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DONALD S. H. DRENNAN.

Summary.

Studies on the Physiology of germination in the genus Avena.

The germination requirements of grains of Avena sativa, Avena fatua and Avena ludoviciana have been investigated, and seed dormancy shown to develop with the attainment of ripeness in these species. The degree of dormancy was found to be transitory in A. sativa, and more intense and more persistent in the other two species.

Promotion of the subsequent germination was observed when grains of A. sativa were allowed to imbibe for several hours, subjected to a dehydration treatment and a period of dry storage before being returned to germination conditions. This was shown to be largely due to the retention of some of the physical and physiological changes which normally occur within the early stages of germination. Embryo damage resulted when the same treatment was given to grains with growing embryos, the severity of the damage being related to the degree of morphological development of the embryo at the time of treatment. It was concluded that there is no initial period of imbibition which can be completely reversed by this treatment, and that there is no distinct lag between the commencement of imbibition and of the changes leading to the onset of growth.

The development of amylolytic enzymes in germinating grains of A. sativa has also been studied. Only b-Amylase was present in the dry grain but concurrently with, or shortly after the commencement of embryo growth a-Amylase began to be developed. General similarities were observed in the pattern of Amylase development in germinating grains of all three species investigated, but dormant grains of A. fatua and A. ludoviciana

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showed no increased amylase development. The development of a-Amylase would appear to take place in the endosperm, but the growing embryo appears to play an essential role in its development.

Analyses of grains of A. sativa after 24 hours in germination conditions showed that a considerable utilisation of seed fats, and of some soluble carbohydrates has occurred. Starch hydrolysis was not evident until 24-48 hours in germination conditions.

Proteolytic activity of dry grains of A. sativa was found to be mainly located in the embryo tissues, and to undergo a several-fold increase during the pre-germination period of imbibition. This development took place mainly in the embryo tissues, with diffusion to the endosperm occurring later. Germinating grains of A. fatua and A. ludoviciana showed a similar pattern of increasing proteolytic activity, but dormant grains showed no capacity for increased activity. Since this increased activity is a pre-germination change, and is potentially capable of restricting embryo growth it must be considered as a possible mechanism by which seed dormancy is enforced.

The presence of germination inhibiting materials in whole grains, and husks of A. sativa has been confirmed, and their activity shown against seed of A. sativa, Hordeum vulgare, Triticum aestivum, Linum usitatissimum, Brassica oleracea and Trifolium pratense. Previous investigators claimed that the action of these materials was due to their inhibition of the amylase enzymes of the grains, particularly a-Amylase. Tests with amylase preparations from germinated grains of A. sativa, and Barley, and fungal and bacterial a-Amylases failed to confirm this finding. The possibility of

retarding germination by inhibiting amylases would appear to be doubtful in the light of our findings on amylase development and starch utilisation. Considerable inhibition of the activity of proteolytic enzymes from grains of A. sativa and Barley was however observed in the extract of A. sativa husks. The same water extracts of whole grains of A. sativa and A. fatua were found to be capable of inhibiting the germination of A. sativa grains and promoting the germination of A. fatua grains. The presence, and the amounts of these germination inhibiting and promoting materials in A. fatua could not be correlated with the degree of dormancy in the grains used for extraction.

STUDIES IN THE PHYSIOLOGY OF GERMINATION IN THE GENUS AVENA.

Thesis presented by

DONALD STEWART HALL DRENNAN, B.Sc.

for the degree of

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in the

University of Glasgow

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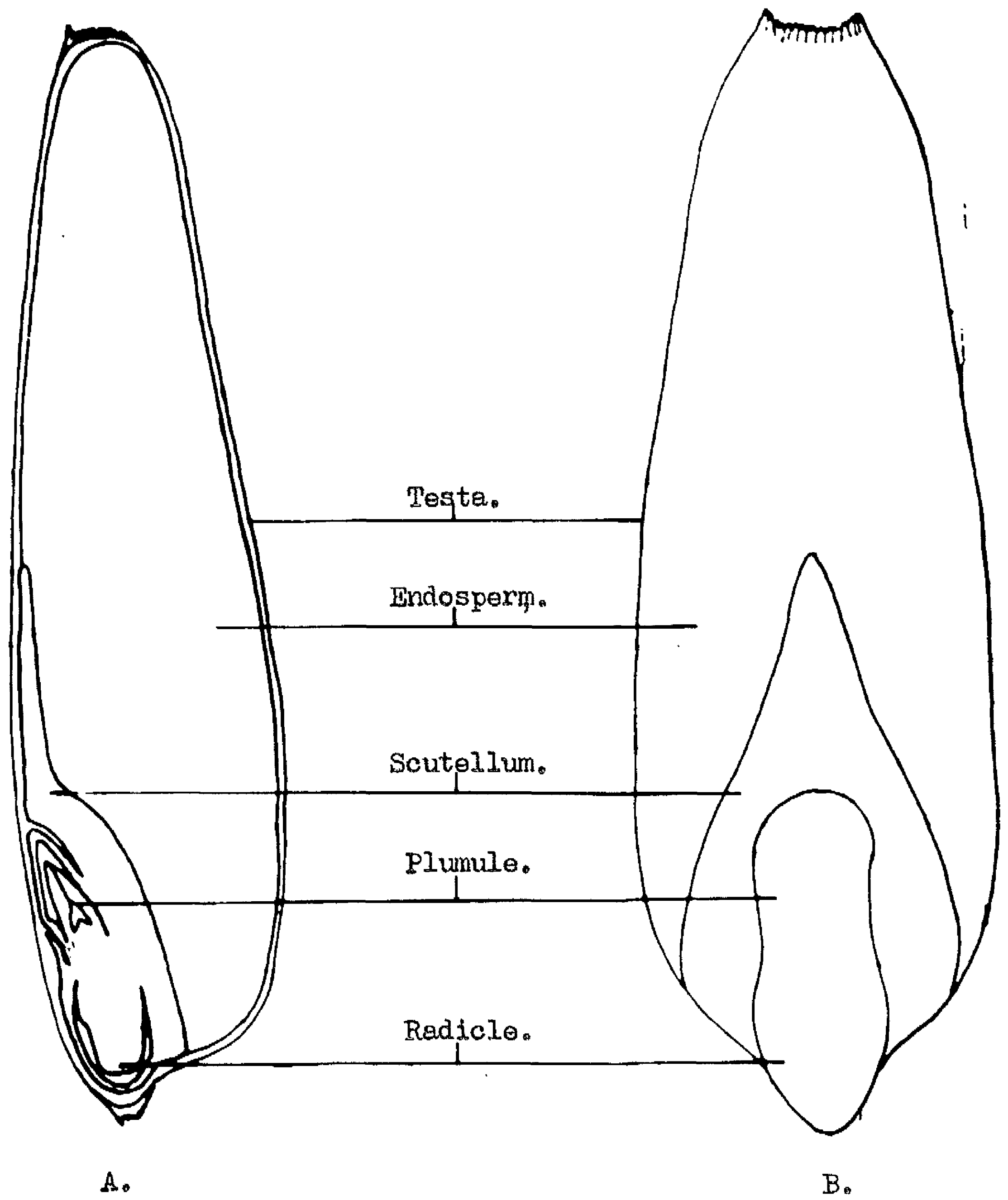


Plate 1. Diagram of the structure of the caryopsis of A. sativa.

A. Longitudinal section.

B. Dorsal view.

STUDIES IN THE PHYSIOLOGY OF GERMINATION IN THE GENUS AVENA.

Introduction, and review of the literature.

The seed is one of the ways by which the higher plant passes through a period of environmental conditions unsuitable for plant growth, and a convenient form by which the species may be disseminated. The structure of the seed and its physiological properties are related to this function. The "seed" of Avena sativa L., the Oat, is representative of most of the Gramineae, and is botanically a fruit - a caryopsis. It is seen to be enclosed in protective layers developed from the fused pericarp and testa (Plate 1.). Within this lies the embryonic axis, with a radicle and several secondary seminal root primordia, and the plumule bud with several primordial leaves enveloping it. This embryonic axis is attached in the median position to a structure called the scutellum, which is usually regarded as the single cotyledon. In many seeds, the cotyledons are the main storage region of the seed reserves of metabolic materials, but in the Gramineae, and some other plants, the endosperm persists and enlarges to fulfil this function. In the resumption of growth in the embryo of A. sativa., following germination, the primordial root and shoot initials commence growth, singly or in pairs, in a regular sequence, over a period of several days. Adventitious roots also form from the first node of the plumule at an early stage of development, and at higher nodes subsequently. The caryopsis of A. sativa is further protected by the persistent pales - the husk, this whole disseminule being the "seed" in commercial usage.

Despite the many adaptions by which the mature plant endeavours to withstand periods of water shortage, those cells which are growing and functioning normally can only do so when they have a high water content. In apical meristems the water content is about 80 - 90% of the fresh weight, while a wide range of plant parts fall between this level and 50% of the fresh weight (Stocking, 1956). A slight reduction in these water contents may seriously upset growth and the physiological activity (Gates and Bonner, 1959), while permanent impairment of the growing parts, and death of the whole plant can result from greater water losses. In a wide range of plants, a reduction of their saturated water contents by 60% was found to be the lethal water deficit level, though in some cases reductions of only 48% were enough (Stocker, 1956). In contrast to this, a wide range of seed can remain viable for periods of many years with water contents as low as 5% of the dry weight (Sifton, 1920; Crocker and Barton, 1957).

In its formation, however, the seed commences as a structure with a high moisture content, about 80% of its fresh weight. This level is maintained during the period of tissue elaboration, and the deposition of the reserve food materials, but as maturity approaches the water content begins to fall, and accompanying this there is a slowing down, and finally a cessation of growth in the seed parts. The entry of translocated food materials stops quite early in this phase, when the water content is about 40 - 45% of the fresh weight (Harlan & Pope, 1923). As the water content falls to the level of the air dry seed, a similar pattern of declining activity, reaching an almost imperceptible level at maturity, is evident in many of the physiological attributes of the seed. Respiratory activity

(Wager, 1957), amylase activity (Nordh and Ohlsson, 1932; Ohlsson and Edtfeldt, 1933; Ohlsson and Uddenbergh, 1933; Chrzascz and Janicki, 1936; Bernstein, 1943 A; Sandstedt and Beckford, 1946; and Ugrumov, 1955), and proteolytic activity all show this trend (Proskuryakov, 1941; Daniellson, 1951). During the period of formative activity and maturation there is also a constant trend towards the formation of the more complex, less mobile, and less physiologically active compounds in each of the main classes of seed constituents (Crocker and Barton, 1957). The mature seed may therefore be characterised by a low water content, suspended embryo development, low physiological activity, and a preponderance of inactive storage compounds over more labile forms. Such a seed is said to be in the <u>resting</u> condition.

Providing the seed is still viable, the availability of sufficient water, at a suitable temperature, and free access to air, is often enough to stimulate the resumption of growth in the embryo of many resting seed. Such a seed is called <u>non-dormant</u>. The water may be present as liquid or vapour, the dry seed being capable of adsorbing both against considerable external forces (Shull, 1916; Owen, 1956). The initial uptake of water by a resting seed is a physical process, dependent on the colloidal properties of much of the seed constituents. In the presence of sufficient water, there is usually a period of several hours rapid imbibition, followed by a gradually slower uptake as the saturation of the colloids is approached (Shull, 1920). A second phase of increased water uptake follows this, usually within a short period, which is more or less continuously maintained throughout the further

growth of the plant. This phase is physiologically regulated by changes in the seed constituents and the development of osmotically functioning tissues during the period preceding and accompanying the resumption of embryo growth (Owen, 1956; Stanley, 1958).

As the water content of the imbibing non-dormant seed increases, an increase in physiological activity also occurs. An increase in the rate of respiration of single seeds has been shown within the first few hours of imbibition, the increase continuing, proportionally to the water content for many hours until the resumption of growth was well under way (Stiles and Leach, 1932, 1933; Leach, 1943). A similar pattern of increasing respiratory activity with increasing water content has also been shown in batch studies with seed of many species (Bailey, 1940; James and James, 1940; Ragai and Loomis, 1954; Milner and Linko, 1958; Stanley, 1958). Studies on seed enzymes during the period of imbibition have also frequently shown evidence of increased activity, with further increases as growth commences. Enzymes investigated in this respect include Invertase (Hora, 1936; Nakamura, 1940), The Amylases, (Nordh and Ohlsson, 1932; Ohlsson and Edfeldt, 1933; Ohlsson and Uddenberg, 1933; Hora, 1936; Luers, 1936; Nakamura, 1940; Popov, 1940; Bernstein, 1943B; Kneen, 1944; Oparin and Kaden, 1945; and Ugrumov, 1955), Proteases and peptidases (Mounfield 1930; Pett, 1936; Luers, 1940; Nakamura, 1940;

Proskuryakoff, 1941; and Daniellson, 1951), Transaminases (Albaum and Cohen, 1943; Linker and Milno, 1958), Lipases (Pett, 1935; Griffiths, 1937; Hutchison, Martin, and Moran, 1951), Phytase (De Turk, Holbert, and Howk, 1938; Albaum and Umbreit, 1943; Peers, 1953), and many respiratory enzymes (Fink, Haen, and Zenger, 1936; Deleano, Popovici, and Jonesco, 1937; Albaum and Eichel, 1943; Das and Sengupta, 1941; Sen, Hsieh, and Chen, 1945). Chemical analyses have also indicated that changes in the composition of the fats, proteins, and carbohydrates of the embryo are evident shortly after the beginning of imbibition (Griffiths, 1937; Albaum and Cohen, 1943; Albaum and Eichel, 1943; Poljakoff-Mayber, and Mayer, 1955; and MacLeod, 1957), while mobilisation and translocation of reserve food material becomes evident some little while later (Hora, 1936; James, 1940; Bernstein, 1943B; Folkes and Yemm, 1958; Malhotra, 1932). In general, it would appear that the changes occurring during imbibition, germination, and early development are the reverse of those already described as occurring during the final stages of formation and maturity (Page 2). Comprehensive reviews on the physiology of germination may be found in Toole et al (1956), Crocker and Barton (1957), and Evenari (1957).

As a consequence of the higher water content, and the physiological activity initiated by this, the final stage in the transformation from the resting seed condition takes place by the onset of growth in the embryo. This shows a similar pattern in most seed. The first signs of growth are observed in the radicle, and the first phase of growth is by the extension of existing embryo cells. Meristematic activity begins some hours after the commencement of extension growth, and is again first

evident in the radicle. The changes in the structure of the embryo, resulting from meristematic activity, and culminating in the development of a seedling capable of independent existence, must be regarded as the recommencement of the normal plant growth cycle, even though it is initially dependent on seed stored material for its maintenance. Germination is accordingly restricted by us to include only those changes involved in the re-establishment of meristematic activity in a resting embryo. Those changes which occur subsequent to this, and more associated with the maintenance of this growth, are defined by the term Development. This restricted meaning of the term is used by Toole et al (1956), and Evenari (1957).

Germination, and development, are dependent on a continuously available supply of water. The effects of a period of severe water shortage on the subsequent growth of mature plants have been described previously (Page 2), but there is little information available concerning the effects of similar water shortages on the imbibed seed, or the developing embryo, before it has reached the stage of an independent seedling, when it would be expected to behave in the same manner as a mature plant. Some investigations on the effects of artificial drying trea tments have been reported using wheat seedlings at various stages of development from a few hours after growth had commenced, to several days old (Milthorpe, 1950). In this study, any seminal root which had commenced elongation, and any leaf which had reached the point of emergence from the coleoptile were killed by a severe drying treatment (90% loss of water). Imbibed primordial initials of both types were able to withstand this drying treatment so long as they had not commenced

growth, but in some cases severe retardation of their capacity for subsequent growth was evident. This indicates that once growth has commenced in an embryo there is no possibility of the water taken up being removed without producing harmful effects on the capacity for subsequent growth, and that the greater the development of the embryo at the time of drying, the more severe are the effects of such a treatment. The effects of drying treatments on non-growing primordia might however indicate that it is possible to remove the water from non-germinated seed without causing any harmful effects. If such behaviour were found, it would indicate that some of the phases of germination were reversible. The capacity of the wheat seedling to show quite a considerable degree of recovery after severe drying treatments at various stages during development, is linked with the previously described form of staggered development shown by the seminal root primordia of this, and other members of the Gramineae (Page 1). Such a form of development can now be seen to be of considerable ecological importance, and increase the value of such seed as successful, resistant, dispersal units.

While the capacity of resting, non-dormant seed to remain viable for long periods in dry storage conditions has important commercial implications, such conditions are not common in nature where seed usually lie in moist soils. In temperate regions germination and seedling establishment are rarely restricted by lack of sufficient water. The more normal restrictions are low temperatures, poor light, waterlogged soil, high winds, and other conditions inimical to seedling establishment over fairly long periods, but often interrupted by short

periods when conditions permit germination to occur. The ability of a seed to remain imbibed and viable, but inactive for considerable lengths of time, including periods permitting germination is due to the presence within these seeds of a mechanism restraining germination. Such seed are said to show Dormancy. It is not perhaps surprising that the majority of plants produce seed which are dormant at maturity. This dormancy will disappear naturally, during varying lengths of time in dry storage, but its disappearance can be speeded up by exposing the imbibed seed to special environmental conditions or treatments. These may include storage at uniform low temperatures, storage at fluctuating temperatures, exposure to illumination by white light, repeated wetting and drying, storage in oxygen-rich atmospheres, breaking or removing the seed coverings, and some chemical treatments.

The phenomenon of seed dormancy is thus seen to be an important factor in the maintenance of the delicate balance which exists between the seasonal growth cycles of plants and the climatic environment in which they grow. The growing cycle of the higher plant begins in one growing season, with the initiation and formation of the seed. A pause in the cycle results from the cessation of growth and the reduction of physiological activity associated with the ripening of the seed at the end of the growing season. The recommencement of the growth cycle by the germination of this seed should preferably not take place until the intervening non-growing season has passed, and conditions amenable to seedling establishment have returned. While the form of the resting seed is a suitable structure for the plant to pass through this unfavourable season, it is only seed dormancy which prevents unseasonable

germination during any period when environmental conditions are temporarily suitable. The restraint imposed by dormancy on the continuance of the growth cycle must first be removed before germination can take place, and the growth cycle carry on to its conclusion, in the development of the next generation of seed. Many of the special treatments which have been shown experimentally to facilitate the removal or the breaking of seed dormancy, are those to which seed would be exposed in soil during the non-growing season, e.g. several months in moist, low temperature, storage. Other treatments would appear to be more related to the environmental conditions present during the transition period from the non-growing to the next growing season. These include temperature alternation during moist storage, whether with a diurnal periodicity, or with a longer time at each temperature level, and repeated wetting and drying treatments. In the case of species which require light for the removal of dormancy, disturbance of the soil by frost heaving, water movements, or animal activities might allow such factors to operate. Degeneration of seed coverings by micro-organisms is also an important factor in the removal of seed dormancy. It would seem, therefore, that the very factors which make the non-growing season unsuitable for seedling establishment are often the same factors which ensure that the dormant seed is freely germinable by the time the next growing season has arrived. If dormancy in some Rosaceaeous seed is not removed by some of these special environmental conditions, but allowed to diminish during dry storage, the seedlings produced on germination of these seed will show abnormal types of growth, usually a partial dwarfness, e.g. Peach (Flemion, 1933, 1934). It is probably true to say that most of the successful annual weed species are only

successful because their seed are dormant, and any elimination programme requires a full knowledge of the extent of such dormancy and the manner in which it can be removed. In crop seed, dormancy can also be troublesome in giving low and uneven germination during seed testing, or brewing procedures. It is generally the case, however, in cereal seeds, at least, that dormancy is not complete and does not operate over long periods after harvesting. This is probably the result of deliberate, and accidental selection against dormancy over many centuries. The subject of seed dormancy is excellently reviewed in Crocker and Barton (1948), and Crocker and Barton (1957).

Seed dormancy has been the subject of much investigation over a long period. Despite this, we are still a long way from understanding its nature, and the mechanisms by which it is produced. One notable exception, perhaps, is the case of "Hard Seed" dormancy in the Leguminosae, in which an impervious seed coat prevents the imbibition of the inner tissues, even when totally immersed in water (Hyde, 1954). In most other cases a more complex, physiological basis must be sought. In some seed, the presence of a semi-permeable seed coat which restricts gaseous diffusion between the embryo and the atmosphere is advanced as the cause of seed dormancy. The mechanism involved is either a restricted rate of aerobic respiration, or the occurrence of anaerobic respiration, with accumulation of some of the products (Atwood, 1914; Davis, 1930; Johnston, 1935). The formation of a normally ripe seed with an immature embryo, which requires a period of further development before being capable of normal germination is another possible cause of dormancy in some seed. In these cases a period of moist storage at

temperatures lower than normal germination temperatures is necessary for the further development of the embryo (Steinbauer, 1937; Stokes, 1953). Another cause to which dormancy is attributed is a chemical imbalance at maturity within the main seed metabolites, to such an extent that the necessary sequence of events during germination is prevented. A period of moist low temperature storage of dormant seed of this type does produce some rearrangements in the types and relative amounts of the seed constituents, and also brings about a loss of dormancy (Pack, 1925; Raleigh, 1930; and Steinbauer, 1937). More recent studies on these lines tend to favour single substances, or types of substances in this controlling role, e.g. amino acids, Vallance (1952); Stokes (1953). There is as yet no acceptable explanation of the effects of light treatments, in addition to normal germination requirements, on those types of dormant seed which will not germinate without it, though physiological responses to such treatments have been observed, within a short period, on the respiration and other metabolic activities of such seed (Gardner, 1921; Kipp, 1929; Grohne, 1952; Leopold and Guernsey, 1954; and Evenari, 1956). Perhaps the most prevalent cause to which seed dormancy is attributed is the occurrence of chemical substances in seed which can be readily extracted and shown to inhibit the germination of known non-dormant seed of the same, or of other species.

The occurrence of germination inhibitors in many species has been recorded, and in some cases, identification of the active principle carried out to a distinct substance or a type of substance. Among these have been listed ammonia, hydrogen cyanide, volatile hydrocarbons, essential oils, mustard oils, alkaloids, unsaturated lactones and

phenolic acids. The mechanisms by which these substances may produce dormancy are unknown in almost every case, but they can produce experimental physiological effects which might be analogous to their biological function. The inhibition of cell elongation (Burstrom, 1954), and prevention of nuclear division (D'Amato, 1954), are two of the physiological effects attributed to the unsaturated lactones similar to Coumarin. Other physiological effects produced by unidentified germination inhibitors on known non-dormant seed are reductions in the respiration rate (De Kock, et al, 1953), upset of normal protein metabolism, and enzyme development (Siegel, 1957), and inhibition of amylase activity (Elliot and Leopold, 1953).

In some seed, there has been reported to be an association between the loss of seed dormancy, and the disappearance of germination inhibiting substances, (Shuck, 1935; Delouche, 1956; Black, 1959) or the development of inhibitor inactivating systems (Black, 1956; Wareing and Foda, 1956). In contrast to this there are other reports in which loss of dormancy can be demonstrated before any change in the amounts of germination inhibitors are evident (Barton and Soult, 1946; Luckwill, 1952; Poljakoff-Mayber, et al, 1957). It should however be remembered that many of the estimations of inhibiting substances are based on whole seed, while the significant changes may only be occurring in a few cells of the embryo and involve total amounts which are barely measurable. Despite this qualification, the biological function of germination inhibitors is still "not proven" as a basis for the control of seed dormancy. That such control might not rest, with substances of inhibitory activity, but with others of germination promotive activity,

or a balance of the two types, is indicated by the recent isolation and identification of substances with such properties from seed of several species, (West and Phinney, 1956; Phinney, et al, 1957; Radley, 1958; Black and Naylor, 1959; and Villiers and Wareing, 1960). Earlier workers had postulated the presence of such substances in order to account for the loss of dormancy in some seed which was not accompanied by a drop in germination inhibiting substances (Luckwill, 1952; Poljakoff-Mayber, et al, 1957). That these germination promoting substances can control seed dormancy has been demonstrated recently, when application of one of them, Gibberellic acid, to seed forming on the parent plant prevented the development of dormancy in the mature seed, in a species in which complete dormancy would have been expected (Black, and Naylor, 1959).

Many of the previous investigations on seed dormancy were carried out with the object of understanding the type of dormancy present, and the special treatments required for its removal, usually to obtain a good sample of growing plants at a similar stage of growth for research or for use in horticulture or forestry. If seed dormancy is however due to an upset of some of the events which constitute germination, the study of the physiology of the dormant seed might prove important, in conjunction with similar studies on non-dormant seed in recognising some of these events, and the way they are inter-related with each other during normal germination. It was this consideration that led to the choice of several species in the genus Avena as experimental material. The widely cultivated "non-dormant" species Avena sativa L., (Oat), is readily available in the form of pure lines from commercial sources.

The species <u>Avena fatua</u> L., and <u>Avena ludoviciana</u>, Durieu, are fairly widespread weeds of arable land being known as Wild Oat, and Winter, or Red Wild Oat respectively. Collected strains of these species are available from several research stations, and in the case of the former, variet ies have been described (Lindsay, 1956). Both these species show dormancy at maturity, and their "Nuisance value" as weeds is often attributed mainly to this feature. By virtue of the presence of seed dormancy the germination of these species in soil conditions takes place over a period of a few years, and at times of year when cereal crops are being sown (Thurston, 1953).

In the following pages, the results of, and the conclusions from, some studies on various aspects of germination on these three species are reported. Most of the studies were carried out with grains of <u>A. sativa</u>, but in many instances parallel studies were also carried out with <u>A. fatua</u> and <u>A. ludoviciana</u>. In these cases the comparative performance of all three species in respect to some property is possible, and in some cases the performance of dormant and non-dormant grains of the same species can be compared. From these studies it is hoped to define the nature and sequence of some of the phases of the germination process, and to gain a clearer understanding of the relationship between dormancy and germination in the species studied.

The studies are grouped into six categories, and are arranged more or less chronologically within each. The nature of these categories is :-

<u>Part I.</u> The germination performance of the three species under different germination conditions, and observations on the commencement of growth and meristematic activity in the embryo of <u>A. sativa.</u>

<u>Part II.</u> The pattern of water uptake, and the effects of dehydrating grains of <u>A. sativa</u> after varying periods of imbibition, on the embryo's capacity to resume further growth.

<u>Part III.</u> The development of Amylase activity in grains of all three species during a 9 - 12 day period in the imbibed condition.

<u>Part IV.</u> Changes in the levels of some of the major seed metabolites during germination and early development of the grains of <u>A. sativa.</u>

<u>Part V.</u> The development of Proteolytic activity in grains of all three species during a period in the imbibed condition.

<u>Part VI.</u> The effects of water extracts of grains and grain parts of all three species on their own germination performance, and that of other species.

Part I. The Optimal Germination Requirements of *Avena sativa* (L.), *Avena fatua* (L.), and *Avena ludoviciana* (Dur.), with Observations on the Changes Occurring with the Onset of Growth in the grain of *Avena sativa*.

In almost any study on germination, it is essential, in order to appreciate the results fully, that the germination performance of the seed concerned should be known under varying germination condit ions. This knowledge involves such matters as germination temperatures, water requirements, maturity and age of the seed at the time of germination, and in the case of dormant seeds, such treatments as might be necessary to bring about germination. In this section, studies on the effects of such factors on the germination capacity of the grains of *A. sativa*, *A. fatua*, and *A. ludoviciana* are reported, along with investigations on the time and nature of visible changes, and the time of onset of meristematic activity in the germinating grain of *A. sativa*.

Materials and Methods.

The samples of *Avena sativa* were obtained commercially, having received a seed-cleaning, but not a seed-dressing treatment. The samples of these used in the studies on the effect of maturity and age on the germination capacity were grown locally during 1958 and 1959.

A sample of *Avena fatua* was obtained from Rothamsted in 1956, and some further amounts grown from this in the University grounds during 1957. This sample proved to be rather a mixture of types, and was only used in some preliminary experiments. A further stock of pure lines of

A. fatua, including three of the four varieties described by Lindsay (1956 was obtained from the University of Alberta, in 1957. Further stocks of this were grown locally in 1958, and 1959.

The Avena ludoviciana sample was obtained from Rothamsted in 1956, and 1957, further stocks being grown locally from 1957 to 1959.

Harvesting of the material grown locally was by hand, the samples being used directly for germination tests in the case of the studies on maturity, or dried in a green-house for 1 - 2 weeks before being stored under laboratory conditions.

The germination tests were carried out in petri dishes, using Whatman Seed testing circles. Distilled water was added in measured quantities as described in each experiment. Germination was taken as having occurred when the first seminal root pierced the husk, a binocular microscope (x 14) being used to aid counting. The germinated grains were removed from the plate as they were counted. In some cases, the husk was removed from the whole grain, and only the caryopsis was germinated. In other cases, pricking of the grain was carried out to give a better germination performance. This involved piercing the dorsal surface of the grain with a mounted needle just below the point of insertion of the awn.

The control of temperature during germination was evenly maintained as far as possible by the use of thermostatically operated incubators. Those tests carried out at 6 - 8°C. were carried out in the warmest part of a refrigerated cabinet. Using a well-insulated wooden box, kept in an unheated outhouse, a temperature of 10 - 12°C. was obtained within

the box by including 1 litre of water at 10°C., and changing it as often as necessary.

Water contents of grain samples were carried out by measuring the loss in weight of samples after two days at 92°C., and expressing this loss as a percentage of the final, dry weight.

To study meristematic activity, samples of whole grains and caryopses were fixed in freshly prepared Carnoy's fluid (3:1, alcohol-acetic acid), for a minimum period of 24 hours. The husk was removed, if necessary, and the length of the radicle from the epiblast to the tip of the coleorhiza, or the extruded seminal root, measured by an eye-piece scale. By means of a scalpel, the terminal -.5 mms of the primary seminal root was removed from within the coleorhiza, and partly crushed on a clean slide. A drop of aceto-carmine was added, and the slide heated gently for a few minutes, when the cover slip was put on, and the full squashing carried out. Examination of the whole slide took place some five minutes later, using a magnification of x 400 and an oil immersion magnification of x 1000 for confirmatory work.

A selection of the results of studies on the germination performance of these three species is given below.

Results.

Experiment 1. The effect of temperature on the germination of *A. sativa*, var. Ayr Bounty, and *A. sativa*, var. Victory.

The stocks of grains used in this experiment were two, and three years old, respectively. Samples of fifty grains were used, 5 mls. distilled water being supplied per plate. Germination tests were made at 6 - 8°C., 20°C. ∓ ½°C., 25°C. ± 1°C., and 30°C. ± 1°C. The % germination counts are given below.

Grain sample.	Germination temperature.	Germination % at 64,	112,	136 hrs.
Ayr Bounty.	6-8°C.	0	0	2
"	20°C.	90	94	96
"	25°C.	96	98	98
"	30°C.	72	90	94
Victory.	6-8°C.	2	48	84
"	20°C.	44	90	92
"	25°C.	58	92	92
"	30°C.	48	78	82

Experiment 2. The effect of varying additions of distilled water on the rate of germination in A. sativa, var. Victory.

The sample of grain used here was one year old at the time of testing. Three, four, or six mls. of distilled water were given to each of four plates of fifty grains, before placing them at 20°C. The mean germination % at various times is given below.

Amount of distilled water, mls.	Mean germination % at,						
	22,	24,	42,	48,	66,	72,	90 hrs.
3	0	0	7.5	13.5	80	90	93
4	0	2.5	67	83	93	96	98
6	2	10.5	55.5	66	72	73.5	73.5

Experiment 3. Changes in the germination capacity of A. sativa var. Victory, and A. sativa var. Ayr Bounty, during the first months of dry storage after harvest.

These samples were grown locally during 1958, and harvested as previously described. For each test, four plates of fifty grains were germinated at 20°C. with 4 mls. distilled water.

Experiment 3 continued.

Date of Testing.	Days from harvest.	Variety.	Mean germination % at,				
			68,	92,	140,	168,	240 hrs.
23/9/58	0	Victory.	0	5	44	57.5	76.5
"	0	Ayr Bounty.	6	45	84	89	-
			48,	72,	96,	120,	168 hrs.
6/10/58.	13	Victory.	2.5	49.5	62.5	77	86
"	13	Ayr Bounty.	8	70.5	87.5	92.5	94
			42,	49,	66,	90,	114 hrs.
10/11/58.	48	Victory.	3	11.5	52.5	85	95
"	48	Ayr Bounty.	13	42.5	92	98	-
			40,	48,	64,	88,	120 hrs.
25/12/58.	94	Victory.	7	24	68	85.5	89.5
"	94	Ayr Bounty.	22	62.5	91	95	96.5
			28,	48,	72,	120 hrs.	
2/3/59.	161	Victory.	5	42	84	97	
"	161	Ayr Bounty.	7	73	96	98	

Experiment 4. The germination capacity of *A. sativa* var. Ayr Bounty, at different stages during ripening.

These samples were grown locally during 1959, being collected as required except for the final sample which was drying in a greenhouse from 14/9/59 to 6/10/59. 4 stages of maturity were chosen - The Milky Stage, when the endosperm was liquid, and grain still green, The Doughy Stage, when the endosperm was doughy and the grain still green, The Harvest Ripe Stage, when the endosperm was moderately firm and the grain yellow green, and the Fully Ripe Stage when the endosperm was flinty

and the grain yellow-brown. One part of each sample was measured for its moisture content, and two plates of fifty grains from the remainder set to germinate at 20°C. with 4 mls. of distilled water. The results of these tests are given in the following table.

Testing date.	Stage of Maturity.	Moisture content.	Mean germination % at,				
			42.	68.	92.	120 hrs.	
4/8/59.	Milky stage.	141%	23	56	65	70	
24/8/59.	Doughy stage.	92%	6	46	81	83	
			42.	72.	96.	120 hrs.	
14/9/59.	Harvest ripe.	31%	0	13	54	91	
			48.	64.	72.	92.	120 hrs.
6/10/59.	Fully ripe.	19%	8	54	66	88	96

Experiment 5. The effect of different treatments on the rate and amount of germination in *A. fatua*.

Some of the germination tests carried out on various samples of *A. fatua* are given below. The tests are based on fifty seeds, or two plates of 25, germinated at 20°C. with 4 or 3 mls. distilled water respectively. The grains were in some cases pricked, in others subjected to husk removal.

Experiment 5 continued.

Sample.	Germination % at, 64.	112.	136.	160.	184 hrs.
Glasgow, 1957.	0	21	24	24	24
" " , pricked.	0	42	48	51	81

	Germination % at, 24.	40.	72.	88 hrs.
Alberta, 1957.	0	70	82	92

	Germination % at, 2.	3.	4.	5.	7	9 days
Glasgow, 1958.	0	22	68	72	80	84
" " , pricked.	0	56	84	92	-	-
" " , husked. and pricked.	72	92	100	-	-	-

Experiment 6. Changes in the amount and rate of germination in A. fatua during the first few months dry storage after harvest.

Samples of A. fatua, varieties pilossissima, vilis, and intermedia, were grown locally during 1958, and harvested as previously described, on or near 23/9/58. During the ensuing months of dry storage germination tests on two plates of 25 grains were carried out at intervals at 20°C. with 3 mls. of distilled water added initially, and further additions as seemed necessary. The results of these tests are tabulated below.

Experiment 6 continued.

A. fatua, var. pilossissima.

Date of Test.	Days since Harvest.	Germination % at, 2.	3.	4.	5.	6.	7.	9.	12 days.
10/10/58.	17	0	12	28	46	-	68	74	-
24/10/58.	31	0	4	10	18	-	20	24	-
26/11/58.	64	0	8	22	32	36	42	44	-
27/12/58.	95	0	6	22	-	42	48	48	-
2/3/59.	153	10	54	74	78	-	84	84	-
6/7/59.	276	8	64	80	92	96	-	-	-

A. fatua, var. vilis.

Date of Test.	Days since Harvest.	Germination % at, 2.	3.	4.	5.	6.	7.	9.	12 days.
19/10/58.	26	0	0	2	6	14	32	48	52
24/10/58.	31	0	0	0	4	8	28	56	64
26/11/58.	64	0	0	4	12	32	56	62	-
27/12/58.	95	0	56	96	-	-	-	-	-
2/3/59.	153	12	72	92	-	-	92	94	-
6/7/59.	276	10	80	96	-	-	-	-	-

A. fatua, var. int ermedia.

Date of Test.	Days since Harvest.	Germination % at, 2.	3.	4.	5.	6.	7.	9.	12 days.
24/10/58.	31	0	2	14	16	18	18	20	-
26/11/58.	64	0	2	20	48	56	60	64	-
27/12/58.	95	0	52	76	-	80	-	-	90
2/3/59.	153	14	78	88	94	-	-	94	-
6/7/59.	276	12	84	98	-	-	-	-	-

Experiment 7. The germination capacity of A. fatua, at different stages of maturity.

Samples of A. fatua, var. pilossissima, were harvested in August 1959 at different stages of development. The development stages, with the exception of the first are similar to those described for A. sativa, (Expt. 4). The first stage consisted of grain in which only half of the full endosperm development had taken place. The size of the sample varied from 30 - 100 grains. Germination followed harvest with the exception of the fully ripe sample which received a few days drying in a greenhouse. Germination took place at 20°C. with requisite amounts of distilled water.

	Germination % at,						
Stage of Maturity.	2.	4.	5.	6.	7.	9.	13 days.
Endosperm underdeveloped.	-	-	4	13	26	52	60*
Milky stage.	-	-	10	14	24	68	88+
Doughy stage.	-	-	12	22	34	59	59+
Harvest ripe.	-	8	15	20	22	27	27+
Fully ripe.	-	-	-	-	-	-	3+
Harvest ripe, pricked.	6	38	64	76	-	84	-
Fully ripe, pricked.	-	56	68	-	84	92	-

* Ungerminated caryopses, small with little endosperm present.

\+ Ungerminated caryopses normal size and appearance.

Experiment 8. The amount and the rate of germination of *A. fatua*, after four days immersion in deoxygenated water.

A sample of approx. 500 grains of six month old *A. fatua* var. *pilossissima* grown in Glasgow during 1958 was placed in a 250 ml. conical flask, which was then completely filled with distilled water, deoxygenated a few hours previously by boiling. A tight fitting cork was inserted, and the flask stored in an inverted position for four days at 24°C. On removal, some of the grains were immediately tested for germination capacity, with and without seed treatments. The remainder was dried under reduced pressure and stored dry in the laboratory, samples being tested at intervals for germination capacity. The germination tests were made with two plates of 25 grains, at 20°C., with 3 mls. distilled water for the dry samples and a slightly damp filter pad for the wet samples. In one treatment 3 mls. of a 25 ppm. solution of Gibberellic Acid was substituted for the distilled water. The germinatio capacity of these grains, and their responses to these seed treatments are given below.

Experiment 8 continued.

Date of Test.	Sample, and Germination treatment.	Germination % at, 1.	2.	3.	4.	5.	6.	8.	10 days
2/3/59.	Original sample.	-	10	54	74	78	-	84	84
	After 4 days immersion								
4/3/59.	No treatment.	0	0	0	0	0	8	8	8
"	Pricked.	0	0	0	22	-	30	32	36
"	Husk removed.	0	6	-	40	-	52	54	56
"	Husk removed and pricked.	22	60	-	68	-	72	74	82
	After dry storage.								
24/4/59.	No treatment.	0	0	4	-	-	42	56	56
"	Pricked.	0	0	48	-	-	74	88	88
"	Gibberellic acid.	0	0	26	-	-	70	84	88
14/5/59.	No treatment.	0	0	0	28	36	52	84	90
"	Pricked.	0	0	0	52	70	76	86	86
6/7/59.	No treatment.	0	0	14	38	64	76	-	86

Experiment 9. To determine the optimal germination temperature of *Avena ludoviciana*.

Several experiments were carried out with different stocks of *A. ludoviciana* to assess its germination performance at different temperatures. In some cases very little germination occurred at any temperature. The results of some tests with one year old samples grown at Glasgow and Rothamsted in 1957 are given below.

Sample.	Temperature and treatment.	Germination % at, 5.	6.	7.	8.	10.	12 days.
Glasgow,1957.	80°C. untreated.	0	0	0	0	0	4
"	10-12°C. "	0	0	0	0	4	12
"	" pricked.	8	28	44	52	56	68
"	20°C. untreated.	0	0	4	4	4	4
"	" pricked.	4	20	28	32	40	56
"	30°C. untreated.	4	4	4	4	4	4
"	" pricked.	0	0	0	0	0	0
Rothamsted,1957.							
	10-12°C. untreated.	2	8	16	22	24	30
	" pricked.	6	14	20	26	36	46

Experiment 10. The effects of pre-treatment at various temperatures on the germination performance of *A. ludoviciana* at 10 - 12°C.

The effects of 2 days at 0°C., 2 days at 4°C., 2, 5, and 7 days at 30°C., and 20 hours at 56°C. was investigated on imbibed untreated, and pricked grains of the one year old *A. ludoviciana* sample from Rothamsted used in the previous experiment. Two plates of untreated and pricked grains, 25 per plate, were placed at the temperatures indicated after the addition of 3 mls. distilled water. After the requisite period, the samples were transferred to 10-12°C., with additional distilled water given in some cases, and the germination capacity followed. The results are given below.

Temperature pre-treatment.	Grain treatment.	Germination % at,				
		4	8	10	12	14 days*
2 days, 0°C.	Untreated.	0	14	18	32	-
"	Pricked.	0	22	40	52	-
2 days, 4°C.	Untreated.	0	14	24	34	-
"	Pricked.	0	18	42	58	-
2 days, 30°C.	Untreated.	0	8	16	18	20
"	Pricked.	2	30	44	68	86
5 days, 30°C.	Untreated.	0	2	10	14	18
"	Pricked.	0	22	36	56	72
7 days, 30°C.	Untreated.	0	4	6	12	12
"	Pricked.	0	16	30	46	58
20 hours, 56°C.	Untreated.	0	0	0	0	0
	Pricked.	0	0	0	0	0

* The period of pre-treatment is included as part of the germination period in these figures.

<u>Experiment 11</u>. To determine the effect of pricking grains of <u>A. ludoviciana</u> before or after a temperature pre-treatment at 30°C., on the subsequent germination performance at 10-12°C.

Samples of the 1 year old <u>A. ludoviciana</u> grown locally in 1957 and used in Expt. 9 were given periods of three or six days at 30°C., preceded or followed by pricking treatments, and then transferred to 10-12°C. for germination to take place. The germination rates measured in terms of the numbers of days at 10-12°C. are given below.

Temperature Treatment.	Pricking Time.	Germination % at 6	7	8	9	12 days.
3 days, 30°C.	Before.	8	56	80	88	92
"	After.	0	4	20	56	76.
6 days, at 30°C.	Before.	0	0	4	12	64
"	After.	0	0	0	0	42.
None.	None.	0	0	0	4	12
"	Beginning.	28	44	52	56	68.

<u>Experiment 12</u>. Changes in the rate and amount of germination during the first few months of dry storage of <u>A. ludoviciana</u>.

Samples of <u>A. ludoviciana</u> were harvested as previously described on 23/9/58, and at intervals during the next nine months dry storage, two plates of 25 basal grains were given a germination test. This test involved pricking, three days at 30°C., and the remainder of the period at 10-12°C. 3 mls. distilled water was added initially, and another 2 mls. after removal from the higher temperature. Further additions were made as necessary. In addition, the germination performance of

untreated grains shortly after harvest and after 1 years dry storage is included. The germination rate, in terms of the number of days at 10-12°C. is given below.

Testing date.	Days from harvest.	Germination % at, .6	.7	.8	.9	.10	.11	.13	.16	.17 days
19/10/58	26	0	0	0	4	8	16	28	60	68
26/11/58	64	0	0	0	0	45	73	87	95	-
27/12/58	95	2	22	62	78	-	86	94	-	-
2/3/59	160	12	32	60	-	-	90	-	96	-
6/7/59*	286	12	28	46	60	72	84	-	-	-
Untreated Grains										
19/10/58	26	-	0	0	0	0	0	0	0	0
20/10/59	366	-	4%	6%	16%	28%	38%	40%	-	-

* Temperature range in this experiment, 10-16°C.

<u>Experiment 13.</u> The time of onset of meristematic activity in the germinating embryo of <u>A. sativa</u>, var. Victory, and its relationship to the development of the embryo.

Preliminary studies indicated that meristematic activity could be found in the germinating grain sometime around 20-26 hours after placing in germination conditions, and that it was first observed in the root-tip of the primary seminal root. To clarify the situation further, plates of fifty whole grains and caryopses of <u>A. sativa</u>, var. Victory, two years old at the time of testing, were germinated at 20°C., with 4 mls.

distilled water present. At intervals, a plate was removed and a random sample of ten grains or caryopses was taken from this for fixing and examination for the presence of nuclear divisions by the technique previously described. The findings of this examination were grouped under four classes of embryo development, for the caryopses and whole grains separately, and in summary form for all the material studied. The four classes of embryo development were defined as :-

Class 1. Testa not burst.

Class 2. Testa burst, primary seminal root less than 1 mm.

Class 3. Testa burst, primary seminal root 1-1.5 mms.

Class 4. Testa burst, primary seminal root more than 1.5 mms.

The results of the examination of these samples for meristematic activity are given below.

A. Caryopses.

Number of meristematic embryos / 10, at,

18.	19.	20.5.	21.5.	22.5.	24.5 hrs.
1	1	5	1	4	7

B. Whole grains.

Number of meristematic embryos / 10, at,

18.	20.5.	22.5.	23.5.	24.6.	25.5 hrs.
0	1	1	0	2	2

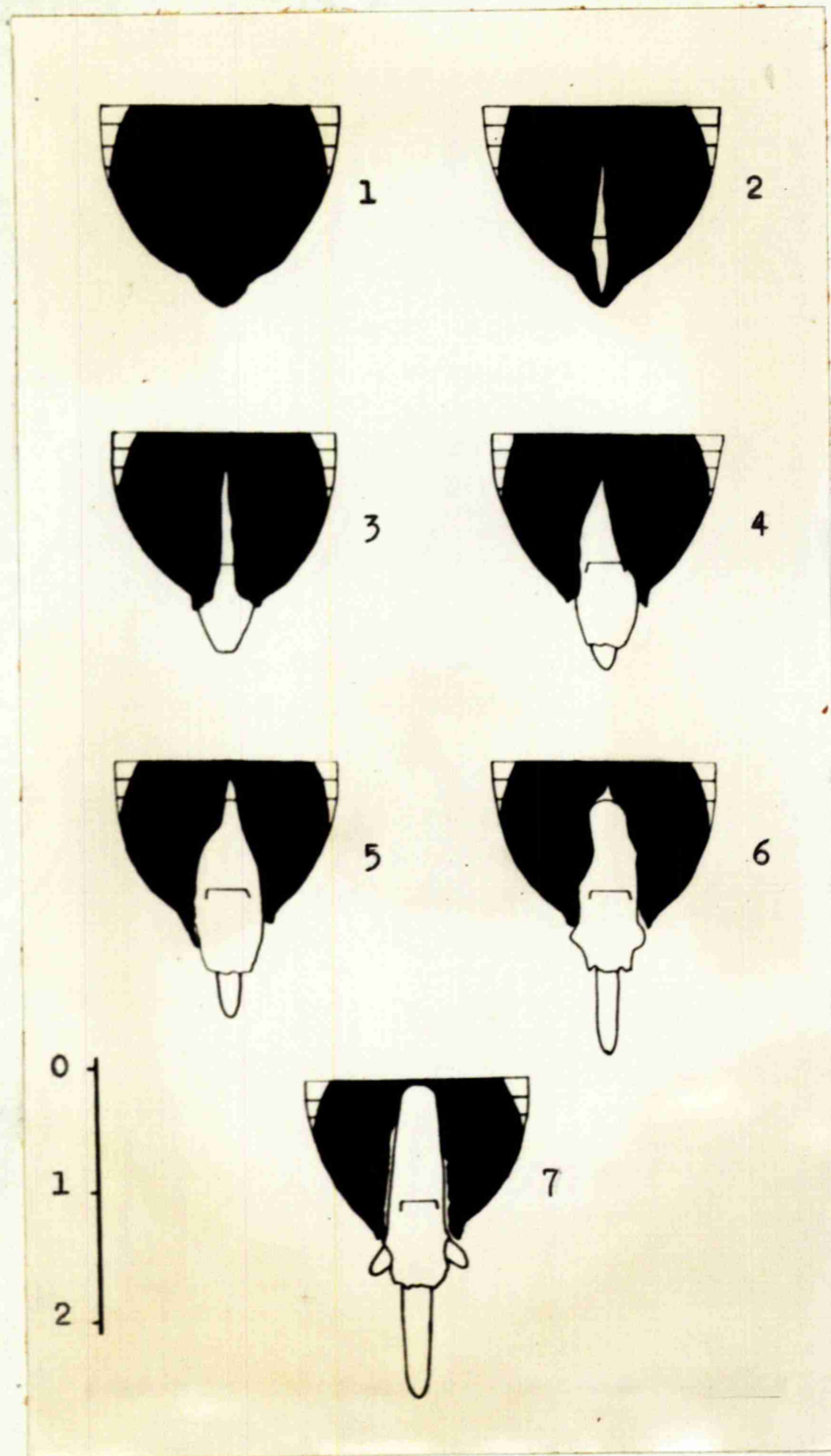
The distribution of meristematic activity within the various development classes is summarised below for all grains examined.

Number in class 1,	47,	Number showing meristematic activity	0.
" " " 2,	29,	" " " "	1.
" " " 3,	32,	" " " "	12.
" " " 4,	12,	" " " "	12.
	120.		25.

Experiment 14. Further observations on the time of onset of meristematic activity in the germinating embryo of A. sativa, var. Victory, and observations on the morphological changes in the embryo during the germination period.

Grains of the same sample and germination conditions as used in the previous experiment were again used, but in this experiment the method of obtaining the sample of ten grains for examination was modified. At sampling times, all the grains in the plate of fifty were examined, husks being removed from the whole grain, and classified into the four development classes described previously. The sample of ten grains was then chosen to show the same proportions in each class as the larger sample. In previous studies a total of 60-80 grains in the class 1 category had been examined without any meristematic activity being observed. In this investigation no further examination of grains in this class was carried out, all being assumed to be non-meristematic for the compilation of the tables. Observations were also made during the classification of the whole grain samples of the times at which various changes in embryo development were first observed. The following time sequence of embryo changes was found to occur.

Plate 2.



Diagrammatic representation of the morphological changes occurring in embryos of A. sativa during the commencement of growth. Stages 1 - 7 correspond to those stages described on page 34. Scale in mms.

Expt. 14 (contd.)

Time of first observation.	Changes in the embryo.
Before 18 hours.	Whole seed swells stretching the testa into a tight membrane.
From 18 hours.	Swelling of the embryo causes disruption of the testa directly above it.
From 20 hours.	Visible extension of the coleorhiza begins, followed by radicle within it.
From 22 hours.	Extension of the radicle bursts the coleorhiza.
From 24 hours.	Radicle extension about 1 mm., first pair of secondary seminal roots begin swelling, piercing the husk in a few cases, i.e. visible germination.
From 26-28 hours.	Radicle about 1.5 mms., piercing the base of the husk in most cases, secondary seminal roots well evident.
From 28-30 hours.	Growth beginning in plumule.

a) Examination of Caryopses.

Development Class.	Numbers of grains in each class at, 20.5.	21.5.	22.5.	24.5.	28.5 hrs.	Total Number in Class.	Number showing nuclear divisions.
Class 1.	6	5	4	4	1	20	0
Class 2.	3	3	3	2	3*	14	1*
Class 3.	0	1	1	1	3	6	4
Class 4.	1	1	2	3	3	10	10
Totals.	10	10	10	10	10	50	15
Number showing nuclear divisions.	1	1	3	4	6	15	

* one radicle meristematic.

Expt. 14 (contd.)

b) Examination of Whole grains.

Development Class.	Numbers of grains in each class at, 20,	22,	24,	26,	28,	30 hrs.	Total Number in Class.	Number showing nuclear divisions.
Class 1.	9	8	8	6	5	3	39	0
Class 2.	1	2	1	3	2	2	11	0
Class 3.	0	0	1	1	2	1	5	0
Class 4.	0	0	0	0	1	4	5	5
Totals.	10	10	10	10	10	10	60	5
Number showing nuclear divisions	0	0	0	0	1	4	5	

Summary of all grains examined in this Expt.

Total number in Class 1,	59,	Number showing nuclear divisions,	0.
" " " " 2,	25,	" " " "	1.
" " " " 3,	11,	" " " "	4.
" " " " 4,	15,	" " " "	15.
	110		20

Summary of all grains examined in two Expts.

Total number in Class 1,	106,	Number showing nuclear divisions,	0.
" " " " 2,	54,	" " " "	2.
" " " " 3,	43,	" " " "	16.
" " " " 4,	27,	" " " "	27.
	230		45

DISCUSSION.

In its germination behaviour, Avena sativa shows the pattern followed by a typical non-dormant seed (Expts. 1 and 2), namely a fairly rapid and complete germination. The optimal temperature for germination is just above 20°C., and no special germination treatments are required. The species is however sensitive to the presence of too much water in the germination medium (Expt. 2, Fig. 1), the optimum amount in our conditions being 4 mls. per 50 grains. Another factor which has a strong influence on the germination behaviour in this species is the age of the sample (Expt. 3, Fig. 2), and its maturity (Expt. 4, Fig. 3). An examination of these results (Fig. 4) shows that with the onset of full ripeness there is the development of a tendency for a much slower rate of germination, this tendency only wearing off after several months in dry storage. There is little indication of an actual restriction on the amount of germination during this period in the samples of A. sativa tested here. Other investigations have found a similar behaviour in Oats of other varieties, and in other Cereals, but frequently the slow rate of germination is also accompanied by a marked reduction of the final germination level (Ducharte, 1852; Harrington, 1923; Larson, et al, 1936; Vines, 1947). This behaviour immediately after harvesting can only be regarded as a mild form of seed dormancy.

