Zinc and Alzheimer's Disease

Ashley I. Bush *et al.* present data from an in vitro study of an interaction of aqueous Zn ions with β-amyloid protein (Aβ) (*1*), the protein that accumulates in neuritic plaques in patients with Alzheimer's disease (AD). Zinc was shown to bind to the protein, inducing aggregates that precipitated from solution (tinctorial amyloid formation). One important question is the specificity of this interaction, and Bush *et al.* partly address this by assaying 14 other metal ions (3 μM) on an equimolar basis.

Although Bush *et al.* state that none of these other metals induced significant aggregation, the data presented show that Cu and Hg effected an approximate 12% decline in filterability of the Aβ solution; the decline with Zn was about 25%, although other data in the report suggest that 3 μM Zn may induce up to 40% reduction. However, almost all of the other experiments described in this report used Zn at 25 μM, presumably to maximize effects [this was the uppermost value in the dose-response graph, figure 2B in (*1*)] and to be operating with concentrations of Zn normally found in plasma. What would the Aβ aggregate-inducing ability of Cu and Hg ions (and indeed the other metals) be if tested at 25 μM, or, more pertinently, if tested at a normal range of concentrations plasma? A review of such concentrations reveals that 3 μM in plasma is unlikely; for Mg and Ca it is about three or four orders of magnitude too low; for Fe and Cu, it is just below their minimums of about 10 μM; for Pb, Al, Hg, Cd, Mn, and Co, it is, respectively, 3, 5, 10, 30, 65, and 100 times the recommended maximum in non–occupationally exposed groups. This information therefore most likely discounts these latter metals as possible effectors of Aβ aggregation, but clearly Mg and Ca (and perhaps Fe and Cu) ions should be tested at concentrations at which they normally exist in plasma.

Bush *et al.* state that elevated (80%) Zn concentrations in cerebrospinal fluid (CSF) have been found in AD cases. However, in the reference they cite, it is evident that these data apply not to confirmed AD cases but to patients with Alzheimer-type dementia (ATD) (*2*). Furthermore, although the average concentrations CSF-Zn in these ATD cases was 80% higher than in age-matched controls, only 21% of these cases (7/33) had the arbitrarily-set, elevated concentration of CSF-Zn. Of a group of six AD cases, verified by autopsy, one had elevated concentrations CSF-Zn as compared with one of eight controls. Hershey *et al.* (*2*) also provide data on Cu, Fe, and Si (an element not tested by Bush *et al.*). In the CSF of the 33 ATD patients, concentrations of Si were elevated in eight patients. The average concentration over all cases was 130% higher than it was in the control group, while analyses of the autopsy-verified AD cases and controls revealed CSF-Si elevated in 5/7 cases versus 0/8 conrols. Also, average concentrations of ATD-CSF-Cu and Fe in patients were 120% and 125% above those in the control group, respectively.

These data may further encourage extended Aβ aggregation studies with Cu and Fe, and suggest that Si (normally present as a complex anion or silicate polymer) should be included. Normal serum concentrations of Si are around 5 μM, but in renal dialysis, concentrations of Si in the plasma of patients can range from 25 to 100 μM, and there are data showing that an elevated concentration of Si has a protective role in limiting Al bioavailability (*3*).

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Bush *et al.* (*1*) report that the aggregation of human AD amyloid peptide, $A\beta_{1-40}$, in tris saline is significantly enhanced by Zn cations. Their qualitative conclusion (that zinc induces Aβ aggregation) confirms our earlier work (*2*) that in either tris buffers or human cerebrospinal fluid at 37°C, Zn was more effective than a variety of other metals tested. Two points must be addressed in studies of this kind: the accuracy and reproducibility of the measurements, and the extrapolation of results obtained at higher concentrations of peptide and metal to the much lower ones found physiologically. Neither of these points has been adequately addressed by Bush *et al.* (*1*). First, their measurements of peptide concentration and aggregation are incorrect because the method used is inappropriate. Second, their extrapolation from data gathered at 1 to 25 μm peptide concentrations to the physiological range (<0.005 μM, a thousandfold lower) is unsubstantiated. Significant aggregation of Aβ is induced by Zn^{2+}, but at concentrations a hundredfold higher than those reported by Bush *et al.* (*1*).

