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# Aqueous Dissolution of Alzheimer's Disease A $\beta$ Amyloid Deposits by Biometal Depletion\*

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**Zn(II) and Cu(II) precipitate A $\beta$  *in vitro* into insoluble aggregates that are dissolved by metal chelators. We now report evidence that these biometals also mediate the deposition of A $\beta$  amyloid in Alzheimer's disease, since the solubilization of A $\beta$  from post-mortem brain tissue was significantly increased by the presence of chelators, EGTA, *N,N,N',N'*-tetrakis(2-pyridyl-methyl) ethylene diamine, and bathocuproine. Efficient extraction of A $\beta$  also required Mg(II) and Ca(II). The chelators were more effective in extracting A $\beta$  from Alzheimer's disease brain tissue than age-matched controls, suggesting that metal ions differentiate the chemical architecture of amyloid in Alzheimer's disease. Agents that specifically chelate copper and zinc ions but preserve Mg(II) and Ca(II) may be of therapeutic value in Alzheimer's disease.**

A $\beta$  is the main component of the amyloid deposits that characterize the neuropathologic lesions of Alzheimer's disease (AD).<sup>1</sup> The mechanism leading to the precipitation of this normally soluble protein is unknown, but it is related to the pathogenesis of the disorder, since all mutations linked to familial AD alter A $\beta$  structure or metabolism (1), and the deposition of  $\beta$ -amyloid in the neocortex of transgenic mice overexpressing A $\beta$  is accompanied by most of the other neuropathological features of AD (2). We have previously found that Zn(II), Cu(II), and, to a lesser extent, Fe(III), at low  $\mu$ M concentrations, induce the rapid aggregation of synthetic A $\beta$  (3). These transition metal ions are highly concentrated in the neocortical regions most affected in AD, and all three metal ions are both

significantly elevated in the neuropil of these regions in Alzheimer's disease and further concentrated within amyloid plaque deposits (4).

We recently reported that Zn(II)- or Cu(II)-induced A $\beta$  precipitation is reversed by treating the aggregate with metal chelators (5, 6). We hypothesized that if the metal ions within brain amyloid mediated the assembly of A $\beta$  aggregates, then treating tissue with metal chelators should induce the solubilization of A $\beta$ . We tested this hypothesis by extracting A $\beta$  amyloid-bearing post-mortem brain tissue in the presence and absence of various metal ion chelators and assaying the distribution of A $\beta$  within the soluble and insoluble phases.

## EXPERIMENTAL PROCEDURES

**Tissue Selection**—Post-mortem tissues, stored at  $-80$  °C, were obtained from the National Health and Medical Research Council-supported Brain Bank at the University of Melbourne together with accompanying histopathological and clinical data. AD was assessed according to Consortium to Establish a Register for Alzheimer's Disease criteria (7). In order to examine the chemical architecture of the A $\beta$  deposition that is observed in non-AD aged brain, A $\beta$  immunohistochemistry was used to select age-matched control (AC) cases that did not reach Consortium to Establish a Register for Alzheimer's Disease criteria and in which amyloid deposition, if present, was detectable only in the form of diffuse plaques but not neuritic plaques.

**Selection of Chelators**—No available chelator is exclusively specific for any particular metal ion; therefore, we surveyed the effects of chelators that display various respective affinities for zinc and/or copper ions relative to more abundant metal ions such as calcium and magnesium. The  $pK_a$  values of *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) are as follows: Al(III), negligible; Ca(II), 3; Cu(II), 20.2; Fe(III), 14.4; Mg(II), negligible; Zn(II), 15.4. The  $pK_a$  values of EGTA are as follows: Al(III), 13.9; Ca(II), 10.9; Cu(II), 17.6; Fe(III), 11.8; Mg(II), 5.3; Zn(II), 12.6. The  $pK_a$  values of bathocuproine (BC) are as follows: Al(III), negligible; Ca(II), negligible; Cu(II), 6.1; Cu(I), 19.1; Fe(III), negligible; Mg(II), negligible; Zn(II), 4.1 (see Ref. 8).

**Sample Preparation**—The cortical meninges were removed, and gray matter (0.5 g) was homogenized using a DIAX 900 homogenizer (Heidolph & Co, Kelheim, Germany) for three 30-s periods at full speed, with a 30-s rest between strokes, in 3 ml of ice-cold phosphate-buffered saline (PBS), pH 7.4, containing a mixture of protease inhibitors (Bio-Rad), with the exception of EDTA, or in the presence of either various chelators or metal ions prepared in PBS. To obtain the PBS-extractable fraction, the homogenate was centrifuged at  $100,000 \times g$  for 30 min, and the supernatant was removed and divided into 1-ml aliquots. Protein within a 1-ml supernatant sample was precipitated using 1:5 ice-cold 10% trichloroacetic acid, and pelleted by centrifugation at  $10,000 \times g$  for 20 min. The pellet was prepared for polyacrylamide gel electrophoresis by boiling for 10 min in Tris-Tricine SDS-sample buffer containing 8% SDS, 10% mercaptoethanol, and 8 M urea. Total A $\beta$  in the cortical samples was obtained by homogenizing in 1 ml of PBS and boiling in sample buffer as above.

