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Biosynthesis of purine derivatives in young wheat plants

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The pathway for the biosynthesis of purines has been established in animal tissues and microorganisms (see reviews given by Böttger 1958; Buchanan 1960; Moat and Friedman 1960; Jeżewska 1961 and papers by Cheeseman and Crosbie 1966; Malathi and Ramakrishan 1966; and others).

The pathway for the biosynthesis of the purine ring in higher plants has been not systematically investigated. Recently Robern, Wang and Waygood (1965), Wang and Burris (1965) demonstrated the incorporation of [2—^{14}C] glycine into the purine derivatives in wheat seedlings.

In the present study the incorporation of [U—^{14}C] glycine, [1—^{14}C] glycine, [^{14}C] sodium formate, [^{14}C] sodium bicarbonate, [^{14}C] carbamoyl--phosphate and [U—^{14}C] sodium acetate into purine derivatives of the acid-soluble and acid-insoluble fraction in wheat shoots was investigated. The influence of excess of adenine on biosynthesis of purines „de novo" in higher plants, as already observed in microorganisms (see review by Moat and Friedman 1960; Gots and Goldstein 1959) and animal tissues (see papers by Mc Fall and Magasanick 1960; Henderson 1962; Henderson and Khoo 1965; Brockman and Chumley 1965 and others) was also studied.

MATERIAL AND METHODS

Reagents: [1—^{14}C] glycine, [U—^{14}C] glycine, [^{14}C] sodium formate, [U—^{14}C] sodium acetate, [8—^{14}C] adenine and K^{14}CNO were purchased from the Radiochemical Centre, Amersham, England. $Na_2{}^{14}CO_3$ was obtained from Instytut Badań Jądrowych, Świerk, Poland. Radioactive carbamoyl-phosphate, dilithium salt, was prepared from K^{14}CNO by the method of Jones, Spector and Lipmann (1955). All non-labelled purine and pyrimidine derivatives were purchased from L. Light Co., Colnbrock, England and Calbiochem, Los Angeles U.S.A., Other reagents were from Fabryka Odczynników Chemicznych, Gliwice, Poland.

Plants: Green, five-day-old wheat plants, variety „Dańkowska 40" were used throughout.

Feeding: Wheat shoots (10g samples) immedietly after cutting, were fed with 1—2 ml of water or buffered solutions of the suitable radioactive substrates. After the feeding period (15 min. — 2 hrs at 20°C) the wheat shoots were washed, surface dried and frozen in dry ice. The uptake of the radioactive substrate by wheat shoots was determined from the difference between total radioactivity of the solution before and after feeding.

Extraction, separation and determination of purine and pyrimidine derivatives

The acid soluble-purine and pyrimidine derivatives. 10g plant samples were ground, extracted with cold $HClO_4$ (10 ml 0.6N + 50 ml 0.3N) and then 35 ml of 0.3N $HClO_4$ were added 4 times. To the combined supernatants 500 mg of activated charcoal were added and the purine and pyrimidine derivatives absorbed during 1 hr at 5°C. The charcoal was filtred off and then washed with cold water until neutral reaction of the filtrate was obtained. Purine and pyrimidine derivatives were eluted from charcoal 4 times with 70 ml of a warm mixture of aceton and 0.1N NH_4OH (4 : 1 vol. vol.) as described by Buchowicz and Reifer (1962). The eluates were freed of ammonia and acetone by heating on a boiling water bath.

The obtained mixture was used for the separation of some individual adenine derivatives either directly or after hydrolysis. The hydrolysis of nucleosides and nucleotides to the corresponding purine and pyrimidine bases was carried out with 9N $HClO_4$ at 100°C for 1 hr. $HClO_4$ was neutralised with KOH and the resulting $KClO_4$ was discarded.

