Plant Physiol. (1984) 75, 181–186
0032-0889/84/75/0181/06/$01.00/0

# Concentrations of Sucrose and Nitrogenous Compounds in the Apoplast of Developing Soybean Seed Coats and Embryos[1]

Received for publication September 15, 1983 and in revised form January 12, 1984

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## ABSTRACT

**The apoplast of developing soybean (*Glycine max* cv Hodgson) embryos and seed coats was analyzed for sucrose, amino acids, ureides, nitrate, and ammonia. The apoplast concentration of amino acids and nitrate peaked during the most rapid stage of seed filling and declined sharply as the seed attained its maximum dry weight. Amino acids and nitrate accounted for 80 to 95% of the total nitrogen, with allantoin and allantoic acid either absent or present in only very small amounts. Aspartate, asparagine, glutamate, glutamine, serine, alanine, and γ-aminobutyric acid were the major amino acids, accounting for over 70% of the total amino acids present. There was a nearly quantitative conversion of glutamine to glutamate between the seed coat and embryo, most likely resulting from the activity of glutamate synthase found to be present in the seed coat tissue. This processing of glutamine suggests a partly symplastic route for solutes moving from the site of phloem unloading in the seed coat to the embryo.**

The developing soybean seed, because of its economic importance and potential for yield improvement, is an attractive system for nutrient translocation studies. The embryo (cotyledons and embryonic axis) is symplastically isolated from the seed coat (4, 7, 18) so that nutrients translocated to the developing seed from the maternal plant and unloaded from the phloem in the seed coat must move apoplastically before entering the cells of the embryo. Kinetic studies of $^{14}C$ photosynthate movement to the developing seed have provided evidence that movement of photosynthate between the seed coat and embryo may be a control point for sucrose accumulation by the developing embryo (17). Other studies have suggested that the physiological factors limiting soybean seed growth rates reside within the developing cotyledon and not within the maternal plant (6).

In theory, it should be possible to predict the rates of movement of assimilates into the developing embryo from the kinetics of uptake and the concentration of the compound in the apoplast of the embryo. Two studies have described the kinetics of sucrose influx into isolated soybean embryos (10, 19) and recently the kinetics of glutamine uptake have been described (2). In this paper we report the apoplastic concentrations of sucrose and nitrogenous compounds in the seed coat and embryo of the developing soybean seed and their temporal variation.

## MATERIALS AND METHODS

**Plant Cultivation and Selection.** Soybean seeds (*Glycine max* cv Hodgson) were planted at the Agronomy Research Farm in Varna, NY, in the summer of 1980. Seeds were inoculated with a commercial *Rhizobium* inoculant (Agway Inc., Syracuse, NY) prior to planting. Row spacing was 75 cm and plant density about 25 plants per meter. In order to minimize variability due to variation in the developmental rate of individual plants, plastochron indexing (20) was used as a criterion for the selection of developmentally uniform plants. Twelve days before our first sample was collected, 500 plants between the plastochron age of 11.02 and 12.99 were tagged, and they formed the population for later samplings. On each sampling date, plants were severed below the first node and brought immediately back to the laboratory for seed collection and sampling. Only pods containing three seeds from nodes 7 through 9 were collected for sampling of apoplastic solutes.

**Washout of Apoplastic Solutes.** The seed coat was removed from the embryo by making a shallow circular incision around the circumference of the seed coat and gently lifting with forceps. Approximately 100 seeds were processed in this manner for each sample. During the processing, the separated seed coats and embryos were kept chilled on ice. Apoplastic solutes were eluted in ice-chilled water (3 ml/g seed tissue) with gentle stirring for 5 min (seed coat) or 7 min (embryo). The elution times for each tissue were chosen to maximize the elution of apoplastic solutes while minimizing elution of symplastic solutes. The eluents were rapidly filtered (Whatman No. 1 paper filters), frozen in an acetone/dry ice mixture and later lyophilized.

