our animal model, we have observed

neurofibrillary tangles, oxidative stress, and apoptosis distributed in the above regions characteristic of their location in AD. We have further shown direct evidence that oxidative stress is directly linked to apoptosis by co-localization studies. We have also observed that neurofibrillary tangles co-localize with evidence of apoptosis in neurons in the same pattern as is seen in the brains of patients with AD. To our knowledge, this is the first animal model showing significant similarities to AD neuropathology.

Study design

1. Choice of Al compound

The preparation of Al solutions from inorganic Al salts at neutral pH is very difficult due to problems of precipitation. At neutral pH, the concentration of *free Al* ($Al(H_2O)_6$) is very low when inorganic Al salts are used for toxicity studies. Further, the addition of Al salts to water reduces the water pH to an acid pH. To overcome these problems, we have for the past decade used Al maltolate, which is a complex that will not undergo hydrolysis from pH 2.0 to pH 9.0. Maltolate enhances the free Al concentration at neutral pH and is non-toxic; hence, there is no synergistic toxicity reaction between Al and maltolate. Thus, Al maltolate is highly suitable for studies of drinking water Al toxicity.

2. Animal model

There have been some studies on animal model systems such as rats and monkeys, but their neuropathological characteristics do not mimic AD pathological lesions. Studies have shown that rabbits are very sensitive to developing Al toxicity. A few groups have studied Al neurotoxicity using young rabbits, and in general their studies have been centred around neuropathological lesions within medulla and spinal cord regions only. Recently, we have studied Al toxicity by the intracisternal administration of Al in aged rabbits (around 5 years old); by sectioning brain coronally from the frontal pole to occipital pole, we observed the presence of neurofilamentous aggregates in hippocampus, subiculum, cortex, and motor regions in these brains and oxidative stress and apoptosis in hippocampus and cortex regions. These brain regions are significantly involved in AD neuropathology. This aged rabbit animal model reflects AD in both the neuropathological changes and, to some extent, behavioural abnormalities. Based on these findings, we feel that the aged rabbit animal model system is increasingly relevant in understanding the mechanism of neurodegeneration of AD, especially since the neurofibrillary tangles in humans and rabbits have been shown to share a number of morphological, neurochemical, and immunohistochemical characteristics (Savory *et al.*, 1995, 1996; Huang *et al.*, 1997). Recent studies have shown that rabbits are highly relevant for understanding human disorders, as they resemble primates more closely than do rodents (Graur *et al.*, 1996).

3. Precautions to avoid external Al contamination while feeding experimental animals

Al is present ubiquitously in nature; hence, contamination is extremely difficult to avoid. In order to overcome such problems, the following measures need to be taken: Animals must be maintained as much as possible in a dust-free environment. Al solutions need to be prepared under laminar flow hoods and to be stored in plastic vials. All plastic ware needs to be selected and cleaned according to the guidelines of Moody and Lindstrom (1977) and stored in a laminar flow hood. The use of talc-free rubber surgical gloves pretreated with 2% quartz distilled nitric acid and washed thoroughly with ultra-pure distilled water (18 M ohm cm) is recommended.

4. Toxicity end-points to be measured in the animal model system

Since we need to correlate the relation between water Al and Alzheimer's condition, we need to choose end-points that are observed in AD brains rather than U.S. EPA standard set guidelines for neurotoxicity studies. Based on recent findings on AD, AD markers could include:

a) neurofilamentous aggregate formation and immunoreactivity for paired helical filament features, hyperphosphorylation, abnormal tau, ubiquitin, Aβ, and alpha-1-antichymotrypsin;
b) oxidative stress and apoptosis and their co-localization studies;
c) CSF tau levels;
d) serum p97 levels; and
e) concentrations of Al, iron, copper, zinc, manganese, and cobalt (stimulants of oxidative stress) in brain regions involved in AD.

Neurotransmitter distribution needs to be included in the study design. Microdialysis studies to confirm Al transport from serum to brain and behavioural studies with reference to motor neuron function, memory, and feeding behaviour also need to be included in the studies.

5. Concentration of Al in water

In view of the recent report from Martyn *et al.* (1997) and based on WHO, U.S. EPA, and EEC set limits, water Al concentrations for neurotoxicological studies can be identified as 0.05 mg/L, 0.2 mg/L, 1 mg/L, and 5 mg/L.

6. Time course

The study needs to be conducted at different time intervals to identify the toxicity time risk phase. The study could be for 4 and 8 weeks as subchronic periods and for 3, 6, 12, and 24 months as chronic periods.

7. Statistical analysis

Analysis may involve correlation coefficients, GLM test, to understand cross-correlations and time, Al concentrations, brain regions, and interaction phases.

8. Storage of rabbit feed and water

The rabbit feed and water should be stored in a laminar flow dust-free chamber in plastic ware.

9. Precautions in brain dissection and blood collection

Blood from rabbits must be collected in plastic vials, perhaps through Teflon tubing. The brain dissection should be performed on a polyethylene plate with stainless steel dissection tools coated with titanium nitrite and immediately frozen at -80°C.

Summary

We have offered some guidelines for the selection of an animal model system and for protocols for investigating chronic neurotoxicity, particularly Alzheimer's-like characteristics, from Al in drinking water. These guidelines are specific to relate water Al levels to possible AD condition.

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APPENDIX 5: Neurotoxicity Testing Guidelines Should Be Used to Conduct a Study that Will Serve as the Basis for an Aluminum Drinking Water Standard

Dr. M. Golub & Dr. C. Keen

What are the appropriate end-points to be measured?

This paper is primarily responsive to question 4 in the list of important issues for the Workshop. Two distinct approaches to aluminum (Al) neurotoxicology research can be identified:

i. establish an animal model for human clinical syndromes and work towards understanding their mechanism; and
ii. screen Al for its neurotoxic properties in a standard format used for any suspect neurotoxicant.

The former approach is typical of biomedical research, while the latter is more typical of regulatory toxicology.

Perhaps the bulk of Al neurotoxicity research performed to date has been directed by the former (biomedical) approach. A predominant theme has been Alzheimer's disease (AD), but there has also been work related to dialysis encephalopathy and, more recently, Al loading from parenteral fluids.

Our own work took the latter (toxicity testing) approach. In examining potential neurotoxic properties of Al, we used a neurotoxicity screening battery for which data had been developed for a number of known neurotoxicants. After identifying sensitive end-points, we then explored such issues as age sensitivity, gender differences, subchronic versus chronic exposure, dose dependence, and reversibility. Most of our work was conducted in mice and used dietary exposure to Al lactate.

Table 1 outlines some of the neurobehavioural effects that have been demonstrated using dietary administration of Al lactate. Many of our studies have demonstrated effects on these end-points using two doses and found dose-dependent effects. Recently, we have completed a seven-dose study, which also demonstrated dose-dependent effects on some variables. Many studies measured brain Al at the end of the dosing period. Table 1 also shows the end-points specified in current U.S. Environmental Protection Agency (EPA) neurotoxicity testing guidelines. *These results can be taken to indicate that Al affects the same standard end-points as other neurotoxicants*.

At the time we began our work, guidelines for neurotoxicity testing (with the exception of organophosphate pesticides) had not yet emerged. There is now a standard set of guidelines adopted by the U.S. EPA, currently being considered for adoption by the Organisation for Economic Co-

operation and Development (OECD). Guidelines for risk assessment of data produced under these testing guidelines are currently in final draft form.

> *It is the position of this paper that, for the purposes of regulatory standard development, an Al neurotoxicity study and developmental neurotoxicity study should be conducted under Good Laboratory Practice conditions using currently accepted international guidelines and drinking water administration.*

The results of the study could then be used for regulatory purposes by any concerned agency.

There are two sets of guidelines, one for a neurotoxicity screening battery to be used in conjunction with a chronic toxicity study, and one for a neurodevelopmental toxicity study. Ideally, both studies should be conducted. Outlines are presented in Table 2.

Advantages and disadvantages

There are both advantages and disadvantages to this approach.

Disadvantages:

i. It does not utilize what we know from biomedical research.
ii. It does not directly address the problem of AD and will not resolve issues in this area.
iii. It will not address the issue of selective cognitive abnormalities seen in occupational and clinical populations.

Advantages:

i. The resulting findings can be readily utilized by government agencies for reference dose (RfD) development.
ii. The validity of such a study will be difficult for industry to dispute.
iii. The study will be performed in an impartial setting under quality control auditing.
iv. The results can be interpreted in the framework of similar studies with widely recognized neurotoxicants, such as lead, mercury, hexane, and acrylamide.

Designing a sensitive study

In order for such a study to be successful, it will have to include a set of design considerations based on previous research that will help ensure its sensitivity and guard against a false-negative result. Many of the design elements (e.g. the number of doses and the duration of dosing) are determined by the testing guidelines. However, a thorough review of the available literature may indicate the most sensitive choices for other parameters, such as form of Al used, the animal model, and the diet. Also important are the parameters of behavioural testing. For example,

spontaneous motor activity is a commonly used end-point. We may know that Al has been shown to influence spontaneous behaviour recorded in the home cage over a 24-hour period, but not spontaneous activity recorded for 1 hour in a novel environment. Thus, the former, rather than the latter, situation should be used in the experiment.

The following are considerations that are critical in designing a sensitive study:

1. Diet
2. Form of Al
3. Use of food motivation

1. Diet

It is essential to consider the daily intake of Al in terms not only of the drinking water component but also of the food component. In order to identify a safe exposure level in drinking water appropriate for humans, the diet used in the study should have an Al concentration similar to that in human diets. Most grain-based commercial rodent chows have an Al content between 100 and 300 µg/g. They are not suitable for use in a study using Al dosing in drinking water.

Because rodents are prandial drinkers (i.e. they drink primarily while they eat), dietary components are as important as drinking water composition in determining Al bioavailability.

Several studies demonstrate that the uptake of Al is highly dependent on the trace metal content of the diet, particularly calcium and magnesium. Commercial rodent chows have high and variable amounts of minerals and essential trace elements and Al; thus, they should not be used. The diet should contain *no more than* the National Research Council recommended amounts of minerals and trace elements using the 1993 AIN guidelines. A commercially available purified or semi-purified diet is the best choice.

The commonly used commercial rodent diets are based on whole grains with other constituents added. Whole grains contain a variety of ligands that interfere with metal absorption. Since human diets are *not* primarily whole grains, a grain-based diet is inappropriate for the study. This is a further argument for using a purified or semi-purified diet.

Human diets contain a number of *organic acids* (lactate, citrate, etc.) that are not commonly found in commercial rodent chows. Since these agents promote Al uptake, they should be included in the diet used for the study. Proportions can be based on those found in human diets.

