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"Metabolic relations of urease in Citrullus Vulgaris Schrad"

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Prafulla Chandra Sharma

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Botany Department,
Bedford College,
London, N.W.1.

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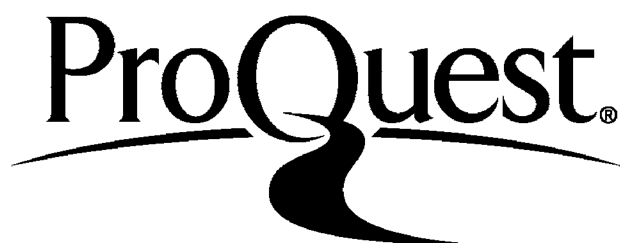

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ABSTRACT.

The enzyme urease is present in considerable quantity in the cotyledons of Citrullus seedlings, though elsewhere in the plant it is present only in traces or is absent; this has been shown by qualitative, quantitative and histochemical methods. The present investigation is aimed at elucidating the possible metabolic role of cotyledonary urease. The enzyme concentration has been found to fluctuate during the course of growth of the cotyledon, showing an initial rise followed by a sudden drop to practically zero.

It has been previously suggested that the urease may be no more than a reserve protein. However, experiments conducted to verify this hypothesis have shown that the protein level in the cotyledons remains practically constant at the time of urease disappearance. Another suggested possibility that the urease in the cotyledon is connected with urea metabolism of the seedling - has also been found to be invalid.

A detailed examination of the biochemical changes in the cotyledon during germination has been made and the possibilities of correlation of urease activity with chlorophyll content, water content, respiration and cell extension have been examined. Experiments were also conducted to ascertain whether the urease activity is affected by a change in nutritional status. These have shown that the cycle of

of urease change is independent of external nitrogen supply but is affected by the addition of glucose, which brings about an early drop in urease activity; the possible reason for this interaction is discussed. Lastly, the changes in urease activity in detached cotyledons have been studied under different experimental conditions.

In general, all the experimental results suggest that urease metabolism is connected solely with the growth and development of the cotyledon; and the very close correlation between cell extension and urease change suggests that the disappearance of urease in the cotyledon is simply one aspect of the "protoplasmic differentiation" that takes place as the cell extends. The high urease content of the young cotyledon is thus probably merely an indication of the state of the immature protoplasm of the unextended cells; there is no evidence that it is concerned in the metabolism of urea.

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I. INTRODUCTION

A. Occurrence.

1. General.

The enzyme urease is widely distributed in many species of bacteria and fungi, and in a number of higher plants. Since the discovery of urease in human urine by Musculus (1876), it attracted the attention of a number of investigators in the early part of the twentieth century. Shibata (1903-4) demonstrated the presence of urease in the mycelium of *Aspergillus niger*. Later, more attention was paid to the occurrence of urease in higher plants, particularly in the families Leguminosae and Cucurbitaceae. A review of the earlier literature shows that at first the investigations were mainly confined to the seeds, but later extended to other plant parts. At a still later stage, quantitative estimations of urease were undertaken and, finally, the distribution of urease in different parts of the same plant and subsequent changes in urease content at different stages of growth of the seedling were demonstrated by histological and quantitative methods respectively.

2. Qualitative and Quantitative investigations.

Takeuchi (1909) discovered urease in the seeds and seedlings of Soy-bean (*Glycine hispida*). This was found

to be more "powerful" than the urease of Aspergillus niger, and Takeuchi noted that the enzymatic action of the seeds was greater than that of the seedlings. He also found the urease reaction in the seeds of two varieties of Phaseolus, Pisum sativum, Triticum vulgare, Avena sativa, Oryza (Paddy rice), Fagopyrum (Buckwheat) and Cucumis melo. Zemplen (1912) demonstrated the presence of urease in the seeds of twenty species in the family Leguminosae, two species of Gramineae and one species in each of the families Moraceae, Rhamnaceae, Polygonaceae, Capparidaceae, Papavaraceae, Linaceae, Compositae, Cruciferae. He also found it in the seeds of Pinus maritima and Taxus baccata. Falk (1913), while investigating the action of neutral substances on the lipolytic activity of castor oil beans, observed the presence of urease in them. He did not attempt the quantitative estimation of urease in castor oil beans, but confirmed its presence by qualitative experiments and found that the enzyme was inactivated by heating to 90 - 100°C.

Annett (1914) studied the urease content of some Indian seeds. He found the urease activity of five varieties of Soy beans to be practically identical and observed that the urease activity of Canavalia ensiformis was many times greater than that of any of the Soy bean varieties tested. Benjamin (1915) noted the occurrence of urease in the nodules of seventeen leguminous plants.

He also found the enzyme in the seeds of *Cucumis melo*, *Abrus precatorius* and *Cucurbita moschata*, in the ovules and pollen of *Hippeastrum*, in the dried immature leaves of *Wistaria*, and in the "tubercles, rootlets and bulb" of *Macrozamia spiralis*. The crushed dried remains of a "saprophytic red alga", a "saprophytic green alga" and the lichens *Ramulina yemensis*, *Xanthoria parietina* and *Usnea* barbata also gave a urease reaction.

Mateer and Marshall (1916), while working on the urease content of certain beans found that the Jack bean (*Canavalia ensiformis*) contains over fifteen times and the Sword bean (*Canavalia gladiata*) about five times as much urease as the Soy bean. They also noted the presence of urease in the seeds of five varieties of *Phaseolus*, *Stizolabium deeringianum* (early florida velvet bean), two varieties of *Dolichos* and *Lupinus albus*. Fosse (1916) attempted but failed to demonstrate the simultaneous presence of urea and urease in *Aspergillus niger*, sprouted wheat, beans, and clover seeds.

Damodaran and Shivaramakrishnan (1937) investigated the urease content of a number of Indian seeds in an attempt to find a suitable source for use in urea determination. They found that urea determination in such materials as whole blood and liver with Soy bean or Jack bean urease gives abnormally high values, but that this effect becomes

negligible when Citrullus vulgaris is used as a source of urease. The urease content of a number of seeds tested is given in Table I.

TABLE I

Urease content in the seeds of a few selected plants. (after Damodaran and Shivaramakrishnan, 1937).

Seeds tested	Urease units (mg. of urea split per hr. per gm. of preparation.
Canavalia ensiformis	8822
" obtusifolia	5826
Cucurbita maxima	1376
Citrullus vulgaris	1152
Trichosanthes anguina	734
Soja hispida	657
Citrullus colocynthis	610
Dolichos biflorus	404
Momordica Charantia	232
Benincasa cerifera	63

3. Distribution.

Granick (1937) published results on the urease distribution in Canavalia ensiformis. Using histological and quantitative methods, he investigated the urease content

in cotyledons, radicle, root, hypocotyl, epicotyl, stem, leaves, flower and fruit. He finds that the cotyledons contain more urease than any other tissues of the plant. The enzyme concentration was found to be greatest in the sub-epidermal cells, especially in the bundle-sheaths and phloem tissue. The enzyme decreased rapidly in the epidermis and in the sub-epidermal parenchyma cells as germination proceeded. The old cotyledons which fell off were found to contain large quantities of urease; as will be shown later, this is in striking contrast to Citrullus, whose cotyledons lose practically all their urease before they fall. He had shown urease to be present in all parts of the plant body, but in relatively small amounts compared with the cotyledons. He also followed the change in urease content in the Jack bean plant as a whole, throughout its life cycle. The urease content decreased with the age of the seedling, but later, when the seeds were formed in the pods, the total urease content of the plant increased as a rapid synthesis of urease took place in the developing beans. The urease content of the nearly mature bean was found to be over 1,000 times that of the very young bean

Granick (1938) next described the urease content of the cotyledons, radicle, root, hypocotyl, stem, leaves, flowers and fruit of Soja Max. As in the Jack bean, the highest concentration of urease was noted in the cotyledons

and there was a gradual decrease of the enzyme as germination proceeded. In 8-day old seedlings, the enzyme was most concentrated in the sub-epidermal layers. The palisade layers gave a stronger urease reaction than the other parenchyma cells. No urease could be detected in the vascular bundles. He found the urease distribution in the etiolated cotyledons to be the same as in the green cotyledons, but with the difference that urease disappeared more rapidly in the etiolated cotyledons.

The total urease content of the cotyledons of 16-day old seedlings was less than the cotyledons of the 8-day old plants, but the nature of distribution was the same in both. In 30-day old seedlings, cotyledons contained very little urease, and all other parts of the seedling showed a low urease content as well. The change in urease activity of the developing beans and of the seedling as a whole was found to be similar to that of Canavalia ensiformis. It was shown for both Soy bean and Jack bean that the urease activity of an organ per grm. of fresh weight was highest when the cells were meristematic and decreased as the cells grew older. The urease activity increased rapidly in the actively dividing cells at first and the rate of increase dropped off when the cells passed into the elongating stage. The urease content was maximal at the end of the elongation stage. After this stage, there was a gradual decrease of the enzyme activity per cell until it attained

a constant low level. Granick regarded the changes in urease distribution and concentration of Soy bean and Jack bean to be of general occurrence in Papilionaceae.

4. Occurrence in animals.

It may be of interest to note the occurrence of the enzyme in animals. Przylecki (1922) found abundant urease in the molluscs Mytilus edulis and Helix pomatia and small amounts in the worm Lumbricus agricola and the crustacean Astacus fluviatilis. Urease was detected in the cells and blood serum of Limulus polyphemus by Loeb and Bodansky (1926). Robinson and Baker (1939) found urease in the larvae of Lucilia sericata and in their secretions.

Luck (1924), Luck and Seth (1925) found urease in the gastric mucosa of sheep, cows, cats, dogs and goats. Fitzgerald (1946) found urease in the gastric mucosa of man, dog, cat, rabbit, pig and rat. He could increase the urease content by feeding a protein or Soy bean diet.

Weil (1944) has shown that the red blood cells of rat, rabbit and man possess urease activity and that this activity is increased by autolytic processes. White blood cells contained very little urease. He was unable to demonstrate the urease activity in kidney, gastric mucosa, pancreas, brain, thymus and muscle of rat even after autolysis.

Most recently, Davies and Kornberg (1951) have investigated the role of gastric urease in acid secretion and have concluded that urea and urease play no direct or catalytic role in the mechanism of acid secretion.

B. Mechanism of urease action.

1. Specificity.

The discovery of a good source of urease in Soya bean by Takeuchi (1909) marked the beginning of a series of investigations into the mechanism of urease action. Tekeuchi tested the action of urease on guanidine, arginine, benzamide, allantoin, leucine, alanine, tyrosine, kreatine, histidine, guanine, glycocoll, uric acid and hippuric acid, but none of these compounds were hydrolysed by urease. Armstrong and Horton (1912) stated that Soya urease had no action on methylurea, S-dimethylurea, as-dimethylurea, ethylurea, s-diethylurea and biuret. According to Werner (1923) mono-butylurea is decomposed by urease at 50° - 60°C., but this could not be repeated by Sumner and Somers (1947). Urease has been found to hydrolyse only urea into ammonium carbonate. Though urease is thus regarded as absolutely specific, it has been recently stated by Shaw and Kistiakowsky (1950) that biuret is hydrolysed by crystalline urease.

2. Path of reaction.

The main argument has centred on the question whether cyanate or carbamate is formed as an intermediate product, and the work must be briefly reviewed, as it has some bearing on the hypothetical functions of urease in plants.

(a) Fawsitt (1902) believed that the hydrolysis of urea was always via cyanate.

(b) Armstrong and Horton (1912), working with Soya urease, believed that cyanate could arise from spontaneous hydrolysis in an aqueous solution of urea but that it did not do so when urea was hydrolysed by urease, the two reactions representing alternative means of decomposing a hypothetical urea hydrate $C(OH)_2\ (NH_2)_2$. They believed that the function of urease was thus to prevent formation of toxic cyanate within the plant.

(c) Lewis and Burrows (1912) found that on heating solutions of ammonium carbamate, about 1% of the carbamate is changed into urea. They therefore suggested the possibility that urea hydrolysis might produce ammonium carbonate in the following stages:-

$$CO(NH_2)_2 + H_2O \rightleftharpoons NH_4O.OC.NH_2$$

$$NH_4O.OC.NH_2 + H_2O \longrightarrow (NH_4)_2\ CO_3$$

(d) Burrows and Fawsitt (1914) repeated the claim that urea hydrolysis goes via cyanate.

(e) Yamasaki (1920) confirmed by quantitative means that carbamate is in fact an intermediate product of hydrolysis of urea by urease.

(f) Mack and Villars (1923) showed that the transformation of ammonium cyanate to urea is not catalysed by urease and that in fact urease is slowly inactivated by

cyanate; they pointed out that it is therefore improbable that the reverse reaction takes place.

(g) The controversy continued and Fearon (1923) again claimed that cyanate is formed as an intermediate product. Kay (1923), Iwanoff (1924) and Sumner (1926) pointed out a number of objections, based on the known characteristics of the reactions concerned, and failed to confirm Fearon's claim. Fearon (1926), using a new colour test, once more claimed to have found cyanate, but Sumner, Hand and Holloway (1931), using Fearon's test, were again unable to confirm.

(h) It is now generally accepted that cyanate is not an intermediate product and that hydrolysis normally goes via carbamate. Sumner, Hand and Holloway (1931) do not rule out the possibility that hydrolysis may go direct to ammonia and carbon-dioxide, and that carbamate may be formed secondarily from these, unless the reaction is carried out in buffered medium.

The precise role taken by urease itself in the reaction has been the subject of suggestions by Bersin and Koster (1937) and Brandt (1937). The possibility that cyanate may be formed from urea solutions in the absence of urease does not seem to have been completely ruled out.

C. Function.

We know very little regarding the function of urease in plants. A review of the work on the urease distribution in higher plants show that most of the workers did not investigate the metabolic activity of urease. The main attention, however, in view of the specificity of the enzyme, has been directed to its possible function in the metabolism of urea in plants. Granick (1938) goes so far as to state that urease performs the function of releasing ammonia from any free urea in the plant cells and makes it available for protein synthesis. The view of Armstrong and Horton (1912), already referred to, is that the presence of urease prevents the formation of cyanate from any free urea. Klein and Taubock (1931) have investigated the presence of urea and ureides in higher plants, and have found urea in a wide range of plants in the families Urticaceae, Leguminosae, Aceraceae, Cucurbitaceae, Gramineae and Orchidaceae. They found that the amount of urea present in any group of plants is very variable and discussed the possibility that it might be formed in plants, as in animals, from arginine. They were unable to find urea in seeds, urea being found only after germination. The ureides of the higher plants are of the aldehyde type - the formaldehyde compound, which is found only in green assimilating plants, and the acetaldehyde compound, which is formed in green unlighted or in chlorophyll-free organs, perhaps as a result of respiratory processes.

It is interesting to note here that Klein (1931) has shown that urea can be absorbed by a legume plant from a sterile culture medium and can presumably be split into ammonia rapidly by the urease present in the cells. Furthermore, he has shown that when large quantities of urea are fed, a considerable amount of ammonia is produced and the plant dies as a result of its toxicity. When urea is no longer supplied, the total urea then present in the plant disappears in a very short time.

The first extensive discussion of the possible metabolic role of urease was due to Granick (1938), who considered urease to be a "growth-bound enzyme". He does not define this term precisely, but evidently implies by it that changes in urease content are closely connected with the growth of the cells in which it occurs. He found rapid synthesis of urease in actively dividing cells, this synthesis continuing during the stage of cell elongation. The maximum amount of urease was found when the cell attained the maximum size. The urease content then decreases to a low level. He compared this change of urease activity with the change of respiratory activity and found that both follow similar courses at different stages of cell development. He considered urease a "growth-bound enzyme" on the basis of the following assumptions:-

(i) Urease, in its active state, is a soluble protein which undergoes changes similar to other proteins

in synthesis and decomposition.

(ii) The amount of soluble proteins, including urease, is a measure of the amount and activity of protoplasm.

(iii) Respiratory activity is related to protoplasmic activity or to the amount of catalytic (protein) surface upon which oxidation-reduction reactions take place.

Granick also discussed the possibility whether the enzyme is synthesised in situ or is transported as such to the growing regions. The rapid increase of urease content in developing beans may be due to its transport from other parts of the plant body. Granick concluded that the enzyme as such is never transported because it has not been detected in secondary phloem tissue and the disappearance of urease from the storage parenchyma cells of a cotyledon bears no observable relation to the proximity of these cells to fibrovascular bundles in their neighbourhood.

Granick's main conclusions appear to be that urease has a possible function in making urea, either from ureides or other sources within the plant, available as ammonia. The reverse reaction, i.e. the synthesis of urea from ammonium carbamate, is not catalysed by urease in plants; carbamate being unstable on the acid side of neutrality

is unlikely to be present in plant sap. In any case, urease is bound up with the process of growth and development and its content fluctuates accordingly.

The great difficulty in interpretation has always been the excessive amount of urease present and because of this it may be argued that urease may have some other function than hydrolysing urea. This, of course, is a dangerous argument, since lavish provision of substances or facilities in organisms is not uncommon, as pointed out long ago by Samuel Butler in his "Evolution, Old and New" (1879).

Williams (1950) made the point that the fact that an enzyme exhibits in vitro certain reactions does not mean that these reactions are necessarily in operation in vivo. He suggests that urease might be no more than a storage protein which only incidentally has the properties of urease. He attempted to support his hypothesis by following the changes in urease activity by quantitative means in *Citrullus* seeds during germination. He found a drop in urease activity as germination proceeded.

D. Scope of the present research.

The earlier researches on urease were mainly confined to kinetic studies or to the production of pure urease preparations for urea determinations. Even Granick's

work on Canavalia ensiformis (1937) and Soja Max (1938) were mainly based on histochemical methods, although a few truly quantitative measurements were made on developing beans and cotyledons of seedlings. The decrease in urease activity in cotyledons and leaves, as these age, was supposed by Granick to be in some way related with the protein catabolism in these organs, but no attempt was made to follow the changes in protein content and urease activity simultaneously. Moreover, Granick's suggestion about the possible relationship of urease activity with respiration was perhaps a speculation, as no quantitative data were presented.

The present research is directed towards the elucidation of the possible metabolic relations of urease in the seedlings of Citrullus vulgaris. The investigation has been mainly confined to the cotyledons, since, as will be shown, all but a trace of the urease present is in these organs. The change in urease activity has been followed by quantitative and histochemical means as the germination proceeds, and an attempt has been made to correlate this change with the protein breakdown in the cotyledons, under a wide range of experimental conditions. Observations have also been made on the structural changes in the developing cotyledons, and on the urease metabolism of cotyledons detached from the plant.

II. MATERIALS AND METHODS.

A. Material.

Citrullus vulgaris Schrad. Seeds of eight varieties were kindly provided by the Ferry-Morse Co. of California, U.S.A; the bulk of the experiments were carried out on the variety "Tom Watson".

B. Methods.

1. Urease estimation - Biochemical.

(a) Qualitative:-

10 cc. of 3 per cent urea solution was taken in a hard glass test tube. A drop or two of phenol red was added and the solution was acidified with sulphuric acid so that it just turned yellow. A suspension of the plant tissue to be tested, prepared by maceration with glass distilled water, was now added. Reddening of the liquid in the test tube indicated the presence of urease. This method is useful as a crude comparative study of the urease activity of the different parts of a plant, the high or low urease content of a constituent part being indicated by the intensity of the red colour of the test solution in a definite time period.

(b) Quantitative:-

A full general account has been given by Van Slyke and Archibald (1944). Three methods are available.

(i) The colorimetric timing method, which is based on the principle that the time required to produce a given amount of ammonium carbonate varies inversely as the urease concentration. The urease acts on urea in a phosphate buffer solution of pH 6·7 and the time required for the pH to rise to 7·7 is measured. This method is not suitable for very small amounts, as the reaction time is then too prolonged.

(ii) The Manometric method. This is based on the principle that in a constant time the amount of carbon dioxide formed is directly proportional to the urease concentration. The change in carbon dioxide tension is determined manometrically.

(iii) The Aeration-titration method. This is based on the principle that in a constant time the amount of ammonia formed is directly proportional to the urease concentration. The amount of ammonia produced is measured by aeration into standard acid. A phosphate buffer is used as vehicle for the reaction. It is of interest to note here that although objections have been raised to the use of

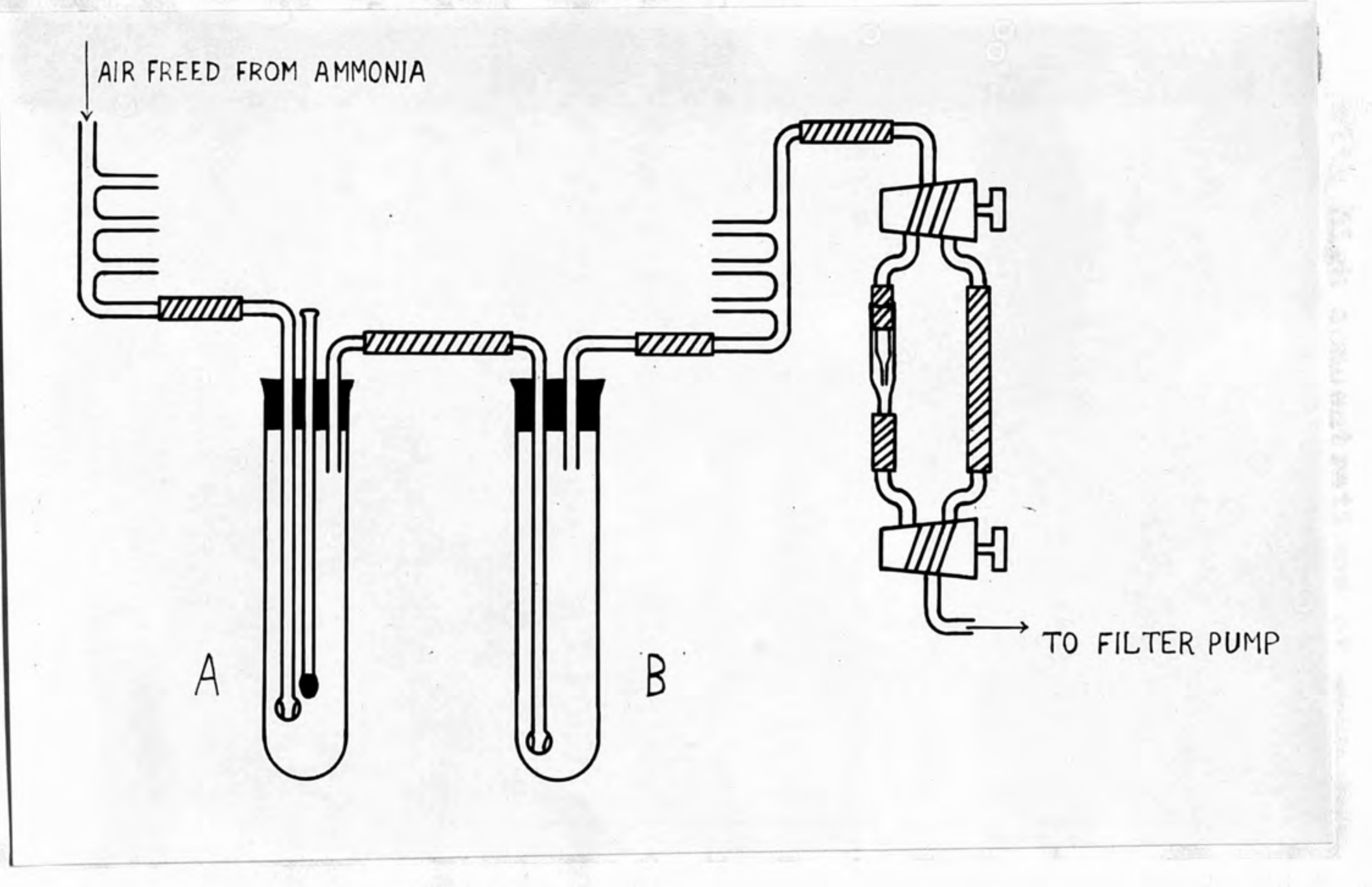


Fig. 1. Arrangement of apparatus used for urease estimation.

