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# NEW $^{14}$C AGES ON CELLULOSE FROM *DIPROTODON* GUT CONTENTS: EXPLORATIONS IN OXIDATION CHEMISTRY AND COMBUSTION

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**ABSTRACT.** We report radiocarbon ages on cellulose isolated from the gut contents of a *Diprotodon* found at Lake Callabonna, South Australia. The maximum age obtained corresponds to a minimum age of >53,400 BP for this extinct giant marsupial. This is older than, and hence consistent with, the generally accepted Australian megafauna extinction window. We argue that dichromate and other strong oxidants are less selective than chlorite for lignin destruction in wood, and our results suggest that ages approaching laboratory background can be obtained using a repeated pretreatment sequence of chlorite-alkali-acid and measurement of the sometimes discarded 330 °C combustion fraction.

## INTRODUCTION

The largest known extinct marsupial, *Diprotodon opatum*, is an Australian megafaunal icon. The largest assemblage of fossil *Diprotodon* remains is at Lake Callabonna, South Australia, a "veritable necropolis of gigantic extinct marsupials and birds which have apparently died where they lie, literally, in hundreds" (Stirling 1894). Callabonna is a small dry lake basin in the arid zone, between the large salt lakes Eyre and Frome (Figure 1). Age estimates for these extinct animals have changed over time. Stirling (1900), for example, accepted then-current geological advice in assigning the fossiliferous lacustrine sediments a Pliocene age. Wells and Tedford (1995) used stratigraphic correlation with the Frome playa to suggest an age of 200–700 kyr, while Roberts et al. (2001) used optically stimulated luminescence (OSL) dating on sand attached to a museum *Diprotodon* bone from Callabonna to estimate an age of 75 ± 9 kyr.

Direct radiocarbon dating of extinct Australian megafauna skeletal remains has yielded a bewildering array of apparent ages (Baynes 1999). All of these are almost certainly incorrect, because with rare exceptions collagen is absent in Australian megafaunal remains, and none of those examples were based on the purified proteins or amino acids, which can yield defendable ages (Gillespie and Brook 2006; Gillespie et al. 2006). To circumvent this difficulty, Grün et al. (forthcoming) sampled a transect of South Australian megafauna sites for electron spin resonance (ESR) and uranium-series (US) dating. The extant and extinct marsupial tooth samples covered a modern annual rainfall range of 250 to 775 mm, and none yielded combined US-ESR ages significantly younger than the 40–51 kyr extinction window proposed by Roberts et al. (2001).

Lake Callabonna was not included in that study, but Stirling (1900) describes a remarkable discovery from the 19th century survey and excavations there:

> "Associated with the skeletons of *Diprotodon*, in a relative position which corresponded with that of the abdominal cavity, were occasionally found loosely aggregated globular masses of what were judged to be the leaves, stalks, and smaller twigs of some herbaceous or arboreal plants. The fragments are very uniform in length, thickness and character, rarely exceeding an inch in length and a line in thickness. They are solid, often irregularly branched, frequently retaining portions of the bark, and have their ends often frayed or crushed, as if by the action of teeth."

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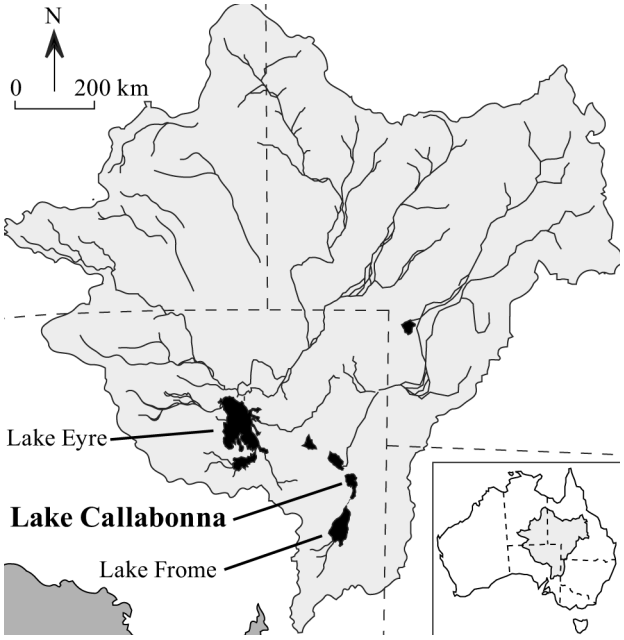


Figure 1 Map of the Lake Eyre basin, with an inset of Australia, showing the location of Lake Callabonna where the *Diprotodon* gut contents were found.