2. Form of Al

The major choices are Al lactate, Al nitrate, Al sulphate, Al citrate, and Al chloride. Al hydroxide is insoluble, and its use in drinking water treatment is no reason to support its use in a toxicity study. Dr. Yokel, Dr. Jose Domingo, and others have compared the bioavailability of these compounds. At least as far as acute toxicity goes, toxicity is correlated with bioavailability. Very few studies have compared the neurobehavioural toxicities of different forms of Al. Because of its powerful chelating effects, we, and others, have found that added citrate alters tissue concentrations of a number of essential trace elements and thus may not be best choice. Nitrate may be considered to have some innate toxicity. The solubility of Al chloride and Al hydroxide may limit doses that can be used. Thus, Al lactate or Al sulphate may be the best choices. Al sulphate has the advantage of previously having been shown to influence a behavioural variable in a drinking water study.

3. Reinforcers for learning/memory tasks

Following up on earlier results, we have recently reported data suggesting that Al uptake leads to enhanced food motivation. The origin of this effect is not known but may be related to findings by others that Al administration sometimes leads to transient increases in food intake and weight gain. There are literally no demonstrations of adverse effects of Al on food-motivated tasks. On the other hand, there are a number of studies reporting effects of Al on shock-motivated tasks and on classical conditioning using aversive reinforcers. Thus, food-motivated tasks should not be used for neurotoxicity testing.

Conclusion

In conclusion, a rigorous and sensitive study of dose-dependent Al neurotoxicity can be designed based on U.S. EPA regulatory testing guidelines. Such a study would receive wide acceptance as the basis for establishing a drinking water reference dose.

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[1] These documents can be downloaded from the U.S. EPA website htttp://www.epa.gov/.

Table 1 ***End-points that are designated for use in the U.S. EPA neurotoxicity testing guidelines and that have been shown to be influenced by oral Al administration in rodents. End-points from both the adult and developmental test guidelines are included.***

End-points shown to be influenced by oral Al administration in repeated-dose studies	End-points designated for use in the U.S. EPA neurotoxicity testing guidelines
grip strength	grip strength
landing foot splay	landing foot splay
thermal sensitivity	sensorimotor response
motor activity	motor activity
righting	righting
auditory startle	auditory startle
shuttle box learning and memory	learning and memory
passive avoidance extinction	

Table 2 ***Outline of U.S. EPA neurotoxicity testing guidelines***

A. Neurotoxicity screening battery	
Species	Rat
Age	>42 days
Sex	male and female
Group size	10/sex; 5/sex for neuropath
Controls	concurrent vehicle positive control for neurotox testing activity testing CNS/PNS pathology
Doses	control + 3; minimally toxic dose “significant toxic or neurotoxic effects”; maximum dose <2g/kg subchronic, 1 g/kg chronic
Route	relevant to humans; practical; bioavailable
Duration	acute; subchronic (90 days); chronic (2 years)
Time of testing	subchronic: 4th, 8th, and 13th week chronic: every 3 months

End-points	FOB autonomic convulsions - tremors - reaction to handling arousal level - posture and gait observations - ranking of posture and gait - grip strength - landing foot splay - sensorimotor response - body weight - "toxic signs" fur, color, discharge, stereotype Additional rearing in the open field - righting - body temperature - vocalizations - respiration - sensorimotor response Motor activity Neuropathology: CNS/PNS brain, spinal cord, and peripheral nerve - stains, H&E, Silver, GFAP

B.	**Developmental neurotoxicity study**
Species	Rat
Age	young adult nulliparous breeders (*in utero* exposure)
Sex	Pregnant females (dosed) male and female offspring studied
Group size	20 litters: 1 male and 1 female per litter; litters culled to 8 on postnatal day 4
Assignment	1 male, 1 female/litter motor activity 1 male, 1 female/litter auditory startle 1 male, 1 female/litter learning and memory 1 male, 1 female/litter brain weight (n=10) and neuropath (n=6), postnatal day 11 1 male, 1 female/litter brain weight (n=10) and neuropath (n=6), end of study
Controls	concurrent vehicle/sham
Doses	control + 3 minimally toxic dose: some maternal toxicity, limited neonatal death or malformation low dose: no maternal or developmental neurotoxicity other doses: equally spaced
Dosing period	day 6 gestation through day 10 postnatal, excluding day of parturition
Route	oral
End-points	Maternal observation; Offspring observation; Developmental landmarks (vaginal opening, preputial separation); Motor activity (PND 13, 17, 21,60); Auditory startle (PND 22 and 60); Learning and memory (PND 21 and 60); Neuropathology

APPENDIX 6: Lifetime Exposure of an Animal Model to Aluminum in Drinking Water to Investigate Possible Chronic Neurotoxicity

Dr. J. Walton

The animal model

Cell and tissue structure and physiological function of organs are considerably different in the aged members of a mammalian species compared with its young members. Thus, to develop a faithful animal model for a chronic neurotoxic disease that primarily becomes apparent in elderly humans, the model should be based on older animals that have been continuously exposed over their life span to low levels of the neurotoxic agent.

To assess chronic neurotoxicity, a defined animal population can be longitudinally analysed for a variety of parameters at succeeding ages over its life span. There is ample opportunity to understand these animals on various levels. However, it is important to confine experimental variability so that there is only *one* control group and *one* experimental group. Otherwise, the study becomes too complicated and confusing. Additional variables can be separately examined in small preliminary experiments that can feed into the main study.

Important considerations in the choice of an appropriate animal model are: 1) length of life span; 2) ease of maintenance; 3) amenability to testing; 4) eating and drinking behaviour (e.g. does the animal usually drink only when it eats or also on an empty stomach?); 5) advantages and disadvantages of using inbred versus outbred strains; and 6) ability for plaques and/or tangles to form in brain tissue of the proposed model.

Approximate life spans of some potential animal models are: rats (3 years); mice (3 years); rabbits (5–6 years); cats (15–20 years), and dogs (10–18 years, depending on size).[2] For the proposed study, consideration should be given to using mice offered by Hsiao *et al.* (1996), which have been genetically altered to produce amyloid.

Importantly, aging laboratory animals require special care and handling with respect to their housing needs, nutrition, bedding, health care, and the laboratory environment. This is more a matter of know-how than of expense.

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[2] If date of birth is known (e.g. from pound records), large animals of older age could be recruited into either the main or preliminary studies. Disadvantages, however, are that the cat/dog pedigrees may widely diverge and their previous health histories will probably be unknown.

^{27}Al in water: pharmacological doses

Using graphite furnace atomic absorption spectrometry (GFAAS) to measure pharmacological dose levels of natural aluminum (^{27}Al) in young rats, we found that plasma Al peaked at approximately 1 hour after oral gavage and then steadily declined. In the urine, the peak occurred at about 2 hours post-gavage. Our preliminary studies in older rats indicated that, after oral dosing, absorption and excretion values were both slower to peak. Hence, blood and urinary Al values strongly depend upon the time when the specimens are taken relative to the time of oral dosing as well as upon the animals' age.

An Al dosage that is too high may produce acute toxicity and premature death, or it may be too high to have obvious human relevance. On the other hand, a dosage too low increases the risk that the study will find no effect.

Unfortunately, Al absorption does not have a strictly linear correlation with Al dosage. Thus, the amount of Al absorbed from a pharmacological dose is not extrapolatable to that absorbed from an ambient dose. Nor do absorption levels in one species necessarily apply to other species.

^{26}Al in drinking water: ambient dosage and brain uptake

^{26}Al can be used to determine absorption, excretion, and tissue uptake values for a known ambient dose of Al in the particular animal model to be used. To my knowledge, combined use of this isotope with accelerator mass spectrometry (AMS) is the only technique sufficiently sensitive to detect relocation of ambient levels of Al across biological barriers.

In our ^{26}Al experiments, rat brains were found to contain a mean fraction of 1×10^{-7} (i.e. 0.00 001%) of an oral aqueous dose of the isotope.

Kobayashi (1990) found that approximately 2×10^{-5} of an intraperitoneally injected bolus of ^{26}Al was taken up into the rat brain. Theoretically, one can subtract the brain Al fraction resulting after bolus injection from the brain Al fraction of the oral dose to estimate the absorbed fraction of the oral dose as 5×10^{-3} or 0.5%.

However, in actuality, blood Al levels are dynamic and more difficult to interpret. Uptake into tissues begins soon after Al ions cross the gastrointestinal tract lining into the bloodstream. Urinary Al content reflects how much Al has been taken up and removed by the kidneys. In order to evaluate how much Al is absorbed from a known oral dose, one would need to measure the residual dose fractions contained in all of the major organs after several days of exposure as well as urinary ^{26}Al content over this period of time. Even so, this would not account for some of the Al fraction returned to the gastrointestinal tract via the bile duct.

Hence, preliminary ^{26}Al experiments and/or analyses provide background to aid interpretation of results from the main chronic neurotoxicity study. It would be desirable 1) to investigate the pharmacokinetic dynamics of ^{26}Al blood and urine levels in the chosen model and how they change with age[3]; 2) to learn how age change affects the tissue distribution of ^{26}Al; and 3) to examine whether ^{26}Al uptake into the brain is reversible or irreversible.[4]

Longitudinal testing and measurable end-points for the main chronic neurotoxicity study

For some test parameters in the main study, Al-treated animals and controls need only be examined at a few ages. I recommend that these ages be equivalent to the human ages of 18, 45, 72, and 90 years (e.g. 6, 15, 24, and 30 months in rats/mice). Examples of these are tests indicative of general health, such as kidney function, bone density, and muscle tone. These tissues change with age and may directly or indirectly affect Al metabolism.

Other tests that are more dependent on neurological status and that depend on learning should be carried out at more regular intervals over the life span (Bilkei-Gorzo, 1993).[5] Examples of these are tests for olfactory function, gait performance and balance (involving bridge traverse), and hippocampal function. Hippocampal tests for short-term memory can be carried out using either a "T" maze or a "Y" maze; a Bilkei-Gorzo (1993) maze (composite of multiple T-mazes); or a radial arm maze. A Morris water maze is useful for assessing position awareness (also mediated by the hippocampus). However, swimming appears to be stressful to many animals, particularly those that are old and frail.

Frequent observations should also be made for the detection of behavioural abnormality. When an animal becomes apathetic, it should be treated to relieve depression prior to suspecting dementia.

After the elderly animals reach the end of their natural life span, various types of assessment can be made on their tissues. Brain and bone Al content can be measured with GFAAS/inductively coupled plasma atomic absorption spectrometry (ICP-AAS) or atomic emission spectrometry (AES).

