

Metabolism of Barley Seed During Early Hours of Germination

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Abstract. The growth process in germinating barley seeds and its inhibition by actinomycin D and puromycin were investigated. Soon after seeds are imbibed, their respiratory activity increases several fold, and the protein- and carbohydrate-synthesizing systems become active. The immediate activation of protein synthesis and its inhibition by actinomycin D and puromycin suggest that the dry seed has all the components necessary for protein synthesis.

Although a good correlation exists between the rate of oxygen uptake and that of protein synthesis during the first 12 hr of germination, respiration appears to be independent of protein synthesis during the first 8 hr, as reflected by the insensitivity of the respiratory process to actinomycin D and puromycin. However, after 8 hr both antibiotics reduce oxygen uptake as well as subsequent seedling growth.

The distribution of ^{14}C , derived from labeled glucose during the early hours of barley germination, among various fractions of metabolites, indicated that 50 to 70% of the utilized glucose appeared in $^{14}\text{CO}_2$. The rest of the incorporated label appeared in hemicelluloses and starch, water-soluble ethanol-insoluble carbohydrates, and to a lesser extent in proteins and cellulose.

The growth process in germinating barley has been under investigation for over a hundred years. Brown and Morris (3) reviewed the work done during the nineteenth century. These early studies concentrated on finding optimum conditions for germination, and on the roles of endosperm and embryo as food source and food consumer respectively, and on the complex interactions between these 2 parts. More recent publications report the interactions between the embryo, which is the natural source of gibberellic acid, and the aleurone cells of the endosperm, which supply enzymes responsible for the degradation and mobilization of the reserve food (2, 5, 7, 19). Most of these studies, however, have been conducted after seeds have been imbibed for 24 to 48 hr; thus the findings reflect the metabolic state of the seeds many hours after imbibition.

The present paper reports on glucose metabolism, protein synthesis, and respiration which take place in barley during the first 12 hr of germination. Knowledge of such early phases of growth could give an insight into the biosynthetic capacity of the seed and thus might be used to determine seed quality and predict seedling performance in much less time than that required by present methods.

Materials and Methods

Barley (*Hordeum vulgare* L.), variety Wisconsin X 691-1, was supplied by Dr. Allen Dixon of the Barley Malting Laboratories¹ in Milwaukee, Wisconsin. DL-Leucine- ^{14}C , specific activity 31 mc/mole, was purchased from New England Nuclear; glucose- ^{14}C , specific activity 73 mc/mole, from Cal-Biochem; actinomycin D (Act D) from Merck, Sharp & Dohme Company; puromycin (Puro), penicillin-G, and streptomycin sulfate from Sigma Chemicals; naphthalene, cab-o-sil, and 2,5-diphenyloxazole from Packard; and 1,4-dioxane from Baker's Chemicals.

Seeds were washed in 1% sodium hypochlorite (NaHClO) for 15 min and aseptically transferred into imbibing and incubation media containing penicillin-G and streptomycin sulfate (20 $\mu\text{g}/\text{ml}$ of each). All pretreatments and incubations were performed in a constant-temperature water bath at 25° with shaking.

Incorporation of Leucine- ^{14}C Into Proteins. The time course for incorporation of leucine- ^{14}C into proteins was determined. Six lots of 60 seeds each were soaked in 1% NaHClO for 15 min, washed 6 times in sterile water, then imbibed in water for 0, 2, 4, 6, 8, and 10 hr. The seeds were then blotted on paper towels for 30 sec, and the incubation was started by transferring them into 50-ml Erlenmeyer flasks containing 5 ml of 10^{-4} M leucine- ^{14}C , 1 $\mu\text{c}/\text{ml}$. After 2 hr incubation, seeds were transferred into

¹ Mention of trade names and the names of commercial companies are used in the publication solely for the purpose of providing specific information.

an ice bucket, washed 4 times with 25 ml of ice-cold water, blotted on paper towels, and frozen at -20° .

