A Research into the Amyloclastic Secretory Capacities of the Embryo and Aleurone Layer of *Hordeum* with Special Reference to the Question of the Vitality and Auto-depletion of the Endosperm.

PART I.

BY

F. STOWARD.

CONTENTS OF PART I.

	PAGE
I. INTRODUCTION : Material. Aims and methods of investigation. Sterilization of material. Preparation of sterilized objects for cultural purposes, and investigation of their relative amyolytic secretory or generative capacity. Method of establishing and conducting cultures	799
II. CULTURE MEDIA AND SUBSTRATA. Determination of relative amyloclastic secretory or generative capacities of the structural parts of the seed. Method of investigation of material and culture media for amylase. Method of determining copper-reducing power. Soluble starches employed	806
III. AMYLOCLASTIC SECRETORY CAPACITY OF THE EMBRYO OF BARLEY.	811
IV. AMYLOCLASTIC SECRETORY CAPACITY OF THE ALEURONE LAYER OF BARLEY	817
IV A. THE RELATIVE AMYLOCLASTIC SECRETORY AND SELF-DEPLETIVE CAPACITIES OF THE ENDOSPERM AND THE INNER ENDOSPERM OF BARLEY	824
IV B. CULTURES OF ENDOSPERMS AND INNER ENDOSPERMS ON MOIST CALCIUM SULPHATE	837

I. INTRODUCTION.

THE statement that the digestion and the ultimate depletion of the storage-reserve materials of the endosperms of cereal seeds, during the progress of the germinative process, is induced by enzymes, meets with universal acceptance.

Considerable divergence of opinion, however, exists as to whether the digestive phenomena observed are to be ascribed solely to the action of enzymes which arise as secretions either of the embryo or aleurone layer, or to both, or whether digestion and ultimate depletion of the reserve contents of the inner endosperm¹ are induced solely by enzymes which originate in or are generated by this tissue itself. That is to say, whether the inner

¹ The term 'inner endosperm', employed throughout in this paper, signifies that portion of the endosperm which remains when the endosperm is completely divested of its aleurone layer.

endosperm, independently of the secretions of the embryo and aleurone layer, is capable of digesting its food reserves.

The subject has been attacked from various standpoints by different investigators. A general survey of the observations and results arising from their investigation and their interpretation bears witness to the divergence of the conclusions arrived at.

The earlier attempts to elucidate the interesting but complex phenomena presented by the physiology of germination as exemplified by cereal and other seeds are due to Gris¹ and Van Tieghem.²

Subsequently, more penetrative examination of certain of the aspects of this many-sided phenomenon was undertaken by Brown and Morris,³ Hansteen,⁴ Puriewitsch,⁵ Linz,⁶ Grüss,⁷ Haberlandt,⁸ Brown and Escombe,⁹ Bruschi,¹⁰ and quite recently by Ford and Guthrie.¹¹

Examination of the literature dealing with these different researches shows that the greatest divergence in the conclusions arrived at by the different investigators mentioned centres itself in the question *whether* or *not* the inner endosperm possesses an auto-depletive capacity.

The adherents of the rival hypotheses advanced to elucidate the observed experimental phenomena range themselves into two distinct groups. On the one hand, Brown and Morris and Brown and Escombe claim that the inner endosperm (barley) possesses neither vitality (or at most only the veriest trace) nor a capacity for self-digestion. On the other hand, Hansteen, Puriewitsch, Grüss (barley), and Linz (maize) suggest that the inner endosperm consists of living cells which are capable of digesting their storage reserves.

Bruschi asserts that the inner endosperm (barley) may possess some residual vitality, that part of the tissue to which this attribute is ascribed being the more peripherally situated cells immediately subjacent to the triple-celled aleurone layer.

The twofold claim made by Hansteen and Puriewitsch that the endosperm possesses vitality and also a self-depletive capacity requires closer examination.

This claim may be at once disposed of by stating that the criterion employed by these authors, viz. the demonstration of enzymatic activity in

¹ Gris : Ann. Sci. Nat., Bot., 5^e sér., ii, 1864, p. 90.

² Van Tieghem : Compt. rend., lxxxiv, 1877, p. 582.

³ Brown and Morris : Journ. Chem. Soc., lvii, 1890, p. 458.

⁴ Hansteen : Flora, lxi, 1894, p. 419.

⁵ Puriewitsch : Jahrb. wiss. Bot., xxxi, 1897, p. 13.

⁶ Linz : Jahrb. wiss. Bot., xxix, 1896, p. 267.

⁷ Grüss : Ber. d. D. Bot. Ges., xi, 1893, p. 286; Jahrb. wiss. Bot., xxx, 1897, p. 644.

⁸ Haberlandt : Ber. d. D. Bot. Ges., viii, 1890, p. 441.

⁹ Brown and Escombe : Proc. Roy. Soc. Lond., lxiii, 1898, p. 3.

¹⁰ Bruschi : Ann. Bot., July, 1908.

¹¹ Ford and Guthrie : Journ. Inst. of Brewing, xiv, 1908, p. 61.

a given tissue is no longer regarded as of value for the determination of the possession or non-possession of vitality by that tissue.

The unqualified statement that the endosperm (barley) is auto-depletive is supported by experimental demonstration of the phenomenon, but the conclusion that the demonstration of an auto-digestive capacity for the endosperm necessarily proves the claim that the inner endosperm is capable of inducing the complete self-depletion of its storage reserves independently of the aleurone layer is (as will be shown) probably inadmissible. Again, demonstration of the capacity of a tissue to augment its enzyme-content (such a capacity the inner endosperm possesses) can hardly be claimed to afford adequate evidence of the possession of a capacity for auto-digestion unless it be shown that digestive changes in the storage materials of sufficient magnitude either accompany or immediately succeed this augmentative capacity.

Apart from the work of Brown and Morris (embryo of barley) the methods of determining relative capacities of isolated endosperm and inner endosperm have been either qualitative or of such kind as to afford little or no means of evaluating the relative capacities for doing enzyme-work possessed by the different anatomical parts of the endosperm.

The study of the phenomenon of endospermic depletion as investigated by the method of experiment of Hansteen and Puriewitsch fails to indicate in what relative degree the aleurone layer and inner endosperm participate in or are contributory to the processes involved and take insufficient cognizance of possible differences in the properties and potentialities of the amyloclastic enzymes which originate in these tissues.

In particular the methods employed by Linz and Grüss in experiments with intact isolated endosperms of maize (although separate estimations of the amylase content of the aleurone layer were made at the termination of the germination experiment) are open to the objection that they fail to take account of the amount of enzyme *secreted* by the aleuronic tissue during the period of cultivation. They amount really to demonstrations of the capacity this or that structural part of the seed possesses of augmenting its amylase-content.

Similarly Grüss's demonstration of the presence of copper-reducing substances in thin sections taken from isolated endosperms under germination conditions, in the absence of the removal of the aleurone layer, fails to show that the apparent hydrolysis of starch implied by these experiments was due solely to an amyloclastic enzyme generated *in situ*.

Material. Two barleys have served as experimental material throughout the course of investigation, the one a North African and the other a brewing Chilian.

Both were mixtures of *H. vulgare* and *H. hexastichum* of the year, possessing the customary characteristics of barleys from these sources

of supply, huskiness and thick skin. Their germinative capacities were satisfactory and were subjected to frequent trials at intervals throughout the course of the inquiry.

Aims and methods of investigation. The principal aims of the investigation have been to endeavour to (1) show whence the amyloclastic enzymes are derived which are operative in influencing digestion of the amyloferous storage reserves of the inner endosperm and to (2) demonstrate in an approximate manner the contributory shares taken by these enzymes in the work of endospermic depletion.

Three possible sources of amylolytic enzyme apparently exist, the scutellar epithelium, aleurone layer, and inner endosperm.

The enzymes furnished by the two former are elaborated by cells which possess a secretory mechanism, and the continuance of their secretory activity depends upon their vitality. The amylases from both these sources are characterized by the possession of a marked power of attack on the *mature starch grains of the inner endosperm* under conditions which are approximately identical with those which obtain during the progress of normal germination.

The increase of enzyme pre-existent in the inner endosperm (augmentation of which occurs under certain experimental conditions to be described) does not with certainty depend upon the possession of *an undoubted secretory capacity* by this tissue; on the contrary, this enzyme possesses but feeble powers of attack on the fully formed starch grains which form by far the greater bulk of the storage reserves of the mature grain.

The augmentation of enzyme and the comparatively restricted amount of amylolytic change it is capable of inducing are therefore preferably referred to as being due to the generative or auto-digestive capacity of this tissue.

At the outset the fact must be clearly emphasized that the amylase which originates solely in the inner endosperm is sharply differentiated from those which are elaborated by the scutellar tissue and aleurone layer by its feebleness of attacking, under comparable experimental conditions, the fully formed starch grains of the mature inner endosperm, and the adequate recognition of the differential powers of attack on the mature starch grain possessed by these amylolytic enzymes is, as we shall see later, of prime importance in any attempt to elucidate and explain the phenomenon of starch digestion of the endospermic starch reserves, and we shall further see that the mere capacity of a given tissue to augment its amylase-content is of subordinate importance unless this augmentation of enzyme is accompanied by evidence of a commensurate increase in the amount of starch digestion.

The interest attached to the work of Ford and Guthrie (l. c.) relates to amylase of the resting grain and the possible rôle it may play in inner

endospermic depletion. Fuller reference to this phase of the question may, however, be deferred until experimental evidence has been adduced which bears on the nature of the amylase existent in the inner endosperm and the behaviour of the isolated tissue under the variable experimental conditions selected.

The chief result of their interesting inquiry is to demonstrate that under certain conditions of extraction the amylase of the resting seed undergoes a tangible *increase in quantity*.

That the embryo secretes an active amyloclastic enzyme, the secretory function being localized in the scutellar epithelium, has been convincingly demonstrated by the investigations of Brown and Morris (l. c.).

The glandular nature and secretory capacity of the aleurone layer has been rendered highly probable by the work of Brown and Escombe (barley) and Haberlandt (rye), and suggested by the researches of Linz (maize) and Grüss (barley), but opposed by Hansteen and Puriewitsch, who contend that its enzyme-secretory or generative capacity is not superior to that possessed by the amyliiferous cells, in fact that it (the aleurone layer) represents physiologically an undifferentiated portion of the reserve system of the seed.

Throughout, the capacity to secrete or generate amylase possessed by the different anatomical parts of the grain (embryo, aleurone layer, endosperm, and inner endosperm) has been investigated as far as possible *quantitatively*. The methods devised and adopted comprised the preparation of sterile material, the establishment of cultures of isolated parts of the seed on nutrient or experimental substrata and their maintenance under *aseptic* conditions, and the subsequent *quantitative* investigation of both objects and media either for amylolytic enzyme or the products or evidence of amylolytic activity.

Sterilization of material. Sterilization of grains was necessarily resorted to to eliminate the possibility of the disturbing influence of micro-organisms, and because of the inadmissibility of the employment in the media of the experiments to be described of antiseptic reagents even in minute concentrations.

Further, in certain instances, notably in the case of experiments with inner endosperms, it was necessary to carry out cultural experiments of lengthy duration in order to afford these objects full opportunity of displaying their presumed powers of auto-depletion.

Intact air-dried seeds were subjected to a preliminary steeping in either¹ 10 % CuSO_4 or ² absolute alcohol for periods of time varying from 24 to 72 hours.

¹ Latterly, in order to provide for the more efficient aeration of the material, the closely fitting stopper was removed and the bottle covered with a sterilized funnel.

² Wherever departure from the method described above has been made reference is made to the fact in the text.

Immediately after removal of the objects from the copper sulphate solution, they were divested of their paleae, and either washed as thoroughly as possible with sterilized tap-water only or washed and re-steeped in tap-water for 24 to 48 hours.

When absolute alcohol served as the antiseptic steeping reagent this re-steeping in water was invariably resorted to, and subsequent experience demonstrated the value of employing two to three changes of water or of placing the seeds under aeration conditions for 24 hours after removal from the water-steep.

