Preliminary observations on the ecology and plant chemistry of some nickel-accumulating plants from New Caledonia

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[Plate 8]

Preliminary studies have been carried out on some nickel-accumulating plants from New Caledonia: *Hybanthus austrocaledonicus* forms A and B, *Hybanthus caledonicus* var. *linearifolia*, and *Psychotria douarrei*. The Australian nickel accumulator *Hybanthus floribundus* was also studied for comparison purposes. The nickel content of all the New Caledonian species was extremely high, and reached nearly 16.0 % (mass of ash basis) in *H. caledonicus* var. *linearifolia*.

Except for *H. floribundus* (29 %), most of the nickel in the plants was soluble in water, and reached 94 % for *P. douarrei* (dry mass basis). Column chromatography with Sephadex G-10 gel showed that nickel existed in most species in two forms: a charged complex (molecular mass < 200) and free nickel ions. Ultracentrifugation experiments and electron microprobe studies showed no evidence for the accumulation of nickel in any specific plant tissues.

High-voltage electrophoresis paper was used to study amino acid patterns and revealed that the nickel was entirely present in a double-charged form (free and complexed) and was not associated with any of the amino acids. The amino acid patterns, together with data on the morphology, elemental content, and ecology of the plants, indicated that *H. caledonicus* form B may be a separate variety of the species.

1. Introduction

Until fairly recently, only two plant species were known to concentrate nickel from their substrate to an abnormal degree. These were: *Alyssum bertolonii* (Minguzzi & Vergnano 1948) and the closely-related *Alyssum murale* (Malyuga 1964). More recently, Severne & Brooks (1972) and Cole (1973) reported a very high accumulation of nickel by the Australian species *Hybanthus floribundus*

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which contained up to 23 % nickel on an ash-mass basis (Severne & Brooks 1972). It appears that accumulation of nickel may be a general feature of the genus *Hybanthus* because other species have also been found to have an elevated nickel content. For example, *H. filiformis*, growing in sandstone in the Sydney area, was found to have over 100 parts/10^6 nickel (ash-mass basis) whereas the substrate had only 5 parts/10^6 of this element (R. R. Brooks, unpublished data). *Hybanthus* species growing in New Caledonia have been found to have nickel contents equal to or surpassing *H. floribundus*. Jaffré, Latham & Quantin (1971) and Jaffré (1973) have reported the discovery of several of these New Caledonian nickel accumulators and have discussed their ecological relationship with their substrate. Brooks, Lee & Jaffré (1974) have given data for the nickel content of the New Caledonian species *Hybanthus austrocaledonicus*, *Hybanthus caledonicus* form A, and *Homalium kanaliense*. Recently yet another nickel-accumulating plant (*Psychotria douarrei*) has been reported from New Caledonia (Jaffré & Schmid 1974) and was reported as having nickel contents in the plant ash as high as 44 %, which is by far the highest value reported in the literature for any element in the ash of a plant species.

The elevated nickel content of the New Caledonian species poses interesting problems in plant physiology, and we have therefore undertaken some preliminary studies on the plant chemistry of these species. The results of these investigations are presented in this paper.

2. MATERIALS AND METHODS

(a) Plants sampled

A list of plants sampled is shown in table 1, which also includes ecological data. Figure 1 shows collection localities on a map of New Caledonia.

A specimen of *H. floribundus* for comparison purposes was collected from the Adelaide Hills about 15 km south of Adelaide, South Australia. Unfortunately it was not possible to obtain specimens growing over ultrabasic rocks where much higher nickel concentrations could be expected.

(b) Inorganic analysis of plants and soils

Plant samples were placed in 100 ml borosilicate glass squat beakers, were dried for 4 h at 110 °C, and were then ashed at 500 °C in a muffle furnace. The ash was dissolved in one hundred times its mass of hot 2 M hydrochloric acid, and the samples were analysed for a number of elements by atomic absorption spectrophotometry. Soils were dried at 110 °C, sieved to −100 mesh size and 0.1 g samples were dissolved in a 1:1 nitric-hydrofluoric acid mixture (10 ml) contained in 50 ml polypropylene beakers. The acid was evaporated to dryness over a water bath, and the residue was redissolved in 10 ml of hot 2 M hydrochloric acid. The solutions were analysed as before by atomic absorption spectrophotometry.

