PHYSIOLOGICAL ASPECTS OF SEED DORMANCY IN AVENA LUDOVICIANA DUR.

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INTRODUCTION

Following imbibition of water by a whole seed, changes occur in the embryo which result in the onset of cell enlargement and division in the radicle. At some point during this process, the enlarging radicle penetrates the testa or other covering tissue. This first appearance of the radicle is commonly thought of as being germination itself, but it can also be regarded as being merely the visual evidence of the previous occurrence of germination. If this view is taken, the expansion and division in the radicle is considered to be post-germinational growth and as such can often be distinguished from germination by different responses to given physical and chemical environments.

The concept of germination subscribed to in this work envisages some controlling activity which occurs prior to, and results in, the onset of cell enlargement. At present, however, the necessary proximity of this germinative step to the initial stages of growth makes the study of germination in isolation very difficult.

An adaptive feature of many plant species is the ability of their seeds to remain ungerminated or dormant under conditions which are favourable for growth and would allow germination of non-dormant seeds of the same species. Dormancy is often manifest as a restriction of the range of conditions permitting germination rather than a total suppression of germination. The causes of dormancy appear to be very variable, and range from gross physical restriction of water uptake

and embryo expansion to fine control of more fundamental cellular activities.

Germination

The initiation of metabolic activity which follows closely on imbibition must depend upon the action of existing enzymes on existing substrates. A study of the enzymes and substrates which are available in the dry seed and which may have a role on imbibition is thus worthwhile. Following from this it is important to attempt to identify the initial enzyme reactions which are of consequence in germination. Finally we may hope to elucidate the steps of the pathways between these initial reactions and the synthetic processes which will lead to the re-establishment of growth.

The Resting Seed

There are many indications that RNA and protein synthesis are intimately involved in germination. Both processes occur during imbibition and inhibitors of these processes also prevent germination. Enzymes concerned in RNA metabolism have been detected in dry seeds (Brawerman and Chargaff, 1955; Ingle and Hageman, 1965; Marre et al., 1965). Marcus and Feeley (1964) have found all the enzymes necessary for the onset of protein synthesis are present in dry seeds, such that synthetic templates can promote amino acid incorporation.

Protein synthesis will be dependent on the level of free amino acids and where this is low, as in dormant cherry (Flemion and De Silva, 1960),

and in dormant Avena fatua (Naylor, 1966), protease may have an important role to play. Proteases are present in dry seeds (Daniellson, 1951; Soedigo and Gruber, 1960). In cereals proteases occur mainly in the aleurone cells along with dipeptidases, while the latter alone occurs in the embryo (Engel and Heins, 1947).

Although protein synthesis is considered a sine qua non for germination, the importance of lipid and carbohydrate metabolism during this early period is less well understood. Work in this laboratory indicates that utilisation of fats occurs in oats before the onset of meristematic activity, but in general there is no clear indication of whether lipid metabolism has a primary role or is the result of germination (Koller et al., 1962). However, esterase activity has been demonstrated in wheat and rye (Engel, 1947a), and isoenzymes of esterase occur in peas (Frankel and Garber, 1965), and in maize (Scandalios, 1964). Isocitritase was found in peanuts by Cherry (1963), but not by Marcus and Velasco (1960). Beevers (1961), could detect no activity in pumpkin seeds. These apparently conflicting results may perhaps be explained by the gradual reappearance of preformed isocitritase which occurs during the germination of some seeds (Presley and Fowden, 1965).

A good example of the qualitative differences which exist between the pre- and post-germination metabolism of the seed is given by the amylases. β-Amylase is found mainly in the aleurone layer of barley (Engel, 1947b), and is presumably responsible for the initial dextrinising activity in cereals on imbibition. After germination β-amylase activity declines. The major enzyme of carbohydrate metabolism follow-

ing germination, α-amylase, is absent in the dry seed of oats (Drennan and Berrie, 1962), although Dure (1960) claims that it exists in the scutellum of barley. However, the immunochemical evidence of Grabar and Daussant (1964) seems to eliminate the possibility of α-amylase in the dry seeds of the variety Aurora.

Other enzymes which occur in dry seeds and are involved in fat and carbohydrate transformations are reviewed by Altschul et al. (1966).

Phosphorus metabolism must have a central role in an energy requiring system such as a germinating seed. In castor bean endosperm the levels of the main high energy respiratory metabolites including ATP, are of the same order as in active tissue (Cocucci, 1964). Koller et al. (1962) believe that the small amount of ATP present in many seeds is sufficient for germination. However, ADP synthesising enzymes are present in wheat germ (Dankert et al., 1964), and the synthesis of ATP and UDP-hexose which occurs early in the imbibition of crested wheatgrass (Wilson and Harris, 1966), implies that existing enzymes are responsible. During the ripening of wheat grains, a method of protein synthesis can be demonstrated which utilises ATP produced from phytate (Morton and Raison, 1963). If this mechanism still exists in the dry seed on imbibition, it could help to explain the rapid onset of protein synthesis. Certainly a similar synthesis of GTP using phytate occurs in mung bean following imbibition (Biswas and Biswas, 1965).

Considering the high phosphoryl transfer potential of phytin and its frequent occurrence in seeds, it is not surprising that it has figured much in germination studies. An active phytase is present in the aleu-

rone of wheat (Peers, 1953), while the bran contains a non-specific phosphomonoesterase (Nagai and Funahashi, 1962). Phytase activity also exists in the wheat embryo at imbibition although this is increased by de novo synthesis (Sartirana and Bianchetti, 1967). However, the number and specificity of the enzymes hydrolysing phytin in seeds remains unclear (Preece and Grav, 1962). Mayer (1958) reports two pH optima for the breakdown of phytin in germinating lettuce seeds.

In the embryo of a non-dormant seed there must exist some respiratory capacity which is already present or is easily obtainable. How this reserve material is maintained in dry storage perhaps for years has long been a subject of speculation. Tchen and Vennesland (1955), suggest that the ability of phosphoenol pyruvate carboxylase to resynthesise very low levels of carbon dioxide may have relevance in this respect. The continued requirement for energy during germination would appear to demand the establishment of some of the respiratory pathways. The range of respiratory enzymes found in dry seeds may be illustrated by reference to wheat. The activity of some of these enzymes will contribute to the first respiratory pathways.

Among the respiratory enzymes found in wheat germ are NAD and NADP linked alcohol dehydrogenases (Stafford and Vennesland, 1953); malic enzyme, isocitric and glucose-6-phosphate dehydrogenases (Anderson et al, 1952); extremely active 6-phosphogluconic and malic dehydrogenases (Barnett et al., 1953; Loewus et al., 1955); phosphoenolpyruvic carboxylase and carboxykinase (Tchen and Vennesland, 1955). An active polyphenol oxidase has also been detected in the aleurone layers (Belderok, 1961).

It is thus possible that the initial energy required for germination in wheat could be derived from the activity of the pentose pathway, from anaerobic respiration, or from some modification of the tricarboxylic acid cycle. This conclusion is supported by numerous other reports of these pathways early in germination. Generally the pentose pathway is considered to operate before the tricarboxylic acid cycle, the latter no doubt being influenced by the abnormal appearance of the mitochrondria present in dry seeds (Cherry, 1963; Briedenbach et al., 1966). Levels of oxidised and reduced NAD and NADP in seeds have been reported by Mukherji, Dey and Sircar, (1968).

The Changes Occurring on Imbibition

Water uptake by viable and non-viable seeds was shown by Atkins (1909) to be initially similar and thus purely imbibitional. The pattern of re-hydration of reserve and enzymic protein is described by Ghetie (1966). Enzymes thus reactivated are bound to display at least some residual activity, dependent upon the availability of substrates. Whether this initial activity is sufficient to effect germination is a matter of conjecture. Marre et al. (1965), believe that enzyme reactivation rather than substrate availability controls the rate of biosynthetic processes during early imbibition.

In lettuce and peanut cotyledons the pentose phosphate pathway has been implicated as the most important respiratory pathway during early germination (Mayer, 1961; Cherry, 1963). The absence of cytochrome oxidase and succinic dehydrogenase in lettuce at this stage seems to remove the possibility of a 'normal' tricarboxylic acid cycle (Mayer et

al., 1957). However, even in castor bean where all the enzymes are present, a glyoxylate cycle predominates (Canvin and Beevers, 1961).

Whatever the method by which the initial energy is supplied, the presence of considerable protein synthesis in wheat embryos after only 15 minutes imbibition is evidence of an efficient system (Marcus et al., 1966). During this imbibitional period the rate of protein synthesis is controlled by the activation of the ribosome-messenger system or, more specifically, by the formation of functional polysomes. The high proportion of polysomes implicates a long-lived messenger RNA: this has also been suggested as the means of initiation of protein synthesis in cotton embryos (Dure and Waters, 1965). As a result of increased RNA-polymerase activity, synthesis of new messenger RNA and polysomes is made possible (Marre et al., 1965).

The Onset of Growth in the Embryo

The second phase of water uptake is active, begins in live wheat embryos before imbibition has ceased in dead embryos, and is inhibited by low temperature or by atmospheres of nitrogen (Wellington and Durham, 1961). In Phaseolus the temperature permitting optimum water uptake is lower at this stage than during imbibition (Walton, 1966). This second surge of water uptake is greatest in the embryo of whole seeds, as indicated by the work of Stiles (1948), with maize and cotton, and Hall and Hodges (1966) with oats. It follows that a clear demonstration of the biphasic nature of water uptake depends on a time lag between imbibition and embryo development. A lag phase can also be detected in the development of respiratory activity during germination (James,

1953); with the onset of cell elongation in the embryo of Phaseolus a sharp increase occurs in the respiratory rate (Walton, 1966). However, in dormant seeds water uptake and respiration do not progress beyond the lag phase until the dormancy is broken (Stiles, 1960a). It is thus evident that important events in the germination sequence occur during this lag phase (Mayer and Poljakoff-Mayber, 1963).

Isolated wheat embryos are fully imbibed in 30 minutes, but the lag phase continues for 6 hours. Protein synthesis begins during imbibition and is thus pre-germinational. The decrease in the rate of protein synthesis which occurs in the lag phase can be explained by depletion of existing long-lived messenger RNA (Marcus et al., 1966). It is possible that germination and the end of the lag phase are a consequence of the complete restoration of the capacity to synthesise messenger RNA in the cells of the embryo. This accords with the report that the breaking of dormancy in potato tubers is accompanied by the ability to synthesise DNA-dependent RNA (Tuan and Bonner, 1964). It may be then that germination is the revival of full nuclear control over the embryonic cell, but at the moment insufficient is known of the metabolism prior to cell enlargement to substantiate this.

Post-germination phenomena include continued protein synthesis, much of which is directed towards the elaboration of new enzyme species designed to degrade the major reserve materials of the cotyledons, endosperm, and aleurone layers. Active transport of these materials to the growing region is characterised by increased scutellar activity in the cereals (Edelman et al., 1959), and transport of protein and

RNA metabolites from the cotyledons of other species (Beevers and Guernsey, 1966). Detailed accounts of these changes are given in the reviews by Drennan (1960), Koller et al., (1962) and Mayer and Poljakoff-Mayber (1963).

In conclusion, germination is restricted here to include only these changes involved in the re-establishment of a metabolic state permitting cell enlargement and division and is thus an extension of the views of Toole et al., (1956) and Evenari (1957).

Dormancy

Dormancy

Dormancy is the inability to germinate except under a special set of environmental conditions. It can be imposed and removed by a variety of treatments and external environments, and this has led to the establishment of many theories concerning its cause. In the future a few fundamental reasons for the failure of an embryo to germinate may emerge, but at the moment, our knowledge is derived largely from description and empiricism and explains few of the basic reactions affected by the agents which break and impose dormancy.

The Inception of the Dormant Condition during Maturation

The level of dormancy in the mature seed is the product of many interacting factors which existed during ripening. Koller et al., (1962) suggest that important dormancy-inducing events may have occurred as early as fertilisation.

Two apparently distinct types of dormancy may develop in cereal seeds during ripening (Belderok, 1961). In the first, germination

is low at the milk stage of development and gradually rises, reaching total germination at maturity or soon after. In the second type, germination is high at the milk stage and falls to its lowest level at full ripeness. The first type occurs in many wheat varieties (Belderok, 1961), and in bamboos, where germination of the short-lived embryo often occurs before dehiscence (McClure, 1966). The second type has been found in barley and in oats (Fuchs, 1942; Bishop, 1944; Thurston, 1962). Fuchs also found a narrowing of the temperature range for germination during maturation.

The appearance of different dormancy levels in the seeds of many closely related species and varieties of cultivated plants indicates a large measure of genetic control of the onset of dormancy. This is the result of the interaction of embryonic and maternal factors. Inter-specific and intraspecific crosses of Papaver species demonstrate that the maternal influence is of overriding importance in determining the dormancy of the seeds, although in a few instances, the genetics of the embryo had greater importance (Harper and McNaughton, 1960). In the light of these results, it is possible that the changes in dormancy during maturation reflect the dynamic interactions which must exist between the developing embryo and the senescing mother plant.

The occurrence of different dormancy levels in the same genetic material in different years and situations indicates that the ripening environment also influences dormancy. It has long been believed that the sprouting of cereal seeds during a wet harvest is related to the previous occurrence of long periods of dry weather, and conversely,

cold damp summers have been linked with increased dormancy levels.

Among the few instances where the variable has been identified are the low temperature induced dormancy of Rosa species (Von Abrams and Hand, 1956), and the inhibition of dormancy induction by high temperature in wheat (Belderok, 1961). In the latter, high temperatures are effective only after the milk stage of development. Humidity and water supply during ripening had no effect on dormancy. That the ripening environment may alter the composition of the seed is shown by the effect of temperature and photoperiod on the fatty acids of flax (Dybing and Zimmerman, 1966): that it may alter the dormancy level by such change is a strong possibility.

Among the changes which occur at this time and which may influence dormancy are changes in the levels of organic acids and in particular malic acid (quoted by Fowden and Moses, 1960). Synthesis of phytic acid in wheat increases rapidly with the onset of water restriction to the seed, and there is a simultaneous fall in ATP production (Jennings and Morton, 1963). In oats, synthesis proceeds only after the milk stage and continues in immature seeds dried off the mother plant (Ashton and Williams, 1958).

During seed development, gibberellin levels are correlated with the growth rate of the seed and appear to fall rapidly on ripening (Radley, 1961; Skene and Carr, 1961; Corocan and Phinney, 1962; Ogawa, 1963). Ripening timothy and perennial ryegrass seeds show similar changes (Stoddart, 1965). The finding that gibberellic acid can prevent the inception of seed dormancy in Avena fatua supports

the belief that the in vivo gibberellin level is an important factor in the determination of the dormancy level (Black and Naylor, 1959). Recently, a correlation between the endogenous gibberellin level and increase in dormancy has been reported in ripening Melampyrum lineare seeds (Curtis and Cantlon, 1968). The idea of Radley (1958), that the disappearance of gibberellins during ripening could be due to their conversion to an inactive 'bound' form, rather than their degradation, is supported by the evidence of McComb (1961). Further studies indicate that endogenous and applied acidic gibberellins decrease while neutral gibberellins increase in developing bean seeds (Hashimoto and Rappaport, 1966). More recently, a biologically inactive glycoside of gibberellin A_8 which can be converted by glucosidase to the active form has been isolated from Phaseolus (Sembdner et al., 1968). It is likely that such changes in active and inactive gibberellins are intimately concerned in the inception of dormancy.

The hormonal aspects of seed development, treated as a facet of the senescence of the mother plant, are reviewed by Carr (1967) and Wareing and Seth (1967).

In conclusion, the dormancy of the seed at maturity is the product of the interaction of the external environment, the maternal influence, and the particular physiology of the embryo as dictated by its genotype.

The Loss of the Dormant Condition during After-ripening

The term 'after-ripening' was first used by Crocker (1916) and is now taken to mean the processes which result in the gradual increase

in germination in a population of seeds which were dormant at harvest, (Stokes, 1965). In a large group of species, after-ripening requires low temperatures and moist conditions. This treatment is known as 'stratification' and leads to the removal of dormancy through physiological change in the embryo which may entail some further growth.

Stratification is required by many Roseaceous seeds and may result in increase in moisture, acidity, soluble nitrogen, sucrose and lipase activity, while fats may decrease (Flemion, 1933). As sugar pine seeds after-ripen, the lag phase of water uptake gradually contracts and disappears due to earlier active uptake (Stanley, 1958). The second rise in respiratory activity also begins earlier.

As yet there are few detailed studies of the metabolic changes which occur during after-ripening, and separation of cause and effect has so far been impossible. The work of Olney and Pollock (1960), has shown that the breaking of dormancy in cherry seeds is preceded by the accumulation of phosphate and nucleotide phosphorus. From this they conclude that dormancy is caused by a block in phosphorus metabolism. In dormant hazel seeds, chilling probably increases the ability of the seed to synthesise nucleotides and possibly promotes the synthetic functioning of the tricarboxylic acid cycle (Bradbeer and Floyd, 1964; Bradbeer and Colman, 1967a). Again in Prunus cerasus, an increase in the activity of the pentose phosphate pathway occurs during after-ripening (Lacroix and Jaswala, 1967). No conclusions can be drawn as to how these respiratory changes affect the dormancy level.

Frankland and Wareing (1962, 1966), detected a small increase in the gibberellin content of hazel seeds during chilling and suggest this as a

possible cause of loss of dormancy. It is not known whether this is the result of synthesis or release of gibberellins. What may be a different system of control of dormancy exists in wild rice, where low oxygen tensions promote after-ripening, and gibberellic acid has no effect on germination (Simpson, 1966).

In a second group of species, after-ripening may be promoted by storage in dry conditions. This effect is typically seen in cereals, but also occurs in clovers, lettuce and cress. After-ripening of this type usually requires much higher temperatures than those for stratification.

Among the first to record this phenomenon was Atterberg (1907), who also found that a short period at 35-40° was as effective as a longer period at room temperature. The gradual release from dormancy is often manifest as a widening of the temperature range for germination as well as increased germination under particular conditions. Dry storage at low temperature was found to be ineffective (Larson et al., 1936). It is interesting that hazel seeds which respond to low temperature stratification, are made dormant by high temperature dry storage (Bradbeer, 1968).

Decrease in dormancy during dry storage has long been thought to be due to the increase in permeability to oxygen of the seed coats (Atwood, 1914; Harrington, 1923; Johnson, 1935). Other workers maintain that it is the drying of the seed during storage which is responsible for the loss of dormancy (Wellington, 1956), but the previous idea has now gained popularity in the form of non-metabolic oxidation of inhibitory

substances (Roberts, 1962). However, although Wareing and Foda (1957) subscribe to the suggestion of oxidation of inhibitors, the inhibitor level in Xanthium seeds does not alter during after-ripening. Other studies have shown that after-ripening in Avena fatua leads to changes in response to exogenous sucrose by the embryo, an increase in a gibberellin-like factor in the embryo (Simpson, 1965), and an increase in availability of protein precursors in the aleurone (Naylor and Simpson, 1961; Naylor, 1966). A positive correlation exists between germinability and citric acid content in several species, including barley (Taufel and Poloudek-Fabini, 1955), and work in this laboratory indicates a similar correlation for malic acid in Avena fatua, but there is no proof that these changes occur in the dry seed during after-ripening.

Nevertheless, the possibility of such metabolic activity occurring during dry storage does exist, but investigation of such changes has been largely neglected due to the belief in non-metabolic oxidation of inhibitors.

In cultivated plants, dormancy at harvest is usually of advantage to the grower, but subsequently non-dormant seeds are required. Dormancy can result in decreased germination and choice of seedlings, lengthening of the time to seedling establishment, and unevenness in the final crop - an important factor in cereals. A knowledge of the factors controlling after-ripening is thus important in agriculture and brewing.

The Regulation of Dormancy by the External and Internal Environments

During ripening and after-ripening, the dormant seed usually retains

the ability to germinate under specific narrow conditions of the physical environment, or under the action of certain promotive substances. Other environments and substances have an inhibitory effect and may serve to deepen the dormant condition or prevent germination of non-dormant seed. The retention of the dormant condition after the unfavourable environment causing it has been removed is known as 'secondary dormancy'.

In this section the physical and chemical factors which have a role in the breaking and imposing of dormancy will be discussed. Where possible, details will be given of the areas of metabolism thought to be affected.

The findings of Atterberg (1907) and Fuchs (1942), concerning the optimum temperatures for germination in cereals, have been extended by many workers. Vegis (1964), has elaborated the concept of restriction and relaxation of a dynamic temperature range for germination during the natural imposition and removal of seed dormancy. This model is similar to that used by Vegis and others to explain bud dormancy, and also shows parallels with diapause phenomena in insects.

As seeds after-ripen, the temperature range for germination widens, either by increase in the maximum, or decrease in the minimum temperature permitting germination. Alternatively, both may occur simultaneously. Examples are given by Vegis (1964) and Toole et al. (1956). Borthwick and Robbins (1928), first showed the inhibitory effect of high temperatures on lettuce germination. A possible explanation of this in terms of temperature-dependent inhibitor formation has been advanced by Berrie (1966). Very little is known of the metabolism involved in temperature-induced dormancy, although the onset of anaerobic conditions which is

said to result from inhibition at high temperatures, may increase the activity of glycolysis relative to the tricarboxylic acid cycle (Vegis, 1964). The large amounts of acetyl-CoA thus produced would then be ~~oxidised~~ reduced to fats; removing the danger of alcohol production, but also increasing dormancy.

The effects of temperature on seed dormancy have been reviewed by Toole et al. (1956), Lang (1965) and Stokes (1965), while Koller et al. (1962) have dealt mainly with thermoperiodic phenomena.

Light sensitivity is a form of dormancy which has received intensive study in recent years. A considerable number of species have a light requirement for germination. This is seldom absolute, in that other treatments such as low temperature, hormones, nitrates, can replace the light effect. The first exact work was performed by Flint and McAlister (1935, 1937), who noted the promotive and inhibitory effects of red and far-red light on lettuce seed germination. These effects are remarkable in that extremely short exposures to light may suffice, and far-red reverses the effect of redlight and vice versa. The work of Borthwick et al. (1952) extended this study and led to the discovery of phytochrome, the pigment which acts as the photoreceptor in this system. This example of light sensitivity in lettuce is in fact one type of light-temperature interaction, as it occurs only at high temperatures. The response of seeds to light is greatly influenced by temperature, and in lettuce, red light or exposure to low temperature will break skoto-dormancy (Ikuma and Thimann, 1964). Other examples of light-temperature interactions are reviewed by Hart (1966).

Less is known of the reactions involved in the inhibition of germination by 'white' light, as distinct from the low energy far-red reversal type of inhibition. Usually much longer exposures to light are required for the operation of this 'High Energy Reaction', activated by blue and far-red wavelengths (Mohr, 1962). Thus in Nemophila insignis, the inhibition is due to the infra-red and blue regions of the spectrum, whereas red light has no promotive effect on germination (Black and Wareing, 1960).

Photoperiodic responses in germinating seeds are noted by Wareing (1963). Reviews of the effects of light on dormancy and germination are provided by Mayer (1960) and Evenari (1965).

As yet, the method by which the primary effect of light is translated into stimulation or inhibition is unknown. However, it has been suggested that in lettuce, red light prevents the occurrence of an inhibitory protein synthesis (Black and Richardson, 1967).

Dormancy imposed by seed coverings has been adequately reviewed (Koller et al., 1962; Vegis, 1964; Barton, 1965; Lang, 1965). For a long time it has been known that cutting or pricking the seed coats will increase germination. It has been suggested that the testa, pericarp-testa, and pales can inhibit germination by mechanically restricting the growth of the embryo (Ikuma and Thimann, 1963); by interfering with the uptake of oxygen by the embryo (Wareing and Foda, 1957); by carrying an inhibitor (Black and Wareing, 1959); or by preventing leaching of inhibitors (Black, 1959). In leguminous seeds, simple impermeability to water imposes dormancy (Hyde, 1954).

A study of the effects of exogenous substances which break and impose dormancy can indicate the type of reaction which is limiting or the area of metabolism involved in dormancy. It is then possible to postulate the presence of similar substances with a similar role in vivo, and to attempt to isolate and identify them.

Among the earliest compounds known to promote germination were thiourea, potassium nitrate, and hydrogen peroxide. Thiourea can overcome the dormancy of a number of light-requiring and cold-requiring seeds (Villiers and Wareing, 1959). It is one of a group of compounds which includes hydrogen sulphide, mercaptans, etc., owing their activity specifically to the presence of the sulphydryl group and not to any reductive ability (Pollock and Kirsop, 1956). Thiourea appears to stimulate the operation of the tricarboxylic acid cycle, but whether this is a primary effect is not known. The effects of nitrates are reviewed by Stiles (1960b). The promotion of germination in rice by hydrogen peroxide has been ascribed to the oxidation of inhibitory substances (Roberts, 1964a).

Development of the concepts of hormonal control of plant growth has led to the investigation of the role of plant growth regulators in seed dormancy and germination. While auxins have little or no effect on germination (Wareing, 1963), gibberellins and kinins often have dramatic effects. In breaking seed dormancy, gibberellic acid can substitute for light (Kahn 1960; Hashimoto and Yamaki 1962), and cold treatments (Frankland, 1961), and can reverse the effect of high temperature (Toole and Cathey, 1961). The effects of gibberellins

on enzymes of seeds has been reviewed by Brian (1966). The work of MacLeod and Millar (1962), Briggs (1963), and Varner and RamChandra (1964), has led to the conclusion that gibberellic acid can stimulate de novo enzyme synthesis. The latter authors believe that this occurs by derepression of the genes regulating the synthesis of the particular enzyme. In dormant Avena fatua gibberellic acid overcomes a block to the hydrolysis of endosperm reserves by inducing maltase synthesis or reactivation (Simpson and Naylor, 1962), and permits sugar utilisation in the embryo (Naylor and Simpson, 1961). Gibberellic acid also substitutes for amino acids in allowing amylase synthesis in the aleurone of dormant wild oats, but synthesis of other proteins occurs 15 hours before amylase (Naylor, 1966). At the moment, therefore, the primary dormancy-breaking function of gibberellic acid is unknown. Although it can not yet be concluded that the increases in gibberellin-like substances reported by Frankland and Wareing (1962) and Simpson (1965) in after-ripening seeds cause loss of dormancy, the involvement of native gibberellins is highly probable. The recent report of gibberellin production as a wound response (Rappaport and Sachs, 1967) may shed new light on the role of pricking, the classical method of breaking dormancy.

The effects of kinetin on seed germination include increased light sensitisation and reduction of heat induced dormancy. These are reviewed by Koller et al. (1962). Germination of Striga species is strongly promoted by kinetin (Williams, 1961), by other 6-amino purines and by 'strigol', an isolate from a host root exudate, while gibberellic

acid has little effect (Cook et al., 1966). Strangely, coumarin derivatives also break dormancy in this species (Worsham et al., 1962).

Naturally occurring compounds which inhibit germination include ammonia, cyanide, unsaturated hydrocarbons, essential oils, mustard oils, alkaloids, unsaturated lactones and phenolic acids, (Toole et al., 1956).

Until recently, the most powerful inhibitor known was coumarin. The dormancy imposed on lettuce seed by coumarin can be overcome by light (Nutile, 1945) and is effective only above 20° (Berrie, 1968). Coumarin has been shown to inhibit phosphorylation in lettuce seed (Ulitzur and Poljakoff-Mayber, 1963). Its mode of action is unknown.