Bush *et al.* (*1*) used ultraviolet spectroscopy at low wavelength, that is, optical density at 214 nm (OD_{214}), to measure concentrations and aggregation of human Aβ (mostly at 1.6 μM). On the basis of this method, they report that Zn^{2+} concentrations above 0.3 μM cause aggregation of Aβ into an insoluble form that can be removed from solution by centrifugation or filtration. At a concentration of about 3 μM Zn^{2+}, half of the Aβ aggregated. In contrast to normal human Aβ, rat Aβ and radioiodinated human Aβ were aggregated at significantly higher (>30 μM) concentration of Zn^{2+}. These Aβ concentrations are at the limits of the sensitivity of the OD_{214} method (*1*, *6*).

OD_{214} is rarely used for peptide quantitation (*8*, *9*). Essentially all organic molecules absorb at 214 nm, producing a high background that is sensitive to artifact. At the concentrations used by Bush *et al.* (*1*), tris buffer has a much greater OD_{214} than 1.6 μM Aβ. Furthermore, the buffer absorbance is highly sensitive to wavelength, pH, temperature, dissolved oxygen, and Zn^{2+}. The optical density of peptides at 214 nm is mostly provided by the peptide bond (*8*, *9*) and as such is dependent on the conformation of the peptide (*8*–*11*) as well as on its concentration. For example, the OD_{214} of polylysine at constant concentration changes by more than 50% as its conformation changes from α helix to β sheet (*11*). The addition of metals such as Zn to peptides and proteins affects their conformation and therefore their optical density. Bush *et al.* have concluded (*6*) that Zn affects the conformation of Aβ and then assume (*1*) that a change in OD_{214} reflects only a change in Aβ concentration, with no effect of the conformational change.

Because the behavior of Aβ is highly dependent on its concentration, extrapolation to physiological (10^{-9} M) concentrations of data gathered at $>10^{-6}$ M (*1*) is not valid (*3*, *4*) without experimental support. We used unlabeled Aβ (tracked by immunoassay), or radiolabeled Aβ (tracked by counting), or mixtures of the two (tracked independently by each method) in various ratios to examine the effect of Zn on Aβ aggregation at Aβ concentrations varying from 10^{-10} to 10^{-6} M. Bush *et al.* (*1*) report that the labeled and unlabeled peptides behave differently, but we found that in all cases ^{125}I-Aβ tracked unlabeled Aβ accurately, validating the use of the radioiodinated tracer in these experiments.

With the use of filtration (*1*) or centrifugation (*1*, *2*) to remove insoluble aggregates, we found that approximately 200 μM Zn^{2+} is required to aggregate half of 1 μM Aβ from solution at pH 7.4 and 37°C; this EC_{50} is about a hundred times higher than that determined by Bush *et al.* (*1*) from optical density measurements. The EC_{50} for Zn varies with Aβ concentration, with

somewhat higher Zn concentrations required to aggregate the same fraction of peptide at physiological concentrations. The deposition of Aβ at physiological concentrations onto plaques in AD tissue sections or homogenates is also enhanced by Zn, but only at Zn^{2+} concentrations above 50 μM (unpublished results). Although the rat and human Aβ peptides are certainly different in their behavior in some systems (*7*), we disagree with Bush *et al.* (*1*) that the peptides differ significantly in the concentration of Zn required to clear them from aqueous solution. In contrast, similar concentrations of Zn were required to aggregate half of either human or rat Aβ at peptide concentrations between 10^{-10} and 10^{-6} M.

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Response: We agree with the interpretation of Maggio *et al.* that Zn appears to induce a major conformational effect on $A\beta_{1-40}$ in vitro, as indirectly indicated by our findings that Zn(II), at physiologically relevant concentrations, provokes the rapid aggregation of human $A\beta_{1-40}$ into particles that are retained by filters of pore sizes 0.1 to 0.65 μm (*1*). In contrast, the rat and mouse form of the peptide is relatively unaffected by Zn, perhaps explaining why these species are spared cerebral amyloid pathology with age. To quantify the retention of $A\beta_{1-40}$, we assayed the amounts of peptide entering the filtrate by measuring optical density at 214 nm (OD_{214}). We validated this technique by preparing standard curves of OD_{214} versus peptide concentration for each of the experimental buffers used (*2*). These curves were linear over the range of concentrations used in these experiments (even after incubation with Zn), which indicated that the changes in absorbance observed in our studies most likely reflect changes in peptide concentration (*3*).

