THE ISOLATION OF WHEAT GERM NUCLEI AND SOME ASPECTS OF THEIR GLYCOLYTIC METABOLISM

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INTRODUCTION

The present report deals with the isolation of wheat germ nuclei and with some aspects of their glycolytic metabolism. It has been the objective of this study to determine whether or not the nucleus has an energy-yielding metabolism of its own. The view here adopted is that a knowledge of the type of metabolism operating in the immediate environment of the chromosomes might furnish pointers to the primary reactions of the chromosomes with their cellular surroundings.

The argument for studying the glycolytic mechanism in nuclei runs as follows: (1) The morphological fact of a nuclear membrane, universal in its distribution, implies an intranuclear environment different from its contiguous cytoplasm. If further proof were needed, it could be supplied from the data already accumulated on the chemical and enzymatic properties of isolated nuclei (1). (2) A special nuclear environment must be associated with the special requirements of chromosome function. (3) If it is true that chromosomes exercise considerable influence in the synthesis of cell components, then such influence should manifest itself in *at least* one of these two ways: (*a*) The synthesis of certain molecular chains requiring a source of energy such as might be obtained through carbohydrate metabolism. (*b*) The mediation of exchange reactions in already complex molecules involving no net energy uptake. Thus, one of the simplest questions which ought to be raised is whether chromosomes function in the immediacy of energy-yielding reactions and/or their products. The choice between an environment equipped to yield primary energy-rich compounds and one obtaining these from a contiguous cytoplasm is perhaps academic; indeed, there is no reason to suppose such patterns to be mutually exclusive. Experimentally, however, the demonstration of active enzymatic mechanisms would appear to be the procedure of choice since important metabolic intermediates, because of their rapid conversion, may easily escape detection. Furthermore, even where spotted, the concentration of such intermediates might provide a rather nebulous index to the significance of their presence.

Enough has been said about the diversity of nuclear types (1) to make clear that the metabolic properties of wheat germ nuclei cannot be regarded as

characteristic of all nuclei. Indeed, the wheat embryo itself is a complex organism housing a number of differentiated tissues. Yet, so fundamental and universal is the glycolytic process that it is hard to imagine its presence in wheat germ nuclei to be without parallel in the nuclei of other tissues. To be sure, the degree of activity may vary widely from tissue to tissue and probably the high activity of wheat germ nuclei is a reflection of the one major property shared by the bulk of the tissues of the wheat embryo—their capacity for rapid proliferation and growth.

The singular advantage in the use of wheat germ for the type of study here undertaken lies in the fact that it is naturally dry. Thus, whatever damaging changes might occur in the otherwise necessary step of freeze-drying a tissue are here eliminated. Furthermore, the tissue is available in large quantities; and as will be shown, with a minimum of effort it is possible quickly to fractionate the tissue into three major fractions, one of which is almost entirely cytoplasmic, another largely nuclear. This rough fractionation provides an excellent guide to the enzymatic properties of the nuclei and spares the use of the less abundant purified nuclear preparation in the many preliminary experiments required.

EXPERIMENTAL

1. The Isolation of Wheat Germ Nuclei

The procedure employed was the non-aqueous one of Behrens as modified in this laboratory (2). For microscopic examination of tissue fractions the acetorcein-fast green mixture, developed in this laboratory by Kurnick and Ris (3), was used; aqueous crystal violet proved unsatisfactory.

A. The Fractionation of Wheat Germ Tissue.—Fresh wheat germ obtained from General Mills, Inc. (Minneapolis, Minnesota) was exhaustively extracted with petrol ether. About 60 gm. of this was suspended in 300 ml. petrol ether and ground in a ball mill for 48 hours. The tissue was then collected and resuspended in about 500 ml. of cyclohexane:CCl_4 mixture adjusted to a specific gravity of 1.395. The supernatants obtained by centrifugation were transferred to clean tubes, vigorously stirred, and recentrifuged. The resulting supernatants were poured into one-third their volume of petrol ether and collected by centrifugation. This fraction is designated as "A." Microscopically, it stains an even green which is the characteristic color of cytoplasm for the dye used. Relatively few nuclei are visible. It will therefore be referred to as the cytoplasmic fraction, although it is to be understood that the cytoplasm thus obtained is a *selected* light fraction. The sediments obtained in the first fractionation were collected and suspended in cyclohexane:CCl_4, adjusted to a specific gravity of 1.447, centrifuged, and the supernatants poured into a beaker containing one-third their volume of petrol ether. The sediments were resuspended, centrifuged, and the operation repeated. The combined supernatants were collected by centrifugation and the fraction designated as "B." Microscopic examination of

this fraction showed it to consist largely of nuclei and starch granules in addition to an appreciable proportion of cytoplasmic fragments and whole cells. It will be referred to as the nuclear fraction. The remaining sediments were similarly collected in petrol ether and the fraction designated as "C." It will be referred to as such. This consisted mainly of starch granules along with cytoplasmic fragments and nuclei. From approximately 65 gm. of defatted tissue the following yields were obtained: A, 8 gm.; B, 21 gm.; C, 31 gm. It is to be noted that beyond the partial separation of nuclei from cytoplasm, no biological significance is attributed to these fractions.

B. Purification of the Nuclear Fraction.—About 100 gm. of defatted wheat germ was treated as in procedure A to obtain first a nuclear-rich fraction. Further purification of this fraction followed the general procedure adopted for animal tissues (2), the nuclei being collected between specific gravities 1.416 and 1.420. The only modification here introduced was the use of higher speed centrifugations (about 6000 *G*) in the latter steps of the procedure since among the contaminants present in the final stages of purification are relatively small fragments which sediment at specific gravities greater than 1.420. The yield was 5.2 gm.