For special purposes endosperms and inner endosperms prepared from air-dried seeds were successively steeped in absolute alcohol and water. In every instance the volume of steeping liquid used was just sufficient to cover the material. Small well-stoppered weighing bottles were employed, and each phase of the operation was carried out under strictly aseptic conditions.

The efficiency of the reagents employed and the method of manipulation followed was amply foreshadowed by the results furnished by several series of preliminary experiments.

Seeds treated as described were placed in tubes containing various nutrient media, and were then incubated at 30° C. for 7 to 14 days. In the majority of instances the contents of the tubes remained absolutely sterile:—in a small percentage only, moulds, but not bacterial forms developed, thus demonstrating the fact that the spores of the former organisms alone survived the toxic action of the reagents employed.

Preparation of sterilized objects for cultural purposes and investigation of their relative amylolytic secretory or generative capacities. The dissection of the steeped sterilized material was carried out in a Hansen inoculation chamber under the following aseptic conditions:—

A flat glass plate was placed on the floor of the chamber (the interior of which, just prior to use, was thoroughly swabbed with 1 % HgCl_2 solution) and covered with an inverted glass funnel. The depaled, steeped, thoroughly washed grains were then introduced *en masse* under the funnel.

Each grain was then successively withdrawn, dissected as required, and returned under the funnel until a sufficient number of objects for a culture experiment were prepared.

Dissections of grains into embryos and endosperms were accomplished by cutting through the integuments in the neighbourhood of the scutellar margin and gently lifting out the embryo with a scalpel: the further dissection of the endosperm into inner endosperm and aleurone layer was carried out by carefully cutting off the latter with a sharp razor in such a manner as to include the merest traces only of the subjacent amyliiferous tissue.¹

¹ This was subsequently controlled by filing off the aleurone layer of a number of air-dried grains and comparing the dry weights of this material after desiccation for 24 hours at 30° C., with

These somewhat tedious operations, affording by far the chief opportunity for reinfection of the material, were carried out with special care and precautions, the apparatus, dissecting instruments, and the operator's hands being, just prior to the commencement of the operation, thoroughly sterilized by passage through the flame of a Bunsen burner.

It is of interest to note that the successful sterilization of the grains turns on the discovery by A. J. Brown¹ of the existence of a semipermeable or selective property possessed by one of the integuments of the seed. The steeping of intact uninjured grains in the reagents mentioned is thereby rendered possible, as neither the copper sulphate nor absolute alcohol penetrates into the interior of the grain.

Method of establishing and conducting cultures. The sterilized material was quickly transferred to the liquid or semi-solid culture medium contained in small Petri dishes; either a definite number (5 or some multiple of 5) of objects (embryos and aleurone layer fragments) were placed on the surface of the medium, scutellar or inner surface respectively downwards, or a definite number of inner endosperms and endosperms were embedded in a thin stratum of semi-solid culture medium of just sufficient depth to cover them completely.

Other methods of establishment of the culture having reference to special experiments are described in the sections dealing with these experiments.

The cultures thus established were conducted *under absolutely sterile conditions*, at the ordinary temperature of the laboratory (15° to 18° C.), for periods of time varying in duration from 3 to 68 days.

In the case of cultures on liquid media the sterility of the culture medium was, at the termination of the culture experiment, controlled by removing a small quantity (0.5 c.c.) and introducing it into a series of tubes containing various nutrient media—dextrose-wort, dextrose-maltose yeast water, &c.)—and subsequently incubating these tubes at 30° C. for 7 to 21 days.

Such inoculations invariably furnished negative results, affording in these instances ample confirmation of sterility of the culture already indicated by macroscopic inspection.

The subsequent employment of semi-solid gelatine and agar medium rendered this system of control unnecessary, and, moreover, provided more favourable conditions for the display of the vital activity of certain of the objects under culture.

Numerous series of petty cultures were also instituted with embryo and aleurone layer fragments for the purpose of ascertaining whether or not the scutellar epithelium and aleurone layer, as stated by Brown and Morris and

that of an equal number of aleurone layers removed by cutting in the manner described above. The difference in no case amounted to more than 4 to 5 %, the former value being the nearer.

¹ Ann. Bot., xxi, p. 79 (1907).

Brown and Escombe respectively, secrete a cytoclastic enzyme, and also to study the mode of attack on starch grains of the amyloclastic enzymes which these tissues secrete.

The data derived from these variously conducted experiments and their discussion are embodied in the separate sections which follow.

II. CULTURE MEDIA AND SUBSTRATA.

Variously constituted culture media and cultural conditions have, during the course of the inquiry, been employed.

Reference need only be made at this juncture to the composition of the medium which has served as the *basis* of those used in the majority of the culture experiments to be described. Wherever modification of its composition, either by the inclusion of other substances or change of concentration of a particular component, has been made, attention is directed to the fact.

The composition of the medium shortly referred to in this paper as the 'mineral salt solution', or more briefly, as 'M.S. solution', is the following:—

Per 100 c.c. of solution.¹

Calcium sulphate	0.100 gm.
Potassium chloride	0.25
Magnesium sulphate	0.25
Potassium dihydrogen phosphate	0.025

2 drops of $N/5 \text{ Fe}_2\text{Cl}_6$ per litre of solution.

Method of investigating material and culture media for amylase. At the termination of the culture experiment the objects and corresponding culture medium from a given plate culture were separately investigated for amylase by digestion with soluble starch under definite and comparable conditions of time, temperature, and concentration of starch solution.

These digestions were carried out in the following uniform manner:— Into each of a series of 50 c.c. conical Jena flasks, previously cleaned by steaming them out, 25–30 c.c. of freshly prepared soluble starch containing 3 gm. starch solids per 100 c.c. of solution were pipetted. The flasks and their contents with the addition of a drop of antiseptic (nitrobenzene or toluene) were at once transferred to a thermostat regulated at 30° C.²

Those required for the *control* starch digestions, before addition of the enzyme-containing material, were rendered distinctly, but not excessively, alkaline by the addition of a few drops of 30 %–40 % NaOH solution.

The objects, previously desiccated for some hours (5–20) at 30° C., were divided into two portions, each consisting of an equal number and representing one-half the total number on the plate, were separately and uniformly

¹ Trans. of Guinness Research Lab., vol. i, Part II, p. 290, Brown, Millar, McMullen, and Escombe.

² Unless otherwise stated, it is to be understood that all starch digestions referred to in the text were carried out at this temperature; the temperature of the thermostat never varied throughout by more than + or – 0.5° C.

ground up with chemically purified sand in a porcelain mortar. When the flasks and their contents had acquired the temperature of the thermostat (usually after an interval of 10–15 minutes) the finely ground material, placed on a small paper chute, was brushed little by little into the digestion flask, thoroughly distributed, and by constant agitation mixed with the starch solution.¹

One half of the material serves as the *experimental* digestion; the other, added to the alkalinized starch solution, as the corresponding *control* digestion. The latter, invariably carried out in all these digestion experiments, affords the means of correcting for the reducing substances pre-existent in the starch solution and added material.²

The culture liquid from a given plate was transferred to a calibrated tube and its volume brought to 20 c.c. with distilled water.³ The tube and its contents were then transferred to the thermostat, and having attained the desired temperature, one half of this volume (experimental digest, 10 c.c.), representing the amount of amylase derived from one-half the total number of objects under culture, was added and thoroughly mixed with the unalkalinized starch solution, the other half (control digest) being added to the alkalinized starch solution. The flasks were then securely corked and the digestion continued for definite but variable periods of time (varying necessarily according to the amylolytic capacities of the material under investigation), at the conclusion of which the experimental digests were arrested by one or other of the methods already described. Finally, the digests were cooled, diluted to 100 c.c., filtered, and the copper-reducing power determined on an aliquot portion of the *clear* filtrate.

The amyloclastic capacity deduced from the experimental data thus determined is expressed throughout in this paper in terms of the weight of copper found, in milligrammes, and by calculation referred to the generative or secretory capacity possessed or displayed by twenty objects, and further reduced to a comparable basis by calculating the output (amylase in the culture medium) or augmentation (amylase in the tissue of enzyme) for a uniform period of digestion of one hour.

Throughout it is to be noted that the evaluation of this capacity refers

¹ Unless this method of transferring the material is adhered to (i.e. if the finely ground material is added to the starch solution *en masse*) 'balling' invariably ensues, and this may be so pronounced as to prevent a portion of the enzyme from gaining access to the starch solution during the earlier phase of the digestion, and may constitute a source of quite considerable error.

² For the purpose of checking this method of arresting amylolytic action, and further to unmask any possible action of the alkali on reducing sugars present, in many instances the controls were also checked by *boiling* the mixture of starch solution and material. No objection can be raised to the former method provided the use of large excess of alkali is avoided. The foregoing statement applies also to the arrests of the experimental digests by the alkali method.

³ The distilled water employed in these experiments in the preparation of starch and nutrient solutions was prepared by means of a copper still modelled specially on the design given by Ford (Journ. Inst. of Brewing, ix, 1907 (5), p. 206).

to a definite number of organisms (embryos) or structural parts (aleurone layers, endosperms, inner endosperms) of the seed. The system adopted appeared to be preferable to that of referring it to *definite weights* of the various objects. If weight of material is taken as the basis of reference, several well-founded objections present themselves. Determination of definite weights of material necessitates desiccation under fixed conditions of time and temperature, and even if these be well below that calculated to be dangerous to the activity of the enzyme present, it does not follow that the enzyme may not undergo, as the result of influence of heat, inactivation or actual destruction. Moreover, the possibility of the desiccation of material of such different origin and constitution in such a manner as to render the weights comparable is excluded by the fact that loss of moisture (if the loss is wholly to be attributed to this) by such diverse material is by no means uniform, and consequently the residual weights of the various structural parts are more or less inadmissible as a basis for calculating their enzymatic capacities.

Were such comparisons confined to objects of the same kind, as, for example, isolated endosperms under cultural conditions to be described, the objection still would remain. The individuality possessed by the objects, the varying degree to which digestive changes progress, leading at every stage to variable proportions of unchanged and partly changed starch, the outward diffusion of products of change, all conspire to render the expression of result on any other than the numerical basis alluded to inadmissible and impracticable.

The manner of expressing results may perhaps be rendered clearer by taking an illustrative example:—a plate culture consisting of ten aleurone layers is established, continued for several days and finally terminated by removal of the cultural objects. Objects and medium are separately digested with soluble starch solution for a period of *half an hour* and the amount of reducing sugars determined and corrected in the manner described. In the experimental and control digestions five objects each are used; in the similarly conducted digestions with the diluted culture medium one half (10 c.c.) serves for the experimental, the other half (10 c.c.) for the control starch digestion, each half-volume of the total diluted culture medium obviously containing an amount of amylase derived from one-half (five objects) the total number of objects on the plate.

Let us suppose that the copper reduction values of the objects and medium after applying the control corrections are x and y milligrammes of copper respectively, then we shall have:—

Amylase per 20 objects per hour

in objects equivalent to $(x \times \frac{20}{5} \times \frac{1}{2} \times 2) = 4x$ milligrammes Cu.

Amylase per 20 objects per hour

in culture medium equivalent to $(y \times \frac{20}{5} \times \frac{1}{2} \times 2) = 4y$ milligrammes Cu.

The above example will serve to explain the mode of expression used in the tabular statements in the different sections descriptive of experimental work.

It is further assumed that, at least in the majority of instances, the augmentations of amyloclastic enzyme unmasked under the conditions of experiment are actually due to an increase in amount of the enzyme itself and not to activation of a non-increasing mass of enzyme. That is to say, production of enzyme as opposed to augmentation of enzymatic activity is implied, although it is conceded that the latter phenomenon may in the ordinary course of events obtrude itself, but purely in a subordinate measure.

It is conceivable that during the various progressive phases of culture, e. g. of isolated endosperms, the chemical and physical changes in residual endospermic substance may at each phase influence its amyloclastic activity, some idea of the extent to which this occurs being rendered evident by starch digestion experiments. The chief factors comprised here are actual augmentation of amylase together with modification of its activity by concomitant changes in the reaction medium induced by changes in the chemical and physical properties of the material under examination.

