INFLUENCE OF AERATION ON THE DIASTATIC ACTIVITY OF BARLEY DURING STEEPING.

BY R. H. RAMACHANDRA RAU, M.Sc.
AND
V. SUBRAHMANYAN, D.Sc., F.I.C.
Department of Biochemistry, Indian Institute of Science, Bangalore.

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ALTHOUGH the process of steeping has long been a recognised item in malting practice, the precise nature of its influence on the germination of seed and the quality of malt has not yet been fully understood. Thus, it is not known whether the seed respires during that period ; or whether the available supply of air has any bearing on the development of the various ferments associated with germination. Since steeping marks the transition period between the resting and the active conditions, it need hardly be pointed out that the study of the associated changes would be of considerable importance in the understanding of certain hitherto obscure aspects of plant life in general.

The effect of steeping on subsequent germination has been studied by many previous workers. Morinaga (1926) concluded that small seeds germinated better when soaked in water than otherwise. Chippindale (1931, 1934) observed that the germination of *Dactylis glomerata* (Cocksfoot) was considerably accelerated if the seeds had at any previous time been soaked in water and subsequently dried. On the other hand, Rudolfs (1925) found that soaking in distilled water was harmful to the germination of all seeds, corn being the least affected. Kidd and West (1918-19) concluded that soaking peas and beans in large excess of water is harmful. Siddappa (private communication) noted that even overnight steeping had an adverse effect on the germination of barley and the development of seedlings raised therefrom.

Baker and Hulton (1923) and Loibl (1923) observed marked increase in the diastatic power of barley during steeping. Ling (1908), Siau and Hodson (1909) and Bleisch and Will (1909) observed no perceptible effect, while Ford and Guthrie (1908) reported distinct decrease in diastatic activity which they attributed to partial inactivation following the removal of salts. The present authors observed that steeping in water or electrolyte for 24 hours prior to electrical treatment (Venkata Giri and Subrahmanyan, 1931) had a distinct depressive effect on the saccharogenic activity of barley amylase. In view of the conflicting nature of the literature on the question and the consistently depressant effects observed during steeping, it was decided to further investigate the problem with particular reference to the development of diastatic activity under different conditions of steeping.

Although plant amylases (especially those from barley and malt) do not respond to salt activation to such a degree as similar enzymes of animal origin (Bierry and Giaja, 1906 ; Bierry, 1912), it was still considered possible that the loss of such activators during steeping may have a bearing on the lowered diastatic activity. About one-tenth of the mineral substance of barley is lost during steeping (Sykes and Ling, 1907) and in view of the suggestion of Ford and Guthrie (*loc. cit.*), it may be of interest to determine the effect of those minerals as also the minute quantities of organic matter (0·6–1·5 per cent.) thus leached out, on the activity of barley amylase.

Baker and Dick (1905) as also Bleisch and Will (*loc. cit.*) showed that sprouting or chitting of the grain is hastened if barley is ærated during steeping. Pollak (1904) observed that intermittent æration of the steep has a beneficial effect on the liquefying and saccharifying action of green malt during drying and couching. These observations do not, however, show how the favourable effects are produced—whether æration facilitates the respiration of the steeped seed or merely assists in removing certain volatile, toxic products ; whether the development of the enzyme is directly associated with æration or is at least partially independent of it.

It has been suggested by some previous workers (Grüss, 1912 ; Detmer, 1883 ; Baranetzky, 1878 ; Mulder, 1858) that oxygen plays a direct part in the metabolic processes leading to the activation of diastase in germinating seeds. Indeed, Lintner (1890) and later, Lüers (1920) attribute the origin of the enzyme to oxidation of proteins. Since many of the earlier studies were conducted without adequate control of acidity, it is difficult to assess the precise significance of those findings (Waldschmidt-Leitz, 1929). Bach and Oparin (1923, 1924) observed that increase of the partial pressure of oxygen had no stimulating effect on the enzymes of germinating wheat ; oxidase was in fact weakened as a result of the treatment. These observations would appear to contradict the earlier theories regarding the rôle of oxygen. On the other hand, the amylase activity of dead cells was found to be greatly stimulated by oxygen—a phenomenon which, in the light of the previous observation, is difficult to explain. In view of these apparently discordant findings, a systematic study of the effect of air on the development of diastatic power under different conditions of steeping was undertaken. The effects of different treatments on germination were also followed.

Experimental.