The mixture of the purine and pyrimidine derivatives was put on a Dowex 1×8 (Cl^-) column (1.2×16 cm). The column was washed with 200 ml of water and the effluent, containing free bases and nucleosides, was evaporated by heating on a boiling water bath. Adenine nucleotides were eluted with 300 ml of 0.02N HCl and the effluent was evaporated under reduced pressure at room temperature. The dry residues were dissolved in small volumes of water and chromatographed on Whatman No. 1 filter paper. The compounds from the water effluent were separated by descending paper-chromatography in solvent system No. 1; n-butanol satd. with water at about 20°C with 5% by vol. of conc. NH_3 soln., added to solvent in bottom of tank. Separated compounds were rechromatographed as above in solvent system No. 2; iso-propanol 170 ml, conc. HCl 41 ml, water to make 250 ml, as described by Wyatt (1955). The compounds from the 0.02N HCl effluent were chromatographed in solvent system No. 2. Spots corresponding to standard samples were eluted with

0.1N HCl (only guanine 1N HCl) for 24 hrs at 37°C. The acid solutions were checked for purity in U.V. — Light (Spectrofotometr SF-4 U.S.R.R.). The quantities of purines and pyrimidines were calculated from the molar extinction coefficients as given by Beaven, Holiday and Johnson (1955). The radioactivity was measured with the window GM counter AAH55, with counting time long enough to reduce the error to less than 5%. No corrections were made for self-absorption, which was negligible under the conditions described.

Chromatographically separated adenosine and the mixture of AMP (adenylic acid) and ADP (adenosine diphosphate) were hydrolysed in 1 N HCl at 100°C for 1 hr and the resulting adenine was separated in solvent system No. 1. The hydrolysis was carried out to establish whether all the radioactivity detected was due to the purine ring only.

Chromatographic procedures were repeated until specific activities of the isolated products were constant and the absorption spectra identical with those of the commercial standards.

The acid-insoluble fraction was hydrolysed with 1 N HCl at 100°C for 1 hr. The resulting purine bases were isolated and determined as described for the acid-soluble fraction.

RESULTS

To compare the incorporation of [U—^{14}C] glycine, [^{14}C] sodium formate, [^{14}C] sodium bicarbonate, [U—^{14}C] sodium acetate and [^{14}C] carbamoyl-phosphate wheat shoots were fed with these substrates under similar experimental conditions (Table 1). After 2 hr feeding the substrates were incorporated into the purine ring. Adenine of acid-soluble fraction had a considerably higher specific activity than guanine. The purine bases isolated from acid-insoluble fraction were barely labelled, but also in that fraction adenine was more radioactive than guanine. From among the used substrates [U—^{14}C] glycine and [^{14}C] sodium formate were efficiently incorporated and [U—^{14}C]sodium acetate only barely so. The quantities of purine bases found in hydrolysed acid-soluble fractions amounted to 1—1.5 μmole of adenine, 0.2—0.5 μmole of guanine per 10 g sample of wheat shoots.

Acid-soluble pyrimidine derivatives, as it was expected, were labelled with ^{14}C from sodium bicarbonate and carbamoyl-phosphate. On the other hand [U—^{14}C] glycine and [^{14}C] sodium formate were incorporated into these products only to a slight extent. Uracil and cytosine isolated from polynucleotide fraction were inactive.

Incorporation of [U—^{14}C] glycine into the various adenine derivatives after 2 hr feeding was almost identical and generally the same was true for [^{14}C] sodium formate incorporation (Table 2). [8—^{14}C] Adenine fed to

Table 1

Specific activities of purine and pyrimidine bases isolated from wheat seedlings fed with [^{14}C] substrates

Wheat shoots (10 g samples) were fed with the indicated substrates dissolved in 2 ml of 50 mM potassium phosphate pH 7.4. for 2 hrs at 20°C. Results are given in imp. /sec/μmole as mean from indicated numbers of experiments.

