*A TEST FOR DE NOVO SYNTHESIS OF ENZYMES: DENSITY LABELING WITH H_2O^{18} OF BARLEY α-AMYLASE INDUCED BY GIBBERELLIC ACID**

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The aleurone cells of barley grain form and secrete α-amylase in response to added gibberellic acid.[1–3] Inhibitors of RNA synthesis or protein synthesis block the development of the enzyme.[3] Radioactive amino acids are incorporated by aleurone cells into the tryptic peptides of α-amylase. Thus some, but not necessarily all, of the α-amylase activity which develops in response to gibberellic acid is due to synthesis *de novo* of enzyme molecules from amino acids.[3]

If only a small fraction of the active α-amylase were new protein, the physiological significance of the synthesis induced by gibberellic acid would be open to question. It is therefore important to determine the origin of the bulk of the activity. The number of amino acid pools, their relative sizes, their variation in time, and their interrelationships in the aleurone system are unknown. Consequently it is not possible at this time to compute from radioactivity data alone the fraction of α-amylase which is new protein. With respect to the current problem, one need only induce α-amylase under conditions where newly synthesized protein will differ in density from pre-existing protein,[4–7] subject the enzyme to isopycnic equilibrium centrifugation,[8] and determine the fraction of the enzyme activity which is due to newly synthesized protein.[5, 7]

In the experiments presented here, O^{18}-labeled amino acids derived from reserve protein and H_2O^{18} *in vivo* (eq. (1)) have been used to generate a density difference between proteins synthesized by barley aleurone cells before and after exposure to gibberellic acid.

$$\text{Reserve protein} + H_2O^{18} \rightarrow (RCHNH_2CO^{16}O^{18}H \leftrightarrow RCHNH_2CO^{18}O^{16}H) \quad (1)$$

It will be shown that α-amylase formed in H_2O^{18} is more dense than enzyme formed in H_2O^{16}. The distribution of the density labeled α-amylase in the CsCl gradient indicates that essentially all of it arises by *de novo* synthesis from free amino acids derived from pre-existing aleurone proteins.

Materials and Methods.—Preparation of aleurone layers: Dry seeds of barley (*Hordeum vulgare*, var. Himalaya) were cut in half and the half containing the embryo was discarded. The embryoless halves were surface-sterilized with 1% commercial sodium hypochlorite for *ca.* 15 minutes, rinsed with sterile distilled water, and pre-incubated on moist sterile sand for 3 days at 23°C. The aleurone layer can then be readily separated from the starchy endosperm.

Induction of α-amylase: The half-aleurone layers were incubated for 36 hr at 25°C on a reciprocal shaker operating at 50 cycles/min. The incubation mixture, normally 0.1 ml per two half-aleurone layers, contains 0.001 *M* sodium acetate pH 4.8, 0.01 *M* calcium chloride, 10^{-6} *M* GA_3, and 5 μg/ml each of streptomycin, mysteclin, and penicillin. At the end of the incubation, the medium was collected and pooled with an equal volume of water with which the aleurone layers had been washed. The liquid was clarified by centrifugation at 1,000 × *g* for 10 min. The supernatant constitutes crude α-amylase.

Preparation of $O^{16}H^3$ α-amylase: The enzyme was induced in 40 half-aleurone layers in the

presence of 200 μc L-lysine-4,5-H^3, 189 μc/μM (Nuclear-Chicago Corp.). To 7.0 ml of crude enzyme was added 4.7 ml 95% ethanol. The precipitate was removed by centrifugation at 10,000 $\times$ g for 10 minutes and discarded. Two mg of rabbit liver glycogen was added to the supernatant, and the mixture was centrifuged at 10,000 $\times$ g for 10 minutes. The sediment was dissolved in 1.0 ml H_2O and dialyzed against 0.001 M D,L-lysine for 36 hours. The yield was 720 μg α-amylase containing 3,000 cpm/μg, as determined by counting an aliquot in 5.0 ml Bray's solution with a Beckman liquid scintillation counter.

