AN ENZYME GARRISON IN WHEAT EMBRYOS

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AN ENZYME GARRISON IN WHEAT EMBRYOS

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CHAPTER I

INTRODUCTION

Mechanisms responsible for the capacity of certain plants and plant parts to withstand varying degrees of desiccation continue to evade interpretation. Information is extensive with respect to descriptive reports of changes in the various components of these plants, in response to conditions of dehydration, but studies directed specifically at an elucidation of crytobiotic phenomena are the exception rather than the rule. The more common lethal effects of water depletion have been attributed to cytoplasmic structural changes, as a consequence of the shrinking of hydrophilic groups of protein molecules, leading to mechanical tensions and the exposure of bonds normally protected and reinforced by hydration shells. The terminal effect is the breaking of bonds and the irreversible denaturation of cellular proteins (59). Inquiries into the nature of desiccation resistance have apparently been limited to studies of structural adaptations and gross physiological responses such as cell and vacuole size, stomatal avoidance responses, etc.

It would seem reasonable to assume that cryptobiotic systems must possess certain resources for the protection and maintenance of enzyme systems. The stability of an enzyme should be expected to be severely tested when subjected to the plausible extremes of solvent changes in cells subjected to progressive desiccation. In the absence of evidence to the contrary it would seem that prolonged periods of intracellular dehydration should expose enzymes to conditions that would denature any protein that lacks some mechanism for shielding its structure. It is, of course, recognized that the cells of resistant plant materials may very well be able to control the ionic strength, pH, and the other factors in their cytoplasmic environments by means of mechanisms that provide for reanimation. If means exist for the control and storage of solutes, as the cell assumes its desiccated state, the possibility that enzymes are stored in some specific manner should also be considered.

The properties of many intensively studied enzymes provide the basis for an inquiry into the habit of an enzyme under conditions of intracellular dehydration. A study of the zymogens stimulates speculation as to the feasibility of the existence of proteins composed of proteolytically controlled enzyme residues. Particulate enzymes as illustrated by those systems of the mitochondria and endoplasmic reticulum suggest the existence of enzymes

having configurations which are maintained by structural lipid, in the absence of water. It is pertinent to refer to the relevant observation that invertase occurs, reversibly, in membrane bound form in at least some plants and can be released from this station by the application of gibberellic acid (38). The responses of succinic dehydrogenase to treatments required for its release into solution reveal a set of properties which might be expected to characterize certain enzymes persisting in desiccated tissues. This extensively studied system is liberated from mitochondrial membranes by treatment with n-butanol. Once in solution it is unstable and, with time, loses much of its activity. Lyophilization is accompanied by extensive to complete inactivation (57). This behavior of succinic dehydrogenase provides a model for a possible mechanism mediating the storage, protection and release of enzymes within the cells of desiccation-resistant plant materials. The maintenance of a potentially active configuration, in the absence of water implies the intervention of complementary factors which contribute structural support to the protein as does lipid in the case of succinic dehydrogenase. Experimental disruption of this association would be expected to nullify the stability of the enzyme with respect to desiccation.

The selection of an enzyme representative of drought resistant material for testing against the

above postulated properties is limited primarily by the obvious criterion that it be present and potentially active in the dormant tissues of the test organism, and that the induction of its activity not be a consequence of its de novo synthesis. Linko and Milner are currently studying glutamic decarboxylase and glutamic-alanine transaminase which they have demonstrated to be active in wheat embryo tissue at extremely low levels of moisture. The decarboxylase and, apparently, the transaminase exhibit activity immediately upon the addition of water to the tissue (25). Obviously, there is no de novo synthesis in the case of these enzymes. Moreover, a capacity for activity at moisture levels of less than 15% should qualify these factors for classification as desiccation-resistant.

The purpose of the present study has been to investigate those properties of the enzymes reported by Linko which can be related to their association with the dry, dormant tissues of wheat embryos. Inquiries have been made in order to determine whether the activity observed can be related to the presence of specific particulate fractions. The postulated participation of factors which buttress enzyme configuration when moisture levels are low has been investigated, as has the possibility that the property of desiccation-resistance resides in a reversible association of

macromolecular enzyme subunits. Finally, the data obtained from studies of dormant embryo tissues have been evaluated in terms of their physiological significance of the germinating wheat grain.

CHAPTER II

REVIEW OF LITERATURE

The most recent survey of literature pertaining to the physiology of seed germination was made by Koller, Mayer, Poljakoff-Mayber, and Klein (20). In the introduction to this review, a serious deficiency in our knowledge of the activities involved in this process was identified. The plentitude of information gathered from studies of the physiological responses of seeds to enviromental variables is counterbalanced by a deficit in our comprehension of the metabolic systems involved, as well as the physics and chemistry of enzyme and hormone activation and inactivation. This deficit is indicative not of any insufficiency in evidence of the presence of specific enzymes in seeds, but of a virtually complete lack of data which clearly demonstrates the metabolic interdependence and the activation sequence of these enzymes during the germination process, as well as the nature of their habits in the dehydrated cells of the dormant seed (66).

Demonstrated Origins of Activated Seed Enzymes

A stimulus to work in the area of enzyme ontogeny in cereal grains has been provided by Paleg (39) and Varner (69) by their recent publications regarding the origin of a-amylase in the aleurone tissue of barley. The investigations of these workers have afforded a composite demonstration of a pathway leading to the mobilization of barley endosperm and represent the most direct disclosure of origin and direction of enzyme activity encountered in the literature to date. Yomo had previously observed that barley endosperm exhibited amylolytic activity, when separated from the embryo, only if endosperm and embryo were cultured, together, in the same flask (38). Results of subsequent investigations of embryo extracts were interpreted by Paleg to indicate that an endosperm mobilization hormone, exhibiting properties characteristic of the gibberellins, is released from the embryo during germination and induces the activation or synthesis of a-amylase in the endosperm (36). This concept was supported by his observation that gibberellic acid (GA_3) would substitute for embryo tissue extracts in eliciting the appearance of enzymes mediating both proteolytic and amylolytic activity. The reported findings of other workers reveal that aspects other than carbohydrate degradation are clearly involved in this response to GA_3, and the possibility

that a hormonal trigger involving many separate but interdependent metabolic pathways is currently under investigation.

Evidence collateral to the problems of maintaining enzyme reserves and shielding enzyme protein during the desiccation phase of seed maturation has ensued from studies of the untrastructural changes taking place in the aleurone tissue of the barley grain during the onset of mobilization (39). Particulate structures composed of spherical inclusions within a confining membrane were observed in the cells of this tissue by means of electron microscopy. The inclusions were reported to be of three types. Two relatively large organelles occupy the central area of the particule, and an indefinite number of much smaller inclusions are arranged along the membrane surface. Changes in the components of these aleurone particles were correlated with the onset of amylolytic activity during the germination process. The particle membrane was observed to become convoluted and extended, ultimately assuming a form resembling that of the endoplasmic reticulum. All inclusions became progressively more diffuse in structure prior to their complete disappearance.

The question of whether the above changes are related to a release of enzyme protein or, rather, to enzyme synthesis has been investigated by Varner (69). By treating barley aleurone tissue with chloramphenicol

and p-fluorophenylalanine he was able to block the onset of amylolytic activity. However, the addition of actinomycin D, azaguanine, thioracil, or thiocytosine had no effect when added to the culture medium of endosperm halves. Analyses of his data and experimental conditions, as well as the results of similar work by Briggs (36) have led him to conclude that a-amylase is synthesized de novo in the cells of the aleurone layer. A consideration of the ontogenic development of aleurone tissue as related to the appearance of a-amylase, however, encourages skepticism regarding Varner's conclusion. Alpha-amylase activity has been recorded in developing barley aleurone tissue as early as ten days following anthesis. No aleurone particles are present at this time. Activity decreases approximately eighteen days later and this decrease is correlated with the appearance of these particles (38). Preliminary investigations into the composition of the inclusions of aleurone particles indicate that some are apparently composed of phytin and the remainder of lipoprotein. The intraparticular matrix of these inclusions seems to be entirely proteinaceous (39). It would appear that the observations reviewed above should stimulate investigation with respect to the possibility that the aleurone particle represents an organized reserve for the storage of enzyme protein, and organic phosphorus in seeds. Varner acknowledged that,

during his study of protein synthesis inhibitor effects on the induction of amylolytic activity, measureable respiratory rates were too low to account for the energy requirements of synthetic processes. Also tempting is the speculation that the matrix material which binds the aleurone particles represents the globulins which have been isolated from cereal grains and demonstrated to exhibit enzymic activity (42). Affinitive mechanisms have been repeatedly demonstrated to be operative in the storage of auxins (22, 62). Consequently, it should not be too venturous to suggest that a systematized design exists which mediates the storage of enzymes. Data have been reported by Yoshida (73) which suggest that the initiation of a-amylase activity in populations of E. coli is not effected by the de novo synthesis of this enzyme from amino acid precursors but is a consequence of the complexing of a protein precursor with small peptide units. In many respects, Yoshida's enzyme appears to be quite similar to the a-amylase in barley aleurone tissue. Other enzymes of E. coli formed by the interaction of two macromolecular components are currently being investigated (72). The existence of another such enzyme, involving proteins associated with ribosomal and soluble cell fractions of lactating mammary glands, has been convincingly demonstrated by Ebner (8).

Enzymes of the Wheat Grain

Information regarding the initiation of metabolic processes in seeds is rarely as comprehensive or direct as that reported for endosperm mobilization in barley. More commonly, evidence for the operation of a given pathway is inferred on the basis of the presence of certain enzymes in the seed, the assumption that those enzymes present are active, and that activities demonstrated in other systems identify native substrates and properties (10). Attempts to characterize sites of enzyme translation are, apparently, even more rare. No reports of work directed specifically at this problem have been encountered in the literature, with the exception of the aforementioned.

Reed and Thorn (47) have reviewed that literature pertaining to the enzymes in wheat grains and have presented a somewhat more cautious appraisal of current knowledge than is typically encountered. It is interesting to note that for many of the enzymes known to occur in the wheat grain, such as glucose-6-phosphate dehydrogenase, these workers consider that no significance has been reported, whereas the occurence of the same enzymes in other seeds has frequently been cited as virtual proof for the operation of some specific pathway.

The significance of the decarboxylase and transaminase

of wheat embryos, studied by Linko, is still uncertain and requires further investigation (26). Enzymic decarboxylation and transamination of glutamic acid were studied in wheat grains and excised wheat embryos by chromatographic and manometric techniques, in relation to water content. It was demonstrated that transamination of a-ketoglutarate with alanine was catalyzed at moisture levels as low as 15%. Almost 25% of the glutamic acid resulting from the transaminase reaction was decarboxylated to gamma-amino butyrate. The manometric technique used in these studies was of particular interest in that the data obtained by this method revealed that activation was immediate upon the addition of water and, therefore, no time for de novo synthesis of enzyme protein was required. This technique was also utilized to demonstrate that the only amino acid substrates entering into significant transamination reactions during the first three hours following the addition of water were alanine and aspartate. An earlier study had revealed that one or more proteases are also active in dormant wheat embryos for which no clear function could be demonstrated (25). The moisture requirement for these proteolytic enzymes approached levels as low as 11.0%. Apparently, these findings were pursued no further. Other investigations of protease activity suggest that proteolytic activity may be vital to the activation of wheat grain enzymes and may possibly

represent one aspect of the seed's hormone and enzyme liberating machinery. Proteinase activity increases by ten-fold during the early stages of germination, concurrent to the activation of increasing numbers and types of enzymes (45). Treatment of wheat endosperm with papain has been demonstrated to increase b-amylase activity in extracts of these seeds (46). The observation that some protein fractions obtained from wheat contain proteinases which cannot be removed by washing encourages speculation that these enzymes may be strategically positioned for liberating metabolically active protein residues (10).

The occurence of at least three decarboxylases, quite active during the early stages of germination (47), assumes a certain degree of significance when it is recalled that carbon dioxide enhances the germination of certain seeds. Efforts by Linko and Milner (25) to determine the significance of glutamic decarboxylase in the wheat embryo have been directed at determining the fate of gamma-amino butyrate which they believe to be an important metabolic intermediate to the germinating seed. Some stimulatory effect by glutamic acid has been reported for the germination of wheat. Aspartate, alanine, oxalacetate, and pyruvate also appear to enhance this germination and enzymes mediating their decarboxylation are also present. It has been observed during the course of gathering information for this study that the pH optima of enzymes known to participate in the

germination process, prior to the initiation of embryo growth, tend to be no higher than 6.0. The possibility of a role for these decarboxylases in the regulation of certain enzymes, via pH regulation would appear to merit consideration.

Decarboxylases and Transaminases of Higher Plants

The decarboxylase activity of extracts obtained from a wide variety of plant materials, including cereal grains, has been extensively studied in the laboratories of Schales and Schales (54). A general pattern was found to prevail involving transamination reactions as requisite preliminary steps to those decarboxylations displayed by the test materials. For these studies, extracts from forty-two different plants were prepared and tested with glutamate, pyruvate, and a-ketoglutarate as substrates. Both keto acids were observed to liberate carbon dioxide. This latter activity was relatively slight however in comparison with the high rate of glutamic acid degradation. Other keto acids were found to respond poorly or not at all. In order to eliminate decarboxylase activity other than that of glutamic acid decarboxylase, the effect of a series of inhibitors was tested. Phenol, in concentrations of 2.0 mg per ml of incubation mixture, was found to be suitable for this purpose. Dialysis of the

plant extracts also abolished their effectiveness against keto acids, but reduced the activity of glutamic acid decarboxylase. The addition of known decarboxylase co-factors restored the activity of pyruvic acid decarboxylase and glutamic acid decarboxylase but none reactivated the plant extracts for a-ketoglutarate. These observations, in addition to the finding that succinic semi-aldehyde does not substitute for a-ketoglutarate in fresh extracts suggested that carbon dioxide evolution from those extracts having added a-ketoglutarate as substrate is not mediated by an a-carboxylase. Analysis of dialyzate concentrates, ultimately permitted the isolation of two factors capable of reactivating dialyzed extracts for a-ketoglutarate. The addition of a mixture of aspartate and alanine exactly duplicated the effect of concentrated dialyzate in restoring full activity. This requirement for aspartate or alanine in an enzymic attack on a-ketoglutaric acid clearly indicated that the first step in the breakdown of this keto acid is a transamination. Coupled transaminase-decarboxylase systems are apparently general in distribution among the higher plants. Subsequent investigations have revealed that variations in this pattern may be observed. The participation of separate transaminases for aspartate and alanine in wheat embryos has been reported more recently by Sanwall, Zink, and Din (50).

The sites of activity of these enzymes is not yet clear.

They are apparently considered to be in solution within the metabolically active cell and to have little functional association with particulate fractions. There may, however be varying degrees to this solubility characteristic. It has not yet been possible to obtain a large proportion of the total pyruvic acid decarboxylase activity in aqueous supernatants, although efforts have been intensive. A beta-globulin fraction possessing oxalacetic acid decarboxylase activity has been obtained in crystalline form from plant sources but it has not been possible to identify the source of activity (68).

Environmental Considerations Pertaining to Enzyme Activity

The existence of catalytically active and inactive states of enzymes is accounted for by the time-honored concept of a specific conformation for a specific activity. The primary structure of an enzyme is genetically determined. The secondary, tertiary, and hence the active conformation of the enzyme is under the control of the environment and may also be genetically determined. Under specific conditions of pH, ionic strength, etc., the enzyme will assume an active configuration spontaneously since, under these conditions, the active configuration is the most stable structure. The primary effect of this specific coiling and folding is that certain residues are brought into a

proximity which confers distinct reactive properties on them as a reactive group or active site. The effect of unfolding is to disrupt this relationship and inactivate the enzyme. Such unfolding is caused by unfavorable temperatures, hydrogen-bonding agents, extremes of pH, ionic strength, etc. Inactivation of enzymes is accompanied by changes in optical rotation, sedimentation values and absorption spectra, suggesting strongly that changes in activity and association affinities are, indeed, a result of changes in molecular conformation (71).