TABLE 1. DESCRIPTION OF PLANTS AND THEIR SAMPLING LOCALITIES

species	morphology	locality	soil type	distribution
Hybanthus austrocaledonicus Schinz. et Guillaumin (Violac.)	shrub 1–3.5 m high; leaves 100 mm × 40 mm	Mt Koghis, near Noumea, New Caledonia	(i) very humic, iron-rich soils over shattered peridotite (ii) iron-rich soils over sedimentary rocks	dense rain forest, found on various substrates but mainly peridotites in south of New Caledonia
Hybanthus caledonicus form A (Turcz.) Cretz. (Violac.)	shrub 0.2–1.5 m high; leaves 70 mm × 25 mm	Massif de Boulinda, New Caledonia	eutrophic magnesium-rich brown soils over serpentinites	forest and scrub at the base of mining massifs in central and northern New Caledonia
Hybanthus caledonicus form B (Turcz.) Cretz. (Violac.)	shrub 0.2–1.5 m high; leaves 130 mm × 40 mm	Plaine des Lacs, Massif du Sud, New Caledonia	residual, moderately humic, colluvial iron-rich soils on peridotites	scrub and semi-forested formation in southern New Caledonia
Hybanthus caledonicus var. *linearifolia* Guillaumin (Violac.)	shrub 0.2–1.5 m high; leaves 65 mm × 10 mm	Massif du Katephié, north of Koniambo, New Caledonia	eutrophic, magnesium-rich brown soil on serpentinites	scrub in the north-central part of New Caledonia
Hybanthus floribundus (Lindl.) F. Muell. (Violac.)	shrub 0.5–1.5 m high; leaves 20 mm × 1.5 mm	Mt Bold, Adelaide Hills, South Australia	sandy soil overlaying siltstones	eucalypt or acacia scrub throughout southern and west-central Australia
Psychotria douarrei (G. Beauvisage) Daniker (Rubiac.)	shrub 0.5–1.8 m high; leaves 150 mm × 60 mm	Mt Koghis, near Noumea, New Caledonia	very humic, iron-rich soils over shattered peridotites	dense rain forest. Species not confined entirely to ultrabasic substrates

(c) Determination of water-soluble nickel

One g samples of pulverized freeze-dried material were shaken with 20 ml of distilled water for 60 min at 0 °C. The containers (100 ml centrifuge tubes) were centrifuged and the supernatant liquid was analysed for nickel by atomic absorption spectrophotometry.

(d) Differential centrifugation

One g samples of pulverized freeze-dried leaves were homogenized for 2 min with 20 ml of tris buffer (0.05 M tris and 0.5 M sucrose adjusted to pH 7.5 with acetic acid). The pulp was filtered off via a 125 µm nylon sieve. The homogenate was successively centrifuged according to the following scheme: 1 min at 500 g, 10 min at 1000 g, 20 min at 20000 g and 100 min at 100000 g. Each residue, at each stage, was taken to dryness, ignited at 500 °C, redissolved in 2 M hydrochloric acid and analysed for nickel by atomic absorption spectrophotometry. The final supernatant was analysed directly.
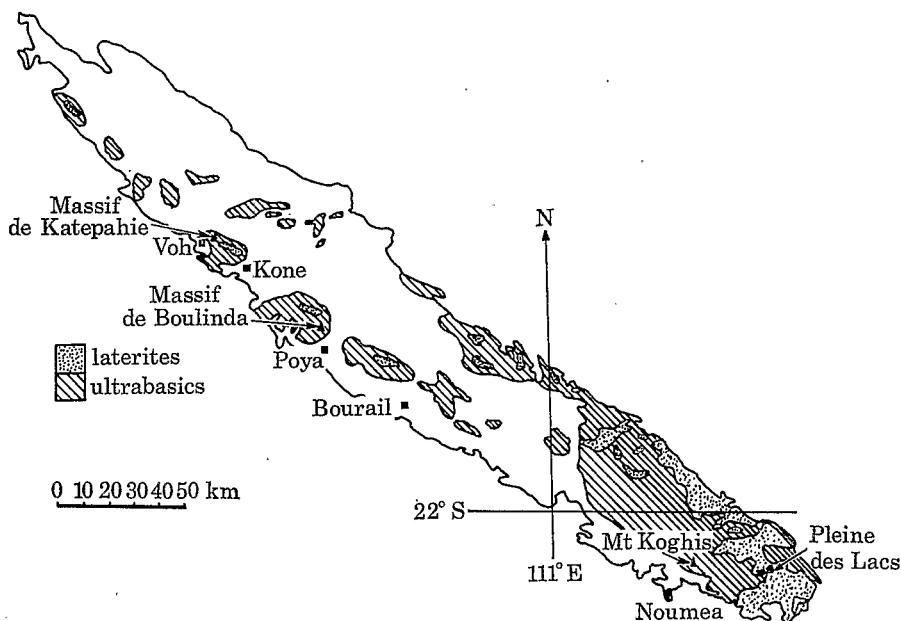