In the samples of A. fatua tested in experiment 7, the full range of germination responses is included. The Alberta, 1957 sample shows no dormancy and speedy germination, the Glasgow, 1957 sample shows marked dormancy, and the Glasgow, 1958 sample shows a high final level, but a very slow rate, of germination. The beneficial effects of pricking the

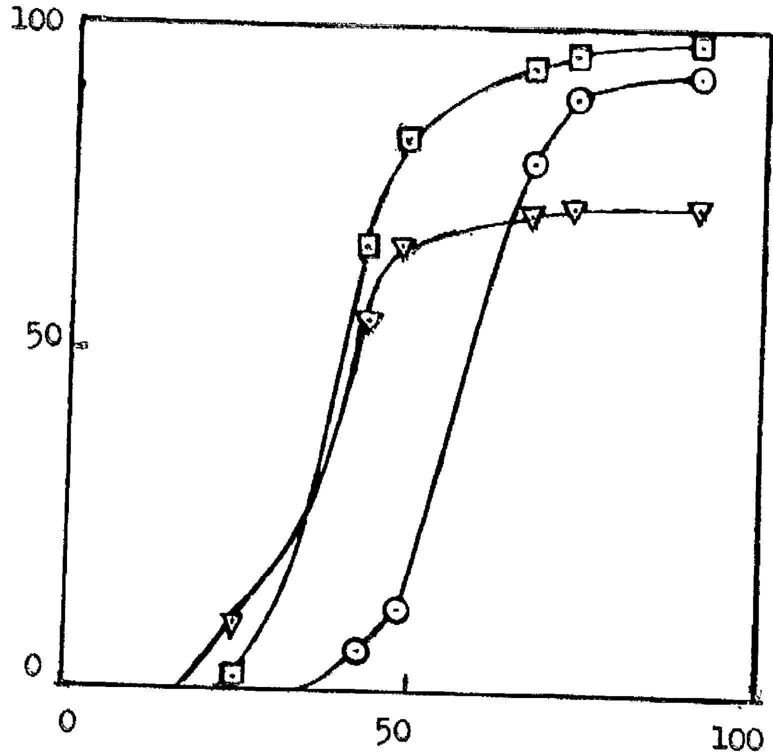
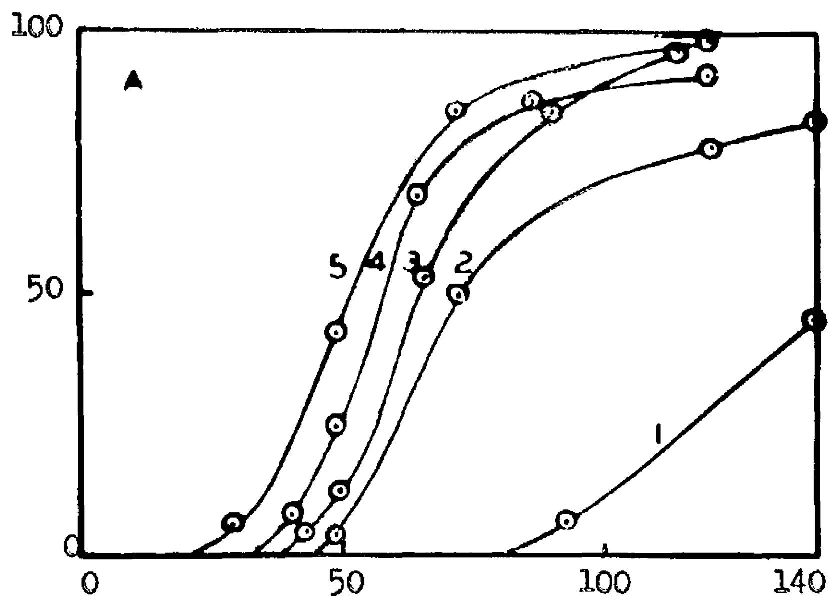


Fig. 1.

Germination rates of samples of grains of A. sativa in the presence of 3 mls.,(⊙), 4 mls.,(⊡), or 6 mls. distilled water / plate of 50 grains,(▽).

Abcissa: Period in germination conditions, hours.

Ordinate: Germination %age,



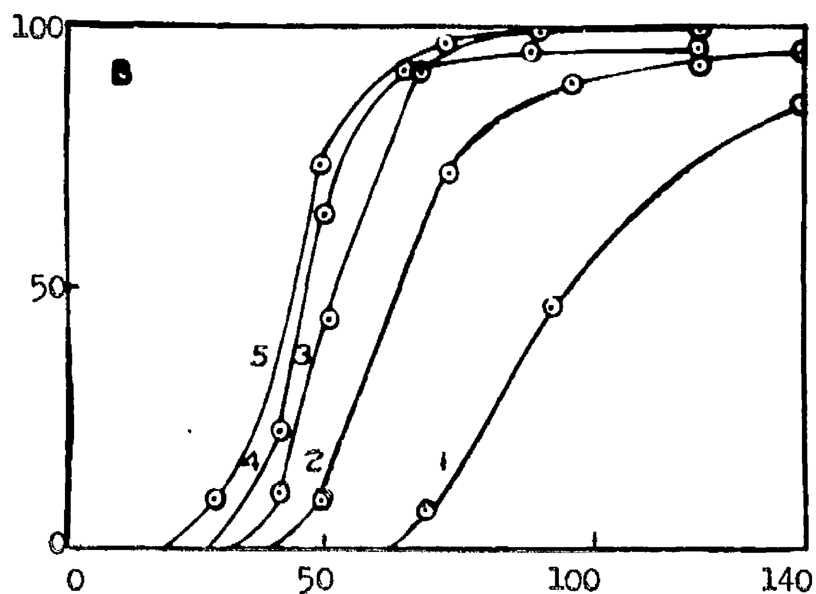


Fig. 2.

Germination rates of samples of grains of A. sativa var. Victory,(A), and A. sativa var. Ayr Bounty,(B), tested at intervals during a period of dry storage after harvesting. Tests carried out at harvesting,(1), and after 13 days,(2), 48 days,(3), 94 days,(4), and 161 days dry storage,(5).

Abcissa: Period in germination conditions, hours.

Ordinate: Germination %age.

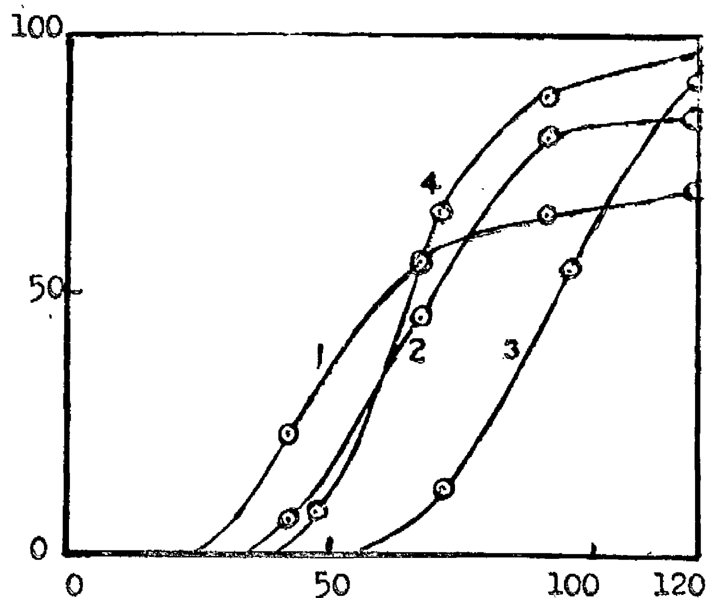


Fig. 3.

Germination rates of samples of grains of A. sativa var. Ayr Bounty tested at different stages of maturity. Milky stage,(1), doughy stage,(2), harvest-ripe stage,(3), and fully ripe stage,(4).

Abcissa: Period in germination conditions, hours.
Ordinate: Germination %age.

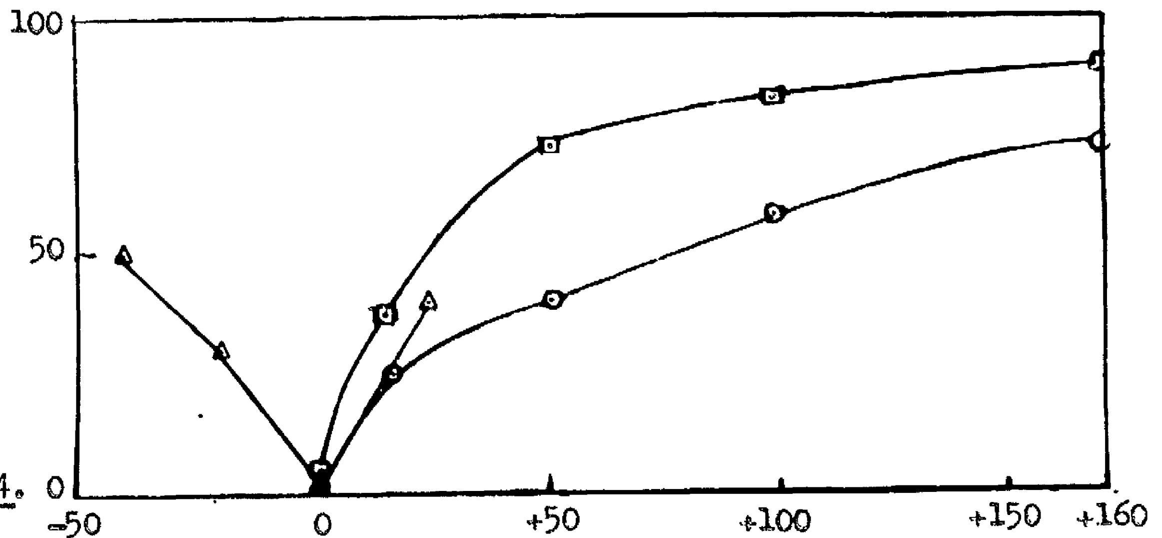


Fig. 4.

Mean %age germination at 60 hours in samples of A. sativa var. Ayr Bounty tested before fully mature,(△), and samples of A. sativa var. Ayr Bounty (⊡), and var. Victory,(⊙), harvested at maturity and tested at intervals during a period of dry storage after harvesting.
Derived from the germination rate curves in Figs. 2 and 3.

Abcissa: Time of testing, days before,(−), and after harvesting,(+).
Ordinate: Germination %age.

grain and removing the husk are well illustrated, these effects having been shown many years previously by Atwood, 1914. One of the factors contributing to this variability is the age of the sample at the time of testing, both in respect to the length of time after the attainment of ripeness (Expt. 6, fig. 5), and to the maturity of the grain (Expt. 7, Fig. 6). The effect of the variety of A. fatua is also of importance in respect to the amount of dormancy present and its duration, and there are some reports that the weather conditions during ripening may have strong modifying effects also. A comparison of the relative germinability of various samples during the period of maturation, and in dry storage, after it, (Fig. 7), shows that in this species also, the restriction of germination only reaches its maximum at full ripeness, and shortly afterwards starts to decline over a period of 6 to 9 months, a situation similar to that already found in A. sativa. There are several observations that dormancy disappears from ripe seed with similar periods of dry storage (Atwood, 1914, and Johnson, 1936) and that immature seed may germinate better than mature seed (Atwood, 1914), but no regular sequence of tests on the same samples over the same length of period are known from previous investigations. The ability to re-establish dormancy, after it had declined during dry storage, and the similarity of this imposed dormancy to natural dormancy in its form, its response to mechanical and chemical seed treatments and its decline during further dry storage has recently been claimed (Hay and Cumming, 1959). These observations were confirmed by our findings (Expt. 8, Fig. 8). The effect of Gibberellic acid on grains made dormant in this manner was also confirmed on a sample six weeks after the re-establishment of dormancy (Expt. 8, Fig. 8B).

Fig. 5.

Germination rates of samples of grains of A. fatua tested at intervals during a period of dry storage after harvesting.

A. A. fatua var. pilossissima.

Samples tested at 17 days,(1), 31 days,(2), 64 days,(3), 95 days,(4), 153 days,(5), and 276 days after harvesting,(6).

B. A. fatua var. intermedia.

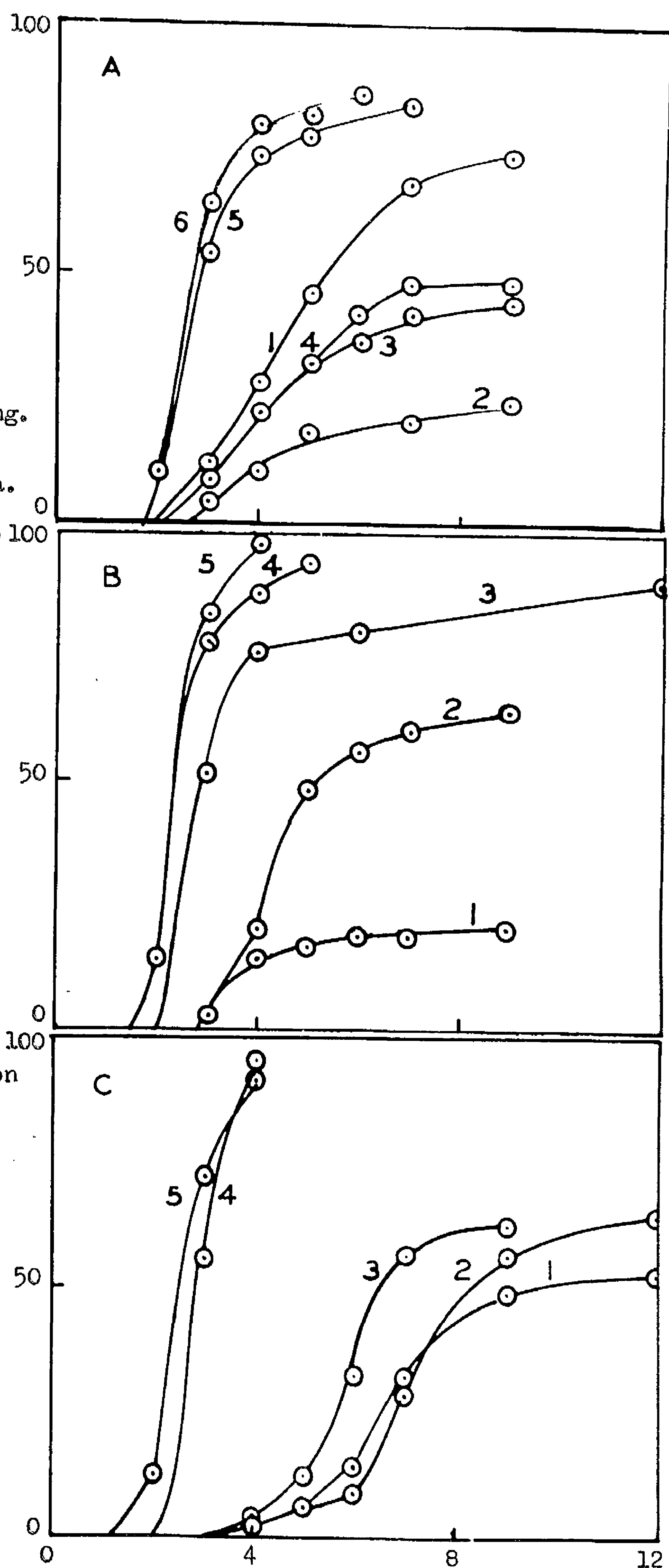
Samples tested at 31 days,(1), 64 days,(2), 95 days,(3), 153 days,(4), and 276 days after harvesting,(5).

C. A. fatua var. vilis.

Samples tested at 26 days,(1), 31 days,(2), 64 days,(3), 95 days,(4) and 276 days after harvesting,(5).

Abcissa: Period in germination conditions, days.

Ordinate: Germination %age.



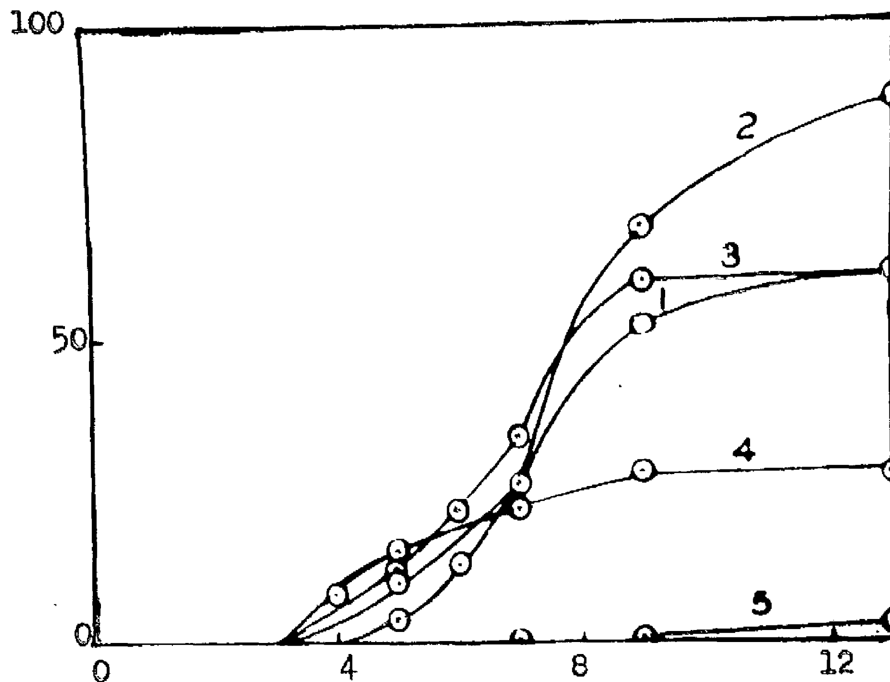


Fig. 6.

Germination rates of samples of A. fatua tested at different stages of maturity - endosperm under-developed,(1), milky stage,(2), doughy stage,(3), harvest ripe,(4), and fully ripe,(5).

Abcissa: Period in germination conditions, days.
Ordinate: Germination %age.

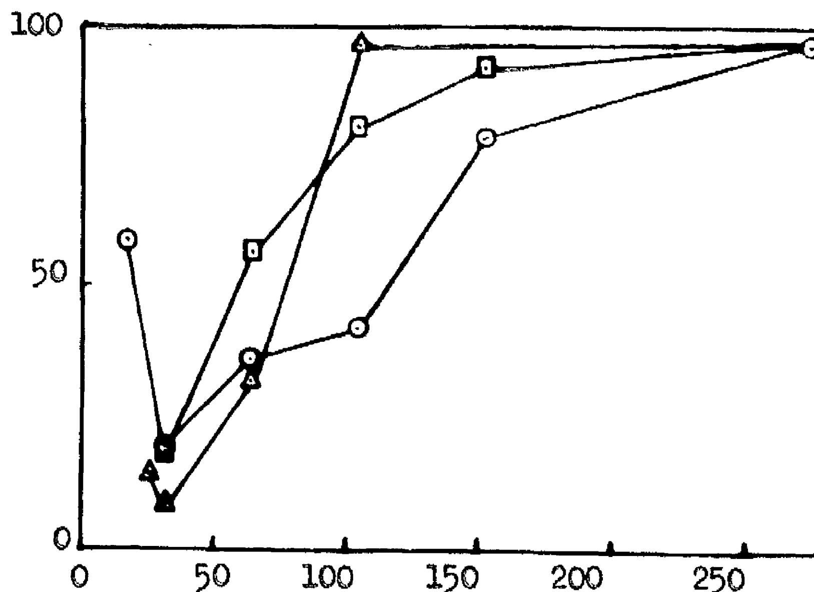


Fig. 7.

Mean %age germination at 6 days in samples of A. fatua var. pilossissima, (⊙), A. fatua var. vilis,(△), and A. fatua var. intermedia,(⊡), tested at intervals during dry storage after harvesting. Derived from data of Fig. 5.

Abcissa: Period after harvesting when tested, days.
Ordinate: Germination %age.

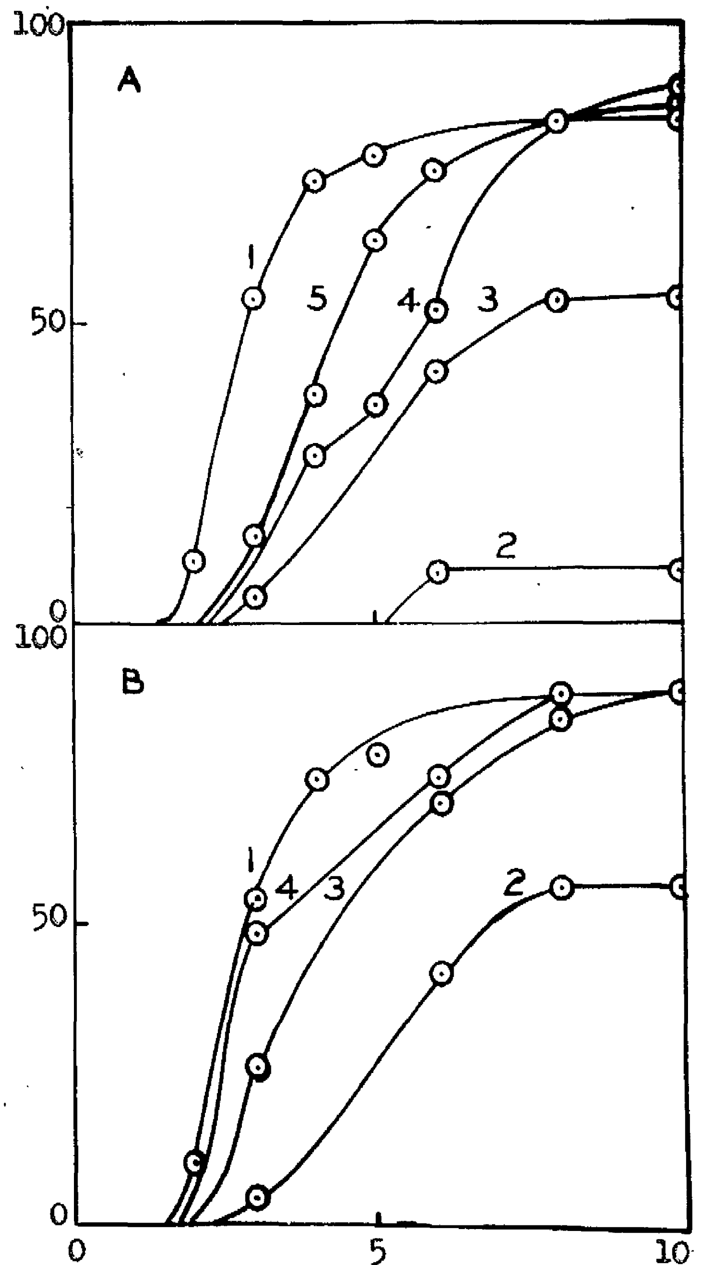


Fig. 8.

Re-establishment of dormancy in grains of A. fatua by water immersion treatments.

A. Germination rates of samples of grains before treatment,(1), immediately after treatment,(2), and after 51 days,(3), 71 days,(4), and 124 days dry storage,(5).

B. Germination rates of samples of grains before treatment,(1), 51 days after treatment,(2), and the same sample germinated in the presence of a 25 p.p.m. Gibberellic acid solution,(3), or given a pricking treatment,(4).

Abcissa: Period in germination conditions, days.

Ordinate: Germination %age.

These findings indicate that dormancy in this species may be determined in response to environmental conditions, and not solely by a particular stage during seed formation. This property might be of considerable significance in the ecology of this species, and in its success as a soil persistent weed.

Observations on the germination capacity of the available samples of A. ludoviciana (Expt. 9) show that dormancy in this species can be very marked and also quite variable. The requirement of a lower optimum germination temperature than for the other species examined was apparent in all the samples investigated, and a temperature of 10-12°C. used throughout these studies. The beneficial effect of pricking the grains is well evident in this species also, but there is also seen to be a considerable benefit resulting from a moist pre-treatment at 30°C., both on the rate of germination on return to the lower temperature, and on the final germination % reached. This beneficial effect is seen to be dependent on the grain having been pricked, the greatest benefit resulting from a pricking treatment before the 30°C. pre-treatment (Expt. 11, Fig. 9). The effect of the temperature treatment by itself is, if anything, unfavourable, and the effect of more than 5 days at the high temperature are less beneficial than shorter periods even with pricked grains. These observations were carried out to confirm previous findings of Thurston (1956) of the beneficial effects of temperature pre-treatments at 27°C. for 1 - 7 days. There also appears to be a change in the response of grains of this species to the combined effects of pricking and temperature treatment during the months immediately following maturity, under dry storage conditions (Expt. 12, Fig. 10), and

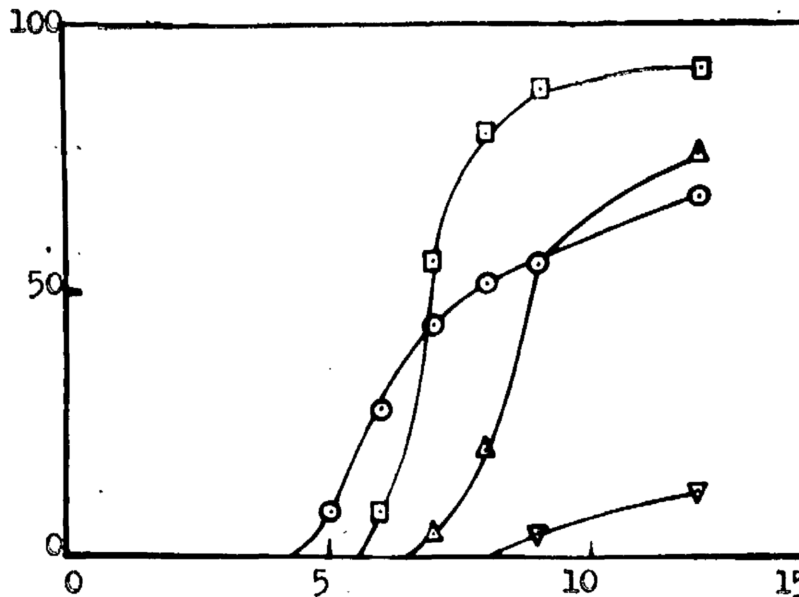


Fig. 9.

Germination rates of a sample of grains of A. ludoviciana at 10 - 12°C.

Untreated grains, (∇), pricked grains, (⊙), and grains pricked before, (⊡), and after, (△), a 3-day pre-treatment at 30°C.

Abcissa: Period in germination conditions, days.
Ordinate: Germination %age.

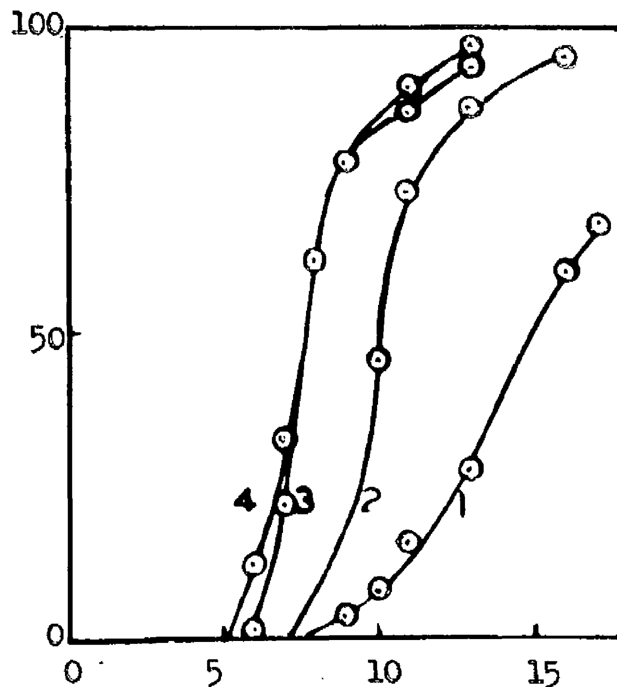


Fig. 10.

Germination rates of samples of grains of A. ludoviciana tested at intervals during a period of dry storage following harvesting.

Germinated at 10 - 12°C. after a pricking treatment and 3 days at 30°C. Samples tested at 26 days, (1), 64 days, (2), 95 days, (3), and 160 days after harvest, (4).

Abcissa: Period in germination conditions, days.
Ordinate: Germination %age.

in the decline of dormancy during the same period in untreated seed. No other experimental observations of the germination behaviour of this species is known.

The studies on the time of commencement of embryo growth in _A. sativa_, and the sequence of morphological changes which it follows, were designed to give information to which reference could be made in later investigations in which the development of the embryo might be involved. The choice of a random sampling technique in choosing the material for root-squash investigation, was changed in the later investigation in favour of a representative sampling technique, because it was felt more confidence could be placed in the sequential nature of these results with the small samples used (Expt. 13). From the later experiment (Expt. 14) meristematic activity can be expected in approximately 10% of a whole grain sample about 26-28 hours after placing to germinate in the conditions used here. The occurrence of meristematic activity in whole grains was noted as early as 20.5 hours in the previous experiment (Expt. 13).

The relationship between the stage of morphological development and the presence of meristematic activity in an embryo should be fairly independent of the sampling method used, except in the relative numbers of each class available at any one time. Accordingly the results of both experiments on this subject will be considered together. The numbers of grains showing meristematic activity in each Development Class, and the %age this represents of the class number is given in Fig. 11. Meristematic activity is found in nearly half of the Class 3 grains and in all of the Class 4 grains. A comparison of the stage of embryo

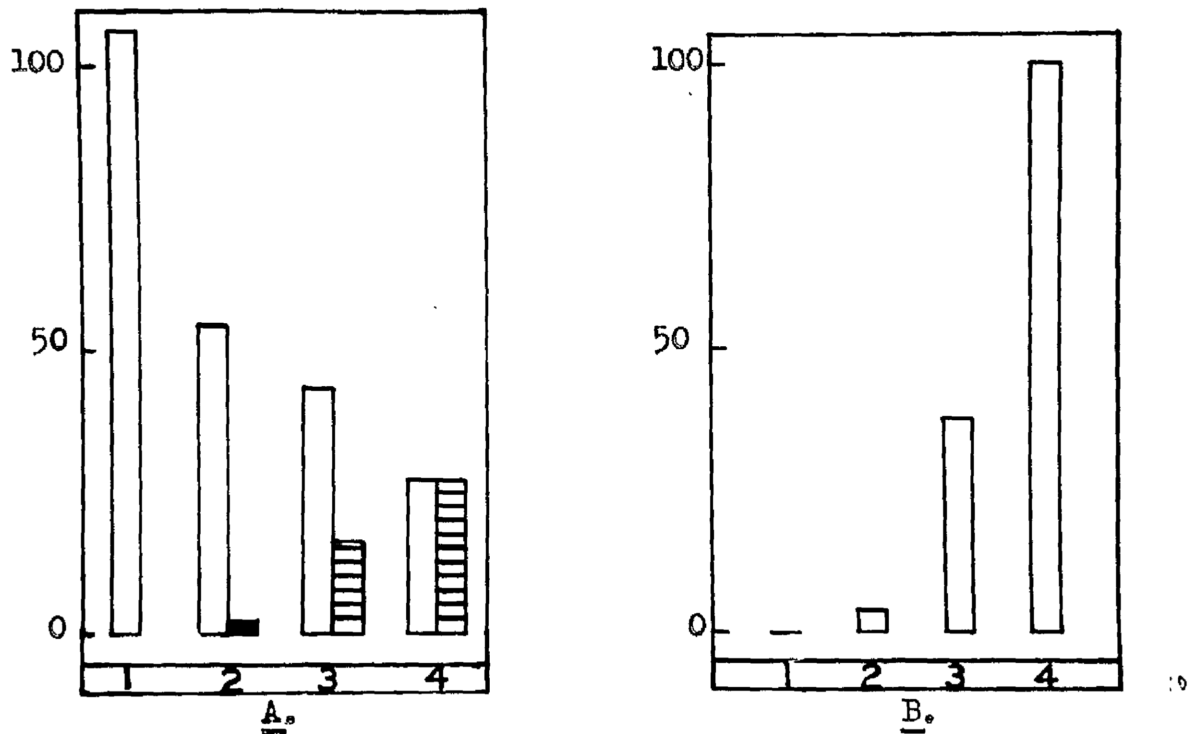


Fig. 11.

The relationship between the morphological development of the embryo and the presence of meristematic activity in germinating grains of A. sativa.

A. The number of grains in each embryo development class, (□), and the number of these showing meristematic activity, (▤).

B. The %age of grains in each embryo development class showing meristematic activity.

Abcissa: Embryo development classes, listed on page 32.

Ordinates: A. Number of grains in each category.
B. %age meristematic activity in each class.

development which these classes represent (page 32) with the time sequence of embryo development observed in this sample of _A. sativa_ (page 34), indicates that there is a close agreement between the commencement of nuclear division and the first signs of visible germination. The useful assumption is made that this is also the case in other samples of _A. sativa_ , used in later experiments. These results are in general agreement with previous studies on Maize (Toole, 1924), and Barley (Smith, 1951).

Summary.

1. The germination performance of various samples of _A. sativa_, _A. fatua_, and _A. ludoviciana_ have been studied under different germination treatments. The most suitable conditions found, and those used in later studies, were 4 mls. of distilled water per plate of 50 grains, a germination temperature of 20°C. for _A. sativa_ and _A. fatua_ or three days at 30°C. followed by transference to 10 - 12°C. for _A. ludoviciana_. Pricking the grain before imbibition has been shown to be a satisfactory method of breaking seed dormancy in these last two species.

2. Seed dormancy in _A. sativa_ and _A. fatua_ has been shown to develop as the seed ripens and to diminish naturally in dry storage over a period of several months. The amount of dormancy developed, and its persistence after harvest seem to be varietal characteristics in both species. A similar trend is indicated in _A. ludoviciana_.

3. Meristematic activity in germinating embryos of _A. sativa_ has been shown to follow a period of embryo growth by the extension of existing embryo cells, and to be apparent at approximately the same time as germination is observed (see page 17).

Part II. The Uptake of Water by Whole Grains of A. sativa during Imbibition, Germination, and Early development, and the Effects of Dehydrating Grains at Various Times During this Period on Their Capacity to Resume Normal Germination and Development.

It is usually considered that germination in a seed follows a sequence of phases - a physical imbibition of water, leading to a stimulation of physiological activity, which in turn leads to the resumption of growth in the resting embryo. How quickly physiological activity increases after the commencement of imbibition is not known, nor is the nature of the activity well understood. The manner in which the necessary conditions for the resumption of growth are brought about is as well understood as the nature of the growth process itself. It is essential, however, for the continuity of these phases of germination, and for the maintenance of growth once it has commenced, that the seed should not only remain imbibed, but that it should continue to have available more water than is required for the mere saturation of the dry seed constituents. This additional water is utilised metabolically in hydrolytic reactions, and in the development of vacuoles in the enlarging embryo cells. The pattern of water uptake in a sample of grains of A. sativa has therefore been studied with the view of finding out if there is any evidence to suggest when this second 'physiological' phase of water uptake commences.

There is also little knowledge available concerning the effects of interrupting the sequence of phases preceding and during germination by means of drying the imbibed seed back to the dry condition - a situation

which must occur fairly frequently in nature. The capacity of such a treated seed to resume germination when subsequently placed in germination conditions has been used here as a means of assessing the effects of this type of treatment. It is possible that a treated seed might show no capacity for further germination at all. This might be due to damage to some essential attribute required in germination, such as the cellular integrity of the embryo, or to a loss of some physiological powers. This same explanation might cover the case where subsequent germination proved abnormal, or exceedingly slow. In the studies on the re-establishment of dormancy in A. fatua (Expt. 8.), this latter effect was probably the reason for the reimposition of dormancy, though it should be remembered that special imbibition conditions were necessary before this would occur. It is also possible that subsequent germination might occur more quickly than in an untreated seed, from a few hours quicker to the whole length of the previous period of imbibition. This is probably due to a retention through the drying treatment of some or all of the progress made towards germination in the first period of imbibition. The third possibility is that the germination of a seed treated in this manner would be exactly similar to that of an untreated seed. This could only mean that any progress towards attaining germination during the first period of imbibition was not only halted, but underwent a regression, like the water content of the grain, to the dry seed condition, i.e. some of the phases of germination would be reversible.

The type of effect produced on a seed by drying it after various times in the imbibed condition would vary with the type of processes occurring in the seed at the time of drying. Growth by its nature is

an irreversible process, so if the seed at the time of drying had commenced embryo growth some residual effect of the previous period of imbibition would be expected. If physiological processes are going on at the time of drying, the results of these activities may not be reversible either, though the drying of the seed might affect the future development of the same processes, This is also true of physico-chemical processes such as occur sometimes when colloids undergo imbibition, and are never afterwards capable of returning to the original structure, i.e. hysteresis. If only physical processes are taking place however, such as the dissolution of crystalline materials, wetting of non adsorbent surfaces, etc., it would seem possible that the drying of the seed could at the same time cause a reversal of these changes to the condition found in the original dry seed. If any residual effects of drying a seed are evident when the seed is subsequently germinated, it would be expected that some evidence of them could be found by examining the germination behaviour of the treated seed, and comparing this, and the seed's composition and physiological activities with untreated seed.

In the following pages, studies on the effects on the subsequent germination of drying grains of A. sativa at various times during imbibition and germination, and early development are reported. The nature of these effects, their persistence, and the probable causes of them were also investigated. From these studies it is hoped to find out if there is any phase of germination in this species which is reversible, and to gain some further knowledge concerning the processes going on in the seed at various times before and during germination.

Materials and Methods.

The grains used in these studies were of A. sativa var. Ayr Bounty, several batches being used at different times, but all conforming to a similar germination performance and giving 90-95% germination within 120 hours under our standard germination conditions.

In the experiments on the reversibility of germination, the first germination treatment took place in enamel trays, 12" x 10", with a weighed batch of seed spread thinly and evenly on a single sheet of Whatman No. 1 filter paper. 40 mls. distilled water was added for each 35 grams of grain present, this being the approx. 1000 seed weight of this sample. The germination of the grains following a dehydration treatment, took place after two weeks storage over $CaCl_2$, 50 grains being germinated per petri dish, with 4 mls. distilled water. Germination counts were carried out using a x 16 binocular microscope, care being taken to observe any abnormal germinations. Each ungerminated grain after 120 hours was carefully examined for signs of embryo damage.

Moisture content was measured as the loss in weight of Surface Dried Grains, (Expt. 16) after two days at 92°C., and expressed as a %age of the final dry weight.

The measurement of Amylase activity was carried out using a modified Smith and Roe technique (1952). 10 mls. of 0.1% soluble starch solution, 5 mls. of Clark and Lub's potassium-acid-pthalate buffer, pH 5.0, and a brei of 10 grains in 5 mls. distilled water were incubated together at 30°C. From samples at various times the rate of starch digestion was

followed by measuring the change in the Starch-Iodine colour at 620 mu spectrophotometrically. Further details are given in a later section (Page 89).

The soluble "tyrosine" level was measured on the supernatant after centrifuging a mixture of 5 grains ground in 10 mls. 6% Trichloroacetic acid, which had been standing for 1 hour at room temperature. A 2 ml. sample was added to 4 mls. 2% NaOH, and 1 ml. of diluted Folin and Ciocalteau's Phenol reagent added with swirling. The colour developed after 10 mins. was measured at 650 mu spectrophotometrically, and compared with known tyrosine standards. This reaction occurs with free tyrosine and tryptophan, and any unprecipitated peptides containing them, and an increase in the value is regarded as evidence of proteolytic activity in the grain. Further details are given in a later section (Page 143).

<u>Results.</u> <u>Experiment 15.</u> The course of water uptake by grains of <u>A. sativa</u>, var. Ayr Bounty, under standard germination conditions.

Batches of 100 grains were germinated in petri-dishes, with 6 mls. distilled water. At suitable intervals, three samples were taken, surface dried, and their moisture content determined as previously described. The values obtained are listed below.

Length of imbibition period.	Moisture content, % of D.W.	Mean Moisture Content.	Length of imbibition period.	Moisture content % of D.W.	Mean Moisture Content.
4 hrs.	36.2		23 hrs.	58.6	
"	37.6		"	57.3	
"	35.8	36.5	"	56.6	57.5
6 hrs.	39.8		27 hrs.*	65.8	
"	40.5		"	65.0	
"	39.2	39.8	"	66.3	65.7
8 hrs.	42.2		29 hrs.	70.5	
"	42.4		"	69.8	
"	43.7	42.8	"	66.3	68.9
12 hrs.	48.6		34 hrs.	75.5	
"	46.8		"	78.8	
"	48.7	48.0	"	76.6	77.0
14 hrs.	49.6		36 hrs.	80.3	
"	48.4		"	79.5	
"	48.8	48.9	"	81.0	80.3
17 hrs.	49.6		41 hrs.	85.8	
"	52.7		"	84.6	
"	51.5	51.3	"	84.1	84.8

* First signs of apparent germination.

Experiment 16. The efficiency of a surface drying technique in removing superficial moisture.

In measuring the moisture content of grains of *A. sativa*, the water which is present on the surface of the seed, and within the husk represents a fraction which is not involved in the processes of the seed itself. A suitable method of removing as much as possible of this superficial moisture was investigated. 4 samples of 100 whole grains were weighed, and then immersed in 10 - 15 mls. distilled water, and shaken in this for several minutes. After weighing the grains were

spread on a sheet of dry blotting paper, and covered by another sheet, and then gently rubbed between both. This process was repeated twice with new sheets of blotting paper each time, following which they were spread out on another dry sheet and exposed to the atmosphere for 5 - 10 mins. After re-weighing the increases in weight were determined, and these are given below as a %age of the initial air-dry weight.

	Increase in weight over original. Range.	Mean.
After immersion.	10 - 15%	12.5%
After surface drying.	2.9 - 4.1%	3.3%

Surface drying by this procedure was used throughout the following experiments.

Experiment 17. The efficiency of a vacuum desiccation technique, using $CaCl_2$, as a quick means of reducing the moisture content of imbibed samples of whole grains of A. sativa.

In a preliminary experiment in which a water pump was used as the source of reduced pressure, the feasibility of obtaining a suitable technique which would dry samples of 100 - 200 whole grains of A. sativa from moisture contents of 60 - 80% to those of 15 - 20% was indicated. Using that technique, a period of more than 12 hours dehydration was necessary. In the present investigation a manually operated vacuum pump was employed which allowed pressures of 10 - 15 mms. of Mercury to be employed, that is a vacuum of approx. 98% of atmospheric pressure. In this experiment batches of approx. 200 grains were allowed to imbibe

at 26°C. for 19 - 20 hours in the presence initially of 8 mls. distilled wate r. After this period they were surface dried as previously described and reweighed. Each batch was then placed in a 300 ml. glass flask, along with a small aluminium foil canister of anhydrous $CaCl_2$. The flask was sealed with a rubber bung pierced with a glass tube, and the internal pressure of the flask reduced to 10 - 15 mms. mercury by connecting the end of the glass tube to the vacuum pump. A screw clip allowed this pressure to be maintained for considerable periods. At various intervals, flasks were removed and the grains inside removed for weighing, after which they were placed at 92°C. for dry weight determinations. By means of various weighings of the same sample, the air dry moisture content, the moisture content after imbibition, and moisture content after various periods of desiccation were determined. These are given below as %ages of the Dry Weight.

Length of dehydration period.	Air-dry Moisture Content.	Imbibed Moisture Content.	"Dehydrated" Moisture Content.
None.	12.7	63.3	-
"	13.4	70.1	-
0.5 hrs.	12.9	70.0	60.5
"	13.0	71.5	64.1
1.0 hrs.	12.7	70.0	51.4
"	13.4	72.5	59.2
1.5 hrs.	13.2	68.1	49.7
"	13.6	70.1	54.1
2.0 hrs.	13.7	67.5	43.2
"	13.9	66.2	38.4
2.5 hrs.	12.2	70.1	40.2
"	13.8	68.5	33.2
3.0 hrs.	13.4	67.5	25.4
"	13.4	69.0	27.6
4.0 hrs.	13.5	68.5	25.6
"	14.1	70.5	22.6
2.0 hrs.*	12.9	68.0	33.3*
" *	16.1	69.0	30.0*

* Two canisters of $CaCl_2$ used instead of one.

This procedure was modified in some respects in its later use, the main modifications being: a) The replacement of aluminium foil containers by 5 ml. pyrex beakers, due to occasional leakage from the former. b). The regular inclusion of two containers of anhydrous $CaCl_2$ per flask. c) The maintenance of the evacuated flasks at 30°C. to avoid condensation of water vapour on the sides of the flask. d) A restriction of the numbers of grains per flask to approx. 100 whenever possible. The normal length of dehydration period used was 3 hours, and following this the grains were then removed to shallow trays in a $CaCl_2$ desiccator for two weeks before further use. The results of such

a treatment, in terms of the moisture content of the grains after a dehydration treatment and two days storage in the desiccator are given below, both the imbibed and the "dehydrated" moisture contents being expressed as a %age of the dry weight.

Length of Imbibition.	Imbibed Moisture Content.	"Dehydrated" Moisture Content.
4 hrs.	32.8	10.0
12 hrs.	48.5	10.1
20 hrs.	58.5	9.8
26 hrs.	66.0	9.8
32 hrs.	69.0	10.0
	Air-dry moisture content,	13.4

In the following experiments, the cycle of treatments on a batch of whole grains from dry grain through imbibed grain, dehydrated grain, and dry storage is referred to as a Germination Reversal treatment, and is defined by the length of the previous imbibed period.

Experiment 18. The subsequent germination performance of grains of *A. sativa*, var. Ayr Bounty after single reversal treatments of up to 36 hours.

Batches of approx. 400 grains were allowed to imbibe and germinate for periods of 1, 2, 4, 6, 8, 12, 16, 20, 24, 30, and 36 hours before receiving the dehydration part of the germination reversal treatment. 8 plates of 50 grains were tested from each sample after the two weeks dry storage, the germination figures being tabulated below as the mean

number germinated per 50 at the stated time. The number of grains ungerminated per plate, and the number of these showing embryo damage was also recorded for each treatment, these being again expressed as mean no. per 50.

Previous Imbibition Period, hrs.	Mean no. germinated / 50 grains at,						Number Ungerm'd. @ 120hrs.	No. with embryo damage
			41.	47.5.	66.	120 hrs.		
0			24.3	35.0	42.9	48.3	1.8	1.3
			42.	48.	65.	120 hrs.		
2			25.6	34.5	42.1	48.1	1.7	1.3
			42.	47.	65.5.	120 hrs.		
4			26.1	34.1	41.5	47.8	2.1	1.3
			41.	47.5.	65.	120 hrs.		
6			27.5	34.0	41.0	47.8	2.3	0.9
		24.	40.5.	52.3.		120 hrs.		
8		3.5	30.1	41.0		47.6	-	-
		23.7.	41.0.	51.7.		120 hrs.		
12		6.0	32.8	39.6		47.0	-	-
		23.2.	41.5.	50.2.	69.2.	120 hrs.		
16		9.4	37.5	40.0	43.8	47.0	3.0	1.5
	18.	23.	42.	51.	69.	120 hrs.		
20	1.5	11.8	37.1	40.0	42.5	46.9	3.1	2.3
	17.5.	22.5.	41.5.	51.2.	68.7.	120 hrs.		
24	9.0	16.0	31.8	35.8	40.0	45.8	4.3	2.0
	17.0.	23.0.	40.5.	51.7.	69.1.	120 hrs.		
30	7.5	15.3	30.5	34.0	38.0	43.0	7.0	4.5
	17.0.	22.0.	42.0.	51.5.	68.5.	120 hrs.		
36	6.9	14.0	25.4	28.0	32.0	34.6	15.4	12.0

Experiment 19. The germination performance of grains of A. sativa, var. Ayr. Bounty after repeated reversal treatments of 12 - 32 hours.

Batches of approx. 1500 grains were started through a series of three germination reversal treatments, with periods of 12, 20, 26, and 32 hours imbibition, respectively. At the end of each storage period, 8 plates of fifty grains were germinated from each sample. The mean number germinated / 50 grains is listed below for each count.

1 Germination Reversal Treatment

Previous Imbibition Period.	Mean germination / 50 grains at,					
	24.	32.3	47.	54.	73.	96 hrs.
None.	0.1	4.8	31.5	38.5	46.3	47.8
	25.3.	31.5.	48.5.	54.5.	72.5.	96 hrs.
12 hours.	7.0	21.6	38.5	42.6	46.9	48.4
	25.8.	30.5.	50.0.	54.8.	72.0.	96.5 hrs.
20 hours.	16.1	24.9	39.4	44.1	46.9	47.5
	24.0.	30.0.	49.5.	55.0.	71.8.	98.0 hrs.
26 hours.	12.4	20.3	31.3	35.1	40.1	42.1
	23.0.	29.5.	49.0.	55.3.	71.5.	96.8 hrs.
32 hours.	8.1	13.3	24.4	29.8	35.5	40.4

Expt. 19 cont'd.

2 Germination Reversal Treatments

Previous Imbibition Period.	Mean germination / 50 grains at,					
	18.3.	24.3.	44.8.	49.4.	64.3.	89.0 hrs.
12 hours.	11.9	26.7	40.4	42.7	45.6	47.0
	17.0.	23.7.	45.0.	49.2.	65.0.	90.0 hrs.
20 hours.	12.9	20.3	33.9	34.4	41.1	43.1
	16.0.	24.0.	41.3.	48.0.	65.6.	89.0 hrs.
26 hours.	5.3	10.6	14.0	15.5	19.1	22.1
	16.5.	23.2.	40.5.	49.0.	66.6.	88.5 hrs.
32 hours.	5.4	8.0	9.0	9.5	9.7	11.0

3 Germination Reversal Treatments

Previous Imbibition Period.	18.0.	23.5.	41.0.	48.0.	66.0.	120 hrs.
12 hours.	6.6	13.4	21.2	31.6	40.0	42.6
	18.5.	24.3.	41.5.	48.3.	67.0.	120 hrs.
20 hours.	3.5	6.1	9.6	10.5	12.4	17.4
	19.3.		45.0.		67.5.	120 hrs.
26 hours.	1.4		1.8		2.0	2.0
	17.5.		45.5.		67.5.	120 hrs.
32 hours.	0		0.3		0.8	0.9

A microscopic examination was carried out of all ungerminated grains at 120 hours, and of any grain showing abnormal germination during the test. The number of grains in which embryo damage was observed, and the number of these with damage extending to the plumule bud was recorded for each sample. These figures, expressed as the mean number / 50 grains, are given in the following table.

Expt. 19 cont'd.

Previous Imbibition Periods.	Number Ungerminated at 120 hours.	Number showing Embryo damage.	Number showing Plumule damage.
None.	2.2	0.5	-
12 hrs.	1.6	0.4	-
20 hrs.	2.5	0.5	-
26 hrs.	7.9	4.4	-
32 hrs.	9.6	6.9	-
2 x 12 hrs.	3.0	2.6	-
2 x 20 hrs.	6.9	6.0	-
2 x 26 hrs.	27.9	20.6	-
2 x 32 hrs.	39.0	26.2	-
3 x 12 hrs.	7.3	3.3	0.5
3 x 20 hrs.	22.6	21.0	8.0
3 x 26 hrs.	48.0	29.1	11.0
3 x 32 hrs.	49.1	42.1	18.8

The surface dry moisture content of the samples at the beginning of each dehydration treatment was also recorded, and is given below as a %age of the dry weight.

Number of Imbibition Periods.	Moisture contents after repeated periods of imbibition of,			
	12 hrs.	20 hrs.	26 hrs.	32 hrs.
1.	48	59	66	69
2.	46	65	74	79
3.	62	73	77	77

Experiment 20. The germination performance of grains of *A. sativa*, var. Ayr Bounty, after repeated reversal treatments of 1 - 6 hours.

A series of germination reversal treatments was begun, involving imbibition periods of 1, 2, 4, and 6 hours, approximately 3000 grains being used in each treatment. Samples for testing the effects of the cumulative reversal treatments were taken at the end of each two week storage period, each sample comprising 8 plates of fifty grains. The germination performance of these samples is given below as the mean number germinated / 50 grains at the various times.

1 Reversal Treatment

Previous Imbibition Period.	Mean germination / 50 grains at,				
	24.	41.	47.5.	66.	132 hrs.
None.	0	24.25	35.0	42.9	48.3
	24.	42.	48.	65.	132 hrs.
2 hours.	0	25.6	34.5	42.1	48.1
	24.	42.	47.	65.5.	132 hrs.
4 hours.	0	26.1	34.1	41.5	47.9
	24.	41.	47.5.	65.	132 hrs.
6 hours.	0	27.5	34.0	41.0	47.8

Experiment 20 (cont'd.)

2 Reversal treatments

Previous Imbibition Period.	Mean germination / 50 grains, at,					
	22.3,	28,	47,	51.7,	75,	130 hrs.
1 hour.	0	2.4	33.1	38.4	45.5	48.3
	22,	27.6,	46,	52,	74.9,	130 hrs.
2 hours.	0	4.4	32.4	38.5	45.8	48.4
	21.7	27.3,	46.5,	52.3,	74.5,	130 hrs.
4 hours.	0.3	7.8	34.5	39.1	45.3	48.3
	21,	27,	45.2,	51.3,	74.7,	130 hrs.
6 hours.	3.3	13.0	34.3	39.5	44.9	47.6

3 Reversal treatments

Previous Imbibition Period.	Mean germination / 50 grains, at,					
	21,	28.7,	47.0	51.1	74.2,	130 hrs.
1 hour.	0	6.0	33.4	38.0	43.4	47.4
	21,	28,	47.3,	52.0,	74,	130 hrs.
2 hours.	0	11.3	34.0	38.6	43.6	47.0
	21.3,	28.5,	44.5,	52.3,	73.5,	130 hrs.
4 hours.	0.8	15.6	32.5	39.4	44.4	47.7
	20.6,	27.8,	44.8,	51.6,	73.8,	130 hrs.
6 hours.	3.0	14.5	33.8	36.6	43.4	47.8

Experiment 20 (cont'd.)

4 Reversal treatments

Previous Imbibition Period.	Mean germination / 50 grains, at,					
	22.	27.7.	46.3.	51.6.	70.	130 hrs.
1 hour.	0	8.3	33.7	39.0	44.4	47.2
	22.3	28.	45.6.	51.2.	70.2.	130 hrs.
2 hours.	1.3	13.1	33.7	38.4	44.9	47.9
	21.2.	28.5.	44.5.	51.8.	69.5.	130 hrs.
4 hours.	4.9	19.0	37.0	40.8	45.0	47.2
	20.5.	28.3.	45.2.	52.0.	69.7.	130 hrs.
6 hours.	6.6	17.5,	34.1	38.4	43.9	46.4

5 Reversal treatments

Previous Imbibition Period.	Mean germination / 50 grains, at,					
	21.3.	27.5.	44.5.	51.3.	76.	130 hrs.
2 hours.	1.8	14.5	34.4	38.6	44.4	47.0
	21.	28.2.	45.	51.5.	75.5.	130 hrs.
4 hours.	7.4	20.8	34.6	38.6	44.3	47.0
	20.5.	28.5.	45.5.	52.3.	75.8.	130 hrs.
6 hours.	8.0	18.2	32.0	36.2	40.6	44.8

Experiment 20 (cont'd.)

6 Reversal treatments

Previous Imbibition Period.	Mean germination / 50 grains, at,					
	22,	28.5,	46.5,	-	70,	130 hrs.
1 hour.	0.8,	10.4,	32.4	-	42.6	47.0
	21.5,	28.0,	44.5,	-	69.5,	130 hrs.
2 hours.	3.4	17.4	33.0	-	42.8	46.4
	21,	27.5,	46.6,	-	69,	130 hrs.
4 hours.	5.3	19.8	31.8	-	41.9	46.0
	20.5,	27.0,	45,	-	69.7	130 hrs.
6 hours.	5.4	15.1	27.0	-	31.5	39.0

8 Reversal treatments

Previous Imbibition Period.	Mean germination / 50 grains, at,					
	21,	27,	41,	48.2,	65,	130 hrs.
1 hour.	1.3	10.8	31.3	35.6	41.9	46.6
	21,	27.3,	42,	47.6	65.3	130 hrs.
2 hours.	3.0	18.0	33.0	35.8	41.1	45.4

The moisture content of the various samples was recorded after the surface drying treatment, on removal from the imbibing conditions. The values are given below as a %age of the dry weight.