The degree of purity of the nuclei thus obtained is indeed subject to question. The presence of some microscopically visible impurities is certain but its magnitude, whether *e.g.*, 5 per cent or 20 per cent, is not clear. For one thing, the nuclei do not all stain uniformly; for another, fragmentation of some of the nuclei leaves the origin of many apparent contaminants in doubt. It has been possible, however, by studying the distribution of β-amylase activity to arrive at a reasonably secure estimate of nuclear purity which would set the outside limit of contamination at about 15 per cent. It will be seen that the relative values of the nuclear properties here studied would be insignificantly affected by a contamination of the above order.

Analytical values obtained by the methods described below are listed in Table I. Acid-insoluble N represents the N content of the tissue fractions after three extractions with cold 2 per cent perchloric acid. All enzymatic activities will be referred to these N values. The determination of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) in these tissues presents something of a problem. Commonly in plant tissues colorimetric estimation of specific pentoses is subject to much error and in wheat germ there is additional interference from ultraviolet-absorbing substances which limits the usefulness of extinction measurements at 260 mμ. A satisfactory, though by no means rigorous procedure, has, nevertheless, been developed. In the case of RNA, it has been applied only to the purified nuclear and cytoplasmic fractions.

RNA.—About 20 mg. of tissue is extracted three times with cold 2 per cent perchloric acid. To the residue is added 5 ml. of H_2O and the suspension adjusted to a pH of approximately 8 by dropwise addition of 0.1 N NaOH. 0.2 ml. of a crystalline ribonuclease solution (1 mg./cc.) is then added to the suspension and the whole incubated for 1½ hours at 37°C. The suspension is centrifuged and aliquots of the

supernatant are analyzed for inorganic P, for ribose (orcinol reaction), and ultraviolet absorption characteristics. For the cytoplasmic fraction the values of RNA calculated from phosphorus and ribose determinations were in excellent agreement (0.485 per cent and 0.481 per cent, respectively); however, the extinction at 260 mμ was 1.36 for a tissue concentration of 1 mg./cc. which would correspond to a value of 0.568 per cent P if interpreted from data available on yeast nuclei acid hydrolysates (4). Whether the latter discrepancy is due to a difference in composition of the two nucleic acids or to interfering factors in the analyses remains unsettled. For nuclei the results were less satisfactory. Phosphorus liberated by RNAase activity was 0.390 per cent; orcinol analysis yielded a value of 0.436 per cent P; the extinction at 260 mμ (1.27), if calculated as for the cytoplasmic fraction, is equivalent to 0.530 per cent. Nevertheless, the ratios nuclear RNA:cytoplasmic RNA correspond

TABLE I

Tissue fractions	(1) Per cent of Tissue by weight	(2) N content	(3) Per cent of tissue N	(4) DNA P	(5) RNA P	(6) Nuclei of total nuclear fraction	(7) Nuclear N in fraction N
		per cent		*per cent*	*per cent*	*per cent*	*per cent*
Cytoplasmic (A)..	13	8.5	23	(0.07)	0.48	(6)	(14)
Nuclear (B).....	35	6.7	48	0.28	—	68	75
Heavy C.....	52	2.3	24	—	—	(26)	(57)
Purified nuclei....	—	8.5	—	0.46	0.41	—	—
Whole tissue.....	100	4.9	100	0.14	—	100	53

The percentage nuclei (6) is obtained by the factor $\frac{(5) \times (2)}{0.14}$.

Parentheses indicate a rough approximation of values due to the difficulties in accurately determining DNA content.

The percentage nuclear N (7) is calculated from $\frac{(6) \times 53}{(3)}$.

if calculated from orcinol measurements (0.91) and from the extinctions at 260 mμ (0.93). Allowing for these discrepancies in analysis, what is here most remarkable is that the RNA content of the nucleus is of an order of magnitude very close to that of the cytoplasm. Within the nucleus its concentration is similar to that of the DNA. Indeed, sections of wheat germ stained with pyronin–methyl green mixture showed no sharp contrast in color between nucleus and cytoplasm (except for peripheral cells); and this was to be expected if enough RNA were present in the nucleus to obscure the staining of the methyl green–specific DNA. It is tempting to conclude that this marked difference in RNA content between the nuclei of the wheat germ and those of many mature animal tissues (2) is associated with the distinctive capacity of wheat germ tissue to undergo a most rapid growth.

DNA.—The residue obtained after removal of the supernatant for RNA analysis is suspended in 5 cc. of 10 per cent perchloric acid and heated for 20 minutes at 70°C. From the supernatant obtained by centrifugation aliquots were taken for (1) diphenyl-

amine reaction, (2) P determination, and (3) absorption spectrum. Concentrations in the cytoplasmic fraction were too small to be evaluated. For nuclei, whole tissue, and nuclear fraction B agreement between the results of (1) and (2) were satisfactory (deviations were no greater than 5 per cent from the mean value) but absorption spectra were most uninforming. Extinctions at 260 mμ were not in respective proportion to the other measured values, and the ultraviolet spectra, apart from showing a peak in the neighborhood of the 260 mμ band, could in no way be regarded as typical of nucleic acid. The values used, therefore, have been based entirely on procedures (1) and (2) which, in view of their concurrence, may be taken reasonably to represent the actual cellular values. From such data the following conclusions may be drawn:—

1. In terms of total mass, nuclei constitute 30.5 per cent of the whole tissue.

2. In terms of acid-insoluble nitrogen, the nuclei constitute 53 per cent of the tissue. These estimates are roughly substantiated by histological inspection. In the larger cells of the tissue, the nuclei, though big, would appear to occupy no more than one-third the cell volume; in the dense tissue of the growing regions, the cells, much smaller, are mainly occupied by the nuclei.

2. β-Amylase Activity

The purpose in studying the distribution of this enzyme was to provide an index, in addition to that offered by microscopic examination, of the purity of the nuclear preparation.