A - Digestion tube.

B - Receiving tube.

phosphate buffer, owing to the competitive inhibition of urease-catalysed hydrolysis of urea by phosphate, Harman and Niemann (1949) have demonstrated that with high concentrations of urea the inhibition is negligible.

This third method has been chosen for the present investigation.

The apparatus is shown diagrammatically in Fig. 1. It consisted of a set of four digestion tubes, each one of which was connected by rubber tubing with a receiving tube at one end and with a bottle containing concentrated sulphuric acid at the opposite end. The acid removed any trace of ammonia from a current of air drawn through the apparatus. The receiving tubes were connected with a suction pump. A control to adjust the current of air through the apparatus and a device to prevent back suction when stopping a current of air were inserted between the receiving tubes and the filter-pump.

The following reagents were used:-

<u>In digestion tubes.</u>

(1) Urea buffer:- 3% urea in $\frac{M}{6}$ phosphate buffer at pH 7 containing approximately 1% egg albumin as a protective colloid. This is made up from:

(i) 50 cc. of a solution of KH_2PO_4 containing 40·827 gms. in 500 cc. glass distilled water.

(ii) 20 cc. $N/_1$ KOH.

(iii) 3 gms. urea.

(iv) 4% egg albumin solution.

(2) Plain buffer:- Same as (1) without urea.

(3) Capryl alcohol.

In receiving tubes.

(4) Saturated solution of boric acid.

Cessation of reaction.

(5) Saturated solution of Potassium carbonate.

For titration.

(6) $N_{/100}$ Hydrochloric acid.

(7) Ma and Zuazaga's indicator:

1 part of 0·1% methyl red in 95% alcohol
+ 5 parts of 0·1% brom cresol green in 95%
ethyl alcohol and adjusted to pH 7. Greeny-grey colour.

The urease solution was prepared by grinding the desired number of cotyledons in glass distilled water and making up to a definite volume. It was found in the course of preliminary experiments that the addition of an abrasive such as powdered glass was unnecessary. 10 cc. of urea buffer and 10 cc. of plain buffer were taken in two digestion tubes respectively; the digestion tube with the plain buffer served as a blank. 25 cc. of saturated boric acid were pipetted into the corresponding receiving tubes. Two drops of capryl alcohol were added to each digestion tube and a drop to each receiving tube, to prevent frothing. The connections of the digestion

tubes on either side were sealed by means of screw clips, which were released (for aeration) only after the digestion was over. The temperature of the buffers was brought to 30°C by means of a water bath and 2 cc. of urease solution was added to each digestion tube. The stoppers were quickly set in place and the digestion was allowed to proceed for exactly 15 minutes at a constant temperature of 30°C. After the digestion was over, a current of air was drawn through the apparatus for 1 minute to remove any free ammonia. 10 cc. of saturated solution of potassium carbonate were then added to each digestion tube, and the stoppers were set in place as quickly as possible to prevent escape of ammonia. A current of air was then passed for a period of twenty minutes and all the ammonia released was aerated into the boric acid in the receiving tubes. The air current was passed slowly for 10 minutes, then rapidly for the remaining ten. The total ammonia nitrogen in each receiving tube was then calculated by titrating two samples, each of 5 cc., of boric acid from each receiving tube with $N/_{100}$ hydrochloric acid. The difference between the two readings for total ammonia gave the mgs. of ammonia nitrogen produced by 2 cc. of urease solution. In a few earlier experiments, $N/_{50}$ sulphuric acid was used in the receiving tubes and titrated against $N/_{50}$ caustic soda, using brom-cresol-green as indicator, but, owing to the unsatisfactory end-point obtained with

very dilute NaOH, in all subsequent experiments boric acid was used, titrated against $^{N}/_{100}$ hydrochloric acid using Ma and Zuazaga's indicator.

The urease content has been expressed in arbitrary units as mgs. of ammonia nitrogen produced in fifteen minutes under the experimental conditions. This unit seems to be arbitrarily decided by different authors. Sumner and Hand (1928) have defined it as that amount of urease which will form one mg. of ammonia nitrogen from 6% urea phosphate buffer at pH 7·0 at 20°C in 5 minutes. Damodaran and Shivaramakrishnan (1937) have defined a unit of urease as that quantity of it which will decompose one mg. of urea per hour under stated experimental conditions.

A few precautions were found necessary. The apparatus must of course be air-tight and the digestion tubes, mortar and pestle, measuring cylinder and pipettes used in connection with the preparation of urease solution should be cleaned with a cleaning solution (H_2SO_4 + Pot. dichromate) before every experiment; otherwise, urease may be carried over from a previous experiment, and, as the turnover number of urease is very high, the slightest trace thus carried over will invalidate a subsequent experiment. A trace of heavy metal may inactivate urease and so the digestion tubes, measuring cylinders, pipettes and mortar and pestle are to be rinsed with glass distilled water two or three times before use. The reagents necessary for the preparation of the

buffers must of course also be made up in glass distilled water. This may present unexpected difficulties; London tap-water was satisfactory after one distillation from glass, but Southampton tap-water appears to be more heavily chlorinated and tended to inhibit the reaction even after distillation. Eventually, for the Southampton experiments, the problem was overcome by using rain-water distilled first over permanganate and then again over caustic soda.

2. Urease estimation - histochemical.

(a) Sen's method (1930).

This is based on the principle that when a solution of urea is added to plant tissues containing urease, ammonium carbonate will be formed in places where urease is present. The location of urease may then be ascertained by double decomposition of ammonium carbonate by various neutral salt solutions of Ca, Cu, Pb, Ni and Co, to give insoluble carbonates which may be detected by appropriate (usually colour) tests.

Sen soaked a piece of Jack bean seed tissue for an hour in the following solution:

Absolute alcohol - 80 cc.

$CO\ (NO_3)_2$ - 1·0 gm.

Distilled H_2O - 20 cc.

The tissue was then transferred and kept in the following

solution for 48 hours.

Absolute alcohol - 80 cc.

$CO(NO_3)_2$ - 0·5 gms.

Urea - 0·5 gms.

Distilled water - 20 cc.

Microtome sections were then cut. The sections were finally washed free of soluble salts with distilled water, after passing through xylol and different grades of alcohol. The sections were then treated with a dilute solution of sodium sulphide or a saturated solution of hydrogen sulphide. The deposits of cobalt carbonate then turned black. When the reaction was complete, the sections were washed and mounted permanently.

This method was tried but with complete lack of success. The main difficulty is that it is impossible to be certain whether the cobalt nitrate is washed out of the sections completely; if any remains, it gives a spurious result. Verbal information has been received from workers in another laboratory in the country that they have also experienced the same difficulty.

(b) Granick's methods (1937).

(i) Indicator method -

Here, the detection of urease depends on the change of hydrogen ion concentration. It consists in the addition of a suitable indicator and a solution of urea to plant

tissues containing urease and the presence of the enzyme can be detected by the change in colour of the indicator, owing to the change in pH due to the production of ammonia. The first molecules of ammonia produced does not affect the pH, due to the buffering capacity of the cells. It is only when sufficient ammonia is produced that the pH changes towards alkaline side. Saturated aqueous solutions of Haematoxylin have been found to be the best for this method. The disadvantage of this method is that the ammonia produced at enzyme points in a particular cell may invade the neighbouring cells or tissues and the indicator test may give spurious results.

(ii) Lake method.

This method has been used in the present investigation. Granick devised this method to overcome the disadvantage of the indicator method. The principle involved is the formation of a non-diffusible precipitate at the enzyme points which are poisoned with the salts of heavy metals to check continuous production of ammonia which may invade the neighbouring tissues. The precipitate should at the same time be visible under the microscope. The heavy metals form their hydroxides when added to a solution whose pH is 6·7 or above. The hydroxides are insoluble precipitates which are not easily visible when formed in the cells, but can be detected when a suitable dye is adsorbed.

Freehand sections of the cotyledons were treated with

a solution of aqueous haematoxylin which was prepared fresh every three or four days, for a few minutes, until it penetrated all the cells. The excess dye was then drained off and a drop of 1% urea solution was added; and immediately afterwards a drop of 0·05M $NiSO_4$ was added. The sections were covered with coverslips after draining off the excess fluid and immediately examined under the microscope to note the distribution of the deep blue colour of the nickel hydroxide haematoxylin lake. The time interval between the addition of urea solution and nickel sulphate solution was adjusted according to the urease concentration in the cotyledons. A longer interval of 4 - 5 minutes was necessary in older cotyledons where urease concentration was very low. The action of the enzyme converted the haematoxylin from yellow to red, which immediately became deep blue on the addition of nickel sulphate. A section was always used as a control, which was treated with haematoxylin and nickel sulphate without urea. It did not give any reaction.

3. <u>Other Estimations.</u>

(a) <u>Total nitrogen and total soluble nitrogen.</u>

These were estimated by the normal micro-Kjeldahl technique. The cotyledons were ground to a fine paste with distilled water and the solution made up to a definite volume. An aliquot of the suspension was used for the

estimation of total nitrogen. To a second aliquot of the same solution, 10% trichloracetic acid (10 cc. of the acid per grm. fresh weight of the material) was added and left overnight. The solution was then filtered with ordinary filter paper (Whatman No. 1) and the filtrate used in the determination of the total soluble nitrogen: it has been assumed for the purposes of this investigation that the material insoluble in trichloracetic acid contained the whole of the protein nitrogen. The digestion was carried out in 100 ml. flasks with Chibnall's catalyst (80 gm. M.A.R. potassium sulphate + 20 gm. M.A.R. hydrated cupric sulphate + 0·34 gm. N-free sodium selenate). Folin's fume absorbers were used as no fume-cupboard was available. An experiment was performed to fix the time period for the digestion. Four green cotyledons were ground up in distilled water and the solution made up to 25 cc. 5 cc. samples of this solution were taken in three Kjeldahl flasks and digested for different time periods after clearance, and their total nitrogen was determined. The results are given in Table II below.

TABLE II.

Effect of digestion period in the determination of Total Nitrogen.

Time in hrs.	Total N. in mgs
(1) 2 hours	1·0115
(2) 4 hours	1·0200
(3) 8 hours	1·0242

A digestion period of four hours has been adopted for the present investigation. The digest was then diluted with water and transferred to a Markham's distillation apparatus. The ammonia, liberated by the addition of sodium hydroxide (40% $^{W}/_{W}$) was passed by steam distillation into 5 cc. of saturated boric acid containing three drops of Ma and Zuzaga's indicator (see above). The amount of ammonia was then found by titration with $^{N}/_{100}$ hydrochloric acid to match the colour of the original 5 cc. boric acid with three drops of the indicator, the solution being made up to the same volume in both before titration. The protein nitrogen was determined as the difference between total nitrogen and total soluble nitrogen.

(b) Dry weights:-

The dry weights of the cotyledons were determined after keeping the cotyledons in weighing bottles for a period of twenty-four hours in an electric oven maintained at a temperature of 80°C.

(c) Respiration measurements:-

The respiration rate of the cotyledons was measured with a Warburg constant volume Respirometer.

4. Experimental Conditions.

The methods of culturing the plants, and the various experimental conditions under which they were grown, are dealt with in the text.

III. VARIATIONS IN UREASE CONTENT UNDER CONSTANT CONDITIONS.

A. Seed.

1. Between varieties.

The variations in urease activity between the seeds of different varieties of *Citrullus* have not been previously recorded. However, some estimate of the variability of the material is clearly desirable in quantitative work; and this investigation was therefore undertaken for the eight varieties available out of the twenty-five or so standard varieties grown in the U.S.A. A sample of five seeds was taken for each determination and the urease activity (in mgs. of ammonia nitrogen) has been expressed both per seed and per grm. fresh weight of the material. The results of two independent sets of determinations, each set being replicated, are given in Table III.

Two points of interest emerge from the data in Table III. First, there is a large difference between sets on different occasions. This phenomenon has often been observed; although the order of results has always been consistent, it has been very difficult to obtain exactly the same results from different batches of seeds and solutions on different occasions. Such discrepancies do not appear to be

TABLE III.

Urease activity in different varieties.

	1st Set				2nd Set			
	1st replicate		2nd replicate		1st replicate		2nd recplicate	
Variety	Urease activity per seed	Urease activity per grm. fresh wt.	Urease activity per seed	Urease activity per grm. fresh wt.	Urease activity per seed	Urease activity per grm. fresh wt.	Urease activity per seed	Urease activity per grm. fresh wt.
Klondyke Blue Ribbon W.R.	2·2	77·3	1·6	53·7	2·3	88·6	2·7	102·7
Florida Giant	3·4	58·8	3·6	67·3	3·6	67·0	6·3	119·0
Klondyke Black seeded	2·6	86·4	2·4	78·2	2·4	85·0	3·2	112·0
Wonder	5·4	92·9	6·2	103·9	8·4	131·2	8·3	151·8
Tom Watson	5·3	112·6	4·2	88·9	5·5	98·4	4·5	93·9
Dixie Queen	2·2	36·2	1·6	26·1	1·7	28·4	1·9	31·0
Klondyke Brown seeded	1·9	63·9	2·4	91·7	3·5	130·0	3·2	123·0
Klondyke R.7 W.R.	1·7	48·8	3·0	93·3	3·2	99·0	3·6	109·5

attributable to any single cause, and it was considered that a complete elucidation of the phenomenon would have been unjustifiably time-consuming. Secondly, "urease per grm. fresh weight" is much less variable than "urease per seed". For this reason, an analysis of variance on the "per grm. fresh weight" data was carried out and is shown below, (Table IV).

TABLE IV.

Analysis of variance.

Source of variance	D.F.	Mean square	Variance ratio
Varieties	7	2727	9·1
Sets	1	4765	15·9
Interaction	7	380) }300	
Error	16	265)	

The interaction is not significant and can therefore be pooled with error to give an error mean square of 300 with 23 degrees of freedom; this has been used in calculating the variance ratio. The analysis indicates a highly significant effect on the P = 0·001 level due to sets. As stated above, the cause of this highly significant difference between the results on different occasions has

not been traced. The variation between varieties is also highly significant on the P = 0·001 level and remains so (F = 5·61) compared with all other sources of variation. However, examination of the results suggests that the bulk of this variance is due to the variety 'Dixie Queen' and so a reanalysis as shown below (Table V) was carried out, omitting the results for 'Dixie Queen'.

TABLE V.

Analysis of variance.

Source of variance	D.F.	Mean square	Variance ratio
Varieties	6	832·17	2·24
Sets	1	5527·30	14·90
Interaction	6	315·657))370·60	
Error	15	392·56)	

The effect due to sets is the same as before, but the intervarietal difference is now not significant. It may therefore be concluded that the variety 'Dixie Queen' has a significantly lower urease content than the other seven varieties examined, which do not differ significantly amongst themselves.

2. Between seeds of the same variety.

The fresh weight and the urease content of ten seeds of the variety 'Tom Watson' were determined and the results are given in Table VI.

TABLE VI.

Variation in urease activity in seeds of the variety 'Tom Watson'.

Fresh weight in grms.	Urease activity per seed	Urease activity per grm. fresh wt.
(1) 0·0522	4·02	77·01
(2) 0·0302	5·80	192·10
(3) 0·0274	5·40	197·10
(4) 0·0334	5·10	152·70
(5) 0·0202	3·50	173·20
(6) 0·0266	3·80	142·90
(7) 0·0500	4·30	86·00
(8) 0·0460	4·70	102·20
(9) 0·0480	4·20	87·50
(10) 0·0340	5·50	161·80

It is desirable to ascertain which method of expressing urease content is the more reliable for seeds of a single variety. The statistical quantities are as follows, (Table VII).

TABLE VII.

Statistical data.

	Urease content per seed	Urease content per gm. fresh wt.
Mean	4·63	137·25
S.E. of mean	0·25	14·42
Coefficient of variation	17·03	33·23

The coefficients of variation ($100S/\overline{X}$), being independent of the unit of measurement, provide the best comparison of variation and it will be seen that the values expressed as "per seed" are considerably more consistent than those expressed as "per grm. fresh weight", although the reverse seems to be true when variation between seeds of different varieties are considered. Evidently, in any one variety, an approximately constant amount of urease is produced per seed irrespective of minor variations in weight of seed. However, between varieties seeds vary greatly in size and in this case the size-variation is evidently reflected in urease content. As it is quite clear from the data that there is no correlation between urease content and fresh weight of the seeds, it seemed possible that there might be some relationship between urease content and total

nitrogen in individual seeds; but preliminary measurements showed that this was not the case. As the present investigation has been carried out on only one variety, urease activity has been expressed on a "per seed" basis throughout the remainder of this thesis.

3. Between cotyledons of the same seed.

The cotyledons of a single seed were carefully separated and their urease activity determined separately. The data are presented in Table VIII.

TABLE VIII.
Variation in urease activity in the cotyledons of a seed.

Seed	Cotyledons	Fresh weight in grms.	Urease activity
I	(1)	0·0232	1·89
	(2)	0·0268	2·42
II	(1)	0·0242	2·49
	(2)	0·0222	2·35
III	(1)	0·0298	2·60
	(2)	0·0316	3·40

It is clear that there may be considerable variation between the two cotyledons of a seed and in any experiment in which these are handled separately this fact must be borne in mind as a possible additional source of variation.

B. Between the different parts of a seedling.

1. Qualitative. The tests were carried out by the method mentioned earlier (p.17), on a 6-day seedling, and the observations are presented in Table IX.

TABLE IX.

Variation in urease activity in different parts of the seedling.

Parts of the seedling	Fresh weight in grms.	Observation.
(1) Small portion of the tip of the cotyledon	0·046	The reddening starts within fifteen seconds and is complete in forty-five seconds.
(2) Top part of the hypocotyl - 3/4"	0·098	The reddening takes place in about fifteen minutes and is completely red within thirty minutes, although the degree of redness is much less than in (1).
(3) Bottom part of the hypocotyl 3/4" (to above the peg)	0·080	Same as (2) but it required forty minutes to attain same deep colour of (2).
(4) Epicotyl	0·005	No change even after one hour and fifteen minutes. Even in the following morning (after about 17 hours) there was no change in the tube.
(5) Roots	0·130	A very slight reddening takes place in about fifty minutes.

2. Quantitative.

The urease content of the different parts of a seedling were estimated quantitatively. This time the investigation

was repeated on seedlings of different ages. The data are presented in Table X: urease content is as usual expressed in mg. ammonia nitrogen.

TABLE X.

Quantitative estimation of urease content in different parts of the seedling.

Seedling	Age	Cotyledons		Epicotyl		Hypocotyl		Roots	
		Fresh weight in grms.	Urease content	Fresh weight in grms.	Urease content	Fresh weight in grms.	Urease content	Fresh weight in grms.	Urease content
(1)	6 days	0·256	8·90	0·0006	-	0·279	0·67	0·117	-
(2)	8 days	0·280	2·04	0·0008	-	0·290	-	0·100	-
(3)	14 days	0·459	0·29	0·0012	-	0·319	0·29	0·170	-
(4)	18 days	0·432	-	0·0060	-	0·279	0·29	0·240	-
(5)	21 days	0·413	0·29	0·0190	-	0·205	-	0·230	-

The results for the 6-day seedling are completely compatible with those obtained by the qualitative method. The striking drop in urease content of the cotyledons with age will not be discussed further in this section.

3. Histochemical.

The distribution of urease in different parts of the seedling was also investigated by Granick's lake method described earlier.

(a) 2- or 3-day seedlings. A section of the cotyledon of a seedling either two or three days old shows the formation of the blue precipitate in all the mesophyll cells within the first minute of the addition of nickel sulphate. A section of the root in either case did not give an immediate reaction after addition of $NiSO_4$ but within 2 - 3 minutes some of the cortical cells developed the blue colour. The period between the addition of urea and nickel sulphate was 3 - 4 minutes in case of roots.

(b) 7-day-old seedlings. A section of the cotyledons showed the blue precipitate to be confined only to the bundle sheaths and a few isolated cells of the mesophyll. A section of the hypocotyl showed the blue precipitate in a few cells near the vascular bundles towards the pith. The blue precipitate was observed in the section of the root in the phloem cells.

(c) 9-day-old seedlings. The blue precipitate in the section of the cotyledon was now confined to the bundle sheaths of the vascular bundles. In the hypocotyl, the blue precipitate developed in a few parenchymatous cells of the cortex as well as in small areas adjacent to the vascular bundles towards the pith. In the root sections, the phloem cells and a few cortical cells showed the blue precipitate.

(d) 10-day-old seedlings. In the case of both roots and hypocotyls, even though the period between the addition of urea and $NiSO_4$ was extended, there was no immediate reaction after the addition of $NiSO_4$ and the blue precipitate developed only after about 4 minutes.