FIGURE 1. Map of New Caledonia showing sample collection sites.

(e) High-voltage electrophoresis and paper chromatography

One g samples of pulverized freeze-dried leaves were shaken with 20 ml of 70 % ethanol for 20 min at 0 °C. After centrifugation, the supernatant was absorbed on to a column of IR 120 cation exchange resin equilibrated with 70 % ethanol. The amino acids were eluted with 30 ml of 25 % ammonia and taken to low volume (1 ml) by use of a rotary evaporator at 30 °C. About 10 µl

of solution was streaked (5 mm) on to Whatman 3MM chromatography paper (90 cm × 27 cm) and high-voltage electrophoresis was carried out for 50 min at 4.5 kV by means of a 'Savant' apparatus comprising a lower buffer layer of 7 % formic acid (pH 1.9) overlain with water-cooled 'Varsol' organic solvent. Paper chromatography (descending) was carried out in the second dimension for 16 h with a 100–150–30–120 mixture of pyridine–butanol–acetic acid–water. Papers were dried in warm air, soaked in 0.2 % ninhydrin in ethanol containing 1 % collidine, and developed for 15 min in a current of warm air (50 °C). The position of nickel on the electrophoretograms was determined by spraying with a 0.5 % aqueous solution of α-furil dioxime which gave a characteristic red colour with this element.

(f) *Determination of the molecular mass of nickel complexes*

One gram samples of material were treated exactly as above in §2(c) except that the supernatant liquid was reduced to about 1 ml by means of a rotary evaporator operated at 30 °C. Sixty grams of Sephadex G-10 gel was swollen with distilled water and packed into a column of 25 mm diameter. This gave a bed height of approximately 200 mm. The column was then washed with 0.05 M ammonium acetate and the void volume was determined by using dextran blue (molecular mass 2000). The aqueous extract was absorbed on to the top of the bed, the nickel was eluted with 0.05 M ammonium acetate, and 3 ml fractions were collected up to a total eluate volume of 150 ml. Each fraction was analysed for nickel by atomic absorption spectrophotometry. Approximate molecular masses were determined from the formula: $-\lg(V_e/V_0 - 1) = 0.0133 \times (\text{molecular mass}) - 0.18$ (Carnegie 1965), where V_e is the volume corresponding to an eluted peak, V_0 is the void volume, and where the constants 0.0133 and 0.18 were obtained from calibration of the column with compounds of known molecular mass.

(g) *Microprobe analysis*

Dried leaf material was mounted on aluminium blocks and coated with a 20 nm thickness in a vacuum evaporator. The samples were analysed via a Cambridge Microscan V microprobe with a lithium fluoride crystal. The unit was operated at 25 kV, 0.03 μA and magnifications of up to × 1000.