Samples of this plant material were collected in 1893 by A H C Zietz, then deputy director of the South Australian Museum, where they have been stored, wrapped in newspaper, ever since. Stirling's opinion changed somewhat after microscopic examination of the plant remains: He found no leaves and very little bark, and concluded that they were intestinal contents.

In 1957, Douglas Mawson, then professor of geology at the University of Adelaide, sent 60 g of one gut contents sample to the Lower Hutt, New Zealand, laboratory for $^{14}C$ analysis, together with teeth from the *Diprotodon* skeleton within which the plant material had been discovered. The gut contents sample was given no chemical pretreatment and a lot of chlorine was evolved during combustion, while the teeth were given a simple acid wash to remove a carbonate coating. Fergusson and Rafter (1959) reported an age of >40,000 yr (NZ-205) for the "crop contents" and 6700 ± 250 yr (NZ-206) for "dentine" from the teeth.

In the intervening 50 yr, $^{14}C$ methodology has moved on and it is now possible to achieve finite ages approaching 60,000 BP with suitably rigorous procedures (e.g. Hogg et al. 2006; Pigati et al. 2007). Given the current debate on the timing of megafaunal extinction, we considered that it would be valuable to revisit the dating of these particular gut contents, to investigate whether a finite age could be obtained, or to improve the lower limit beyond the original >40,000 BP.

Here, we report new $^{14}C$ measurements on some of the remaining *Diprotodon* gut contents sampled for NZ-205, located in the Callabonna collections at the South Australian Museum in 2003. No collagen has been found in Callabonna bones, and no megafauna dung, which can yield reliable $^{14}C$ dates (e.g. Martin et al. 1961; Mead and Agenbroad 1998), has been reported, so the gut contents provide a rare opportunity for $^{14}C$ to be relevant for Australian extinct megafauna. This twiggy plant material, midway between diet and dung, is the next best thing to dating the animal itself. The photo

in Figure 2 shows untreated fragments of the gut contents, and others after soaking overnight with 20% sodium hypochlorite in 0.05M NaOH. In the course of this work, we explored simpler alternatives to the standard α-cellulose extraction, and the extent to which different stages of the procedure remove contamination.


Figure 2 Photograph of plant fragments from the *Diprotodon* gut contents: upper 2 samples untreated, lower 2 samples after overnight soaking in alkaline hypochlorite bleach.

## METHODS AND RESULTS

Pretreatments were simplified from the Jayme-Wise method for α-cellulose (e.g. Loader et al. 1997; Gaudinski et al. 2005) and the ABOX method (Bird et al. 1999). Chemical processing was carried out in a fume hood in an over-pressurized, hepa-filtered chemistry laboratory at the ANU Department of Nuclear Physics. We used 10-mL loosely capped PTFE tubes with ultrasonic agitation in a controlled-temperature water bath. Insoluble residues were separated by centrifugation at 2000 rpm, and MilliQ™ water was used for all washing steps. Three separate pretreatment regimes were investigated as follows:

1. About 40 mg of gut contents sample GC-1 was digested at 80 °C in 0.01M HCl, with 100 mg $NaClO_2$ added at 0, 1, 2, and 3 hr; washed; heated with 2M HCl for 1 hr; and washed again. The oxidized (bleached) residue was treated with 1M NaOH for 1 hr at 80 °C, washed, heated in 1M HCl for 1 hr, and washed to neutrality.
2. A subsample of the chlorite-alkali-acid residue from step 1 was digested at 60 °C with 0.1M $K_2Cr_2O_7$ in 2M $H_2SO_4$ for 1 hr, then washed to neutrality.
3. A fresh 170-mg sample was given the chlorite-alkali-acid sequence twice at 80 °C, using 100 mg $NaClO_2$ in 6M HCl for each 2.5-hr oxidation, 1M NaOH for 1 hr, and 2M HCl for 2 hr, with washes between each stage, and final washing to neutrality. When dry, about 36 mg of very thin white fibers remained, resembling a tiny piece of handmade paper.

The residues remaining after procedures 1 to 3 were step-combusted at 330, 650, and 850 °C according to the Bird et al. (1999) procedure, then converted to graphite for AMS measurement; where $CO_2$ yields were inadequate, the 650 and 850 °C fractions were combined for graphitization. Blanks for the combustion/graphite preparation system were made from untreated Ceylon graphite, which was step-combusted and the 850 °C fraction reconverted to graphite in the same apparatus used for the gut contents samples (negligible $CO_2$ was recovered from the 330 and 650 °C fractions).

$^{14}$C measurements on the *Diprotodon* gut contents sample and combustion blanks are given in Table 1. A weighted mean of the 5 blanks (0.093 ± 0.012 pMC) was subtracted from sample measurements before age calculation. Figure 2 shows the pretreatment chemistry, combustion temperature, and $^{14}$C age of fractions prepared from sample GC-1.

