AN APPARENT SELENIFEROUS LEAF WAX FROM STANLEYA BIPINNATA*

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The accumulation of selenium (Se) by certain indicator plants and their apparent obligate requirement for Se are well known (Trelease and Beath, 1949). All of the Se accumulated in Stanleya plants is in organic form (Beath and Eppson, 1947). It has been generally assumed that Se follows the same metabolic pathways as does sulfur and substitutes for sulfur in sulfur-containing compounds such as methionine, cysteine, etc. (Shrift, 1958).

In the course of studying various organic Se-compounds in Stanleya bipinnata (a member of the mustard family), a fraction was found in various preparations which was very soluble in organic solvents and which moved close to the ether front during counter-current distribution between ethyl ether and 0.05N aqueous HCl.

This fraction was obtained in small amounts and selenium detection and measurement by the modified method of (Klein, 1947) was not satisfactory. In order to facilitate Se detection and measurement it was decided to incorporate Se^{75} into the plants. Stanleya plants, from the field (Shirley Basin, Wyoming), were transplanted to large pots and grown in the greenhouse. Approximately 10% of the plants survived. When visible growth was evident they were given a Se^{75} label by watering at intervals with a solution of Se plus Se^{75} in the form of H_2SeO_3.

After about two months growth, individual leaves were cut from the labeled plants and separately extracted by macerating with different non-polar organic

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solvents. The radioactivity and infrared spectra were run on the extracts to evaluate the efficiency of each solvent to extract Se^{75} and determine the relative purity of the extracts obtained. Solvents were then evaporated from the extracts and the residues were pelleted in KBr and infrared spectra again taken.

At this point, although the spectra did not indicate any degree of purity, it was noted that all the spectra of pelleted samples showed a prominent doublet at 13.75 and 13.95, which was lacking in all the solvent-run spectra. This suggested that the samples contained long hydrocarbon chains whose transition point was above room temperature. This information, along with the presence of prominent acid and ester carbonyl absorption peaks suggested that the extracts were rich in waxes.

Reasoning that waxes should be present in fair amount and in fairly pure form on the external surfaces of the Stanleya leaves, a number of individual leaves were harvested and individually rinsed by dipping in Skelly F for 30 seconds. The Skelly F rinses and the rinsed whole leaves were then counted with the result that an amount varying from 5% to 10% of the total leaf selenium had been extracted into the Skelly F rinse.[1] Upon evaporating the Skelly F and pelleting the residues in KBr, infrared spectra were obtained which were typical of the spectra of waxes.

The spectrum shown in Figure 1 contains the expected hydrocarbon absorptions, free acid and ester carbonyl absorptions, and the prominent doublet at 13.75 and 13.95 characteristic of high molecular weight hydrocarbons.

These data imply that a significant amount of seleniferous wax is produced in Stanleya. However, proof of this has been beset with difficulties and has not been completely successful.

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1 Subsequent ratios of rinsable count to total leaf count on new regrowth of harvested plants are lower; therefore, no "typical" values can be assumed.

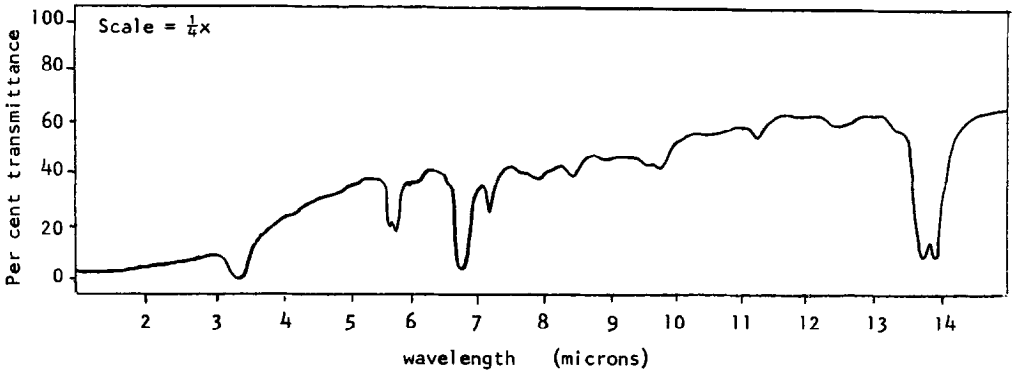

Fig. 1. Infrared spectrum of the potassium bromide pellet of the Skelly F Stanleya leaf extract.

Specific activity could not be determined on the present samples because of the loss of 60% to 90% of the selenium (radioactivity) during digestion, using the method of (Klein, 1947). That this loss is a characteristic of the material is believed to have been shown by attempting digestion with perchloric acid, using the test tube method of (Feigl, 1961). In this method, it was possible to determine radioactivity in the sample during various stages of digestion. When digestion was complete, according to the method (and appeared visibly complete, judged by clearing of the sample), 25% of the radioactivity had been lost. Furthermore, the remaining Se was apparently not yet oxidized, since the radioactive material in the sample filtered through an asbestos mat after treatment with hydrazine sulfate.

All the remaining plant material was harvested and most of the wax preparation obtained by rinsing the leaves with Skelly F was sacrificed in an attempt to chemically determine Se to evaluate specific activity. Although a trace of Se was obtained, the amount was only slightly higher than the reliability of the titration and, therefore, has little significance.

