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THE INCORPORATION OF $H_3P^{32}O_4$ AND P^{32}LABELED RNA INTO VARIOUS CELLULAR FRACTIONS OF RICE SEEDLINGS

By

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Introduction

Gale and Folkes (1) found that the ability to incorporate C^{14}-amino acids into protein can be restored by the addition of Staphylococcal DNA or RNA. The restoration of activity is apparently specific for Staphylococcus nucleic acids.

Webster et al. (2) have obtained the results that the incorporation of amino acids into the proteins of disrupted pea seedlings is promoted by added RNA which is not specific for the kinds of nucleic acid.

The above results raise many questions concerning the mode of action of the nucleic acids in bacteria and plants.

There are many reports that protein synthesis is closely associated with nucleic acid metabolism in plants. Since Hevesey's research (3), many reports have been obtained about the absorption, distribution and exchange of inorganic phosphorus with P^{32} in plants. It is well known that inorganic phosphate is utilized by plants and metabolized into organic phosphates, but there are some problems about the absorption and metabolism of organic phosphorus compounds in plants.

How are added organic phosphates utilzied by plants? From the results mentioned above, if added RNA is absorbed in intact plants and utilized for protein synthesis, it may be of interest to investigate the action of organic phosphates as a matter of organic nutrition of plants. Webster (2) reported that promotion of amino acid incorporation is greater if the ribonucleic acid molecule has been hydrolyzed to its constituent nucleotides. These results suggest that the apparent need for added RNA in pea seedlings is actually a need for ribonucleotides or related compounds.

It is supposed that organic phosphorus as described has an action on plants different from the inorganic phosphorus if it is not decomposed in plants or in nutrient solution.

Rice seedlings have the action of RNase and phosphatase. Therefore, how are organic compounds utilized in rice seedlings? Added RNA may be decomposed by these enzymes and the organic phosphorus of RNA may become inorganic phosphours.

It is thought that there is a difference in the action by plants between organic phosphorus contained in RNA and inorganic phosphorus.

This paper reports on the incorporation of P^{32} labeled RNA and $H_3P^{32}O_4$ into various cellular fractions of intact rice seedlings.

Material and Methods

Plant Material and Culture: Rice seedlings (Norin, No. 16) were used. Seeds were sown on vinyl net with wooden frame in water for three weeks and 120 seedlings on one liter polyethylene beaker were grown in water culture medium in a green house. This experiment was carried out from August 1 to 11 in 1961.

Preparation of P^{32} labeled RNA: P^{32} labeled RNA was prepared from yeast by the method of Dicarlo et al. (4) The product contained 13,9% N and 7,2% P. ε(P) was 9530. ε(P): atomic extinction coefficient with respect to phosphorus, viz. $\varepsilon(P)=30.98E/cl$, where E=extinction (log I_0/I), c=concentration of phosphorus in the solution in g. per liter, and l=thickness of the absorbing layer in cm. (5). Its purity was ca. 95% according to ultraviolet absorption measurements. It was free from protein and deoxyribose.

Preparation of P^{32} labeled RNA solution: Two hundred milli-gram of the above P^{32}-RNA was dissolved in water and 20 ml of 0.1N sodium hydroxide. This solution was adjusted to pH 6-6.5 and filled up to 200 ml. One milli-liter of this solution contained the radioactivity of 4.4×10^2 cpm.

Design of Experiments: Thirty milli-liter of P^{32}-RNA solution that had a radioactivity of 1.32×10^4cpm. was injected into each nutrient medium in one group and $H_3P^{32}O_4$ solution containing the same amount of radioactivity of P^{32}-RNA solution was injected into each nutrient in another group. One hundred and twenty seedlings in each nutrient medium were respectively collected at 48, 120, 144 and 240 hours to exmaine the action of each phosphorus compound on the rice seedlings.