The properties of wheat germ β-amylase have been broadly explored (5). It is sufficient for this study to indicate (1) that the range of optimum activity lies in the region of pH 4.6–6.3 and (2) that full activation of the enzyme is achieved through preincubation of the tissue with papain.

Method.—Tissues were homogenized in 0.9 per cent NaCl; enough M acetate buffer (pH 5) and a suspension of commercial papain were added so that in the final dilution of the tissue (1 mg./ml.) the acetate would be 0.5 M and the papain 0.25 mg./cc. The suspension was incubated at 37.5°C. for 2 hours; longer periods of incubation did not significantly increase amylase activity. For assays, 1 to 5 ml. of tissue homogenate were added to 5 cc. of a 1 per cent solution of starch (in 0.05 M acetate buffer) and when necessary the total volume brought to 10 ml. with additional buffer. All incubations were performed at 37.5°C. 1 cc. samples were removed at 5 minute intervals and treated with dinitrosalicylic acid as described by Meyer *et al.* (6).

The values listed in Table II were derived under these conditions (*a*) The time course of the reaction was linear for the first 20 minutes. (*b*) Except for nuclei, fractions with low activity were incubated in high enough concentration to yield significant colorimetric readings. In the case of nuclei, excessive concentrations would have been required.

From the values listed in Table II the following may be inferred:—

1. Since total activity is recovered from the three tissue fractions the low activity in nuclear fraction B cannot be attributed to the concentration of an

inhibitory factor but must be due to the nuclei which constitute 75 per cent of the N content. In all probability, the very small amylase activity of the purified nuclear preparation is due to cytoplasmic contamination. It would, therefore, follow that almost all types of nuclei in the wheat germ are deficient in amylase activity.

2. β-Amylase activity is not exclusively associated with light or heavy cytoplasm. Activity of the light cytoplasm is much the same as that of the heavy in fraction C. Since the fractionation method employed is based entirely on specific gravity differences, the absence of β-amylase activity in the nuclei may be taken to mean that the nuclear preparation is relatively free of both light and heavy cytoplasm.

3. The lower activity calculated for the cytoplasm in fraction B, though not surprising in view of the mechanical method of fractionation, makes evaluation of nuclear purity less precise. If it is assumed that in the further purification of fraction B, there is a random removal of cytoplasmic fragments with

TABLE II

β-Amylase Activity of Wheat Germ Fractions

Milligrams of maltose liberated/milligram of N in 20 minutes at 37.5°C.

	Cytoplasm (A)	Nuclear fraction (B)	C	Tissue	Nuclei
Activity	24	3.3	12	10.5	(0.85)
Total activity, *per cent*	54	17	30	100	—
Activity/mg. non-nuclear N	27	13	25	22	—

respect to amylase activity, the nuclear preparation would be 93 per cent pure; if, on the other hand, it is assumed that half of the cytoplasmic fragments in fraction B are inactive with respect to amylase activity and that none of these is removed in the course of purification—a rather unlikely possibility—then the purity of the nuclei would approximate 84 per cent. Even so, it will be readily seen that the main conclusions drawn in these studies remain unaffected. The high RNA concentration of the nuclei would only be slightly lowered in view of the near equivalence of its concentrations in fraction A cytoplasm and in the nuclei; the measured glycolytic activity of the nuclei would have to be revised upward, since for all enzymes studied they were the most active centers of metabolism.

3. Enzymatic Activities in the Glycolytic Cycle

The oxidizing enzyme systems of wheat have been broadly reviewed by Sullivan (7), and although to the writers' knowledge, no detailed studies have been made of the enzymes here listed, the demonstration of their activities is simple. There has been no attempt to isolate, or even to purify, the enzymes in

question; for the purpose of this investigation it has been sufficient to determine the "*in vitro*" enzymatic activities of the various tissue fractions.

In the present study the sequence of reactions starting with fructose diphosphate as substrate and ending with the formation of free pyruvate was followed:

(*a*) Aldolase: hexose diphosphate $\rightleftharpoons$ 2 triosephosphate

(*b*) Phosphoglyceraldehyde dehydrogenase: phosphoglyceraldehyde + H_3PO_4 + DPN + adenosinediphosphate (ADP) $\rightleftharpoons$ phosphoglyceric acid + adenosinetriphosphate (ATP) + DPN·H_2

(*c*) Enolase: phosphoglyceric acid $\rightleftharpoons$ phosphoenol pyruvate

(*d*) Pyruvate kinase: phosphoenol pyruvate + ADP $\rightleftharpoons$ pyruvate + ATP

Aldolase.—During the initial rapid growth of the wheat embryo, in common with the behavior of higher plants, the energetic requirements are satisfied by the endogenous food stores of the seed. One of the main storage substances—starch—is not to be found in the nuclei; neither is one of the principal enzymes active in degrading it to simpler and utilizable saccharides—β-amylase—present in detectible quantities. Thus, whatever the enzymatic route furnishing the product fructose diphosphate, and however the intracellular distribution of enzymes concerned with the transformation, it is clear that a most simple feature of nucleocytoplasmic relations is the furnishing to the nucleus by the cytoplasm of substrate for glycolytic activity. While it is true that aldolase, which acts on hexose diphosphate to yield triosephosphates, is present at much lower concentrations in plant than in animal tissue (8), the generality of its occurrence in plants would point to an importance of function, although it is conceivable that the triosephosphates produced by photosynthetic activity might obviate in large measure the need for this hexose-splitting mechanism.

Method.—Tissues were homogenized in 0.9 per cent NaCl and the concentrations adjusted to about 10 mg./ml. The suspensions were dialyzed against distilled water overnight and aliquots of these used to determine aldolase activity according to the procedure described by Stumpf (8). Components of the reaction mixture were as follows: (A), 2 ml. tissue suspension, 2 ml. 0.1 M veronal buffer (pH 8.5), and 2 ml. KCN (0.25 M, adjusted to pH 8.5 with 6 N HCl); (B), 0.1 M hexose diphosphate (a commercial product used without further purification). 0.85 ml. was pipetted out of solution (A) for a control run and to the remainder was added 0.85 ml. (B). The mixture was incubated at 37.5°C. for 40 minutes, 1 ml. samples being pipetted into tubes containing 1 ml. trichloroacetic acid (10 per cent), at 10 minute intervals.