Further evidence of the lability of the Se in the wax preparation is that tracer Se^{75} is rapidly lost when the wax is dissolved in chlorinated solvents, CS_2, and alcohols, and after pelleting in KBr. Loss of count from CS_2 solutions should rule out metallic Se, which is soluble and stable in this solvent.

Infrared spectra have not yet been helpful in locating a functional Se group. This is not surprising, since R-Se-H absorption is weak and theoretically lies at 4.25μ where CO_2 interferes; R-Se-R and R-Se-Se-R absorptions theoretically lie beyond the 15μ range of our instrument and are also weak. Other possible organic Se bond absorptions are not known with sufficient certainty to be of much assistance.

The results of two experiments lend strong circumstantial evidence to our belief that Se is chemically a part of Stanleya wax molecules. The wax forms excellent cystalline urea clathrates (Trutter, 1956), and the tenacity with which the Se^{75} label stays with the clathrate indicates that it is chemically bound in the hydrocarbon molecules. Table I gives the results of two types of clathrate formation experiments. The indicated MeOH washed clathrate was formed by taking the wax up in 50:50 MeOH-Benzene and adding it to an equal volume of urea-saturated MeOH. The mixture was warmed and the clathrate allowed to crystallize at room temperature, after which it was filtered and washed three times with three times the precipitate's volume of MeOH. The benzene-washed clathrate was formed by adding the wax (dissolved only in benzene) to an equal volume of urea-saturated MeOH and by repeating the above procedure by washing only with benzene.

The washed clathrates were subsequently decomposed with hot water and the water-insoluble waxy material taken up in Skelly F.

TABLE I.

Fraction	MeOH washed clathrate counts/m/total sample	Benzene washed clathrate counts/m/total sample
Washed clathrate	696	2,147
Washings from clathrate	503	33
Aqueous phase from decomposed clathrate	108*	0
Hexane soluble wax from decomposed clathrate	497*	2,163

* Label is unstable in MeOH

From the well-known requirements for and specificities of urea clathrate formation described by (Trutter, 1956), the data of Table I are consistent with a high molecular weight fairly straight-chain hydrocarbon material containing chemically bound Se. Infrared spectra of the wax recovered from the clathrates were no different than the spectrum of Figure 1.

Chromatography of the seleniferous wax on silica gel by the (Lis, 1961) microadaptation of the method of (Hirsch and Ahrens, 1958) proved destructive in terms of loss of Se^{75} but furnished further evidence that we were dealing with a seleniferous wax. In this procedure, 70% of the radioactivity eluted in the 1% ethyl ether in Skelly F fraction (I) and 22% eluted in the absolute MeOH fraction (IV). Most of the dry matter was eluted in fractions II and III, which had negligible count. According to (Hirsch and Ahrens, 1958), fraction I should contain sterol esters, carotenoids, hydrocarbons, and vegetable waxes. The ability to form urea clathrates practically eliminates sterol esters as constituents of fraction I, and carotenoids are ruled out by U.V. and visible spectral determinations. Therefore, fraction I should contain hydrocarbons and/or vegetable waxes.

The infrared spectrum of fraction I was the expected spectrum of a hydrocarbon wax with prominent ester carbonyl absorption at 5.74 and only slight free-acid carbonyl absorption at 5.84. Fraction IV should contain phospholipid, but the infrared spectrum did not support this. The infrared spectrum of fraction IV indicated only free fatty acid. The infrared spectrum of fraction II showed hydrocarbon, but neither acid or ester carbonyl and the spectrum of fraction III showed large amounts of free fatty acid.

Re-chromatography of a portion of fraction I resulted in a recovery of 89% of the radioactivity in the new fraction I, but 10% of the radioactivity appeared in new fraction IV. Again, the infrared spectra characterized fraction I as predominantly hydrocarbon ester and fraction IV as free fatty acid. Based on the unreliable chemical determination of Se and specific activity, the second fraction I had a Se-to-dry-matter ratio of 270:1 compared with a theoretical

ratio of 7:1 for R-C(=O)-Se-R of a molecular-weight equivalent to beeswax. This would imply that only one in 40 wax molecules contained selenium or that the molecular weight is very high.

Many other physical and chemical tests have been made mostly with inconclusive results or complete loss of label.

In general, it is significant that in any procedure which produces fractions, not all of which have label, the label is always lacking in those fractions lacking ester and acid carbonyl spectra. Fractions which, because of various treatments, should be devoid of any free organic acid, nevertheless always show some free-acid carbonyl absorption at 5.84μ. Since, according to (Renson, 1962), this is also the location of the displaced ester carbonyl of seleno-esters, one may strongly suspect that at least some of the Se is present as R-C(=O)-Se-R.

Since waxes are in themselves complex mixtures, one may expect that Se will occur in various functional groups. We have already separated at least one high-molecular-weight waxy material from the crude-wax preparation and recrystallized it five times to nearly constant count. A larger share of the label, however, remains in the residual crude-wax fraction, which is of considerably lower molecular weight than the crystallizable constituents. Much larger amounts of material may be needed before further significant studies can be made of structure.

The data presented make it clear that a fair amount of Se is intimately associated with Stanleya leaf wax. It suggests, but does not conclusively prove, that Se is a chemical part of the wax molecules.

The mere appearance of Se absorbed through the roots, in a leaf-wax preparation, seems worthy of reporting at this time. If, as seems likely, Se proves to be part of the wax molecules, then an interesting biochemical adaptation of Stanleya is indicated, and the metabolism of Se in these plants may differ significantly from that of sulfur.

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