Our aggregation assay compares the OD_{214} of an incubated peptide solution to the OD_{214} of the same solution that has been filtered after the same incubation. Maggio *et al.* maintain that the changes in OD_{214} that we have reported in comparing a filtered to an unfiltered solution may be reflecting changes not only in concentration, but also in the peptide consequent to the act of filtration. Their comment does not take into account our other findings that support the interpretation that Zn(II) at ≤25 μM induces the rapid and abundant aggregation of Aβ. These findings (*1*) include (i) retention by filtration of zinc:Aβ complexes (with 1:1 stoichiometry) after the incubation of the peptide with 25 μM Zn(II), confirmed by the lack of retention of the tracer ^{65}Zn in the absence of peptide; and (ii) the ability of Zn(II) at 25 μM (and not EDTA) to rapidly induce the production of tinctorial amyloid from soluble $A\beta_{1-40}$. The observation of visible amyloid after treatment of $A\beta_{1-40}$ with Zn is independent of assays that depend on measurements of OD_{214}.

In this rebuttal, we present results of other experiments corroborating those obtained (*1*) with the use of OD_{214} measurments. To confirm that the reduction in OD_{214} after the filtration of Zn-incubated Aβ solutions is a result of retention of the aggregated peptide by the filtration membrane, we assayed both the filtrate and the retentate for Aβ by immunoassay. To measure the amount of Aβ that enters the filtrate after the filtration of a peptide solution that has been incubated with Zn, we developed an ELISA that uses immobilized Zn or Cu to capture Aβ peptide from solutions or suspensions (*4*). The results (Table 1) confirm our earlier observations that >60% of a 1 μM solution of $A\beta_{1-40}$ incubated with Zn(II) (≤8 μM) is lost after filtration. When we applied heated SDS sample buffer to the filter following the peptide filtration, and then assayed the eluted peptide by protein immunoblot (*5*), we confirmed (Fig. 1) that the peptide was retained by the filter in the presence of Zn, but not in the presence of EDTA.

Maggio *et al.* state that they do not detect precipitation of Aβ at peptide concentrations of ≤1 μM unless they incubate the peptide with Zn concentrations approximately an order of magnitude (>200 μM) greater than those that we used. Thus, the techniques used by these investigators appear to be unable to demonstrate precipitation of a 1 μM Aβ solution by 25 μM Zn(II), a strongly positive result that we have now confirmed by three different assay techniques (OD_{214} measurement, ELISA, and western blot). Since Maggio *et al.* are unable to reproduce a result that is, for us, a positive control, it is unlikely that they will be able to detect precipitation of Aβ at lower peptide concentrations.

We are presently examining the effects of physiological concentrations of zinc upon Aβ at peptide concentrations below the limit of detectability of 214 nm spectrophotometry. Because iodinating the peptide at a critical tyrosine residue (position 10) (*6*) abolishes the zinc-induced precipitation of Aβ (*1*), we do not believe that ^{125}I-Aβ is a suitable tracer for such studies (*7*). Therefore, we are exploring alternative means of labeling the peptide without damaging its physicochemical properties.

Fitzgerald questions the specificity of metal-induced $A\beta_{1-40}$ aggregation, point-

Table 1. Confirmation by immunoassay of zinc-induced precipitation of Aβ. $A\beta_{1-40}$ (1 μM, 400 μl) was brought to the metal concentrations indicated in tris-buffered saline (100 mM NaCl, 20 mM tris-HCl, pH 7.4), incubated, and filtered through 0.2 μm pore-size filters as previously described (*1*). The filtrate was diluted 1:100 in TBS and then an ELISA was performed for Aβ immunoreactivity against a standard curve (*4*). The data indicate the amount of peptide assayed in the filtrate as a fraction of peptide in the starting material (n = 3). Zn(II) chloride induced loss of $A\beta_{1-40}$ whereas Fe(III) citrate had no effect, confirming the results of the OD_{214} measurements.

Filtrate (μM)	Filtered fraction	
	Mean	SD
No metal	0.90	0.01
Zn(II) 8	0.39	0.08
Fe(III) 8	0.91	0.02
Zn(II) 25	0.20	0.13
Fe(III) 25	0.91	0.03

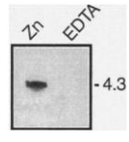

Fig. 1. Confirmation of zinc-induced retention of $A\beta_{1-40}$. $A\beta_{1-40}$ was brought to 1 μM (800 μl) in TBS ± 50 μM EDTA or 25 μM $ZnCl_2$, incubated, and filtered through a 0.2 μm pore-size filters as previously described (*1*). The membrane was washed twice with the incubation buffer (500 μl). Sample buffer (8% SDS, 68 mM tris-HCl, pH 6.8, heated to 95°C, 100 μl) was applied to the membrane and the filter centrifuged (10,000g × 5 min). Filtered sample buffer was collected in a clean vessel, 10 μl was loaded onto a tricine gel and a protein immunoblot was performed with 6E10 (gift of K. S. Kim and H. Wisniewski). Migration of the band relative to the molecular size markers (low M.W., Amersham, Arlington Heights, Illinois) is indicated. Figure illustrates n = 7 experiments.