Proteins were extracted by grinding the seeds in an ice-cold mortar in 10 ml of water for 12 min and centrifuging at $9000g$ for 10 min. The precipitate was washed 3 times, centrifuged, and the washings were combined with the original supernatant. A 0.5 ml aliquot of the supernatant was saved and the radioactivity determined. Cold perchloric acid (PCA) was added to the rest of the supernatant to make the final concentration 10% (v/v); this was refrigerated overnight. It was centrifuged, and the precipitate was washed twice in 10 ml of 10% cold PCA containing 10^{-3} M unlabeled leucine, then resuspended in a small volume of 0.3 N KOH. This fraction is referred to as soluble proteins.

Incorporation of Glucose- ^{14}C Into Various Components. The metabolic balance during the early hr of germination was studied using glucose- ^{14}C . Seeds were soaked in 1% NaHClO for 15 min, washed 6 times, imbibed in sterilized water for 6 hr, then blotted, and incubated in a medium containing 10^{-3} M glucose- ^{14}C , 1 μ C/ml, for 3 hr at 25° with constant shaking. During incubation the system was flushed with a stream of air (30 cc/min). The $^{14}CO_2$ was trapped in saturated $Ba(OH)_2$. Incubation was terminated by washing the seeds 4 times in ice-cold water and freezing at -20° .

The extraction procedure (Fig. 1) is a modification of that described by Robinson (16). Seeds were ground for 12 min in water in an ice-cold mortar and centrifuged at $9000g$ for 10 min. The precipi-

tate was washed 3 times with 10 ml of cold water; the washings and the original supernatant were combined.

The precipitate (Fraction II) was hydrolyzed with 17.5% (w/v) NaOH for 24 hr at room temperature with occasional stirring. The hydrolysate was centrifuged. The residue (Fraction IV), which consisted mainly of cellulose, was washed 4 times in water until neutral, centrifuged, and the washings combined with the supernatant. The alkaline supernatant (Fraction V) was brought to pH 4.5 with HCl, then brought to 80% (v/v) ethanol, and left in ice. The precipitated starch and hemicelluloses (Fraction VI) were washed twice with 95% ethanol.

The supernatant (Fraction III) contained water-soluble proteins, polysaccharides, amino acids, and organic acids. Soluble proteins (Fraction IX) were precipitated in 10% PCA, held in an ice bath for several hr, centrifuged and the precipitate washed twice in 10% cold PCA. The precipitate was suspended in 2 ml 0.3 N KOH. Water-soluble, ethanol-insoluble polysaccharides (Fraction XI) in the acid-soluble fraction were precipitated in 80% ethanol after adjusting the pH to 6.2 and eliminating the perchlorate. The 80% ethanol precipitate was further washed twice in 95% ethanol. The ethanol supernatant fraction (Fraction X) contained the ethanol-soluble sugars, organic acids, and amino acids.

Each fraction was counted by liquid scintillation (Packard-Tricarb) in a solution containing 100 g naphthalene, 6 g 2,5-diphenyloxazole brought to 1