Keto-aldehydes occur natually and have been implicated as the cancerostatic agent in non-dividing animal cells. Theyappear to owe their activity to combination with sulphydryl groups (Szent-Gyorgyi et al., 1967). A potent inhibitor of germination occurs in oat hulls (Elliott and Leopold, 1953) and is also inhibitory to amylase activity and growth of etiolated pea sections. This inhibitor and the keto-aldehyde type are inactivated by glutathione, which suggests that binding of sulphydryl groups may have a role in dormancy.

As can been seen from the reviews of Evenari (1949), and Wareing (1965), inhibitory substances are considered important in the imposition of seed dormancy, although little is known of their mode of action. The most inhibitory component of the 'β-inhibitor' complex is d-abscisic acid, previously abscisin or dormin. Its ability to accelerate abscission and inhibit growth was first studied by Okhuma et al. (1963), whose

proposed structure was confirmed by Cornforth et al., (1965). Abscisic acid has now been identified in a number of plants, and it has been suggested by Cornforth et al. (1966), that it contributes most of the inhibition found in acid fractions of plants (Hemberg, 1961). Inhibition of germination has been reported in hazel (Bradbeer, 1968), and this is overcome by gibberellic acid, whereas in lettuce, gibberellic acid is ineffective but kinetin can reverse the inhibition (Sankhla and Sankhla, 1968). In vivo, abscisic acid can inhibit the synthesis of α-amylase induced by gibberellic acid (Chrispeels and Varner, 1967).

The positive identification of this naturally occurring inhibitor has considerably strengthened the concept of the state of dormancy being determined by the interaction of inhibitors and stimulators (Naylor and Simpson, 1961; Eagles and Wareing, 1963; Villiers, Frankland and Wareing, 1963; Bradbeer, 1968). From the information now being amassed on abscisic acid, it appears that the role of the inhibitors is as complex as that of the growth promoting substances.

The theory that dormancy is determined by levels of inhibitory and stimulatory substances or interactions of these is also given credence by the wide range of dormancy levels which can exist within a species and between closely related species. Such a situation exists within the genus Avena where Avena sativa L. is virtually non-dormant while Avena fatua L. and Avena ludoviciana Dur. display two distinct types of dormancy. The germination behaviour of A. sativa and the dormancy of A. fatua have already been investigated by Drennan (1960) and by Hart (1966).

The present study was aimed at further characterisation of the particular dormancy displayed by seeds of A. ludoviciana.

The first part of the study consists of observations on responses to given physical and chemical treatments. The second part follows from the observation by Hart (1966) that malic acid has a role in the physiology of dormancy in A. fatua. The importance of malate metabolism with respect to dormancy in A. ludoviciana was investigated. This involved the study of the enzymes and substrates of the malate 'system' in seeds of varying dormancy levels both before and after maturation.

PART II MATERIALS AND METHODS

Seed Material and Germination Conditions

Seed Material

In A. ludoviciana the natural dispersal unit is the whole spikelet consisting of two or three grains (caryopsis and pales). In this study, the unit of caryopsis plus pales (lemma and palea) is referred to as the seed, while the use of the term caryopsis denotes the absence of pales. The Proximal or lower seeds are larger than the Distal seeds and these have been considered separately in experiments. The very small third seeds which occur occasionally have been included in one experiment only.

Stocks of A. ludoviciana were originally obtained from Rothamsted in 1956 and 1957 and have been grown at Glasgow since then. Due to a poor harvest in 1965, a further supply had to be obtained from Rothamsted to allow preliminary experiments to continue. This seed had been harvested in 1954.

According to the classification presented by Thurston (1957), all populations consisted of one variety, namely Avena ludoviciana Durieu var. typica Malzew, which is characterised by very hairy brown pales, long hairs at the callus and scabrid awns.

Standard Germination Procedure

Standard germination tests were performed as follows:

(a) Three replicates of 25 or 50 seeds in 9 cm. petri dishes on Whatman Seed Test Paper (9 cm. x 0.4 mm.) plus 4 ml. (25 seeds) or 5 ml. (50 seeds) deionised water. Petri dishes were placed in light-tight cans.

(b) Five replicates of 20 seeds in 4.5 cm. petri dishes on Seed Test Paper cut to 1.5" squares plus 1.5 ml. deionised water. Petri dishes were placed in light-tight metal biscuit boxes.

Seeds were exposed to the stated temperature ± 1°C in thermostatically controlled incubators at 7, 10, 15, 20, 25 and 30° and in a deep freeze at -20°. A cold room at approximately 4° and a constant temperature room at 26° were also used on occasion.

Germination was counted as having occurred when the radicle was first observed. Such seeds were removed from each dish. Counting of dark-treated seeds was carried out under a green safelight containing two fluorescent tubes (WWX, 8w.) covered with green gelatin (Withrow & Price, 1957) previously tested for lack of physiological effect on Grand Rapids lettuce and wild oat seeds. Unless otherwise stated, figures refer to percentage germination after 14 days. Seeds remaining ungerminated after this time were pricked with a needle. This treatment caused almost all to germinate in a few days. Germination is then given as a percentage of viable seeds. Except in one case, which is detailed, there was no essential difference between this figure and germination as a percentage of total seeds.

Since the degree of dormancy in wild oat populations varies with the position of the seed in the panicle (Johnson, 1935), the stage of

ripening (Drennan, 1960) and with particular genotypes (Imam and Allard, 1965), allowance has been made for this by repetition of experiments and statistical treatment of results. The results reported refer to germination in a representative experiment.

The statistical significance of the differences between treatments was determined either by a contingency chi-squared test or an analysis of variance, the latter also indicating the reliability of replicates and the significance of interactions. For the chi-squared test, numbers of germinated and ungerminated seeds in the totalled replicates were used, while the analysis of variance required the angular transformations of the individual germination percentages.

Seeds were irradiated by an incandescent bulb (6v. 15w.), 30 cm. from the seed, and after passing through the petri dish lid, giving radiation at a value of 1.1×10^{-5} cal./cm.^{2}/sec. at seed level. Alternatively, a unit of two fluorescent tubes (8w.) was employed giving 5.5×10^{-6} cal./cm.^{2}/sec. at seed level. The radiant energy was measured by a Kipp and Zonen compensated thermopile.

Studies on Enzymes and Soluble Proteins

Extraction Procedures

Slightly more than 1g. of caryopses were weighed and counted, powdered in a Moulinex grinder, and exactly 1g. of material used. The equivalent number of caryopses used was calculated. In germination experiments, replicates of 50 proximal seeds were weighed at the beginning. 1g. of powder or 50 wet seeds were ground in a mortar with 3 ml. 0.02M Tris-HCl buffer pH 7.1 0.4M in sucrose in a cold room at 5°C. The homogenate was centrifuged at 500 g for five minutes, the pellet was removed, re-ground in 2 ml. buffer and re-centrifuged. The 500 g supernatants were combined. An aliquot of this was designated Total fraction. The remainder was spun at 10,000 g for 15 minutes giving a pellet designated Mitochondrial fraction and a supernatant - the Soluble fraction. The mitochondrial pellet was mixed with 2 ml. buffer. The Total and Mitochondrial fractions were then frozen twice in liquid nitrogen and all fractions were spun at 190,000 g for 15 minutes in an MSE Superspeed 50 centrifuge. The supernatants were used for spectrophotometry.

Although the above extracts could be used for electrophoresis, a ratio of 2 ml. buffer per g. of dry seed was found to be superior, while the final centrifugation was increased to 1 hour at 190,000 g.

Protein Determination

Protein was assayed spectrophotometrically with a Unicam SP 800

using the absorbance at 280 and 260 mμ.

$$\text{Protein (mg/ml)} = (1.45 \times A_{280}) - (0.74 \times A_{260})$$

This method was found to be sufficiently accurate for electrophoresis where 250-500 μg. of protein per tube is required, and after centrifugation at 190,000 g the results were sufficiently reproduceable for the calculation of specific activities.

Spectrophotometry

NAD-Malate Dehydrogenase, E.C. 1.1.1.37, activity was determined by a modification of the method of Wolfe and Neilands (1956). The oxidation of malate was followed by measuring the reduction of NAD at 340 mμ. using a Unicam SP 800 recording spectrophotometer with the cell compartment held at 25°C by connection to a water bath. The presence of a double beam system allows automatic subtraction of a control value (minus substrate). The reaction mixture contained the following in quartz cuvettes of 0.5 cm. optical path length.

Experimental Cuvette	Control Cuvette
0.1 ml. NAD (10 mg/ml)	0.1 ml. NAD
0.55 ml. buffer *	0.65 ml. buffer
0.1 ml. M L Malate	-
0.9 ml. buffer (containing 0.025 ml. enzyme)	0.9 ml. buffer and enzyme

* 0.55 ml. 0.1M NaOH - glycine NaCl buffer pH 10.3

To ensure equal volumes of enzyme solution, an aliquot of enzyme was mixed with buffer and a larger volume of this was added to each

cuvette which was then stoppered and shaken.

The rate of reaction was determined from the tangent of the angle formed by the region of linear increase in absorbance at 340 mμ. To obtain a measurable linear increase, a concentration of enzyme was desired which would not reduce the NAD too rapidly. The molar extinction coefficient of $NADH_2$ is 6220, so that an increase of 1.0 in E_{340} mμ. corresponds to 0.48 μ. mole of malate oxidised (Pierpoint 1963). Results are expressed as μ. mole malate oxidised/min/g. seed or /min/ mg. protein (Specific Activity).

The assay of NADP - malate dehydrogenase (malic enzyme) E.C. 1.1.1.40 was attempted using the method of Walker (1960) which is essentially similar to the above except that the pH is 7.4 and $MnCl_2$ is required.

Polyacrylamide Gel Electrophoresis

Electrophoresis of the soluble proteins of Avena ludoviciana was carried out in polyacrylamide gel following the method of Davis (1964) and Ornstein (1964), in an apparatus supplied by Shandon Limited, having provision for 8 gels. 7.5% acrylamide was used as the small pore solution and was evacuated for a few seconds before being placed in the Precibor tubes (diam. 5 mm.). Evacuation was necessary to prevent bubbles forming during gelation at 30°C. The large pore gel was photo-polymerised, either by sunlight or by 2, 8-watt fluorescent tubes arranged in a special housing. Water overlayers were applied to the gel surfaces to prevent meniscus formation. This was done with a very finely drawn out Pasteur pipette so that no mixing of the layers occurred.

Protein solutions were applied from a 0.1 ml. pipette, directly on to the large pore gel, and not in large pore solution as this had been found to give inferior results. The inclusion of 0.4M sucrose in the extraction medium allowed the introduction of the reservoir buffer solution to the remainder of the tube without any mixing with the protein solution. When the 8 tubes were inserted in the upper cathodic reservoir and the latter filled with buffer, no mixing with the protein layer occurred. Tris-Glycine buffer pH 8.3 was used in both reservoirs.

The 8 tubes were subjected to 32mAmp and 160V at 5°C in a cold room for 35 minutes. This was generally sufficient time to allow the front to progress about 3 cm. down the small pore gel which was found to result in the optimum balance between separation and diffusion of the bands. The electrophoretic front was marked by including some bromo-phenol blue in the upper reservoir, but this was unnecessary as an obvious interface formed as a matter of course. Gels were removed from the running tubes using syringes fitted with fine needles and filled with reservoir buffer.

Visualisation of Enzymes and Proteins on Gels

Protein was detected by standing the gels in a 1% solution of Amido Black in 7% acetic acid for a period in excess of 1 hour. The acetic acid fixes the protein in situ while the dye forms a dark blue complex with the protein. The gel is then washed repeatedly in 7% acetic acid which gradually removes the dye from the areas of gel without protein. Electrophoretic destaining was found to alter the gel characteristics

and was not used.

Methods of staining for NAD-malate dehydrogenase were developed from the principles given in Pearse (1960). These methods were later found to be very similar to those used by Fottrell (1966) for starch gel electrophoresis. Gels were incubated in 25 ml. Erlenmeyer flasks in a small volume of the following reaction mixture. The flasks were sealed with Parafilm and shaken in a shaking incubator. This permitted the use of very small volumes (2.8 ml. per gel) of reaction mixture and largely prevented the generalised staining caused by diffusion of the enzymes on to the surface of the gel.

Reaction mixture. NAD-malate dehydrogenase. E.C. 1.1.1.37.

for 8 gels	3.6 ml. M L malate (Sigma)
	4.0 ml. Nitro-Blue Tetrazolium (1 mg/ml) (Curr)
	18.8 ml. 0.2 M Tris-HCl buffer pH 8.0
	16 mg. NAD (Sigma)
	6.4 mg. Phenazine methosulphate (Sigma)

The intensity of staining was generally sufficient in 15-30 minutes depending upon the temperature. The gels were then fixed and stored in 7% Acetic acid to prevent overstaining. It is important to recognise that due to the variety of factors which can alter during the preparation of the gels, during electrophoresis and staining, quantitative differences in staining can be attributed to differences in enzyme activity only within a group of 8 gels.

An attempt to locate NADP-malate dehydrogenase on polyacrylamide gel used the method of Henderson (1966) for starch gels.

Reaction mixture. NADP-malate dehydrogenase E.C. 1.1.1.40

8.0 ml. M L Malate

5.0 ml. NBT (1 mg/ml)

5.0 ml. PMS (1.6 mg/ml)

1.0 ml. NADP (10 mg/ml)

0.2 ml. 0.25M $MnCl_2$

45.0 ml. 0.2 M Tris-HCl buffer pH 8.0

Gels were examined, measured and densitometer traces prepared using a Joyce Loebel Chromoscan Densitometer. A red filter of maximum transmittance at 660 mμ. was used to scan soluble proteins and to scan the gels stained for dehydrogenase activity.

Studies on Organic Acid Levels

Extraction Procedures

Air dry seed were weighed, counted and then powdered in a Moulinex grinder as for enzyme extractions. 1 or 2g. of powder was extracted three times (1 hr., 5 hrs. and 18 hrs. duration) with 80% ethanol (100 ml.) at 75° (water bath) in covered polythene centrifuge tubes. Following low speed centrifugation the supernatant was decanted, combined and reduced in volume by heating on a steam bath. The remaining aqueous solution was then filtered.

Amino acids and other basic materials were removed by passage through Zeocarb 225 cation exchange resin in the H-form (15 x 0.8 cm. columns). The acidic fraction was absorbed on Amberlite IR-4B anion exchange resin in the OH form (12 x 0.8 cm. columns). After washing with deionised water to remove sugars and other neutral substances, the acids were eluted from the anion exchanger with 2N ammonium hydroxide. The free acids were liberated from the ammonium salts by passage through a second Zeocarb column and elution by 0.1N HCl. Alternatively the final ion exchange step was omitted and the ammonium salts themselves were used. (See Appendix III). Total volumes of eluate were measured and a fraction used for thin layer or gas-liquid chromatography. This is essentially the method of Ranson (1955).

Gas-liquid Chromatography

Solutions of acids or ammonium salts were evaporated to dryness on

a steam bath under a stream of nitrogen for formation of derivatives for gas-liquid chromatography.

Formation of trimethylsilyl esters and trimethylsilyl ethers. The dry material was dissolved in pyridine (0.25 ml.), and hexamethyldisilazine [Koch-Light] (0.15 ml.) and trimethylchlorosilane [Hopkin and Williams] (0.05 ml.) were added. The reaction was allowed to proceed overnight at room temperature. (See Appendix Ia). Alternatively, the same products were formed by the addition of bis-trimethylsilyl acetamide [Pierce Chemical Co.] (0.2 ml.) to the dry acids, and latterly this procedure was adopted.

Formation of methyl esters and trimethylsilyl ethers. Methyl esters were prepared by the addition of freshly prepared ethereal diazomethane solution (1-2 ml.) to the dry acids and immediately evaporating the ether on a steam bath under nitrogen. This procedure was repeated to ensure complete esterification. Hydroxyl groups on the methyl esters were then treated by either of the above methods to form trimethylsilyl ethers (See Appendix Ib). These methods have been described by Dalgliesh et al. (1966).

Formation of O-methyloxime derivatives. O-methyloxime derivatives of acids containing aldehyde or keto groups were formed after heating for 1 hour in a small volume of pyridine containing methoxyamine hydrochloride [Eastman Kodak] (3 mg.). The solution was then evaporated to dryness under nitrogen and silylated as described above. (See Appendix Ic).

Gas chromatography was carried out on a Pye 104 or 105 chromato-

graph using 9 ft. glass columns packed with either 1 or 5% SE-30 coated on 100-120 mesh acid washed and silanised Gas Chrom P prepared according to the method of Horning et al. (1963). The instruments were temperature programmed at 3°/min. from 75°-200°. Carrier gas (nitrogen) inlet pressure was 30 p.s.i. The signal produced by the ionisation of material passing the hydrogen flame was recorded on a Honeywell recorder with a chart speed of 20"/hr. Volumes in the range of 1-5 µl. were injected using a Hamilton micro-syringe.

Quantitative estimates of the trimethylsilyl (TMS) derivatives were made by measuring the area of the peak produced. This area is proportional to the amount of material present, subject to the conditions and using the methods detailed in Appendix II.

Methylene Unit Valves. These values are an aid to identification and are used to designate unidentified peaks appearing on the chromatogram (Dalgliesh et al., 1966). They are a measure of the relative retention on the column compared to the reference compounds consisting of the homologous series of straight chain hydrocarbons. These compounds emerge at time intervals which are approximately equal during linear temperature programming. The peaks produced by the hydrocarbons are given a value corresponding to the number of methylene units present, and the value for any peak lying between two hydrocarbon peaks can be derived by interpolation. These values are equivalent to retention indices (Wehrli and Kovats, 1959; Ettre, 1964).

Mass Spectrometry. The instrument used for combined gas-liquid chromatography-mass spectrometry was an L.K.B. 9000 installed in the University of Glasgow, Chemistry Department. Analyses were carried out using a

1% SE-30 column, temperature programmed at 3°/minute for plant acids and run isothermally at 124° for standard acids. The molecular separator was run at 250°, the ion source at 270°, and a scan voltage of 70eV was used. Line diagrams were drawn with the largest intensity peak being given a value of 100, and other peaks ascribed proportionate values. These are presented in Appendix IV.

Thin Layer Chromatography

Following reduction to suitable volumes, samples were chromatographed along with known standards on silica gel G. (0.2 mm. thickness). Plates were developed in either:

(i) 96% ethanol : water : 20% ammonia solution (100:12:16) (Ganshirt et al. 1965), or

(ii) n-butanol : formic acid : water (120:20:20) (Wall et al., 1961),

and acids were located by spraying with bromocreosol green.

For preparative chromatography, thicker plates (1 mm.) were used and after development, acids were eluted from the silica with 80% ethanol, following location by spraying a 1 cm. vertical strip along each edge with bromocreosol green.

Studies with $^{14}CO_2$

Two Quickfit flasks (100 ml.) were connected with right-angle adaptors and rubber tubing (total volume 280 ml.) Seeds of A. ludoviciana were placed in one flask and 0.0123g. $BaCO_3$ in the other. Neutralisation of the $BaCO_3$ with 0.2 ml. N HCl. gave an atmosphere

containing 0.5% CO_2. Since the radioactivity of the carbonate as Ba $^{14}CO_3$ was 0.0062 mc./mg. the atmosphere in an experimental flask contained 0.0382 mc. as $^{14}CO_2$.

Twenty-four hours later the generator flasks were removed after sealing the experimental flasks. The latter were then stored in the dark at various temperatures for periods of four and six months.

Radioactivity in the total ethanol-extractable material and acidic, basic and neutral fractions was determined on an I.D.L. low beta counter. Fractions were obtained by the ion exchange methods previously described and samples were evaporated on aluminium planchettes (dia. 2.5 cm.).

PART III. THE GERMINATION BEHAVIOUR OF AVENA LUDOVICIANA

Introduction

The first part of this thesis records attempts to obtain more detailed information on the germination behaviour of *Avena ludoviciana* than has previously been reported.

The initial section permits a general assessment of the relative importance of various physical factors in determining the germination response, while the other two sections provide information on the effect of high temperature and gibberellin A_3. In all, the purpose of the information is to serve as a basis for the study of the metabolism of dormancy and germination.

As used in the experimental sections of this work, the term 'dormant' refers to the seed population, and the level of dormancy in the population is described by the percentage germination in a given environment. Changes in dormancy due to different treatments are reflected in changes in the composition of the population in respect of dormancy.

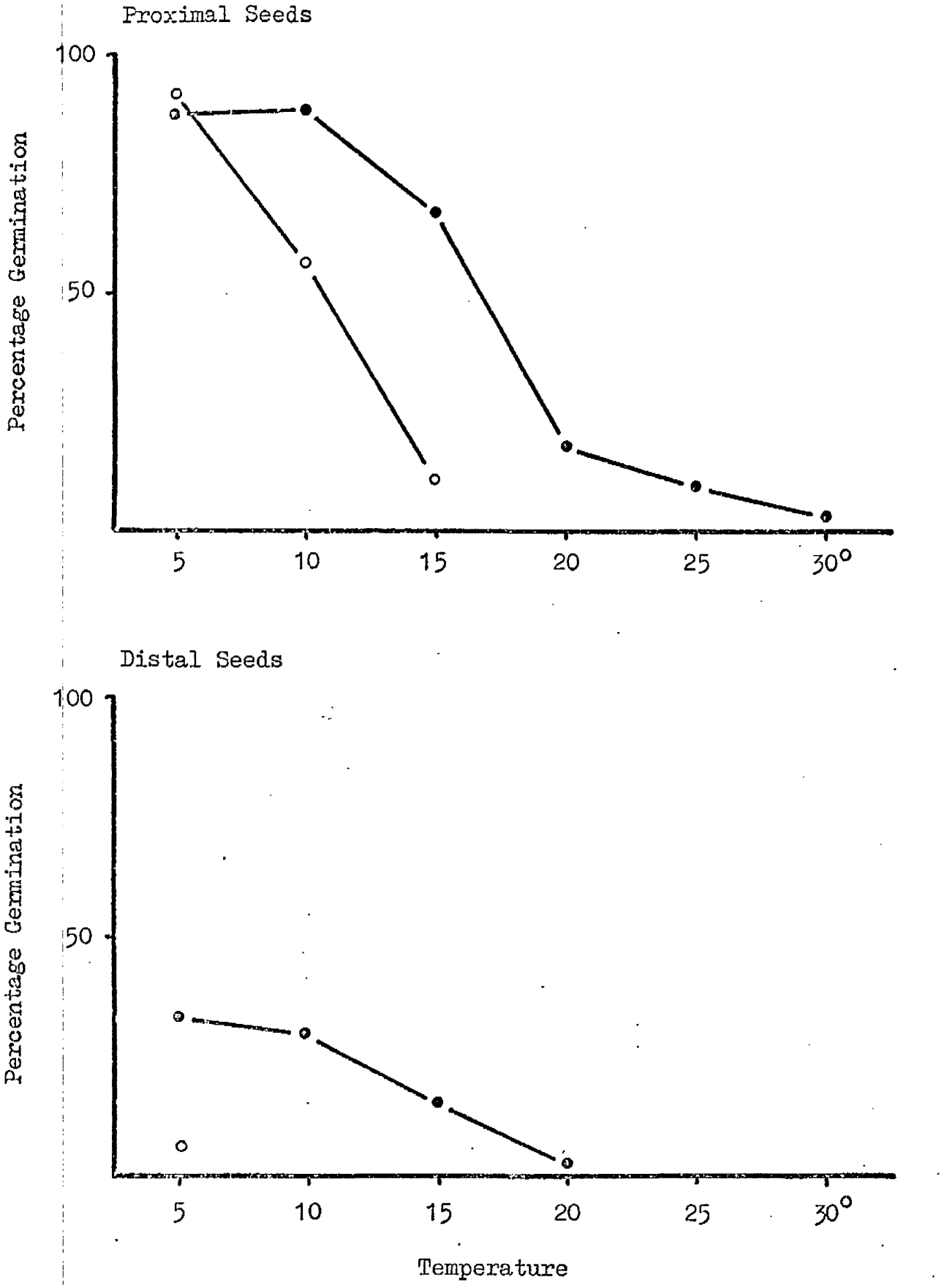


Figure 1. Germination at a range of temperatures after storage for different periods.

Section I. A Preliminary Survey of Germination Behaviour

Results

Experiment 1. The effect of temperature on germination in two seed samples

A batch of seed was stored at room temperature and samples were removed after 2 and 14 months. These were set to germinate according to Standard Procedure (b), at a range of temperatures. Germination patterns are illustrated in Figure 1 and germination percentages after 18 days are reported in Table 1. It is evident that both the deeply dormant distal seeds and the less dormant proximal seeds are progressively inhibited by higher temperatures, especially during the initial period of after-ripening.

Table 1.

Final percentage germination at a range of temperatures

Seed type	Storage period	Temperature					
		5	10	15	20	25	30
Proximal	2 months	90	56	10	0	0	0
	14 months	87	88	67	17	10	3
Distal	2 months	5	0	0	0	0	0
	14 months	34	30	15	2	0	0

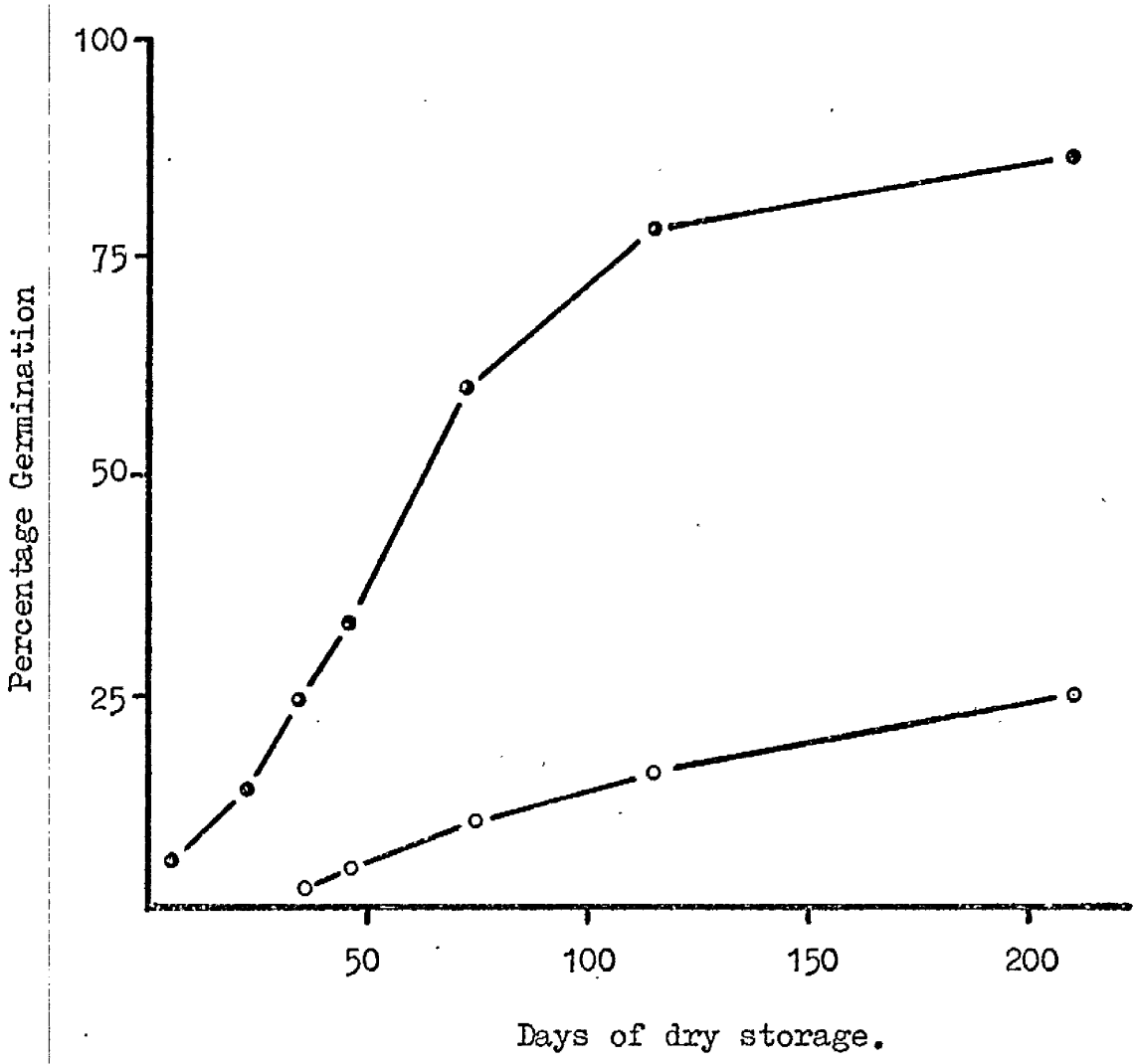


Figure 2. Percentage germination of Proximal and Distal Seeds after different periods of dry storage.