Table 1. Composition of the nutrient medium

Component	Concentration (ppm)	Form
N	40	$(NH_4)_2SO$
K_2O	40	KCl
MgO	6	$MgSO_4\cdot7H_2O$
Mn	2	$MnCl_2\cdot4H_2O$
Ca	2	$MnCl_2\cdot2H_2O$
Fe	5	EDTA-Fe
P	2	$NaH_2PO_4\cdot2H_2O$ + RNA

Table 2. The green weights of 120 rice seedlings by treatments (g)

Treatment \ Time (hr)	0	48	120	144	240
$H_3P^{32}O_4$	5.0	5.4	6.1	7.0	8.6
P^{32}-RNA	5.0	5.3	6.2	6.9	8.7

The composition of nutrient medium is shown in Table 1. Green weights of 120 seedlings at each period during growth are shown in Table 2.

Fractionation Procedure for Homogenate of Rice Seedlings: Leaves of 120 rice seedlings at each period in two treatments were ground in a mortar with 20ml of 0.4M sucrose and 0.05M phosphate buffer. The material was then filtered through a double layers of cotton cloth and the filtrate was made up to 25 ml. Twenty milliliters of this filtrate were introduced into the centrifugal tube and centrifuged as shown in Table 3 according to the procedure of Martin (6). The final superantant was made up to 20 ml.

For ultracentrifugation a Hitachi model 40 P centrifuge was used and all operations were carried out in a low temperature (0-5°C)

Table 3. Fractionation procedure for homogenates of rice leaves

Fraction	Relative centrifugal force (×g)	Time of centrifugation (min)
A Nuclei, cell debris	1,500	15
B Chloroplast Chloroplast fragement Mitochondria	10,000	15
C Microsomes	50,000	90
D Cytoplasmic solution		

In Table 3, A is thought to be unbroken cell and cell debirs, B cholorplast fragment and mitochondria, C microsome, and D cytoplasmic solution. Each fraction except the cytoplasmic solution were dried in a vacuum of 1 mm Hg pressure. The changes in dry weights of each fraction at each period are shown in Fig. 1.

Determination of Nitrogen: Nitrogen was determined by the semi-micro Kjeldahl method.

Estimation of Radioactivity: Distribution of radioactivity among each fraction was determined by G.M. counter.

Estimation of P^{32} labeled nucleotides in Cytoplasmic Solution: Ten milliliters of cytoplasmic solution was added with an equal volume of 10% perchloric