3. RESULTS AND DISCUSSION

(a) *Elemental concentrations in plants and soils*

Table 2 summarizes data for elemental concentrations in plants and soils. All the New Caledonian species showed abnormally high concentrations of nickel. The highest value shown in table 2 was 15.83 % in the ash of *H. caledonicus* var. *linearifolia*. The specimen of *P. douarrei* had 14.00 % nickel in the ash and 1.99 % on a dry mass basis (compared with 1.03 % dry mass for the specimen of *H. caledonicus* var. *linearifolia*).

TABLE 2. ELEMENTAL CONCENTRATIONS (%) IN PLANT ASH AND ASSOCIATED SOILS

element	H. austro-caledonicus on peridotites		H. caledonicus form A on serpentinites		H. caledonicus form B on peridotites		H. caledonicus var. linearifolia on serpentinites		H. floribundus on siltstones		P. douarrei on peridotites	
	plant	soil	plant	soil	plant	soil	plant	soil	plant	soil	plant	soil
Ca	17.2	1.36	26.0	0.02	9.4	0.008	13.1	0.03	17.3	—	17.2	1.36
Co	0.030	0.020	0.060	0.14	0.030	0.07	0.121	0.14	0.144	0.0004	0.020	0.02
Cr	0.020	1.230	0.020	3.69	0.007	2.26	0.007	3.71	0.002	—	0.065	1.23
K	15.2	0.02	19.2	0.01	6.5	0.002	19.6	0.02	28.6	—	8.8	0.02
Mg	8.0	2.29	10.4	2.05	2.8	0.15	10.0	1.45	18.5	—	4.0	2.29
Mn	0.25	0.21	0.72	0.92	3.69	0.75	1.33	0.90	0.70	—	0.09	0.21
Ni	13.20	0.33	11.00	0.55	1.58	0.63	15.83	0.56	0.11	0.0005	14.00	0.33
ash (%)	11.1	—	8.0	—	10.9	—	6.5	—	5.8	—	14.2	—
Ni dry mass (%)	1.46	—	0.88	—	0.17	—	1.03	—	0.006	—	1.99	—
Ni (plant/soil)	40.0	—	20.0	—	2.5	—	28.3	—	220	—	42.4	—
Ni/Co (plant)	44.0	—	183	—	548	—	130	—	0.76	—	7000	—
Ca/Mg (plant)	2.15	—	2.50	—	3.36	—	1.31	—	0.94	—	4.30	—

FIGURE 2. Leaf forms of New Caledonian nickel-accumulating plants. *Hybanthus caledonicus* form B, *H. austrocaledonicus, H. caledonicus* form A, *H. caledonicus* var. *linearifolia* (top to bottom). Front of leaves shown on the right. Back of leaves shown on the left.

The New Caledonian *Hybanthus* species are also accumulators of cobalt. This has also been noticed by Severne & Brooks (1972) and Cole (1973) for the West Australian *H. floribundus*. The ash of the South Australian specimen (table 2) contained a large amount of cobalt (1445 parts/10^6) relative to nickel (1100 parts/10^6). It will be noted from the nickel/cobalt ratio for *P. douarrei* in table 2, that this species differed from *Hybanthus* in being unable to concentrate cobalt to any extent.

The nickel accumulating capacity (plant/soil ratio) in the New Caledonian species was highest in *H. austrocaledonicus* and *P. douarrei* and lowest in *H. caledonicus* var. *linearifolia*. Although the South Australian *H. floribundus* had the lowest nickel content, it also had the highest nickel accumulating factor (220).

It has been shown by Krause (1958) that calcium/magnesium ratios in plants are a measure of their suitability for colonizing ultrabasic substrates, i.e. the lower the ratio, the greater their suitability. *P. douarrei* is distinguished by having an appreciably higher ratio (4.30) than the *Hybanthus* species, and therefore seems to be atypical of serpentine plants.

The very high accumulation of nickel in the New Caledonian species led to the following experiments designed to determine the way in which nickel was bound to the plant tissues. It was also hoped that the data would assist in the chemotaxonomic differentiation of the various polymorphs of *H. caledonicus* which include: var. *linearifolia*, with long narrow leaves, form A with small broad leaves and form B with very large leaves. Leaves of these three forms are illustrated in figure 2, plate 8, together with leaves of *H. austrocaledonicus*.