Radioactive substrates in μmoles	Specific activities of substrates imp/sec/ /μmole	Uptake* imp/sec	Number of experiments	Acid-soluble fraction **				Acid-insoluble fraction	
				Adenine imp/ /sec/ /μmole	Guanine imp/ /sec/ /μmole	Uracil imp/ /sec/ /mole	Cytosine imp/ /sec/ /μmole	Adenine imp/ /sec/ /μmole	Guanine imp/ /sec/ /μmole
3.8 [U-^{14}C] sodium acetate	17 500	37 800	4	0.9	<0.1	<0.1	—	—	—
2.3 [U-^{14}C] glycine	20 000	20 200	4	58.6	5.4	0.3	—	—	—
2.1 [^{14}C] sodium formate	20 000	18 800	8	67.0	4.4	0.4	0.5	1.6	0.4
1.9 [^{14}C] sodium formate (1 hr feeding)	20 000	9 000	2	31.8	3.0	—	—	0.5	0.1
2.0 [^{14}C] sodium bicarbonate	16 000	—	2	2.4	0.1	0.8	—	—	—
54.0 [^{14}C] sodium bicarbonate	6 000	—	4	21.7	2.5	5.2	0.7	0.4	<0.1
38.0 [^{14}C] carbamoyl-phosphate	1 000	12 500	4	3.2	0.8	2.4	0.6	<0.1	<0.1

* Difference between total radiactivity of the solution before and after feeding.

** Compounds of the acid-soluble fraction were hydrolysed to the bases before isolation (see methods).

Table 2

Specific activities of some purine derivatives isolated from wheat seedlings fed with [^{14}C] substrates

Wheat shoots (10 g samples) were fed with indicated substrates dissolved in 2 ml of water for 2 hr at 20°C. Results are given in imp./sec./μmole as mean of two separate experiments

Radioactive substrates in μmoles	Specific activities of substrates imp/sec/ /μmole	Acid-soluble fraction *			Acid-insoluble fraction	
		Adenine imp/sec/ /μmole	Adenosine imp/sec/ /μmole	AMP+ADP imp/sec/ /μmole	Adenine imp/ /sec/ /μmole	Guanine imp/ /sec/ /μmole
3.0 [U-^{14}C] glycine	10 000	50.0	45.0	47.0	0.4	<0.1
5.5 [^{14}C] sodium formate	20 000	178.0	80.0	174.0	4.5	0.8
0.36 [8-^{14}C] adenine	44 000	1637.0	1566.0	877.0	30.0	6.1

* Specific activity was determined after hydrolysis to adenine.

Table 3

Specific activities of adenine derivatives isolated from wheat seedlings fed with [1—^{14}C] glycine for various length of time

Wheat shoots (10 g samples) were fed with 0.74 μmole [1—^{14}C] glycine of specific activity 32.000 imp./sec./ μmole dissolved in 1 ml of water for various periods at 20°C. Results are given in imp./sec./μmole as mean of two separate experiments

Feeding period in minutes	Uptake* of [1-^{14}C] glycine imp/sec.	Adenine imp/sec /μmole	Adenosine ** imp/sec /μmole	AMP+ ADP** imp/sec /μmole	Adenine of acid-insoluble fraction imp/sec/μmole
15	9 000	5.7	4.4	4.8	<0.1
30	13 700	10.0	11.4	7.0	0.1
60	15 200	23.0	21.6	—	0.2
120	17 600	28.0	16.6	—	0.4

* Difference between total radioactivity of the solution before and after feeding.
** Specific activity was determined after hydrolysis to adenine.

Table 4

The effect of adenine on the incorporation of [1—^{14}C] glycine into purine derivatives

Wheat shoots (10 g samples) were fed with (1-^{14}C] glycine of specific activity 32.400 imp./sec./μmole for 2 hr at 20°C. Where adenine was applied it was used in quantity of 5 μmoles. The compounds were dissolved in 1 ml of water. Results are given in imp./sec./μmole as mean of two separate experiments

Substrates in μmoles	Uptake* of [1-^{14}C] glycine imp/sec	Adenine		Adeno-sine** imp/sec /μmole	AMP+ +ADP** imp/sec /μmole	Adenine of acid-insoluble fraction imp/sec/μmole
		μmole	imp/sec /μmole			
0.28 [1-^{14}C] glycine	7 500	0.4	9.3	11.1	7.1	0.2
0.28 [1-^{14}C] glycine+ 5.0 adenine	7 700	2.0	0.2	0.8	0.6	<0.1
0.74 [1-^{14}C] glycine	19 100	0.4	59.5	80.0	55.0	1.4
0.74 [1-^{14}C] glycine+ 5.0 adenine	20 200	1.8	0.5	2.6	3.6	0.1

* Difference between total radioactivity of the solution before and after feeding.
** Specific activity was determined after hydrolysis to adenine.

wheat shoots was converted into adenosine, AMP, ADP as well as incorporated into the acid-insoluble fraction.