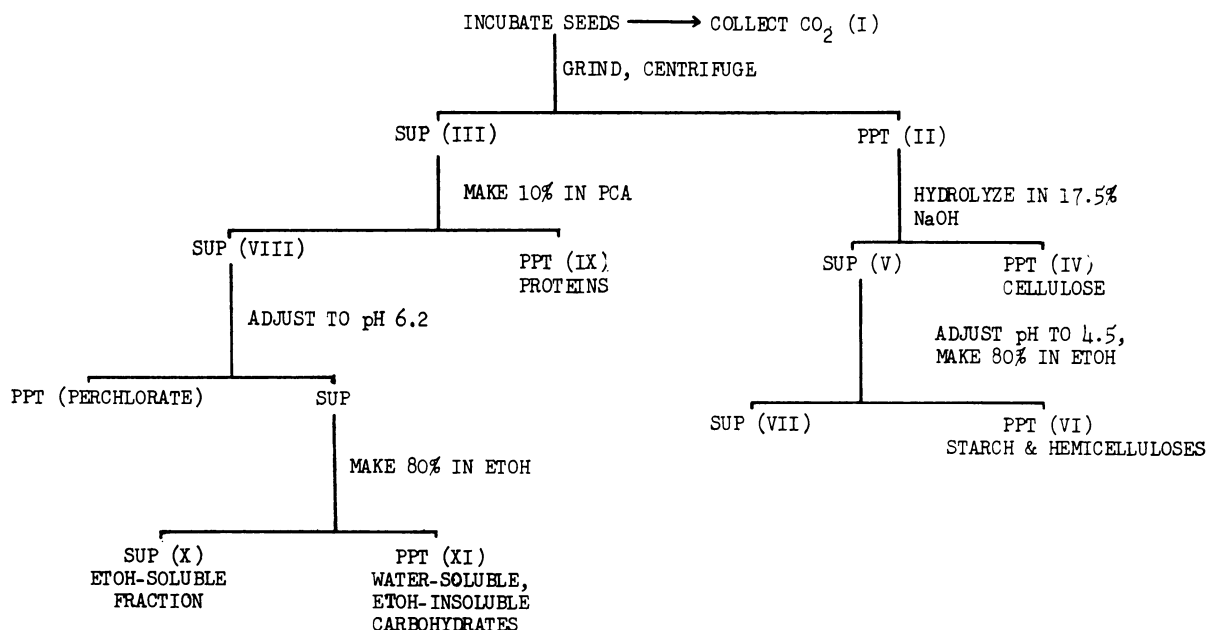


FIG. 1. Outline of procedure for extracting major organic constituents of germinating barley. Seeds were imbibed for 6 hr in water then incubated for 2 to 3 hr in glucose- $U-^{14}C$ at 25° before being extracted. The abbreviations PPT., SUP., ETOH, and PCA refer to precipitate, supernatant, ethanol, and perchloric acid respectively.

liter with 2,4-dioxane. Cab-o-sil was added to samples which contained insoluble material to keep it in suspension. This method gave a counting efficiency of approximately 41 % for ¹⁴CO₂ and 61 % for the other fractions.

Respiratory Measurements. Oxygen uptake was determined manometrically under both septic and aseptic conditions in a Gilson-type respirometer at 25° with constant shaking. Samples of 10 seeds were used in 17-ml flasks. Two ml of imbibing medium (water, sugars, puromycin, or actinomycin D) was placed in the main compartment, and 0.2 ml of 14 N KOH was placed in the center well with a filter paper wick to provide more surface area for CO₂ absorption. Readings were taken at 1-hr intervals without opening the system.

Germination and Growth Tests. Percent germination and average shoot growth were determined on the fourth day on seeds grown in the dark in a germinator at 25° constant temperature and 100 % RH. Seeds were germinated on blotters in 15-cm petri dishes. An average of 30 seeds and 17 ml of solution were placed in each dish.

Results and Discussion

Relationship of Respiration to Protein Synthesis.

Time courses for respiration (O₂ uptake) and incorporation of leucine-¹⁴C into soluble proteins during the first 11 hr of imbibition are shown in Fig. 2. The oxygen-uptake curve shows 4 distinct phases: (1) a quiescent phase of very low activity which exists in the dry seed; (2) a lag phase (0-2 hr) which is initiated by placing the seed in water and during which water uptake and respiration gradually increase; (3) a fully imbibed steady-state phase (2-7 hr) during which the respiration rate is 5-fold that of the first phase; and (4) a rapidly accelerating phase (9-11 hr) during which oxygen uptake is 12-fold that of the first phase. Initiation of the last phase seems to be associated with emergence of the radicle.

