MOLECULAR EXCLUSION CHROMATOGRAPHY OF NITRITE AND HYDROXYLAMINE REDUCTASES FROM PLANTS WITH REFERENCE TO ELECTRON DONOR SYSTEMS

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Reduction of nitrite, hydroxylamine, and also of sulphite, is considered to depend on a single enzyme in Escherichia coli (Mager 1960, Lazzarini and Atkinson 1961, Kemp et al., 1963) and in yeast (Naiki 1965). However a multiplicity of hydroxylamine reductases has been revealed by density gradient centrifugation of proteins obtained from Neurospora crassa (Leinweber et al., 1965, Siegel et al., 1965). Betts and Hewitt (1966) reported a partial separation of nitrite and hydroxylamine reductase activities in spinach preparations by passage through a "Sephadex" G 200 column.

We have found evidence for the occurrence of multiple hydroxylamine reductase enzymes but only one nitrite reductase in preparations from higher plants. The hydroxylamine reductases (HR) show different electron donor specificities. One of these (HR 1) is close in M wt. to nitrite reductase (NR). Both HR 1 and NR function equally well with ferredoxin (Fd) or with reduced benzyl viologen ($BV^{\cdot}$). Activity of HR 1 but not NR is equally as good with FMN as with the other donors. HR 2 functions with $BV^{\cdot}$ but not appreciably with Fd and substantially less with FMN, and is not associated with NR activity. Other HR components of lower activity apparently occur and differ with respect to specificity with Fd or FMN as donors.

Experimental Leaf extracts (Cresswell et al., 1965) of marrow (Cucurbita pepo) or spinach (Spinacea oleracea) were fractionated by ammonium sulphate 47-70% saturation. Protein was resuspended in 0.05 M K_2HPO_4 (pH 7.5), centrifuged and desalted on a G25 "Sephadex" column equilibrated with 0.1 M KCl 0.03 M K_2HPO_4 (pH 7.5). For gel filtration G100 or G200 "Sephadex"

columns as specified in the figures were packed with gel previously swollen and equilibrated in KCl/K_2HPO_4 buffer at 1°C and calibrated for M wt. distribution as described by Andrews (1964, 1965) using mixtures of well characterized proteins ranging in M wt. from mammalian cytochrome C to human γ globulin. Fractions (3 ml.) were collected at 15-30 ml./hr. Each standard showed normal distribution and a linear relationship between peak elution volume and log M wt. Plant preparations (6-18 ml.) were run through the columns and each 3 ml. fraction was subdivided, immediately frozen for storage, and later assayed for NR and HR by various procedures below.

Assays with BV• for NR and HR were done as described by Cresswell et al., (1965). Assays for HR were also carried out with FMN (4×10^{-4}M), or Fd (3×10^{-5}M) which was prepared as described by Betts and Hewitt (1966) from the same species as that from which the enzyme extract was made. These donors were reduced in Thunberg tubes under argon by glucose-6-phosphate, its dehydrogenase, NADP and chloroplast diaphorase (Avron and Jagendorf 1956). This was separated from NR and HR by elution from DEAE cellulose with 0.08 M NaCl. The electron donor was reduced by pre-incubation (15 min.); substrate was tipped and incubation continued for 25 min. Assays for NR when using Fd were done either as described above or, where only 0.1 - 0.5 ml. of fraction had been reserved, by the photo-chemical system of Betts and Hewitt (1966). Illumination was for 3-5 min. under argon with 45,000-90,000 lux tungsten light, in presence of ascorbate and indophenol, Fd (3×10^{-5}M) from the relevant species, and 700-800 μg chlorophyll as thrice-washed spinach grana heated 5 min. at 55°C which were devoid of reductase activities. Nitrite and hydroxylamine were determined according to Cresswell et al., (1965) and Hewitt and Betts (1965).

Results and Discussion When assayed with BV• or Fd, activity of NR always occurred in a single symmetrical peak (Fig. 1) corresponding to M wts. of about 60,000 to 64,000 for marrow and spinach. When assayed with BV•, marrow HR showed 2 major peaks; one at approximately 60,000 (HR 1) and

