UREASE DISTRIBUTION IN PLANTS: GENERAL METHODS

S A M  G R A N I C K

Introduction

Although numerous studies have been made on enzymes with the purpose of elucidating the intermediary metabolism of various substances, such as the fats, carbohydrates, and proteins, the metabolic changes undergone by the enzymes, that is, their synthesis, storage, and decomposition have received scant attention. Of course the metabolism of an enzyme could be discussed only superficially until a few years ago, because its composition was totally unknown. The discovery by SUMNER (22) that an enzyme, urease, possessing the properties of a globulin, could be obtained in a crystalline state, marked the beginning of a new era in enzyme chemistry. Likewise, the methods developed by LINDERSTRÖM-LANG and HOLTER (13) for the application of quantitative histochemistry to enzymes, have led away from a study of gross structures to a more intimate study of tissues and even of cells.

The purpose of the present investigation was to follow the changes in the content and distribution of urease throughout the life cycles of the two leguminous plants which, so far as known, are the richest in their content of this enzyme, namely, the soy bean and the jack bean. The most sensitive tests for most proteins are valueless below a dilution of one part in 10,000. Urease can be detected in much greater dilutions than this. Advantage was taken of the catalytic nature of the enzyme to select certain histological staining reagents which would reveal the presence of the enzyme directly within the cells. By means of these reagents it is possible to study the changes of urease activity taking place during the histological development of the plant. Since the number of cells per organ (for example, the leaf) does not change greatly after an early stage in the development of that organ, one can study, by analyzing organs of different ages, the progressive changes in urease content of cells of increasing age.

It was hoped that the data would not only reveal the course of urease metabolism but would also clarify a number of other problems as well. Considering urease as a protein, one can follow the metabolism of a protein of specific constitution throughout the life history of a plant. If the assumption be made that the main portion of the protoplasmic framework of a cell

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This is the first of a series of three papers on urease distribution in Canavalia ensiformis and Soja max. More extensive data and discussion of this subject may be found in the writer's thesis in the University of Michigan library.

2 Newcombe Fellow in Plant Physiology, University of Michigan.
consists of proteins, it might be possible, by following the changes in urease activity of the cells under various environmental conditions, to get some information about the changes occurring in the protoplasmic framework. Also, since urease has been found in plants of every division of the plant kingdom (4, 27), it becomes interesting to determine what fundamental relation the enzyme has to the nitrogenous metabolism of the plant.

The reaction catalyzed by urease, as shown by Mack and Villars (15) and by later investigators, is the hydrolysis of urea to ammonium carbamate. Urea itself is very slightly ionized. Ammonium carbamate is alkaline; it is unstable, especially in acid solutions, and undergoes spontaneous hydrolysis to ammonium carbonate. Two general methods have been used in this investigation: a histological method which depends on the increase in alkalinity as urea is hydrolyzed by urease; and a quantitative method based on the determination of the ammonia produced.

**Histological urease determinations**

An attempt was made to find a method which would give direct visual evidence of the exact location of urease in the tissues. Wagenaar (25), Sen (20), and Wasicky and Krach (27) used a number of reagents. The last-named investigators found that reagents for the determination of NH$_4^+$ such as Nessler's reagent, chloroplatinic acid, etc., were much less sensitive than pH indicators, and suggested the use of an alcoholic haematoxylin solution as a suitable indicator. After testing a number of methods, the writer came to the same conclusion as Wasicky and Krach, namely, that the change of hydrogen-ion concentration was the most sensitive method upon which to base the detection of urease.

Two histological methods were used, the indicator and the lake methods, both of which depend upon the detection of urease by an increase in alkalinity.

**Indicator method**

A number of factors which would influence the sensitivity and accuracy of the indicator method had to be considered. These may best be appreciated by imagining an enzyme point in a cell at which urea molecules are undergoing decomposition. The first molecules of ammonium hydroxide released at this point will react with various buffers in the immediate vicinity of the enzyme point. This ammonium hydroxide will therefore not show up as a change in hydrogen-ion concentration and the indicator will not be affected. After sufficient ammonia has been produced the pH of that region will shift toward the alkaline side. The rapidity of the pH change will then partially depend on buffer capacity. It is readily seen that the most sensitive indicator to choose will be one whose midpoint (pK) will be that of the
hydrogen-ion concentration of the cells. It is therefore necessary to know the hydrogen-ion concentration of the tissues examined. The series of indicators recommended by Small (21) was found suitable for an approximate determination of the pH of the tissues.

