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# THE ISOLATION OF NUCLEI AND OXIDATIVE PARTICLES FROM VIABLE WHEAT EMBRYOS[1,2]

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This paper describes a procedure for the preparation of pure suspensions of cell nuclei from viable wheat embryos. Hitherto, no satisfactory method for the isolation of plant nuclei has been reported although the procedures tested have been those successfully applied to animal tissues. In aqueous media, which are the media commonly used for cell fractionations, plant nuclei break and their contents disperse. In non-aqueous media, although nuclei and nuclear fragments remain coherent, final purification of the nuclear fraction is difficult; even with wheat germ, which is rich in nuclei, it cannot be freed from considerable cytoplasmic contamination (12). One of the contaminants is cytochrome oxidase but the others have not been identified.

A good fractionation procedure for the whole of the plant cell is desirable, but this is unlikely to be achieved until cytoplasmic structures can be morphologically identified in vitro. The procedure here described is therefore aimed largely at the isolation of nuclei, for which wheat germ is obviously the material of choice. Furthermore, the limitations of milled wheat germ for physiological studies do not apply to viable wheat embryos which can now be isolated in quantity (6).

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## METHODS

The following procedure was finally adopted for isolation of the nuclei. Unless otherwise indicated, all steps were performed within the range of 0 to 5° C. Solutions were made up to concentration at approximately 20° C and then chilled for use. For microscopic examination, a drop of tissue suspension was mixed on a slide with a drop of iodine solution which stained the cell fractions quickly and effectively. Starch granules were easily distinguished by their blue coloration.

1. (a) About 10 gm of viable embryos are collected just after being floated off the 2 M sucrose solution (6), and without further washing are cut into sections of 25 microns thickness on a freezing microtome. The common type of microtome may be modified to accommodate 5 gm of tissue at a time by enlarging the yoke and replacing the 1-inch head with a 2-inch one. Or, (b) about 10 gm of embryos obtained by flotation on a cyclohexane-carbon tetrachloride mixture (6) are ground in an agate mortar. The use of smooth agate rather than rough porcelain is advised because the objective is more to spread than to grind the tissue. The powder so obtained consists largely of intact, but separated cells.

2. The embryo sections or powder are suspended in 100 ml of 2 M sucrose which is 0.001 M with respect to $CaCl_2$. The suspension is ground in a stainless steel blendor ("Omnimixer") for 15 minutes at a

setting of 35 volts. The metal container is immersed in an ice-bath during this interval.

3. The homogenate is filtered successively through a single thickness of muslin, a layer of 10 XX mill silk, and two thicknesses of flannelette which are first wetted with 2 M sucrose. The filtrate is spun in an anglehead centrifuge for 15 minutes at 12,000 × g (centrifugal forces are calculated for the maximum radius of the head). The supernatant, if desired, is set aside for the isolation of oxidative particles; the residue is suspended by means of a rubber policeman in a sucrose solution containing 205 gm of sucrose per 100 gm of water. Although supersaturated this solution did not deposit crystals after standing at 0° C for several days. The suspension is centrifuged at 56,000 × g for 15 minutes. The residue contains starch and cell wall debris, and is discarded; the supernatant is diluted to approximately 2 M concentration with one half volume of 0.5 M sucrose, and is centrifuged at 5,400 × g for 30 minutes. The use of water for this dilution step is not recommended because the nuclei tend to hydrate and swell.

The residue, which consists mainly of nuclei, is suspended in 2 M sucrose and centrifuged at 5,400 × g for 30 minutes to remove small particles. This last step is repeated once to complete the procedure for nuclear isolation.

Oxidative Particles: If the solution previously set aside is centrifuged at 140,000 × g for 15 minutes, a residue consisting of a few nuclei and a variety of particles is obtained; this fraction shows moderate oxidative activity. If the supernatant is then diluted to 1 M with water and centrifuged at 36,000 × g for 20 minutes a very active oxidase fraction sediments.