Inactivation of an enzyme can readily be induced, the agent and its approximate amount being readily enough determined; it is otherwise, however, when we turn to the question of the activation of an enzyme. The view held here is that a given enzyme possesses a finite capacity for work which does not exceed certain limits, and the display of this maximal capacity depends upon the provision of optimal working conditions. Wherever apparent activation occurs it is because in the previous conditions of experiment a retarding factor or factors were included.

In dealing with material possessing such variable amyloclastic capacities as structural parts of seed, the adoption of the foregoing method of expressing results is permissible. In order to meet the changes in magnitude of this capacity modifications in the method of starch digestion are unavoidable. Moreover, for the purpose of rendering the results of one series of experiments comparable with those of others, the conditions must be selected in such manner that the *R* of the mixed starch digestion products does not exceed the limiting value indicated by Kjeldahl's 'proportionality law', and this condition has been strictly adhered to throughout the course of this inquiry.

Experiments such as those briefly outlined afford striking evidence of the individuality of the objects which have been subjected to examination, and this factor alone probably overshadows those which may intervene during starch digestion, conditioned by alteration in number of objects, time, and concentration of starch employed.

The selection of what may appear to be comparatively small numbers

of objects for plate cultures turns on the fact, fully recognized at the very commencement of the inquiry, that the risks of reinfection increase greatly with increase in the number of objects handled. This refers more especially to objects like aleurone layers and inner endosperms, which have to be prepared by careful dissection of the aleurone layer. The successful preparation of sterile cultures of endosperms and embryos is a far easier task by reason of the more rapid handling of these objects.

Method of determining copper-reducing power. Many of the earlier determinations were made by the standard gravimetric method, the cuprous oxide being weighed as such, and the Cu calculated by applying the appropriate factor or the oxide reduced in a current of dry H and weighed directly.

The greater number of estimations have, however, been made by the extremely convenient and rapid method of *Bertrand*.¹

In every instance these reductions were carried out in duplicate. The following comparative results show how closely estimations by the two methods approximate each other:—

1.1875 gm. of cane sugar were hydrolysed with HCl, neutralized with Na₂CO₃, cooled and diluted to 250 c.c.; duplicate determinations by the two methods made on 10 c.c. of the dilution gave—

<i>Gravimetric method.</i>		<i>Bertrand's method.</i>	
97.2 mg. Cu, 49.5 mg. invert sugar		93.58 mg. Cu, 49 mg. invert sugar	
95.6 ,, Cu, 48.5 ,, ,, ,,		92.59 ,, Cu, 48.5 ,, ,, ,,	
Theoretically 10 c.c. of dilution contains		49.97 ,, ,, ,,	

Soluble starches employed. The soluble starches used in the digestions represent three separate specimens prepared by precisely the same method from one and the same stock of potato starch. They were prior to use and during the progress of the inquiry subjected to critical control, either with a standard malt of known amylolytic power kept for the purpose or by means of a standardized enzyme solution.

The enzyme solutions employed in the following digestions were taken from a single embryo-plate culture.

COMPARISON OF SOLUBLE STARCHES.

<i>Soluble starch.</i>	<i>Starch solids per 100 c.c. of solu- tion.</i>	<i>Amylase per 20 objects per hour equivalent to mg. of Cu.</i>
A ₁	2.62	90.8
A ₂	2.67	85.2
A ₃	2.63	94.7

These values not only show how little these starch preparations differ

¹ Bertrand: Bull. Soc. Chim., t. xxxv, 1906, p. 1285.

from, but also how closely the amyloclastic secretory powers of the objects parallel each other; an approximation of sufficient nearness for ordinary biological investigation.

III. AMYLOCLASTIC SECRETORY CAPACITY OF THE EMBRYO OF BARLEY.

The salient features of the general anatomy and histology of the barley embryo have been so frequently described that this portion of the subject calls for limited reference only.

The organ of special interest, the scutellum, is a shield-shaped expansion which over its greater area is in close apposition, but not organically united to the proximal end of the inner endosperm, the limiting layer forming the boundary between the scutellum and endosperm consisting of a single layer of columnar cells.

The functions of the scutellum are twofold—absorptive and secretory. It is regarded from the standpoint of morphological botany as a cotyledonary organ, and from the physiological standpoint as a glandular tissue, the secretory function being localized, at least principally, in the columnar or epidermal layer; the enzymes arising as a result of activity of secretory mechanism, amylase, cytase, and probably others being largely destined for extra-cellular work.

While it is broadly accepted that the scutellum possesses and exercises both absorptive and secretory functions it is perhaps not so generally recognized how important is the former function in the regulation of the complex processes which have their seat of action in the inner endosperm during the progress of normal germination, not only in regard to the removal of simplified products of enzymatic action, but to the accumulation of enzymes and the continuance of their specific activity.

It has been, and still is, a question of controversy whether the various degradation changes of the complex storage substances in inner endosperms can be or are induced solely by the action of enzymes which are elaborated by the embryo, or by enzymes which arise in or are generated by the inner endosperm independently of the embryo.

Demonstration of the possession by the embryo of a distinct amyloclastic secretory function and its localization in the columnar or secretory epithelium is due to Brown and Morris (*l.c.*), but this particular aspect of the subject, as far as can be ascertained, does not appear to have been the object of any subsequent independent reinvestigation.

The secretory function ascribed to the embryos by Brown and Morris, although generally accepted, has been more recently challenged by Ling.¹

The investigation of the amyolytic secretory power of the embryo in this inquiry embraces two lines of attack: (1) cytological, (2) biochemical,

¹ Journ. Inst. Brewing, xv, p. 651.

and to the consideration of evidence afforded by application of these methods of investigation we must now turn.

1. *Cytological.* Embryos, removed from seeds after adequate steeping, were germinated for varying periods of time in Coldewe germinators at laboratory temperatures, 15° C.—20° C.; the material, after fixation, was embedded, microtome sections prepared, and the stained preparations examined and studied.

Briefly stated, cytological study of the scutellum, and particularly of the columnar epithelial cells during progressive phases of germination, reveals the fact that these cells are, as far as cytological evidence permits this statement to be made, distinctly secretory in function.

During the earlier phases of germination, 0–4 days, their cytoplasmic contents undergo marked changes—solubilization of specific granular inclusions which are probably destined to furnish enzymatic as well as nutritive material,—changes which are accompanied by significant and comparatively well-defined changes in the nucleus and its structures.

The succeeding interval, 4–8 days, presents features which, if unsupplemented by collateral biochemical evidence, are not so easy to follow or interpret. There is during this interval a gradual but complete disappearance of those granular contents (which various authors in other fields of cytological research have regarded as either enzymatic or pre-enzymatic in constitution) which are never renewed. Nevertheless, nuclear and cytoplasmic activity persists, as shown by the more or less continued production of chromatin and nucleolar substance, and by the vacuolate condition of the cytoplasm.

The final interval, 8–11 days, is marked by the gradual disorganization of cytoplasm and degeneration of the nucleus.

Judged solely by the cytological results of study, the most active phase of secretory activity would be ascribed to the earlier phase comprised within the 0–4 days' interval.

As we shall see, the biochemical evidence to be adduced not only supports the original contention of Brown and Morris that the columnar epithelium consists of cells which are secretory in function, but helps to elucidate the cytological evidence in a significant manner.

2. *Biochemical.* Embryos from sterilized steeped seeds were placed on various kinds of media, and after the lapse of definite time periods, either the culture medium or both culture medium and objects were investigated under uniform conditions for amylase.

Preliminary experiments in which embryos were cultivated on (1) water (starvation conditions), (2) cane sugar plus mineral salt solution, (3) 0.55 % asparagin solution, evidenced that the latter solution yielded the most favourable results. The amounts of amylase were exceedingly small, however, and as the seed from which the embryos were derived had been steri-

lized in CuSO_4 solution, it was surmised that this reagent, manifesting its influence more especially during the digestion with soluble starch, constituted a probable disturbing factor. This suggestion was, however, negatived by similarly established cultures with embryos derived from seeds which had been steeped successively in absolute alcohol and water yielding equally low results.

Asparagin and KH_2PO_4 are amphoteric substances, and, as shown by Ford¹ and Ford and Guthrie,² such substances may function in a protective manner towards barley amylase by maintaining the (1) extraction or (2) digestion medium in a state of comparative neutrality.

Whether this holds during the different phases of culture on nutrient substrata in the experiments to be described presently, or whether they serve mainly as nutritive substances and partly by stimulating secretory activity, lies beyond the actual scope of this inquiry.

The fact that in general enhanced secretion of amylase by the embryo follows on their use warrants their employment.

The results given in the following table may be taken as typical examples of possession by the embryo of this secretory capacity, of its variation with different concentrations of asparagin, and of its progressive increase with prolongation of the culture period.

TABLE I.

CULTURES OF EMBRYOS ON ASPARAGIN-MINERAL SALT MEDIUM.

<i>Concentration of asparagin per 100 c.c. of solution.</i>	<i>Duration of culture.</i>	<i>Digestion period.</i>	<i>Amylase in culture medium per 20 embryos (equivalent to mg. of Cu).</i>
(African barley.)			
Experiment 1. Seeds 48 hours absolute alcohol, 48 hours water.			
1.65	4 days	2½ hours	20.1 mg.
1.10	"	"	16.1
0.55	"	"	30.5
0.055	"	"	32.2
(African barley.)			
Experiment 2. Seeds 48 hours CuSO_4 .			
1.65	8 days	2½ hours	50.9
1.10	"	"	31.9
0.55	"	"	75.7
0.055	"	"	95.9
(African barley.)			
Experiment 3. Seeds 48 hours CuSO_4 , 24 hours water.			
0.55 (asparagin only)	8 days	20 hours	5.7

The results show clearly, as already demonstrated by the work of Brown and Morris, that the barley embryo undoubtedly possesses the power of secreting amylase.

¹ Journ. Soc. Chem. Ind., 1904, xxiii. 414; Journ. Chem. Soc., 1906, lxxxix. 76.

² Journ. Inst. Brewing, 1908, xiv, i. 61-87.

An obvious increase in the amount of enzyme secreted with prolongation of the culture periods is evident, and the optimal output of amylase evidently does not coincide with the higher concentrations of asparagin, but lies between the limits comprised in the 0.55 and 0.055 concentrations, if not beyond this latter.

When asparagin only is used, as shown by the results of Experiment 3, the secretory activity of the embryo falls enormously, and in comparison with Experiments 1 and 2 the result points to the probable influence exercised by the mineral salts or by certain of them under the particular conditions selected.

The results of experiments with embryos on mineral salt medium with and without asparagin, comprised in the following table, lend support to this suggestion.

TABLE II.

CONTEMPORANEOUS CULTURES OF EMBRYOS ON MINERAL SALT
MEDIUM, WITH AND WITHOUT ASPARAGIN.

<i>Composition of culture medium.</i>	<i>Duration of culture.</i>	<i>Digestion period.</i>	<i>Amylase per 20 embryos in medium, equivalent to mg. of Cu.</i>
Experiment 1. Seeds 48 hours 10 % copper sulphate.			
0.55 % asparagin- mineral salts.	4 days	2½ hours	61.7 mg.
Mineral salts only.	"	"	16.0
Experiment 2. Seeds 48 hours 10 % copper sulphate.			
5 % gelatine, 0.55 % asparagin-mineral salts	7 days	2½ hours	172.7 mg.
5 % gelatine, mineral salts only.	"	"	114.7

These experimental results indicate that the mixture of asparagin and mineral salts influences the secretory function more favourably than either alone.

This influence is, however, only apparently shown in Experiment 1. In order to prevent misapprehension it must be stated that the gelatine medium employed in Experiment 2, by affording mechanical support to the plantlets, eliminates a disturbing effect which is liable to ensue in Experiment 1, namely, partial submersion of the embryo, which at once interferes with the exercise of its normal functional activities as a whole.

The general conclusion, therefore, to be derived from these experiments is that the secretion of amylase by the embryo is principally influenced in some way by the presence of mineral salts, and that that induced by the inclusion of asparagin in the culture substratum is adjunctive.

The following experiment throws some light on the question as to whether mineral salts or certain of them stimulate the elaboration and excretion of amylase by the embryo; that is to say, whether their influence is to be regarded as physiological or otherwise.