Number of Imbibition Periods.	Moisture contents after repeated periods of imbibition of,			
	1 hr,	2 hrs,	4 hrs,	6 hrs.
1	31.6	-	35.6	40.1
2	35.6	39.4	39.2	45.4
3	36.6	41.3	45.0	48.0
4	40.3	43.4	52.5	50.4
5	43.6	44.7	52.5	55.6
6	No samples available.			

Plate [illegible].

Class 1. Class 2.

Class 3.

Class 4.

Class 5.

Typical samples of the grains of <u>A. sativa</u> in each of the development classes outlined in page 59.

Experiment 21. The effect of a single germination reversal treatment on grains of A. sativa, var. Ayr Bounty, at different stages of development.

Batches of grains were germinated under standard conditions for 72, and 96 hours, by which time they were showing 86% and 92% germination respectively. The grains in each sample were then grouped into classes on the basis of their degree of development, and each group separately given a germination reversal treatment. The development classes chosen were :-

Class.	Root development.	Plumule development.
1	2 or more seminal roots.	Longer than husk.
2	2 or more seminal roots.	Not evident.
3	1 seminal root, $>$ 2 mms.	" "
4	1 seminal root, $<$ 2 mms.	" "
5 and 5a	Ungerminated (see text below).	

There is present in this untreated sample of A. sativa a small proportion of grains which do not germinate, or germinate abnormally. This amount would naturally be classified among those in Class 5, as defined above. In this sample of grains this fraction amounts to 4 - 5% of the total. Therefore, with the numbers present it would amount to 16 - 20 grains in the 72 hour sample, and 9 - 12 gfains in the 96 hour batch. A Class, 5a, is therefore included in the following tables by subtracting these figures from the numbers in Class 5 showing embryo damage after 120 hours subsequent germination. It is felt that this is a true assessment of the ability of ungerminated, but fully germinable, grains to respond to a germination reversal treatment. At

the end of the two week storage period, the batches were allowed to germinate for 120 hours, at which time the number showing normal germination, the number showing embryo damage, confined to the root system, and the number showing complete embryo damage was recorded. Grains which did not g erminate at this time but showed no obvious sign of embryo damage are grouped with those showing only root damage.

The results of these investigations are given below, for the two separate batches.

a). 72 hours germinated sample.

		Subsequent Germination performance.					
	Number	Normal Germ'n.		Root Damage only.		Complete Embryo Damage.	
Class.	in Class.	Number of Grains.	% of Class.	Number of Grains.	% of Class.	Number of Grains.	% of Class.
1	24	0	0	1	4.2	23	95.8
2	105	0	0	10	9.5	95	90.5
3	140	0	0	14	10.0	126	90
4	70	14	20	8	11.4	48	68.5
5	57	28		6		13	
5a	28	28	100	0	0	0	0
	396	42		39		305	

b). 96 hours germinated sample.

		Subsequent Germination performance.					
	Number	Normal Germ'n.		Root Damage only.		Complete Embryo Damage.	
Class.	in Class.	Number of Grains.	% of Class.	Number of Grains.	% of Class.	Number of Grains.	% of Class.
1	30	0	0	0	0	30	100
2	145	0	0	11	7.5	134	92.5
3	34	1	3	6	17.5	27	80.5
4	8	2	25	1	12.5	5	62.5
5	20	10		2		8	
5a	10	10	100	0	0	0	0
	237	13		20		204	

<u>Experiment 22.</u> The persistence of the effects of a single germination reversal treatment on the grains of <u>A. sativa</u>, after 15 weeks in dry storage.

Sample of 100 - 200 grains of the batches given a single germination reversal treatment of 8, 12, and 30 hours in Experiment 18, were stored dry in the laboratory for a period of 15 weeks. Two plates of fifty grains from each sample were tested for their subsequent germination performance, the results being listed below as the mean number germinated / 50 grains at the various times.

Previous Imbibition Period.	Mean number germ'd / 50 grains, at, 17.	27.	42.	120 hrs.
8 hours.	0	8	32	48
12 hours.	0	14	34	48
30 hours.	7	18.5	31.5	44

<u>Experiment 23.</u> The effects of the handling procedures in a series of repeated germination treatments on the subsequent germination performance of <u>A. sativa</u>.

The possibility that some of the effects of repeated germination reversal treatments might be due to the handling received by the grains during the treatment was investigated. Observation indicated that there was an occasional time when a few grains had their husks displaced by the drying treatments. Accordingly, a batch of 300 - 400 grains were put through a series of treatments consisting of an imbibition

period of two minutes shaking in distilled water, the normal surface drying technique, and a dehydration period of five minutes for the first three cycles, and the fifth, sixth and seventh cycles. The dehydration treatments after the fourth and eighth cycles were of the normal duration. Storage treatments were missed out completely. Samples of three plates of fifty grains were taken after the fourth and eighth dehydration treatment and their germination performance tested under standard conditions. The mean number germinated / 50 grains is given below for the various times.

Number of cycles received.	Mean number germ'd. / 50 grains, at, 20.	30.	44.	120 hrs.
4	0	7.3	31	47
8	0	9.3	33.3*	46.6
None	0	3.0	30.0	47.8

* Counted at 46 hours.

Experiment 24. The rate of moisture uptake by grains of *A. sativa* which have previously experienced a 20-hour germination reversal treatment.

A batch of grains of *A. sativa* were given a germination reversal treatment including 20 hours imbibition. During the two week storage period, they were stored in the same desiccator as a similar sized batch of untreated seed, in order to allow equilibration of moisture contents between the two. Both batches were germinated in trays as previously described, care being taken that equivalent amounts of water were added,

and after five hours imbibition the surface-dry, moisture content of both batches was determined on 10 samples of 50 - 100 grains. These moisture contents, as a %age of the dry weight are given below.

	Moisture Contents.					Mean.
Untreated Grains.	31.6,	34.6,	32.8,	31.6,	28.4,	
	32.7,	30.3,	31.3,	32.6,	31.2,	31.6%
Treated Grains.	34.8,	35.2,	38.1,	38.4,	38.7,	
	36.1,	36.8,	35.5,	37.2,	33.8,	36.5%

A t-test on these values showed significance between these means at the 0.05% level of significance.

Experiment 25. The Free Amylase activity, and the Soluble Tyrosine level in grains of *A. sativa* after a dehydration treatment following various periods of imbibition.

These investigations were made on samples of the grains which were used in Experiment 22, to examine the persistence of the effects of a dehydration treatment after imbibition periods of 8, 12, and 32 hours. These tests were carried out after the grains had been in dry storage for 17 weeks, the methods used having been previously described. The rate of starch digestion, which is directly proportional to the free amylase activity, is expressed as the number of mgms. of starch digested at the various times. The soluble tyrosine level is expressed as the concentration of tyrosine in the supernatant in p.p.m.

Experiment 25 (cont'd.)

Previous Imbibition Period.	Amylase activity Mgms. starch digested, at, 20.	50.	90.	150 min.	Tyrosine content of supernatant, p.p.m.
None	2.10	3.64	4.47	5.25	94
8 hours.	2.10	3.60	4.27	5.45	110
12 hours.	2.24	3.92	4.47	5.30	115
30 hours.	3.64	5.32	6.08	6.32	142.

Discussion.

The form of the water uptake pattern by grains of A. sativa during the first 40 hours under standard germination conditions has been investigated (Expt. 15, Fig.12). This shows a period of initial quick increase in the moisture content, starting to level off about 8 hours after commencement, with a period of slow increase from about 12 - 17 hours imbibition. After 18 - 20 hours imbibition the rate of increase picks up again and this faster rate is maintained for the rest of the period. From the investigations previously reported (Expt. 14), the beginnings of embryo growth would be expected about 18 - 20 hours after commencement, and this is seen to correspond to the second phase of water uptake quite well. The first signs of apparent germination in these samples was observed in the 27 hour sample, which compares quite favourably with the 26 - 28 hour value for the same stage in the previous investigation.

In the experiments on the effects of dehydration on grains of A. sativa, after various periods of imbibition and development, the employment of a technique giving a quick reduction in the moisture content

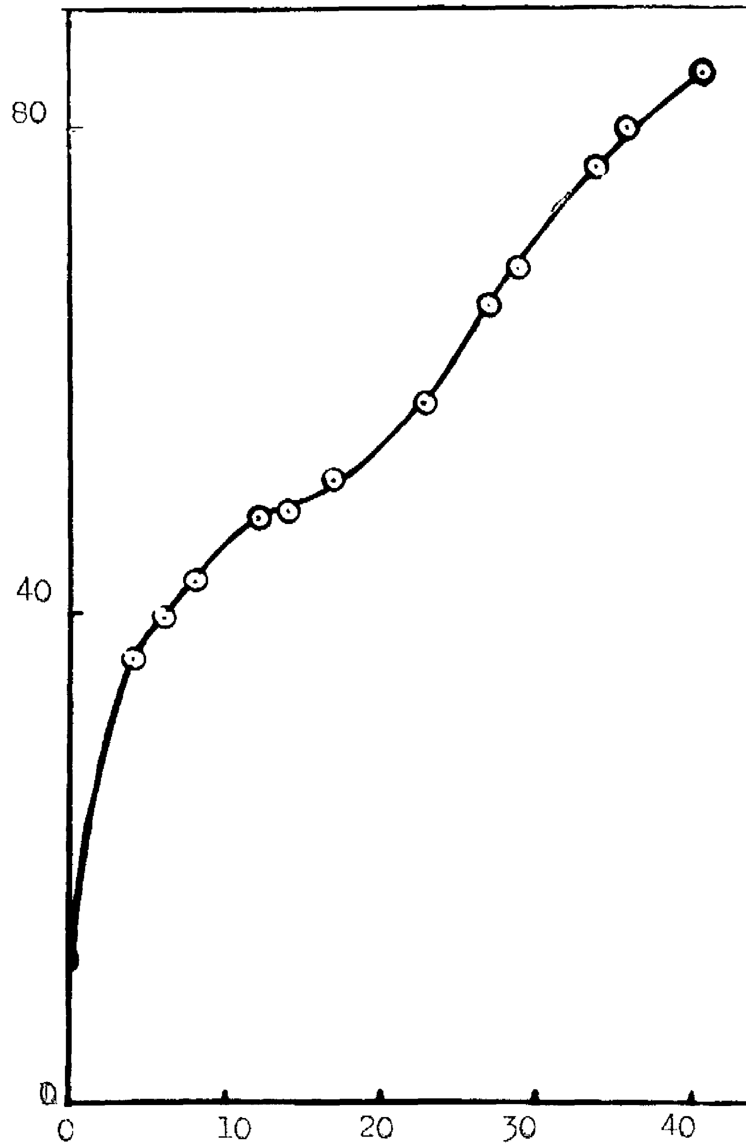


Fig. 12. Water Uptake by grains of A. sativa under standard germination conditions. Abcissa: Period of Imbibition, hours. Ordinate: Moisture content of grains as a %age of dry weight.

of the grains, at ordinary temperatures is necessary if the term "period of imbibition" is to have any finite meaning. This was obtained by surface drying to remove superficial moisture, and the use of reduced-pressure storage in the presence of anhydrous $CaCl_2$ to remove internal moisture (Expts. 16 and 17). By this means the moisture content of treated grains could be brought within a few % of normal air dry weight within about four hours, from moisture contents of up to approx. 70% of the dry weight.

The effects of such a dehydration treatment on the subsequent germination of grains of A. sativa was first investigated using grains that had imbibed for up to 36 hours before the treatment (Expt. 18). The germination curves of the various samples (Fig. 13) showed residual effects of the previous treatments in all samples which had received a previous imbibition period of more than 6 hours. This took the form of an earlier appearance of germination in these samples, with little or no change in the rate of germination in those samples with previous imbibition periods of less than 24 hours. With imbibition periods longer than this, the rate of germination was markedly reduced. These points are brought out in a comparison of the mean number germinated / 50 grains after 30 hours, and 40 hours in germination conditions (Fig. 14). Accompanying this reduction in germination rate, there was also a reduction in the final germination level, after 120 hours in germination conditions. A microscopic examination of the ungerminated grains at 120 hours, and of any grain showing abnormal germination previous to this, showed that in the majority of cases obvious signs of embryo damage were

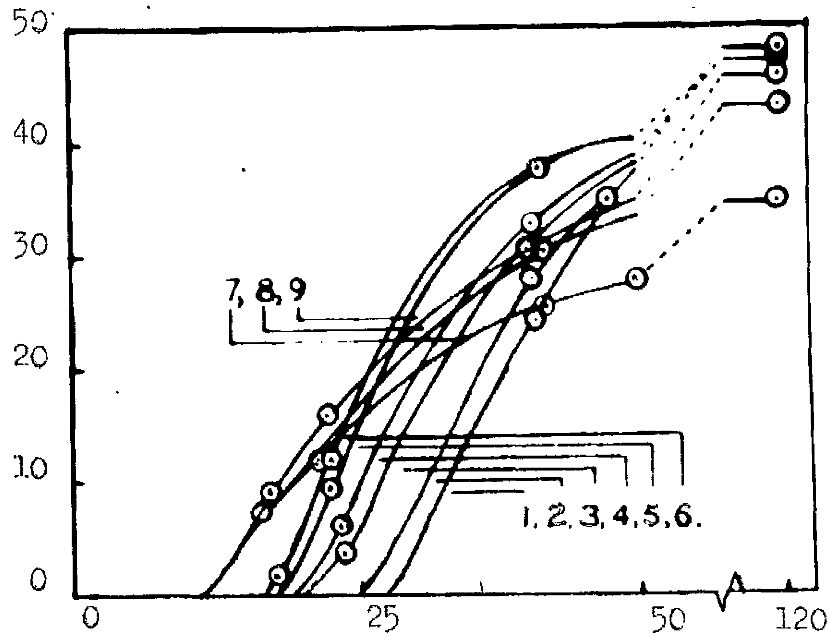


Fig. 13. Germination rates of A. sativa after single germination reversal treatments. Abcissa: Period in germination conditions, hours. Ordinate: Mean no. of grains germinated / 50. Length of previous imbibition period, 1= 0-4 hours, 2= 6 hours, 3= 8 hours, 4= 12 hours, 5= 16 hours, 6= 20 hours, 9= 24 hours, 8= 30 hours, 7= 36 hours.

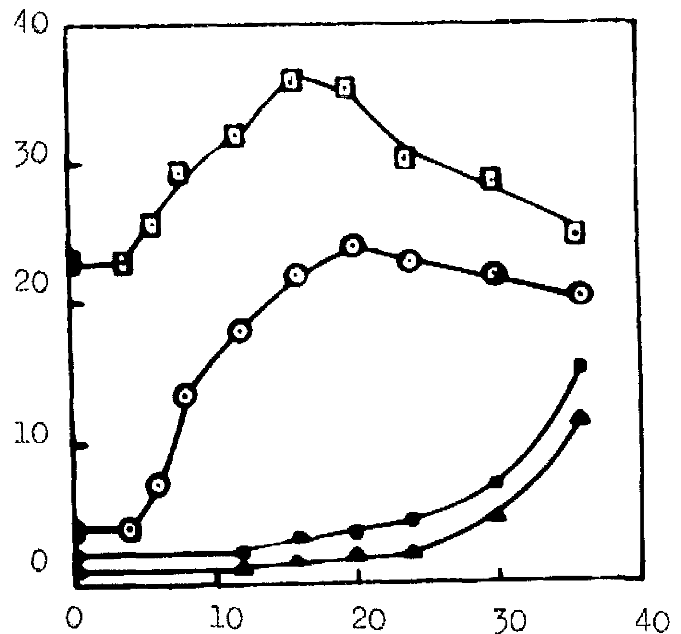


Fig. 14.
Germination of A. sativa after single germination reversal treatments, II.
Mean no. of grains germinated / 50 at 30 hours, (⊙), and at 40 hours, (⊡). Mean no. of grains ungerminated / 50 at 120 hours, (■), and showing embryo damage at 120 hours, (▲).
Abcissa: Length of previous imbibition period, hours.
Ordinate: Mean no of grains / 50.

Discussion cont'd.

evident (Fig. 14). In this experiment, the embryo damage was almost entirely due to necrosis of all or some of the seminal root primordia. The promotion of the onset of germination described here is later referred to in further discussion as the beneficial effect, and the reduction in germination rate and final level as the harmful effects, of dehydration treatments. In this series of tests, the sample which had received 20 hours previous imbibition showed the optimum development of the beneficial effects, associated with no sign of the harmful effects. The general trends shown in this investigation have been found consistently in four previous experiments covering a similar length of imbibition, and including another variety of A. sativa.

Some of the samples used in this experiment were stored in dry conditions to see if any change occurred in the nature, and amount of the residual effects described above, in the interval. The results of germination tests, 15 weeks after the dehydration treatment, showed that no change had occurred in either respect (Expt. 21, Fig. 15).

The effects of multiple reversal treatments with periods of imbibition from 12 - 32 hours, followed by dehydration treatments, were next examined (Expt. 19). The germination curves of the samples after each of three treatments show a further occurrence of the effects described previously (Fig. 16). The single treatment shows the same results as those outlined above. The effects of multiple treatments are to bring forward the optimal beneficial effects to the 12 hour sample, and to introduce or increase the magnitude of the harmful effects in all samples. This is shown in a comparison of the mean number germinated /

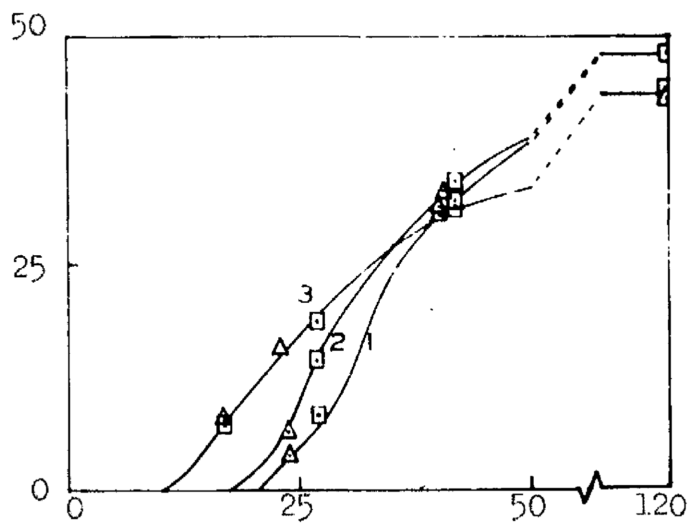


Fig. 15.

The persistence of the effects of a single germination reversal treatment on grains of A. sativa after 2 weeks, (△), and 15 weeks dry storage, (⊡). Length of previous imbibition period, 1= 8 hours, 2= 12 hours, 3= 30 hours.

Abcissa: Period in germination conditions, hours.

Ordinate: Mean no. of grains germinated / 50.

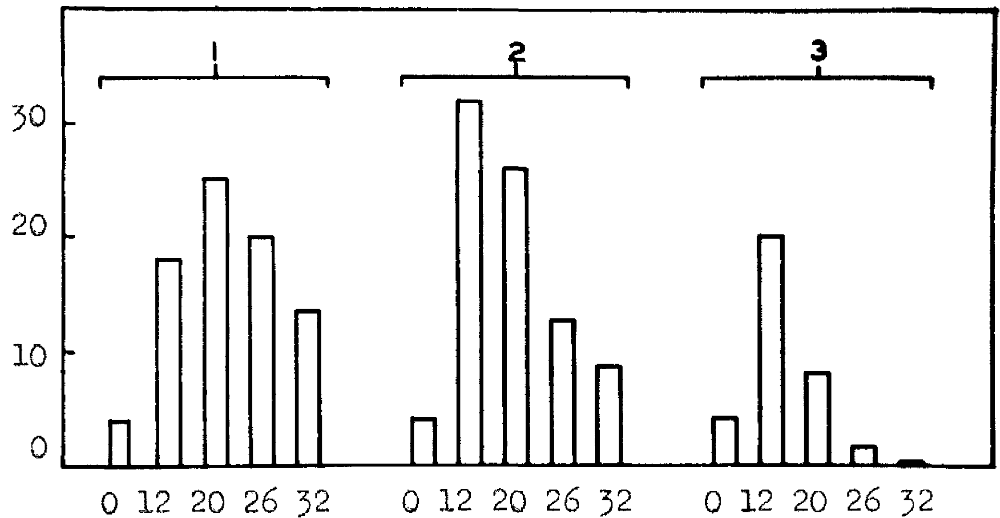


Fig. 17.

The effects of multiple germination reversal treatments on grains of A. sativa, II. Mean no. of grains germinated at 30 hours after 1, 2 and 3 germination reversal treatments.

Abcissa: Length of previous imbibition period. hours.

Ordinate: Mean no. of grains germinated / 50.

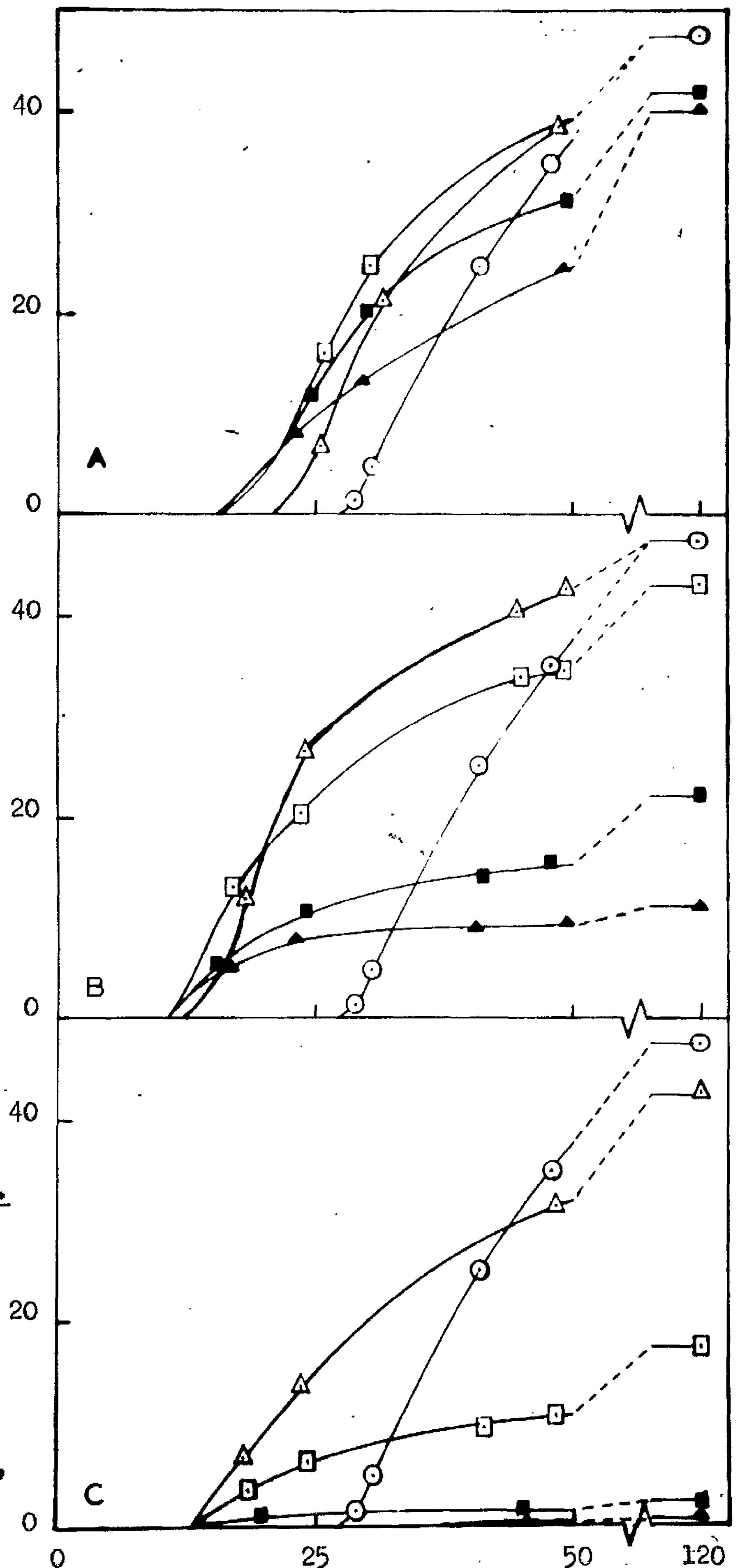
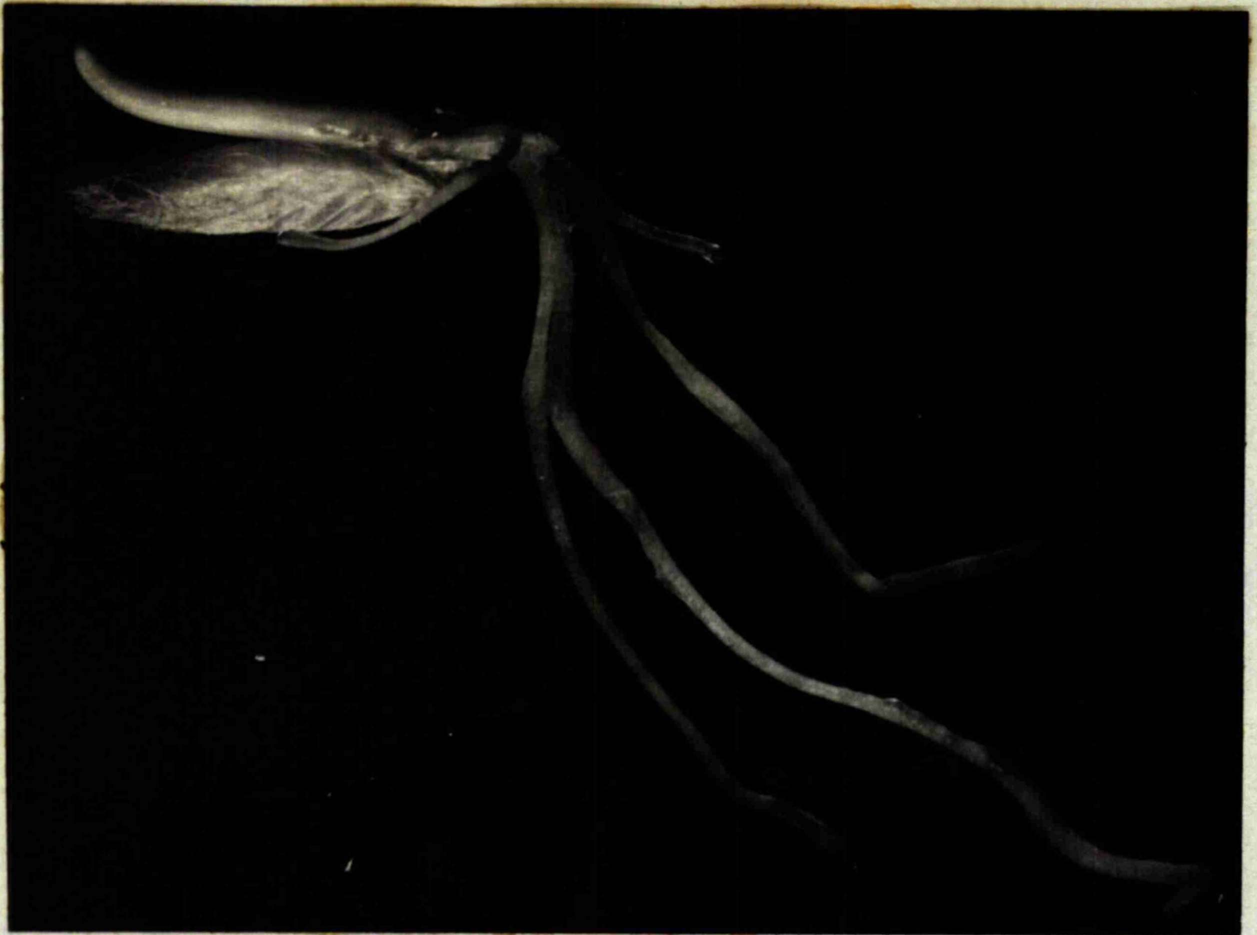


Fig. 16.
The effects of multiple germination reversal treatments on grains of A. sativa.
I. The germination rates of samples after 1, 2 and 3 germination reversal treatments including imbibition periods of 0 hours, (⊙), 12 hours, (△), 20 hours, (⊡), 26 hours, (◫), and 32 hours, (▲).

Abcissa: Period in germination conditions, hours.

Ordinate: Mean no. of grains germinated / 50

Plate 4.



No Damage - Untreated.

Partial Root Damage.

Complete Root Damage.

Complete Embryo Damage.

Types of embryo damage observed on subsequent germination of grains of A. sativa given a germination reversal treatment.

Discussion cont'd.

50 grains after 30 hours in germination conditions (Fig. 17), and even more so by a comparison of the number of seed ungerminated after 120 hours. An examination of these ungerminated grains and of the abnormal types occurring previously, showed again that embryo damage was present in the majority of cases (Fig. 18). As in the previous experiment, much of this damage took the form of necrosis of the seminal root primordia, but after three dehydration treatments in the longer imbibitional periods, there was also a considerable amount of necrosis evident in the plumule (Fig. 18, and accompanying plate).

An examination of these grains ungerminated after 120 hours in germination conditions show the presence in some treatments of considerable numbers of grains which show no evident sign of embryo damage. In order to gain further information on the possible viability of these grains a sample of 26 were replaced in germination conditions for a further 7 days. At the end of this period a further examination showed that 1 of them had developed a single seminal root, 4 were showing slight extension of the plumule but the remaining 22 could only have been classified as showing complete embryo necrosis. This indicates that, while the dehydration treatment is the factor responsible for causing the embryo damage, the obviously visible, blackened necrotic appearance is probably due to autolytic or microbial disintegration of the damaged tissues during the subsequent germination period.

Whatever the direct cause of this necrosis, the result is an impairment of the embryo's capacity for growth and development into a normal seedling. In the case of those grains showing complete embryo

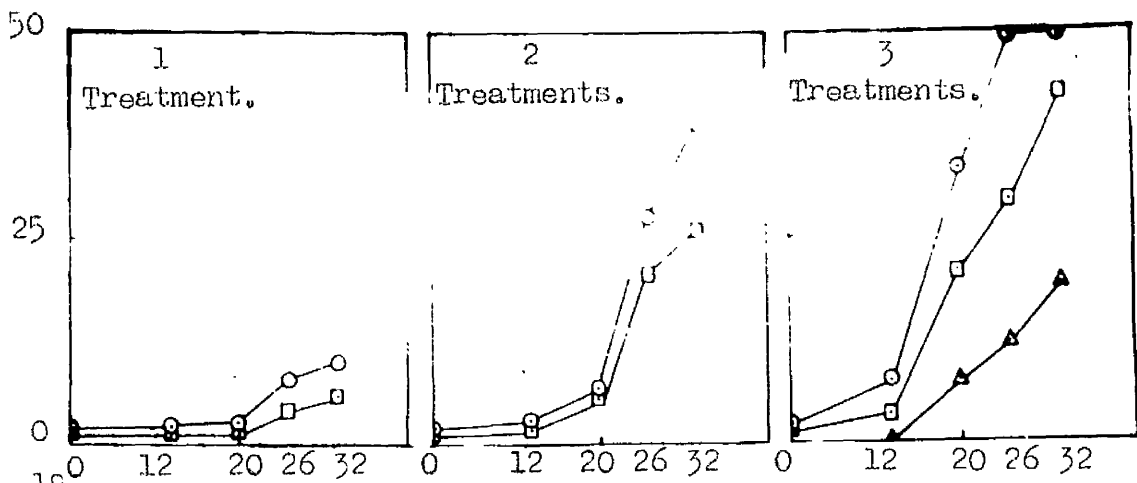


Fig. 18.

The effects of multiple germination reversal treatments on grains of A. sativa. III. The mean no. of grains ungerminated, (⊙), the mean no. showing embryo damage, (⊡), and the mean no. showing plumule damage, (△), after 120 hours subsequent germination.

Abcissa: Length of previous imbibition period, hours.

Ordinate: Mean no. of grains / 50.

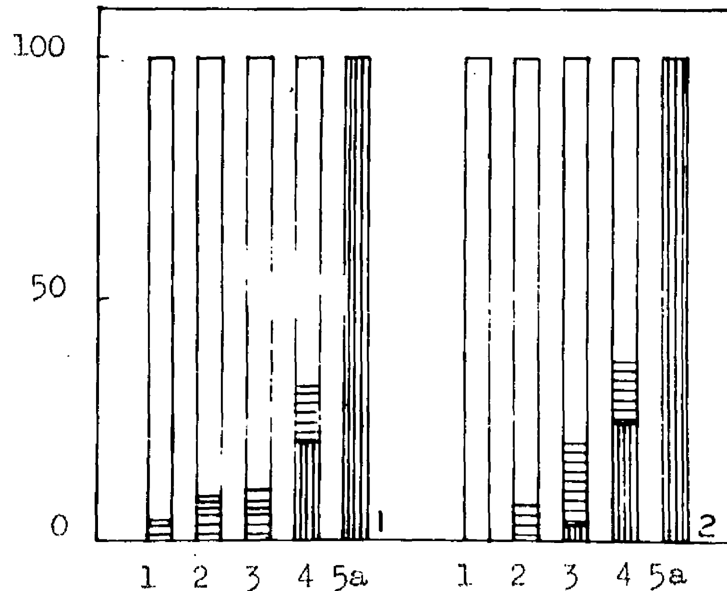


Fig. 19.

The effect of a single germination reversal treatment on grains of A. sativa at different development stages. %age of each class showing normal germination, (▥), root damage only, (☰), and complete embryo damage, (□), after 120 hours subsequent germination. Samples derived from batches of grains after 72 hours, (1), and 96 hours germination prior to treatment, (2).

Abcissa: Development classes.

Ordinate: %age of each class in the previous categories.

Discussion cont'd.

necrosis, this impairment is probably a permanent incapacity for further growth to occur. In those grains in which some of the seminal roots have developed within 120 hours subsequent germination, the chance of a near normal seedling being produced is quite good, though the amount of root development will be considerably restricted for a period. In a large number of cases, however, there was no seminal root development evident within 120 hours, but the plumule was apparently unaffected. The capacity of such grains to establish themselves as viable seedlings was investigated by replacing 26 such grains into germination conditions for a further seven days. Of these, 8 produced no roots by the end of the period, 12 produced one or two seminal roots, and 6 produced one or two seminal roots, and a pair of adventitious roots which formed from the first node of the extending plumule. This indicates a considerable chance of recovery in grains showing this type of embryo damage after 120 hours, depending on the presence of some undamaged seminal root primordia, and the speed at which the adventitious root system becomes developed.

A consideration of the results of these dehydration treatments in respect to the time at which these harmful effects of subsequent germination are first seen (Fig. 14 and 17) indicates that there might be a correlation between a particular stage of development and a susceptibility to dehydration damage. Since these effects are first apparent with previous imbibition periods of 24-26 hours, and our previous observations (Expt. 14) have shown that the embryo by this time has undergone a certain amount of extension growth and is nearing

Discussion cont'd.

the stage of apparent germination, a comparison of the mean number of grains showing the harmful effects of a desiccation treatment and the mean number that would be expected to show visible germination at the time of treatment was carried out using the data of Experiment 19.

The figures of the number of grains exposed to a drying treatment in each sample were derived from the numbers of grains germinated within the period of imbibition in the untreated control, and in the first and second subsequent germination tests of the same sample. The number of seed showing embryo damage is recorded in Expt. 19.

Number of dehydration treatments.	Attribute compared.	Length of imbibition period, 12.	20.	26.	32 hrs.
1	Apparent germination.	0	0	1	7.5
1	Embryo damage.	0.4	0.5	4.4	6.9
2	Apparent germination.	0	5	18	23
2	Embryo damage.	2.6	6.0	20.6	26.2
3	Apparent germination.	3	22	30	31.5
3	Embryo damage.	3.3	21.0	29.0	42.1

While these figures do indicate a high degree of correlation between these two events, they do not prove that the grain showing embryo damage after a dehydration treatment is necessarily that which is showing apparent germination before it. This consideration led to a study of the effects of dehydration treatments on samples of known embryo development (Expt. 21). An examination of these results (Fig. 19) shows a general similarity between the same development classes in the two different samples, and shows quite convincingly that the only grains not

showing embryo damage after a dehydration treatment are those which have not reached the stage of apparent germination (Class 5a), and a few which have just reached it (Class 4). The correspondence between the amount and severity of the embryo damage with the increasing degree of embryo development before treatment is also striking.

The effects of multiple imbibition and dehydration treatments with periods of 1 - 6 hours in germination conditions, was next examined. This gave more information concerning the beneficial effects already described as resulting from some dehydration treatments. The single treatment shows the behaviour already found with these periods of imbibition, viz. a slight beneficial effect with a 6 hour imbibition period, and no difference with the shorter ones (Fig. 20). With another treatment, a beneficial effect is found after two four, and two two hour periods of imbibition, and with three treatments, the one hour sample shows the presence of this effect also. In general, therefore, it would seem that with increasing numbers of treatments, and consequently, increasing total periods of imbibition, there is an increase in the magnitude of the beneficial effects on subsequent germination. This is made more apparent by comparing the mean numbers of germinated grain / 50 after 30 hours germination for each sample in each treatment (Fig. 21). As previously found with single treatments, those samples reaching a total imbibition period of 20 hours show the optimal development of this beneficial effect consistent with the absence of the harmful effects of these treatments. These harmful effects are observed in this experiment, but only to a limited extent, and where

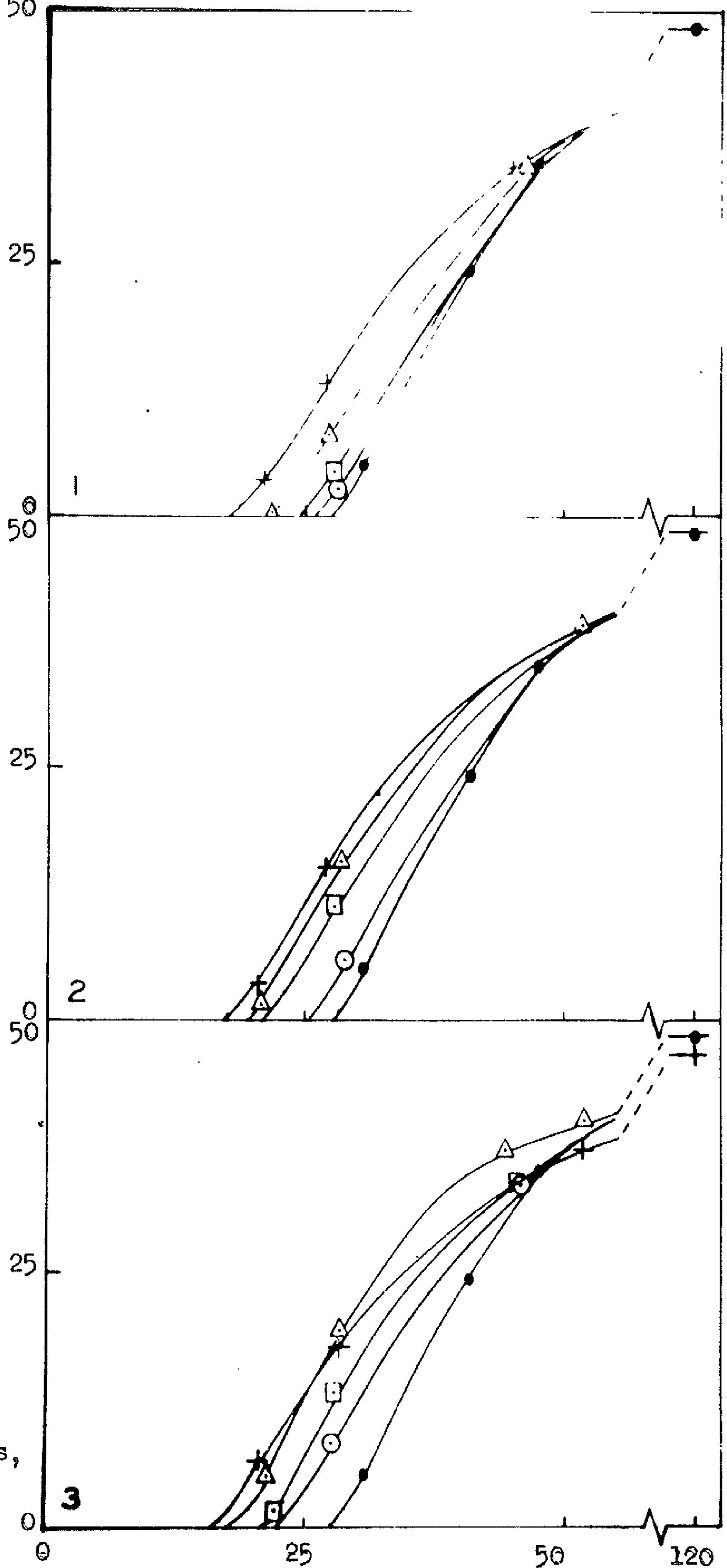


Fig. 20.

The effects of multiple germination reversal treatments on grains of A. sativa.

IV. The germination rates of samples after 2,(1), 3,(2), 4,(3), 5,(4), 6,(5), and 8,(6) germination reversal treatments including imbibition periods of 0 hours,(•), 1 hour, (⊙), 2 hours,(⊡), 4 hours,(△), and 6 hours, (+).

Abcissa: Period in germination conditions, hours.

Ordinate; Mean no. of grains germinated / 50.

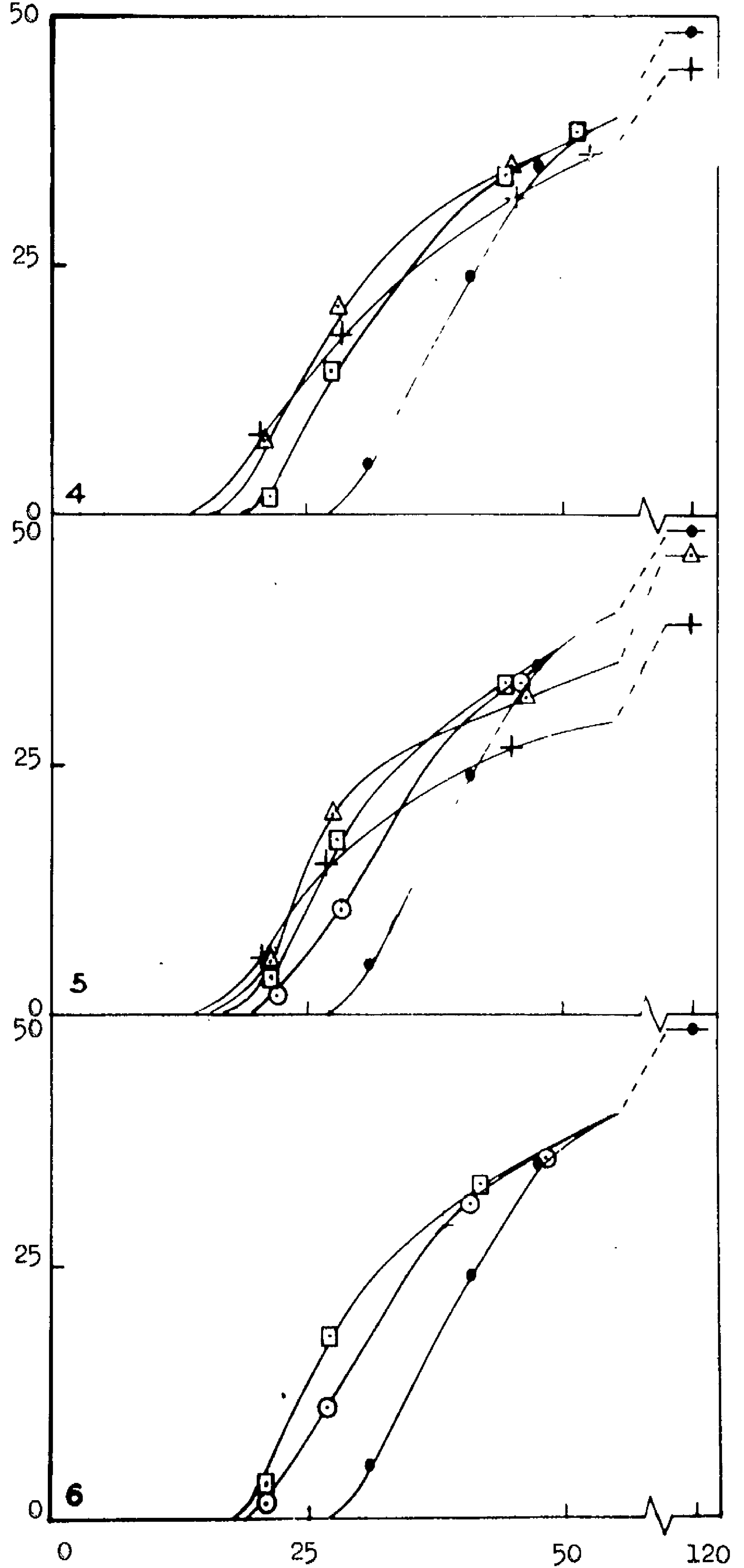


Fig. 20, (cont'd.).

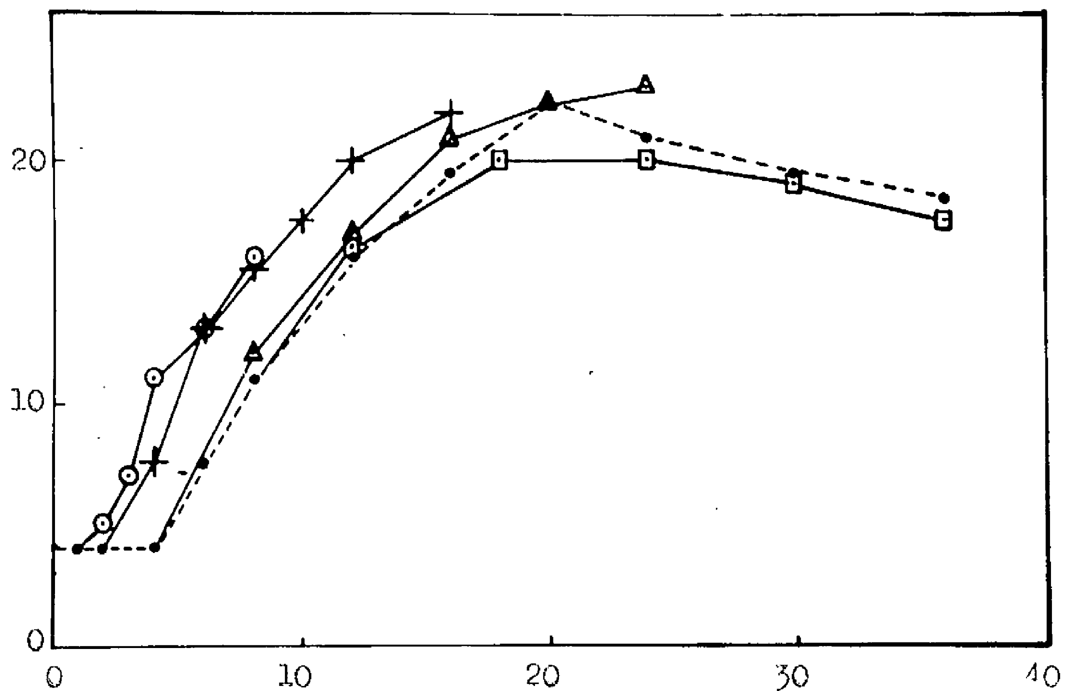


Fig. 21.

The effects of multiple germination reversal treatments on grains of *A. sativa*. V. The mean no. of grains germinated at 30 hours after multiple germination reversal treatments including imbibition periods of 1 hour, (⊙), 2 hours, (+), 4 hours, (△), and 6 hours, (⊡). Broken line represents similar values obtained from samples given a single germination reversal treatment of the same total duration of imbibition.

Abcissa: Total length of previous imbibition period, hours.

Ordinate: Mean no. of grains germinated / 50.

Discussion cont'd.

they do occur, (e.g. 6th treatment, Fig. 20), they show a similar behaviour to that already encountered.

Since this residual beneficial effect is evident with previous imbibition periods of 6 hours, and is showing a considerable magnitude before any apparent phase of embryo growth is seen (Expt. 14), its nature and mode of action must be related to some change preceding the growth phase of germination. There is the possibility that it might be an artefact, produced by the handling techniques used, but it might also be due to those normal changes in the physiological or physical constitution of the grain which occur at this period of imbibition, and whose retention following a dehydration treatment allows for the accelerated germination behaviour which is observed.

It was noted that where single dehydration treatments were employed after imbibition periods of more than 6 hours, the magnitude of the residual beneficial effect increased proportionately to the length of period up to at least 20 hours (Expt. 18, Fig. 14). In this case the same amount of handling was received by all samples, so that the participation of causes other than handling of the grains must be expected. Nevertheless, the possible beneficial effects of the handling treatments alone were investigated (Expt. 23, Fig. 22), and the results showed that some promotion of germination can be caused by effects produced by handling. A comparison of the mean number of germinated grains / 50, after 30 hours in germination condition in these tests, and in some of the tests carried out on samples from previous experiments, shows that the magnitude of the beneficial effect due to handling

treatments is not enough to account for the amounts observed when even a short period of imbibition is involved, e.g.

	Mean germ'n at 30 hrs.
Untreated.	4.0
Handling treatments x 4.	7.5
" " x 8.	10.0
1 x 24 hours imbibition.	24.0
2 x 12 hours imbibition.	30.0
4 x 1 hour imbibition.	12.0
8 x 1 hour imbibition.	15.5

These values do not discount the possibility that a considerable proportion of the beneficial effects resulting from multiple dehydration treatments involving short imbibition periods are the result of the amount of handling treatments received. This might be one explanation of the relatively better performance found in the multiple treatments with 1 and 2 hour imbibition periods, when compared to single treatments of th e same total duration (Fig. 21).

The nature of any possible physical changes in the constitution of the grain which might be operative in bringing about the beneficial effects of a previous imbibition - dehydration treatment can only be a matter for conjecture. Among reasonable possibilities might be included hysteretic effects on colloidal proteins, changes in the permeability of seed membranes, changes in the structural properties of the tissues enclosing the embryo, and irreversible displacement of the husks produced by the imbibed seed swelling. The mode of action of such changes might be operative in the rate of water uptake by the grain, the balance of

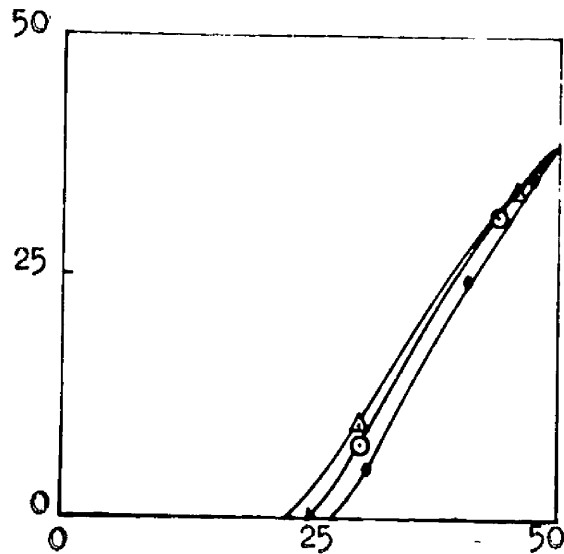


Fig. 22.

The effect of handling treatments on the germination of grains of A. sativa. Untreated samples, (•), samples given 4 handling treatments, (⊙), and 8 handling treatments, (△).

Abcissa: Period in germination conditions, hours.

Ordinate: Mean no. of grains germinated / 50.

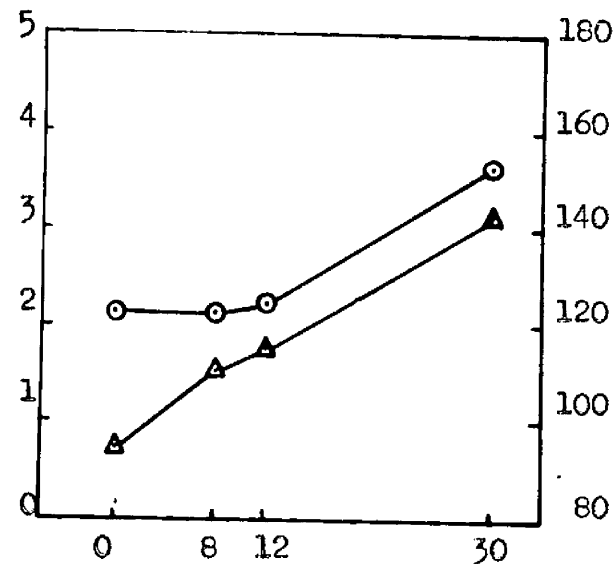


Fig. 23.

Residual physiological changes in grains of A. sativa after a single germination reversal treatment and 17 weeks dry storage. Free Amylase activity in the dry grain, (⊙), and the soluble Tyrosine level in the dry grain, (△).

Abcissa: Length of previous imbibition period, hours.

Ordinates: Left scale, Mgms. starch digested at 20 mins.

Right scale, Concentration of tyrosine, ppm. in the T.C.A. extract of 10 dry grains.

gaseous exchange between the embryo and the atmosphere, or a reduction in the mechanical restrictions to embryo enlargement. The only observation with relevant information on these possibilities are the moisture contents of the various samples after the same period of imbibition with increasing numbers of dehydration - imbibition treatments (Expts. 19, 20). Since these observations were made as a check on the general similarity of the treatments within one series, only one sample of grains was taken on each occasion. An experiment was however carried out to confirm the trends indicated by these figures in which a sufficient number of samples were tested for the results to be statistically acceptable (Expt. 24). These combined observations do show that an enhanced capacity to take up water is evident after an imbibition - dehydration treatment as used here, and the probability is that it is due to some physical change in the constitution of the grain, possibly some of those discussed above.

The possible participation of residual physiological changes following an imbibition-dehydration treatment in producing the beneficial effects on subsequent germination was investigated mainly to observe if any such changes could be demonstrated, rather than to give an exhaustive survey of them and to evaluate those most likely to be operative. For this reason only th ose physiological attributes already being studied with respect to other considerations were investigated. The grains used for these examinations were those in which the beneficial effects of the previous treatment had been shown to remain unchanged for a period of 15 weeks, so that any differences found are of a more than temporary nature. A study of the Free Amylase activity and the Soluble Tyrosine

content in these grains (Expt. 25, Fig. 23) showed that these two attributes could both show a persistent higher level than that found in the dry seed. In the case of the two shorter treatments, the occurrence of a considerable residual beneficial effect is not accompanied by any increase in the Free Amylase level, so this particular physiological change must be discounted as a contributory factor to the beneficial effect. The increase in the Soluble Tyrosine content in all three samples and the implication that other soluble protein degradation products are also present, indicates that this is a change which might be of considerable advantage in accelerating the subsequent germination processes.

