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Deuterium Oxide as a Density Label of Peroxidases in Germinating Barley Embryos[1]

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ABSTRACT

Density labeling with deuterium oxide, gel electrophoresis, and isopycnic equilibrium sedimentation were used to study the appearance and disappearance of individual peroxidases in the embryos of germinating barley. No detectable label was incorporated into those peroxidases which are present in the embryo of the dry seed and disappear during germination. Deuterium was incorporated into the additional peroxidases which appeared in the embryo during germination. This incorporation is not due to deuterium-hydrogen exchange into preformed proteins. The results indicate that the newly appearing peroxidases arise by synthesis during germination.

Differentiation requires quantitative and qualitative control of the cells' enzymic apparatus. A detailed study of the control mechanism therefore requires an unequivocal determination of whether the appearance of a new enzyme activity is the result of activation, that is, some change in molecules synthesized at an earlier stage of development, or whether it is due to *synthesis* of the enzyme protein at the time of the appearance of the enzyme activity. To do this one can (*a*) show whether the increase in enzyme activity is prevented by inhibitors of protein or RNA synthesis; (*b*) attempt to label the enzyme in question by administration of radioactive amino acids, and show that the specific radioactivity of the purified labeled protein is the same as the specific radioactivity of the amino acid added; or (*c*) attempt to density label the enzyme with stable heavy isotopes instead of radioactive isotopes. The density labeling procedure, adapted from Hu *et al.* (7), has been described by Fan (4) and by Filner and Varner (5). This procedure consists of supplying the tissues to be studied with a compound containing a heavy isotope which can be incorporated into amino acids used for protein synthesis. This isotope incorporation causes an increase in the mass of the protein which can be detected by isopycnic equilibrium centrifugation (7).

$H_2{}^{18}O$ is (except for its high cost) the label of choice in tissues in which the pool of free amino acids is low and synthesis of new protein molecules requires the hydrolysis of existing proteins or the synthesis of amino acids. In either case 1 or 2 ^{18}O atoms would be introduced into the α-carboxyl group of each amino acid, and any protein synthesized from these amino acids would have a correspondingly greater mass and buoyant density.

However, a less expensive density label would obviously be welcome. Deuterium oxide appears to be such a label. Density labeling of proteins with deuterium oxide has been used successfully in microorganisms (3, 4, 7) and to a limited extent in plants (6, 11). General applicability in plants, though, remains to be established. In this paper we report the results of using deuterium oxide as a means of labeling the peroxidases of the embryos of germinating barley seedlings.

MATERIALS AND METHODS

Growth and Extraction of Seedlings. Barley seeds (*Hordeum vulgare* L. cv. Himalaya kindly supplied by R. A. Nilan, Washington State University, Pullman) were germinated by placing them on sand moistened with either H_2O or D_2O and incubating them in the dark at 25°. Fifty seedlings were harvested at the specified times, and the desired tissue was dissected out and homogenized in a porcelain mortar with sand and 1 ml of buffer, pH 8.3 (0.076 M tris, 0.051 M citric acid) (14), until smooth suspension was obtained. This step and all subsequent steps were performed at 5°. The resultant suspension was clarified by centrifugation at 12,000*g* for 10 min.

Electrophoresis. The supernatant (0.5–1.0 ml) from the tissue homogenate was applied to strips of Schleicher and Schuell No. 470 filter paper, and these were inserted into an 11% horizontal starch gel. The starch gel was prepared according to the procedure of Scandalios (14). Electrophoresis was conducted in the cold for 15 to 20 hr at a voltage gradient of 6.0 v cm^{-1}. A discontinuous buffer system was used (lithium borate and tris-citric acid, pH 8.3), as described by Ashton and Braden (1). Following electrophoresis, a horizontal section 2 mm thick was sliced from the gel and stained for peroxidase with 1% H_2O_2 and benzidine solution (13).