C. Conclusions.

The investigation into the variation in urease content between different seeds of the same variety ('Tom Watson') has shown that, surprisingly, there is less variation in urease content than in fresh weight. The significance of this observation is obscure, particularly since the relationship breaks down strikingly when seeds of different varieties are compared; but it justifies the use of the very convenient measure of "urease content per seed" throughout the investigation.

The results of the investigations into young seedlings (c. 6 days or younger) show that almost the whole of the urease is present in the cotyledons, no more than a trace being detectable in the hypocotyl or roots and apparently none in the epicotyl. The quantitative method is insufficiently sensitive to detect the small quantity in the roots; this small quantity, whose significance is considered later (p.102), is however detectable by Granick's histochemical method. It is evident that studies in the metabolic relationships of urease can be practically confined to the cotyledons.

The disappearance of urease from the cotyledons of older seedlings is the most important aspect -- since it is the most striking change observed -- of this investigation, and is dealt with in detail in the subsequent sections.

IV. MORPHOLOGICAL CHANGES DURING GERMINATION.

A. Introduction.

In order that changes in urease content in the developing seedling may be related to the stage of differentiation reached at any time, it is essential to know the time-course of development, both of the morphology of the seedling as a whole, and of the anatomy of the cotyledons in particular, with reasonable accuracy. An account of the germination of Citrullus vulgaris has been given by Hufford (1938); but this account does not provide the information of day-by-day changes which is required for the present work, and a further investigation has therefore been necessary.

The seeds are relatively large, which has simplified the work. The testa is usually smooth, though it may be rough in the varieties with a spotted testa, and the seeds are flat and more or less ellipsoidal in shape. The seed-size varies considerably with the variety, as Table XI shows; in this table the mean dimensions of ten seeds, together with their standard errors, are given for each of the eight varieties available. It should be noted that the testa also varies markedly in thickness, so that the external dimensions give little guide as to the size of the embryo within.

TABLE XI.

Size and colour of seeds in different varieties.

Variety	Average length in mms.	Average breadth in mms.	Colour
Florida Giant	13·9 ±0·31	8·45 ±0·17	Black spotted
Klondyke R.7. W.R.	10·6 ±0·16	6·25 ±0·13	Light brown
Klondyke Brown seeded	10·0 ±0·20	6·30 ±0·11	Yellowish brown
Klondyke Blue Ribbon W.R.	9·7 ±0·19	6·55 ±0·17	Yellowish brown
Dixie Queen	12·3 ±0·32	7·20 ±0·24	White
Tom Watson	12·0 ±0·24	7·50 ±0·13	White
Klondyke Black seeded	8·9 ±0·10	5·45 ±0·12	Black
Wonder	13·35 ±0·22	8·00 ±0·11	White

B. Methods.

The germination was very erratic when the seeds were sown in soil. The seedlings generally emerged between ten and fifteen days after sowing. This procedure being unsuitable for determining the age of the seedlings, as the exact date of germination was not known, a different method of germinating seeds was adopted. Seeds were first soaked in water for twenty-four hours, then transferred to wet filter paper and kept in an incubator which was maintained at a constant temperature of 25°C. With such treatment, it was

always found that nearly seventy per cent of the seeds germinated within the first 24 hours of incubation, and the rest within forty-eight hours. In later experiments, a different batch of seeds was used; 70 - 75% of these seeds germinated within forty-eight hours of incubation and the rest within seventy-two hours. On the first day of germination, which was taken as the day when the radicle was 1 - 5 mms. in length, the seeds were transferred to pots containing soil. A mixture of sand, loam and peat in equal proportions was used. The pots were then put under artificial light. In most experiments, eight 80-watt daylight fluorescent tubes were fitted to a frame in the form of an arc and this could be moved up and down and so maintained at a desired height over the pots. The light intensity was 750 ft. candles at the tubes. The pots were exposed to a daily light period of sixteen hours and a dark period of eight hours, the lights being operated by a time-switch. This light was found to be suitable for the growth of the seedlings. The pots were watered on every alternate day. It was observed that under conditions of low light intensity, as found after long usage of the fluorescent tubes, the seedlings showed signs of etiolation, the hypocotyl being unusually elongated and the expansion of the cotyledon checked to a certain extent. It was therefore necessary to replace the fluorescent tubes about every four months. For the later experiments carried out at Southampton, such a lighting system was not available and a 1000-watt

incandescent lamp was used.

The changes in the external morphology of the seedlings were recorded daily. Simultaneously, other seedlings were examined for changes in the internal structure of the cotyledon by the following method. The material was fixed in formalin aceto-alcohol and embedded in paraffin by the tertiary-butyl-alcohol method. Transverse sections were then cut at 12μ with the microtome and stained with safranin and Delafield's haematoxylin. The central part of the cotyledon was always used for fixing to get the best comparison at different stages of growth. In the early stages of growth, the tissues did not take up any safranin at all, thus showing that lignification was negligible. The sections were then drawn with a projection microscope under a magnification of x56 to show the outline of the tissues of the mesophyll and the central part of the section with a vascular bundle was also drawn under a magnification of x240. The central vascular bundle was chosen as the bundles towards the margin of the cotyledon were always less developed.

C. Results.

1. Summary of changes.

Changes in external morphology and cotyledon anatomy are summarized in Table XII and figures 2, 3 and 4.

TABLE XII.

Changes in external morphology and cotyledon anatomy during germination.

Day	External Morphology	Anatomy of the cotyledons	Figs.
0	-	Mesophyll may be distinguished into palisade and spongy parenchyma; the palisade consists of 2-3 layers; no discernible intercellular spaces; five pro-vascular strands are present in the spongy parenchyma; the strands are parenchymatous and no differentiation into xylem and phloem is visible.	
1	Seedlings below the soil surface, and cotyledons were inside the seed coats, radicle 1-5 mms. in length.	————	2E and 3E
2	Seedlings below the soil surface and radicle 10-12 mms. in length.	Few initial cells immediately below the palisade layer have begun to divide, forming groups of small cells which will ultimately form the traces of the veinlets.	
3	Seedlings below the soil surface, and radicle 25-35 mms. in length; the "peg" is visible.	————	
4	Seedlings nearly on surface of the soil; hypocotyl greenish and 5-6 mms. in length; sometimes the lower part of the cotyledons	Intercellular spaces visible in spongy parenchyma. Few xylem vessels embedded in a mass of parenchyma are now visible in the pro-vascular strands, but phloem cannot be clearly distinguished. Veinlet traces	

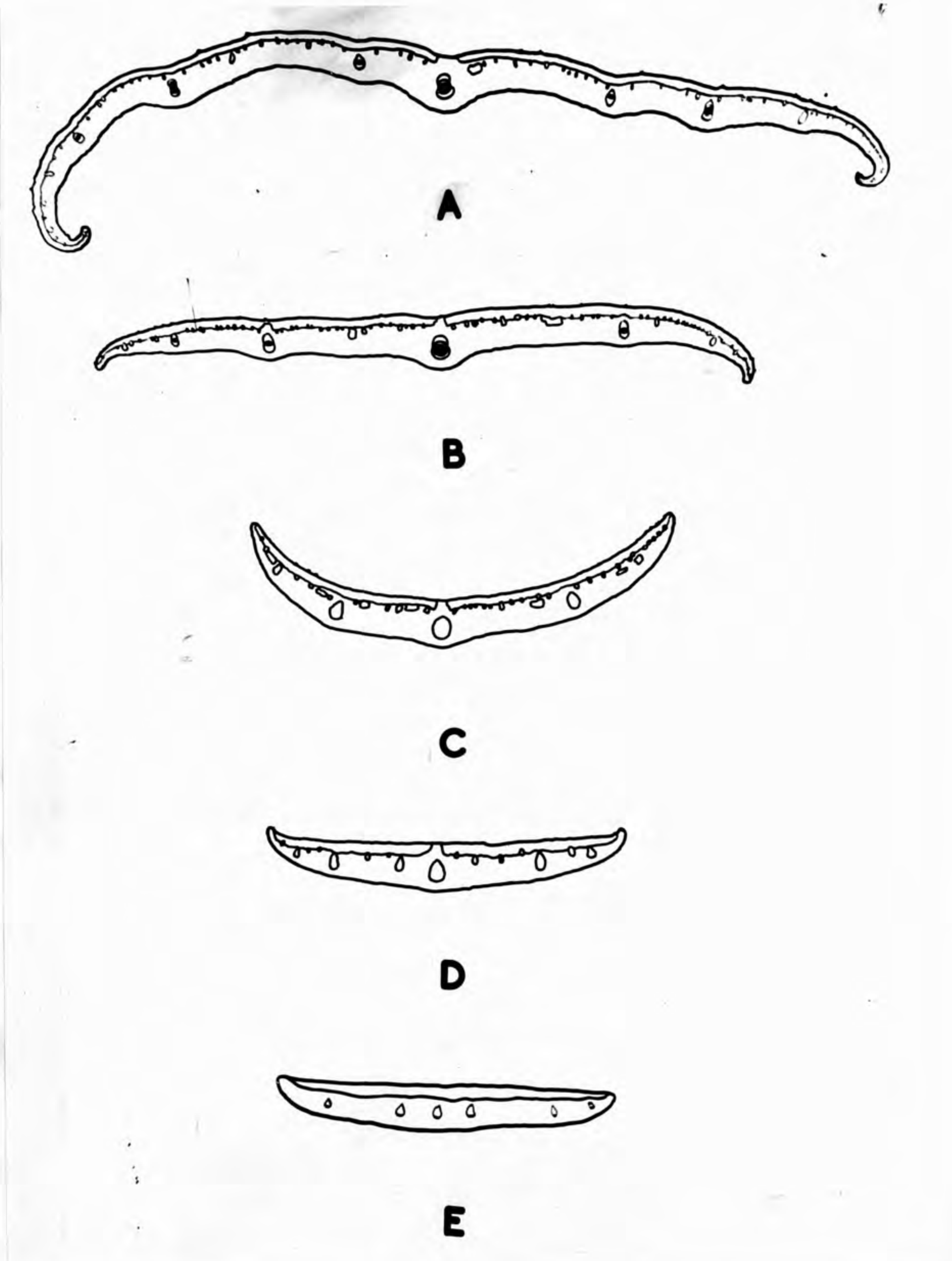


Fig. 2. Outlines of the transverse sections of the cotyledons of different ages.

A - 13 days
B - 10 days
C - 7 days
D - 5 days
E - 1 day.

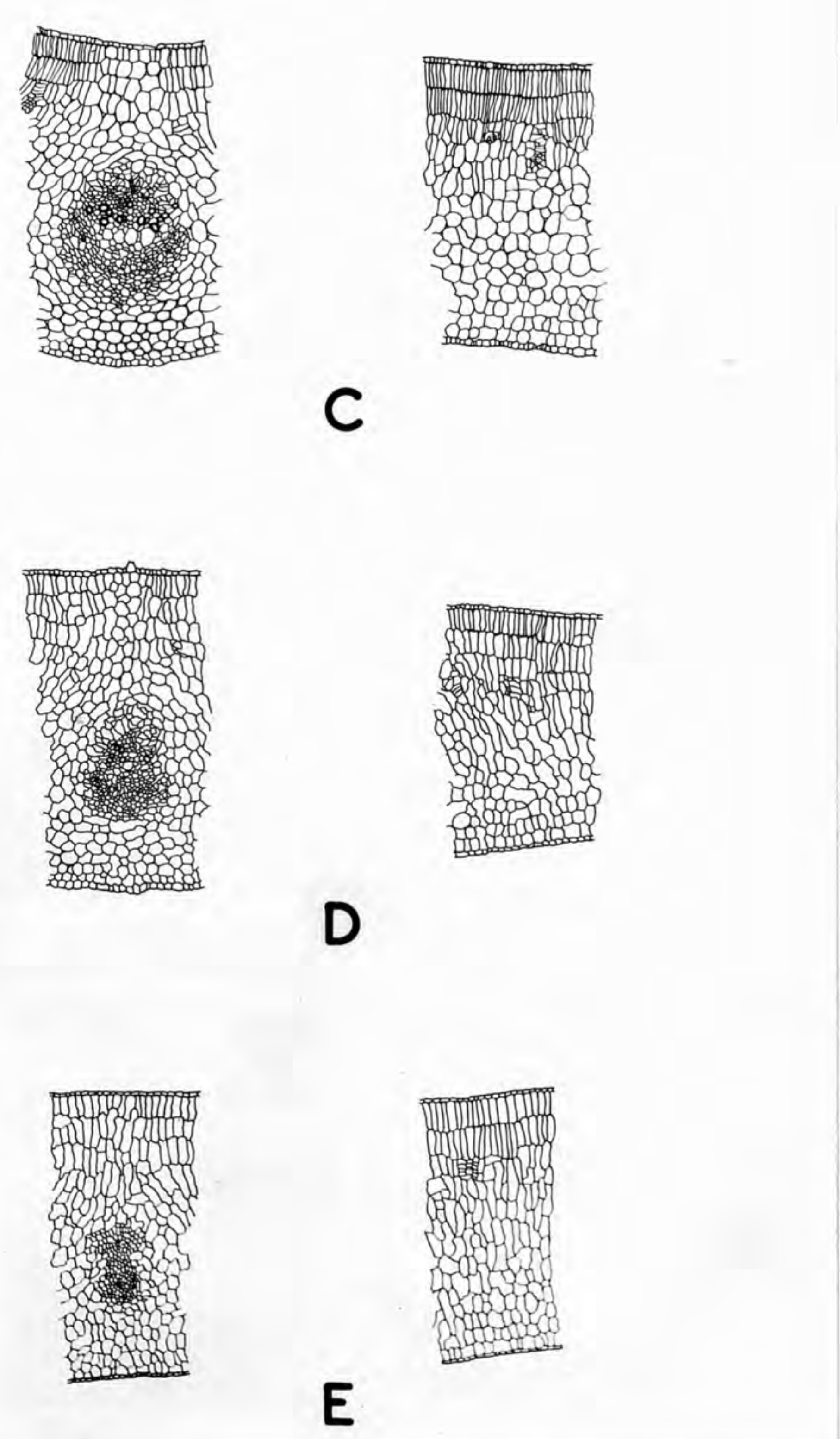


Fig. 3. Transverse sections of the cotyledons of different ages.

C - 7 days

D - 5 days

E - 1 day.

Day	External Morphology	Anatomy of the cotyledons	Figs.
	which were by now half way out of the seed coats were also greenish. Radicle 50-65 mms. in length and small lateral rootlets have begun to develop.	have increased in number, but without any development of xylem in it.	
5	Seedlings emerged from the soil; cotyledons greenish, hypocotyl 20-30 mms. in length; minute plumule was observed and lateral rootlets are increasing in number.	Intercellular spaces are visible in the palisade as well.	2D and 3D
6	Cotyledons became deep green in colour and were increasing in size. The cotyledons of an individual seedling were parallel to each other and the plumule was developing.	The number of veinlet traces have greatly increased. In the vascular bundle, on either side of the xylem can be observed a mass of phloem, that on the lower side being separated from the xylem by parenchyma.	
7	In some seedlings the cotyledons were still parallel to each other and in others these were spreading apart from each other.	Stomata visible in the lower epidermis. Compact cells of the mesophyll form more or less a sheath round the vascular bundle.	2C and 3C
8	Cotyledons now separated and horizontal; plumule consisted of a small folded first lamina enclosing inside a much smaller second lamina; hypocotyl 65-75 mms; root system well developed.	Differentiation into xylem and phloem is visible in bundles corresponding to side veins. Veinlet traces have nearly doubled in number.	

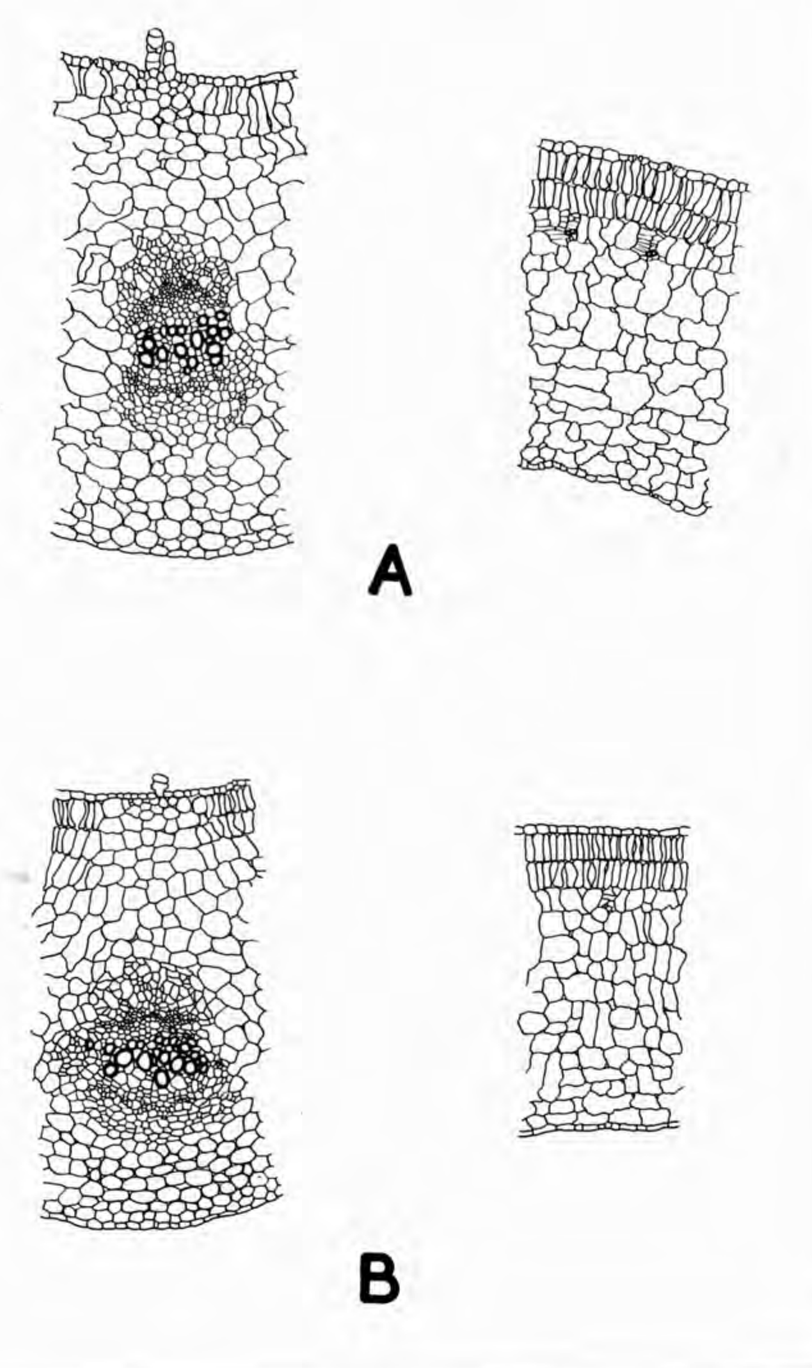


Fig. 4. Transverse sections of the cotyledons of different ages.

A - 13 days

B - 10 days

Day	External Morphology	Anatomy of the cotyledons	Figs.
9	Cotyledons were extending and the first leaf still folded.	————	
10	First leaf expanded.	————	2B and 4B
12	Cotyledons were extending; first leaf expanded and the second developing.	Besides the central vascular bundle, all other bundles corresponding to side veins have differentiated xylem and phloem. Veinlet traces have also developed xylem and stomata are visible in both the upper and lower epidermis.	
13	————	————	2A and 4A
15	Cotyledons practically ceased to extend; first and second leaves expanded and third, fourth and fifth developing.	Intercellular spaces are much wider.	

2. External morphology of seedling.

The growth of the seedling was observed for five weeks. On the third day of germination, the "peg" was visible as a parenchymatous outgrowth at the base of the small hypocotyl which, during the two following days, grew considerably, forming an arch. The "peg" aided in

splitting the valves of the seed coat and the arch of the hypocotyl gradually straightened out, withdrawing the cotyledons from the seed coat, which remained attached to the peg in the soil. This function of the "peg" has already been discussed by Crocker, Knight and Roberts (1910), who described it as a feature common to all the seedlings of Cucurbitaceae. The extension of the cotyledons which began about the fifth day continued to the fifteenth day and thereafter their area remained more or less constant at about 1·2 sq. cms. The first leaf expanded on the twelfth or the thirteenth day and from this period onwards the growth of the seedling was mainly confined to the development of the leaves. In five weeks, the seedlings developed 10-12 leaves and, either at the end of the fourth week or the beginning of the fifth week, the cotyledons became yellow and fell off.

3. Anatomy of cotyledon.

A survey of the anatomy of the cotyledon from young to the mature stage shows that, even in the seed stage, the tissue of the cotyledon is differentiated into palisade and spongy parenchyma; a few pro-vascular strands whose number is variable in different seeds are present in the spongy parenchyma. With the growth of the cotyledons the pro-vascular strands are gradually differentiated into xylem and phloem, though even in the mature condition there are

only a few xylem elements with phloem on either side of them. On the second or the third day of germination, some initials of the veinlet traces immediately below the palisade begin to divide and form small groups of cells which are transformed into traces of the veinlets. The development of xylem in these traces takes place on or about the twelfth day of germination. The number of these traces increases with the extension of the cotyledon. The growth of the cotyledon is also accompanied by the development of the intercellular space and of stomata on both the upper and lower epidermis. Hufford (1938) also presented the anatomy of a mature cotyledon of Citrullus vulgaris, but he neither showed the structure of the veinlet traces nor discussed its mode of development. The other features of the anatomy of the cotyledon presented here agree with those of Hufford.

4. Mechanism of cotyledon growth.

The great increase in size of the cotyledon during the growth of the seedling may be due either to rapid cell division or the mere extension of the cells. Cell division in the cotyledon does occur during the development of the vascular bundles from the pro-vascular strands and in the formation of the veinlet traces, but the number of cells so produced seem to be negligible in comparison with the total number of cells in the whole mesophyll. This suggests that the overall process of growth of the cotyledon may be

connected with the extension of the cells rather than with cell division. In order to investigate this, the cell counting technique of Brown and Rickless (1949) was used. A cotyledon was allowed to remain in 5 cc. of 5% chromic acid for a period of 15-16 hours (in the case of the cotyledon of the seed it was necessary to leave it in the acid for 24 hours). At the end of this period, the whole mass of the liquid was vigorously shaken by hand. The few lumps that were left after this treatment were dispersed by forcing a jet of the liquid with a 2 cc. pipette against the side of the vessel. After maceration, a drop of the suspension was introduced below the coverslip of a Haemocytometer slide with a pipette. The Haemocytometer slide used had a depth of 0·1 mm. and the grid of the slide had an area of $^1/_{16}$ sq. mm. The slide was placed on the stage of a microscope fitted with a $\frac{2}{3}$" objective and x10 eyepiece. A single cotyledon was used for each maceration and a duplicate reading was taken for each suspension. The results are given in Table XIII.