Indicators Used.—Because of their visually favorable change in color from yellow at the acid end to dark blue at the alkaline end, the indicators brom-cresol purple, brom-thymol blue, brom-cresol green, etc., were used in a 0.2 per cent. aqueous solution to detect enzymic action. They were adjusted to their midpoint with 0.05N acid or alkali. Although these indicators showed the presence of the slightest traces of enzyme their colors were not intense, but quite diffuse, so that only a vague notion could be obtained as to the enzyme location. Haematoxylin, on the other hand, was found to be excellent for the purpose. Saturated aqueous solutions of haematoxylin were prepared fresh every three or four days since changes take place in this indicator on standing. It is yellow at the acid end and intensely red at the alkaline end of its range, with a midpoint around pH 6.5. Besides showing a marked color change with change in pH, the dye in its alkaline form appears to be quite localized in a tissue. It was found upon further study that the permeability of the cells is much less to alkaline haematoxylin than to the neutral or acid haematoxylin. The yellow-colored (acid) haematoxylin could be washed out readily with water, without as readily removing the red-colored haematoxylin.

Permeability of Cells to Indicators and Urea.—If one is to judge the location of the enzyme by a change in color of an indicator within the cells, one must know that the indicator and urea solutions have penetrated all the different cells of a tissue section. The penetration of the indicator is quite readily determined by noting its distribution. Generally, the dye colors all of the cells of a tissue section. However, in certain instances, as, for example, in sections of the collet region of jack bean seedlings, some pith cells appear to be less readily permeable to the dye than their neighbors. In such instances, the dye solution is allowed to remain on the section for a few minutes longer until these cells also are colored.

It has generally been considered that cells are readily permeable to urea. Höfler and Stieglcr (8), Höfler and Weber (9), and Weber (28) have shown, however, that the penetration of urea into the cells of various tissues of the same plant and even of the same tissue was different; permeability toward urea was also found to differ with the age and the activity of the cells. By treating cells of various tissues with 20 per cent. ethyl alcohol in order to destroy the plasma membranes and permit unhindered diffusion of urea into all of the cells, it was found that the controls and alcohol-treated sections showed no marked differences in permeability toward urea. In a few instances the alcohol-treated sections appeared to contain slightly less
enzyme, but the distribution even in these sections was the same as in the controls. Perhaps even the least permeable cells allowed sufficient urea to enter from the aqueous urea solution. SMALL (21) reports an increased permeability and outward diffusion of salts from cells treated with 20 per cent. alcohol. The enzyme apparently is not rapidly destroyed in 20 per cent. alcohol. Indeed, sections of jack bean cotyledons immersed for a few minutes in a 95 per cent. alcoholic solution still possessed active urease.

BUFFER CAPACITY OF CELLS AND REAGENTS.—As already indicated, it is necessary to take into account the buffer capacity of the cells studied because, if ammonia is released at the enzyme points, the buffers of the cell will neutralize the ammonia; the rate of indicator change toward the alkaline side will therefore depend upon the buffer capacity of the cell. Although one cell may have a greater enzyme content than another, yet the amount of enzyme in the first cell may be masked by its greater buffer capacity. Likewise, it is possible that a cell may possess both the enzyme and a high buffer capacity, whereas a neighboring cell may contain no enzyme but have a low buffer capacity; in this instance ammonia would diffuse to the neighboring cell containing no enzyme and change the indicator to the alkaline side and apparently indicate the presence of the enzyme. It was found that the meristematic tissues, including the cambium, are most highly buffered, next come the smaller parenchyma cells, then the larger parenchyma cells of the cortex and pith, and finally the xylem cells.