Induction of α-amylase in H_2O^{18}: Two half-aleurone layers, pre-incubated on moist sand in H_2O^{16} were blotted dry (estimated H_2O^{16} remaining: 5.25 μg/half-aleurone layer) and incubated in 0.1 ml H_2O containing 91.3 atom % O^{18}, deuterium content "normalized" (Yeda Batch 1553), plus 0.004 ml H_2O^{16} containing the usual reagents at 25-fold the final concentration. The incubation mixture thus contained 0.114 ml H_2O with 80.1 atom % O^{18}. The incubation was carried out in a sealed tube with a volume of *ca.* 5 ml.

Assay of α-amylase: Boil for 1 min a 0.15% suspension of native (not solubilized) potato starch in 0.04 M KH_2PO_4, 0.2 mM $CaCl_2$. After cooling, centrifuge at 3000 $\times$ g for 10 minutes and discard sediment. The supernatant is used as substrate. Prepare an iodine stock solution containing 6% KI, 0.6% I_2 in H_2O. Dilute the stock solution 100-fold with 0.05 N HCl for use as iodine reagent. To assay α-amylase, dilute an aliquot of the enzyme to 1.0 ml with water. Add 1.0 ml of starch solution and incubate at 25°C for 1–10 min. Stop the reaction and form the blue starch-iodine complex by addition of 1.0 ml of iodine reagent. Dilute with 5.0 ml of H_2O and measure absorbancy at 620 mμ. The decrease in absorbancy relative to a zero time reaction mixture is proportional to the time of incubation and the amount of α-amylase present in the range of 20–80% decrease in absorbancy. The absolute rates vary, however, with the batch of starch used as a substrate.

Assay of peroxidase: Purified horseradish peroxidase was purchased from Calbiochem. Activity was determined by measuring the rate of oxidation of benzidine to benzidine blue in the presence of H_2O_2 and peroxidase. Dissolve 1 gm benzidine in 9.0 ml glacial acetic acid and add 36 ml H_2O. Add a solution containing 1–2 nanograms peroxidase in 1.0 ml to 6.0 ml 0.003% H_2O_2 in a colorimeter set at 610 mμ. Add 0.05 ml of benzidine reagent, mix, and follow the increase in absorbancy from 15 to 30 sec. The rate of absorbancy increase is proportional to peroxidase concentration.

Assay for carbohydrate: The anthrone method was used. Dissolve 0.2 gm anthrone in 95 ml H_2SO_4 plus 5.0 ml H_2O. Add 0.5 ml anthrone reagent to 0.2 ml of unknown. Heat at 100°C for 15 min. After cooling, measure absorbancy at 625 mμ.

Preparation of tobacco cell proteins labeled with N^{15}, N^{14}, C^{14}, and H^3: The XD strain of cultured tobacco cells was allowed to grow exponentially for 10 generations on a medium M-1D containing either $N^{14}O_3^-$ or 99.3 atom % $N^{15}O_3^-$ (Volk Chemicals).[9] The cells were then subcultured in the above media and allowed to grow exponentially for another 4 days, at which time each 500-ml culture contained 2.0 grams fresh weight of cells. Five μc L-lysine-C^{14}, 10 μc/μM (Schwarz BioResearch, uniformly labeled, 240 μc/μM diluted with unlabeled L-lysine), or 100 μc L-lysine 4,5-H^3 (Nuclear-Chicago, 189 μc/μM) was added, and after 14 hr, the cells were collected on a Miracloth filter. They were suspended in ice-cold 0.1 M tris, 0.001 M cysteine, pH 7.5, 5.0 ml per gram fresh weight cells, and homogenized by 30 strokes of a Teflon-glass homogenizer at 4°C. The homogenate was centrifuged at 10,000 $\times$ g for 20 min and the sediment was discarded. An equal volume of saturated ammonium sulfate adjusted to pH 7.5 was added to the supernatant and after 1 hr at 4°C, the precipitate was collected by centrifugation at 10,000 $\times$ g for 10 min. The precipitate was dissolved in 5.0 ml 0.01 M KH_2PO_4, 0.001 M cysteine pH 7.5 and stored at −70°C. Average yields were 5 mg protein according to the Lowry method[10] with a specific activity of 500 dpm/μg for C^{14}, or 10,000 dpm/μg for H^3.