Nucleic Acids: The Schmidt-Thanhauser-Schneider method was followed for the extraction and separation of RNA and DNA (13). The Ogur-Rosen procedure (7) gave similar results although it was not used in calculating the values reported here. Neither method was satisfactory for an accurate determination of RNA in the tissue fractions. Interfering substances in the RNA extract rendered useless not only ultraviolet absorption measurements, but also ribose determinations by the orcinol method of Bruckner (2). Thus, there was no good means of checking on the specificity of the phosphorus determinations. P/N ratios of the RNA extracts were approximately 0.05, one-twelfth the expected value for pure RNA, indicating a strong contamination by other nitrogenous compounds. The analyses for DNA, on the other hand, appeared to be satisfactory. The P/N ratios of the nuclear extracts were of the order of 0.5. Although UV spectra were too impure for quantitative comparisons, desoxyribose and phosphorus measurements agreed closely. The phosphorus contents of the extracted DNA as calculated from desoxyribose measurements were 8.2 % for the whole tissue and 8.9 % for the nuclei. The calf thymus DNA used for standardizing the desoxyribose test had a P content of 9.2 %. The modified diphenylamine procedure of Burton (3) was followed for desoxyribose determinations and the effects of non-specific absorption at the 600 mμ peak were eliminated by means of a second optical density reading at 540 mμ, the difference between these being used for construction of the calibration curve. No DNA was found in the RNA extracts.

Cytochrome Oxidase: This was measured spectrophotometrically by the procedure of Hogeboom and Schneider (5). The cuvette chamber was maintained at about 25° C by means of a cooling attachment. Readings were taken every minute and rates were calculated from the first three minutes, the reaction being linear for that interval. The amounts of tissue used were such that the activities measured were directly proportional to concentration. For the oxidized and reduced forms of cytochrome c, extinction coefficients of 0.9 and $2.87 \times 10^{-7}$ $cm^2$ per mole, respectively, were used to express the activities in terms of micromoles of ferrocytochrome c oxidized.

## Results and Discussion

Isolation of Nuclei: There are three critical steps in the procedure outlined, all of which are important in preserving the intactness of the cell-free nuclei: fragmentation of the embryos, suspension of the fragments in concentrated solutions of sucrose, and addition of $CaCl_2$ to the blending medium. It is of considerable advantage that the embryo is a highly desiccated tissue so that hypertonic solutions do not shrink or distort the cells.

A partial fragmentation of the embryos prior to blending is necessary for a good yield of nuclei. Intact embryos blended at a setting of 35 volts do not release nuclei at all. Large fragments, obtained by grinding embryos with dry ice in a Wiley mill, release relatively few nuclei after 3 or 60 minutes of blending. Sectioned embryos yield a maximum number of free nuclei after 15 minutes, and although a large number of unbroken cells remain in the embryo sections no additional nuclei are released even after an hour of blending. It is important to note that the techniques of fragmentation keep artifacts to a minimum. Apart

Table I

The Effect of Calcium Ion in Preventing Rupture of Nuclei in Sucrose Media

| M conc. $Ca^{++}$ in 2 M sucrose | No. nuclei/gm starting material |
|---|---|
| 0.0 | 22,000 ± 15 % |
| 0.001 | 400,150 |
| 0.01 | 357,900 |

Fifty grams of commercial germ were washed free of starch by stirring in a 2 M sucrose medium (with or without calcium) and filtering through flannelette. The residue was ground in an "Omnimixer" for 30 min at 30 volts, and filtered to remove unbroken tissue. The nuclei obtained by centrifugation were suspended in a known volume of sucrose and counted by means of a hemocytometer. In this procedure less than 5 % of the tissue cells were broken, but the same differences with respect to calcium were readily apparent in preparations using the procedure described under Methods.