One of the main reasons for carrying out this whole group of investigations has been to find out to what extent germination in this species can be reversed, by returning the moisture content of the imbibed grain back to that of the air-dry grain. If a definition of reversability is taken, involving only the capacity to produce a normal seedling, then obviously those grains showing partial or total embryo damage have already progressed beyond any point of reversability. These studies have shown that the occurrence of this damage is related to the stage when germination is first apparent, but that previous to this there is some degree of embryo growth occurring which is not harmfully affected. This phase of embryo growth begins with the extension of non-vacuolated embryo cells, so we may conclude that the embryo damage resulting from a dehydration treatment is produced by effects either on vacuolated cells, possibly undergoing extension, or on meristematic cells in which nuclear

division is commencing. By this same definition of reversibility, those grains receiving imbibition-dehydration treatments of less than 20 hours are fully reversible. If a more restricted definition of reversibility is taken that the subsequent rate and time of onset of germination is to be exactly similar to that of untreated grains, and that multiple treatments should be accommodated over and over again without any persistent change, then there is no evidence for any germination reversibility in this species. Even 1 hour imbibition periods show a pre-disposing effect towards non-reversibility when the treatment is repeated once or twice. This can only mean that within the first few hours of commencing imbibition, irreversible changes leading to the accomplishment of germination are under way.

In order to ascertain whether the beneficial and harmful effects resulting from dehydration treatments pass through a series of treatments to give similar values to a single treatment of the same total length of imbibition, a comparison of the relative amounts of both these effects in a series of samples from the previous experiments has been carried out (Fig. 24). In the case of the beneficial effect, there is found to be a general concordance between these values. This indicates that the sum of the effect in a series of dehydration treatments is fairly additive, and that little or no reversal of the factors concerned occurs. In the case of the harmful effects, however, there is found to be a considerable divergence between these values, the severity of the effects being much less when multiple shorter treatments are given than with a single treatment of the same duration. This surely indicates that some phases

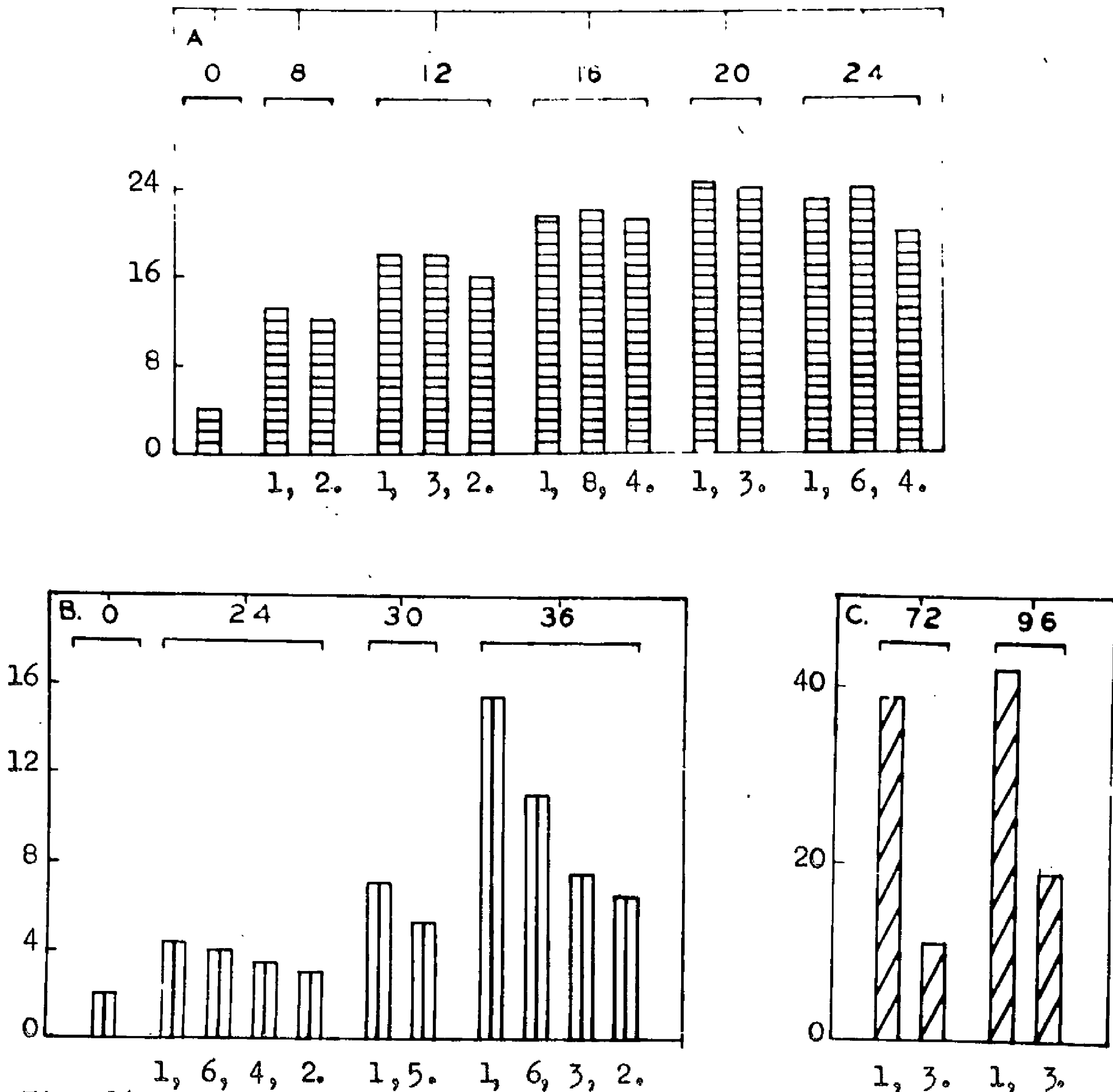


Fig. 24.

The effect of single and multiple germination reversal treatments of the same total imbibition period on the subsequent germination of grains of A. sativa.

A. Beneficial effects. The no. of grains germinated at 30 hours.

B. Harmful effects. I. The no. of grains ungerminated at 120 hours.

C. Harmful effects. II. The no. of grains showing plumule damage at 120 hours.

Abcissae: Top, Length of total imbibition period, hours.
Bottom, No. of treatments to give total imbibition period.

Ordinate: Mean no. of grains / 50.

of germination directly involved with the processes of growth are reversible to a limited extent at least.

A survey of previously published literature dealing with the effects of soaking and drying seed on their subsequent germination performance showed a dearth of knowledge of the precise effects involved and the factors which were responsible for them.

In the grain investigated here, the uptake of water during germination has shown a typical bi-phasic nature, similar to that shown previously in seeds of other species (Shull, 1920; Owen, 1952; Stanley, 1958). The observation that residual beneficial and harmful effects on the subsequent germination of the grains of this species could be shown by drying imbibed grains back to dry weight is in accord with some previous investigations. Kidd and West (1918) did not themselves study these effects but they summarise and quote tables from some early German investigators whose work is not now readily available. These investigators showed that residual beneficial effects could be shown on the subsequent germination of seeds of Bean, Pea, Lupin, Vetch, Spring Rye, Maize, Barley and Oats when soaking was carried out with limited amounts of water, and the seed were allowed to dry slowly (Krauss, 1880; Wollny, 1885; Eberhardt, 1907; and Schleh, 1907). If the drying is carried out quickly (no details given), then a marked slowing of the germination rate and a reduction of the final germination level was the usual result. Since the periods of soaking ranged from 24-100 hours, these findings are in agreement with those found here. It is also mentioned in the reports on these early investigations that a quicker

rate of uptake of water on being again allowed to imbibe was characteristic of these treated seed. No information is available on the effects shown by shorter periods of imbibition or on the nature or the cause of the reduction in final germination level.

In the use of a drying treatment as a means of forcing the germination of newly harvested grains, including A. sativa, which have moisture contents higher than at maturity the occurrence of a beneficial effect on their subsequent germination is reported, accompanied by an enhanced capacity for water uptake in the treated grains (Harrington, 1923). This may not be quite in the same category as the effects being investigated here.

The absorption of water by grains of Lolium perenne and some other seed of the Gramineae has been shown to be restricted by the closely adherent pales, and the waxy nature of the cuticle (Brown, 1931). After a certain stage of imbibition, however, the swelling of the seed loosens these pales and increases the permeability of the cuticle, probably by stretching the waxy layer. No examinations were carried out to see whether a residual beneficial effect was present after drying these grains, and whether it was accompanied by an enhanced capacity for water uptake, but the types of changes produced by the first imbibition period seem unlikely to revert to the dry seed condition if these seed were dried.

In a later investigation, the occurrence of a residual beneficial effect was observed on the subsequent germination of grains of Dactylis glomerata after "floating" for 17 hours in distilled water, and air drying for 24 hours (Chippingdale, 1933). In this case, the occurrence

Discussion cont'd.

of an enhanced capacity for water uptake was also evident, which was also claimed to be due to irreversible changes in the fatty or waxy layer on the pales, giving increasing permeability to water, and a reduction of surface tension. The same worker later extended these studies to other grains of pasture grasses, including Lolium perenne, and to A. sativa (Chippingdale, 1934). In most of the grasses a residual beneficial effect of the previous soaking-drying treatment was evident but not to the same extent as in Dactylis glomerata. No comment on the water uptake potential of these treated grains is given. In the case of A. sativa, the occurrence of a residual beneficial effect on subsequent germination, proportional to the length of soaking is shown for periods of 6 - 48 hours at $3^{\circ} - 5^{\circ}$C. There is said to be no difference between the water uptake rates in these treated grains and in untreated grains. The occurrence of detrimental effects on prolonged soaking is hinted at in this investigation in grains which have reached the stage of apparent germination, but no figures or further information is given on these last two points. These results in general bear out the general findings of our experiments if allowance is made for the probable slower rate of germination at the low temperature employed by this investigator, and the soaking conditions used. The cause of the beneficial effect in the case of A. sativa, and perhaps in some of the other grains investigated is said to be "The early metabolic processes of germination ... initiated during the period of soaking, and ... merely arrested and not reversed by subsequent drying", though no evidence is brought forward to support this claim.

Discussion cont'd.

The occurrence and nature of embryo damage has however been investigated in a recent study on the effects of drying wheat grains at different stages of embryo development, from the first stages of seminal root elongation to a seedling with its first leaf emerged (Milthorpe, 1950). The drying technique used was confined storage over concentrated sulphuric acid, which proved to be much slower than the rates of drying obtained in these experiments. The grains were also partly immersed in the soaking solution and the figures for water contents indicate that they have imbibed more water than was necessary for optimal development to occur, which would also restrict the effectiveness of a drying treatment. For example, the moisture content at 24 hours is given as 294% of the dry weight, whereas the moisture content of grains of A. sativa at this same stage in our experiment was in the region of 65 - 70% of the dry weight. The wheat sample mentioned required 10 hours drying to reduce its moisture content to 17% of the dry weight, and another 20 hours to reduce it to the level of 10% of the dry weight. One interesting observation on the rate of drying showed however that the root tissues were much more subject to water loss than plumule tissues, the differences being as much as 100% in the first hours of drying. Despite the differences in the techniques used, some of the results of these investigations compare favourably with those of the present experiments. This is particularly the case in respect of the root damage. It was found that seminal roots which had elongated to more than 2 mms were fairly readily killed by only moderate drying treatments, while those which had elongated less than this could only be killed by more severe desiccation (to approx. 20% of the dry weight). Undeveloped primordia proved resistant to damage.

by drying to within 6% of the dry weight. These results show a good comparison with our present findings. The occurrence of plumule damage was neither so early in evidence nor so severe as that found in these experiments. The slower rate of drying of these parts of the embryo, already mentioned, might be one of the reasons for this phase of embryo damage not showing the same agreement with our results while the occurrence of root damage did show good agreement. Nevertheless, a considerable reduction in the capacity for subsequent plumule growth, and the occurrence of areas of plumule cells which seemed to show irreversible structural damage from the drying treatment was observed fairly frequently after the stage of elongation to about 3 mm. had been reached. The more developed the embryo became, the more severe and easier produced such retardation became. A complete incapacity to undergo further growth could be shown when an embryo at the point of leaf emergence was dried to a level of 10% of the dry weight, though this required a drying period of about 80 hours to bring it about. The cause of embryo damage in these studies is attributed to the death of the protoplast in vacuolated cells following the rapid changes in volume on drying and resoaking. This conclusion is not at variance with the conclusions drawn from our present studies, but there is no evidence to completely neglect actively dividing meristem cells as the possible location of damage. This protoplasmic effect might be the same phenomenon described as being the basic cause of frost or drought damage to mature vacuolated cells (Levitt, 1956). In these, the shrinking of the protoplasm, if severe enough, can produce physical changes causing it to become brittle. On moisture again becoming available, only a partial

de-plasmolysis can occur before the semi-rigid protoplast ruptures itself. There is no evidence from the results of this investigation on Wheat seedlings to indicate the possible occurrence of residual beneficial effects on the subsequent germination of the short period samples, nor of a quicker rate of water uptake in any treated sample.

Summary.

1. The uptake of water by grains of A. sativa has been shown to follow a bi-phasic course under certain germination conditions.

2. A residual beneficial effect of a previous imbibition period on the time of onset of subsequent germination in grains of A. sativa has been shown to persist after a dehydration treatment, and a considerable period of dry storage. This has been shown to be due to a combination of physical and physiological changes occurring normally in the grain during imbibition, and to a limited extent to the handling received by the grains during treatment.

3. In grains which have imbibed for periods of 24 hours or more the occurrence of harmful effects of the dehydration treatment has been observed. These effects are mainly due to damage of embryo cells which have undergone some degree of elongation growth, the severity of the damage increasing with increased embryo development. Inactive primordia do not seem affected by such treatments.

4. The relevance of these findings to previous investigations is discussed, and a considerable amount of agreement found in most cases.

Part III. The Development of Amylase Activity in germinating and Developing grains of *Avena sativa* L., and in Dormant and Germinating grains of *Avena fatua* L., and *Avena ludovicana* Dur.

Introduction.

Starch is the main carbohydrate storage compound of the grains of the Gramineae, and may constitute up to 60 - 65% of the total dry weight in the cereal grain. It functions in the seed as a source of carbon compounds for use in respiration or in the synthesis of other metabolites for the growing embryo until the established seedling is capable of providing these requirements by its own photosynthetic activity.

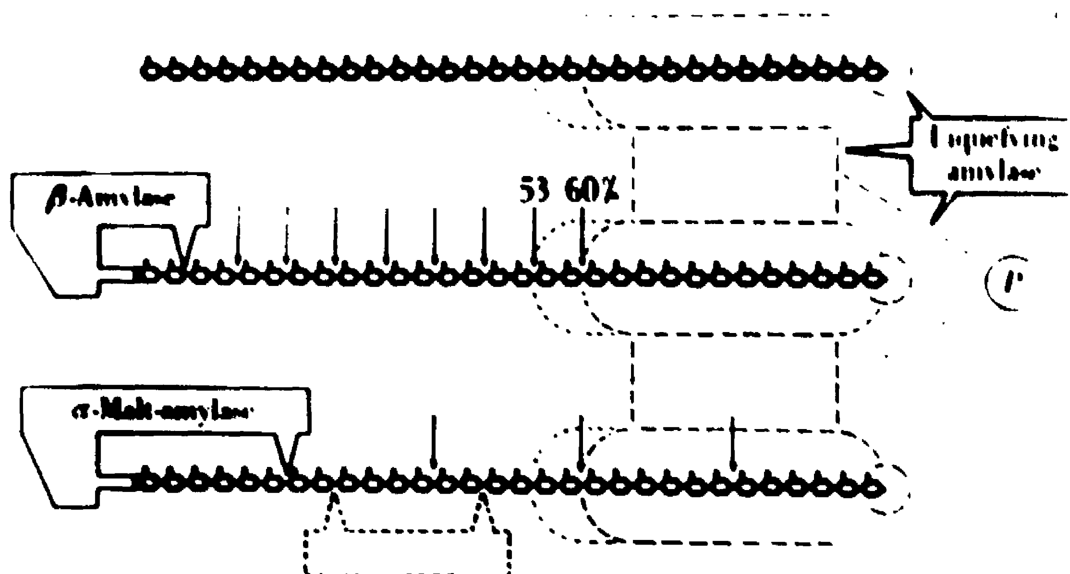
Starch is chemically a polymer of glucose, in which the glucose molecules are joined into long chains through α-1:4 linkages, with side chains attached to these through α-1:6 linkages. In addition to this there may be Hydrogen bonds and Phosphoric Acid ester links joining adjacent chains, so that it is present in nature as a very complex macro-molecule. In the seed of the cereals it is mainly confined to the cells of the endosperm, in which it is aggregated into discrete structures called Starch Grains. These form within plastids, but the current view is that when the starch grain has reached its full size the plastid membrane is either no longer present, or is reduced to a sparse network of protein fibrils. When starch in this form is removed from plants, it is found to be virtually insoluble in cold water. If the water is moderately warm however the starch grains swell, and gradually gelatinize, though not undergoing solution. This change probably involves the hydration and breaking of the hydrogen bonds between chains

and is the first stage in starch solubilisation. The rate of swelling is increased by the presence of surface active substances, or dilute acids, and we may presume that there are ways in which gelatinisation is brought about in the plant at normal temperatures. In order to be utilised in the processes mentioned previously the starch must be broken down into small soluble compounds which can be translocated to the embryo where they can be utilised.

Starch in the form of gelatinized starch grains can be digested by α-Amylase into smaller fractions, but it is doubtful if β-Amylase by itself can accomplish any digestion of such starch material, though it will freely act on the cleavage fractions resulting from α-Amylase activity, and in this way aid in reducing the complex macro-molecule to small fractions of several glucose molecules in size. Other enzymes with the properties of "disjointing" the α 1:6 branching linkage, or the phosphate bonds between chains may also play quite an important role in the solubilisation and digestion of native starch, largely by making it more accessible to amylase digestion, e.g. R-Enzyme, Z-Enzyme, Limit Dextrinase, and Amylophosphatase. In this study, soluble starch preparations have been used, and since this material has undergone a considerable degree of de-polymerisation in its preparation from native starch, it is readily digested by both Amylases, and the possible limiting effects of the other starch liquefying enzymes on amylase activity can be neglected.

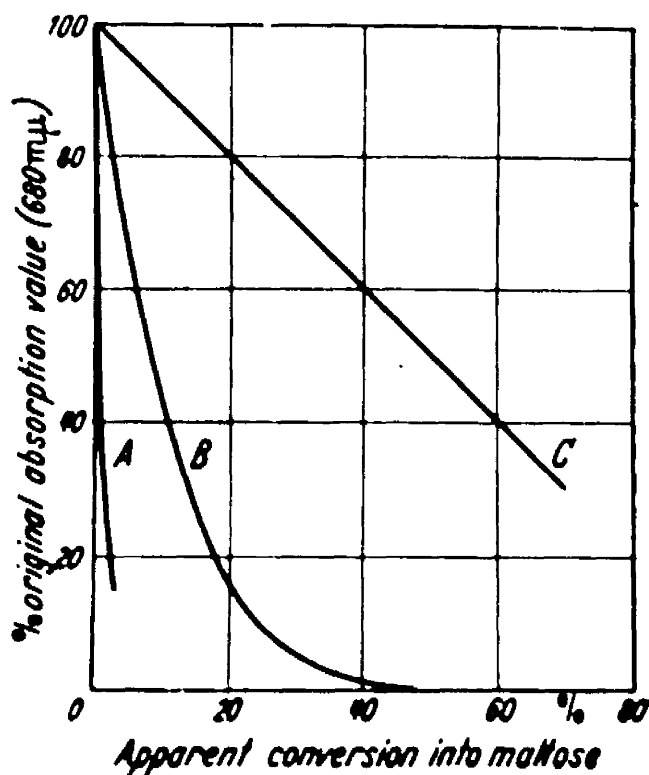
By merit of their commercial importance in the brewing and food industries, the amylases have been very extensively studied. Crystalline preparati ons of both types have been prepared of a high degree of purity,

and their properties and mode of digestion of various starch preparations carefully studied. Both enzymes digest starch by hydrolysing the α-1:4 link between adjacent glucose molecules, but while α-Amylase can attack this point at any part of the molecule, and can straddle branch linkages without any difficulty, β-Amylase can only remove the last two glucose molecules, in sequence, from the non-reducing end of a chain, and is prevented from digesting the whole of such a chain by the presence of a branching linkage. (See accompanying Plate.) Because of these differences in their mode of attach the changes produced in a soluble starch preparation by both enzymes are entirely different. During an α-Amylase digestion, the Starch is quickly reduced into small chain fragments, called dextrins, which may be further broken down until they are all less than 8 glucose units in length. Because of this there is a quick drop in the viscosity of the starch solution, and a rapid change in the iodine staining capacity from blue, through red, to colourless. The end products of such a digestion consist of only 20 - 40% of glucose and maltose, the remainder being dextrins. In a β-Amylase digestion on the other hand, there is a parallelism between the amounts of maltose formed, the amount of decline in the iodine staining property, and the decline in viscosity of the starch solution, until all the available free chains of the starch molecule have been converted into maltose, usually about the level of 60% conversion. At this point the remaining parts of the starch molecules within the branching points still display a considerable violet iodine staining capacity, this residual part being called the β-limit dextrin. In the presence of α-Amylase this dextrin is further digested to maltose, and the β-Amylase can complete the digestion of most of the dextrins



Hypothetical schema of the mode of action of amylases in relation to the constitution of starch. (From Hanes, 1937.).

Plate 6.



Amylose Value / Conversion curves for human salivary a-Amylase,(B), and sweet potato b-Amylase,(C). (From Whelan, 1958.).

resulting from an α-Amylase digestion. When both types of amylase act together they therefore tend to complement each other's action rather than compete for substrates, and may in some proportions show a definite synergistic action (Freeman and Hopkins, 1936; Preece and Shadakhsharaswamy, 1949).

If the relative amounts of Starch digested, measured by the change in Iodine staining capacity, and the amount of sugar formed, by direct measurement, are compared at the same times during an amylase digestion a graphical representation of the type of starch digestion occurring is obtained. This is called the Amylase Value/Conversion Curve (A.V. Conversion Curve). In the accompanying plate, the typical results of an α- and a β-Amylase digestion are shown.

Because of their distinctly different modes of digestion it is possible to get different values if the amylase activity of the enzyme solution is measured in several different ways. Measures of changes in viscosity, or reduction in Iodine staining capacity favour α-Amylase, while measurement of the sugars formed favours β-Amylase activity. Since both amylases have some capacity for all three properties, however, and they may be showing a synergistic action, it is impossible to measure the activity of any one of the components, in a mixture of the two, by a single measure of this type. The most convenient way of getting such a value is to inactivate or remove one of the components from the mixture. This can be accomplished either by heating the mixed solution to 70°C for 15-20 mins. when the β-Amylase will be preferentially destroyed, or by lowering its pH to 3.0 when the α-Amylase is inactivated first (Ohlsson, 1925).

Since the Starch of the grain requires to be mobilised by the action of these amylases, particularly α-Amylase, it is impossible for such a change to occur if these enzymes are absent, or not active. In this present investigation the role of Starch in the seed's metabolism is investigated by studying the presence, amount, and nature of the amylase activity during the germination and development of grains of A. sativa. The localisation of this activity during this period has also been studied on dissected grain parts. Similar studies have also been carried out on the amylase activity of dormant and non-dormant grains of A. fatua, and A. ludoviciana in order to compare the amylase activity pattern of all three species, and to investigate the possibility that dormancy in the latter two might be related to the mobilisation of the endosperm carbohydrate materials.

The aspects of the structure of starch, and the action of the amylases and other starch liquefying enzymes discussed in this section are adequately reviewed in Hanes (1937), and more recently in Badenhuizen (1958), and Whelan (1958).

Materials, and Methods.

The grains employed in these studies were of Avena sativa (L.), var. Victory, Avena fatua (L.), var. pilossissima, and Avena ludoviciana (Dur.). Dormant and non-dormant samples of the last two were available, or could be produced, as described in part I. Germination conditions employed were those found suitable for each species in part I, viz. 4 mls. distilled water / 50 grains, 20°C for the

first two species, and 3 days at 30°C, followed by transfer to 10-12°C for the third species.

Whenever available, "Analar" grade chemicals were employed.

<u>0.1% Soluble Starch</u> solutions were prepared by making a thin paste of 1 gm. Soluble Starch in 30 mls. cold distilled water. This was washed, with continuous stirring into approx. 500 mls. boiling distilled water, and kept at this temperature for 20 mins. Approx. 400 mls. cold distilled water were then added and the volume made up to 1 litre in a volumetric flask, the temperature being approx. 60°C at this time. When cooled, 1 - 2 mls. Toluene were shaken through it and the solution stored in the refrigerator until required. Fresh solutions were prepared as needed, approximately every three weeks, or as soon as there was any sign of reduced iodine staining capacity.

The <u>Iodine</u> reagent used for starch determinations contained 0.6% iodine and 6% potassium iodide, in distilled water (Smith and Roe, 1949).

Clark and Lubs' potassium acid phthalate - sodium hydroxide buffer solution was employed to control the pH of the enzyme digestion mixture (Documenta Gei gy, p. 105).

<u>Free Amylase</u> solutions were prepared by grinding 50 grains to a coarse paste with a little sand and distilled water. This brei was made up to 100 mls., a few drops of toluene shaken through it, and placed to extract at 4°C for two hours. The coarser materials were then removed by suction filtration through Whatman No. 1 paper, and the filtrate used directly for enzyme activity determinations.

<u>α-Amylase</u> solutions were prepared by adding 1 gm. of calcium sulphate

to 30 mls. of a Free Amylase solution and placing in a 70°C water bath, for twenty minutes, after which it was cooled in an ice-water mixture, and centrifuged to remove the particles. The supernatant was used directly for enzyme activity determinations (see Expt. 32).

The difference in enzyme activity between a Free Amylase solution, and the α-Amylase solution prepared from it is sometimes given as a measure of the β-Amylase component of the Free Amylase solution (Bernstein, 1943; Graesser and Dax, 1946). This assumption has been shown previously to be valid only in certain proportions, however, since both amylases acting together tend to have a synergistic interaction. For this reason we prefer to define this difference as non-β-Amylase activity.

The enzymic digestion of soluble starch solutions was carried out in 100 ml. flasks immersed in a water bath at 30°C ± ½°C. The reaction mixture consisted of 10 mls. 0.1% soluble starch solution (i.e. 10 mgms. Starch), 5 mls. pH 5.0 buffer solution (see Expt. 31), and usually 4 mls. distilled water and 1 ml. of the enzyme solution being tested. In some cases 5 mls. of enzyme solution were employed. The starch and buffer solutions, and distilled water were mixed together and equilibrated for ten minutes separately from the enzyme solution, until both had reached water bath temperature. The time of commencement of the digestion was taken when 50% of the amount of enzyme had been added. The flasks were frequently shaken through a digestion period, and toluene was added at all stages.

The activity of the Amylase solutions was measured in two ways, as Dextrinising Activity, i.e. the amount of Starch (mgms) digested beyond

forming a colour compound with iodine, or as Sugar Forming Activity, i.e. the amount of sugar formed (mgms).

The procedure adopted to measure Dextrinising Activity was to remove 1 ml. samples of the digestion mixture at appropriate intervals, and add these to two mls. N. HCl to prevent further enzyme digestion (see Expt. 26). At the end of a digestion all the samples were made up to 10 mls. with distilled water, and 0.1 ml. of the standard iodine reagent added and thoroughly mixed (see Expt. 25). The optical density of these samples was read within 5 - 10 mins. at 620 mμ against a blank sample from a digestion mixture with the starch solution replaced by distilled water. The optical density reading was converted to mgms. of Starch by reference to a calibration curve of known starch amounts in the same initial volume and diluted and tested in the same manner as described above (see Expt. 26). This procedure is substantially that of Smith and Roe (1949).

Sugar Forming Activity was determined on 4 ml. samples of the digest mixture, which were taken at appropriate times, added to 12 mls. of hot distilled water, and immediately boiled for 1½ mins. to destroy enzymic activity. At the close of a digestion, each sample was made up to 20 mls. by adding 4 mls. absolute ethanol and distilled water, giving an ethanol concentration of 20%. 1 gm. of "Norit" activated charcoal was added to each sample, and the mixture shaken mechanically for 10 minutes, after which the charcoal was removed by 10 mins. centrifugation, and filtration of the supernatant through Whatman No. 1 filter paper (see Expt. 29). The sugar content of the supernatant was estimated on a 2 ml. sample, by adding 0.15 mls. of 80% aqueous Phenol, followed as quickly as

possible by 5 mls. concentrated Sulphuric Acid (Dubois, et al, 1951; see Expt. 27). The optical density of these solutions was read at 490 mμ after 30 mins. at room temperature. The optical density reading was converted to mgms. of Maltose by reference to a calibration curve, prepared in a similar manner, from solutions containing known amounts of maltose and given the same procedural treatment (see Expt. 28). This reagent will react with any saccharide which is present in the supernatant, but the charcoal adsorption procedure described above should ensure that any dextrins of more than 4 glucose units will be removed (Whelan, et al 1953).

Experiments relating to the suitability of these procedures, and the amounts of reagents required for their best usage are included in the following section.

Optical density determinations were carried out on the same S.P. 600 spectrophotometer throughout, using optically matched test-tubes.

In making comparisons between the activities of various enzyme solutions the "initial slope" technique of Hanes (1932) was employed, in which the values to be compared are shown to lie on the linear part of the digestion curve, or a projection of this to a suitable time intercept.

Results.

Experiment 25. The relation between Iodine Concentration and the intensity of the Iodine-Starch Colour.

Solutions of soluble starch containing 2, 10, 20, and 40 mgms starch / 20 mls. were prepared, and 1 ml. samples of each diluted to 10 mls with 2 mls. N. HCl, and distilled water. 0.1 mls. of iodine solutions containing varying amounts of iodine from 0.033 - 1.05 mgms. I_2 were added to one tube from each starch concentration, and thoroughly mixed. The optical density of the resultant starch-iodine solution was read at 620 mu, and is given below in optical density units.

Mgms. I_2. added.	Optical Density of Starch-Iodine Solution with increasing amounts of Iodine. 2.	10.	20.	40 mgms. Starch / 20 mls.
0.033	0.075	0.27	0.35	0.47
0.066	0.087	0.39	0.56	0.90
0.10	0.098	0.46	0.65	1.08
0.30	0.100	0.65	0.90	1.60
0.45	0.100	0.69	0.98	2.00
0.60	0.100	0.715	1.00	-
1.05	0.120	0.75	1.06	-

These values are graphed in figure 25, and from this, the use of 0.6 mgms. of Iodine was judged to be near optimal for the range of starch concentrations likely to be met in later work.

Experiment 26. The relation between Starch Concentration and the intensity of the Starch-Iodine Colour.

Solutions of soluble starch were prepared to contain 0.5 - 30 mgms. of starch in 20 mls. 1 ml. samples of these were diluted and treated with iodine as described above, and the optical density of the resultant starch-iodine solution determined at 62o mμ. The results of three

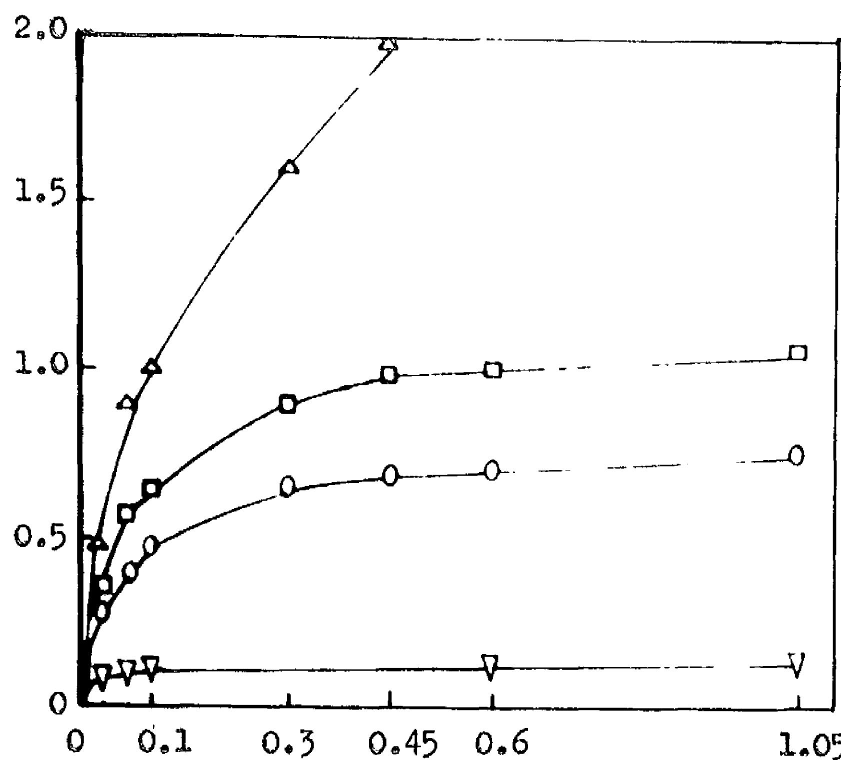


Fig. 25.

Relation between the amount of iodine and the intensity of the starch-iodine colour.

Optical density of samples from starch solutions containing 2 mgms., (▽), 10 mgms., (⊙), 20 mgms., (□), and 40 mgms., (△), starch / 20 mls. with graded amounts of iodine.

Abcissa : Mgms. Iodine added.
Ordinate: O.D. at 620 mu.

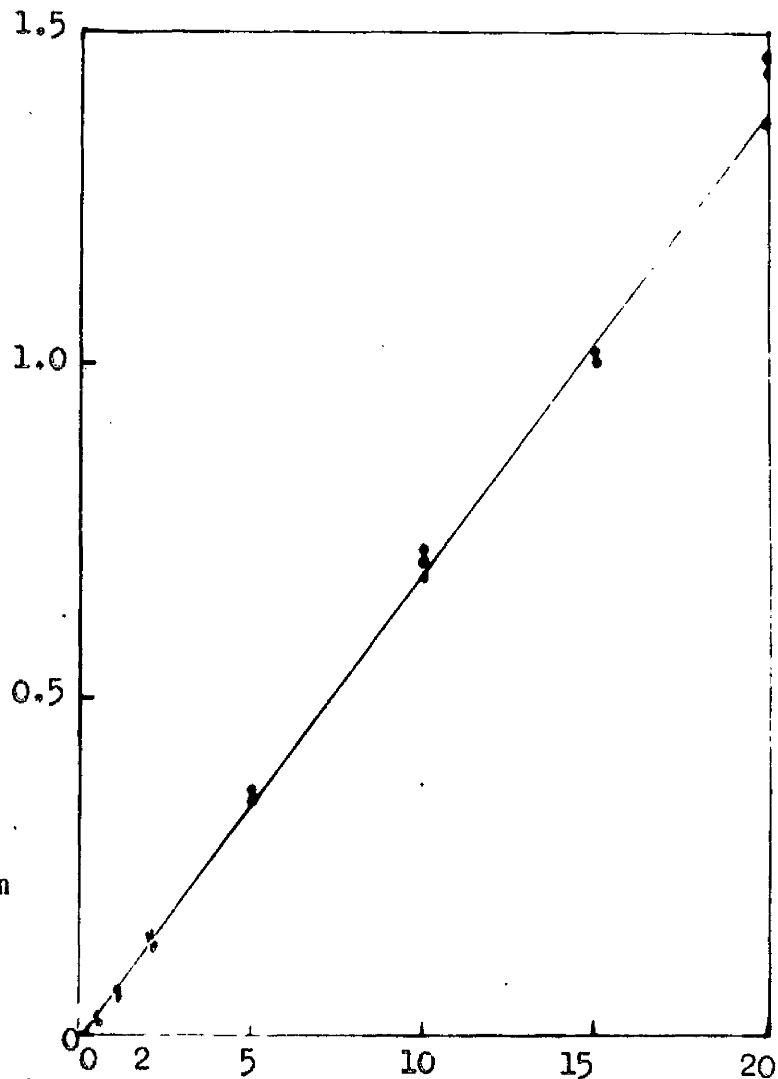


Fig. 26.

Relation between starch concentration and the intensity of the starch-iodine colour.

Optical density of samples from starch solutions containing from 0 - 20 mgms. starch / 20 mls. with 1ml. of stock iodine solution.

Abcissa: Concentration of starch solutions, mgms. / 20 mls.
Ordinate: O.D. at 620 mu.

determinations from different stock starch solutions is expressed below in optical density units.

Mgms. Starch / 20 mls.	Optical density of starch-iodine solution with increasing amounts of starch.		
	1.	11.	111.
0.5	0.02	0.022	0.02
1.0	0.065	0.07	0.06
1.5	0.112	0.106	0.100
2.0	0.140	0.145	0.137
5.0	0.350	0.360	0.362
10.0	0.72	0.724	0.71
15.0	1.06	1.08	1.09
20.0	1.44	1.47	1.36
25.0	1.75	1.80	1.78

These values are graphed in figure 26. The optical density of the iodine-starch colour shows a linear relation with the concentration of starch over a wider concentration range than will be met with in later experiments. This curve is used as a calibration curve for starch determination in later experiments.

Experiment 26. The effectiveness of N.HCl as an Amylase Inactivator, and the effects of standing in HCl for varying periods of time on the development of the Iodine-Starch Colour.

An enzyme digestion mixture, containing an enzyme solution capable of digesting all the starch present to a non-iodine staining condition in 30 mins. had a 4 ml. sample removed 1 min. after adding the enzyme. This was added to 8 mls. N. HCl in a small flask, and after shaking once or twice, two 3 ml. samples were taken from this solution and added to test tubes containing 7 mls. of distilled water. 0.1 mls. of iodine was added to one, and after thorough mixing its optical density was read at 620 mμ. After 10 mins, a further 3 ml. sample was taken from the

flask, diluted, and iodine added to this tube, and the second tube of the previous pair, and their optical densities determined. After a further 10 mins, the remaining 3 mls. in the flask was treated similarly, and the optical density determined then, and again 10 mins. later. The optical densities of the other tubes were also read at these ten minute intervals. The length of time from the removal of the sample from the digest mixture to the first optical density determination was just over 1 minute. The results of these determinations, in optical density units, is given below.

	Optical density at,			
Sample.	0.	10.	20.	30 mins.
Tube 1.	0.63	0.64	0.64	0.62
Tube 2.	-	0.64	0.64	0.63
Tube 3.	-	0.63	0.62	0.63
Tube 4.	-	-	0.64	0.62

These results confirm that enzymic activity is destroyed extremely quickly by N. HCl, and that no alteration in the intensity of the starch-iodine colouration results from storage under these acidic conditions before carrying out the colour determination.

Experiment 27. The effect of inwreasing amounts of 80% Aqueous Phenol on the colour development of a Maltose-Phenol-Sulphuric Acid Mixture.

Maltose solutions containing 1, 2.5, 5, and 10 mgms. in 20 mls. distilled water were prepared, and 4 mls. samples of each diluted with ethanol and water to give 20 mls. of 20% ethanol. Amounts of 80% aqueous Phenol from 0.02 mls. to 0.24 mls. were added to 2 ml. samples of these solutions, followed by 5 mls. concentrated Sulphuric Acid. The amount

of colour development after 30 mins. at room temperature, in terms of the optical density at 490 mµ, is listed below for each sample.

Initial Maltose Concentration, Mgms. / 20 mls.	Optical density with additions of 80% Phenol of 0.02,	0.06,	0.10,	0.14,	0.18,	0.24 mls.
1	0.18	0.27	0.28	0.30	0.41	0.34
2.5	0.32	0.59	0.66	0.74	0.77	0.77
5	0.64	1.08	1.30	1.36	1.35	1.40
10	0.89	1.50	+	+	+	+

(+ too dense to read.)

These values are graphed in figure 27. The addition of 0.14 mls. is seen to be nearly optimal for these maltose concentrations, the use of greater amounts tending to give an unduly high blank value without increasing accuracy.

Experiment 28. The relation between Maltose Concentration and the development of the Maltose-Phenol-Sulphuric Acid Colour Reaction.

Maltose solutions containing from 0.2 to 6 mgms. in 20 mls. were prepared and 4 ml. samples of these diluted to 20 mls. in 20% ethanol. 0.14 mls. of 80% aqueous Phenol was added to two ml. samples of these solutions, followed immediately by 5 mls. aqueous Phenol, and the colour development after 30 mins. at room temperature read as the optical density at 490 mµ. Two completely separate series of initial maltose solutions were tested in this way, the results of the determinations, in optical density units being given below.

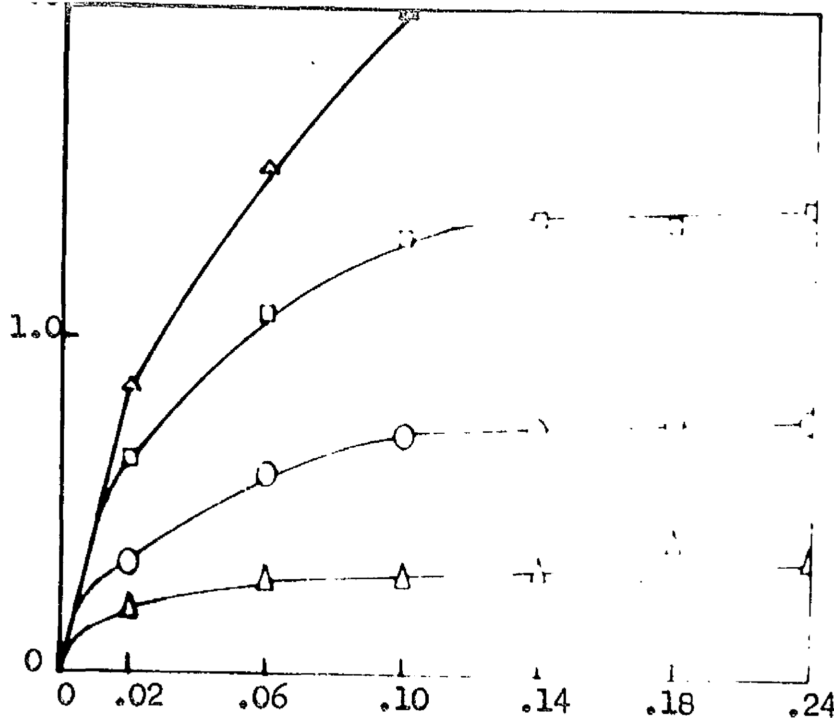


Fig. 27.

The relation between the amount of Phenol and the intensity of the Maltose-phenol colour.

Optical density of samples from Maltose solutions containing 1 mgm., (△), 2.5 mgms., (○), 5 mgms., (□), and 10 mgms. maltose / 20 mls., (△) with graded amounts of phenol.

Abcissa: mls. 80% aqueous phenol added.

Ordinate: O.D. at 490 mu.

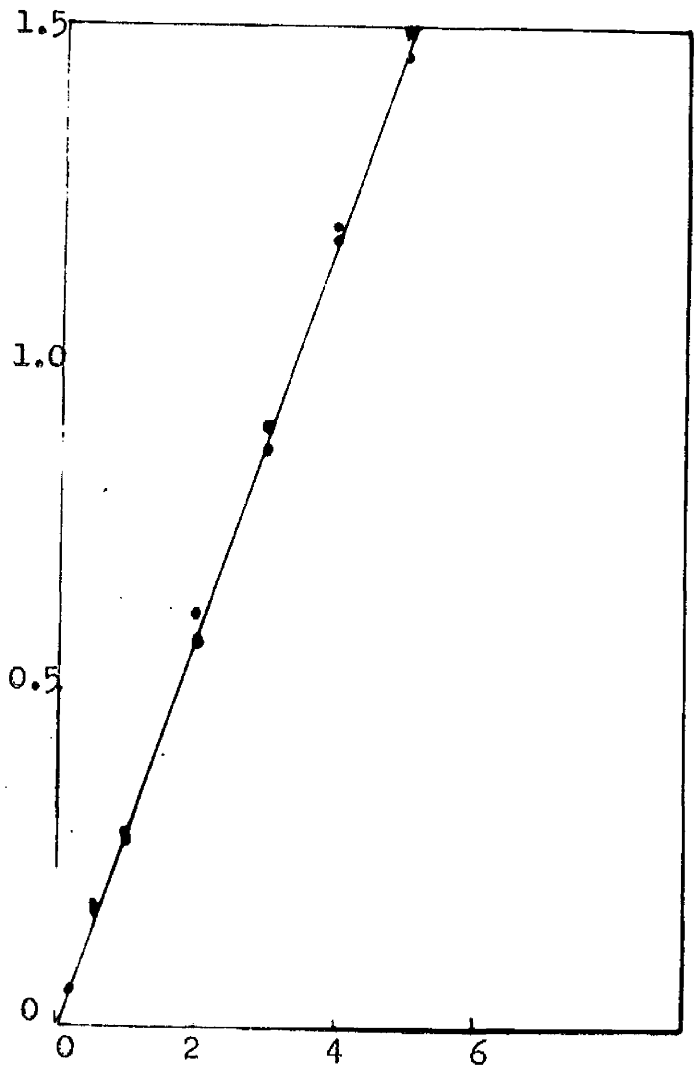


Fig. 28.

The relation between Maltose concentration and the intensity of the maltose-phenol colour.

Optical density of samples from maltose solutions containing 0 - 5 mgms. maltose / 20 mls. with 0.14 mls. phenol solution.

Abcissa: Maltose concentration, mgms. / 20 mls.

Ordinate: O.D. at 490 mu.

Initial Maltose Concentration, (Mgms. / 20 mls.)	Optical Density at 490 mu.	
	1.	11.
0.2	0.05	0.05
0.5	0.18	0.17
1.0	0.27	0.275
2.0	0.62	0.58
3.0	0.90	0.86
4.0	1.18	1.20
5.0	1.45	1.50
6.0	1.80	1.76
7.0	Too dense.	

These values are graphed in figure 28. It is seen that a linear relation exists between colour intensity and concentrations of maltose up to 5.0 mgms. in 20 mls. Above this level accurate reading becomes difficult by the compression of the logarithmic Optical Density scale. In later work, any maltose determination of concentrations greater than this was carried out in a diluted sample in order to bring it down onto the lower part of this curve. This curve is used in later experiments as a calibration curve, including some of the experiments in the following section. In these, the optical density is related to the concentration of maltose in the 1/5th diluted testing solution, not the original 20 mls. digestion mixture. It was also shown in this experiment that the presence of toluene and time intervals of 5 - 60 seconds between the additions of the Phenol and the addition of the Sulphuric Acid were without effect on the development of colour intensity.

Experiment 29. The removal of Starch from Solution in 20% Ethanol, by adsorption on Charcoal.

10 flasks were prepared containing 10 mgms. of soluble starch in 20 mls. 20% ethanol. To pairs of these "norit" activated charcoal was added at the rate of 0.0, 0.1, 0.25, 0.5, and 1.0 grams. Each flask was then given 10 mins. mechanical shaking, and the charcoal removed by centrifuging for 10 mins. and filtration of the supernatant through Whatman No. 1 paper. After dilution of a 10 ml. portion of the supernatant to 100 mls., 1 ml. of the standard iodine reagent was added, and the optical density of the iodine-starch colour determined. The mean value of the two determinations at each level of charcoal addition is given below, with the relevant level of starch concentration, and the % absorption this represents.

Charcoal Added, Gms.	Optical Density at 620 mu.	Starch Concentration Mgms./ 20 mls.	% Absorption.
0.0	0.71	10.0	0
0.1	0.305	4.25	57.5
0.25	0.16	2.2	78.0
0.5	0.012	0.25	97.5
1.00	0.00	0.0	100.0

These figures are graphed in figure 29.

In subsequent tests, 1 gm of charcoal was used in the standard procedure since no more than 10 mgms. of starch were ever met with experimentally.

Experiment 30. The determination of Maltose in the presence of Starch.

Since the Phenol-Sulphuric Acid method of determining saccharides gives a colour reaction with Starch, and Starch cleavage fractions, it is necessary to ensure that such material is removed from a digest mixture before the test is carried out, and that the method of removing these substances does not remove any significant amounts of Maltose. As described above, complete removal of starch is possible by adsorbing it on charcoal, and the same should hold with dextrins of sizes greater than 4 glucose units, when the adsorption is carried out in 20% ethanol (Whelan, et al, 1953). To ensure that there was no considerable loss of maltose in this procedure some flasks were set up containing from 1 to 10 mgms of maltose in 20 mls. 20% ethanol. A similar set of flasks containing the same amounts of maltose, but with the addition of starch, to make up a total of 10 mgms added materials was also prepared. 1 gm. of charcoal was added to each flask and the normal shaking, centrifuging, and colour determination procedure carried out. The results of these determinations, in mgms. Maltose, and the %age recovery of Maltose in the presence of a starch solution is given below.

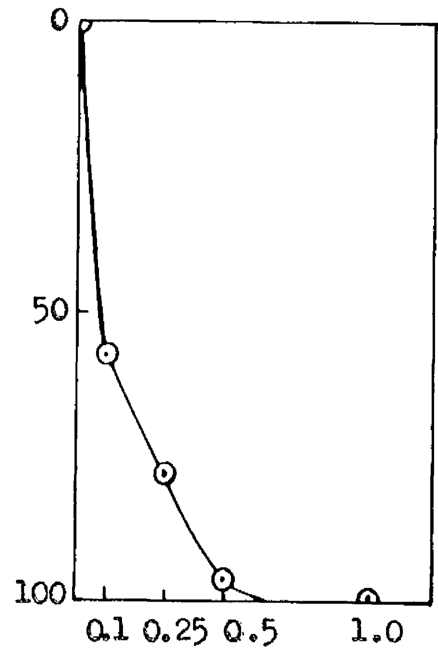
Maltose Originally present, mgms.	Maltose Determined (Starch absent) mgms.	Maltose Determined (Starch present) mgms.	% Recovery Maltose in Starch sol'n.
1	1	0.5	50
2	2	1.9	95
4	4	3.9	97.5
5	5	4.7	94
6	6	5.6	92.5
8	7.8	7.5	96
10	9.0	8.8	96

Fig. 29.

Adsorption of Starch by charcoal.
10 mgms. starch present in 20 mls.
20% ethanol.

Abcissa: Gms. activated charcoal added.

Ordinate: % Starch adsorbed.



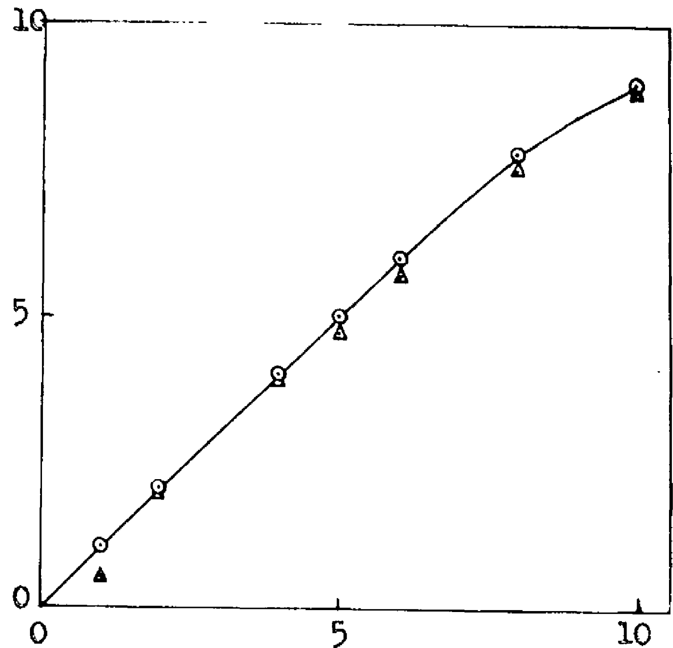


Fig. 30.

The recovery of maltose in solution alone,(⊙), and in a mixture with starch,(△), in 20% ethanol after a charcoal adsorption treatment.

Abcissa: Mgms. of maltose originally present.

Ordinate: Mgms. of maltose recovered

These values are graphed in figure 30. There would appear to be some loss of Maltose in the more concentrated solutions, but this is not much greater in the presence of Starch than in its absence. Such concentrations would only rarely be found experimentally.

Experiment 31. The effect of pH on the rate of digestion of Soluble Starch by a Free Amylase Solution.

Free amylase solutions were prepared from grains of *A. sativa* after 2, and 5 days in germination conditions. The Dextrinising Activity of these solutions in dig estion mixtures containing buffer solutions ranging from pH 4.2 - pH 5.8 was determined on two samples of each enzyme solution, the means of which are given below in mgms. of starch digested, in 20 mins. for the first, and in 3 mins. for the second.

Amylase Solution.	pH of digestion mixture. 4.2	4.4	4.6	4.8	5.0	5.2	5.4	5.8
2 days, germ'd.	1.82	-	2.8	-	3.5	-	3.35	2.4
5 days, germ'd.	3.8	4.8	5.35	6.05	6.5	6.2	5.9	4.55

These values are graphed in figure 31. The optimum pH for activity would appear to be approx. pH 5.0. This agrees with the published pH optima of similar preparations from wheat and barley, which range from pH 4.7 - pH 5.4 according to the buffer employed (Whelan, 1958, p. 203).

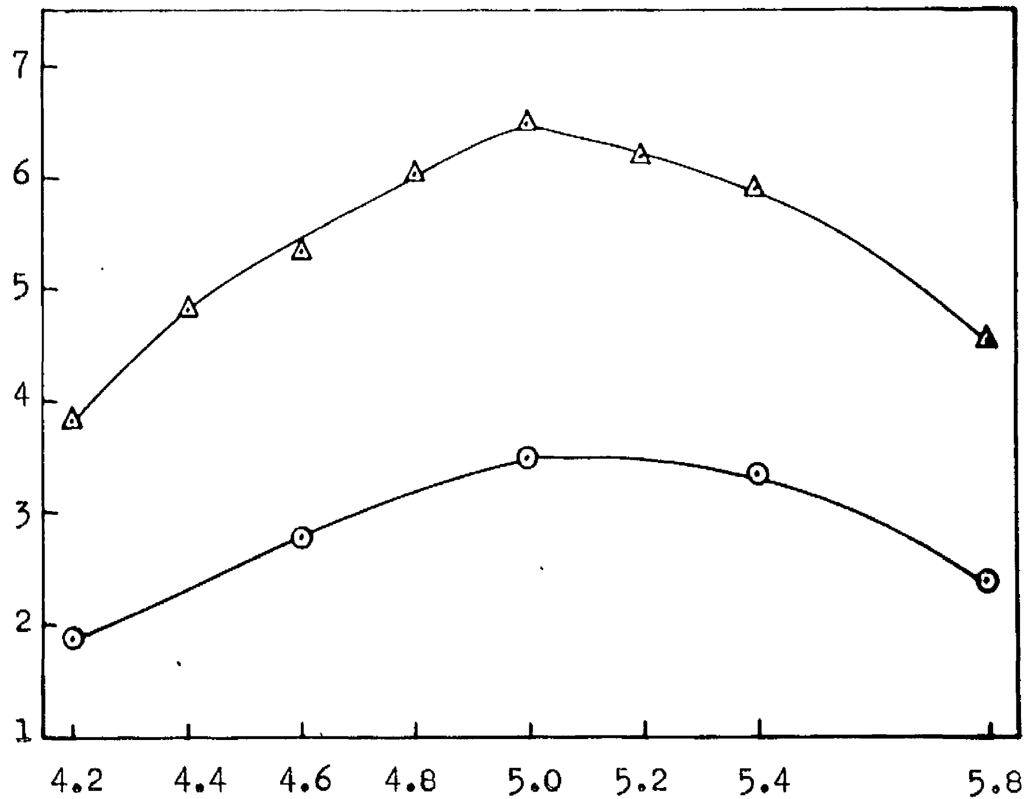


Fig. 31.