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[3] For example, to monitor change over time in absorption and excretion abilities, ambient levels of water-derived ^{26}Al in the blood and urine of living animals could be measured by AMS in young adulthood, middle age, and late age.

[4] It should be noted that we already have prepared samples, now in storage, that could answer points 2 and 3 if funds were available for converting the tissue samples to Al oxide and making the AMS measurements.

[5] Testing should be sufficiently frequent to detect whether poor performance is of a temporary nature or progressively deteriorating.

Antibody staining can be used to assess the presence of amyloid and/or amyloid precursor protein (bearing in mind species differences for enzyme processing of amyloid precursor protein). Brain tissue can be silver-stained (and counter-stained) to reveal any neurofibrillary tangles that may be present. Other, less specific, histological stains may also yield useful information, such as congo red for amyloid and solochrome azurine or morin for tissue Al. If appropriately prepared, tissue sections can also be examined by scanning transmission electron microscopy using X-ray analysis or X-ray mapping for Al and other elements.

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APPENDIX 7: Aluminum in Drinking Water as a Risk Factor for Alzheimer's Disease: Animal Models

Dr. D. McLachlan

Alzheimer's disease (AD) is a unique, progressive, neurodegenerative condition that occurs only in humans. AD is unique in both the behavioural changes observed and the neuropathological changes associated with the disease. The natural history and characteristics of the clinical dementia, although variable among individuals, are sufficiently distinct to separate AD from other brain diseases. However, attempts to design experiments to reproduce the constellation of cognitive deficits that occur in AD in any subhuman species do not appear to be feasible because of the very different neural organization of the human brain. In testing for an environmental risk factor for AD, it will be necessary to establish which disorders in cognitive performance in laboratory animals constitute reliable supporting evidence. Deficits in performance of memory tasks, short term and/or remote, may be considered essential, but disorders in reasoning, attention, and the organization of higher-order motor behaviours are also important attributes of the clinical manifestations of AD.

Similarly, the complete constellation of histopathological changes of AD are not found in the brains of any other subhuman species. Even today, 6 years after the genetic defect in the B-amyloid precursor protein was discovered, transgenic mice bearing any of the four known AD susceptibility genes do not reproduce AD histopathology. What A. Alzheimer described in 1907 were the combined findings of a particular brain distribution of amyloid plaques, neurons with neurofibrillary degeneration, neuron loss, and gliosis. Similar histopathological findings in brains of a transgenic strain would constitute conclusive evidence that the test gene is necessary and sufficient to cause AD. This has not been observed. Some transgenic mice exhibit an increase in brain amyloid and increased phosphorylation of tau, but all animal models to date have failed to exhibit a convincing picture of AD histopathology.

In view of the very strong epidemiological and other evidence already available that implicates aluminum (Al) in drinking water as a risk factor for AD, it could be argued that it is highly improbable that further animal studies will yield useful additional evidence. Indeed, further delay in instituting the recommendations of the arms-length 1993 review committee of a regulatory Al standard of 100 µg/L in finished drinking water for the Province of Ontario could be viewed as an unacceptable public health risk.[6]

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[6] Commissioned by Public Health Branch, Ontario Ministry of Health, Toronto. Submitted 4 June 1993. Entitled: *Health Effects of Aluminum: A Critical Review with Emphasis on Aluminum in Drinking Water*. Project Sponsor: Institute of Environment and Health, University of Toronto and McMaster University; p. 88, section 5.3: Public Health Implications...."The level of concern identified justifies a regulatory standard equal to but not lower than the current ODWO" — i.e. 100 µg/L.

Notwithstanding the strong evidence already available, further animal studies are justified, because it is probable that AD is a multifactorial degenerative process requiring the interaction of both genetic and environmental factors. Among environmental factors, the most likely candidate is Al. Transgenic AD models of AD susceptibility genes have only been developed in mice. Unfortunately, mice are very resistant to an induced Al encephalopathy, even following direct brain application of soluble Al salts. Furthermore, the amount of Al taken up and the concentration deposited in brain from drinking water differ considerably among mouse strains (Fosmire *et al*., 1993). The experiments of Fosmire *et al.* (1993) have, in part, been reproduced by Westaway *et al.* (unpublished). In two strains of non-transgenic mice, Westaway *et al.* have demonstrated that after 103 days of exposure to 1000 ppm Al lactate in drinking water, the mean concentration of Al in brain tissue rose 5- to 7-fold. However, there was approximately a 2.5-fold higher brain Al concentration in DBA/2 than in strain FVB/N. (FVB/N strain was chosen because this strain has been engineered to carry the human AD mutation on chromosome 14, presenilin 1, the most common cause of the genetic form of AD.) Their data confirm that a 5- to 7-fold increase in mean brain Al concentration may follow drinking water exposure and that the extent of brain deposition is strain dependent. The critical question is whether the brain concentration that results from oral exposure might ever reach a neurotoxic range. To compound the difficulty, the neurotoxic range has not been established in rodents. However, in rabbits and cats, several different end-points indicate an alteration in information processing by neural nets at concentrations exceeding the range 4.5–5.5 µg/g dry weight. Examination of the raw data generated by Westaway *et al*. reveals that 1 in 8 of the DBA strain reached a brain concentration exceeding 4 µg/g. Thus, with longer drinking water exposure times, the appropriate selection of strain, and/or more appropriate selection of the Al species or ligand in water, it is probable that brain Al concentrations could be achieved in mice that are known to be neurotoxic in at least two other species.

Al alone does not induce AD histopathology in laboratory animals, but Al does interact with several brain neurochemical steps associated with the AD process. For example, Al alters certain metabolic steps in the processing of the amyloid precursor protein and the phosphorylation of tau (reviewed in: McLachlan, 1995; McLachlan *et al*., 1996). In order to test further the hypothesis that Al is a risk factor for AD, presenilin FVB transgenic mice could be crossed with a high-Al-uptake strain, a strain that must also be suitable for behavioural testing, to look for an interaction of risk factors that might occur below the Al neurotoxic range.

Experimental proposals

To test the hypothesis that Al in drinking water is a risk factor for AD, at least three selection criteria are required. First, a mouse strain, possibly DBA, should be sought that deposits at least 4–6 µg/g dry weight of Al in brain following exposure to high concentrations of Al in drinking water. Second, the high-uptake strain must be suitable for behavioural testing. Third, the selected strain must be crossed with FVB strains carrying genetic risk factors for AD that are driven by a well-

documented promoter such as the hamster prion protein gene promoter. It will be necessary to establish which species and ligands of Al are most appropriate. Following selection of an appropriate strain and water Al species/ligand, four Al concentrations should be tested: 0, 100, 250, and 750 ppm. Exposure times of 8 and 16 months would be required. The selected mouse test strain should include the following groups: 1) mice with wild type genetic complement, 2) transgenic mice with presenilin 1, 3) transgenic mice with presenilin 1 plus the human amyloid mutation, AB 1-48, and 4) transgenic mice with the human amyloid mutation only.

The end-points for the assessment of toxicity should include both behavioural and brain biochemical analysis. Behavioural tests such as water or other maze testing for learning/memory performance and motor control would be suitable, but the repertoire of behaviours tested should be expanded to plausibly embrace those encountered in AD. Brain tissue analysis should include histopathological examination for AD pathology and chemical analysis for brain tissue Al concentration, AB 1-40 amyloid accumulation, and measures of the extent of tau phosphorylation and tau conformation.

While there is evidence that the oral administration of Al results in both altered behaviour and increased brain Al concentration in mice (Oteiza *et al.*, 1993), the present experiments address the interaction of a common environmental factor with AD susceptibility genes and therefore address the human condition in a more realistic fashion.

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APPENDIX 8: Background to Issues for Discussion at the Workshop

Dr. R. Yokel

In an animal study with ^{26}Al or ^{27}Al, how much Al is absorbed?

Studies suggest that the fraction of oral aluminum (Al) that is absorbed (F) is up to ~0.01 (1%), although there is a very wide range of estimates. Studies conducted with ^{27}Al use ≥1000 times the human daily oral dose of Al in water. Most studies deliver the Al as chemical species not representative of drinking water. Few studies determine F by the most accurate method: area under the plasma Al concentration time curve after oral compared with intravenous administration. Some results suggest increased F with increased Al dose and increased F in the absence of stomach contents. However, the potential presence of food and/or faeces in the animal's stomach has not been adequately avoided when an empty stomach was intended. The relative F of Al from food versus water has not been determined.

The F of oral Al appears to positively correlate with solubility of the Al species, decreased gastric pH, carboxylic acid (especially citrate) concentration, and the presence of uraemia. Increased calcium appears to decrease the F of oral Al. There are mixed results concerning the effect of silicon and fluoride on the F of Al: both a decrease in F and no effect on F have been suggested. Therefore, the chemical species appears to be important.

Where does Al accumulate?

In the absence of greatly elevated exposure, lung, liver, and bone Al concentrations are higher than measured concentrations in other tissues. Owing to their Al content and sizes, the skeleton, lung, muscle, and liver contain most of the body burden. Brain Al concentration is lower than concentrations in most tissues. After the increased Al exposure that produces the dialysis encephalopathy syndrome, the liver, spleen, and kidney have the greatest elevations. The percent increase in brain Al is relatively less.

Some results suggest that a fraction of Al that enters the brain is permanently retained. Several studies report a similar fraction of a single ^{26}Al dose in the brain for up to 1 month after dosing. One study found no decrease in brain Al from 5 to 270 days after a single injection.

What are the pathways for Al accumulation?

In the gastrointestinal tract, Al may adhere to intestinal mucus with either subsequent release back into gut lumen and elimination or passive paracellular permeation of the jejunal and duodenal

gut wall into systemic circulation. This may be consistent with dose-dependent uptake. A fraction is cleared in the first pass through the liver. Circulating Al is ~80% bound to transferrin; the remainder is associated with citrate, phosphate, and other low-molecular-weight ligands.

The pathway or pathways of brain Al uptake are unknown. Diffusion through cell membranes and receptor-mediated endocytosis of transferrin-bound Al appear to be too slow to account for the ability of Al to rapidly appear in the brain in studies using pharmacological concentrations or doses. Al may enter the brain as a result of Al-induced opening of the blood–brain barrier, although this has not been shown. The monocarboxylic acid transporter appears to move Al from brain to blood. Perhaps it also transports Al into the brain.

What are the effects of age?

