STUDIES ON THE RESORPTION OF UREA BY ROOT OF ZEA MAYS-SEEDLINGS IN STERILE CULTURE

By

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With 3 Text-figures

INTRODUCTION

Since the studies of DE VRIES (1899) it is generally well known that urea permeates very easily through the plasma membrane of plant cells. This fact can be demonstrated by the deplasmolytic (FITTING, 1917) or plasmometric method (HÖFLER and STEIGLER, 1921; HÖFLER and WEBER, 1926) using urea in surrounding solution.

The existence of urea in a plant body was reported for the first time in fungi by BAMBERGER and LUNDSIEDEL (1903). IWANOFF (1923a, 1923b, 1925) recognized that urea exists in some species of Lycopodiaceae, and from the result of his quantitative and qualitative studies he concluded that the occurrence of urea in a plant body depends in some degree on the nitrogenous compounds in the culture solution and the stage of maturity of the fungi. He discussed also the physiological meaning of these substances. WEYLAND (1912) reported that urea occurs in the mycorrhiza of Orchidaceae, Equisetaceae and some special fern plants which grow in marsh land, but WEISSFLOG (1917) denied the occurrence of urea in mycorrhiza of Orchidaceae in the natural condition. KLEIN and TAUBÖCK (1927) proved that urea exists in Angiosperms, for example, in Leguminoceae, Orchidaceae, Urticaceae and Cucurbitaceae.

FOSSE (1916) and IWANOFF (1923a) explained hypothetically the assimilation of urea in a plant body as a nitrogenous organic matters.

but these explanations were not proved experimentally. According to FOSSE (1923), urea can be resorbed by higher plants from soil, but not assimilated without being decomposed into ammonia in plant body, therefore, he did not attach importance to it as a nitrogenous source. But from the frequent occurrence of urease in plant body and its catalytic action in chemical processes, we can recognize that urea plays a role in nitrogenous metabolism and it is good as a nutritive substance for plant growth. On the other hand, in a plant body arginase can be widely found. It decomposes arginin into urea and ornithin. Already KLEIN and TAUBÖCK (1927) found urea in plant bodies when they were cultured in culture solution containing urea as the nitrogen source in the sterile cultures. According to IWANOFF (1923b) arginase has also catalytic activity for the synthetic chemical reaction reverse to that mentioned above.

From these facts we may suppose that urea has relation with that part of synthesis by which arginin, purin basis and higher complex protein compounds are built up, that it remains as a temporary form of nitrogenous organic compounds in the metabolism of higher plants, aud that on the other hand urea is decomposed into ammonia by the aid of urease, and ammonia thus formed is used in the synthetic process of the nitrogenous organic compounds.

Concerning the nutritive value of urea for plant growth, TRUFFANT and BEZSSONOFF (1924) state that it is better than ammonium and nitrate salts, because urea can enter very easily through the plasma membrane into the inner part of cell, or it is decomposed into ammonia in the culture soil, and ammonia thus formed can prevent the increase of acidity in soil. KOSTYTSCHEW (1926, p, 145) says that urea is very available for plant growth on account of its character for absorption and chemical synthesis. For these reasons it is necessary to study the resorption of urea by plants, and its nutrient value as a nitrogenous source.

In studying the nutrient value of urea as a nitrogenous source for higher plants, and its resorption by them, it is very important to carry

out experiments in sterile condition, because urea can be easily attacked and decomposed by bacteria and fungi, before the cultured plants utilize it.

If the resorption of urea aud its nutrient value for higher plants were studied only by the method of the ordinary water culture, it follows that such a study may sometimes bring about insufficient conclusion. Therefore, the present work was carried out in sterile water culture using the seedlings of *Zea Mays* (starch corn, Sapporo eight lines).

A part of the present work was done at the Botanical Institute of the Agricultural Faculty of Hokkaido Imperial University. Further studies were continued at the Botanical Department of the Science Faculty of the same University.