Experiment 2. Loss of dormancy during dry storage at room temperature

A batch of seeds was stored at room temperature for different periods before being tested under standard germination procedure (a) at 10°. The percentage germination after various periods is shown in Table 2 and illustrated in Figure 2.

Table 2.

Germination at 10° after different periods of dry storage at room temperature

Seed type	Days of Storage						
	5	22	34	45	73	116	210
Proximal	5	13	24	32	60	78	86
Distal	0	0	1	4	10	15	24

Experiment 3. The effect of removal of the pales on germination at 10°

Seeds after-ripened for 1 year at room temperature were set to germinate under standard procedure (b) at 10°. Percentage germination of proximal, distal and third seeds or caryopses is shown in Table 3. It is seen that the pales exert an inhibitory effect on germination. In other experiments, the effect of the proximal pales was significant, but was always slight in comparison to the effect of the distal pales.

Table 3.

Germination of the different seed types from the spikelet of A. ludoviciana as whole seed or caryopses

Seed type	Condition		χ^2 (1df) C v. S
	Caryopses	Seeds	
Proximal	97	89	3.76
Distal	76	23	54.08 **
Third seed	14	2	8.22 **

Experiment 4. The effect of a pricking treatment on whole seed and caryopses

Seeds after-ripened for 1 year were germinated under standard procedure (b) at 10°. Proximal and distal seeds and caryopses were pricked with a mounted needle on the dorsal surface, above the embryo, prior to imbibition. Increases in germination percentages due to pricking are seen in Table 4.

Table 4.

Germination of whole seed or caryopses after a pricking treatment

Seed Type	Caryopses		χ^2 (1df) I v. P	Seed		χ^2 (1df) I v. P
	Intact	Pricked		Intact	Pricked	
Proximal	97	99	0.25	89	100	9.62 **
Distal	76	92	8.37 **	23	81	65.08 **

Table 5b.

Analysis of variance in the populations in Table 5a.

Source	D.F.	Sum of Squares	Mean Square	Variance Ratio
Replicates	3	6.124	2.041	0.043
Incand. v. Fluor. (I)	1	2,665.270	2,665.270	57.307 **
Light v. Dark (L)	1	9,314.421	9,314.421	200.275 **
Proximal v. Distal (P)	1	9,852.796	9,852.796	211.851 **
Seeds v. Caryopses (S)	1	225.187	225.187	4.841 *
I x L	1	1,297.711	1,297.711	27.902 **
I x P	1	0.329	0.329	0.007
I x S	1	232.830	232.830	5.006 *
L x P	1	1,696.719	1,696.719	36.482 **
L x S	1	1,251.834	1,251.834	26.916 **
P x S	1	2,427.903	2,427.903	52.204 **
I x L x P	1	32.299	32.299	0.694
I x L x S	1	624.302	624.302	13.423 **
I x P x S	1	105.700	105.700	2.272
L x P x S	1	287.675	287.675	6.185 *
I x L x P x S	1	186.248	186.248	4.004
Residual	42	1,953.346	46.508	
Total	63	32,160.676		

Experiment 5. The effect of light on germination

Proximal and distal seeds and caryopses, after-ripened for 8 months, were germinated under standard procedure (a) at 10° in light or dark. In the first part of the experiment, seed material was irradiated with incandescent light (1.1×10^{-5} cal/cm^2/sec. Red : far-red ratio 1 : 1.17). After 14 days, the first experiment was terminated and a second experiment set up in the same incubator with seed from the same sample but irradiated with fluorescent light, (5.5×10^{-6} cal/cm^2/sec. Red : far-red ratio 1 : 0.60).

The germination percentages from these experiments are recorded in Table 5a. Analyses of variance were performed on the combined data (Table 5b) and on the separate parts of the experiment (Table 5c,5d). It is evident that light inhibits germination in this species. In seed after-ripened for longer periods, less inhibition resulted, but incandescent light remained more effective.

Table 5a.

The germination of seeds and caryopses in light and dark

Light Source	Seed Type	Condition	Light	Dark	Total
Incandescent	Proximal	Caryopses	17	92	334
		Seeds	33	90	
	Distal	Caryopses	20	58	
		Seeds	2	22	
Fluorescent	Proximal	Caryopses	34	97	470
		Seeds	86	88	
	Distal	Caryopses	38	61	
		Seeds	34	32	
Total Germination			264	540	

Table 5c.

Analysis of variance in the populations under incandescent light in Table 5a.

Source	D.F.	Sum of Squares	Mean Square	Variance Ratio
Replicates	3	20.543	6.847	0.139
Light v. Dark (L)	1	8,782.763	8,782.763	178.634 **
Proximal v. Distal (P)	1	4,983.513	4,983.513	101.360 **
Seeds v. Caryopses (S)	1	457.985	457.985	9.315 **
L x P	1	1,098.633	1,098.633	22.345 **
L x S	1	54.028	54.028	1.098
P x S	1	1,773.399	1,773.399	36.069 **
L x P x S	1	5.495	5.495	0.111
Residual	21	1,032.494	49.166	
Total	31	18,208.853		

Table 5d.

Analysis of variance in the populations under fluorescent light in Table 5a.

Source	D.F.	Sum of Squares	Mean Square	Variance Ratio
Replicates	3	33.214	11.071	0.266
Light v. Dark (L)	1	1,829.368	1,829.368	43.995 **
Proximal v. Distal (P)	1	4,869.611	4,869.611	117.111
Seeds v. Caryopses (S)	1	0.032	0.032	0.001
L x P	1	630.392	630.392	15.160
L x S	1	1,822.118	1,822.118	43.820 **
P x S	1	760.207	760.207	18.282 **
L x P x S	1	468.410	468.410	11.265 **
Residual	21	873.220	41.581	
Total	31	11,286.572		

Discussion

Populations of Avena ludoviciana are composed of three sub-populations on morphological and physiological grounds. The fact that distal seeds are more dormant than proximal seeds (Experiments 1 - 5) has already been reported by Thurston (1957). It is now seen that the third seed has an even deeper dormancy than the distal (Experiment 3), indicating that a considerable gradient of dormancy exists within the spikelet. This necessitates separate consideration for the different seed types. Differences between first and second have also been reported in A. fatua (Hart, 1966) and in Xanthium pennsylvanicum (Shull, 1911; Wareing and Foda, 1957).

Dormant seeds of A. ludoviciana respond to temperature in the manner typical of freshly harvested cereal seeds (Atterberg, 1907; Harrington, 1923) in that germination occurs readily only at low temperatures (Experiment 1). As a result of dry storage, the ability to germinate at higher temperatures increases and the temperature optimum rises (Experiments 1,2). However, the range for germination in this species remains unusually low (Thurston, 1962), and is distinct from that of A. fatua, where maximum germination in after-ripened samples occurs at 21° (Friesen and Shebeski, 1960), and 80% germination can be obtained at 30° (Hart, 1966).

Breaking of dormancy by removal of husks or pales has been demonstrated in A. fatua (Atwood, 1914; Kommedhal et al., 1958; Black 1959), in barley (Pollock, 1959) and in rice (Roberts, 1961b).

In common with these results, increases in germination of

A. ludoviciana follow removal of the pales (Experiment 3), but the relative contribution to dormancy made by the pales differs for the three seed types. Black (1959) speculated that the pales prevent leaching of inhibitors from the caryopses of A. fatua but was unable to demonstrate differences in levels of inhibitors in dormant and non-dormant seeds.

A. ludoviciana will germinate more fully following a pricking treatment (Experiment 4). This effect was also noted by Atwood (1914) for A. fatua and Hay (1962) has postulated that increased entry of air or exit of a volatile inhibitor is responsible. However, Hart (1966) found that covering the pricked area with lanolin could not prevent some stimulation. This increases the possibility of a stimulation resulting from the pricking itself, and in this context the wound induced gibberellins demonstrated in potato tubers by Rappaport and Sachs (1967) may have importance.

Light inhibits germination of A. ludoviciana (Experiment 5). Incandescent light appears more inhibitory than fluorescent light, but the energy differences do not permit excessive confidence in this result. White light inhibited germination in partially dormant A. fatua (Cumming and Hay, 1958) and inhibition was attributed to the blue and infra-red regions of the spectrum. Red light had no effect. Similar inhibitions by blue and far-red occur in dormant Hordeum spontaneum (Burger, 1965), but white, green yellow or red light were stimulatory in this case. Hart (1966) found that light was stimulatory in A. fatua at high temperatures, but inhibitory at low temperature. No such stimulation was

ever noticed in A. ludoviciana. Light inhibits germination of caryopses of A. ludoviciana more than whole seeds when compared to germination in the dark, and a marked ability of the pales to reduce the light inhibition on the caryopses is evident for proximal seeds. This is in contrast to A. fatua, where removal of the pales led to increased light stimulation in some samples (Hart, 1966).

The results of other experiments involving light indicated (a) that no photoperiodic effect existed, short days being inhibitory, and long days more inhibitory, and (b) that inhibition required considerable periods of illumination.

No conclusions can be reached concerning the light reactions involved in the inhibition, but the long periods of illumination required eliminate the phytochrome system as it is normally envisaged.

Conclusions from Section I

(1) The population of A. ludoviciana is composed of three seed types, each having different dormancy characteristics.

(2) The caryopses are covered by pales which inhibit germination.

(3) Dormancy levels are high immediately after harvest, and the optimum temperature for germination is very low.

(4) Dormancy is lost in dry storage, but the optimum temperature for germination remains about 10°, and high temperatures prevent germination.

(5) Pricking breaks dormancy and overcomes the inhibitory effects of the pales.

(6) Light inhibits germination, but this can be reduced by the presence of the pales.

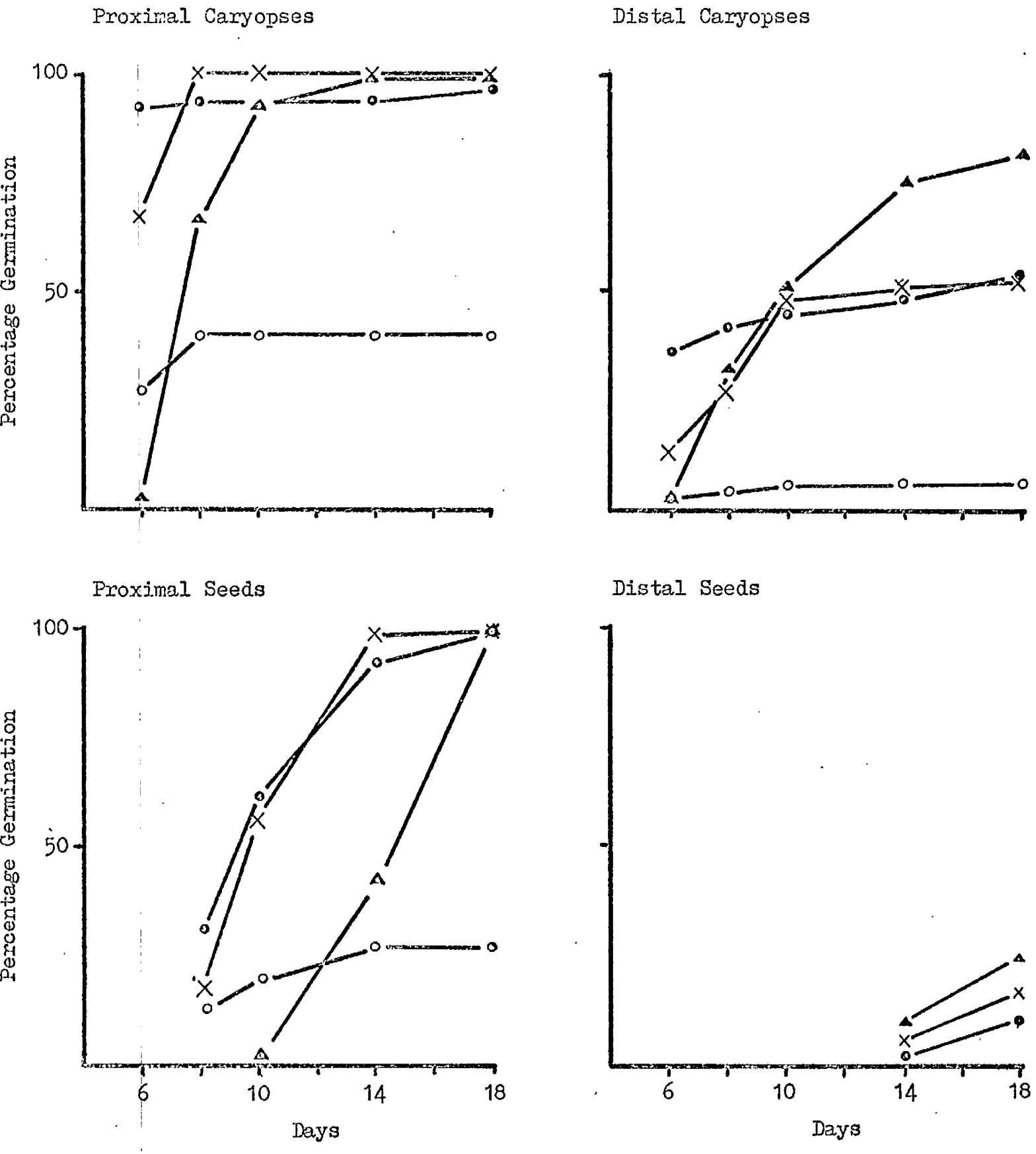


Figure 3. Germination of Proximal and Distal Seeds and Caryopses after high temperature pre-treatments.

△ = 10° o = 30° (1 day) → 10° × = 30° (3 days) → 10° o = 30°

Section II. High Temperature Effects on Germination

Results

Experiment 6. The effect of imbibition at high temperature on subsequent germination at low temperature

Proximal and distal seeds and caryopses after-ripened for 11 years were germinated according to Standard Procedure (a), in the following temperature regimes:-

(a) 10° continuously

(b) 30° (1 day) followed by 10°

(c) 30° (3 days) followed by 10°

(d) 30° continuously

Germination percentages after 18 days in a typical trial are recorded in Table 6 and illustrated in Figure 3. High temperature pre-treatments permit a more rapid achievement of complete germination in proximals but reduce final germination in distals.

Table 6.

Germination at 10° following periods at 30°

Seed type	Seed Condition	Period at 30°				χ^2 (1df)	
		0	1 day	3 days	Continuous	0 v. 1	0 v. 3
Proximal	Caryopses	99	96	100	40		
	Seeds	99	99	100	27		
Distal	Caryopses	81	54	52	6	12.12 **	13.23 **
	Seeds	22	10	16	0	4.01 *	0.68

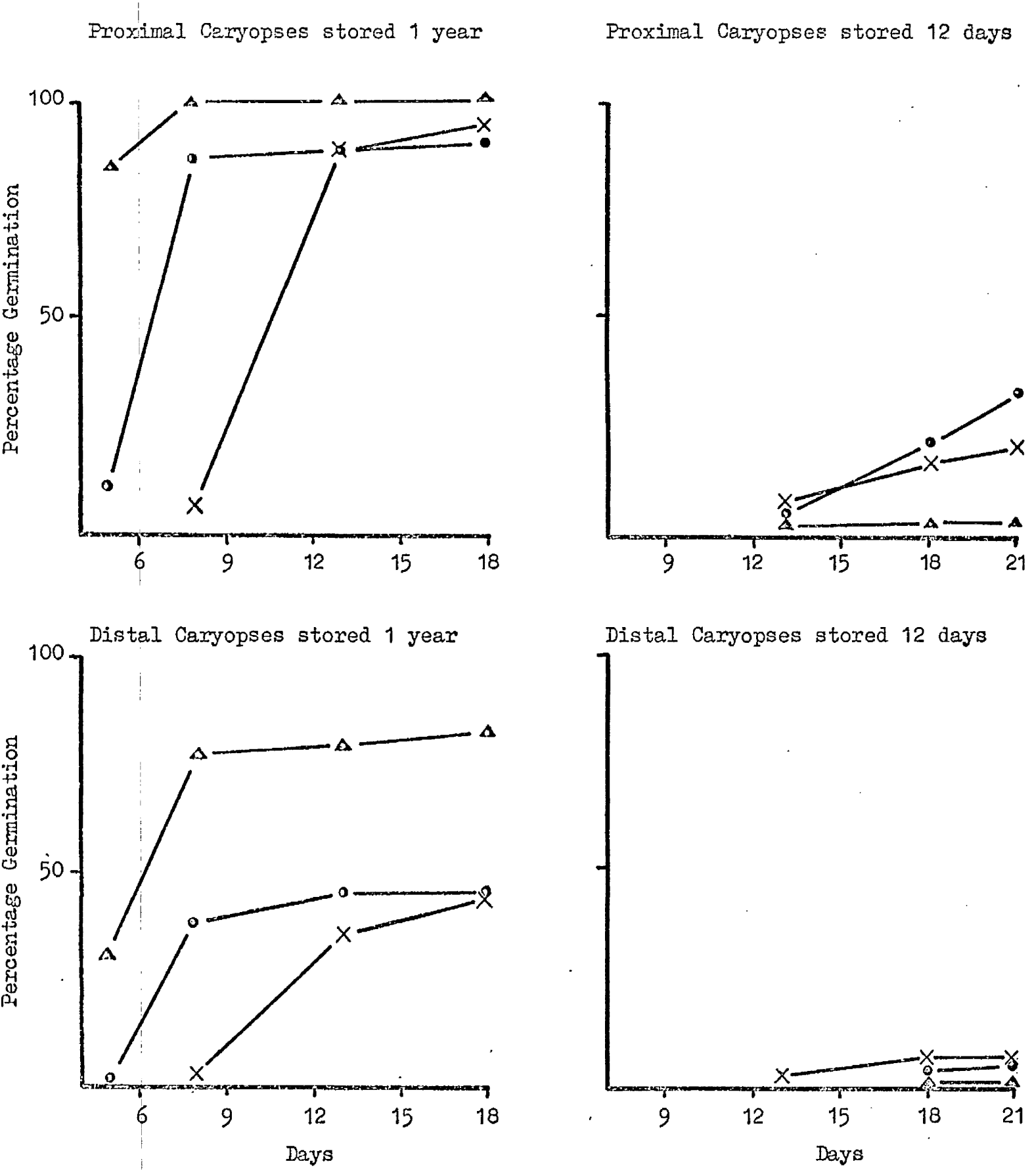


Figure 4. Germination of after-ripened and non after-ripened caryopses following high temperature pre-treatments.

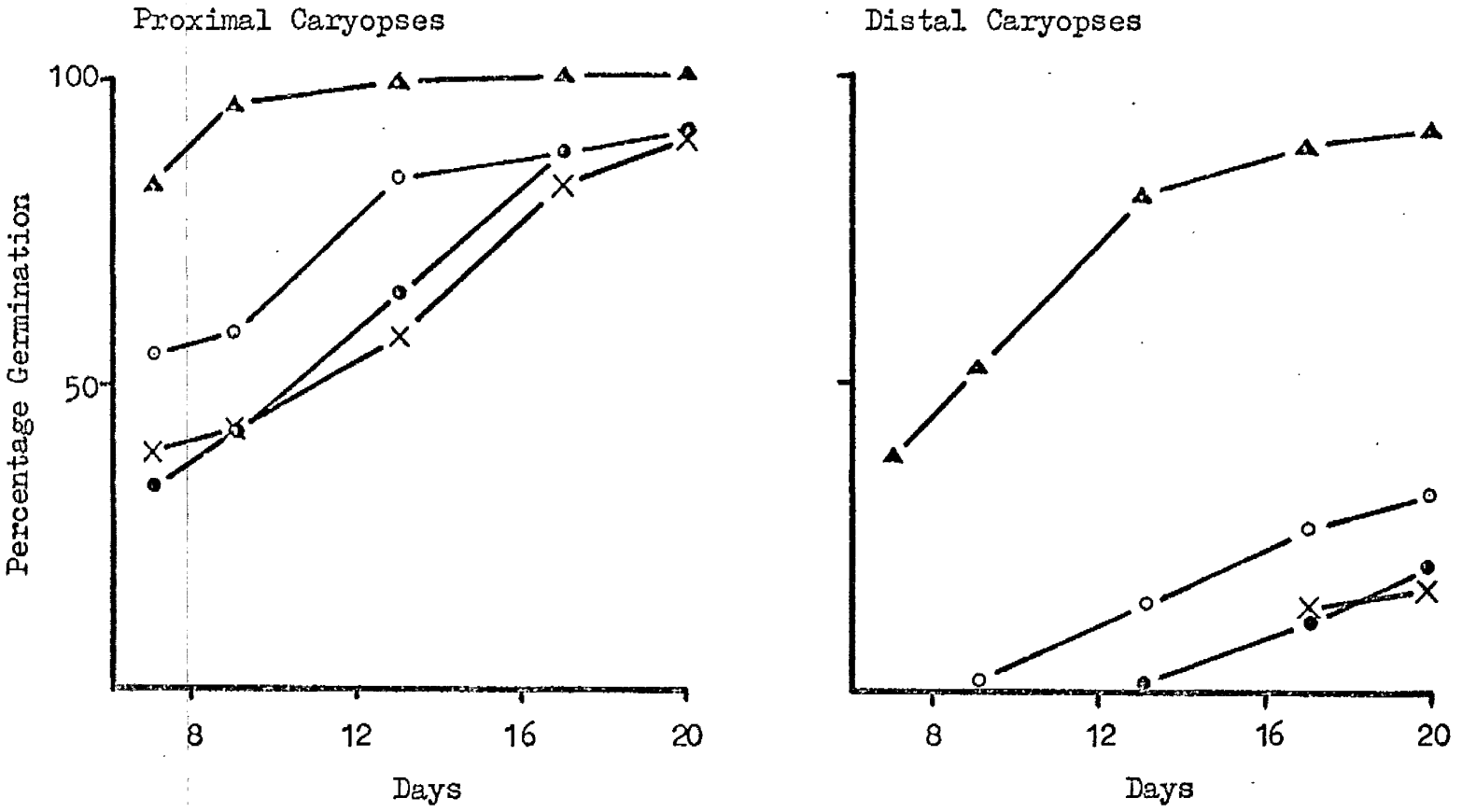
Experiment 7. The effect of after-ripening on the response to high temperature pre-treatments

Caryopses of seed which had been stored for 12 days or 1 year at room temperature were germinated under Standard Procedure (b) at 10° following pre-treatment at 30° for 1 or 3 days. The germination percentages recorded at intervals (Figure 4) and final germination at 18 days presented in Table 7 indicate that high temperature pre-treatments may inhibit or promote germination depending on the stage of after-ripening.

Table 7.

Germination of after-ripened and non-after-ripened caryopses at 10° following pre-treatments at 30°

Seed type	Storage period	Days at 30°			χ^2 (1df)	
		0	1	3	0 v. 1	0 v. 3
Proximal	12 days	3	22	16	14.81 **	8.37 **
	1 year	100	89	95	9.62 **	3.28
Distal	12 days	1	3	5	0.25	1.54
	1 year	81	44	42	27.64 **	30.49 **



△ = 10° continuously

○ = 10° except day 1 (30°)

× = 10° except day 2 (30°)

○ = 10° except day 3 (30°)

Figure 5. Percentage germination at 10° after 30° treatments at different stages of imbibition.

Experiment 8. The effect of a high temperature treatment at different stages of imbibition

Proximal and distal caryopses from seed stored at room temperature for six months were germinated under Standard Procedure (a) at 10°, with a 24 hour period at 30° interjected at 0, 24 and 48 hours. Germination patterns, and final percentage germination after 20 days are shown in Figure 5 and Table 8. High temperature treatments reduce final germination in all cases in this seed sample, and in distal caryopses the reduction is more severe if exposure occurs on day two rather than day three.

Table 8.

Germination percentages of proximal and distal caryopses at 10° following 30° treatments at various times during imbibition at 10°

Caryopsis type	30° Treatment				χ^2 (1df)		
	-	Day 1	Day 2	Day 3	- v. 1	1 v. 3	2 v. 3
Proximal	100	91	89	89	5.39*	-	-
Distal	91	20	16	33	72.09**	2.21	4.42*

Experiment 9. The ability of a pricking treatment to overcome the high temperature inhibition of germination

Proximal and distal seeds and caryopses after-ripened at room temperature for one year were germinated under Standard Procedure (b) at 30° following pricking with a mounted needle. In Table 9 it is seen that the pricking treatment overcomes the inhibitory effect of high temperature.

There were no significant differences between seeds and caryopses in any treatment at this temperature.

Table 9.

Germination of proximal and distal seeds and caryopses following a pricking treatment

Seed type	Seed condition	Treatment	
		Intact	Pricked
Proximal	Seeds	6	90
	Caryopses	15	95
Distal	Seeds	0	73
	Caryopses	4	82

Discussion

Acceleration of germination by high temperature pre-treatments has previously been reported in A. ludoviciana (Thurston, 1962). Similar results were obtained with an 11 year old sample (Experiment 6), but germination was delayed in 1 year old material. This delay was observed repeatedly when other after-ripened material was investigated.

In addition to the alteration in the time of germination, high temperature treatments also affect dormancy levels. In most after-ripened samples dormancy is induced and this is most obvious in the distal material (Experiments 6, 7, 8). However, soon after harvest, when dormancy is still displayed at low temperature, high temperature pre-treatments may stimulate germination to some extent(Experiment 7). This latter situation may account for a similar report of high temperature stimulation by Thurston (1960).

No difference was ever observed between the effects of one or three days at high temperature, and 24 hours exposure on the second day has a similar effect (Experiment 8). The inhibition is slightly smaller if the treatment is given on the third day, which implies that after 48 hours at 10°, some of the material has already passed into a stage of development which is insensitive to high temperatures.