Human brain, lung, heart, and blood Al concentrations have been reported to increase with age. The prolonged elimination of Al after occupational and experimental Al exposure indicates the presence of one or more very long half-lives of elimination. One study in rats reported a positive correlation between half-life and age. An age-related increase of Al is consistent with very long retention of a fraction of the absorbed Al. There are no studies under conditions modelling Al in drinking water to indicate if age influences oral absorption, distribution into the brain, or brain Al retention.

Drinking water contributes only a small percentage of total Al consumption. Can it significantly contribute to Al body burden?

The lack of reports of increased brain Al concentration or higher incidence of Al-induced neurotoxicity in consumers of Al-based antacids argues against the hypothesis that stomach acid converts all consumed Al to similar chemical species. The potential for greater absorption of Al from water than from other sources argues against the hypothesis that Al in drinking water cannot significantly contribute to body (brain) Al burden because it is only a small percentage (~1–2%) of total Al intake.

Among three mammals (rat, rabbit, and human), brain Al is generally reported to be highest in the human. Dietary Al intake is greater in the rabbit (~100-fold) and rat (~10-fold) than in the human. If 1) diet is a major contributor to brain Al, 2) steady-state brain Al concentrations have been reached in these studies (after exposure periods of months in the rat and rabbit, decades in the human), 3) F is comparable among rat, rabbit, and human, and 4) the fraction of systemic Al that is retained by the brain is comparable among these mammalian species, then food must not be the major contributor to brain Al. This suggests that Al in drinking water may significantly contribute to brain Al accumulation. On the other hand, the permanent retention of a fraction of Al may explain the age-related increase in Al and the higher brain Al in humans than in experimental animals.

What is an appropriate method to use to avoid contamination from Al during an animal study?

When using ^{27}Al for pharmacokinetic studies, which is not recommended to address Al absorption from drinking water, samples should not contact glass or stainless steel. Alternatives are quartz, Teflon, and titanium. For sample digestion, ultra pure acids should be used. All work should be conducted in an ultraclean environment. All labware that contacts samples should be rigorously cleaned and rinsed.

When using ^{26}Al, avoid re-use of labware. Frequently swipe the laboratory to monitor contamination. Include experimental and analytical blanks to detect contamination. In a whole-animal dosing study, because nearly all Al will be excreted in faeces after oral dosing or urine after intravenous dosing, avoid sample contamination from excrement.

Practical issues to consider

Pharmacokinetic studies should be conducted with ^{26}Al. We should consider a study with concurrent ^{26}Al and ^{27}Al exposure: ^{26}Al to determine pharmacokinetic end-points, and ^{27}Al to enable determination of toxicity end-points. Statistical tests should be conducted to determine relationships between these two, to ascertain if Al neurotoxicity relates to Al presence or to an Al-induced effect, independent of brain Al concentration.

Doses should model human exposure. Studies intended to model Al in water should use Al species representative of drinking water. If stomach contents are a concern, this must be controlled.

APPENDIX 9: Is an Animal Study Crucial in Human Risk Assessment?

Dr. K. Bailey

Is an animal study an appropriate next step?

No, I do not believe that an animal study is the appropriate next step. Rather, I believe the next step is to answer the following two questions in sequence:

1) Have we "wrung" the last bit of information from the dialysis patient data?

2) Is it possible to reach a consensus on what the parameters (e.g. diet, chemical tested, etc.) of an "acceptable" aluminum (Al) study would be? There is little point in conducting a study unless there is agreement that, whatever the result, the study is likely to answer the question asked.

I. Dialysis patient data

Undoubtedly, this meeting is being held because of public concern about Al in drinking water. At least in part, this concern is based on the fact that Al is neurotoxic (e.g. dialysis patients). Thus, at least one of the public's questions concerning Al is: Are the Al levels in my drinking water low enough to protect me against neurotoxicity of whatever kind?

Typically, animal studies are conducted after we have "wrung" the last bit of information from the human data. We have human data, for that is how we know that Al is neurotoxic.

Have we "wrung" the last bit of information from the dialysis patient data? I suspect that we have not. Specifically, I do not believe that we have answered the question: What is the lowest level of Al, ingested in drinking water, needed to produce the dementia that has been seen in dialysis patients (lowest-observed-adverse-effect level, or LOAEL)? Alternatively, what is the highest level of Al, ingested in drinking water, that fails to produce the dementia that has been seen in dialysis patients (no-observed-adverse-effect level, or NOAEL)? While the answer to either of these questions will not answer all of the public's questions about Al, I believe that it will answer some of them.

Possibly, the risk assessment procedures applied to lead could be applied to Al as well. We normally relate blood lead levels to health effects (e.g. decrease in IQ). In turn, lead levels in drinking water are related to blood lead levels and thus indirectly to health effects.

If the data are adequate, we could do the same thing for Al that we do for lead. Specifically, what is the highest blood Al level not associated with dementia, and, in turn, what level of Al in ingested drinking water would be needed to produce that blood Al level?

It may well be that the data are not adequate to answer this question about the relationship between Al in blood and drinking water. However, such a conclusion would, in part, constitute an argument that an animal study is an appropriate next step.

II. "Acceptable" study parameters

Before we consider whether an animal study is or is not the next step, I believe that we should attempt to reach a consensus on what the parameters of an "acceptable" study would be. In short, is it possible to conduct a meaningful study? Specifically:

A. Diet

Animal chow typically contains relatively high levels of Al (e.g. 297 mg Al/kg chow, in Fulton and Jeffery, 1990). What is the highest level of Al that is acceptable in chow?

It has been argued that fluoride and silicates likely have significant effects upon Al toxicity (Yokel and Golub, 1996). What are the highest levels of both fluoride and silicate that are acceptable in chow?

B. Compound tested

Al is used to decrease phosphate absorption in humans. Thus, Al phosphate is probably not a suitable test compound. What is a suitable test compound?

C. Route of administration

Since we are concerned about drinking water, that is presumably the desired route of administration. However, at least as an initial pilot study, a gavage study may be a more sensitive measure of toxicity. What is a suitable route of administration?

D. End-points

If a study is conducted, certain end-points will be measured (e.g. behaviour, histology, tissue levels, etc.). What end-points should be measured, and how, as appropriate, can you minimize contamination and/or investigator biases?

What are the problems in assessing the toxicity of Al in drinking water for humans?

Flaten *et al.* (1996) have stated that there are two "trenches" in the scientific community; the "non-believers" have tended to be skeptical towards Al as a health hazard in general.

In relation to drinking water, I would modify this statement to: While the "non-believers" believe that Al is toxic (e.g. dialysis dementia), they are not convinced that, except for certain medical uses, Al in drinking water constitutes a health risk.

In answer to the question posed in the title, the problem in assessing the toxicity of Al in drinking water for humans is that there is no consensus in the scientific community that there is a problem.

What would be needed to reach a consensus? Put another way, what would be needed to derive a "sensible" health-based Al drinking water standard? Roughly, I believe that the following are needed:

Human data — Data quantitatively linking Al levels in ingested drinking water to adverse blood Al levels (e.g. from dialysis data) or adverse effects in the normal population

and/or

Animal data — An animal study that demonstrates a convincing relationship between Al levels in ingested drinking water and some adverse effect(s).

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APPENDIX 10: Notes for Workshop
Dr. T. Flaten

1. What are the problems in assessing the toxicity of aluminum (Al) in drinking water for humans?

Perhaps the most difficult problem involved in deriving a health-based guideline for Al in drinking water is which end-point to use. In order to derive any health-based guideline, there have to be adequate data on the effects of the substance (Al) on humans or laboratory animals. The only situation where we know that Al in drinking water is a human health problem is when such water is used to prepare dialysate for intravenous use in renal failure patients, but obviously this fact is of little value in setting a general guideline. For Al in drinking water, our concern for the last 10–15 years has mainly been with Alzheimer's disease (AD), which could be the end-point. Alternatively, the end-point could be cognitive impairment in general or other neurological effects, based upon the literature on such effects among occupationally exposed subjects.

It seems logical to base a guideline upon published epidemiological studies linking drinking water Al to AD. If this is the choice, the obvious problem is which animal model(s) to use (Workshop issues 3 and 4). One further problem in using AD as the end-point is that in order to do so, there should be scientific consensus that Al from any source is causally related to AD. Undoubtedly, no such scientific consensus exists.

If the guideline is to be based on the (subtle) neurological effects seen in workers, then it is easier to choose an animal model or models. In this case, a major problem would be to choose which neurological effect(s) to focus on, since the published occupational studies are far from consistent in this respect. There are already several reports in the literature on different neurobehavioural effects on animals orally exposed to Al. There is great need for a better and more comprehensive study, including larger groups of animals, more than one animal species, and several chemical forms of Al in drinking water (see 2 below).

2. How important are Al speciation and substances that affect speciation (silica, fluoride, pH…)?

I believe that this issue is very important for the planned study. Drinking water contains a wide variety of Al species. For example, it is conceivable that drinking water contains organic Al complexes that have the critical combination of water solubility, lipid solubility, and thermodynamic stability to pass through the gastrointestinal system and be taken up into the bloodstream and into the target tissue (in our case, the central nervous system) without the Al ion being transferred to other

ligands in the body. In this respect, there are several aspects that could possibly be incorporated into the study:

i. Use different Al compounds in the drinking water: a simple inorganic salt (chloride or sulphate; only one of these is necessary) and 2–4 organic compounds (the most relevant are citrate, maltolate, lactate, and acetylacetonate). Acetylacetonate is being much used by Zatta's group in Italy, and I really think it is worthwhile to test this.

ii. Other constituents in drinking water may interact with Al, usually through complexation. The most relevant are fluoride (F^-) and silicic acid (H_4SiO_4). These would be well worth including as extra variables, although personally I would place a higher priority on testing the different compounds described in (i).

iii. One could consider interactions with other (mainly essential) elements in the body by placing the animals on diets adequate and deficient in such elements as calcium, magnesium, iron, copper, and zinc. There are indications in the literature that the nutritional status of these elements in experimental animals influences the animals' handling of Al. However, inclusion of this aspect would add greatly to the complexity and resources needed for the study, and I do not think that it is worth the effort. One possibility could be to use a diet slightly deficient in key elements, to increase Al uptake and thus the sensitivity of the study (cf. Golub's paper).