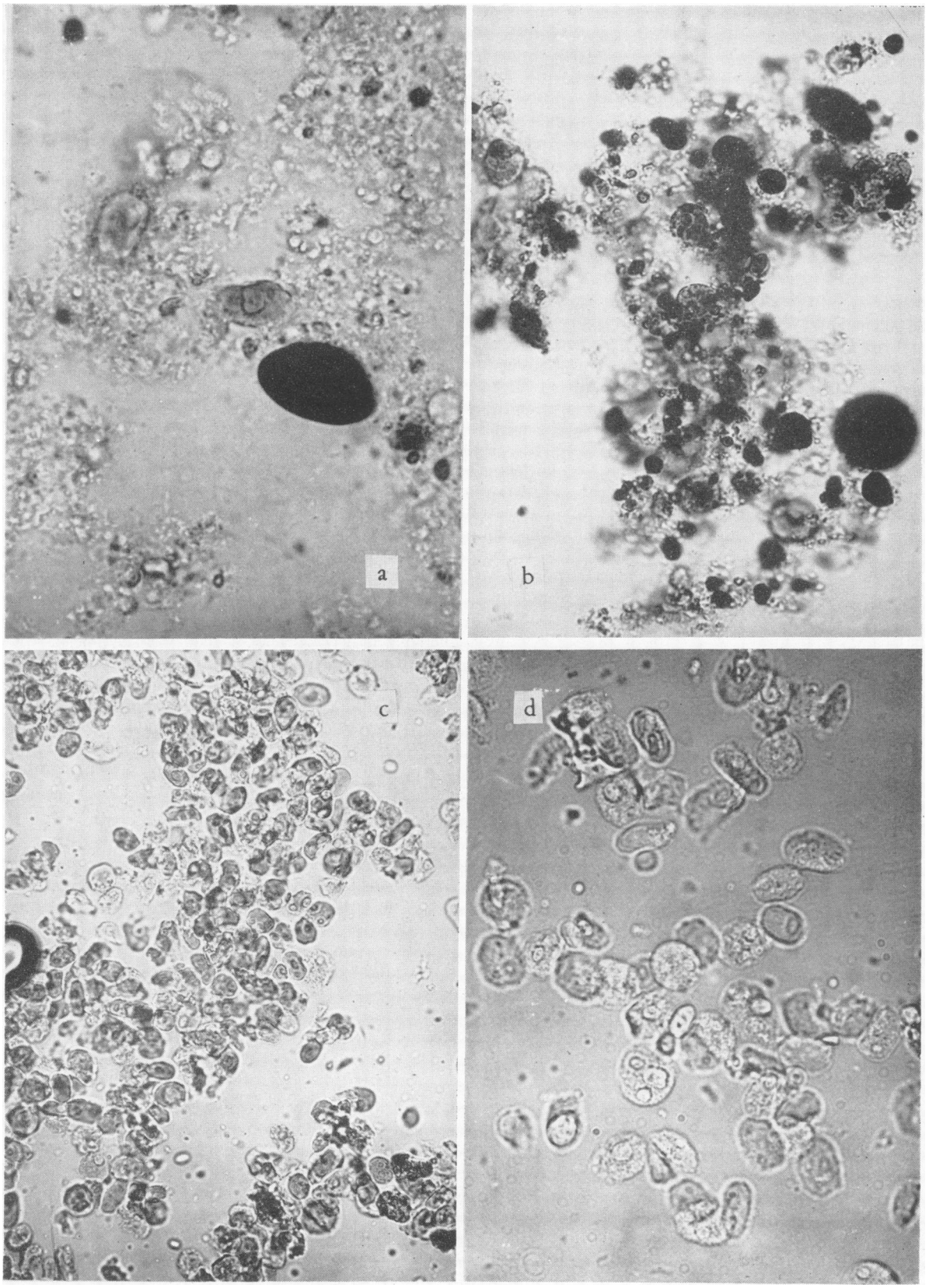
a
b
c
d

TABLE II

NUCLEIC ACID CONTENT OF WHEAT EMBRYOS AND OF ISOLATED NUCLEI

| FRACTION | ACID-INSOLUBLE N IN SAMPLE | TOTAL P | RNA-P | DNA-P | RNA-P / N | DNA-P / N |
|---|---|---|---|---|---|---|
| | *mg* | *mg* | *mg* | *mg* | % | % |
| Whole tissue | 103 | 10.2 | 7.4 | 1.6 | 7.2 | 1.55 |
| Purified nuclei | 3.6 | 0.58 | 0.1 | 0.38 | 2.8 | 10.55 |