Drennan (1960) reported that maximum germination of A. ludoviciana was obtained by allowing pricked seeds to imbibe at 30° for 3 days prior to germination at 10°. As pricking stimulates germination even at 30° i.e. creates non-dormancy (Experiment 9), and high temperature pre-treatments accelerate germination of non-dormant seeds (Experiment 6),

the combined effects of the treatments used by Drennan may be explained.

The inhibitory effects of supra-optimal temperatures on germination are well documented, especially with regard to their role in thermo-periodicity (Toole et al., 1956). It has been postulated that high temperature is conducive to formation of an inhibitory situation (Vegis, 1964). In lettuce seed, the net accumulation of an inhibitory substance at temperatures above 27° has been suggested (Berrie, 1966). It is interesting that the minimum temperature for dormancy induction in proximal seeds of A. ludoviciana is also 27° (Thurston, 1962).

Inhibitors might also be involved in dormancy induction in A. ludoviciana. As the endogenous dormancy level in the population is in some manner related to the capacity for dormancy induction, it is possible that after-ripening involves the loss of the ability to form inhibitors on imbibition. This would provide an alternative to the view that existing inhibitors are inactivated during after-ripening.

Conclusions from Section II

(1) High temperature pre-treatments accelerate germination in samples with low dormancy.

(2) High temperature pre-treatments may induce dormancy and are more effective if administered during the first or second day of imbibition.

(3) Stimulation by high temperatures can be demonstrated soon after harvest.

(4) Pricking stimulates germination at high temperatures.

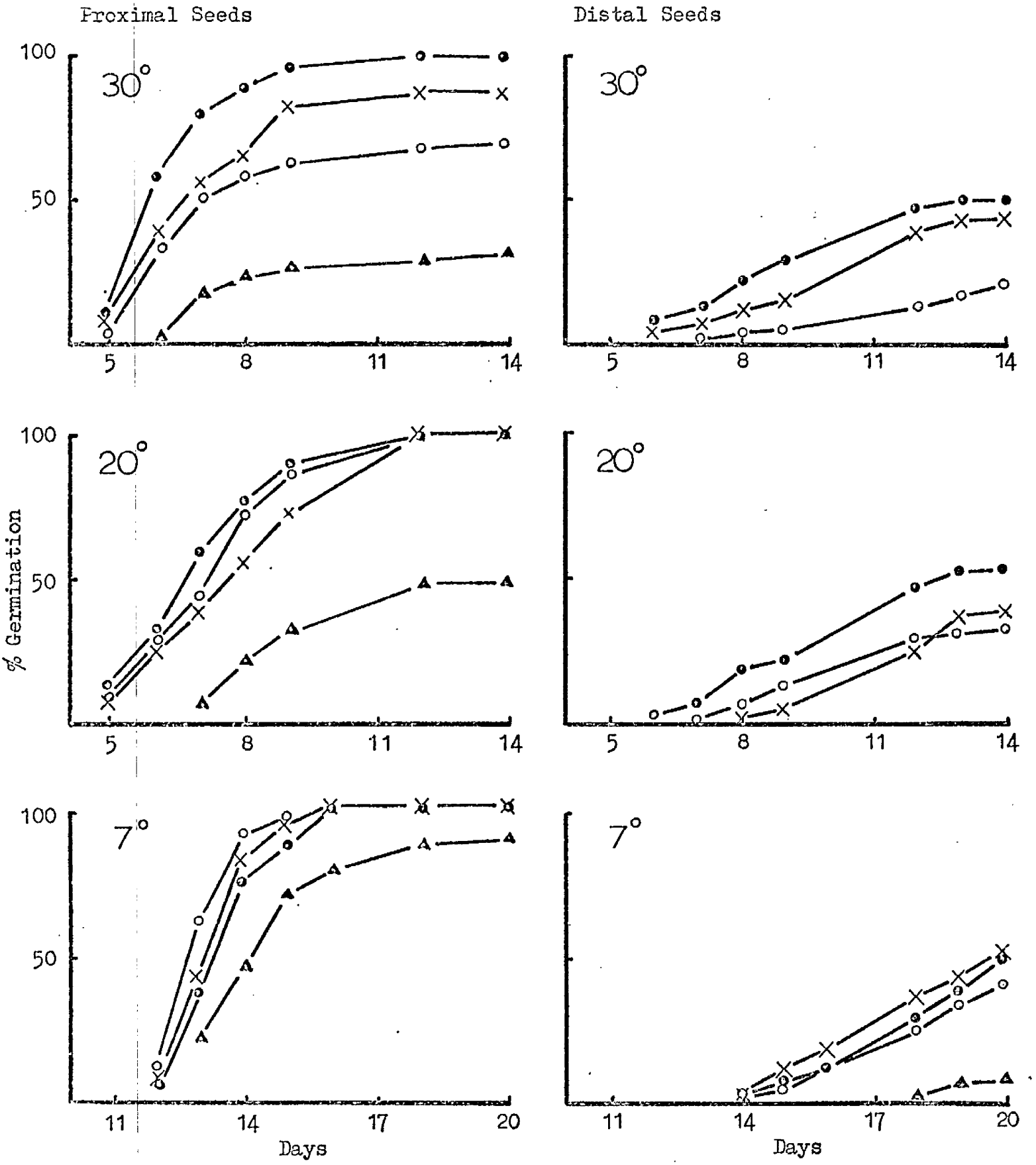


Figure 6. Germination of Seeds at different temperatures in 0, 50, 100 or 200 µg/ml GA. Note difference in time intervals at 7°.

Section III. The Effects of Gibberellic Acid on Germination

Results

Experiment 10. The effect of temperature on the ability of gibberellin A_3 (GA_3) to promote germination in an 11 year old sample

An 11 year old seed sample was germinated according to Standard Procedure (a) at 7°, 20° or 30° in water or GA_3 (50, 100 or 200 µg/ml). Final germination percentages after 14 days (20° and 30°) and 20 days (7°) are presented in Table 10a and germination patterns are illustrated in Figure 6. As supplies of this sample were limited, only two replicates, each of 25 proximal and 25 distal seeds, were used. An analysis of variance (Table 10b) indicates that a significant interaction exists between the effects of GA_3 and temperature.

Table 10a.

The effect of temperature on promotion by gibberellin A_3

Proximal Seeds

Temperature	GA_3 (µg/ml)			
	0	50	100	200
7°	90	100	100	100
20°	48	98	98	100
30°	32	70	88	100

Distal Seeds

Temperature	GA_3 (µg/ml)			
	0	50	100	200
7°	6	40	50	48
20°	0	32	36	52
30°	0	20	42	50

Table 10b.

Analysis of Variance in the results presented in Table 10a.

Source	D.F.	Sum of Squares	Mean Square	Variance Ratio
Replicates	1	11.573	11.573	0.53
Proximal v. Distal (P)	1	22,898.366	22,898.366	1,061.73 **
G.A. (G)	3	11,267.708	3,755.902	174.15 **
Temperature (T)	2	1,815.188	907.594	42.08 **
P x G	3	40.94	13.64	0.63
P x T	2	496.816	248.408	11.51 **
G x T	6	1,122.833	187.138	8.67 **
P x G x T	6	237.780	39.63	1.83
Residual	24	517.613	21.567	
Total	48	38,408.817		

Experiment 11. The effect of temperature on the ability of gibberellin A_3 to break dormancy in a seed sample after-ripened for 2 months

Seeds, 2 months from harvest, were germinated according to Standard Procedure (a) at 5°, 10°, 20° or 30° in 5 ml. water or GA_3 (10, 33, 100 or 333 µg/ml. present as the K-salt). Final percentage germination (20 days) is presented in Table 11a. An analysis of variance indicates that a significant interaction occurs between GA_3 and temperature.

Table 11a.

The effect of temperature on the ability of gibberellin A_3 to break dormancy

Proximal Seeds

Temperature	GA_3 (µg/ml)				
	0	10	33	100	333
5°	90	97	97	100	99
10°	56	80	99	100	100
20°	0	3	4	8	17
30°	0	0	0	0	11

Distal Seeds

Temperature	GA_3 (µg/ml)				
	0	10	33	100	333
5°	5	45	49	79	92
10°	0	8	28	69	88
20°	0	1	4	5	11
30°	0	0	0	0	0

Table 12.

Germination in GA_3 previously exposed to seeds or pales at different temperatures

Petri dish pre-treatments				Resulting Germination	
Temperature	Addition	GA	Drying	Proximal	Distal
10°	Seeds	+	+	95	24
10°	Pales	+	+	92	36
10°	-	+	+	89	25
30°	Seeds	+	+	79	20
30°	Pales	+	+	87	25
30°	-	+	+	87	27
-	-	+	+	97	61
-	-	+	-	95	49
-	-	-	-	63	3

Experiment 12. The effect of temperature on solutions of gibberellin A_3 in contact with non-sterile seeds

Gibberellin A_3 (5 ml; 250 µg/ml as K-salt) was incubated at 10° or 30° in petri dishes (9 cm.) with seed test paper, in the presence of spikelets (50) or pales (from 50 spikelets) of A. ludoviciana or no addition. Spikelets and pales were removed after 9 days, the petri dishes plus papers dried at 65° and fresh seeds (25 proximal; 25 distal) and water (5 ml.) added. Freshly prepared GA_3 was similarly dried and prepared with seeds and water. All dishes were then placed at 10° in the dark, accompanied by controls of seeds in water and in fresh (non-dried) GA_3. Percentage germination after 18 days is presented in Table 12.

It is evident that in the absence of any additions, similar reductions in effectiveness of the GA_3 occurred during incubation at 10° and 30°. In the presence of seeds or pales, slightly more reduction in effectiveness occurred at 30° than at 10°, but a high level of effective GA_3 nevertheless remains, as indicated by a comparison with the germination obtained in water. Some loss of effectiveness of GA_3 has therefore occurred, but the effect of high temperatures on this loss was slight.

In conclusion, it appears more likely that the absence of germination at 30° results from a change in response to GA_3 within the seed itself rather than inactivation outside the seed, but the use of a proven gibberellin assay such as the lettuce hypocotyl extension test would have given a better estimate of the residual gibberellin.

Experiment 13. The effect of the presence of the pales on the ability of gibberellin A_3 to promote germination

Seeds and caryopses after-ripened for 7 months were germinated under Standard Procedure (b) at 10° in water or GA_3 (10 µg/ml). Percentage germination (14 days) is presented in Table 13. Significant differences between seeds and caryopses are indicated by asterisks. It is evident that GA_3 at this level cannot overcome all of the inhibitory effect of the distal pales.

Table 13.

Germination of seeds and caryopses in water or gibberellin A_3

Seed type	Seed condition	Germination medium	
		Water	GA_3
Proximal	Seeds (S)	86	95
	Caryopses (C)	93	99
χ^2 (1df)	S v. C	1.91	1.54
Distal	Seeds (S)	7	40
	Caryopses (C)	50	89
χ^2 (1df)	S v. C	43.28**	50.31**

Experiment 14. A comparison of the promotive effects of a pricking treatment and a high level of gibberellin A_3 on germination at high temperature

Seeds and caryopses, 7 months after harvest, were germinated entire or following a pricking treatment, in water or in GA_3 (1,000 µg/ml. present as the K-salt) at 30° under Standard Procedure (b). Percentage germination (14 days) is recorded in Table 14. Significant differences between the effect of pricking and GA_3 are indicated by asterisks. It is evident that for caryopses, promotion by GA_3 is more effective than pricking, whereas for whole seeds, pricking is considerably more effective than GA_3 at this concentration.

Table 14.

Percentage germination at high temperature following pricking or treatment with gibberellin A_3

Seed type	Seed Condition	Treatment			χ^2 (1df)
		-	Pricked	GA_3	Pricking v. GA_3
Proximal	Seeds	0	84	64	9.38 **
	Caryopses	7	91	100	5.84 *
Distal	Seeds	0	59	14	41.76 **
	Caryopses	0	79	91	4.74 *

Discussion

In well after-ripened seeds, GA_3 can overcome both the dormancy displayed by distal seeds at low temperature and the dormancy imposed by high temperature (Experiment 10). Soon after harvest, high levels of GA_3 also promote germination of distal seeds at low temperature, but high temperature dormancy is not removed (Experiment 11). GA_3 inter-acts with temperature such that at progressively higher temperatures, progressively more GA_3 is required to effect the same germination.

At low temperature the effect of GA_3 on seeds or caryopses is similar insofar as the differential between germination in seeds and caryopses remains the same as in water (Experiment 13). However, at high temperature, the pales markedly reduce the effect of GA_3 (Experiment 14). A similar interaction of GA_3 with seed coats has been reported in Datura ferox (Sanchez et al., 1967). Pricking seeds is a more effective method of overcoming the inhibition of the pales, but is less effective than high concentrations of GA_3 in breaking dormancy of caryopses.

The ability of the pales to reduce the effect of GA_3 at high temperature could be the result of restricted entry of the hormone, but it is more likely that the pales create a particular environment within the caryopses, perhaps by preventing leaching of inhibitors (Black, 1959) and that this environment promotes degradation or prevents utilisation of the GA_3. Alternatively, the reduced effect of GA_3 could be due to interaction with inhibitors present in the pales, similar to those which occur in the pales of A. fatua (Kommedahl et al., 1958; Black, 1959).

If pricking does stimulate germination by the production of gibberellins as a wound response, it is possible that its effectiveness is due to the production of high levels at active sites, whereas imbibition in GA_3 solution may be an inefficient method of satisfying the requirements of these sites. Alternatively, pricking may stimulate production of other gibberellins or a range of gibberellins, which may be more effective than GA_3. An increase in a gibberellin-like factor in the embryo of A. fatua accompanies the loss of dormancy during after-ripening (Simpson, 1965) and is possibly responsible for the removal of the block to metabolism.

From the observations on the effect of GA_3, it is possible to speculate that the opposing effects of high temperature and the pales on the one hand and low temperature and pricking treatments on the other, operate through control of the levels of interacting inhibitors and stimulators within the caryopses. In an attempt to elucidate the role of such substances in seed dormancy, Bradbeer (1968) has studied the effects of the interaction of abscisic acid and gibberellin A_3 on hazel seed.

Conclusions from Section III

(1) Gibberellin A_3 promotes germination and can overcome the high temperature inhibition.

(2) The promotive effect of gibberellin A_3 and the inhibitory effect of high temperatures interact so that higher concentrations of hormone are required at higher temperatures to achieve similar germination.

(3) At high temperatures, pricking is more effective in breaking dormancy of whole seeds than high concentrations of gibberellin A_3.

General Conclusions from Part III

The dormancy displayed by seeds of A. ludoviciana has characteristics similar to those reported for other temperate grasses and cereals, but includes certain distinguishing features. Immediately after harvest, germination is minimal and requires low temperatures. Dry storage increases this germination and extends the range to higher temperatures, but the optimum temperature remains comparatively low. High temperature inhibition which may be accompanied by induction of dormancy, can be overcome by GA_3. The concentration required is higher at higher temperatures or in the presence of other circumstances normally inhibiting germination. The puncturing of the pericarp-testa remains the most effective method of promoting germination in an inhibitory environment. The response to these environments is quantitatively distinct in the three types of caryopsis and in general is markedly reduced by the presence of the pales. An exception occurs in light however, where the pales may prevent inhibition of germination.

Explanations of these phenomena in terms of destruction and synthesis or binding and release of controlling substances are mere speculation at this stage, but a basis for the investigation of some of these concepts is provided by the production of populations with varying degrees of dormancy. From one seed sample it is possible to create six subpopulations each with distinct dormancy levels by separating the seed types and dehusking half of them. The material thus lends itself to a quantitative examination of the causes of dormancy.

PART IV. MALATE METABOLISM AND DORMANCY : ENZYME STUDIES DURING AFTER-RIPENING AND GERMINATION

Introduction

During low temperature after-ripening of seeds in moist conditions, changes occur in enzyme activities. It has been suggested that these changes may be responsible for the loss of dormancy (Bradbeer and Colman, 1967a,b; Lacroix and Jaswala, 1967). A similar correlation between enzyme activity and loss of dormancy has not yet been demonstrated in seeds which after-ripen in the dry state. A possible example of the latter is afforded by the reduction of tetrazolium salts in non-dormant whole caryopses of A. fatua (Hay, 1962) and rice (Roberts, 1964a), but not in dormant caryopses. Hay visualised the apparent lack of dehydrogenase activity in dormant wild oats as a cause of dormancy, but Roberts presented evidence that the reductive activity in non-dormant rice seed merely indicated the imminence of germination. Thus the role of dehydrogenase enzymes in dormancy is undecided.

In studies with A. fatua, Hart and Berrie (1968) demonstrated that the malic acid content of dry seeds was inversely correlated with the degree of dormancy; malic acid levels fell during dormancy induction; carbon dioxide could stimulate germination. It was later demonstrated that carbon dioxide was fixed into malate during germination. As this evidence indicates the involvement of malate metabolism in dormancy, an investigation of the enzymes likely to be important was included in the

present study. As these are mainly dehydrogenases, the previous results of Hay (1962) and Roberts (1964a) are of importance here.

Therefore, the levels of activity of NAD-malate dehydrogenase and NADP-malate dehydrogenase (malic enzyme) were examined in dormant and non-dormant caryopses during after-ripening and in the early stages of the germination process using spectrophotometric and electrophoretic methods.

The use of polyacrylamide gel electrophoresis for visualisation of the multiple forms of the enzymes studied, also makes it possible to determine changes in the soluble protein fraction which may be associated with particular dormancy states. This technique has been used to demonstrate changes in the proteins of wheat embryo during vernalisation (Teraoka, 1967) and of alfalfa roots at the onset of cold hardiness. It therefore seemed worthwhile to investigate the proteins of A. ludoviciana during after-ripening.

SECTION I : SPECTROPHOTOMETRY

Results

Experiment 15 The effect of pH on the oxidation of malate by NAD-Malate dehydrogenase

A total fraction (500 g 190,000 g supernatants) of the soluble proteins of dry caryopses was prepared as described.

Aliquots were assayed in 1 cm. cuvettes at a range of pH values close to pH 10, as recommended by Wolfe and Neilands (1956). The results of two such experiments are shown in Table 15.

Table 15

mμ moles malate oxidised/min/ml. by preparations of dry caryopses at different pHs

pH	9.5	9.9	10.3	10.9	11.3
Experiment A	310	390	418	185	177
Experiment B	207	264	408	385	329

Experiment 16 The influence of volume of enzyme solution on the rate of oxidation of malate

A total fraction was prepared from 50 dry proximal caryopses after-

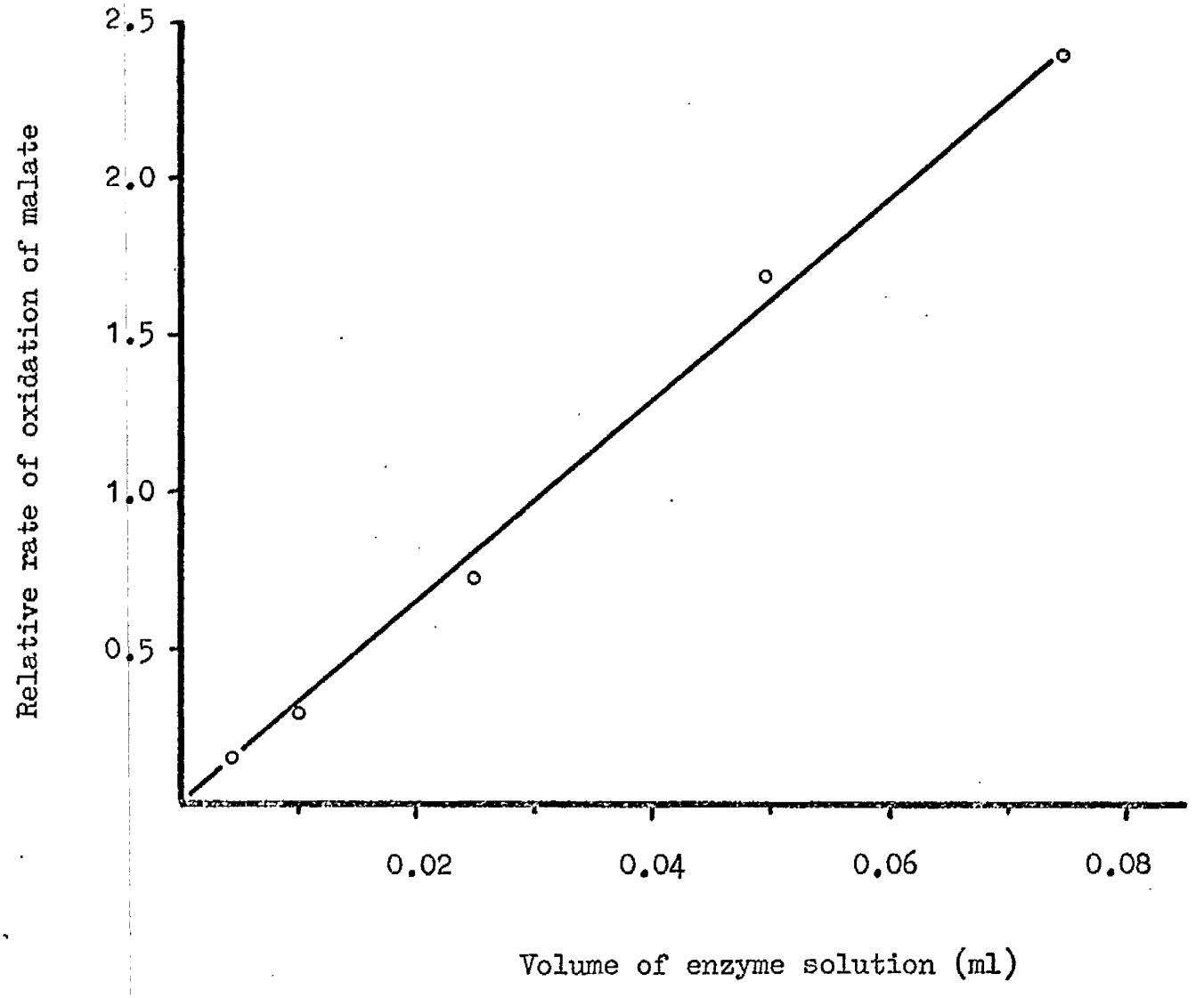


Figure 7. The effect of volume of enzyme solution on the rate of oxidation of malate.

ripened at room temperature for one year. A range of volumes of this enzyme solution was then assayed. To minimise error in the addition of very small volumes, a range of concentrations was prepared and the same final volume added in each case. The results of a typical experiment are seen in Table 16 and Figure 7.

Table 16

The activity (μmoles malate oxidised/min) per ml. of total fraction obtained by the addition of different volumes of enzyme solution

Volume	Angle of Slope	Tangent	Activity/ml.
0.005	8° 24'	0.1477	2.646
0.010	17° 36'	0.3172	2.842
0.025	36° 6'	0.7292	2.612
0.050	59° 12'	1.6775	3.006
0.075	67° 18'	2.3906	2.855

Experiment 17 The NAD-malate dehydrogenase activity in proximal and distal caryopses

Caryopses (1g.) were subjected to standard treatment and the equivalent number present was calculated. The enzyme activity in the total (500 g 190,000 g supernatants) mitochondrial (10,000 g pellet, 190,000 g supernatant) and soluble (10,000 g 190,000 g supernatants) fractions is expressed as activity per g. dry caryopses (Table 17). In addition, the sum of the mitochondrial and soluble activities is

similarly expressed, and the mitochondrial activity calculated as a percentage of this value. The results of four typical experiments are given. The results of two germination tests at 10° carried out with the same seed in the same month as Experiments a and c are also given.

Table 17

The NAD-malate dehydrogenase activity in the Total, Mitochondrial and Soluble fractions per g. of proximal or distal caryopses

Expt.	Caryopses	No/g.	% Germ	Total	Soluble	Mito	Soluble + Mito	% Mito	Activity per Seed
a	Prox	50	92		9.470	1.352	10.822	12.4	0.216
	Dist	83	36		9.282	1.789	11.077	16.1	0.133
b	Prox	56		12.647	12.107	1.214	13.321	9.1	0.238
	Dist	90		12.525	11.862	0.864	12.726	6.7	0.142
c	Prox	49	95		10.735	1.506	12.242	12.3	0.250
	Dist	85	39		10.943	1.304	12.247	10.6	0.144
d	Prox	53			9.317	1.020	10.338	9.8	0.196
	Dist	84			10.020	1.304	11.324	11.5	0.135

Experiment 18 The NAD-malate dehydrogenase activity of proximal caryopses at different stages of after-ripening

Three replicates of 25 proximal carypses were subjected to standard

extraction treatment following after-ripening at room temperature for one year or one month. Table 18 shows the total activity extracted and total activity per g. dry caryopses. Germination tests at 10° were carried out as part of this experiment.

Table 18

The NAD-malate dehydrogenase activity of proximal caryopses after-ripened for different periods

After-ripening Period	% Germination	Total Activity per 25 Caryopses	Activity per gram
12 Months	98	6.267 6.673 6.779	10.880 11.852 12.986
1 Month	7	6.851 6.587 6.396	11.592 11.617 10.989

Experiment 19 The NAD-malate dehydrogenase activity of proximal caryopses after 2 or 4 days imbibition at 10° with or without a pricking treatment

Samples of 50 proximal caryopses were set under standard germination conditions at 10° with or without a prior pricking treatment. After 2 and 4 days, two replicates were withdrawn and a soluble and mitochondrial extract prepared. Two replicates of dry caryopses were

similarly treated. All caryopses were weighed dry and fresh weights of imbibed caryopses were recorded before homogenisation. Results are expressed as activity per 50 caryopses or per g. dry caryopses (Table 19a,b) Water uptake has been calculated as a percentage of the original air dry weight (Table 19c).

Table 19a.

NAD-malate dehydrogenase activity from samples of 50 caryopses after periods at 10° with or without a pricking treatment

Fraction	Soluble			Mitochondrial			Soluble + Mitochondrial		
Days at 10°	0	2	4	0	2	4	0	2	4
Whole Caryopses	9.626	9.912		0.600	1.096		10.226	11.008	
	9.506	9.984		0.680	1.076		10.186	11.060	
Pricked Caryopses		10.118			1.134			11.252	
		10.404			1.154			11.558	
Whole Caryopses	8.524		9.624	0.828		1.134	9.352		10.758
	8.936		9.930	0.852		1.086	9.788		11.016
Pricked Caryopses			9.352			0.892			10.244
			9.746			1.112			10.858

Table 19b.

NAD-malate dehydrogenase activity per g. dry caryopses after periods at 10° with or without a pricking treatment

Fraction	Soluble			Mitochondrial			Soluble + Mitochondrial		
Days at 10°	0	2	4	0	2	4	0	2	4
Whole	10.62 9.70	9.90 10.44		0.66 0.69	1.09 1.12		11.28 10.39	10.99 11.56	
Pricked		10.31 11.12			1.15 1.23			11.46 12.35	
Whole	8.51 9.24		9.67 10.59	0.82 0.88		1.14 1.15	9.33 10.12		10.81 11.74
Pricked			9.69 10.16			0.92 1.16			10.61 11.32

Table 19c.