**Apoplastic Volume Determination and Compartmental Analysis.** Apoplastic volumes are often determined by allowing equilibration of tissue with a radioactive impermeant solute and simply quantifying the radioactivity associated with the tissue. With developing soybean embryos, we found that all solutes which freely entered the apoplast also penetrated the cell. Consequently, it was necessary to estimate the apoplastic volume by compartmental analysis of efflux kinetics of a radioactive permeant solute. In addition to an estimate of apoplastic volume, compartmental analysis also allowed quantification of the contribution of different cellular compartments (*i.e.* apoplast, cytoplasm, and vacuole) to each washout sample.

Efflux kinetics were determined for either seed coat or embryo tissue by first incubating the tissue in α-[*methyl*-$^3H$]aminoisobutyric acid (New England Nuclear, 10 Ci/mmol) for 5 min (seed coat) or 2 h (embryo) and then monitoring the efflux of radioactivity. Efflux was measured at 4°C by placing tissue in a 5-ml syringe fitted with a stopcock and aeration tube and collecting eluent through the stopcock at specified intervals. The

[1] Supported by United States Department of Agriculture, Competitive Research Grants Office grant No. 5901-0410-9-0346-0.

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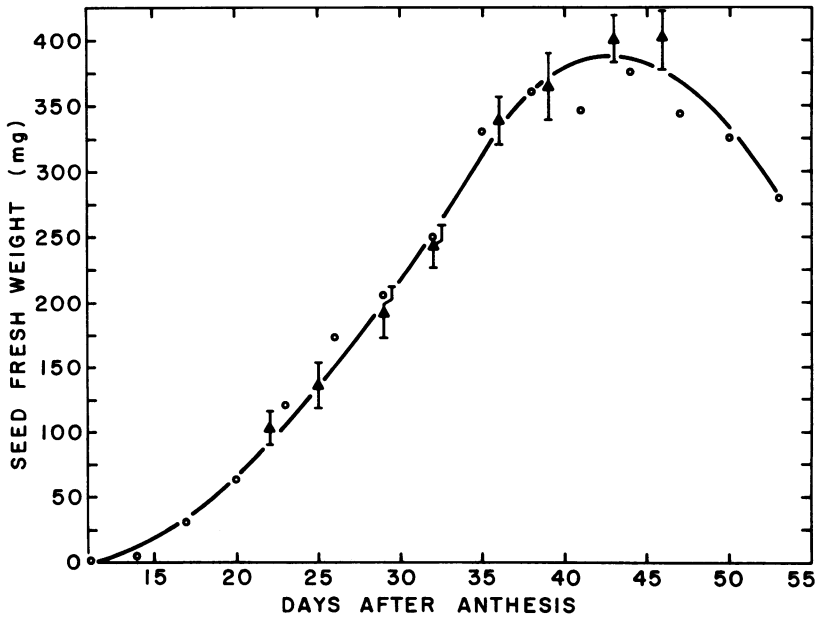


FIG. 1. Growth curve for soybean seeds (*Glycine max* cv Hodgson). Seeds were collected from pods beginning at 11 d after anthesis and fresh weight determined for 15 individual seeds and the mean values plotted (O). The seeds collected for apoplastic sampling were also weighed and the mean ± SD are plotted for each sampling date (▲).

