SHORT COMMUNICATIONS

IRON IN THE DEVELOPING SOYBEAN NODULE*

By F. J. Bergersen†

The metabolism of iron in legume root nodules is of particular interest because of the presence of relatively large amounts of the myoglobin-like pigment, leghaemoglobin. This pigment appears when the nodules are about 2 weeks old and its appearance coincides with the onset of nitrogen fixation. There are also quite large amounts of bacterial cytochromes present (Appleby and Bergersen 1958) and, because of the active metabolism of molecular hydrogen (Hoch, Schneider, and Burris 1960; Bergersen 1963), probably appreciable amounts of ferridoxin as well (Tagawa and Arnon 1962).

In mammalian liver, iron from degraded haemoglobin is conserved in the iron-containing protein, ferritin, from which it can be released for incorporation into newly synthesized haem or other compounds (Granick 1943; Green and Mazur 1956). Cultured tumour cells can be induced to form ferritin when iron is added to the medium (Richter 1961). Recently, Hyde, Hodge, and Birnsteil (1962) reported iron-containing protein particles 106 Å in diameter with an electron-dense central core 56 Å in diameter in subfractions of microsome preparations of pea seedlings. These authors assigned the term "phytoferritin" to this material. During the course of an electron microscope study of developing soybean nodules (Bergersen and Briggs 1958), plastid-like bodies were observed in thin sections of osmium-fixed, 1-week-old, central tissue cells which, later in development, would have contained bacteroids and haemoglobin. These plastid-like bodies contained numerous electron-dense granules about 60 Å in diameter which seemed to resemble ferritin (Plate 1, Figs. 1 and 2). These granules were seen in nodules aged up to 7 days but were not seen in nodules aged 2 weeks. Attempts have subsequently been made to confirm the existence of ferritin in young soybean nodules (Bergersen 1960).

Experimental

Lincoln soybeans were inoculated and grown in nitrogen-free medium as previously described (Bergersen 1958). The time of appearance of the first nodule was noted as day 1. In the first series of experiments, samples of nodules of about 3 g fresh weight were gathered at intervals and analysed for iron. In later experiments nodules aged 1–3 days were harvested from 3000 seedlings and extracted with 0·25M sucrose according to Kuff and Dalton (1957).

(i) *Iron Extraction.*—The methods used were based on those of Brukman and Zondek (1940) in which iron was extracted from ferritin by a mixture of hot sodium pyrophosphate and trichloracetic acid (TCA). Nodules aged from 2 to 14 days were

* Manuscript received June 24, 1963.

† Division of Plant Industry, C.S.I.R.O., Canberra.

divided into 1-g samples. (*a*) *Extraction of soluble and loosely bound iron:* The first sample was ground with a little powdered "Pyrex" glass and 2 ml 10% (w/v) TCA at 0°C. The slurry was centrifuged and the deposit washed twice with 2 ml cold TCA. Iron was estimated in the combined extracts. (*b*) *Extraction of non-haemin (ferritin-like) iron:* The residue from (*a*) was suspended in 2 ml saturated sodium pyrophosphate +2 ml 10% (w/v) TCA and extracted at 70°C for 20 min. The slurry was centrifuged and the deposit washed twice with the mixed reagents. The combined supernatants contained non-haemin iron corresponding to that extracted from ferritin

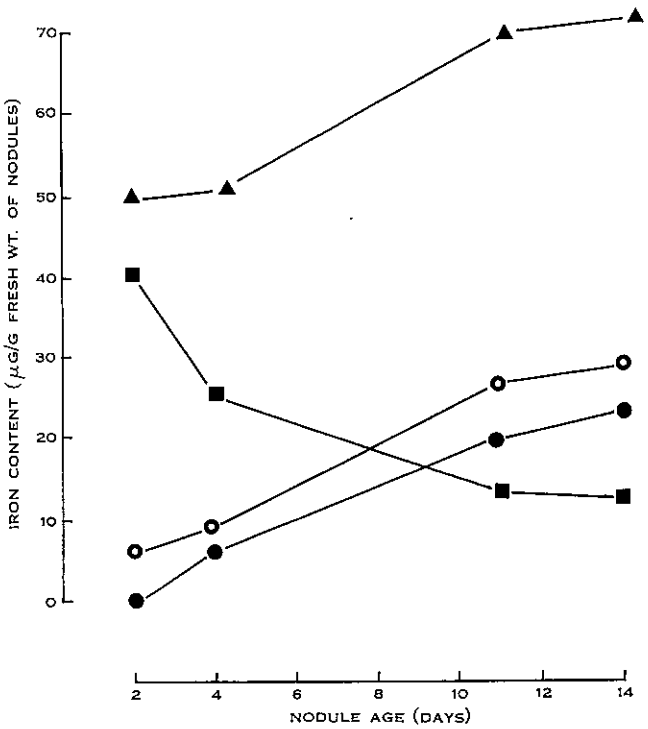


Fig. 1.—Distribution of iron among nodule fractions of various ages. ▲ Total iron. ■ Non-haemin iron (ferritin-like iron). ○ Cold TCA-soluble iron. ● Haemin iron.