The following conclusions may be drawn from the results summarized in Table III:—

1. With respect to enzyme content of the whole tissue, wheat germ is at

least as active, if not more so, than other plant tissues examined. Direct comparisons are, however, not possible since other studies of aldolase have been conducted at 30°C. (8). It may be held, therefore, that the significance of this enzymatic mechanism for carbohydrate metabolism in wheat germ is much the same as for other plants.

2. There is no evidence for any localized factor interfering with aldolase activity. The sum of activities in the three fractions equals that for the whole tissue.

3. The highest activity, referred either to dry weight or to acid-insoluble nitrogen, is in the nuclei. The nuclei in fraction C do not appear to share this high activity; for that matter, the cytoplasmic portions of fractions A and C are not comparable. Since C contains no more than 25 per cent of all the nuclei of the germ, it may be concluded that the bulk of wheat germ nuclei are the most active sites of aldolase activity.

TABLE III

Aldolase Activity in the Wheat Germ

Micromoles of triosephosphate produced/milligram of N in 10 minutes at 37.5°C.

	Cytoplasm (A)	Nuclear fraction (B)	C	Tissue	Nuclei
Activity	2.16	2.75	0.55	1.97	3.04
Total activity, *per cent*	24	67	7	—	—

Phosphoglyceraldehyde Dehydrogenase.—The classic example of "oxidative" phosphorylation is to be found in Warburg's resolution of the reaction:

$$\text{D-3-Phosphoglyceraldehyde} + H_3PO_4 + \text{DPN} + \text{ADP} \rightleftharpoons \text{D-3-phosphoglyceric acid} + DPNH_2 + \text{ATP}$$

To be sure, under aerobic conditions this pathway would appear to be a relatively poor source of high energy phosphate, but under anaerobic conditions there can be little doubt that this medium constitutes a singular and important source of the energy-rich pyrophosphate linkage. It can easily be shown that the wheat germ is equipped with the necessary enzymes to convert either hexose diphosphate or D-3-phosphoglyceraldehyde to the reaction products indicated above.

Method.—In line with the observations of Cori *et al.* (9) on the instability of this dehydrogenase isolated from rabbit muscle, it will later be shown that the instability of the enzyme is most marked in wheat germ tissue. Since the conditions of measurement require a minimal concentration of solutes of tissue origin (most emphatically so in the case of wheat germ in which the solutes absorb strongly in the ultraviolet portion of the spectrum), and since dialysis even over relatively short periods of time (2 hours) strongly decreases enzymatic activity, it has been found most convenient

to proceed in the following manner: A weighed amount of tissue is homogenized in 0.8 saturated $(NH_4)_2SO_4$, centrifuged in plastic tubes, and the supernatant discarded. The operation is repeated, care now being taken to pack the residue tightly in the course of centrifugation. The walls of the centrifuge tube are wiped well with filter paper (first dry, then moistened) to remove as much of the adhering $(NH_4)_2SO_4$ as possible. The residue is then extracted with 0.06 M Na pyrophosphate buffer (pH 8.5) in the presence of 0.01 M cysteine, the concentration of original tissue with respect to extractant being in the range of 10 to 40 mg./ml. In the complete incubation mixture the following volumes of the components (in milliliters) are present: Diphosphopyridine nucleotide (1 mg./ml.), 0.05; sodium pyrophosphate buffer (0.06 M, pH 8.5), 0.675; cysteine (0.08 M), 0.05; NaCN (0.01 M), 0.05; sodium arsenate (0.06 M), 0.1; D-3-phosphoglyceraldehyde, a sample synthesized by Dr. E. Shaw of this Institute, (0.05 M), 0.05. Total volume = 1 ml. In the experiments with hexose diphosphate as substrate, a 0.2 M solution replaced the phosphoglyceraldehyde. It is important to assay the extract as soon after preparation as possible.

TABLE IV

Reduction of DPN by Wheat Germ Tissue Extract in the Presence of Hexose Diphosphate (37.5°C)

Values are expressed as ΔE_{340}.

Time	−Arsenate	+Arsenate	$+CN^-$	$+F^-$ (0.02 M)	+ iodoacetate (0.002 M)
min.					
5	0.040	0.155	0.110	0.150	0
15	0.090	0.285	0.280	0.280	—
20	0.105	0.315	0.345	—	—

Activities are measured by the increase in extinction at 340 mμ resulting from the reduction of the pyridine nucleotide. Experiments designed to test for the destruction of DPN by the tissue either in the presence or absence of pyrophosphate yielded negative results. It may be assumed, therefore, that the concentration of DPN remains more or less constant during the time of incubation.

Evidence for Identity of the Enzyme.—On the basis of the following results, summarized in Table IV, we conclude that the observed reduction of DPN is due to the action of the enzyme D-3-phosphoglyceraldehyde dehydrogenase.

1. In the absence of arsenate (or phosphate), the reduction of DPN is relatively slow. The incomplete inhibition of activity under these conditions is possibly due to the presence of some free phosphate. It is unlikely, however, that the source of inorganic phosphate is the buffer, sodium pyrophosphate. Incubation of tissue with sodium pyrophosphate did not yield significant amounts of orthophosphate.

2. Neither cyanide nor fluoride inhibits DPN reduction. In fact, the presence of cyanide is useful in that it appears to prevent oxidation of the accumulating reduced DPN.