We have seen that the secretory activity of the embryo cultivated upon a 0.55 % solution of asparagin is minimal.

If we take such culture solutions upon which embryos have been growing for some days and digest them separately with (1) soluble starch alone, or with (2) soluble starch containing mineral salts in the concentrations in which they occur initially in the small quantity (10 c.c.) of culture medium employed for each plate culture, the results should indicate whether the influence of these salts takes place externally, i. e. merely protects the amylase secreted during the culture period, or whether they influence the general metabolism of the plant and, consequently, its secretory activity.

The following experimental results, taken in conjunction with those of Tables I and II, favour the latter alternative, namely, that the influence they exercise is mainly physiological.¹

TABLE III.

DIGESTIONS OF CULTURE MEDIUM WITH SOLUBLE STARCH IN THE PRESENCE AND ABSENCE OF MINERAL SALTS.

<i>Digestion period.</i>	<i>Composition of digestion mixture.</i>	<i>Amylase per 20 objects in culture medium (equivalent to mg. of Cu).</i>
1. 20 hours	Starch only	6.0
2. ,,	Starch and mineral salts	4.0

The culture liquids investigated were derived from two cultures of 40 embryos each, cultivated on 0.055 % asparagin for seven days.

It is obvious from the above results, by comparison with Experiment 3, Table I, that the mineral salts used in the experiments previously described enter in some way into the general metabolism of the embryo, and thereby influence its secretory functions, thus negating the suggestion that under the conditions of culture employed they—or certain of them, notably the phosphate—function as protective agents to the enzyme excreted into culture medium. It is not denied that they do not function in the manner indicated during certain phases of the culture experiment, but it is suggested that they principally influence secretion by entering into or influencing the general metabolic activities of the plantlet.

In order to demonstrate further the possession of an amylo-secretory function by the embryo, cultures of these objects were established on a medium consisting of 5 % gelatine and finely ground barley endosperm

¹ H. M. Richards : Ann. Bot., xxi, 1907, p. 501.

substance; the latter being effectively sterilized and the pre-existent amylase completely annihilated (as controlled by inoculation and digestion experiments respectively) by heating in a Koch's steam sterilizer for two hours at 100° C.

In order to avoid gelatinization of the starch grains, the finely ground sterilized endospermic substance was thoroughly mixed with the cooled, still mobile gelatine prior to the transference of the mixture to the Petri culture dish.

The results are embodied in the following table:—

TABLE IV.

CULTURES OF EMBRYOS ON 5 % GELATINE-BARLEY ENDOSPERM SUBSTANCE.

Seeds (African barley) 48 hours CuSO_4 , 48 hours water.

<i>Exp.</i>	<i>Duration of culture.</i>	<i>Digestion period.</i>	<i>Amylase per 20 objects in medium (equivalent to mg. of Cu).</i>
1.	6 days	1 hour	80.5
2.	8 "	"	96.7

As already evidenced (Table I), the secretory capacity of the embryo augments as duration of culture period is extended.

The following typical experiments afford further evidence of this feature:—

TABLE V.

CULTURES OF EMBRYOS ON 0.55 % ASPARAGIN-MINERAL SALT MEDIUM.

Seeds 48 hours 10 % CuSO_4 , 1 day under aseptic germination conditions.

<i>Exp.</i>	<i>Objects.</i>	<i>Duration of culture.</i>	<i>Digestion period.</i>	<i>Amylase per 20 objects in medium (equivalent to mg. of Cu).</i>	<i>Relative augmentation of secretory capacity, 24 hours.</i>
1.	40 embryos	2 days	2 hours	11	
2.	"	3 "	1 hour	10	
3.	"	4 "	"	69	60 mg.
4.	"	6 "	"	119	25 "
5.	"	8 "	"	150	14 "

There is an obvious progressive augmentation of the secretory capacity of the embryo accompanying extension of the period of culture.

From the results afforded by these experiments under the conditions selected, it may reasonably be assumed (in the absence of direct experimental investigation) that they are really the net result of two series of factors which are opposed to each other. On the one hand, production of enzyme by the embryo; on the other, destruction or inactivation of enzyme by the operation of factors in and external to the medium, such as changes

in composition of the medium and oxidation by atmospheric oxygen. The relative extent to which these two series of opposed factors operate varies with the particular phase of the culture experiment considered. Presumably it is most marked during the period 0-3 days; from this period onward the relative augmentation of the secretory capacity is well maintained.

Although in the majority of instances, especially in gelatine media, the embryo exhibits good growth accompanied by considerable increase in its root and plumular development, there is reason to believe that under these conditions development is by no means strictly normal. In particular is this reflected on its amyloclastic secretory capacity; the results given in the foregoing tables distinctly under-estimate the potentiality of its secretory functions. The values of the secretory capacity of the embryo are of practically the same order as in the preceding experiments on purely artificial media.

The preliminary treatment to which the barley endosperm substance was subjected probably induces changes in it of both a physical and a chemical nature which render it less readily attackable by the embryo.

The experiment furnishes an example of the difficulty of preparing a culture substratum which shall serve as an adequate substitute for the natural inner endosperm.

The positive nature of the evidence adduced furnishes direct proof that the barley embryo secretes an amyloclastic enzyme. Initially the embryo, as stated by Brown and Morris (*loc. cit.*, p. 492) and confirmed by direct examination by myself, contains little (not more than 1-2 mg. per 20 objects) or no pre-existent amylase at the moment of removal from the seed at the termination of the steeping operation carried out under the conditions described. The amounts of enzyme found in the medium at the termination of the cultural experiment therefore represent the magnitude of its varying secretory capacity under the conditions of experiment selected.

IV. AMYLOCLASTIC SECRETORY CAPACITY OF THE ALEURONE LAYER OF BARLEY.

The earlier researches of Brown and Morris (*l. c.*) indicate that these authors regarded the aleurone layer not as a glandular tissue, but as functioning in some serviceable way in the general economy of the seed during the later stages of the germinative process.

It was shown by Haberlandt (*l. c.*) that the aleurone layer of rye possesses amyloclastic secretory powers, and it was suggested by him that the possession of this capacity was probably a common attribute of the aleurone layer in other members of the Grasses,

Hansteen and Puriewitsch (l. c.) contend that the aleurone layer does not constitute a differentiated glandular tissue, and that the secretory capacity of the aleurone layer cells is not, unit for unit, superior to that possessed by those of the subjacent amyloferous tissue.

In other words, they regard the aleurone layer as forming an undifferentiated portion of the reserve storage system of the seed.

Re-investigation of the work of Brown and Morris by Brown and Escombe led the latter authors to consider that the aleurone layer of barley represents a secretory tissue.

The assertion that the aleurone layer consists of units which are distinctly living receives general acceptance. In the barley grain it consists of a triple layer of cubical cells with highly cuticularized cell-walls (probably the thickest and most resistant in the entire seed) which form an almost complete envelope to the inner endosperm.

Each cell possesses a conspicuous nucleus, centrally disposed, and a cytoplasmic network which, prior to the inauguration of the germinative process, is densely crowded with, for the most part, large and conspicuous protein grains—the so-called aleurone grains.

Cytological investigation shows that the aleurone layer cells closely parallel the columnar epithelial cells in many important respects. Fixed, embedded, stained microtomed material from seeds in various stages of germination presents features which bear close similarity to those described for the columnar cells of the scutellum.

In particular, the nuclear and cytoplasmic changes and those undergone by the granular contents of the cytoplasm not only closely resemble but synchronize with these same general phenomena in the epithelial cells.

Were judgement by analogy strictly permissible, the deduction might at once be made that, although morphologically distinct, these cells are similar in function to those of the columnar epithelium.

It is noteworthy that hitherto experiments destined to demonstrate the possible possession of amylolytic secretory powers by the isolated aleurone layer have been entirely qualitative.

Investigation of the enzymatic secretory capacity of a given tissue which confines itself to the determination of the capacity which that tissue (such as the aleurone layer) may possess of augmenting its enzyme content intracellularly is necessarily incomplete because it takes no account of the quantity of enzyme excreted, and is therefore inadequate as a criterion of its total secretory powers.

Throughout this inquiry the treatment of the subject has been as far as possible distinctly quantitative, and the apparent non-recognition of the desirability of quantitative investigation has led to misapprehension regarding (1) the secretion of amylase, (2) its sources of origin in the germinating seed, and (3) the general trend of endospermic depletion.

As in the case of the embryo, before it is possible to evaluate its secretory capacity it is necessary to determine the initial amylase content of the aleurone layer directly after its removal from the steeped seed. The results of such a determination are given in the following table:—

TABLE VI.

INITIAL AMYLASE CONTENT OF ALEURONE LAYER OF STEEPED SEED.

Chilian barley. Seeds 48 hours absolute alcohol, 48 hours water.

<i>Experiment.</i>	<i>Amylase per 20 aleurone layers (equivalent to mg. of Cu).</i>
1. 2.5 aleurone layers	506
2. " "	675
3. " "	675

Typical examples of the secretory capacity of the aleurone layer are given in the following tabulations:—

TABLE VII.

SECRETION OF AMYLASE BY ALEURONE LAYER.

Cultures of dorsal aleurone layers (African barley) on liquid and on solid substrata.
Seeds 24 hours 10 % CuSO_4 .

<i>Exp.</i>	<i>No. of objects on culture medium.</i>	<i>Duration of culture period.</i>	<i>Medium.</i>	<i>Amylase per 20 objects in culture medium (equivalent to mg. of Cu).</i>
1.	20 dorsal aleurone layers	2 days	0.55 % asparagin solution	365
2.	" " "	3 "	" " "	362
3.	" " "	4 "	" " "	414
4.	" " "	6 "	" " "	269
5.	" " "	7 "	10 % gel., 0.55 % asparagin-M.S.	852
6.	" " "	7 "	10 % gel.-M.S.	552

The above experiments, it is to be noted, were carried out with material prepared from seeds sterilized by steeping in copper sulphate solution, as will be shown later; there are certain objections to the use of this reagent in the case of aleurone layers, endosperms, and inner endosperms.

The superior results in Exp. 5 and 6 are not wholly due to prolongation of the culture period, but to substitution of a solid for a liquid substratum. In the latter circumstances the aleurone layer fragments are apt to be drowned, and this seriously interferes with their secretory functions.

The results comprised in the following table (VIII) may be taken as typical examples of the amyloclastic secretory powers of isolated aleurone layers prepared from alcohol-water steeped seeds.

TABLE VIII.

SECRETION OF AMYLASE BY THE ALEURONE LAYER (CHILIAN BARLEY).

Cultures of isolated aleurone layer on 5 % gelatine subs. Seeds 48 hours absolute alcohol, 48 hours water (aerated).

Exp.	No. of objects on culture medium.	Culture period.	Amylase per 20 objects (equi- valent to mg. of Cu) in :—			Augmentation of amylase.
			Medium.	Objects.	Total.	
1.	5 aleurone layers	4 days	2222	396	2618	1943
2.	„ „	6 „	2172	198	2370	1675
3.	„ „	7 „	1428	634	2062	1387

Amylase initially present in 20 aleurone layers . . 675

The augmentation of amylase observed in these and similarly conducted experiments depends principally on the substitution of absolute alcohol for CuSO_4 in the sterilization of the seeds, and also on the employment of a semi-solid medium which affords the fragments adequate mechanical support and provides for their aeration.

The examples given afford sufficient justification for regarding the aleurone layer as a glandular tissue, one of the functions of which is the secretion of an amylolytic enzyme, and they further furnish evidence that the amylolytic capacity of the aleurone layer, *as suggested by Brown and Escombe* (l. c.), is greatly superior to that of the embryo.

In order to show definitely that passage of amylolytic enzyme from the objects cultivated (embryos and aleurone layers) under the conditions described actually represents a secretory act, and not a mere passive straining out of enzyme pre-existent in the tissues of the embryo and aleurone layer, the following experiments were instituted :—

Embryos and dorsal aleurone layers were removed from steeped seeds and anaesthetized by immersion for forty-eight hours, at laboratory temperature, in a saturated aqueous solution of either chloroform or nitrobenzene contained in closed vessels. They were then cultivated on 0.55 % asparagin-mineral salt solution for seven days under the usual conditions. Examination of the culture media at the termination of the experiments afforded the following data :—

TABLE IX.