ing out that our experiments (*1*) were conducted at a concentration (3 μM) that is lower than the physiological concentrations of Mg, Ca, Cu, and Fe. We have addressed this question by performing studies of the effects of metal ions at the maximum total concentrations at which they are found in biological fluids (Table 2). Out data indicate that Ca(II) and Mg(II) at 1 mM, and Fe(III) at 25 μM (Tables 1 and 2), do not significantly induce retention of $A\beta_{1-40}$ in the filtration assay. Cu(II) at 25 μM induces a 20% loss of OD_{214} in the filtrate, and Fe(II) at ≥8 μM induces a 40% loss (Table 2).

These findings confirm that the effect of 25 μM Fe(II) on $A\beta_{1-40}$ solubility resembles that of Zn(II) at the same concentration (*2*). To characterize this reaction, we incubated $A\beta_{1-40}$ with EDTA, Zn(II), or Fe(II) and examined the content of the pellets after centrifugation by immunoblot and Congo Red staining. Protein immunoblot analysis confirmed that abundant $A\beta_{1-40}$ precipitate was produced only when the peptide was incubated with Zn(II) at ≥8 μM (Fig. 2). A much smaller amount of $A\beta_{1-40}$ precipitate was induced by Fe(II), and sparse unstained particles, of much smaller average size than those induced by Zn, were observed under the microscope in the resuspended pellet. In contrast, Zn induces the formation of abundant particles that are visible by light microscopy, frequently as large as 10 to 40 μm in diameter, and stained by Congo Red (*1*). These observations were corroborated by capture ELISA assay of the filtrates, protein immunoblots of the filter retentates, and by OD_{214} measurements of the centrifuged supernatants (data not shown). The observation that filter retention of $A\beta_{1-40}$ exceeded pellet formation after incubation with Fe(II) indicates that there are significant qualitative differences between the reaction products induced by Fe(II) as opposed to Zn(II).

The most likely explanation for our findings is that the Zn(II)-induced aggregation of $A\beta_{1-40}$ produces a larger and denser reaction product that therefore sediments more readily under these conditions. Moreover, we have observed Zn(II)-induced $A\beta_{1-40}$ aggregation at metal ion concentrations of <3 μM, where similar concentrations of Fe(II) have no apparent effect. Therefore, Zn(II) and Fe(II) appear to exert their aggregating effects on $A\beta_{1-40}$ by differing mechanisms. Fe(II) has been reported to induce $A\beta_{1-40}$ aggregation by an oxidative mechanism that is abolished by the presence of antioxidants like ascorbate (*8*). We found that, whereas ascorbate abolished the filter retention of $A\beta_{1-40}$ incubated with Fe(II), it did not inhibit Zn(II)-induced aggregation (*9*). In summary, Zn appears to induce Aβ aggregation by structural interaction and the formation of assemblies which have a 1:1 stoichiometry, whereas Fe(II) induces Aβ aggregation by oxidation. Maggio *et al.* refer to their work on the effects of metal salts upon $A\beta_{1-40}$ solubility (*10*), which also noted the effects of iron on the precipitation of the peptide. They reported that iron induced ^{125}I-$A\beta_{1-40}$ precipitation, but only at metal concentrations >100 μM, and that the effects of Fe(II) and Fe(III) were indistinguishable in their experiments. As with the effects of Zn, we observe the aggregating effects of Fe(II), but not Fe(III), and at much lower metal ion concentrations than Maggio and coworkers reported for their studies of the solubility of ^{125}I-Aβ. This is further evidence of the apparent unsuitability of iodinated Aβ as a valid tracer for these kinds of experiments.

With regard to the inquiry of Fitzgerald on the effects of Si on amyloid formation, we have shown that Aβ readily adheres to a suspension of aluminum silicate (*2*), a major component of laboratory glassware, but is not precipitated by aluminum chloride (*1*). Contact with glass may artefactually contaminate AD-affected tissue preparations with aluminum silicate (*2*).