The modern concepts of conformational requirements for enzyme activity are not as limiting as the earlier hypothesis that an individual enzyme molecule, under a given set of conditions possesses either full activity or is completely devoid of activity. Thermodynamic considerations have initiated a pattern of cogitation, the essence of which contends, that the degree of activity depends upon a reversible, configurational equilibrium between active and inactive states. Secondary structure is dynamic as a consequence of variations in the interaction between molecular side chains and solvent molecules, as mediated by physical factors. Under circumstances such as these an equilibrium involving a large number of configurations exists, as opposed to the concept of a static configuration. This viewpoint has been set forth in thermodynamic terms on the basis that the free energy of unfolding of the enzyme may be small, whereas the enthalpy and entropy

requirements may be large (23).

Amid the multitude of agents which are believed to contribute to the configuration of an enzyme there are those which appear to be particularly pertinent to the present study. The water dipole is a major factor in enzyme configuration, not only via its participation in tertiary structure but also due to its participation in linkages contributing to patterns of coiling. It is believed that many of the linkages stabilizing secondary structures are composed of charged groups that are, at least partially, surrounded by water. Eisenberg and Schwert have reported evidence of "frozen water" trapped in the interior of active chymotrypsinogen, the release of which is accompanied by irreversible inactivation (9). Functional patterns such as these are additions to the more conventionally accepted roles for water in forming dynamic hydration shells and in bridging distances between charged groups as reviewed by Stocker (59). A tertiary linkage involving serine and threonine connected via phosphate bonds has been reported by Perlmann (43). It has been independently observed that compounds donating phosphoric ester groups change the activity of several proteinases (23). The possible significance of these findings to the frequent requirement of phosphate buffers for dialyzing, washing, etc., crude enzyme preparations was not discussed. Finally, in regard to agents required for specific configurations, the reported partici-

pation of carbohydrates and lipids in stabilizing the configuration of certain enzymes is of direct significance to the present investigation. There is recurring evidence that a carbohydrate-protein combination is of frequent occurence (60). An alanyl-xylopyranose compound has been isolated under conditions indicating its function as a carbohydrate-protein bridge. Unfortunately very little is known concerning the nature of these bonds in enzymes (23) although Neurath (34) has investigated the properties of a strongly hydrophobic bond in trypsin and concluded from his data that the activation of the enzyme is effected by the fission of a lipid-enzyme bond, permitting the catalytic site to be assembled. It is conceivable that such a mechanism may be operative in controlling the release of enzyme reserves. The hydrophobic bonds alone could augment designs for maintaining reserves. Hydrophobic regions composed of paraffin chains are believed, in a number of instances, to encompass and protect reactive groups of enzymes. Removal of such groups is sometimes required for <u>in vitro</u> activation of these enzymes (34).

In the absence of compensating mechanisms, which have not been demonstrated, the enzymes of seeds should be subjected to extreme changes of the cellular environment as the maturing cells assume their dormant state. Increases in ionic strength or changes in the nature of

the ions present alter hydrogen bonding patterns and usually impose a net positive or negative charge on the enzyme molecule. Internal repulsions are believed to be responsible for subsequent disruption of the secondary structure. In this unfolded state the enzyme is highly susceptible to degradation by proteolytic enzymes (23). With the exception of the demonstrated effect of proteinases on enzymes, mechanisms responsible for irreversible denaturation are not clearly understood. This phenomenon is believed to be, most frequently, a consequence of factors creating stresses which lead to the breaking of covalent bonds, permitting irreversible formation of others. The ultimate effect is an irreversible hyperfolding of the protein molecule (15).

CHAPTER III

MATERIALS AND METHODS

Source of Embryo Tissue

The problem of obtaining excised wheat embryo tissue in the quantities required for physiological investigations has commonly been avoided by the use of wheat germ. This material is made readily available in fresh, granular or flaked form by modern milling methods and is considered appropriate for enzyme-centered studies of the wheat grain embryo. As a source of preparations exhibiting specific enzyme activities wheat germ has been highly popular. Its utilization as a basis for investigating the intermediary metabolism of the embryo itself has been more recent (25).

Fresh wheat germ prepared by the flaking process was provided by the International Milling Company, Minneapolis, Minnesota, as the source of embryo tissue for this study. The material was stored at 4° C in closed glass containers. Contamination due to the presence of traces of endosperm material has been demonstrated by Linko and Milner to be insignificant. They have not considered, however, the possible presence and significance of minute quantities

of aleurone tissue which, as previously detailed, is a site of intense metabolic activity during the germination of wheat and barley.

Isolation of Crude Preparations Displaying Decarboxylase Activity

The procedure of Suzuki and Takakuwa (61) for isolating glutamic acid decarboxylase preparations from Scopolia japonica root tissue, with modifications, provided the basis for separating a crude water soluble fraction from wheat germ. The decarboxylase activity of the preparation relative to the preparation reported by Suzuki and Takakuwa, was quite high. The detailed procedure follows:

In a Lourdes homogenizer 100 grams of wheat germ were blended with 400 ml of M/15 phosphate buffer, pH 5.8, for a period of 4 minutes. The resulting homogenate was centrifuged for 30 minutes at 3,000 x g and the sediment discarded. Ammonium sulfate was added to 50% saturation and a period of 20 hours was allowed for precipitation (61). The precipitate thus obtained was collected by centrifuging at 3,000 x g for 10 minutes and the supernatant discarded. Nineteen and two-tenths grams of a dry, hygroscopic powder were obtained upon drying the collected precipitate in vacuo from the frozen state. The powder thus obtained was stored in vacuo over calcium chloride at room temperature and

designated as fraction A (fig. 1). All manipulations other than the lyophilization process were performed under temperature conditions of 4° C and all solutions employed were stored at this temperature prior to their utilization.

During a subsequent operation 30 gram samples of tissue homogenate, which had served as source material for preparing fraction A were re-extracted by the method described. The previously extracted homogenate was blended with fresh buffer, centrifuged, extracted for 2 hours and precipitated with ammonium sulfate. Subsequently, the homogenate was re-blended and extracted for a period of 17 hours. The ammonium sulfate precipitates corresponding to the 2 hour and 17 hour extractions were designated as fraction B and fraction C, respectively (fig. 1).

The isolation procedure described above was followed as a step preliminary to obtaining preparations with higher activities than that exhibited by fraction A. It has since been observed, however, that homogenization of the original embryo tissue with distilled water, rather than buffer, at room temperature, alters neither the yield nor the decarboxylase activity of the preparation.

Analytical Procedure

All assays were carried out in a Warburg Apparatus at 37° C. The main compartment of the flask received a 2.0 ml of 50 mg/ml suspension of the enzymically active

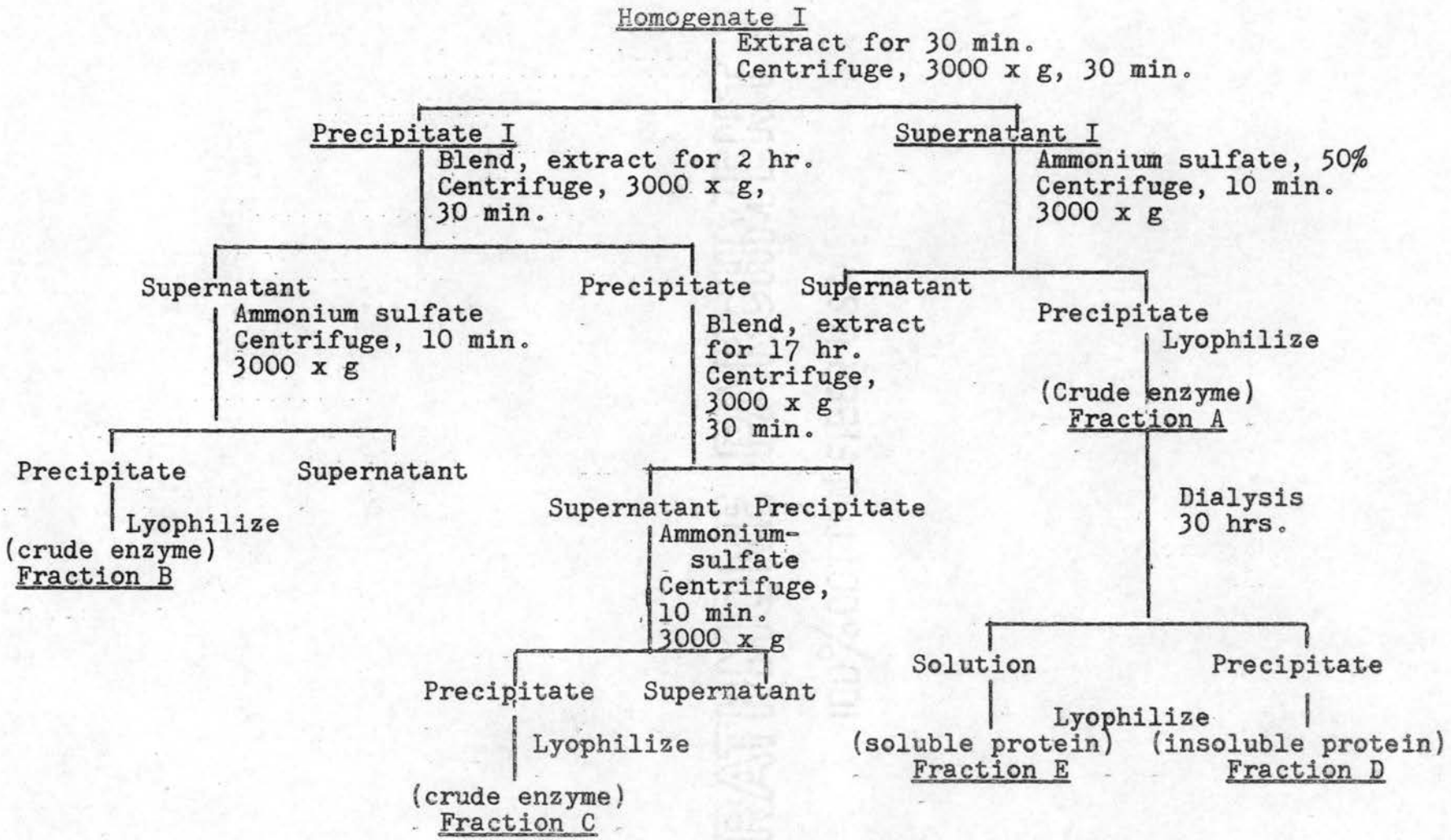


Figure 1. Isolation of soluble wheat germ fractions.

preparation in M/15 phosphate buffer, pH 5.8, and 0.5 ml of a solution containing 50 micrograms of pyridoxal phosphate. Into the side arm was pipetted 0.5 ml of a 1% solution of glutamic acid, adjusted to pH 7.0. A blank vessel, with water substituting for substrate was employed, permitting correction for any carbon dioxide which might be contained in the buffer or released by the enzyme preparation in the absence of substrate. Manometer readings were taken at five minute intervals for a period of sixty minutes. The gas phase was nitrogen. This procedure is essentially that recommended by Schales with the exception of the added pyridoxal phosphate (54).

In all assays, one activity unit signifies the release of one microliter of carbon dioxide in one hour at 37° C. Specific activity denotes activity units per 100 mg of the preparation to be tested for decarboxylase activity.

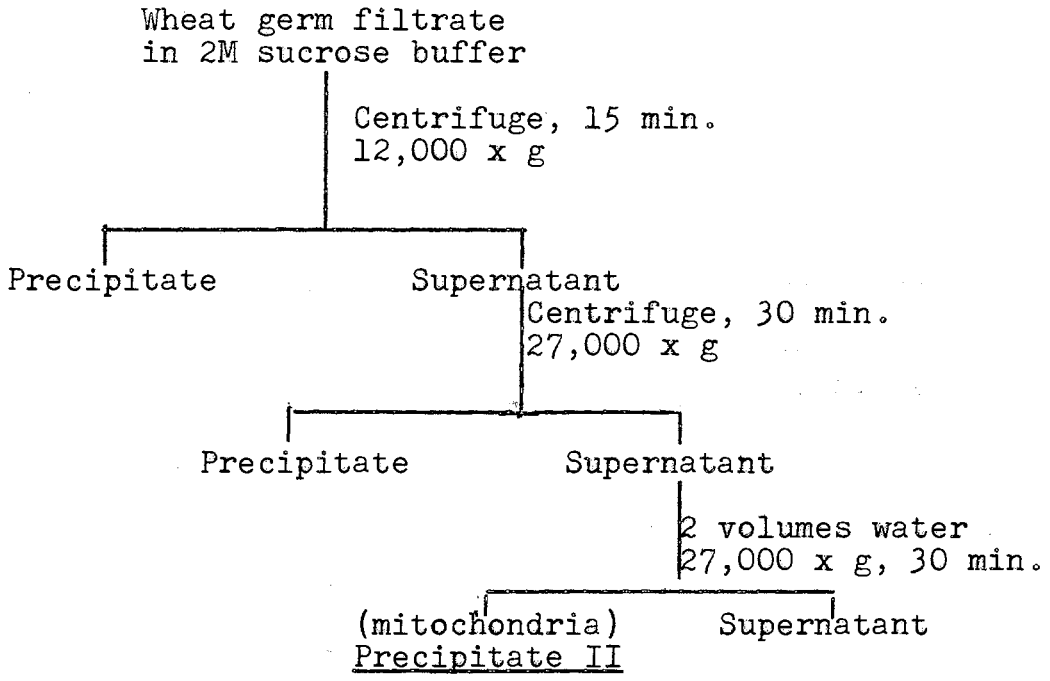
Differential Centifugation of Embryo Homogenates

The possible association of glutamic decarboxylase with particulate material in latent or permanently bound form was investigated by assaying nuclear, mitochondrial, and microsomal fractions separated by differential centrifugation. Two procedures, differing with regard to the medium employed in obtaining the individual fractions,

were followed. The method of Johnston and Stern (21) has been developed specifically for the isolation of oxidative particles from wheat embryo tissue and was utilized in obtaining one of the mitochondrial fractions tested. The details of this procedure follow:

Ten grams of embryo tissue were homogenized in 100 ml of 2M sucrose solution prepared in M/15 phosphate buffer, pH 6.0. The temperature was maintained at 0° C by means of an ice bath. The homogenate was filtered through a single layer of cheesecloth and centrifuged at 12,000 x g for 15 minutes. The pellet was discarded and the supernatant centrifuged at 27,000 x g for 30 minutes. The supernatant obtained in the latter step was then diluted by addition of two volumes of distilled water and, again, centrifuged at 27,000 x g. The sediment obtained was lyophilized, stored in vacuo over calcium chloride at 4° C, and designated as precipitate II (fig. 2).