At first glance it would seem that, because of the impossibility of controlling the tissue buffers, the indicator method would be useless for the exact localization of the enzyme in specific cells and tissues. Fortunately, this is not generally so. In sections of the apical bud, where buffer capacity must be considered, it is found that the apical primordium and the axillary primordia, which are the most highly buffered tissues, are also the tissues containing the most enzyme. On the other hand, the buffer capacity in the cambium does not permit a comparison of the enzyme content of that tissue with the content of neighboring parenchyma cells. This is true in the stem below the third node (first node above the cotyledonary node) of the seedlings. In these parts, the enzyme content of the pith and cortex is relatively high, but none can be detected with certainty in the cambium or its derivatives. Above the third node, however, the pith and cortical parenchyma contain no enzyme detectable by the indicator method, and the cambium also gives a negative reaction. That the cambium and its derivatives contain no urease is a generalization that may be made, while keeping the limitations of the indicator method strictly in mind.

Since the enzyme is detected by a change in the hydrogen-ion concentration, it is essential that the reagents used should also have little or no buffering power. Determinations of the buffer capacity of the 0.2 per cent.
dye solutions and the 1 per cent. urea solution showed it to be very slight in these solutions. As contrasted with the buffer capacity of any of the parenchyma cells examined, that of the indicators and of the urea solutions is negligible.

**Time factor.**—It is evident that if urease is permitted to act for a longer time more ammonia will be formed and the tissue will become more alkaline. The maximum time for observation is limited by the following considerations: In the first place, carbonic acid is constantly being produced, and neutralizing a portion of the ammonia released. In certain tissues, the enzyme content is so slight that more CO₂ than ammonia is produced and the acidity of the tissue increases, as has been determined in a few experiments with a sensitive glass electrode apparatus. In the second place, the enzyme appears to be slowly inactivated. Thirty minutes was found to be the maximum time that could be used for these histological determinations.

**Tissue sections.**—For approximate estimates of the enzyme, sections were made with a hand razor. If delicate structures were to be examined or accurate comparisons of sections were to be made, a freezing microtome was used at first. It was soon found, however, that small pieces could be imbedded in paraffin of low melting-point and rapidly cooled, without appreciable destruction of the enzyme. The time, from removing the material from the plant until the section was ready for study under the microscope, varied between 15 and 20 minutes.

**Lake method**

Although a particular indicator will permit the detection of urease by the first appearance of color change, the ammonia generated at the enzyme points diffuses so rapidly into the surrounding cells that the color of the alkaline indicator becomes quite diffuse. The ammonia may also invade tissues containing no enzyme and the indicator test may then give spurious results. It is therefore necessary, for sharp localization of the enzyme, to secure a reagent which will form a non-diffusible precipitate at the enzyme points. At the same time it is desirable to poison the enzyme points in order to prevent ammonia from being continuously liberated and invading the neighboring tissues. It is necessary, moreover, that the precipitate be readily visible within the cells. Of the large number of reactions tested, the formation of a lake (an adsorption complex of a dye with the hydroxide of a heavy metal) satisfies these three requirements.

The inactivation of a urea solution with various metals has been studied by Schmidt (19), and more recently by Jacoby (11). The following was found to be the order of poisoning effectiveness: Cu⁺⁺ > Hg⁺⁺ > Ag⁺ > Ni⁺⁺ > Fe⁺⁺⁺ > Zn⁺⁺ > Pb⁺⁺. The last elements had only comparatively slight effect on urease activity.
The formation of metallic hydroxides at various hydrogen-ion concentrations has been studied by Barron (3) and others. They find that the hydrogen-ion concentrations at which the hydroxide will form is a characteristic of that metal. For example, Ni\(^{++}\) will form its hydroxide when added to a solution whose hydrogen-ion concentration is pH 6.7 or above. The hydroxides of the heavy metals are generally very insoluble gelatinous precipitates which would be difficult to discern when formed within the cells. With the proper dye adsorbed, it is possible to detect traces of metallic hydroxides.

The application of the principle outlined is best illustrated by a specific example. Microtome sections of an apical meristem are treated with aqueous haematoxylin. This is allowed to remain on the sections for a few minutes until it has penetrated all of the cells. Excess fluid is drained off and a drop of a 1 per cent. urea solution and a drop of 0.05 M NiCl\(_2\) are now added. A cover slip is placed over the sections, excess fluid is drained off, and the slide is examined immediately with a microscope to note the distribution of the deep blue color of the nickel hydroxide-haematoxylin lake.