Elution from Gel. The stained section served as a reference to locate peroxidase on the unstained gel. The individual peroxidase bands were cut out from the gel, ground in a porcelain mortar with sand, and centrifuged at 40,000*g* for 15 min. The supernatants were immediately decanted and assayed for peroxidase activity. In several instances part of the supernatant was reapplied to a starch gel for electrophoresis to assure that complete separation of the single peroxidase band had been achieved.

Isopycnic Equilibrium Centrifugation. The centrifugation procedure was essentially that used by Filner and Varner (5). *Bacillus subtilis* α-amylase (Sigma Chemical Co.) was used as an internal

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marker. This marker was mixed with the solution containing the peroxidase and layered over a CsCl solution to make a final volume of 3.0 ml and a mean density of 1.31. The tubes were centrifuged at maximum speed for 65 to 72 hr in a swinging bucket rotor, either SW-39 or SW-65L Ti (Spinco). In some experiments ^{14}C-amylase from barley rather than α-amylase from *B. subtilis* was used as an internal marker.

After centrifugation the tubes were punctured with a No. 22 stainless steel needle, and fractions were collected. Aliquots were pipetted from each fraction for determination of refractive index, scintillation counting, and enzyme assays. The refractive indices were measured on a Bausch and Lomb refractometer and were converted to density units by means of a standard curve. Scintillation counting was done in Bray's solution (2) with a Beckman scintillation counter. Peroxidase and amylase assays, scaled down by a factor of four, were performed as described by Filner and Varner (5).

RESULTS AND DISCUSSION

Exchange of Deuterium between Protein and Medium. Most of the protons attached to oxygen, nitrogen, and sulfur in proteins can freely exchange with deuterium in the medium (8). During the grinding and centrifugation to which our material had to be subjected deuterium atoms in these positions would re-exchange and be fully replaced with protons from the medium. Thus, in attempts to density label proteins in living cells with deuterium oxide, there should be no increase due to exchange into existing protein molecules. However, folding of the protein molecules may trap some deuterium on nitrogen, oxygen, and sulfur in regions inaccessible to solvent under normal conditions (10). This may result in density increases in pre-existing molecules and in labeling of new proteins after synthesis. The following experiments were devised to estimate the amount of deuterium introduced by exchange into the molecules and trapped in slowly exchangeable positions of the protein molecule.

In the first experiment purified malt amylase was incubated at 25° (pH 4.8) for 15 hr in 8 M urea in 99.7% D_2O. The urea was removed by dialysis in D_2O. The D_2O was removed by further dialysis in H_2O. In the control an aliquot of the same amylase solution was subjected to the same treatment in a water solution. Equilibrium density determinations were made using ^{14}C-amylase from barley (5) as a marker. The amylase treated in urea-D_2O had a buoyant density 0.0008 unit higher than the amylase treated in urea-H_2O. This apparent density shift is within experimental error. We conclude, therefore, that amylase under the conditions of this experiment acquires no detectable slowly exchangeable deuterium.

In the second experiment, peroxidase (Calbiochem) was heated to 70° (pH 4.8) for 20 min in 90% D_2O, then dialyzed against H_2O at 0°. Again equilibrium density determinations were made with ^{14}C-labeled barley amylase as a marker. The density shift (Fig. 1) was 0.0020 unit. This corresponds to 1 slowly exchangeable deuterium per 5 amino acid residues and is in the range of slowly exchangeable deuteriums reported for several proteins (8).

Density Labeling of Peroxidases of Barley Seedlings. It is known that new peroxidase enzymes appear in the embryos of germinating rye grain (16). Our preliminary experiments showed that new peroxidases also appear in barley embryos during germination and that barley will germinate in deuterium oxide. The barley embryo is therefore a suitable system in which to test the usefulness of deuterium oxide as a density label.