Because Aβ is normally a soluble component of cerebrospinal fluid (*11*) a systematic appraisal of the physiological factors that induce aggregation of the soluble peptide may yield clues to the pathological events that lead to Aβ deposition, the major feature of the neuropathology of AD and Down syndrome, and a complication of head injury (*12*). To this end, we have shown that the peptide is unusally vulnerable to the presence of Zn(II) and Fe(II). The task ahead is to determine whether any of these factors play a role in the pathophysiology of these disorders and hence interact with Aβ to form amyloid. Zn is the most likely of these factors in cerebral amyloidosis because it is highly concentrated in neurons, achieves high extracellular concentrations (>300 μM) with neurotransmission, is important for memory functions, and because there is growing evidence of a lesion of cerebral Zn metabolism in AD [reviewed in (*1*)]. Furthermore, rapid induction of $A\beta_{1-40}$ aggregation by Zn differentiates the behavior of the human from the rat form of the peptide (*1*), perhaps explaining the scarcity with which aged rats form cerebral amyloid. Meanwhile, precipitation by aluminum silicate does not differentiate the two forms (*9*).

Fitzgerald also points out that the report by Hershey *et al.* (*13*) of elevated concentrations Zn in the CSF in Alzheimer's dementia patients may not be valid. We agree that this sole report is insufficient to argue for the existence of a lesion of cerebral zinc metabolism in AD. The more compelling of the data in this regard are the consistent observations of decreased concentrations of Zn in cortical tissue from AD-affected hippocampus or temporal lobe (*14*). This suggests that Zn is not entering or being retained by these cells normally.

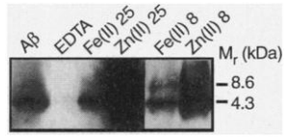

Fig. 2. Specificity of zinc-induced $A\beta_{1-40}$ centrifugal sedimentation. $A\beta_{1-40}$ (25 μM, 400 μl) was incubated in tris-buffered saline in the presence of the salts indicated (in μM), then centrifuged at 10,000g (20 min) as previously described (*1*). Supernatant was removed and pellet washed with the corresponding incubation buffer (1 ml), repelleted, and washed again (×2). Before final centrifugation, suspensions were removed to a new clean vessel so that the subsequent extraction step would not be extracting peptide that had adsorbed to the vessel wall. After centrifugation, pellets were dried and extracted into 40 μl of sample buffer (containing 4% SDS and 50 mM EDTA), heated to 95°C (10 min), and analysed by protein immunoblot with the use of 6E10. Aβ indicates 200 ng of synthetic $A\beta_{1-40}$ blotted as a standard.

Table 2. Specificity of zinc-induced $A\beta_{1-40}$ aggregation. $A\beta_{1-40}$ (1.6 μM) was incubated in TBS in the presence of the metal ion concentrations indicated and filtered as previously described (*1*). The fraction of peptide entering the filtrate is represented by the OD_{214} of the filtrate expressed as a fraction of the OD_{214} of the unfiltered incubation mixture (n = 3). All salts are chloride except Fe(III), which was citrate. Similar results for Fe(II) were obtained with the ammonium sulfate salt at the same concentration.

Filtrate (μM)		Filtered fraction	
		Mean	SD
No metal		0.92	0.07
Zn(II)	8	0.38	0.04
Zn(II)	25	0.32	0.01
Fe(II)	8	0.52	0.12
Fe(II)	25	0.50	0.01
Cu(II)	25	0.69	0.12
Fe(III)	25	0.88	0.01
Mg(II)	1000	0.89	0.06
Ca(II)	1000	0.91	0.07

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3. We account for the contribution of buffer absorbance by blanking the spectrophotometer on the incubation buffer. In our hands, the OD_{214} of carefully prepared 25 μM $ZnCl_2$, 100 mM NaCl, 20 mM tris-HCl, pH 7.4, is 0.056 (blanked on H_2O), which rises to 0.169 in the presence of 1 μM Aβ. Therefore, unlike Maggio *et al.*, we find that the OD_{214} of this buffer is not greater than the OD_{214} of the peptide alone. However, contamination with bacteria and organics will artefactually increase the OD_{214} of the buffers.
4. R. D. Moir, A. I. Bush, L. Rhodes, K. M. Rosenkranz, R. E. Tanzi, manuscript in preparation. This assay is capable of validly measuring Aβ that has been aggregated by Zn.
5. We have previously reported (*2*) that zinc-induced polymerization of Aβ is at least partially reversible by treatment with SDS.
6. This tyrosine residue is a phenylalanine in the rat species, and lies within the zinc-binding domain of the peptide. Hence, this residue may be critical for coordinating the high-affinity binding of Zn found in the human peptide, but absent in the rat peptide (*1, 2*).
7. ^{125}I-Aβ does not co-migrate with its unmodified precursor on native gel electrophoresis (A. I. Bush, unpublished observations), indicating that properties such as charge and conformation, that could relate to the peptide's ability to interact with Zn, have been altered by the modification.
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