Table 1 $^{14}$C measurements on *Diprotodon* gut contents sample GC-1 from Lake Callabonna, South Australia, showing pretreatment chemistry, step-combustion temperature, and graphite yield. A weighted mean combustion/graphite preparation blank (0.093 ± 0.012 pMC) based on the 850 °C fraction of 5 step-combusted Ceylon graphites has been subtracted from each of the measured pMC values.

| Lab # ANUA- | Chemistry sequence | Combust. temp. (°C) | Graphite (mg) | pMC (background subtracted) | $^{14}$C age (yr BP) | +1 σ | –1 σ |
|---|---|---|---|---|---|---|---|
| 31210 | chlorite-alkali-acid | 330 | 0.6 | 0.25 ± 0.03 | 48,130 | 1000 | 910 |
| 31211 | | 650 + 850 | 0.3 | 0.31 ± 0.04 | 46,400 | 1110 | 970 |
| 32712 | chlorite-alkali-acid-dichromate | 330 | 0.7 | 0.25 ± 0.03 | 48,130 | 1000 | 910 |
| 32709 | | 650 + 850 | 0.2 | 0.64 ± 0.08 | 40,580 | 1070 | 950 |
| 32711 | chlorite-alkali-acid ×2 | 330 | 1.0 | 0.08 ± 0.02 | 57,280 | 2310 | 1790 |
| 32708 | | 650 | 0.3 | 0.43 ± 0.03 | 43,775 | 580 | 540 |
| 32710 | | 850 | 0.4 | 0.12 ± 0.02 | 54,025 | 1470 | 1240 |

## DISCUSSION

### *Diprotodon* Age

We regard the oldest $^{14}$C age determined for *Diprotodon* gut contents sample GC-1 as a minimum age of >53,400 BP. Although the background-subtracted pMC value in Table 1 differs by more than 3 σ from zero, the Ceylon graphite blanks used for background subtraction are a measure only of contamination introduced during combustion and graphitization. These blanks do not capture any residual contamination that may remain in the wood samples after pretreatment. It is clear from Table 1 that such contamination is an issue, with the various pretreatments removing more or less of it, and we cannot be confident that even the oldest, apparently finite age is not affected by residual contamination. The only way of obtaining such assurance would have been to obtain similar material to the sample of interest that was known to be >70 kyr, and to put this through the entire procedure. Such material was not available to us at the time. Consequently, a strict limit on the $^{14}$C age of the gut contents at 2 σ is >53,400 BP.

This is consistent with, but more stringent than, the earlier minimum age of >40,000 yr obtained almost 50 yr ago by Fergusson and Rafter (1959). As noted by Stirling (1900) and Tedford (1973), the *Diprotodon* and other extinct megafauna became mired in the surface sediments of Lake Callabonna, and the OSL age of 75 ± 9 kyr determined by Roberts et al. (2001) reflects regional drying ~75–70 kyr following the oxygen isotope stage 5a wet phase (DeVogel et al. 2004; Magee et al. 2004). The local giant marsupials and birds died because the waterhole to which they were tethered vanished, but as several other megafauna sites are reliably dated to younger ages, they did not become extinct then (Roberts et al. 2001; Grün et al., forthcoming). Our >53,400 BP result is significantly older than the Roberts et al. (2001) extinction window of 40–51 kyr, and beyond all but the most optimistic first arrival times for humans in Australia. As Stirling (1900) observed on Lake Callabonna megafauna excavations, “no indication whatever was met with of the contemporaneous presence of man.”

### Sample Pretreatment

The rationale behind α-cellulose preparation from wholewood is that organic solvent extraction removes resins and lipids; chlorite oxidation selectively targets the lignin fraction for removal, leaving "holocellulose"; and finally, strong alkali extraction removes "hemicellulose"—carbohydrates other than cellulose. This has become a favored pathway for the isotopic analysis of wood, although the first 2 stages are very time consuming (Loader et al. 1997; Cullen and Grierson 2005; Gaudinski et al. 2005).

To simplify the procedure, we followed Bird et al. (1999) and Santos et al. (2001) by not using solvent extraction on these ancient woody fragments, and an inert gas blanket was not used during alkali extractions because a hot 2M acid wash should take care of any absorbed atmospheric $CO_2$ (e.g. Hoper et al. 1998; Hatté et al. 2001).

Previous work (e.g. Chappell et al. 1996) has shown that holocellulose prepared with chlorite oxidation alone does not generally yield reliable ages for very old samples. Hence, our first experiment already incorporated an alkali extraction as well as a subsequent hot acid wash. This yielded a $^{14}C$ age of 48,000 BP. The addition of a dichromate oxidation step, as used in the ABOX method for wood by Bird et al. (1999) and Santos et al. (2001), did not change the age. Further, the reaction had to be stopped after 1 hr because of rapid sample destruction, as noted for cellulose by Wolbach and Anders (1989). Finally, the oldest age of 57,000 BP was obtained from the sample subjected twice to a chlorite-alkali-acid chemistry sequence.