CULTURE OF ANAESTHETIZED EMBRYOS AND ALEURONE LAYERS.

Seeds (African barley) 24 hours 10 % CuSO_4 , 24 hours water.

Exp.	Objects.	Anaesthetic reagent.	Digestion period.	Amylase in culture medium per 20 objects.
1.	40 embryos	Chloroform	20 hours	2 mg.
2.	„	Nitrobenzene	„	4 „
3.	20 dorsal aleurone layers	Chloroform	1 hour	42 „
4.	„ „ „	Nitrobenzene	„	130 „

The embryos exhibited no signs of growth. These results, on the one hand, show that the treatment described is fatal alike to the vitality and secretory functions of the embryo, and inferentially, and by comparison with results of experiments (Tables I, II, &c.), the same deduction may be drawn from the results furnished by the aleurone layer experiments.

The anaesthetic reagents employed at first suspend and then completely annihilate the secretory mechanism of both the embryo and aleurone layer.

One probable reason for the comparatively small reduction of the secretory capacity of the aleurone layer is the difficulty offered to rapid penetration of the anaesthetic reagents through or across the highly cuticularized walls of the cells of this tissue.¹

That this is probable is shown by the fact that when immersion of aleurone layer fragments in aqueous solutions of these reagents was restricted to twenty-four hours the reduction in the amount of amylase found in the *culture medium* was still *less* than in the foregoing experiments.²

It is equally probable that these agents in small quantity may stimulate secretion during the earlier stages of anaesthesia.

The results are also of interest because they serve to explain certain anomalies in the work of Brown and Morris,³ dealing with the alleged non-glandular nature of the aleurone layer.

The statement is made that, in contrast to the embryo, fragments of the aleurone layer, after treatment with chloroform vapour, retain their diastatic powers quite unimpaired. Such fragments placed upon starch grains attacked these quite as quickly and completely as non-chloroformed fragments; that is to say, treatment which was found to be fatal to the embryo and completely arrested its amyloclastic functions, failed to do so in the case of fragments of the aleurone layer. Both tissues are regarded by the authors as possessing vitality, yet in one case (embryos) arrest of vitality means arrest of the secretory mechanism, in the other (aleurone layers) arrest of vitality signifies apparent non-arrest of the secretory function. The anomaly is evident, and the qualitative method of investigation used by the authors is largely responsible for the interpretation placed upon the phenomenon observed, as the foregoing experiments and those which follow show that treatment with chloroform is fatal to the life of both the embryo and aleurone layer. The embryo excised from the steeped seed contains little, if any, preformed amylase, and consequently when it or sections of the scutellum are placed on moistened starch grains no action occurs. Fragments of aleurone layer taken from the steeped or germinating seed invariably contain a considerable quantity of preformed enzyme, and

¹ Stoward, Ann. Bot., xxii, 1908, pp. 87, 442.

² The probable meaning of this is that with shorter steeping *less* amylase diffuses from the fragments of killed tissue into the steeping medium, and hence *more* enzyme is found subsequently in the culture medium.

³ Journ. Chem. Soc., lvii, 1890, p. 525.

this diffusing from the chloroformed and killed tissue attacks the starch grains placed at its disposal.

The evidence adduced by Brown and Morris therefore is valueless as a proof of the non-possession of an amyloclastic secretory capacity by the aleurone layer.

The experimental data given in the following table furnish additional evidence on this important point.

TABLE X.

CULTURES OF ALEURONE LAYERS ON 5 % GELATINE-MINERAL SALTS
IN THE PRESENCE OF (1) NITROBENZENE, (2) CHLOROFORM.

Chilian barley: seeds steeped successively in absolute alcohol and water; 48 hours in each reagent. Culture period, 7 days.

Exp.	Objects.	Anaesthetic reagent.	Amylase per 20 objects per hour (equivalent to mg. of Cu) in:—		
			Medium.	Objects.	Total.
1.	10 aleurone layers	Chloroform ¹	1160	105	1265
2.	" "	Nitrobenzene	1202	85	1287
	Amylase initially present in 20 aleurone layers . . .				675

When fragments of aleurone layer are removed from steeped seeds (loss of amylase during steeping is thereby eliminated), and at once placed on the surface of gelatine media with which are mingled copious amounts of chloroform or nitrobenzene, there are found, just as in experiments with similarly prepared and similar fragments of aleurone layers, considerable quantities of amylase in both medium and objects. Apparently, then, the fragments of aleurone layer are either non-living, or if living, these anti-septics behave in an anomalous manner.

Neither alternative, however, is correct. Under the conditions selected, although secretion of amylase apparently takes place, it occurs prior to the complete annihilation of the secretory mechanism. The amylase found *quantitatively in medium and objects on comparison with that found in experiments on gelatine media without the addition of an antiseptic* (compare experiments, Table VIII) is observed to have *undergone marked reduction in amount*. As a result of the influence of the antiseptic the augmentative capacity of the tissue falls. The amounts of amylase found represent the amounts of amylase pre-existent in and secreted by the tissue prior to the complete cessation of the activities of the secretory mechanism. The comparatively large amounts of enzyme are probably due to the fact that the action of the antiseptic is not abrupt because of the resistance to its rapid entry offered by the highly cuticularized cell-walls of this tissue.

¹ Chloroform renewed daily; both cultures remained absolutely sterile throughout the course of the experiment.

It is to be noted in these experiments that *at least two* different anaesthetic agents have been employed.

In short, the action of these agents on the embryo is signalized by the complete and rapid arrest of its secretory functions, minimal amounts only of enzyme being pre-existent in its scutellar tissues, and consequently only minimal amounts of enzyme are found in the culture medium. On the other hand, the inhibitory and fatal action of the same agents on the aleurone layer is marked by comparative slowness, the secretory function being more tardily impaired. Although considerable amounts of amylase are thus accumulated by the anaesthetized aleurone layer, its augmentative capacity, *as compared with similar objects not subjected to the influence of such agents, suffers marked reduction.*

This reduction of amyloclastic enzyme, as the result of the interference with and arrest of the secretory mechanism of a *living* tissue, is the kind of behaviour we should anticipate in the case of the aleurone layer. Complete and prompt arrest of its secretory powers (as in the case of the embryo as observed by Brown and Morris) could not occur; the *apparent* secretion of enzyme by the aleurone layer under complete anaesthesia, described in this section, really represents for the most part the passive diffusion of the accumulation of pre-existent enzyme¹ in its cells—a fact to be elicited only by the employment of quantitative methods of inquiry.

The augmentative capacity of the aleurone layer under anaesthetic conditions of culture suffers marked reduction, and this is taken to signify that the aleurone layer is a living tissue—a fact fully supported by other considerations.

Had its augmentative capacity been equal to or greater than that exhibited by similar aleurone layer fragments cultivated on similar media without the addition of antiseptics recognized as agents fatal to living protoplasm, the claim that this tissue possesses vitality would be rendered extremely difficult to maintain. The inner endosperm, as we shall see (Section IV A, pp. 824–37), does actually exercise its augmentative capacity as rapidly and to the same extent in the presence or absence of antiseptics.

The argument may be advanced that the method of treatment to which the isolated aleurone layers were subjected does not preclude the possibility of loss of amylase by the enzyme diffusing from the fragments of tissue into the steeping medium. The fact remains, however, that neither the aleurone layer nor the embryo after such preliminary treatment any longer possesses the power of exercising its secretory powers to the same extent as non-anaesthetized objects.

The following experiments will serve to show that in the foregoing experiments with anaesthetized aleurone layers (Tables IX and X) the

¹ The chloroformed embryos examined by Brown and Morris were probably taken from seeds immediately after steeping; at this stage they would contain little or no amylase. If taken at later stages from germinating seeds, the accumulated amylase would probably diffuse out and action upon barley starch would have been demonstrable, and unless quantitative examination were resorted to confusion similar to that instanced in the case of the aleurone layer would have arisen.

amylase found in the culture medium really represents that present initially in the tissue at the moment of the removal of objects from the anaesthetic steep solution.

Three series of ten dorsal aleurone layers each, removed from seeds steeped for forty-eight hours in water, were prepared. Of them, one was dried at 30° for forty-eight hours, and the other two were steeped for the same period in a saturated aqueous solution of either chloroform or nitrobenzene contained in closed vessels. The amounts of amylase were then determined in the steep media and also in the steeped and unsteeped objects after short desiccation at 30° C.

The results are given in the following table:—

TABLE XI.
LOSS OF AMYLASE BY ISOLATED DORSAL ALEURONE LAYER
DURING STEEPING IN ANAESTHETIC SOLUTIONS.

<i>Exp.</i>	<i>Objects.</i>	<i>Anaesthetic steep solution.</i>	<i>Duration of steep.</i>	<i>Amylase per 20 objects (equivalent to mg. of Cu) in:—</i>	
				<i>Steep medium.</i>	<i>Objects.</i>
1.	10 dorsal layers	5 c.c. sat. aq. chloroform	48 hours	258.5	126.6
2.	„ „	5 c.c. sat. aq. nitrobenzene	„	237.0	85.0
3.	„ „	Desiccated at 30° C.	„	—	211.0

The general conclusions to be derived from these experiments with the embryo and aleurone layer may be briefly summarized:—

Both the embryo and aleurone layer possess an amyloclastic secretory capacity.

The magnitude of the aleurone layer capacity is considerably greater than that of the similar capacity of the embryo.

Prolonged action of saturated aqueous solutions of chloroform or nitrobenzene annihilates both the vitality and secretory functions of the embryo and aleurone layer. The amylase found in the culture liquids upon which anaesthetized objects have been placed represents merely the enzyme pre-existent in these objects at the commencement of the culture experiments.

The secretion of amylase by the embryo and aleurone layer is notably influenced (1) by the inclusion in the culture substratum of certain mineral salts, the action of which appears to be chiefly physiological; and (2) by physical conditions, notably by substitution of a semi-solid for a liquid medium.

IV A. THE RELATIVE AMYLOCLASTIC SECRETORY AND SELF-DEPLETIVE CAPACITIES OF THE ENDOSPERM AND THE INNER ENDOSPERM OF BARLEY.

The endosperm of barley constitutes the great mass of the resting mature seed and consists of an inner portion—the inner endosperm—composed of large irregularly isodiametric cells, in each of which there

is a much attenuated network of cytoplasm, the meshes of which are densely crowded, principally with variously sized starch grains, and among the other far less abundant inclusions are those of a protein nature.

Immediately external to the amyliiferous cells is the aleurone layer, forming as already stated, with the exception of that portion of the inner endosperm which abuts on the scutellum, an investing envelope to the entire inner endosperm. External to the aleurone layer is the spermoderm comprising the different external integuments of the seed.

The principal point of interest in the present inquiry refers to the question as to whether or not the inner endosperm, independently of the amyloclastic secretions of the embryo and aleurone layer, possesses a capacity for self-depletion, and if so, in what degree transformation of the starch storage reserves is induced solely by the pre-existent amylase of the resting seed.

The conclusions of the different investigators who have attacked this aspect of the question are marked by their extreme divergency.

Brown and Morris (loc. cit. p. 482) state that the inner endosperm consists of a mass of non-living cellular units which are unable to induce self-digestion of their starch contents.

Hansteen (loc. cit.) and also Puriewitsch (loc. cit.) contend that the endosperm of barley is self-digesting and that each cell of the inner endosperm is a living functionary unit.

The claim of vitality for the inner endosperm rests almost entirely on the demonstration of enzyme action in this tissue, this constituting a criterion which, as already stated, is no longer regarded as valid in determining whether a tissue possesses vitality or otherwise. Although Puriewitsch's view is opposed to Haberlandt's conclusion that the aleurone layer possesses a secretory function, yet many of his experimental results really suggest and support the correctness of Haberlandt's contention.

Brown and Escombe,¹ as a result of a reinvestigation of the work of Brown and Morris (loc. cit.), modify the views of the latter authors materially.