The writer wishes to express his sincere gratitude and hearty thanks to Prof. T. SAKAMURA for his suggestions and guidance throughout the present work.

Method and Material

The total amount of urea in the culture solution at the beginnig and the end of experiments was determined, and the difference between these two determinations was regarded as the resorbed amount of urea.

All cultures were conducted in the green house. At the end of each culture, the plants were cut off from the residual seed at the point of attachment; the lower part was regarded as the root, the upper part as the shoot. These two parts were separately dried in a dry oven of 80°C, and the dry weight was determind by balancing until the constant weight was reached.

As culture medium, throughout this work, use was made of a modified KNOP's solution which contains urea and calcium chloride instead of calcium nitrate as nitrogenous and calcium source. The amount of calcium in this modified KNOP's solution, is equivalent to that in the ordinary KNOP's solution, but the amount of nitrogen is two thirds that

in the ordinary KNOP's solution. This culture solution is called "urea culture solution," hereafter, for convinience. The urea culture solution was prepared as follows:

Urea ($CO(NH_2)_2$)	0.2402 gm
Calcium chloride ($CaCl_26HO$)	1.3352 gm
Potassium biphosphate (KH_2PO_4)	0.25 gm
Magnesium sulphate ($MgSO_47H_2O$)	0.25 gm
Ferric chloride ($FeCl_32\%$)	3 drops
Distilled water	1000 cc

pH=4.6

The distilled water used in the present work was prepared by treating it with KAHLBAUM's blood charcoal. That the distilled water thus treated was free from copper and other oligodynamically toxic substances is shown by the fact that *Spirogyra*, a very sensitive plant, could grow in it quite well for a day or so, while the untreated distilled water proved decidedly poisonous to that alga (SAKAMURA, 1922).

The vessels used in this work were made of non alkaline glass, some of them being of Jena glass. In some other cases porcelain cylinders were used. They were washed with chrombisulphuric acid and then throughly rinsed with tap and distilled water.

To determination of the final and initial pH value of the culture solution, the colorimetric method of CLARK and LUBS was employed using both their standards and indicators.

The seed for experiments were delivered to the writer from the First College Farm of this University, and from the Agricultural Experimental Station, Hokkaido Government. The writer wishes to express his thanks for the kindness of these offices.

Estimation of Urea: For the estimation of urea, the micro-KJELDAHL method for ammonia was used, combined with hydrolysis with aid of urease. Free ammonia which is produced in consequence of decomposition of urea by the catalytic action of urease was distilled and collected by hydrochloric acid (N/100), the excess of the hydrochloric acid was titrated with sodium hydroxide (n/100), using sodium alizarin

sulphonic acid as indicator. From the amount of ammonia that of urea was calculated.

Preparation of Urease Extract: Urease extract was prepared from Soy bean. In the urease extract thus prepared a small quantity of free ammonia was contained. Therefore, the amount of free ammonia in the urease extract was estimated, whenever it was used, and subtracted from the amount of total ammonia in this mixture. The free ammonia from urea was determined in this manner, viz., 4–5 cc. of urease extract, 0.5–1.0 of phosphate buffer solution, and five drops of a phenol red solution were mixed with a measured amount of sample and this mixture was placed in a thermostat of 30 C° for 30–60 minutes. The pH value of this mixed solution was nearly 7.3, which is approximately the optimum pH for the urease action (VAN SLYKE, 1914). After perfect decomposition of urea, this mixed solution was diluted properly and a suitable amount was distilled, using the micro-KJELDAHL apparatus.

Method of Sterile Water Culture

The sterile culture of higher plants is very difficult compared with the pure culture of bacteria or fungi. Therefore no generally available method is known. It should be variable according to the plant species and the purpose of the investigation. The attached parts on the surface of the seed were removed, and then the seeds were submerged in sterilized water under low pressure for 5–10 minutes in order that water may soaks into every nook of the seed surface and drive out air bubbles.