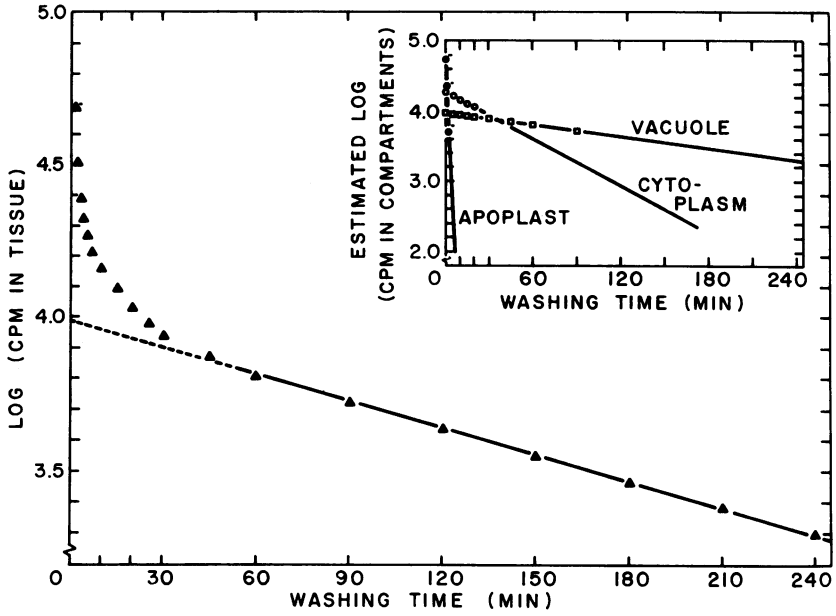


FIG. 2. Efflux kinetics of [$^3$H]AIB from a soybean seed coat. After sequential subtraction of the slower exchanging compartments the efflux from three tissue compartments, presumably the apoplast, cytoplasm and vacuole, can be resolved (inset).

radioactivity in each eluent was counted using a Beckman LS-100C liquid scintillation counter. At the end of the efflux collections, the remaining radioactivity in the tissue was determined after combustion of the sample in a Packard Tri-Carb B306 sample oxidizer.

**Solute Quantification.** Sucrose was hydrolyzed by invertase (Sigma) in acetate buffer (pH 4.6) at 55°C and the resulting glucose assayed by reaction with *O*-toluidine reagent (Sigma technical bulletin No. 635). Allantoin and allantoic acid were determined as previously described (8). Nitrate was assayed by nitration of salicylic acid and colorimetric determination of its product (3). Amino acids and ammonia were analyzed with a Beckman amino acid analyzer on a physiological fluid program.

**Assay of GOGAT.**[4] Seed coats were separated from embryos as described above and the layer of nucellus tissue (referred to as embryo sac in Ref. 18) was peeled from the inner surface of the seed coat. Both parts were immediately frozen on dry ice and saved separately. The tissues were powdered in a dry ice bath with a mortar and pestle and then homogenized in 50 mM Tris-HCl, 1 mM EDTA, 5 mM mercaptoethanol and 1% (w/v) BSA (pH 7.5) using a Brinkman Polytron homogenizer. The homogenate was centrifuged at 20,000*g* for 10 min and the supernatant passed through a Bio-Gel P-10 column. Column eluents were either assayed for glutamate synthase activity directly or after concentration in a Minicon B-15 concentrator. Assay conditions were the same as Beevers and Storey (1). The blanks consisted of assay mixtures without NADH. Only NADH-dependent GOGAT activity was assayed.

**Microscopy.** Soybean seeds (approximately 30 mg fresh weight) were fixed by the method of Karnovsky (9). Fixed tissue was then dehydrated in a graded acetone series and embedded in epoxy resin. Thick sections (1 μm) were prepared on a Sorvall MT-1 microtome equipped with glass knives. Sections were stained with 0.05% toluidine blue in 0.1 M $NaHCO_3$ and 0.05 M $Na_2CO_3$ (pH 9.5) and photographed with a light microscope.

[4] Abbreviations: GOGAT, glutamate synthase; AIB, α-aminoisobutyric acid.

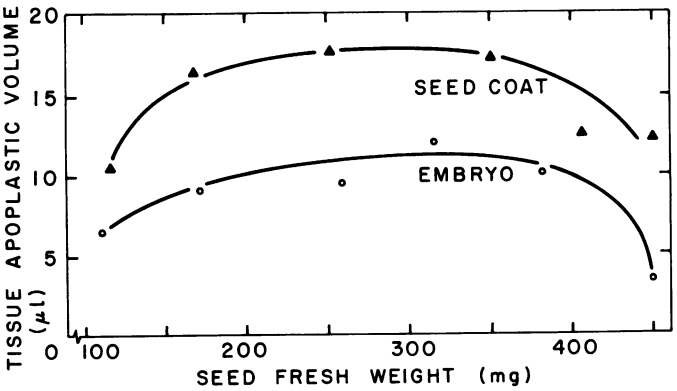


FIG. 3. Apoplastic volumes of seed coats and embryos throughout seed development. Values shown are expressed in terms of μl/seed coat or μl/embryo.

## RESULTS

**Seed Development.** Under the field conditions in 1980, soybean seed development was complete in about 53 d after flowering, with physiological maturity (maximum seed dry weight) attained after 42 d (Fig. 1). The sampling period for apoplastic solutes, between the 22nd and 46th d after flowering covered more than two thirds of the rapid phase of seed-fill and extended to the time of physiological maturity.