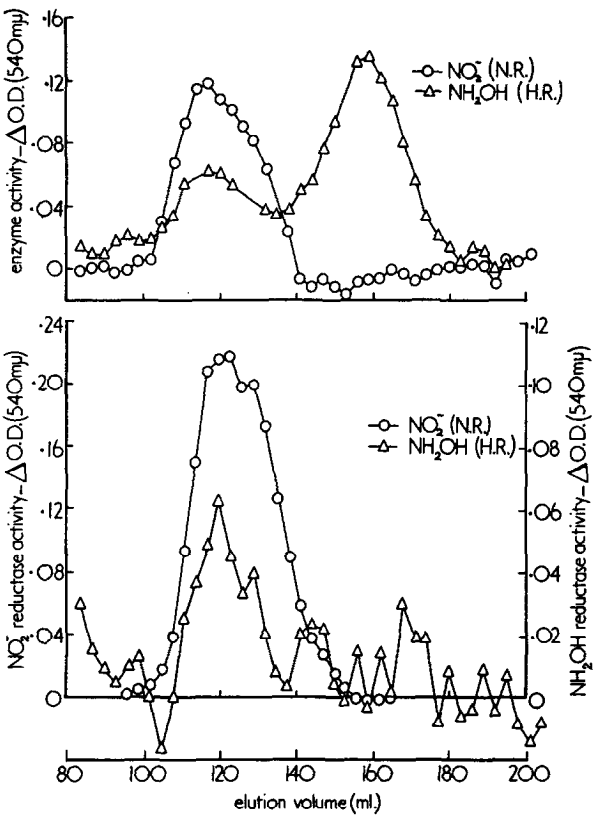

Fig.1 (Upper figure) Spinach preparation from "Sephadex" G100 column (280 ml.) assayed for NR and HR activities in 3ml. fractions with BV• as electron donor. Activities as loss of nitrite or hydroxylamine in terms of Δ E 540 mμ. (See Experimental).

Fig.1 (Lower figure) Spinach preparation (as used above) from "Sephadex) G100 column (280 ml.) assayed for NR and HR activities with ferredoxin (Fd) as electron donor. The Fd was reduced with glucose-6-phosphate and NADP(H) for the HR assay and with light and chloroplast grana for the NR assay as described in Experimental.

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another at about 32,000 M wt. (HR 2). Their activity ratio varied between 0.8 and 1.4 to 1. Spinach HR also showed 2 peaks occurring in variable ratios of 0.4 to 1 or less for HR 1 at about 65,000, and HR 2 at about 32,000 M wts. respectively.

When assayed with FMN or Fd (Fig. 2), the HR 1 peak was always present as the major component with M wt. about 59,000 to 61,000 whereas HR 2 was either absent or greatly diminished in both species. There was however an

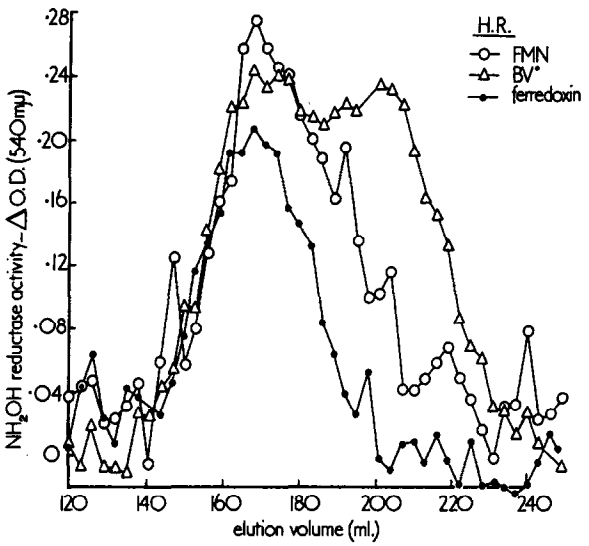

Fig.2 Marrow preparation from "Sephadex" G200 column (285 ml.) assayed for HR activity in 3ml. fractions with BV*, FMN or Fd as electron donors. The BV* and FMN assays were carried out on sub-divided samples of each fraction from the same experiment. The Fd assays were carried out on fractions from an identical column with the same preparation. The BV* assay results for this run (not shown here) coincided with those obtained in the first experiment.

apparent manifestation of other sub-peaks not revealed with BV* probably because of the high activity of HR 2 with this donor. Several sub-peaks and "shoulders" observed with FMN assays occurred at fairly regular intervals of about 16,000 M wt. multiples for marrow. Many of these sub-peaks were less evident with Fd, suggesting even greater specificity. For spinach, (Fig. 1) up to 3 sub-peaks appeared with Fd, those about 22,000 and 40,000 M wts. being the most consistent.

The question of the identity or independence of HR 1 and NR proteins is of interest. Consistent small separations were obtained when the BV* assay was used. It is possible however that the HR 1 peak was broader or was "pulled" slightly towards HR 2 in relation to the true M wt. because of mutual supplementation in the intervening fractions. When Fd was used, (Fig.1) such possible artefacts were minimized, and no consistent resolution of NR and HR 1 peaks was observed even with columns of 570 ml. Nevertheless ratios

of NR and HR 1 changed in successive fractions across the "front", and peak ratios varied widely in different preparations. Furthermore, as shown elsewhere whereas FMN gives only 10 to 25% of the activity obtained with Fd in the NR assay (unpublished work with G. F. Betts) these donors are equally efficient with HR. 1. It is conceivable however that a single "multiheaded" protein might possess different electron donor specificity for reduction of different substrates if partially denatured after extraction. It is possible that different aggregations of an oligomeric system might have different catalytic properties.

In many systems which utilize Fd physiologically, $BV^{\cdot}$ functions as an artificial donor, often with substantial or comparable activity. It was therefore interesting to find that in both species HR 2 reacted with $BV^{\cdot}$ but scarcely at all with natural donors such as Fd or FMN. Work is in progress to test other natural compounds as possible donors for the HR 2 system, and to study the kinetic characteristics of the different HR fractions. Our preparations containing NR and HR activities do not reduce sulphite at appreciable rates with $BV^{\cdot}$ used as donor under the conditions described here.

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