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<sup>1</sup> The abbreviations used are: AD, Alzheimer's disease; AC, age-matched control; BC, bathocuproine disulfonic acid; TPEN, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine; PBS, phosphate-buffered saline; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

**Polyacrylamide Gel Electrophoresis and Western Blotting**—Tris-Tricine polyacrylamide gel electrophoresis was performed by loading samples onto 10-well, 10–20% gradient gels (Novex, San Diego, CA), followed by transfer onto 0.2-mm nitrocellulose membrane (Bio-Rad). The A $\beta$  was detected using monoclonal antibodies WO2 (which detects A $\beta$ 40 and A $\beta$ 42 at an epitope between 5–8), G210 (which is specific for A $\beta$  species that terminate at carboxyl residue 40), or G211 (which is specific for A $\beta$  species that terminate at carboxyl residue 42) (9), in conjunction with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako, Denmark) and visualized using chemiluminescence (ECL, Amersham Pharmacia Biotech). Each gel included two or more lanes containing known quantities of synthetic A $\beta$  (Keck Laboratory, Yale University, New Haven, CT) as reference standards.

**Blot Scanning and Transmission Densitometry Assay for A $\beta$** —Blot films were scanned using a Relisys scanner with transparency adapter (Teco Information Systems, Taiwan), and densitometry was performed using Image 1.6 software (National Institutes of Health, Bethesda, MD). The dynamic range of the film/scanner was determined using a step tablet (catalog no. 911ST600, Eastman Kodak Co.), a calibrated film exposed by the manufacturer to provide steps of known increasing intensity. The quantifiable range of signal intensity for densitometric analysis of our A $\beta$  bands was based on the comparison with a curve obtained by scanning and densitometry of the step tablet. The dynamic range of the scanner was increased by using a transparency adapter rather than reflection.

For the survey comparing levels of A $\beta$  in post-mortem brain samples from AD cases and controls (Fig. 3), the combined signals generated from 4.3-kDa immunoreactive A $\beta$  (apparent monomer) and 8.6-kDa immunoreactive A $\beta$  (apparent dimer) were quantified. Successive ECL exposure times of 2 min, 5 min, 10 min, 15 min, and 30 min were routinely performed to establish the optimal exposure for each individual blot, so that the relative amounts of A $\beta$  measured by transmission densitometry remained in the linear response range of the assay, while determining at what point the signal from the A $\beta$  standards had reached saturating intensity. Preliminary blots were routinely performed to determine how the samples were to be subsequently diluted in order to try to ensure that the A $\beta$  signals fell within the quantifiable portion of the A $\beta$  standard curve. All of the experimental samples extracted from the same brain specimen were initially diluted to the same degree and included on the same blot for analysis (as in Fig. 3A). However, it was usually not possible to determine all of the A $\beta$  readings from one blot at one dilution. The A $\beta$  content varied broadly between the extracted samples (note the range in A $\beta$  intensity between the various extracts of the same brain specimens illustrated in the blots in Fig. 3A), and therefore it was usually necessary to perform subsequent individual blots on specific samples that had been further diluted in order to generate A $\beta$  signals that fell within the linear range of the standard curve.

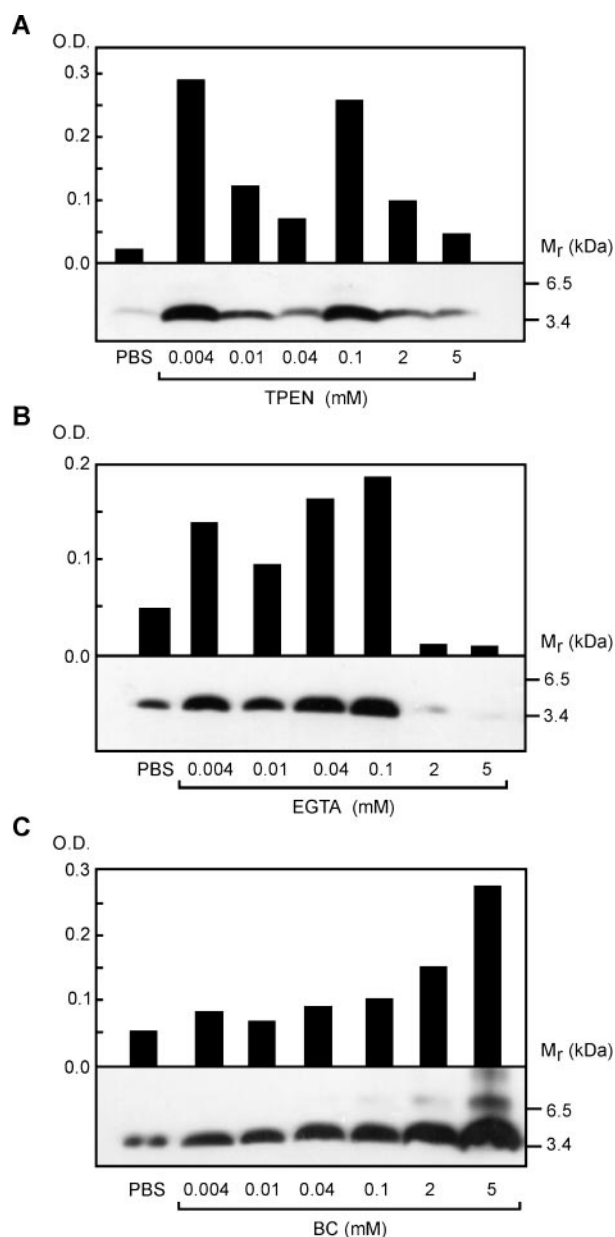
This technique was chosen for the A $\beta$  assay in preference to enzyme-linked immunosorbent assay, since it has the advantage of discriminating the  $M_r$  of the A $\beta$  immunoreactivity and therefore is less likely to inappropriately detect non-A $\beta$  species such as APP fragments, like those that have recently been found to have been inadvertently cross-reacting with APP in an assay that previously had been considered to be well characterized (10).