The values listed are based on two separate preparations and duplicate measurements of each. The average amount of tissue used for each series of analysis is indicated in the first column as total acid-insoluble N.

from mechanical breakage, the procedures followed in preparing the tissues for disintegration do not impair the viability of the cells. Embryos kept frozen in 2 M sucrose for twenty minutes and then thawed, or those floated off cyclohexane-carbon tetrachloride mixture, all germinated when sprinkled on agar plates. Of considerable interest is the fact that organic solvents, although they do not affect germination of the embryos, do affect their physical quality. Embryos isolated in this way are easily ground into a powder consisting largely of separate cells, whereas the cells from sucrose-floated germ disintegrated when similarly ground. The advantage of grinding over sectioning is that the yield of nuclei is increased some 5-fold; the disadvantage is that a small percentage of whole cells collects with the nuclei throughout the isolation.

The high concentration of sucrose serves two purposes. First, it helps to prevent rupture of the free nuclei. If, for example, the 2 M sucrose used in blending is replaced with 0.25 M, very few, if any, intact nuclei remain. Second, it is possible to separate cell debris from the nuclei by use of supersaturated solutions of sucrose. The effectiveness of such separation depends to some extent on the degree of hydration of the embryos prior to fragmentation. Storage of the embryos over $H_2SO_4$ (specific gravity = 1.20) at 4° C for 24 hours has proved satisfactory as a means of assuring a good yield of nuclei.

Calcium chloride is introduced to reduce further the breakage of nuclei. Low concentrations of this salt have been used to prevent clumping of rat liver nuclei (8), but the phenomenon has not been observed in these nuclei, perhaps because of their lower DNA concentration. The effectiveness of $CaCl_2$ in reducing fragmentation of wheat nuclei is demonstrated in table I.

The product obtained by the procedure outlined is illustrated in the accompanying figure. If embryo sections are used as the starting material, about 10 to 20 % of the DNA present in the flannelette filtrate is recovered in the final nuclear preparation. The percentage recovery of intact nuclei is much greater, however, since a large fraction of the DNA present in the filtrate is derived from nuclei which have been broken and dispersed in the course of sectioning. Clearly, the method is unsatisfactory for a quantitative fractionation of the germ, but qualitatively, the method is good. The only contaminants present in the nuclear preparation are small particles. The extent of this contamination may be judged from figure 1d. Occasional particles are starch and these can be spotted by their reaction with iodine. Others may be oxidative particles, but it will be shown that these can constitute no more than 3 % of the total nitrogen of the preparation.

NUCLEIC ACID CONTENT: A summary of analytical values is given in table II. From the percentage (DNA-P)/N in the nuclei it may be calculated that about 6 to 7 % of the total weight of nucleic acid plus protein is DNA (taking nucleic acid to be 10 % P and 16 % N and protein to be 16 % N). This value is small compared to those obtained for many species of mammalian nuclei isolated in non-aqueous media (1), but it is twice that previously obtained for wheat germ (12). The discrepancy between the two preparations of wheat nuclei may in part be accounted for by the probable loss of soluble protein from the sucrose-isolated nuclei and by their higher degree of purity. Obviously, a more certain comparison would require a better procedure for non-aqueous isolation of wheat nuclei. Yet, despite the differences between the preparations, the results agree on the point that compared with mammalian nuclei, wheat germ nuclei have a low DNA and a high protein content. To what extent this is a characteristic of plant nuclei remains to be explored.

The proportion of embryo N which is nuclear may be calculated from the percentages of DNA-P in whole tissue and nuclei, respectively. (DNA is restricted to the nuclei so that the concentration measured in the whole tissue may be regarded as a dilution of the nuclei by non-nuclear nitrogen.) Thus calculated, nuclei account for 15 % of embryo N, a very low figure compared to the 50 % reported for the non-aqueous preparation (12). A loss of soluble pro-

FIG. 1. Stages in the isolation of nuclei from viable wheat germ tissue. The preparations are all contained in 2 M sucrose to which a drop of conc iodine solution was added in order to stain the cell fragments. a (*upper, left*). Initial homogenate (×540). b (*upper, right*). First sediment obtained by centrifuging homogenate at 12,000 × g for 15 min (×294). c (*lower, left*). Final nuclear preparation (×215). d (*lower, right*). Nuclear preparation (×500).

tein from animal nuclei isolated in sucrose media has been established (11), and wheat nuclei probably behave in much the same way. Such loss of protein and the higher degree of purity of the sucrose nuclei are undoubtedly two of the reasons for the difference. The improved procedure for DNA determination is probably another. To judge frrom the cytological picture, the real proportion of nuclear nitrogen is somewhere between 15 and 50 %.