Incorporation of leucine-¹⁴C into soluble proteins proceeds slowly during the first 5 hr of imbibition,

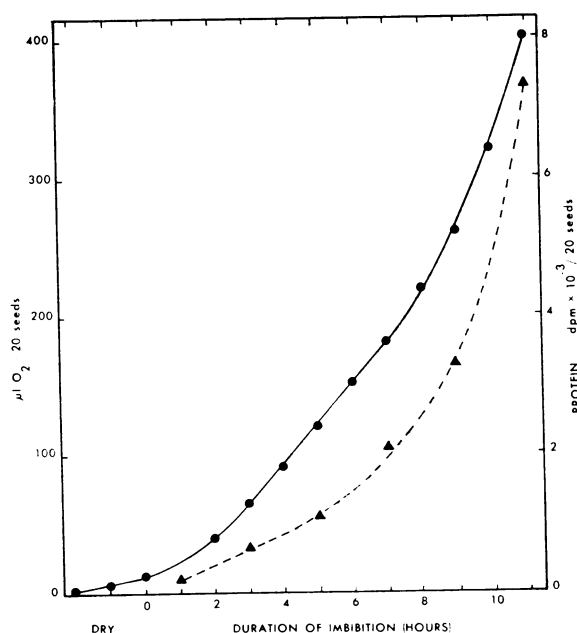


FIG. 2. Time course for O₂ uptake (circles) and incorporation of leucine-¹⁴C into soluble proteins (triangles). Each point is the average of 2 experiments with 3 to 4 replicates per treatment.

then accelerates and reaches the maximum rate after 11 hr. The rates of oxygen uptake and protein synthesis follow a similar pattern. For example, at 2, 6, and 10 hr of imbibition, the rates of oxygen uptake are 13, 32, and 80 μl/20 seeds/hr and those of leucine incorporation by the same seeds are 200, 525, and 2600 dpm respectively. However, changes in respiration rates appear slightly earlier than those of leucine incorporation. This may reflect the importance of respiration to provide energy for the synthesis of new proteins as well as for other energy-requiring reactions.

Experiments with inhibitors were conducted to investigate the need for RNA-dependent protein synthesis during the early hr of imbibition. Actinomycin D inhibits protein synthesis by inhibiting DNA-dependent RNA synthesis (10), whereas puromycin

Table I. Inhibition by Actinomycin D and Puromycin of Incorporation of Leucine-¹⁴C into Protein

Samples of 80 seeds were imbibed at 25° for 5 hr in water, antibiotic (10 μg/ml Act D or 10⁻³ M Puro), or water followed by antibiotic (2.5 hr in each). They were then incubated for 2 hr in 10⁻⁴ M leucine-¹⁴C ± antibiotic (same concn), and extracted in cold water. Soluble proteins were precipitated in 10 % cold perchloric acid and washed in 10 % cold perchloric acid containing 10⁻³ M unlabeled leucine.

Pretreatment	Treatment 2 hr	Total incorporation	Total uptake	Incorporation Inhibition	
		dpm/20 seeds	dpm/20 seeds	%	%
5 Hr H ₂ O	Leu	10,900	33,440	32.6	0
2.5 Hr H ₂ O → 2.5 hr Act D	Leu	10,370	30,850	33.6	0
5 Hr Act D	Leu + Act D	6,530	22,870	28.6	12.3
2.5 Hr H ₂ O → 2.5 hr Puro	Leu	7,800	24,090	32.4	0.6
5 Hr Puro	Leu	5,560	24,470	22.7	30.4
2.5 Hr H ₂ O → 2.5 hr Puro	Leu + Puro	4,910	23,080	21.3	34.7
5 Hr Puro	Leu + Puro	3,650	17,490	20.9	35.6

mycin inhibits the transfer of aminoacyl residues from soluble RNA into long-chain polypeptides (13). Table I shows the effects of 10 $\mu\text{g}/\text{ml}$ of actinomycin D and 10^{-3} M puromycin on the uptake and incorporation of leucine- ^{14}C into soluble proteins. When seeds are first imbibed in water then in actinomycin D, the uptake and incorporation of leucine are unaffected. On the other hand, when the water pretreatment is eliminated and the dry seeds are imbibed in actinomycin D, both uptake and incorporation of leucine are reduced. In contrast to actinomycin D, puromycin inhibits the uptake but not the incorporation of leucine when applied to seeds which were already imbibed in water for 2 and one-half hr. However, it inhibits both uptake and incorporation of leucine in seeds which did not receive a water pretreatment.