**Efflux Analysis.** In order to measure apoplastic volumes and to select appropriate elution times for the collection of apoplastic solutes, compartmental analysis of the efflux kinetics of preloaded [$^3$H]AIB was carried out. AIB was used in these experiments since it is taken up but not metabolized by soybean embryos (2), which allowed the establishment of stable internal pools of AIB for subsequent elution. The results of a typical elution of a seed coat is shown in Figure 2. The data plotted in this manner for both seed coats and embryos could be analyzed in terms of efflux of AIB from three compartments in series. From a linear regression analysis of each set of efflux data as detailed by Cram (5), the radioactivity in each compartment could be determined at each time point (Fig 2, inset). From the intercept of the efflux from each compartment, the radioactivity at time zero in each compartment could be estimated. Using this estimate of the apoplastic radioactivity, the apoplastic volume could be calculated by:

$$\text{apoplastic volume } (\mu l) = \frac{\text{apoplastic radioactivity (cpm)}}{\text{cpm}/\mu l \text{ of AIB solution}}$$

Using this method, apoplastic volumes of seed coats and embryos at various stages of development were determined (Fig. 3). Both seed coat and embryo apoplastic volumes increased with increasing seed weight, with the maximum volume reached near physiological maturity, after which time the apoplastic volume decreased during seed desiccation. The apoplastic volume of the seed coat was consistently larger than that of the embryo even though the fresh weight of the embryo exceeded that of its companion seed coat by a factor of 2 to 10 throughout development (data not shown). Light micrographs of soybean seeds (Fig. 4) indicate that the large apoplastic volume of the seed coat is attributable to the large air spaces present in this tissue but not in the embryo or cotyledonary tissue. Infiltration of these airspaces during loading of the tissue with [$^3$H]AIB would result in their inclusion in the apoplastic volume determination during subsequent elution. It is likely that solutes unloaded from the phloem within the seed coats are restricted to movement within