3. Is an animal study crucial in human risk assessment? That is, is an animal study an appropriate next step?

Based on the problems discussed under (1) above, it is not easy to give a clear answer to this question. As far as I know, it is not common that drinking water guidelines are based primarily on human data. The most common situation is that such guidelines are derived mainly from data from studies on laboratory animals, so animal studies are crucial. Of course, it seems very improbable that one single animal study, no matter how good and how big, will be enough to set a drinking water guideline. However, whether or not we can agree upon the feasibility of setting a guideline, I do hope we can agree upon recommending that this study be carried out. It seems clear to me that a high-quality animal study on drinking water Al will bring us closer to being able to determine whether a health-based guideline can and should be set, and, if so, the study will be helpful in the future derivation of such a guideline. But to really make a difference, the study should probably be much larger than the animal studies carried out to date. A final point in this respect is that the World Health Organization has set a provisional tolerable weekly intake (PTWI) of 7 mg Al/kg body weight based mainly on studies on Al-containing food additives in laboratory animals.

4. Which is the best animal model…?

I would very much like to see rabbits used in this study, because rabbits are among the most susceptible species to Al intoxication, and they develop neurofibrillary pathology, while rats and mice do not. Furthermore, I am not aware that Al toxicokinetics have been studied in rabbits using ^{26}Al/accelerator mass spectrometry. It should be noted, however, that long-term, low-level intracisternal Al exposure in rabbits seems (for unknown reasons) to produce different clinico-pathological pictures: Savory and co-workers (cf. Savory's short paper for this meeting) stress that intracisternal Al maltolate in aged rabbits produces Alzheimer-like pathology and symptoms, while in Garruto's laboratory, repeated low-level intracisternal Al chloride produces almost exclusively motor pathology and symptoms more reminiscent of amyotrophic lateral sclerosis (ALS).

We need to use at least two species of animals, so rats or mice should also be included. Perhaps a comparison of how an Al-susceptible species (rabbit) and an Al-resistant species (rat or mouse) metabolize Al (studied using ^{26}Al) could give valuable and interesting results.

Finally, perhaps one could use some transgenic mouse species, to determine if such mice (e.g. with mutations in the amyloid precursor protein) handle Al differently from normal mice. This could be interesting in light of the mounting evidence of interactions between Al and amyloid.

APPENDIX 11: How Important Are Aluminum Speciation and Substances that Affect Speciation, such as Silica, Fluoride, and pH?

Dr. W.F. Forbes

There is no question that the toxicity of aluminum (Al) will depend on the chemical form in which it exists. Al can exist in many different forms, both stable and unstable. Generally, Al will not exist in a "free" form, because of the basic principle that any substance will form as many bonds as possible in order to reduce the energy of the system. What we would like to know is the chemical form of the neurotoxic Al compound(s).

Another general point is that there will be a number of factors that determine the neurotoxicity of Al. This multifactorial process will include genetic and environmental factors, and the latter will include the speciation of Al and the environmental conditions that will affect its neurotoxicity. Also, because of this multifactorial situation, the clinical and histochemical manifestations of the neurotoxicity are likely to vary depending on which particular factors predominate under specific conditions.

Various groups have attempted to identify "free" Al, and a number of different definitions have been used, such as "dissolved Al," "readily reactive species of Al," "inorganic Al," and "monomeric Al," but the definitions and the methods of determining these species have not been generally agreed upon. Moreover, there are likely to be various equilibria between different Al-containing species that again will depend on the chemical environment. For example, there will be equilibria between toxic and non-toxic species.

Considering the Al-containing species in drinking water, these will therefore be affected by a number of variables. One of these is pH, which appreciably affects the solubility of Al hydroxide; it is also well known that different Al-containing species are formed under acidic and basic conditions. In addition, there is epidemiological evidence that pH affects the neurotoxicity of Al (Forbes *et al.*, 1995a).

Next, there is evidence that the presence of silica affects the neurotoxicity of Al-containing compounds (Birchall, 1993). This is not surprising, since Al and silica react with each other. Again, the epidemiological evidence also indicates that silica can be important (Forbes *et al.*, 1995b).

Fluoride is another substance often present in water that can affect the neurotoxicity of Al (Radic and Bralic, 1995). The evidence is similar to that for silica, except that perhaps the epidemiological evidence is more consistent than for silica (Forbes *et al.*, 1995a).

The above-mentioned water constituents are not the only ones that can affect the neurotoxicity of Al. That is, the presence of iron, calcium, and other substances may all be important. Perhaps of particular interest is turbidity, representing suspended plus colloidal Al, and the epidemiological evidence (Forbes and McLachlan, 1996) suggests that the neurotoxic form of Al is not present to an appreciable extent in the colloidal or insoluble form of Al. Hence, a complete analysis of Al speciation is unlikely to be feasible, particularly since any treatment carried out during the chemical analysis is likely to change the sample, affecting the equilibria between the various species and thus the speciation. It follows that, at least in the short term, the use of total Al present in water is the most appropriate measure on which any guideline has to be based. This is not the most desirable situation, but, because of the association reported in a number of studies between total Al and various manifestations of cognitive impairment, together with the evidence obtained from animal experiments, this is most likely the appropriate course to take.

Since Al speciation is important, animal studies have the potential of providing information about the relevant interactions, as briefly outlined above. If such interactions in animal experiments, preferably in more than one species, are consistently obtained and are consistent with evidence involving human subjects, it can probably be assumed that these interactions are also relevant for establishing a guideline. Consequently, it may be possible in the future to provide more definitive guidelines rather than those that will rely on total Al concentrations.

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APPENDIX 12: Importance of Aluminum Speciation to the Design and Interpretation of Toxicity Studies

Dr. M. Gardner & Dr. E. Dixon

Abstract

Aluminum speciation in water is discussed in relation to the design of studies of the metal's toxicity. The need to monitor speciation as part of any investigations of toxicity and to relate the conditions of the test to those in waters of interest is emphasized.

Introduction

Trace metals are present in biological systems and natural waters in a variety of forms or species. The total amount of metal present in a given system will be divided between particulate and dissolved forms. (Particulate species can be present as a precipitate of a salt or compound of the metal or as metal ions adsorbed to suspended solid material.) The dissolved forms might be present in association with organic molecules, or they may be in the form of free ions or inorganic complexes.

It is accepted that the form of a metal can have an important influence on its toxicity, fate, and behaviour. The fact that contaminants can be present in various forms, or species, can explain differing behaviour under changing system conditions. In the case of aluminum (Al) in drinking water, speciation is likely to play a key part in determining the metal's potential bioavailability and toxicity. If a study of Al toxicity is to reflect the behaviour and potential effects of the metal, the study design should take account of the forms of Al likely to be present in drinking water (Gardner and Gunn, 1995). It is necessary to consider not only the forms of metal that might be present in potable waters of different qualities, but also the potential for exchange between one form and another.

Similarly, in interpreting existing toxicity studies, it is necessary to consider the implications for speciation of the chosen experimental approach. An experimental design for which the speciation of the Al is not the same as that in drinking water is unlikely to provide a reliable estimate of the metal's toxicity in potable waters.

Key Issues Relating to Al Speciation

a) Dissolved-phase species

Al is present in solution in a wide range of chemical forms or species. These may be categorized as follows:

- free hydrated Al^{3+} ions;
- Al ions complexed by inorganic ligands (e.g. hydroxide, fluoride); and

- Al ions complexed by organic ligands (e.g. humic acids, citrate).

In water, Al complexes with inorganic and organic ligands exist in a state of equilibrium with one another. The position of the various equilibria, and therefore the species that predominate over the rest, can be calculated if the equilibrium constants for the complexes and the respective concentrations of ligands are known. For Al and its inorganic complexes, this is usually possible. Figure 1 illustrates the range of species in possibly the simplest situation — a solution of Al ions in pure water. The calculation of dissolved monomeric species has been carried out using the MINTEQ program for chemical equilibria (Felmy *et al.*, 1984). The main feature revealed in Figure 1 is that the free (hydrated) Al ion, Al^{3+}, is likely to constitute only a small proportion of the total at pH values larger than approximately 5.0. For pH values in the range 5–9, a variety of hydroxy complexes predominate. These differ from Al^{3+} in that they are of higher molecular weight, one is uncharged, and one is negatively charged. These differences are likely to have important consequences for the solubility and, possibly, for the bioavailability of the metal (see below).

Figures 2 and 3 illustrate the effect of a competing ligand, fluoride, on the distribution of hydroxy-Al species. When the concentrations of Al and fluoride are the same (both 100 µg/L; Figure 2), fluoride complexes are important only at pH values below 6.0. If the fluoride concentration is 20 times that of Al (Figure 3), the influence of fluoride complexes is extended up to pH values near 7.0.

The importance of a particular ligand is determined by the strength and stoichiometry of the complex formed and the concentration of the ligand. Thus, a relatively weak complex can still be significant, provided the ligand concentration is sufficiently high. In the examples shown in Figures 2 and 3, the weaker ligand (hydroxide) predominates over the stronger (fluoride) at pH values where the concentration of the former results in more effective competition. Similar considerations apply to other ligands, organic and inorganic.

In the case of organic ligands, the issues are complicated by the fact that the nature and concentration of the ligands in waters and biological systems are not usually known. Potts *et al*. (1985) reported that complexation occurs with humic substances mainly in the pH range 3–5. Above this range, hydroxy species tend to predominate.

b) Dissolved/particulate division

The dissolved fraction of Al in water is often an insignificant proportion of the total quantity of metal. Particulate forms, ranging from colloidal species containing fewer than 10 Al atoms to Al-containing mineral particles, may constitute the major fraction of the total. Al-hydroxy complexes tend to be of low solubility at neutral pH values. The influence of species charge is shown in Figure 4, where thermodynamic equilibrium calculations of complexation and solubility have been applied to a pure water containing 100 µg of Al (nitrate was used in the calculations as a non-complexing counter-ion to achieve overall charge balance). The calculations, based on measured solubilities of

Al-containing minerals, indicate that in water of pH between 5.5 and 9, there is essentially no Al in solution. This is primarily a consequence of the tendency of the dominant Al species over this pH range (the uncharged $Al(OH)_3$) to precipitate as the mineral diaspore.