The statement by Brown and Morris that the accumulation of amylase which occurs in the inner endosperm during the germinative process is largely due to the secretory activity of the embryo they amend by conceding that the contributory share in the sum total of the amylase found in the endosperm furnished by the embryo was, no doubt, considerably over-estimated. On the other hand, they substantiate Haberlandt's suggestion that the aleurone layer possesses glandular functions and claim that it plays a much greater rôle in endospermic depletion than had hitherto been assigned to it.

Experiments devised to ascertain if the isolated inner endosperm

¹ Proc. Roy. Soc., lxiii, 1898, p. 3.

was capable of inducing self-digestion of its starch contents afforded negative results only, and the authors adhere to the opinion of Brown and Morris that this tissue possesses neither vitality nor a self-digestive capacity.

Brown and Morris recognize the existence of two amyloclastic enzymes, the one, the more active amylase designated 'secretion' amylase, the other, a feebler type which they regard as being similar to, if not identical with, the 'translocation' variety generally met with in plant tissues.

The former readily erodes starch-grains and liquefies and saccharifies starch-paste; the latter dissolves, but not by erosion, solid starch-grains and liquefies gelatinized starch much less readily than does the 'secretion' variety, which the authors contend is principally responsible for the dissolution and digestion of the *solid* starch reserves of the endosperm.

The amylase of ungerminated barley, according to Brown and Morris, is an unused residue of the 'translocation' variety and probably owes its origin to the action of an acid produced by the developing embryo upon a zymogen in the endosperm cells, and in their opinion plays a subordinate rôle only in endospermic depletion.

It is to this enzyme and its augmentation that the work of Ford and Guthrie refers specially; extracts of finely ground ungerminated barley prepared under certain conditions, i. e. the auto- and papain pre-digestion methods of these authors, exhibit higher amylolytic activity than extracts prepared by the customary short method of extraction. The general conclusion derived from their investigation appears to be that the results obtained are due, not to an increase of amylolytic activity, but to an augmentation of the enzyme itself, and the whole question implied, though not specifically raised, is whether the pre-existent enzyme of the resting grain does not contribute, in greater measure than was supposed by Brown and Morris, to the dissolution and depletion of the starch reserves of the inner endosperm.

The experimental work described in this section, therefore, represents an attempt to ascertain whether the amylase, which is pre-existent in the inner endosperm (and which augments under the varying conditions of experiment selected), is capable of inducing the depletion of its starch storage materials.

Perhaps no portion of the subject presents such difficulties of attack as the investigation of the relative amyloclastic secretory and self-depletive capacities of the endosperm and inner endosperm of the barley seed.

First, the difficulty encountered in the preparation of sterile material is greatly enhanced by the fact that the employment of copper sulphate as an antiseptic steep-reagent is inadmissible because of its tendency to adhere in small traces to these objects even after subsequent steeping in water for some time, and therefore the use of absolute alcohol, which is admittedly a less powerful agent, has to be resorted to.

Secondly, complexity is introduced into any attempt at solution of the question by the fact that the isolated endosperm (just as occurs when similarly prepared objects are investigated by the auto-digestion method) is capable of autonomously augmenting its amylase content. In comparable experiments with isolated endosperms, therefore, the augmentation of enzyme which invariably takes place cannot be wholly assigned to the secretory and excretory activity of the aleurone layer.

Thirdly, the induction and maintenance of endospermic depletion processes, either in the *absence* or *presence* of the aleurone layer, demand the provision of conditions which must at least parallel in some measure those provided by the embryo, namely, ready means for the outward diffusion of the mobilized products of amyloclastic activity.¹

CULTURE EXPERIMENTS WITH ENDOSPERMS AND INNER ENDOSPERMS IN 5 % GELATINE WITH AND WITHOUT MINERAL SALTS.

In the various experiments described in this section the sterilization, preparation of material, establishment and maintenance and subsequent examination of the culture medium and objects, &c., were carried out in the manner previously described.

In the following table are shown typical examples of the relative augmentation capacities of endosperms and inner endosperms :—

TABLE XII.

CULTURES OF ENDOSPERMS AND INNER ENDOSPERMS ON 5 % GELATINE WITH AND WITHOUT MINERAL SALTS.

Chilian barley. Culture period, 11 days. Seeds 48 hours absolute alcohol, 48 hours water.

Exp.	No. of objects on plate.	Medium.	Amylase per 20 objects (equivalent to mg. of Cu.) in :—		
			Medium.	Objects.	Total.
1.	10 endosperms (whole)	Gelatine	2474	1190	3664
2.	10 " " "	Gelatine-M.S.	2634	1349	3980
3.	10 endosperms (halved) ²	Gelatine	2914	1349	4236
4.	10 inner endosperms (halved) ²	Gelatine-M.S.	1508	396	1904
5.	10 " " "	"	1239	396	1635
	Amylase present initially in 20 endosperms	.	.	.	1181
	" " " 20 inner endosperms	.	.	.	844

We may for the present confine our attention solely to the relative capacities these two classes of objects, differing only by removal of the

¹ The important influence which the embryo has on endospermic depletion during the progress of the germinative processes can be more readily realized by the morphological changes these cells undergo. Their elongation lengthwise and partial separation laterally increase their absorptive capacity very considerably.

Similar changes occur in the secretory epithelial cells of tea, as shown by Reed (Ann. Bot., xviii, 1904, p. 267). In addition deep glands are conspicuous features in the scutellum of tea, described by Misses Sargent and Robertson (Ann. Bot., xix, 1905, p. 115) and wholly confirmed by the writer's observations.

² Objects halved longitudinally to facilitate diffusion processes.

aleurone layer, possess of augmenting their amylase content. The salient point of difference to be noted is that under precisely parallel external conditions and quite apart from any possible differences in the attributes of the amylase from the two available sources (aleurone layer and inner endosperm), the amounts of enzyme which have *diffused* from the objects into the culture medium and naturally the total amounts of enzyme in objects and medium in Experiments 1, 2, 3 (endosperms) are decidedly superior to those in Experiments 4, 5 (inner endosperms).¹

If certain provisional assumptions are made, certain tentative evaluations of the relative augmentative capacities of the endosperm and inner endosperm under the selected conditions of experiment may be formed. These are that (1) destruction of enzyme falls equally on the enzyme from either source; (2) both enzymes have similar properties or attributes; (3) in both series of experiments the inner endosperm comports itself in precisely the same manner as regards its enzyme augmentative capacity, i.e. the presence of the aleurone layer does not influence the augmentative capacity of the inner endosperm; in short, the inner endosperm retains its individuality. Then, from the data given in Table XII, an approximate calculation may be hazarded of the relative augmentative capacities of the endosperm, inner endosperm, and of the aleurone layer.

The total augmentation of enzyme in each experiment is readily arrived at by deducting the initial amylase content from the total amylase content. Thus in Experiments 1 and 4 we have:—

$$\text{Exp. 1. } 3664 - 1181 = 2483 \text{ mg.}$$

$$\text{Exp. 4. } 1904 - 844 = 1060 \text{ mg.}$$

The relative contributory shares of the aleurone layer and inner endosperm may be computed by deducting the augmentation of enzyme in the inner endosperm from the augmentation in the endosperm. Thus in Experiments 1 and 4 we have:—

$$2483 - 1060 = 1423 \text{ mg.}$$

For the convenience of survey these results are given in tabulated form.

¹ These experiments afford clear evidence of the diffusibility of one colloidal substance into another (see Brown and Morris, *Chemistry and Physiology of Foliage Leaves*, Journ. Chem. Soc., lxiii, 1893, p. 657). Diffusion of enzyme across the aleurone layer cell-walls and spermoderm probably only takes place to a very limited extent. In Experiments 1 and 2 (endosperms), therefore, the enzyme must pass principally if not wholly via the 'germ bed' or exposed proximal end of the amyloferous tissue. Although the conditions in Experiments 4 and 5 (inner endosperms) for outward diffusion of enzyme apparently are greatly improved by removal of the aleurone layer, yet the amount of enzyme found in the medium is (as compared with Experiments 1, 2, 3) considerably less. The result is hardly that which anticipation would lead one to expect, if the units of the inner endosperm and aleurone layer possessed equal capacities for augmenting their content of amyloclastic enzyme.

Apart from the provision of external conditions favourable to outward diffusion, however, as we shall see later, the presence of a cytoclastic enzyme in the endosperm modifies certain internal conditions which probably favour diffusion. Removal of the aleurone layer means elimination of one of the principal sources of this cytoclastic enzyme.

TABLE XIII.

RELATIVE AMYLASE AUGMENTATIVE CAPACITIES OF ENDOSPERMS
AND INNER ENDOSPERMS.

<i>Exp.</i>		<i>Total augmentation of amylase. (equivalent to mg. of Cu.)</i>	<i>Relative augmentative capacities of:— Aleurone layer. Inner endosperms.</i>
1.	Endosperms	2483	1423
2.	"	2802	1742
3.	"	3082	2022
4.	Inner endosperms	1060	—
5.	" "	791	—

The tentative values given in the foregoing table show that the contribution of amylolytic enzyme derived from the secretory activity of the aleurone layer forms no mean proportion of the total augmentation of amylase found in the isolated endosperm under the conditions of experiment described.

In order further to investigate the augmentative capacity possessed by the inner endosperm, and for the purpose of comparison with similar objects under other experimental conditions, a further series of experiments was instituted with inner endosperms.

In this series of experiments the copper-reducing powers of the control digestions of the culture medium and object were specially noted, and careful macroscopical and microscopical examination was made of the condition of the starch contents taken from various parts of the objects under culture at the termination of their sojourn on the plates. The object was to ascertain whether any marked evidence suggestive of enzymatic action, the presence of reducing sugars, evidence of the corrosion or dissolution of starch grains, or the friability of the objects was demonstrable in the inner endosperm contents.

In the following table are grouped the results of the separate digestions of the culture medium and objects with soluble starch:—

TABLE XIV.

CULTURES OF INNER ENDOSPERMS ON 5 % GELATINE-MINERAL
SALTS.

Chilian barley. Culture period, 13 days. Seeds absolute alcohol 48 hours, water 48 hours.

<i>Exp.</i>	<i>Objects on plate.</i>	<i>Amylase per 20 objects (equivalent to mg. of Cu):—</i>			<i>Augmentation of amylase.</i>
		<i>Medium.</i>	<i>Objects.</i>	<i>Total.</i>	
1.	5 inner endosperms (halved)	1587	634	2221	1371
2.	" " "	1031	396	1427	583
3.	" " "	1507	634	2141	1297
4.	" " "	2304	238	2542	1698
Amylase present initially in 20 inner endosperms .				844	

In the objects themselves there were no reducing sugars in any of the experiments, showing that although augmentation of amylase had taken place it was unaccompanied by any demonstrable evidence of hydrolysis of the starch reserves.

Reducing sugars equivalent to 35 mg. maltose were found in Exp. 4 only, representing at best a very feeble amount of amylolytic action.

Macroscopically the objects were devoid of any outward indications of change; they had retained their intact form and mass, and microscopical examination of specimens of starch taken from different parts of an object failed to reveal any indication of action on the starch grains.

Thin sections of objects taken in various planes and freed from their starch contents by digestion at 30° C. with filtered, diluted saliva, and then examined microscopically, showed clearly that their cell-walls and cytoplasmic contents were quite intact.

After desiccation at 30° C. for twenty-four hours the objects were subjected to physical examination, with the result that *friability* of the inner endospermic substance, one of the usual and invariable accompaniments of recognizable change in the cell-walls of the amyliferous cells, was found to be entirely absent.

The results so far adduced, while they show that the inner endosperm is capable of generating and accumulating amylolytic enzyme in its tissue, negative the view that this enzyme very readily or vigorously acts upon the semi-solid barley starch at its disposal.

In the following table the data derived from similarly established comparative experiments with inner endosperms and endosperms are summarized, the only difference in the experimental conditions between these and the preceding experiments being the doubling of the concentration (indicated by the symbol P_2) of KH_2PO_4 .