These chemistry variations suggest that there may be quicker ways to isolate cellulose from wood. In our experience with GC-1 and with modern wood samples (not shown), a hot alkali extraction after chlorite oxidation removes yellow-brown material falling within the operational definition of humic acids—organic material insoluble in acid but soluble in alkali. Removal of this partly oxidized substrate, which is likely to be carboxyl-enriched, allows fresh chlorite to focus on oxidizing the remaining polymeric lignin. Alkali extraction after dichromate oxidation of wholewood has a similar outcome, i.e. humic-like substances are removed, but dichromate is less selective than chlorite because it attacks the desired cellulose as well as lignin and other components of wholewood.

Gillespie (1990, 1997) found that strong oxidation of charcoal with 10% $KClO_3$ in 6M $HNO_3$ removed contamination from humic acids better than standard ABA chemistry, and that alkali extraction after the oxidation removed more humic-like material, yielding older ages for the residue. Bird et al. (1999) similarly demonstrated that strong dichromate oxidation was beneficial for charcoal, but did not employ alkali extraction after the oxidation. For wood samples, however, the application of strong non-selective oxidants (e.g. chlorate/nitric acid or acetic acid/nitric acid) has been less successful and can actually increase the contaminant to sample ratio (Gillespie 1997; Gaudinski et al. 2005). Bird et al. (1999) found with the ABOX-SC method applied to charcoal that the 330 °C combustion fractions were often too young, and suggested that they could be discarded. Santos et al. (2001) discarded the 330 °C combustion fractions from wood samples, obtaining similar maximum ages from 910 °C fractions of step-combusted residues after ABOX chemistry and the Loader et al. (1997) α-cellulose method on the same wood. In our gut contents sample, we found that about 2/3 of the residues burned at 330 °C in pure $O_2$ gas, which is not unreasonable for cellulose. We also found that the 330 °C combustion fractions of cellulose prepared by our 3 different chemistry sequences yielded older ages than all higher-temperature fractions (Figure 3).

Our gut contents sample is unusual, probably consisting of young plant tissue that may not have the well-developed lignocellulose structure of mature wood, and it has been incompletely digested by a

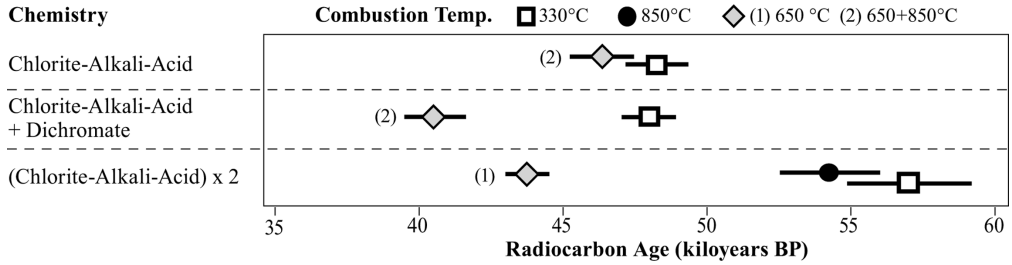


Figure 3 Pretreatment chemistry, stepped-combustion temperatures, and AMS $^{14}C$ ages for cellulose prepared from the *Diprotodon* gut contents sample.

*Diprotodon*. Some high-temperature fractions may be compromised by their small size, and several details of both chemistry and combustion require further experimentation to optimize the procedures. Nonetheless, the results reported here suggest that chlorite has the advantage over dichromate for analyzing wood samples because it is a more selective oxidant that targets lignin for removal, and that the strategy of discarding the 330 °C combustion fraction from wood may not be justified. The identity of the plant species from the last meal of this *Diprotodon* is being pursued using microscopy and X-ray difraction.

## CONCLUSIONS

We obtained a minimum age of >53,400 BP from well-preserved cellulose isolated from the gut contents of a *Diprotodon* found at Lake Callabonna. An efficient decontamination procedure, using a repeated sequence of chlorite oxidation-alkali-acid chemistry, with low-temperature combustion, produced the oldest results. This places the death of one particular *Diprotodon* from Lake Callabonna beyond the earliest human arrival in Australia, and older than the currently accepted megafauna extinction window. We suggest that refinement of the chemistry for our shortcut to cellulose, and a simpler combustion procedure, may offer a more fruitful approach than the ABOX-SC method for isotopic analysis of wood.

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