Ribonucleic acid measurements indicate that the bulk of embryo RNA lies in the cytoplasm. Per mg N, RNA-P is 2.5 times as concentrated in the whole tissue as in the nuclei, and since the latter account for only 15 % of the tissue nitrogen, nuclear RNA may be estimated as 6 % of the total. Within the nucleus 20 % of the nucleic acid is of the ribose form. Since 50 % of the nucleic acid of non-aqueous nuclei of wheat was found to be RNA (12), the possibility arose that a soluble "ribonucleic acid" might have been leached out of the sucrose nuclei. A preliminary check on this possibility was performed in the following way: The supernatant remaining in 2 M sucrose after sedimentation of the nuclei was diluted to 1 M with water and the pH adjusted to 5.0 with 5 M acetate buffer. Without acidification, a slightly turbid supernatant remained even after centrifugation at 140,000 × g. Some soluble "RNA" might have been precipitated in this step but acidification appeared to be preferable to analyzing for RNA in the presence of microsome-like particles. The acidified suspension could be clarified by centrifugation at 12,000 × g for 30 minutes. It was then diluted with ¼ volume of 25 % TCA (1.8 M) and the resultant precipitate extracted by the Schmidt-Thanhauser-Schneider procedure (13). The RNA content of the extract was found to be approximately 1 % of the total RNA of the tissue. Since nuclear RNA is about 6 % of the total, the whole of the RNA measured in the supernatant would, if derived from the nuclei, increase the concentration of nuclear RNA by ⅙, raising it to 23 % of the total nucleic acid of the nucleus. This value would still be far removed from the 50 % found in the non-aqueous nuclei. The discrepancy needs to be resolved by further experiment, and until then, the earlier conclusion that wheat germ nuclei have a very high content of RNA along with a high nitrogen content must be regarded as suspect.

TABLE III

CYTOCHROME OXIDASE ACTIVITIES OF VIABLE WHEAT GERM PREPARATIONS

| FRACTION | $-\Delta E_{550}$/MG N | MICROMOLES CYT. C OXIDIZED IN 3 MIN/MG N |
|---|---|---|
| Whole tissue homogenized | 132 | 6.2 |
| Whole tissue less nuclear sediment | 192 | 9.7 |
| First particulate sediment | 225 | 11.5 |
| Second particulate sediment | 960 | 48.5 |
| Nuclear preparation | 48 | 2.4 |
| Purified nuclear preparation | 27 | 1.4 |

The changes in extinction values ($\log_{10} I_o/I$) are calculated for a 3-min period for 3 ml of reaction medium with a light path of 1 cm. Four or more measurements were made for each tissue fraction. Conditions of reaction were as described under Methods.

CYTOCHROME OXIDASE: There are three principal points of interest in connection with the oxidase measurements listed in table III.

First, the activity of the whole tissue homogenate is about nine times the activity of a number of samples of commercial germ tested by the same spectrophotometric procedure. In his original work with commercial germ, Goddard (4), using a manometric technique, obtained an oxygen uptake of 240 $\mu$l/hr × 50 mg of germ. If the germ is estimated as 5 % protein N, then this value may be rewritten as 8 microatoms of oxygen/hr × mg N or 0.8 micromoles of cytochrome c/3 min × mg N. This is one-eighth the value obtained with viable wheat embryos.