Sterilization of Seed Surface: For the sterilization of the seed surface, corrosive sublimate, Usplun, Zonite and calcium hypochloride were used, but only sublimate was satisfactory. Seeds of *Zea Mays* were sterilized with 0.1% alcoholic sublimate solution (solvent: 20% alcohol) for thirty minutes. According to WOLF (1927) an alcoholic solution is more effective for this purpose than aqueous solution.

The sterilization method and apparata were reported by HUTCHINSON and MILLER (1908) and HEMMI (1926) and others. The writer used the following method, because this is very simple to practice and seed can be perfectly sterilized by it (Fig. 1).

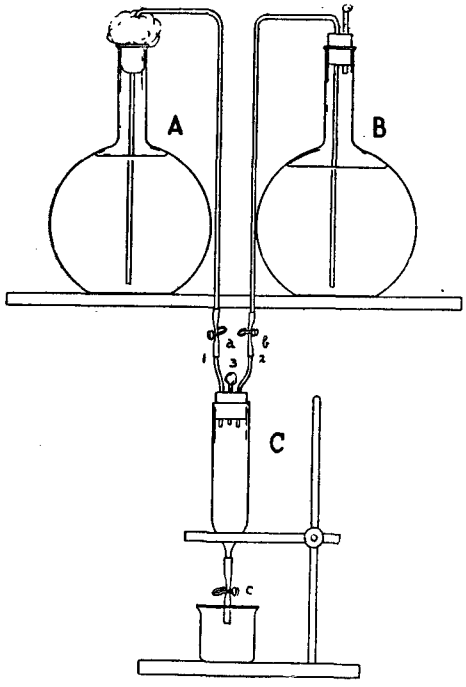

Fig. 1.

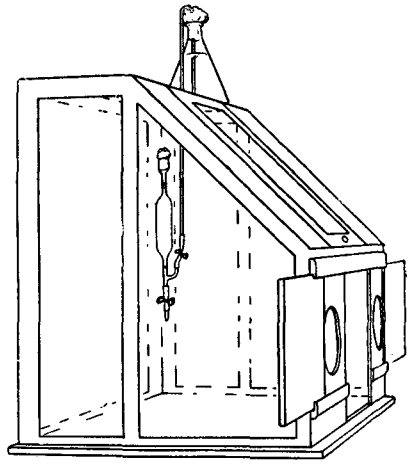

Fig. 2.

The apparatus consists of three vessels. A and B are long shaped flasks each with a capacity of 2 liters. Flask A is filled with water and stopped with cotton and then sterilized in KOCH's sterilizing apparatus. Flask B is filled with corrosive sublimate solution. Seeds are sterilized in vessel C. Through the tube 1 sterile water is conducted from flask A into C, and through the tube 2 the corrosive sublimate solution is conducted from flask B. By opening of the pinchcock c waste water and sublimate solution are drawn away. After the seeds were sterilized in tube C for a definite time, sublimate solution was thrown away, and

seeds were washed out with sterilized water repeatedly in this vessel. The seeds thus treated were transferred into a sterilized PETRI-dish. This entire operation was conducted in a sterile box (Fig. 2).

The sterilized seeds in a PETRI-dish were placed in a thermostat of 30°C. until the seeds germinated moderately.

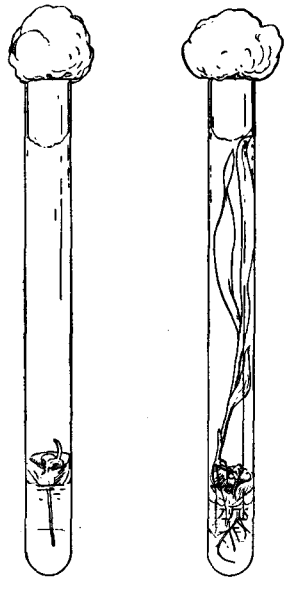

Fig. 3. The lower part of this culture tube was covered with one thickness each of black and white paper in order to prevent the entrance of sunlight.

