THE NATURE OF URANIUM OCCURRENCE IN THE LEAVES OF *COPROSMA AUSTRALIS* (A. RICH.) ROBINSON

By N. E. WHITEHEAD,* R. R. BROOKS,* and P. J. PETERSON†

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Summary

The distribution and chemical form of uranium was investigated in leaves collected from plants of *C. australis* (Rubiaceae) growing in a mineralized soil in the Buller Gorge, N.Z. Only small amounts of uranium (< 10%) were found in a low molecular weight form. The predominant occurrence of uranium (65%) was as a uranium–RNA complex, which was isolated by high-voltage electrophoresis from an aqueous extract of the freeze-dried leaves. Uranium (25%) was released from the solvent-extracted leaf residue by pepsin, thus revealing the presence of a uranium–protein complex. However, in view of the known dissociation constants for these two complexes, and other tests, it is clear that the majority of the uranium *in vivo* is in the form of a uranium–protein complex. This finding is confirmed by a differential centrifugation experiment, in which it was shown that at least 50% of the total uranium was bound to cell wall proteins.

I. INTRODUCTION

We have recently investigated the accumulation of uranium by indigenous plants in the vicinity of an ore deposit in the Buller Gorge, N.Z. (Whitehead and Brooks 1969*b*). The uranium content in some of these plants was as high as 0·5% dry weight, which is of considerable physiological interest, since this element has been described as a potent inhibitor of several enzyme systems (Singer *et al.* 1947). Uranium is known to be toxic to many plants yet its application to certain species collected from uraniferous areas of the Colorado Plateau actually stimulated their growth and induced earlier flowering (Cannon 1960*a*). These two apparently contradictory statements may be rationalized by a study of the distribution of uranium in plants. It has been shown earlier that selenium-accumulator plants and zinc-tolerant plants exhibit different distributions of the elements compared with their respective non-accumulator and non-tolerant species (Peterson and Butler 1967; Peterson 1969).

Although the accumulation of uranium by various plants has been known for many years, especially from the work of Cannon (1960*a*, 1960*b*) and Kleinhampl (1962), information is lacking on the chemical nature of the accumulated uranium and the location of uranium in the cell. This communication describes our research on the location and chemical form of uranium in leaves of a common indigenous shrub, *Coprosma australis* (A. Rich.) Robinson (Rubiaceae), collected from mineralized ground. In addition, we report the results of a differential centrifugation study of a homogenate of leaves collected from seedlings grown in soil watered with uranyl acetate.

* Department of Chemistry and Biochemistry, Massey University, Palmerston North, N.Z.

† Applied Biochemistry Division, DSIR, Palmerston North, N.Z.

II. Materials and Methods

(a) *Collection and Preparation of Samples*

Leaves were collected from *C. australis* plants growing in a mineralized area on the south bank of the Buller Gorge. The ecology as well as primary and secondary mineralizations of this area have been described elsewhere (Whitehead and Brooks 1969*a*, 1969*b*). The samples were washed with distilled water to remove any surface contamination and freeze-dried. The dried tissue was powdered in a hammer mill to pass a screen of 0·5 mm mesh size.

Twelve young *C. australis* seedlings with attached soil were collected from the above area and grown in a glasshouse for 4 months. To increase the uranium content of these plants, so that uranium determinations could be carried out on small aliquots available from a differential centrifugation experiment, 1·5 mg of uranyl acetate in water was added to the soil of each plant over a period of 2 weeks. Leaves were cut from the plants and washed before use.

(b) *Uranium Determination*

The technique for uranium determination has been described in an earlier paper (Brooks and Whitehead 1968). Briefly, it involves the ashing of the material under investigation, dissolution of the ash, solvent extraction to remove uranium from interfering cations, and a fluorimetric assay involving fusion in gold dishes at 700°C with pellets containing sodium fluoride. Using a fluorimetric attachment to an atomic absorption spectrophotometer the limit of detection of the method was 10 ng.