The fact that the calculated dissolved concentration is considerably lower than concentrations measured in similar waters (e.g. drinking waters after treatment by Al coagulation) requires consideration. The probable reasons for this are as follows: (i) in practice, it is very difficult to measure the truly dissolved species — low-molecular-weight colloidal forms of Al are invariably included in the dissolved fraction when dissolved and particulate forms are separated (e.g. by filtration); (ii) real waters may contain other ligands, inorganic and organic, which tend to stabilize the dissolved concentration at a higher level than that predicted by calculation; and (iii) the calculations used to derive Figure 4 are based on the attainment of thermodynamic equilibrium — in real waters, the speed of reactions in dilute solution may be so slow that equilibrium is not reached.

c) Kinetic effects

Knowledge of thermodynamic equilibria does not necessarily provide an insight into the conditions prevailing in natural systems. Although thermodynamics can be used to understand the ultimate equilibrium conditions that a system may reach, there is no guarantee that sufficient time will be available for equilibrium to be achieved. In the case of Al speciation, kinetic considerations may be important in:

i) *The establishment of solution equilibria.* Inorganic equilibria may be established in a few seconds or less; organic equilibria may take minutes or hours to complete;

ii) *The precipitation of Al-containing solids.* At trace concentrations of dissolved Al, it is common to observe that the low dissolved-phase concentration predicted by thermodynamic calculations is not achieved. This may be due to solution-phase complexation or to slow attainment of the predicted equilibrium. Loss of dissolved Al as a precipitate is probably the most important change in speciation that might occur during a toxicity test. This process could lead to exposure of test organisms to lower concentrations than intended, with consequent underestimation of toxicity. In the case of studies in mammals, this is an important consideration in test solution storage if the test material is drinking water.

Tests carried out at the authors' laboratory on the toxicity of Al to tadpoles illustrate this point. These tests were undertaken as part of work intended to establish environmental quality criteria for Al in surface waters (Dixon, 1997). The tests were intended to assess Al toxicity at two pH values, 6.0 and 7.5. A range of water samples of Al concentrations from 0 to 320 µg/L was prepared in an uncontaminated groundwater. These solutions were replenished every 2 days for the 7-day duration of the test. Chemically labile monomeric Al (see below for discussion and a description of

the method used) was determined at intervals as a check on the forms of Al to which the tadpoles were exposed. Figures 5 and 6 show the results of these determinations. At pH 6.0, the concentration of monomeric Al was found to be substantially less than the total spiked level (Figure 5). Even immediately after spiking, the concentration was much lower than expected; the test solution with a total concentration of 320 µg/L was of a monomeric Al concentration of less than 35 µg/L. After 24 hours, the monomeric Al concentrations of all test solutions were less than 20 µg/L. At the higher pH value of 7.5 (Figure 6), the difference between monomeric Al concentrations and the corresponding spiked levels was large (although not as large as at pH 6.0). The 24-hour values for monomeric Al were similar to those measured initially, indicating greater stability than that observed for samples at pH 6.0.

Analytical implications

The relatively vague distinction between reactive and unreactive dissolved and undissolved forms of Al has led to the development and use of analytical techniques designed to determine the reactive species of the metal. These techniques are based on determination of a chosen size fraction or on the speed with which species can undergo reactions. The species determined are referred to as "labile" or "labile monomeric" forms and are considered to constitute the most biologically and environmentally significant fraction. It is important to recognize that, in principle, each analytical technique for labile Al or each variant of a given approach is empirical — the technique defines the fraction determined. In practice, comparisons among several of the most common approaches have revealed a moderately good degree of agreement (Lazerte, 1984; Clarke *et al.*, 1996). The procedure described below (based on the method described by Barnes, 1975) has been used by the authors for studies of Al speciation in drinking waters and surface waters.

The use of ^{26}Al in toxicity tests has certain advantages, but it is by no means the complete solution to the problems of speciation, fate, and behaviour discussed above. ^{26}Al is relatively easy to detect and provides a means of assessing the nature of uptake and following the fate of the metal. Nevertheless, the validity of conclusions drawn from such studies depends crucially on the need for the added isotope to be in the same form as the naturally occurring isotope. This may be difficult to achieve and still more difficult to demonstrate.

The use of measurements of ^{26}Al as a means of determining total Al will be subject to many of the problems of sample handling and contamination that beset testing and measurements involving only ^{27}Al.

Separation of dissolved labile monomeric Al by fast oxine extraction

The procedure described below is intended as a method for the determination of reactive forms of Al in water. The separation technique, which is based on that described by Barnes (1975),

is intended to isolate reactive low-molecular-weight Al species that are most likely to be of environmental or health significance. Sample types for which the procedure is applicable include raw and potable waters, surface water, groundwater, and saline waters. The approach is an empirical one — the proportion of total Al determined is dependent on the details of the procedure used.

a) Sample collection and pre-treatment

It is not possible to guarantee the stability of Al species or speciation. Hence, every effort should be made to process samples as soon as practicable. If it is not possible to filter and extract samples immediately after collection, a 1-L sample should be collected and stored in an acid-washed polyethylene bottle. Samples should be transported in the dark and stored at 4°C. Once filtered, samples should be extracted right away.

b) Reagents

- Oxine solution: Dissolve 1.2 g of 8-hydroxyquinoline in 2.5 mL of acetic acid (Aristar or equivalent grade) and dilute to 100 mL with deionized water.

- Oxine/acetate reagent: Mix 30 mL of oxine solution (above) with 30 mL of 1 M sodium acetate solution (82 g of sodium acetate made up in 1 L of solution) and 150 mL of deionized water.

- Buffer: Add 2.5 M hydrochloric acid dropwise to 20 mL of 5 M ammonia solution until a pH value of 8.3 is reached. Add an additional 32 mL of 5 M ammonia solution and dilute to 100 mL with deionized water.

- Concentrated nitric acid, density 1.42 (Merck Aristar or equivalent grade).

- Methyl isobutyl ketone (4-methyl pentan-2-one), or MIBK.

c) Equipment

- 50-mL polypropylene Oak Ridge screw-capped centrifuge tubes — soaked for 48 hours in 5% nitric acid (v/v) and then rinsed thoroughly with deionized water.

- 25-mL polypropylene screw-capped centrifuge tubes — soaked for 48 hours in 5% nitric acid and then rinsed thoroughly with deionized water.

- 50-mL polyethylene syringe — soaked for 48 hours in 5% nitric acid and then rinsed thoroughly with deionized water.

- 0.45-µm cellulose acetate cartridge filter — washed with 5% nitric acid and then rinsed thoroughly with deionized water.

d) Separation of labile monomeric Al

Filter a 50-mL portion of sample through an acid-washed 0.45-µm filter. Process at once, as described below.

Transfer 12 ± 0.3 mL of filtered sample to a 50-mL centrifuge tube. Add 2.5 ± 0.05 mL of oxine acetate reagent and a volume of the buffer solution sufficient to produce a pH value of 8.3 ± 0.2 units. (This volume should be determined by trial on a separate portion of the sample(s) concerned, with the reagents as prepared. In most cases, a volume of approximately 100 µL of buffer will be required.) Mix and add 5 mL of MIBK. Cap the tube and shake vigorously for 15 ± 2 seconds. Allow at least 10 seconds for the phases to separate, and remove 2.5 mL of the MIBK layer by pipette. Transfer to a 25-mL centrifuge tube containing 2.5 mL of deionized water and 200 µL of concentrated nitric acid (Aristar or equivalent grade) (or 2.5 mL of 8% v/v nitric acid). This bottle should be capped and shaken for 20 seconds. It can then be stored until analysis. Determine Al in the aqueous layer using an analytical technique with a limit of detection of 1 µg/L or less.

It is recommended that each sample be extracted in duplicate. Standard solutions and spiked samples should be taken through the extraction procedure as a quality control check. Blank samples (deionized water) should always be included in any batch of extractions as a check on contamination.

Conclusions

Although the importance of speciation is accepted widely, it is relatively rare for the determination of key species to form part of toxicity testing, standard setting, or routine quality monitoring. There are two principal reasons for this. First, the measurement techniques to determine particular metal species have yet to be developed and refined to the stage where they can be accepted widely and applied on a routine basis. Secondly, water quality legislation is usually framed in terms of the total quantity of a metal, rather than as limit values for individual, important forms. As long as this is the case, there will be a strong incentive to determine only total metal.

Recommendations

1. Speciation needs careful consideration in the design of toxicity tests.

2. The Al used in toxicity tests should be in a form similar to that present in drinking water.

This implies that:

i) the conditions of the toxicity test, including the Al species present and possible ligands that may affect speciation, should be monitored;

ii) the test should be carried out using a water sample of matrix (or matrices) similar to that of the potable water(s) of interest (N.B.: the speciation in the chosen matrix must be confirmed by analysis to ensure a valid comparison with the species used in the toxicity test); and

iii) attention should be paid to possible changes in speciation during the toxicity test.

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Figure 1 - Speciation of Aluminum in Pure Water

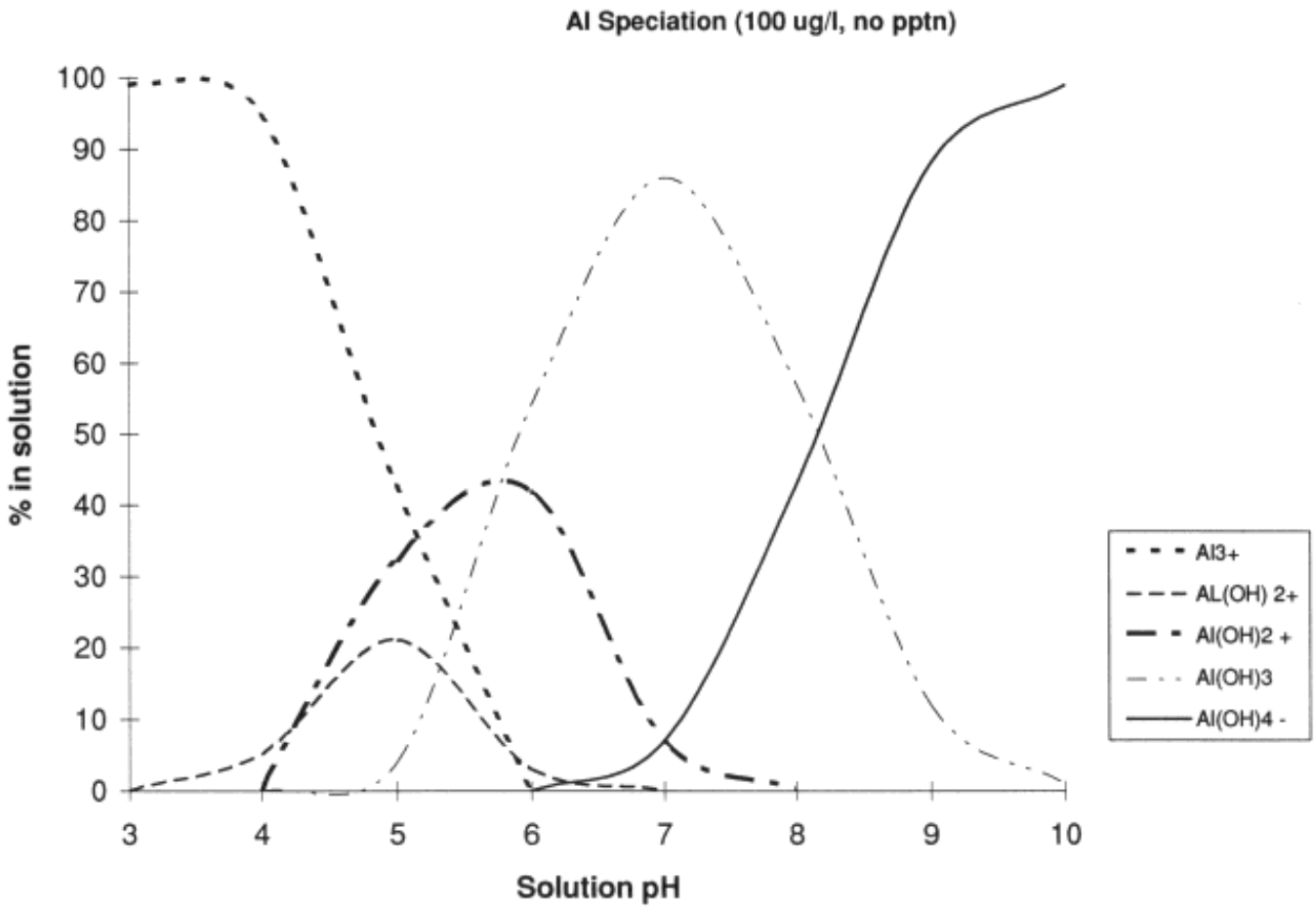


Figure 2 - Effect of Fluoride Complexation on Aluminum Speciation

(equivalent concentrations of Al and F)