The effect of the pH of the digestion mixture on the activity of Free Amylase preparations of A. sativa.
Mgms. of starch digested at 20 mins,(⊙), and at 3 mins.,(△), by Free Amylase preparations from grains after 2 days and 5 days in germination conditions, respectively.
Abcissa: pH of digestion mixture.
Ordinate: Mgms. starch digested.

Experiment 32. The preparation of a-Amylase Solutions.

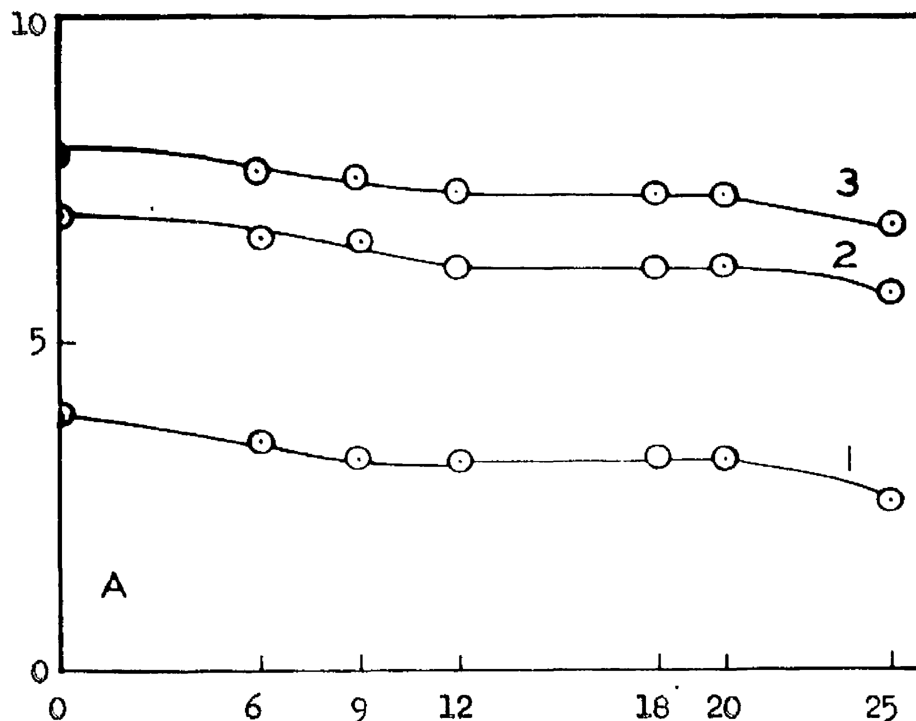
A solution of Free Amylase was prepared from grains of A. sativa after 4 days in germination conditions. 30 ml. portions of this were added to boiling tubes containing 1 gm. of Calcium sulphate, and thoroughly shaken. The tubes were then placed in a water bath at 70°C ± 1°C for lengths of time, and then removed to a beaker of ice-water. The temperatures inside the tubes during the period of heating up to 70°C, and cooling down from it are given below.

	Temperature, °C., at times of					
	1.	2.	3.	4.	5.	6 mins.
Heating.	-	-	44	58	68	70
Cooling.	61	37	25	18	15	-

After centrifuging to remove the flocculent precipitate, the enzymic activity of all samples was determined with respect to their Dextrinising Activity. Some of the samples were also tested for Sugar Forming Activity. These results are given below.

a). Dextrinising Activity, (mgms. of starch digested).

Length of digestion, mins.	Length of period in water bath, 0.	6.	9.	12.	18.	20.	25 mins.
5.	3.78	3.5	3.22	3.22	3.22	3.18	2.52
10.	6.93	6.6	6.6	6.15	6.15	6.15	5.75
15.	7.85	7.63	7.55	7.3	7.3	7.3	6.85



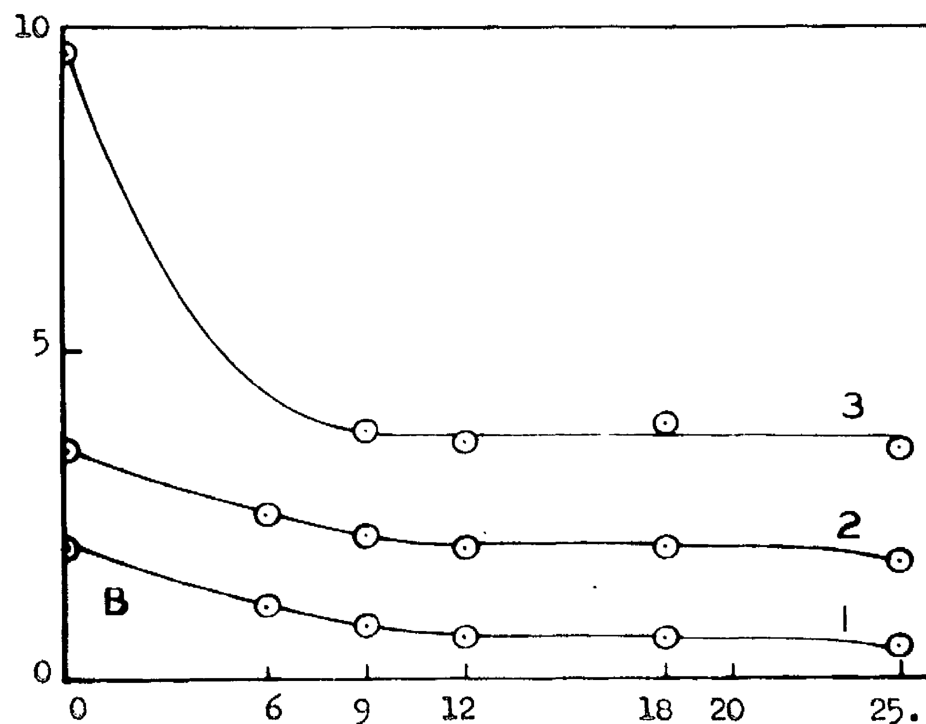


Fig. 32.

The effect of heating a Free Amylase preparation from germinated grains of A. sativa at 70°C. on its Dextrinising, (A), and Sugar Forming activities, (B).

1, 2 and 3 in A correspond to the mgms. starch digested at 5, 10, and 15 mins., respectively. 1, 2, and 3 in B correspond to the mgms. of sugar formed at 10 mins., 30 mins., and 20 hours, respectively.

Abcissa: Period in 70°C. water bath, mins.

Ordinate: Mgms. starch digested, or sugar formed.

b). Sugar Forming Activity, (mgms of sugar formed).

Length of digestion period, mins.	Length of period in water bath, 0.	6.	9.	12.	18.	25 mins.
10.	2.0	1.1	0.8	0.58	0.58	0.50
30.	3.5	2.5	2.2	2.0	2.0	1.8
hours.						
20.	9.6	-	3.8	3.6	3.9	3.5

These values are graphed in figure 32. From the points of inflexion on these curves the initial drop in amylase activity is over within 9-12 minutes of commencing the heat treatment, and no further inactivation is evident from this time till after 20 mins. heating has taken place. This behaviour, and the sugar producing capacities of the different samples, is entirely consistent with the early inactivation of the b-Amylase present leaving only a-Amylase activity relatively unaffected. In later investigations the normal procedure was to heat the enzyme preparations for 20 mins.

Experiment 33. Free Amylase Activity in grains of A. sativa during Imbibition, Germination, and Development.

Free Amylase preparations were made from three samples of fifty grains of A. sativa taken at intervals during a period of 12 days while the samples were maintained in germination conditions, with supplementary distilled water added from the fifth day. The Dextrinising Activity of 1 ml. samples of these enzyme solutions was determined, the mean values observed being given below. The germination rate of the same sample is also given.

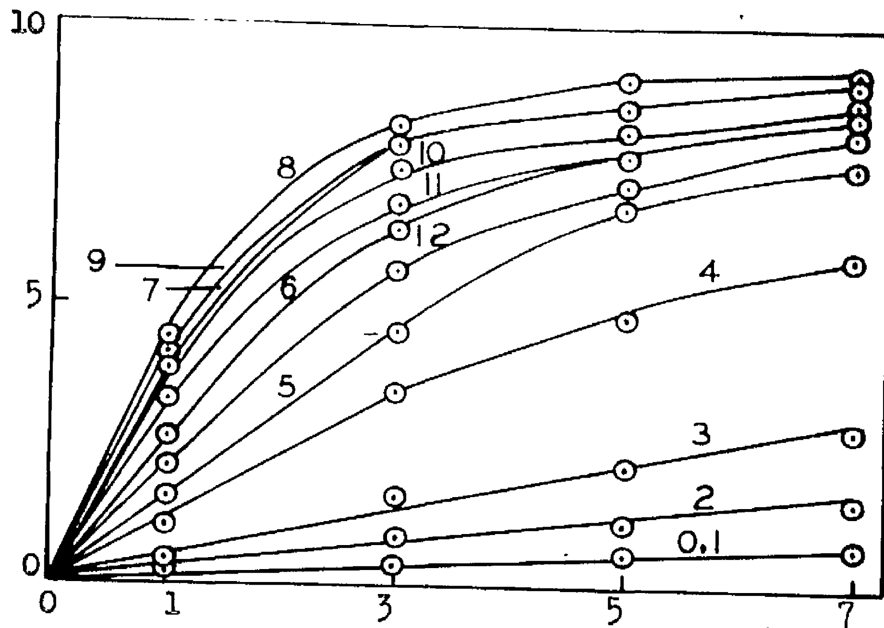


Fig. 33.

Digestion rate curves of Free Amylase preparations from grains of A. sativa during 12 days in germination conditions.

Numbers on curves correspond to the number of days the samples have spent in germination conditions.

Abcissa: Length of digestion period, mins.

Ordinate: Mgms. of starch digested.

No. of days in Germination Conditions.	Germination %	Mgms. Starch Digested at, 1.	3.	5.	7 mins.
0	0	0.19	0.34	0.56	0.62
1	10	0.14	0.26	0.56	0.62
2	72	0.22	0.80	1.15	1.50
3	94	0.48	1.58	2.19	2.80
4	100	1.08	3.50	4.75	5.70
5	-	1.59	4.56	6.75	7.45
6	-	2.06	6.34	7.70	8.45
7	-	3.92	7.86	8.60	9.00
8	-	4.40	8.20	9.10	9.20
9	-	4.20	7.75	8.55	8.78
10	-	3.82	7.38	8.13	8.65
11	-	3.32	6.85	7.87	8.50
12	-	2.15	5.52	7.15	8.05

The digestion rate curves of these values are given in figure 33. This experiment was repeated on other two occasions, similar results being obtained each time.

Experiment 34. a-Amylase activity in grains of *A. sativa* during Imbibition, Germination and Development.

Free Amylase, and a-Amylase solutions were prepared from two samples of fifty grains at intervals during a period of nine days, during which time the samples were maintained in germination conditions, with supplementary additions of distilled water from the fifth day. The Dextrinising, and Sugar Forming Activities of 1 ml. samples of these enzyme solutions was determined, the mean value of two determinations being given below.

Experimeht 34 (cont'd.)

a). Dextrinising Activity.

Days in germ'n conditions.	Enzyme Solution.	Mgms. Starch Digested, at,				
		60.	120.	180.	240 mins.	20 hrs.
0	Free Amylase.	0.56	1.1	1.6	2.2	-
0	a-Amylase.	0.00	0.00	0.00	0.00	0.00
1	Free Amylase.	0.70	1.40	2.10	2.80	-
1	a-Amylase.	0.08	0.22	0.36	0.48	1.40
		5.	15.	30.	60.	120 mins.
2	Free Amylase.	0.98	3.50	5.60	7.30	8.7
2	a-Amylase.	0.56	0.98	2.24	4.75	7.3
		3.	5.	15.	30.	60 mins.
3	Free Amylase.	1.12	2.94	7.15	8.55	8.85
3	a-Amylase.	0.98	2.10	6.65	7.85	8.85
4	Free Amylase.	3.7	5.95	8.40	8.95	9.25
4	a-Amylase.	2.60	3.92	7.85	8.80	9.25
5	Free Amylase.	5.3	7.0	8.80	9.10	9.40
5	a-Amylase.	3.5	5.87	8.75	9.25	9.25
		1.	2.	4.	8.	15 mins.
7	Free Amylase.	4.30	7.15	8.82	9.10	9.80
7	a-Amylase.	3.15	5.8	8.70	9.25	9.80
9	Free Amylase.	4.05	7.00	-	9.10	-
9	a-Amylase.	3.42	6.10	-	9.15	-

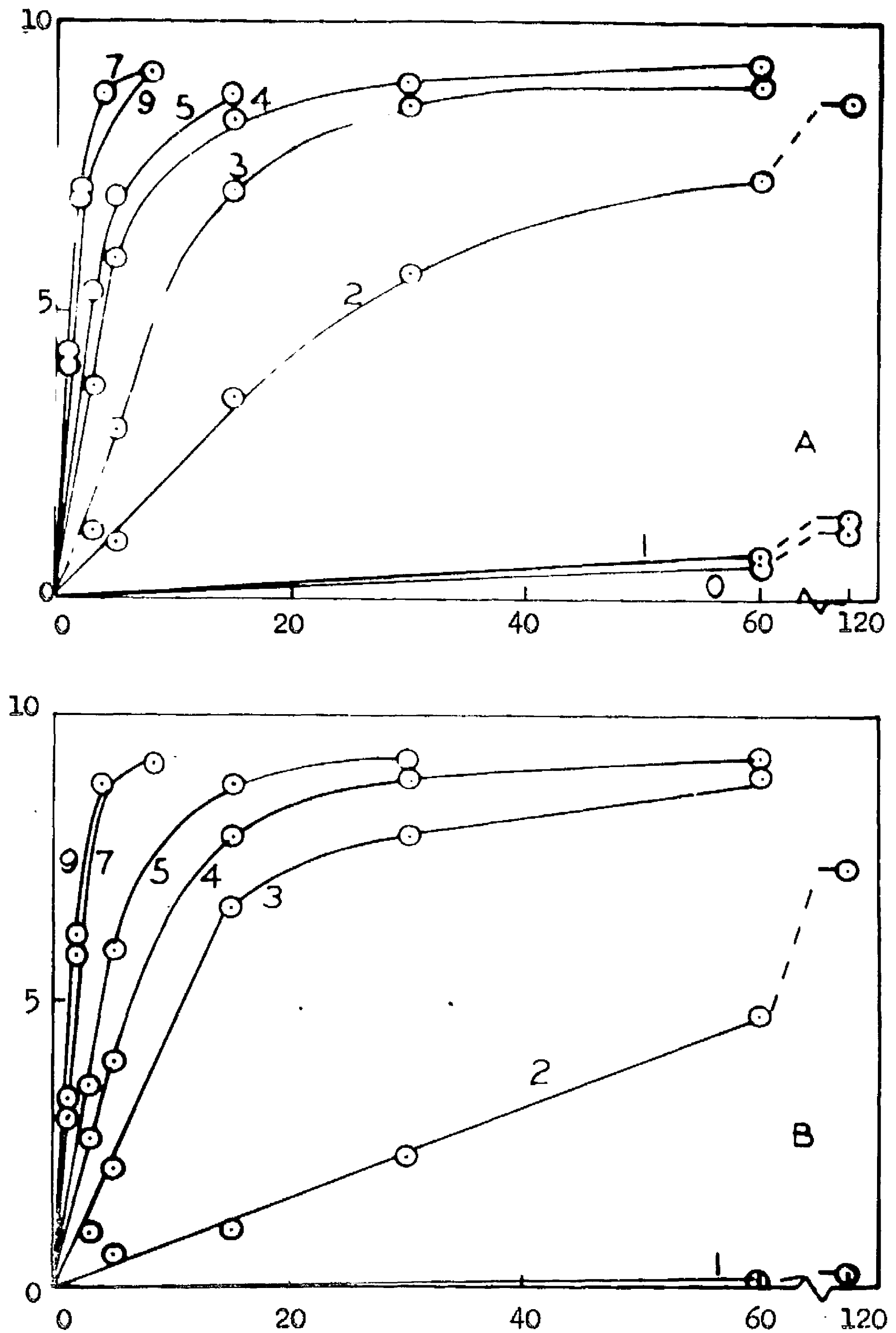


Fig. 34.

Digestion rate curves of Free Amylase,(A), and a-Amylase preparations, (B), from grains of A. sativa during 9 days in germination conditions.

I. Dextrinising activity.

Numbers on curves correspond to the number of days the samples have spent in germination conditions.

Abcissa: Length of digestion period, mins.

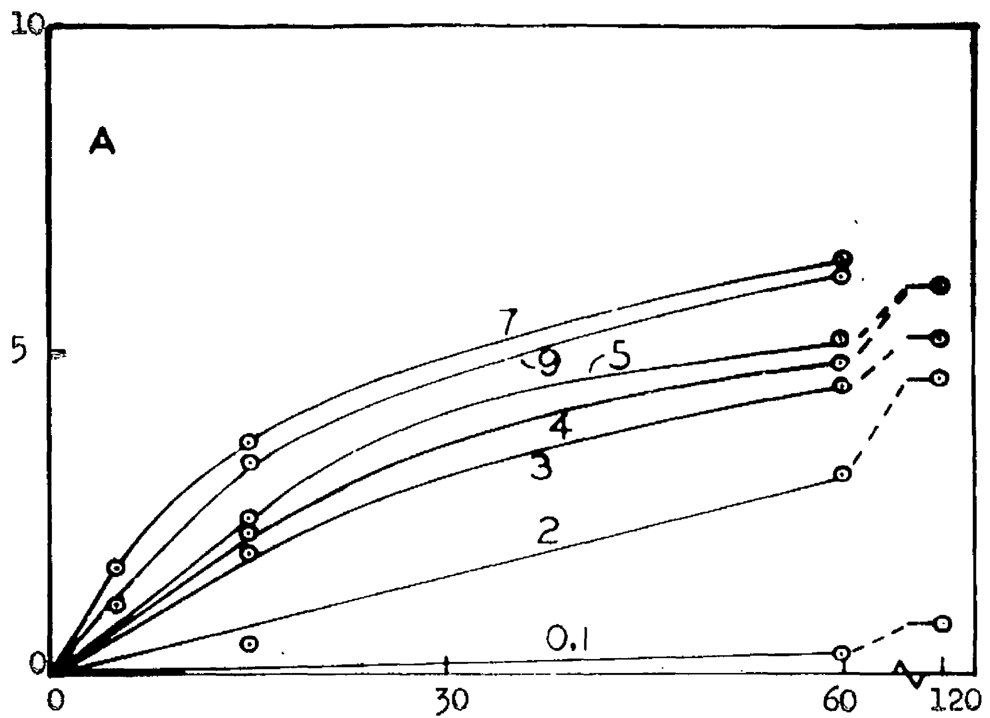
Ordinate: Mgms. of starch digested.

Experiment 34 (cont'd.)

b). Sugar Forming Aotivity.

Days in germ'n conditions.	Enzyme Solution.	Mgms. Maltose Formed, at, 60.	120.	180.	240 mins.	20 hrs.
0	Free Amylase.	0.36	0.76	1.16	1.54	6.70
0	a-Amylase.	0.00	0.00	0.00	0.00	0.00
1	Free Amylase.	0.40	0.80	1.20	1.65	7.20
1	a-Amylase.	0.00	0.00	0.00	0.04	0.10
		15.	60.	120.	180 mins.	20 hrs.
2	Free Amylase.	0.40	3.05	4.60	6.10	8.00
2	a-Amylase.	0.00	0.30	0.40	0.60	1.80
3	Free Amylase.	1.90	4.50	5.20	6.20	9.00
3	a-Amylase.	0.04	0.80	1.00	1.60	3.80
4	Free Amylase.	2.15	4.80	6.00	6.60	9.00
4	a-Amylase.	0.15	0.90	1.40	2.60	3.40
5	Free Amylase.	2.40	5.20	6.20	6.60	9.20
5	a-Amylase.	0.60	1.40	2.40	3.00	4.10
		5.	15.	60.	180 mins.	20 hrs.
7	Free Amylase.	1.6	3.6	6.4	7.2	9.2
7	a-Amylase.	0.6	1.0	2.4	3.6	4.1
9	Free Amylase.	1.0	3.2	6.2	7.2	9.1
9	a-Amylase.	0.7	1.35	2.6	3.6	3.9

The digestion rate curves of these enzyme preparations is given in figure 34.



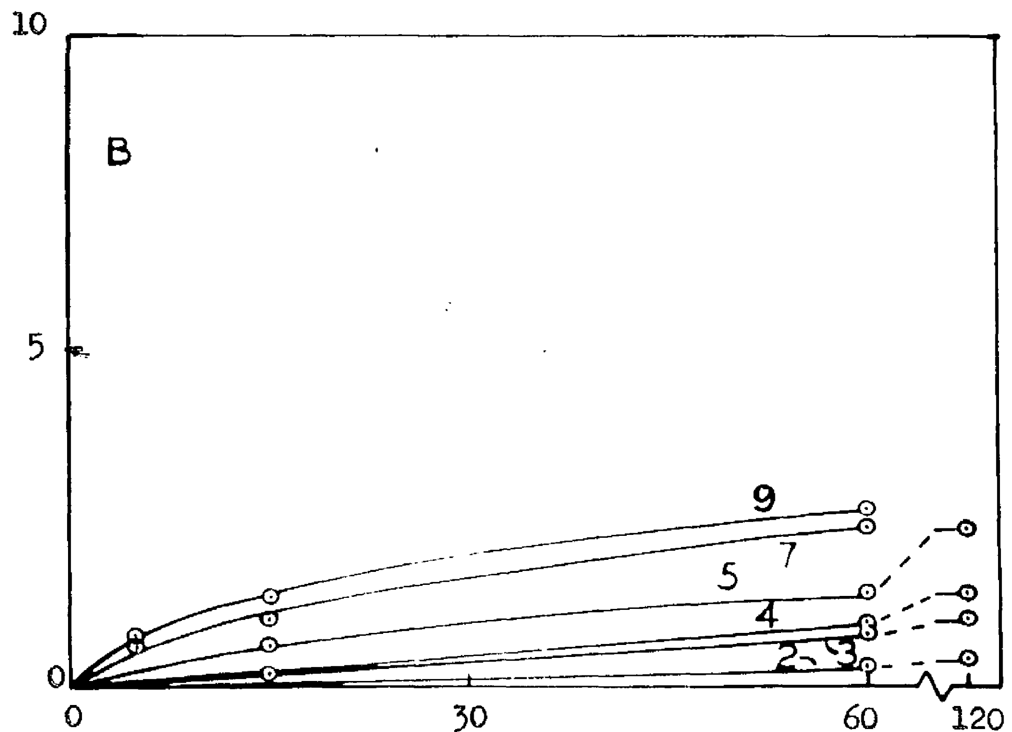


Fig. 34, (cont'd.)

Digestion rate curves of Free Amylase,(A), and a-Amylase preparations, (B), from grains of A. sativa during 9 days in germination conditions.

II. Sugar Forming activity.

Numbers on curves correspond to the number of days the samples have spent in germination conditions.

Abcissa: Length of digestion period, mins.

Ordinate: Mgms. of sugar formed.

Experiment 35. Further studies on the Free Amylase and a-Amylase activity in grains of A. sativa during imbibition.

Free Amylase solutions were prepared from samples of grains of A. sativa in the dry condition and after 16 and 22 hours imbibition. 100 grains were extracted in 50 mls. distilled water, and 5 ml. samples used for determining the Dextrinising Activity. The enzyme component of these digestion mixtures is therefore some twenty times that of the previous experiment s. a-Amylase solutions were prepared from these enzyme preparations and used in the same manner. Two determinations of each enzyme preparation's activity were carried out, the mean values being given below.

a). Free Amylase Activity.

Period of Imbibition.	Mgms. of Starch digested at, 0.25.	0.5.	0.75.	1.0.	3 hrs.
None.	3.2	4.2	5.0	5.6	6.6
16 hrs.	3.2	4.2	5.2	5.6	6.7
22 hrs.	5.7	7.0	7.6	7.8	8.5

b). a-Amylase Activity.

Period of Imbibition.	Mgms. of Starch digested at, 5 hrs.	20 hrs.
None.	0.0	0.0
16 hrs.	0.0	0.0
22 hrs.	2.8	8.4

Experiment 36. The development of Free Amylase Activity in dormant and non-dormant grains of Avena ludoviciana.

Dormant grains of A. ludoviciana were allowed to imbibe for several days at 10 - 12°C, while another similar sample was first pricked, and allowed to imbibe at 30°C for 3 days, and then placed at 10 - 12°C. Free Amylase solutions were prepared from two samples of each batch at various times during a period of nine days, and the Dextrinising Activity of 1 ml. samples of the enzyme solutions determined. The number of grains showing embryo growth after removal of the husk was also recorded. These values are given below.

a). Dormant Sample.

No. of days in Imbibed condition.	Germ'n. %	Mgms. Starch digested at, 15.	25 mins.
0	0	0.0	0.0
1	0	1.4	1.68
2	0	1.38	2.03
3	0	1.40	1.68
4	0	1.40	1.96
5	0	1.22	1.89
6	0	1.34	1.96
7	0	1.26	1.75
8	0	1.30	2.03
9	0	1.34	1.89

b). Non-dormant sample.

No. of days in Imbibed condition.	Germ'n. %	Mgms. Starch Digested at, 15.	25 mins.
4	0	1.22	1.89
5	38	1.40	1.96
6	68	1.32	1.96
7	88	1.40	2.80
8	100	2.42	4.06
9	100	4.8	6.44

The digestion rate curves of these enzyme preparat ions is given in figure 35.

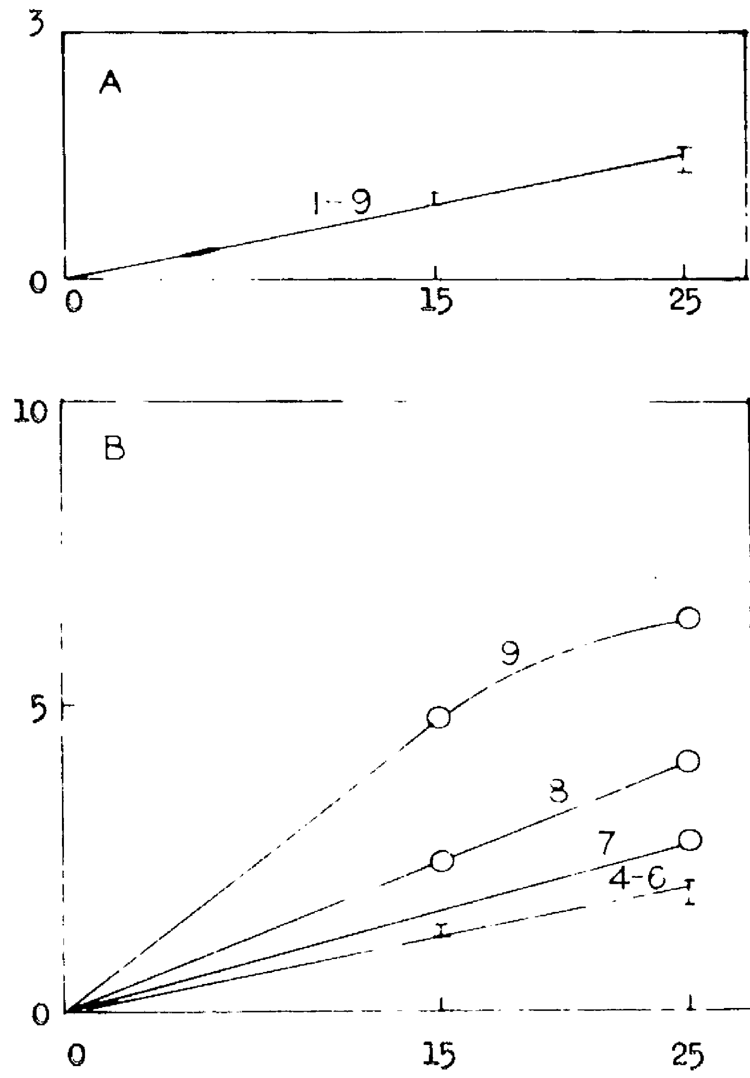


Fig. 35.

Digestion rate curves of Free Amylase preparations from dormant, (A), and non-dormant grains of A. ludoviciana, (B), during 9 days in imbibed conditions. Numbers on the curves correspond to the number of days the sample has spent in imbibed conditions.

Abcissa: Length of digestion period, mins.

Ordinate: Mgms. of starch digested.

<u>Experiment 37.</u> The development of Free Amylase Activity in dormant and non-dormant grains of *Avena fatua*.

Batches of grains of a non-dormant, a partially dormant, and a pricked, partially dormant sample of *A. fatua* were placed to germinate at 20°C. Free Amylase solutions were prepared from two samples of each batch at various times during a period of nine days, and the Dextrinising activity of 1 ml. samples of these solutions determined. The mean of these values, and the number of grains showing embryo growth after the r emoval of the husk is recorded below.

a). <u>Non-dormant Sample.</u>

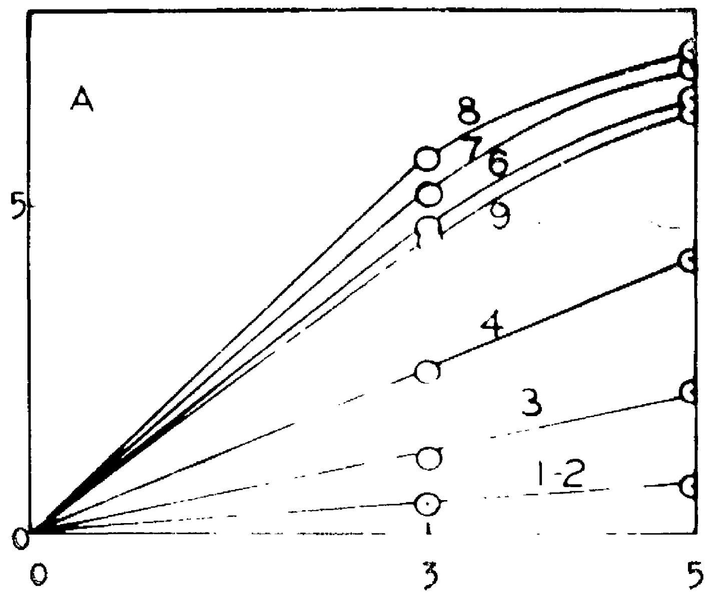
No. of days in Imbibed condition.	Germ'n. %	Mgms. Starch digested at, 3.	5 mins.
1	0	0.49	0.74
2	38	0.45	0.70
3	86	1.12	2.16
4	92	2.45	4.20
6	96	4.70	6.70
7	96	5.20	7.20
8	100	5.75	7.35
9	100	4.62	6.50

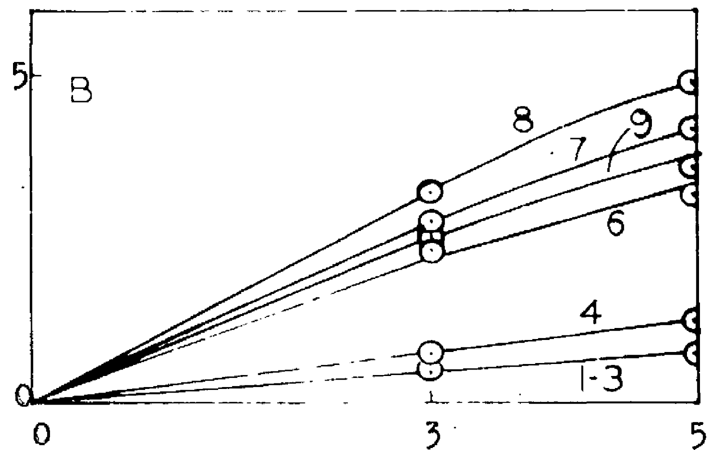
b). <u>Partially Dormant Sample.</u>

No. of days in Imbibed condition.	Germ'n. %	Mgms. Starch digested at, 3.	5 mins.
1	0	0.46	0.69
3	40	0.49	0.70
4	48	0.70	1.22
6	64	2.35	3.60
7	64	2.74	4.12
8	62	3.20	4.95
9	64	2.54	3.20

c). <u>Partially Dormant Sample, Pricked.</u>

No. of days in Imbibed condition.	Germ'n. %	Mgms. Starch digested at, 3.	5 mins.
3	86	1.02	1.98
6	96	4.15	6.25
8	98	4.83	6.90





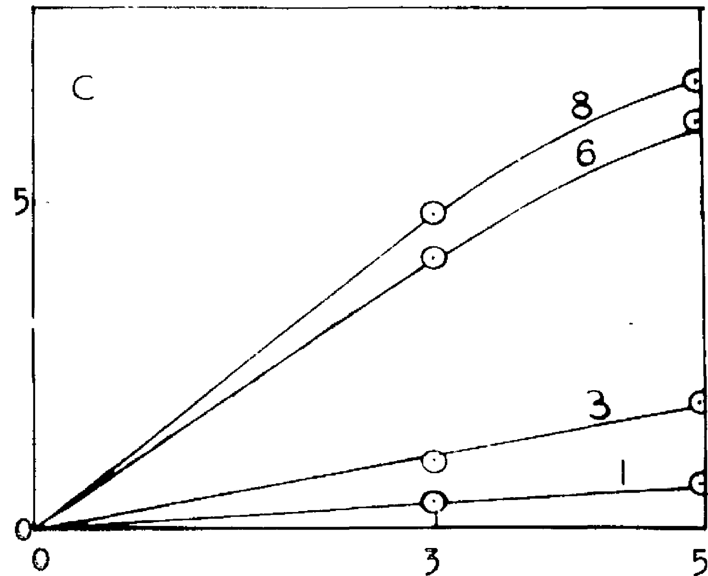


Fig. 36.

Digestion rate curves of Free Amylase preparations of non-dormant, (A), partially dormant, (B), and pricked partially dormant grains of A. fatua, (C), during 9 days in germination conditions. Numbers on curves correspond to the number of days the samples have spent in germination conditions.

Abcissa: Length of digestion period, mins.

Ordinate: Mgms. of starch digested.

Digestion Rate Curves of these preparations are given in Fig. 36.

A sample of the partially dormant grains after 8 days in the imbibed condition was separated into those showing embryo growth, and those showing no embryo growth, and Free Amylase solutions prepared from each group. The Dextrinising Activity of these enzyme solutions was determined on two samples, the mean values being given below.

	Mgms. Starch Digested at,	
	3.	5 mins.
Dormant Grains.	0.38	0.65
Germinated Grains.	5.30	7.24

Experiment 38. The distribution of Free Amylase activity within grains of *Avena sativa* during Imbibition, Germination, and Development, and in grain parts cultured independently.

Some husked grains of *A. sativa* were surface sterilised by 20 mins. shaking in 0.1% Mercuric Chloride, rinsed three times in sterile distilled water, and placed to germinate in sterile petri dishes under standard conditions. A similar batch of grains was allowed to imbibe for 12 hours after sterilisation, and then the embryo and scutellum were carefully dissected out, rinsed in three washings with st erile distilled water, and cultured on a nutrient agar medium already shown to be capable of maintaining the growth of these parts, (Harris, 1958). Distal-halves of dry grains were also prepared, carefully freed of scutellum tissues, sterilised, and washed as above, and maintained in petri dishes under the same conditions as grains receive for germination. At intervals, during

a 10 days period, Free Amylase solutions were prepared from equal numbers of whole grains, embryos and scutellums, and endosperms separated that day from whole grains, and embryos and scutellums, and distal half-grains, cultured independently from the commencement. The Dextrinising Activity of 1 ml. samples of two Free Amylase solutions from each type of preparation was determined, the mean values of these being given below.

a). Whole Grains.

No. of days in Imbibed condition.	Mgms. Starch Digested at, 2.	3.	5.	10 mins.
2	0.20*	0.21	0.63	1.05
3	1.50	2.40	4.07	6.10
4	2.60	4.05	6.60	9.30
5	4.80	7.02	8.40	9.30
6	5.40	8.10	8.90	9.30
7	6.80ø	8.50	9.00	9.40
9	5.10	7.20	8.15	9.25

b). Embryos and Scutellums from Whole Grains.

No. of days in Imbibed condition.	Mgms. Starch Digested at, 2.	5.	20 mins.
2	0.04*	0.70	0.28
3	0.60*	1.65	5.20
4	1.20	1.80	6.75
5	1.80	4.22	9.30
6	1.20	2.80	8.75
7	0.85	2.10	6.30
10	0.40	-	3.84

c). Endosperms from Whole Grains.

No. of days in Imbibed condition.	Mgms. Starch Digested at, 2.	3.	5.	10 mins.
2	0.21*	0.35	0.64	1.05
3	1.10	2.10	2.52	5.05
4	1.60	2.84	4.20	7.00
5	3.10	4.90	7.82	9.30
6	4.80	7.55	8.75	9.40
7	5.50ø	7.80	8.58	9.25
10	4.3	6.7	8.5	9.25

d). Cultured Embryos and Scutellums.

No. of days in Imbibed condition.	Mgms. Starch Digested at, 2.	20.	40.	60 mins.	22 hrs.
5	0.00*	0.00	0.00	0.00	1.50
6	0.00*	0.00	0.00	0.00	3.30
7	0.03*	0.64	-	1.20	7.90
10	0.22*	3.84	6.14	-	-

e). Distal Half-grains.

No. of days in Imbibed condition.	2.	3.	5.	20.	60 mins.
3	0.00*	-	-	-	0.14
4	0.00*	-	0.10	0.47	-
5	0.02*	-	0.14	0.70	-
6	0.02*	-	0.14	0.70	1.40
7	0.10*	-	0.56	1.68	4.50
10	2.20	6.72	8.50	-	-

The digestion rate curves of this data were prepared and it was confirmed that the observed 2 min. digestion values lay on the linear portion in each case. Where no value was observed, an intercept value for this digestion time was taken, these being marked above by an *. Where the observed value lay beyond the linear portion of the curve, an extrapolated value was taken, these being given above accompanied by an ø.

Experiment 39. The effect of removing grain parts on the development of Free Amylase Activity in *Avena sativa*.

Samples of dry grains of A. sativa were carefully dissected so that some had the embryo removed, and others the embryo and scutellum. These mutilated grains were placed in the same conditions as whole grains for seven days under standard germination conditions. On the second, fourth and seventh days Free Amylase solutions were prepared from two samples of

each treatment, and the Dextrinising Activity of 1 ml. samples of these determined. The mean values of these determinations are given below.

a). Whole Grains.

No. of Days in Imbibed condition.	Mgms. Starch digested at, 10,	20,	30,	40,	60 mins.
2	1.47	2.80	4.35	5.60	7.60

No. of Days in Imbibed condition.	2,	5,	10,	15,	20 mins.
4	2.80	4.35	7.00	7.40	7.70

No. of Days in Imbibed condition.	1,	3,	5,	10 mins.
7	4.05	7.25	8.65	9.25

b). Grains with Embryo Removed.

No. of Days in Imbibed condition.	Mgms. Starch digested at, 10,	30,	60,	90 mins.
2	0.07	0.21	-	0.84
4	-	0.84	2.24	3.36
7	0.39	1.05	2.86	4.35

c). Grains with Embryo and Scutellum Removed.

No. of Days in Imbibed condition.	Mgms. Starch Digested at, 10,	30,	60,	90 mins.
2	-	0.22	-	0.63
4	-	0.28	0.56	0.84
7	-	0.28	0.56	0.92

The reaction rate curves of these preparations is given in figure 37.

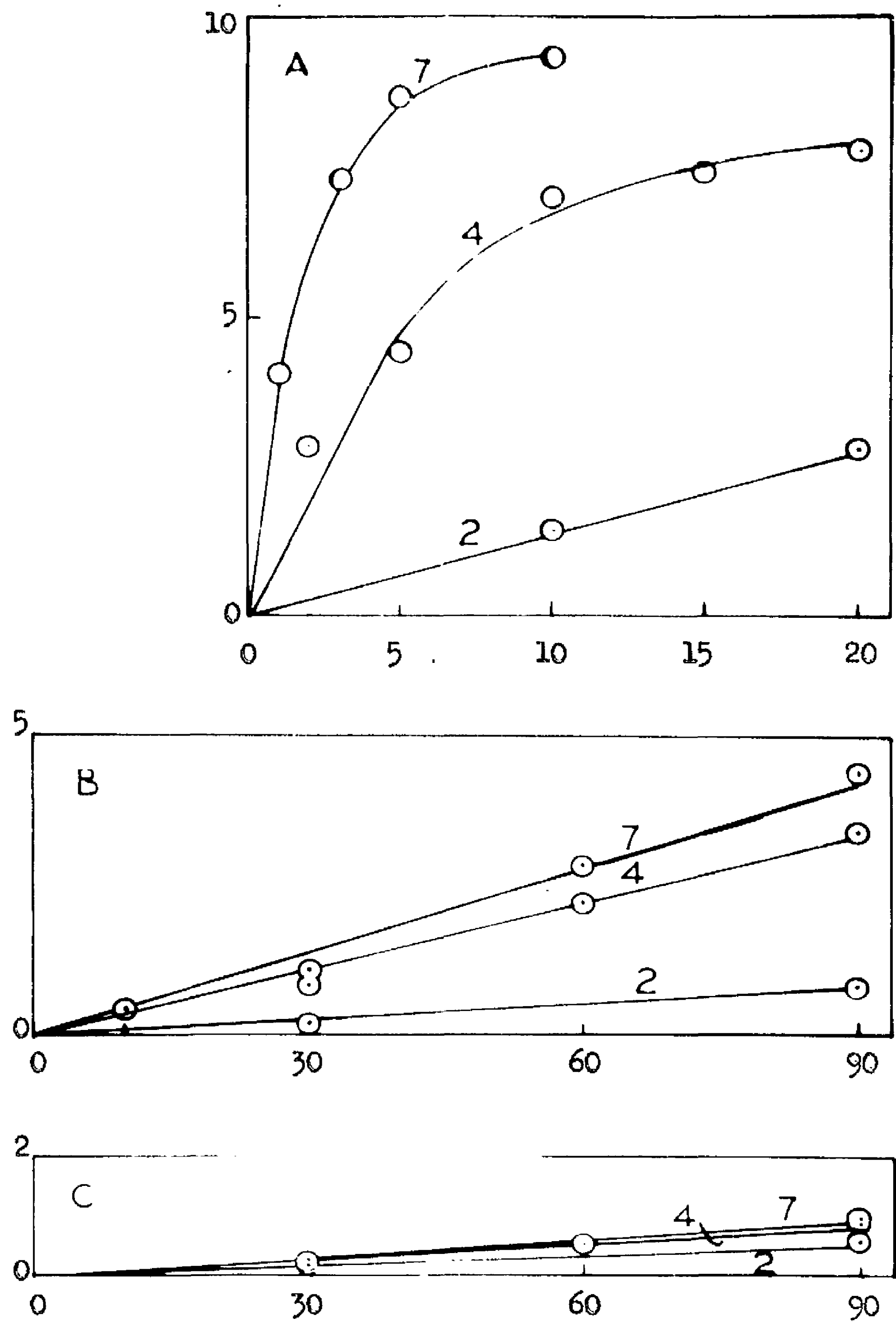


Fig. 37.

Digestion rate curves of Free Amylase preparations from whole grains of A. sativa, (A), caryopses minus embryos, (B), and caryopses minus embryos and scutella, (C), during 7 days in germination conditions.

Numbers on curves correspond to the number of days spent by the sample in germination conditions.

Abcissa: Length of digestion period, mins.

Ordinate: Mgms. of starch digested.

Discussion.

In the dry grain of A. sativa, the presence of a small amount of Free Amylase has been found (Expts. 33-35). A comparison of the 2 min. digestion values of similar preparations (Expt. 33) during the period of imbibition, germination and embryo development of these grains indicates that there is a considerable increase in this activity, reaching a maximum of more than a 20 fold increase by the 8th day, and decreasing from then on (Fig. 38). In this experiment the first signs of increased activity were found with the two day preparation, but in later studies, where longer digestion periods, or more concentrated enzyme preparations, were employed a slight increase in Free Amylase activity was evident within 22 hours of commencing imbibition, (Expt. 34, 35).

The nature of the Amylase Activity present during this period was investigated by determining the α-Amylase activity present (Expts. 34, 35). Dry grains, and grains imbibed for 16 hours showed no activity, but the presence of traces of α-Amylase could be shown after 22 hours imbibition, with prolonged digestion periods, or more concentrated enzyme preparations. A comparison of the 2 minute digestion values for Dextrinising Activity, and the 15 min. values for Sugar Forming Activity (Fig. 39), shows that the α-Amylase activity increases from this time right through the nine day period covered by the experiment, though the Free Amylase activity had passed its optimum development within this time. The non-α-Amylase activity, which is the difference between these two, also shows its optimum development before the end of the experimental period. Both Dextrinising Activity and Sugar Forming Capacity show these

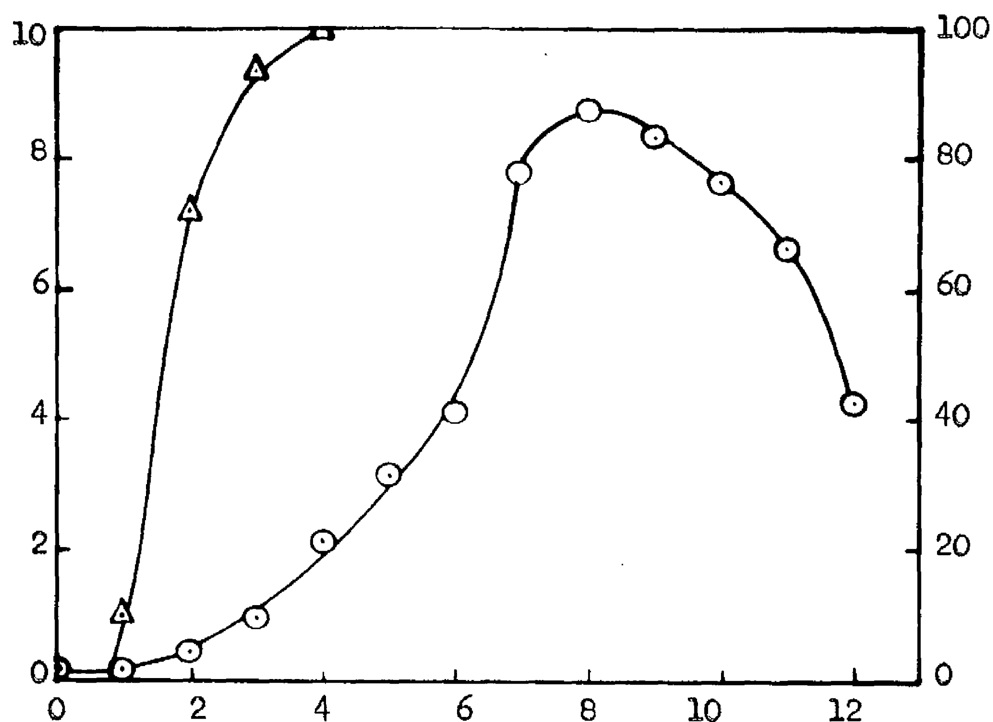


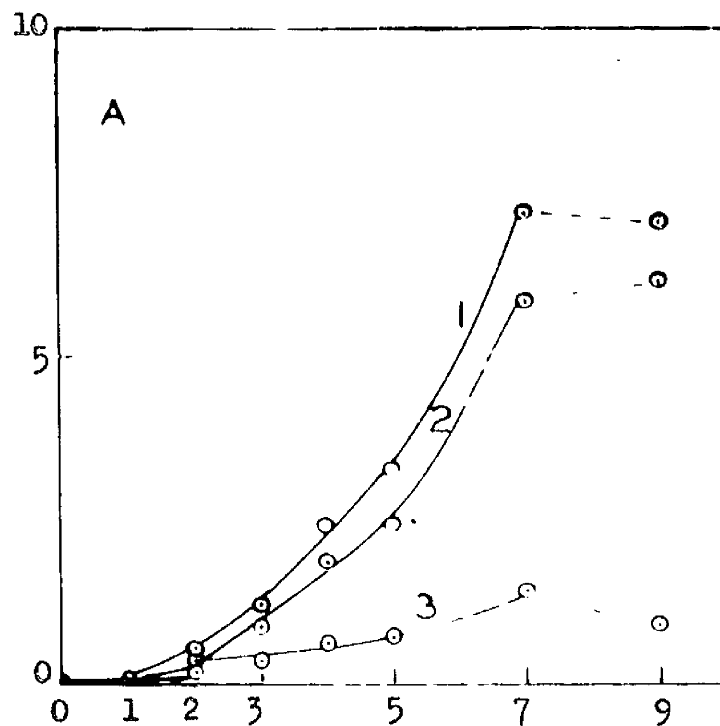
Fig. 38.

Changes in the Free Amylase activity of grains of A. sativa during 12 days in germination conditions.

Mgms. of starch digested at 2 mins., (⊙), and germination rate, (△).

Abcissa: Period in germination conditions, days.

Ordinates: Left. Mgms. starch digested at 2 mins.
Right. Germination %age.



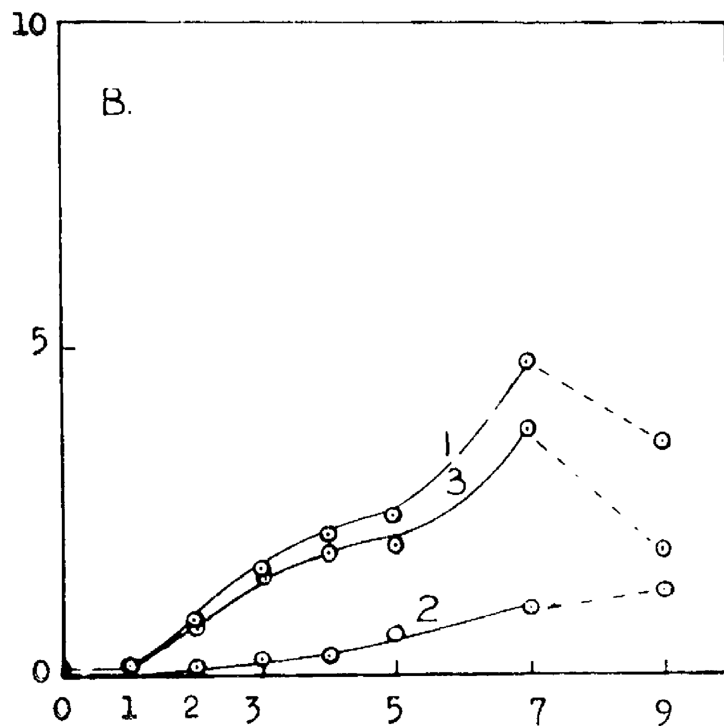


Fig. 39.

Changes in Free Amylase,(1), a-Amylase,(2), and non-a-Amylase activity,(3), in grains of A. sativa during 9 days in germination conditions. Measured as Dextrinising activity,(A), - mgms. of starch digested at 2 mins. - and Sugar Forming activity,(B), - mgms. of sugar formed at 15 mins.

Abcissa: Period in germination conditions, days.

Ordinate: Mgms. starch digested, or sugar formed.

trends. As would be expected, α-Amylase activity accounts for most of the Dextrinising Activity of the Free Amylase preparations, but only accounts for a small proportion of the Sugar Forming Capacity. Conversely, non-α-Amylase only contributes 20% of the Dextrinising Activity, but 80% of the Sugar Forming Capacity of the Free Amylase preparations, at its optimum development, on the seventh day.

Taken together, these results point to a pattern of Amylase development in which β-Amylase is initially present alone, followed by a period when both Amylases are present and increasing in activity, then a period when β-Amylase is decreasing while α-Amylase is maintaining, or slightly increasing its activity. The very considerable drop in the Dextrinising Activity of Free Amylase solutions prepared from grains after 9 - 12 days in germination conditions (Fig. 38), suggests that α-Amylase activity decreases during this period also. The preparation of A.V. / Conversion curves, from the data of Expt. 34, brings out these trends of changing composition of the Free Amylase solutions (Fig. 40). The α-Amylase preparations conform consistently to the pattern expected from them (cf. Page 85, and Fig. 40), while the Free Amylase preparations show all gradations from the pure β- type curve of the dry grain, to the almost pure α-type of the 9 day sample. The development of α-Amylase by the first day, and the change in proportion of it between the seventh and ninth days are well evident.

In <u>A. ludoviciana</u>, the activity of a Free Amylase preparation from dry grains was too low to give a value within the experimental period (3 hours), but weak activity was observed with a digestion period of 20 hours (Expt. 36). After 1 day, however, the activity increased and

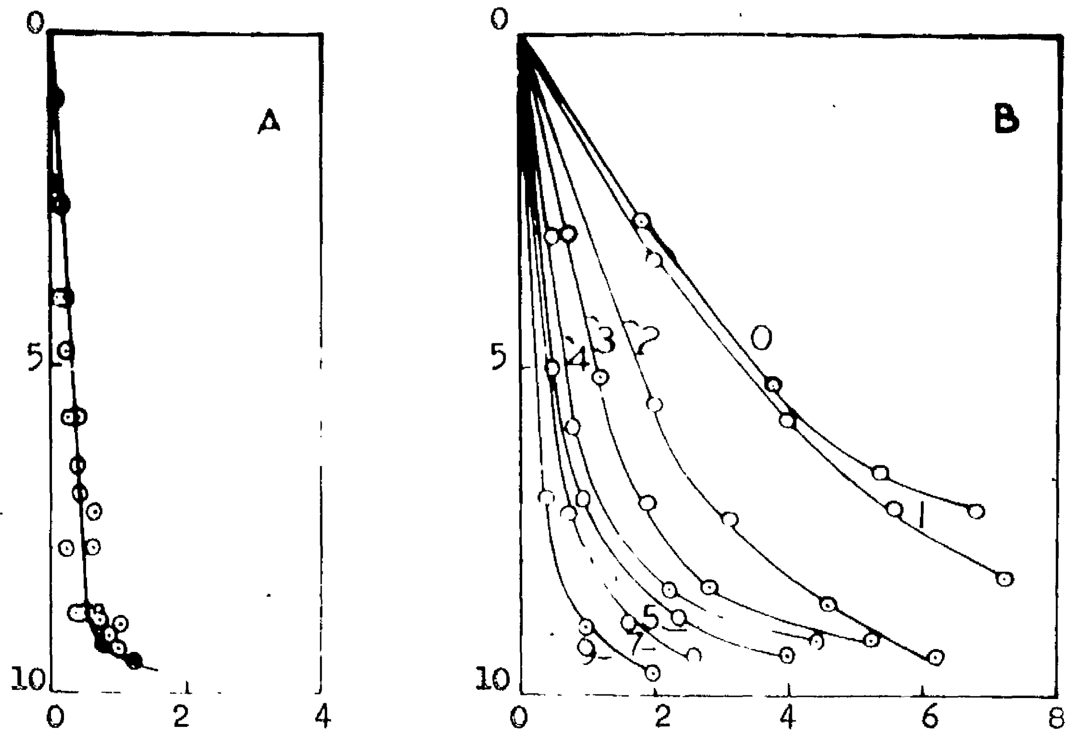


Fig. 40.