(b) *Water-soluble nickel in plant material*

Experiments to measure the proportion of water-soluble nickel in freeze-dried material (see §2(c)) showed that except for *H. floribundus*, most of the nickel in the plant material was water soluble. The data are shown in table 3. It is probable that there is some relation between the solubility of nickel in water and the total nickel content of the freeze-dried material since the highest solubility (94 %) was found in the case of the highest nickel content (1.99 % in *P. douarrei*) and by far the lowest solubility (29 %) was found in *H. floribundus* which had easily the lowest nickel content (0.006 % dry mass).

The high solubility of nickel in all species investigated is not surprising in view of the work of Timperley (1971) who has reported solubilities of 57–86 % for four species of New Zealand plants. The inference of the high solubility of nickel in plant material is that the element is present either as free ions or as highly polar complexes.

(c) *The distribution of nickel within the cell fractions*

The differential centrifugation experiments (see §2(d)) were carried out on individual samples of *H. austrocaledonicus* and *H. caledonicus* var. *linearifolia*. The data are summarized in table 4. Only 10–14 % of the total nickel in the

plant tissue was found in the cells. When account is taken of the dry mass of the cells, the residual nickel bound to them, after breakdown of the cells, is of the same order of magnitude for all cell fractions except the microsomes, which have very little nickel. It is clear from table 4 that there is little or no tendency for nickel to be concentrated in any of the cell fractions.

The above pattern of uniform concentration of nickel in cell fractions was confirmed by the electron microprobe studies which showed a very uniform distribution of nickel within leaf tissue of all the species studied.

TABLE 3. PERCENTAGE OF WATER-SOLUBLE NICKEL IN PLANTS
ON DRY MASS BASIS

species	total Ni (%)	ratio free/complexed	total (%) as Ni^{2+}
H. austrocaledonicus	65	0.61	25
H. caledonicus form A	90	3.05	68
H. caledonicus form B	82	2.54	59
H. caledonicus var. linearifolia	83	0.50	28
H. floribundus	29	0.40	8
P. douarrei	94	∞	94

TABLE 4. NICKEL IN CELL FRACTIONS (DRY MASS) OF *Hybanthus* SPECIES

fraction	g	min	H. austro-caledonicus % of total Ni	Ni in tissue (%)	H. caledonicus var. linearifolia % of total Ni	Ni in tissue (%)
unhomogenized residue	—	—	26.3	—	17.8	—
supernatant	—	—	63.3	—	68.2	—
cell wall	500	1	3.7	0.90	8.5	1.72
chloroplasts	1000	10	5.5	2.34	4.6	1.75
mitochondria	20000	20	1.0	1.46	0.8	0.75
microsomes	100000	100	0.2	0.29	0.1	0.07
total Ni in cells (%)	—	—	10.4	—	14.0	—
total Ni in intact plant material (%)	—	—	100.0	1.46	100.0	1.03

(f) *Free amino acids in plant material*

Table 5 shows data from an electrophoretogram for 70% ethanolic extracts of each species studied. The position of nickel ions and complexes was determined on samples not subjected to column chromatography which was used to purify material before determining amino acids, but data from the 'nickel' electrophoretograms and the 'amino acid' electrophoretograms are combined together in the table.

Although the position of nickel was evident from the red spots obtained with α-furil dioxime (see §2(e)), the electrophoretograms were cut into 20 × 20 mm squares and analysed for nickel in case the red colour had not developed because

of the presence of strong organic complexes of nickel. Each square of paper was ignited at 500 °C, and the ash was dissolved in 2 M hydrochloric acid and analysed for nickel by atomic absorption spectrophotometry.