TABLE XIII.

Cell number ($\times 10^{-5}$) per cotyledon.

Age of the cotyledon in days	1st Reading	2nd Reading
0	696	656
4	680	640
7	680	656
16	664	688
19	656	688

It is clear that the difference in the number of cells between cotyledons of different ages is negligible, and does not even call for statistical treatment. We may, therefore, say with some confidence that the growth of the cotyledon is entirely due to the extension of existing cells and not to continued cell division. This is once more extremely convenient for the presentation of quantitative results, since it implies that a "per seed" estimate of urease content may be treated without danger of serious error as if it were a "per cell" estimate.

V. BIOCHEMICAL CHANGES DURING GERMINATION.

A. Introduction: a preliminary experiment.

The stage of germination at which the urease content in the cotyledons changes has not been previously recorded. Williams (1950) showed that urease tends to disappear ten to twelve days after sowing, but this was insufficiently definite for the purpose of the present investigation, as the date of germination of the seeds was not known. Therefore, as a preliminary experiment, measurements were carried out daily after germination of urease content, total nitrogen, protein nitrogen and water content in the cotyledons. Three seedlings were used in each experiment and the cotyledons were divided into two samples, each sample having one cotyledon from each seedling. One of these samples was used for the dry weight measurement and the other for urease content and the nitrogen fractions. At this stage, the supply of material was very limited and more extensive replication was not possible; moreover, the extent of variability of urease activity in different seeds was unknown. This has made the results, as will be seen, somewhat unreliable in certain details though still useful as a basis for subsequent work, in that they showed the period of time over which the important changes occur. The results are shown graphically in figure 5.

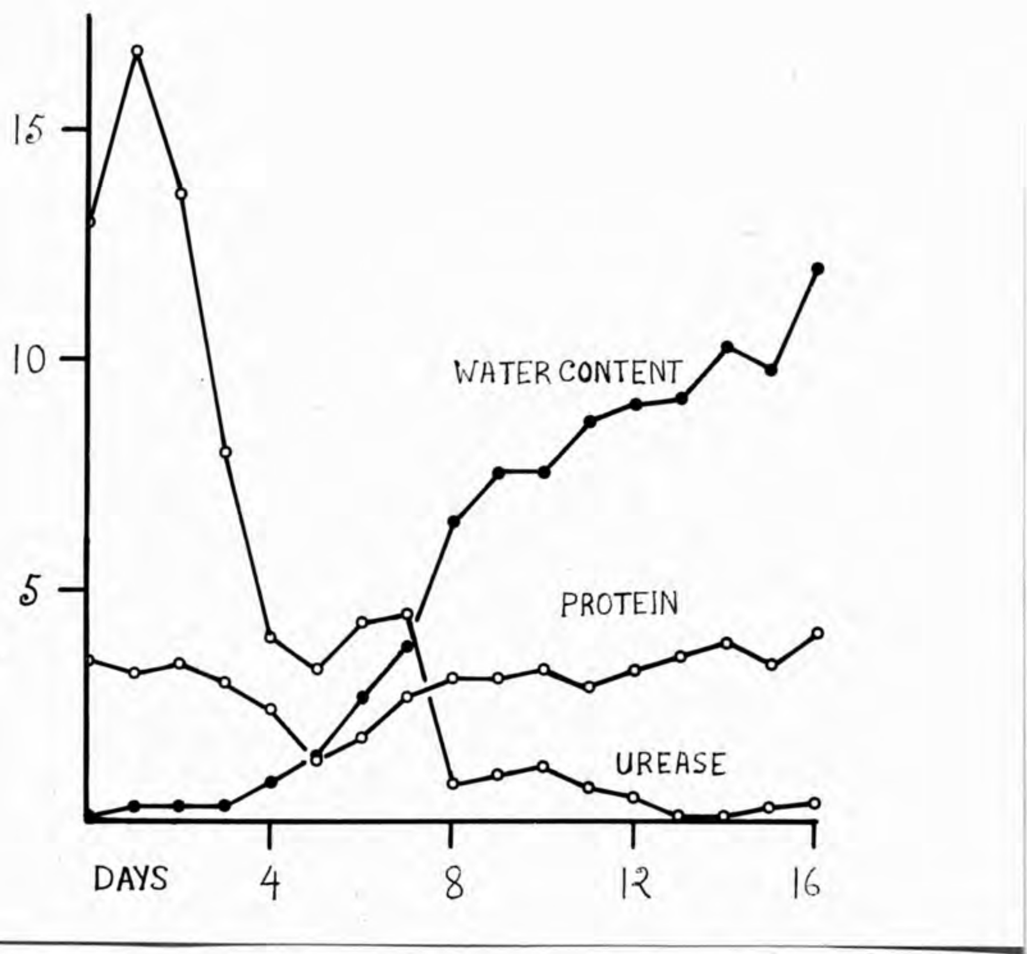


Fig.5. Day to day change in urease activity, protein and water content during germination.

Scales: urease and protein, 1 scale division = 1 mg. ammonia N.

water content, 1 scale division = 0·1 grm.

The total nitrogen gradually decreases to the fifth day and then increases up to the eighth day, thereafter remaining more or less constant. The initial drop in total nitrogen is apparently due to the breakdown of protein in the cotyledons and the total soluble nitrogen during the same period was found to be constant, which shows that the excess total soluble nitrogen due to protein breakdown in the cotyledons is translocated away into the rest of the seedling. The changes in total nitrogen followed a parallel course to that of protein nitrogen and are therefore not shown in the figure. The urease content, after a preliminary rise, shows a sharp fall between the second and the fourth day; what little remains after this fall practically disappears between the sixth and the ninth day. All later experiments have shown that the drop in urease on the second day was anomalous; the fall normally begins about the sixth day. The changes in urease do not appear to be related to changes in nitrogen fractions. The water content increases strikingly from about the fourth day, presumably due to cell extension (see later). This increase, occurring at about the time when urease is dropping, suggests a possible connexion between urease changes and cell-extension.

B. Nitrogen fractions.

1. Methods.

The plants were grown in soil under artificial light as mentioned earlier. A sample of eight seedlings, selected at random, was used in each experiment. The

cotyledons were detached at their bases with a razor blade and divided into two samples of eight cotyledons, each sample having one cotyledon from an individual seedling. Fresh weight and dry weight were determined from one of these samples and the urease activity and the different nitrogen fractions were estimated from the other. The fresh weight was taken immediately after detachment. The fresh weight and dry weight were also determined for the rest of the seedling including roots, hypocotyl and the small plumule (indicated as "Rest" in the figures). A replicate determination was made in each case. The changes were followed over a period of twelve days after germination.

2. Total nitrogen (fig. 6A).

The total nitrogen in the cotyledons shows a steady drop after germination to the sixth day, remains more or less at the same level to about the ninth day, and increases slightly again up to the twelfth day. The total nitrogen in the rest of the seedling increases very markedly between the third and the sixth day and then shows little change. It is quite obvious that the cotyledonary nitrogen store is drawn on at the beginning but that the rest of the seedling gains more nitrogen than the cotyledon loses after about the third day. Lateral root formation begins about the fourth day and therefore the seedling is now presumably gaining nitrogen from outside. It appears from one of the

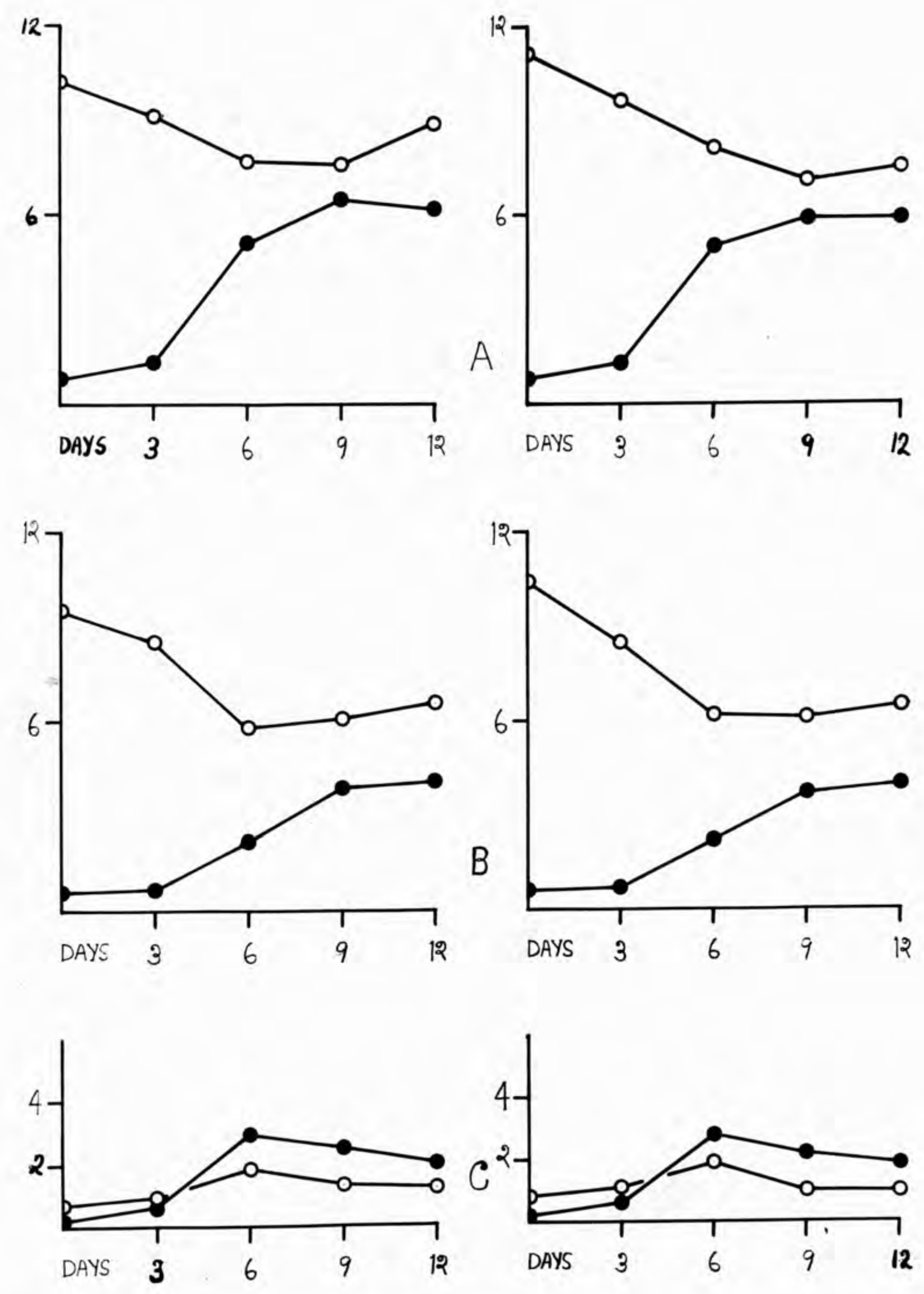


Fig. 6. Changes in total nitrogen, protein and total soluble nitrogen with time during germination.

Open circle, cotyledons.

Closed circle, "Rest" of the seedlings.

A - Total nitrogen (Two replicates)

B - Protein (Two replicates)

C - Total soluble nitrogen (Two replicates).

Scales: In every case, 1 scale division = 1 mg. ammonia N.

replicates in fig. 6A that a little nitrogen may still be drawn from the cotyledons between the sixth and the ninth day, but that the nitrogen-content of the cotyledons after this period tends to increase again slightly.

3. Protein nitrogen (fig. 6B).

The changes in the protein are more or less parallel to the changes in total nitrogen. The protein breakdown in the cotyledons is complete by the sixth day, after which there is practically no change except a tendency to slight increase between the ninth and the twelfth day. It is evident from this that the small drop in total nitrogen in the cotyledons between the sixth and the ninth day is due to the draining off of soluble nitrogen. The protein content in the rest of the seedling continues to increase, especially from the third to the ninth day. The flattening off of the curve after the ninth day is likely to have been due partly to further increase in the root system's being hindered by the pot, or perhaps to the exhaustion of the available nitrogen supplies.

4. Total soluble nitrogen (fig. 6C).

The total soluble nitrogen, in both cotyledons and the rest of the seedling, shows its main increase after the third day; so that in the plant as a whole soluble nitrogen is maximal on the sixth day, when the source of nitrogen is passing from the cotyledons to the external medium.

The soluble nitrogen gradually drops to a relatively steady level after the sixth day in the cotyledons as well as in the rest of the seedling. The increase in soluble nitrogen between the third and the sixth day in cotyledons is obviously due to the breakdown of protein; a small amount is still being drawn upon between the sixth and the ninth day and this parallels the slight drop of total nitrogen during the same period, as mentioned earlier.

5. Urease.

(a) total quantity (fig. 7).

The urease content shows an initial rise to the third day and is not in any way correlated with the protein fraction which is falling during this period. This rise may perhaps be considered to be paralleled by a small rise in soluble nitrogen, but even this is not maintained as urease begins to fall slowly between the third and the sixth day when soluble nitrogen is still rising. The urease practically disappears between the sixth and the ninth day when there is no change in the protein content of the cotyledons. Thus urease is definitely not being drawn upon as a reserve protein of the cotyledons during germination, and the suggestion to this effect by Williams (1950) cannot be sustained.

(b) distribution

The distribution of urease in the cotyledons of different ages has been studied by Granick's lake method.

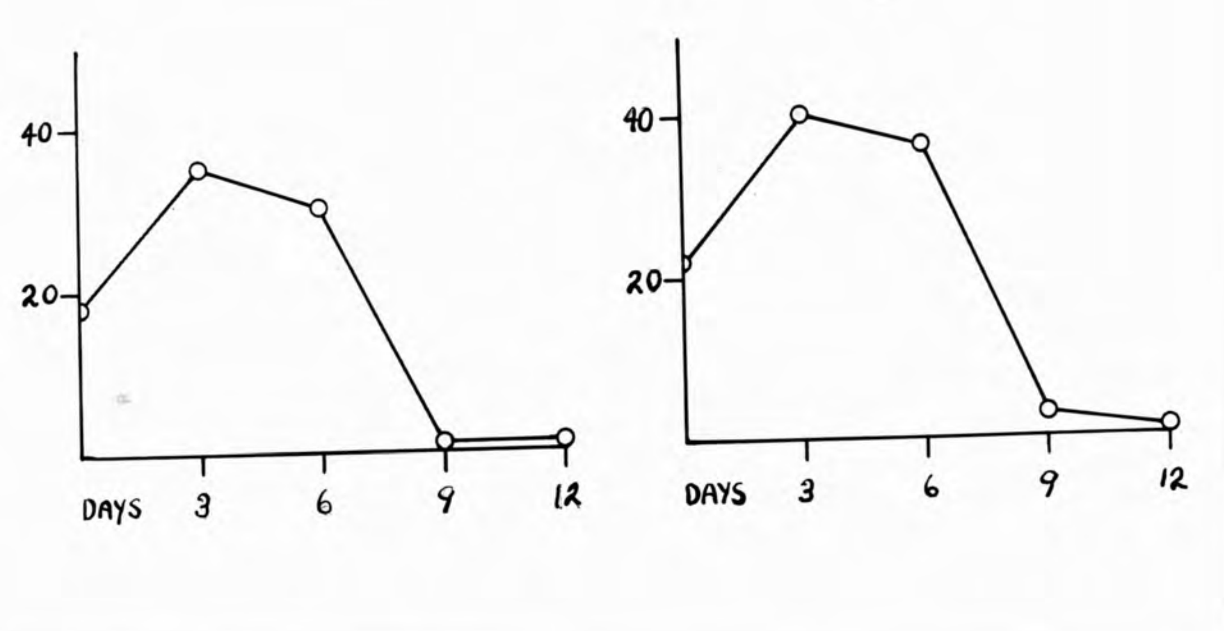


Fig. 7. Change in urease activity in cotyledons during germination.

(Two replicates)

Scales: 1 scale division = 1 mg. ammonia N.

The central part of the cotyledon was always used for freehand sectioning. The sections were treated with aqueous haematoxylin for 10 minutes and the time interval between the addition of urea solution and nickel sulphate was one minute in sections of 0 - 6 days old cotyledons, 2 minutes in seven day old cotyledons and 4-5 minutes in 8-10 day old cotyledons. In young cotyledons (age 0-6 days), the deep blue colour of the nickel-hydroxide-haematoxylin lake was found to be widely spread over the whole section. Sometimes the blue precipitate was distributed in definite patches in the mesophyll. In 7-day old cotyledons, the precipitate was confined mainly to the bundle sheaths of the vascular bundles and to a few cells of the spongy parenchyma. In 8-10 day old cotyledons, the precipitate was observed only in the bundle sheaths. The histochemical investigation shows that urease is present in almost all the cells of the palisade and spongy parenchyma, although more in the latter, in the early stages of growth. Gradually, with the extension of the mesophyll cells, urease disappears and remains confined only to the cells of the bundle sheath which undergo practically no extension. It should be noted here that the disappearance of the enzyme from the mesophyll cells as the cotyledons age, as indicated by the absence of the blue precipitate, is very abrupt; no gradual disappearance from any particular tissue could be observed.

6. Nitrate.

Nitrate has been estimated by a comparison of a Kjeldahl determination in which reduced iron was used with a similar determination in which it was omitted; both determinations being carried out on aliquots from the same sample. The amount of nitrate present per eight seedlings is shown in Table XIV. As in previous cases, the amount of nitrate in the cotyledons shown in the table is only for a sample of eight cotyledons.

TABLE XIV.

Nitrate storage in seedlings.

Age in days	Nitrate in mgs.	
	Cotyledons	Rest of the seedling
3	Absent	Absent
6	0·64	0·45
9	0·49	1·16
12	0·70	2·40

It is clear from these results that nitrate is present only in very small amounts in the cotyledons, forming roughly one-thirteenth of the total nitrogen over the period of twelve days after germination, whereas in the rest of the seedlings nitrate tends to accumulate, its proportion being

one-tenth of the total nitrogen on the sixth day and thereafter gradually increasing to one-sixth up to the twelfth day. Evidently Citrullus seedlings store nitrate, probably in the growing root system. As the present investigation is mainly concerned with the cotyledons, in which the amount of nitrate is virtually negligible, the reduction of nitrate has not been carried out in the rest of the experiments.

C. Other changes.

1. Dry weight. (fig. 8A).

The dry weight of the cotyledons drops slowly to the sixth day, remains more or less constant to the ninth day and then shows a tendency to increase again. The drop is no doubt due to the protein breakdown in the cotyledons and the translocation of nitrogen compounds to the remainder of the seedling, and the slight rise may be explained as due to synthesis of carbohydrates and proteins. The dry weight of the rest of the seedling increases mainly between the third and the ninth day, during which period maximum root formation takes place. This increase in dry weight is no doubt due to synthesis of protein and cell-wall material; the cotyledons become green at about the sixth day and active photosynthesis is therefore presumably in operation from this time.

2. Water content. (fig. 8B).

The water content of only the cotyledons is

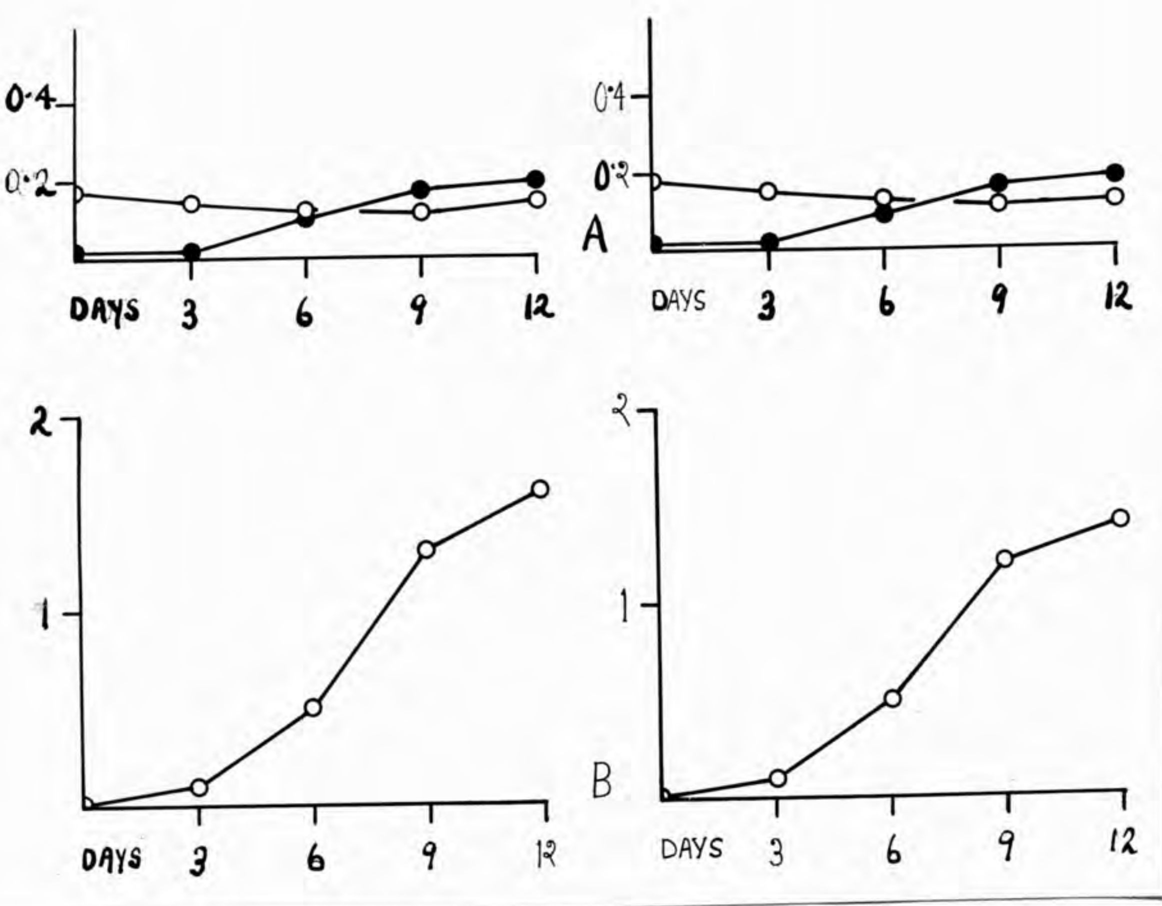


Fig.8. Changes in dry weight and water content during germination.