Nuclear and mitochondrial fractions were isolated from 0.25M sucrose in M/15 phosphate buffer, pH 5.8, by subjecting homogenates to a centrifugal force of 100 x g and the supernatant thus obtained to 27,000 x g. A force of 140,000 x g was applied to the supernatant obtained by the latter treatment in order to obtain a microsomal fraction. Each particulate fraction was washed with buffer, lyophilized and stored as described previously for decarboxylase preparations. The nuclear, mitochondrial, and micro-



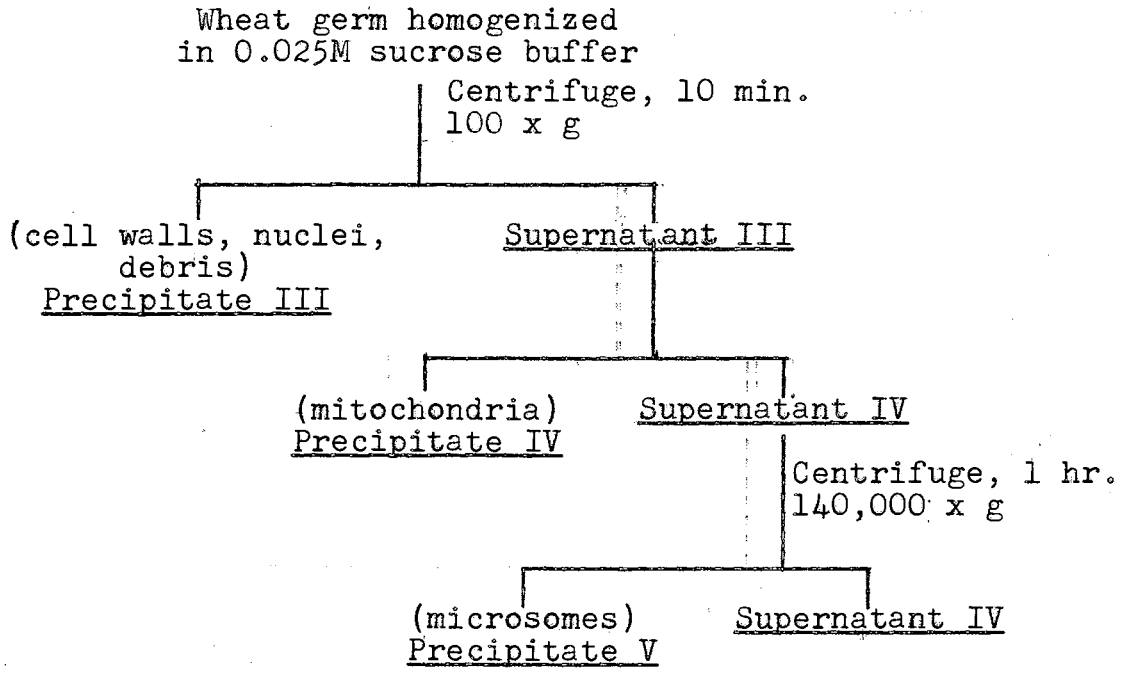


Fig. 2. (top). Isolation of wheat embryo mitochondria in medium of 2M sucrose.

Fig. 3. (bottom). Centrifugal separation of wheat germ fractions in medium of 0.025M sucrose.

somal specimens obtained by this procedure were specified as precipitates III, IV, and V respectively, and the corresponding supernatants as supernatants III, IV and V (fig. 3). Assays were performed for the purpose of determining whether decarboxylase activity is associated with a soluble or a particulate fraction. This effort, which should be described as exploratory, was directed at collecting data suggesting the participation of membranous surfaces as sites for decarboxylase activity.

Suspensions of each particulate fraction were prepared consisting of 100 mg of lyophilized material combined with 2.0 ml of M/15 phosphate buffer, pH 5.8, in a hand homogenizer. The suspensions were assayed according to the procedure described for decarboxylase preparations. The supernatants associated with each particulate fraction were tested by combining 2.0 ml aliquots with 0.5 ml of pyridoxal phosphate solution in the main compartment of each Warburg flask and proceeding as previously detailed. Suspensions of pellet material in 2.0 ml of buffer or supernatant were tested for activity indicative of surface catalysis, unknown interactions and the effects of the lyophilization treatment.

Measurements of Glutamic Decarboxylase Activity as a Function of pH

Investigations of the glutamic decarboxylase activity of preparations obtained from plant materials consistently

involve reaction mixtures adjusted to a pH within the range of 5.6 - 6.0. The criteria for determining conditions of pH is not recounted in the literature which has been surveyed concurrent to this study. However, the extensive investigations of Schales (54) have yielded data which should justify a conclusion that, in plant materials, glutamic decarboxylase enzymes may be characterized by a common pH optimum. Since the response of this enzyme to a specific treatment is to be measured on the basis of variations in the volume of carbon dioxide evolved, it is particularly desirable that support for the assumption of identity of glutamic decarboxylase activity with carbon dioxide evolution be obtained. The response to varying conditions of pH by fraction A (fig. 1) of the crude decarboxylase preparations was determined by measuring the relative yield of carbon dioxide in the usual manner but at pH values of 3.0, 4.0, 5.0, 5.6, 5.8, 6.0, 7.0 and 8.0. The reaction mixtures were adjusted within the pH 5.6 - pH 8.0 by suspension of the dry preparation in phosphate buffers, and within the pH 3.0 - pH 5.6 range by suspension in citrate-phosphate buffer. The estimated maximum, minimum, and optimum values were plotted for comparison with the results of Schales.

Dialysis of Decarboxylase Preparations

Previous studies have shown that aqueous extracts of

plant materials containing glutamic decarboxylase are quite stable to prolonged dialysis, with regard to this enzyme (53). In order that the activity of fraction A be concentrated and undesirable cofactors, possibly altering measurements assumed to be a function of test enzyme activity, be removed, the crude preparation was subjected to dialysis against distilled water. Ten grams of the enzymically active powder were combined with 20 ml of distilled water and dialyzed for 30 hours at 4° C against a sequence of 2 liter volumes of water which were renewed at 10 hour intervals. The resulting precipitate and soluble fractions were separated by centrifugation at 3,000 x g and lyophilized. The total recovery was 4.47 grams, accounted for by 4.10 grams of powdery precipitate, designated as fraction D (fig. 1), and 0.28 grams of the translucent, apparently crystalline material, fraction E, obtained from the soluble phase. These same fractions, obtained from subsequent dialysis treatments of crude preparations were dried <u>in vacuo</u> from the liquid state in order to test the resistance of the enzyme, at this stage, to drying under conditions allowing for chemical interaction between the various constituents of the fractions. The activities of 100 mg samples of each fraction and of a mixture consisting of 100 mg of precipitate and 10 mg of crystalline material were measured manometrically in the usual way.

The above procedure was varied with respect to dialyzing medium for the purpose of obtaining information suggesting

the advisability of a more extensive inquiry, regarding possible roles for sugar and phosphate in contributing to the desiccation resistance of glutamic decarboxylase. Separate 2 gram samples of fraction A were suspended in 10 ml volumes of 0.025M sucrose, 0.25M sucrose and M/15 phosphate buffer, pH 5.8, and dialyzed against their respective suspension media for 17 hours. The soluble and insoluble fractions thus obtained were not separated, but were dried together and assayed in 100 mg quantities representative of each medium. From the sucrose-dialyzed materials 1.28 grams and 2.31 grams were recovered from 0.025M and 0.25M conditions of concentration respectively. The phosphate treatment yielded 1.17 grams of dialyzed enzyme preparation. Reference to "the dialyzed enzyme preparation" in the procedures yet to be described will relate to the combined D and E fractions resulting from water-dialysis of fraction A.

Disruption of Associated Lipid and Protein

Investigations of many enzymes have been limited by the interaction of these enzymes with lipid. A number of methods have been employed for separating such complexes. Autolysis and digestion, with lipases, trypsin, and papain have permitted varying degrees of separation. Sonic vibration and treatment with sodium deoxycholate have achieved some disruption of cellular membranes, with liberation of enzymes

structurally bound to these membranes, as has the technique of alternate freezing and thawing. These methods have been considered to be more or less undesirable and to have limited application (31). Proteolytic procedures have been observed to destroy, or commonly, to alter the enzyme under investigation, and to consistently interpose contaminating products. With regard to sonic vibration and the freeze-thaw method the possibility of denaturation is considerable. Sodium deoxycholate often promotes apparent solution through micelle formation.

The extraction of lipid from dry enzyme preparations under the controlled utilization of n-butanol alone appears capable of effecting the desired separations of lipoprotein complexes without risk of losing enzyme activity, in the case of enzymes so far investigated (32).

For the separation of lipid from the dialyzed enzyme preparation, 50.0 ml of reagent grade n-butanol were added to one gram of enzymically active material. Dispersion was achieved by means of the Lourdes Homogenizer at room temperature. The suspension obtained was mechanically stirred for 20 minutes and the n-butanol subsequently removed by centrifuging at 3,000 x g for 10 minutes. This procedure was repeated twice with n-butanol and finally with reagent grade acetone at -27° C. Rapid removal of organic solvents from the precipitated material with its cargo of enzyme was accomplished by means of the vacuum desiccator. Samples of

100 mg mass were assayed for decarboxylase activity, again by the described manometric procedure. The resistance of the enzyme to drying, in the absence of its postulated lipid garrison was then tested by subjecting the sample to *in vacuo* desiccation immediately after suspending it in buffer, and then assaying a distilled water dispersion of the resulting preparation.

The suggestion that the effects of the latter treatment might be reversed, to some extent, by treating the material thus obtained with the butanol utilized during the extraction procedure was also explored. A 200 mg quantity of the defatted desiccated preparation was held in contact with 150 ml of the butanol solution recovered from the lipid extraction. A fluted filter paper was employed as a supporting container for the sample and was positioned in a manner allowing the substances in solution to pass through the test material and the butanol to evaporate from a progressively increasing area of the paper. Recovery of 196 mg made possible the manometric assay of 100 mg of the, possibly, refatted material.

Estimation of Glutamic Decarboxylase Activity in Tissue Homogenates

The activities of enzyme preparations exposed to the described levels of purification were related to the effects of dialysis, butanol extraction, and desiccation on homogenized samples of unpurified embryo tissue. This

phase of the present study was motivated by recognition of the possibilities for interaction by enzyme protein with soluble and insoluble components of the fractions separated during the initial extraction procedure. In addition to previously employed precautions a general decarboxylase inhibitor, phenol, demonstrated to have no effect on glutamic decarboxylase activity was utilized in an effort to minimize activity of enzymes, other than the test decarboxylase, mediating the release of carbon dioxide (54).

Untreated homogenates of embryo tissue were prepared in a hand homogenizer by mincing 200 mg samples of germ in 3.0 ml of buffer and 1.0 ml of a solution containing 8.0 mg/ml of phenol in distilled water. An estimated 2.0 ml quantity of homogenate was withdrawn and assayed manometrically. Treated homogenates were obtained by subjecting 1.0 gram samples of germ to: (1) thirty hour dialysis against distilled water at 4° C; (2) dialysis, followed by butanol extraction; and (3) dialysis, butanol extraction, and desiccation <u>in vacuo</u>. Each treated sample was tested for activity after homogenizing 200 mg quantities in phenolic buffer as described above. Butanol treatment of 1.0 gram of dialyzed homogenate yielded 0.67 grams of defatted material. One gram of lyophilized pellet material obtained by centrifuging the original homogenate yielded 0.87 grams when extracted with butanol.

Estimation of Glutamic Acid-Alanine Transaminase

Observations reported by Linko (24) encouraged the speculation that the same techniques utilized in obtaining glutamic acid decarboxylase preparations would also yield materials mediating transminations between a-ketoglutaric acid and alanine, and between a-ketoglutarate and aspartic acid. Procedures for the isolation of crude powders, dialyzed preparations and treated specimens to be assayed for transaminase activity, were identical with those previously described. However, the selection of a method for estimating activity was coupled with unexpected difficulties. Two procedures, tested for relative applicability, will be described in order of consideration.

Spectrophotometric Measurement of Oxalacetic Acid Formation. This method has been detailed by Cohen (4) for determining the transaminase activity of crude tissue extracts and was the method of first choice in this study because of the relative specificity of the data obtainable. One gram of fraction A was dialyzed in the usual manner in order to minimize side reactions requiring coenzymes. The dialyzed material was frozen and dried in vacuo and 100 mg were suspended in 4 ml of chilled M/15 phosphate buffer, pH 7.2, with a hand homogenizer. The suspension was centrifuged at 27,000 x g for 30 minutes and 1.0 ml of the supernatant incubated with 1.0 ml of a solution containing

30 micrograms per milliliter of pyridoxal phosphate for 30 minutes and added to the quartz cell of a Perkin-Elmer Model 202 spectrophotometer. This mixture was further incubated with 1.0 ml of a 20 micromole per milliliter solution of aspartic acid and 0.2 ml of a 100 micromole per milliliter solution of a-ketoglutaric acid in M/15 phosphate buffer, pH 7.2. The blank employed consisted of the complete system from which a-ketoglutarate had been omitted. The reaction mixture was assayed at 280 mμ. Density readings were recorded every two minutes for thirty minutes for the purpose of measuring the rate of oxalacetate formation. Deviations from Cohen's procedure were necessary for the preparation of the enzymically active supernatant since the material to be assayed had not been prepared as an aqueous extract and, in the conditions of assay since the spectrophotometer employed did not allow for the maintenance of a constant 38° C temperature. All operations were performed at room temperature. Extensive protein coagulation was observed to occur in the reaction mixture during the period of assay and this method was therefore abandoned.

Manometric Assay of Glutamic Acid-Alanine Transaminase. The presence of glutamic acid decarboxylase in preparations, for which an estimation of transaminase activity is sought, suggests that an assay of decarboxylase activity, as limited by the rate of a-ketoglutarate amination might reveal a functional relationship allowing for the collection of the

desired measurements. It is immediately apparent, however, that any treatment significantly inhibiting the activity of the decarboxylase cannot reliably be tested for its effect on transaminase activity, which is to be determined on the basis of carbon dioxide release.

Assays were carried out manometrically, as described in the procedures for glutamic decarboxylase with minor, but necessary modifications. Alanine and a-ketoglutarate solutions, made up to contain 100 micromoles per milliliter and 20 micromoles per milliliter, respectively, in M/15 phosphate buffer, pH 6.0 were added to the side arm in 0.5 ml quantities. It was observed that adjustment of the pH of all solutions and reaction mixtures was highly critical to the activity of the coupled systems, and that frequent checks for changes in this variable were necessary. Phenol was added to the main chamber as described for assays of embryo homogenates. Utilizing the above modifications in substrate and pH, all tests described for the various glutamic acid decarboxylase preparations were repeated, excluding those for which no decarboxylase activity had been obtained or exploratory observations confirmed that no activity could be expected with a-ketoglutarate and alanine as substrates.

Evaluation of Glutamic Decarboxylase Activity in Germinating Wheat Grains

The physiological significance of the desiccation-resistant property of glutamic decarboxylase to the germinating wheat grain was explored. The activity of this enzyme, as exhibited by grains of Triticum aestivum L. variety Concho which had been germinated for varying periods of time was related to the activity displayed by grains which had been germinated and then dried in vacuo, and with grains which had been germinated, dried, and extracted with butanol.

Four gram quantities of seeds, previously dusted with Arasan, were placed in each of four large petri dishes containing 20 ml of distilled water and three thicknesses of Whatman No. 1 filter paper. Germination was allowed to proceed for periods of 17, 30, 48, and 72 hours. From each petri dish, corresponding to a given germination period, a specimen consisting of fourteen seeds was removed and assayed, individually, for its total glutamic decarboxylase activity without further treatment. The remaining seeds were dried in vacuo from the unfrozen state and 2 gram quantities were then treated with butanol in the usual manner. The responses of the glutamic decarboxylase of germinating seeds, to desiccation and to defatting treatments were then determined by the manometric method.

Assay procedures were identical to those previously detailed for estimating glutamic decarboxylase with the exception of the homogenization step. Test specimens, consisting of fourteen seeds each, were first washed three times by immersing briefly in distilled water. Each sample was then homogenized with 4.0 ml of a solution, consisting of 3.5 ml of phenolic buffer and 0.5 ml of pyridoxal phosphate solution, in a mortar and pestle. This homogenate then occupied the reaction chamber of the Warburg flask. To the side arm 0.5 ml of glutamic acid solution was added. Grains which had been treated by dusting with Arasan and washing with distilled water prior to assaying served as the zero-time control for experimental samples which had been germinated and assayed directly. For samples which had been subjected to the desiccation treatment, grains which had also been desiccated were used as controls. The control for the butanol-treated experimentals consisted of seeds which had been dusted, washed, dried, and then extracted with butanol. The gas phase for all assays was nitrogen.

Investigation of Potential Decarboxylase Subunit Sources

Certain observations, to be discussed in a later section, were considered at this stage of the present investigation to be suggestive of a glutamic decarboxylase system consisting of more than a single apoenzyme component and

its cofactor. For reasons, also to be discussed, the properties of the enzyme system resolved by Ebner (8) encouraged speculation as to the possibility that, in glutamic decarboxylase, the property of desiccation-resistance resides in one or more subunits.