The efficiency of the trichloroacetic acid precipitation procedure was validated by testing samples of whole human serum diluted 1:10 to which had been added 2 mg of synthetic A $\beta$  1–40 or A $\beta$  1–42. A $\beta$  recovery was assessed by extracting the precipitate into SDS sample buffer and performing Western blot analysis against synthetic A $\beta$  standards as above. Protein in the trichloroacetic acid pellet was estimated by resuspending the pellet in water and assaying the protein recovery using a BCA assay (Pierce). This indicated that the efficiency of protein and A $\beta$  precipitation was approximately 90%. The efficiency of the 8 M urea solubilization was found to be higher and less variable than that of formic acid in a parallel, blinded assay conducted independently. All chemicals were obtained from Sigma unless otherwise indicated.

**Analysis of Metals**—The postcentrifugation pellets were dissolved in 4 ml of 3 N HNO<sub>3</sub> plus 1 N HCl for 24 h and then assayed by inductively coupled plasma atomic emission spectroscopy.

## RESULTS

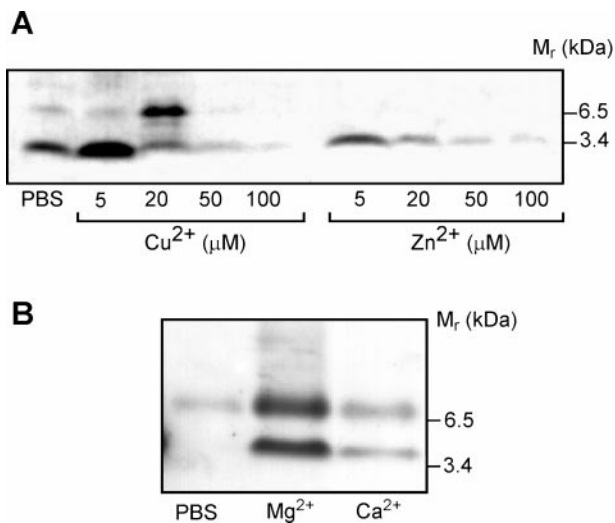
AD frontal cortex was compared with tissue from the same region of AC. A survey of the effects of the chelators at a range of concentrations (0–5 mM) on six AD cases confirmed that the solubilization of A $\beta$  was specifically enhanced by the presence of chelator (Fig. 1), although total trichloroacetic acid-precipi-



**FIG. 1. Release of A $\beta$  from sedimentable deposits by chelators.** Frontal cortex from an AD brain was homogenized in PBS, pH 7.4, with or without increasing concentrations of TPEN (A), EGTA (B), or BC (C). Following centrifugation, A $\beta$  in the supernatants was visualized by Western blot using anti-A $\beta$  monoclonal antibody WO2 (lower panels), and quantified by densitometry (graphs above corresponding blots). Although there is considerable variation in the optimum chelator concentration for the maximal recovery of A $\beta$  from case to case, these data are representative of 17 AD cases.

table protein was not affected by any of the chelators at the concentrations tested (data not shown).

Extraction of AD brain into PBS alone liberated a small amount of A $\beta$  into the soluble phase in every case, confirming previous reports (11–13). In contrast, homogenization in the presence of either EGTA or TPEN at concentrations between 0.004 and 0.1 mM significantly increased soluble A $\beta$  extraction. The optimum concentrations of EGTA or TPEN for the resubilization of A $\beta$  varied considerably from case to case and did not show linear concentration dependence. Typically, as illustrated in Fig. 1A, there was a biphasic response in A $\beta$  extraction as concentrations of EGTA or TPEN were increased. One peak typically occurred when homogenization was performed in the presence of 0.004 mM of either chelator. A second peak in