(c) *Solvent Extraction*

The freeze-dried powder (3 g) was refluxed with 70% ethanol (250 ml) for 20 min, cooled, and filtered. The extracted residue was then refluxed for the same length of time with water. The aqueous fraction was concentrated *in vacuo* and centrifuged to clarify it (26,000 *g*; 15 min) before use.

(d) *Electrophoretic Separation*

The concentrated aqueous extract was streaked across washed Whatman 3MM chromatography-grade paper dipped in 0·7M pyridine–acetic acid buffer, pH 5·3. High-voltage electrophoresis (apparatus manufactured by Miles Hivolt Ltd.) was then carried out for 5 min at 300 mA. After electrophoresis the paper was dried in a current of warm air.

The position of uranium-containing compounds on the sheet was determined by cutting the paper into strips perpendicular to the current flow and ashing them before carrying out the fluorimetric assay. Alternatively, the uranium could be quantitatively eluted with two successive quantities of water.

(e) *Molecular Weight Estimations*

Aqueous uranium-containing samples were ultrafiltered at 3·5 kg/cm^2 pressure in an ultrafiltration apparatus (Amicon Corporation, model 50) using a series of Diaflo molecular weight cut-off membranes.

(f) *Qualitative Assays*

The resorcinol reaction of Svennerholm (1957) was used as a test for RNA, while the indole reaction of Ceriotti (1952) was used for DNA. Perchloric acid digestion of RNA and DNA following the method of Ogur and Rosen (1950) was also used. Ultraviolet spectra were made using a spectrophotometer (Unicam SP800). The procedure outlined by Feigl (1947) was used as a test for phosphate. Proteolytic digestion using pepsin (50 mg in 40 ml 0·1M HCl) was also carried out.

(g) *Differential Centrifugation*

Seedling leaves (21 g fresh wt.) were homogenized at 4°C in a Waring blender for 1 min with a Tris–HCl buffer (300 ml, 0·05M, pH 7·4) containing sucrose (0·5M). The solution after filtration through nylon mesh was centrifuged at 100 *g* for 10 min to give a "cell wall" fraction. The supernatant was centrifuged at 1000 *g* for 10 min to yield a "chloroplast" fraction; at 20000 *g* for 20 min to give a "mitochondria and nuclei" fraction; and at 78,000 *g* for 2·5 hr to give a "ribosome" fraction.

III. Results

(*a*) *Uranium Content of Leaves*

The uranium content of leaf ash from 21 *C. australis* plants and from soil ash collected in the experimental area are summarized in the following tabulation:

Samples	Uranium Contents (p.p.m.)		
	Lowest	Mean	Highest
Leaf ash	1·4	46	146
Soil ash	27	628	2560

The uranium determinations were carried out in triplicate and, although the amounts present in the samples varied, there was extremely good correlation ($r = 0{\cdot}79$, significant at $P<0{\cdot}001$) between leaf ash and soil ash. A composite sample of leaves with a total uranium content of 145 p.p.m. was taken and used for all subsequent chemical fractionations.

(*b*) *Preliminary Fractionation*

The percentage of uranium in successive extracts of two samples of freeze-dried leaves is given in the following tabulation. Analyses were carried out in quadruplicate on each sample:

	Uranium Content (%)	
	Sample 1	Sample 2
70% ethanol extract	8	5
Water extract	72	67
Extract residue	20	28

Both samples showed a predominance of uranium in the water-soluble fraction, there being reasonable agreement between the samples. Leaf samples collected at a later date from plants growing in the same mineralized area also showed a predominance of uranium in the water-soluble fraction.

Aqueous extracts were concentrated and subjected to high-voltage paper electrophoresis. The distribution of uranium along a typical electrophoretogram is shown in Figure 1. One principal compound which was consistently detected had a charge of approximately −3 to −4, as shown by the simultaneous electrophoresis of suitably charged markers.