Amylose Value / Conversion curves of the a-Amylase,(A), and Free Amylase preparations,(B), of Expt. 34.

Abcissa: Mgms. of sugar formed.
Ordinate: Mgms. of starch digested.

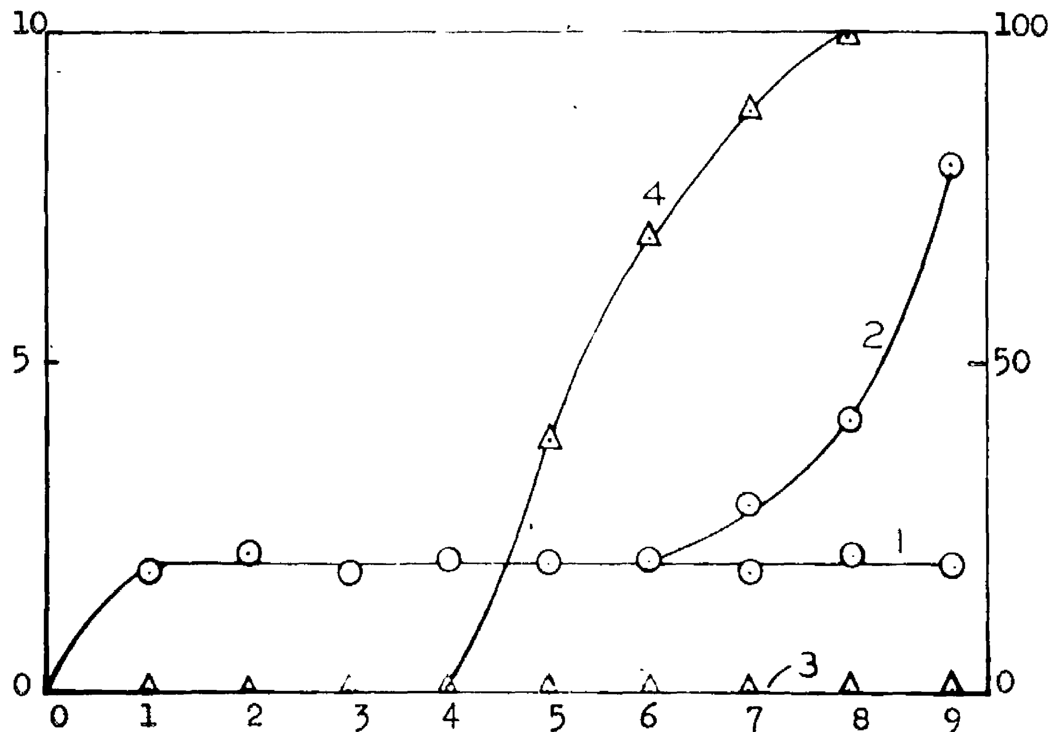


Fig. 41.

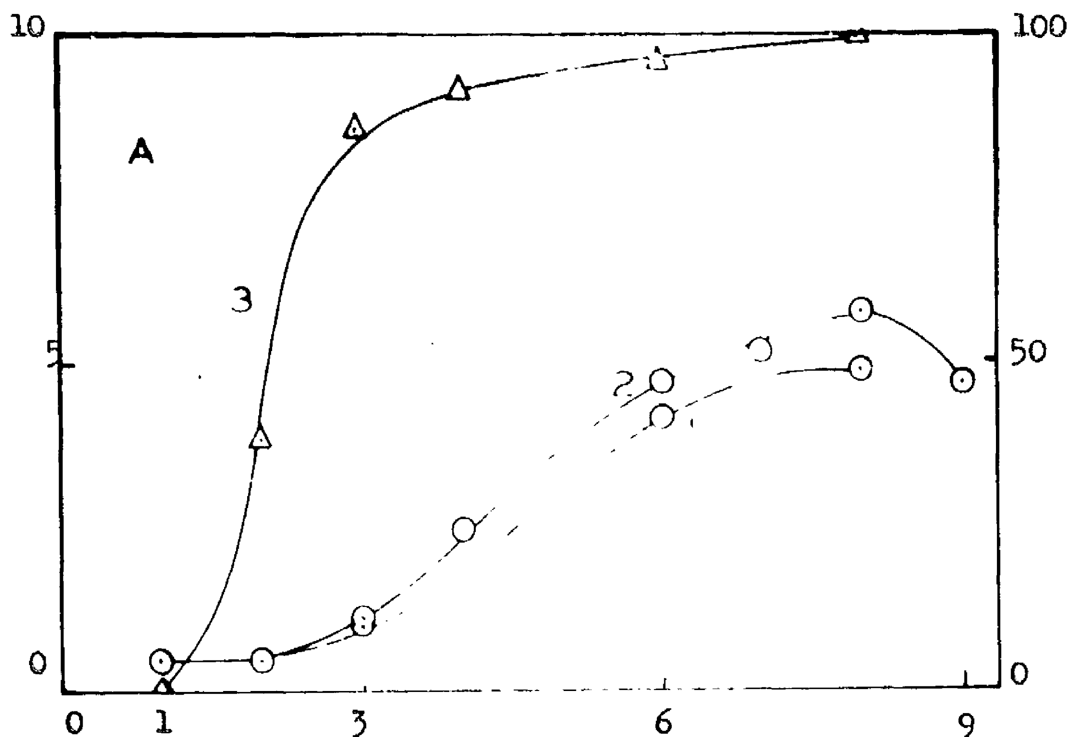
Free Amylase activity in dormant and non-dormant grains of A. ludoviciana during 9 days in imbibed conditions.

Mgms. starch digested at 25 mins. by preparations from dormant,(1), and non-dormant grains,(2). Germination rates of dormant,(3), and non-dormant samples,(4), in conditions described in Expt. 36.

Abcissa: Period in imbibed conditions, days.
Ordinate: Left . Mgms. starch digested at 25 mins.
Right. Germination %age.

in Fig. 41 the 25 mins. digestion value is compared for dormant and non-dormant samples throughout the remaining period of the experiment. No further signs of increased Free Amylase activity was found in the dormant sample. In the non-dormant sample, an increased activity was manifest by the seventh day, continuing to the ninth. Germination in this sample commenced on the fourth day, and was completed by the eighth. Some grains in the non-dormant sample were pricked after nine days, placed at 30°C for three days, and then returned to 10-12°C. Almost all of them proved to be viable after this treatment.

The investigations on Amylase development in A. fatua (Expt. 37) showed a similar trend to those reported previously, as shown in a comparison of the 3 mins. digestion values (Fig. 42). Both non-dormant samples show a quick commencement of germination, followed one day later by an increase in Free Amylase Activity. This increased activity continued to a maximum level at the eighth day, and dropped a little by the ninth. A similar pattern was found in the partially dormant sample, but in this case the level of activity was much less, reaching only some 66% of the activity shown by the same grains given a pricking treatment. This pricking treatment also had the property of increasing the germination %age from 64% to 100%. The striking similarity between the proportion of germination, and maximum Free Amylase development in these two samples suggested that Free Amylase development in a partially dormant sample was restricted only to those grains which germinated. The separation of such a sample into germinated and dormant grains after imbibition, and the determination of the relative Free Amylase Activity confirmed this suggestion (Expt. 37). The activity found in the dormant



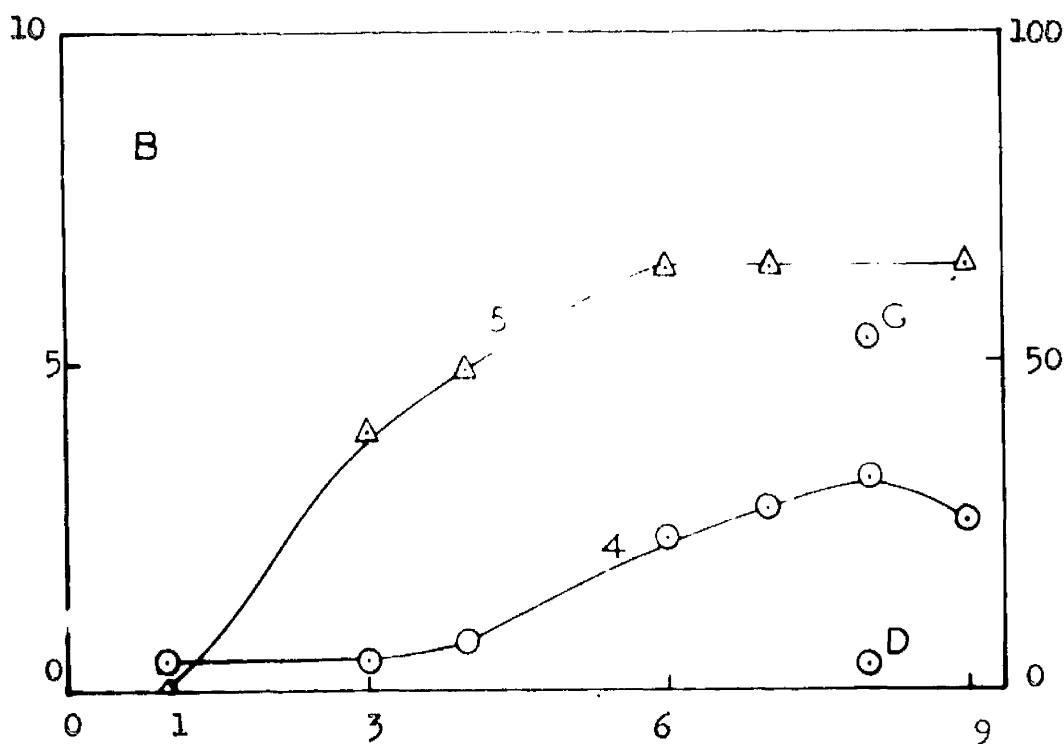


Fig. 42.

Free Amylase activity in non-dormant,(A), and partially dormant grains of A. fatua,(B), during 9 days in germination conditions.

Mgms. starch digested at 3 mins. by preparations from pricked partially dormant grains,(1), naturally non-dormant grains,(2), and partially dormant grains,(4). Germination rates of non-dormant,(3), and partially dormant samples,(5). Points "G" and "D" in B are explained in text, page 114.

Abcissa: Period in germination conditions, days.
Ordinates: Left, Mgms. starch digested at 3 mins.
Right, Germination %age.

grains is similar to that of the 1 day samples (Point D. Fig. 42B), while the germinated grains show an activity of the same order as the pricked sample of the same grains (Point G. fig. 42B).

A comparison of Figs. 38, 41, and 42, indicates that there is a general similarity in the Free Amylase development pattern in non-dormant grains of these three species. The greatest activity per grain is found in A. sativa, with A. ludoviciana showing the least activity. In A. fatua and A. ludoviciana, however, the increase in free Amylase activity is definitely a post-germination phenomenon while in A. sativa, both events begin simultaneously. In this species, however, we can discount the increase in Free Amylase activity, or the development of α-Amylase activity as events necessary before germination can occur. We know that no sign of either is present up to the 16th hour of imbibition, by which time the second phase of water uptake is commencing (Fig. 12). By the 22nd hour of imbibition when traces of α-Amylase and increased Free Amylase activity were present, many of the embryos in the more advanced grains would be undergoing extension growth (Expt. 14) and some would be on the threshold of germination (Fig. 38). These findings, in relation to the development of Amylase activity, support the view that Starch derived products are not the foundation of metabolism in the embryo during the period preceding, during, and possibly shortly after germination in these Avena species. This implies that the embryo and scutellum are sufficiently well endowed with other materials capable of sustaining their needs for this period. Studies on the isolated embryo and scutellum of Maize (Toole, 1924) and Barley (James, 1940) bear out this statement, since in these structures normal development and metabolism can be

maintained for periods up to 24 hours at least without external supplement.

Our studies on Amylase development in A. sativa, in terms of the general pattern of Free Amylase Activity, the development of α-Amylase activity, the relative times of commencement of germination and increased Amylase activity, are fairly well supported by most earlier workers who investigated Amylase development in the cereals. These include studies on Barley (Luers, 1936; Hora, 1936), Wheat (Popov, 1940; Ugrumov, 1955), Maize (Bernstein, 1943 B) and Rye (Ohlsson and Uddenbergh, 1933). In some cases, in Barley (Nordh and Ohlsson, 1933), and in Oat (Ohlsson and Edfeldt, 1933), the data show no sign of a decrease in Amylase activity during the 7 - 14 day period. The germination conditions employed in these investigations were not specified and it may be that the method of estimation was not sufficiently reliable to pick up small changes in high levels of α-Amylase activity which might be occurring during this period.

The studies of Amylase Development in A. fatua and A. ludoviciana show that no increase in Amylase activity occurs in dormant grains of these species. In these studies it has not been found possible to break dormancy without inducing the development of Amylase activity. This indicated that there is an association between the mechanisms controlling dormancy and germination, and the processes involved in the development of Amylase in these species. In view of our conclusions concerning the participation of starch derived products in the pregermination metabolism of these grains, it is considered highly unlikely that the lack of the increased Amylase activity in the dormant grain is the factor responsible for the dormant condition.

In comparing the relative distribution of Free Amylase activity within the grain of A. sativa, the two minute digestion values were chosen as suitable, (Expt. 38, Fig. 43). It would be expected that the first signs of increasing Amylase activity would be found in that part of the grain in which Amylase was being developed. An examination of our results (Fig. 43) shows that the first part to show an increased activity was the endosperm, which continued to retain the largest proportion of the activity throughout.

If all the factors involved in the development of the increased Amylase activity were located within one part of the grain, and it could be separated and cultured independently, this part would be expected to show a similar pattern of Amylase development to the whole grain. In the case of the two grain parts studied here, (Expt. 38), neither the Embryo and Scutellum, nor the Distal Half-grain showed any approximation to the Amylase development of the whole grain, or to the same parts detached from the whole grain at the time of testing (Fig. 43). This is substantiated by the studies in which the embryo alone, and the embryo and scutellum were removed from whole grains, and the Amylase development of the mutilated grains compared with whole grains (Expt. 39, Fig. 37). A comparison of the relative amounts of Starch digested by the 7 day preparations during 30 mins. unlimited digestion shows an activity ratio of 120 : 1 : 0.3 mgms. Starch, (400 : 4 : 1), for whole grains, grains minus embryo, and grains minus embryo and scutellum. In no case did the mutilated grain show any development of Amylase activity which approached the whole grain activity, though the two types of mutilated grains show different levels of Amylase activity. The belated development of amylase

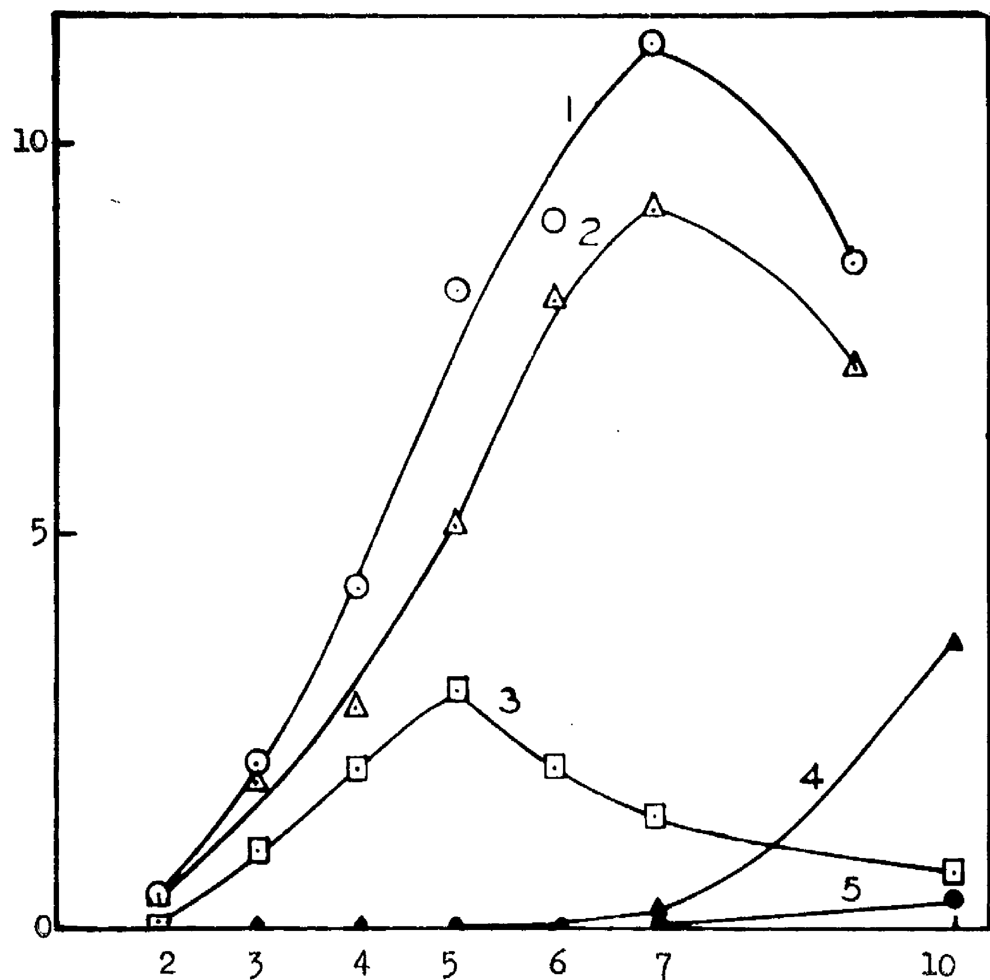


Fig. 43.

Distribution of Free Amylase activity within the grain of A. sativa and in grain parts cultured independently for 10 days in germination conditions.

Mgms. starch digested at 2 mins. by Free Amylase preparations from whole grains,(1), endosperm portions,(2), and embryo and scutellum portions of whole grains,(3), isolated distal half-grains,(4), and isolated embryo and scutella, (5).

Abcissa: Period in germination conditions, days..

Ordinate: Mgms. starch digested at 2 mins.

activity in the isolated distal half-grains observed here (Fig. 43) agrees with similar findings with isolated endosperms of Rye, Wheat, Barley, and Maize (Bruschi, 1908; Horning & Petrie, 1927), and from studies on naturally occurring embryoless Wheat grains (Lyon, 1928). These findings indicate a high degree of inter-relationship within the parts of the grain, and the necessity for the maintenance of its structural integrity in order that the normal pattern of Amylase development may occur.

Our previous investigations have shown that most of the increase in the Dextrinising Activity of the Free Amylase preparations during imbibition and after germination is due to the development of α-Amylase. Our finding that the endosperm is the first part of the grain to show this increase is indicative that the α-Amylase develops there. This agrees with the results of Elion (1945), who found it mainly in the cells adjoining the aleurone layer and the scutellum, and Linderstrom-Lang and Engel (1937), who showed α-amylase activity in the regions adjoining the aleurone layer. They did not investigate the scutellar region. Both workers also found β-Amylase activity in the embryo and scutellum of the grains investigated, mainly in the epithelial layer of the scutellum. This agrees with our finding that the endosperm alone has less free amylase activity than the endosperm plus scutellum (Expt. 39). It might also explain the amount of amylase activity found in the cultured embryo and scutellum fraction after 7 days (fig. 43), though this activity is much less than in the same fraction removed from the whole grain on the third day. This difference between the amylase activities of the same grain part cultured independently, and removed

from the whole grain, suggests that either the cultured embryo and scutellum is unable to develop to the full its potential amylase activity, or that the same fraction detached from the whole grain has been having its activity supplemented from the endosperm fraction. Soluble nitrogenous material has been shown to be reaching the embryo by the second day in germinating Barley (Folkes and Yemm, 1957), but whether amylases can be translocated in the same way is unknown.

While both fractions separated from the whole grain show an increasing amylase activity for the period up to the fifth day, the embryo and scutellum fraction shows a considerable decrease from then on (Fig. 43). The continuing increase in the endosperm fraction from the fifth to the seventh day is much greater than can be accounted for solely by the transference of the activity present in the embryo at this time, to the endosperm, and studies on the movements of nitrogenous materials within the seed at this stage of development indicate that such a movement would be in opposition to the main direction of flow (Folkes and Yemm, 1957). Though the translocation of this amylase from the embryo to the endosperm cannot be disproved, it would appear more likely that the drop in amylase activity in the embryo fraction is due to inactivation of the enzyme. If this is the case, the continued decrease in amylase activity would be due to a rate of inactivation greater than the rate of amylase development in this part of the grain, or the rate of amylase entry into it, from the endosperm. The general drop in activity observed in the whole grain from the eighth day might be due to a development of the amylase inactivating mechanism in the endosperm, or to a greater loss of amylase by translocation to the embryo and

inactivation there, than could be met by the processes of amylase development elsewhere in the grain. Previous investigators have also found evidence for the presence of amylase inactivating systems in the embryo fractions of Wheat (Oparin and Kaden, 1945) and Barley (Dax and Graesser, 1946) after several days germination.

The source from which the increased amylase activity develops during the germination of seed has been the subject of several previous investigations. It is generally agreed that the increase in β-Amylase which accompanies and follows germination is due to the formation of this enzyme in comparable amounts during seed development, with its apparent disappearance during ripening, to be again developed during germination. This sequence has been demonstrated in Wheat, Barley, Rye, Oat and Maize, (Chrzaszcz and Janicki, 1936; Bernstein, 1943 A; Sandstedt and Beckford, 1946; and Ugrumov, 1955). The low activity in the dry grains of these species is shown to be only an apparent loss by the fact that most of the β-Amylase which develops later can be extracted from dry grains by special extraction methods. The use of H_2S, Peptone, Papain, and some baking chemicals, like cysteine, glutathione, etc., in the extraction procedure causes the release of the total β-Amylase, (Chrzaszcz and Janicki, 1936, A & B; Ford & Guthrie, 1908, and Davidson, 1945). It is felt that these treatments simulate, or stimulate, a natural process which occurs during the germination of the grain, and since these substances are either proteolytic enzymes, stimulators of proteolytic activity, or products of proteolysis, it is generally agreed that the β-Amylase in the dry seed is inactive due to its adsorption or incorporation with other proteinaceous substances. Since these substances are also potential sulphydryl donors,

and β-Amylase has been shown to require a free sulphydryl group for its activity (Caldwell, 1945), it is possible that the action of these substances might also include a regeneration of any of the enzyme's sulphydryl groups which were oxidised during the period of inactivation of the enzyme in the ripening seed.

The situation with regard to the development of α-Amylase during germination is a bit more uncertain. The presence of small amounts of α-Amylase in immature grains of Wheat, Barley, Rye, and Oat has been claimed, or inferred, but these amounts disappeared well before ripening, and could not be extracted from the ripe grains by extraction with papain or peptone (Chrzaszcz and Janicki, 1936; Sandstedt and Beckford, 1946; and Ugrumov, 1955). In Maize no α-Amylase activity could be found at any stage during seed formation (Bernstein, 1943 A). Even where α-Amylase activity was present before ripening, the amounts present in every case were found to be only a small fraction of the total activity which developed after germination had taken place. This suggests that the bulk of the α-Amylase activity following germination is due to synthesis of the enzyme in the germinating seed. Few studies have been carried out on seed or seed parts to ascertain the parts responsible for the synthesis of α-Amylase, and the factors which must be available before the synthesis can take place. Oparin and Kaden, (1945) have shown that incubation of ground dry grains of Wheat with a brei of sprouted embryos led to a development of α-Amylase activity, while a brei of unsprouted embryos did not have this property.

Some studies on the formation of the same α-Amylases by other plant tissues have however been reported. These include cultured virus tumour

tissues of Rumex sp. (Brakke, et al, 1951), and submerged cultures of Aspergillus spp. and Penicillium spp. (Shu and Blackwood, 1951 A & B.; Goodman, 1950). In these, the greatest amounts of Amylase were formed when the carbohydrate present was Starch, or Maltose, the enzyme only forming in some cases by adaption to the presence of Starch. Most sources of nitrogen were capable of being utilised, ammonium acetate proving as good as hydrolysed protein. This is not the case however with animal preparation, in which the requirement of at least 10 amino acids has been shown to be necessary before Amylase synthesis occurs (Hokin, 1951). The synthesis of α-Amylase in cell free preparations from animal tissues has also been demonstrated recently, and in this case, the additional requirements of A.T.P. and R.N.A. were shown. The presence of sub-cellular particles in these preparations was also claimed as necessary for the synthesis to take place, though no attempt to isolate those responsible was reported (Straube, et al, 1955). These studies on plant preparations and animal systems indicate the need for a fairly high level of metabolic activity to be going on in the tissues in which α-Amylase is being synthesised, which fits in with our findings that it only develops in grains of Avena spp. in which germination has occurred or is about to occur.

An association between amylase activity and mitochondria was advanced by Horning and Petrie (1927). Using cytological methods, these workers found the development of large numbers of mitochondria, in the scutellum of germinating cereals, and subsequently in the neighbouring endosperm cells where they seemed to be involved in the process of starch digestion. They concluded that a migration of these particles occurred from the

scutellum to the endosperm. No evidence of a similar behaviour was found in the aleurone cells. This increase in the numbers of mitochondria has been confirmed in studies of the scutellum of germinating Maize using modern ultra-centrifugal techniques (Hagemann and Hanson, 1955). This mitochondrial preparation also had starch digesting capacities, but a similar preparation from the embryonic axis lacked them. A later study of similar preparations to these by electron micrography showed that a considerable amount of endoplasmic reticulum was also present in these cases, so no direct association between the mitochondria and amylase activity could be made (Hanson, et al, 1959). Engel and Bretschneider (1949) had come to the same conclusion when they tried to correlate the numbers of mitochondria and the amylase activity of various parts of Wheat grains; Stafford (1951) found the amylase activity remaining in the supernatant after centrifuging down the particulate matter of homogenized Peas; while Daniellson and Sandegren (1942) found that both amylases were present in the water soluble albumin fraction of Barley grains. Millerd and Bonner (1953), and Goddard and Stafford (1954), in reviewing the enzymic properties of mitochondria from plants and animals make no mention of an association between amylase activity and mitochondria in either.

While the movement of mitochondria within living cells is now well substantiated (Gay, 1953), there has never been any evidence to bear out the theory of Horning and Petrie (1927) that they can move from one cell to another. The finding of these same authors that a belated increase in mitochondrial numbers take place in isolated endosperms is itself one argument against their theory of mitochondrial migration, though in this

case too, evidence of increased amylase activity at the same period was also present. This finding is substantiated by our studies on distal half-grains (Expt. 38, Fig. 43). The simplest explanation of the observations of these investigators is that the increase in numbers of mitochondria in the scutellum and subsequently in the endosperm, is due to their development there in response to some inducing factor. The presence, and functioning of the mitochondria might however be necessary for the synthesis of α-Amylase. The formation of α-Amylase in the animal preparations mentioned previously would seem to be dependent on the presence of sub-cellular particles of some sort, and the presence of A.T.P. is itself indicative of the implication of mitochondria. The mitochondria have also been described as the main location of peptide bond synthesis in plant cells (Webster, 1953), a property which would be necessary in the formation of a complex protein like α-Amylase.

Our findings that the structural integrity of the seed must be retained for the normal development of α-Amylase activity is largely in agreement with these considerations on its mode of development, and in complete agreement with the view that α-Amylase develops in the endosperm of the grain in response to some diffusible factor from the growing embryo or its scutellum.

Summary.

1. The Free Amylase activity of non-dormant grains of A. sativa, A. fatua, and A. ludoviciana has been measured during imbibition, germination and development. A low activity was found in the resting seed, which increased after imbibition to a maximum value about the 8th day, and decreased thereafter.

2. The main reason for the increased activity in A. sativa has been shown to be due to the development of α-Amylase, after 20 - 22 hours imbibition in germination conditions, and its increase thereafter for a period of some nine days. The non-α-Amylase fraction also increases during the first seven days in the same conditions.

3. The increase in Free Amylase activity has been shown to be a post-germination phenomenon in A. fatua, and A. ludoviciana, and concurrent with germination in A. sativa. No increased activity occurred in dormant grains of the former two species unless first stimulated to germinate.

4. The first signs of Increased Free Amylase activity were found in the endosperm of A. sativa but it only developed when the structural integrity of the grain was maintained, indicating a participation of the other seed parts in its development. Some indication of an amylase inactivating system in the embryo and scutellum fraction was evident from the fifth day.

5. The relevance of these findings to the participation of endospermic food reserves in the metabolism of the germinating grain, to the nature of the dormancy mechanism in A. fatua, and A. ludoviciana, and to previous literature in this field has been discussed.

Part IV. Changes in the Amounts of some of the Major Metabolites during the first few days in Germination Conditions.

The nature of the substrates used by the respiring embryo during the early stages of growth and germination have been investigated by studying the changes in the levels of fats, sugars, and starch within the seed during this period. Changes in the level of soluble nitrogen compounds were determined in relation to the studies reported in the next part and will be discussed there. Changes in the fat content were determined by measuring the amount of ether soluble material extracted from ground samples. Soluble carbohydrates were extracted with 80% ethanol, and determined colorimetrically as Maltose. This fraction would be expected to be very heterogeneous, so a chromatographic analysis of the types of sugars present at the various times was also carried out. Starch determinations were carried out using hot water, and perchloric acid extraction procedures and determing the iodine staining capacity of the extract, and by acid hydrolysis and colorimetric determination of the sugars as Maltose.

Methods and materials are given in each experiment.

<u>Experiment 40</u>. Changes in the Ether Soluble materials in grains of <u>A. sativa</u> during the first three days in germination conditions.

Batches of 200 grains were germinated in groups of 50 in standard conditions as previously described (Part I). Three batches of 200 grains were taken as dry grains, and after 1, 2, or 3, days in germination conditions, dried for about 20 hours at 80°C in a ventilated oven. The batches were then finely ground in a coffee mill, stored in a soxhlet

why?

extraction thimble for a further 20 hours at 80°C, and weighed before extracting with ether in the normal soxhlet procedure. After extraction the thimble was again dried for 20 hours at 80°C and reweighed.

The first time this experiment was carried out an extraction period of 3½ hours was used. This was subsequently felt to be insufficient so 6 hours extraction was used on the repeat experiment.

No. of days in germination conditions.	First Experiment.		Second Experiment.	
	Loss on extraction, mgms.	Mean.	Loss on extraction, mgms.	Mean.
0.	0.515		0.614	
	0.598		0.585	
	0.527	0.546	0.590	0.596
1.	0.480		0.516	
	0.507		0.506	
	Lost.	0.493	0.518	0.513
2.	0.454		0.489	
	0.478		0.484	
	0.435	0.455	0.482	0.485
3.	0.418		0.460	
	0.444		0.453	
	0.399	0.420	0.462	0.458

Per 200 grams?

<u>Experiment 41.</u> Changes in the carbohydrates soluble in 80% ethanol during the first three days in germination conditions.

The material used in this determination was the ether extracted residue from the second of the previous experiments. This material was further ground after drying until it all passed through a 100 mesh sieve. 0.5 gm. samples of this were ground to a fine paste with a little sand and few drops 80% ethanol and then suspended in 20 mls. 80% ethanol and shaken mechanically for 20 minutes. This solution was centrifuged, the supernatant recovered and the pellet extracted twice more with the same volume of ethanol. The combined volume of the supernatants was made up to 100 mls. The Phenol-Sulphuric acid method of determining sugar concentration was utilised on samples of this solution (see Part III). A 2ml. portion of the testing solution was found to give too high a reading for accurate determinations, so portions of the first three sets of testing solutions were diluted by a factor of 2, and the three day solution by a factor of 4. The maltose calibration curve (Fig. 28) as used in this experiment was read in terms of the mgms. maltose per 100 ml. testing solution, so the only corrections required in this case were the dilution factors utilised in giving a sample within the suitable reading range. These determinations were carried out twice on a total of five samples of each material. Some of these samples were lost in the first experiment through broken centrifuge tubes, but those determined agreed with the results of the second experiment given below as the mean of two samples at each stage.

No. of days in germination conditions.	Observed Maltose Conc., mgms. / 100 ml.	Dilution factor.	Corrected Maltose Conc., mgms. / 0.5 gms.	%age Maltose in Sample.
0.	3.13	x2	6.26	1.23
1.	3.05	x2	6.10	1.20
2.	3.30	x2	6.6	1.32
3.	3.7)	x4	15.4	3.08

Wt. Calcn shd. be based . 0·5 gm.

<u>Experiment 42</u>. Changes in the Starch content of grains of <u>A. sativa</u> during the first three days in germination conditions - I. By Hot Water Extraction.

The material utilised in these determinations was the same as that used in the previous experiment. Two 0.5 gm. samples from each day were boiled in 50 mls. distilled water for 40 minutes, filtered through a Buchner filter (Whatman no.1) and the residue washed with approx. 50 mls. hot water. The residue and filter paper were again boiled for another 10 mins. and this solution filtered and washed in the same manner as the first. The combined volume of filtrate was made up to 500 mls. with distilled water. The starch content of this solution was determined by the Iodine-Starch colour developed by the procedure described previously (Part III). The samples used were 0.5 mls. instead of the 1 ml. sample of the standard procedure in order to give a density which could be accurately determined. Since the total volume of this test solution is 500 mls., and the concentration of the starch is determined in mgms. / 20 mls. from the calibration curve (Fig. 26), a multiplication of the observed concentration by 25 for this factor, and by 2 for the dilution factor, is needed to give the amount of starch in the total

extract. The results given below are the mean of two determinations.

No. of days in Germination Conditions.	Observed Starch concentration mgms. / 20 mls.	Total starch extracted. mgms. / 0.5 gms.	%age Starch in sample.
0.	7.10	355	71
1.	6.95	345	69.5
2.	6.60	330	66
3.	5.90	2.95	59

<u>Experiment 43</u>. Changes in the Starch content of grains of <u>A. sativa</u> during the first three days in germination conditions, - 11. By Perchloric Acid Extraction (McReady et al, 1950).

The materials used in these determinations were the same as that used in the two previous studies. Two 0.5 gm. samples from each day were extracted three times with 20 mls. freshly diluted 30% Perchloric acid. 15 minutes extraction were allowed each time, followed by centrifuging and collection of the supernatant. The residue after the third extraction was washed four times with cold distilled water on a Buchner filter, the total volume of extract being made up to 600 mls. with cold distilled water. 1 ml. samples were used to determine the starch concentration by the Iodine-Starch colour as described previously. The total amount of starch extracted was determined by multiplying the observed starch content in mgms / 20 mls by 30.

No. of days in Germination Conditions.	Observed Starch concentration mgms. / 20 mls.	Total Starch extracted. mgms. / 600 mls.	%age Starch in sample.
0.	10.4	312	62.4
1.	10.6	318	63.6
2.	10.0	300	60
3.	6.85	205.5	41.1

<u>Experiment 44</u>. Changes in the Starch content of grains of <u>A. sativa</u> during the first three days in germination conditions, - 111. By Acid Hydrolysis.

The material used in these determinations was the same as that used in the three previous studies. Two 0.5 gm. samples from each day were boiled for 2.5 hours in N. HCl. The solution was filtered through a Buchner filter (Whatman no.1 paper), and the residue washed three times with boiling water, the total volume of the filtrate being made up to 500 mls. In order to determine the sugar content of this extract by the Phenol-Sulphuric Acid method (see Part III) it was necessary to dilute samples of it to 1/40th concentration. Since the reading of the sugar concentration of two ml. samples of this diluted solution are given in mgms. Maltose / 100 mls. test solution, it is necessary to multiply this value by 200 to get the total maltose extracted since the testing solution in this case is 500 x 40 mls. The means of the two determinations are given below. To convert Maltose to Starch a conversion factor of x 0.95 was used.

No. of days in Germination Conditions.	Observed Maltose concentration mgms. / 100 mls.	Total Maltose extracted. mgms. / 0.5 gms.	%age Maltose in sample.	%age Starch in sample.
0.	1.67	334	66.8	63.4
1.	1.65	330	66.0	62.9
2.	1.55	310	62.0	58.9
3.	1.35	270	54.0	51.3

Experiment 45. The chromatographic analysis of the 80% ethanol extracts of grains of *A. sativa* during the first five days in germination conditions.

Five grams of the materials used in Expts. 41 - 44 were extracted three times with 30 mls. 80% ethanol, each extraction consisting of 30 mins. mechanical shaking. The suspension after this treatment was centrifuged for 10 mins., and the combined supernatants evaporated to dryness at approx. 40°C. The dry material was dissolved in 5 mls. 80% ethanol and 0.02 mls. of these extracts drop loaded on Whatman No. 1 chromatography paper. Marker spots of Xylose, Fructose, Glucose, Sucrose, Maltose, Maltotriose, and Maltotetraose were also included on the chromatogram. Chromatograms were run with Propyl alcohol : Ethyl acetate : Water (6 : 1 : 3) as the solvent for periods of 24 - 30 hours, at room temperature. During this period the solvent front had usually run to 35 - 40 cms. After air drying, the papers were sprayed with Benzidine - Trichloracetic acid reagent (0.5 gms. Benzidine dissolved in 10 mls. glacial Acetic acid, added to 10 mls. 40% Trichloracetic acid, and then to 80 mls. absolute Ethanol), and the spots developed with heat. Examination by ordinary light was supplemented by the use of ultra-violet light to bring out weak or invisible spots.

The distances moved by the marker substances, relative to the solvent front, (R_f.), were,

Sugar.	R_f.
Xylose.	0.44 - 0.47
Fructose.	0.40 - 0.42
Glucose.	0.37 - 0.38
Sucrose.	0.32 - 0.30
Maltose.	0.22 - 0.23
Maltotriose.	0.165
Maltotetraose.	0.11

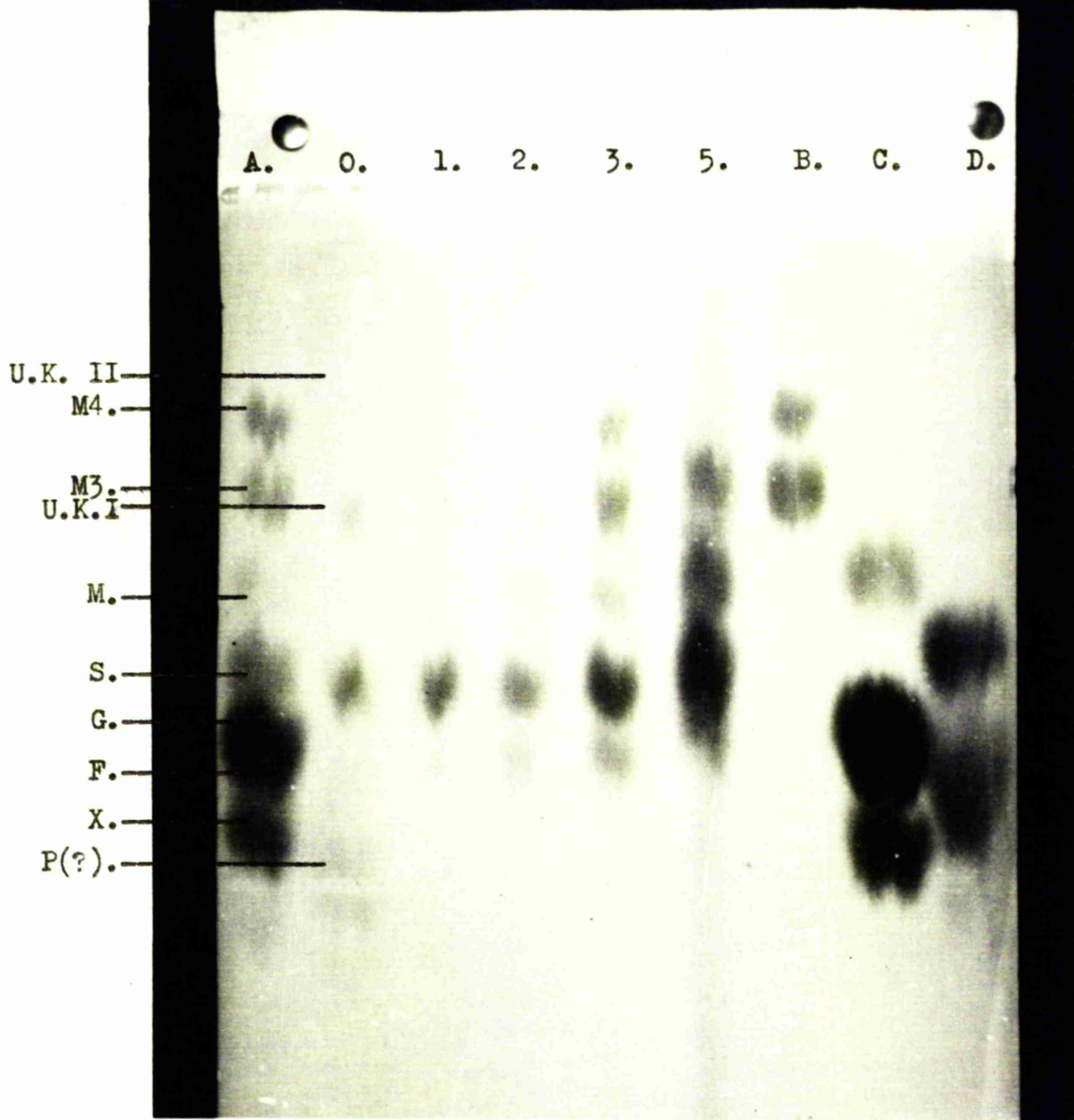
Five runs of these extracts were carried out, on two different extractions with similar results each time. A typical result is shown in the accompanying plate. Using a scoring system, with the following symbols, the amount of the sugars present in these extracts is indicated below.

- Absent.
+ Only visible under U.V.
++ Faint spot.
+++ Dense spot.

Sugars.	Dry Grains.	Extract Source, 1,	2,	3,	5 days
Xylose.	+(?)	-	-	-	-
Fructose.	-	-	-	-	-
Glucose.	-	+	+	+++	+++
Sucrose.	+++	+++*	+++	+++	+++
Maltose.	-	-	+	++	+++
Maltotriose.	-	-	+	+++	+++
Maltotetraose.	-	-	+	+++	++
Unknown I.	+++	++	-	-	-
Unknown II.	+	-	-	-	-

* Consistently less dense than others.

Unknown I, with an R_f of 0.18 - 0.19 is probably a trisaccharide which runs faster than Maltotriose. Unknown II, with an R_f of 0.095 might be a slow moving tetrasaccharide, or a sugar of larger dimension.



Chromatographic analyses of the carbohydrates soluble in 80% ethanol in grains of A. sativa during 5 days in germination conditions.

Marker spots run together,(A), and in groups of Maltotetraose and Maltotriose,(B), Maltose, Glucose, and Xylose,(C), and Sucrose and Fructose,(D). Extracts of grains number to correspond to the period spent in germination conditions. Symbols used, M4 = Maltotetraose, M3 = Maltotriose, M = Maltose, S = Sucrose, G = Glucose, F = Fructose, X = Xylose, U.K. I & II = Unknown spots, P(?) = Possible pentose.

Discussion.

The amount of ether soluble material in grains of A. sativa shows a marked decrease in the first 24 hours in germination conditions, continuing to decrease at a slower rate thereafter (Expt. 40, Fig. 44). This result is in complete agreement with a similar investigation on the same species by Albaum and Eichel (1943).

The carbohydrates soluble in 80% ethanol show a slight fluctuation in amounts during the first 24 - 48 hours, which probably represents a slight decrease followed by an increase in amount, which is well marked by 72 hours (Expt. 41, Fig. 45). Studies on the composition of this fraction during this period are more revealing, however (Expt. 45). A slight trace of a Pentose sugar was found in the dry grain but not subsequently. Fructose was not evident to any extent during the first five days. Glucose, absent from the dry grain, made its appearance within 24 hours and increased from then on. Sucrose was present all the way through but seemed to diminish slightly in the first 24 hours to increase again later. Maltose, Maltotriose, and Maltotetraose were all absent from the grain until the 48 hour stage and increased thereafter. The two unknown sugars were both only present in the early stages. These trends indicate that changes are occurring within the soluble carbohydrates within the first 24 hours in germination, and that some sucrose and possibly some of the unknown sugars were being metabolised during this period. The total amounts involved however are so small, less than 0.5% of the total sample, that it is impossible to imagine these compounds being the sole respiratory metabolic substance in the first 24 hours. From the 48 hour stage on, these studies on the whole grain are only explainable

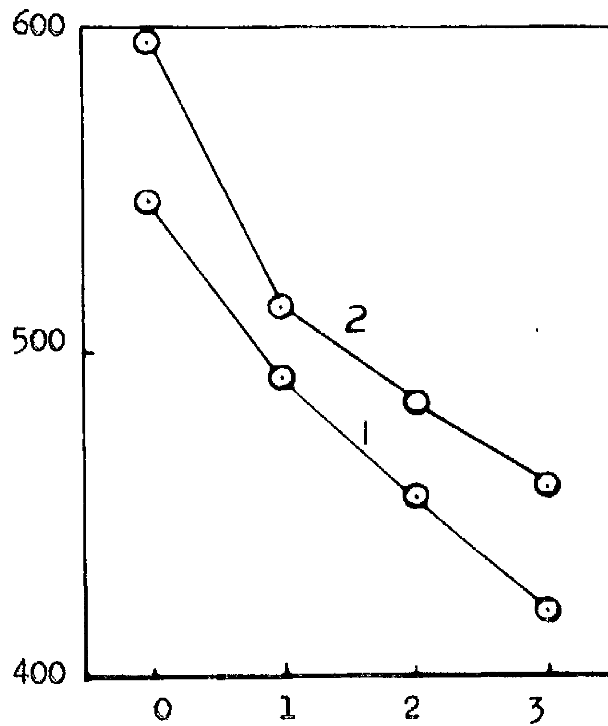


Fig. 44.

Changes in the level of ether soluble materials in grains of A. sativa during 3 days in germination conditions.
Estimated after $3\frac{1}{2}$ hours,(1), and 6 hours extraction,(2).

Abcissa: Period in germination conditions, days.
Ordinate: Mgms. ether soluble materials extracted / 200 grains.

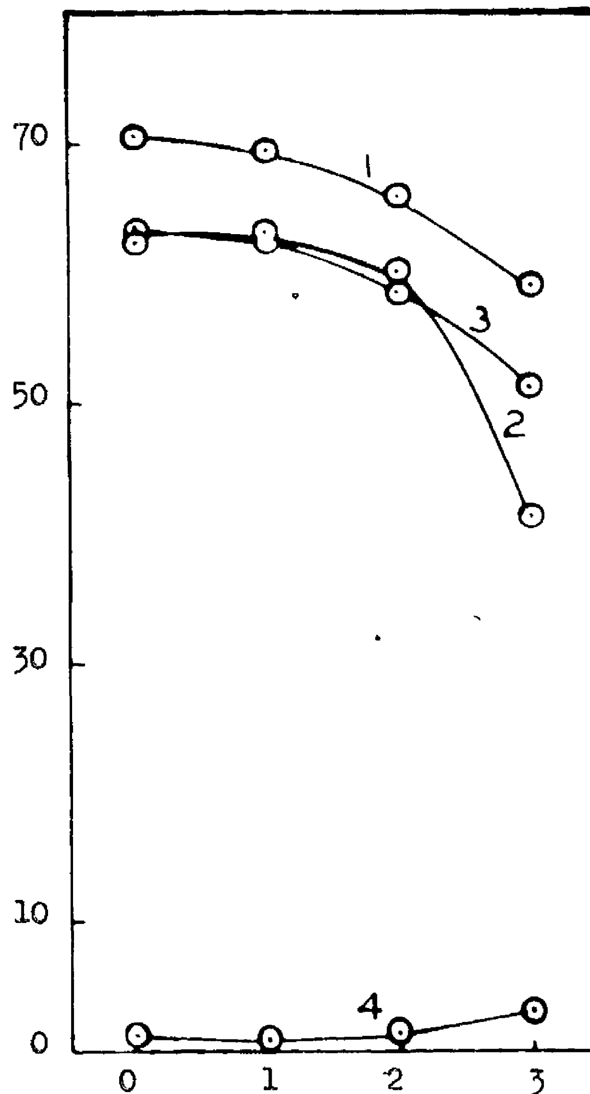


Fig. 44.

Changes in the level of starch and soluble carbohydrates in grains of A. sativa during 3 days in germination conditions.
Starch measured by hot water,(1), or perchloric acid extraction,(2), and estimated by iodine colouration, or by acid hydrolysis and estimated as maltose. Soluble carbohydrates extracted with 80% ethanol and estimated as maltose.

Abcissa: Period in germination conditions, days.

Ordinate: Starch or soluble carbohydrate as a %age of the dry weight of the sample.

by the commencement of starch digestion in the endosperm with the formation of starch derived sugars and sucrose. These qualitative studies show a good agreement with earlier quantitative studies on the carbohydrate metabolism of germinating Barley (James and James, 1940).

The identity of the unknown sugars in the dry grains is purely conjectural but James and James (1940), and MacLeod (1957), have both shown the presence of the trisaccharide Raffinose in dry grains of Barley, and its disappearance in 1st and 2nd days in germination conditions. Chromatographic evidence for the presence of Raffinose in dry grains of Wheat and other members of the Gramineae is also available, and in addition, the presence of a small amount of the pentose Arabinose, and of several sugars of size greater than four hexose units has been noted (Williams and Bevenue, 1952, MacLeod and McCorquodale, 1958). These observations support our finding of the two unknown sugars in Experiment 46, and the doubtful presence of a pentose sugar in the dry grain.

The studies on Starch changes support the conclusions arrived at in relation to the soluble carbohydrates (Expts. 42 - 44, Fig. 45). These show an insignificant change in the level of the starch fraction in the first 24 hours, but obvious decreases by 48 hours and later. Similar results are to be found in the studies of Hora (1936) on Barley, and of Bernstein (1943 B) on Maize.

The comparison of these changes in the major carbon containing metabolites carried out in these experiments leads to the general conclusion that the metabolism of the seed during the pre-germination period, and shortly after, is based on materials other than Starch. The component

showing the biggest and earliest decrease is the Ether Soluble material, but the participation of Soluble carbohydrates, and possible protein derivatives in the carbon metabolism of the seed cannot be neglected. This conclusion is in general agreement with the views of Albaum and Eichel (1953), who also studied the metabolism of germinating Oat grains.

Summary.

1. Changes in the levels of Ether Soluble material, Carbohydrates soluble in 80% ethanol, and Starch have been investigated during the first few days in germination conditions, and the composition of the soluble carbohydrate fraction examined.

2. It is concluded that digestion of endospermic Starch materials does not occur before the embryo has commenced growing, and consequently Starch does not supply the main source of carbon metabolites during the first 24 hours or more.

3. The participation of seed Fats and soluble carbohydrates in supplying these compounds is strongly supported.

4. The relevance of these findings to those of previous investigators is discussed.

Part V. Studies on the Proteolytic Enzymes of A. sativa, A. fatua and A. ludoviciana in dormant and germinating grains.

The physiological diversification of the embryo cells of a germinating grain is dependent on the capacity of these tissues to synthesise proteins. These materials constitute the primary part of all the cytoplasmic structures, so the multiplication of these structures in cell formation depends on the development of additional, similar proteins. The enzymes which have been purified and examined so far have also been shown to be proteins, frequently in conjugation with other organic molecules or ions. Proteins are macromolecular nitrogenous substances, which can be broken down into gradually smaller fractions by hydrolytic treatments, the basic component of which are the amino acid, and the amide. In the protein, these compounds are joined together by peptide bonds, the sequence and proportion of the individual types determining the physical and physiological properties of particular proteins.

The processes by which proteins are synthesised in plants is however a subject on which divergent views are held. The most widely accepted theory is that proteins synthesis takes place essentially by the reverse of protein hydrolysis, viz. by the conjugation of free amino acids and amides in a particular sequence until a macromolecule of the dimension, composition, and properties of the particular protein is obtained. In growing whole plants, the free amino acids are probably synthesised from ammonia, and α-ketoglutaric acid, giving glutamic acid. From this glutamic acid, the whole range of other amino-acids can be derived. In a seed germinating in distilled water, however, other considerations are necessary, since we are dealing with a closed system with respect to external sources of nitrogen. In this case,

the free amino acids and amides must be already present in the dry seed, or there must be a means of producing them from other sources in the seed. The hydrolysis of existing proteins is one obvious source, since this will give a direct pool of these compounds. If there is protein synthesis occurring in the immediate neighbourhood, then there is no reason to suppose that a direct incorporation of such amino acids into new protein might take place. The bulk of the evidence obtained so far does not support the view however that there is a widespread translocation of all the amino acids and amides released in protein hydrolysis from a considerable distance to the site of protein synthesis. It would appear that a certain few forms, and especially amides, are formed from the hydrolysate, and that these are the main mobile nitrogen compounds translocated to the sites of protein synthesis. Once there, the process is reversed and the elaboration of the many forms from the few translocated compounds takes place. The many enzymes required for these transformations, transaminations, and interconversions are widely present in plant tissues, and many of them have been isolated and characterized. The other main theory is more a variant of this one than a contradiction of it and is rapidly losing ground in the face of new information. In this theory, the main carbon framework of the new protein is synthesised separately, and then the amino groups are inserted into it at appropriate positions. The amino-group donors are thought to be the amides. In this scheme the amino acid is therefore solely a product of protein hydrolysis, and must have its amino group transferred to an amide in order that the nitrogen may recirculate once more. In both theories, however, it is implicit that a recirculation of combined nitrogen can occur, in what is termed the protein cycle. Also, in both cases, there is a release of a considerable part of

the carbon framework of the existing protein, and the incorporation of other carbon compounds into the framework of the new protein. Such changes are entirely consistent with the observed relationships between protein hydrolysis and respiration on the one hand, and protein synthesis and carbohydrate metabolism on the other.