In the first experiment 20 barley seeds were germinated in the dark either 60 hr in water or 7 days in 80% D_2O. Seven days in D_2O is required to obtain growth comparable to that in H_2O in 60 hr. The embryos were homogenized in 2.5 ml of 10^{-2} M acetate buffer (pH 4.8), and the extracts were subjected to equilibrium centrifugation. From the results (Fig. 2) it is apparent that the

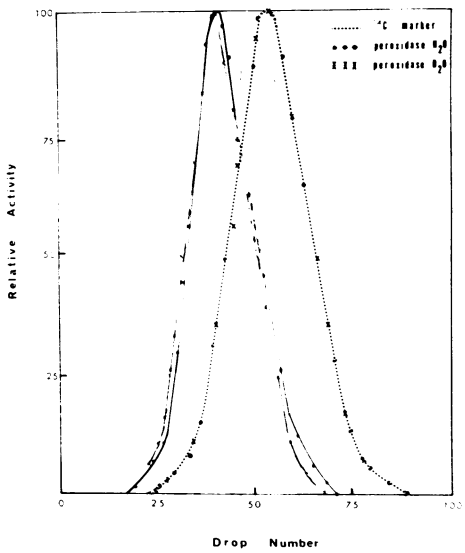

FIG. 1. Equilibrium distribution in CsCl gradients of horseradish peroxidase enzyme activities after heating in H_2O and 90% D_2O. The gradients were centrifuged at 65,000 rpm in a SW-65 L Ti rotor at 4°. The distribution curve of the radioactive barley ^{14}C-amylase markers of both gradients were superimposed. Density increases to the left in all figures.

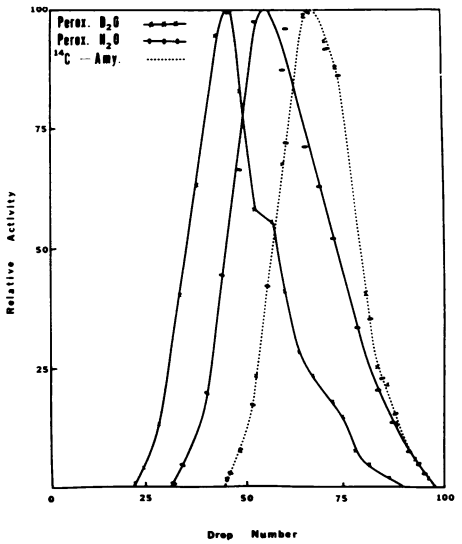

FIG. 2. Equilibrium distribution in CsCl Gradients of peroxidase enzyme activity from barley embryos grown in H_2O and 80% D_2O. Centrifugation conditions were the same as in Figure 1. See text for other experimental conditions.

peroxidases from the 80% D_2O-grown embryos increased in density and that the distribution of the enzyme activity in the gradient is asymmetrical. However, the distribution of the enzyme activity from the H_2O-grown embryos is also asymmetrical. Although a symmetrical distribution of an enzyme on a CsCl gradient doesn't necessarily indicate homogeneity, an asymmetrical distribution always indicates heterogeneity. It is impossible to draw meaningful conclusions about possible density labeling from a density distribution of multiple molecular species. The nature of the heterogeneity must first be understood.

Our preliminary experiments showed that barley contains several forms of peroxidase. Therefore, the most likely explanation for the asymmetry observed on the gradient is that different per-