Depression of Diastatic Activity during Steeping.—Samples (50 g.) of malting barley from Cawnpore were steeped in water (100 c.c.) for 24 hours in loosely stoppered conical flasks. At the end of that period, the grains in one set were transferred to solution of sodium nitrate (0·1 per cent.) in which they were steeped for a further period of 2 hours. The supernatants in both the sets were then drained off and the grains washed free from adhering electrolyte (as shown by the absence of the phenol-disulphonic acid reaction in the leachate). The two sets of specimens were then freed from adhering moisture by filter paper and finally dried at 30° for 24 hours. As a control, unsoaked barley was also dried under similar conditions. The three sets of specimens were powdered separately, passed through 40 mesh and their diastatic activities determined according to Ling's volumetric method as modified by Hind, Threadgold and Arnold (1926). The results were as follows :—Untreated, 43·3 ; steeped in water, 31·8 ; steeped in nitrate solution, 29·8.

The kinetics of the hydrolysis of starch by enzyme extracts from different sets of specimens was also followed. To mixtures of 40 c.c. of 2 per cent. soluble starch, 10 c.c. of Walpole's acetate buffer (pH 4·6) and 25 c.c. of distilled water, 10 c.c. lots of the enzyme extracts were added and the mixtures maintained in a thermostat at 30°. Aliquots (10 c.c.) of the reaction mixture were taken out at stated intervals, dropped into alkali to stop the enzyme action and the sugar formed estimated according to Bertrand (1910). The following are the velocity constants (K) calculated according to the formula for a monomolecular reaction :—Untreated, 0·02440 ; steeped in water, 0·01660 ; steeped in nitrate solution, 0·01542.

From the above figures it is evident that soaking barley in water or electrolyte has a depressant action on diastatic activity.

Attempted activation of steeped barley by the mineral matter dissolved out in the steep.—After steeping the seed (50 g.) in water for 24 hours, the supernatant liquid was transferred to a tared silica dish and evaporated to dryness. The steeped seeds were then dried and powdered as before. The diastatic activity of the preparation was again determined after addition of the appropriate aliquot of the solution of the salts washed out (10 c.c. for every 5 g. of powder). The value thus obtained was 32·2 which is about the same as that of the specimen steeped in water. It was concluded therefore that leaching out of mineral matter was not responsible for the lowered diastatic activity.

Effect of æration of the steep on diastatic activity of barley.—Further specimens of barley were steeped in 100 c.c. lots of water in a diffuser. Compressed air was bubbled through for all the 24 hours for which the steeping was continued. The drying, extraction and determination of diastatic power were carried out as before. The activity of the preparation was 40·8, which, allowing for the slight inactivation consequent on prolonged drying, is about the same as that of the untreated control. The observations would thus show that the lowered activity resulting from steeping was due to either inadequate æration or the presence of certain volatile substances which paralysed enzyme activity.

Influence of air on the evolution of diastatic activity in barley.—The seeds were steeped under conditions corresponding to (*a*) complete absence of air, (*b*) continuous supply of air in dissolved form and (*c*) continuous æration and the related changes in diastatic power followed at intervals.

It is well known that, when in the wet condition, enzymes are susceptible to inactivation by heat. Previous observations having also suggested that prolonged drying of the steeped seeds prior to extraction leads to partial inactivation of the enzyme, it was decided to carry out subsequent studies on the wet material itself. On adoption of this procedure, determination of enzyme activity on the basis of the weight of the seeds became impossible since different seeds absorb and retain water to different extents. So the activity was determined on the basis of the number of seeds. This method had the additional advantage that the percentage germination could also be followed at the same time. The success of the method obviously depends on an adequate grading of the seeds, the range of whose individual weights ought to be narrow enough so that the weights of large aggregates (say 100 seeds) would not differ so much as to show a significant difference in enzymic activity

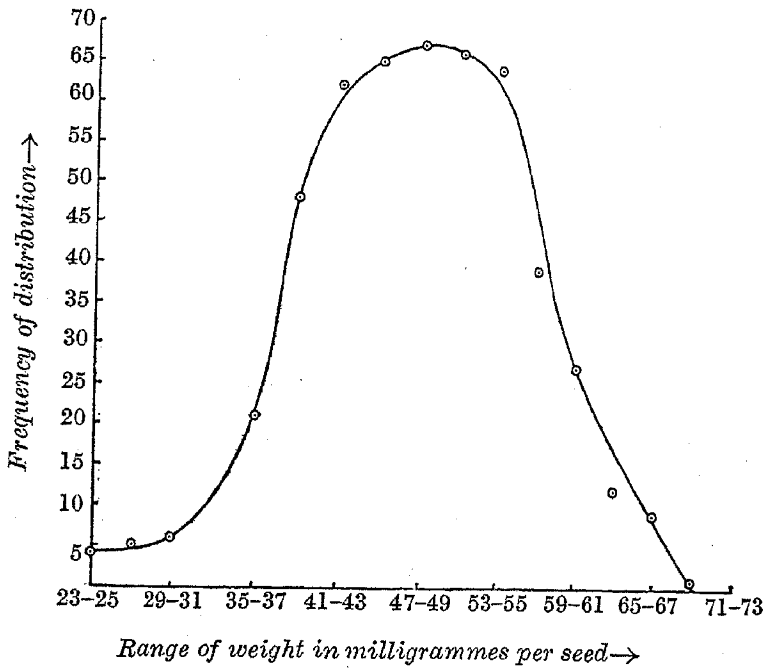

FIG. 1. Frequency Distribution of seed weights.