3. Iodoacetate inhibits completely. Cysteine does not reverse this effect.

4. In other experiments it is shown that (*a*) D-3-phosphoglyceraldehyde can replace hexose diphosphate. In this way the activity of the tissue is enormously increased. Obviously, aldolase activity which provides triosephosphate for the reaction is the limiting factor when hexosediphosphate is used as substrate. (*b*) In the absence of cysteine, the reaction proceeds much more slowly.

Activity of Tissue Extracts in the Presence of D-3-Glyceraldehyde Phosphate.— In the presence of 0.0025 M triosephosphate, the reduction of DPN is so rapid that measurements must be made at room temperature over a period no longer than 5 minutes. In Fig. 1 are drawn typical assay curves, and for comparative purposes, calculated values are summarized in Table V. It is at once instructive to compare the activity of wheat germ tissue with that of the crystalline en-

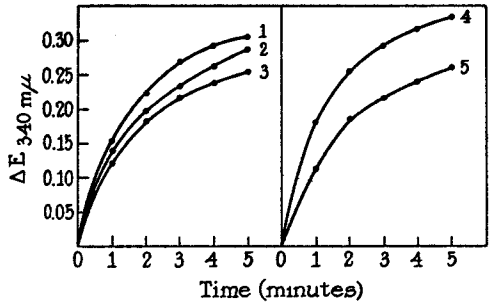

FIG. 1. Phosphoglyceraldehyde dehydrogenase activity of wheat germ extracts. Incubations were performed directly in 1 ml. Beckman cuvettes at a temperature of approximately 23°C. The incubation mixture was that described under Method with the exception of triosephosphate which was added at zero time. The extracts assayed were derived from the following masses of tissue (in milligrams): (1) 0.25 nuclei. (2) 0.25 nuclear fraction B. (3) 0.25 cytoplasmic fraction A. (4) 0.5 whole tissue. (5) 1.0 fraction C.

zyme obtained from rabbit muscle. From extrapolation of their curves to zero time, Cori *et al.* (9) calculate the turnover number of the enzyme to be 67 μM DPN reduced per minute per milligram of protein at 27°C. Since the nitrogen content of the enzyme protein is 16.4 per cent, the number may be expressed as 408 μM/mg. N/minute at 27°C. Ignoring the lower temperature (23°C.) at which our measurements were made, the activity of the whole tissue (1.16 μM DPN reduced 1 minute/23°C./mg. N) would signify an enzyme content of 0.3 per cent of the total acid-insoluble N. This undoubtedly represents a value well below the actual one since (1) some of the enzyme is destroyed in the course of extraction and (2) the rate values here used for calculation are not maximal ones. Nevertheless, it is apparent that the concentration of this dehydrogenase in wheat germ is of considerable magnitude and even approaches that of so specialized a glycolytic tissue as muscle.

From Table V it can be seen that recovery of dehydrogenase activity from the several fractions is incomplete. This is not surprising in light of these conditions:

1. The rates measured are not linear even over the first 2 minutes of incubation.

TABLE V

Glyceraldehyde Phosphate Dehydrogenase in Wheat Germ Extracts

Micromoles of DPN reduced/minute/milligram of N at 23°C.

	Cytoplasm (A)	Nuclear fraction (B)	C	Tissue	Nuclei
Activity	1.03	1.16	0.80	1.16	1.14
Total activity, *per cent*	20	48	17		

In calculating the amount of DPN reduced, the $E_{m(340m\mu)}$ was assumed to be 6.3×10^3.

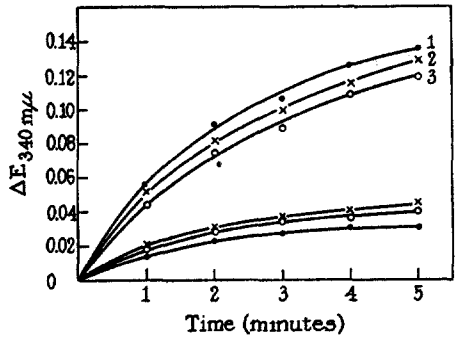

FIG. 2. Activation of phosphoglyceraldehyde dehydrogenase in wheat germ extracts. Extracts were made with pyrophosphate buffer in the absence of cysteine and aliquots of these directly assayed, or assayed after the addition of cysteine. The upper curves represent the readings obtained in the presence of cysteine. Curve 1, nuclei; curve 2, whole tissue; curve 3, cytoplasmic fraction A.

2. Activation of the enzyme by cysteine is not uniform for all fractions of the tissue. This is illustrated in Fig. 2. Cysteine added to the incubation medium increases the rate of whole tissue extract by 260 per cent, but that of nuclei by 400 per cent. Equally to the point is the fact that nuclei or cytoplasm, if extracted in the absence of cysteine, lose half their activity which cannot be restored by the cysteine present in the incubation medium. In general, these fractions appear to be more labile than the tissue as a whole. That this lability is manifested largely in the course of extraction is pointed to by the failure of combined extracts to yield activities equivalent to the tissue as a whole. There is no reason to believe that the discrepancies in Table V are due

to any fundamental differences in enzyme properties of the various fractions. It would appear rather that some special conditions obtaining in wheat germ tissue interfere with a proper assay of activity. Indeed, in view of the extremely high capacity for dehydrogenase activity in both nuclei and cytoplasm, the exact ratio of their reductive activities is, for the present, a problem of minor proportion.

Enolase.—One of the main pathways by which phosphoglyceric acid—the product of phosphoglyceraldehyde dehydrogenation—is further metabolized is its conversion by enolase to phosphoenolpyruvic acid as demonstrated by Lohmann and Meyerhof (10). This step would appear to be of some importance since the energy of the phosphate linkage is thereby increased and its incorporation into the pyrophosphate linkage of the adenylic acid system made possible. Again, it can be demonstrated that this enzyme is part of the wheat germ equipment and that, furthermore, it is a most active component of the nuclei.