Isopycnic equilibrium centrifugation: In experiments with the swinging bucket rotor (Spinco SW-39), 0.96 ml of water saturated with CsCl (Harshaw optical grade) was mixed with 2.04 ml of water and buffer, less the volume of the solution of proteins to be banded. The protein solution was layered over the CsCl solution, and the tube was filled by layering 2.0 ml of paraffin oil over the protein solution. The mean density of the aqueous phase is 1.30. The tubes were centrifuged at 39,000 rpm for 65 hr at 4°C in a Spinco L-2 ultracentrifuge. After centrifugation, the tubes were punctured through the bottom and *ca.* 270 drops were collected in 3-drop fractions. Alter-

nate fractions were collected directly into 5.0 ml of Bray's solution for scintillation counting. The other set of fractions was used for enzyme or chemical assays. In addition, the density gradient was measured by determining the refractive index of every tenth fraction in a Bausch and Lomb Abbe-type refractometer, and converting the refractive index readings to densities from a standard curve.

In some experiments, isopycnic equilibrium centrifugation was performed in the Ti-50 angle rotor.[11] The procedure was essentially the same. In this case, however, the gradient volume was 4.5 ml, and an overlay of 7.5 ml of paraffin oil was used. In the one case where peroxidase was banded, the initial density was 1.32 rather than 1.30.

Results.—Highly purified O^{16} α-amylase formed a symmetrical band with mean buoyant density of 1.30 ± 0.002. At half-maximum protein concentration, the band width was $.064 \pm 0.002$ density units, or 64 ± 2 drops out of 270 drops in a 3.0-ml gradient (Fig. 1). (It is fortuitous that 1 drop corresponds to 0.001 density unit.)

The distribution of crude O^{16} α-amylase located by enzyme activity was virtually identical to that of purified O^{16} α-amylase located by radioactivity (Fig. 2). The extraneous proteins in crude α-amylase clearly do not aggregate with the enzyme molecules, since such interactions would have made the distribution of the crude enzyme narrower than that of the purified enzyme.[8]

During early seedling development, storage proteins of seeds are hydrolyzed to amino acids which are then available for re-use in protein synthesis. If these events occur in H_2O^{18}, the carbonyl oxygens of the peptide chain should contain one-half the atom % O^{18} present in the H_2O^{18} (see equation 1). Assuming that these carbonyl oxygens account for 15% of the mass of proteins, and that the hydrolysis and protein synthesis proceed in 100 atom % H_2O^{18}, the proteins should have 0.95% higher mass than when synthesized in H_2O^{16}.

Experiments with soluble N^{15} protein and N^{14} protein prepared from cultured tobacco cells showed that a density difference of *ca.* 1% could be readily detected in the preparative equilibrium density gradient. The distributions of N^{15},H^3-protein and N^{15},C^{14}-protein were superimposable (Fig. 3), while that of N^{14},C^{14}-

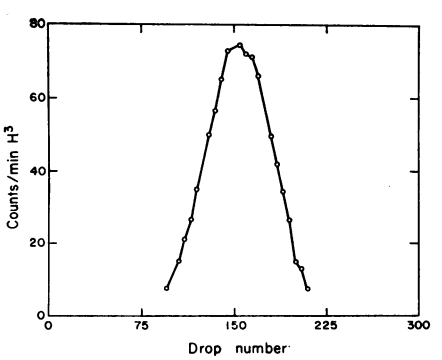

FIG. 1.—Equilibrium distribution of radioactivity (—○—) after centrifugation of purified $O^{16}H^3$ α-amylase in 3.0 ml CsCl solution, $\bar{\rho}$ = 1.30, at 39,000 rpm in a Spinco SW-39 rotor for 65 hr at 4°C. Density increases to the left in all figures.