The reduction by actinomycin D and puromycin of leucine- ^{14}C incorporation into the acid-insoluble fraction when dry seeds are imbibed suggests that protein synthesis takes place as early as the first hr of imbibition (see Fig. 2). These results indicate that dry barley seeds have an active protein-synthesizing system which becomes operative when imbibition begins.

Apparently the protein-synthesizing system in barley seed resembles that in the wheat embryo. Marcus and Feeley (12) reported that the dry quiescent wheat embryo possesses a protein-synthesizing system which is activated by imbibition. On the other hand, the protein-synthesizing system in germinating barley appears to differ from that in the germinating cottonseed embryos in its response to actinomycin D. Dure and Waters (8) reported that with cottonseed even 20 $\mu\text{g}/\text{ml}$ of actinomycin D failed to reduce the incorporation of leucine- ^{14}C into soluble protein during the first 16 hr of germination. The authors suggested that the failure of actinomycin D to inhibit protein synthesis in cottonseed embryos was due to the existence of a long-lived messenger RNA in that tissue. The possibility still exists that the antibiotics do not inhibit protein synthesis in certain tissues because they fail to reach the active sites.

Effects of Actinomycin D and Puromycin on

Table II. Inhibition by Actinomycin D and Puromycin of Germination and Shoot Growth

Lots of 400 seeds were germinated in 15-cm petri dishes on germinating paper, using water, 10 $\mu\text{g}/\text{ml}$ actinomycin D or 10^{-3} M puromycin. Seeds were grown in the dark in a germinator at 25° and 100% RH. Percent germination and seedling shoot length were recorded on the fourth day.

Treatment	Germination	Seedling shoot length
H ₂ O	100	48.5
Act D 10 $\mu\text{g}/\text{ml}$	71	43.4
Puro 10^{-3} M	29	29.7

Germination and Seedling Growth. The effects of 10 $\mu\text{g}/\text{ml}$ actinomycin D and 10^{-3} M puromycin on barley germination and shoot growth are presented in table II. Both antibiotics reduced percent germination and shoot growth, presumably through their effects on RNA and protein synthesis during the early hr of germination. The reduction in percent germination and growth of the seedling shoot was greater in puromycin- than in actinomycin D-treated seed. These results agree with the early effects of the 2 antibiotics on protein synthesis during imbibition (table I).

The effects of actinomycin D on germination and growth in barley seed differ from those on cotton and lettuce seeds. Dure and Waters (8) found no difference in growth rate of cottonseed embryos germinated in water as compared to those germinated in 20 $\mu\text{g}/\text{ml}$ actinomycin D for 36 hr. In barley 10 $\mu\text{g}/\text{ml}$ actinomycin D reduced shoot growth by 11%. This reduction in shoot growth was also accompanied by 29% reduction in germination. Seeds that failed to germinate as a result of treatment with the antibiotic were not killed; they imbibed the solution but failed to grow. Actinomycin D, however, failed to reduce germination of Grand Rapids lettuce seed even at a concentration of 500 $\mu\text{g}/\text{ml}$ (14). Such differences in the responses of these 3 kinds of seeds to actinomycin D reflect the diversity among tissues with respect to the regulation and control of the complex growth process.