The amount of free amides and free amino acids in dry cereal grains is not very large, Bailey (1944) quoting values of 8 - 10% of the total nitrogen of wheat grains as being present as soluble amino acids, amides, and peptides. Judging by analyses of flour samples, (Sullivan and Payne, 1951) a considerable proportion of this is present in the endosperm.

The number of amino acids present are also limited to some 15-18, while protoplasmic proteins may consist of 22 or more (Biserte and Scribban, 1950; Sullivan and Payne, 1951). These considerations suggest that there must be a speedy inauguration of the hydrolysis of existing proteins in the germinating cereal in order to maintain protein synthesis in the germinating embryo. Observations on changes in the protein and non-protein nitrogenous compounds of germinating Wheat and Barley bear this out. There is an increase evident in the total amounts of free amino acids and amides in the first 48 hours in germination conditions, accompanied by an increase in the number of amino acids to include all those normally present in proteins, and by changes in the relative proportions of the individual types. There is also an overall increase in the protein content of the embryo, at the expense of the protein content of the other grain parts (Folkes, Willis, and Yemm, 1958; Biserte and Scribban). These changes are entirely consistent with a papain-type hydrolysis of existing storage proteins, mainly in the

endosperm, with the conversion of the amino nitrogen into amides for transportation to the sites of protein formation in the embryo.

The Proteins of the cereals have however proved to be a very complex mixture, and the particular proteins, which are true reserve proteins, are difficult to distinguish. Most of these previous studies have been carried out on Wheat and Barley, but it appears that the general types of protein found are similar in all the common cereals. At present these can only be separated into broad groups by differential solubility, but some indications of the individual components of these are now being obtained by sedimentation, and electrophoretic investigations. The presence of at least two different enzyme proteins - α- and β-Amylase, in the albumin fractions of Barley has been mentioned previously (Daniellson and Sandegren, 1947). The occurrence of at least two similar globulin components in all the common cereals and of an additional two in Barley is also fairly well established (Daniellson, 1949). The prolamin of Barley has also recently been shown to consist of at least five components, whose proportions change at different rates during germination (Biserte and Scriban, 1950). The glutelin proteins of the cereals are the most difficult to obtain in a reasonably pure state, but there is no evidence to suggest that a pure fraction would be any more homogeneous than the others. In terms of amino acid composition, the albumin proteins of Barley have been shown to resemble protoplasmic proteins of Barley leaves while the other components show differences from this and from each other, (Folkes and Yemm, 1956). The distribution of the proteins is also found to be localised to a considerable extent in the grain. The embryo contains most of the albumin and one of the globulin components which are common to all the cereals, while the endosperm contains the other globulin components,

and the prolamin and glutelin proteins. The relative amounts of the different protein classes is also different in the common cereals. Wheat, Barley, Rye and Maize all contain about 80% of their protein in the form of prolamin and glutelin, but in Oat there is only 15 - 20% of these components, and 80% of globulin protein (Brohult and Sandegren, 1954). A consideration of the relative amounts of these protein fractions, their amino acid composition, and distribution in the seed, would seem to support the conclusion of Osborne (1909) that the albumins are not storage proteins but probably the physiologically active proteins of the seed. Analyses of the changes in the various protein fractions in Wheat and Barley bear this out. It is found that the prolamin-glutelin fractions and the small amounts of albumin and globulin in the endosperm undergo a speedy hydrolysis between the second and the sixth day, while the albumin component of the whole seed is increasing (Folkes, Willis and Yemm, 1958; Biserte and Scribban, 1950). This suggests that the proteins in the endosperm, particularly the prolamin-glutelin proteins, are the main seed reserve proteins of the grain. A closer investigation of the globulins of Barley during germination, however, showed that the globulin component in the embryo had almost completely disappeared before the endosperm components showed much change (Saverborn, Daniellson and Svedberg, 1944). This indicates that the embryo tissues may also be involved in the storage of reserve proteins, probably in the cells of the scutellum.

The same considerations discussed with regard to the mobilisation of reserve starch in the germinating grain, viz. that a substrate cannot be utilised in the absence of the enzymes involved in that utilisation, led to a survey of the presence and distribution of the enzymes responsible for

hydrolysing proteins in A. sativa. In addition to this, estimations of the increase in free tryptophan and free tyrosine in whole grains and parts of grains were carried out at different times during the first few days in germination conditions, this being taken as evidence of protein hydrolysis in the part concerned. From these studies it may be possible to ascertain the possible role of the various seed parts in supplying these requirements of the growing embryo at different periods during imbibition, germination, and development. Studies on the proteolytic activity of grains of A. fatua and A. ludoviciana were also carried out to compare the performance of dormant and non-dormant grains of these species in this respect.

The material discussed in this section has been recently reviewed in Yemm and Folkes (1958), Steward and Thomson (1954), Brohult and Sandegren, (1954), and Pace (1955).

Materials and Methods.

The first protein solutions used in determining proteolytic activity were prepared according to Anson and Mirsky (1934) by dissolving Edestin in Urea. When a crude enzyme extract from *A. sativa* was added to this a considerable amount of precipitation was observed and it was subsequently found that this reduced the rate of enzyme activity and in some cases prevented it completely. This was overcome by making up the edestin solution in 0.2M. Acetic Acid, and using this solution in preparing the Buffer. This was the method employed by Mounfield (1936). In this case too, some precipitation could be shown if sufficient seed extract was added, but there was no observable effect of the rate of action of dilute seed extracts initially at least (see Expt. 51). This same observation was made by Mounfield in his study on proteolytic activity in Wheat grains.

The choice of an Acetic acid - sodium acetate Buffer (Walpole, 1914), was due to the observation by Mounfield that buffers containing chloride, citrate, or phosphate ions proved inhibitory to his Wheat preparations. In making up this buffer solution 41 mls. of 0.2 M. Acetic acid were added to 9 mls. 0.2M. Sodium Acetate, and 50 mls. distilled water to give a pH of 4.0. 0.5 gms. of Edestin was dissolved in the acetic acid before this so that a final concentration of 0.5% Edestin, and 0.1m Acetate ions was present in the digestion mixture. In preparing a digestion mixture, the distilled water portion of the buffer solution was omitted and replaced by an equivalent amount of the enzyme preparation. Both were equilibrated to the digestion temperature before mixing. The volume of the digestion mixture was usually 20 mls., but in some cases larger volumes were used. The digestion

temperature was 40°C, and the pH was initially 4.0 (see Expt. 50).

The rate of proteolytic activity was determined by the increase in the level of free tyrosine and tryptophan in the digestion mixture. To measure this samples were removed, at appropriate intervals, either of 2 or 4 mls. digestion mixture, and added to 4 mls. of 6%, or 2 mls. of 12% Trichloroacetic acid. Proteinaceous materials precipitable in 4% T.C.A. were precipitated by this treatment and could be removed by centrifuging and filtering the supernatant after standing for at least 30 mins. at room temperature (Expt. 48). The tyrosine content of the supernatant was determined by adding 2 mls. of filtrate to 4 mls. 2% Sodium Hydroxide, and adding 1 ml. of 1/3rd diluted Folin and Ciocalteau Phenol Reagent, slowly and with swirling. The blue colour produced after 10 minutes at room temperature was determined spectrophotometrically at 650 mμ against a blank of distilled water, and the concentration of the tyrosine and tryptophan present estimated by reference to a calibration curve of a standard Tyrosine solution (Expts. 46, 47). This solution contained 0.1gm of D-L Tyrosine in 100 mls. 0.2M HCl, containing 5% Formalin, giving a stock solution of 1,000 p.p.m. from which requisite concentrations could be prepared, (Anson and Mirsky, 1934). In later experiments this procedure is described as the standard procedure, the volume of the digest solution tested being specified in each case. The results of some of the procedural experiments are given in optical density units, but in the main studies the results are expressed as the increase in the tyrosine concentration (p.p.m.) of the T.C.A. filtrate at the stated digestion times.

This estimation procedure is substantially the spectrophotometric version of the colorimetric procedure of Anson and Mirsky (1934) which is described in Davis and Smith (1955).

Analar, or Fine Grade chemicals were used throughout, the Folin and Ciocalteau reagent being obtained commercially (B.D.H.).

The grains used were A. sativa var. Victory, A. fatua var. pilossissima, and A. ludoviciana.

In preparing the enzyme solutions, grains of A. sativa were ground to a fine paste with a little sand and distilled water. Usually 10 mls. of distilled water were added for each ten grains, and the brei allowed to extract for two hours at $4^{o}C.$ before being centrifuged, and used directly to determine its proteolytic activity. The grains of A. fatua and A. ludoviciana were husked before grinding to remove any microorganisms growing over the hairy coverings. Toluene was used liberally during extraction and digestion procedures. Unless stated otherwise in the later experiments enzyme activity was determined on 10 mls. of this extract, this corresponding to the proteolytic activity of 10 grains.

In the following pages some experiments relating to the use of these materials and procedures are reported.

Results.

Experiment 46. The stability of the colour formed by Folin and Ciocalteau's reagent with Tyrosine.

Two solutions of 50 p.p.m. Tyrosine were prepared from the standard solution and two ml. samples treated for colour development as previously described. The optical density of the colour produced was determined at intervals from two minutes on. These values are given below in terms of optical density units.

	Length of colour development, mins.							
	2	3	5	9	12	18	26	36
Solution I.	0.40	0.41	0.41	0.41	0.40	0.385	0.375	0.355
Solution II.	0.42	0.435	0.435	0.435	0.430	0.42	0.41	0.38

These values indicate that the colour produced is stable for the period of 3 - 10 mins. after producing it, and that all readings should be determined during this period. A period of 10 mins. was chosen for the standard procedure.

Experiment 47. The relation between optical density and concentration of Tyrosine using the standard procedure.

Solutions of tyrosine were prepared from the stock solution to give concentrations ranging from 0 - 100 p.p.m. Samples of these were tested in the manner described previously, and the optical density determined at 650 mu. The values found for two different series of dilutions are given below.

	Tyrosine concentration, ppm.								
	0	2.5	7.5	10	20	40	60	80	100
I.	0.02	0.045	0.10	0.12	0.195	0.33	0.46	0.65	0.78
II.	0.03	0.06	0.095	0.115	0.195	0.355	0.48	0.63	0.80

These values were obtained using distilled water as a blank, the 0 p.p.m. value being determined on a sample of distilled water given the full colour development procedure. The 60 ppm. solution with the optical density of 0.48 was also used as a blank value and the higher concentrations determined relative to this. The observed readings are given below.

	Tyrosine concentration, p.p.m.	
	80	100
I.	0.140	0.305
II.	0.134	0.285

Both of these sets of figures are graphed in figure 46. From this it can be seen that there is a linear relation between optical density and tyrosine concentration over the range tested, and that values slightly over this range can be determined accurately by using a less concentrated solution as the blank. In samples substantially more concentrated than this, further dilution of the filtrate with T.C.A. was found to be satisfactory for determination of the Tyrosine concentration. These figures are used as the Tyrosine calibration curve in further experiments.

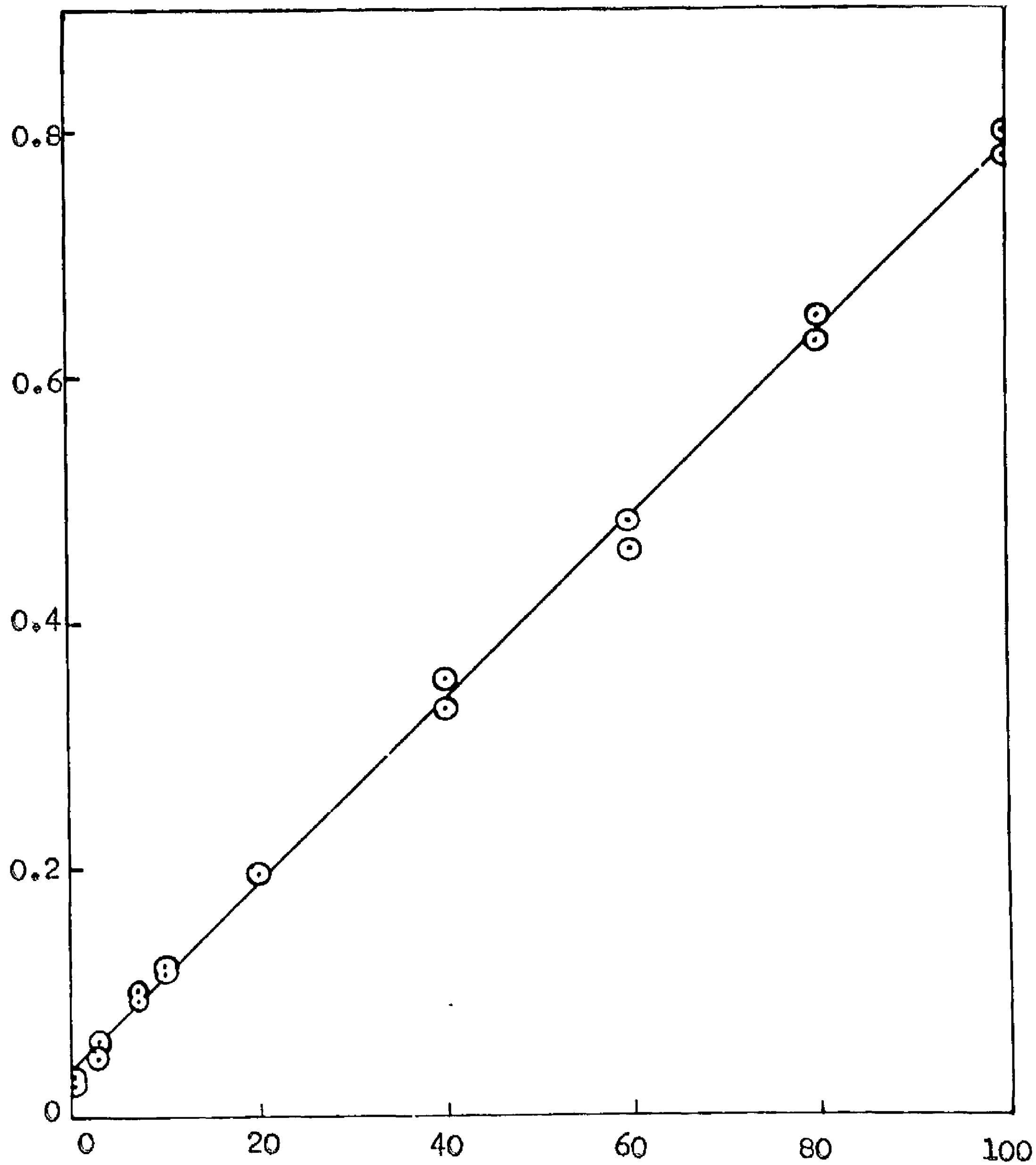


Fig. 46.

The relation between tyrosine concentration and the intensity of the tyrosine - phenol colour.

Abcissa: Concentration of tyrosine, p.p.m., in test solution.
Ordinate: Optical density at 650 mu.

Experiment 48. The Precipitation of Edestin by 4% T.C.A.

Two ml. portions of an 0.5 M. solution of Edestin in acetic acid were added to four mls. of 6% T.C.A., and allowed to stand for various periods room temperature or 70°C. before being filtered. A similar addition of Edestin solution to 4 mls. distilled water, and a 6 ml. sample of distilled water were given 10 mins., at room temperature and filtered. The tyrosine content of the various filtrates was determined in the manner previously described, the Edestin-distilled water samples being diluted one-fourth before testing. Duplicate determinations of each treatment were made, the optical density of these being given below, along with the mean tyrosine concentration of the test solution, in p.p.m.

Source of Test Solution	O.D. @ 650 mu. I	O.D. @ 650 mu. II.	Tyrosine Concentration p.p.m.
Distilled water.	0.04	0.03	0.
" " + Edestin.	0.68	0.72*	348.
T.C.A. + Edestin, 5 mins. R.T.	0.18	0.16	17.
" " , 10 " "	0.06	0.07	4.
" " , 5 mins. 70°C.	0.13	0.12	12.
" " , 10 " "	0.08	0.06	4.5

(* Diluted ¼ concentration of test solution. Tyrosine value corrected.)

No advantage was found from carrying out these precipitations at the higher temperature, so in the standard procedure thirty minutes standing at room temperature were allowed for precipitate development, following which the sample was centrifuged for a few minutes, and filtered through Whatman No. 1 filter paper. The occurrence of a small trace of non-precipitable tyrosine staining material is indicated above, in this Edestin preparation, and has been confirmed several times. The zero time determination of free

tyrosine in the digestion mixture takes account of this, however, and of the amount of similar materials added with the enzyme extracts.

<u>Experiment 49.</u> The Recovery of Tyrosine from Edestin solutions by the standard procedure.

A series of Tyrosine solutions were prepared, ranging from 80 - 320 ppm. concentration, and 5 ml. samples added to either 5 mls. 0.5% Edestin in acetic acid, or 5 mls. distilled water. After thorough shaking, 2 ml. samples of each mixture were added to 4 mls. 6% T.C.A. and the tyrosine concentration of the supernatant determined as previously described. The results of duplicate determinations of each treatment are given below in optical density units.

Tyrosine Solutions	Tyrosine conc'n. in Mixture,			
	40.	80.	120.	160 p.p.m.
With Dist. Water. I.	0.38	0.69	0.98	1.30
" " " II.	0.36	0.68	0.95	1.31
With Edestin. I.	0.375	0.69	0.93	1.26
" " II.	0.35	0.67	0.99	1.31

These figures show a satisfactory high recovery of tyrosine over a wide range of concentrations from a joint solution with Edestin, using the method described previously, and used in further studies.

Experiment 50. The effect of pH on the rate of Digestion of Edestin by proteolytic enzymes of A. sativa.

Solutions of Edestin in Acetic acid buffer solutions covering the range of pH 3.6 to pH 4.8 were prepared. Enzyme preparations were made from grains of A. sativa after 6 days in germination conditions. Extracts from two separate batches of grains, and a half-concentration extract of one of them, were tested over this pH range, ~~including~~ samples being removed after 1½ hrs. digestion. The increases in free tyrosine in the T.C.A. filtrate during this period, given below, in p.p.m., are the means of duplicate determinations.

Enzyme Source.	pH of Digestion Mixture. 3.6	3.8	4.0	4.2	4.4	4.8	No Buffer, pH 6.5*
Equiv. to 10 grains, I.	31.6	42.8	56.0	50.5	42.8	36.4	3.9
" " " " II.	36.4	-	61.0	49.2	39.0	28.5	2.6
Equiv. to 5 grains.	19.5	23.4	32.0	29.8	21.2	14.2	-

(* Immediate precipitation.)

These values are graphed in figure 47. The optimum activity is seen to be in the region of pH. 4.0, which agrees very well with the optimum value of pH 4.1 found for similar preparations from Wheat, acting on the same substrate, and using the same buffer components (Mounfield, 1936).

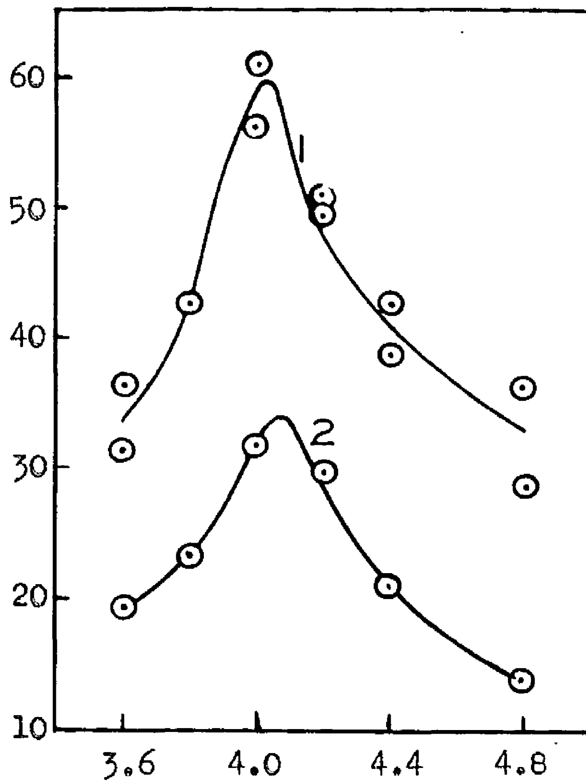


Fig. 47.

The effect of pH on the activity of proteolytic enzyme preparations from grains of A. sativa.

Enzyme preparations from grains after 6 days in germination conditions and containing the activity present in 10 grains,(1), or 5 grains,(2), in the sample tested.

Abcissa: pH of digestion mixture.

Ordinate: Tyrosine concentration of T.C.A. filtrate, p.p.m., after $1\frac{1}{2}$ hrs. digestion.

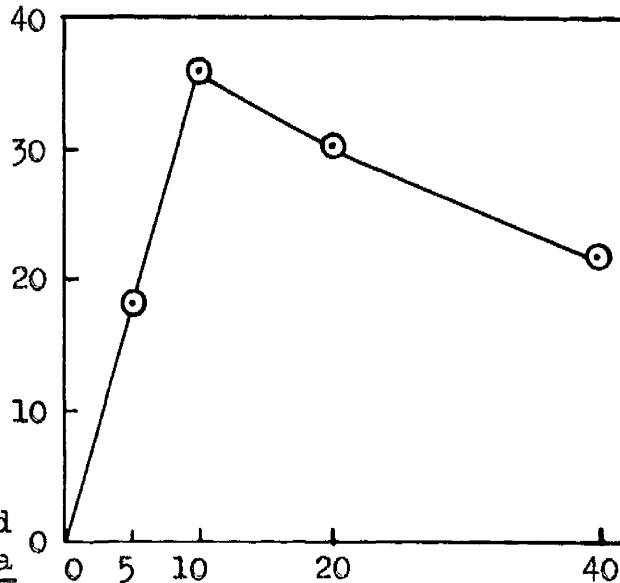


Fig. 48.

The effect of the concentration of the proteolytic enzyme preparation of 4-day germinated grains of A. sativa on the rate of enzyme action.

Abcissa: Number of grains whose activity is present in the enzyme sample.
Ordinate: Tyrosine concentration of T.C.A. filtrate, p.p.m., after 3 hours digestion.

<u>Experiment 51.</u> The effect of increasing concentration of Enzyme Extract from *A. sativa* on the rate of enzyme activity.

Enzyme extracts were prepared from grains of *A. sativa* after four days in germination conditions, such that 10 mls. of the extracts were equivalent to 5 - 40 grains, and duplicate determinations of the proteolytic activity of each concentration carried out by the standard procedure. The results of these determinations are given below in optical density units, and the mean increase in tyrosine concentration of the T.C.A. filtrate.

Enzyme Concentration.	O.D. @ 650 mu, after 0	1.5	3 hrs.	Increase in Tyrosine conc'n @ 1.5	3 hrs.	Nature of ppt.
Equiv. to 5 grains.	0.50	0.625	0.72	8	18	None.
	0.54	0.61	0.68			
Equiv. to 10 grains.	0.67	0.83	0.99			
	0.635	0.825	1.03	18	36	Slight.
Equiv. to 20 grains.	0.90	1.07	1.25			
	0.94	1.095	1.20	16	30	Dense.
Equiv. to 40 grains.*	0.94	0.97	1.03			
	0.93	0.945	1.02	7	22	Very Dense.

(* These solutions were diluted by half for determination of the O.D. The tyrosine values are given as the corrected amount.)

The 3 hr. increase in the tyrosine concentration of the T.C.A. filtrates are graphed in Figure 48. This illustrates the effect of the interaction between the seed extract and the Edestin solution mentioned previously and observed by Mounfield (1936) with his Wheat preparations. In the standard procedure the use of enzyme extracts equivalent to 10 grains was adopted, but in preparations showing weak activity the digestion mixture sample was increased from 2 mls. to 4 mls. and accordingly, the T.C.A. solution was

reduced from 4 mls. of 6% to 2 mls. of 12% concentration. This effectively doubles the concentration of the testing solution without altering its volumes or T.C.A. concentration.

Experiment 52. Proteolytic Activity in grains of *A. sativa* var. Victory during 14 days in germination conditions.

Batches of 50 grains were germinated under standard germination conditions for a period of 14 days, with supplementary distilled water added from the fifth day. Proteolytic activity was determined in the manner previously described, 2 mls. of digestion mixture being added to 4 mls. 6% T.C.A. for this determination. Duplicate or triplicate determinations of each sample were carried out, the results being given below as the mean increase in the tyrosine concentration of the T.C.A. supernatant, in p.p.m., at the stated times.

Days in Germination Conditions.	Tyrosine Conc'n. (p.p.m.) at, 1.5.	3.	4.	5.	20 hours.
0	-	-	-	5.8	28.0
1	-	-	-	12.3	53.5
2	7.8	15.6	-	22.6	62.5
3	14.2	28.6	-	44.0	108.0
4	24.0	46.0	-	76.0	142.0
5	34.0	69.0	92.0	-	-
6	58.0	103.0	126.0	-	-

Days in Germination Conditions.	0.5.	0.75.	1.0.	1.5.	2.0.	3.0.	3.5 hrs
7	-	50.5	-	88.0	-	117.0	-
9	56.0	-	86.0	-	112.0	-	-
10	50.5	-	81.0	-	120.0	-	-
12	26.5	-	47.0	85.0	-	-	-
14	13.0	-	-	42.0	-	-	91

The digestion rates of these preparations are graphed in figure 49.

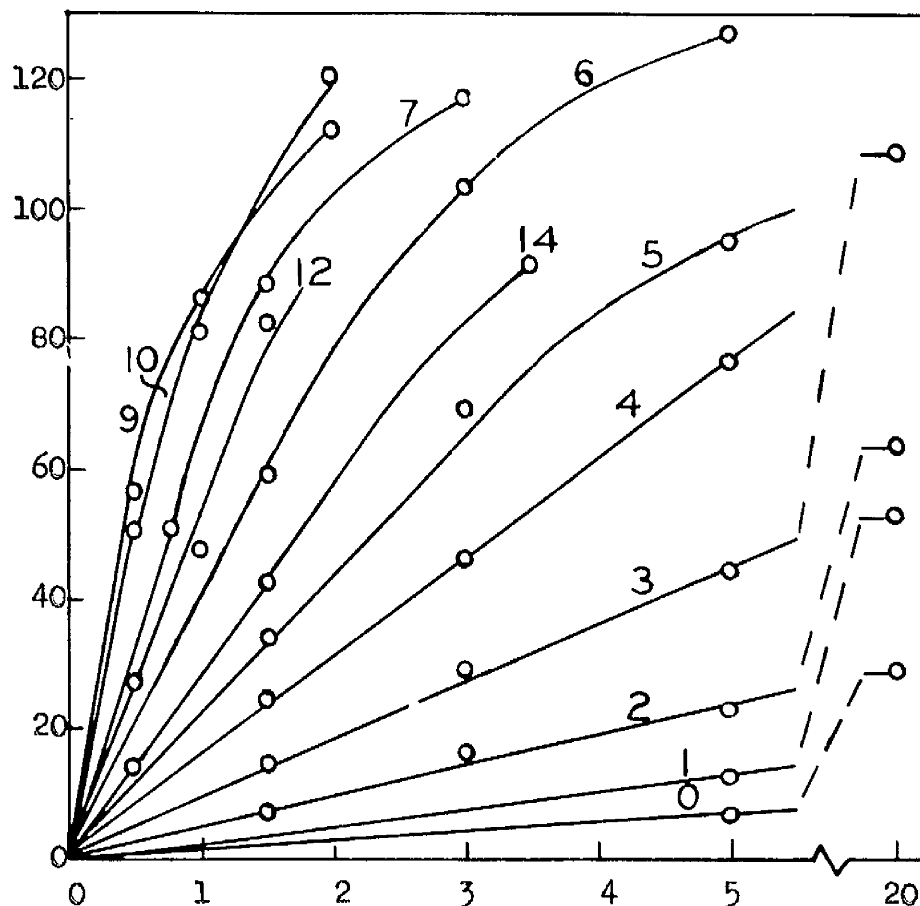


Fig. 49.

Digestion rate curves of proteolytic enzyme preparations from grains of A. sativa during 14 days in germination conditions.

Numbers on curves correspond to the number of days the samples have spent in germination conditions.

Abcissa: Length of digestion period, hours.

Ordinate: Tyrosine concentration in T.C.A. filtrate, p.p.m.

<u>Experiment 53.</u> The distribution of Proteolytic Activity within grains of <u>A. sativa</u>, var. Victory during the first few days in germination conditions.

Samples of dry grains, and of grains removed from germination conditions at daily intervals, were dissected into the endosperm portion and the embryo and scutellum portion. The proteolytic activity of whole grains and of these separate parts were determined by the standard procedure. Similar studies were carried out shortly after these on endosperms isolated from dry grains, sterilized with 0.1% Mercuric Chloride, and placed in germination conditions for a few days.

4 ml. samples of the digestion mixture were added to 2 mls. 12% T.C.A. for the determination of the tyrosine content. Duplicate determinations of all treatments were carried out, the results being given below as the mean increase in the concentration of tyrosine, in p.p.m., in the T.C.A. filtrate.

Grain Part Tested.	Time in Germination Conditions, Days.	Tyrosine Conc'n. (p.p.m.), at 2.5	5.0	7.5 hours.
Whole grain	0	12.5	-	39.0
	1	35.0	69.5	-
	2	52.0	95.0	-
	3	74.0	128.0	-
Endosperm	0	2	-	9
	1	8	18.5	-
	2	21	43	-
	3	42	88	-
Embryo and scutellum	0	9	-	32
	1	28.5	52.5	-
	2	29.5	60.5	-
	3	34.0	68.0	-
Isolated Endosperms		2	4 hours	
	1	4.0	9.0	
	2	7.5	13.0	
	4	12.0	25.0	

The Digestion Rate curves of these preparations are given in Figure 50.

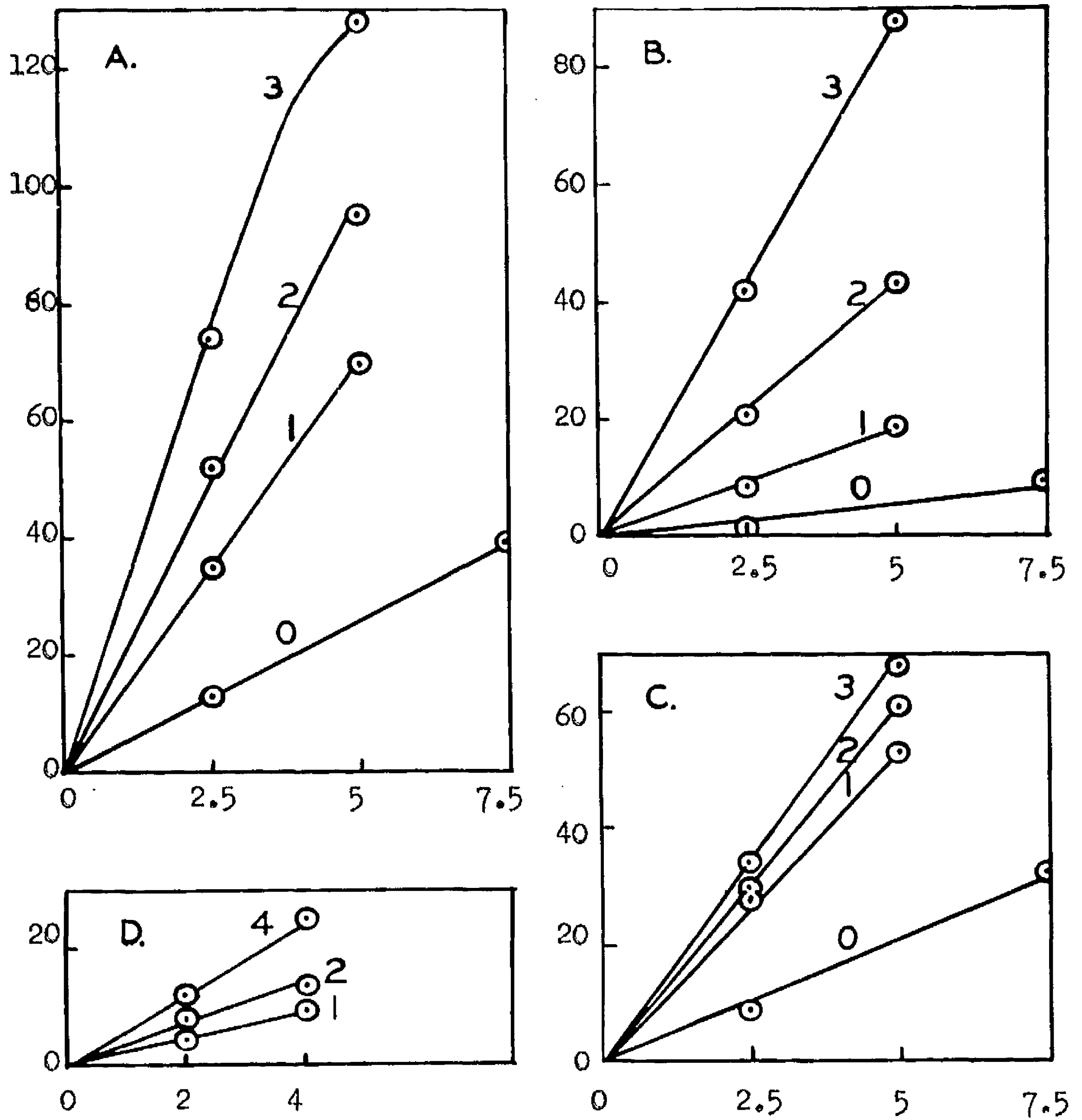


Fig. 50

Digestion rate curves of proteolytic enzyme preparations from whole grains of A. sativa, (A), endosperm parts, (B), and embryo and scutellum parts, (C), of whole grains, and isolated endosperms, (D).

Numbers on curves correspond to the number of days the samples have spent in germination conditions.

Abcissa: Length of digestion period, hours

Ordinate: Tyrosine concentration of T.C.A. filtrate, p.p.m.

Experiment 54. Proteolytic Activity in dormant and germinating grains of A. fatua.

Samples of A. fatua var. pilossissima were germinated at 20°C. with and without a pricking treatment. The pricked sample showed a speedy and full germination while the unpricked sample proved to be partially dormant. 25 ungerminated grains from this sample, and a random sample of 25 grains of the pricked sample were used in the preparation of proteolytic enzyme extracts at the times stated below, and duplicate determinations of the proteolytic activity carried out by the standard procedure. 4 ml. samples of the digestion mixture were added to 2 ml. samples of 12% T.C.A. for the determination of the mean increase in tyrosine concentration of the T.C.A. filtrate. These results are given below in p.p.m. tyrosine.

Germinating Grains.

Days in Imbibed Condition.	Germination %	Tyrosine Conc'n. (p.p.m.) at 1	2	2.5	4 hours.
0	0	-	-	5.5	9.5
3	78	-	-	42	66
5	92	39.5	90.5	-	160
8	100	-	-	140	208

Dormant Grains.

Days in Imbibed Condition.	Germination % *	2.	4 hours.
5	36	9	17.5
8	64	12	22

(* only ungerminated grains were used in these determinations.)

The digestion rate curves of these preparations are given in figure 51.

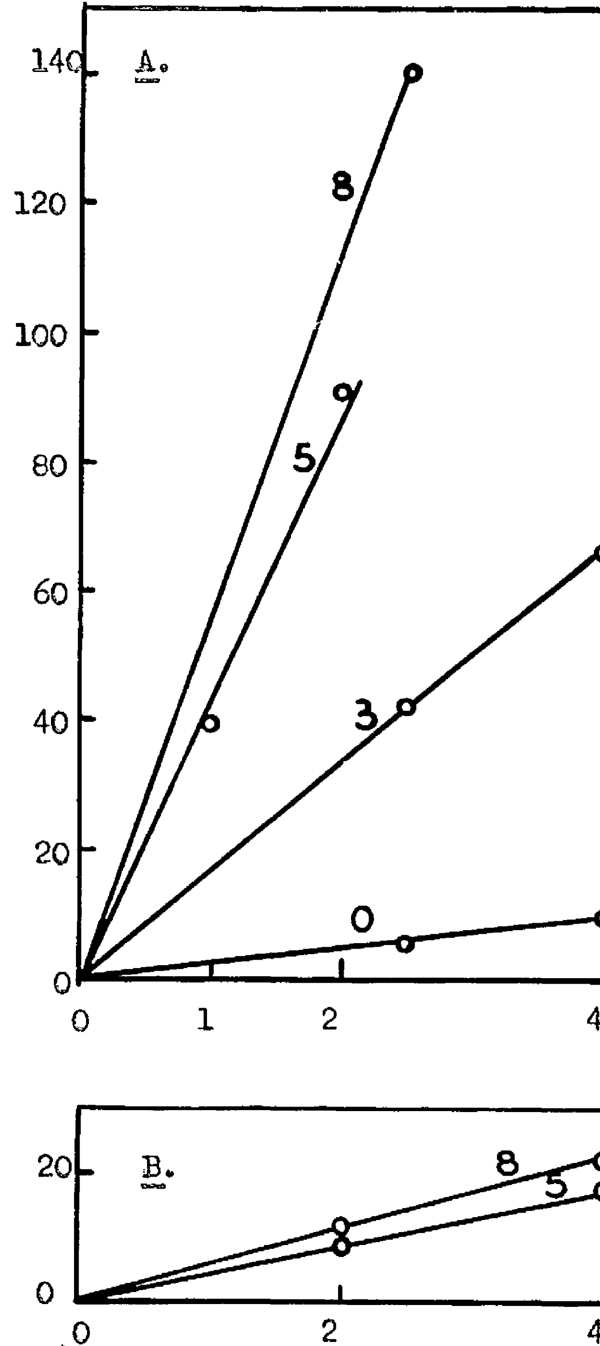


Fig. 51.

Digestion rate curves of proteolytic enzyme preparations from non-dormant,(A), and dormant grains of *A. fatua*,(B).

Numbers on curves correspond to the number of days the samples have spent in germination conditions.

Abcissa: Length of digestion period, hours.

Ordinate: Tyrosine concentration of T.C.A. filtrate, p.p.m.

Experiment 55. Proteolytic Activity in dormant and germinated grains of *Avena ludoviciana*.

Samples of *A. ludoviciana* were germinated at 10 - 12°C. without pricking, and others pricked, placed at 30°C. for three days, and then at 10 - 12°C. for a further period. The first samples contained mainly dormant grains, there being only 10 - 15% germination by the 10th day, while the other samples showed a quick and fairly complete germination within 7 days of being placed at the lower temperature. Proteolytic enzyme preparations were made from batches of 25 grains from both samples at various times, and the proteolytic activity determined by the standard procedure, 4 mls. of digestion mixture being added to 2 mls. 12% T.C.A. for this purpose. The mean of duplicate determinations of the increase in tyrosine concentration, in p.p.m., of the T.C.A. filtrate are given below.

Non-Dormant Grains.

Days at 10 - 12°C.	Germination %	Tyrosine conc'n. (p.p.m.), at 1.	2.	2.5.	4 hours.
0	0	-	-	8	12
2	10	-	-	12.5	20.5
5	84	41	79	-	119
7	96	72	134	-	220

Dormant Grains.

Days at 10 - 12°C.	Length of Digest. Period, 2	4 hours.
8	10.5	19.5
10	9	21.5

The digestion rate curves of these preparations are given in figure 52.

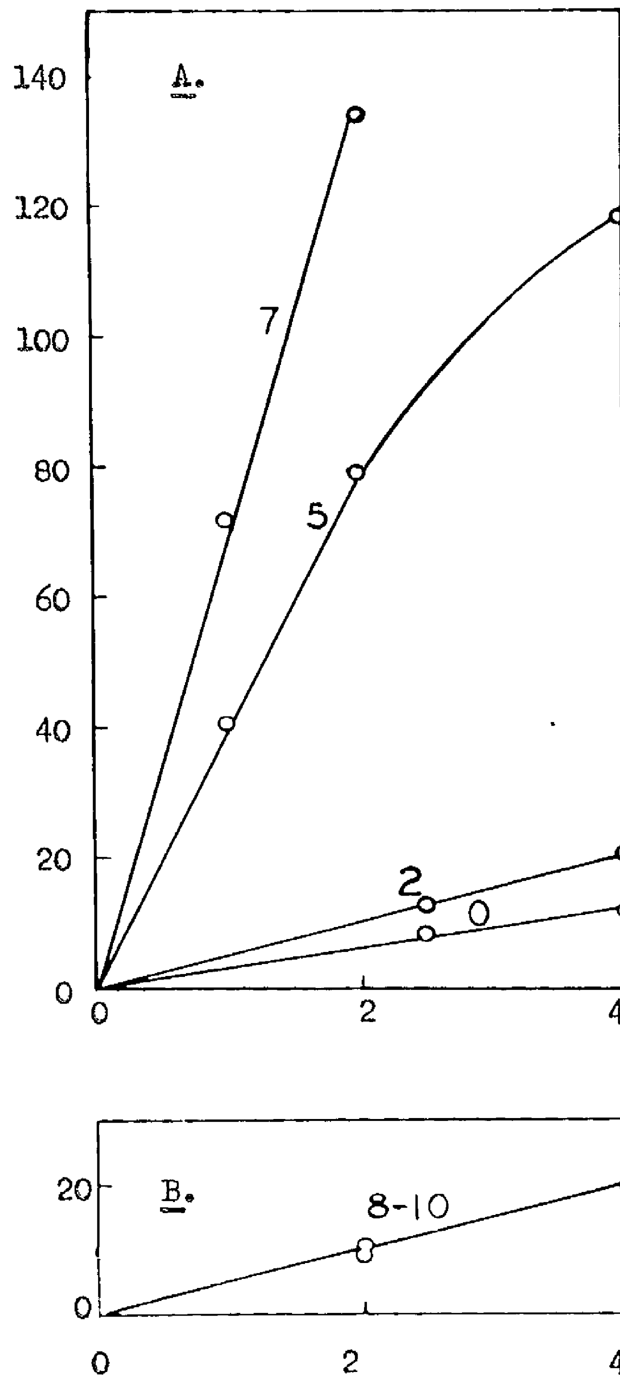


Fig. 52.

Digestion rate curves of proteolytic enzyme preparations from non-dormant, (A), and dormant grains of *A. ludoviciana*, (B).

Numbers on curves correspond to the number of days the samples have spent in germination conditions.

Abcissa: Length of digestion period, hours.

Ordinate: Tyrosine concentration in T.C.A. filtrate, p.p.m.

<u>Experiment 56.</u> The changes in the Free Tyrosine level in grains of <u>A. sativa</u> during the first few days in germination conditions.

A batch of grains of <u>A. sativa</u> were placed in standard germination conditions, and at daily intervals for the first three days four samples of 10 whole grains and four samples of embryos and scutellums separated from whole grains were prepared. Each sample was ground to a fine paste with some sand and a little 6% T.C.A. The brei was taken up in more T.C.A. to give a final volume of 10 mls. These were shaken at intervals and allowed to extract at room temperature for one hour. Following centrifuging and filtering, the tyrosine concentration of 2 mls. of the filtrate was determined by the standard procedure. The results of all four determinations, in p.p.m. tyrosine, are given below.

Grain Part Tested.	Days in Germ'n. Conditions.	Tyrosine Concentration of Extract (p.p.m.)				Mean
Whole Grain	0	82,	79,	79,	78,-	79.5
	1	83,	84.5,	84.5,	83,-	83.8
	2	99,	101,	99,	104,-	101
	3	122,	127,	122,	120,-	123
Embryo and scutellum	0	30,	28.5,	26,	26.5,	28.0
	1	23.3,	24,	25.3,	24.6,	24.3
	2	31,	33.5,	32.5,	31,-	32.0
	3	53,	50.5,	50.5,	49,-	51.0

Discussion.

In setting out to determine the activity of the proteolytic enzymes i[n] *Avena sativa* the suitability of the colorimetric method devised by Anson an[d] Mirsky (1934) was first tested using Edestin as the substrate. Due to interaction between the enzyme preparation and the substrate it was found necessary to modify this method, this being carried out by supplying the substrate in the form used by Mounfield (1936), in which the Edestin was dissolved in the acetic acid portion of the buffer system employed. In this way a suitable colorimetric method of high sensitivity was developed which was entirely suitable for the material concerned (Expts. 46 - 51).

Using this method, the presence of proteolytic activity was found in dry grains of *A. sativa* (Expts. 52, 53). When placed in germination conditions the proteolytic activity in the grain was found to increase continuously for a period of nine days, after which it decreased (Fig. 53). The level of activity on the 5th and 9th days was 10 times, and 100 times that of the dry grain, respectively. A fairly similar state of affairs was also found when the proteolytic activity of non-dormant grains of *A. fatua* and *A. ludoviciana* was examined (Expts. 54 and 55, and Figs. 54 and 55). In these the level of proteolytic activity on the 5th day was also approximately 10-fold that of the dry grains. This same trend of increasing proteolytic activity with length of time in germination conditions has been found previously in *Wheat* (Mounfield, 1936), and *Barley* (Luers, 1936) and can be inferred in *Pea* (Daniellson, 1951). The optimal pH for activity of the *A. sativa* preparations was found to be the same as that found in *Wheat* by Mounfield (1936).

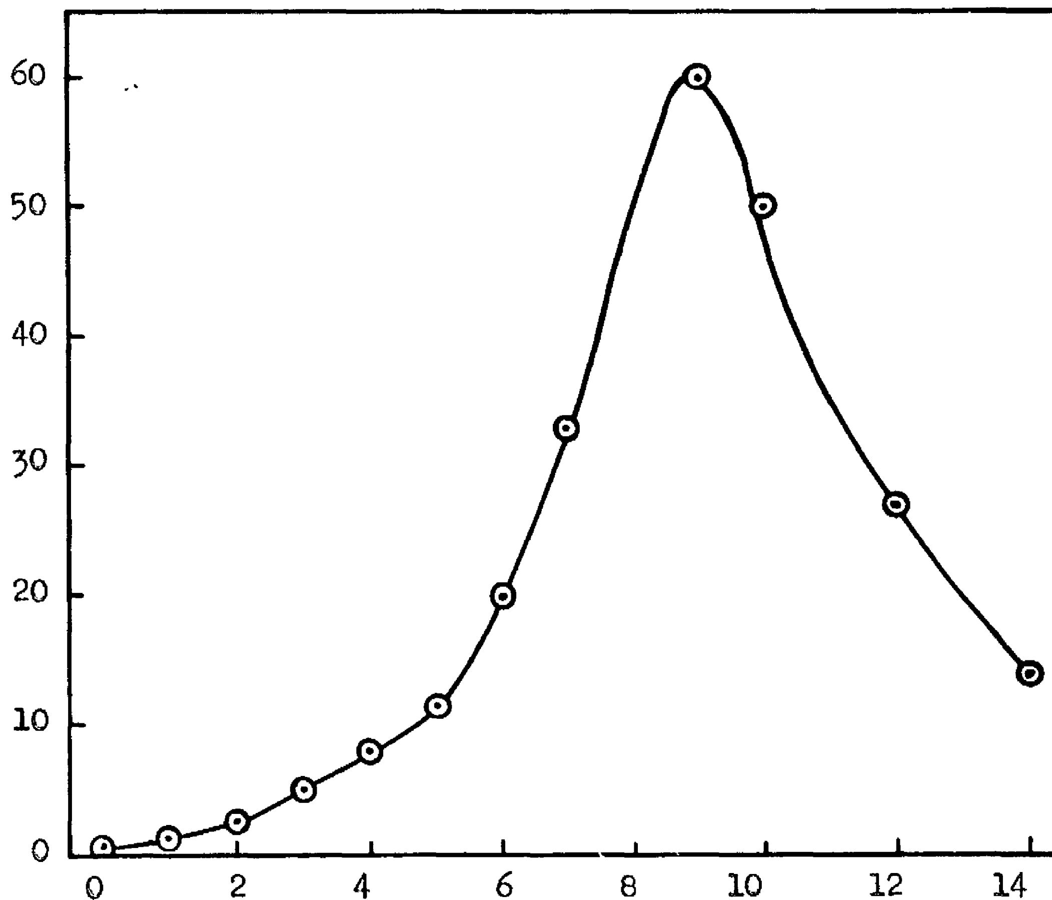


Fig. 53.

Changes in the level of proteolytic activity in grains of A. sativa during 14 days in germination conditions.

Measured as the increase in the tyrosine concentration of the T.C.A. filtrate after 1 hour's digestion.

Abcissa: Period in germination conditions, days.

Ordinate: Increase in tyrosine concentration of the T.C.A. filtrate, p.p.m.

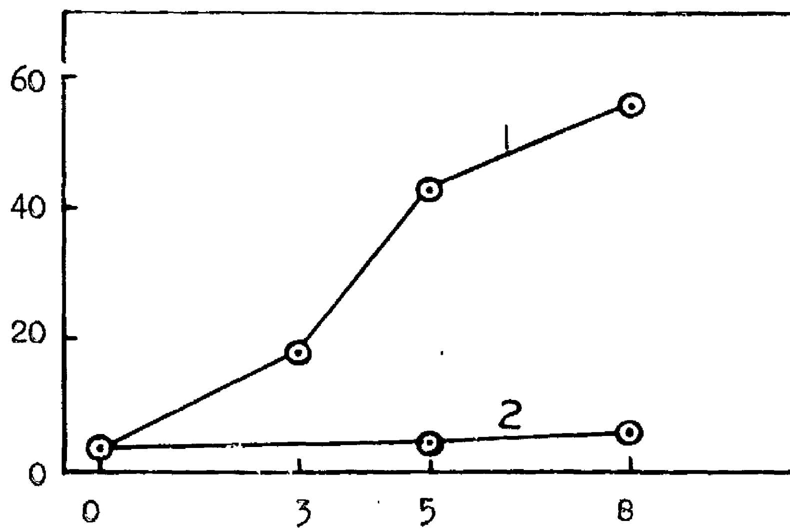


Fig. 54.

Changes in the level of proteolytic activity in non-dormant,(1), and dormant grains of *A. fatua*,(2). Measured as the increase in the tyrosine concentration of the T.C.A. filtrate after 1 hour's digestion.

Abcissa: Period in germination conditions, days.
Ordinate: Tyrosine concentration, p.p.m.

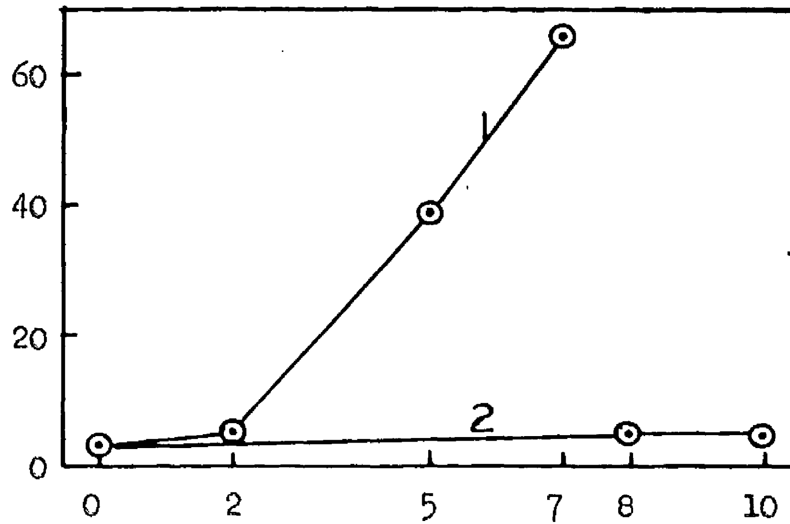


Fig. 55.

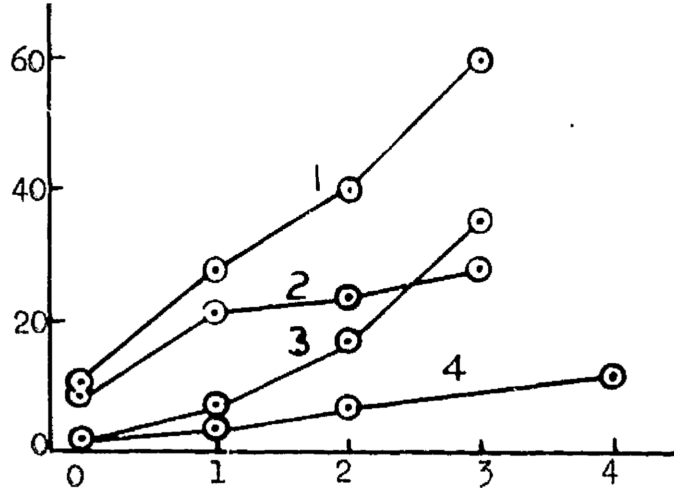
Changes in the level of proteolytic activity in non-dormant,(1), and dormant grains of *A. fatua*,(2). Measured as the increase in the tyrosine concentration of the T.C.A. filtrate after 1 hour's digestion.

Abcissa: Period in germination conditions, days.
Ordinate: Tyrosine concentration, p.p.m.

In dormant grains of A. fatua and A. ludoviciana there was no sign of any considerable increase in proteolytic activity within a period of 8-10 days in germination conditions (Figs. 54, 55). This suggests a connection between the processes involved in the formation of the increased proteolytic activity in these species, and the processes involved in the resumption of growth in the embryo. It is not possible from this evidence to say that this lack of a physiological property is the basis of the dormancy mechanism in these species, but it is at least a feasible possibility that the commencement of embryo growth is indeed dependent on changes in the level and distribution of such an enzyme system as this.

An examination of the distribution of proteolytic activity within dry grains of A. sativa showed that the bulk of the activity - approximately 85% - was present within the embryo and scutellum part of the grain (Expt. 53, Fig. 56). Similar distribution was reported within grains of Wheat, Barley, and Rye by Pett (1935), and Engel and Heins (1947). As found previously, there is a considerable increase in the proteolytic activity of the whole grain during the period investigated, including the first 24 hours when a threefold increase over the dry seed level is found. This increase is occurring while embryo growth is just commencing and before meristematic activity has commenced (Expt. 14), so it is definitely a pre-germination phenomenon. The distribution of proteolytic activity within the grain parts in the first few days in germination conditions shows a different pattern in the two parts studied. In the embryo and scutellum there is a considerable increase in activity within the first day, and smaller increases thereafter. The endosperm on the other hand shows only a slight increase in the first day, but considerable increases thereafter, and contains almost half of the total

Fig. 56.



The distribution of proteolytic activity within grains of A. sativa during the first few days in germination conditions. Whole grains,(1), embryo and scutellum parts,(2), and endosperm parts of whole grains,(3), and isolated endosperms,(4).

Measured as the increase in tyrosine concentration of the T.C.A. filtrate after 2 hours digestion.
Abcissa: Period in germination conditions, days.
Ordinate: Tyrosine concentration, p.p.m.