Open circle, cotyledon.

Closed circle, "Rest" of the seedlings.

A - Dry weight (Two replicates)

Scales: 1 scale division = 1 gm.

B - Water content (Two replicates)

Scales: 1 scale division = 1 gm.

represented in fig. 8B. The graph for the rest of the seedling has not been included owing to difficulties of scale; moreover, the main interest is in the cotyledons where the urease is present. However, it may be noted that as soon as the roots begin to form (after the third day) there is an enormous increase in water content in the remainder of the seedling. The water content of the cotyledons shows a small rise to the third day (when the urease content is also rising), a comparatively larger increase between the third and the sixth day, when the urease tends to drop, and a very large increase between the sixth and the ninth day, exactly corresponding to the main urease fall. The increase in water content between the ninth and the twelfth day is by comparison very small, probably due to the fact that the extension of the cells of the cotyledon is virtually finished by the ninth day.

It is reasonable to postulate that the increase in water content closely parallels cell-extension; but the progress of extension can easily be measured approximately. The results of such measurement are shown in fig. 9A. Freehand transverse sections of the central part of the cotyledon were cut and the diameter of the cells measured with an eyepiece micrometer. Every point in the graph represents an average of twenty measurements. The measurement of any particular cell in the section was made in two planes, one parallel to the upper or lower epidermis

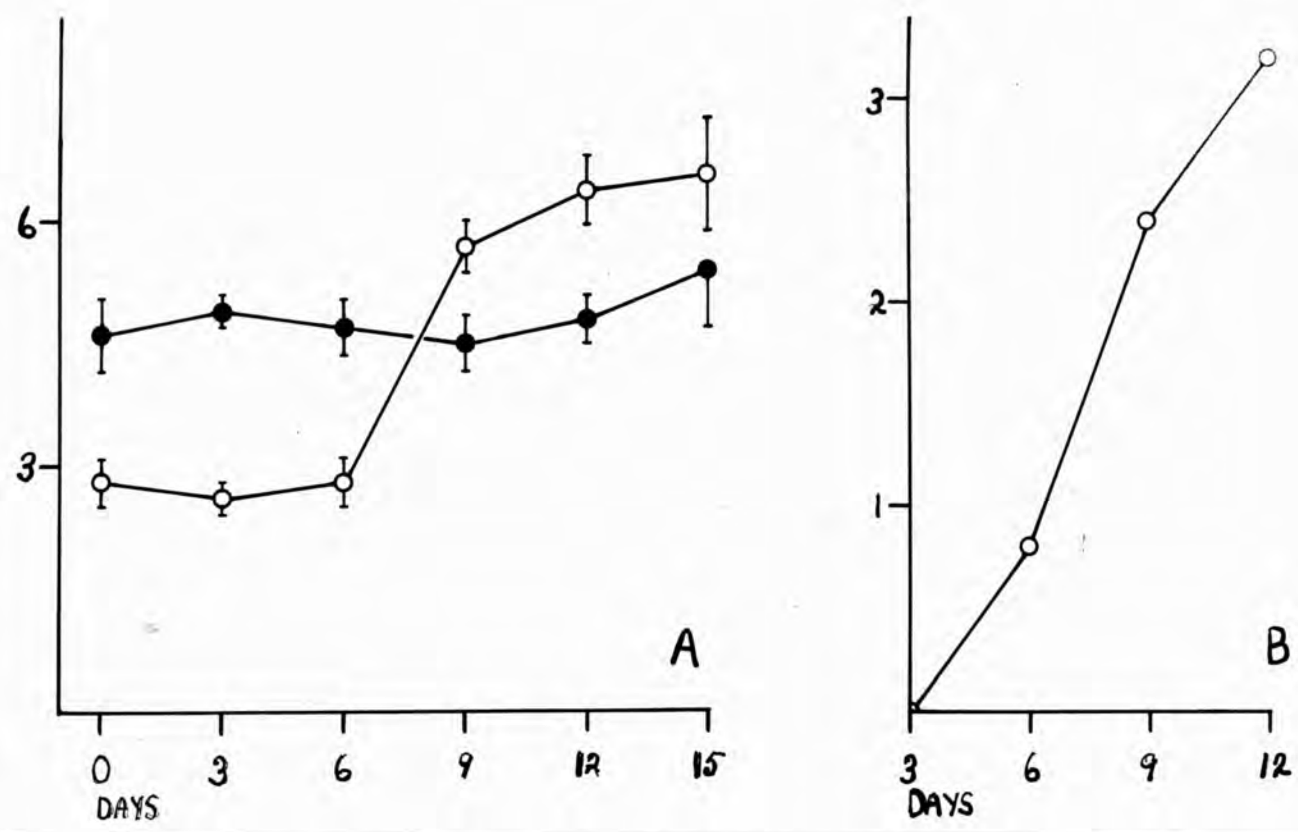


Fig.9. Cell extension and chlorophyll content in the cotyledons during germination.

A - Cell extension ($x10^{-1}$)

open circle, lateral extension;

closed circle, vertical extension.

Scale: 1 scale division = 1 μ

B - Chlorophyll content.

Scale: 1 scale division = 1 mg.

(indicated as lateral extension in the graph) and the other being at right angles to the epidermis, (indicated as vertical extension). No measurement was made in the third (longitudinal) direction.

Fiducial limits of ± twice the standard error of the means have been included, and it is clear that with the sample-size here used the only significant change is that in lateral extension between the sixth and ninth days. Evidently this period represents a critical phase in the growth of the cotyledons; during these three days the cells of the mesophyll undergo their main extension, the water-content increases enormously, and the urease falls almost to zero.

3. Chlorophyll (fig. 9B).

Observation of the growing seedling shows that the cotyledons are not only expanding between the sixth and ninth days; they are becoming progressively greener. It therefore seemed desirable to measure the change in chlorophyll content of the developing cotyledons.

Eight cotyledons were used for the extraction of chlorophyll in every experiment. The cotyledons were ground into a fine pulp with 80% acetone and transferred to a Buchner funnel and filtered. 80% acetone was gradually added to the mass on the filter paper until the green colour was removed. The solution was then transferred to a

separating funnel and ether was added. On adding a little distilled water two layers were separated and the lower acetone water mixture was discarded. The chlorophyll solution in ether was then treated with 10-15 cc. of saturated solution of KOH in methyl alcohol. After addition of a little distilled water the mixture was shaken for twenty minutes and the solution of saponified chlorophyll was separated from an ethereal layer containing the yellow pigments. The saponified chlorophyll solution was run out and made up to 100 cc.

The concentration of chlorophyll in the solution was determined by comparing the saponified chlorophyll solution with the inorganic colour standard of Guthrie (1928) in a Spekker photometer. A red filter (Ilford) was used in taking the Spekker reading. The colour standard was diluted with distilled water in proportions of 3:1, 1:1, 1:3 and the Spekker readings of these three solutions, together with the pure standard, were noted. The colour of Guthrie's standard made up to 100 cc. is equivalent to 8·5 mgs. of chlorophyll. From this, the concentrations of chlorophyll equivalent to the colour of the diluted solutions were calculated and plotted against the Spekker readings and thus a standard curve was obtained. The extracted saponified chlorophyll solution in each experiment was then compared with the standard curve and the amount of chlorophyll present was thus ascertained.

It will be seen from fig. 9B that there is a sharp rise in chlorophyll content between the sixth and ninth days, paralleling the changes in water-content and urease. The parallelism is, however, less exact in this case, since the chlorophyll continues to rise fairly steeply for the next three days. It must also be remembered that histochemical investigation (p. 39) showed that urease is retained in the bundle-sheaths long after it has disappeared from the mesophyll of the cotyledon; and that not only do the cells of the sheath expand far less than those of the mesophyll, they also fail to develop chlorophyll. Some connexion between the development of chlorophyll and the disappearance of urease cannot be ruled out.

With only eight cotyledons, the amount of carotenoid pigments was insufficient for accurate estimation. So far as could be ascertained, the final concentration was reached very quickly and no changes comparable in magnitude with those exhibited by chlorophyll could be detected.

4. Invertase (fig. 10).

Brown (1952) noted a close connexion between invertase activity and cell extension in root tips. He found that as soon as cell extension stopped, the invertase activity also decreased. Since it seemed conceivable that the same relationships might hold for the cells of the cotyledon, the invertase activity was followed during the extension

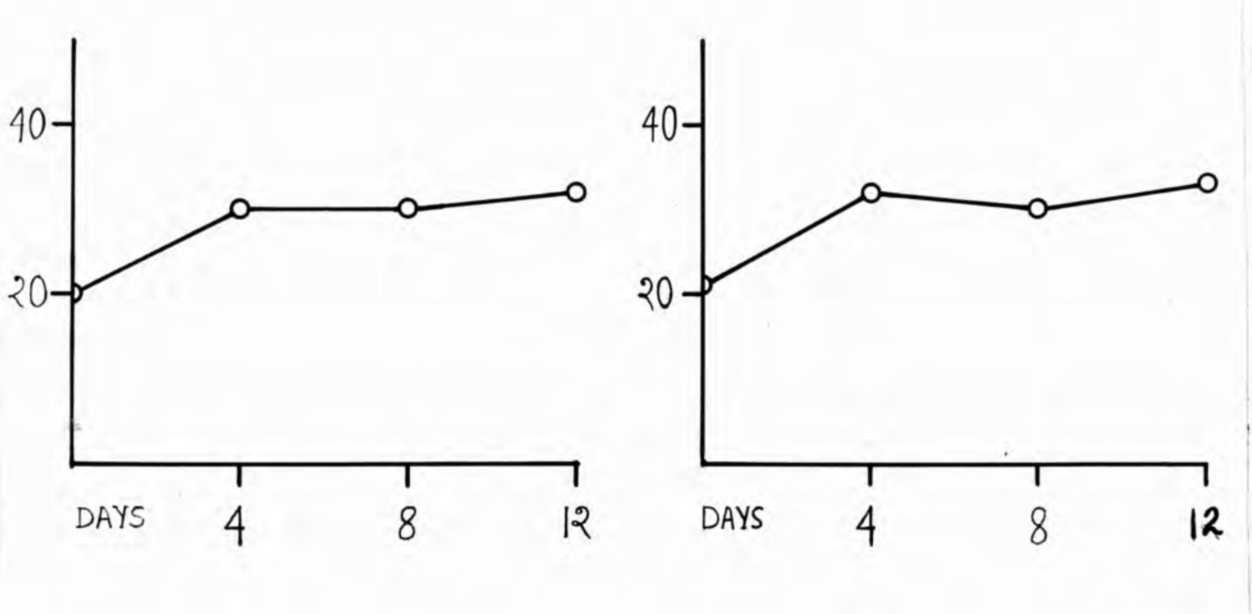


Fig.10. Change in invertase activity in the cotyledons during germination.

Two replicates.

Scales: 1 scale division = 1 mg. of invert sugar.

phase of the cotyledon. The method of Sumner and Howell (1935) was adopted for the determination of invertase activity and the titration was carried out by Cole's ferricyanide method. Eight cotyledons were used in each experiment and a replicate determination was made in each case. The results represented in fig. 10 show that the invertase activity gradually increases after germination to the fourth day and then remains more or less at the same level throughout. The increase in invertase activity corresponds with the increase in urease activity in the early stages, but it differs strikingly from the urease activity in that it does not show the sharp fall which is characteristic of urease between the sixth and the ninth day. The continuity of the invertase activity at the same level, even after the extension phase, suggests that the metabolic behaviour during the extension phase of green photosynthetic organs like cotyledons is different from that of the root-tips used by Brown. It is interesting to note here that Granick (1938), while following changes in urease activity in developing Soy beans, also investigated the protease and lipase activity, but found that these followed a quite different course.

D. <u>Respiration</u>. (Fig. 11).

The rate of respiration of the cotyledons was followed for a period of eleven days after germination. In every experiment the O_2 uptake and CO_2 output were measured

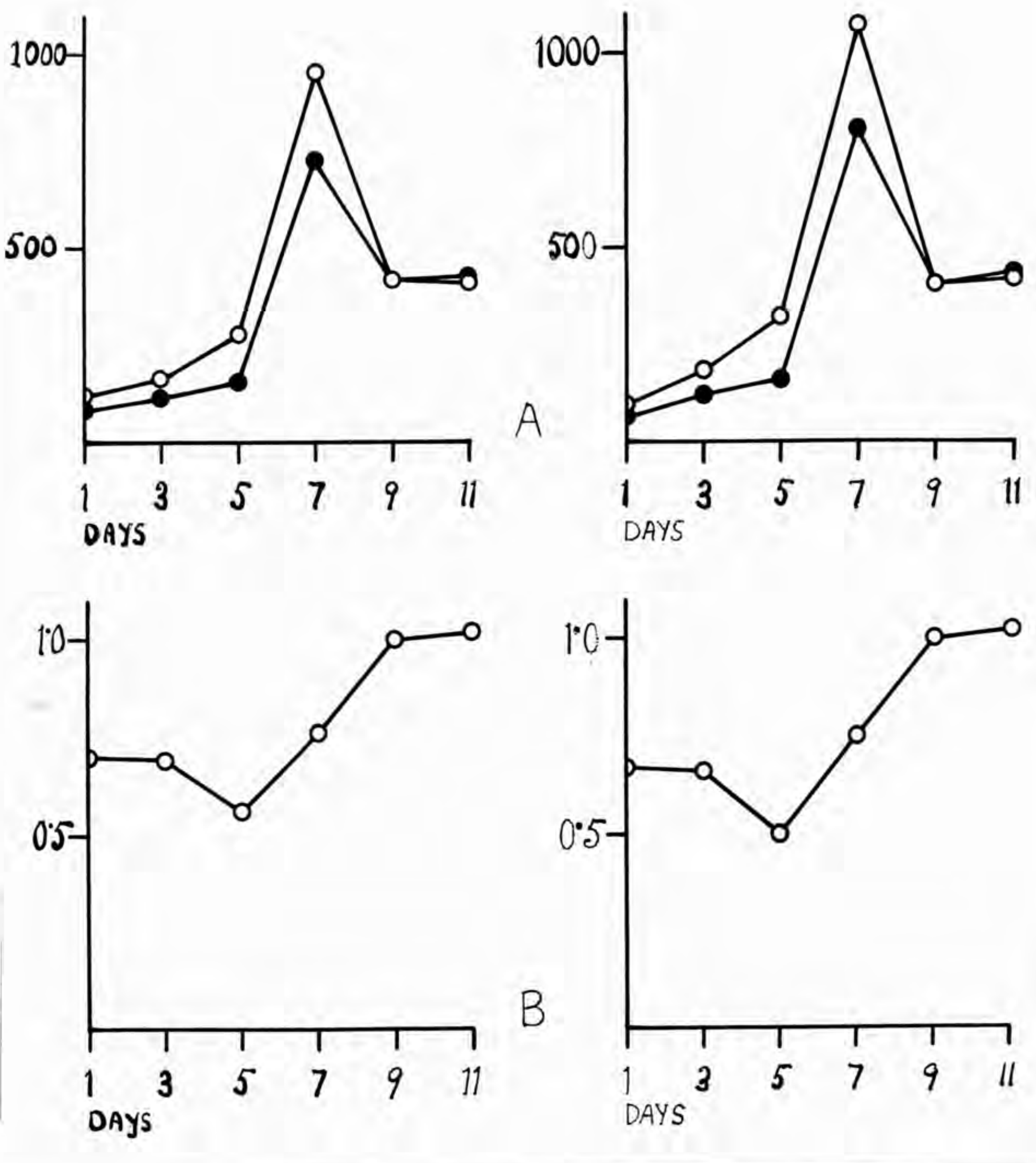


Fig.11. Changes in respiration rate and respiratory quotient in cotyledons during germination.

A - Respiration rate (Two replicates) open circle, oxygen uptake; closed circle, carbon dioxide output.

Scales: oxygen uptake and carbon dioxide output, 1 scale division = 1 μl per hour.

B - Respiratory quotient (Two replicates)

Scales: 1 scale division = 1·0.

simultaneously and from this the respiratory quotient was calculated. Eight cotyledons were used in each experiment which was carried out in complete darkness. The cotyledons after detachment were immediately put in Warburg flasks and the rate of respiration was followed for five hours. The results are represented in fig. 11A. It was observed in each experiment that the rate of respiration was high in the first hour, perhaps due to the handling of the material, but then the rate dropped and remained more or less at the same level. Every point in fig. 11A was taken from this steady level.

The oxygen uptake gradually increases up to the fifth day and then shows a sharp rise so that by the seventh day the uptake is three times more than that on the fifth day. It drops sharply from the seventh up to the ninth day and then remains constant. The CO_2 output follows more or less a parallel course to the O_2 uptake but is always lower than O_2 uptake to the ninth day, after which it equals O_2 uptake. The CO_2 output on the fifth day is in fact only about half the oxygen uptake. The respiratory quotient (fig. 11B) is below unity even on the first day of germination, being then 0·7, and remains at this level to the third day. On the fifth day the R.Q. falls to about 0·5 and thereafter gradually increases so that by the ninth day it becomes unity and then remains constant.

The rate of respiration which has been expressed here per eight cotyledons may be treated as if it were measured

on a "per cell" basis, since it has been pointed out earlier (p.54) that the number of cells in the cotyledon during its growth remains more or less constant. The sharp rise in the rate of respiration appears to be associated with the extension of the cells, which commences about the fifth day, and the fall in the rate of respiration is probably due to the slowing down of the process of extension. The extension of the cells is virtually over by the ninth day and this may be the reason for a constant rate after this period. Brown and Broadbent (1950) have also shown that, in the growing zones of the pea root, the rate of respiration per cell increases sharply during the period of extension, after which it drops to a constant level. The respiratory quotient follows a similar course to the R.Q. of Ricinus seeds during germination (Stiles 1936, p.126-127). The water melon seeds, like those of Ricinus, contain a very high percentage of oil, 53·67% of the dry weight of the kernel (Langueld, 1930). The low R.Q. in the early stages of germination is presumably due to the oxidation of fats. Stiles (1936) also found the R.Q. as low as 0·5 in Ricinus after about 120 hours of germination and pointed out that if at any time half the fat which disappears is oxidised to carbon dioxide and water and the other half converted into sugar, the apparent R.Q. would be 0·57. The gradual increase of the R.Q. from the fifth to the ninth day is probably due to the

oxidation of sugars derived partly from the oxidation of fats and partly from photosynthesis, as the cotyledons become green about the sixth day. From the ninth day onwards, only carbohydrate has been used as a respiratory substrate.

E. Conclusions.

It is clear from the foregoing detailed investigation of the biochemical and related changes associated with the development of the cotyledons that the change in urease content is virtually independent of changes in the major nitrogen fractions. Whatever the function of urease may be, it clearly cannot be considered as a "reserve protein" (Williams, 1950), since protein-N and urease-N show practically no parallelism in behaviour; and the total quantity of urease present, though very great by enzyme-concentration standards, is too small for it to act alone as a reserve food-substance.

There is, however, a very striking connexion between the characteristic fall in urease and the process of cell-extension. As the cells undergo their main extension, their water-content rises, they exhibit a marked respiratory peak, and their urease-content falls. This suggests very strongly that changes in urease content are no more than what Brown (1952) has called "protoplasmic differentiation" -- changes in the enzyme-complement of the protoplasmic structure

as the cell develops into its mature form. The urease-change perhaps represents no more than a stage in the differentiation of the mature protoplasm, but a stage which happens incidentally to show the special chemical properties which we associate with the *in vitro* activity of urease. Granick's (1938) conclusion that urease is a "growth-bound enzyme" may reasonably be regarded as an early, and thus less explicit, statement of the same conception.

There remains the possibility of some connexion with the development of chlorophyll, which is taking place at the same time. This is hardly likely -- after all, urease is not detectable in the epicotyledonary leaves at any stage -- but on the basis of the results of the preceding section alone cannot be entirely excluded. This possibility will receive further attention in the subsequent sections.

VI. THE EFFECTS OF NUTRITIONAL STATUS.

A. Introduction.

At this stage in the investigation it seemed improbable that much more could be learned from further study of the changes occurring under normal conditions. It was therefore decided to subject the plants to abnormal metabolic conditions, in the hope that the interdependence of urease and other changes might thereby be further clarified. Three forms of interference with normal metabolism were chosen, viz:-

(1) Withholding completely the nitrogen supply. This might be expected to cause a heavier drain on the nitrogen reserves of the cotyledons.

(2) Adding glucose. This may also be expected to affect protein changes. Paech (1935) suggested that the level of protein synthesis or breakdown in an intact cell is determined by the relative amounts of "monoses" and of active nitrogenous compounds present. Although the interpretation of his "monoses" is open to doubt, it is generally agreed (_vide_, e.g. Chibnall, 1938) that glucose may act in a similar manner.

(3) Growing the plants in darkness. This may be expected to prevent chlorophyll-formation and, to a large extent, extension of the cotyledons.

B. <u>Experimental method and design</u>.

As soil is obviously unsuitable for such nutritional studies, preliminary experiments (in light) were carried out using water cultures (Knop's solution). The changes in urease content in the cotyledons and the changes in the nitrogen fractions in both cotyledons and the rest of the seedling were determined. The data are not presented here as they were found to be very similar to those for plants grown in soil. However, the final drop in urease content was delayed, presumably owing to the reduced extension of the cotyledons in water culture. On the whole, it was observed that water culture was unfavourable for the growth of the seedlings. The difficulty in aerating the culture solution was suspected to be one of the causes of unhealthy growth. It was later found that plants were comparatively healthier in sand cultures, although the rate of growth was still less than under the optimum conditions of the soil. The experiments were therefore carried out in sand cultures on a three factor (2 x 2 x 2) basis, the three factors being:-

(1) <u>light and darkness</u>: the 'light' experiments were carried out under the bank of fluorescent lamps

described earlier, the 'dark' experiments in an incubator at 25°C.