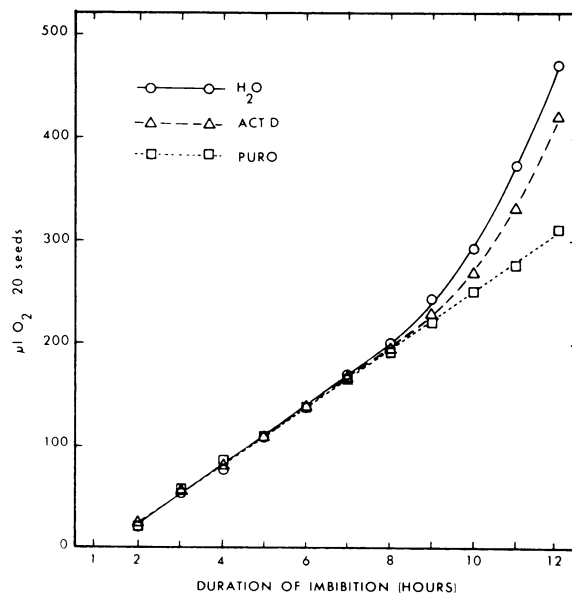


FIG. 3. Inhibition by actinomycin D and puromycin of O₂ uptake in germinating barley. Seeds were placed in 17-ml respiration flasks (10 seeds/flask) containing 2 ml of water or antibiotic (10 $\mu\text{g}/\text{ml}$ actinomycin D or 10^{-3} M puromycin). KOH (0.2 ml, 14 N) was placed into the center well with a paper wick for absorption of liberated CO₂. Reaction temperature was 25°. Each point represents the average of 3 experiments with 3 to 4 replicates per experiment.

Effect of Actinomycin D and Puromycin on Respiration. The respiratory rates of barley seed imbibed in actinomycin D or puromycin (10 $\mu\text{g}/\text{ml}$ and 10^{-3} M respectively) during the first 12 hr of germination are shown in Fig. 3. Although these concentrations of antibiotics inhibit leucine incorporation (table I) they do not inhibit respiration during the first 8 hr of imbibition. However, after 11 hr the reductions in oxygen uptake due to actinomycin D and puromycin treatments are 12% and 67% respectively. Puromycin, which was more inhibitory than actinomycin D to leucine incorporation as well as to germination and shoot growth, was also more inhibitory to seed respiration after 8 hr. It would appear that respiration during the first 8 hr of imbibition does not depend on protein synthesis.

Relationship of Glucose- ^{14}C Uptake to Incorporation. Among the factors that affect incorporation of glucose- ^{14}C into seed components are the rate of isotope uptake and pool size. Fig. 4 shows the relationship between uptake and incorporation of glucose- ^{14}C by germinating barley seed. The times required to reach a steady state of uptake and incorporation of glucose- ^{14}C by the seed are approximately 4 and 6 hr respectively. However, the rate of percent incorporation ($100 \times$ total incorporation/total uptake) remains the same whether or not the uptake rate is in a steady state.

High concentration of glucose- ^{14}C (10^{-3} M) was used in the incubation medium in order to compensate for 2 major dilutions of the isotope (unpublished data). The first stemmed from the leakage of sugars from the seed into the imbibing medium; the second was dilution inside the seed by endogenous sugar pools. Such glucose concentration had no effect on seed respiration. Glucose concentrations as high as 1 M which are known to alter the metabolic activity of plant tissues (1, 4, 6, 9, 15, 17) did not change seed respiration during the first 10 hr of imbibition.

The Metabolic Balance. These experiments were undertaken in order to determine the fate of glucose- ^{14}C in the germinating seed and in the embryo and endosperm portions. Whole seeds, embryo and