Method.—Suspensions of tissue were made in either distilled water or 0.9 per cent NaCl. The enzyme appears to be quite stable, but since dialysis did not alter the activities of the tissues significantly, undialyzed suspensions were directly assayed. The composition of the medium was as follows:—0.035 to 0.1 ml. tissue suspension (containing 10 to 40 mg./ml.), 0.1 ml. of 0.1 M phosphoglyceric acid (the Ba salt produced by Nutritional Biochemicals was used), 0.1 ml. of 0.05 M $MgCl_2$, and 0.1 ml. 1 per cent $NaHCO_3$. The total volume was made up to 1 ml. In later experiments the $NaHCO_3$ was omitted and instead the mixture adjusted to pH 7.4 with KOH. There was little pH effect noted in the range 7.3 to 7.8. Below pH 7.0, activities began to drop. Incubations were generally for 10 minutes at 37.5°C. The reaction was stopped with 0.5 ml. of 10 per cent trichloroacetic acid and analyzed in either of these ways: (1) Two 0.5 ml. aliquots were removed from the clear supernatant and one of these treated with iodine-KI solution according to the method of Lohmann and Meyerhof (10). The other was treated in exactly the same way except for the omission of the iodine. The difference in phosphorus released under these two conditions was taken as equivalent to the phosphopyruvate present. Alternatively, a 1 ml. sample was removed from the supernatant and the amount of iodine used in formation of iodoform from the phosphopyruvate was determined titrimetrically with sodium thiosulfate (10). Both methods yielded equivalent results.

Evidence for Identity of the Enzyme.—Inasmuch as the substrate is phosphoglyceric acid and the product phosphopyruvate the nature of the enzyme is certain. Its similarity to enolases examined in other tissues is indicated by these properties: (1) 0.001 M iodoacetate has no effect on reaction rate. (2) 0.025 M fluoride inhibits the reaction completely. (3) In dialyzed suspensions Mg^{++} accelerates the reaction.

Activity of Wheat Germ Fractions.—The results obtained in this study are summarized in Table VI. In all cases two different concentrations of a tissue were used and activities were found to be in respective proportion. The re-

covery of total activity from the three fractions points to an absence of interference in the enzyme assays. The most outstanding feature of the results is the very high activity of the nuclei; the significance of such activity, however, remains unclear.

Pyruvate Kinase.—The enzymatic catalysis of the reaction

$$\text{Phosphoenol pyruvate} + \text{ADP} \rightleftharpoons \text{pyruvate} + \text{ATP}$$

has been broadly studied by Lardy and Zeigler (11) whose chief contribution has been the demonstration of its reversibility. The significance of such reversibility does not concern us here; our main interest lies in the possible fate of the phosphopyruvate produced in consequence of enolase activity. It has been pointed out that the phosphate of phosphopyruvate can be transferred only to adenosinediphosphate and that the frequent use of adenosine-5-phosphate (AMP) with tissue extracts is successful because of the following equilibrium (12).

$$\text{ATP} + \text{AMP} \rightleftharpoons 2\ \text{ADP}$$

TABLE VI

Enolase Activity in Wheat Germ Tissue Suspensions

Activity: micromoles of phosphopyruvate/milligram of N/10 minutes at 37.5°C.

	Cytoplasm (A)	Nuclear fraction (B)	C	Tissue	Nuclei
Activity	16.4	35.8	25.2	27.2	43.5
Total activity, *per cent*	14	64	23	—	—

The presence of trace amounts of either ATP or ADP in the tissue suspensions used is probable. The kinase activity of wheat germ tissue here measured in presence of AMP may thus be due to the operation of an additional enzyme; this possibility has not been investigated.

Method.—Tissues were homogenized in distilled water neutralized with dilute KOH. At acid pH's a variable proportion of enzymatic activity was destroyed. The following concentrations of tissues were prepared; whole tissue, 40 mg./cc.; A and nuclei, 46 mg./cc.; B, 56 mg./cc.; C, 83 mg./cc. If equivalent concentrations of N were retained the values would read 40, 23, 28, and 83 respectively; the lability of fractions A, B, and nuclei made the more concentrated suspensions preferable. Each tissue was assayed immediately after homogenization by incubation at 37.5°C. in a mixture of the following composition: 0.1 ml. of 0.1 M K phosphoglycerate (converted from the commercial Ba salt); 0.1 ml. of 0.03 M adenosine-5-phosphate (a "Sigma" product); 0.1 ml. of 0.05 M $MgCl_2$; 0.05 to 0.10 ml. of tissue suspension. Total volume was made up to 1.0 ml. Prior to addition of tissue suspension the pH of the medium was adjusted to 7.3–7.4 with KOH. The K^+ introduced into the mixture by the pro-

cedure just described was adequate. Enolase activity was used as a source of substrate for kinase activity. It will later be shown that this arrangement did not prove limiting under the experimental conditions specified.

Incubations were stopped by the addition of 0.5 ml. of 10 per cent trichloroacetic acid. 0.5 ml. was used to determine the pyruvate concentration by the method of Friedemann and Haugen (13). With the exception of fraction C the controls showed extremely small blanks. Whether fraction C contains free pyruvate or some other compound capable of reacting with 2,6-dinitrophenylhydrazine has not been determined.

TABLE VII

The Effect of Adenosine-5-Phosphate on the Production of Free Pyruvate from Phosphoglyceric Acid

Figures represent gamma of pyruvate produced in 10 minutes at 37.5°C.

	−AMP	+AMP
Whole tissue	4.8	43.2
Nuclear fraction (B)	5.7	135.6

TABLE VIII

The Effect of Standing at 0°C. on Pyruvate Kinase Activity of Wheat Germ Suspensions

Figures are for extinctions measured at 520 mμ, the absorption peak of the hydrazone used in measuring activity.