In order to test this subunit postulate, a sample of wheat germ was homogenized and separated into a series of fractions. These fractions then served as members of a series of recombinants which were assayed for decarboxylase activity. The procedures employed in achieving the various separations (fig. 4) were as follows:

A wheat germ homogenate was prepared in the manner previously described (fig. 1) and separated into fractions, which have been denoted as Precipitate I and fraction D and E, by methods summarized in figure 1. A fraction F (fig. 4) was prepared by boiling 500 mg of the insoluble dialysis fraction D for 5 minutes in 300 ml of water and then isolating the precipitated material by centrifugation at 100 x g. The sediment thus obtained was vacuum-dried from the frozen state and stored over calcium chloride at room temperature. Ten grams of the pellet material identified as Precipitate I were treated with butanol in the usual manner. The defatted particulate material obtained by this treatment was designated as Precipitate B. By extracting 1.0 gram of Precipitate B with 20 ml of chilled buffer for one hour and then centrifuging at 100 x g, a solution was obtained which was identified as Extract I.

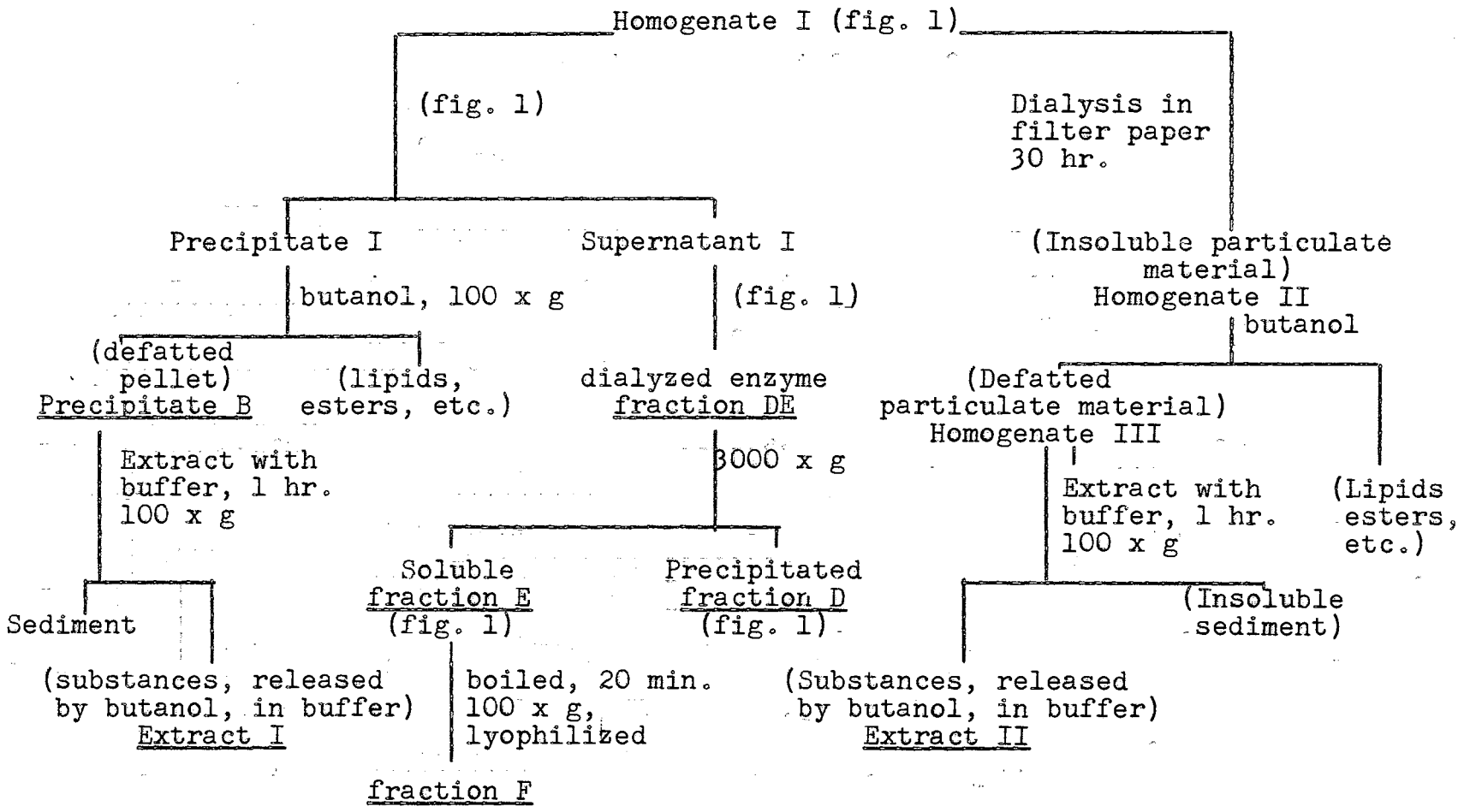


Figure 4. Separation of wheat germ fractions to be tested for glutamic decarboxylase subunit activity.

A particulate fraction, free of soluble material and corresponding to Precipitate I, was prepared by "dialyzing" 10 grams of Homogenate I from filter paper. The homogenate specimen was confined within an enclosure consisting of one thickness of filter paper and one layer of cheesecloth. The dialysis procedure previously employed (fig. 1) was then followed for 30 hours. The particulate material recovered was lyophilized and denoted as Homogenate II. By means of butanol and buffer extraction procedures identical to those applied to Precipitate I, two fractions specified as Homogenate III and Extract II were separated (fig. 4).

The capacity for carbon dioxide evolution was measured for each fraction individually and in combination with one or more other fractions. Recombinants and assay results will be described simultaneously under "Results." All reaction mixtures included 1.5 ml phenolic buffer, 0.5 ml pyridoxal phosphate solution, and 0.5 ml glutamic acid solution, prepared as previously detailed.

CHAPTER IV

RESULTS

Activity of Crude Decarboxylase Preparations

From 100 grams of the initial excised embryo tissue 19.2 grams of dry hygroscopic powder (fraction A, fig. 1) were obtained. The enzyme activity of this type of preparation has been reported to decline rapidly in air but to retain its activity indefinitely when stored *in vacuo* over calcium chloride at room temperature (52). Upon combining test quantities of the powder with phosphate buffer a suspension was obtained from which a visibly large proportion of insoluble materials settled to the base of the reaction chamber. The apparently stable dispersion remaining was only slightly turbid.

The total and specific activities recorded for this fraction are listed in Table I with the values derived from assays of other fractions prepared from Supernatant I (fig. 1). Activity was immediate upon addition of substrate, and proceeded at a constant rate for the one-hour period of assay when the reaction was permitted to advance in an atmosphere of nitrogen. Activity was erratic when the gas

TABLE I

GLUTAMIC DECARBOXYLASE ACTIVITY OF PREPARATIONS OBTAINED FROM SOLUBLE WHEAT GERM FRACTIONS

Preparation	*Total Activity Units	**Specific Activity	Yield %
HOMOGENATE	104,700	104.7	
1. Fraction A (fig. 1)	23,385	121.8	22.3
a. Dialyzed against water (D + E)	19,510	208.2	18.9
b. Dialyzed against 0.025M sucrose	22,828	184.1	21.8
c. Dialyzed against phosphate buffer, pH 5.8	9,831	87.0	9.4
d. Dialyzed against 0.25M sucrose***	12,058	52.2	11.5
e. Dialyzed, butanol treated	13,467	156.6	12.9
f. Dialyzed, butanol treated, desiccated	0	0	0
g. Immersed in butanol extract after treatment f	-	25.1	-
2. Fraction D (fig. 1)	0	0	0
3. Fraction E (fig. 1)	346	69.6	0.35
a. Butanol treated	346	69.8	0.35

*One unit of activity specifies the release of one microliter of CO_2 in a period of one hour at 37° C.
**Specific activity is designated as the number of activity units per 100 mg of test material.
***Preparations assayed were more than 55% sucrose.

phase was air and was denoted by small positive and negative fluctuations in gas pressure for the duration of the assay.

The total activity obtained under anaerobic conditions, was determined to be 23,385 units for this preparation. This exceeds by greater than two-fold the activity observed by Suzuki and Takakuwa (61) for preparations obtained from subterranean stem tissues of Scopolia japonica. The conditions of assay employed by these workers were identical to those of the present study with exception that preparations from S. japonica exhibited maximal activity in an atmosphere of either nitrogen or air. It is of interest to note that the constant rate of reaction observed for the wheat germ preparation differs from the observations reported by Suzuki and Takakuwa not only in magnitude but also as regards duration of peak activity.

Previously extracted homogenate (Precipitate I, fig. 1) was adjudged to be a poor source of decarboxylase protein on the basis of the data presented in Table II. Preparations obtained from re-extracted precipitate I were observed to be, relatively, devoid of capacity for decarboxylating glutamic acid, regardless of the time allowed for materials to enter into solution (Table II: a, b, c).

Decarboxylase Activity of Centrifugal Fractions

Efforts directed toward associating the activity of

TABLE II

GLUTAMIC DECARBOXYLASE ACTIVITY OF WHEAT GERM FRACTIONS PREPARED BY TREATMENT WITH WATER AND BUTANOL

Preparation	*Total Activity Units	Specific Activity	Yield %
Soluble fractions (fig. 1)			
a. Fraction A	20,936	121.8	21.1
b. Fraction B	178	4.2	0.17
c. Fraction C	62	3.3	0.07
Homogenate I (fig. 1)	104,680	104.7	
d. Butanol treated	190,677	279.3	182.0
e. Dialyzed	33,782	53.2	51.8
f. Dialyzed, butanol treated	84,773	191.4	81.0
g. Dialyzed, butanol desiccated	38,715	87.0	37.1
h. Precipitate I (fig. 1)	0	0	0

*Total activity is based on 100 grams of wheat germ, for comparison with fractions separated from this quantity of source material.

glutamic decarboxylase with a particulate cellular component yielded the results listed in Table III. The activities of the various supernatants, precipitates, and recombined supernatants and precipitates associated with different centrifugal forces appear to reveal rather definite trends. Relative to the low degree of activity expected for unconcentrated extracts, the rate of carbon dioxide release mediated by each supernatant fraction, regardless of the centrifugal force to which it had been exposed, was high and generally the same for all supernant fractions. Particulate fractions, whether washed or unwashed, lyophilized or immediately assayed, exhibited little or no decarboxylase activity. The maximum activity obtained following the recombination of 50 mg samples of the dried mitochondrial fractions with 2.0 ml specimens of Supernatants III and IV was no greater than that generally observed for the supernant itself.

Deviations from the generalization that supernatant fractions are uniformly active and particulate fractions inactive are represented by the behavior of the mitochondrial supernatant, in 0.25M sucrose, and the mitochondrial pellet material (Precipitate II) separated from 2M sucrose. The total activity displayed by Supernatant IV from mitochondria was observed to be less than that of analogous fractions obtained at other centrifugal forces. Since the activity mediated by reaction mixtures containing both Supernatant IV

TABLE III

DISTRIBUTION OF GLUTAMIC DECARBOXYLASE ACTIVITY IN SOLUBLE AND PARTICULATE WHEAT GERM FRACTIONS

Fraction	Total Activity Units	Specific Activity	Yield %
HOMOGENATE	104,700	104.7	
a. Precipitate II (fig. 2)	56	3.7	0.054
b. Precipitate III (fig. 3)	0	0	0
c. Precipitate IV (fig. 3)	0	0	0
d. Precipitate V (fig. 3)	0	0	0
e. Supernatant III (fig. 3)	17,775	22.3	16.9
f. Supernatant IV (fig. e)	9,075	12.1	8.7
g. Supernatant V (fig. 3)	14,400	19.2	13.8
h. Precipitate IV+ Sup. III	17,325	23.1	16.7
i. Precipitate IV+ Sup. IV	16,500	21.9	15.8

and mitochondrial material was virtually identical to that displayed by other supernatants tested, it is assumed that the activities of all supernatants should actually be equivalent. The procedure utilizing 2M sucrose as a medium for isolating mitochondria yielded the only particulate material which exhibited any suggestion of decarboxylase activity. When assayed in the usual manner a very feeble activity, 0.61% of that observed for Supernatant IV, was recorded. It is immediately obvious that minute procedural errors would be expected to significantly alter responses mediated by preparations displaying such feeble activities as those assayed in this phase of the present inquiry.

The data recorded in Table III reveal, perhaps, the most significant information afforded by these embryo tissue fractions. Most of the carbon dioxide-liberating capacity of the unresolved homogenate is lost when the homogenate is separated into its component supernatant and precipitated fractions, by centrifugation at 3000 x g. The sum of the activity units of these two fractions is but 16.9% the total activity recorded for the homogenate.

Effect of pH on Decarboxylase Activity

Crude, ammonium sulfate-precipitated powders (fraction A, fig. 1) were assayed for specific activity as a function of pH (fig. 5). This parameter was experimentally varied over a

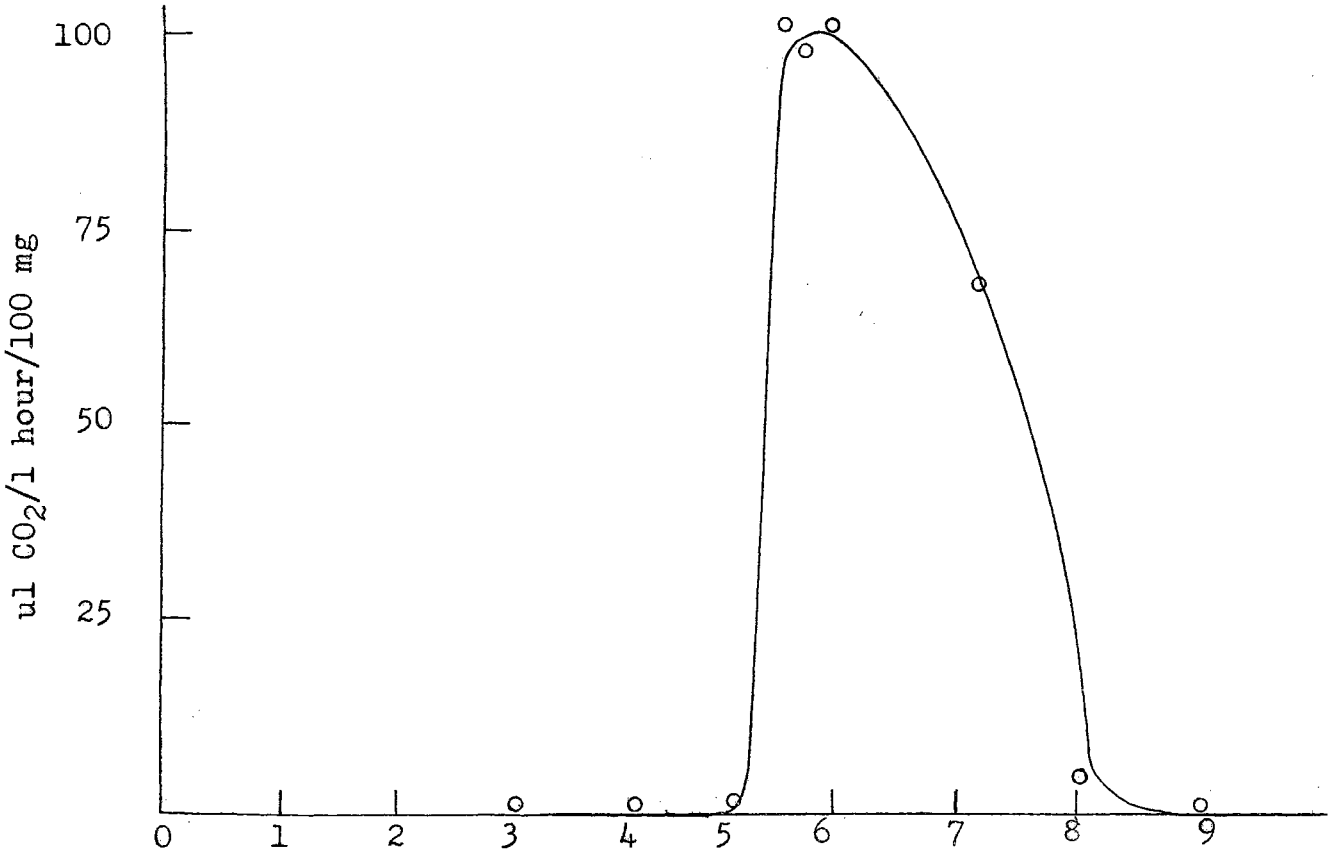


Figure 5. The effect of pH on the specific activity of ammonium sulfate precipitated glutamic decarboxylase preparations.

range of values extending from a minimum pH of 3.0 through a maximum value of 9.0. At pH 3.0 to pH 5.0 the preparations were inactive with respect to decarboxylase activity. Maximal activity was recorded at pH 5.6, 5.8, and 6.0. Reaction rates declined sharply at higher values, approaching zero at pH 9.0. This response to variation in pH is apparently identical to responses reported by Schales (52) for glutamic acid decarboxylase preparations obtained from seeds, fruits, and tubers of plants representing a wide range of plant species. Response to variation in pH frequently varies as an enzymically active preparation is purified. Only the behavior of the initial, crude preparation was investigated with respect to this parameter since the primary purpose of characterizing the enzyme in this way was to associate it with enzymes which have been extensively studied, and can be recognized, with a high degree of confidence, on the basis of their activity when conditions for reaction are specifically controlled.