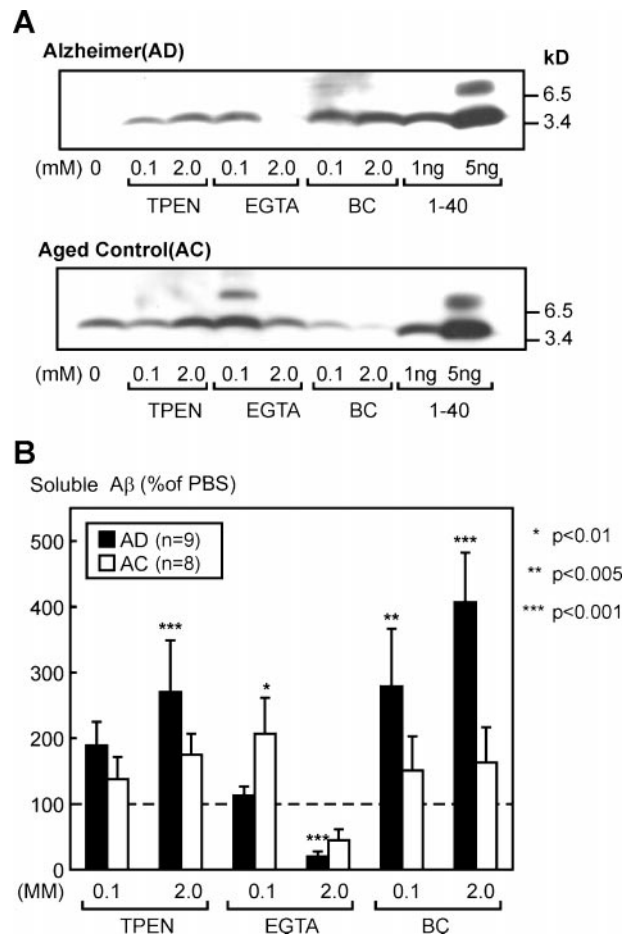


**FIG. 2. The effect of metals upon the solubility of brain-derived A $\beta$ .** *A*, Zn(II) and Cu(II) inhibit the solubilization of A $\beta$  by PBS extraction. Specimens of AD frontal cortex were homogenized in the presence of PBS or varying concentrations of Cu(II) (as sulfate) or Zn(II) (as sulfate). After centrifugation, A $\beta$  in the supernatants was visualized as described in Fig. 1. *B*, A $\beta$  in metal-depleted deposits is liberated into the soluble phase by Mg(II) and Ca(II). Samples of AD frontal cortex were homogenized in 2 mM EGTA, a condition that consistently abolishes the release of A $\beta$  (see Figs. 1 and 3) and removes Zn(II), Cu(II), and other metal ions from the solid phase of the homogenate. After centrifugation, the remaining (metal-depleted) pellets were treated with PBS (pH 7.4) alone, 2 mM Mg(II) in PBS, or 2 mM Ca(II) in PBS and then centrifuged again. Data shown are representative of A $\beta$  in the soluble fraction of the three treated samples, visualized as in Fig. 1.

A $\beta$  soluble extraction occurred at about 0.1 mM for EGTA and 2 mM for TPEN (although there was considerable case-to-case variation, and the case illustrated in Fig. 1A had an extraction peak in response to 0.1 mM TPEN). Both TPEN and EGTA were less effective at extracting A $\beta$  when present at concentrations in the millimolar range, and EGTA at  $\geq 2$  mM abolished the signal for A $\beta$  (Fig. 1B). In contrast, BC elicited a concentration-dependent increase in A $\beta$  extracted from AD tissue (Fig. 1C) plateauing at 10 mM. This finding is of interest because BC is highly selective for Cu(I), and the result is compatible with our recent finding that A $\beta$  rapidly binds and reduces Cu(II) to Cu(I) (6, 23), suggesting that a proportion of A $\beta$  assembly is mediated by Cu(I).

Insulin-degrading enzyme, a zinc-metalloproteinase, has been reported to cleave A $\beta$  in the brain and in biological fluids (15). To determine whether chelator-mediated augmentation of A $\beta$  solubilization was due to inhibition of this enzyme, we also performed homogenizations in the presence of 1 mM *N*-ethylmaleimide, a potent inhibitor of insulin-degrading enzyme. Enhancement of A $\beta$  signal was not observed above that of PBS alone (data not shown). To determine whether other enzymatic activities may be artifactually modifying the data, we compared extraction of the brain A $\beta$  at 4 °C to extraction at 37 °C. There was no decrease in A $\beta$  signal to suggest enhanced degradation at the higher temperature. These controls suggest that inhibition of A $\beta$ -cleaving enzymatic activities by chelators does not contribute to the generation of soluble A $\beta$  under these conditions.

To characterize the metal ions participating in the precipitation of brain-derived A $\beta$  and to investigate the nonlinear response of A $\beta$  extraction in the presence of EGTA or TPEN, we added additional metal ions to the extraction system. The presence of Cu(II) or Zn(II) in the PBS homogenization buffer abolished the increased extraction of A $\beta$  caused by chelator treatments (data not shown). Also, the presence of additional



**FIG. 3. Patterns of chelator-mediated release of brain A $\beta$  in AD and age-matched, non-AD tissue.** Post-mortem samples of AD frontal cortex ( $n = 9$ ) and age-matched controls ( $n = 8$ ) were treated with PBS, TPEN, EGTA, or BC (chelators at 0.1 and 2 mM), and soluble A $\beta$  was assayed by Western blot. *A*, the soluble material from the seven treatment conditions of each individual case were initially compared on the same blot. An iterative process was used to arrive at the final A $\beta$  concentration for each sample (per g, wet weight) that involved multiple blots quantified by densitometry with reference to two standards of synthetic A $\beta$ 1–40 (1 and 5 ng) as well as an 8  $\mu$ M urea extract of the starting tissue. The upper panel of *A* shows a representative blot of soluble A $\beta$  extracted from an AD case. The lower panel of *A* shows a representative blot of soluble A $\beta$  extracted from a control (AC). For the purposes of the illustration, similar densities of A $\beta$  signal in both the AC and the AD cases shown were achieved by loading more sample onto the AC blot and by slightly prolonging its exposure. However, when normalized against synthetic peptide standards, the amount of A $\beta$ /g of brain sample in typical AC specimens was less than AD (see Table II). *B*, a graphical representation of the effects of chelator-mediated release of brain A $\beta$  derived from data in Table II, which summarizes averaged ( $\pm$ S.E.) data from the AD and AC groups, where the amount of soluble A $\beta$  extracted by the six chelator treatments is expressed as a proportion of the amount of A $\beta$  solubilized by treatment with PBS alone (normalized to 100%) for each individual case.