Water uptake of caryopses as a percentage of original dry weight after periods at 10° with or without a pricking treatment

Days	Whole Caryopses	Pricked Caryopses
2	28.07 25.62	39.04 36.16
4	36.13 38.73	48.79 45.91

Experiment 20 The NAD-malate dehydrogenase in caryopses set to germinate in light and dark at 10°

Fifty proximal caryopses were placed under standard germination conditions at 10° in darkness and incandescent light. After 4 days, when germination in the dark was 4% and nil in the light, a standard extraction was performed, giving total, mitochondrial and soluble fractions. Activity was calculated per 50 caryopses and per g. dry caryopses (Table 20). Protein values values and activity per mg. protein are also recorded. The activity values in this experiment are slightly lower than in others, possibly due to the use of a NAD solution which had been kept in a deep freeze for a few days.

Table 20

The NAD-malate dehydrogenase activity in different fractions of caryopses after 4 days at 10° in light or darkness

		Activity per 50 seeds	Activity per gram	% Mito Activity	Protein mg/ml	Activity/ mg. Protein
Total						
	Light	7.048	7.459		10.66	82.64
		7.554	7.836		10.01	94.30
	Dark	7.004	7.176		12.51	69.94
		7.354	7.861		12.92	71.13
Soluble						
	Light	6.836	7.438		9.07	94.23
		6.864	7.116		9.39	93.42
	Dark	6.384	6.539		10.49	79.65
		6.742	7.196		10.85	80.89
Mitochondrial						
	Light	0.980	1.067	14.31	1.52	169.10
		1.054	1.093	13.95	1.63	169.92
	Dark	0.848	0.869	12.11	1.84	120.78
		0.956	1.021	12.99	1.84	136.50

NADP-malate dehydrogenase. No activity of this enzyme could be demonstrated in any of the extracts tested, using the method of Walker (1960).

Discussion

The spectrophotometric method of assay of NAD-malate dehydrogenase (MDH), employed by Wolfe and Neilands (1956) has been found easy to use and is adequately reproduceable within one experiment. The increase in absorbance at 340 mμ in the control cuvette (minus malate) was always extremely small, even after some hours at 25°. This indicates that a comparatively negligible amount of malate was added with the enzyme solution. Boiled enzyme solutions gave no increase in absorbance.

The optimum pH for measuring the activity of this enzyme in extracts of A. ludoviciana was found to be 10.3 (Experiment 15), which is slightly higher than the pH used by Wolfe and Neilands. As the reoxidation of NADH is completely prevented at this pH, the major difficulty in assaying this enzyme is removed, without using cyanide or other agents to trap oxalacetate. However it must be stated that the pH for optimum expression of the forward reaction in isolation is not the physiological pH optimum. The results of Experiment 16 show that the volume of enzyme solution chosen for standard assay lies within the range where enzyme concentration is directly proportional to the activity.

Despite the fact that the germination percentages of the two seed types are so distinct, no differences are evident between the malate dehydrogenase activities of equal weights of proximal and distal caryopses. Thus the differences in rate of loss of dormancy does not reflect differences in MDH activity. The activity in a proximal caryopsis is greater than that in a distal caryopsis in the ratio of their weights (Experiment 17) From the results of Experiment 18 it is highly unlikely that the activity

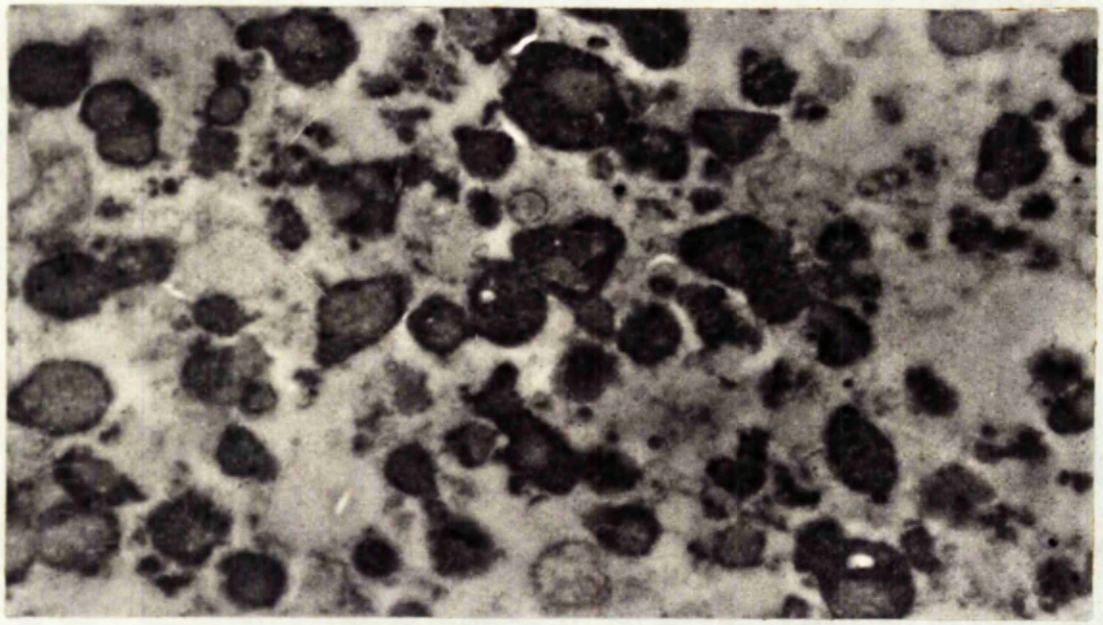


Plate Ia. The mitochondrial pellet from dry caryopses, magnification 5,000X, enlarged to 12,500X.

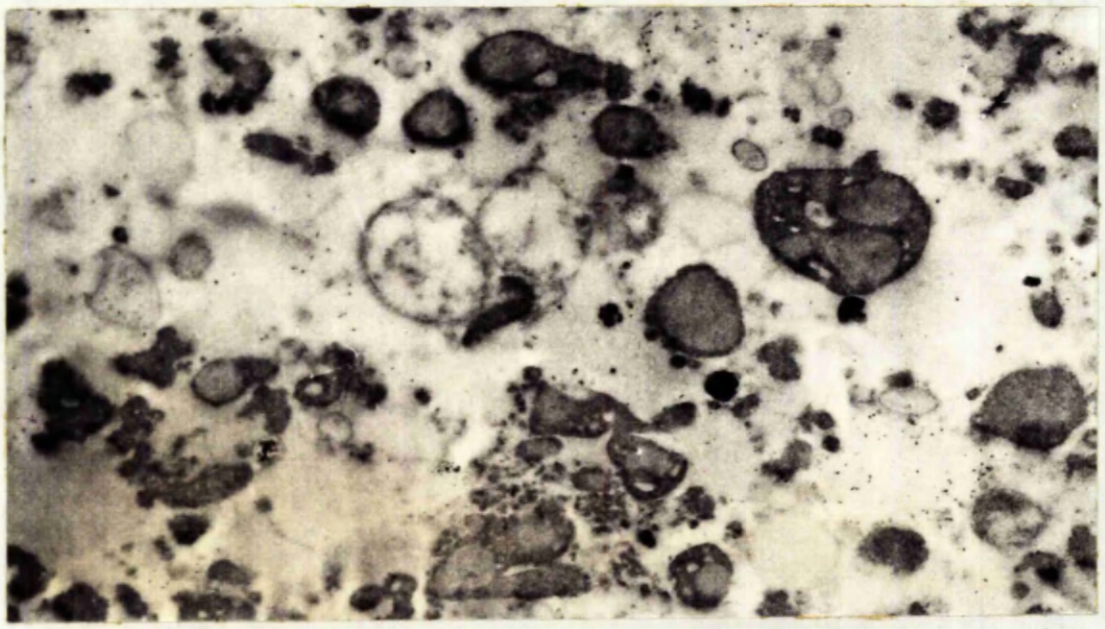


Plate Ib. The mitochondrial pellet from caryopses imbibed 5 days at 10°, magnification 5,000X, enlarged to 12,500X.

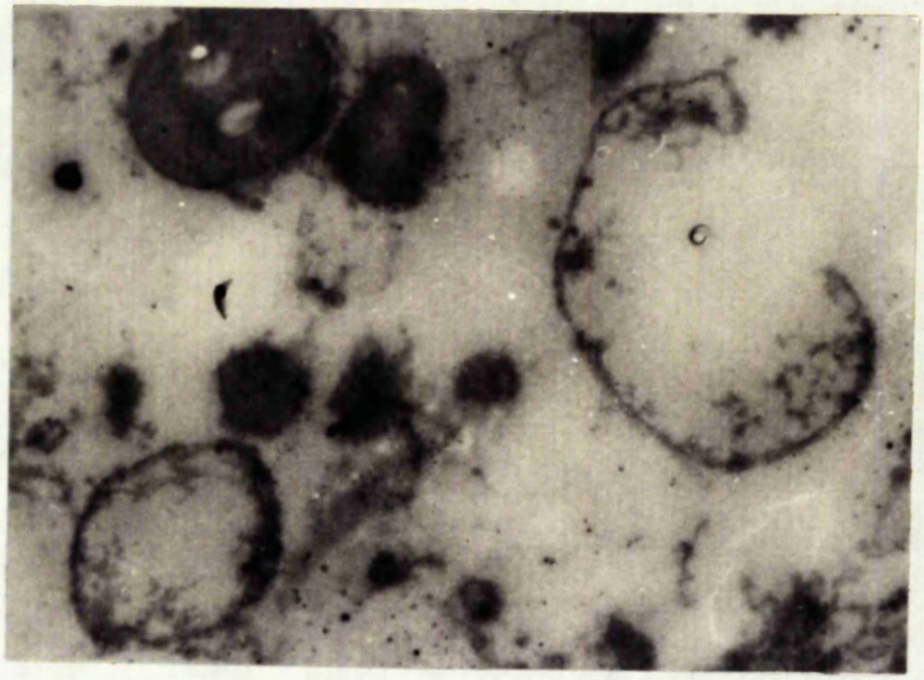
For details of fixation see Appendix V.

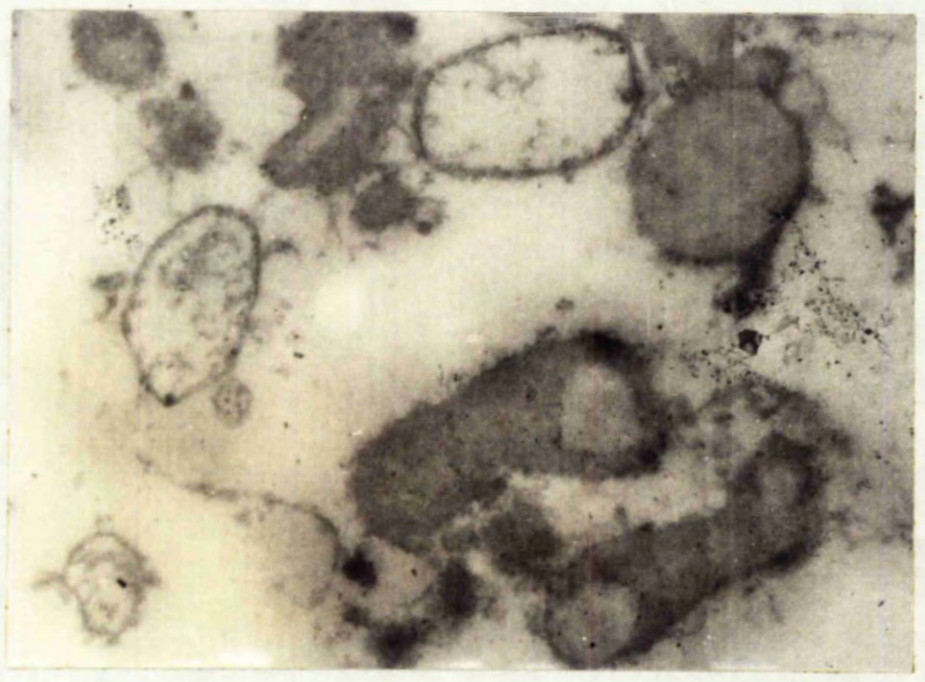
of this dehydrogenase alters during after-ripening in this species.

During 2 or 4 days imbibition at 10° there is very little increase in soluble or total activity, but there is an increase in the mitochondrial activity when considered alone, (Experiment 19). Also, as a percentage of the total activity, the mitochondrial contribution rises after 2 days. It is possible that these higher values reflect greater resistance of imbibed mitochondria to physiological and physical stress during extraction. Pricking the caryopses normally stimulates germination, but did not increase the activity of the total or mitochondrial fraction relative to imbibed whole caryopses.

Light reduces the germination percentage of naked caryopses in this species (Experiment 20), but does not affect the MDH activity relative to that in darkness even to the point when visible germination is beginning in the dark. The effect of light in lowering the amount of soluble protein in the seed extracts results in higher activity per unit of protein in the light. This is perhaps due to the solubilisation of the reserve proteins having commenced in the dark, but not in the light.

It is interesting that the range of mitochondrial activities as a percentage of total activity (6-16%) is similar to the values given by Pierpoint (1963) for tobacco leaves (7-19%), and by Yue (1966) for barley seedlings (15%). Electron micrographs prepared by Miss M. Cox of the mitochondrial pellet from A. ludoviciana (Plates 1 and 2) show that a large number of the mitochondria present lose much of their contents before freezing, and much of this must be present in the soluble fraction. Since the number of mitochondria which burst would be expected to vary between





Plates IIa. and b. Mitochondria from caryopses imbibed for 5 days at 30°. stained with lead citrate
Magnification 10,000X enlarged to 25,000X

experiments, experimenters and material, it is strange that these figures are so similar. Perhaps this represents residual activity associated with membranes.

The levels of activity of MDH found in this species accord well with the high activity already recorded in oats (Berger and Avery, 1943), lettuce (Mayer et al., 1957) and wheat (Honold, Farkas and Stahmann, 1967). The last authors estimate that this enzyme is at least 1,000 times more active than any of eight other dehydrogenases they examined. This may in part explain the present inability to detect NADP-malate dehydrogenase.

The results presented here indicate that the level of malate dehydrogenase does not alter during after-ripening or during the important early stages of germination, and appears to be unrelated to particular dormancy states and unresponsive to a pricking treatment. It is not known which dehydrogenases are responsible for the production of formazan observed by Hay (1962) and Roberts (1964a). As MDH appears to have general high activity in cereals, it is likely to be involved. If this is the case, the lack of tetrazolium reduction in dormant seeds is probably due to inhibition of existing dehydrogenases rather than a lack of synthesis of dehydrogenase enzyme protein.

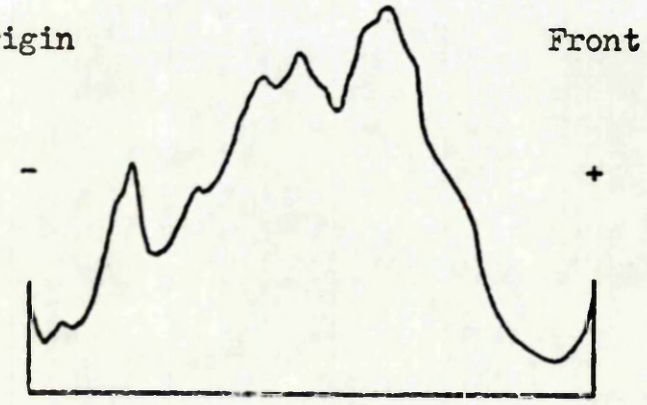


Figure 8.

Plate III.

Acrylamide gel and densitometer trace of the water soluble proteins from A. ludoviciana

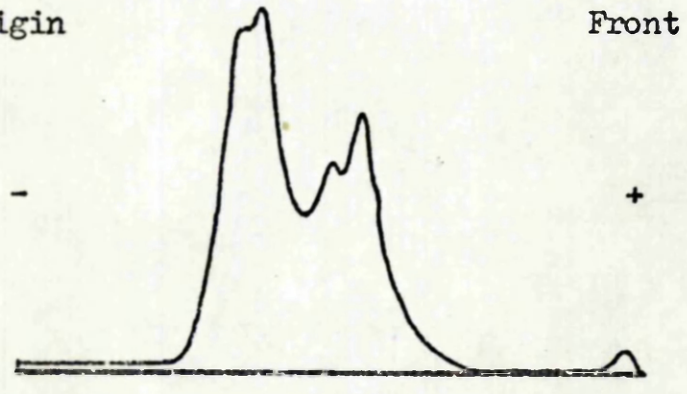


Figure 9.

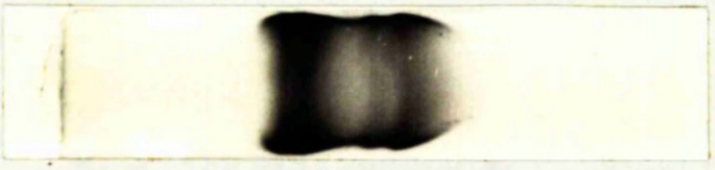


Plate IV.

Acrylamide gel and densitometer trace of the MDH isozymes from A. ludoviciana

SECTION II : POLYACRYLAMIDE GEL ELECTROPHORESIS

Results

Experiment 21 The extraction of protein and malate dehydrogenase for electrophoresis

Total extracts were prepared from proximal caryopses (1.6g.) of A. ludoviciana. Extraction was carried out in 3.2 ml. of water, 0.2M tris-HCl buffer pH 7.4 or 0.06M phosphate buffer pH 7.4. A volume of solution containing 500 µg. protein, assayed spectrophotometrically was added to each gel. Following electrophoresis, gels were stained for protein and for MDH activity. Typical densitometer tracings of protein and enzyme activity in water extracts are shown in Figures 8 and 9, Plates 3 and 4.

Experiment 22 The soluble proteins and malate dehydrogenase from separated embryos and endosperms after 3 days at 10°

Total extracts were prepared from embryos and endosperms (plus aleurone etc.) from 60 proximal caryopses. Embryos (0.2g.) and endosperms (1.3g.) were extracted with 0.8 and 2.6 ml. of 0.02M tris-HCl buffer pH 7.4 respectively. Typical gels are seen in Plate 5.

Experiment 23 A comparison of the malate dehydrogenase isoenzyme complement in whole caryopses, plumules plus coleoptiles and seminal roots of A. ludoviciana

Dry proximal caryopses (1.6g.) were extracted with 3.2 ml. 0.02M

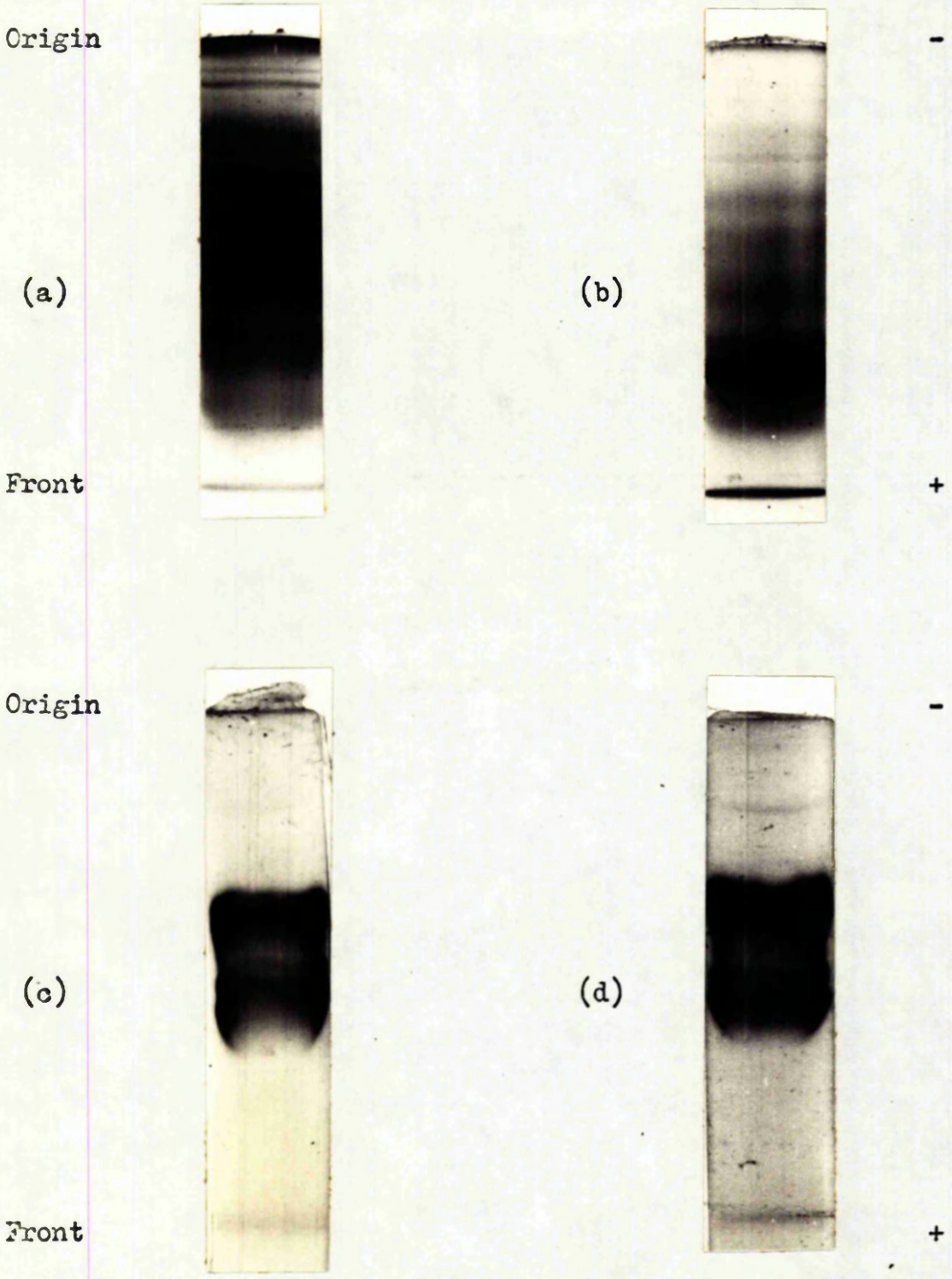


Plate V. The soluble proteins from the embryo (a) and the endosperm (b) of A. ludoviciana separated on acrylamide gel, and the formazan staining produced by the malate dehydrogenase isozymes present in the embryo (c) and the endosperm (d).

tris-HCl buffer pH 7.4. Roots (2.1g.) and green plumules plus coleoptiles (3.2g.) were extracted with 1.05 and 1.6 ml. of the same buffer. The coleoptiles were about 2.5 cms. long, and the roots about 7.5 cms. Total fractions were prepared and 500 µg. protein added to each gel tube. Typical gels are seen in Plate 6.

Experiment 24 The NAD-malate dehydrogenase isozymes present in total, mitochondrial and soluble fractions of A. ludoviciana after imbibition at 10° for 3 days

Proximal caryopses (1g.) were extracted in 2 ml. 0.02M tris-HCl buffer pH 7.4, 0.4M in sucrose. Fractions were prepared as described previously and 250 µg. protein was applied to each tube. Typical gels are seen in Plate 7.

Attempts were made to stain gels for NADP-malate dehydrogenase activity, but no activity could be detected with the extracts used, including those from the previous experiment.

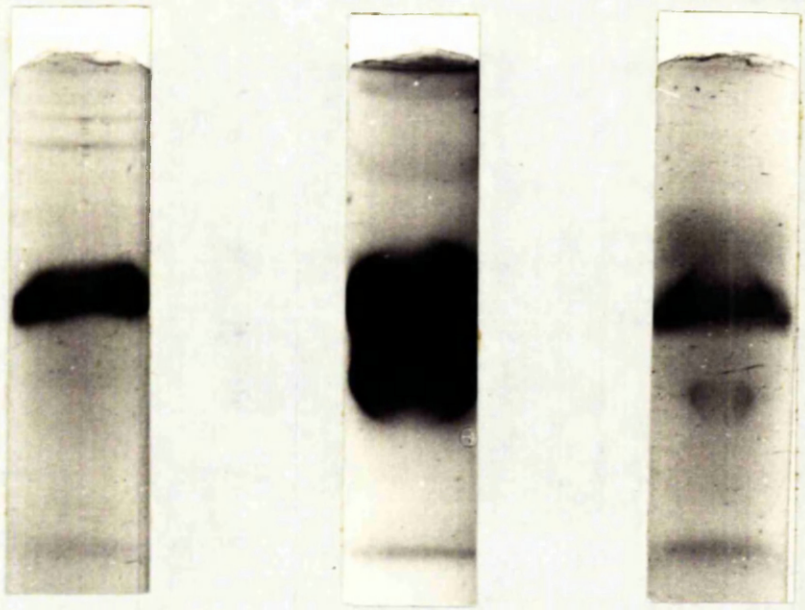


Plate VI. Formazan staining on acrylamide gels produced by the malate dehydrogenase isozymes present in the Total fractions from green plumules (a), whole caryopses (b) and roots (c) from A. ludoviciana.

Discussion

The results of Experiment 21 indicated that the electrophoretic patterns obtained from extracts in water, tris or phosphate buffer were virtually identical, and no apparent advantage could be attributed to any. It was decided to use tris buffer routinely. Typical densitometer traces of the soluble proteins from whole dry caryopses show many bands (Figure 8). This trace and the gel shown in Plate 3 are also typical of the soluble protein patterns from dry caryopses at any stage of after-ripening and from proximal and distal caryopses. No apparent changes in soluble proteins occur during after-ripening in the dry state.

There are four main bands of MDH activity corresponding to electrophoretic variants termed isozymes (Figure 9 and Plate 4). Preliminary experiments indicated the reliability of the staining procedure for MDH. No formazan bands were produced in the absence of malate or NAD, and none of the characteristic bands appeared in the presence of other substrates. The use of boiled enzyme solutions, or the inclusion of 10^{-3}M p-chloromercuribenzoate in the staining solution also prevented the development of bands.

While there are striking differences between the soluble protein fractions from embryos and endosperms (Plate 5), it is seen that all four isozymes occur in both tissues. Many examples of tissue-specific isozymes occur in animal tissues, and in plants the work of Scandalios (1964) and Macko et al. (1967) affords many examples from maize and wheat.

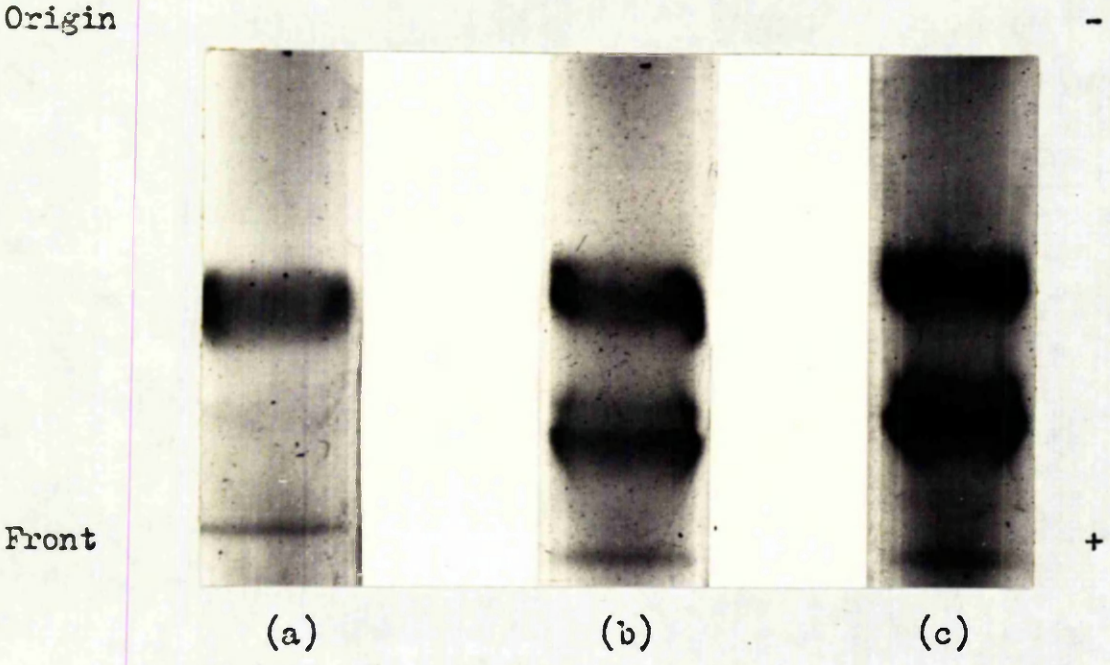


Plate VII. Formazan staining on acrylamide gels produced by the malate dehydrogenase isozymes present in the Mitochondrial (a), Supernatant (b) and Total (c) fractions from caryopses of A. ludoviciana.