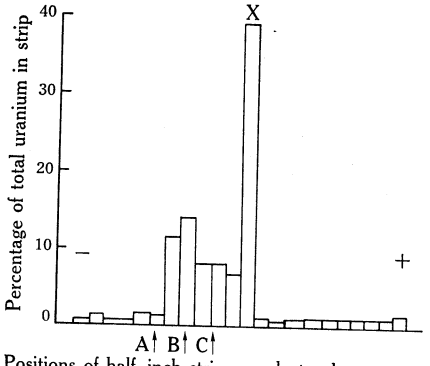

Fig. 1.—High-voltage electrophoresis separation at pH 5·3 of uranium compounds present in the aqueous extracts of *C. australis* leaves. Each vertical bar in the histogram represents the uranium percentage in a half-inch strip of the electrophoretogram. Sample application position is indicated by arrow *A* (origin), while *A*, *B*, and *C* refer to the positions of leucine, uranyl acetate, and uranyl citrate with charges of zero, −1, and −2 respectively. Peak *X* is the uranium–RNA complex studied in this report.

(c) *Examination of the Principal Uranium-containing Compound*

The principal uranium-containing compound was eluted from the paper with water and subjected to a series of tests to establish its identity. The ultraviolet spectrum of the compound showed substantial absorption at 260 nm which is indicative of nucleic acids but there was an absence of a protein peak at 280 nm. RNA was shown to be present by the resorcinol reaction of Svennerholm (1957). The characteristic spectral peak at 450 nm was also observed. A test for DNA was carried out by the indole reaction of Ceriotti (1952) but the result was negative. To confirm the occurrence of a uranium–RNA complex, the sample was incubated with 1M perchloric acid at 4°C following the method of Ogur and Rosen (1950), which hydrolyses RNA, and was ultrafiltered through a membrane of molecular weight 1000. Uranium was located in the diffusate along with the RNA fragments. The residue not ultrafilterable was treated with 0·5M perchloric acid at 70°C, which hydrolyses DNA, and the hydrolysis products were ultrafiltered. No uranium was associated with the DNA hydrolysis fragments. Finally, the procedure outlined by Feigl (1947) was used as a test for phosphates after hydrolysis of the uranium complex. Substantial amounts of phosphate were detected, which is consistent with the conclusion that uranium occurs in the aqueous fraction predominantly as a complex with RNA.

A molecular weight estimation was made on the uranium–RNA complex isolated from the high-voltage electrophoresis papers by uranium assays of the diffusate from a series of Diaflo filtration membranes which are able to exclude particles of known molecular weight. Estimates from such a series of sequential filtrations are given in the following tabulation:

Molecular Weight Estimation	Percentage of Total Uranium Found in Each Fraction	
	Manipulation for 2 Days	Storage for 7 Days
>100,000	22	18
20,000–100,000	48	2
10,000– 20,000	0	1
1,000– 10,000	30	66
<1,000	0	13

Much of the material was of molecular weight 20,000 or higher, but one-third was in the range 1,000–10,000. Due to the lengthy nature of the initial extraction, concentration, electrophoretic separation, elution of the compound, and sequential filtration, the occurrence of uranium in the low molecular weight fraction was considered to arise by degradation of the high molecular weight polymers. Indeed, after allowing a sample to stand for 7 days, the greatest uranium concentration was found in fractions in the low molecular weight range. It is concluded therefore that virtually all of the uranium–RNA complex occurs naturally with a molecular weight of >20,000.

(d) *Uranium in Extracted Leaf Residue*

Incubation of solvent-extracted residue with pepsin and monitoring the solution showed that all of the uranium in this fraction, i.e. about 25% of the total uranium in the leaves, was protein-bound. The released uranium, however, was not able to

be ultrafiltered, which was unexpected. To check whether released uranium was being bound to the added enzyme, an incubation mixture containing pepsin plus UO_2^{2+} was treated in a like manner to the solvent-extracted residue. It was clearly established that pepsin indeed binds uranium up to 1% (w/w).