Zn(II) ( $\geq 5$   $\mu$ M) or Cu(II) ( $\geq 50$   $\mu$ M) in the homogenization buffer without chelator abolished extraction of A $\beta$  due to treatment with PBS alone (Fig. 2A). Therefore, these metal ions can modulate the solubility of A $\beta$  in this system.

The presence of Cu(II) at 5  $\mu$ M in the PBS homogenization buffer without chelator increased the extraction of A $\beta$  by PBS (Fig. 2A). At pH 7.4, Zn(II) induces far more A $\beta$  aggregation than Cu(II), hence this result may be due to Cu(II) displacing Zn(II) from A $\beta$ . At 20  $\mu$ M, Cu(II) induces the appearance of an apparent SDS-resistant A $\beta$  dimer, which may be due to an oxidative modification of the peptide or may represent an intermediate produced during the process of A $\beta$  aggregation.

Because millimolar concentrations of TPEN or EGTA unex-

TABLE I  
Residual metal levels following treatment of brain homogenates with TPEN

Frontal cortex from AD ( $n = 10$ ) was homogenized in the presence or absence of 0.1 mM TPEN and metal levels in postcentrifugation pellets were determined using inductively coupled plasma atomic emission spectroscopy, normalized for the starting wet weight of the tissue sample. An asterisk indicates significant difference from treatment with PBS alone on  $t$  test ( $p < 0.01$ ). Results are shown  $\pm$ S.E.

	Zinc	Copper	Iron	Calcium	Magnesium	Aluminum
PBS	50.7 $\pm$ 4.9	11.9 $\pm$ 1.5	227 $\pm$ 28.8	202 $\pm$ 28.3	197 $\pm$ 39.1	44.0 $\pm$ 46.2
TPEN	33.2 $\pm$ 4.1*	9.8 $\pm$ 1.7	239 $\pm$ 31.7	210 $\pm$ 37.0	230 $\pm$ 39.2	65.0 $\pm$ 45.0

TABLE II  
Concentrations of A $\beta$  extracted by PBS, chelator, and 8 M urea treatment of AD and AC specimens

See legend of Fig. 3. Concentrations of A $\beta$  (in  $\mu$ g/g, normalized for specimen wet weight) extracted from AD and AC specimens by 8 M urea (representing estimated "total" A $\beta$  content), PBS, and two chelators (0.1 and 2.0 mM) were compared. To illustrate the variation, data from each case are shown. Percentage of the total is shown in parentheses.

	AD subject									X $\pm$ S.E.
	1	2	3	4	5	6	7	8	9	
Total A $\beta$	22	77	12	80	15	24	8	33	10	31 $\pm$ 9.3
PBS	0.2 (0.9)	1.3 (1.7)	0.1 (0.8)	0.3 (0.4)	0.9 (6.0)	0.5 (2.1)	0.4 (5.0)	1.6 (4.8)	0.6 (6.0)	0.7 $\pm$ 0.2 (3.1 $\pm$ 0.8)
TPEN, 0.1 mM	0.6 (2.7)	2.8 (1.7)	0.4 (3.4)	0.3 (0.4)	0.8 (5.3)	0.9 (3.8)	0.8 (10)	1.6 (4.8)	0.7 (7.0)	1.0 $\pm$ 0.3 (4.3 $\pm$ 0.9)
TPEN, 2 mM	0.3 (1.4)	1.8 (2.3)	0.9 (7.5)	0.8 (1.0)	2.0 (13)	1.3 (5.4)	1.2 (15)	2.9 (8.8)	0.5 (5.0)	1.3 $\pm$ 0.3 (6.6 $\pm$ 1.7)
EGTA, 0.1 mM	0.3 (1.4)	1.5 (1.9)	0.04 (0.3)	0.5 (0.6)	1.1 (7.3)	0.5 (2.1)	0.7 (8.8)	1.7 (5.2)	0.4 (4.0)	0.8 $\pm$ 0.2 (3.5 $\pm$ 1.0)
EGTA, 2 mM	0.1 (0.5)	0.8 (1.0)	0 (0)	0 (0)	0.2 (1.3)	0 (0)	0.1 (1.3)	0.5 (1.5)	0.1 (1.0)	0.2 $\pm$ 0.1 (0.7 $\pm$ 0.2)
BC, 0.1 mM	0.14 (0.6)	1.8 (2.3)	0.9 (7.5)	1.4 (1.8)	1.2 (8.0)	0.9 (3.8)	1.6 (20)	3.1 (9.4)	0.5 (5.0)	1.3 $\pm$ 0.4 (6.5 $\pm$ 1.9)
BC, 2 mM	0.8 (3.6)	3.3 (4.3)	0.8 (6.7)	1.6 (2.0)	1.7 (11.3)	1.5 (6.3)	2.3 (28.8)	3.7 (11.2)	1.5 (15.0)	1.9 $\pm$ 0.3 (9.6 $\pm$ 2.7)