(2) <u>with or without nitrogen</u>: either complete Knop's solution, or Knop's solution in which the calcium nitrate was replaced by calcium phosphate.

(3) <u>with or without glucose</u>: in the 'with glucose' experiments glucose was simply added to the Knop's solution to a concentration of 1%.

In all cases the sand was washed several times in tap-water before use, and the pots with the sand placed in crystallizing dishes containing the culture solution. The pH of the culture solution was 6·76.

In the experiments with glucose, the culture solutions, sand, pots and crystallizing dishes were all sterilised before use, but even then butyric acid fermentation could not be avoided under the relatively high temperature of the experimental conditions. This occurred particularly in the pots with Knop's solution without nitrate in light and consequently the growth of these seedlings was checked to a certain extent.

The pots were supplied with culture solutions on every fourth day and tap water was used on the intervening days; in experiments with glucose sterile tap water was used. Three seedlings were taken for each experiment and the cotyledons used for the determination of fresh

weight, total nitrogen and urease content over a period of either fifteen or sixteen days. The size of the cotyledons was also measured with a planimeter, but it was observed that the size always paralleled the fresh weight of the cotyledons and therefore only the fresh weight is presented in the figures 12, 13, 14 and 15.

C. Experimental Results.

1. Results.

The results are presented graphically in Figs. 12, 13, 14 and 15, and the main effects may be conveniently summarized as follows:-

(a) Effect of nitrogen.

The four treatments (with and without light, with and without glucose) are given as separate figures: in each case (A) represents the results obtained with nitrate, (B) those obtained without. The effect of nitrogen can thus be ascertained by a comparison of fig. 12(A) with 12(B) and similarly for the three remaining figures.

In all four cases the changes in urease content and the amount of total nitrogen drawn upon have been found to be identical. It may, however, be observed that total nitrogen tends to increase slightly after the ninth day of germination only in plants grown in complete Knop's solution in light. The same effect was observed in plants grown in soil. Apart from this small modification, we may therefore infer that the metabolism of urease and nitrogen in the cotyledon during the early stages of germination is independent of external nitrogen supply.

(b) Effect of etiolation (without glucose).

The most striking effects (in the plants grown without added glucose) are, first, the initial rise of urease content no longer takes place; and, secondly, although extension

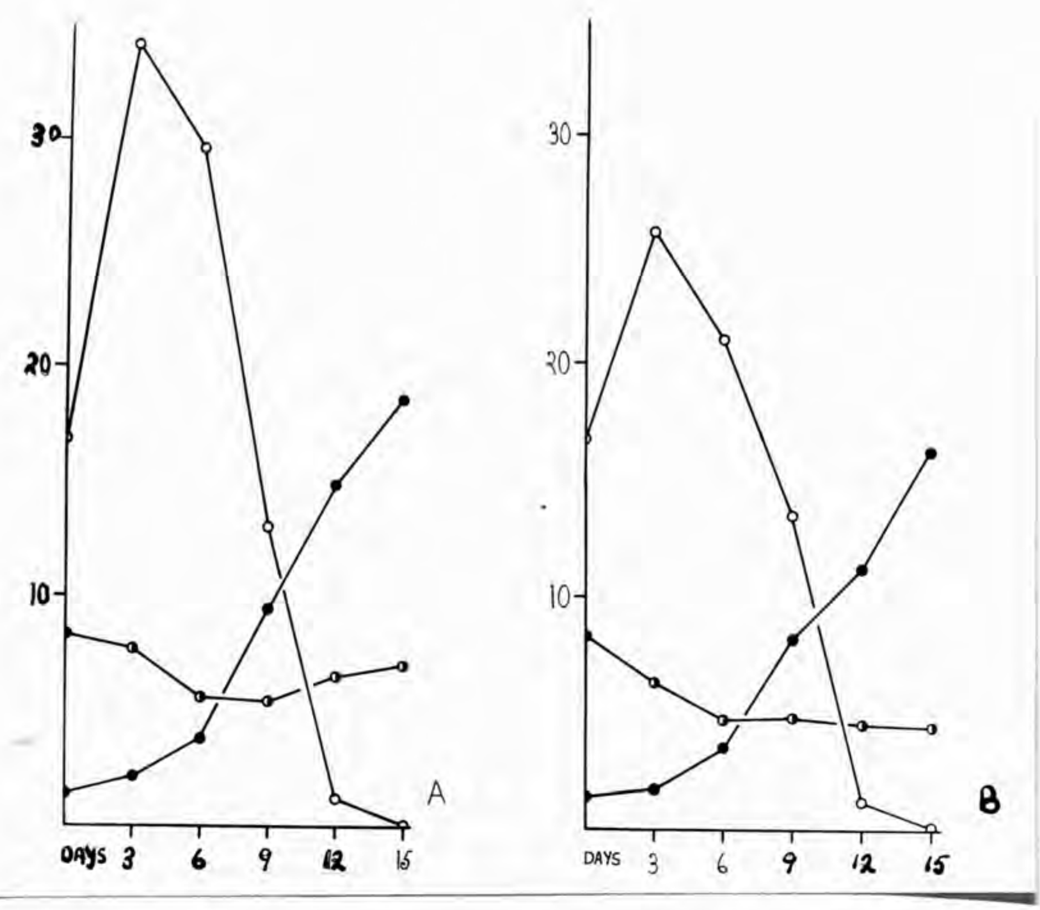


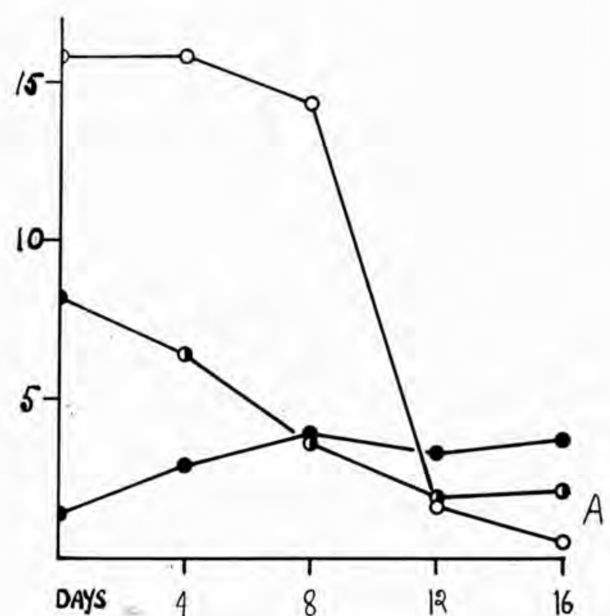
Fig.12. Effect of nitrogen supply on urease activity, total nitrogen and fresh weight of the cotyledons of seedlings grown under artificial light.

Open circle, urease activity;
half circle, total nitrogen;
closed circle, fresh weight.

Scales: urease and total nitrogen, 1 scale division = 1 mg. ammonia N.
Fresh weight, 1 scale division = 0·1 gm.

A - seedlings grown in full Knop's solution

B - seedlings grown in Knop's solution without nitrate.



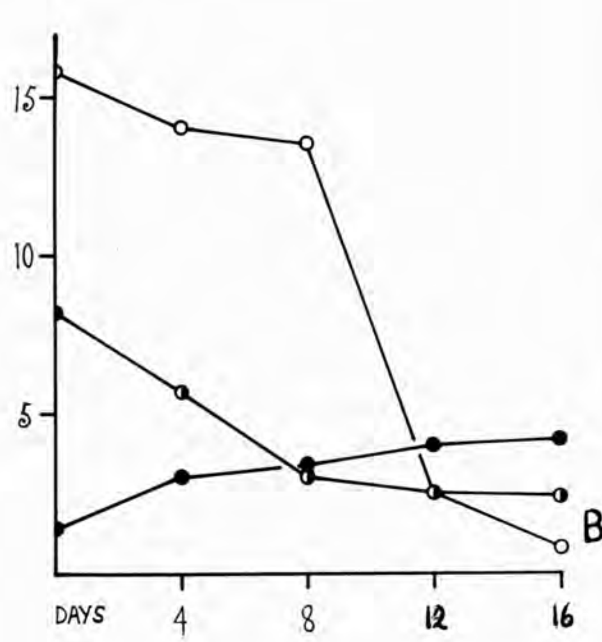


Fig.13. Effect of etiolation on urease activity, total nitrogen and fresh weight of the cotyledons.

Open circle, urease activity;
half circle, total nitrogen;
closed circle, fresh weight.

Scales: urease and total nitrogen,

1 scale division = 1 mg. ammonia N.

Fresh weight, 1 scale division = 0·1 gm.

A - Seedlings grown in full Knop's solution.

B - Seedlings grown in Knop's solution without nitrate.

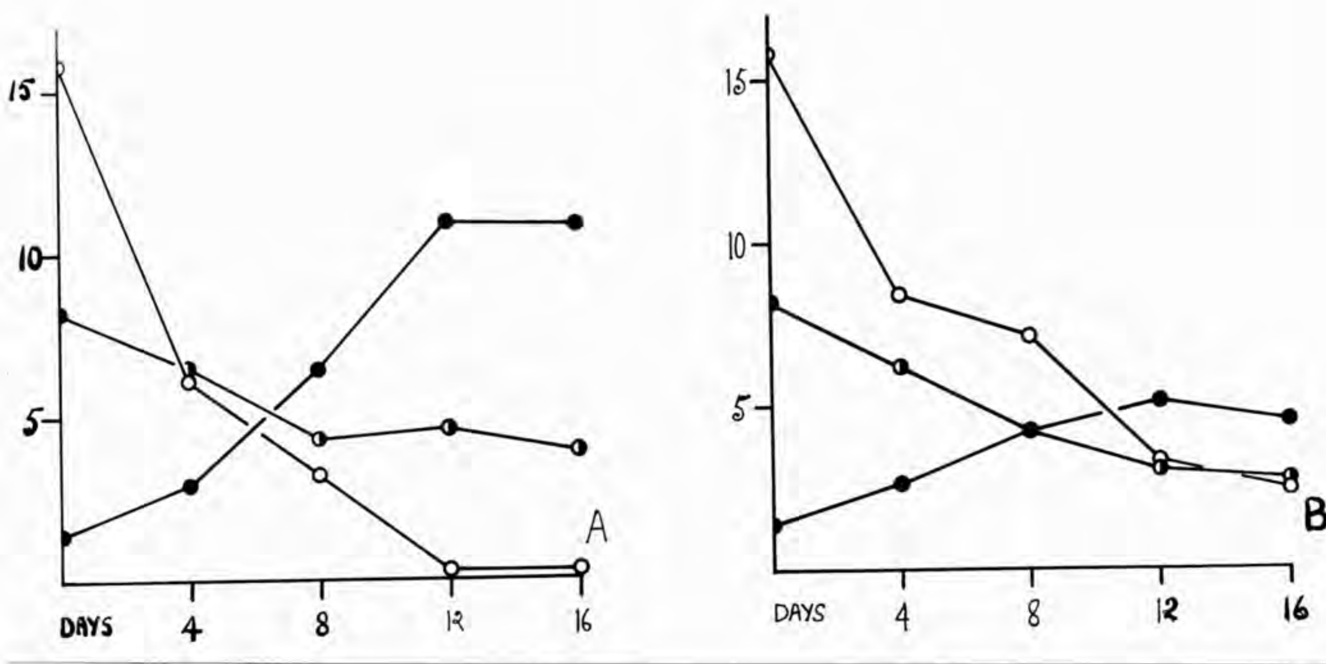


Fig.14. Effect of glucose supply on urease activity, total nitrogen and fresh weight of cotyledons of seedlings grown under artificial light.

Open circle, urease activity;
half circle, total nitrogen;
closed circle, fresh weight.

Scales: urease and total nitrogen, 1 scale division = 1 mg. ammonia N.
fresh weight, 1 scale division = 0·1 gm.

A - Seedlings grown in full Knop's solution.

B - Seedlings grown in Knop's solution without nitrate.

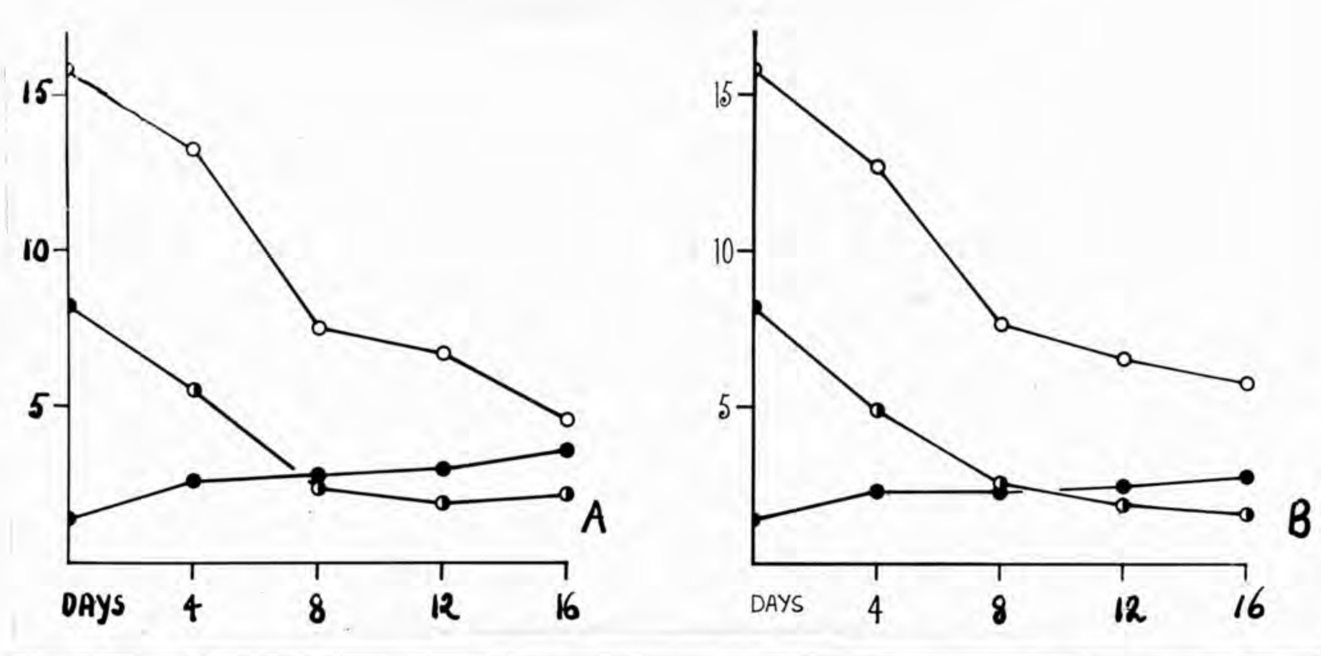


Fig.15. Effect of glucose supply on urease activity, total nitrogen and fresh weight of the cotyledons of etiolated seedlings.

Open circle, urease activity;
half circle, total nitrogen;
closed circle, fresh weight.

Scales: urease and total nitrogen, 1 scale division = 1 mg. ammonia N.
fresh weight, 1 scale division = 0·1 gm.

A - Seedlings grown in full Knop's solution.

B - Seedlings grown in Knop's solution without nitrate.

is very greatly reduced, the fall in urease is delayed, though not prevented. Moreover, the amount of total nitrogen drawn upon is more than in plants grown in light, and the total nitrogen in plants supplied with nitrate does not show the tendency to increase again in the later stages of germination as observed in plants grown in light. The effect of etiolation with glucose is complex and is considered below.

(c) Effect of glucose.

The effect on changes in total nitrogen is slight. In light it causes a little more drainage of the cotyledonary nitrogen store compared with plants grown without glucose, but in darkness the amount of total nitrogen drawn is practically the same whether glucose is supplied or not. There are, however, two striking positive effects:-

(i) In light, glucose not only suppresses the initial rise in urease content but actually causes a sharp fall.

(ii) In darkness, it causes an increased drop of urease at the beginning (compared to plants grown without added glucose), but then delays its further disappearance.

D. Conclusions.

It is evident that the nitrogen-metabolism of the cotyledons is, in the early stages of germination at least,

virtually independent of external nitrogen supply. This is not unexpected, and we may profitably devote our main attention to the light-glucose interaction phenomena.

The invariable effect of glucose is to cause an early fall of urease. This might be due to the fact that the ordinary progressive rise and fall of urease is an energy-requiring process; the addition of respiratory substrates such as glucose might then in effect hasten the process. At the time of cell extension, formation of new cellulose takes place; this must involve metabolism of glucose in some form, so that there may be a connection between urease and glucose metabolism, and this in turn may account for the fact that if glucose is provided at an early stage there is a corresponding change in urease activity. In this connection, it is interesting to note that we have been informed by Dr. D. F. Cheesman of Bedford College, London, that there appears from his experiments to be some mutual antagonism between A.T.P. and urease. In etiolated seedlings, although cell extension and chlorophyll formation are almost prevented, urease still disappears. The fall in urease in etiolated seedlings between the eighth and the twelfth day might be due in part not to the same cause as in the normal seedlings but to protoplasmic respiration (Blackman 1908); the addition of glucose might provide an alternative substrate, thus "protecting" the urease from being metabolised as a respiratory substrate.

It is to be expected that, if there is any close relationship between urease disappearance and glucose metabolism, this would be reflected in the rate of respiration. It was therefore decided to investigate the rate of respiration of the cotyledons in glucose-fed plants. Eight cotyledons were used in each experiment. The rate of O_2 uptake and CO_2 output were determined, from which the R.Q. was calculated; the results are represented in fig. 16. It is evident from the figure that the rise and fall in the rate of respiration follow a more or less similar course to those in plants grown under normal conditions, but on supplying glucose the maximum point in the rate of respiration is reached earlier, i.e. it occurs on the fifth day of germination instead of the seventh under normal conditions. It is interesting to note here that Brown and Sutcliffe (1950) have reported increased rates of respiration as a result of the provision of sugar in root fragments. They suggest that sugar provides the necessary substrate for cellulose synthesis which is an energy-consuming process and that a high rate of respiration provides this energy. The earlier high rate of respiration due to supply of sugar in the present case may perhaps be explained on the same basis. The R.Q. does not fall on the fifth day to the same extent as was observed in plants under normal conditions, and this comparatively higher R.Q. may well be due to the presence of glucose, which probably prevents oxidation of fats to a certain extent. The increase of R.Q. to unity is also

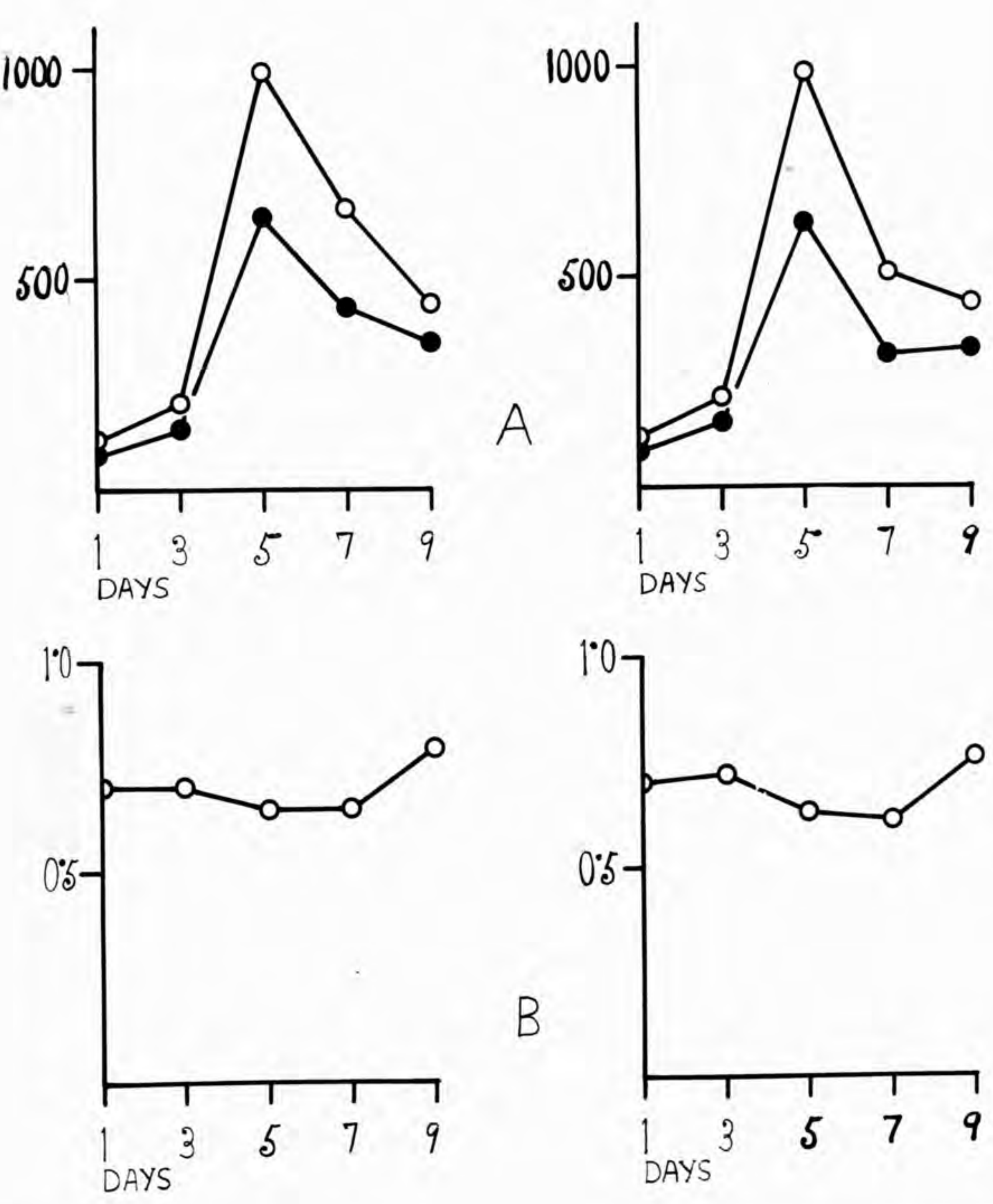


Fig.16. Effect of glucose supply on respiration rate and respiratory quotient of the cotyledons of seedlings grown in full Knop's solution under artificial light.

A. Respiration rate (Two replicates)
open circle, oxygen uptake;
closed circle, carbon dioxide output.

Scales: oxygen uptake and carbon dioxide output,
1 scale division = 1 μl per hour.