between parallel samples. With this object in view the frequency distribution of the weight was determined for a large number of seeds. From the curve thus obtained (Fig. 1) it was observed that the maximum number of seeds were found to be distributed between 44–52 mg. per seed. It was decided, therefore, to choose seeds coming within this range. For each experiment, 100 seeds were employed. The variation in activity of a series of 100 seed lots of the graded specimens was next determined. It was found to be a little high and hence the range of weight per seed was further narrowed. Seeds weighing between 47 and 52 mg. were then chosen. This proved to be satisfactory as may be seen from the results given in Table I. This range was therefore adopted.

TABLE I.

Lot Number	1	2	3	4
Maltose formed in terms of c.c. 0·8132 N/10 thiosulphate	2·50	2·60	2·45	2·55

Conditions of steeping.—The seeds were soaked in (1) absence of air, (2) flowing water and (3) water through which air was diffused.

In absence of air.—The graded seeds (100) were placed in a 100 c.c. conical flask which was then completely filled with water which had been first boiled vigorously to expel as much air as possible followed by bubbling of carbon dioxide, reboiling to drive off the gas and finally cooling out of contact with air. The diastatic powers of the steeped seeds were determined at daily intervals.

In flowing water.—A second batch of 100 seeds was placed in a flask through which a gentle stream of water was kept continuously flowing. The steeped specimens were examined as before.

In a diffuser.—The seeds were placed in a small cylinder made of wire-gauze closed at one end. The open end was fitted with a cork. The whole was placed under water in a diffuser and compressed air passed through it for varying periods of time.

Extraction of enzyme.—The conditions of crushing and extraction of the wet seeds had to be carefully standardised, as, otherwise, widely divergent results were obtained for parallel specimens. The following was the procedure finally adopted:—At the end of the period of soaking, the seeds were pressed between folds of filter paper to remove adhering water. They were then crushed about ten at a time in a porcelain mortar. The whole operation was confined to exactly half an hour. The crushed seeds were transferred to a 100 c.c. measuring flask with a little water and the residual contents of the mortar finally washed well with distilled water. The volume of the suspension was made up to 100 c.c. and the flask kept at 30° in a thermostat for 3 hours with occasional shaking. The contents of the flask were then centrifuged, the almost clear supernatant filtered through dry filter paper. The first 20 c.c. of the filtrate was rejected after which the rest of the filtrate was preserved with a little toluene and used for determining diastatic activity.

Estimation of diastatic activity.—In every case, the kinetics of hydrolysis of starch was followed for one hour. The reaction mixture (150 c.c.) consisting of 2 per cent. soluble starch and Walpole's acetate buffer (pH 4·6) was maintained at 30° in a thermostat. To the mixture, 10 c.c. of the enzyme extract was added. Suitable aliquots of the mixture were removed at stated intervals, dropped into decinormal alkali to stop the enzyme action and the sugar contents estimated iodimetrically according to the method of Willstätter and Schudel (1918). From the numbers for the milligrammes of maltose formed, the mono-molecular constant K was calculated for the first 15 minutes of reaction since it is known that the hydrolysis of starch by barley diastase proceeds as a mono-molecular reaction only upto the stage when about 40 per cent. of the theoretical quantity of maltose is produced ; in every case, that stage was attained in about 15 minutes. The results have been presented in Table II.

TABLE II.

Treatment	$K \times 10^4$			
	First day	Second day	Third day	Fourth day
Steeping in absence of air	90·9	123·0	154·1	160·6
,, in flowing water	103·4	132·1	168·0	180·9
,, in the diffuser	110·6	158·1	194·1	205·2
Fresh unsteeped barley	108·5			

Germination.—It was observed that the seeds steeped in the absence of air failed entirely to germinate. In the case of those soaked in flowing water and in the diffuser, germination commenced after about 36 hours; it was 100 per cent. in both those cases.

Discussion of Results.