	AC subject								X $\pm$ S.E.
	1	2	3	4	5	6	7	8	
Total A $\beta$	0.7	0.5	1.0	4.2	2.7	3.2	3.6	0.5	2.1 $\pm$ 0.52
PBS	0.17 (24)	0.16 (32)	0.03 (3.0)	0.13 (3.0)	0.18 (6.7)	0.11 (3.4)	0.66 (18.3)	0.06 (12)	0.19 $\pm$ 0.07 (12.8 $\pm$ 3.9)
TPEN, 0.1 mM	0.12 (17)	0.17 (34)	0.10 (10)	0.29 (6.9)	0.10 (3.7)	0.10 (3.1)	0.60 (16.7)	0.08 (16)	0.19 $\pm$ 0.07 (13.4 $\pm$ 3.7)
TPEN, 2 mM	0.22 (31)	0.17 (37)	0.10 (10)	0.38 (9.0)	0.26 (9.6)	0.09 (2.8)	1.1 (30)	0.09 (18)	0.30 $\pm$ 0.12 (18.4 $\pm$ 4.5)
EGTA, 0.1 mM	0.39 (55.7)	0.22 (44)	0.17 (17)	0.28 (6.7)	0.15 (5.5)	0.12 (3.8)	1.0 (27.8)	0.10 (20)	0.30 $\pm$ 0.1 (22.6 $\pm$ 7.0)
EGTA, 2 mM	0.15 (21.4)	0.03 (6.0)	0.03 (3.0)	0 (0)	0 (0)	0.04 (1.25)	0.2 (5.5)	0 (0)	0.06 $\pm$ 0.03 (4.6 $\pm$ 2.7)
BC, 0.1 mM	0.09 (12.9)	0.15 (30)	0.15 (15)	0.20 (4.8)	0.18 (6.7)	0.08 (2.5)	0.98 (27.2)	0.08 (16)	0.23 $\pm$ 0.11 (14.3 $\pm$ 3.7)
BC, 2 mM	0.03 (4.3)	0.04 (8.0)	0.15 (15)	0.24 (5.7)	0.30 (11)	0.08 (2.5)	1.16 (32)	0.10 (20)	0.26 $\pm$ 0.14 (12.3 $\pm$ 3.5)

pectedly suppressed A $\beta$  resolubilization, we suspected that Mg(II) or Ca(II) may participate in the resolubilization of A $\beta$ . Mg(II) and Ca(II) are more abundant than Cu(II) and Zn(II) in brain samples. Therefore, given the relative affinities of the chelators used, sequestration of Mg(II) and Ca(II) would require higher chelator concentrations than those necessary to complex Zn(II) and Cu(II). Samples of frontal cortex (0.5 g) from AD were homogenized in 2 mM EGTA, a condition that consistently abolishes the solubilization of A $\beta$  (see Fig. 3) while removing Zn(II), Cu(II), and other metal ions from the solid phase of the homogenate. The homogenates were centrifuged at 100,000  $\times$   $g$  for 30 min, and the supernatants were discarded. The remaining (metal-depleted) pellets were rehomogenized in a further 2 ml of PBS (pH 7.4) alone, 2 mM MgCl<sub>2</sub> in PBS, or 2 mM CaCl<sub>2</sub> in PBS, and the homogenates were subjected to centrifugation again at 100,000  $\times$   $g$ . A $\beta$  in the soluble fraction was visualized by Western blot with W02 as described. When Mg(II) (2 mM) or Ca(II) (2 mM) was added to the homogenization buffer, there was no appreciable alteration in the extraction of soluble A $\beta$  (data not shown). However, when supplemented to the pellet fraction of a brain homogenate previously depleted of metals by treatment with 2 mM EGTA during homogenization, Mg(II), and to a lesser extent Ca(II), both resolubilized the sedimentable A $\beta$  (Fig. 2B). Taken together, these data indicate that although removal of metal ions like Zn(II) and Cu(II) may be necessary for the resolubilization of A $\beta$  deposits, the presence of Mg(II) and Ca(II) is required for the sedimentable A $\beta$  to resolubilize. Therefore, the optimal chelator concentration for the resolubilization of A $\beta$  deposits depends upon an interplay of antagonistic factors, which may explain the nonlinear response of A $\beta$  extraction to increasing chelator concentrations (Fig. 1, A and B) and the case-to-case variability of the chelator concen-

trations required to achieve maximal extraction of A $\beta$ .

In order to investigate which metal ions are removed by chelator treatments, we measured the amounts of various metals (aluminum, iron, magnesium, calcium, copper, and zinc) remaining in the brain pellet after treatment with PBS with or without chelator. Analysis of the effects of 0.1 mM TPEN was performed first, since this treatment induced an increase in soluble A $\beta$  in the first six AD samples analyzed and because complete complexation of Mg(II) and Ca(II) was unlikely at that concentration of chelator.