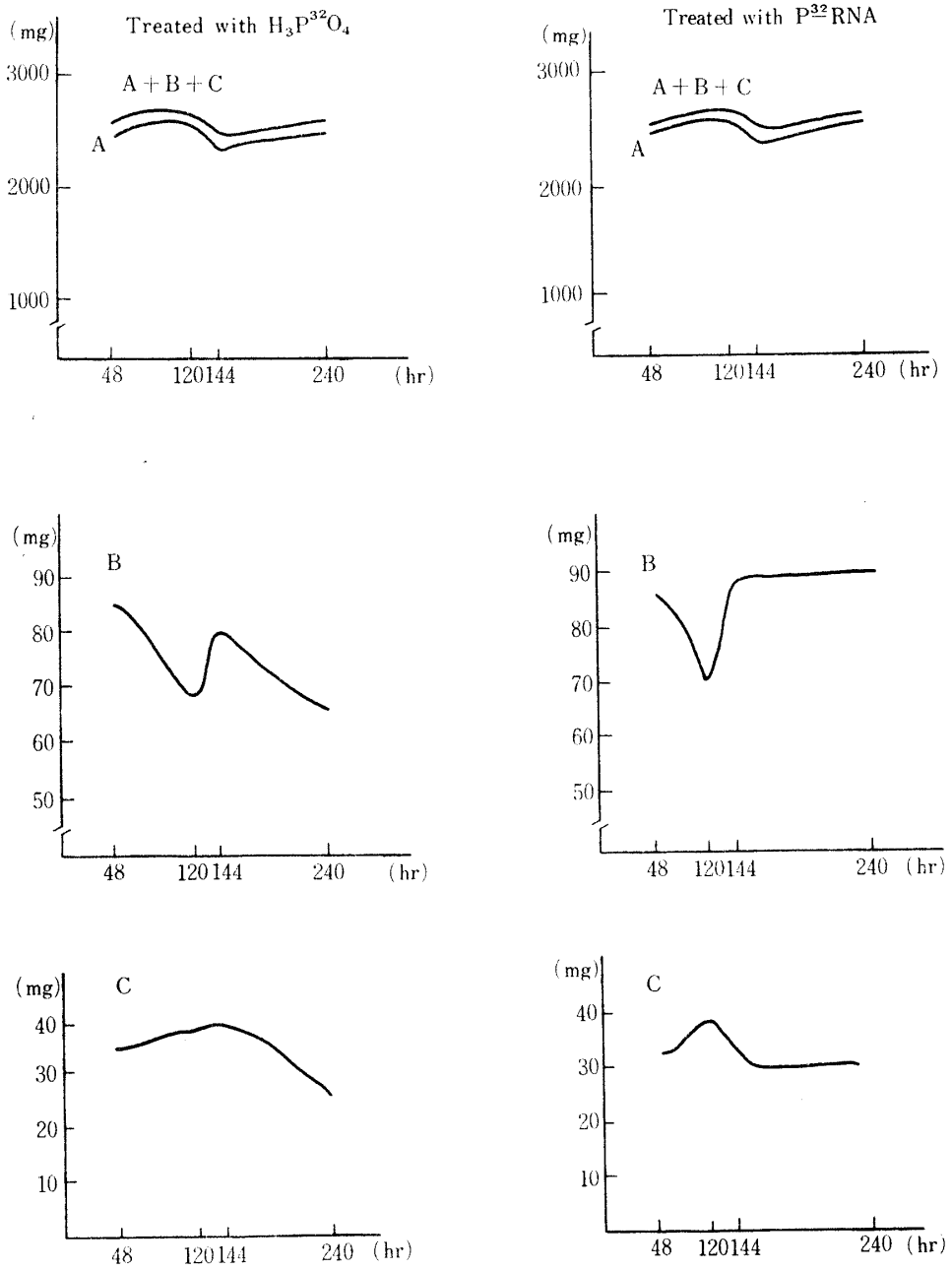

Fig 1. The change of dry weight of each fraction at each period

acid and cytoplasmic solution was then separated into acid soluble and acid insoluble fractions. The acid soluble fraction was further adsorbed in column (2×7cm) which was filled up by the mixture of 1g of refined active charcoal and 500mg of Celite on glass wool packed with 500mg of Celite. And, the adsorbent was eluted with 10 ml of 50% ethanol containing 1% ammonium hydroxide. Contaminated inorganic P^{32} was then precipitated by Fiske's reagent (7) from the eluate and the superantant was determined as P^{32}-nucleotides.

Results and Discussion

Changes of the green weights are shown in Table 2. This indicates that green weights increased with time and showed no difference under the two treatments.

Changes of the dry weight of each fraction are illustrated in Fig. 1. The same trend was shown in the amounts of A+B+C and A in both treatments, but the difference between the two treatments was found in the amount of B fraction after 144 hours. In the dry weight in B fraction by the two treatments after 240 hours, a greater increase (about 30%) by treatment of P^{32}–RNA than that of $H_3P^{32}O_4$ was seen. A difference in the dry weight of the C fraction of two treatments after 240 hours was also seen. These results indicate that a difference of dry weight in various cellular fractions between H_3PO_4 and RNA treatment can be found clearly in B and C fractions with a lapse of time.