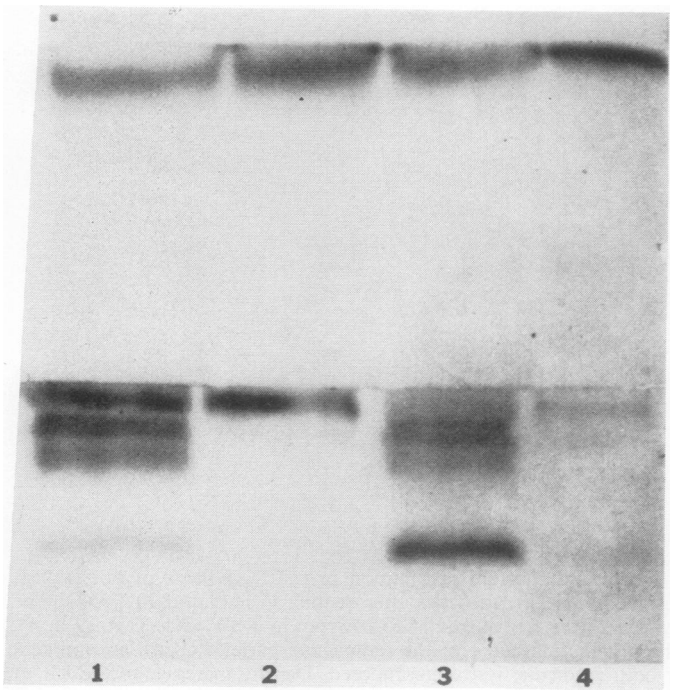

FIG. 3. Starch gel zymogram of peroxidase from CsCl gradients of Figure 2. From left to right: 1: trailing edge of 80% D_2O peak; 2: leading (high density) edge of 80% D_2O peak; 3: trailing edge of H_2O peak; 4: leading edge of H_2O peak. Electrophoresis was conducted at 4° for 6 hr. See text for other experimental conditions.

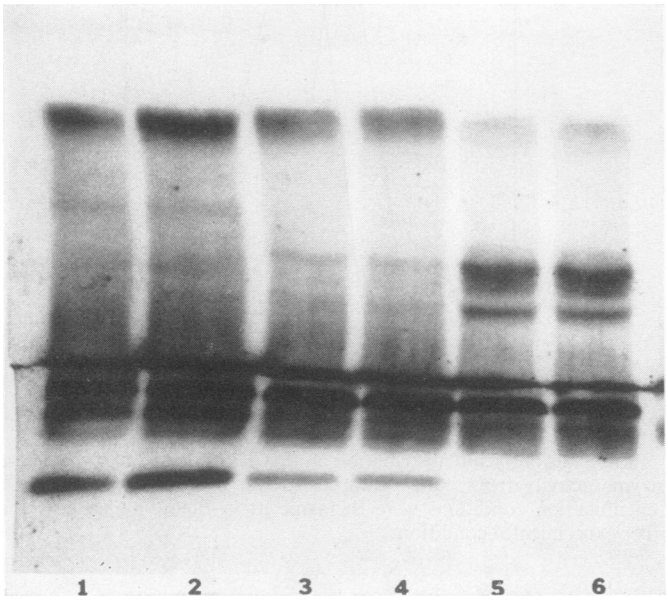

FIG. 4. Starch gel zymogram of peroxidase from barley embryos grown in H_2O and D_2O. From left to right: 1 and 2: H_2O; 3 and 4: 75% D_2O; 5 and 6: 99.7% D_2O. Electrophoresis conditions were the same as for Figure 3.

oxidases have different buoyant densities. We examined this possibility by combining the gradient fractions from the experiment shown in Figure 2 into two samples, one from the leading edge and one from the trailing edge of the peak. The samples were dialyzed to remove CsCl, lyophilized to reduce the volume, and subjected to starch gel electrophoresis. Figure 3 shows that the asymmetrical distribution of the peroxidase activity on the gradient does result from the presence of several peroxidases with different buoyant densities. The leading (high buoyant density) edge of the peroxidase distribution curve is composed predominantly of two peroxidases while the trailing edge consists of at least five.

These differences in buoyant density might be due to differences in carbohydrate content. As much as 18% carbohydrate has been reported in some horseradish peroxidases (15). Proteins have buoyant densities in the range of 1.27 to 1.35 while carbohydrates have buoyant densities of 1.50 to 1.70. Therefore, glycoproteins with much carbohydrate would have a greater density than glycoproteins with little carbohydrate.

Density Labeling of Individual Peroxidases. In density labeling studies each peroxidase must be examined separately. To do this we used gel electrophoresis as a preparative procedure for obtaining single peroxidase bands and studied these individual enzymes by means of isopycnic equilibrium sedimentation.