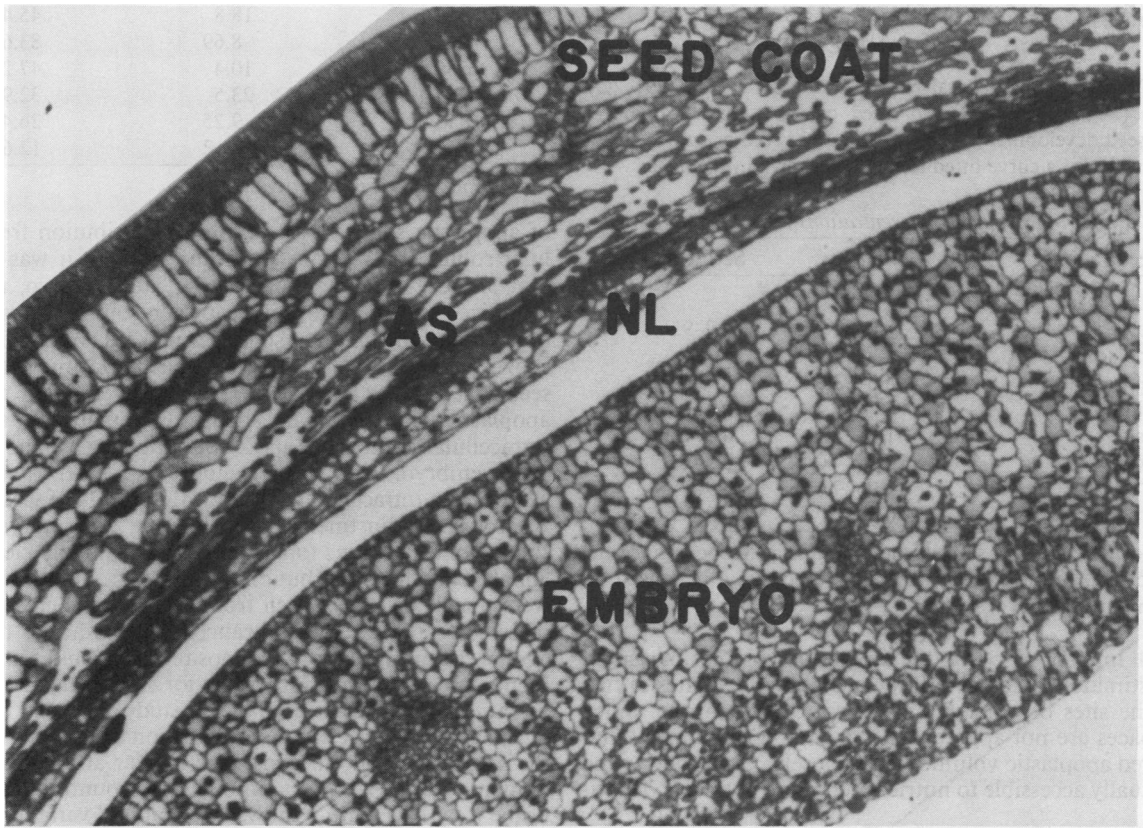


FIG. 4. Light micrograph of the seed coat and embryo tissue taken from seed weighing approximately 30 mg fresh weight. Note large air spaces (AS) and densely cytoplasmic nucellus layer (NL) in the seed coat.

Table I. *Contribution of Different Compartments to Solutes in 170-mg Seed-Free Space Wash Out Samples*

| Variable | Seed Coat (5-Min Wash) | | | Embryo (7-Min Wash) | | |
|---|---|---|---|---|---|---|
| % Free space cpm cleared | 99.7 | | | 73.1 | | |
| % cpm of different compartments | Free space | Cytoplasm | Vacuole | Free space | Cytoplasm | Vacuole |
| | 95.6 | 3.6 | 0.79 | 76.8 | 23.1 | 0.16 |

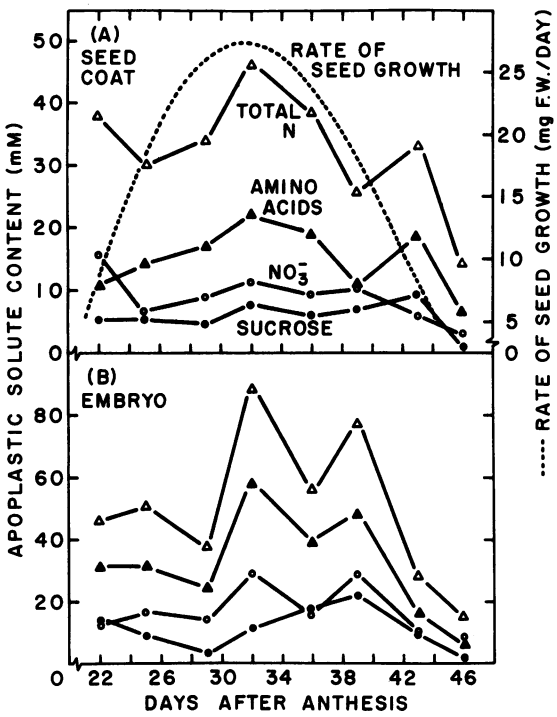


FIG. 5. Concentrations of total N (△), amino acids (▲), $NO_3^-$ (○), and sucrose (●) in the apoplast of seed coat (A) and embryo (B) throughout seed development. The plot of rate of seed growth (——) is the first derivative of a curve fitted to the data shown in Figure 1.

Table II. *Range of Apoplastic Concentrations for Major Nutrients*

| Concentrations | Embryo | Seed Coat |
|---|---|---|
| | *mM* | |
| Sucrose | 3.4–22.2 | 4.9–9.9 |
| Amino acids | | |
| Neutral | 8.6–23.8 | 8.7–20.0 |
| Acidic | 8.9–17.4 | 0.5–1.7 |
| Basic | 1.0–2.9 | 0.8–1.8 |
| Total[a] | 20.0–44.0 | 10.5–22.5 |
| $NO_3^-$ | 13.0–29.6 | 5.9–15.9 |
| $NH_3$ | 0.4–2.2 | 0.2–1.5 |

[a] Totals do not equal the sum of the amino acid fractions since the minimums or maximums for each fraction did not occur in concert on the same sampling date.

the cell wall interstices so that the apoplastic volumes measured here overestimate the actual volume accessible to solutes diffusing from the sites of phloem unloading to the embryo. Since large air spaces are not apparent in the embryo, it is likely that the measured apoplastic volumes more closely correspond to the volume actually accessible to nutrients en route to the cotyledonary cells.