Vessels and Method of Sterile Culture: The urea culture solutions was used for the sterile water culture. If urea and other salts in this culture solution are sterilized together, urea is decomposed into ammonia by heating, so that the C_H of this culture solution decreases and precipitations are produced, which are chiefly ammonium magnesium phosphate. In order to prevent such C_H-change and precipitation, urea and salts solutions were sterilized separately, and after sterilization these two parts were mixed in the sterile condition. As vessels for sterile culture large non-alkaline glass test tubes were used. Filter paper rolled conically was put in the tube to hold the seedling, as used for the first time by YOSHII (1925) (Fig. 3).

Verification of Sterile Condition: It is very important to verify the sterile condition of the culture solution and the cultivated plant at the end of experiments. SCHLOW (1911) has ascertained the sterile condition of the culture solution by inoculation of a small drop of the culture solution into a bouillon culture medium (bouillon, 2 % grape sugar, 1/2 % asparagin).

FRED (1912) has examined unexpected contamination by microorganisms in the sterile soil culture by inoculation of the culture soil into agar or bouillon culture medium at the end of the culture.

KOTTE (1926) has proved sterile condition by inoculation of a small drop from the surface and inner part of the culture solution into agar or bouillon culture medium. In the present work the sterile condition was proved by microscopical examination, staining the micro-organisms with acid fuchsin and methylene blue. If in a few cases a very small number of micro-organisms exists in culture solution, it does not, however, cause measurable chemical change of the culture solution and can be practically neglected.

Results of Experiments with Sterile Water Culture

EXPERIMENT 1. (Table I)

30 cc. of the urea culture solution which contains 9.09 mg. of urea (0.004 mol solution) and 0.3 mg. of free ammonia were placed in each test tube. Sterile culture was carried out in the green house for 10, 15, 20, 25, and 30 days. At the end of each culture, urea in the culture solution was estimated.

In the determination of the dry weight, endosperm was removed from seedling at the scutellum. Duration of culture: Oct. 9—Nov. 8, 1927. Temp: 12–28°C.

All cultures shown in Table I were perfectly sterile. From these results it will be seen that the total resorbed amount of urea and dry weight increased in each series according to the duration of the culture. The C_H in the culture solution changed to more acidic side than that in the control solution in the ten and fifteen day cultures. The C_H in the twenty and twenty five day cultures increased than at the beginning. In the longest duration (thirty days) the acid reaction remained unchanged. Generally speaking the C_H always increased. The amount of free ammonia was less at the end of the experiment than at the beginning.

Free ammonia seems not to have been formed in the culture solution by micro-organisms and other causes during the experiment. From

TABLE I.

Duration of Culture (days)	Series	pH		Dry Weight (mg.)		Resorption of Urea			Free Ammonia ± 0.34 mg.		Remarks
		Initial	Final	Root	Root and Shoot	Resorbed Urea ± 0.6 mg. (mg.)	Percentage of resorption (%)	Resorbed Urea per 1 mg. of dry weight (mg.)	Total Amount (mg.)	Increase and Decrease (mg.)	
10	1	4.6	5.5	21.5	35.0	1.8	18	8.5	0.51	0.21	Sterile
	2	,,	5.5	41.0	56.4	2.4	24	5.9	0.34	0.04	,,
	Average					2.1		7.2			
15	1	4.6	5.2	57.5	87.4	4.2	42	7.3	0.34	0.04	Sterile
	2	,,	5.4	36.2	54.1	3.9	39	10.1	0.34	0.04	,,
	Average					4.0		8.7			
20	1	4.6	3.8	54.3	102.9	3.9	39	7.2	0.17	0.13	Sterile
	2	,,	3.6	76.6	94.6	3.9	39	5.1	0.34	0.04	,,
	Average					3.9		6.2			
25	1	4.6	3.8	70.5	127.0	3.3	33	4.7	0.17	0.13	Sterile
	2	,,	3.6	76.4	125.2	3.9	39	5.1	0.17	0.13	,,
	Average					3.6		4.9			
30	1	4.6	4.6	50.1	117.4	5.7	57	11.3	0.34	0.04	Sterile
	2	,,	4.6	54.0	103.5	7.5	75	13.8	0.34	0.04	,,
	Average					6.5		12.5			