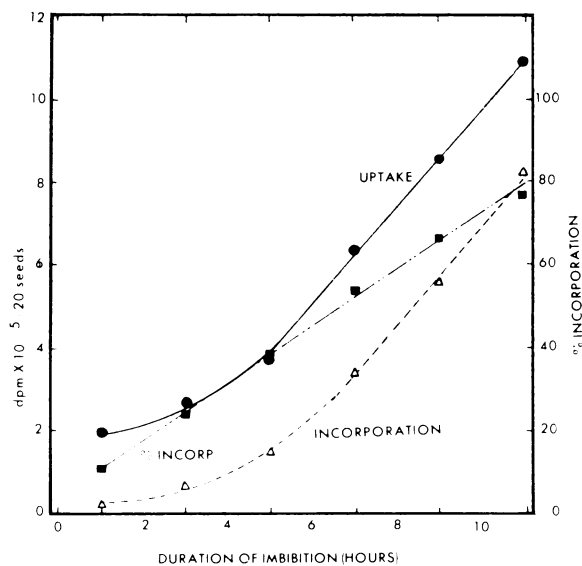


FIG. 4. Relationship between uptake and incorporation of glucose- ^{14}C into germinating barley seed. Seeds were imbibed in water for 0, 2, 4, 6, 8, and 10 hr and then incubated in 1.3×10^{-5} M glucose- ^{14}C ($1\mu\text{c}/\text{ml}$) for 2 hr at 25° . $^{14}\text{CO}_2$ was collected in saturated solution of $\text{Ba}(\text{OH})_2$ during incubation. Total incorporation includes activity in protein, water-soluble carbohydrates, hemicelluloses and starch, cellulose, and CO_2 .

endosperm halves (prepared by cutting the seed into 2 equal portions along the equatorial axis), and dead seed (10-year-old seeds that failed to germinate) were used. The uptake of glucose- ^{14}C by whole seeds does not appear to be an active process since the uptake rate of the label is not higher by live than by dead seed (table III). On the other hand, the greater uptake rates of the label by both embryo and endosperm half-seeds, as compared to that by whole seeds, reflects the extent to which wounding increases the rate of glucose entry and transport into the sites where it is utilized. Because, as shown in Fig. 4, incorporation of glucose depends upon uptake, a 3.3-fold increase in total uptake by half seeds over

Table III. Uptake and Incorporation of Glucose- ^{14}C Into Germinating Barley

Seeds were imbibed in water for 6 hr, then incubated for 3 hr in 10^{-3} M glucose ($1\mu\text{c}/\text{ml}$) at 25° . CO_2 was collected in saturated $\text{Ba}(\text{OH})_2$ during the incubation period. Each treatment consists of 3 replicates which were incubated and extracted separately according to procedure outlined in Fig. 1.

Extracted component	Dead	Viable	Embryo	Endosperm
	(whole)	(whole)	halves	halves
	<i>dpm/20 whole seeds or 40 halves</i>			
$^{14}\text{CO}_2$ (in BaCO_3)	130	6340	71,750	59,360
Water-soluble proteins	50	280	6420	500
Water-soluble; ethanol-insoluble carbohydrates	320	2070	7800	19,940
Hemicellulose and starch	70	3400	11,100	7360
Cellulose	0	250	340	490
Deproteinized ethanol-soluble	204,680	155,580	451,110	460,730
Total uptake	205,250	167,920	548,520	548,380
Total incorporation	570	12,340	97,410	87,650
% Incorporation	0.3	7.4	17.8	16.0

whole seeds resulted in a 7.5-fold increase in total incorporation.

By far the largest part of the incorporated label appears as CO_2 which represents approximately 50 % of the total label incorporated by whole seeds and 75 % of that incorporated by half seeds. These results reflect the active respiratory metabolism in the germinating seed and its importance in the production of both energy and various metabolites which are used for synthesis of cellular polymers. The low incorporation rate of glucose- ^{14}C into cellulose during the early hours of germination is to be expected, since at this stage very little cellular elongation and maturation are taking place. A shift in the metabolic balance towards cell-wall synthesis begins as soon as shoots and roots emerge and start to grow (11).

Although agreement is fair between embryo and endosperm halves, with respect to percent of the label which is incorporated into CO_2 , cellulose, and hemicelluloses, the 2 halves differ greatly in their ability to incorporate the label into proteins and water-soluble, ethanol-insoluble sugars. More than 90 % of the labeled proteins appear in the embryo half where growth is localized. On the other hand, about 75 % of the labeled water-soluble sugars are located in the endosperm half. The lower level of these unidentified water-soluble sugars in the embryo half could be in part due to their rapid utilization by the growing embryo for the synthesis of macromolecules. Absorption of the ^{14}C -label by the different fractions extracted from dead seeds is insignificant and can be regarded as passive absorption.