In addition to the determination of apoplastic volumes, efflux analysis also provided a basis for selecting an appropriate elution time for both seed coats and embryos to maximize the collection

Table III. *Major Amino Acids in Apoplast of the Soybean Embryo and Seed Coat*

| Amino Acids | Embryo | Seed Coat |
|---|---|---|
| | % | |
| Glu | 35.4 | 1.0 |
| Gln | 0 | 27.8 |
| Asn | 18.5 | 10.5 |
| Ala | 14.1 | 7.6 |
| Ser | 5.9 | 8.3 |
| B-Ala | 4.9 | 5.4 |
| Asp | 4.1 | 2.5 |
| Arg | 3.6 | 3.4 |
| Gly | 2.3 | 3.1 |
| Cys | 2.3 | 4.2 |
| Total | 91.1 | 73.8 |

Table IV. *Seed Coat and Nucellus GOGAT Enzyme Activity*

| Seed Fresh Weight | GOGAT Activity | |
|---|---|---|
| | Seed coat | Nucellus tissue |
| *mg* | *nmol NADH oxidized* $min^{-1}$ $g^{-1}$ | |
| 167 | 5.60 | 27.1 |
| 194 | 18.8 | 45.4 |
| 217 | 8.69 | 83.6 |
| 280 | 10.4 | 47.2 |
| 368 | 23.5 | 32.9 |
| 415 | 9.25 | 26.4 |
| 410[a] | 6.12 | 12.6 |

[a] Postphysiological maturity.

of apoplastic solutes with minimal contribution from intracellular solute pools. From the efflux analysis it was determined that 5 and 7 min for the seed coat and embryo, respectively, optimized the collection of apoplastic solutes. A typical breakdown of the collection of solutes from seed coats and embryos is shown in Table I. It is apparent that the 5-min elution of the seed coat seeds of this size yields a nearly complete collection of apoplastic solutes with very small contamination by solutes from intracellular compartments. Due to the larger tissue dimensions of the embryo, the apoplastic elution is neither as complete nor as free from intracellular solutes as the elution of seed coat tissue. Appropriate adjustments in calculating the embryo apoplastic solute concentrations to account for incomplete clearing of the apoplast and for contributions of solutes from intracellular pools were made. As can be seen from the example given in Table I, these corrections tended to cancel one another as they were of similar magnitude but in opposite directions. Similar adjustments in calculations were made for seed coats and embryos at different growth stages used in this study.

**Apoplastic Solutes.** The composition of apoplastic solutes in the seed coats and embryos were similar at all stages of development with $NO_3^-$ and amino acids accounting for 80 to 95% of the total N. Figure 5 shows the temporal variation in concentrations of the major apoplastic solutes. Each sample was collected at 10 AM on the indicated day after flowering. A peak of apoplastic amino acids, $NO_3^-$, and total N concentrations in

both the seed coat and embryo coincided with the most rapid phase of seed growth, while sucrose concentration was relatively constant over the entire period of seed development. The second peak of amino acids occurred after the seed growth rate started to decline and could be caused by the influx of remobilized nitrogenous compounds from senescencing vegetative tissues. The ureide (allantoin or allantoic acid) content of the samples was detectable on only one sampling date (25 d after flowering) were ureides contributed only 2.5% of the total N. Ureides were conspicuously absent in all other samples.

Diurnal variations in apoplastic solute content were monitored by sampling at 6-h intervals over a 24-h period on four occasions throughout the period of seed development. Solute content was fairly stable in the seed coat and embryo apoplast over the diurnal cycle (data not shown) in agreement with the observations of Patrick and McDonald (15).

Figure 5 also indicates that the solute concentrations in the seed coat were consistently lower than in the embryo. This is contrary to the accepted notion that solutes diffuse down a concentration gradient from the seed coat to the embryo (18). This, most likely, results from the large volume estimate of the seed coat apoplast due to infiltration of the large air spaces (discussed above) and may not reflect the actual concentration of solutes restricted to the cell wall matrix.