To do this it was necessary to determine whether the patterns of the peroxidase enzymes of barley seedlings grown in H_2O and in D_2O are comparable. Their slow growth rate is indicative that the barley embryos in D_2O are under isotopic stress. Does this stress alter the normal pattern of peroxidases in the embryo? Figure 4 shows that there are quantitative changes in the normal

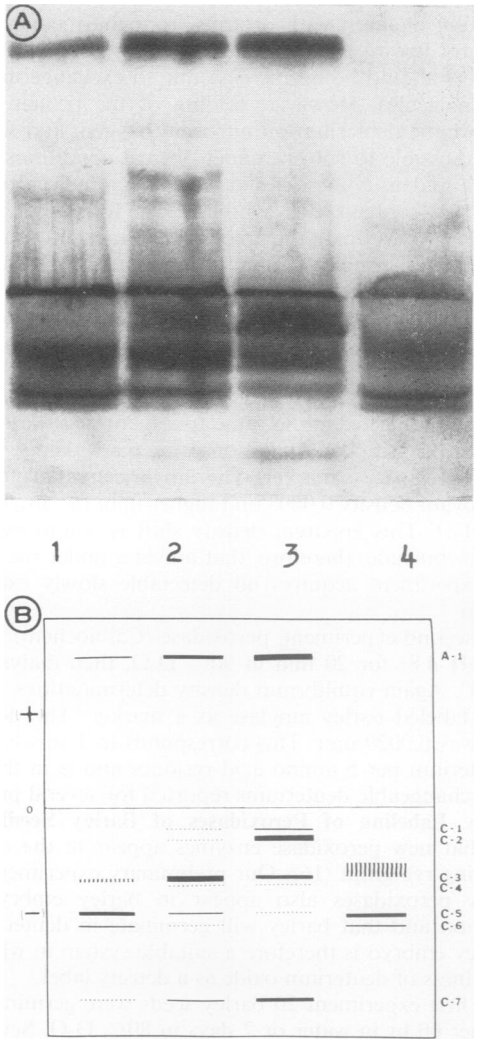

FIG. 5. A: Starch gel zymogram of peroxidase from barley embryos germinated in H_2O. 1: One day; 2: 2 days; 3: 3 days; 4: 0 days (dry seed embryo). Electrophoresis conditions were the same as for Figure 3. B: Schematic diagram of part A.

pattern but no detectable qualitative differences. We did not find the changes in the electrophoretic mobility of peroxidases from D_2O-grown embryos as have been reported *for* D_2O-grown rye embryos (16).

The dry, resting barley embryo contains three major peroxidase bands (Fig. 5). During normal germination two of these bands slowly disappear and several new bands appear (Fig. 5). Before the physiological meaning of these changes can be understood, we need to know whether the appearance of the new bands represents *de novo* synthesis and whether there is turnover (some synthesis accompanied by a more rapid destruction) of the two pre-existing bands which disappear. There was no detectable increase in buoyant density of the pre-existing peroxidase, band C-6, in comparison with the H_2O control of this band during 35 hr of germination of the seed in 75% D_2O (Fig. 6). Therefore, we conclude that these two pre-existing bands disappear without turnover.

The newly appearing band, C-2, extracted from roots of embryos germinated for 5 days in 75% D_2O shows an increase in density over the H_2O-germinated control of 0.010 density unit (Fig. 7). Because the entire band shifts (*i.e.*, there is no band broadening), one must conclude that all of the molecules are density labeled to the same extent. Additional studies with peroxidases extracted from the scutella of 72-hr embryos confirm these results. Density shifts were not observed in those peroxidases present in the scutella of dry seed but were observed in the newly appearing peroxidases.