of mammalian liver, spleen, and kidney. (*c*) *Extraction of haemin iron:* A further 1 g sample was ground with 4 ml methanol +1 ml 1N HCl at 0°C. The slurry was centrifuged and the deposit washed twice with methanol. The combined extracts were again centrifuged after standing over 1 g anhydrous magnesium sulphate for 30 min at 4°C. The extract contained haemin iron which came mainly from the leghaemoglobin of the nodules. (*d*) *Total iron:* This was estimated on a further 1 g sample. Iron was estimated colorimetrically in all extracts and in whole nodules, after digestion in 1 ml H_2SO_4–$HClO_4$ (1 : 1 v/v), by adding 3 ml conc. NH_4OH to the digest, making the volume to 10 ml, and adding two drops thioglycollic acid; the optical density at 495 mμ was determined after 30 min.

Results

Results are shown in Figure 1 from which it is seen that non-haemin iron (ferritin-like iron) accounted for virtually all the iron in 2-day-old nodules, but rapidly decreased with age as the amount of cold TCA-soluble iron increased; a rise in haemin iron then followed reaching a maximum value at about 14 days. Although not shown in Figure 1, the amount of cold TCA-soluble iron fell to a very low value between 14 and 18 days, and all other fractions remained approximately constant.

(ii) *Ferritin Extraction.*—10 g (fresh weight) of nodules aged 1–3 days were ground with 1 g alumina powder in 25 ml 0·25M sucrose (Kuff and Dalton 1957). The slurry was centrifuged at 4°C at 2500 *g* for 10 min and the extraction repeated three times. Intact bacteria and large particles were removed by centrifugation at 16,000 *g* and the supernatant centrifuged successively for 15 min in a Spinco model L ultracentrifuge at 70,000 *g* and 125,000 *g*, and at 144,000 *g* for 90 min. The deposits from the three ultracentrifugations were each suspended in 1 ml 0·25M sucrose and heated to 80°C for 5 min. Coagulated protein was removed by centrifuging at 3000 *g* for 5 min and the supernatants were then centrifuged at 144,000 *g* for 1 hr. All three fractions yielded red-brown pellets, the most being in the 125,000 *g* fraction. These pellets were then examined in the Spinco model E ultracentrifuge at 50,000 r.p.m. A diffuse peak was seen momentarily but it rapidly disappeared presumably due to inhomogeneity of the particles. The material was recovered and found to contain 0·16 μg iron per μg protein.

Discussion

The following points which have been established suggest that very young soybean nodules contain a ferritin-like material:

(1) Plastid-like bodies in the central tissue cells contained electron-dense granules about 50 Å in diameter which resembled ferritin granules found in mammalian organs.