Dialysis of Crude Decarboxylase Preparations

Ten gram quantities of crude decarboxylase preparation, (fraction A) were observed to dissociate into two fractions when combined with 20 ml of distilled water and dialyzed for 30 hours (fig. 1). These fractions were assayed for their individual activities and for their combined activity (Table I: 1a, 2, 3). The specific activity of preparations containing

both fraction D and fraction E was 208.2, as compared to 121.8 for the undialyzed fraction A. Unexpected results were obtained when the fractions were segregated and tested individually. It was anticipated that the activity mediated by the combined moities of the dialyzed decarboxylase preparation should be localized in either the soluble or the precipitated fraction. Repeated tests for CO_2-liberating capacity by the insoluble material disclosed, convincingly, a complete absence of decarboxylase capacity when glutamic acid was the substrate. The specific activity of the soluble E fraction was found to be approximately 30% of that when fraction E was combined with fraction D. No concentration of activity within either component of the dialyzed preparation was indicated by the results of numerous attempts to identify such a localization. Glutamic acid decarboxylation, as mediated by the combined D and E fractions, proceeded at a rate exceeding, by virtually threefold, the sum of the rates of the individual elements.

Preparations obtained by dialyzing fraction A specimens against 0.025M sucrose were characterized by a slight increase in total activity, when compared with preparations dialyzed against distilled water (Table I: 1b). When the dialyzing medium was 0.25M sucrose, the specific and total activities of the resulting preparation were both considerably lower than those for water-dialyzed materials (Table I: 1d). The greatest loss in total activity resulting from a

dialysis treatment was observed for preparations dialyzed against the M/15 phosphate buffer solution used in all assays. The data recorded in Table I reveal that the total activity of fraction A preparations dialyzed against water exceeds, by approximately two-fold, the total activity of buffer-dialyzed preparations. Assays of the butanol treated, water-dialyzed enzyme preparation disclosed a considerable reduction in both specific and total activities, relative to the untreated powder (Table I: 1e). The stability of this defatted preparation under conditions of desiccation, was tested by drying in vacuo a suspension of the treated material in phosphate buffer, pH 5.6. Repeated assays of materials subjected to this treatment revealed a total loss of decarboxylase activity when glutamic acid was the substrate (Table I: 1f). However, the semi-crystalline fraction E, obtained by lyophilization of the soluble fraction from dialyzed material, was unaffected by treatment with butanol (Table I: 3a). A small, but repeatedly confirmed, amount of activity was restored to preparations which had been rendered inactive by the latter drying treatment, when samples were immersed in the extracted butanol solution and the butanol, subsequently, evaporated. Carbon dioxide was evolved by these previously inactive preparations at a rate 16% that of the original defatted material (Table I: 1g).

Decarboxylase Activity of Embryo Tissue Homogenates

The responses of unpurified wheat germ to dialysis, butanol, and desiccation procedures were tested for comparison with the decarboxylase activities of preparations representing the previously described levels of purification. The behavior of the original source tissue was examined by direct assay of wheat germ samples which had been combined with cofactor and buffer in a hand homogenizer. When the results presented in Table II are interrelated it is apparent that CO_2 is liberated by the unpurified Homogenate I at a rate comparable to that of the fraction A preparation, precipitated with ammonium sulfate. Data yielded by homogenates, which were subsequently dialyzed, butanol extracted, and desiccated, as described previously for enzyme powders, are also represented in Table II. The usual dialysis procedures decreased the specific activity of homogenates by 49.2% (Table II: e). When the dialyzed tissue was further subjected to butanol extraction its response to conditions of assay was both unexpected and pronounced. A four-fold increase in rate of activity was achieved by this treatment (Table II: f). This increase represented a near two-fold augmentation of the rate observed for the untreated homogenate and an activity exceeding that of the most highly active preparation isolated by the procedure which Schales has recommended (Table II: a).

The behavior observed when samples of the latter, butanol extracted, tissues were tested for the stability of their decarboxylase system toward conditions of desiccation was no less striking. The results, as represented in Figure 6 illustrate the nature and the magnitude of a response not previously encountered during the course of this study. When substrate was introduced into the reaction mixture, a lag in excess of 10 minutes intervened prior to the initiation of CO_2 liberation. This delay might be regarded as a component of a longer lag period, beginning at the moment when buffer is added to the test material and continuing as procedures preparatory to the actual assay are performed. The total activity recorded over the standard period of assay was only slightly less than that observed for untreated homogenate specimens.

Butanol treatment of undialyzed homogenate samples yielded materials exhibiting the greatest specific and total decarboxylase activities assayed. The yield, in units of activity, was increased by 82% when untreated homogenate samples were subjected to the usual butanol extraction procedure (Table II: d).

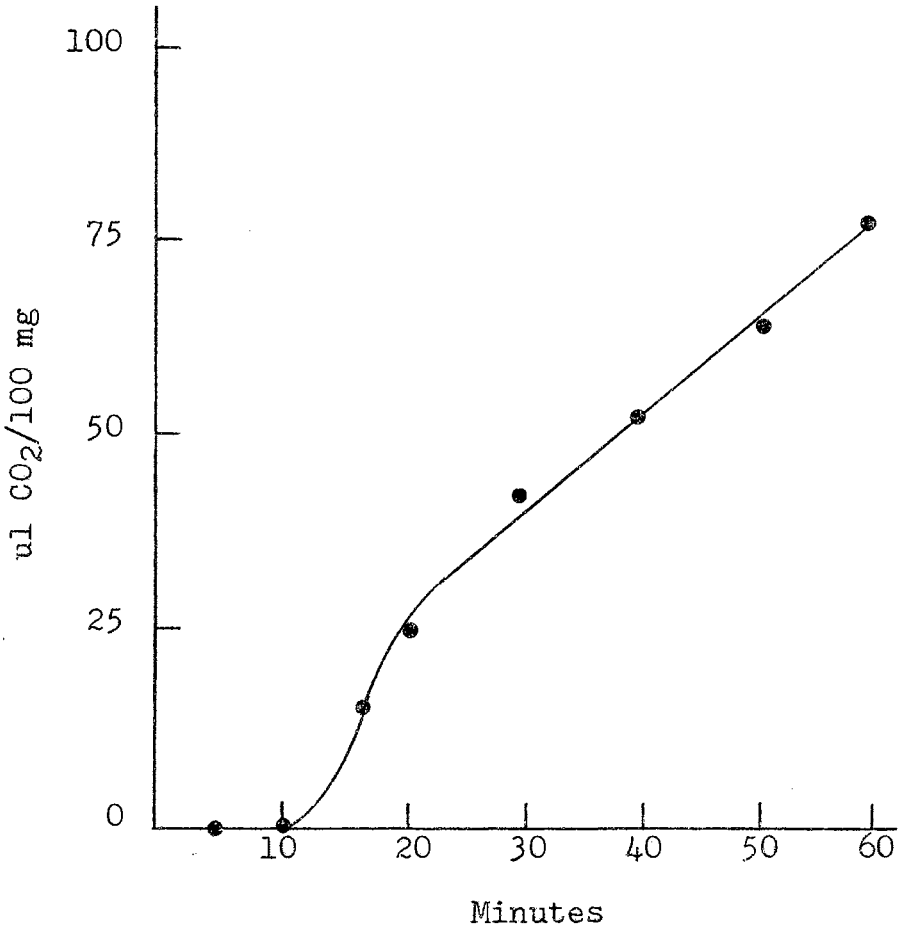


Figure 6. Decarboxylase activity of dialyzed wheat germ homogenate following extraction with butanol and desiccation.

Carbon Dioxide Production Mediated by Dry Enzyme Powders with Alanine and Alpha-Ketoglutaric Acid as Substrates

The presence of an alanine-glutamic acid transaminase system in materials studied by Cohen (4), and by Schales (53), for their activity in decarboxylating glutamic acid, allowed for the estimation of this transaminase by manometric techniques. The activity observed is ascribed to the rate of the transmination reaction proceeding between alanine and a-ketoglutaric acid, which is the rate limiting step of a coupled transaminase-decarboxylase reaction sequence. It is obvious that responses to treatments already demonstrated to decrease the decarboxylase activity of the preparations under investigation may only reflect the degree to which the decarboxylase step has become limiting. The concentration of transaminase activity achieved by the dialysis of crude fraction A preparations is illustrated in Table IV: a. The specific activity mediated by crude ammonium sulfate precipitates was increased by a factor of 3.34 at this level of purification, as compared to the approximate two-fold increase, noted previously, for decarboxylase activity.

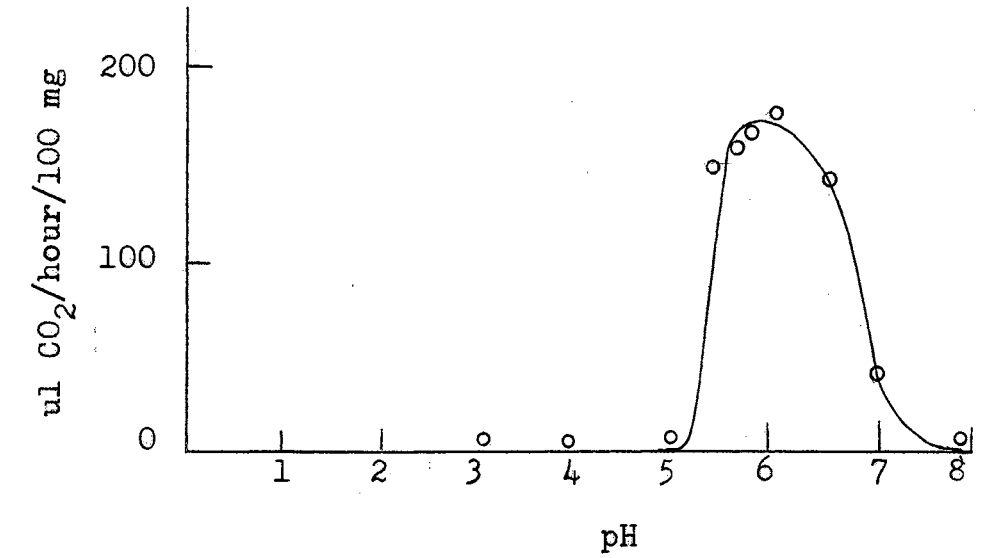
Responses to change in pH by the coupled reaction system paralleled, to a large extent, those observed with glutamic acid as substrate (fig. 7). The maximum rate observed was at pH 6.0. No activity could be measured at pH 5.0 or 8.0.

The transaminase system of fraction A displayed a greater

TABLE IV

CARBON DIOXIDE PRODUCTION BY EXTRACTED PREPARATIONS AND HOMOGENATES OF GERM WITH a-KETOGLUTARATE AND ALANINE AS SUBSTRATES

Preparation	Total Activity Units	Specific Activity	Yield %
Homogenate I (fig. I)	139,200	139.2	
a. fraction A (fig. I)	10,224	52.2	7.3
b. fraction A (dialyzed)	16,008	174.0	11.5
c. fraction A (dialyzed, butanol treated)	5,796	69.0	4.1
d. fraction A (dialyzed, butanol desiccated	0	0	0
e. fraction A (refatted)	-	14.5	-
Homogenate I			
f. Untreated	139,200	139.2	
g. Butanol treated	235,840	352.0	169.0
h. Butanol treated and desiccated	81,405	121.5	58.5
i. Dialyzed	32,340	49.0	23.7
j. Dialyzed and butanol treated	32,340	49.0	23.7



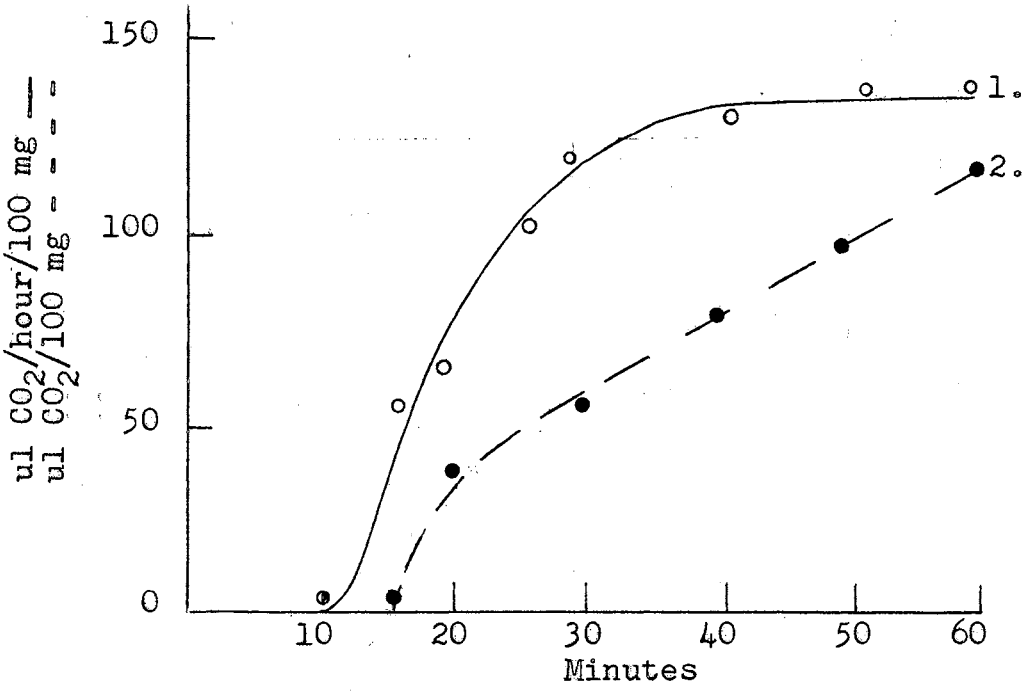


Figure 7 (top). The effect of pH on transaminase activity of crude enzyme preparations.

Figure 8 (bottom). Transaminase activity by: (1) untreated and, (2) butanol treated, desiccated wheat germ homogenates.

sensitivity toward the effects of butanol, in comparison with glutamic decarboxylase. When dialyzed preparations were extracted with butanol a considerable decrease in activity was observed (Table IV: c). This loss exceeds, by a considerable margin, the reduction recorded for decarboxylase activity in response to the same treatment. Table IV: d and e details the total loss in activity observed following the vacuum-drying of a suspension of the butanol extracted preparation in phosphate buffer, and the subsequent restoration of minor activity by the re-fatting procedure. The latter two responses are rendered difficult to interpret, however, due to corresponding responses by reaction mixtures containing glutamic acid.

Carbon Dioxide Production by Embryo Homogenates with Alanine and Alpha-Ketoglutaric Acid as Substrates

The CO_2-liberating capacity displayed by homogenized wheat germ was altered with respect to pattern, and degree of activity, when a-ketoglutarate and alanine were substituted for glutamic acid as substrates. A change, with time, in reaction rate was observed which contrasts with the constant rates of reaction mediated by the enzymically active materials described earlier (fig. 8). The initial low degree of activity increased sharply about 12 minutes following the introduction of substrate. A

progressive increase was then observed over a period of 30 minutes and a constant rate was not attained until 50 minutes had elapsed, following the addition of substrates. The sigmoid form of the function presented in Figure 8 is suggestive of responses expected by reacting substrates in the presence of a steadily increasing concentration of enzyme since substrate was not limiting (54).