The observed increase in extractable A $\beta$  correlated with significant depletion (30%) in zinc and, to a lesser extent, copper, in each of 10 AD cases examined, when compared with PBS-treated tissue. No other metal measured was significantly influenced by treatment at this concentration (Table I). A survey of the metal content of pellets taken from AD brain homogenates ( $n = 2$ ) treated with the complete range of chelator concentrations described in Fig. 1, confirmed that EGTA treatment at  $\geq 2$  mM depleted (>30%) the sample of zinc, calcium, and magnesium, whereas treatment with TPEN at similar concentrations depleted zinc, copper, calcium, and iron. Measurement of metals remaining in the pellet following treatment of these samples over the range of BC concentrations studied indicated that none of the metals was depleted (data not shown). Since BC has an affinity for Cu(I) that is 13 orders of magnitude greater than for Cu(II), the lack of detectable total copper depletion caused by treatment with BC is not unexpected, since copper levels were relatively low in these preparations and the proportion of copper that exists as Cu(I) is likely to be small.

To determine the consistency of chelator effects upon A $\beta$  extraction from brain, we surveyed a larger sample of speci-

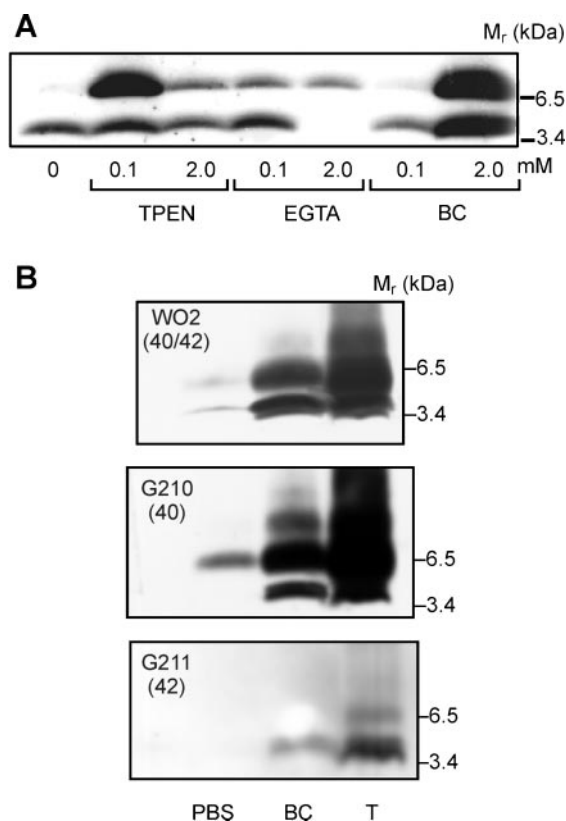
mens using two chelator concentrations (0.1 and 2 mM), and also measured the total amount of A $\beta$  in the samples by 8 M urea solubilization. After measuring the effects of treatment with the three chelators upon AD ( $n = 9$ ) and AC ( $n = 8$ ) brain samples, a significant pattern emerged (Fig. 3). For AD cases, significant increases of solubilized A $\beta$ , compared with the baseline amount liberated by PBS treatment, were induced by TPEN at 2 mM (2.7-fold,  $p < 0.001$ ) and BC at 0.1 mM (2.8-fold,  $p < 0.005$ ) and at 2 mM (4.1-fold,  $p < 0.001$ ). The effects of chelators upon the release of A $\beta$  from the AC group were markedly attenuated and therefore did not reach significance with the exception of the effect of 0.1 mM EGTA, which induced a significant increase (2-fold,  $p < 0.01$ ). These data support the possibility that Zn(II) and Cu(I) maintain the aggregated state of A $\beta$  in AD brain but are less important in the architecture of A $\beta$  aggregates in AC. EGTA (2 mM) inhibited the extraction of A $\beta$  in both AD (decreased 80%,  $p < 0.001$ ) and AC (decreased 50%, not significant) groups. This result is compatible with the extraction of Ca(II) and Mg(II) from the tissue homogenates, since these are metal ions that are required for the release of A $\beta$  from deposits that have been depleted of zinc and copper (Fig. 2). The cases analyzed in Fig. 3 were also assayed with reference to the total amount of A $\beta$  extracted from the individual brain specimens (Table II). The concentration of total A $\beta$  in the AD specimens was much greater (31  $\mu\text{g/g}$ ) than the total amount in the AC samples (2.1  $\mu\text{g/g}$ ). The concentration of A $\beta$  in AD brains that was extracted by PBS alone was  $0.7 \pm \mu\text{g/g}$ , representing 3.1% of total A $\beta$ . The amount of A $\beta$  in AD brain extracted by a single treatment with 2 mM BC increased significantly to  $1.9 \pm \mu\text{g/g}$ , representing 9.6% (range 2.0–28.8%) of total A $\beta$ . This proportion is likely to be an underestimate of the amount of A $\beta$  that is assembled by biometals, since the result was achieved by exposing the individual brain specimens to only one brief chelator treatment. Repeated extraction cycles resulted in further A $\beta$  release, up to 50% of the starting values. We limited the highest concentration of BC to 2 mM for comparison with other chelators at equimolar concentrations, because our initial data (Fig. 1) indicated that millimolar concentrations of TPEN and EGTA suppressed A $\beta$  solubilization.