Table 4. P^{32} distribution of each fraction (c/10min)

Time (hr)	48		120		144		240	
Treatment / Fraction	$H_3P^{32}O_4$	P^{32}-RNA	$H_3P^{32}O_4$	P^{32}-RNA	$H_3P^{32}O_4$	P^{32}-RNA	$H_3P^{32}O_4$	P^{32}-RNA
A	474 (48.5)	545 (56.6)	1495 (29.8)	2130 (24.0)	2215 (29.5)	3448 (17.9)	4490 (43.5)	4580 (27.5)
B	62 (6.4)	56 (4.8)	179 (3.6)	260 (2.9)	283 (3.7)	514 (2.7)	443 (4.3)	693 (4.2)
C	41 (4.2)	29 (2.4)	145 (2.8)	104 (1.1)	204 (2.8)	478 (2.5)	107 (1.0)	157 (1.0)
D	400 (40.9)	540 (46.2)	3200 (63.8)	6400 (72.0)	4814 (64.0)	14800 (76.9)	5280 (51.2)	11200 (67.3)
Total	977 (100)	1170 (100)	5019 (100)	8894 (100)	7520 (100)	119240 (100)	10320 (100)	16630 (100)

Figures in the brackets represent percentage of the total.

The distribution of P^{32} absorbed in each fraction by two treatments is shown in Table 4. Total activities of absorbed P^{32} were enhanced with time by two treatments. After 48 hours, total activities of absorbed P^{32} were almost the same as with two treatments. But, after that, they differed greatly. Total activities of P^{32} absrobed by treatment of P^{32}-RNA were larger than by treatment of $H_3P^{32}O_4$ after 120, 144 and 240 hours. From Table 4 we see that the activities of P^{32} of each fraction after two treatments were largest in the D fraction. When the order in the amount of P^{32} is shown by a sign of inequality, it is D>A>B>C with two treatments. The most remarkable difference in the amount of P^{32} by two treatments was seen in the D fraction.

The mechanism of the alkaline digestion of RNA has been explained by Todd

and his collaborators (8) and they showed that alkaline hydrolysis yields nucleoside 2'- and 3'-phosphates, respectively (9).

As the RNA used in this experiment was dissolved in a weak alkali, it is thought that RNA has been degraded to the nucleoside 2'- and 3'-phosphates and nucleosides by alkaline hydrolysis. Therefore, it was supposed that P^{32}-RNA was absorbed in the form of nucleosides phosphates and nucleosides.

As phosphatase was found in roots of rice seedlings (10), P^{32}-RNA might be partly absorbed as inorganic phosphorus besides nucleotides.

Low molecular nucleotides were known to cross the cell membrane of roots, so it was presumed that P^{32}-RNA with alkaline digestion was absorbed as low molecular nucleotides and was related to phosphorus metabolism in its own way different from inorganic phosphorus.

Table 5 shows the distribution of nitrogen contents of each fraction after two treatments. The trend of the distribution of nitrogen contents of each fraction was similar to that of the activity of P^{32}.

Table 5. Nitrogen distribution of each fraction (mg)

Time (hr)	48		120		144		240	
Treatment / Fraction	$H_3P^{32}O_4$	P^{32}-RNA	$H_3P^{32}O_4$	P^{32}-RNA	$H_3P^{32}O_4$	P^{32}-RNA	$H_3P^{32}O_4$	P^{32}-RNA
A	12.5 (42.0)	12.7 (43.5)	19.5 (48.2)	21.7 (53.5)	17.5 (40.9)	18.6 (39.6)	18.7 (49.5)	20.6 (47.9)
B	3.2 (10.8)	3.2 (11.0)	3.3 (8.2)	3.3 (8.2)	3.8 (8.9)	4.4 (9.4)	3.0 (7.9)	3.6 (8.1)
C	1.1 (3.8)	1.1 (3.7)	1.5 (3.8)	1.4 (3.5)	1.5 (3.4)	1.6 (3.2)	0.7 (1.7)	0.8 (0.4)
D	12.9 (43.4)	12.2 (41.8)	16.2 (39.8)	14.1 (34.8)	20.0 (46.8)	22.4 (47.8)	15.4 (40.8)	19.3 (43.6)
Total	29.7 (100)	29.2 (100)	40.5 (100)	40.5 (100)	42.8 (100)	47.0 (100)	37.8 (100)	44.3 (100)

Figures in the brackets represent percentage of the total.