Time	Whole tissue	Nuclear fraction (B)	Cytoplasm (A)	Nuclei
hrs.				
—	0.305	0.275	0.235	0.255
4	0.290	0.120	0.116	0.095

Evidence for Identity of Enzyme.—Critical evidence for the activity of a pyruvate kinase is this: That in the absence of an adenylic acceptor for phosphate no free pyruvate is formed. Data supporting this are summarized in Table VII.

Notes on Instability of Enzyme.—Two principal conditions underlie the instability of pyruvate kinase in tissue suspensions.

1. Time.—On standing at 0°C. suspensions rapidly lose activity. Similar to their behavior with respect to phosphoglyceraldehyde dehydrogenase, the fractions appear to be more labile than the tissue as a whole, but unlike the latter enzyme inactivation appears to be continuous and not marked by a rapid drop in the course of tissue homogenization. Evidence for such instability is presented in Table VIII. It should be noted that although in this particular experiment the whole tissue appeared to be stable on standing, in all cases measurements were made immediately after homogenates were prepared.

2. Dilution.—Increase of tissue concentration in the incubation medium

results in a disproportionate increase in activity. Whether this is actually due to a reduced destruction of the enzyme or to some other factor has not been established. For the purpose of this study it has been considered sufficient to use relatively high concentrations of tissue in which discrepancies between the activities at two concentrations were less marked. It should be noted from Table IX that again the nuclei are the more aberrant with respect to this property.

Activity of the Enzyme in Various Tissue Fractions.—Calculations from experimental data are given in Table X. From these the following conclusions may be drawn:—

TABLE IX

Effect of Dilution on the Activity of Pyruvate Kinase in Wheat Germ Suspensions

Figures are for extinctions measured at 520 mμ×.

Tissue	Cytoplasm (A)	Nuclear fraction (B)	Nuclei
ml.			
0.1	0.870	0.946	1.070
0.05	0.396	0.400	0.428

TABLE X

Pyruvate Kinase Activity in Wheat Germ Fractions

Activity: micromoles of pyruvate/milligram of N/10 minutes at 37.5°C.

	Cytoplasm (A)	Nuclear fraction (B)	C	Tissue	Nuclei
Activity	3.93	4.54	—	3.47	4.93
Total activity, *per cent*	26	62	—	—	—

1. Enolase and pyruvate kinase activity are not parallel in their intracellular distribution. The deficit in recovery of activity from the three fractions (12 per cent) could not account for the differences in the ratio of enolase to kinase activity between nuclei and cytoplasmic fraction A.

2. Nuclei are the most active centers of kinase activity.

3. Fraction C appears to be inactive. This may be due to a destruction of the enzyme in the suspension but there is no evidence for it. However, even if the entire deficit in recovery were due to this fraction, its kinase activity would, with respect to enolase, be disproportionately low.

4. Fraction C apart, the distribution of kinase activity would appear to be much more even than that of enolase.

The Effect of Enolase Concentration on the Measurement of Kinase Activity.—The following experiments have been performed to test the possibility of enolase limiting the measurement of kinase activity:

1. Equal amounts of cytoplasm, which has a low enolase activity, and of

nuclei, which have a high enolase activity, were incubated together. The results were these:

Tissue	Pyruvate
	γ
0.05 ml. cytoplasm + 0.05 ml. nuclei	33 (calculated sum = 30.5)
0.1 ml. cytoplasm	28
0.1 ml. nuclei	33

There is thus no evidence for a serious limitation of kinase activity in the cytoplasmic fraction. The difference between the calculated and observed values is within the range of experimental error.

2. Fraction C with little, if any, kinase activity but with appreciable enolase activity was added to nuclear fraction B with these results:

$$0.1 \text{ ml. B} = 0.265 \ (E_{520})$$

$$0.1 \text{ ml. B} + 0.05 \text{ ml. C} = 0.286$$

Here again the evidence suggests no appreciable limitation of kinase activity by enolase.

TABLE XI

Recovery of Pyruvate in Wheat Germ Tissue Suspensions Incubated at pH 7.4 for 10 Minutes

	Pyruvate added	Pyruvate recovered	Pyruvate lost
	γ	γ	*per cent*
Nuclear (B)	52.5	50	4
Cytoplasm (A)	117	113	3

Pyruvate added equals the value for control runs in which pyruvate was added at the end of the incubation period.

Pyruvate Breakdown and Kinase Activity.—The possibility that under the conditions of these experiments free pyruvate might be broken down was tested by substituting sodium pyruvate for the phosphoglycerate in the incubation mixtures. The results of two such tests are given in Table XI. From these it does appear probable that a very small fraction of the pyruvate is attacked; its magnitude could not, however, seriously alter the pattern of results obtained.

DISCUSSION

The main question set out in the introduction to this report—Do chromosomes function in an energy-yielding environment?—can now definitively be answered. They do, at least in the nuclei of wheat germ. To be sure, such nuclei represent a particular type which, if identified by no other mark than a high ribonucleic acid content, would be set apart from the type of animal nuclei commonly isolated and studied (2). That wheat germ nuclei are, however, in respect to composition without parallel is doubtful; the intense pyro-

nin staining of nuclei in the apex of roots would suggest that RNA-rich nuclei may be a common feature of vigorously growing plant cells. Some hint as to the generality of a relatively high glycolytic activity in diverse nuclei may be obtained from an analysis of the diphosphopyridine nucleotide (DPN) content of nuclei previously isolated from tissues of calf (2). The results are listed in Table XII and have been obtained in the following way: About 30 mg. of tissue was extracted with 0.2 M nicotinamide at 100°C. for 2 minutes, cooled, and the DPN content assayed with a sample of crystalline alcohol dehydrogenase. These values may be considered as pointers, but not proofs, that the concentrated glycolytic mechanism of wheat germ nuclei is no extraordinary instance, but that despite the deep-seated intranuclear changes which must occur in the course of growth and differentiation, the need for a vigorous glycolytic mechanism remains.