The initial effect of steeping on diastatic power is one of depression, the effect being most marked in the case of seeds soaked in the absence of air (Fig. 2). The ærated seeds are not adversely affected though even in that case an initial "lag-phase" is definitely noticeable. The velocity constants for the later stages would show that the development

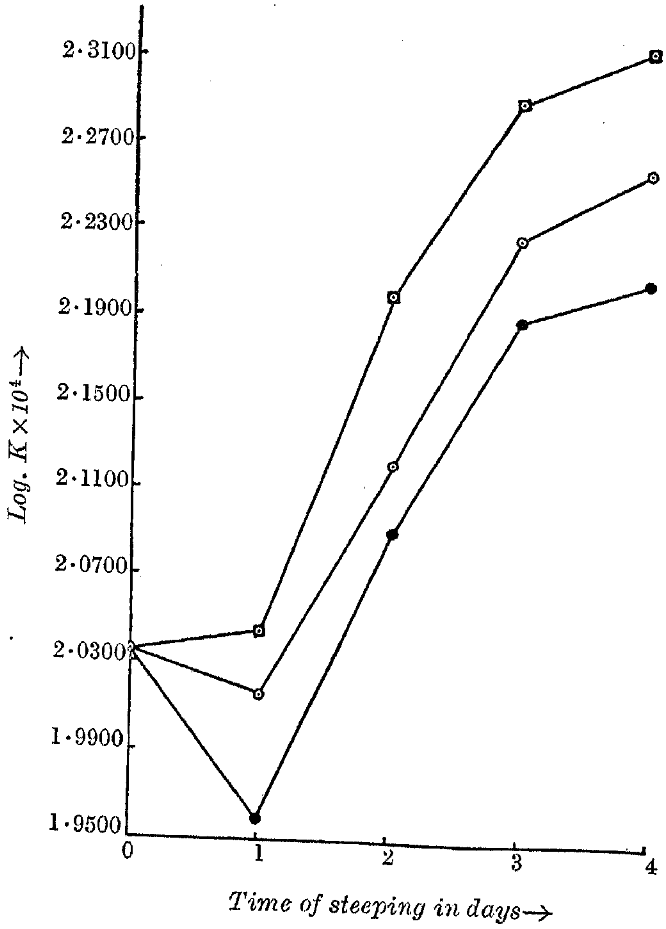

FIG. 2. Development of diastatic activity under different conditions of steeping.
▫—▫ Steeped in diffuser. ⊙—⊙ Steeped in flowing water.
●—● Steeped in absence of air.

of diastatic activity in steeped barley is not dependent on germination; nor is the presence of air essential. These observations are supported by those of Bach and Oparin (*loc. cit.*) who noticed increase in the diastatic power of germinated wheat meal even in an atmosphere of nitrogen. The penetration of water into the resting seed would appear to have led to certain internal changes resulting in increased diastatic power. It is possible that the proteases already present in the seed acted on the protein complex releasing increasing quantities of the strongly adsorbed diastase (Waldschmidt-Leitz, 1931). This explanation would suffice to show why increased diastatic power is observed even in the absence of air. It does not, however, explain the distinctly greater diastatic activity observed in the other two cases.

The flowing stream of water as well as the diffusion of air help to supply fresh quantities of oxygen to the steep. The former brings in dissolved oxygen which, though useful, is obviously not so helpful as the latter which provides liberal amounts of gaseous oxygen. The related observations would show that though not absolutely essential, the supply of oxygen is definitely beneficial to the development of diastatic power in the steeped seed.

It is well known that seeds are capable of internal respiration even in the absence of oxygen. This respiration would appear to be independent of the ability of the seed, as a whole, to germinate and to be stopped only by the complete dismemberment of individual cells or the presence of certain poisonous substances. Assuming that the function of diastase is a part of the respiratory mechanism of the seed, it should be possible to explain not only the foregoing observations but also those of some of the previous workers. On steeping in water, the respiratory activity of the seed is stimulated. If the air supply is adequate, the respiration proceeds by direct oxidation of the soluble carbohydrates made available by diastatic activity. If the air supply is cut off, the seed does not germinate but the life activities continue for a time though not so efficiently as in the presence of abundant supply of air. Instead of direct oxidation, the cells resort to internal decomposition of the soluble carbohydrate matter as observed in the case of yeasts during alcoholic fermentation. Even crushed seed or seed powder is capable of internal respiration because the individual cells are not killed out by the treatment. It is only when the cells are dismembered or the enzymes separated out by extraction that the diastatic power may be expected to remain stationary. It may then decrease owing to ageing and other conditions but not increase of its own accord as observed in all the cases in the present study. This theory though helpful in indicating the nature of the relation between air supply, germination and diastatic power is not fully supported by facts. Further evidence regarding the nature and extent of respiration under different conditions and its bearing on diastatic power is still wanting. The study of these and related aspects of the problem is in progress and will form the subject of a later communication.

Summary.

1. The immediate effect of steeping barley is one of depression of diastatic activity. This is due to inadequate supply of air in the steep and not to removal of any soluble diastase activator as has been suggested by some previous workers.

2. With extended steeping, the diastatic power of the seed shows definite increase, the effect being observed even in the absence of air,

3. Development of diastatic power in the steeped seed is independent of its capacity to germinate. It is favoured by æration but is not entirely dependent on it.

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