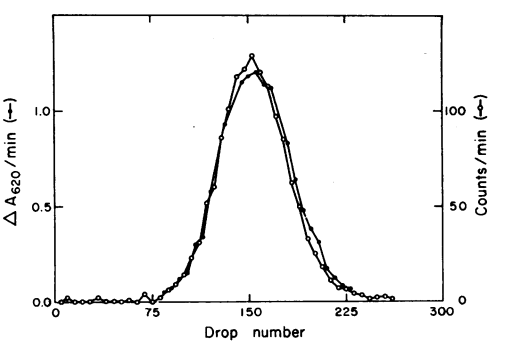

FIG. 2.—Equilibrium distributions of radioactivity (—○—) and α-amylase enzyme activity (—●—) after centrifugation of a mixture of 2 μg purified $O^{16}H^3$ α-amylase and *ca.* 25 μg crude α-amylase induced in H_2O^{16}. The enzyme activity contributed by the radioactive protein was negligible with respect to that contributed by the crude enzyme. Centrifugation conditions were the same as in Fig. 1.

protein was displaced 12 ± 1 drops in the direction of lower density (Fig. 4). Substitution of N^{15} for N^{14} in protein should increase the mass *ca.* 1%. The observed increase in density of soluble protein from tobacco cells was 0.92 ± .08%. It is noteworthy that the N^{15} and N^{14} protein molecules banded independently of each other, since the protein was a total crude soluble protein preparation. This means that very little mixing of N^{15} and N^{14} subunits occurred, either because proteins with subunits are a minor component of the soluble proteins, or the subunits do not dissociate and reassociate in the gradient.

The results of the key experiment, in which α-amylase was induced in H_2O^{18}, can be seen in Figure 5. The presumed O^{18}-enzyme was located by enzyme assay, while the trace of O^{16},H^3 α-amylase was located by radioactivity. The band shapes were very similar, but the entire distribution of O^{18}-enzyme was displaced 15 drops in the direction of higher density, which represents a 1.1% increase. As calculated from the amino acid composition of α-amylase, the peptide carbonyl oxygens account for 14.4% of the mass. The final atom % O^{18} in the incubation mixture was 80 atom %. The expected increase in mass of α-amylase is therefore 0.72%.

The difference between the expected mass increase and the observed density increase may have either a physical or biochemical explanation. The buoyant density of a protein is known to be a function of the water activity, and water activity is a function of position in high salt gradients.[12] It is therefore possible that the expected mass increase was achieved, but the effect of the lower water activity at the higher density position in the gradient was to increase the buoyant density.

The mass increase may in fact have been greater than expected. There are a variety of biochemical explanations possible. Oxygens other than the peptide carbonyl oxygen may have been labeled with O^{18}, or the peptide carbonyl oxygens may have attained a level of O^{18} labeling greater than one-half the atom % of the H_2O^{18}.

The greater than expected increase in density does not affect the basic interpre-

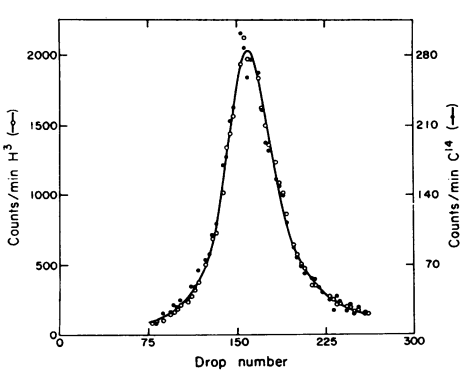

FIG. 3.—Equilibrium distributions of H^3 radioactivity (—○—) and C^{14} radioactivity (—●—) after centrifugation of a mixture of 150 μg each of soluble $N^{15}H^3$ and $N^{15}C^{14}$ tobacco protein. Centrifugation conditions were the same as in Fig. 1.