Recently, from a combination of two kinds of analyses—ATPase activity, and the production of lactic acid in presence of hexose diphosphate—Lang and Siebert (14) have concluded that nuclei meet their energetic requirements

TABLE XII

DPN Content of Some Calf Tissues

Gamma of DPN/milligram of dry weight of tissue.

	Pancreas	Liver	Heart
Whole tissue	560	800	570
Nuclei	740	1100	680

by ATPase activity. The argument: In the nuclei of liver and kidney isolated in sucrose media from the tissues of rat and pig, lactic acid production is relatively small and ATPase activity relatively high. So radical a conclusion deserves the best attention. Unfortunately, the conclusion is as probably wrong as the facts are certainly doubtful. The use of an aqueous medium for nuclear isolations opens the assay of soluble enzymes to most serious question (2) and that, quite apart from the undesirability of a procedure which measures over-all glycolytic activity in disrupted cells. There is no reason to believe that the glycolysis of hexose diphosphate to lactic acid constitutes a unitary system; there is evidence that it doesn't. As for ATPase activity, enough has been said of its paucity in the nuclei in a previous publication (1).

A second question which must be brought to bear is this: Of what significance can the glycolytic cycle be to cellular economy if the great yield of high energy phosphate is a function *par excellence* of the oxidative metabolism of the mitochondria? The following remarks are therefore pertinent.

1. One may leave aside glycolysis as an obvious source of trioses, and consider oxidative phosphorylation in this light: It is by no means an unmixed blessing and for the reason that the c-c linkages which it consumes in the course

of yielding pyrophosphate bonds are an integral part of cell structure—pentoses, polysaccharides, cellulose. The complete combustion of carbohydrate in a cell makes sense when the excess would otherwise be lost. When, however, c-c linkages are required (and this could be true of the nucleus), the vigorous and complete destruction of carbohydrate would prove fatal since so far as we know, the building of carbon chains is most efficiently accomplished by the photosynthetic mechanism (15).

2. Considerations of relative efficiency aside, there is a fundamental distinction to be made between the relation of glycolytic enzymes and that of mitochondrial enzymes to the structural organization of a cell. The mitochondria appear to be true subcellular units, autonomously organized and marvellously specialized in converting reduced c-c linkages into high energy phosphate; enzymes of the glycolytic system, on the other hand, appear to be part of the warp and woof of protoplasm. The argument for this is simple. There is no evidence for a particulate organization of the glycolytic enzymes. Also, the facts of their intracellular distribution point against any type of subcellular organization, which if it were to have any meaning at all, would require that the relative activities of the various components of the system have a constancy in whatever part of the cell they appear. To take one example, the gross differences in the ratio of enolase to kinase activity in nucleus and cytoplasm weigh heavily against a conception of some coherent ultracellular structure. From these considerations it would follow that there is no cellular organelle functioning specifically to furnish glycolytic products to the cell as a whole; glycolysis would appear rather to be generally associated with the structure of the cell and possibly, to be present where needed. The mitochondria, on the other hand, stand out not only as remarkable but equally as singular units bearing by analogy, if not homology, a closer structural relation to the plant plastids than they do to the enzymes of the metabolically associated glycolytic system.

3. In terms of nucleocytoplasmic relations the fate of mitochondrial metabolism remains an open question, that of glycolytic metabolism is at least faintly delineated. Whether the nucleus is a truly anaerobic organ, incapable of directly assimilating atmospheric oxygen, remains to be studied but a few limited—and partly speculative—inferences can be drawn with respect to nuclear glycolysis.

(*a*) The extent of glycolytic activity in the nucleus makes it most unlikely that reoxidation of reduced DPN would proceed as in a closed system, *via* the reduction of pyruvic to lactic acid. Such a scheme would result in a tremendous accumulation of lactic acid in the sprouting wheat seed, and for it there is no evidence. If, as is generally assumed, the nucleus is lacking in oxidases, then there must be a route of nucleocytoplasmic interaction by which the reduced coenzymes are reoxidized.

(*b*) The yield of high energy phosphate in consequence of glycolysis leaves open these questions:

1. To what extent does its production meet the needs of the nucleus? Alternatively, are the phosphorylated products of mitochondrial activity required for nuclear function?

2. Although it would be unreal to translate the absolute values of "*in vitro*" enzymatic activity as near equivalent measures of "*in vivo*" behavior, the relative values of the activities of different parts of the cell probably have a parallel in the functioning intact cell. If so, the disproportionate concentration of enolase in the nucleus is puzzling. It could mean that additional phosphoglyceric acid furnished by the cytoplasm (or for that matter by some other intra nuclear metabolic route) is converted to phosphoenolpyruvate, thus serving as an additional source of pyrophosphate for the adenylic acid system. Alternatively, it could mean that, compared with the cytoplasm, the nucleus transfers less of phosphopyruvate P to ADP and utilizes it in some other direction.

3. The fate of the trioses themselves remains to be studied. Probably part of these are converted to desoxypentose (16). The further breakdown of pyruvate is an unsolved question. Conceivably, but not necessarily, pyruvate may be largely returned to the cytoplasm.

SUMMARY

1. A procedure for isolating nuclei of the wheat germ in non-aqueous media has been described.

2. Such nuclei were shown to constitute about 50 per cent of the protoplasmic mass and to have a ribonucleic acid content of an order equivalent to that of the cytoplasm.

3. Studies of the distribution of the enzymes—aldolase, phosphoglyceraldehyde dehydrogenase, enolase, and pyruvate kinase—have revealed that the nuclei are the most vigorous sites of glycolytic activity.

4. Analysis of the DPN content of the nuclei in calf tissues—liver, pancreas, and heart—pointed to the probability that glycolytic activity is a characteristic common to many nuclei.

5. The significance of glycolytic activity to nuclear function has been discussed and some suggestive comparisons made between the two energy-yielding systems of glycolytic and oxidative phosphorylation.

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