The order of adding the reagents depends on the amount of enzyme present. If the enzyme is highly concentrated, as it is in the mature cotyledons, it is advisable to use a solution of nickel chloride and in addition a trace of cupric or mercuric chloride to poison the enzyme more rapidly. This solution is added immediately after the urea. If, on the other hand, the enzyme content is very low, it may be desirable to add a very dilute nickel chloride solution some time after the urea solution is added. The enzyme action will convert the yellow haematoxylin to a red-purple color. On addition of the dilute nickel chloride solution, this color will become deep blue and quite sharply delimited from the yellow of the surrounding tissues.

Quantitative urease determinations

An excellent discussion of various quantitative methods for the determination of urease is given by Euler (4). After several methods had been tested, a colorimetric method was chosen. The ammonia produced by a piece of tissue containing urease, after the latter has acted on a urea solution for a certain time, is driven over with a rapid current of air into an acid solution in which the ammonia is determined. With some modifications the method is essentially that described by Van Slyke and Cullen (29).

A weighed portion of fresh tissue is finely ground in a porcelain mortar with 1 to 2 cc. of M phosphate buffer (pH 7.2), with quartz sand when necessary. The mixture is transferred to a volumetric flask of appropriate capacity, made up to volume with distilled water at 30° C., and the flask placed in the water bath (30° C.). Into each of the several reaction tubes
are pipetted 1 cc. of phosphate buffer, 1 cc. of freshly prepared 2 M urea solution, and the requisite amount of distilled water. The amount of distilled water is so chosen that the final volume of solution in the reaction tube is exactly 10 cc. The tubes are also placed in the water bath. After they have come to the temperature of the bath, an aliquot of tissue solution is pipetted into each tube. The time from the beginning of grinding until the tissue is added to the reaction tube varies between 10 and 15 minutes. Exactly 30 minutes (stop watch) after the tissue is placed in the tube the urease is inactivated by the addition of 0.5 cc. of 2.5 N H₂SO₄. The acid solution serves a twofold purpose. It not only inactivates the enzyme solution completely, but also decomposes the ammonium carbamate which was produced by the hydrolysis of urea. (Alkali does not inactivate urease completely.) After 5 minutes, the reaction tube is placed in the aeration apparatus. A few drops of caprylic alcohol and 5 gm. of anhydrous K₂CO₃ are rapidly added, the tubes stoppered, and suction from the water pump is applied. Control tubes containing tissue but no urea are treated in all respects like those described above. After all the ammonia has been driven over into the tube containing acid, it is determined colorimetrically with a Duboseq colorimeter. If the ammonia content is above 0.05 mg., the customary Nessler’s reagent is used. For determinations of less than 0.05 mg. NH₃, the sodium phenate reagent as modified by Van Slyke and Hiller (30) was used.

The sum of the ammonia present in the tissues and in the reagents, subtracted from the total ammonia values obtained, gives the ammonia produced through the decomposition of urea by the urease of the tissues.

Using a maximum of 0.5 gm. of tissue and a time-period of 30 minutes for reaction the method permitted the determination of a wide range of enzyme activity. In actual practice, the enzyme values ranged from 30 U.U. down to 0.0001 U.U. where U.U., the urease unit, is a quantity of urease which will produce one milligram of NH₃ per minute under the experimental conditions employed.

**Factors concerned in quantitative urease determinations**

The quantity of urease is determined indirectly by measuring the rate of formation of ammonia from urea under certain standardized conditions. These conditions were chosen and various other factors examined in relation to their influence on urease activity. Although numerous publications have dealt with the quantitative measurements of the activity of a particular tissue containing an enzyme few bear upon the difficulties that arise in an attempt to make comparisons of the quantity of enzyme in the same tissue at different stages of development, or in different tissues. A number of factors cannot be adequately controlled. However, an approximation to the true values of enzyme activity can be obtained.
A number of methods have been used by investigators to prepare the plant material for enzyme analysis. For determinations of the total enzyme content it is obviously useless to extract the enzyme, since extraction is never complete; it is different for different materials; and during extraction inactivation of the enzyme occurs.