Data collected from transaminase estimations of butanol-extracted wheat germ were characterized by a directional similarity to values recorded when decarboxylase activity was under investigation. The rate of CO_2 liberation by wheat germ homogenates was increased by a factor of 2.55 when the homogenized tissue was extracted with butanol (Table IV: f). When the butanol treated material was dispersed in phosphate buffer and then dried in vacuo the response was qualitatively identical to that observed when the substrate was glutamic acid (fig. 8). The assay period could again be subdivided into lag, acceleration, and stationary phases with respect to changes in rate of activity. The response pattern exhibited a resemblance to the behavior of the untreated homogenate with respect to transaminase activity.

The final examination of glutamic acid-alanine transaminase behavior was directed toward a determination of the effects of dialysis on homogenates of embryo tissue with respect to this activity. A decrease in activity representing 64.1% that observed for untreated homogenate specimens

was recorded for the dialyzed germ (Table IV: i). That this decrease was not due to a reduced decarboxylase activity was indicated by the subsequent finding that the rate of CO_2 production could not be increased by extraction with butanol (Table IV: j).

Carbon Dioxide-Releasing Capacity of Germinating Wheat Grains

Wheat grains which had been germinated for periods of time ranging from 0-78 hours were observed to differ with respect to their relative carbon dioxide-liberating capacities when glutamic acid was provided as substrate. Germinating seeds which were assayed without further treatment (2 fig. 9) displayed little change in decarboxylase activity over periods as long as 44 hours. A rather sharp increase in rate of activity occurred between 44 and 54 hours of germination. The rate attained after 54 hours was not exceeded by seed specimens which had been allowed to germinate for 78 hours.

The decarboxylase activity of seeds representing all germination periods considered was decreased following <u>in vacuo</u> desiccation treatment (3 fig. 9). The increase in activity of desiccated seeds, germinated for 54 hours, was not as pronounced as for untreated seeds. However, the general pattern of change in rate with germination time is still apparent in figure 9. Line 1 of figure 9 is a graphic representation of the per cent difference between the

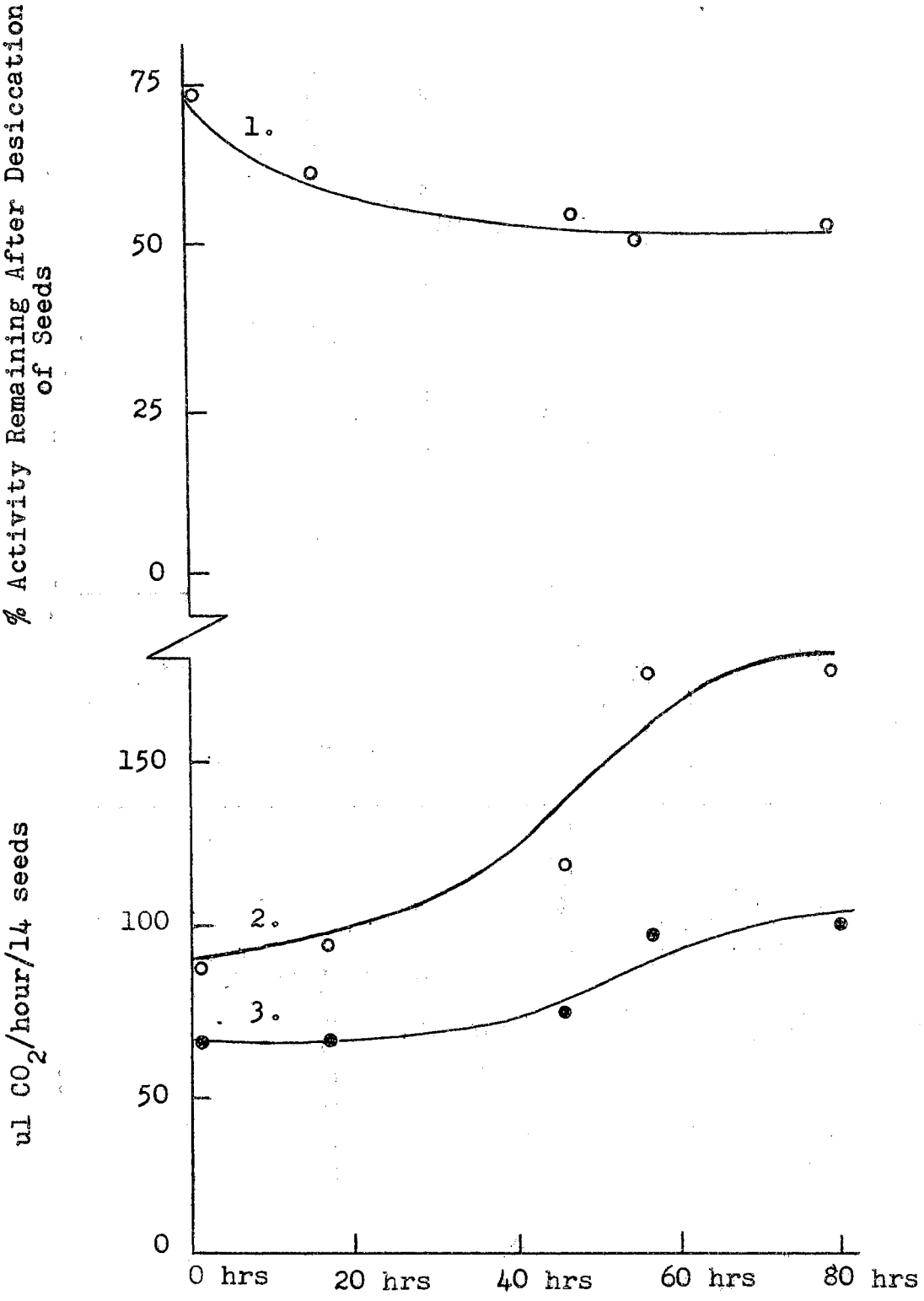


Figure 9. Decarboxylase activity of germinating wheat grain. (1) % activity remaining after desiccation. (2) Activity of seeds prior to desiccation. (3) Activity of desiccated seeds.

activity displayed by desiccated seeds and that displayed by untreated seeds representing each germination period. The variables of: germination time, rate of activity, and desiccation effects appear to be functionally associated in the data presented.

Seeds representing each germination period were also extracted with butanol. No variation in response was observed during the subsequent assays. The rates of activity were in the range of 34.4-34.8 microliters CO_2 per hour per 14-seed specimen for all but the 17 hour germination period sample which released CO_2 at the rate of 30.3 microliters per hour.

Glutamic Decarboxylase Activity of Individual and Recombined Wheat Germ Fractions

The fractions designated as Precipitate B and Extract I, obtained by defatting the inactive Precipitate I (fig. 4), were observed to be totally devoid of any capacity for decarboxylating glutamic acid under the usual conditions of assay (Table V). The time required for obtaining D and E fractions of the dialyzed enzyme preparation (fig. 4) permitted the execution of an assay which had not been planned. Ninety milligrams of an inactive fraction E preparation, which had been stored at room temperature for eight months, were intermixed with 10 mg of Precipitate B. The data compiled in Table V reveal that, when these two inactive

TABLE V

GLUTAMIC DECARBOXYLASE ACTIVITY OF REACTION MIXTURES COMPOSED OF RECOMBINED WHEAT GERM FRACTIONS

	Reaction Mixtures		Activity Units
No.	Components	Quantities	
1.	Precipitate B (fig. 4)	100 mg.	0
2.	Fraction E (stored)	100 mg.	0
3.	Fraction E (stored) Precipitate B	90 mg. 10 mg.	40.9
4.	Fraction E. (fig. 1)	100 mg.	57.3
5.	Fraction D	100 mg.	0
6.	Fraction D Fraction E	100 mg. 10 mg.	109.3
7.	Fraction D (boiled) Fraction E	100 mg. 10 mg.	26.3
8.	Extract I (fig. 4)	2 ml	0
9.	Fraction E Extract I	100 mg. 2 ml	58.3
10.	Fraction E Precipitate B	50 mg. 50 mg.	52.7
11.	Fraction E Precipitate B Extract I	50 mg. 50 mg. 2 ml	64.1
12.	Fraction D Fraction E Extract I	100 mg. 10 mg. 2 ml	106.7
13.	Fraction D Fraction E Precipitate B	100 mg. 10 mg. 50 mg.	109.3

TABLE V - Continued

Reaction Mixtures			Activity Units
No.	Components	Quantities	
14.	Fraction DE (fig. 4)	100 mg.	121.5
15.	Fraction DE	100 mg.	
	Extract II	2 ml	126.0
16.	Fraction DE	50 mg.	
	Homogenate II (fig. 4)	50 mg.	
	Extract II	2 ml	79.3
17.	Fraction DE	50 mg.	
	Homogenate III (fig. 4)	50 mg.	
	Extract II	2 ml	81.0

fractions were combined, a reaction mixture was obtained which liberated carbon dioxide at a moderate rate.

The specific activity of freshly prepared samples of fraction E was observed to be increased when relatively large quantities of the inactive fraction D were added. This stimulatory effect of fraction D was destroyed by subjecting it to a 5 minute period of boiling in distilled water. The results thus far described are illustrated quantitatively by Reaction mixtures 1-7 in Table V.

Recently prepared fraction E specimens were characterized by a specific activity which could be increased only by recombination with fraction D. Reaction mixtures 8-17, assembled by intermixing fraction E with one or more of each of the wheat germ fractions prepared, mediated the release of carbon dioxide at rates which could be accounted for by the presence of fractions D and E. Precipitate B, which had been effective in stimulating activity in stored samples of fraction E was ineffective when combined with freshly prepared samples. With the exception of the data recorded for Reaction 3, the results illustrated by Table V indicate that glutamic decarboxylase activity is confined to fractions D and E.

CHAPTER V

DISCUSSION

The responses of materials mediating the decarboxylation of glutamic acid, toward the experimental treatments imposed upon them in this study, indicate that certain decisions are warranted regarding the properties of glutamic acid decarboxylase in wheat embryo tissues. It appears highly probable that the activity displayed by preparations extracted from wheat germ may be exclusively associated with components which are immediately water-soluble. Variations in the time allowed for extraction of enzymically active protein from the source material yielded increases in neither mass nor activity of ammonium sulfate precipitates, indicating that the release of the catalytic material into water is not dependent upon a time limited cleavage of some protein precursor. This conclusion is further supported by the observation that the subsequent extractions of previously extracted homogenates yielded preparations with negligible decarboxylase capacities, and suspensions of centrifugal precipitates exhibited a generally consistent void with regard to this activity. Minute volumes of CO_2 were

released from the reaction mixture containing test quantities of mitochondrial material sedimented from 2M sucrose. However, this activity may be accounted for by a decrease in solubility of the enzyme protein in this extraction medium or by significant alteration in the feeble activity mediated by this preparation as a result of some procedural error. The importance of particulate material in enhancing the rate of glutamic acid decarboxylation is suggested by the high rate of activity of the unresolved homogenate, relative to the extremely low rate mediated by the associated supernatant and the total void in activity displayed by the corresponding insoluble material. The CO_2-liberating capacity of supernatant fractions remained constant, regardless of the gravitational force employed for separation. Particulate material capable of enhancing this catalysis was sedimented at a force of 3000 x g. Organelles requiring greater forces for precipitation, apparently, do not contribute to the activity of this system. It must also be considered that this apparent contribution could be interpreted as a consequence of the adsorption of decarboxylase protein, which is no longer active following centrifugation.

On the basis of currently accepted criteria for associating the observed gas evolution with the activity of glutamic decarboxylase, it may be reasonable to assume that the activities displayed by the preparations assayed during the course of this inquiry do, in fact, relate

specifically to glutamic decarboxylase. In addition to maintaining the conditions for assay detailed by Cohen (4), the precautionary measures recommended by Schales (54) were observed. Blank vessels in which water was substituted for glutamic acid as substrate yielded net changes in gas volume of zero following thermobarometric corrections. The response by crude preparations to changes in pH conformed to that reported for extensively studied glutamic acid decarboxylase preparations obtained from many different plant sources.

The effects of dialysis, as a procedure for concentrating the activity of ammonium sulfate precipitates, varied according to the medium employed. When the dialyzing medium was 0.025M sucrose the yield, in units of activity, was 100% relative to the total activity of the crude precipitate. The increase in specific activity resulting from this treatment may be rated as moderate. With distilled water as the dialyzing medium the per cent yield was only slightly less than with the above sucrose solution and the enhancement of specific activity slightly greater. The frequently observed protective effect of sucrose on the stability of proteins toward denaturation, in addition to the results obtained by dialysis against water, may rule out a role for sucrose as a molecular species which protects enzyme configuration via specific bonding patterns. It would appear that dialyzable factors must play, at best, a minor

role in protecting glutamic decarboxylase against the effects of vacuum drying, when the total activity of fraction A is compared with equivalent values determined for dialyzed fractions. There remains, however, the possibility that significant differences might be obtained by varying the molarity or ionic strength of the solution employed during the extraction procedure.

Dialysis against M/15 phosphate buffer yielded results which contrast with those discussed above for water and dilute sucrose solutions. Decreases in both total and specific activities were recorded. Interpretation of the effects of the latter dialysis treatment must be based on an assumption, fundamental to the present study, that the constellation of the active decarboxylase is protected by the configuration or attitude assumed by the enzyme as it reverts to the inactive form. In the presence of phosphate buffer, and under conditions of dialysis for 30 hours, it would be expected that the individual components of this constellation should be susceptible to the activities of potent proteolytic enzymes reportedly active in the embryo tissue of germinating wheat grains (26). Extensive degradation of enzyme protein in either water or sucrose solutions would tend to be evaded due to conditions of pH which are considerably above the optimum for this protease activity and which impose an inactive, protected, configuration upon the enzyme. Materials treated with 0.25M

sucrose as the dialyzing medium diverged, with regard to activity, in the same direction as that observed for phosphate buffer dialyzates. The results obtained, however, were of questionable validity. The large proportion of sucrose present in the vacuum dried preparation reduced the quantity of enzymically active material contained in the standard 100 mg test sample, considerably. In addition, the concentration in sucrose of the reaction mixture would be expected to alter protein solubilities.

The individual and combined activities exhibited by the two fractions separated by dialysis revealed the existence of another interrelationship which may be of utmost significance with regard to the direction of future inquiries. The rapid liberation of CO_2 mediated by preparations consisting of an inactive precipitate in combination with a sparingly active soluble component is difficult to interpret unless consideration is given to the possibility that the more active form of the decarboxylase system, represented by this preparation, consists of at least two, reversibly associated, components. The plausibility of such a phenomenon has been suggested by Gholson (13), as a potential explanation for the events observed, on the basis of evidence collected by Ebner. Ontogenic studies of a-amylase in E. coli have convinced Yoshida (73) that the activity of this enzyme is a consequence of interactions between a protein precursor and certain, unidentified,

peptides which are either synthesized or released during the logarithmic phase of population growth. Although the occurrence of similarly constituted enzymes in higher plants has not been demonstrated (8), the findings reported by Varner (69), regarding the ontogeny of a-amylase in barley aleurone tissue could be interpreted as support for the speculation that this enzyme is structurally related to the a-amylase of E. coli. A capacity for dissociation into precursory protein and activator units would be expected to increase the possibilities for organization of seed protein into molecular assemblages providing for the maintenance of critical enzyme configurations. This capacity should also negate, to some extent, the stresses imposed upon the secondary and tertiary protein structures during seed maturation. Attention is directed at this point to the analogous responses of unresolved homogenates and dialyzed enzyme preparations when separated into their component fractions. It is possible that traces of the particulate material contributing to the activity observed for homogenized tissue specimens may account for the effect of the insoluble fraction in dialyzed preparations.