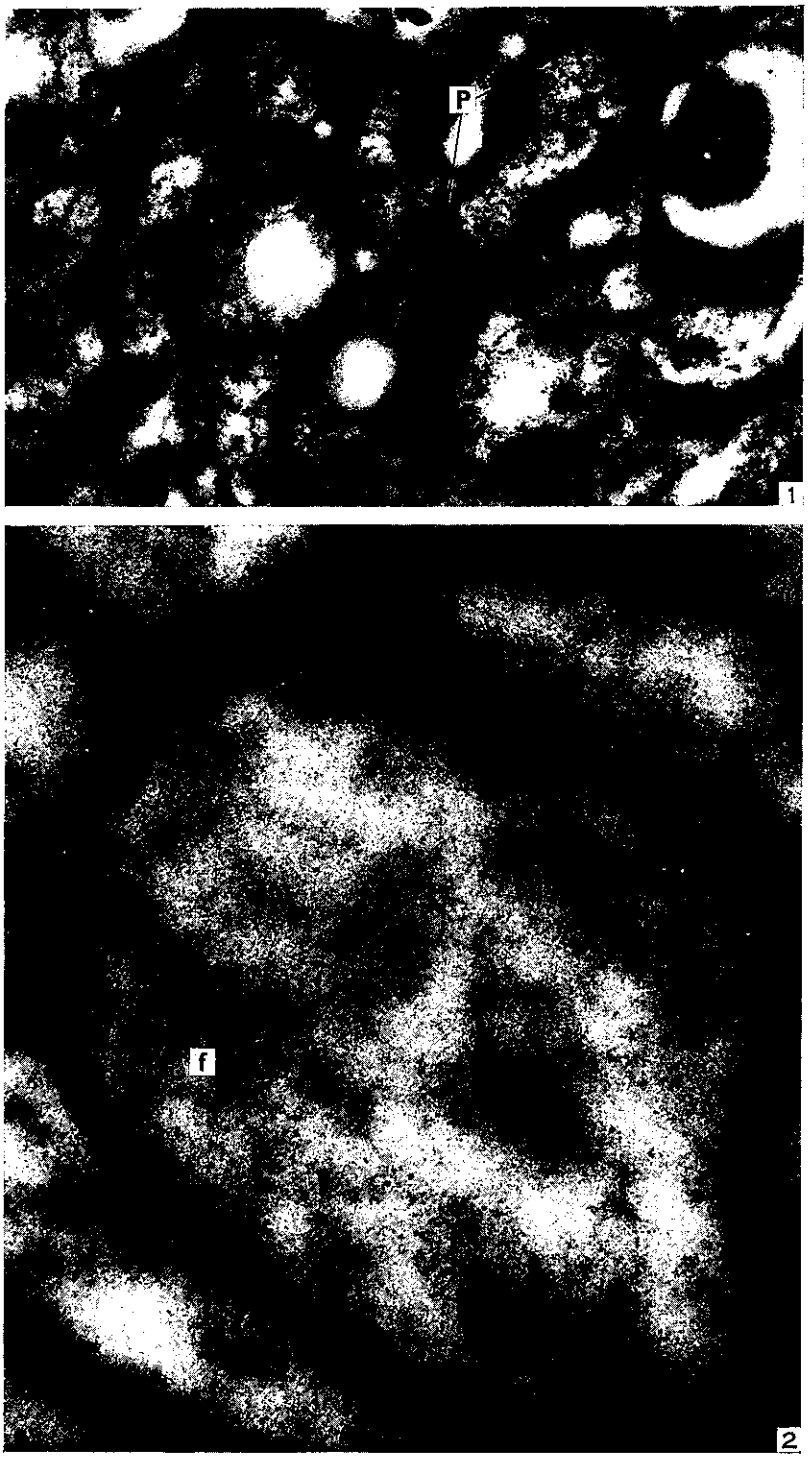
(2) Almost all the iron of 2-day-old nodules was in a form which was extracted by a hot pyrophosphate–trichloracetic acid mixture. Ferritin iron is extracted from mammalian tissue by this procedure.

(3) Ultracentrifugation of a sucrose extract of young nodules yielded red-brown material sedimenting in 15 min at 125,000 *g* and with an iron : protein ratio of 0·16. These procedures (Kuff and Dalton 1957) yield ferritin with an iron : protein ratio of about 0·2 from mammalian liver.

The small amount of 10 g fresh weight of 1–3-day-old nodules represented the maximum yield obtainable with the facilities available. This quantity of nodules gave such minute amounts of ferritin-like material that full characterization by crystallization with cadmium sulphate (Granick 1943) and other methods was impossible. However, the amount obtained should have been sufficient to allow determination of a sedimentation coefficient in the analytical ultracentrifuge. That this was not possible (due to inhomogeneity of the material) may have been due to non-uniform loss of iron during preparation as is indeed suggested by the somewhat low iron content and as has been reported by Rothen (1944) for mammalian ferritin.

The data of Figure 1 suggest that the ferritin-like iron of young nodules is a reserve from which this element is mobilized for synthesis of haemoglobin and other compounds as part of the maturation of the tissue which precedes the onset of nitrogen fixation.

IRON IN THE DEVELOPING SOYBEAN NODULE



The author is indebted to members of the Microbiology Section, Division of Plant Industry, C.S.I.R.O., Canberra, for help in harvesting nodules, to Mrs. M. Stiller for technical assistance, and to Mrs. Margaret Wright (née Briggs) who made the initial electron microscope observations.

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EXPLANATION OF PLATE 1

Fig. 1.—A plastid-like body (*P*) in an electron micrograph of a thin section of a central tissue cell of a 7-day-old nodule. Osmium fixation. ×49,000.

Fig. 2.—Ferritin-like granules (*f*) within a plastid-like body. ×110,000.