In A. ludoviciana, although the embryo as a whole possessed four isozymes, green plumules and coleoptiles appear to have only the two slower-moving less anodic isozymes. Roots never gave satisfactory results on any occasion. Difficulties in the electrophoresis of root protein have been noted by R.M.M. Crawford (personal communication). The low protein content of such solutions demands the addition of larger volumes per gel tube which may be responsible for the effects seen in Plate 5.

Compartmentation within the cell is demonstrated by the presence of four isozymes in the total and soluble fractions, while only two of these isozymes occur in the mitochondrial fraction. These appear to be associated with the slower migrating pair in the total and soluble fractions, but have a tendency to travel slightly further. This compartmentation has been known for some time. Price and Thimann (1954) showed that MDH activity occurs in both soluble and particulate fractions of plant material. Differences between these two main forms of the enzyme have been demonstrated in their response to oxaloacetate concentrations (Davies and Kun, 1957), to pyridine nucleotide analogues (Kaplan and Ciotti, 1961) and oxaloacetate analogues (Kun and Volfin, 1966).

Ting et al. (1966) have shown that maize roots contain three isozymes, of which two occur in the particulate fraction, and, as in A. ludoviciana, appear to travel slightly further than their apparent counterparts in the total extract.

By the methods employed, and with the evidence from electron micrographs (Plate 2), it is reasonable to assume that a soluble isozyme

could not be isolated free from mitochondrial contamination. Ting et al. (1966) suggest that the two slow isozymes in the soluble fraction are mitochondrial or particulate contaminants and the faster isozyme is the major, if not the only MDH of the soluble compartment of the cell. Yue (1966) obtained one mitochondrial and two supernatant isozymes in barley seedlings. One of the supernatant isozymes had chromatographic characteristics and Michaelis Constant Values similar to the single mitochondrial isozyme, but different from the other supernatant isozyme. In these and the present studies, it seems probable that the two isozymes which occur only in the soluble fraction are the only ones originally present, but this is not proven.

The occurrence of only two isozymes in green tissue and the similarity of these to the mitochondrial isozymes from whole seeds is interesting in the light of the similarity of mitochondrial and chloroplast isozymes found in Vicia faba by Laycock et al. (1966). The identity of chloroplast and mitochondrial isozymes would be of great consequence, but has not been conclusively demonstrated.

Lipsand Beevers (1966 a,b) have distinguished two separate malate pools in corn roots, in which the products of CO_2 fixation are not in complete equilibrium with the mitochondrial pools. It is now suggested (Ting et al., 1966, Danner and Ting, 1967) that the important function of the mitochondrial isozymes is in the TCA cycle, while the isozymes present only in the soluble fraction are active in the metabolism of CO_2 fixation. The possibility of separate functions is supported by the kinetic differences reported above. The presence of two bands in the mitochondrial

fraction and two in the soluble phase only, adds further complexity in A. ludoviciana. Lieberman and Baker (1965) have presented evidence for the occurrence of two separate pathways oxidising and reducing NAD within the mitochondria and requiring separate isozymes. It is tempting to postulate that a similar explanation may account for the two mitochondrial isozymes found in this study.

However, in broad terms the implication of Ting's work is that in the cell the mitochondrial isozymes degrade malate while the soluble isozymes synthesise it. This means that increases in total activity in vitro cannot be interpreted in terms of malate metabolism in vivo unless it is certain that the increase is due to a particular isozyme of known function. Equally it is quite possible that any given assay system favours some isozymes in preference to others.

Therefore, the importance of electrophoretic studies lies in the provision of information which allows the validity of such assays and the reliability of extraction procedures to be questioned.

If the evidence of Lips and Beevers (1966 a,b) and Ting et al. (1966) is accepted, then any involvement of MDH in the formazan staining reaction must be due to the mitochondrial isozymes, since the soluble isozymes are thought to synthesise malate. The latter may, however, have importance in the CO_2 stimulation and fixation in A. fatua (Hart and Berrie, 1968).

At the moment it can be said that the mitochondrial isozymes are given much greater relative importance in the densitometer traces of total fractions than in spectrophotometric assays of mitochondrial fractions.

Conclusions

(1) The caryopses of *A. ludoviciana* contain a high level of easily extractable malate dehydrogenase.

(2) There are no differences in the malate dehydrogenase activities of dormant distal caryopses and non-dormant proximal caryopses, and the activity does not alter during after-ripening.

(3) There are no increases in malate dehydrogenase during early germination except for a slight increase in the mitochondrial fraction. No increases could be attributed to pricking the caryopses and no changes occurred during imbibition in light.

(4) The soluble protein fraction is markedly different in embryo and endosperm but does not alter during after-ripening.

(5) The malate dehydrogenase activity exists as four principal isozymes.

(6) All four isozymes occur in the total and soluble fractions whereas only two occur in the mitochondrial fraction. These two appear to have identity with the least anodic pair in the other fractions.

(7) The possibility that the presence of the slower least anodic pair of isozymes in the soluble fraction is due to leakage from damaged mitochondria is discussed.

(8) The possible roles of the isozymes of malate dehydrogenase are discussed.

PART V. MALATE AND PHOSPHATE LEVELS DURING AFTER-RIPENING

Introduction

Hart and Berrie (1968) have reported a correlation between malate levels and dormancy in *A. fatua*. The logical extension of this discovery is that malate accumulates during after-ripening of the dry seed, but this had not been demonstrated conclusively.

In Part IV it was shown that enzymes of two possible pathways of malate metabolism occur in the dry seed of *A. ludoviciana* but the levels of these enzymes were not correlated with dormancy in any way. Furthermore, no proof of their activity *in vivo* during after-ripening has yet been obtained.

It was therefore of interest to see whether such a correlation of malate levels and dormancy existed in *A. ludoviciana* and, if so, whether these changes occurred during the after-ripening process. Considerable amounts of malate occur in the pales of *A. fatua* (Hart, 1966) and in view of this it was clearly necessary to examine the caryopses of *A. ludoviciana* separately in order that any changes in malate levels may be attributed to metabolic activity within the caryopses themselves.

In a procedure involving a lengthy extraction, separation on two ion exchange columns, formation of TMS derivatives and gas-liquid chromatography of an extremely small volume of material, reproduceability is sufficiently adequate for analyses performed concurrently, but comparison of absolute figures is not recommended between separate experiments. In the comparison of malate levels during after-ripening, it was

therefore preferable to obtain a range of dormancy levels in material from a single harvest, available for analysis on one day, rather than to analyse a single batch of material repeatedly during after-ripening.

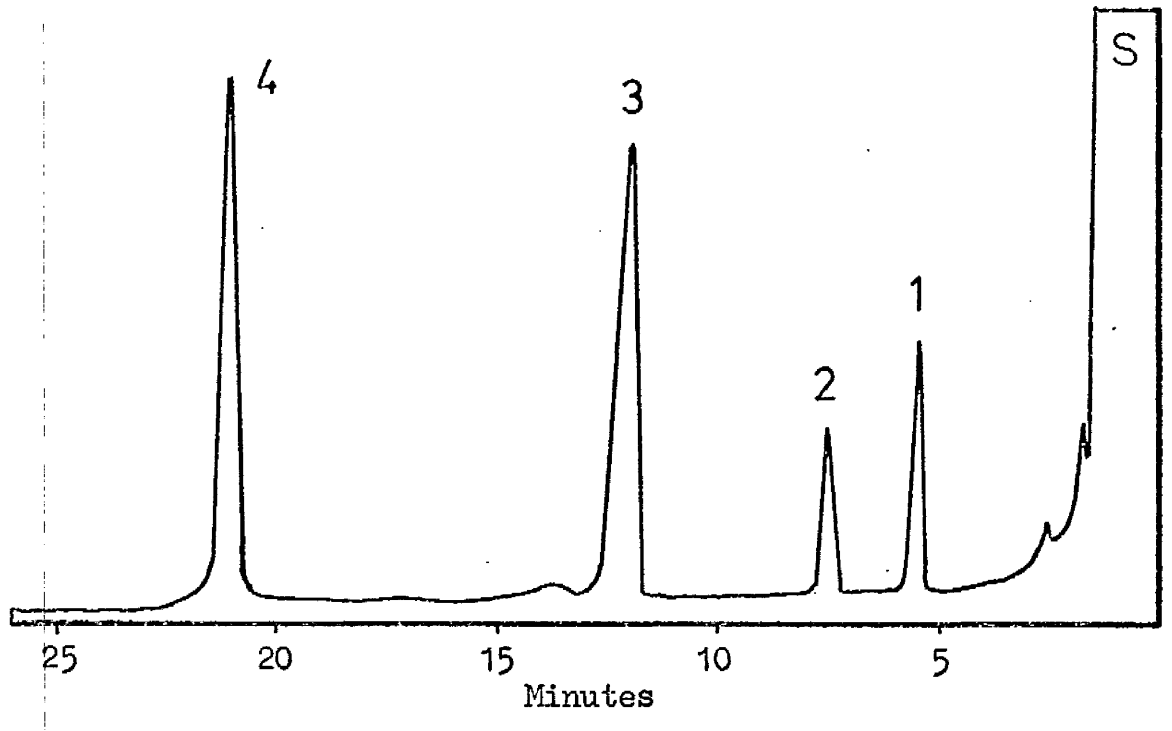


Figure 10. GLC trace of peaks from TMS derivatives of the acid fraction from seeds on 5% SE-30.

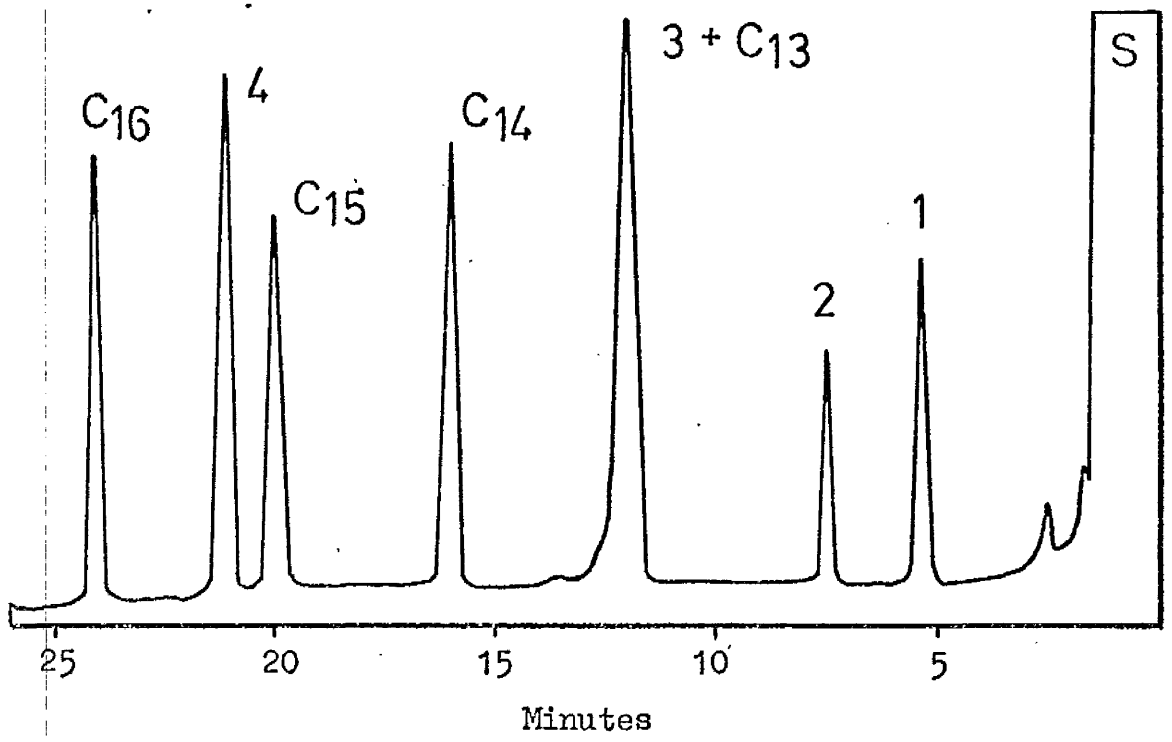


Figure 11. GLC trace of peaks from TMS derivatives of the acid fraction from seeds and standard hydrocarbons C_{13}-C_{16} on 5% SE-30

Section I : Identification of the major acidic components present in ethanolic extracts from seed of A. ludoviciana

Results

Experiment 25. Gas-liquid chromatography of the trimethylsilyl (TMS) derivatives of acids extracted from A. ludoviciana

The acid fraction from an 80% ethanol extract of seed was isolated by ion exchange chromatography. TMS derivatives were prepared using hexamethyldisilazine and trimethylchlorosilane (Dalgliesh et al., 1966). Gas-liquid chromatography on 5% SE-30 produced a trace of the type shown in Figure 10. Four major peaks were present and, of these, only peaks 3 and 4 were attributable to the seed extract; 1 and 2 were also detected following injection of the reagents used for silylation (the reagent blank).

Following co-injection of the standard hydrocarbons C_{13}-C_{16}, peaks 3 and 4 were assigned approximate methylene unit values of 15.30 and 13.00 respectively (Figure 11) using the method described by Vanden Heuval et al. (1964) and Dalgliesh et al. (1966) following a technique similar to that used by Woodford and van Gent (1960).

Identification of Peak 4

On 10% F-60 TMS-malate is reported to have a methylene unit value of 15.26 (Dalgliesh et al., 1966). This is very close to the value for peak 4. Since F-60 and SE-30 columns are known to exhibit similar properties, authentic TMS-malate was chromatographed on 5% SE-30. The

resulting peak position was identical to that of peak 4, and when TMS-malate was co-chromatographed with TMS derivatives from seed, only one peak appeared in that region. It was assumed that peak 4 was due to TMS-malate, and this was later confirmed by mass spectrometry (Appendix IV).

Identification of Peak 3

Peak 3 was originally believed to be TMS-succinate, but co-chromatography with authentic material produced two peaks, TMS-succinate having a slightly higher methylene unit value (13.22). TMS-phenylacetate was considered as it has a methylene unit value of 13.00 on 10% F-60 (Dalgliesh et al., 1966), but separation from peak 3 could be achieved on 5% SE-30 by programming at 1°/min. from 50° 150°. Many other organic acids were examined, but none matched peak 3.

The methylene unit values of the methyl (Me)-ester, TMS-ether derivatives of organic acids (Appendix Ib) are lower than those of the corresponding TMS-ester, TMS-ether derivatives by approximately 1.00 - 1.27 units per esterifiable group (Dalgliesh et al., 1966). Reductions in retention of this order were noted on 5% SE-30 for the Me-ester TMS-ether derivatives of many known acids and of malate in the seed extract, but no peak appeared which could be assumed to be the Me-ester TMS-ether of the unknown compound.

Aldehyde or keto groups do not readily form TMS derivatives under the conditions used, and quantitative gas-liquid chromatography of the keto acids is unsatisfactory. Peak area and shape can be improved by the formation of the O-methyloxime derivatives with methoxyamine hydrochloride (Appendix IIc). A slight alteration in retention time also

results. When this technique was applied to seed extracts however, no increase in peak area or change in methylene unit value occurred with reference to peaks 3 or 4. Of other acids tested, only TMS-glyoxylate responded. It was thus unlikely that peak 3 contained aldehyde or keto groups.

Attempts were made to isolate the unknown acid by thick layer preparative chromatography to allow characterisation by infra red spectroscopy. Following separation of the acid fraction from seeds in an ethanol ammonia solvent system, malic acid could be easily eluted from the gel with 80% ethanol, and the TMS derivative prepared for gas-liquid chromatography. Approximately 50% recovery was possible. Despite the presence of other acid bands, in particular at the origin, and various fluorescing bands near the solvent front, the unknown acid could not be successfully located and eluted, even when the total extract from 2g. of caryopses had been chromatographed on one plate.

During gas-liquid chromatography of the TMS derivatives of the acid fraction, mass spectra were obtained at 12 positions on the trace. Spectra of the TMS derivatives of 10 aromatic and mono- and di-carboxylic aliphatic organic acids were also obtained. Comparison of the mass spectrum from peak 3 (Appendix IV) with those from known acids, suggested that the acid was not aromatic, but no definite association with any particular aliphatic acid was evident. The prominence of the peaks at 314 and 299 increased the likelihood of their representing M (the molecular ion) and M-15 (loss of Me), in which case the molecular weight would be 314.

Thus the acid under investigation was not aromatic, lacked aldehyde or keto groups, had a methylene unit value of about 13.00 on 5% SE-30, was destroyed or bound on silica gel plates and seemed likely to have a molecular weight of 314.

Canvin (1965) reported a situation, using gas-liquid chromatography of the Me-esters of acid fractions from plant extracts, where a compound was eluted just ahead of Me-succinate and which interfered with the assay of the latter. This compound was more prominent in roots and seeds than in leaves, was not a carboxylic acid (infra-red data) and was later identified as trimethyl phosphate (personal communication from Dr. P.K. Agrawal).

The TMS derivative of phosphoric acid was prepared and could not be separated from peak 3 when co-chromatographed with seed derivatives. A mass spectrum of TMS-phosphate was obtained (Appendix IV) which was identical to that from the compound corresponding to peak 3. Peak 3 therefore represents the phosphate extractable from A. ludoviciana using 80% ethanol.

The highly polar phosphoric acid is relatively immobile on silica gel and in an ethanol ammonia system remains at the origin. Presumably this explains the inability to elute it with 80% ethanol. Subsequently Rf values of about 0.33 were obtained in the butanol-formic system and elution was possible using that solvent.

Table 21.

The malate and phosphate levels in proximal and distal caryopses

Expt.	Caryopses				µg. Malate			µg. Phosphate			Phosphate Malate Ratio
	Type	Germ.	Number	Weight	Extract	/Caryopsis	/Gram	Extract	/Caryopsis	/Gram	
a	Proximal	92	105	2.109	926	8.81	439	2,820	26.85	1,337	3.04
	"	"	107	2.213	876	8.18	395	3,153	29.46	1,424	3.60
	Distal	58	163	2.196	856	5.25	389	2,866	17.58	1,305	3.35
	"	"	172	2.213	860	5.00	388	2,784	16.18	1,258	3.24
b	Proximal	56	133	2.420	1,231	9.25	508	3,581	26.92	1,479	2.91
	"	"	127	2.267	1,020	8.03	449	3,234	25.46	1,426	3.17
	Distal	8	200	2.396	954	4.77	398	2,927	14.63	1,221	3.06
	"	"	209	2.428	988	4.72	406	3,284	15.71	1,352	3.33

Section II : Malate and phosphate levels in A. ludoviciana

Results

Experiment 26. The levels of malate and phosphate in the 80% ethanol extractable material from proximal and distal caryopses after-ripened for 8 months

2g. lots of proximal and distal caryopses were weighed, counted, ground and a known weight of powder extracted in 80% ethanol as described in Materials and Methods. The equivalent number of caryopses taken was calculated. The acid fraction was silylated using hexamethyl disilazine and trichlorosilane. 2 µl. samples were chromatographed on 5% SE-30 along with 2 µl. of TMS-laurate thus permitting quantitation according to the methods detailed in Appendix II. The levels of malic and phosphoric acids per caryopsis and per gram of dry material are recorded in Table 21. The results of two separate experiments, a and b, involving samples after-ripened for 3 and 8 months respectively are reported, along with germination percentages obtained for these samples at the time.

Table 22a

The malate and phosphate levels in proximal caryopses and pales from seed after-ripened for 1 and 12 months

Caryopses

Harvest	% Germination	Replicate	Weight g.	µg. Malate			µg. Phosphate		
				/Extract	/Caryopsis	/Gram	/Extract	/Caryopsis	/Gram
1966	95	A	0.969	420	8.4	434	1,342	26.8	1,384
		B	0.977	369	7.3	378	1,391	27.8	1,423
		C	1.025	301	6.0	294	1,313	26.2	1,280
		Average	0.990	363	7.2	368	1,348	26.9	1,362
1967	17	A	1.022	486	9.7	476	1,251	25.0	1,224
		B	1.130	371	7.4	328	981	19.6	821
		C	1.124	378	7.5	336	1,227	24.5	1,090
		Average	1.092	405	8.2	380	1,153	23.0	1,045

Pales

Harvest	Replicate	Weight g.	µg. Malate			µg. Phosphate		
			/Extract	/Unit of pales	/Gram	/Extract	/Unit of pales	/Gram
1966	A	0.613	264	5.2	430	972	19.4	1,585
	B	0.610	393	7.8	644	921	18.4	1,510
	C	0.626	406	8.1	649	984	19.6	1,571
	Average	0.616	354	7.0	574	959	19.1	1,555
1967	A	0.795	673	13.4	802	709	14.1	892
	B	0.786	717	14.3	912	753	15.0	959
	C	0.721	860	17.2	1,192	785	15.7	1,089
	Average	0.744	750	14.9	968	749	14.9	980

Experiment 27. Analysis of the 80% ethanol extractable malate and phosphate in two seed samples

Caryopses and pales from three replicates of 50 proximal seeds harvested in 1966 and 1967 and after-ripened at room temperature for 12 and 1 month respectively were extracted in 80% ethanol. Following separation of the acid fraction the ammonium salts were silylated and the TMS derivatives chromatographed on 5% SE-30. The amounts of malic and phosphoric acid extracted were derived from the peak area ratios produced, and from these values, extractable acid per unit of caryopsis or pales or per g. of air dry material was calculated (Table 22a). The ratios of phosphate/malate are presented in Table 22b.

Extraction and chromatography of all replicates was conducted simultaneously, but ion-exchange chromatography was carried out in three groups, so that all replicates designated A were chromatographed at the same time and so on. It is evident that both the pales and caryopses of the 1966 sample had more extractable phosphate than the 1967 sample. There was less malate in the pales of the older sample, but levels in the caryopses were inconclusive.

Table 22b.

Phosphate/malate ratios in proximal caryopses and pales from seed after-ripened for 1 and 12 months

Harvest	Replicate	Phosphate/Malate Ratio	
		Caryopses	Pales
1966	A	3.18	3.68
	B	3.76	2.34
	C	4.35	2.42
1967	A	2.57	1.11
	B	2.50	1.05
	C	3.24	0.91

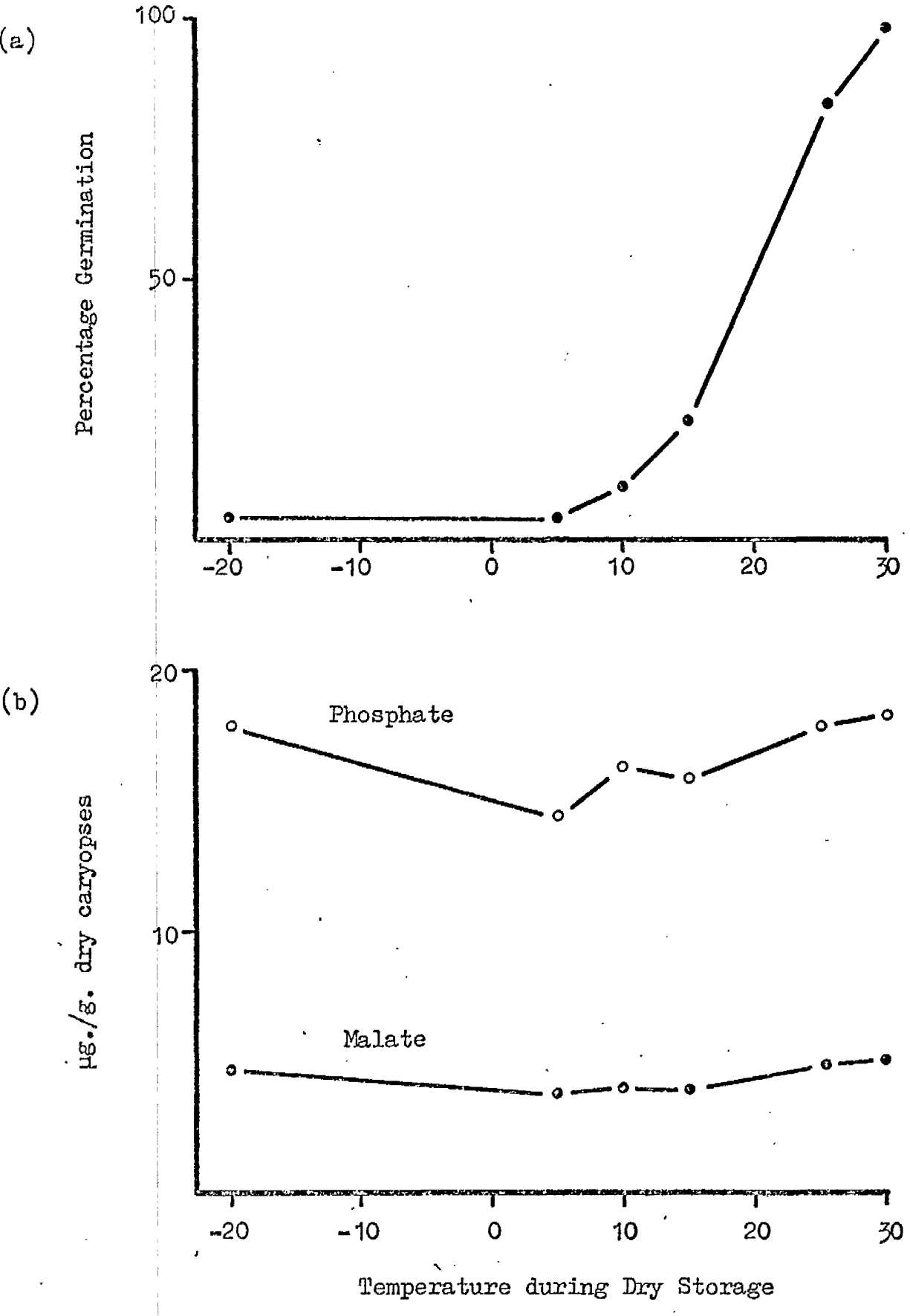


Figure 12. Percentage germination (a) and levels of malate and phosphate acid (b) in proximal caryopses stored at at a range of temperatures.