'Soluble' nickel in the alcoholic extracts existed almost entirely in the form of double-charged nickel ions or nickel complexes because the 'spots' coincided very nearly with a Ni^{2+} standard. The spots were slightly retarded and this implied the existence of complexes with a greater molecular mass than free

TABLE 5. DATA FROM ELECTROPHORETOGRAM SHOWING FREE AMINO ACIDS IN NICKEL-ACCUMULATING PLANTS COMPONENTS LISTED IN ORDER OF OCCURRENCE WITH THE ORIGIN (POSITIVE) ON THE TOP

	H. austro-caledonicus	*H. austro-caledonicus* var. *linearifolia*	*H. cale-donicus* form A	*H. cale-donicus* form B	*H. flori-bundus*	*P. douarrei*
cysteine + hydroxyproline	−	−	−	−	−	+
aspartic acid	+	+	+	+	+	+
unidentified	+	+	+	+	+	+
glutamic acid	+	+	+	+	+	+
proline	−	+	+	−	−	+
methionine	+	+	+	+	+	+
isoleucine	+	+	+	+	−	+
valine	+	+	+	+	+	+
β-alanine	+	+	+	+	+	+
unidentified	+	−	−	−	−	+
unidentified	+	+	+	−	−	+
α-aminobutyric acid	+	+	+	+	+	+
nickel complexes	+	+	+	+	+	+
ethanolamine	−	−	−	−	−	+

nickel ions. The experiments were of course carried out with 70% ethanolic extracts, whereas the experiments to measure the extractability of nickel (see §2(c)) had been carried out on pure water extracts. However, electrophoresis carried out on water-soluble extracts, indicated nickel spots with the same positions and intensities as those derived from the alcoholic extracts.

Characteristic and highly reproducible amino acid patterns were obtained for each species studied. Table 5 represents the results of four replicate analyses of each sample. The identity of the amino acids was established by running standards and comparing them not only in the electrophoretograms but also in paper chromatograms in the second dimension. Further proof of identity was established by specific colour reactions with ninhydrin-collidine or isatin developer.

Analysis for nickel on the paper containing each spot showed that there was no correlation whatsoever between nickel and amino acids. It was, however, hoped that the amino acid patterns would assist in the possibility of chemo-taxonomic differentiation of the *Hybanthus* species. It is clear from table 5 that

P. douarrei is differentiated from all other species by the presence of ethanolamine and two other amino acids which are tentatively identified as cysteine and hydroxyproline. This identification is tentative because these amino acids are only rarely found in plant material in the free state. Cysteine is normally oxidized to cystine or cysteic acid but its apparent presence in *P. douarrei* could be a residue from an initially high concentration. The *Hybanthus* species have a fairly common pattern with *H. floribundus* being deficient in isoleucine.

The three polymorphic forms of *H. caledonicus* are particularly interesting because of previous doubts as to their taxonomic classification. The two small-leaved forms: *H. caledonicus* var. *linearifolia* and *H. caledonicus* form A have identical amino acid patterns. Form B differs from the other two species by being deficient in proline and an unidentified amino acid adjacent to α-amino butyric acid.

H. austrocaledonicus is differentiated from the other New Caledonian species by the presence of an unidentified amino acid adjacent to β-alanine. This same amino acid is also present in *P. douarrei*.

Whereas it is obviously not possible to make firm chemotaxonomic conclusions from the limited data presented here, the studies have at least indicated that further taxonomic work should be carried out on the large-leaved form (form B) of *H. caledonicus*. When form B is compared with the other two polymorphs in table 2, it is strikingly apparent that it is unique in having the lowest concentrations of calcium, cobalt, magnesium, nickel and potassium. It also has the highest manganese content as well as the highest nickel/cobalt and calcium/magnesium ratios. The relatively high calcium/magnesium ratio (3.36) indicates that this form is not particularly suitable for colonizing ultrabasics. The nickel-accumulating power of this plant is particularly low.

It may well be that the elemental content and morphology of form B of *H. caledonicus* is influenced by the nature of its substrate: i.e. specimens of *H. caledonicus* growing over periodotites tend to develop large leaves and have a lower uptake of several elements, but this does not, however, explain the deficiency of two amino acids found in both of the other polymorphs. Form B may therefore be a distinct ecotype of *H. caledonicus*, and if further work shows that this is indeed so, it might justifiably be named *Hybanthus caledonicus* var. *grandifolia*.