these points it can be concluded that urea is resorbed in the form of urea itself by plants. However, it is hardly tenable forthwith to conclude that urea resorbed by plants in the form of urea itself is very good for plant nutrition as the inorganic nitrogen source, because the experiment was carried out using seedlings bearing endosperm which can still deliver necessary nutrients. Therefore, in order to make clear this point the following experiments were carried out.

Experiment 2. (Table II)

At the beginning of the experiment the endosperm of sterilized seedlings was removed at the scutellum as perfectly as possible. The method of the experiment was the same as in Experiment 1. By removing of residual endosperm from seedling the supply of C-source becomes insufficient, though CO_2- assimilation already more or less occurs in them. In order to make good this lack grape sugar was added to the culture solution.

The composition of the culture solutions was as follows:

Urea culture solution.

Urea ($CO(NH_2)_2$)	0.2402 gm.
Calcium chloride ($CaCl_2 \cdot 6H_2O$)	1.3352 gm.
Potassium biphosphate (KH_2PO_4)	0.258 gm.
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.25 gm.
Ferric chloride ($FeCl_3$ 2%)	2 drops
Grape sugar anhydrous ($C_6H_{12}O_6$)	1.801 gm.
Distilled water	1000 cc.

Culture solution without urea and other nitrogenous sources.

Calcium chloride ($CaCl_2 \cdot 6H_2O$)	1.3352 gm.
Potassium biphosphate (KH_2PO_4)	0.25 gm.
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.258 gm.
Ferric chloride ($FeCl_3$ 2%)	2 drops
Grape sugar anhydrous ($C_6H_{12}O_6$)	1.801 gm.
Distilled water	1000 cc.

TABLE II.

Culture solution	Endosperm	pH		Length (cm.)		Dry weight (mg.)			Resorption of Urea			Free ammonia ± 0.02 mg.	
		Initial	Final	Root	Shoot	Root	Shoot	Total	Resorbed urea ±0.15 mg (mg.)	Percentage of resorption (%)	Resorbed Urea per 1 gm. of total dry weight (mg.)	Total amount (mg.)	Increase and Decrease (mg.)
Urea culture solution	Without	4.8	4.0	15.0	23.0	29.0	35.0	64.0	6.0	41.2	93.8	2.05	+1.7
	,,	,,	4.0	11.0	31.0	11.0	58.0	69.0	7 1	49.3	102.0	0.17	−0.17
	With	,,	3.9	20.0	27.0	60.0	109.0	169.0	9.0	62.5	53.1	0.22	−0.12
	,,	,,	4.9	17.0	38.0	49.0	150.0	199.0	9.8	68.4	49.3	0.56	+0.22
Culture solution without urea	Without			22.0	5.0	13.0	14.0	27.0					
	,,			32.0	5.5	13.0	13.0	26.0					
	With			35.0	32.0	54.0	74.0	128.0					
	,,			42.0	14.0	55.0	44.0	99.0					

Experiments were conducted with the following two kinds of culture solution:

1. Urea culture solution containing grape sugar.
   a. Without endosperm
   b. With endosperm

2. Culture solution containing grape sugar without urea and other nitrogenous sources.
   a. Without endosperm
   b. With endosperm

Duration of culture: Dec. 17, 1928—Jan. 4, 1929, 18 days. Temp: 12—28°C. Experiments were carried out in the green house. Concentration of urea in the culture solution was 0.004 mol. Each 60 cc. of the urea culture solution contained 14.4 mg. of urea and 0.34 mg. of free ammonia. One seedling in each culture tube.