Because at least some of the peroxidases are glycoproteins (15) containing galactose and arabinose or xylose and fucose, it is necessary to consider whether the density labeling occurs in the carbohydrate moiety only, in the protein moiety only, or in both. There are three ways to account for the density labeling observed in our experiments: (*a*) the deuterium was introduced into the amino acids before polymerization, in which case the density labeling demonstrates *de novo* synthesis of the polypeptide parts of the peroxidase; (*b*) the deuterium was introduced into the monosaccharides before the oligosaccharide was formed, in which case the density labeling demonstrates *de novo* synthesis of the carbohydrate part of the peroxidase; and (*c*) the deuterium was introduced into both polypeptide and carbohydrate moieties before polymerization. Assuming that only the protein is density labeled, a maximum increase of 0.045 density unit can be expected with 75% D_2O (7). However, with proteins from the germinating embryo a smaller increase would be expected because most amino acids used for protein synthesis arise by hydrolysis of reserve protein and are directly utilized without further conversion (12, 17). Thus, a density increase of 0.010 unit could very well result if only the polypeptide parts of the peroxidase are synthesized *de novo*. A 0.010 density unit increase in this case corresponds to the incorporation of 1 deuterium per amino acid residue.

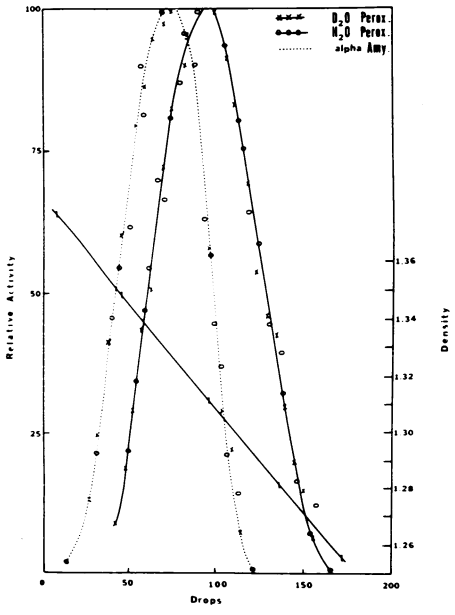

FIG. 6. Equilibrium distribution in CsCl gradients of peroxidase band C-6 (see Fig. 5) eluted from starch gel from barley embryos grown in H_2O and 75% D_2O. The distribution curve of enzymic activity of *B. subtilus* α-amylase markers from both gradients were superimposed. Gradients were centrifuged at 31,000 rpm in SW-39 rotor for 72 hr at 4°.

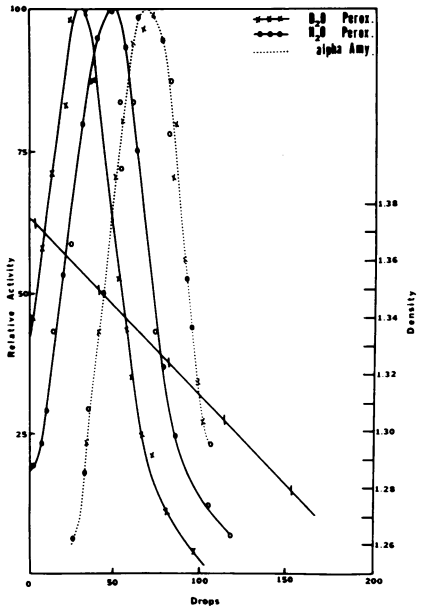

FIG. 7. Equilibrium distribution in CsCl of peroxidase band C-2 (see Fig. 5) eluted from starch gel from barley embryos grown in H_2O and 75% D_2O. Centrifugation conditions were the same as for Figure 6.

If, in this system, the glucose used for the synthesis of galactose, arabinose, xylose, and fucose were derived from endosperm starch rather than from gluconeogenesis, there would be little opportunity for the introduction of deuterium from the medium into the carbohydrate of the peroxidases. Until the biogenesis of the carbohydrates attached to the peroxidases is elucidated in the barley embryo, only tentative conclusions can be drawn from these density labeling experiments. Even keeping these reservations in mind, we feel that it is highly significant that in this developmental system a pre-existing peroxidase does not become density labeled, while newly appearing peroxidases do. The density labeling technique, in conjunction with gel electrophoresis, is clearly an effective tool for investigating the appearance and disappearance of enzyme activities during development.

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