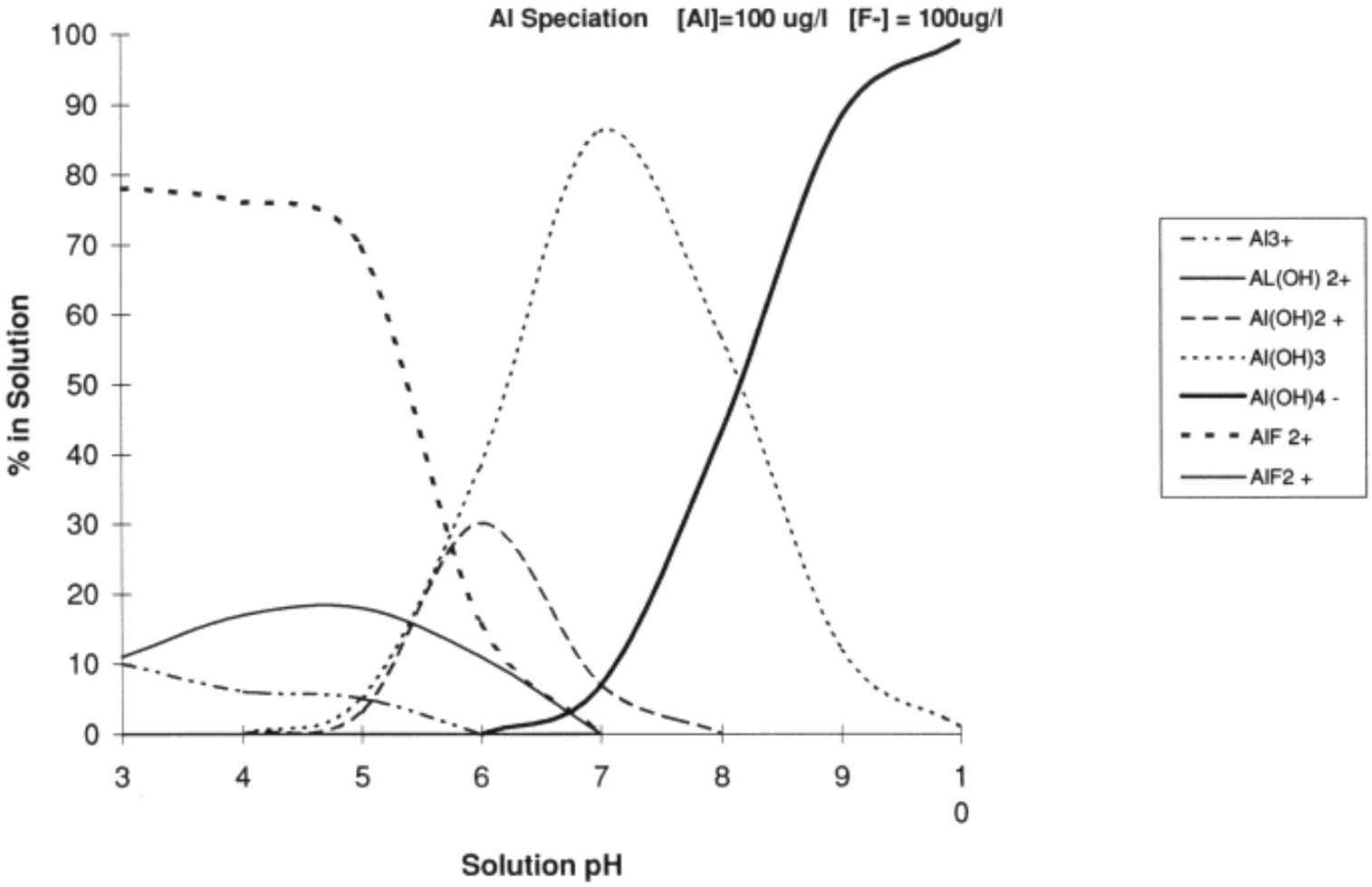


Figure 3 - Effect of Fluoride Complexation on Aluminum Speciation
(F in excess over Al)