Experiment 28. The percentage germination and levels of malate and phosphate in proximal caryopses stored at a range of temperatures

Harvest-ripe seed was dried at room temperature for 15 days, at which point 3% germination of proximal caryopses occurred at 10° using standard procedure (b). Batches of approximately 500 spikelets were then stored in sealed containers in the dark at -20, 5, 10, 15, 26 and 30°. The percentage germination at 10° and levels of malate and phosphate in proximal caryopses (approximately 1g.) following 110 days storage are presented in Table 23, and illustrated in Figure 12. It is evident that high temperatures promote after-ripening in dry seed.

Table 23.

Germination percentages and levels of malate and phosphate in proximal caryopses dry stored for 110 days at different temperatures

Storage Temp.	% Germ.	Malate		Phosphate		Phosphate /Malate
		µg./Caryopses	µg./g.	µg./Caryopses	µg./g.	
-20	4	9.67	474	36.51	1,790	3.77
5	4	8.00	380	30.27	1,438	3.78
10	10	8.76	399	35.91	1,636	4.09
15	23	8.11	397	32.28	1,581	3.97
26	84	10.83	493	39.27	1,789	3.62
30	99	11.11	506	40.15	1,829	3.61

Table 24b.

Radioactivity in the total, basic, neutral and acidic fractions of 80% ethanol extractable material from seeds stored at a range of temperatures in the presence of $^{14}CO_2$.

Storage Temperature	Radioactivity (counts/min.)			
	Total	Basic	Neutral	Acidic
-20	2.20	0.49	0.09	0.00
10	3.91	0.26	0.54	1.21
20	6.45	0.00	0.42	3.76
30	7.54	0.34	2.03	9.52

Table 24c.

The levels of malate and phosphate in the acidic fraction of seeds dry stored at different temperatures in air or 0.5% CO_2.

Atmosphere	Storage Temp.	Malate		Phosphate		Phosphate /Malate Ratio
		µg. extracted	µg./g.	µg. extracted	µg./g.	
0.5% CO_2	-20	4,322	720	9,222	1,536	2.13
	10	3,615	602	8,041	1,339	2.22
	20	3,952	658	8,652	1,443	2.19
	30	3,714	619	8,649	1,441	2.32
Air	-20	4,167	697	8,923	1,403	2.01
	10	3,693	615	8,361	1,393	2.26
	20	4,261	710	9,162	1,685	2.37
	30	3,998	666	8,587	1,431	2.14

Experiment 29. Uptake of $^{14}CO_2$ by seeds in dry storage at a range of temperatures

6g. lots of seeds were placed in 100 ml. Quickfit flasks and exposed to an atmosphere of air or air plus 0.5% CO_2, incorporating 0.0382 mc. as $^{14}CO_2$ as described in Materials and Methods. Seeds were stored in darkness at -20, 10, 20 and 30°. After 140 days, percentage germination of proximal and distal seeds at 10° was determined by standard procedure (b) [Table 24a]. At the same time, 80% ethanol extracts were prepared from complete 6g. lots of seeds, and radioactivity in samples of the total, basic, neutral and acidic fractions was measured on an automatic recording low beta counter (IDL). Each group of samples was subjected to at least 10 complete cycles, and the average results are reported in Table 24b. Levels of malate and phosphate were also determined following silylation and gas-liquid chromatography of part of the acidic fractions (Table 24c).

Table 24a.

Percentage germination of proximal and distal seeds after storage at different temperatures in air or 0.5% CO_2

Storage Temperature	Proximal Seeds		Distal Seeds	
	Air	CO_2	Air	CO_2
-20	4	25	2	2
10	18	46	3	5
20	69	74	17	17
30	99	87	26	36

Discussion

The procedure described in this section detected malate and phosphate in seed extracts of A. ludoviciana. Small amounts of succinate, oxalate and citrate were occasionally present, but the appearance of the resulting peaks was very erratic. Nevertheless, the system is capable of detecting virtually all the common organic acids. Holton and Noll (1955) detected phosphate and oxalate in the acid fraction of A. sativa using paper chromatography, but failed to detect malate. The present study and the previous work of Hart (1966) indicates that malate is the major organic acid extractable from wild oats, while in barley, malate is easily the most abundant (Wall et al., 1961).

38-69% of the malate in a whole seed of A. ludoviciana is in the pales (Experiment 27). A comparison of populations with different dormancy levels showed that more malate can be extracted from the less dormant proximal caryopses than from the distal caryopses (Experiment 26). However, year old non-dormant samples may have less extractable malate than dormant samples 1 month after harvest (Experiment 27). Thus in these two experiments, no consistent correlation exists between dormancy and malate level. It is noteworthy that the latter result is in complete contrast to the results of Hart (1966) with A. fatua.

In contrast to the effects of temperature during imbibition (Experiment 1) when seeds are placed in dry storage at a range of

temperatures, dormancy is broken by high temperature storage while the process of after-ripening is retarded by low temperatures (Experiments 28, 29). This agrees with previous work on cereals (Harrington, 1923; Larson et al., 1936; Essery and Pollock, 1956; Roberts, 1962). Malate levels from batches after-ripened to varying degrees by this procedure are seen in Tables 23 and 24c. These are difficult to interpret since high malate values occur in both after-ripened and non after-ripened material.

The presence of 0.5% CO_2 had an inexplicable stimulatory effect on the subsequent germination of proximal seeds (Table 24a). Radioactivity in the fractions indicates that the uptake of CO_2 was temperature dependent and at high temperatures, the bulk of the ^{14}C was in the acid fraction. No correlation between uptake of CO_2 and rise in malate has been demonstrated. The figures presented in Table 24b. are anomalous in that when summed, the radioactivity in the fractions exceeds the total radioactivity in one case, and is lower in the other three cases. It is assumed that these errors are due to the very small amounts of radioactivity present, but do not detract from the trends shown in the total and acidic fractions.

36-43% of the phosphate extractable in 80% ethanol in the seed occurs in the pales. As with malate, slightly more phosphate occurs in the less dormant proximal caryopses (Experiment 26), but in contrast to the malate values, more phosphate was extracted from non-dormant, year old caryopses and their pales than from dormant month-old material (Experiment 27). The phosphate/malate ratios for the dormant and non-

dormant material are thus quite different.

In Experiments 28 and 29, however, the changes in phosphate paralleled those of malate so closely that the ratios remained constant irrespective of temperature treatments. This suggests the possibility that virtually no changes in the level of malate and phosphate occurred during the course of the experiments, unless malate and phosphate levels are related.

Correlations between germinability and acidity have been reported in the review by Fowden and Moses (1960). In A. fatua, Atwood (1914) first recorded a rise in acidity accompanying after-ripening, and in cereals, high citric acid has been correlated with high germinability (Taufel and Pohlondek-Fabini, 1955). These findings are supported by the report of Hart and Berrie (1968) in A. fatua.

Similar increases in malate, malonate and citrate during the after-ripening of Corylus avellana in moist conditions have been reported by Bradbeer and Colman (1967a), while in Prunus cerasus phosphate accumulates (Olney and Pollock, 1959). The breakdown of phytin and the release of inorganic phosphorus characterises the germination of A. sativa (Hall and Hodges, 1966) and cotton (Ergle and Guinn, 1959), while in A. fatua, Simpson (1965) reported that dormant embryos had restricted levels of free phosphate following imbibition. The interaction of inorganic phosphate and GA_3 in seed germination (Hashimoto and Yamaki, 1962) suggests an important role for available phosphate in early germination.

However, the inability to detect such changes in A. ludoviciana

when seed were deliberately after-ripened, suggests that whereas changes in acid levels may accompany after-ripening, they are not pre-requisites for non-dormancy in this species. Nevertheless, the large amounts of available malate and phosphate must have important roles in germination, and the considerable quantities in the pales must be an important factor in determining the environment to which the enclosed caryopsis is subjected.

The uptake of $^{14}CO_2$ may reflect fixation into the organic acid and this suggests that some metabolic activity exists within the seed even in dry storage at high temperature. Investigations on this point are continuing.

Conclusions

(1) The major components in the acidic fraction from an 80% ethanol extract of caryopses were identified as malic and phosphoric acids by combined GLC/MS of the TMS derivatives.

(2) A large proportion of the malate and phosphate in the whole seed is in the pales.

(3) The larger proximal caryopses contain slightly more malate and phosphate per gram of air dry material than the smaller distal caryopses.

(4) The rate of removal of dormancy in dry storage increases with increase in temperature.

(5) No convincing correlation could be detected between malate or phosphate levels and the state of dormancy during after-ripening.

(6) Carbon dioxide is fixed into the acid fraction during after-ripening in dry storage.

PART VI. THE INCEPTION OF DORMANCY DURING SEED MATURATION

Introduction

There are two main methods of investigating the problem of seed dormancy. In the first, the dormant seed is examined in an attempt to characterise both the response to various environments and the physiological state of the dormant condition. This method, involving attempts to alter and remove the dormancy and accompanied by analyses of substances considered to be important, has been used in the first three experimental parts of this work.

The second method involves investigation of the inception of the dormant condition as the seed ripens, by characterisation of the environments which alter the resulting dormancy and by analysis of the changes occurring during ripening. Such methods have been used in this part of the work.

Previous reports concerning the inception of dormancy in this species have included investigation of the effect of neighbouring seeds on dormancy, and the observation that dormancy increased during ripening. These investigations have been repeated. Since some confusion existed as to whether in previous experiments seeds had received a drying treatment before germination, the effect of such a treatment on the resulting dormancy was also examined.

An analysis of the malate and phosphate levels during ripening was carried out to check the possibility of a malate/dormancy correlation and to see if the disappearance of free phosphate, typical of ripening seed, was evident. It was also decided to extend the investigation of

the effect of the ripening environment on dormancy by using the method of Black and Naylor (1959), to examine the effect of substances likely to have importance in the malate system.

Results

Experiment 30. The effect of the absence of one seed, either proximal or distal, on the dormancy of the remaining seed in the spikelet

In a batch of seeds, the majority of spikelets contain both proximal and distal seeds with well developed caryopses. Due to natural causes, a small proportion have no distal caryopses, and a smaller proportion lack proximal caryopses. A batch of seeds stored at room temperature for 4 months was divided into these three categories, and complete spikelets were further separated into their component seeds. Samples of proximal and distal seeds which had had neighbours or had lacked neighbours were then set to germinate at 10° according to standard procedure (b). The results of a typical trial are recorded in Table 25.

Table 25.

Percentage germination of seeds which developed alone or with neighbours

Seed type	Proximal		Distal	
Neighbour	Present	Absent	Present	Absent
Germination	67	81	27	65
χ^2 Present v. Absent	4.39 **		27.56 **	

Experiment 31. The inception of dormancy during ripening

Seeds were collected at various stages of ripeness, and classified according to an arbitrary system using caryopsis and pale scar colour. Proximal caryopses were germinated immediately at 10° (standard procedure b) without a drying treatment. Whole seeds were considered unsuitable as the unripe pales supported vigorous fungal growth.

The results presented in Table 26 indicate that dormancy increases as the caryopsis matures, until at harvest, no germination will occur at 10°. As viability was low in the most immature class, germination is presented as a percentage of all caryopses as well as of viable material.

Table 26.

Percentage germination of proximal caryopses at different stages of ripeness

Class	Colour		Germination as % of		% Viability
	Caryopsis	Pale scar	All caryopses	Viable caryopses	
1	Green	Green	34	49	69
2	Yellow/Green	Green	61	66	92
3	Yellow	Green	31	34	91
4	Brown	Green	4	4	95
5	Brown	Brown	0	0	97

Experiment 32. The effect of a drying treatment on the dormancy of unripe caryopses

Caryopses judged to be in classes 2 and 4 were selected and germinated at 10° according to standard procedure (b), either directly after dehusking, or after drying for 6 days at room temperature. In Table 27, it is seen that the percentage germination of proximal and distal caryopses is significantly lowered by a drying treatment.

Table 27.

Percentage germination of unripe caryopses following a drying treatment

Class	Colour		Proximal		χ^2	Distal		χ^2
	Caryopsis	Pale scar	Not dried	Dried	N.D. v D.	Not dried	Dried	N.D. v D.
2	Yellow/Green	Green	44	12	23.83**	31	4	23.41**
4	Brown	Green	16	6	4.13*	8	1	4.18*

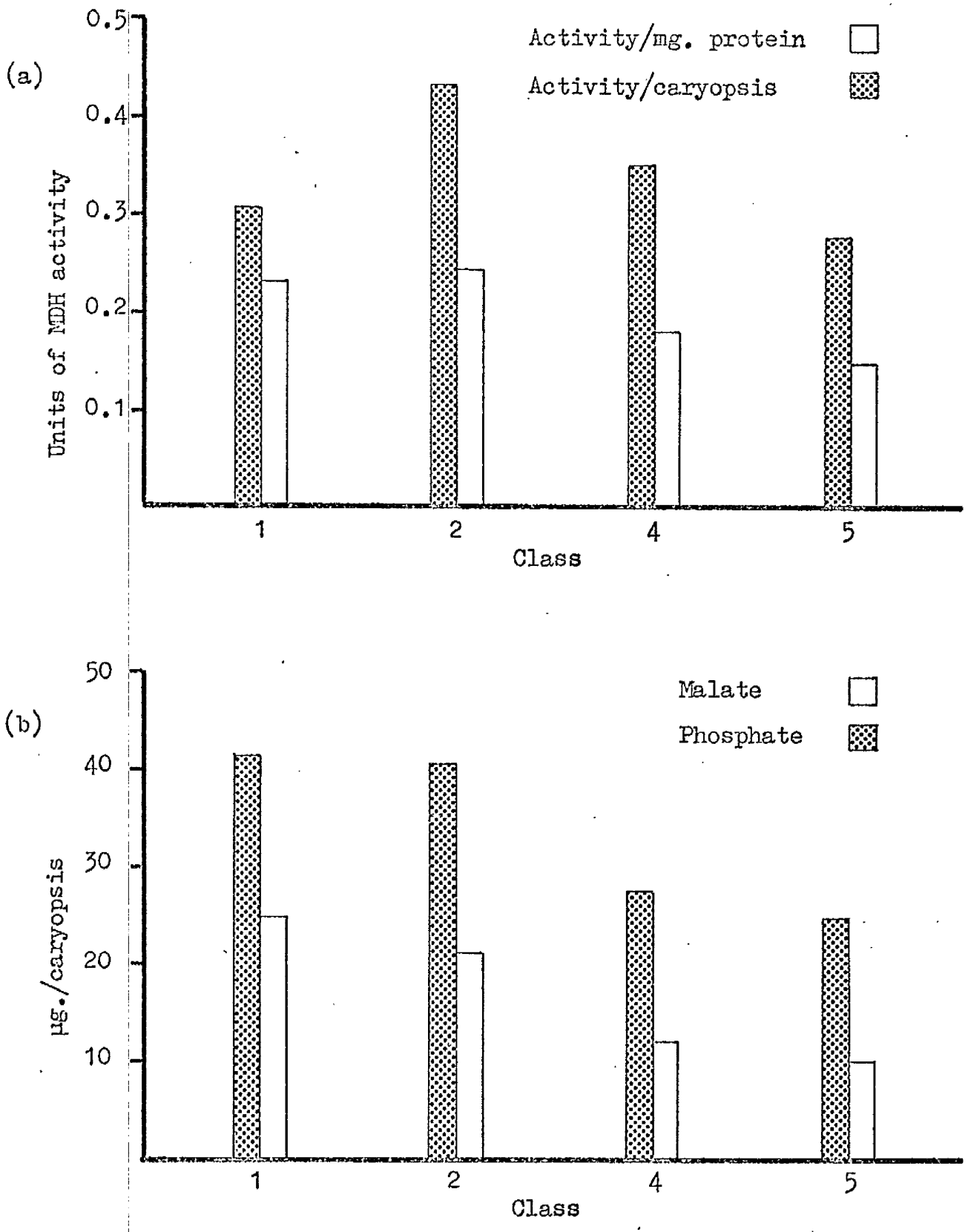


Figure 13. The malate dehydrogenase activity (a) and the 80% ethanol extractable malate and phosphate from ripening caryopses (b).

Experiment 33. Malate dehydrogenase activity and ethanol extractable malate and phosphate levels in ripening caryopses

Lots of 50 proximal caryopses from the seed samples used in Experiment 31 and belonging to classes 1, 2, 4 and 5, were analysed for total fraction malate dehydrogenase activity and for 80% ethanol extractable malate and phosphate levels by the methods previously described. The results of typical trials are recorded in Tables 28a and b and Figure 13.

Table 28a.

Malate dehydrogenase activity in ripening proximal caryopses

Class	Colour		Fresh wt.(g.) /50 caryopses	Activity/ caryopsis	Activity/ g. fresh wt.	Activity/ mg. Protein
	Caryopsis	Pale scar				
1	Green	Green	1.060	0.308	14.535	0.231
2	Yellow/Green	Green	1.366	0.435	15.890	0.242
4	Brown	Green	1.510	0.349	11.572	0.177
5	Brown	Brown	1.388	0.276	9.959	0.144

Table 28b.

Malate and Phosphate levels in ripening proximal caryopses

Class	Fresh wt.(g)/ 50 caryopses	µg. Malate			µg. Phosphate			Phosphate /Malate
		/Extract	/Caryopsis	/g.	/Extract	/Caryopsis	/g.	
1	1.245	1,238	24.76	994	2,067	41.34	1,661	1.67
2	1.406	1,066	21.33	758	2,051	41.02	1,459	1.92
4	1.503	605	12.09	402	1,382	27.64	919	2.28
5	1.364	510	10.20	374	1,241	24.82	958	2.43

Table 29.

Percentage germination of caryopses from panicles ripened in various solutions

Treatment	Concentration		χ^2 treatment v. water	
	10^{-4} M	10^{-3} M	10^{-4} M	10^{-3} M
Malic acid	18	35	0.24	6.99**
Succinic acid	11	24	0.06	2.04
Oxaloacetic acid	18		0.27	
GA_3	43	100	14.24**	110.22**
Coumarin		36		8.52**
PCMB	42	83	13.49**	66.64**
Iodoacetate	12	95	0.00	98.87**
Sodium fluoride	3ϕ		4.04*	
Water	14			

ϕ No reduction in viability occurred in sodium fluoride : all seeds germinated after pricking.

Experiment 34. The effect of various substrates and inhibitors on the inception of dormancy in ripening seed

Panicles of A. ludoviciana in which the uppermost seeds were almost ripe were collected, cut in water below the final node, and placed randomly in 500 ml. Erlenmeyer flasks containing 250 ml. of one of the following: malic acid, succinic acid, GA_3 (as the K-salt), sodium p-chloromercuribenzoate (PCMB) or sodium iodoacetate at 10^{-3} and 10^{-4} M; oxaloacetic acid or sodium fluoride at 10^{-4} M or coumarin at 10^{-3} M. Each flask received 15 panicles, which were then ripened in an unheated glasshouse. Seeds were collected on dehiscence and stored at room temperature. After 30 days, remaining seeds were collected and dried. Little dehiscence or loss of chlorophyll occurred in those in 10^{-3} M GA_3. An abnormally large proportion of one-seeded spikelets occurred, but the usual procedure was followed of accepting only those containing two well formed seeds. Germination of proximal caryopses after a further 15 days at room temperature is recorded in Table 29. It is to be noted that this experiment was performed in late summer 1967, and has not been repeated.

Experiment 35. Germination of caryopses in malic acid, GA_3 and metabolic inhibitors

Caryopses from proximal seed dry stored for three months at room temperature were set to germinate by standard procedure (b) at 10° in the following solutions: malic acid (pH 3.8) GA_3 (as the K-salt), sodium p-chloromercuribenzoate (PCMB), sodium iodoacetate, sodium fluoride, coumarin, all at 10^{-3} M, and water. Germination percentages are recorded in Table 30. Only GA_3 and coumarin had an effect significantly different from that of water.

Table 30.

Percentage germination of proximal caryopses in various solutions

Treatment	% Germination	χ^2 treatment v. water
Malic acid	59	0.33
GA_3	97	32.61**
Coumarin	40	10.59**
PCMB	55	1.32
Iodoacetate	67	0.08
Sodium fluoride	60	0.19
Water	64	

Discussion

Thurston (1962) has shown that proximal seeds of A. ludoviciana become less dormant if the distal seed is removed at an early stage of development. The natural loss of companion seeds also decreases dormancy in the survivors, but the effect is more stiking in lone distal seeds (Experiment 30). This non-dormant material, as a source of heterogeneity, will have increased importance when seed death rates are high, as in Experiment 34. Fortunately, the morphology of A. ludoviciana permits exclusion of neighbourless seeds, but in A. fatua disarticulation of the spikelet means that these less dormant seeds will be included in experimental material.

A plausible explanation for the reduction in dormancy is that during ripening, both seeds act as sinks for a limited supply of a promoting factor. Being larger, and nearer the source, the proximal seed normally receives more than the distal, and thus removal of the proximal has a greater effect on the surviving distal than removal of the distal has on the proximal.

The method used here to measure maturity was chosen both for convenience and because material of the same chronological age from anthesis displayed a range of physiological development, possibly reflecting different rates of ripening on different parts of the panicle. The method adopted considers physiological development only.

As seeds ripen, dormancy increases (Experiment 31). This has previously been reported for A. ludoviciana and A. fatua (Thurston, 1962), and for cereals (Fuchs, 1942; Bishop, 1944). Lack of dormancy in

unripe seeds is most obvious when fresh material is set to germinate. If such seeds are first allowed to dry, dormancy develops (Experiment 32), but the after-ripening process removing dormancy may also begin earlier, as in rice the total time required for after-ripening of unripe seed is similar to that for ripe seed, (Roberts, 1961a).

Ethanol-extractable phosphate levels fall during ripening (Experiment 33). Similar results have been reported in ripening peas (Rowan and Turner, 1957) and rice (Asada and Kasai, 1962). In ripening wheat, a rapid synthesis of phytic acid and decline in inorganic phosphate coincided with the times of restriction of water to the endosperm and pericarp-testa (Jennings and Morton, 1963). Similar changes occurred in A. sativa and A. strigosa after the milk-ripe stage of seed development (Ashton and Williams, 1958). Phytin continued to increase in unripe seed off the mother plant, but slow drying of the seed gave greater increases than rapid drying.

It is not known whether these compounds affect dormancy, but it is evident that as dormancy increases during drying, metabolic activity continues, and that part connected with dormancy is able to function without any translocated influence from the mother plant.

The decline in levels of malate during ripening (Experiment 33), is in agreement with the correlation between malate levels and dormancy advanced by Hart and Berrie (1968). It is not known whether there is a connection between this fall in malate levels and the high level of MDH which occurred prior to this, as ripening began.

The possibility that the increase in dormancy during ripening is due to binding of gibberellins has been discussed in Part I. The dormancy level resulting in the ripened seed of A. fatua can be reduced by ripening

the inflorescences in GA_3 (Black and Naylor, 1959). Dormancy in A. ludoviciana can be similarly reduced by GA_3 (Experiment 34), and as GA_3 promotes germination when present in the petri dish (Experiment 35), the simplest explanation is that the effect is due to the presence of promotive levels of GA_3 in the seed when set to germinate.

More complex explanations are required for malic acid and the metabolic inhibitors. At 10^{-3} M, malic acid reduced the level of dormancy in the ripened seed, and may have resulted in higher malate levels in the seed, although this has not yet been determined. It is noteworthy that neither succinic nor oxaloacetic acids affect the level of dormancy. Exogenous malic acid had no visible effect on germination per se. (Experiment 35). This had previously been demonstrated with A. fatua (Hart, 1966). The absence of an effect is not meaningful however, following the discovery by Hart and Berrie (1968) that no uptake of labelled malic acid occurred during imbibition of A. fatua.

Seeds ripened in PCMB and iodoacetate displayed dramatic reductions in dormancy compared to those ripened in water, whereas sodium fluoride significantly increased the dormancy. No explanation is available for these opposite effects of compounds which generally affect metabolism in a broadly similar manner. When supplied during germination, these three inhibitors had no effect. However, in rice, although neither sodium fluoride nor iodoacetate have effects, PCMB inhibits germination (Roberts, 1964b). Sodium fluoride has no effect on germination of A. fatua (Hart, 1966). It has not yet been demonstrated that any of these compounds can enter the seed during imbibition of A. ludoviciana. Coumarin reduced germination in the petri-dish, but surprisingly, acted like the respiratory

inhibitors PCMB and iodoacetate in reducing dormancy in the ripened seed.

In conclusion, part of the metabolism by which seeds of A. ludoviciana are rendered dormant during ripening includes steps which can be inhibited by some respiratory poisons, and by malic acid. Sodium fluoride, on the other hand, must inhibit different reactions, perhaps preventing the formation of compounds which normally aleviate dormancy, or, by its presence in the seed, inhibiting any after-ripening processes.

The possibility that sulphydryl groups are of consequence at this time suggests the involvement of dehydrogenase enzymes in the inception of dormancy. The effect of malate on the dormancy level considered alongside the fall in malate during the normal imposition of dormancy, further suggests that redox reactions involving malate may have importance in this process.

Conclusions

(1) The absence of either a proximal or a distal seed in a spikelet renders the remaining seed less dormant.

(2) During maturation, the level of dormancy is initially low, but increases until harvest, when dormancy is complete.

(3) Unripe seeds can be made dormant by removal from the mother plant followed by a drying treatment.

(4) Malate dehydrogenase activity is highest as ripening commences, and falls thereafter during ripening.

(5) Malate and phosphate levels are initially high, but decrease during ripening.

(6) GA_3, malic acid, p-chloromercuribenzoate, iodoacetate and coumarin can prevent the inception of dormancy during ripening, but except in the case of GA_3, do not increase germination *per se*.

GENERAL CONCLUSIONS AND DISCUSSION

As discussed in the Introduction the work of Belderok (1961) indicates that the level of dormancy in a harvest-ripe seed population is to some extent dependent on the environment which existed during seed development. These environmental influences appear to have greatest effect when received during early ripening. However in wheat the level of dormancy is initially high and decreases during ripening (Belderok, 1961): in A. ludoviciana the seeds are non-dormant as they become viable and dormancy increases rapidly during ripening, reaching a climax at harvest.

The level of dormancy imposed during ripening depends upon the position of the seed in the spikelet, there being a gradient of dormancy which increases distally. The absence of either a proximal or distal seed renders the remaining seed less dormant.

At harvest, a high level of dormancy is evident, but this dormancy gradually diminishes in dry storage and as an increasing amount of germination is attained, three distinct levels of dormancy become evident in the three types of caryopsis. The dormancy of the caryopsis is reinforced to varying extents by the presence of the pales.

The process of removal of dormancy in dry storage (after-ripening) is dependent on temperature, high temperatures being promotive. The temperature range for germination, although restricted to low temperatures at first, widens during after-ripening to permit some germination at slightly higher temperatures, but the optimum remains extremely low.

Imbibition at high temperature (30°) may accelerate germination in non-dormant samples, but usually prevents germination and may induce dormancy. Light inhibits germination but the inhibitory effect is reduced by the presence of the pales.

The experiments on ripening of neighbourless seeds suggest that the seeds may be regarded as sinks competing for a supply of substances which affect seed development. These substances would enter the seed by translocation from the mother plant. It can be assumed that a larger supply of such substances would be available to single seeds and yet a lower level of dormancy results in these populations. It is thus possible that it is translocation of promoting substances which is influenced by the presence of a neighbour and results in reduction in the dormancy level. Whether or not a factor derived from the mother plant can influence the final dormancy level, the inception of dormancy can occur in the absence of a maternal influence. When immature seeds are ripened off the mother plant, the inception of dormancy and the loss of water are advanced in time compared to natural ripening. How these factors are related to the seed metabolism is unknown.