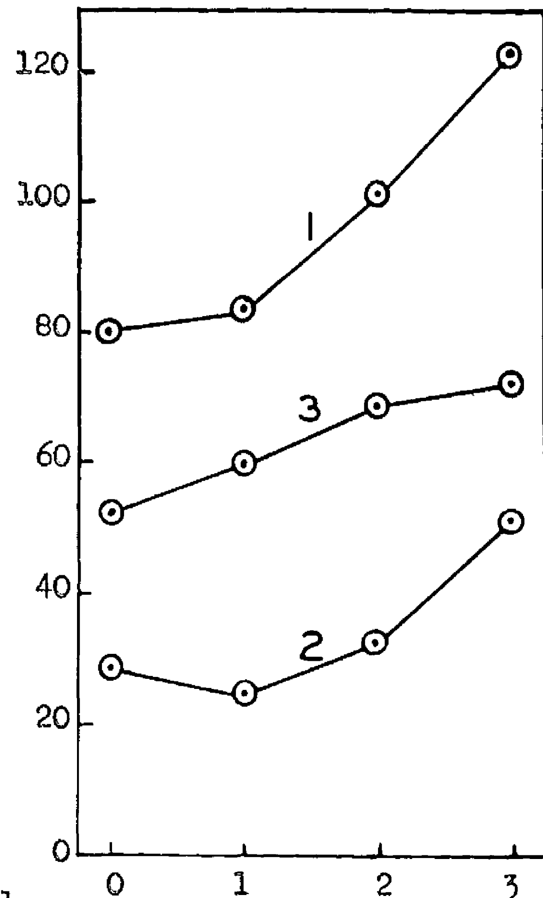


Fig. 57.

The free tyrosine level in grains of A. sativa during the first few days in germination conditions.

Measured as the tyrosine concentration of a T.C.A extract of 10 whole grains,(1), and the embryo and scutellum parts,(2), and the endosperm parts of 10 whole grains,(3)

Abcissa: Period in germination conditions, hours.
Ordinate: Tyrosine concentration, p.p.m.

activity present in the whole grain by the second day. Endosperms isolated from dry grains and maintained under the same germination conditions do not show the same pattern of increasing activity as the same part of whole grains, there being only a five-fold increase in activity within four days in the one case, and an eighteen-fold increase within three days in the other (Fig. 55). It would appear therefore that the endosperm has a low natural capacity to increase its own proteolytic activity, but that this is supplemented in the whole grain by the translocation of proteolytic enzymes from the other grain parts, or by the transmission of some stimulus by which its own capacity to produce proteolytic enzymes is much increased. The first explanation is felt to be the most likely, indicating that there is a capacity for protein synthesis in the embryo and scutellum in the pre-germination and immediate post-germination period of imbibition. The necessity of maintaining the structural integrity of the whole grain for the development of the normal pattern of proteolytic activity is obvious whatever the explanation.

In studying the level of free Tyrosine in grains of A. sativa (Expt. 56, Fig. 57), the amount of tyrosine and tryptophane present in the form of free amino acids and small peptides soluble in 6% T.C.A. has been measured. This is regarded as an indication of the general level of similar nitrogen compounds within the grain. It is found that there is an increase in the amounts of these compounds within the first 24 hours imbibition in whole grains, with greater increases thereafter. Similar trends in soluble nitrogen compounds have been described in A. sativa (Albaum and Cohen, 1943) and Barley (Folkes and Yemm, 1958). These changes are consistent with the presence, and activity of proteolytic enzymes as described previously. When these figures for whole grains are broken down into those of the different

grain parts, a departure from these trends is obvious. The embryo and scutellum part shows a decrease in the level of these compounds in the first 24 hours, which gives way to an increase from then on. In the endosperm the level increases considerably for the first 48 hours, and then tends to level off. These changes are consistent with the occurrence of protein synthesis in the embryo and scutellum within the first 24 hours, discussed in the previous paragraph, and the development of protein hydrolysis in the endosperm and the translocation of soluble nitrogen material from it to the embryo from the first day on. Albaum and Cohen (1943), also found that there was an increase in the amount of protein in the embryo of A. sativa during the first day in germination conditions, while Folkes and Yemm (1958) found a similar situation in germinating Barley, and obvious signs of hydrolysis of endosperm proteins, and translocation of soluble nitrogen to the embryo by the second day. Our findings would therefore appear to be in agreement with these more detailed quantitative studies.

It would appear from these studies that the embryo and scutellum part of the whole grain is well endowed with the capacity to hydrolyse proteins during the first 24 hours, in addition to the capacity to synthesise proteins which has been discussed previously. Whether any use can be made of this first capacity will of course depend on the presence of a suitable substrate. It has been shown in a previous part that grains submitted to an imbibition - dehydration treatment, involving 8 - 12 hours imbibition, and retaining a beneficial effect of this treatment for a considerable period, were found to contain a higher level of free tyrosine than untreated dry grains of the same sample (Pages 63 and 74). This is consistent with the occurrence of protein

hydrolysis within this period, probably within the embryo and scutellum. This infers that there is indeed a certain amount of "suitable substrate" present within this part of the grain, and that it is possibly utilised in the early phases of physiological development within the imbibed seed. The studies of Saverborn, et al (1944) on changes in the level of globulins in the different parts of Barley grains have been discussed in the introduction to this part. These and the studies on isolated embryos of Maize (Toole, 1924), and Barley (James and James, 1940), bear out the case that there is indeed some amount of reserve protein materials within the embryo and scutellum of the cereal grain, which is utilised at an early stage of imbibition, possibly before the transport of soluble materials from the endosperm gets under way.

The findings that the soluble nitrogen compounds in the embryo fraction show decreases in amount within the first 24 hours imbibition, and the inference that protein hydrolysis also occurs in this part of the grain within the same period, allow for the possibility that some of these nitrogenous compounds might be utilised in the respiration of the germinating embryo, in addition to those other compounds discussed in the previous part (Page 134).

Summary.

1. Proteolytic activity was found to be present in dry grains of *A. sativa*, and to increase 100-fold during the first 9 days in germination conditions. A similar pattern was evident in non-dormant grains of *A. fatua* and *A. ludoviciana*.

2. In dormant grains of these last two species, no obvious increases in proteolytic activity were found during 8 - 10 days in germination conditions. A connection would therefore appear to exist between the processes involved in developing proteolytic activity and the dormancy mechanism in these species.

3. The bulk of the proteolytic activity of the dry grain is present in the embryo and scutellum. A considerable increase in the level of proteolytic activity was observed in the pre-germination period of imbibition. The pattern of increase, and the distribution of activity within the grain indicate that the increase results from the synthesis of proteolytic enzymes within the embryo and scutellum, and their distribution to the endosperm from the first day on.

4. Studies of the level and distribution of free Tyrosine within the grain indicated that there was protein synthesis occurring within the grain in the first day in germination conditions, and that hydrolysis of endosperm proteins and transportation of soluble nitrogen compounds was occurring from this time on. The participation of embryo and scutellum reserve protein in the early stages of embryo growth is indicated.

5. The relevance of these findings to previous work in this field is discussed.

Part VI. The Occurrence and Role of Germination Inhibitors in Grains of *A. sativa* and *A. fatua*.

Ruge (1939) found that filter papers on which successive batches of grains of *A. sativa* were germinated contained materials which retarded the germination of *A. sativa* and *Helianthus annuus* (Sunflower). The inference was that germination inhibiting substances were leached from the grains and gradually accumulated. This experimental technique is open to many objections, however, on the basis of microbial contamination, and on the grounds that the inhibiting materials might be a metabolic product of the germinated grains and not present in the resting grain. Stout and Tolman (1941) confirmed that there were inhibiting materials in a water extract of whole grains of *A. sativa* which retarded the germination of *Beta vulgaris* (Beet), and *Lactuca sativa* (Lettuce). In 1953, Elliot and Leopold investigated this matter further and claimed the presence of a water soluble germination inhibitor in grains of *A. sativa* var. Victory, located chiefly in the hulls, though some activity was also present in the other grain parts. The same extract retarded the growth of etiolated Pea stem segments in auxin, and inhibited the activity of preparations of an a- and a b-Amylase. Since the amylase inhibition could be reversed by the addition of sulphydril group donors, they concluded that the inhibition of germination was due to the inhibition of enzymes such as the amylases which require free sulphydril groups for their activity, amylases being regarded by them as enzymes involved in germination. The presence of a considerable number of poly-hydroxy phenolic acids, and coumarin has also been demonstrated in grains of many of the Gramineae, including *A. sativa* (Van sumere and Massart, 1959).

These naturally occurring substances were mainly present in the husk and seed coats of the grains and are all potent germination inhibitors. The physiological action of these materials is still conjectural at present.

In view of our conclusions based on the studies carried out in Parts III and IV that the amylases play an insignificant role in germination due to the fact that starch is not the respiratory substrate of the germinating embryo in A. sativa, it was decided to confirm these findings of Elliot and Leopold. Some experiments were carried out to confirm the presence of germination inhibiting materials in grains and husks of A. sativa, and to examine the range of seeds affected by such materials. The action of this extract on various enzyme preparations in vitro was also carried out, including preparations from grains of A. sativa. The presence of germination inhibitors in A. fatua has also been studied, with a view to assessing their possible role in the dormancy exhibited by this species.

Materials and Methods.

With the exception of Expt. 57, the grains used in these studies were the same batches of A. sativa var. Victory, and A. fatua var. pilossissima used in Parts III and V. The seed of other species used in Expt. 58 were available in the department, some being several years old. No attempt was made to find the optimal germination conditions of these species, all germination tests being carried out at 20°C., with the addition of 4 mls. distilled water, or test solution to batches of 50 seed.

Husks were prepared by hand for the initial experiments, but samples were obtained commercially for some of the later experiments. These husks

were obtained from grains of A. sativa in preparing Oatmeal after the kilning of the grains. The extracts prepared from the commercial husk samples appeared to be identical with those from hand separated husks.

The preparation and testing of enzyme preparations used in this section was carried out according to the methods described in Parts III and V.

Results.

Experiment 57. The role of the husk in the germination of A. sativa.

In this experiment a three year old sample of A. sativa, var. Victory was employed, having a rather slower germination rate than the other one year old samples used later. The husks were removed manually from approximately 400 grains, 4 plates of fifty of these caryopses being germinated with distilled water, and a similar number with 4 mls. of a husk extract. This extract was prepared by grinding 250 husks and extracting this material with 25 mls. distilled water, at 2°C. for 16 hours. A 4 ml. portion of this extract contains the materials extractable from approximately 50 husks. In addition to these caryopses, 20 plates of fifty whole grains were also set to germinate. At intervals, 4 plates of 50 whole grains were taken, the apparent germination counted, and the husks removed. The number of these caryopses showing any sign of embryo growth was then determined, and similar counts made on the caryopses treatments described previously. The mean of these counts at the various times of counting are given below.

Sample.	Germ'n medium.	Attribute.	%age of attribute at, 17.	21.	41.	47.	65 hrs.
Caryopses.	Dist. water.	Embryo growth.	1	6.5	94.5	96.5	97.
Caryopses.	Husk extract.	Embryo growth.	0	5.5	91	94	95.5
Whole grains.	Dist. water.	Embryo growth.	0	0.5	77.5	90.5	95.
Whole grains.	Dist. water.	Apparent germination.	0	0	3	10	50.

These values are graphed in Fig. 58.

Experiment 58. The effect of an aqueous extract of A. sativa husks on the germination of seeds of several species.

30 grams of husks separated manually from grains of A. sativa var. Victory were extracted overnight at 2°C. with 200 mls. water, in the presence of a few drops of toluene. The liquid expressed after pressing in a cheese-cloth was evaporated to 100 mls. at 45°C. The effects of this extract were observed on the germination of the following species: A. sativa, var. Victory, Hordeum vulgare (L.), var. Abed. Kenia (Barley), Triticum aestivum (L.) var. Squarehead Master (Wheat), Linum usitatissimum (L.), (Flax), Brassica oleraceae var. caulo-rapa (De Cand.), (Kohl-rabi), Trifolium pratense (L.), (Red Clover). 4 mls. of the husk extract were given in place of 4 mls. of distilled water. Four plates of fifty seed were given each treatment and the germination counted at intervals, the presence of visible radicle growth being the criterion employed. The mean germination %ages observed are given below.

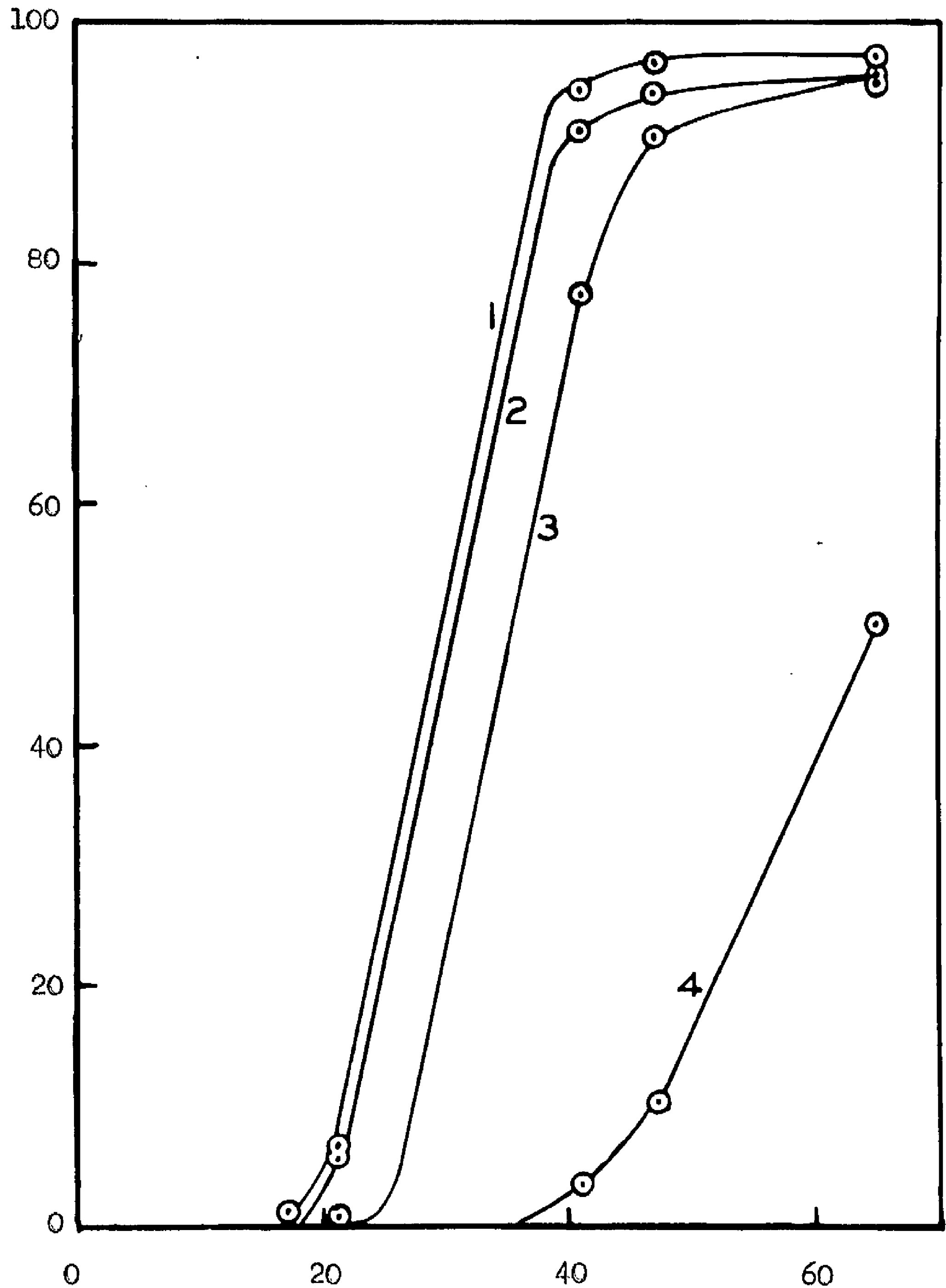


Fig. 58.

The role of the husk in the germination of grains of A. sativa.

The rate of onset of embryo growth in caryopses germinated in distilled water,(1), in a water extract of the same number of husks,(2), and removed from whole grains germinated in distilled water at the time of counting,(3). Germination rate of whole grain samples,(4).

Abcissa: Period in germination conditions, hours.
Ordinate: %age embryo growth or germination.

Species Tested.	Germination medium.	Germination % at, 27.	44.	51.	68.	76.	92.	115 hrs.
A. sativa.	Dist. water.	16.5	63	79	92.5	97.6	97.6	98.5
"	Husk extract.	0.5	29	55.5	79.5	83.5	89.0	96.0

Species Tested.	Germination medium.	24.	29.	44.	71 hrs.
H. vulgare.	Dist. water.	75	93.5	97	97.5
"	Husk extract.	58.5	83	93.5	96
T. aestivum.	Dist. water.	50.5	85	98.5	99
"	Husk extract.	31.5	68	96	99

Species Tested.	Germination medium.	23.	39.	47.	63.	71.	87.	120.	144 hrs
L. usitatissimum.	Dist. water.	5	52	66	80	84	90	-	-
"	Husk extract.	1	19	45	73	83	89	-	-
B. oleracea.	Dist. water.	-	-	3	19	29	42	62	68
"	Husk extract.	-	-	1	10	13	25	40	55
T. pratense.	Dist. water.	3	30	34	53	58	66	87	92
"	Husk extract.	0	10	16	29	37	47	76	88

The germination rates of these samples is given in Fig. 59.

Fig. 59.

The effects of an aqueous extract of A. sativa husks on the germination of seed of several species.

Germination rates of samples given 4 mls. distilled water, (A), or 4 mls. husk extract, (B).

Species tested,:-

1. A. sativa.
2. Hordeum vulgare.
3. Triticum aestivum.
4. Linum usitatissimum.
5. Brassica oleracea.
6. Trifolium pratense.

Abcissa: Period in germination conditions, hours.
Ordinate: Germination %age.

Experiment 59. The effect of extracts of whole grains of A. sativa and A. fatua on their germination.

Samples of 100 grams of whole grains of A. sativa var. Victory, and of A. fatua var. pilossissima at one month (fresh), and thirteen months after harvest (old) respectively, were ground to a fine powder, and extracted with 300 mls. distilled water at 2°C. for 20 hours. A little toluene was shaken through each sample. The samples were then centrifuged until the supernatant was a clear yellow brown colour. Samples of these extracts were tested for their effects on the germination of A. sativa, and the one year old sample of A. fatua, being used at full strength or at half concentration. Four samples of fifty grains of the first species, and four samples of twenty-five grains of the second, were tested for each treatment, the mean germination %age being given below.

a) Effect on the germination of A. sativa.

Extract Source.	Extract Concentration	Germination % at, 26,	32,	48,	72,	96 hrs.
Distilled water.	-	6	40	87	95	97
A. sativa.	Half strength.	3	16	61	79	93
"	Full strength.	0	3	32	64	85
A. fatua, fresh.	Half strength.	0	20	67	87	94
" "	Full strength.	0	4	56	77	93
A. fatua, old.	Half strength.	0	10	70	84	92
" "	Full strength.	0	1	34	69	87

b) Effect on the germination of A. fatua (one year old).

Extract Source.	Extract Concentration	Germination % at, 3.	4.	5.	6.	7.	9* days.
Distilled water.	-	16	30	43.5	52	63	79
A. sativa.	Half strength.	38	48	58	60	65	78.5
"	Full strength.	35	45	54	61.5	67	80
A. fatua, fresh.	Half strength.	45	55	63	67	72	83
"	Full strength.	26.5	40	48	55	59	77
A. fatua, old.	Half strength.	46.5	58.5	65	69	74	84
" "	Full strength.	35	42	52	54	61.5	73

(* Husk removed from remaining grains and those showing signs of embryo growth regarded as germinated.)

Germination rate curves of these counts are given in Fig. 61.

These results have been confirmed with similar extract preparations in repeat experiments.

Fig. 61.

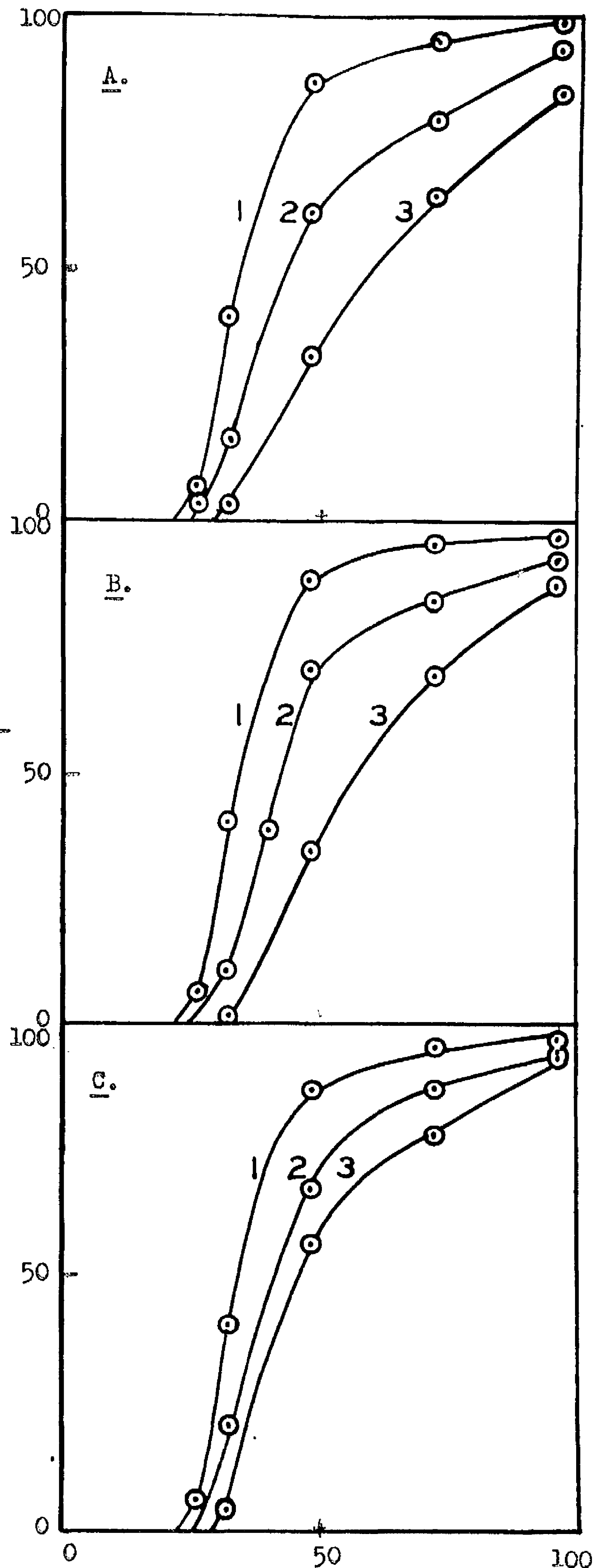
The effects of extracts of whole grains of A. sativa and A. fatua on their germination.

I. The effects of extracts of whole grains of A. sativa, (A), newly harvested, (B), and one year old grains of A. fatua, (C), on the germination of grains of A. sativa.

Samples germinated with distilled water, (1), half strength, (2), and full strength grain extracts, (3).

Abcissa: Period in germination conditions, hours.

Ordinate: Germination %age.



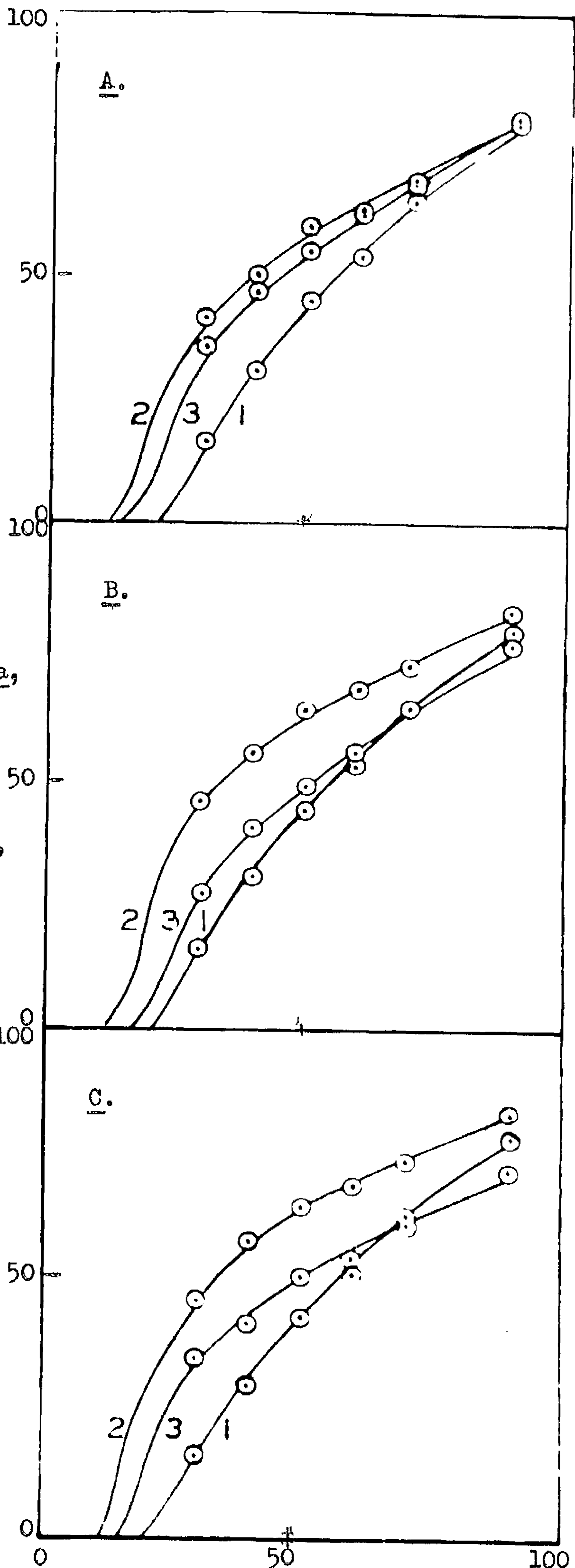


Fig. 61, (Cont'd.).

II. The effects of extracts of whole grains of A. sativa, (A), newly harvested, (B), and one year old grains of A. fatua, (C), on the germination of one year old grains of A. fatua.

Samples germinated with distilled water, (1), half strength, (2), and full strength grain extracts, (3).

Abcissa: Period in germination conditions, hrs.
Ordinate: Germination %age.

Experiment 60. The effect of an aqueous extract of husks of A. sativa on the activity of several amylase preparations.

70 grams of the commercially supplied husk material were extracted with 350 mls. distilled water for 20 hours at 4°C. This extract is called the dilute extract, a portion of it being evaporated at 4°C. to 1/6th the original volume and used later as the concentrated extract. Both extracts were filtered under vacuum through a Seitz filter using asbestos "Sterimat" pads, and used directly for the germination inhibiting test. The remainder was stored at 2°C. for 2 days before being tested for enzyme effects. Four plates of fifty grains were tested in each treatment, with 4 mls. distilled water or extract, the mean germination %age being given below at the counting times.

Germination Medium.	Germination %age at, 30.	46.	70.	94 hrs.
Distilled water.	30	69	87	91
Dilute extract.	14	60	78	90
Concentrated extract.	0	6	50	85

Germination Rate Curves of these figures are given in Fig. 62.

Free amylase extracts were prepared from grains of A. sativa and Hordeum vulgare (Barley), after four days in germination conditions, and solutions prepared from a commercial preparation of Taka-diastase, and a crystalline a-Amylase from Bacillus subtilis. The enzyme activity of these preparations was adjusted to give a suitable digestion rate. The effects of the husk extracts on the Dextrinising activity of this preparation was tested in a digestion system containing 10 mls. 0.1% soluble starch, 7 mls. buffer, 1 ml. enzyme preparation, and either 2 mls. distilled water, or

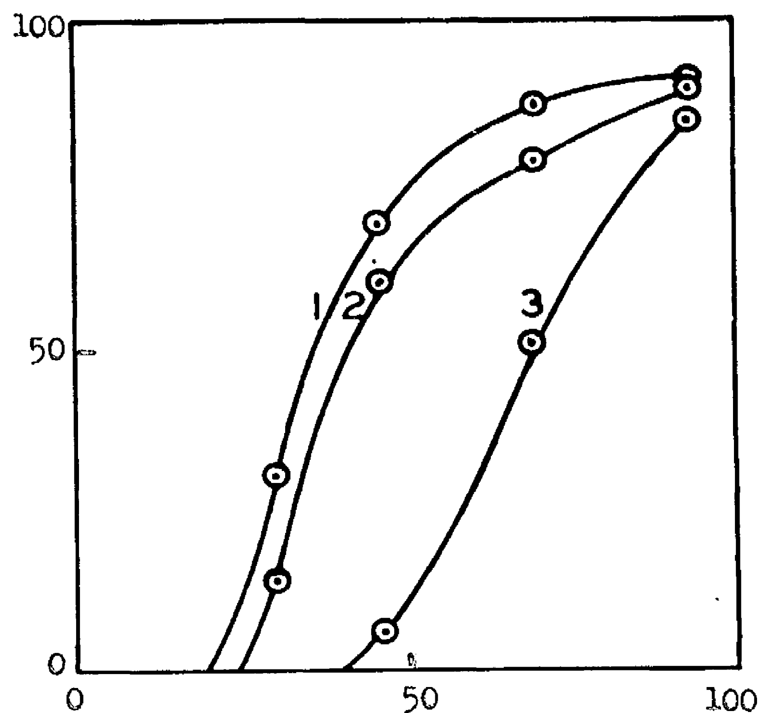


Fig. 62.

The effect of the aqueous extract of A. sativa husks used in the enzyme inhibition studies on the germination of grains of A. sativa.

Samples germinated with distilled water, (1), dilute extract, (2), and concentrated extract, (3).

Abcissa: Period in germination conditions, hours.
Ordinate: Germination %age.

2 mls. husk extract. The procedure used was that described in Part III. The mean of two determinations of each treatment is given below.

Amylase source.	Treatment.	Mgms. Starch digested at, 5.	10.	20 mins.
A. sativa.	None.	1.96	4.6	8.15
"	Dilute extract.	2.1	4.7	7.85
"	Conc. extract.	2.25	4.7	8.05
H. vulgare.	None.	2.45	4.8	8.25
"	Dilute extract.	2.45	4.75	8.2
"	Conc. extract.	2.52	4.55	8.2
Taka-diastase.	None.	7.45	9.4	9.6
"	Conc. extract.	7.55	9.25	9.55
B. subtilis.	None.	3.6	7.0	9.15
"	Conc. extract.	3.6	6.85	9.50

Similar results to these have been obtained on two other occasions when the effects of husk extracts on the dextrinising activity of *A. sativa* amylase preparations were previously investigated.

Experiment 61. The effect of an aqueous extract of husks of *A. sativa* on the activity of proteolytic enzyme extracts of *A. sativa* and *H. vulgare*.

The same concentrated husk extract used in the previous experiment was employed in these studies, both investigations being carried out simultaneously. The proteolytic activity of extracts of grains of *A. sativa* and *H. vulgare* after 5 days in germination conditions was determined in the same manner as described in Part V, with a digestion system of the same composition plus the addition of 2 mls. distilled water or concentrated husk extract. The 10 ml. enzyme extract used was equivalent to the proteolytic activity present in 10 grains. No obvious sign of precipitation was observed on adding the husk extract. The Tyrosine content of the T.C.A. filtrate was determined on 2 samples of each treatment, the mean values in p.p.m. being given below.

Enzyme source.	Treatment.	Conc'n. of Tyrosine in T.C.A. filtrate, p.p.m. at, 1½.	3 hrs.
A. sativa.	None.	46	92.5
"	Husk extract.	8.5	19.5
H. vulgare.	None.	68	137
"	Husk extract.	42	90

The effect of a similar husk extract on the activity of proteolytic enzyme preparations from 2 day germinated grains of *A. sativa* confirmed these results.

Discussion.

The presence of the husk has been found to retard the onset of embryo growth in the whole grain by some six hours, compared to the caryopsis alone (Expt. 57, Fig. 58). This has been observed previously in the studies on the time of commencement of meristematic activity in the germinating embryo (Part I, Expt. 14). The cause of this retarding effect of the husk is not however due solely to the presence of a water-soluble germination inhibitor, since commencement of embryo growth in those caryopses germinated in a husk extract containing the materials extracted from the same number of husks is very similar to that in caryopses germinated with distilled water, though a slight retarding effect does seem to be present. It would seem therefore the main retarding effect is due to the physical and structural properties of the husk.

That there is indeed a water soluble, germination retarding material or materials in the husk, is borne out by the studies reported in Expt. 58, and by many other similar studies not included here. In this experiment, the four mls. of husk extract used in the germination tests contains the materials extracted from approximately 120 husks. With this amount of extract all the species tested showed a considerable retardation of germination compared to the distilled water controls (Fig. 59). The retardation takes the form of a delay in the onset of germination rather than a reduction of the rate of germination, or a decrease in the final level of germination. The very slow germination of the control samples of B. oleracea and T. pratense are probably the result of too much liquid in the germination medium, so the amount of germination retardation in the presence of the husk extract might be increased

by these less favourable germination conditions. This combined action has been observed previously in samples of A. sativa germinated in the presence of 6 mls. distilled water / 50 grains and the same amount of a husk extract.

In the studies on the effects of extracts of whole grains (Expt. 59, Fig. 60), germination retarding properties were also found in the extract of grains of A. sativa. The 100 gram sample from which this extract was prepared would contain approximately 2,500 grains, so the four mls. full strength extract should contain the materials extracted from approximately 30 - 35 whole grains. The retardation produced by this extract is greater than that produced by the husk extract equivalent to 120 husks described previously (Compare Fig. 59, I, with Fig. 60, A.). There would also appear to be a reduction in the rate of germination in the presence of this whole grain extract which was not evident in the husk extract described above. From this we may conclude that there are germination inhibiting materials present in other parts of the grain than the husk, and possibly that these materials are not necessarily the same as those present in the husk.

When extracts of whole grains of A. fatua were tested on the germination of A. sativa, the presence of germination inhibiting properties in these extracts was also found. There appeared to be little difference whether the extract was prepared from a sample one month after harvest, and showing about 70 - 80% dormancy, or from a sample from the previous years harvest showing over 80% germination (Fig. 60, B. & C.). There would not appear to be any correlation therefore between the presence of these inhibiting materials, and the dormancy of the grains of this species.

When extracts of whole grains of A. sativa and A. fatua were tested on

the germination of grains of the one year old sample of A. fatua, a similar trend was evident in each case. A promotion of the time of onset of germination, and a speeding up of the rate of germination was found with extracts of both concentrations. Again, virtually no difference was found in extracts of freshly harvested, and one year old samples of A. fatua, in this respect. Whether the response of these two species of Avena to the same extracts is due to different responses to the same materials in the two species, or a response to a different material in each species cannot be judged on the present evidence.

Some possible physiological basis for the action of the germination inhibiting extract from the husk of A. sativa have been investigated. The pH of several extracts of different concentrations have been determined, and all found to lie in the range of pH 5.7 - 6.0. No determinations of osmotic pressure have as yet been carried out. No evident effects were found on the dextrinising activity, when a husk extract showing marked germination inhibiting properties was added to digestion mixtures containing amylase preparations from A. sativa, H. vulgare, Taka-diastase, or crystalline B. subtilis a-amylase (Expt. 60, Fig. 63). Since these preparations are predominantly, or purely a-amylase, this enzyme would appear to be unaffected by the husk extract. While dextrinising activity is not the best method of determining b-amylase activity in a mixture of the two amylases, it has been noted previously that the A. sativa preparation described above contains approximately 25% non-a-amylase, in terms of dextrinising activity measurements (Expt. 34, Fig. 39), and the preparation from H. vulgare is probably similar. Since neither preparation shows any sign of comparable inhibition, we may conclude that this enzyme is also unaffected, or at most

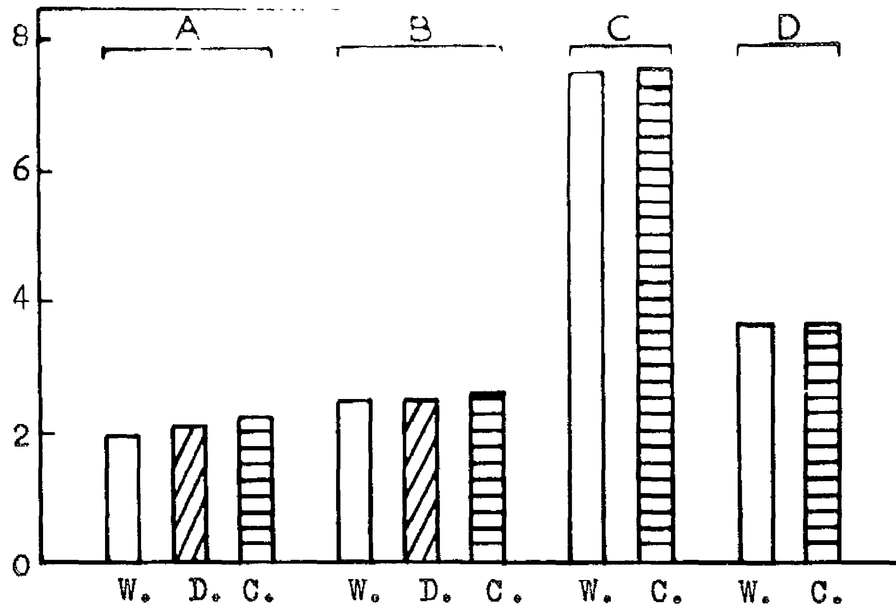


Fig. 63.

The effect of an aqueous extract of A. sativa husks on the activity of Free Amylase preparations from four-day germinated grains of A. sativa, (A), H. vulgare, (B), Taka-diastase, (C), and crystalline B. subtilis a-Amylase, (D).

Abcissa: Distilled water, (W), dilute, (D), or concentrated husk extract added, (C).

Ordinate: Mgms. starch digested at 5 mins.

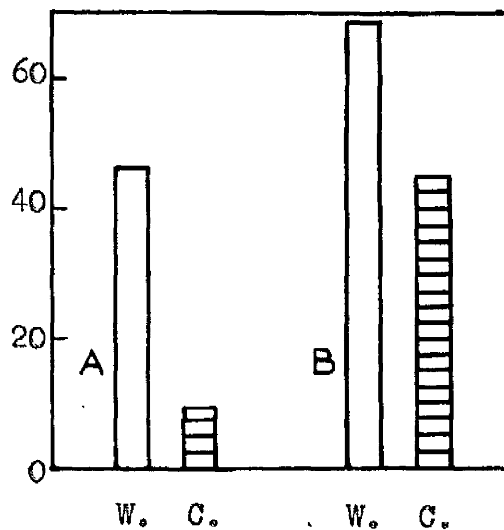


Fig. 64.

The effect of an aqueous extract of A. sativa husks on the activity of proteolytic enzyme preparations from 5-day germinated grains of A. sativa, (A), and H. vulgare, (B).

Abcissa: Distilled water, (W), or concentrated husk extract added, (C).

Ordinate: Tyrosine concentration of the T.C.A. filtrate, p.p.m., after 1½ hours digestion.

slightly affected, by the materials present in the husk extract. When added to proteolytic enzyme preparations, however, a considerable inhibition of enzyme activity was noted in both preparations examined. The A. sativa preparation showed some 80% inhibition, and the H. vulgare some 40% inhibition (Expt. 61, Fig. 64).

Our findings that there is a germination inhibiting effect when a water extract of whole grains or husks of grains is added to untreated grains of this species, and to seeds of other species is in accord with the views of Ruge (1939) and Massart et al (1959). A closer examination of the data of Elliot and Leopold (1953) indicates that they do not demonstrate the presence of a germination inhibitor at all. In no case is the effect of a water extract of whole grains, or grain parts, on the germination of untreated seed discussed. The leaching experiments designed to establish the presence of a germination inhibitor, are at best unconvincing, and open to several different interpretations. This is largely due to a lack of sufficient information on such points as the age of the seed samples used, the criterion of germination employed, the temperature at which germination was carried out, and the amount of water available to the germinating grains in the petri dishes. They found that untreated grains showed no germination after four days in their germination conditions, while samples of the same variety used in these experiments show 90% germination after 2½ days, and complete germination after 4 days in our germination conditions (Fig. 60). Grains which they soaked for 24 hours in still water showed only 5-15% germination, again after four days in their germination conditions. Similar treatments carried out with the samples of grains used in some previous experiments showed that 80% germination occurred after 2½ days, and 96% germination after

4 days in our standard germination conditions, and 60% germination if germinated at 10°C. instead of 20°C. The results of the leaching experiments obtained by Elliot and Leopold may indeed be due to the inception of changes within these grains analogous to those produced by Hay and Cummings (1959) in the induction of dormancy in grains of <u>A. fatua</u> by similar soaking treatments (Expt. 8, Fig. 8).

No amylase inhibiting properties were evident in the water extract of husks of <u>A. sativa</u> tested here, but Elliot and Leopold report an inhibition of <u>B. subtilis</u> a-amylase and <u>Barley</u> b-amylase with materials present in a water extract of whole grains, and of the same a-amylase, by materials extracted from husks of <u>A. sativa</u> with ether, and then dissolved in water. The activity of this inhibitor was found to withstand up to 20 mins. boiling without inactivation. Kneen and Sandstedt (1946) found no trace of amylase inhibitors in a water extract of grains of <u>A. sativa</u> (variety unspecified), which had been boiled for a few minutes, and then tested against salivary and <u>B. subtilis</u> a-amylases. The assumption of Elliot and Leopold that inhibition of one a-amylase is evidence of the inhibition of other a-amylases is not borne out to any extent by studies on the naturally occurring amylase inhibitors. The water insoluble inhibitors found in <u>Fagopyrum</u> <u>esculentum</u> L. (Buckwheat), (Chrzaszcz and Janicki, 1933), and Navy Bean, (Bowman, 1944) were thought to show a complete spectrum of amylase inhibition, but some doubt has been thrown on this point with regard to the former (Kneen and Sandstedt, 1946). In all the water soluble amylase inhibitors, some degree of specificity has been found. That present in the endosperm of <u>Wheat</u> inhibits a-amylases of animal origin, some of bacterial origin, but none of the cereal amylases tested (Kneen and Sandstedt, 1946). The inhibitor,

present largely in the germ of Sorghum, inhibits cereal amylases, and bacterial amylases, but not fungal amylases (Miller and Kneen, 1947).

The finding that amylases of the same type from different sources may show different responses to inhibitors is substantiated by studies on the essential groups required for amylase activity. b-amylase of Barley has been shown to require free sulphydril groupings in order to show enzymic activity (Weill and Caldwell, 1945), and a similar claim is made for the a-amylase of B. subtilis (Di Carlo and Redfern, 1947), but pancreatic a-amylase was not found to be dependent on the presence of this grouping for its activity, (Little and Caldwell, 1942, 1943).

The inhibition of B. subtilis a-amylase, and its reversal by chemicals with sulphydril reactivating properties as demonstrated by Elliot and Leopold (1953), is therefore consistent with the conclusion that the inhibitor acts by blocking free sulphydril groups in this enzyme. To infer from this that germination of A. sativa can be inhibited by this same material blocking the sulphydril groups of Oat a-amylase is however a premature conclusion, since no information is available concerning the nature of the chemical groups necessary for the activity of the cereal a-amylases.

The inhibition of growth in etiolated pea stem sections by the same seed extracts, while not being regarded by these investigators as evidence of the blocking of sulphydril-requiring enzymes connected with respiration and growth, is at least consistent with this theory of inhibitor action. Our findings that proteolytic enzymes from A. sativa and H. vulgare are inhibited by a husk extract might also be indicative of the presence of a sulphydril blocking material in this, since proteolytic enzymes of all types seem to require the presence of free sulphydril groups to show activity, and to be

extremely sensitive to agents blocking these groups (Barron, 1951).

On the basis of our studies in previous sections (Parts III and IV), it was concluded that the amylases play a very insignificant role in the physiology of the grain of A. sativa preceding and during germination, and that materials other than starch were utilised in this pre-germination metabolism. The possibility of inhibiting the germination of this species by inhibiting a-amylase activity would therefore be strongly doubted. Our observation that proteolytic activity is evident in the embryo considerably before the occurrence of germination (Part V), and that enzyme preparations from the seed being investigated show a strong inhibition in vitro in the presence of the germination inhibiting extract, is more in tune with the expected influence that such an inhibition would exert in vivo on the embryo's capacity for growth. If the inhibiting material is however a universal sulphydril inhibiting material, interaction with many other enzymes in respiration and other metabolic functions is possible. In this non-specifi view of the action of the inhibiting materials present in A. sativa, general agreement is shared with the findings of Elliot and Leopold (1953).

The behaviour of the extracts of A. fatua in inhibiting the germination of grains of A. sativa and promoting the germination of non-dormant grains of A. fatua may be partly explainable on the basis of some recent studies carried out by Black (1959), and Black and Naylor (1959). In their studies they also found that loss of dormancy was not correlated with a decrease of inhibiting materials in the grains, but that it depended on the relative levels of germination inhibiting and promoting materials, germination occurring when the inhibitors were removed from the seed by leaching. The results found

here (Expt. 59, Fig. 61) are consistent with the presence of two materials with these opposing properties, acting in different concentrations or of one material showing different properties at different concentrations.

Summary.

1. The presence of germination inhibiting properties has been demonstrated in water extracts of whole grains of A. sativa and A. fatua, and in the husk of the former species.

2. No loss of inhibiting capacity could be correlated with the loss of dormancy in A. fatua.

3. Extracts of whole grains inhibiting the germination of A. sativa were also observed to promote the germination of a non-dormant sample of A. fatua.

4. No amylase inhibiting properties were observed in the water extract of A. sativa husks, but considerable inhibition of proteolytic enzyme preparations from A. sativa and H. vulgare was shown.

The relevance of these findings to previous work has been discussed.

General Conclusions.

Perhaps the most interesting conclusion from the studies carried out on the reversability of germination in grains of A. sativa is that the changes by which the resting embryo is converted to a growing seedling are initiated within a few hours of commencing imbibition. The stimulus bringing about these changes is of course the hydration of the embryo tissues, bringing about the resulting changes in the physical nature of the cell proteins, and the concentration of soluble materials in the cell fluids. In the studies reported here several distinct aspects of the physiology of the imbibed grain have been observed to be actively changing during the period before meristematic activity has commenced, i.e. pre-germination by our definition. These consist of a considerable utilisation of seed fat, alterations in the amount and nature of soluble carbohydrates, indications of protein hydrolysis, and evidence of the synthesis of enzymic proteins. Some aspects of the seeds physiology which were observed to be initiated concurrently with, or post-germination, were the hydrolysis of endosperm starch and proteins, and the translocation of soluble derivatives of these to the growing embryo tissues. This sequence of changes is indicative that the embryo of the resting seed is fairly independent of the other parts of the grain during the early stages of its conversion to the actively developing seedling, and leads to the conclusion that sufficient materials to meet the needs of this activity must already be present in these tissues, along with the capacity to utilise them. The observation that synthesis of a-amylase, and of proteolytic enzymes must take place before the endosperm food reserves can be utilised is evidence of a definite restriction to this utilisation, which

requires to be removed by the positive action of synthesising the enzyme. That this is one of the primary activities set in motion by the growing embryo in conjunction with the non-growing parts of the seed, and not independently by either, is borne out by the observation that the integral unity of the seed must be maintained for the development of these enzymes. This suggests that the development of the means of utilising the endosperm food reserves, i.e. enzyme synthesis, is dependent on the onset of growth, and not merely with the attainment of a certain level of hydration.

The view that the metabolism of the germinating grain of A. sativa consists of the two phases indicated above - an early embryo based metabolism, and a later endosperm dependent metabolism, with a considerable degree of overlap in the 24 - 48 hours period after commencing imbibition, is substantiated to a considerable degree by previous investigators. The capacity for independent growth shown by excised cereal embryos has been mentioned previously in relation to this (Toole, 1924; James and James, 1940). The several studies by Albaum and co-workers on metabolic activity in germinating Oat grains have also been mentioned in relation to the particular aspects studied here, but taken together they bear out the general conclusions stated above. They indicate a fat based, cyanide sensitive respiration during the first 48 hours in germination conditions, changing then to a carbohydrate based respiration fairly insensitive to cyanide, with protein and organic phosphorus metabolism related to this changing pattern.

In comparing the development of amylase and proteolytic enzymes in non-dormant grains of A. sativa, A. fatua, and A. ludoviciana, an essential

similarity was apparent in all three species. This encourages the belief that most other aspects of the physiology of these species may also be similar. If this is indeed the case, then the physiological basis of dormancy in these last two species must be related to some aspect of the early embryo based metabolism, and not the later endosperm dependent metabolism. A restriction acting on some of the phases of this latter pattern, might constitute a suitable means of delaying the rate and nature of embryo development, but it would be an unlikely means of preventing the onset of embryo growth.

Of the differences observed in these studies between dormant and non-dormant grains of *A. fatua* and *A. ludoviciana*, the lack of development of the increased amylase activity characteristic of non-dormant grains can be disregarded as a potential cause of dormancy on the grounds discussed above. The non-development of the increased proteolytic activity observed in the dormant grain must however be admitted as a potential cause of dormancy, on these grounds,

a) The increase in non-dormant grains commences in the embryo tissues in the pre-germination period of imbibition.

b) It is a physiological difference between dormant and non-dormant grains of the same species.

c) It is at least potentially capable of depriving the embryo tissues of some of the primary materials required for the synthesis of enzymes and other proteins involved in the resumption of growth by the embryo.

It is however evident that this physiological difference can be regarded as no more than a potential cause. A considerable amount of investigation on many other aspects of the physiology of these grains still requires to be

carried out before any definite assignment of causality can be made. For the present it is probably better to regard both these differences in the physiology of dormant and non-dormant grains of these species, as an expression of deficiency in, or a restriction of, some earlier unknown phase of the metabolism of the imbibing embryo. The capacity to synthesise enzymic proteins immediately suggests itself as one possible common link between these two physiological differences.

It has been observed in these studies that dormancy in grains of A. fatua and A. ludoviciana, and the transitory dormancy observed in grains of A. sativa is a change which comes about in the ripening grain as maturity approaches, and which wears off gradually during dry storage. Physiological differences have been described above in these dormant grains compared with non-dormant grains of the same species. The theory that dormancy is due to the presence of a chemical material in these grains preventing germination would require,

1) That such materials could be demonstrated as being present.
2) That they should decrease as dormancy decreases, and
3) That any interference with the metabolism of the embryo should be with some aspect of the early embryo based metabolism.

The presence of germination inhibiting properties has been demonstrated here in water extracts of whole grains of A. sativa and A. fatua, and of the husk of A. sativa. No correlation was found between the amount of inhibition and the amount of dormancy in dormant and non-dormant grains of A. fatua. The husk extract proved to have no effect on the amylases of A. sativa, and several other preparations, but a considerable inhibition of the proteolytic enzyme of A. sativa and H. vulgare. This last property could well fit the

third requirement given above. Allowing this, it would appear that two of these three requirements are satisfied, though this is insufficient evidence to advance the theory that dormancy was due solely to the presence and activity of a germination inhibiting substance.

If the original theory is modified to state that dormancy is due to the relative amounts of a germination inhibiting substance and a germination promoting substance, similar requirements should still apply to the germination promoting substance, though one would expect it to increase, relatively to the inhibitor, as dormancy wore off. It has been observed that germination promoting effects were found when the extracts of whole grains of these two species were tested against non-dormant grains of A. fatua. As with inhibiting properties, however, there was no substantial difference between an extract prepared from dormant or non-dormant samples of A. fatua, and no physiological action can yet be attributed to the presence of such materials. Since both inhibiting and promoting effects were present in the same extract it would appear that the effect of the two properties is not a simple cancellation of each other.

On the basis of the knowledge gained from these studies it would therefore appear that dormancy in A. fatua cannot be explained

a) by the development of a germination inhibiting substance at maturity, and its disappearance during storage,

b) by the lack of a germination promoting factor at maturity and its formation during storage, or

c) a combination of these two.

These conclusions are in general agreement with those reached by

Black (1959), that dormancy is related to the ease with which an inhibiting substance leaves the grain of <u>A. fatua</u> and the presence of germination promoters within the grain. While a possible role may be envisaged for the germination inhibitor present in <u>A. fatua</u>, it is difficult with our present knowledge to do so for <u>A. sativa</u>, and we feel that this species should be grouped with those investigated by Barton and Solt (1949), in which the function of the germination inhibiting materials present was considered to be of little or no biological importance.

Since dormancy has been observed to be associated with the attainment of ripeness in these studies, and elsewhere, the possibility exists that these so-called "germination inhibitors" may be persistent "ripening inducers". By restricting the development of the immature seed, in particular ways, and bringing about the resting condition, the many secondary changes resulting in seed dormancy could be brought about. The correction of such changes to bring the metabolism into the proper alignment for germination to take place would then constitute the basis of natural after-ripening, associated with which might well be the development of natural germination promoting substances.

The general picture emerging from all these studies on grains of these three species is that of a well balanced, sequential pattern of physiological changes by which a quiescent non-dormant embryo is transformed to an active seedling capable of every independent aspect of growth and development. The upsets of this balance which constitute the basis of dormancy have been further characterised in some respects, but their fundamental nature is still unresolved.

Summary.

1. Seed dormancy in Avena sativa, Avena fatua, and Avena ludoviciana has been found to develop at maturity and to wear off gradually during a period of several months dry storage.

2. No evidence for a completely reversable phase of germination was obtained when the period of imbibition of grains of A. sativa was interrupted by a dehydration treatment. Initially germination promoting effects were observed, but after some degree of embryo growth had occurred embryo damage resulted from such treatments.

3. The high level of Free Amylase activity characteristic of germinated grains of A. sativa, A. fatua and A. ludoviciana has been shown to be mainly due to the development of a-Amylase, concurrently with, or shortly after the initiation of embryo growth. The site of development is the endosperm, but an essential role for the growing embryo is indicated, since mutilated grains, separate grain parts, and dormant grains of the last two species show little or no capacity for such development. The participation of amylases in the pre-germination metabolism of A. sativa is therefore doubtful.

4. Studies on the levels of fats, starch, and on the amounts and nature of soluble carbohydrates in germinating grains of A. sativa indicate that materials other than starch form the foundation of the pre-germination metabolism.

5. An actively increasing proteolytic activity was found to be present in the embryo of grains of A. sativa during the pre-germination period of imbibition. The high level of activity characteristic of the germinated

grain was found to be due to the formation of these enzymes within the embryo, and their diffusion to the endosperm. An essential role for the growing embryo is again indicated by the lack of similar behaviour in isolated endosperms of A. sativa and dormant grains of the last two species.

6. Water extracts of whole grains and husks of A. sativa, and whole grains of A. fatua were found to exhibit germination inhibiting properties against A. sativa and several other species. The same whole grain extracts promoted the germination of non-dormant grains of A. fatua.
No correlation could be drawn between the presence and amount of these properties, and the dormancy status of grains of A. fatua. The husk extract was found to have no effect on several amylase preparations but produced a considerable inhibition of proteolytic enzymes.

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