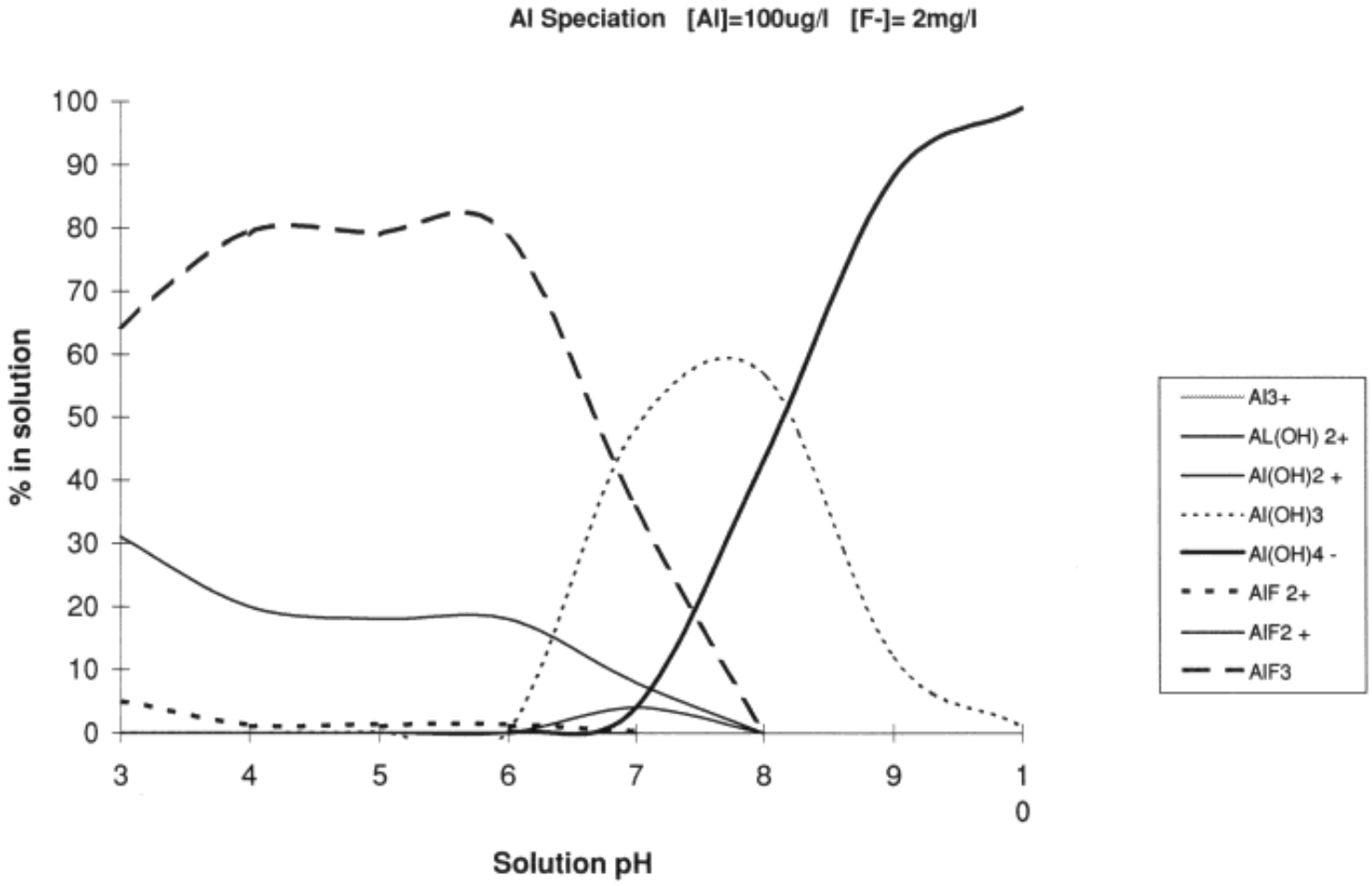


Figure 4 - Calculated Concentrations of Dissolved Aluminum

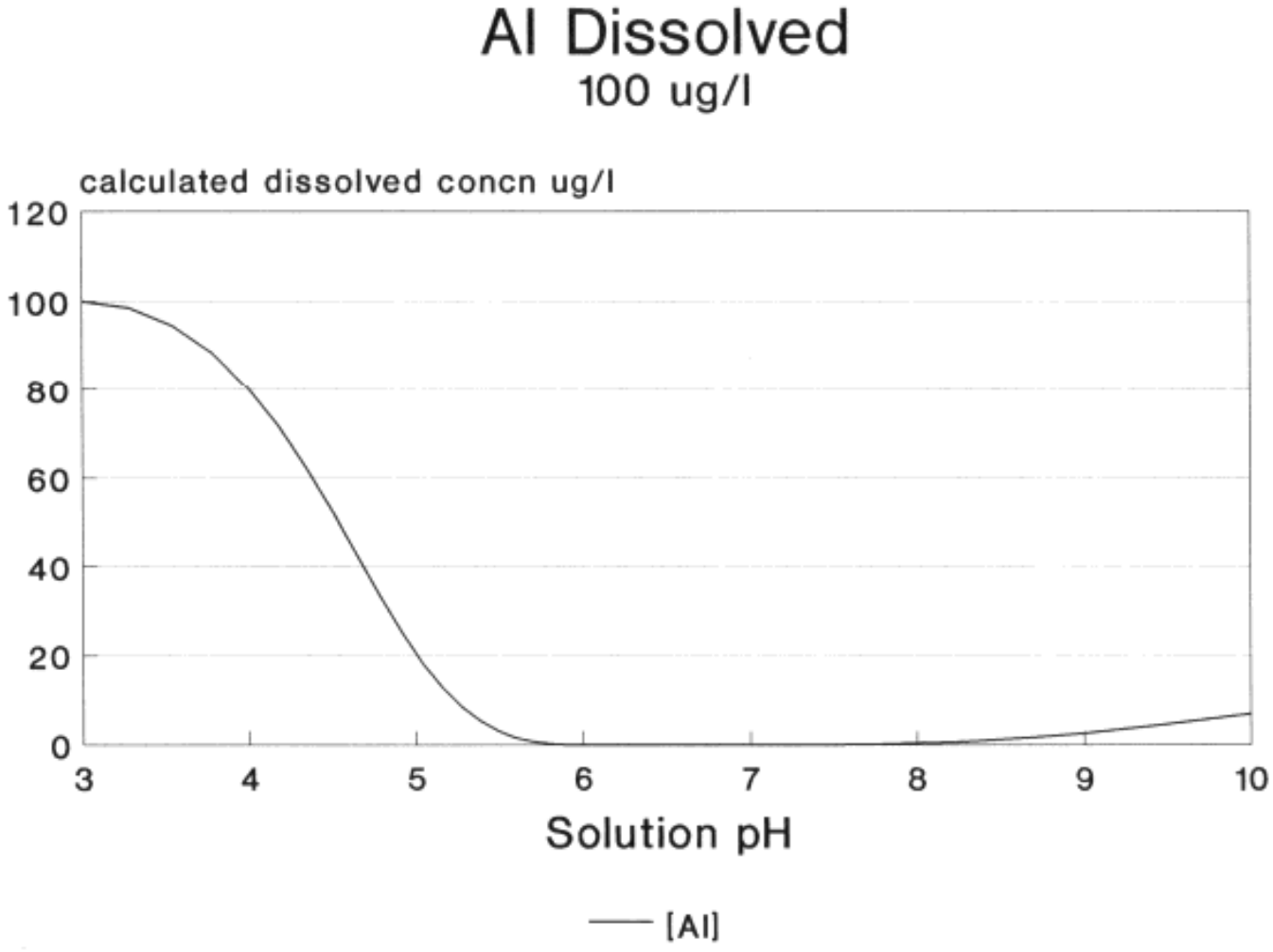


Figure 5 - Measured Monomeric Aluminum at pH 6.0

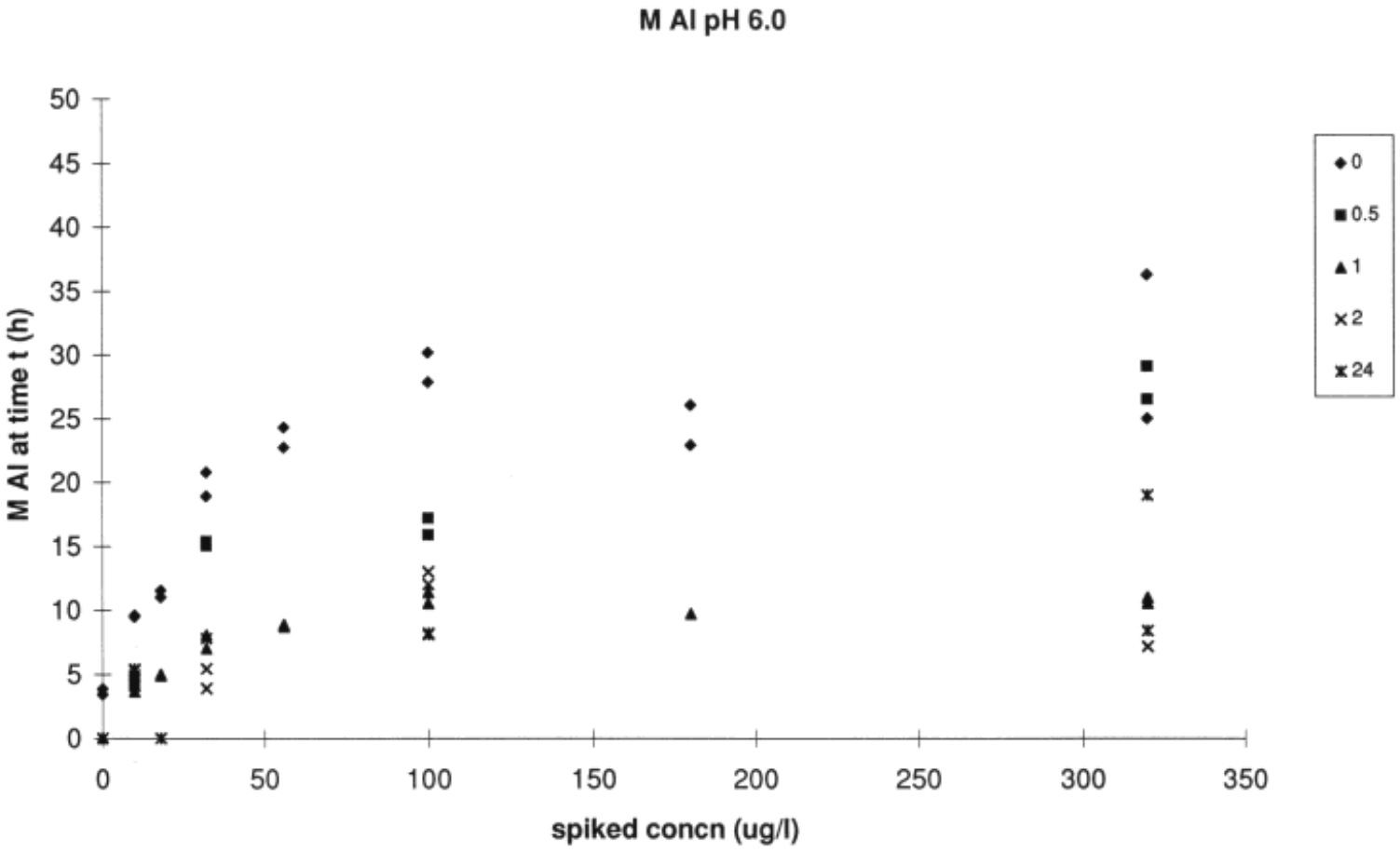
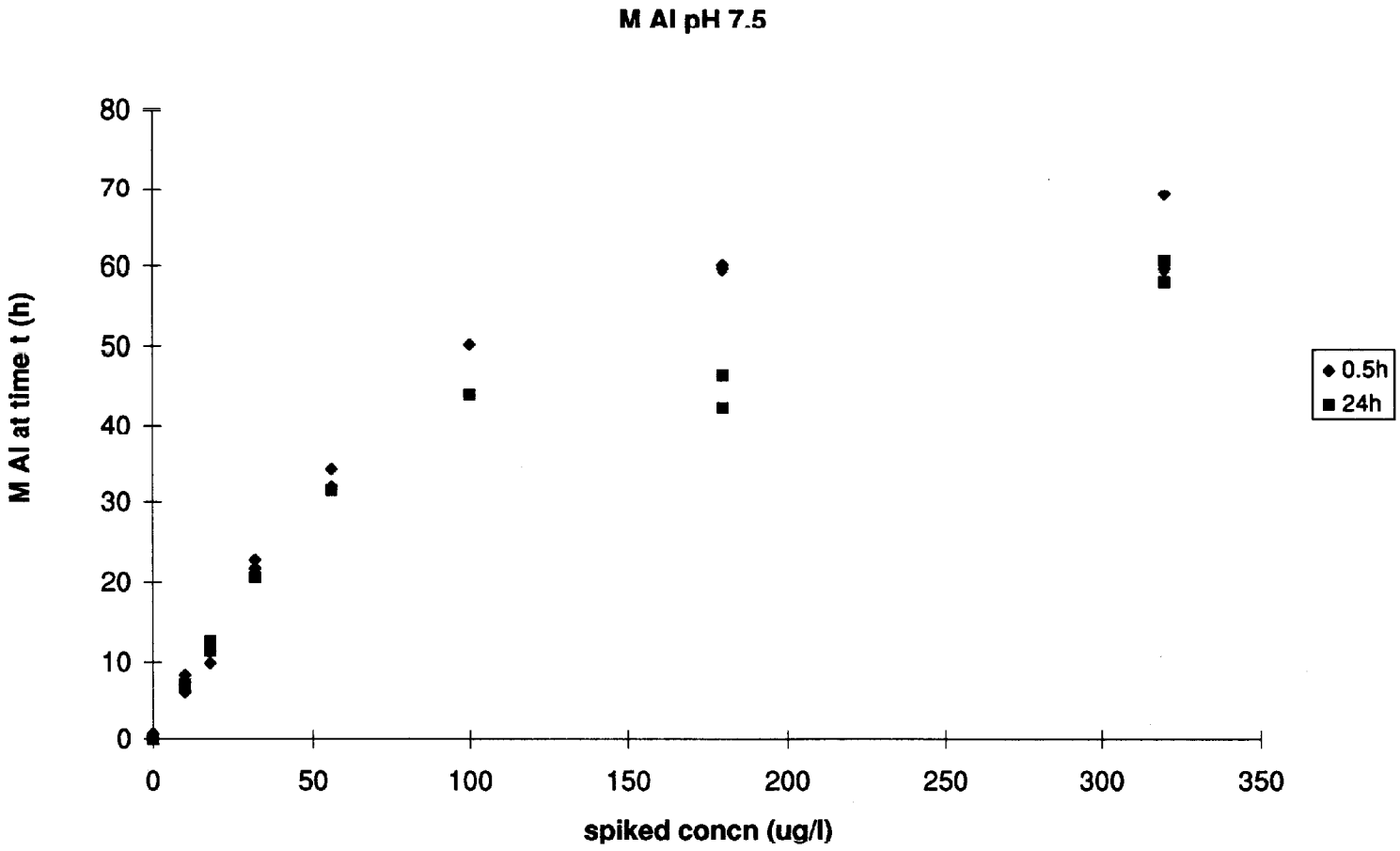


Figure 6 - Measured Monomeric Aluminum at pH 7.5



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