All cultures shown in Table II were perfectly sterile. From this result it will be seen that plant growth in the urea culture solution had the advantage over that in the N-free culture solution, regardless of existence of endosperm. In the N-free culture, a large quantity of anthocyan was formed in the lower part of the shoot and at the base of adventive root, while in the urea culture no such anthocyan formation occurred in any part. This may mean that in the former case resorbed sugar was accumulated in plant body without being utilized for the synthesis of nitrogenous organic compounds. In the latter case it may be very probable that resorbed urea is soon used for such synthesis, and sugar can not be reserved for the formation of anthocyan. This relation indicates more probably that resorbed urea can be easily assimilated to higher nitrogenous compounds.

Judging from these points mentioned above it is very probable that urea is resorbed by plants as itself and it can be utilized as a nitrogenous source by plants.

Resorbed Urea in Plant Body

It will seem more probable that urea is resorbed in unchanged form, if it is found in plant body even in the ordinary urea culture.

EXPERIMENT 3. (Tables III, IV & V)

The seed of *Zea Mays* were sowed on saw-dust, and the seedlings cultured for one week in tap water. Each urea culture solution contained respectively 0.1 mol, 0.02 mol and 0.004 mol of urea. The whole vessels were covered with bell-jars in order to cause guttation from tip of leaves by increasing the humidity of the air surrounding the plants. After a definite time drops of guttation were gathered for test, and also all sap was pressed out from the shoot for the same purpose. Urea in this drop and cell sap was qualitatively tested microchemically by the xanthydrol method of FOSSE (1913).

In the presence of urea, crystals of dixanthyl urea were easily formed by heating, after the addition of reagent. According to KLEIN and TAUBÖCK (1927) the reaction occurs most easily between 180° and 230°C. Tables III, IV and V show the results of the microchemical tests.

TABLE III.

The culture began Oct. 10, 1927.

Concentration of urea in culture solution (mol.)	Observed		
	Oct. 11.	Oct. 11.	Oct. 15.
0.004	−	−	−
0.02	+	−	+
0.1	+	+	+

TABLE IV.

The culture began Feb. 18, 1828.

Concentration of urea in culture solution (mol.)	pH of culture solution		Observed	
	Initial	Final	Feb. 20	Feb. 28
0.004	4.6	5.8	−	+
0.004	,,	5.8	−	+
0.02	,,	5.9	+	+
0.02	,,	6.9	+	+
0.1	,,	6.8	+	/
0.1	,,	7.2	+	/

TABLE V.

(Urea in cell sap of shoot)

Concentration of urea in culture solution (mol.)	Observed Mar. 8, 1928.	Remarks
0.004	+	Plant growth and root development good
0.004	+	,,
0.02	++	,,
0.02	++	,,
0.1	+++	Plant growth poor
0.1	+++	,,

From the results of these observations, it will be seen that the resorbed urea is transported to leaves without change. The amount of urea excluded is variable in accordance with concentration of urea in the culture solution.

Urease in *Zea Mays*-Seedlings

Studies on the occurrence and activity of urease were made by TAKEUCHI (1907) KIESEL (1911, 1927) FOSSE (1916) and ISHIBASHI (1927).

PIRSCHLE (1929) has studied the relation between the urease action of many higher plants and quantity of urea in the culture solution, and said that there is an intimate relation between them, that is, the root which is cultured in the urea culture solution shows increase of the action of urease. Considering the distribution of urease in plant body and its quantitative relation, it may be naturally said that urease has a close physiological relation with the nitrogen metabolism in plant body, and this may be especially the case in which urea occurs in plant body or is resorbed. IWANOFF (1924) studied the relation between the occurrence of urease and the amount of urea of *Bolbitius* and he said that this lower plant does not keep urea as such in its body for a long time, though it can resorb urea in unchanged form, because *Bolbitius* has urease in its own body. From the results of the above experiments, it can be recognized that a part of the resorbed urea appeared in the drops of guttation unchanged. It is not the principal purpose of the present work to discuss the urease action in the process of protein synthesis in plant body. In the investigation on the resorption of urea it is, however, necessary to determine the presence or absence of urease secretion from the root of *Zea Mays*, because, if it should occur, it is not improbable that urea can be resorbed by root only after its hydrolysis.