Treatment of AD specimens with chelators generated an apparent SDS-resistant A $\beta$  dimer (immunoreactivity migrating at approximately 8.6 kDa) that was not evident when the specimen was treated with PBS alone in over 60% of cases (Fig. 4A). Frequently, the appearance of an 8.6-kDa A $\beta$  species was not accompanied by a proportional increase in the amount of apparent A $\beta$  monomer (Fig. 4A). These findings are relevant because SDS-resistant dimeric forms of A $\beta$  purified from AD brain have been reported to possess increased neurotoxic properties (16). The possibility that there is a specific metal ion-mediated abnormality of neurotoxic A $\beta$  dimer assembly is being investigated further.

We analyzed Western blots of brain extracts with antibodies that are specific for A $\beta$ X-40 (G210) and A $\beta$ X-42 (G211) (Fig. 4B), since the latter A $\beta$  subspecies is enriched in AD amyloid plaques (17). We found that treatment with BC significantly increased the solubilization of both A $\beta$  subspecies in AD samples, indicating that A $\beta$ X-42, while less soluble than the more abundant A $\beta$ X-40 (18) is nonetheless released by chelation of Cu(I).

#### DISCUSSION

These data indicate that there is a pool of A $\beta$  within the affected neocortex in AD that is held in sedimentable aggregates by metal ions, likely to be Cu(I) and Zn(II), and that these aggregates are solubilized by treatment with chelators. Mg(II) and Ca(II) were found to be essential for the release of A $\beta$ . The microanatomical site of these collections cannot be determined



**FIG. 4. Dissection of some components of metal ion-assembled brain A $\beta$  deposits.** A, extraction of soluble, SDS-resistant A $\beta$  dimers by chelator treatment. Shown is a representative Western blot of the AD samples that exhibit the release of a soluble A $\beta$  dimer when treated, as in Fig. 3, with 0.1 and 2 mM TPEN, EGTA, or BC. B, treatment with chelators promotes the solubilization of A $\beta$ 40 and A $\beta$ 42 from AD brain tissue. A representative AD specimen was divided and treated with PBS  $\pm$  5 mM BC, or 8 M urea to estimate total A $\beta$  content (T). Western blots of the extracts were probed with monoclonal antibodies WO2 (raised against residues 5–16, recognizing many A $\beta$  subspecies including A $\beta$ 40 and A $\beta$ 42), G210 (raised against residues 35–40, recognizing A $\beta$ 40), or G211 (raised against residues 35–42, recognizing A $\beta$ 42).

by our methods, but it is likely to be extracellular, since this is where A $\beta$  deposition in AD is readily demonstrable by morphological techniques and because chelator treatment of AC tissue (possessing much less extracellular plaque deposit) did not release as much A $\beta$ . The possibility of the artifactual combination of cellular metal ions with soluble A $\beta$  leading to A $\beta$  precipitation as a consequence of the tissue homogenization must also be considered. However, since the precipitated fraction of A $\beta$  in AD neocortex is much greater than the soluble cellular pool, this possibility is unlikely to contribute substantially to the phenomenon that we have observed. Other recent observations detecting enrichment of zinc, copper, and iron in amyloid deposits by histological means (4) support the likelihood that our observations reflect the chemical structure of A $\beta$  assembly in amyloid deposits. A $\beta$ -associated, Zn/Cu-metalloproteins apolipoprotein E (19) and  $\alpha$ -2-macroglobulin (20–22), may also participate in the reactions we have described.

Our data support the development of chelator compounds as chemotherapeutic agents for AD. One previous clinical trial of a chelator compound, desferrioxamine, was reported to significantly arrest the progression of the disease (14), but no further attempts to reproduce this finding have been reported. Desferrioxamine, like all chelators, is not perfectly specific for a particular metal ion, and although the desferrioxamine trial was thought to target Al(III), it is possible that the beneficial effect of the treatment was due to chelation of Fe(III), Cu(II),

and Zn(II). Our current findings indicate that an ideal therapeutic to dissolve A $\beta$  amyloid would involve a compound that is relatively selective for Cu(I), Zn(II), and possibly Fe(III); that does not sequester Mg(II) or Ca(II); and that coordinates metal ions in the cerebral amyloid mass but not systemically.

We have recently concluded a larger study comparing soluble and insoluble A $\beta$  in AD and AC brains and have found a significant correlation between the PBS-extractable A $\beta$  component and disease severity.<sup>2</sup> Although representing only a small portion of the total A $\beta$  load, an approximate 3-fold difference in the levels of the most readily mobilized A $\beta$  fraction distinguished AD from non-AD in an age-matched population. The present study suggests that 4–7-fold increases in PBS-extractable A $\beta$  can be achieved by direct chelation. At the concentrations used, this effect is observed without apparent impact upon the solubility of other proteins. We have observed that chelator concentrations as low as 4  $\mu$ M were effective at resolubilizing A $\beta$  deposits from AD brain samples, which indicates that delivering an effective biometal-depleting compound to the amyloid load *in vivo* may not necessitate biologically incompatible doses. Clearly, compounds targeted to the dissolution of aggregated amyloid only have promise as therapeutic agents if the resolubilized and potentially toxic A $\beta$  can be effectively cleared from the AD brain.

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<sup>2</sup> C. McLean, R. A. Cherny, F. Fraser, S. J. Fuller, M. J. Smith, K. Beyreuther, A. I. Bush, and C. L. Masters, submitted for publication.

## **Aqueous Dissolution of Alzheimer's Disease A $\beta$ Amyloid Deposits by Biometal Depletion**

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