Table 6. Percent of acid soluble P^{32} and acid insoluble P^{32} to total P^{32} in cytoplasmic solution

Time (hr)	48		120	
Treatment / Fraction	$H_3P^{32}O_4$	P^{32}-RNA	$H_3P^{32}O_4$	P^{32}-RNA
Acid soluble	95.7	95.4	94.2	95.3
Acid insoluble	4.3	4.6	5.8	4.7

The parallelism in the changes of total P^{32} and total nitrogen contents with time was seen by the P^{32}-RNA treatment but not by the $H_3P^{32}O_4$ treatment.

The cytoplasmic solution to which the bulk of P^{32} was incorporated after two treatments was further investigated concerning the quality of the phosphorus fraction. The percent of acid soluble P^{32} and acid insoluble P^{32} to total P^{32} in cytoplasmic solution is shown in Table 6.

No difference in percent of acid soluble P^{32} and acid insoluble P^{32} to total P^{32} in cytoplasmic solution was seen by two treatments after 48 and 120 hours.

As it was presumed that P^{32}-RNA might be absorbed and utilized as P^{32}-nucleotides in rice seedlings, the amount of P^{32}-nucleotides in the cytoplasmic solution after two treatments was investigated after 48 and 120 hours.

Table 7 shows the percent of P^{32}-nucleotides to acid soluble P^{32} and percent of protein-N to total N in cytoplasmic solution. The percent of P^{32}-nucleotides was greater with a treatment of P^{32}-RNA than with $H_3P^{32}O_4$.

Table 7. Percent of P^{32}-nucleotides to acid soluble P^{32} and percent of protein-N to total N in cytoplasmic solution

Time (hr)	48		120	
Treatment	$H_3P^{32}O_4$	P^{32}-RNA	$H_3P^{32}O_4$	P^{32}-RNA
Nucleotides	6.2	12.3	9.5	21.0
Protein-N	12.0	12.0	15.8	21.7

This result indicates that there was a large amount of nucleotides in the cytoplasmic solution under the P^{32}-RNA treatment. Protein-N in cytoplasmic solution was also greater after treatment of P^{32}-RNA.

Webster (2) showed that nucleotides are necessary to protein synthesis in pea root homogenate and a mixture of nucleotides of purines and pyrimidines increases glutamate incorporation into protein.

He said that the promotion of amino acid incorporation by nucleotides may be due to their acting as efficient precursors of RNA.

The increase of nucleotides and protein-N in cytoplasmic solution of rice seedlings with a treatment of P^{32}-RNA indicates that nucleotides from P^{32}-RNA might take part in the synthesis of RNA and protein.

These results suggest, then, that the RNA applied in rice seedlings was utilized as nucleotides besides inorganic phosphorus and that it participated in protein synthesis as a highly efficient precursors of RNA different from inorganic phosphorus.

Summary

(1) The incorporation of P^{32}-RNA and $H_3P^{32}O_4$ into various cellular fractions of intact rice seedlings were investigated for 240 hours. The green weight of rice seedlings during the stage of growth was the same after two treatments, but the dry weight in cellular fractions differed in the mitochondria, chloroplast and microsomes fraction.

(2) The distirbution of P^{32} into cellular fractions by two treatment was greater in the cytoplasmic solution, and the amount of P^{32} in the cytoplasmic solution was from 40% to 70% of the total P^{32}. P^{32} absorbed by treatment with P^{32}-RNA was greater than that by treatment with $H_3P^{32}O_4$ after 120, 144 and 240 hours.

(3) Parallelism was found in the activity of total P^{32} and total nitrogen of rice seedlings cultured in P^{32}-RNA solution but was not found in rice seedlings cultured in the solution of $H_3P^{32}O_4$.

(4) A cytoplasmic solution of rice seedlings applied with P^{32}-RNA contained more P^{32}-nucleotides than that applied with $H_3P^{32}O_4$. It is supposed that these P^{32}-nucleotides were utilized different from inorganic P and took part in protein synthesis during this stage of rice seedlings.

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