(g) The form of nickel in the plant material

It has already been established that most of the nickel in the plant material was water soluble. When aqueous extracts were concentrated to low volume (see §2(f)), absorbed on to a column of Sephadex G-10 gel and eluted with 0.05 M ammonium acetate, two eluted peaks were obtained for most of the species studied. The shape and position of the peaks was remarkably consistent for each species. The elution peaks are shown in figure 3. Among the polymorphs of *H. caledonicus*, form B was again readily distinguished from the other forms, this time by its lowest proportion of complexed nickel. There seemed to be

some relation between the free/complexed nickel ratio and the solubility of the plant-bound nickel in water (see table 3). *P. douarrei* with the highest free/complexed ratio (∞) had the greatest amount of soluble nickel (94%), whereas *H. floribundus* with the lowest ratio (0.40) had only 29% soluble nickel. All other species, with the exception of *H. caledonicus* var. *linearifolia*, followed a similar order of ratios and solubilities. Such a behaviour is not unexpected in view of the greater likelihood of free nickel ions being more soluble in water than complexed nickel.

If nickel exists mainly in the Ni^{2+} form in the species studied, it might be expected to have a relatively uniform distribution within the plant tissues. This was confirmed, as previously stated, by the results of the electron probe determinations.
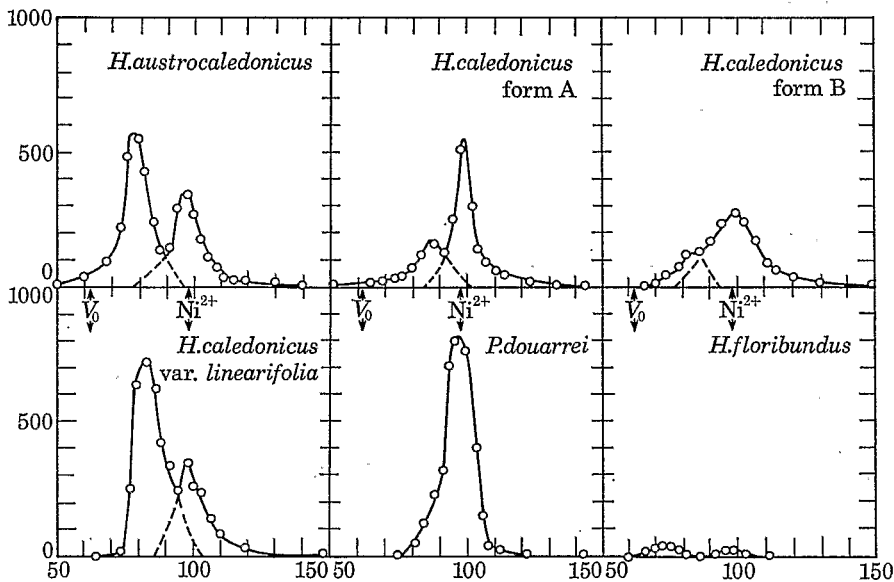

FIGURE 3. Elution curves from a Sephadex G-10 column showing the molecular mass distribution of nickel complexes and free nickel ions in acqueous extracts of nickel-accumulating plants.

4. GENERAL DISCUSSION AND CONCLUSIONS

Because of the preliminary nature of this study, and because identification of the structure of nickel complexes was outside the scope of this investigation, conclusions must necessarily be restricted. It does, however, appear that most of the nickel in the New Caledonian species was soluble in water, and the nickel was found either as free ions or as a low molecular mass complex with a molecular mass of under 200.

None of the nickel was associated with amino acids. However, the amino acid patterns for the three forms of *H. caledonicus*, together with the data on elemental

uptake from the substrate, and the ecology of the species, indicated that *H. caledonicus* form B may be a distinct variety or ecotype meriting further investigations.

The structure of the nickel complexes and the role of nickel in the metabolism of the plants are still open questions, but it is hoped that this preliminary study will serve to stimulate further work in this direction.

Of the nine unusually high accumulators of nickel so far reported in the literature (including this paper), five are members of the genus *Hybanthus*. It may well prove that accumulation of nickel is a characteristic of many of these species, and further investigations into the genus should result in the discovery of further nickel accumulators.

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