Efforts directed toward disrupting possible associations of lipid and protein in decarboxylase preparations, by extraction with n-butanol, yielded expected results. The activity of the combined fractions of dialyzed materials was

decreased whereas the activity of separately treated samples of the soluble fraction was unaltered, indicating a greater sensitivity, to treatment with butanol, for the insoluble component. A role for lipid, as a factor contributing to the stability of the protein decarboxylase toward conditions of desiccation was indicated by the observation that the defatted preparations could no longer be subjected to in vacuo drying treatments without a total loss in activity. The amount of lipid present in stable preparations accounted for only 8.1% of their mass. It is possible that butanol imposes its effect upon the reactivity of the enzyme subunits by disrupting a strategic positioning of lipophilic groups, consequently exposing sites on molecules which then complex irreversibly when the preparation is desiccated. Localized lipoprotein associations could account for the occlusion of potentially interacting sites, as originally postulated. However, the extremely small decrease in mass resulting from extraction with butanol suggests the possibility that hydrophobic groups on the enzyme protein itself may participate in shielding sites having high affinities for complementary sites on other molecules. The butanol solution obtained via the extraction procedure was utilized for treating samples of preparations rendered inactive by extraction with butanol and subsequent desiccation. A restoration of activity to the "refatted" material should

support the alternative that a lipid factor does, in fact, participate in protecting the decarboxylase fractions against irreversible associations with other components of the preparation. The rate of activity mediated by the refatted, materials may be regarded as interesting by virtue of the results obtained through repetitions of the above treatment which were consistent with regard to both presence and magnitude of activity. However, an evaluation of the relative contribution of lipids toward decarboxylase stability is difficult to make on the basis of the activity observed.

The relative abundance of diverse molecular species in embryo tissues suggests that the effects of treatments, observed to decrease the stability of glutamic acid decarboxylase in partially purified preparations, may be more strikingly manifested in unfractionated homogenates of the source material, when the fundamental effects of these treatments is to alter the consequences of molecular interaction. The assumption that the CO_2 liberated by the homogenized tissue is a measure of glutamic decarboxylase activity is based on the previously detailed precautions observed and the investigations performed by Linko (24) who assayed both intact and dissected wheat grains for glutamic decarboxylase activity.

The evolution of gas mediated by untreated homogenates was observed to proceed at a rate comparable to that of ammonium sulfate precipitates, demonstrating that separation

of decarboxylase activity by the ammonium sulfate precipitation method employed was far from complete. The sharp decline in activity exhibited by dialyzed homogenate specimens was unexpected in view of the stability observed for extracted preparations. The succeeding enhancement response by this dialyzed material to extraction with butanol was even more surprising. The consideration that butanol may release latent decarboxylase enzymes, liberated in nature by action of lipolytic factors, was eliminated by the negative results obtained from assays of test quantities of homogenates which had been extracted, first with water and then with butanol.

The development of a postulate permitting a tentative interpretation of the behavior observed for embryo tissue homogenates may be facilitated by considering certain previously discussed findings. The specific activity of homogenate specimens, which compares favorably with that displayed by crude preparations of soluble protein, is virtually lost when the homogenate is separated into its component soluble and insoluble fractions. The most highly purified soluble protein preparation also consists of two phases, analogous in behavior to the homogenate fractions. Relatively inactive homogenates, which have been dialyzed, assume a level of activity equivalent to that manifested by the most highly active preparation extracted when treated with butanol. However, homogenized tissue (Homogenate I)

is rendered inert, with respect to decarboxylase capacity, by a short period extraction with water. No further activity may then be extracted with water, and, no further activity may be obtained by extraction with butanol. These findings suggest that this decarboxylase system involves an activating factor, incapable of mediating activity directly, which may be associated with particulate material and, therefore, probably affiliated with lipoprotein elements. The factor is apparently active in bound form, as indicated by its participation in the activity manifested by "intact" homogenates only. The acceleration of activity by butanol is accounted for by the release of this activation factor, concurrent to the disruption of lipid and protein associations, as reported for other particulate systems (57).

The relative inertness of the butanol treated homogenate following the subsequent desiccation treatment was expected. The unusual pattern of activity displayed by samples of this material, however, may be of possible significance. Results obtained by treating the dialyzed enzyme preparation with butanol reveal that the soluble component is not directly affected by this solvent, but is apparently denatured in the presence of a molecular species which <u>is</u> attacked. It is, tentativey, inferred that the lipid supports the configuration of the activating component of this enzyme system. The configuration thus imposed permits this component to enter into a readily

reversible association with its complementary member (fig. 10). In the absence of lipid an increased affinity between the two enzyme residues may shift equilibrium far in the direction of association. The decline in activity of butanol treated homogenates following desiccation would, by this interpretation, be a measure of a corresponding decrease in the capacity for dissociation by the enzyme complex. The intervention of a lag period is then accounted for by the time required for re-association, in equilibrium proportions, of factors which were released from their respective complexes during the preceding desiccation period.

On the basis of the above interpretation it is possible to relate the experimentally derived findings to the properties of the decarboxylase system as it exists in the cells of the developing seed embryo. The model presented (fig. 10) is proposed in the spirit of encouraging critical consideration of the problems involved in an analysis of cryptobiotic phenomena, and with the justification that it is always instructive to develop one (11, 56).

Figure 10 is a diagramatic representation of the concept that glutamic decarboxylase is a catalytic system composed of two or more reversibly associated components, synthesized during the maturation phase of seed development. One factor, associated with the soluble intracellular protein fraction, is activated by interaction with a particulate component of the system which is individually inert, with

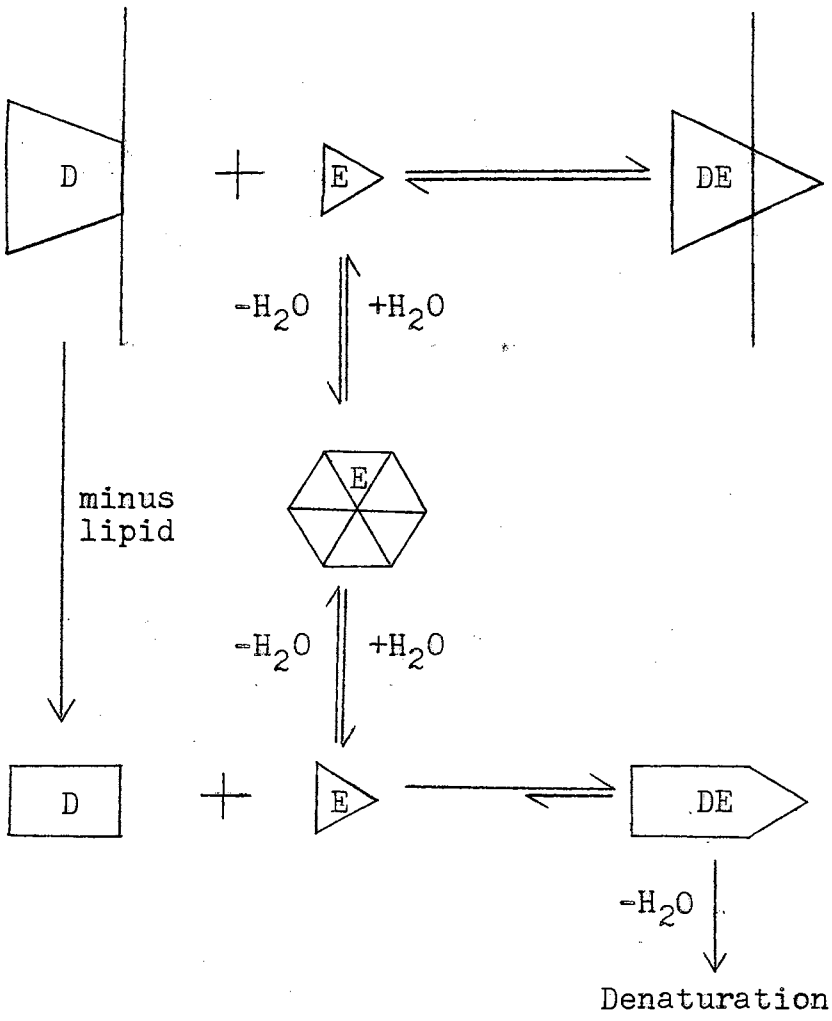


Figure 10. The postulated interaction of aggregate (D) and soluble (E) decarboxylase subunits. In the maturing seed and unresolved homogenate (upper) interaction is readily reversed by changes in intracellular environment. In the germinating seed and defatted homogenate (lower) equilibrium is shifted toward subunit association due to changes in configuration and reactivity of the lipid-free D subunit.

respect to decarboxylase activity. Changes in the cellular environment, in response to desiccation, during the maturation phase of the wheat grain, alter the relative affinities of the enzyme components, permitting them to dissociate. The soluble units participate as protein residues in the synthesis of crystalline seed protein (44). The most significant property of the system, in relation to its stability in desiccated tissues is apparently this capacity for dissociation and reorganization into associations of complementary molecules which, mutually, provide critical configurational support, in the absence of water. Lipids may play a significant role in determining the reversibility of association between the two factors, and, therefore, the relative proportion of enzyme protein which is disrupted under conditions of desiccation. With the initiation and progress of germination, lipolytic factors may release the aggregate subunit from its association with lipid. The consequence of this release would be a decrease in the capacity of the component members of the system for reversible association and, therefore, a decrease in resistance to conditions of water stress.

The presumptuous anticipation that the above mechanism might represent a new concept accounting for the resistance of enzymes toward environmental stresses proved to be too optimistic. The results of recent investigations conducted by Sadoff and coworkers (49) in an effort to understand the

mechanism of heat resistance in bacterial spores, strongly indicate that the property of heat resistance resides in the capacity of enzyme protein to undergo a reversible dimer-monomer conversion. Their data suggest, further, that the monomer is capable of disaggregation into smaller units exhibiting even greater heat resistance. The problem of maintaining enzyme stability in an intracellular environment characterized by drastic increases in factors such as ionic strength and hydrogen ion concentration may also be resolved by the findings of these workers. Maximum heat resistance was displayed by the enzyme investigated when heated in a solution which was 5M in NaCl. The greatest degree of disaggregation also took place in this solution. It would appear that an increase in ionic strength may actually be a requirement for the initiation of events leading to enzyme storage and protection. The bacterial enzymes which have been extensively studied in connection with their heat-resistant properties are glucose dehydrogenase, a-amylase, and catalase. These factors are also present in seeds. Basler (2) has observed that the glutathione reductase activity of cultured cotton cotyledons is virtually lost when its soluble and particulate fractions are separated. The possibility that this enzyme consists of a system of subunits has not been investigated.

In the model proposed for glutamic decarboxylase in the wheat embryo it was suggested that the system which is

active in the vegetative plant may be derived directly from the system operating in the embryo as a result of the release of particulate subunits. This postulate was explored by measuring the activity of germinating seeds. The increase in rate of activity displayed by seeds representing longer germination periods corresponds with results which would be expected if an activating factor were being released. It would also be expected that the greatest loss in activity, resulting from desiccation treatments, would be recorded for seeds which had been germinated for longer time periods. This prediction was also confirmed. Finally, as the release of the activating factor progresses during the course of germination, the effects of butanol treatments in increasing decarboxylase activity should be progressively less effective. This latter assumption could not be verified. The activity of seed specimens representing all germination periods was markedly decreased. Possibly a lipid-bound factor in the endosperm inactivates one or more of the decarboxylase components when released into solution.

The physiological significance of glutamic decarboxylase to the phenomenon of drought resistance in living systems may be evaluated by interrelating certain findings. The decarboxylase capacity of seeds which had been germinated for longer than 44 hours was notably decreased when the seeds were subjected to a desiccation treatment. Unpublished

results obtained by Todd (63) reveal that seeds germinated for a period of 48 hours or longer are also less resistant to conditions of drought than seeds germinated for shorter time periods. The conclusion that a causal relationship exists between the desiccation responses of the enzyme and the seedling is tempting. However, proportionality does not necessarily imply causality (35). In another investigation regarding the effects of drought stress on the in vivo activity of wheat leaf enzymes, Todd and Yoo (65) demonstrated that the activity rates of five of the six enzymes studied were decreased significantly when the leaves were desiccated prior to assay. It would seem logical to conclude that the glutamic decarboxylase present in wheat embryos should be regarded as one model of a desiccation-resistant enzyme and not as a single critical factor which regulates the entire mechanism of desiccation-resistance in the wheat grain.

The data obtained in efforts directed at locating the sites occupied by decarboxylase subunits tended to confirm the existence of such factors but did not support certain conclusions which have been offered. The insoluble fraction D of dialyzed enzyme preparations failed to stimulate the rate of activity of the soluble fraction E following a 20 minute boiling treatment of the insoluble material. It was therefore concluded that the factors responsible for the previously observed activating effect

were protein in nature. Results obtained by combining a sample of defatted particulate material with a fraction E specimen were regarded as both unexpected and promising. This latter preparation was unique in that a somewhat moderate activity was mediated by a reaction mixture assembled from individually inactive fractions. No complementary effect was achieved by recombining fraction E specimens, which had been recently prepared, with any of the various fractions prepared from butanol treated particulate material. The series of assays performed did not yield results supporting the concept of a lipid-bound particulate subunit. These findings were quite surprising in view of the response recorded earlier for butanol-treated homogenates and the results obtained when eight month old fraction E was employed as one of the recombinants. The possibility that changes had taken place in the wheat germ, which had been stored in closed containers at 4° C for longer than nine months, was considered. New fraction A specimens, prepared by the previously employed procedure, mediated the decarboxylation of glutamic acid at a rate of 243.6 microliters of carbon dioxide per hour per 100 mg of enzyme. This rate exceeds by two-fold that obtained for fraction A preparations nine months earlier and encourages the speculation that the source material had undergone changes. Changes in the lipid composition of wheat grains which had been stored for six months at 4° C have been

studied by Daftary and Pomeranz (5). Their results reveal that both polar lipids and triglycerides disappear during storage. This deterioration may account for the change in activity observed for fraction A and the negative results of efforts to separate a lipid-bound subunit from its particulate site by defatting procedures.

Considering that the estimation of glutamic acid-alanine transaminase was achieved by coupling the reaction with the system catalyzing the decarboxylation of glutamic acid, the results obtained must be analyzed with caution, since the magnitude of the responses measured can be limited by treatments reducing the catalytic capacity of glutamic decarboxylase as well as those altering transaminase activity. A guarded assumption for the purposes of the immediate study is that responses, deviating from those observed when glutamic acid was the substrate, to treatments which have been demonstrated to spare the decarboxylase are probably significant. On the basis of these criteria the data collected from assays of extracted preparations are of questionable significance, differing from those of decarboxylase assays only in degree of activity. The behavior of unextracted homogenates, however, is indicative of potentially pertinent differences between the two enzyme systems. The steady increase in rate of activity displayed by the homogenate transaminase system could be a consequence of a corresponding accumu-

lation of glutamic acid, or a manifestation of a gradual liberation of enzyme protein or activators. The participation of activating factors is indicated once more by the increase in activity, stimulated by butanol treatment and, reversed by *in vacuo* desiccation. The results obtained are generally indicative of similarities between the transaminase and decarboxylase systems, with regard to the properties investigated during this study. Nevertheless, the response of dialyzed homogenate to extraction with butanol can not be accommodated by inferences based upon the results of assays for decarboxylase activity. Whatever is responsible for the transaminase response to butanol treatment it is removed by dialysis and therefore differs from the corresponding decarboxylase factor with regard to one or more properties.

CHAPTER VI

SUMMARY

The effect of various treatments on the stability of glutamic decarboxylase and glutamic-alanine transminase from wheat germ was investigated in an effort to identify those properties which contribute to the desiccation resistance of these enzymes. Subcellular fractions were obtained by differential centrifugation of wheat germ homogenates and assayed for activity. Enzymically active fractions prepared from the soluble protein fraction of the source material were dialyzed against distilled water, sucrose solutions, and phosphate buffer. The preparation obtained by dialysis against water was extracted with n-butanol. Homogenized wheat germ was subjected to identical dialysis and butanol treatments and the pellet material obtained by centrifugation at 3000 x g was also defatted. Each of the fractions prepared was assayed for its individual carbon dioxide-releasing capacity by manometric techniques and then recombined with other fractions and, again, assayed.