The rapid conversion of glucose- ^{14}C into CO_2 , polysaccharides, and protein during the first few hr of germination provides ample evidence that metabolic processes are active in the seed. Since these processes are different segments of the entire growth complex, they might serve as a useful index for predicting the quality or vigor of seeds.

Literature Cited

- BERTSCH, W. F. AND W. S. HILLMAN. 1961. The photoinhibition of growth in etiolated stem segments. I. Growth caused by sugars in *Pisum*. *Am. J. Botany* 48: 504-11.
- BRIGGS, D. E. 1963. Biochemistry of barley germination: action of gibberellic acid on barley endosperm. *J. Inst. Brewing* 69: 13-19.
- BROWN, H. T. AND G. H. MORRIS. 1890. Research on the germination of some of the gramineae. *Chem. Soc. J.* 57: 438-528.
- BROWN, R. 1943. Studies in germination and seedling growth. I. The water content, gaseous exchange and dry weight of attached and isolated embryos of barley. *Ann. Botany N. S.* 7: 93-113.
- CHRISPEELS, M. J. AND J. E. VARNER. 1967. Gibberellic acid-enhanced synthesis and release α -amylase and ribonuclease by isolated barley aleurone layers. *Plant Physiol.* 42: 398-406.
- CHRISTIANSEN, G. S. AND K. V. THIMANN. 1950. The metabolism of stem tissue during growth and its inhibition. I. Carbohydrates. *Arch. Biochem.* 26: 230-47.
- COHEN, D. AND L. G. PALEG. 1967. Physiological effects of gibberellic acid. X. The release of gibberellin-like substances by germinating barley embryos. *Plant Physiol.* 42: 1288-96.
- DURE, L. AND L. WATERS. 1965. Long-lived messenger RNA: Evidence from cotton seed germination. *Science* 147: 410-12.
- GALSTON, A. AND M. HAND. 1949. Studies on the physiology of light action. I. Auxin and the light imbibition of growth. *Am. J. Botany* 36: 85-94.
- GOLDBERG, I. H. AND E. REICH. 1964. Actinomycin in inhibition of RNA synthesis directed by DNA. *Federation Proc.* 23: 958-64.
- JAMES, A. L. 1940. The carbohydrate metabolism of germinating barley. *New Phytologist* 39: 133-44.
- MARCUS, A. AND J. FEELEY. 1964. Activation of protein synthesis in the imbibition phase of seed germination. *Proc. Natl. Acad. Sci.* 51: 1075-79.
- NATHANS, D. 1964. Inhibition of protein synthesis by puromycin. *Federation Proc.* 23: 984-89.
- NEUMANN, J. 1964. The effect of actinomycin D on lettuce seedlings and its differential uptake by roots and shoots. *Physiol. Plantarum* 17: 363-70.
- PURVES, W. K. AND W. S. HILLMAN. 1958. Response of pea stem sections to indoleacetic acid, gibberellic acid, and sucrose as affected by length and distance from stem apex. *Physiol. Plantarum* 11: 29-35.
- ROBINSON, T. 1964. In *Organic Constituents of Higher Plants*. Burgess Publishing Company 1964. p 29.
- SCHNEIDER, C. L. 1938. The interdependence of auxin and sugar for growth. *Am. J. Botany* 25: 258-70.
- VARNER, J. E. AND G. R. CHANDRA. 1964. Hormonal control of enzyme synthesis in barley endosperm. *Proc. Natl. Acad. Sci. U.S.A.* 52: 100-06.
- VARNER, J. E. 1964. Gibberellic acid controlled synthesis of α -amylase in barley endosperm. *Plant Physiol.* 39: 413-15.