EXPERIMENT 4. (Table VI)

Young seedings of *Zea Mays* germinated on the saw-dust were taken, and after sufficient washing with distilled water, each of them was divided into four parts, shoot, root, embryo and scutellum, and endosperm. These four parts were separately ground with 50 cc. of 30% alcohol in a mortar, and this gruel was left alone for twenty minutes, and then filtered. Five cc. of this alcohol extract mixed with 5 cc. of phosphate buffer solution were added to 25 cc. of a 0.01 mol solution of urea. 5 drops of phenol red as indicator and a small piece of thymol as aseptic were used. The pH value of this mixed solution was 7.3 and kept unchanged during the experiment. After keeping this mixture

TABLE VI.

Part of seedling	No. of experiment	Duration of urease action	Amount of Ammonia ± 0.01 cc.				Remarks		
			Total ammonia(1)	Free ammonia in urea solution(2)	Free ammonia in control solution(3)	Free ammonia formed by decomposition of urea	Number of seedling	Average length (cm.)	Fresh weight (gm.)
Shoot	1	16 hours	0.62	0.12	0.60	0.10	100	5	20
	2	,,	0.63	0.12	0.58				
	3	,,	0.58	0.12					
	Average	,,	0.61	0.12	0.59				
Root	1	16 hours	0.61	0.12	0.45	0.05	100	20	195
	2	,,	0.62	0.12	0.45				
	3	,,	0.62	0.12					
	Average	,,	0.62	0.12	0.45				
Embryo and scutellum	1	16 hours	0.53	0.12	0.32	0.11	100	/	13.5
	2	,,	0.54	0.12	0.31				
	3	,,	0.54	0.12					
	Average	,,	0.54	0.12	0.31				
Endosperm	1	16 hours	0.43	0.12	0.31	0.01	100	/	35
	2	,,	0.43	0.12	0.31				
	3	,,	0.43	0.12					
	Average	,,	0.43	0.12	0.31				

(1) Total ammonia in sample.
(2) Amount of free ammonia in 25 cc. of a 100 urea solution.
(3) Amount of free ammonia in the control solution without urea.

for 16 hours in the thermostat of 30°C., total free ammonia was estimated by the micro-KJELDAHL method.

As control the mixed solution was used with as much distilled water as a 0.01 mol urea solution added. Table VI shows the results of Experiment 4.

From these results it will be seen that the extract from the part of embryo and scutellum and from root is active for the decomposition of urea only in such a degree as to be practically almost negligible. The extract from other parts shows very weakly such an action, and the quantity of free ammonia is within the limits of experimental errors.

PIRSCHLE (1929) reported that the root of *Zea Mays* decomposes urea in the culture solution containing urea. It is very probable that in such a long lasting culture as PIRSCHLE's the secretion of urease from root becomes more remarkable than in the writer's experiment. So far as the writer's experiments are concerned, urea found in plant body, should be, therefore, certainly resorbed as urea itself, but not after attacked by urease.

SUMMARY

1. By sterile culutre it was found that urea is resorbed in unchanged form by seedlings of *Zea Mays* and can be available for plant growth as a nitrogenous source.

2. A part of urea which is resorbed by seedlings of *Zea Mays* remains for a while in the plant body in this form.

3. The more urea is contained in the culture solution, the more it is resorbed by seedlings of *Zea Mays*.

4. Occurrence of a very small amount of urease is ascertained in the part of embryo and scutellum in the seedlings of *Zea Mays*, but in other parts such occurrence can not be detected.

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The present work is a part of investigations carried out under the title: "Absorption of electrolytes and non-electrolytes by plant cells," with the aid granted me by the Imperial Academy for the promotion of scientific researches, for which I wish to express my acknowledgement. Tetsu SAKAMURA.

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