The objects were not macroscopically and microscopically examined, but for present purposes it is only necessary to direct attention to the quantities of reducing sugars in the culture media in the endosperm experiments of this series, since this criterion affords a far more accurate and critical means of forming an opinion of the relative amylolytic activities possessed by the amylases (whatever may be their other specific attributes) which originate in the inner endosperm and aleurone layer respectively, than do measurements of the capacities possessed by these tissues to increase their amyloclastic enzyme content.

TABLE XV.

CULTURES OF ENDOSPERMS AND INNER ENDOSPERMS ON 5 %
GELATINE-MINERAL SALTS (P₂).¹

Chilian barley. Culture period, 7 days. Seeds 72 hours absolute alcohol, 48 hours water.

Exp.	Objects.	Amylase per 20 objects (equivalent to mg. of Cu) in:—			Reducing sugars per 20 in medium.
		Medium.	Objects.	Total.	
1.	5 inner endosperms	1111	317	1428	0.00
2.	5 endosperms	2222	974	3196	159
3.	„	2761	1349	4111	238

The amounts of reducing sugars found in the culture media in the case of the endosperm experiments, 2 and 3, afford convincing proof that when the aleurone layer is left intact, not only is there proof of augmentation of amylolytic enzyme by the endosperms, but there is also evidence of marked amylolytic action.

In Experiment 1, just as in the preceding experiments with similarly prepared objects, there is undoubtedly an increase of amylase, but there is an *entire absence of reducing sugars in demonstrable amount in the culture medium, over and beyond that initially present in the objects.*

The suggestion may be advanced that in the experiment under consideration the period of time to which the experiment was restricted may have been insufficient for the normal display of the presumed amylolytic function possessed by the inner endosperm.

The experimental data summarized in the following table fail to lend support to such a suggestion, as shown by the results of Experiment 3, with inner endosperms, given in the table below.

TABLE XVI.

CULTURES OF ENDOSPERMS AND INNER ENDOSPERMS ON 5 %
GELATINE-MINERAL SALTS (P₂).

Chilian barley. Culture periods, 3 and 17 days. Seeds 48 hours absolute alcohol, 48 hours water.

Exp.	Objects.	Duration of culture.	Amylase per 20 objects in:—			Reducing sugars in medium (equivalent to mg. of Cu).
			Medium.	Objects.	Total.	
1.	5 endosperms (halved)	3 days	1070	1130	2200	0.00
2.	„ „	17 „	2202	634	2836	313
3.	5 inner endosperms (halved)	17 „	1309	317	1626	0.00

It may be noticed in passing that as a general *result of increased amylolytic activity during prolongation of the culture period, in these culture experiments a diminution of enzyme occurs.* That is, enzyme activity in

¹ KH₂PO₄ concentration doubled.

these conditions entails enzyme destruction; only by prolongation of the experiment can this feature be rendered apparent, because during earlier phases of the culture experiment enzyme elaboration probably overshadows the opposed processes of enzyme inactivation and destruction. During normal germination within the limits of the malting period the accumulation of amyloclastic and other enzymes must necessarily be greatly in excess of the demand in order to ensure finally a substantial amount of residual enzyme.

These experimental results afforded by cultures of inner endosperms and endosperms on gelatine mineral salt medium may be briefly summarized.

Both types of experimental objects possess the capacity of augmenting their amylase-content, *the capacity of the endosperm, by virtue of exercise of the secretory functions of the aleurone layer, being distinctly superior to that of the inner endosperm.*

These experiments therefore furnish indirect collateral confirmation of the results already afforded by independent investigation of the isolated aleurone layer.

The secretion of amylase in the endosperm culture experiments is, if the experiment is of sufficient duration, invariably accompanied by the appearance of reducing sugars in the culture medium in easily demonstrable amounts.

In the inner endosperm experiments on this medium, this phenomenon is either absent or barely demonstrable, the amount of amylolytic action being *minimal*.

Macroscopically and microscopically the two series of objects, i. e. endosperms and inner endosperms, at the termination of the culture experiment, present features which are in marked contrast to each other.

There is invariably, in the case of the endosperm, reduction in the mass of culture objects, marked erosion of starch grains, and considerable alteration in the cell-walls of the amyloferous cells, features which are entirely absent in the inner endosperm cultivated and examined under identical conditions. The mass of the inner endosperm in these latter experiments remains practically unchanged, and there is an entire absence of any visible or readily recognizable change in either the starch contents or the cell-walls of the storage tissue.

Since in the one case the aleurone layer remains, and in the other it is removed, and further since direct experiment demonstrates that the aleurone layer possesses the capacity of secreting an active variety of amylase, it is difficult to avoid the conclusion that the observed phenomena are primarily due to the amylase which arises as a product of the secretory activity of the aleurone layer cells.

CULTURES OF ENDOSPERMS AND INNER ENDOSPERMS ON
AGAR MEDIA.

It appeared most desirable not to confine this important division of the inquiry to *one particular type of experimental substratum*.

Experiments were therefore instituted with endosperms and inner endosperms upon a 0.8% agar medium, this percentage of agar being chosen as the result of several preliminary trials, because of the additional facilities such a medium might offer in the promotion of the complex diffusion processes which present themselves in the type of experiments under consideration.

In the following table are summarized the results of a typical experimental series:—

TABLE XVII.

CULTURES OF ENDOSPERMS AND INNER ENDOSPERMS ON 0.8%
AGAR WITH AND WITHOUT MINERAL SALTS (P₂).

Chilian barley. Culture period, 19 days. Seeds absolute alcohol 72 hours, water 48 hours (2 changes).

Exp.	Objects.	Medium.	Amylase per 20 in:—			Reducing sugars per 20 in:—	
			Medium.	Object.	Total. (equivalent to mg. of Cu.)	Medium.	Objects.
1.	10 endosperms (halved)	Agar-M.S.	1358	1707	3065	465	0.00
2.	„ „ „	Agar only	776	1552	2328	465	0.00
3.	10 inner endosperms (halved)	„ „	1435	232	1667	174	0.00

Again the fact is evident that the augmentative capacity of the endosperm distinctly surpasses that of the inner endosperm. The results further bear evidence that the enzyme generated by the inner endosperm does possess a certain capacity for attacking its *amyliferous storage reserves*,¹ but this capacity is decidedly inferior to that exhibited by the endosperm, as the comparison of Experiments 1 and 2 with Experiment 3 very clearly indicates.

In these experiments examination of the two types of objects at the termination of the culture period reveals the same differential features as those presented by the gelatine cultures, and their significance does not require further detailed reference.

¹ If specimens of barley starch taken from various parts of the inner endosperm are examined microscopically, it is invariably found that intermingled with the large fully formed starch grains are a considerable number of very minute grains which stain blue on treatment with iodine, and therefore consist of starch. It is highly probable that these minute grains are more readily attacked than are the larger ones by the amylase of the inner endosperm. These minute grains are probably either of the nature of or very similar to 'transitory' starch.

Often repeated and searching microscopical examination of specimens of the starch contents from inner endosperms after removal from the culture medium in which reducing sugars were demonstrable, as in Experiment 3 above, fail to reveal any evidence of visible dissolution of the larger fully formed mature starch grains.

In the following series of experiments, Experiment 4 calls for special notice because it was designed to show, under conditions which are strictly comparable as regards external circumstances, the very different behaviour of the two halves of the endosperm under the following conditions:—

The dorsal portion of the aleurone layer was completely removed from each endosperm, and the latter was divided into two longitudinal halves by a plane traversing it midway between its dorsal and ventral halves; on each plate, therefore, in Experiments 3 and 4 there are ten dorsal halves of endosperms deprived of their aleurone layers, and ten corresponding ventral halves with their aleurone layers still adherent.

The numerical data furnished by these experiments are embodied in the following table:—

TABLE XVIII.

CULTURES OF ENDOSPERMS ON 0.8 % AGAR-MINERAL SALTS (P₄).¹

Chilian barley. Culture period, 19 days. Seeds 48 hours absolute alcohol, 48 hours water.

Exp.	Objects.	Amylase per 20 in:—		Reducing sugars in medium (equivalent to mg. of Cu).
		Medium. (equivalent to mg. of Cu.)	Objects.	
1.	10 endosperms (whole)	0.00	620	378
2.	10 endosperms (halved)	0.00	698	543
3.	10 endosperms deprived of dorsal aleurone layers	0.00	124	494
4.	10 endosperms, halved, and dorsal halves deprived of their aleurone layers	0.00	232	378

Just as in the experiments of Table XVII, there is a considerable amount of reducing sugar present in the culture medium in each experiment. The absence of amylase in the culture medium in this series apparently turns on the presence of the higher concentration of KH₂PO₄ employed. Whether this reagent is operative in the culture medium or during digestion with soluble starch is undecided and remains a question for future investigation.

The results show on the one hand (Exps. 3 and 4) that even when the area of the aleurone layer is reduced to practically one-half its original extent, and the reduction in the area of the amylase-secretory tissue is very considerable, the amylolytic activity of the remaining secretory tissue is still able to effect a considerable amount of transformation in the starch storage materials, as shown by the quantities of reducing sugars in the medium of these experiments.

It is, however, the *macroscopical and microscopical features presented by the ventral and dorsal halves in Exps. 3 and 4 which require mention.*

The former show the usual phenomena, already enumerated in the case of endosperm cultures: reduction in the mass of and rounding off

¹ Concentration of KH₂PO₄ fourfold.

of the contours of the inner endosperm tissue, erosion of starch granules, and evidence of marked change in the cell-walls of the amyliferous tissue.

The latter objects, on the contrary, exhibit little if any evidence of visible change either in mass or condition. In one or two instances, however, where a minute fragment of the aleurone layer was inadvertently left attached, the amyliferous tissue immediately underneath and on its margins when viewed by transmitted light was distinctly less opaque, and the fragment had literally sunk into the subjacent tissue as dissolution of the starch had progressed.

Invariably, in the endosperm of the intact seed under normal germination conditions, in the isolated endosperm under the cultural conditions here employed, action on the starch contents proceeds most rapidly, in point of time, in *the tissue subjacent to the dorsal aleurone layer*. The experiments just referred to demonstrate that the ventral portion of the aleurone layer is equally efficient in inducing transformation of the starch reserves placed at its disposal. The important and crucial point is that marked depletive change takes place, under the conditions selected, only in those halved objects which were not deprived of their aleurone layers. Where a minute fragment was all that remained, there was striking evidence of its influence in determining dissolution of the subjacent starch, an observation rendered possible only by the lengthy duration of these culture experiments.

The demonstration of the amylolytic capacity of the inner endosperm in Experiment 3, Table XVII, p. 833, was confined to the examination of cultures of these objects on agar alone. An additional series of experiments was therefore undertaken to ascertain whether the inclusion of mineral salts with variation of the concentration of the phosphate would influence the process of self-digestion exhibited by the inner endosperms.

These experiments are summarized in the table below, and additional data of interest are also included, namely, direct and indirect estimations of the amounts of reducing sugars in both objects and medium, and the weights of the objects after removal from the culture medium and subsequent desiccation for twenty-four hours at 30° C.

Two methods of preparing the objects were adopted :—

(1) The aleurone layers were filed off the air-dried endosperms, and the inner endosperms thus prepared were then steeped successively in absolute alcohol and water, forty-eight hours in each reagent (Experiments 1, 2, 3, and 4).

(2) *Endosperms* were steeped as in (1), and the aleurone layer removed with a razor (Experiments 5 and 6).

Method (1) excludes the possibility of enzyme passing by diffusion from the aleurone layer and embryo to the subjacent tissue during steeping.

Method (2) eliminates the possibility of the embryo secretion diffusing into the endosperm.

TABLE XIX.

CULTURES OF INNER ENDOSPERMS ON 0.8 % AGAR-MINERAL SALTS (P_2 AND P_4).¹

Chilian barley. Culture periods, 14 and 20 days.

Exp.	Objects.	Concentration of KH_2PO_4 in culture medium.	Duration of culture experiment.	Amylase per 20 objects per hour in:—		Reducing ² sugars per 20 objects in:—		Weight per 5 objects at termination of culture experiment. ³
				Medium.	Objects.	Medium.	Objects.	
				(equivalent to mg. of Cu.)				
1.	5 inner endosperms	P_2	14 days	0.00	379 ⁴	124	—	—
2.	" "	P_4	14 "	0.00	221 ⁴	103	—	—
3.	" "	P_2	20 "	—	—	26	0.00	167 mg.
4.	" "	P_4	20 "	—	—	26	0.00	142 "
5.	" "	P_2	20 "	85	2532 ⁵	113	—	139 "
6.	" "	P_4	20 "	0.00	3798 ⁵	29	—	125 "

Average initial weight of 5 inner endosperms, 175.