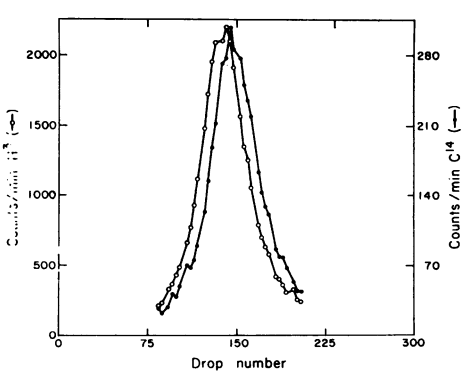

FIG. 4.—Equilibrium distributions of H^3 radioactivity (—○—) and C^{14} radioactivity (—●—) after centrifugation of a mixture of 150 μg each of soluble $N^{15}H^3$ and $N^{14}C^{14}$ tobacco protein. Centrifugation conditions were the same as in Fig. 1.

tation of the experiment: that α-amylase is synthesized *de novo* in response to gibberellic acid.

There are two possibilities which must be ruled out however. If α-amylase had a nonprotein, oxygen-rich component, namely a polysaccharide, then mere attachment of the polysaccharide would increase the buoyant density, if the amount of polysaccharide were substantial. Alternatively, an amount of polysaccharide too small to increase the buoyant density by mere attachment could do so by adding O^{18}. Only 4% of the mass of α-amylase need be O^{18}-polysaccharide to account for the observed increase in buoyant density.

The anthrone reaction is a convenient means to measure carbohydrate associated with protein in gradient fractions. The superposition of horseradish peroxidase enzyme activity and anthrone positive material is shown in Figure 6. The buoyant density is ca. 1.35, which is about what is to be expected for a glycoprotein containing 16% carbohydrate, the figure reported for this glycoprotein.[13]

On the basis of its buoyant density alone, it may be inferred that α-amylase has very little associated carbohydrate. The anthrone assay (Fig. 7) indicated that there is at most 0.4% carbohydrate. The observed density increase in α-amylase cannot be attributed to carbohydrate.

Still another explanation is that O^{18} exchanges with O^{16} in pre-formed protein. Such exchange would have to be enzymatic, since back-exchange would occur otherwise during centrifugation in H_2O^{16} solutions. In order to rule out this possibility, α-amylase was induced in H_2O^{18}, and then the medium, into which the enzyme is secreted, was diluted fivefold with H_2O^{16}. After incubation for 12 hours at 22°C, the enzyme was banded with some N^{14},C^{14}-tobacco protein, which has the same buoyant density as O^{16} α-amylase. No indication of enzyme-catalyzed exchange in the medium was found (Fig. 8).

Such exchange is also rendered unlikely by the successful use of H_2O^{18} to label

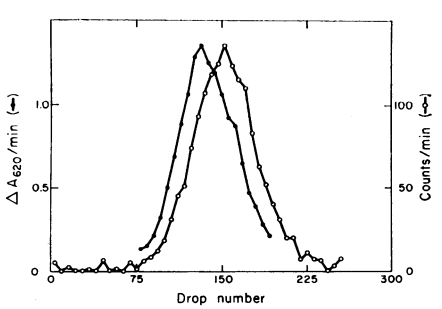

FIG. 5.—Equilibrium distributions of radioactivity (—o—) and α-amylase enzyme activity (—●—) after centrifugation of a mixture of 2 μg purified $O^{16}H^3$ α-amylase and *ca.* 25 μg crude α-amylase induced in H_2O^{18}. Centrifugation conditions are the same as for Fig. 1.

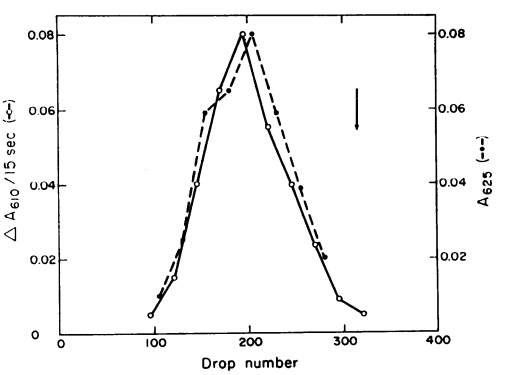

FIG. 6.—Equilibrium distributions of peroxidase enzyme activity (—o—) and anthrone-positive material (—●—) after centrifugation of 1 mg of horseradish peroxidase in 4.5 ml CsCl solution, $\bar{\rho}$ = 1.32, at 49,000 rpm in a Spinco Ti 50 angle rotor for 65 hr at 4°C. The arrow indicates the approximate mean position of either O^{16} α-amylase or N^{14} tobacco protein, when they are banded under these conditions.

proteins in measurements of protein turnover in *E. coli*.[14] The data of Boyer and Stulberg[15] also suggest that such exchange is nonexistent or very slow.