(*e*) *Chelating Ability of Protein and RNA for Uranium*

It is known from the work of Rothstein and Larrabee (1948) and Zubel and Beer (1961) that the stability constants for uranium complexes with nucleic acids are about $1{\cdot}4\times10^7$, whereas the corresponding value for proteins is 2×10^6. Thus it is possible that, under the vigorous extraction conditions previously described, transfer of uranium from one compound to another might occur, and this would tend to be in favour of the uranium being finally attached to nucleic acids. Experiments were hence necessary in order to determine whether transfer could occur, and if so, the quantitative extent of this process.

To determine the percentage uranium bound to nucleic acids under much milder extraction conditions, the procedure of Ogur and Rosen (1950) was followed. This entails extracting nucleic acids from tissues with 0·5M perchloric acid for 40 min at 70°C, a process which is known to extract negligible amounts of protein (Wannemacher, Banks, and Wunner 1965). In all, 46% of the total uranium was extracted compared with 65% under the more vigorous conditions described earlier. Another extraction designed to remove RNA only (incubation with 1·0M perchloric acid for 24 hr at 4°C), succeeded in extracting only 23% of the total uranium. It is thus clear that the value of 65% for uranium bound to RNA does not represent accurately the true binding state in the plant and that substantial transfer from some other compound to RNA is occurring.

(*f*) *Other Substances Capable of Chelating Uranium*

To determine whether any other macromolecules were capable of chelating significant quantities of uranium, leaf tissue was extracted with perchloric acid as previously described, and then incubated with buffer solution (0·1M Tris–HCl, pH 7·4, 40 hr, 37°C) under conditions which previous experiments had shown would remove all significant protein. It is known that no carbohydrates except starches extract under these conditions. The residue was extensively dialysed and 400 p.p.m. uranyl acetate was added, following which the dialysis was repeated. Analysis showed that only negligible amounts of uranium were bound on to this fraction, suggesting that protein and nucleic acids are the two main substances capable of chelating uranium in the living cell.

(*g*) *Differential Centrifugation*

To overcome the possibility of uranium–RNA or uranium–protein complexes being produced during the lengthy extraction procedures being employed, leaves were collected from *C. australis* seedlings and homogenized with buffer solution for 1 min and differential centrifugation was carried out on the resulting homogenate. Uranium determinations were carried out in duplicate on these fractions and the percentage uranium distribution was found to be as follows: cell wall fraction 44%

(largest amount), chloroplast fraction 25%, mitochondria and nuclei fraction 23%, supernatant 8%. No uranium was detected in the ribosome fraction.

IV. Discussion

Only 7% of the total uranium in leaf tissue was soluble in 70% ethanol. Although the nature of this fraction was not investigated, it is likely that it exists as a small molecule or the simple uranyl ion. Indeed, the differential centrifugation method revealed that 8% of the uranium occurred in the high-speed supernatant, and it seems reasonable to surmize that these two fractions may be the same.

As 70% ethanol removes most small ions, the remainder of the uranium must be mainly chelated in the tissues, and is only extracted after prolonged or vigorous conditions. Differential centrifugation revealed that the uranium was indeed distributed throughout the various cell particles.

About one-half of the uranium in the leaves occurred in the cell wall fraction, and since the lack of material prevented more detailed examination of the fractionation of cell components under the conditions used, it is very likely that this value is a minimum, and that possibly most of the uranium is found in this fraction. Since nucleic acids do not occur in cell walls to any marked extent, it follows that the main locus of binding is cell wall protein. The exact extent of natural occurrence of nucleic acid complexes of uranium is difficult and perhaps impossible to determine, owing to the necessity of extraction procedures being employed, but it is unlikely to be substantial.

The only other significant studies in the literature (though on yeast cells and not higher plants) have concluded that most of the uranium was chelated by the cell wall (Muntz, Singer, and Gusman-Barron 1947; Rothstein and Meier 1951; Rothstein and Hayes 1956). This undoubtedly has some effect in reducing the toxic effects of the uranyl ion.

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