Possible objections to these methods of preparation will be discussed under the next subsection (IV B). It may, however, be noted here that very little, if any, diffusion of enzyme appears to take place when intact seeds are steeped as previously described.

The general character of these results, minimal depletive action, absence of obvious evidence of action on the cell-walls and contents, so closely parallels in every respect those yielded by similar objects prepared from intact steeped seeds that further reference to them is unnecessary.

In Experiments 3 and 4 the objects were directly investigated for reducing sugars, to test whether, in the absence of demonstrable cytohydrolysis of the cell-walls and the consequent presumed hindrance to the ready outward diffusion, the products of amylohydrolytic action had accumulated in the objects themselves. The foregoing experimental evidence, however, negatives this presupposition, and it is evident that the inferior auto-depletive capacity of the isolated inner endosperm cannot be attributed to this factor.

To confirm the statement frequently employed in previous sections, that

¹ The 0.8 % agar solution *per se* was invariably faint alkaline to alizarin. The phosphate employed in the concentration P_2 was apparently just sufficient to render the 0.8 % agar-M.S. neutral.

² The value given for the reducing sugars here and elsewhere is corrected for the small amount of reducing sugars present initially.

³ The reactions of the culture medium towards alizarin at the termination of the culture experiment were—Experiments 1, 3, and 5, neutral, and Experiments 2, 4, and 6, acid.

⁴ Determined by direct-digestion method.

⁵ Determined by papain-digestion method.

no appreciable reduction in the mass of inner endosperms takes place even when the culture period covers 14 to 20 days, the actual weights of the objects in Experiments 3 to 6 after 24 hours' desiccation at 30° C. were determined, and also for comparison the initial average weight of 5 inner endosperms similarly prepared and steeped only.

The influence of the phosphate in concentrations ranging from P_2 to P_4 appears to be in some way detrimental to the amylase which passes by diffusion into the medium.

Inner endosperms (Table XVII, p. 833) on agar *alone* apparently, as judged by the amount of reducing sugar in the medium, exhibit greater depletive power than on the same medium with the addition of mineral salts.

The evidence so far accumulated again tends to show that, while the inner endosperm does exhibit an auto-depletive capacity, that capacity is, as compared with that possessed by the endosperm, relatively limited.

IV B. CULTURES OF ENDOSPERMS AND INNER ENDOSPERMS ON MOIST CALCIUM SULPHATE.

Under the experimental conditions described in the preceding sections the quantitative method employed elicited two important facts, viz. that the isolated inner endosperm possessed a capacity for augmenting its amylase content, and an auto-depletive capacity, both of which, however, are inferior to the similar capacities possessed by the endosperm. Since the endosperm for our purpose consists of inner endosperm and aleurone layer, and since evidence has been adduced showing that the latter tissue is to be regarded as a secretory tissue, the marked signs of depletion which the endosperm exhibits may fairly be attributed to the action of an active amylolytic enzyme elaborated by the cells of the aleurone layer.

General considerations derived from a survey of the foregoing experimental results indicated that, as in all biological problems, the relation of the object ¹ to its substrate is an important consideration.

The employment of gelatine or agar media involves the use of substrata whose chemical *reaction* may vary from time to time within quite small limits, yet slight changes in this respect may introduce factors whose influence it is at once difficult to detect and control. It was therefore necessary to choose a single and preferably neutral inorganic substance in order to ensure the uniformity of the substrate.

¹ As will be pointed out later, we are probably dealing, in the case of inner endosperms, with the relation of an enzyme to its substrate.

Calcium sulphate appeared to offer many advantages: it was used successfully by Hansteen, by Puriewitsch, and latterly by Miss Bruschi, in their studies of endospermic depletion, and Ford and Guthrie have shown it to be one of those salts which solubilize the 'latent' amylase of the resting grain.

Experiments with endosperms and inner endosperms were therefore carried out on this substrate in the following manner:—

In each of a series of Petri dishes was placed a layer of calcium sulphate 15 mm. in depth; these were sterilized and, after cooling, sufficient sterilized tap-water was added to form a semi-solid stratum. The objects (endosperms and inner endosperms) were then inserted by their proximal ends, for a distance of a few millimetres, into the substratum, and finally water added in such quantity that, on tilting the dish slightly, water was visible at the lower margin of the semi-solid stratum. Under these conditions the objects received sufficient mechanical support to retain their vertical positions throughout the course of the experiment, and were virtually partially immersed by their proximal ends in a saturated solution of calcium sulphate. The conditions selected probably afford better conditions for the diffusion processes, both inward and outward, than the use of the *solid* gypsum columns adopted by Hansteen and others.

Typical examples of the results furnished by experiments established on these lines are given in the table which follows. They are not illustrative of the results furnished by one series of experiments merely, but of many similarly conducted essays.

After transferring to a beaker, the calcium sulphate substrate was treated with almost boiling water to arrest further amylolytic action, the mass well stirred and thrown on a filter, washed, cooled, and the filtrate and washings diluted to 100 c.c., and duplicate copper reductions carried out by Bertrand's method.¹

The sterility of the medium was controlled by inoculating small quantities of liquid and solid portions of the substratum into yeast-water-dextrose-maltose medium and incubating for 14 days at 30°.

¹ The residues from these filtrates were each evaporated to small bulk, refluxed with 90% alcohol, the alcoholic extract filtered and after expulsion of the alcohol examined polarimetrically; each was found to be dextro-rotatory. On treatment with phenylhydrazine each preparation yielded apparently a mixture of two osazones. These facts leave no doubt as to the nature of the reducing substances found in the various media after termination of the culture experiment.

TABLE XX.

CULTURE OF ENDOSPERMS AND INNER ENDOSPERMS ON MOIST CALCIUM SULPHATE (Kahlbaum).

Chilian barley. Culture period, 10 days.

Exp.	Objects.	Total reducing sugars in medium equivalent to milligrams of Cu.
1.	20 endosperms	601
2.	20 inner endosperms	133
3.	„ „	123

Experiment 1. Endosperms of air-dried seed steeped successively in (1) absolute alcohol; (2) water; 48 hours in each reagent.

Experiment 2. Aleurone layers filed off endosperms of air-dried seed; inner endosperms, thus prepared, then steeped as in Experiment 1.

Experiment 3. Intact seeds steeped successively in (1) absolute alcohol; (2) water; 48 hours in each reagent; embryos excised and aleurone layers removed with razor.

The contrast in the results afforded by Experiment 1 and Experiments 2 and 3 is most marked. In the space of 10 days 75 % of the endosperms in Experiment 1 were almost completely emptied of their inner endosperm contents; in the remaining 25 % the contents were in a semi-solid condition, and gentle pressure only was necessary to cause free ejection of their contents.

The inner endosperms (Experiments 2 and 3), on the contrary, had suffered little or no reduction in mass, and had the semi-solid consistency characteristic of similar objects which had been simply steeped and subsequently maintained in a moist condition.

These experimental results afford most convincing evidence, on the one hand, of the capacity of the endosperm, and, on the other, of the relative incapacity of the inner endosperm to induce complete and veritable depletion of the inner endosperm contents. The former result is conditioned by the presence of the aleurone layer, the latter by its absence.

The relative order of magnitude of the two capacities is also well shown by the relative amounts of reducing sugars in the substrata given in the above table.

The points of difference are still further emphasized by the contrast revealed by microscopical examination of starch granules from the two types of objects. These, in Experiment 1, exhibited extensive erosion, which is so closely similar to that hitherto attributed exclusively to the amylase secreted by the embryo ('amylase of secretion') that there is full justification for the statement that the enzyme secreted by the aleurone layer is *identical* with that elaborated by the embryo.

Microscopical examination of starch grains taken from various parts of the inner endosperms (Experiments 2 and 3) fails to reveal either any signs of erosion or any visible evidence of dissolution of the larger mature starch granules; they retain their characteristic lenticular shape, and the opinion is reiterated that in all probability the attack of the amylase on the inner

endosperm is restricted to the immature starch granules of minute size, which are always present in all parts of this tissue.

Invariably, in endosperm experiments such as Experiment 1, not only are the starch reserves degraded, but there is overwhelming evidence of advanced cytolysis. The cell-walls of the amyliferous cells exhibit every gradation of disintegrative change, from the initial swelling up and splitting into laminae to their complete dissolution. Careful, often repeated, examination of the cell-walls of objects 'cultivated' as in Experiments 2 and 3 fails to afford any conclusive evidence of cell-wall disintegration. Thin sections of such objects, treated with diluted saliva at 30° C. for several hours in order to dissolve out their starch contents, do not exhibit any recognizable sign of having undergone even partial cytolysis.

Physical examination of endosperms and inner endosperms removed after periods (varying from 7 to 20 days) of cultivation on calcium sulphate and subsequent desiccation for 24 hours at 30° C. shows that, while the former objects are distinctly friable, the latter do not exhibit this attribute, and the almost unavoidable inference to be derived from these observations is that cytolysis of the cell-walls, even where it does not result in their complete disintegration, is correlated in some way with acquisition of friability by the endospermic contents.

Finally, examination of the action, either of these two types of objects directly or of aqueous extracts prepared from them, on gelatinized starch affords a further means of emphasizing points of difference in the attributes of the amylolytic enzymes, which take their origin in the aleurone layer and inner endosperm respectively.

The objection may be raised that the method of preparing inner endosperms in Experiment 2 does not preclude, on the one hand, possible coagulation of amylolytic enzyme by absolute alcohol; on the other, considerable loss of enzyme by aqueous extraction during the steeping in water.

This objection is partly met by the results of Experiment 3, in which, by the method of steeping adopted, the inner endosperm was not exposed to these influences. In order to dispel this objection, inner endosperms were prepared as described in Experiment 2, and determinations were made of the amount of amylase which had diffused into the steep-water during steeping, and of the amylase present in the objects immediately after removal from the steep and subsequent desiccation at 30° for 24 hours.

The results were:—

	<i>Amylase per 20 per hour (equivalent to mg. of Cu).</i>
Amylase in steep water	261
Amylase in inner endosperms (direct digestion)	1055
Amylase in inner endosperms (0.5 % papain digestion)	2966

These data show that loss of amylase does occur under the circumstances, as one would have surmised ; but as direct digestion, and particularly papain digestion, demonstrate, very considerable amounts of amylase, both pre-existent or 'free' and 'latent', are still present in the tissue, prior to the objects being placed in the experimental substrate.

The general conclusion to be deduced from the experimental results comprised in this section, in which the relative powers of the endosperm and inner endosperm to induce auto-depletive change have been further examined, is that the endosperm, by virtue principally of the secretory functions of the aleurone layer, is capable of veritable auto-depletion. The inner endosperm, on the contrary, possesses this capacity in a restricted sense only, the process it is capable of inducing being one of limited auto-digestion, which never attains or approaches that of complete depletion.

Stoward, Frederick. 1911. "A research into the amyloclastic secretory capacities of the embryo and aleurone layer of *Hordeum* with special reference to the question of the vitality and auto-depletion of the endosperm. Part I." *Annals of botany* 25, 799–841.

<https://doi.org/10.1093/oxfordjournals.aob.a089355>.

View This Item Online: <https://www.biodiversitylibrary.org/item/239487>

DOI: <https://doi.org/10.1093/oxfordjournals.aob.a089355>

Permalink: <https://www.biodiversitylibrary.org/partpdf/319863>

Holding Institution

Smithsonian Libraries

Sponsored by

Biodiversity Heritage Library

Copyright & Reuse

Copyright Status: Not in copyright. The BHL knows of no copyright restrictions on this item.

This document was created from content at the **Biodiversity Heritage Library**, the world's largest open access digital library for biodiversity literature and archives. Visit BHL at <https://www.biodiversitylibrary.org>.