B. Respiratory quotient (Two replicates)

Scales: 1 scale division = 1·0.

delayed and here again the slow oxidation of fats resulting in a lessened production of sugar may be the reason for the slow increase in R.Q. Although it was expected that the R.Q. would increase to unity earlier than in normal plants, this was not the case; probably because the bulk of the glucose present in the cotyledons has been diverted to some other process such as cellulose synthesis.

The fact that urease disappears at a time when the rate of respiration is nearly maximum lends further support to the suggestion already made that rise and fall of urease is an energy-requiring process. It seems, too, that the disappearance may be connected with increased production of A.T.P. when the rate of respiration is high.

VII. UREASE AND UREA METABOLISM.

A. Introduction.

If, as seems to have been the generally accepted view of the earlier writers, cotyledonary urease is in fact concerned with the metabolism of urea, certain results might be expected to follow, viz:-

1. The provision of urea as the sole source of nitrogen might cause a change in the time-course of urease disappearance; although, since urease is not destroyed in the course of the reaction it promotes, this is by no means certain.

2. Since the hydrolysis of urea by urease goes via ammonia, then if urea is acceptable as a source of nitrogen, ammonium salts should be equally effective.

3. If the cotyledons, and therefore the bulk of the urease supply, are removed at an early stage, urea may be expected to accumulate in the seedling if no other nitrogen source is available.

Experiments have been undertaken to investigate these possibilities.

B. Urea and ammonia as sources of nitrogen.

Plants were grown in sand cultures supplied with Knop's solution in which nitrate was replaced by urea in one

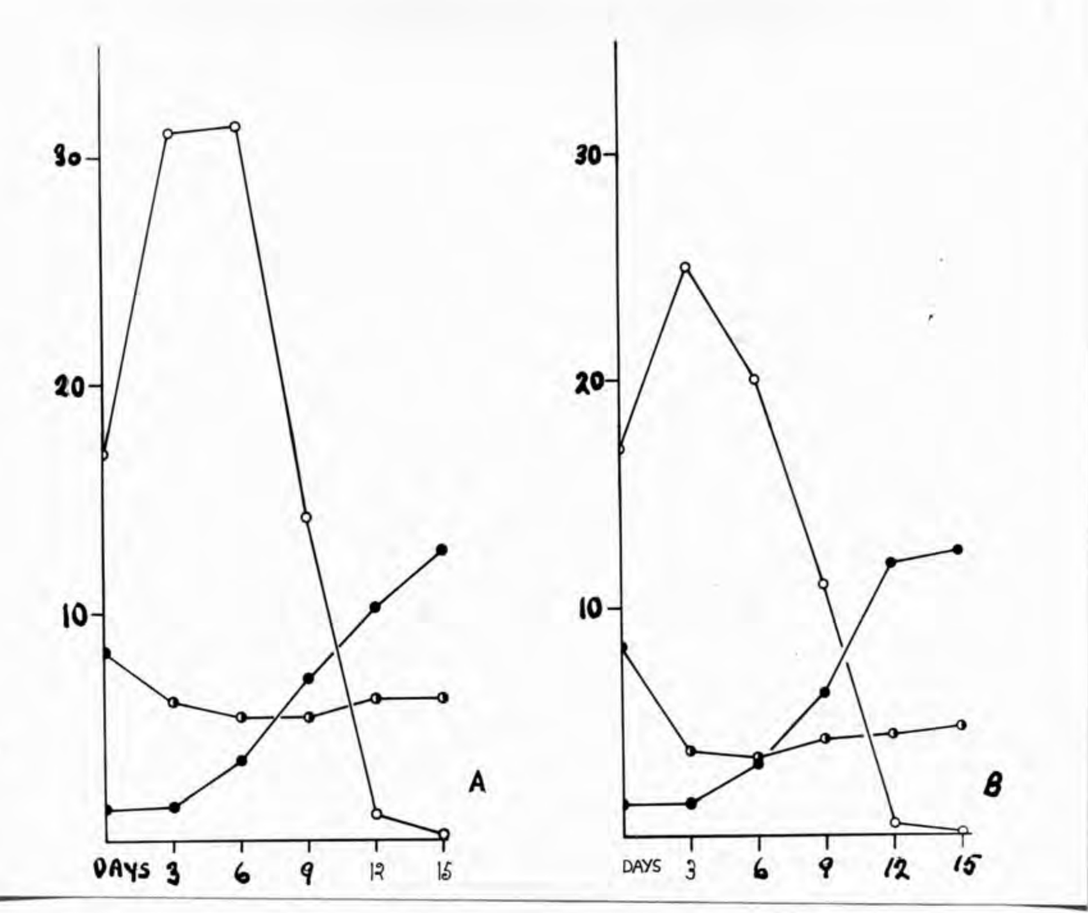


Fig.17. A comparison of changes in urease activity, total nitrogen and fresh weight of the cotyledons of seedlings supplied with either ammonia or urea and grown under artificial light.

Open circle, urease activity;
half circle, total nitrogen;
closed circle, fresh weight.

Scales: urease and total nitrogen, 1 scale division = 1 mg. ammonia N.
Fresh weight, 1 scale division = 0·1 gm.

A - Seedlings grown in Knop's solution where nitrate was replaced by ammonia.

B - Seedlings grown in Knop's solution where nitrate was replaced by urea.

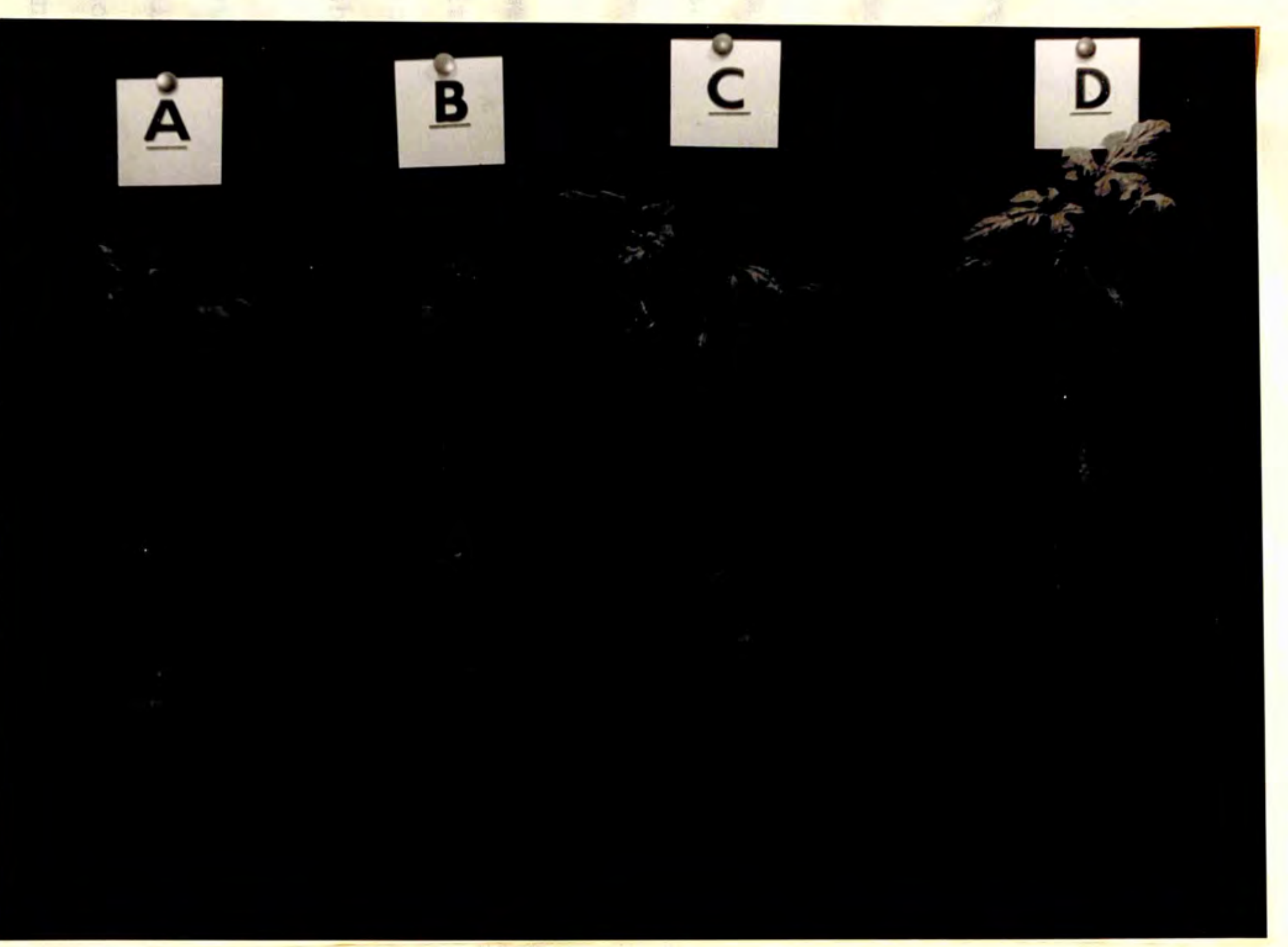


Fig.18. A comparative study of seedlings grown for five weeks with nitrate, without nitrate, with ammonia and with urea.

A - with nitrate

B - without nitrate

C - with ammonia

D - with urea.

C. Effect of removal of cotyledons.

1. Experimental methods

(a) Design of experiment

One set of plants was grown in sand culture supplied with the modified Knop's solution in which nitrate was replaced by urea; a second set was grown in sand culture supplied with full Knop's solution. On the sixth day of germination, the cotyledons of half of the plants in each set were detached at their bases with a razor blade. The purpose of removing the cotyledons was, as explained above, to ascertain whether urea accumulates in the rest of the seedling when the main source of urease is absent; or whether, on the other hand, urea is metabolised elsewhere in the seedling even without the cotyledonary source of urease. It was, however, feared that whether or no the plants without cotyledons could assimilate urea, they might die either through the loss of photosynthetic surface in the early stages of germination or as a result of the absence of the cotyledonary store of nitrogen. It is for this reason that one set of plants was supplied with nitrate, these merely serving as control. The plants were supplied with culture solution on every fourth day and with tap water on the intervening days. The pH of the culture solution with ammonia was 6·70 and that of the culture solution with urea was 6·74.

(b) Methods of estimation

The estimation of urea and ammonia was carried out in the same apparatus as that used for the determination of urease activity. Urea was determined only in the plants fed with urea; in nitrate-fed plants only ammonia was estimated. Two plants were taken for each experiment. The attached cotyledons of the plants in each set were removed before every experiment, and the estimations carried out as follows:-

The seedlings were ground up in glass distilled water and the suspension made up to 20 cc. Two 5 cc. samples of this solution were taken in two digestion tubes together with 10 cc. of plain buffer (as used in the determinations of urease activity) in each tube and 25 cc. of saturated boric acid were taken in the corresponding receiving tubes. The solution in one of the digestion tubes was maintained at a constant temperature of 30°C by means of a water bath, 5 cc. of urease solution (made by grinding two B.D.H. urease tablets in glass distilled water) was added, and the digestion was allowed to proceed for 15 minutes. At the end of the digestion period, 10 cc. of saturated K_2CO_3 was added and the ammonia formed was aerated into the boric acid in the receiving tube for 20 minutes. Similarly, saturated K_2CO_3 was added to the second digestion tube and any ammonia formed was aerated into the corresponding receiving tube. The amount of

ammonia in each receiving tube was determined by titrating a 5 cc. sample of the boric acid against $^{N}/_{100}$ HCl using Ma and Zuazaga's indicator. The amount of ammonia in the receiving tube corresponding to the digestion tube to which urease solution was added is derived both from the conversion of urea into ammonia and from ammonium salts present as such in the seedling; whereas the ammonia in the second receiving tube has been derived only from ammonium salts. The difference between the ammonia nitrogen in the two receiving tubes gives the amount of urea nitrogen from which the amount of urea was calculated. The ammonia in the nitrate fed plants was similarly estimated by adding saturated K_2CO_3 directly to 5 cc. samples of solution and subsequent aeration into boric acid.

Estimates were therefore obtained of both the urea and ammonia accumulated by the seedlings (excluding cotyledons) of the plants provided with urea as nitrogen source; and of ammonia only in the case of the corresponding control (nitrate-fed) plants.

2. Experimental Results.

(a) Morphology

After the cotyledons were removed on the sixth day, the growth of the plumule and the root system was checked as compared with plants with cotyledons; the hypocotyl, however, continued to grow to at least a further 2 cms. These differences can be seen clearly in 14-day old

Fig.19. Effect of detaching cotyledons in the early stages of germination on the morphology of the seedlings.

A - Seedlings supplied with full Knop's solution and with cotyledons intact.

B - Same as A, but without cotyledons.

C - Seedling supplied with Knop's solution where nitrate was replaced by urea and with cotyledons intact.

D - Same as C, but without cotyledons.

seedlings in fig. 19. The hypocotyl of the urea-fed seedlings without cotyledons has already become soft by this period and shows signs of decay. This seems to suggest that the cotyledons are in fact more important when urea is used as a source of nitrogen; the biochemical results below, however, do not bear this out, and it may be no more than a reflection of the fact that urea is in general a less suitable source of nitrogen than is nitrate, the difference being exaggerated by the adverse conditions of growth.

The probable cause for the reduced formation of roots in plants whose cotyledons were detached might be the loss of photosynthetic surface at such an early stage of germination. The effect of detaching the cotyledons on the morphology of the seedlings might, however, also be explained on the basis of Went's hypothesis. Went (1938) has suggested that the growth of the root, stem and leaf depend not only on auxins but on certain specific factors called "calines". He has postulated that the factor "rhizocaline" responsible for the growth of the roots and "phyllocaline" responsible for the growth of the leaves are stored in the cotyledons. These factors are supposed to disappear during their action so that a constant supply is needed. Therefore, when the cotyledons are detached the main source of these factors is removed and as a result growth of the root and leaf cannot proceed further. However, some rhizocaline is present in the stem as well,

and formation of roots will continue till it is depleted. Went has further shown that the growth of the stem is possible even without cotyledons, as the factor "caulocaline" responsible for the elongation of the stem is formed in roots. The experiments here reported are at least not in conflict with this hypothesis.

(b) Biochemical changes

The results, given in Table XV, show that even after removal of the main source of urease in the plant, urea does not accumulate in the rest of the seedling; this must be due to the fact that the slight traces of urease present in the tissues of the root and hypocotyl are quite sufficient to cope with the urea metabolism of the plant. However, a small amount of urea may accumulate in the early stages of urea feeding, even in plants with cotyledons, as can be seen in 6-day old plants (Table XV); this urea subsequently disappears gradually. Klein (1931) reported that urea can be absorbed by a legume plant from a sterile culture medium and split into ammonia by the urease present in the cells; he further stated that if large amounts of urea are fed the plants die of ammonia poisoning. However, under our experimental conditions no case of ammonia poisoning was observed even though some of the plants grown in cultures with urea for five weeks. He also observed that if urea was later withheld, the total urea already taken up disappeared in a very short time.

TABLE XV.

Accumulation of urea and ammonia in plants.

	Urea fed plants				Nitrate fed plants	
	Urea content in mgs.		Ammonia content in mgs.		Ammonia content in mgs	
Age in days.	Plants with coty-ledons	Plants without coty-ledons	Plants with coty-ledons	Plants without coty-ledons.	Plants with cotyledons	Plants without cotyledons.
6	0·39	-	0·18	-	0·17	-
9	0·03	0·09	0·18	0·21	0·32	0·32
12	absent	absent	0·21	0·25	0·23	0·18
14	"	"	0·20	0·23	0·17	0·22

D. Conclusions.

The non-accumulation of urea in the seedling when the cotyledons are removed suggests that the urease in the cotyledon has no relationship with the metabolism of urea in the rest of the seedling. There is no reason to doubt that urease is responsible for the hydrolysis of urea absorbed as nitrogen-source: but it appears, as stated above, that the traces of urease known to be present in root and hypocotyl suffice for

this purpose. In view of its high turnover number, and the fact that it is not destroyed in the course of the reaction, this is not surprising; and the function of the cotyledonary urease, if any, must be sought elsewhere.

VIII. DETACHED COTYLEDONS.

A. Introduction.

From what has been shown earlier, it is becoming abundantly clear that the changes in cotyledonary urease are virtually concerned only with the development of the cotyledons. It was therefore decided to observe the cycle of changes in urease activity in detached cotyledons cultured in water as this would lend further support to the same idea if changes similar to those exhibited by cotyledons attached to seedlings were found. Moreover, it might be possible to affect the rate of growth of the detached cotyledons by different experimental treatments; and this in its turn might affect the urease behaviour.

B. Experimental methods.

1. First investigation.

Two sets of cotyledons were used in these experiments. In the first set, cotyledons were detached with a razor blade on the first day of germination and in the second, the cotyledons were detached on the sixth day of germination after growing the seedlings in soil. The cotyledons were then grown either in water or in nutrient solutions in small specimen tubes with only the bases dipping in the

solutions. Those detached on the first day were cultured in distilled water and under stronger light (1000 watt lamp), whereas the cotyledons detached on the sixth day were divided into three lots and cultured respectively in tap water, Shive's nutrient solution mixed with α-naphthylamine and Shive's nutrient solution mixed with adenine, using a comparatively lower light intensity (four 80-watt fluorescent tubes). The growth substances were mixed in the nutrient solution in the proportion of 0·5 mgs. per litre. The growth substances were used with a view to accelerate the growth of the cotyledons, since Kruyt and Veldstra (1947) have noted a considerable increase in the fresh weight of shoots of Cosmos plants treated with Shive's nutrient solution mixed with either α-naphthylamine or adenine. Since this effect was largely due to increased leaf growth, it was considered possible that these growth substances might have the same effect on the cotyledons. However, it was observed that within a few days of treating the cotyledons with nutrient solutions the cotyledons curled up and such solutions thus seemed to be unfavourable for their growth. The nutrient solutions were therefore replaced by distilled water mixed with adenine or α-naphthylamine in the same proportion as mentioned above. At regular intervals, eight cotyledons from each culture were taken for urease determination.

2. Second investigation.

The cotyledons of both sets were also treated in a

different way to prevent extension. Immediately after detachment the cotyledons were smeared with a thin film of vaseline to prevent transpiration (it is of course realized that this may have interfered with gaseous exchange), and their bases sealed with wax to prevent any uptake of water. Those detached on the first day of germination were kept in specimen tubes under stronger light and the others detached on the sixth day were exposed to low light intensity. Here also eight cotyledons were used for each determination of urease activity. Before every experiment the wax was removed from the bases of the cotyledons which were then dipped in toluene for a few seconds to remove as much vaseline as possible.

It may be noted here that the experiments with the cotyledons detached on the sixth day and cultured in water and growth substances were carried out on one occasion and those treated with vaseline on another, and the initial urease in either case was slightly different. The differences in light intensity were not intended as part of the experiment, but were imposed by the facilities available in two different laboratories (Bedford College, London, and University College, Southampton).

C. Experimental results.

1. First investigation: effect of culturing in water or water with growth substances.

It is clear from the figs. 20 A, B and D that the growth substances do not in any way accelerate the growth

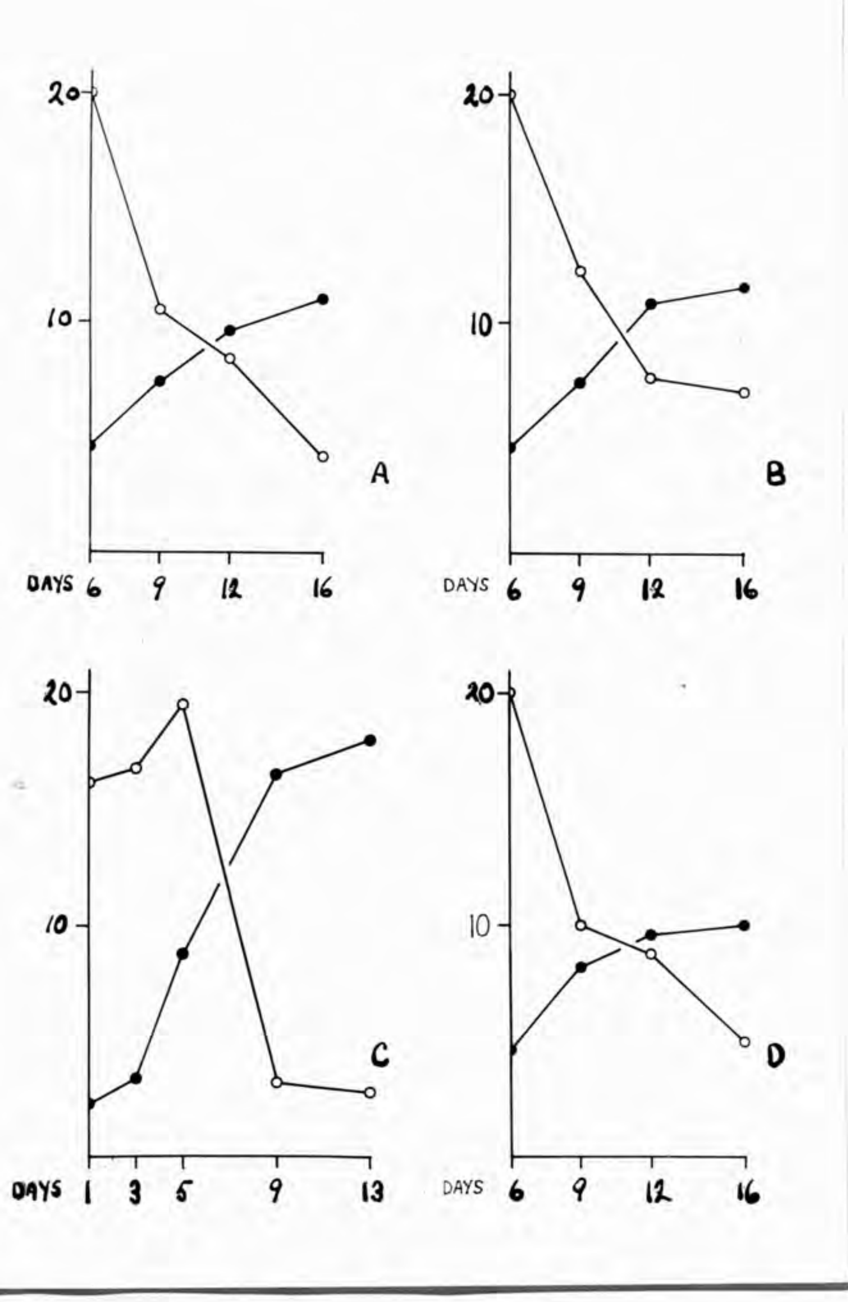


Fig.20. Changes in fresh weight and urease activity of detached cotyledons with time under different experimental conditions.