With respect to predicting rates of solute influx from uptake kinetics, it was of interest to tabulate the physiological range of concentrations for the major seed nutrients. These are shown in Table II for both the seed coat and embryo apoplast. The high levels of $NO_3^-$ and $NH_3^+$ suggest that a study of their uptake by isolated embryos, or of their interaction with the uptake of sucrose or amino acids may be of considerable importance.

The amino acid composition of the apoplastic solution is shown in Table III for samples collected during the period of the greatest rate of seed growth. The amino acid composition of all other samples was similar to that shown in Table III. Methionine, an essential amino acid deficient in soybean seed protein represented less than 1% of the total amino acids present. Of special interest here is the nearly quantitative conversion of glutamine to glutamate between the seed coat and embryo. The conversion was evident in every sample collected. Since samples were prepared and analyzed identically, it is presumed that this conversion did not result from the preferential, nonenzymic deamination of glutamine in samples originating from the embryo but instead resulted from processing of glutamine *in vivo*. Since enzymes catalyzing the conversion of glutamine to glutamate, most notably GOGAT, are intracellular, this suggested that solutes in the seed coat apoplast moved to the embryo by a partly symplastic route.

**GOGAT Identification.** The innermost layer of the seed coat (nucellar tissue) is densely cytoplasmic (Fig. 4) and seemed to be a likely place for the conversion of glutamine to glutamate. This inner tissue layer was separated from the rest of the seed coat, and GOGAT activity was assayed in soluble homogenates from both tissues (Table IV). The nucellar layer contained levels of GOGAT that were higher than the rest of the seed coat on a fresh weight basis, supporting the possibility that glutamine conversion took place in this inner tissue layer of the seed coat. The high glutamate to glutamine ratio in the embryo free space was consistent with observations on these two amino acid pools in maturing pea cotyledons (Fig. 7 in 11).

## CONCLUSIONS

The work presented here was undertaken with the long range goal of elucidating mechanisms responsible for solute translocation into developing soybean seeds. Specifically, the data presented on apoplastic solute concentrations provide information as to what solutes get into the seed, and what kind of processing these solutes undergo between the site of unloading and the site of uptake by the embryo. As indicated in Table I, there are errors associated with sampling apoplastic solutes. Eluent from the embryo apoplast is slightly contaminated by cytoplasmic solutes, which may contribute to error in the compositional analysis. On the other hand, volume determination of the embryo apoplast is not confounded by large air spaces so that the calculated concentrations of solutes are not underestimated in this tissue. For seed coat tissue the situation is reversed with the apoplastic eluent being essentially free of solutes from intracellular compartments, but having large air spaces which most likely result in an overestimation of the apoplastic volume available to solutes and, consequently, an underestimation of solute concentrations in the seed coat apoplast. We do not feel that these complications invalidate the results presented here but should be considered when interpreting the data. As a consequence, we do not feel that the apparent reversal of the solute concentration gradient between the seed coat and embryo should be construed to suggest that fluxes of solutes between the seed coat and embryo are the result of active processes.

Other features of the results which deserve comment are the low ureide content and the glutamine to glutamate conversion in the seed coat. Since our plants were well nodulated and ureides are the major *N*-compounds translocated from soybean root nodules (12, 13, 16), we were surprised that ureides were not detected in the seed apoplast. Although the field was treated with fertilizer containing $NO_3$ at the time of seeding, nodule growth was vigorous and N fixation was initially normal as monitored by acetylene reduction in soybean plants adjacent to ours (R. Denison, personal communication). However, at the time of flowering, the Ithaca area was subjected to a period of sustained drought which severely reduced N fixation. This might explain the low ureide levels we observed.

The presence of glutamate synthase in the soybean seed coat suggests that this enzyme was responsible for the observed conversion of glutamine to glutamate between seed coat and embryo samples. While this conversion may have taken place at any point between the seed coat and embryo (14), the high concentration of GOGAT activity in the nucellus suggested that solutes may pass through the symplasm of this tissue layer. The transport properties of this strategically located tissue deserve further study.

*Acknowledgments*—We thank T. Galgano for technical assistance and Dr. R. Zobel of the United States Department of Agriculture Rhizobotany Project for providing field facilities and planting the soybeans.

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