**Effect of Drying.**—It is important to learn whether tissues may be dried without causing changes in enzyme activity. Pett (17) has studied the effect of drying germinated wheat seeds and concludes that the dipeptidase activity increases on drying. Since he used a glycerol extract of the material for his enzyme determinations, it may be that drying merely increased the amount of enzyme extracted by the glycerol, and not the dipeptidase activity.

In the following experiments the material was spread out on filter paper and allowed to dry at room temperature (approximately 22° C.). The urease content was determined immediately and after several days of drying.

**TABLE I**

_Effect of drying on urease activity_

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>Time after collecting</th>
<th>Moisture</th>
<th>U.U. per bean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy beans, nearly mature (beans removed from pods; 10 used for each determination)</td>
<td>Immediate</td>
<td>61.0</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>35.2</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td>10.1</td>
<td>0.976</td>
</tr>
</tbody>
</table>

**TABLE II**

_Effect of drying on urease activity_

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>Time after collecting</th>
<th>U.U. per gm. of original fresh weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jack bean leaflets (young; 5–8 cm. long; 15 used for each determination)</td>
<td>Immediate</td>
<td>0.0542</td>
</tr>
<tr>
<td></td>
<td>6 days</td>
<td>0.0344</td>
</tr>
</tbody>
</table>

The data in table I show only a slight decrease in urease content during slow drying of nearly mature soy beans. In the young leaflets of jack bean (table II), however, air-drying decreased the urease activity 37 per cent. Because of these results it was decided that all analyses should be made on fresh tissues.

**Effect of Macerating.**—To determine what effect crushing and macerating the tissues would have on urease activity, opposite leaflets (with petiolules removed) of jack bean plants were used. These leaflets possess numerous small stomata on the under surface but none on their upper surface. Whole leaflets, leaflets cut into 5-mm. squares, and macerated leaflets were compared
as to their urease activity. It was found that with a 0.2 M urea solution the whole leaf showed an activity one-thirtieth that of the macerated tissue. The 5-mm. squares of leaflets had an intermediate value. Infiltration of whole leaflets with urea by means of suction, and increasing the length of time for reaction on the urea solution to 60 minutes, increased the indicated urease values of whole leaflets to one-fourth that of macerated leaflets. It appeared probable that the lower value of urease activity, if whole leaflets were used, was because of the inability of the urea solution to come into contact with all of the available enzyme points in the cells. It was thought possible that the permeability of the plasma membranes might be increased by the addition of 1 and 2 per cent. ethyl ether or ethyl chlorhydrin to the urea solution. Determinations on macerated, as well as on whole tissue, showed, however, that these chemicals, at the concentrations used, inhibited urease activity from 15 to 25 per cent. No increase in urease activity was observed with leaf-tissue mash suspended in 5 per cent. sodium chloride. Another series of experiments was performed to determine whether very high urea concentrations would not give values for urease activity of the whole leaf approximating those of macerated leaves. Using 1 to 4 M urea solutions, it was found that the activity of urease in whole leaves, especially at the highest urea concentrations, approximated that of macerated leaves. It may be concluded that maceration of the jack bean leaves causes no detectable inactivation of urease. In this connection it is interesting to note that Lindström-Lang and Holter (13) report no difference in the activity of peptidase present in whole sections of barley roots as compared with crushed sections.

Urea Concentration.—An attempt was made to choose a sufficiently high urea concentration so that the amount of decomposition produced in a given time would be independent of the concentration. The data of Van Slyke and Cullen (29) and of Lövgren (14) on soy bean extracts indicate that the rate of decomposition does not increase above a concentration of 2 M urea, although the increase above 0.2 M is slight. Results were obtained on soy bean cotyledons that were in accord with the findings of these investigators; that is, there was no appreciable increase in the decomposition rate above 0.2 M concentration of urea. A concentration of 0.2 M was then chosen for the quantitative investigations. It is a convenient concentration to handle, because a 2 M urea solution can be made up, and 1 cc. of this pipetted into a reaction tube, tissue mash and buffer added, and the whole finally diluted to 10 cc.