Second, the specific activity of the isolated oxidase particles is higher than that generally recorded for plant preparations. A recent measurement of cytochrome oxidase activity in particles prepared from germinating peas (9) gave an E of 78/mg N × 3 min, a value lower than that obtained for homogenates of whole embryo. Stafford (10), using manometric techniques for measurement of oxidase activity in particles of pea seedlings, found an uptake of 260 $\mu$l $O_2$/hr × mg protein. This is equivalent to about 14 micromoles of cytochrome c oxidized/3 min × mg protein N, which is one-third of that obtained for the wheat germ particles. The closest in oxidase activity to the wheat particles are the highly purified preparations of rat liver mitochondria which, measured under similar conditions, oxidize 56.7 micromoles of reduced cytochrome c/3 min × mg N (5). Thus Goddard's speculation that the oxidase of the embryo is fully developed even though respiration rises markedly with germination is consistent with the evidence here obtained for highly active oxidase particles. Whether the high activity of the wheat germ particles becomes higher yet in the germinating embryo cannot be determined in the absence of a morphological criterion of purity of particulate preparations.

Third, wheat germ nuclei, like isolated nuclei from a diversity of animal tissues, do not possess cytochrome oxidase activity. The conclusion is based on the decreasing oxidase activity per mg protein N with increasing purification of the nuclei, and on the very high activity of the oxidase particles which can best account for the residual activity in the purified nuclear preparation.

## SUMMARY

A method has been described for the isolation and purification of nuclei from viable wheat embryos. A highly active preparation of oxidative particles has also been obtained. Tissue and nuclei were analyzed for nucleic acids and the significance of the measurements discussed.

Comparable preparations of isolated nuclei have since been obtained from viable embryos of oats, barley and rye by means of the procedure described here.

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# INDUCTIVE CONTROL OF INDOLEACETIC ACID OXIDASE ACTIVITY BY RED AND NEAR INFRARED LIGHT [1,2]


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Many plant processes, including seed germination, photoperiodic flower induction, and the development of stems and leaves, can be affected by brief exposures to low energies of light. Action spectra for such light effects resemble each other closely, with red light (ca 660 mμ) showing maximum effectiveness. In addition, the effects of red light can usually be prevented by exposure to near infrared[3] radiation (ca 730 mμ) sufficiently soon after red light treatment. These characteristics have led to the belief that one photoreaction and pigment system may control widely diverse processes (2, 3, 9, 19). Although the relation between this photoreaction and a biochemical system which might control plant growth is unknown, there is considerable evidence that auxin metabolism may be involved (5, 7, 11, 12, 13). Since the indoleacetic acid (IAA) oxidase system may be a controlling factor in auxin metabolism (6), its relation to red light action has been investigated in the present work. The results indicate that the IAA oxidase activity of certain tissues is controlled by a low energy photoreaction apparently typical of those controlling photoperiodism and the other processes mentioned.

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[3] Wassink and Stolwijk (19) have pointed out why "infrared" is preferable to the term "far-red" to designate this region of the spectrum.

## General Procedures

Most of the experiments were conducted with peas (*Pisum sativum* L. var. Alaska) obtained from Associated Seed Growers, Inc., New Haven, Connecticut. The seedlings were grown in vermiculite for 7 days at 26° C in darkness, with occasional dim green light during handling. Some were also exposed to 2 to 2500 kiloergs/cm$^2$ of red light during a brief period 5 minutes to 40 hours before harvest. Red light was supplied by Sylvania red fluorescent tubes with maximum emission at 640 mμ and none below 580 mμ. Energy measurements were made with a phototube light meter whose construction and calibration is described elsewhere (5).

Tissues harvested for IAA oxidase assays were weighed, placed immediately at −16° C, and stored at that temperature until use (1 to 10 days) with little or no resultant loss in activity. For assay, the frozen tissue was rapidly reduced to a powder with a chilled mortar and pestle and ground further with 0.02 M phosphate buffer, pH 6.1. The preparation was centrifuged in the cold to remove cellular debris and the clear supernatant extract made to volume and assayed. Usually 600 mg fresh weight of tissue were made to a final volume of 25 ml.

The assay used, described in more detail elsewhere (10), consists of colorimetric determinations of residual IAA in reaction mixtures incubated at 26° C. Each 10-ml reaction mixture contained 0.5 to 5 ml extract, 2 ml 0.01 M phosphate buffer, pH 6.1, and a