The removal of dormancy in dry storage infers alteration of substances affecting dormancy within the dry seed. This has mainly been envisaged as the occurrence of oxidative processes involving inhibitors of germination (Wareing and Foda, 1957; Roberts, 1962), but proof of this awaits isolation of active reduced and inactive oxidised forms from seed samples. Further examination of the role of high temperature in this process of after-ripening is required.

It is equally likely that rather than a substance being altered, it is the ability to produce inhibitory compounds which changes during after-ripening.

The opposite concept of alterations in the levels of available promotive compounds has been advanced (Frankland and Wareing, 1962; Simpson, 1965), and it has been demonstrated that GA_3 can prevent the formation of the dormant condition during ripening and can stimulate germination in the petri dish. The interaction of GA_3 with temperature during germination indicates the possibility that gibberellin antagonists may be natural causes of dormancy. Binding of gibberellins is well documented, but no evidence is available for the release of gibberellins during dry storage, other than that of Simpson (1965).

The effectiveness of pricking in overcoming dormancy imposed by high temperature or the pales is obviously due to the release of a powerful stimulatory system within the seed. Explanations may be in stimulation of release of gibberellins as a wound response (Rappaport and Sachs, 1967) or in provision of an oxidative internal environment (Wareing and Foda, 1957).

It is thus possible that during ripening, a supply of promotive substances reaches the spikelet and is distributed unevenly therein. Further ripening or drying alters the situation within the seed with the result that on imbibition, either inhibitory substances are present, or can be produced. Following dry storage, either these substances are removed, perhaps by oxidation, or alternatively the capacity to produce them on imbibition is lost and if sufficient promoting substances are

present, depending on the seed type, germination will proceed. However, imbibition at high temperature or in the presence of the pales may continue to favour the exercise or production of these inhibitory substances.

The level of malic acid in ripening caryopses is initially high, but falls during maturation. It is thus inversely correlated with dormancy. MDH activity is also initially high, and again falls during ripening to the level found in dry caryopses. Malic acid was capable of reducing the formation of dormancy during ripening, whereas succinic acid was unable to do so. The metabolic inhibitors PCMB, iodoacetate and coumarin also prevented the inception of dormancy, but sodium fluoride increased it. In the petri dish however, neither malic acid nor any of the inhibitors save coumarin, had an effect on germination.

During after-ripening malic and phosphoric acids, the major components of the acid fraction, did not alter in a manner which permitted conclusions to be drawn, although slightly more of these acids was recovered from the proximal caryopses than the distal caryopses. MDH activity although present in quantity in dry caryopses did not alter during after-ripening and was not correlated with dormancy in other ways. Separation of four isoenzymes was possible however, and classification of two of these as mitochondrial and two as being derived from the supernatant is described and possible roles in acid metabolism are discussed.

Malic acid and MDH levels are thus correlated with dormancy during ripening but not convincingly in the mature caryopsis. It is possible therefore that the differences noted by Hart (1966) do not in fact

represent changes which occurred during after-ripening, but rather are those which existed from harvest, although perhaps still reflecting the dormancy level. The results presented here for malic acid levels, the effect of malic acid on ripening and the fall in MDH during ripening permit some speculation that malate metabolism has a role in dormancy. The effects of the metabolic inhibitors whether increasing or decreasing dormancy further support the involvement in dormancy of SH-containing enzymes, among which the dehydrogenase are well represented. The inability of malate or other compounds to affect germination in the petri dish could be explained by a lack of entry (Hart and Berrie, 1968), and this may be the result of an effect of pH on weak acids (Simon and Beevers, 1952). The effect of malic as opposed to succinic acid on the inception of dormancy may reflect a situation in which malic differs from other organic acids, as occurs in the selective entry of malic acid to the 'cytoplasmic' pool rather than the mitochondria (Steer and Beevers, 1967).

It is considered that these results warrant -

(a) a further investigation of malate metabolism during ripening in the presence of PCMB, iodoacetate, sodium fluoride and other inhbitors,

(b) a quantitative study of the isoenzymes of MDH and malic enzyme during ripening,

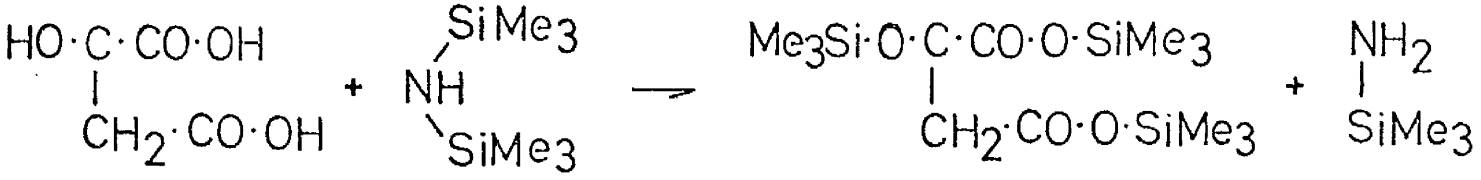
(c) an investigation of malate metabolism during high temperature dormancy induction, and

(d) a search for a method of obtaining entry of malic acid to mature caryopses.

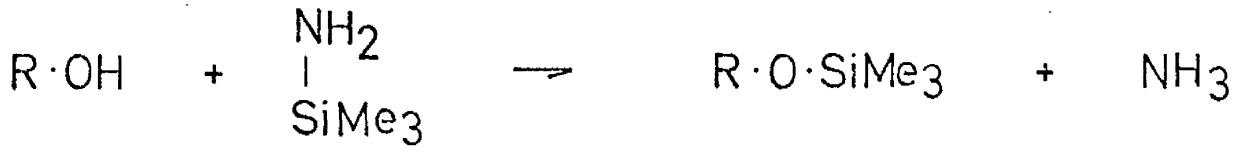
Appendix Ia.

Formation of Trimethylsilyl Esters and Ethers

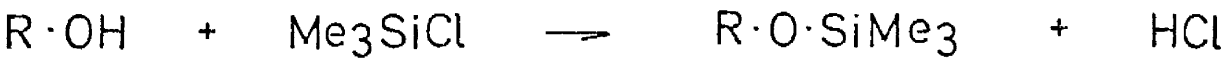
(i) With Hexamethyldisilazine (HMDS) and Trimethylchlorosilane (TMS-Cl)



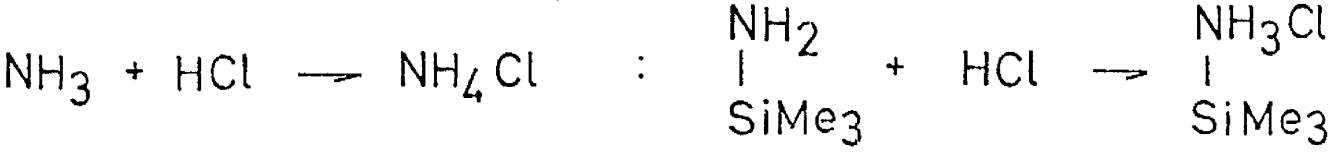
Malic acid HMDS TMS-Malate + Trimethylaminosilane



Trimethylaminosilane TMS-derivative



TMS-Cl TMS-derivative



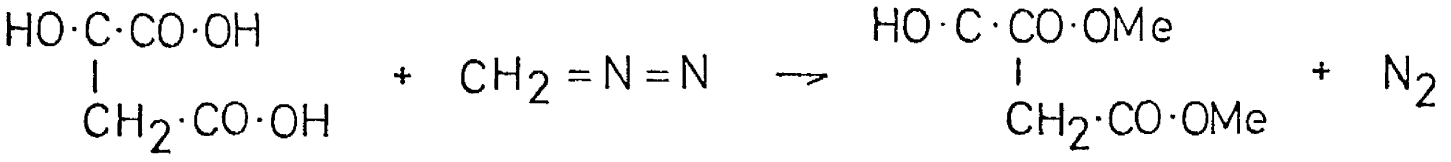
Ammonium chloride and trimethylaminosilane-hydrochloride form a white precipitate which does not interfere with gas-liquid chromatography.

(ii) With Bis-trimethylsilylacetamide (BSA)

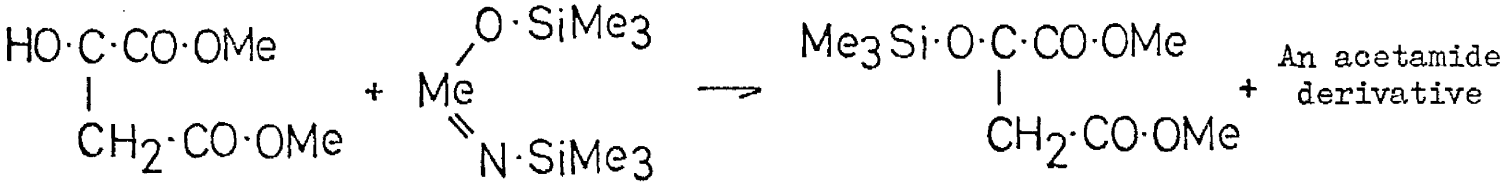
Phosphoric acid BSA TMS-Phosphate

Appendix Ib.

Formation of Methyl Esters and Trimethylsilyl Ethers



Malic acid Diazomethane Malic acid, Me-ester Nitrogen

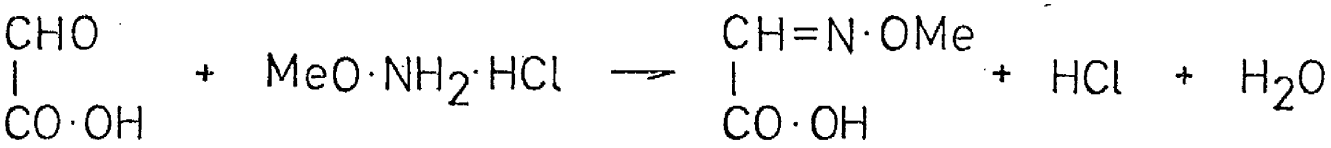


Malic acid, Me-ester BSA Malic acid, Me-ester TMS-ether

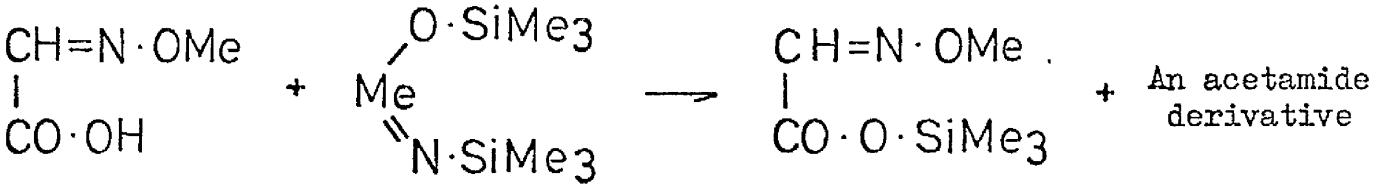
Complete methylation with diazomethane is necessary to prevent the formation of the TMS-ester and ether on the addition of BSA.

Appendix Ic.

Formation of O-methyloxime derivatives



Glyoxylic acid Methoxyamine HCl Glyoxylic acid, O-methyloxime



Glyoxylic acid, O-methyloxime BSA TMS-Glyoxylate, O-methyloxime

Appendix II.

Quantitation of TMS derivatives of malate and phosphate from the peak areas produced in gas-liquid chromatography

In gas-liquid chromatography, ionisation detectors produce an electrical response proportional to the quantity of a compound passing the detector in unit time (measured in gram moles/second). This response, as recorded on the usual strip chart potentiometric recorders, produces a peak, the area of which is directly proportional to the quantity of compound leaving the column and passing the detector.

Calibration of peak area against size of sample introduced for quantitative analysis is rendered difficult since variation can arise from the non-reproduceability of the size of injections at the top of the column, adsorption and decomposition on the support material and variation in detector response to different compounds (Horning et al., 1963). To overcome these problems, a known quantity of internal standard is included in each injection, and the results obtained are expressed relative to the putative quantity of this standard (Horning et al., 1963, Creech, 1964). If the standard chosen has similar chemical structure, functional groups and retention characteristics to those it is intended to analyse, the assumption may be made that both are eluted from the column and detected with equal efficiency.

Consequently, Peak Area Ratios, i.e. peak area of malate or phosphate/peak area of standard, are directly comparable between chromatograms. Differences should then reflect real differences in amounts injected.

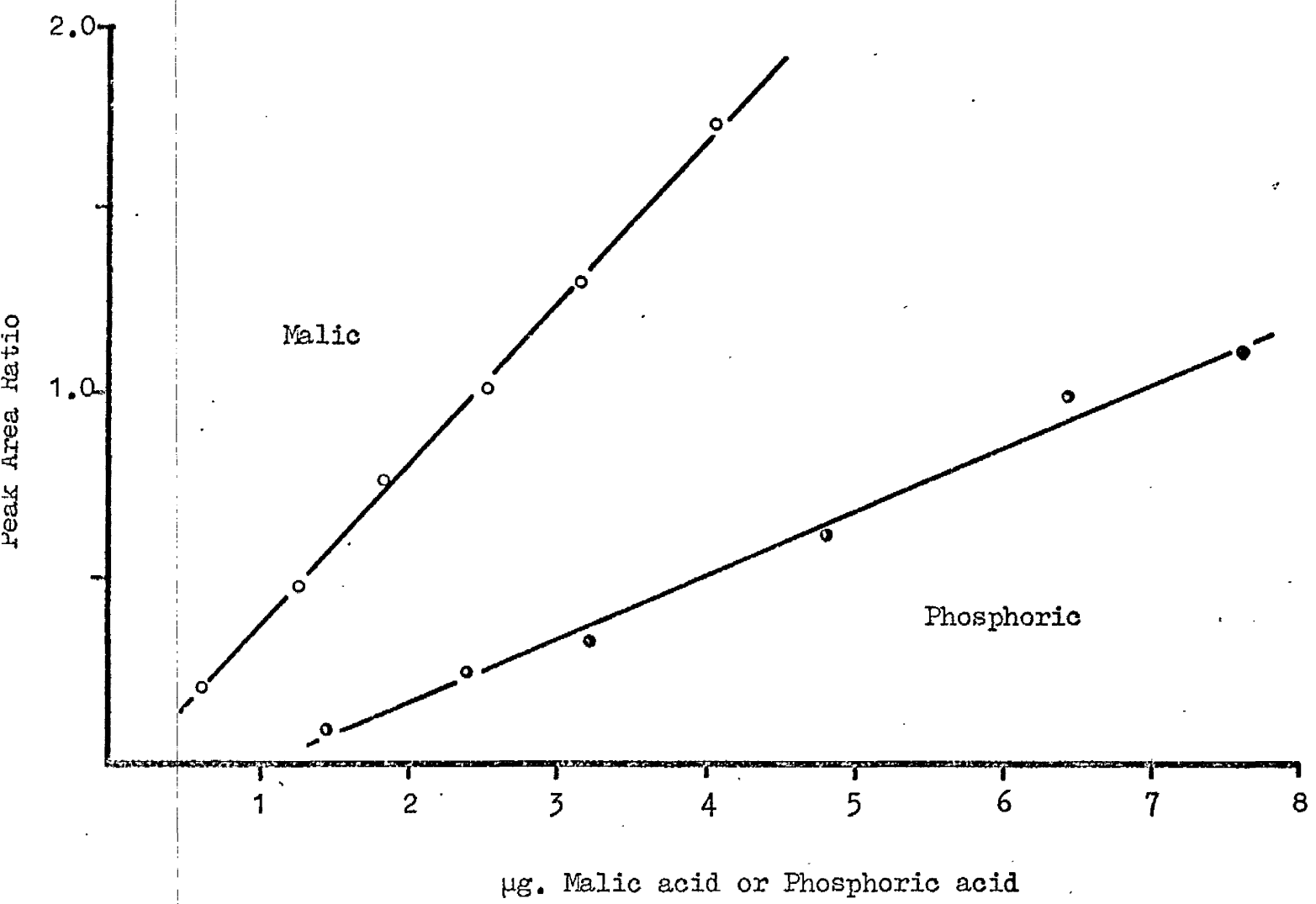


Figure 14. The peak area ratios derived from GLC traces produced following injections of the TMS derivatives of a range of malate or phosphate concentrations, along with a constant amount of TMS-laurate.

Peak area ratios resulting from co-chromatography of a range of concentrations of TMS malate or phosphate with a constant amount of TMS laurate (7 μg.) were calculated, and plotted against the amount of malate or phosphate injected. The resulting curves (Figure 14) permit expression of peak area ratios as μg. of malate or phosphate.

As it is not known how these values reflect the absolute amounts actually present in the seed, they are used for comparative purposes only. However, reconstruction experiments, in which known amounts of acids were subjected to the full extraction process, gave recovery values ranging from 65-78%.

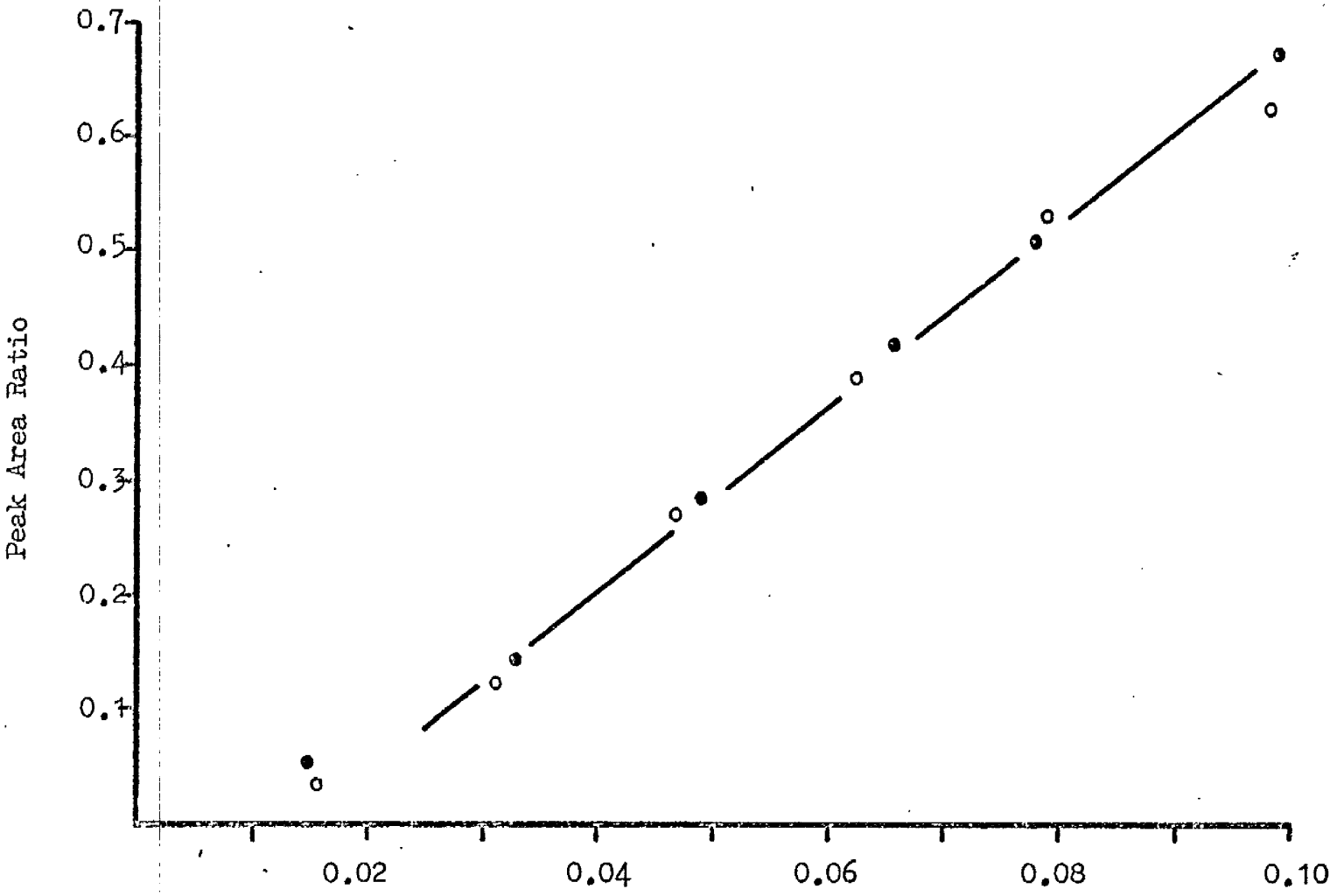


Figure 15. The Peak Area Ratios obtained following gas-liquid chromatography of the TMS derivatives of mono- and di-basic ammonium phosphate.

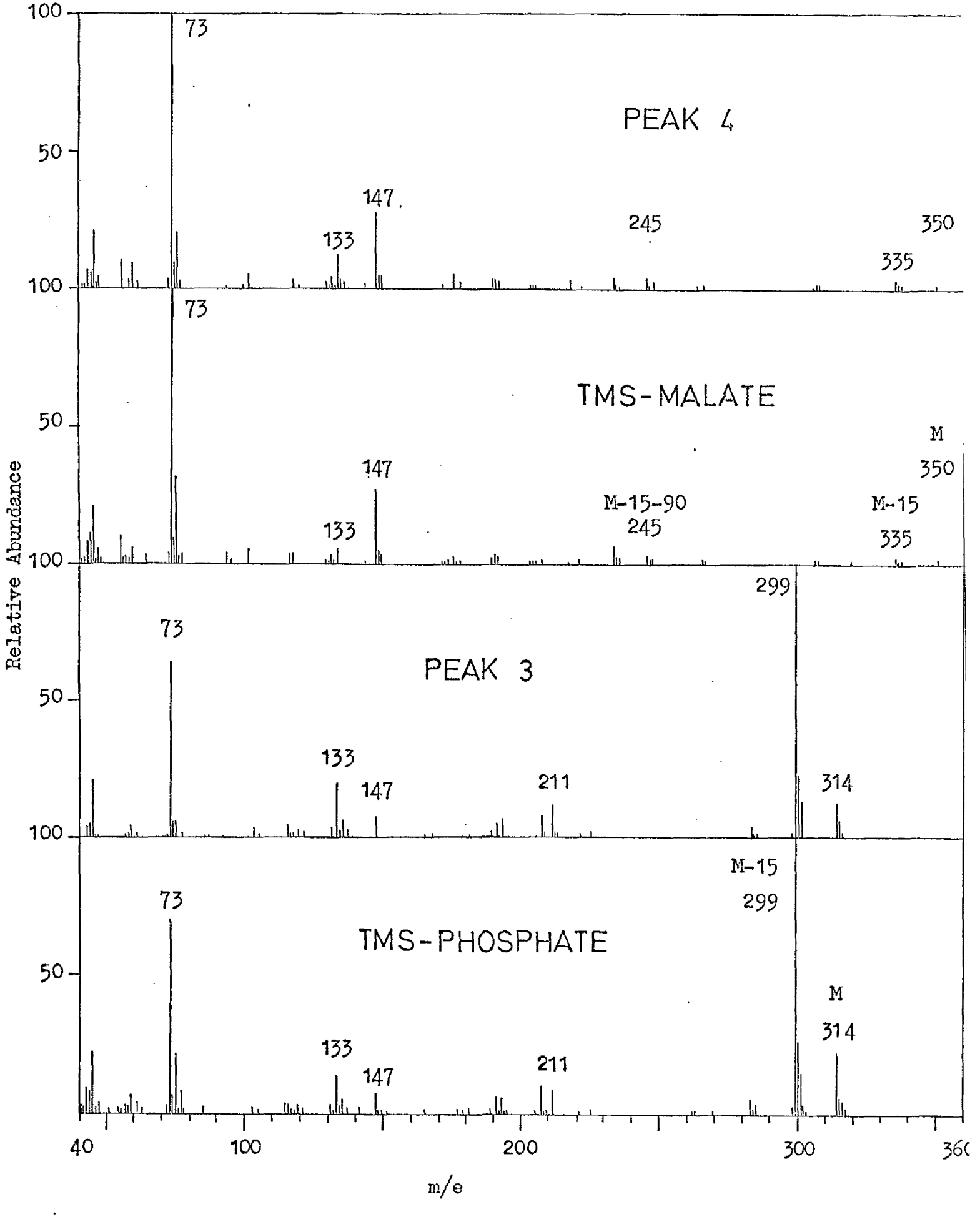
Appendix III.

Silylation of Ammonium Salts

It was discovered that the ammonium salts eluted from the anion exchange column could be silylated with BSA or with HMDS and TMS-Cl as easily as the free acids obtained from a further passage through the cation exchanger. Aliquots of a range of organic acids dissolved in ethanol were evaporated to dryness. Half of the samples were then dissolved in 2N-Ammonium hydroxide and were then dried on a steam bath under a stream of nitrogen. Gas chromatography of the TMS derivatives from the acids and the ammonium salts gave peaks with similar areas. It was thus possible to omit the final step in ion-exchange chromatography to save time and to prevent the small but otherwise unavoidable losses incurred in chromatography.

Silylation of a range of concentrations of ammonium dihydrogen orthophosphate and diammonium hydrogen phosphate was followed by chromatography on 1% SE-30, performed isothermally at 114°. A constant amount of TMS-fumarate was included.

The peak area ratios (TMS-phosphate/TMS-fumarate) for a range of phosphate concentrations are seen in Figure 15. It is evident that equimolar amounts of the two compounds produce equimolar amounts of TMS-phosphate on silylation. No silylation was possible with K, Na, Mg or Ca-salts of phosphoric acid.


PEAK 4
73
147
133
245
350
335
TMS-MALATE
73
147
133
M-15-90
245
M
350
M-15
335
PEAK 3
299
73
133
147
211
314
TMS-PHOSPHATE
M-15
299
73
133
147
211
M
314
Relative Abundance
100
50
40
100
200
300
m/e

Appendix V. Fixation and Embedding of Mitochondrial Pellets for Electron Microscopy

Fixation

Mitochondrial pellets were separated as described previously in phosphate buffer (0.067 M pH 7.3) 0.4 M in sucrose, soaked in this medium containing 2.5% gluteraldehyde at 4° (4 hours) and washed in buffered medium (2 x 6 hours). Buffered medium was replaced by 1% osmium tetroxide in 0.4 M sucrose at 4° (3 hours), the pellet was cut up, returned to osmic medium (2 hours) and finally washed in water (2 x 1 hour).

Embedding

Broken pellets were dehydrated in an alcohol series:- (a) 30% ethanol (15 mins. at 4°), (b) 70% ethanol (12 hours at 4°), (c) 90% ethanol (30 mins. at 4°) and (d) 100% ethanol (2 x 1 hour at room temperature). Alcohol was removed in propylene oxide (2 x 25 mins.) and araldite was introduced in the following series -

Propylene oxide	:	Araldite G + 2% Accelerator
4	:	1 (4 hours)
1	:	1 (12 hours)
1	:	3 (8 hours)

Pellets were then transferred to Araldite G + 2% Accelerator.

Blocks were allowed to harden at room temperature (24 hours), 30° (48 hours), 50° (24 hours) and 60° (at least 24 hours). Araldite G is prepared from Resin CY212 (35 parts) and Hardener DDSA (40 parts).

The above method was devised by Dr. T. Oliver and Miss M. Cox. Sectioning and microscopy were performed by Miss M. Cox.

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