It was later found that the conclusion arrived at for the urea concentration of soy bean cotyledons does not hold for jack bean leaf tissue. Even above a 2.1 M substrate concentration there still is an appreciable increase in the rate of urea decomposition. With 0.1 M urea solution, 0.61 mg. NH₃ was produced; with 0.7 M urea solution, 0.68 mg. NH₃; with 2.1 M urea
solution, 0.72 mg. NH₃; and with 3.5 M urea solution, 0.76 mg. NH₃. Obviously, one cannot speak of a maximum concentration above which all the enzyme points are being readily supplied with urea without specifying the tissue-preparation used. One may conclude that, if different tissues or plant parts, such as roots and leaves, are to be compared as to total urease content, an error of 25 per cent. may arise merely because of insufficient urea concentration; that when the same tissues or plant parts are being compared this error is eliminated.

The temperature chosen was 30° C. This is sufficiently high temperature to effect rapid enzymic hydrolysis, but not high enough for inactivation of the enzyme to become an important factor.

The reaction time was chosen as 30 minutes. This was selected after a number of preliminary trials had been made. Because the rate of inactivation of different enzyme preparations could not be controlled, the shortest time was chosen in which determinations of the lowest enzyme concentrations could be determined accurately. This of course depended on the sensitivity of the method selected for the ammonia determinations.

Some idea of the rate of urease inactivation was obtained from the following experiments. The moment that the maceration of the tissues was begun was taken as zero time. After a thorough grinding and proper dilution, the mash was placed at 30° C. and overlaid with toluene. At various intervals, an aliquot of the mash was taken for analysis, and allowed to react on the urea for 30 minutes at 30° C.

| TABLE III |
| Rate of inactivation of mash containing urease at 30° C. |

<table>
<thead>
<tr>
<th>Material used</th>
<th>Time after beginning of maceration</th>
<th>U. U. per gm. fresh wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min. hr.</td>
<td></td>
</tr>
<tr>
<td>Soy bean cotyledons (germinated 36 hours)</td>
<td>55</td>
<td>3.92</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>3.86</td>
</tr>
<tr>
<td></td>
<td>295</td>
<td>3.54</td>
</tr>
<tr>
<td>Jack bean leaflets (5–8 cm. long)</td>
<td>49</td>
<td>0.0547</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>0.0550</td>
</tr>
<tr>
<td></td>
<td>194</td>
<td>0.0603</td>
</tr>
<tr>
<td></td>
<td>361</td>
<td>0.0605</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0.0574</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>0.0069</td>
</tr>
<tr>
<td>Jack bean leaflets (5–8 cm. long; dried at room temperature 6 days)</td>
<td>66</td>
<td>0.0344</td>
</tr>
<tr>
<td></td>
<td>396</td>
<td>0.0041</td>
</tr>
</tbody>
</table>
From table III it is seen that the decrease of urease activity in the soy bean cotyledons is negligible during the first few hours. It is interesting to note that the mash, made from fresh leaflets of jack bean, actually increases in urease activity during the first three hours. Here again the change of activity, during the first hour after addition of urea, is negligible. With leaflets of jack bean, dried at room temperature for six days, the rate of inactivation appears to be especially marked; no change in urease activity was observed on the addition of 0.5 cc. of saturated aqueous H₂S to the reaction tubes containing an aliquot of mash. These experiments indicate that two simultaneous processes are going on: an inactivation of the enzyme by denaturation, coagulation, or poisoning; and a dispersion, peptization, or activation of the enzyme.

The hydrogen-ion concentration of pH 7.2, which was produced by a phosphate buffer, was used for the urease determinations. Lövgren (14), using phosphate buffers, reported an optimum pH between 7.2 to 7.8 for soy bean extracts. Howell and Sumner (10) found that the urease activity depended on the type of buffer and on the concentration of urea, the pH optimum increasing with decreasing urea. Bach (1) found an optimum hydrogen-ion concentration of pH 8.6 for a urease preparation extracted from an Aspergillus. It is quite possible that the optimum hydrogen-ion concentration for a urease preparation may depend to some extent on its concomitant impurities, as has already been found for certain enzymes. No experiments were conducted to determine whether the hydrogen-ion concentration optimum is different for different structures of the same plant. With the dipeptidases of the wheat grain Pett (17) found about the same pH-activity curve for all portions of the wheat grain that were studied.