The data collected have been interpreted as indicating that particulate material is incapable of mediating the decarboxylation of glutamic acid but that pellet material, sedimented by a gravitational force of 3000 x g, does enhance the decarboxylase activity of supernatants obtained by centrifuging at forces as high as 140,000 x g. The specific and total activities catalyzed by unresolved homogenates were greatly increased by butanol treatments which had no effect on the isolated, inert, pellet material. Two fractions were obtained when preparations, precipitated from the supernatant with ammonium sulfate, were dialyzed. The precipitated fraction was inactive with respect to decarboxylase activity and the soluble fraction was only weakly active. When the two fractions were combined the resulting preparation exhibited the highest specific activity of all the protein preparations assayed. The stimulatory effect of the inactive fraction was destroyed by boiling. Butanol totally eliminated the desiccation-resistance of the dialyzed preparation when applied to the combined fractions but had no effect on that of the weakly active member. Defatted particulate material, separated from wheat germ homogenate by centrifuging at 3000 x g, initiated decarboxylase activity in a previously inactive specimen of the soluble dialysis fraction which had been stored for longer than eight months. The defatted material did not alter the activity of analogous

fractions prepared from stored wheat germ nor did it enhance the activity of any other fraction. The glutamic decarboxylase activity of germinating wheat grains increased sharply after a period of 44 hours. Desiccation-resistance decreased up to this period. Butanol extraction of whole seeds reduced their capacity for decarboxylase activity regardless of the length of the germination period.

It is concluded that the enzymic decarboxylation of glutamic acid in wheat embryo tissue is effected by a catalytic system composed of two or more reversibly associated protein subunits. The property of desiccation-resistance in this enzyme resides in the capacity of these subunits for aggregation and disaggregation. The reversibility of this aggregation is dependent upon the presence of lipid which, in this way, lends support to the protection of the system against conditions of drying. A particulate subcellular fraction participates in the mediation of activity by enhancing its rate. The nature of the factors responsible for the effect of this particulate material is unknown, but they are apparently released or activated by treatments which disrupt associations of lipid and protein. The decrease in resistance to conditions of desiccation observed for glutamic decarboxylase in germinating seeds is probably an indication of a similar loss of resistance by seed enzymes in general.

Conclusions as to the nature of the transaminase system investigated do not seem justified at this time. The data collected do tend to indicate an organizational similarity to glutamic decarboxylase.

BIBLIOGRAPHY

(1) Basler, E. "Interaction of 2, 4-Dichlorophenoxyacetic Acid, Ethylene Chlorohydrin and Glutathione in the Stability of Microsomal Ribonucleic Acid of Excised Cotton Cotyledons." Okla. Acad. Sci. Vol. 43. (1963) 35-40.

(2) _____. Unpublished glutathione studies. Dept. of Botany and Plant Pathology, Oklahoma State University, 1966.

(3) Ben-Zeev, N., and S. Zamenhof. "Effects of High Temperatures on Dry Seeds." Plant Physiol. Vol. 37. (1962) 696-699.

(4) Cohen, P. P. "Estimation of Animal Transaminses." Methods in Enzymology. Vol. II. Ed. S. Colowick and N. O. Kaplan. New York: Academic Press, 1955. pp. 178-194.

(5) Daftary, R. D., and Y. Pomeranz. "Changes in Lipid Composition in Wheat During Storage Deterioration." J. Agr. Food Chem. Vol. 13. (1965) 442-446.

(6) Dixon, M., and E. C. Webb. Enzymes, 2nd ed. Academic Press, New York, N. Y. (1964).

(7) Duerre, J. A., and E. Ribi. "Enzymes Released from Escherichia coli with the Aid of Servall Cell Fractionator." Appl. Microbiol. Vol. II (1963) 467-471.

(8) Ebner, K. E. Personal Communication. Department of Biochemistry, Oklahoma State University.

(9) Eisenberg, M. A., and G. W. Schwert. "The Reversible Heat Denaturation of Chymotrypsinogen." J. Gen. Physiol. Vol. 34 (1951) 583.

(10) Engel, C., and J. Heins. "The Distribution of Enzymes in Resting Cereals." Biochem. Biophys. Acta. Vol. 1. (1947) 190-196.

(11) Evenari, M. "A Survey of the Work in Seed Physiology by the Department of Botany, Hebrew University, Jerusalem (Israel)." Proc. Int. Seed Testing Assoc. Vol. 26. (1961) 597-658.

(12) Fruton, J. S., and S. Simmonds. General Biochemistry, 2nd ed. John Wiley & Sons Inc., 1958.

(13) Gholson, R. K. Personal communication. Department of Biochemistry, Oklahoma State University.

(14) Gunsalus, I. C. Experimental Biochemistry, 2nd reprint. Stipes Publishing Company, Champaign, Ill. (1959).

(15) Harris, J. I. "Effect of Urea on Trypsin and Alpha-Chymotrypsin." Nature. Vol. 177. (1956) 471.

(16) Haurovitz, F. Chemistry and Biology of Proteins. Academic Press, New York, N. Y. (1950).

(17) Heber, U. W. and K. A. Santarius. "Loss of Adenosine Triphosphate Synthesis Caused by Freezing and Its Relationship to Frost Hardiness Problems." Plant Physiol. Vol. 39. (1964) 712-719.

(18) Hird, F. J. R., and E. V. Rowsell. "Additional Transaminations by Insoluble Particle Preparations of Rat Liver." Nature. Vol. 166. (1950) 517-518.

(19) Holme, J. "Characterization Studies on the Soluble Proteins and Pentosans of Cake Flour." Cereal Chem. Vol. 39. (1962) 132-144.

(20) Koller, D., A. M. Mayer, A. Poljakoff-Mayber, and S. Klein. "Seed Germination." Ann. Rev. Plant Physiol. Vol. 13. (1962) 437-461.

(21) Johnston, F. B., M. Nasatir, and H. Stern. "The Isolation of Nuclei and Oxidative Particles from Viable Wheat Embryos." Plant Physiol. Vol. 32. (1957) 124-128.

(22) Leopold, A. C. Plant Growth and Development. McGraw-Hill, New York, N. Y. (1964).

(23) Linderstrom-Lang, K. U., and J. A. Schellman. "Protein Structure and Enzyme Activity." The Enzymes. Ed. P. D. Boyer, H. Lardy and K. Myrback. New York: Academic Press, 1959, pp. 443-510.

(24) Linko, P., and M. Milner. "Gas Exchanges Induced in Wheat Embryos by Wetting." Cereal Chem. Vol. 36. (1959) 274-279.

(25) _____, and _____. "Enzyme Activation in Wheat Grains in Relation to Water Content. Glutamic Acid-Alanine Transaminase, and Glutamic Decarboxylase." Plant Physiol. Vol. 34. (1959) 394-396.

(26) _____, and L. Sogn. "Relation of Viability and Storage Deterioration to Glutamic Acid Decarboxylase in Wheat." Cereal Chem. Vol. 37. (1960) 489-499.

(27) Mayer, A. M., and A. Poljakoff-Mayber. The Germination of Seeds. The MacMillan Company, New York, N. Y. (1963).

(28) Miyamoto, T., N. E. Tolbert, and E. H. Everson. "Germination Inhibitors Related to Dormancy in Wheat" Plant Physiol. Vol. 36. (1961) 739-746.

(29) Mills, G. L. "Some Observations on the Electrophoresis of Gliadin." Biochem. Biophys. Acta. Vol. 4. (1954) 274-281.

(30) Morino, Y., and W. Hiroshi. "The Differences Between the Glutamic-Oxalacetic Apotransaminases of Mitochondria and the Supernatant Fraction of Beef Liver." International Symposium on Biological and Chemical Aspects of Pyriodixal Catalysis. Ed. E. E. Snell. New York: The MacMillan Company, 1963, pp. 175-184.

(31) Morton, R. K. "Separation and Purification of Enzymes Associated with Insoluble Particles." Nature. Vol. 166. (1950) 1092-1095.

(32) _____. "Methods of Extraction of Enzymes from Animal Tissues." Methods in Enzymology. Vol. I. Ed. S. P. Colowick and N. O. Kaplan. New York: Academic Press, 1955, pp. 25-50.

(33) Naylor, J., and G. M. Simpson. "Dormancy Studies in Seeds of Avena fatua." Can. J. Botany. Vol. 39. (1961) 281-295.

(34) Neurath, H., J. A. Gladner, and E. W. Davis in: The Mechanism of Enzyme Action. Ed. McElroy and Glass. Baltimore: Johns Hopkins Press, 1954, p. 61.

(35) Ostle, B. Statistics in Research. The Iowa State University Press, Ames, Ia. (1963).

(36) Paleg, L. G. "Physiological Effects of Gibberellic Acid. I. On Carbohydrate Metabolism and Alpha-Amylase Activity of Barley Endosperm." Plant Physiol. Vol. 35. (1960) 293-299.

(37) _____. "Physiological Effects of Gibberellic Acid. III. Observations on Its Mode of Action on Barley Endosperm." Plant Physiol. Vol. 36. (1961) 829-837.

(38) _____. "Physiological Effects of Gibberellins." Ann. Rev. Plant Physiol. Vol. 16. (1965) 291-322.

(39) _____, and B. Hyde. "Physiological Effects of Gibberellic Acid. VII. Electron Microscopy of Barley Aleurone Cells." Plant Physiol. Vol. 39. (1964) 673-680.

(40) _____, D. Sparrow and A. Jennings. "Physiological Effects of Gibberellic Acid. IV. on Barley Grain with Normal, X-Irradiated, Excised Embryos. Plant Physiol. Vol. 37. (1962) 587-593.

(41) Peers, F. G. "The Phytase of Wheat." Biochem. J. Vol. 53. (1953) 102-110.

(42) Pence, J. W., D. K. Mechan, and H. S. Olcott. "Review of Proteins in Wheat Flour." J. Agr. Food Chem. Vol. 4. (1956) 712-716.

(43) Perlmann, G. E. "Enzymic Dephosphorylation of Pepsin and Pepsinogen." J. Gen. Physiol. Vol. 41. (1958) 441.

(44) Perner, E. "Patience with Peas." Time, Vol. 87, No. 2. (January 14, 1966) 54.

(45) Pett, L. B. "Studies on the Distribution of Enzymes in Dormant and Germinating Wheat Seeds." Biochem. J. Vol. 29. (1935) 1898-1902.

(46) Pollack, J. R. A., and A. A. Pool in: Wheat Chemistry and Technology. Ed. I. Hlynka. St. Paul: American Assoc. of Cereal Chemists, Inc., 1964.

(47) Reed, G., and J. A. Thorn. "Enzymes." Wheat Chemistry and Technology. Ed. I. Hlynka. St. Paul: American Assoc. of Cereal Chemists, Inc., 1964, pp. 397-434.

(48) Sadoff, H. L. "Some Properties of the Spore Catalase and Some Heat Resistant Enzymes in Bacterial Glucose Dehydrogenase." Spores II. Ed. H. O. Halvorson. Washington D. C.: Am. Inst. Biol. Sci., 1961, pp. 181-193.

(49) _____, J. A. Bach, and J. W. Kools. "Significance of Multiple Forms of Glucose Dehydrogenase in Relation to Its Heat Resistance." Spores III. Ed. L. L. Campbell and H. O. Halvorson. Ann Arbor, Michigan: American Society for Microbiology, 1965, pp. 97-109.

(50) Sanwall, B. D., M. W. Zink, and G. Din. "Transaminases and Racemases." Modern Methods of Plant Analysis. Vol. VII. Ed. H. F. Linskens, B. D. Sanwall, and M. V. Tracey. Berlin: Springer-Verlag, 1964, pp. 361-379.

(51) Schales, O., and S. S. Schales. "Glutamic Acid Decarboxylase of Higher Plants. III. Enzymic Determination of l (+) - Glutamic Acid." Arch. Biochem. Vol. 10. (1946) 455.

(52) _____. "Coupled Transaminations and Decarboxylations in Extracts of Higher Plants." Federation Proc. Vol. 12. (1953) 264.

(53) _____. "Amino Acid Decarboxylases of Plants." Methods in Enzymology. Vol. II. Ed. S. P. Colowick and N. O. Kaplan. New York. Academic Press, 1955, 190-199.

(54) _____. "Decarboxylations and Transaminations in Extracts of Higher Plants." Arch. Biochem. and Biophys. Vol. 69. (1957) 378-389.

(55) Scheraga, H. A. Protein Structure. Academic Press, New York, N. Y. (1961).

(56) Simpson, G. G., C. S. Pittendrigh, and L. H. Tiffany. Life: An Introduction to Biology. Harcourt, Brace and Company, New York, N. Y. (1957).

(57) Singer, T. P. "Newer Knowledge of Succinic Dehydrogenase." Advances in Enzymology. Vol. 18. (1957) 65-111.

(58) Sizer, I. W., and T. W. Jenkins. "The Reactivity of the Pyridoxal Phosphate Group of Glutamic Aspartic Aminotransferase." International Symposium on Biological and Chemical Aspects of Pyridoxal Catalysis. Ed. E. E. Snell. New York: The MacMillan Company, 1963.

(59) Stocker, O. "Physiological and Morpholigical Changes in Plants Due to Water Deficiency." Plant-Water Relationships in Arid and Semi-Arid Conditions XV. UNESCO, 1960, pp. 63-103.

(60) Strobel, R. G., and J. Holmes. "Electrophoretic Separation of the Water-Soluble Constituents of Bleached Cake Flour". Cereal Chem. Vol. 40. (1963) 351-360.

(61) Suzuki, Y., and N. Takakuwa. "Decarboxylation of l-Glutamic Acid in Scopolia japonica." Naturwiss. Vol. 44. (1957) 353-354.

(62) Thimann, K. V., and F. Skoog. "The Extraction of Auxin from Plant Tissues." Am. J. Botany. Vol. 27. (1940) 951-960.

(63) Todd, G. W. Unpublished studies on plant-water relationships. Department of Botany and Plant Pathology, Oklahoma State University.

(64) _____, and E. Basler. "Fate of Various Protoplasmic Constitutents in Droughted Wheat Plants." Phyton (Argentina). Vol. 22. (1). (1965) 79-85.

(65) _____, and B. Y. Yoo. "Enzymatic Changes in Detached Wheat Leaves as Affected by Water Stress." Phyton (Argentina). Vol. 21 (1). (1964) 61-68.

(66) Toole, E. H., S. B. Hendricks, H. A. Borthwick, and V. K. Toole. "Physiology of Seed Germination." Ann. Rev. Plant Physiol. Vol. 7. (1956) 299-324.

(67) Umbreit, W. W., R. H. Burris, and J. F. Stauffer. Manometric Techniques, 3rd ed. Burgess Publishing Company, Minneapolis, Minn. (1959).

(68) Utter, M. F. "Oxidative Carboxylations and Decarboxylations." The Enzymes, 2nd ed. Vol. 5. Ed. P. D. Boyer, H. Lardy, and K. Myrback. New York: Academic Press, 1961, pp. 319-366.

(69) Varner, J. E. "Gibberellic Acid Controlled Synthesis of Alpha-Amylase in Barley Endosperm." Plant Physiol. Vol. 39. (1964) 413-415.

(70) Westheimer, F. H. "Enzyme Models." The Enzymes. Vol. 1. Ed. P. D. Boyer, H. Lardy, and K. Myrback. New York: Academic Press, 1959, pp. 260-277.

(71) White, A., P. Handler, and E. L. Smith. Principles of Biochemistry. McGraw-Hill Book Company, Inc., New York, N. Y. (1959).

(72) Wilson, D. A., and I. P. Crawford. "Purification and Properties of the B-Component of Escherichia coli Tryptophan Synthetase." J. Biol. Chem. Vol. 240, No. 12. (1965) 4801-4808.

(73) Yoshida, A. "Biological and Chemical Properties of Alpha-Amylase Precursor." Biochim. Biophys. Acta. Vol. 44. (1960) 388-390.

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