There appears to be no other explanation for the result than that α-amylase is synthesized *de novo* in barley aleurone cells in response to gibberellic acid. Furthermore, within the limits of detection, all of the α-amylase is newly synthesized, since no light α-amylase is evident after induction in H_2O^{18} (Fig. 5).

Discussion.—The technique of isopycnic equilibrium centrifugation is of limited value for preparative separation of proteins because of the broadness of the bands and the closeness of the buoyant densities in most cases. Proteins conjugated with carbohydrate, such as horseradish peroxidase, are made sufficiently dense by the carbohydrate to render them completely separable from unconjugated proteins, such as α-amylase (see Fig. 6).

If two proteins do not interact when in the density gradient, and if they can be assayed independently of each other, then the density gradient technique can be applied analytically to mixtures of proteins in spite of extensive overlapping of the bands. Under the conditions used in this work, a difference in mean band positions of as little as 3 drops out of 270 is detectable. On the basis of the results with crude soluble tobacco proteins, it appears to be true that interaction among proteins strong enough to perturb the banding of the proteins is rare.

The successful application of the density shift test for *de novo* synthesis to α-amylase should encourage its use as a general method for determining whether a new enzyme activity arises by synthesis or activation.

Summary.—The α-amylase formed in barley aleurone layers in response to gibberellic acid was induced in the presence of H_2O^{18}. The enzyme was more dense than enzyme induced in H_2O^{16}. It was concluded on the basis of the distribution of the enzyme in a CsCl gradient that essentially all of the α-amylase induced by gibberellic acid is synthesized from amino acids which arise primarily by hydrolysis of pre-existing seed proteins.

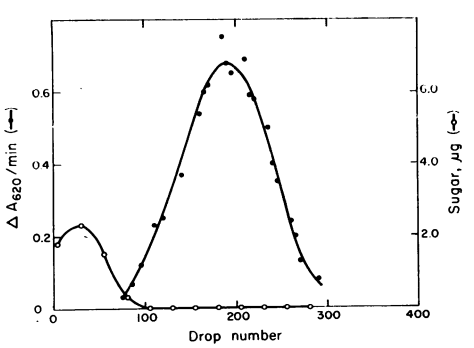

FIG. 7.—Equilibrium distributions of α-amylase activity (—●—) and anthrone-positive material (—○—) after centrifugation of 1 mg of purified O^{16} α-amylase in 4.5 ml CsCl solution, $\bar{\rho}$ = 1.30, at 49,000 rpm in a Spinco Ti 50 angle rotor for 65 hr at 4°C.

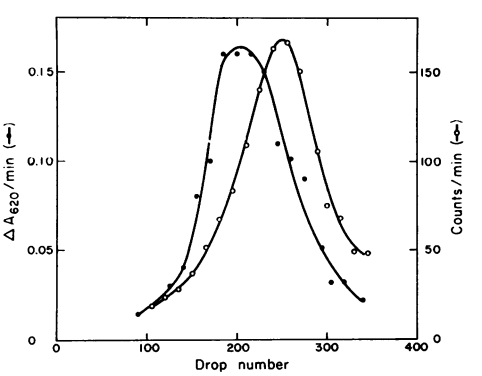

FIG. 8.—Equilibrium distributions of radioactivity (—○—) and α-amylase enzyme activity (—●—) after centrifugation of 2 μg $O^{16}H^3$ α-amylase and *ca.* 25 μg crude α-amylase which had been induced in H_2O^{18}, then post-incubated in H_2O^{16} in the presence of the medium for 12 hr. Conditions of centrifugation were the same as for Fig. 7.

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