Since ammonia is produced during urea hydrolysis and the solution becomes alkaline, it is necessary to have a sufficient amount of buffer present to prevent any marked change in the hydrogen-ion concentration. In this investigation, a quantity of enzyme material has been taken which will generally produce less than 1 mg. of NH₃ in 30 minutes. A concentration of 0.1 M phosphate buffer (prepared from Na₂HPO₄ and KH₂PO₄) was used, which when present in the enzyme substrate mixture was found to keep the hydrogen-ion concentration from increasing more than three-tenths of a pH unit.

The recovery of ammonia with the aeration apparatus was determined, using standard solutions of ammonium sulphate, and also using mash plus standard ammonium sulphate solutions. Although 90 per cent. of the ammonia was recovered after 30 minutes of aeration at room temperature, recovery was complete only after two hours of aeration. The presence of mashed tissue did not interfere with recovery.

Sampling of plant material.—Analyses were made on the basis of the fresh weight of plant organs or parts of organs. The organs were removed
from the plants in the morning, then weighed and analyzed immediately. For each analysis generally ten organs were selected from ten different plants. The tissues were ground and duplicate determinations were run on aliquots of the mash, including also a control for the free ammonia present in the mash. In selecting samples for analysis, an attempt was made to choose plants that were similar in number and length of leaves and internodes. Plants either more or less vigorous than the average were discarded.

Discussion and conclusions

It is necessary to clarify the term "quantitative" in reference to enzyme determinations. An enzyme is detected only through the catalysis of a more or less specific reaction. This criterion is, however, not sufficient to establish the absence or the total quantity of enzyme, but merely the amount of "active" enzyme present.

An extensive review by Lövgren (14) of the inactivation of urease by various substances indicates that inactivation, in most instances, can readily be accounted for by a denaturization or coagulation of the protein. As for activation by specific substances, Waldschmidt-Leitz and Steigerwald (26) report that urease activation with glycine or hydrocyanic acid is observed only with crude enzyme preparations, and not with purified or crystalline enzymes. There is some evidence (2, 5, 7) that oxidation inactivates and reduction reactivates urease but the meaning of this is not clear.

The data at present available on crystalline hydrolytic enzymes, including urease, favor preponderantly the contention (24) that these enzymes are proteins containing in their make-up, and holding by chemical linkages, peculiar arrangements of atoms which constitute the active groups. Since urease can be detected only in its active state, that is, by the number of active groups with which the urea molecules can come in contact, it becomes important to examine under what conditions and to what extent these groups are available for action.

In the cells, proteins are present in various states of aggregation, some protein molecules being monomolecularly dispersed, others clustered into larger groups, and still others aggregated into ergastic protein materials such as the globoids, aleurone grains, and crystals. The protein urease likewise can be considered to be present in the cells in these various states of aggregation. Grabar and Reiger (6) have recently investigated the activity of urease as related to particle size. Using four different urease preparations they found, by means of membranes of graded porosity, that the particles of urease were in different states of aggregation. Crystalline urease in aqueous solution was the most homogeneous preparation examined, and had dimensions near those of serum globulin. On digestion of the urease preparations with trypsin it was found that those particles small enough to pass through pores 15 m\(\mu\) in diameter possessed no urease activity. Under the same
conditions serum albumin passed through pores 30 μ in diameter but not through 9 μ pores. Crystalline urease was the most active preparation. From these data, it appears that the activity of a urease preparation is at its maximum when the urease particles are of the dimensions of serum globulin particles (mol. wt. 150,000); presumably the urea molecules are now able to come into contact with all the available active groups of the urease particles. The work of Kirk and Sumner (12) may be cited as evidence that the decrease of urease activity occurs with agglomeration of urease particles. These workers noted that the inhibiting effect of anti-urease (antibody) on urease consists, largely but not entirely, in decreasing the dispersion of urease.

From the above, it may be seen that when larger groups of urease molecules, as in the form of ergastic materials in the cell, are dispersed, an apparent synthesis may occur. If urease is decomposed proteolytically below a certain molecular weight there will be a loss of urease activity; but there may also be a decrease in urease activity or apparent decomposition when the enzyme becomes denatured, agglomerated, or stored in some form in which it is not readily dispersed. The urease that is detected is, in the main; in a highly dispersed condition. The data on the rate of inactivation of mash of jack bean leaf show an increase in urease activity during the first three hours after maceration, indicating that more of the active groups have become available than are being removed by autolysis or denaturization.