Open circle, urease activity;
closed circle, fresh weight.

Scales: urease, 1 scale division = 1 mg. ammonia N.
Fresh weight, 1 scale division = 0·1 gm.

A - Cotyledons treated with α-naphthylamine.

B - Cotyledons treated with adenine.

C - Cotyledons treated with distilled water.

D - Cotyledons treated with tap water.

of the cotyledons, as the fresh weight of cotyledons remains more or less the same throughout the experiment in every case. This might perhaps be the reason for the observation that in all cases the rate of disappearance of urease is identical. The urease in the 6-day cotyledons seems to disappear much more slowly than in attached cotyledons. The cotyledons detached on the first day of germination, however, (fig. 20(C)) show that in this case the change in urease content follows a parallel course to that in attached cotyledons. This difference in the result of the two sets is evidently due to the difference in light intensity which has markedly affected the growth of the cotyledons. It is interesting to note here that the fresh weight of the cotyledons under high light intensity on the ninth day of germination is on the same level as those of the attached cotyledons.

2. Second investigation: effect of smearing the cotyledons with vaseline (fig. 21).

The extension of the cotyledons has in both cases been completely suppressed, and the urease content has also remained constant throughout the experimental period in both. The cotyledons detached on the first day did not develop any chlorophyll and thus virtually remained dormant in a state comparable with that of the freshly-soaked seed. The difference in urease behaviour between these cotyledons and the etiolated ones which remained attached to the

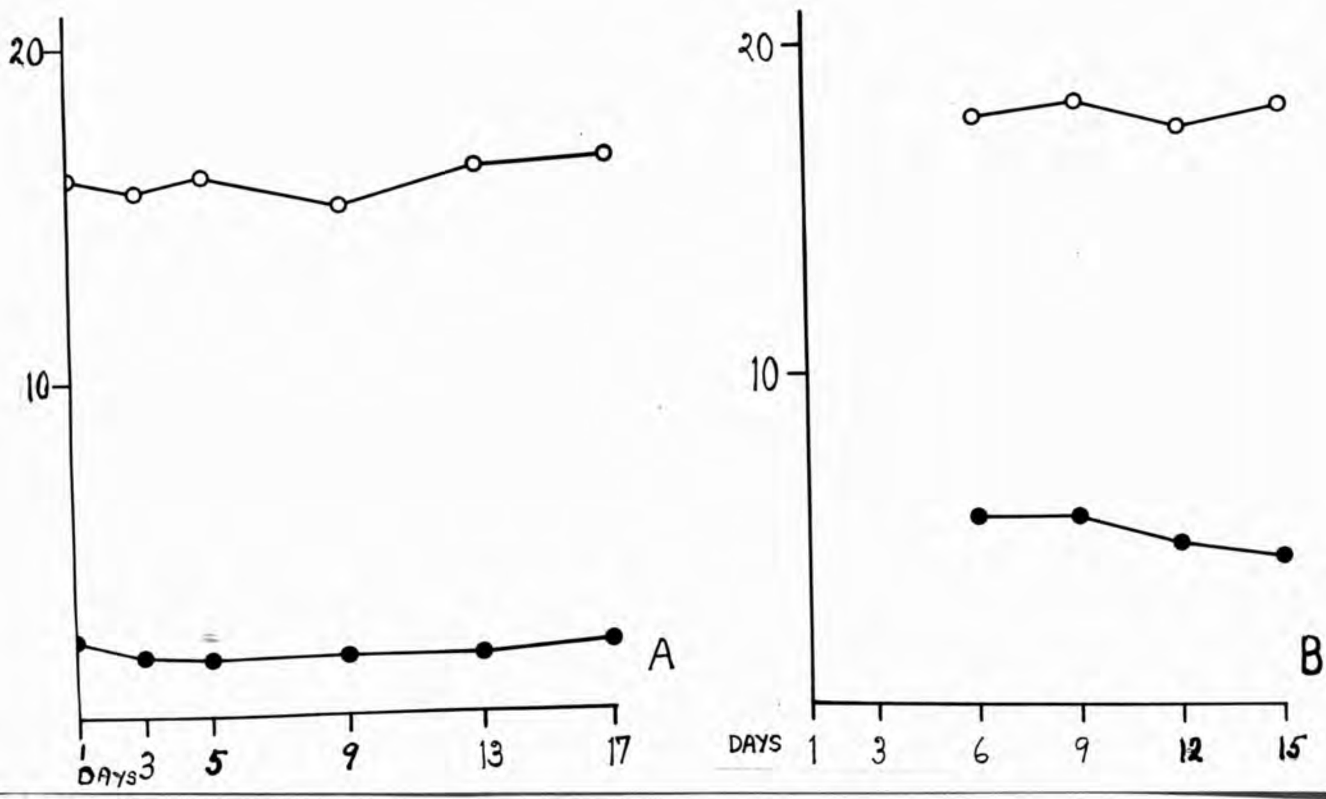


Fig.21. Effect of smearing the detached cotyledons with vaseline and sealing the bases with wax on urease activity and fresh weight with time.

Open circle, urease activity;
closed circle, fresh weight.

Scales: urease, 1 scale division = 1 mg. ammonia N.
Fresh weight, 1 scale division = 0·1 gm.

A - Cotyledons detached on the first day of germination.

B - Cotyledons detached on the sixth day of germination.

seedlings presumably lies in the active respiration of the latter and thus lends support to the view that in etiolated seedlings urease is ultimately used up in "protoplasmic respiration". The cotyledons which were detached on the sixth day were already green and it cannot definitely be said whether more chlorophyll formation took place after smearing with vaseline.

D. Conclusions.

First, the fact that urease behaviour follows a normal course in detached cotyledons under suitable experimental conditions lends further support to the view that the metabolism of urease is connected solely with the development of the cotyledons. The slower rate of disappearance of urease, on this view, may be explained as due to the reduced growth of the cotyledons under certain unfavourable experimental conditions.

Secondly, the fact that the urease does not disappear from cotyledons which are prevented from extending suggests definitely that urease disappearance and cell extension are intimately linked.

IX. UREASE IN DEVELOPING SEEDS.

This investigation has so far been concerned with the disappearance of urease in germinating seeds, and so it is naturally of interest to consider the reverse process - its appearance in developing seeds. Granick (1938) studied the activities of enzymes urease, protease and lipase in developing Soy beans. He found that with the increase in weight of the beans the urease activity gradually increased; but this was not paralleled by an increase in either lipase or protease activity. It was therefore decided to attempt to ascertain whether urease in developing _Citrullus_ seeds follows the same course as in developing Soy beans and further, if possible, to find out whether there is in this case any correlation between urease and other nitrogen fractions. With this in mind, plants were grown in pots in greenhouses in the Chelsea Botanic Garden in the summer of 1951. The plants reached the fruiting stage but, unfortunately, the fruits only attained a small size with few seeds before they decayed and fell. This might have been due partly to the humid conditions of the greenhouse or lack of space for the root system in the pots. However, seeds of two sizes were taken from one such small fruit and their urease activity and the other

nitrogen fractions were determined. In each lot of the same size, three seeds were used for experiment. The results are shown in Table XVI.

TABLE XVI.

Changes in urease activity and the nitrogen fractions in developing seeds.

Wt. of seeds in grms.	Urease activity in mgs.	Total nitrogen in mgs.	Total sol. nitrogen in mgs.	Protein nitrogen in mgs.
(1) 0·0238	0·0525	0·1554	0·1323	0·0231
(2) 0·1032	0·1838	0·3423	0·2247	0·1176

The results indicate the possibility that, in developing Citrullus seeds, increase in size is paralleled by increasing urease activity; but it is not possible to draw any conclusions as to correlation either with protein nitrogen or with total soluble nitrogen as both are increasing. It was clear that no reliance could be placed on the possibility of obtaining a supply of developing seed in this climate without considerable greenhouse resources; and it was therefore decided to postpone this aspect of the problem to some future date.

X. GENERAL DISCUSSION.

A. Introduction - Urease behaviour in Citrullus.

Urease is present in considerable amounts only in the cotyledons of Citrullus seedlings; this has been shown clearly by qualitative, quantitative and histochemical methods. The experiments carried out in this investigation were therefore aimed at the elucidation of the possible metabolic role of urease in the cotyledons. The urease content fluctuates in the germinating cotyledons, showing an early rise and then a sudden drop to practically zero. Such fluctuations have been already noted in leguminous plants such as _Canavalia ensiformis_ and _Soja Max_ by Granick (1937-38). He has further shown that the urease activity of an organ per gram fresh weight is highest when the cells are meristematic and decreases as the cells grow older. The histochemical investigation has shown that urease in the cotyledon is very evenly distributed in the cells of the palisade and spongy parenchyma and that when the urease content falls with the ageing of the cotyledon, no gradual disappearance of the enzyme is observed from any particular tissue. It is convenient at this point to recapitulate the relationships exhibited between urease and other changes in the germinating seedling.

B. Metabolic relationships.

1. Urease - a reserve protein.

Granick (1938), while following the changes of urease content in the cotyledons of _Soja Max_, suggested that the decrease of urease activity may be caused by the proteolytic enzymes splitting the proteins (including urease) into smaller, more soluble transportable products; he also discussed the possibility of the synthesis of urease in the early stages of germination. Similarly, Williams (1950) suggested that urease in _Citrullus_ cotyledons might be no more than a storage protein which incidentally possesses the properties of urease. In order to ascertain the validity of these suggestions, experiments were carried out to examine the protein level in the cotyledons when the urease disappears. The results, however, were contrary to the above-mentioned views. It was observed that the protein breakdown in the cotyledons is complete by the sixth day after germination, after which it remains more or less at a constant level apart from a slight tendency to increase later. The fact that urease disappears only after the sixth day of germination, when protein level is practically constant, shows that urease is definitely not drawn upon as a reserve protein during germination. It can, moreover, be noted that there is rather an increase in urease content when protein breakdown is vigorous. Similarly, correlation of urease activity with total soluble nitrogen is not possible.

The initial rise in urease content is found to be paralleled by a small rise in soluble nitrogen, but this is not maintained later as urease begins to fall slowly between the third and the sixth day when soluble nitrogen is still rising. Thus urease is not related to changes in nitrogen fractions. Further experiments have also shown that the metabolism of nitrogen and urease in the cotyledons is unaffected by external nitrogen supply in the early stages of germination. It may be noted here that the earlier theory of Williams (1950) that urease is a reserve protein has already been reported to be untenable by Williams and Sharma (1951).

2. Urease and urea metabolism.

The possibility of urease being connected with the urea metabolism in plants has to be discussed before assigning any other role to the enzyme. As urease hydrolyses urea *in vitro*, it is generally assumed that the same function is in operation *in vivo*. This aspect has been further strengthened by the investigations of Klein and Taubock (1931), who found urea in a wide range of plants, either in the free state or as ureides. These authors have also discussed the possibility of arginine being the precursor of urea in plants. Klein and Taubock have found that very little free urea is present in higher plants and so it is believed that any urea released from

ureide combinations is decomposed by urease into ammonia. Granick (1938) in his studies in Soy bean and Jack bean suggested that urease has the function of releasing ammonia for protein synthesis. Granick has further emphasised that urease in plants does not function in the synthesis of urea, as ammonium carbamate, from which synthesis of urea is possible, is unstable on the acid side of neutrality and, plant tissues being acidic, no ammonium carbamate would be formed. However, experiments designed to elucidate any possible correlation between urea metabolism and urease in *Citrullus* seedlings have completely failed to confirm the generally accepted view. It was expected that when cotyledons, which are the main sources of urease, are detached in the early stages of growth in urea-fed *Citrullus* seedlings, urea would accumulate in the rest of the seedling; or that urea-fed plants with intact cotyledons would show some discernible changes in the nitrogen content of the cotyledons. However, neither the accumulation of urea nor any marked change in the nitrogen fractions of the cotyledons could be observed in the experiments. It may therefore be concluded that the traces of urease that are present in the tissues of the root and hypocotyl of the seedlings are quite sufficient to cope with the amount of urea absorbed by the root system and the urease in the cotyledon bears no relationship to the urea metabolism in the rest of the seedling.

3. Urease and growth.

The increase in fresh weight has been used as a measure of growth of the cotyledons. It has been observed that when the plants are grown under optimum conditions in the soil, the maximum growth of the cotyledons takes place during the period when urease disappears. However, the data for fresh weight has not been presented and instead, increase in water content has been shown in fig. 8B; this runs parallel to increase in fresh weight. When the plants were grown in sand cultures, the growth of the cotyledons was comparatively slow, and at the same time the urease disappearance was also slowed down. In the experiments with detached cotyledons (figs. 20 A, B and D) where growth is slower than with attached cotyledons, the urease seems to disappear very gradually and when the growth of the cotyledons is completely checked, as it has been by smearing the cotyledons with vaseline, the urease does not drop but remains at a constant level. All these observations suggest that the urease activity is in some way connected with the growth and development of the cotyledons. Granick (1938) also considered the possibility of urease being a "growth bound enzyme" by which he means to imply that the urease activity is intimately bound up with the growth of the parenchyma cells. He observed that the urease activity of the meristematic cell increases rapidly during the period of elongation, and as soon as the cell

reaches maturity the urease declines to a constant low level. He came to this conclusion from a comparative study of the different parts of Soy bean and Jack bean seedlings, wherein he observed a decrease in urease content with increasing physiological age.

The growth of the cotyledon in Citrullus has been found to be due to the extension of the existing cells rather than to any cell division. This has been shown by the cell counting technique of Brown and Rickless (1949). The data in Table XIII show that the number of cells remain practically constant throughout the developmental stages of the cotyledon. However, cell division does occur to a certain extent in connection with the development of the vascular bundles and veinlet traces, but the number of cells so produced seems to be negligible in comparison with the total number of cells in the whole mesophyll. A study of the extension of cells in the cotyledon during growth, represented in fig. 9A, shows that cell extension occurs only between the sixth and the ninth days after germination and can therefore be closely correlated with urease disappearance. It is interesting to note that cell extension and increase in water content (fig. 8B) do go together. Brown (1952) investigated the activities of the enzyme invertase, dipeptidase and phosphatase during cell extension in roots, and found all three enzymes increasing during the period of cell extension and then

decreasing about the time when growth ceases. He found the relative increases in the three enzymes to be different and therefore the ratios of the activities of any two of the enzymes to change during the course of growth. He therefore concludes that, since the ratios between the enzyme activities change, the metabolic pattern must change during the course of growth; during the growth and differentiation of the cell changes occur in the metabolic state such that the relative intensities of different reactions change in the course of the process. It seems probable that this "protoplasmic differentiation" during extension of the cells may be one of the reasons for the disappearance of urease in the cotyledon.

4. Urease and respiration.

If urease is intimately connected with the growth of the cell, it might be expected that the changes in urease activity would parallel changes in respiratory activity. Granick (1938) reported from a comparison of the data of the urease activities of parenchymatous tissues of Soy bean with the data of the respiratory activity of legumes, that both run parallel. As urease forms a part of the protoplasmic framework, he suggests that one can study the protoplasmic activity in a cell by observing the changes in urease activity. The changes in the respiratory activity during the growth of the cotyledon were observed for eleven days after germination.

It was found that the rate of respiration increased sharply during the period of extension and by the time the extension of the cells was over the rate also dropped down to a constant level. Brown and Broadbent (1950) have also shown that in the growing zones of the pea root the rate of respiration per cell increases sharply during the period of extension, after which it drops to a constant level. This drop in the rate of respiration, according to Brown and Broadbent, may be due to the change in the metabolic situation of the mature cells. The respiration data presented in fig. 11 may also be taken as on a per cell basis as cell division in cotyledons is negligible, as pointed out earlier. The R.Q. was found to be as low as 0·5 on the fifth day after germination, and this is presumably due to the oxidation of fats. Langueld (1930) has reported the oil content of Citrullus seeds to be 53·67% of the dry weight. On the whole, the respiratory activity follows the course of changes in urease activity in the expected manner.

5. Urease and other enzymes.

Brown (1952) noted the close connection of the changes in the enzymes invertase, dipeptidase and phosphatase with cell extension, all increasing during extension, and all tending to decrease when growth ceases. It was therefore interesting to see whether other enzymes in

Citrullus cotyledons follow the same course of changes as does urease during extension; and with this idea, the invertase activity was followed simultaneously with that of urease. The results represented in fig. 10 show that there is a parallelism between invertase and urease during early stages of germination when both enzymes increase, but later on invertase remains at a constant level and urease falls. Such dissimilarity in enzymic behaviour during extension has been also noted by Brown (1952). He found that, in certain instances, there was in root fragments a decrease in invertase activity and an increase in acid phosphatase activity. Such changes in the activities of enzymes during growth, according to Brown, may be due to the production of specific inhibitors or to a transformation of one enzyme into another. It should also be noted here that Granick (1938) found a marked increase in urease activity in the developing seed; he measured at the same time lipase and protease activities which, however, followed a different course. His view is that these enzymes are not "growth-bound".

6. Urease and glucose metabolism.

The experiments conducted in plants supplied with glucose seem to suggest that there may be an indirect relationship between urease activity and the metabolism of glucose. The immediate effect of supplying glucose

was reflected in the depression of urease activity in the cotyledons whether the plants were grown in light or in darkness. This could be due, as has already been pointed out, to the fact that the change in urease activity may be an energy-requiring process, in which case the addition of a respiratory substrate like glucose might hasten the process. It has been pointed out by Brown and Broadbent (1950) that the increase in dry weight of the cell during extension is mainly due to the laying down of cell-wall material; this must involve metabolism of glucose in some form, and so there may well be a connection between urease and glucose metabolism. Consequently, when glucose is provided at an early stage, cellulose synthesis, which normally takes place only during extension, might set in earlier and consequently bring about a corresponding change in urease activity. It is interesting to repeat here that we have been informed by Dr. D.F. Cheesman that he has obtained experimental results which suggest the existence of some mutual antagonism between A.T.P. and urease. The measurement of the respiration rate of the cotyledons in glucose-fed plants also shows that urease disappears at a time when rate of respiration is very high. The effect of glucose on respiration was to bring about an early rise in its rate. Such increased rate of respiration by provision of sugar has been reported by Brown and Sutcliffe (1950) in root fragments. These authors suggest that sugar provides the substrate for cellulose synthesis,

which requires energy, and that a high rate of respiration provides the necessary energy. The R.Q. was also found to be, though not unity, a little higher than in cotyledons of plants grown under normal conditions on the fifth day after germination. This also suggests that the bulk of the sugar has been used up in some other process such as cellulose synthesis. The fact that urease disappears at a time when the rate of respiration is very high, both under normal conditions as well as when glucose is supplied, suggests that the disappearance of urease may be connected with the production of specific inhibitors like A.T.P.

7. Urease and Chlorophyll.

A correlation of urease activity with chlorophyll content, as has been pointed out earlier from the histochemical location of urease in the cotyledon, does not seem to be very probable. The data of the chlorophyll content presented in fig. 9B show that some chlorophyll is formed before the onset of the extension period although urease content during the same period remains very high. Moreover, studies with etiolated seedlings have shown that urease disappears even without chlorophyll formation. However, the great increase in chlorophyll content during the extension period may indirectly affect the urease activity through carbohydrate synthesis in a way similar to that suggested above.

8. Urease in etiolated seedlings.

The metabolism of urease under abnormal conditions, such as in etiolation, may be involved in processes other than those occurring in normal plants. Although extension is negligible in etiolated cotyledons, the drop in urease content is not prevented, though delayed. This drop, as pointed out earlier, may be due to protoplasmic respiration (Blackmann, 1908). It is interesting to note that urease may be "protected" from being used as a respiratory substrate by the supply of glucose. The initial rise in urease content, observed in plants under normal conditions, is not found in etiolated cotyledons.

9. Urease in detached cotyledons.

The urease content in detached cotyledons has been found to follow the same cycle of changes as in attached cotyledons, thus showing that the process is independent of the rest of the seedling. The initial increase in urease content which is a common feature in attached cotyledons, and which is also noted to some extent in detached cotyledons cultured in distilled water, may be due to the structural changes in protoplasm brought about by the uptake of water. Such increases in enzyme systems during growth have already been noted by the investigators whose work is referred to above. It may be observed that in experiments where the cotyledons were cultured in tap water together with growth

substances, the rate of growth of the cotyledons was very slow. This might have been due to the low light intensity under the fluorescent tubes, and this seems to be true from the experiments where the cotyledons were cultured in distilled water under high light intensity and where the fresh weight of the cotyledons was found to be practically double that of those cultured under fluorescent tubes by the ninth day. This difference in growth rate is interesting, as it has produced a difference in the rate of disappearance of urease. When the growth is slower, urease disappearance is also slower, and when rapid, the urease disappears very quickly. Moreover, in the experiments where growth of the cotyledons was completely checked by smearing with vaseline, the urease content also remained constant throughout. All these observations suggest that the metabolism of urease is bound up with the development of the cotyledon.

C. Conclusions.

The tendency to regard enzymes as separate entities, lying in the protoplasm as if they were protoplasmic inclusions such as are calcium oxalate crystals, belongs to an earlier stage in plant physiology. It is becoming increasingly evident that enzymes are intrinsic parts of the protoplasm, whose activity therefore shows changes as the protoplasm differentiates into its mature form. All the evidence presented in this thesis points to the view that the urease present in such remarkable quantity in *Citrullus* cotyledons is no more than an indication of the particular state of the protoplasm attained by the cotyledons before their growth ceases in the developing seed. There seems little doubt that functional urease, capable of exercising its familiar *in vitro* properties, does occur in roots and hypocotyl; but there seems equally little doubt that the cotyledonary urease is unconnected with any such processes. The fundamental problem which emerges from this investigation is perhaps the elucidation of the reasons why the protoplasm in different plants at similar stages of development (in this case the resting cotyledons) should have such very different biochemical properties; why, in fact, development of protoplasmic structure in *Citrullus* cells goes through the "urease" stage, whereas that in many other plants does not. This problem, however, must wait on a much greater knowledge of the structure of protoplasm.

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