There is no way at present of determining the total quantity of enzyme present (16, 18). The methods used in this investigation have been chosen to determine as nearly as possible the relative amounts of available active groups present in the cells at the time of examination. In all instances, where comparisons could be made of the histological and the quantitative methods with the same tissue, it was found that the results obtained with both methods checked each other nicely. One may infer, therefore, that in the macerated tissue, the enzyme does not differ in activity to any extent from the enzyme in the cells of tissue sections. The experiments in which "whole" and "macerated" leaf tissues were compared showed that macerating caused no detectable inactivation of the enzyme. Further experiments (table III) indicate that urease inactivation within the first 30 minutes after crushing the tissue is slight or negligible; and this has been the length of time chosen for the enzyme to act on urea.

Although, with the cotyledonary tissue of soy bean, there is no increase in the rate of urea decomposition above 0.2 M urea concentration, this is not true for the tissue of the jack bean leaf. In this leaf tissue, there still appears to be an appreciable increase in the decomposition rate of urea even above a 2.1 M concentration. This may be another instance of making the active groups of urease available, since it is known that urea in high aqueous concentrations has a powerful solvent action on many proteins, and appears to split certain
protein molecules. Edestin, for example, is split into particles whose molecular weight is about one-fourth of that determined by Svedberg for edestin in neutral salt solutions. For convenience, a concentration of 0.2 M urea was used in these determinations. When different plant structures are to be compared as to total urease content, an error of 25 per cent. may arise because of an insufficient urea concentration; when the same tissues or plant structures are being compared, however, this error is eliminated. Fortunately, the differences of urease content of different structures are much greater than 25 per cent. so that this factor does not influence any of the conclusions that have been drawn from the data.

Although the limit of sensitivity of the quantitative method is 0.0001 U.U. per gram of fresh weight of tissue (where a urease unit, U.U., is that quantity of urease which will produce 1 mg. of ammonia per minute), the histological method is not as sensitive. From comparative determinations it has been found that the indicator method cannot detect urease in tissues having a lower activity than 0.02 U.U. per gram fresh weight.

By making certain assumptions, it may be calculated that there are about 25 urease molecules contained in a cell, at the limits of sensitivity of the quantitative method. Since the indicator method is only one-two hundredth as sensitive as the quantitative method, at least 5000 urease molecules must be present in a cell in order to change the indicator color. It is therefore impossible to determine the positions of the enzyme in the cells by the indicator method unless the urease molecules are sharply localized in specific regions.

Summary

1. Two methods for the determination of urease are described. The first, a histological method, depends on detecting the increase in alkalinity of the cells as urea is being hydrolyzed by the urease present in the cells. This increase in alkalinity is made evident either by the use of a suitable pH indicator or by the formation of a lake. The second, a quantitative method, depends on the determination of the ammonia produced when urea is hydrolyzed by the enzyme.

2. The following factors were considered in applying the histological method: The hydrogen-ion concentration of the tissue; the suitability of various indicators; the permeability of cells to indicator dyes and to urea; the buffer capacities of cells and reagents; the time factor for the method; and the preparation of tissue sections for analysis. In the lake method, reagents were chosen which would form insoluble and highly colored precipitates at the enzyme points.

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3 Assumptions: Dimensions of a parenchyma cell 20 × 50 × 50 μ; density of cell 1.0; there is one active group per urease molecule; urease has a molecular weight of 150,000; 1 gm. of crystalline urease can produce 26,000 mg. NH₃ per minute, which is the highest activity reported by SUMNER (23).
3. The following factors were considered in applying the quantitative method to various tissues: Effect of drying the tissues; effect of macerating the tissues; effect of various urea concentrations on the maximum urease activity; effect of the temperature; effect of the time; the rate of inactivation of urease; the effect of the hydrogen-ion concentration; the choice and concentration of the buffer; the recovery of ammonia by aeration; and the sampling of tissues for analysis.

4. A concept of urease activity, based on the number of available active groups of urease, is discussed.

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University of Michigan
Ann Arbor, Michigan

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