Since there were no differences in the specific activities of the investigated adenine derivatives after 2 hr feeding, the next experiment was carried out with shortened feeding periods (Table 3). It was observed that [1—^{14}C] glycine fed to wheat plants for 15 min. was incorporated into all investigated acid-soluble adenine derivatives with similar

efficiency. With prolongation of feeding time their specific activities were correspondingly higher and the appearance of weak radioactivity in adenine of polynucleotide fraction was observed.

Then the influence of adenine on the incorporation of [1—^{14}C] glycine into the purine derivatives was investigated (Table 4). The activity of purine derivatives in plant samples fed with [1—^{14}C] glycine only and with [1—^{14}C] glycine plus adenine differed considerably. Specific activities of isolated radio-adenine were 50—100 times lower at total quantity of adenine only 5 times higher. Specific activities of adenosine, AMP, ADP and also adenine of the polynucleotide fraction were about 10 times lower in the samples with added adenine.

DISCUSSION

The above investigation proves that [^{14}C] sodium bicarbonate, [^{14}C] sodium formate and [U—^{14}C] glycine are specific precursors of purine derivatives also in higher plants. These substrates contrary to [U—^{14}C] sodium acetate were intensively incorporated into acid-soluble purine derivatives and adenine of polynucleotide fraction in young wheat shoots. The hydrolysis of adenosine, AMP, ADP proved that glycine and formate were incorporated into the purine ring only. It was confirmed that [^{14}C] carbamoyl-phosphate, one of the pyrimidine precursors, was incorporated into the purine ring as previously reported by Rybicka, Buchowicz and Reifer (1967). As was to be expected [U—^{14}C] glycine and [^{14}C] sodium formate were not incorporated into pyrimidine derivatives to any significant extent.

The individual adenine derivatives isolated from the acid-soluble fraction had a similar specific activity even after short feeding with [1—^{14}C] glycine (15 min.). The above observation is not consistent with the generally accepted view that adenine and adenosine may arise only through degradation of AMP. If this were so AMP should show, after a short period of [1—^{14}C] glycine feeding, higher specific activity than adenine and adenosine.

It is known that adenine is incorporated into nucleotides of various organisms and the same is true for wheat plants fed with [8—^{14}C] adenine. However adenine applied in greater quantity (5mM solution) strongly inhibits „de novo" biosynthesis of adenine derivatives. Similar observations were made on other material (Gots and Goldstein 1959; Mc Fall and Magasanick 1960; Henderson 1962; Henderson and Khoo 1965; Brockman and Chumley 1965) see also review given by Moat and Friedman (1960).

The obtained results would indicate that in higher plants the general

mechanisms of purine biosynthesis and its inhibition are similar to those in other groups of organisms.

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SUMMARY

Incorporation of [U—^{14}C] glycine, [^{14}C] sodium formate, [^{14}C] sodium bicarbonate, [^{14}C] carbamoyl-phosphate, [U—^{14}C] sodium acetate and [8—^{14}C] adenine into purine derivatives in 5-day-old wheat shoots was investigated. These substrates (except for acetate) were intensively incorporated into purine derivatives of the acid-soluble fraction and to a much smaller extent into purines of the acid-insoluble fraction. Guanine had a considerably lower specific activity than adenine.

Adenine, adenosine, AMP with ADP had similar specific activities even after short time of feeding with [1—^{14}C] glycine.

The conversion of [1—^{14}C] glycine into purine derivatives was almost completly inhibited by excess of adenine.

Results would indicate that the purine biosynthesis and its inhibition in higher plants are in general similar to those in other organisms.

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Biosynteza pochodnych purynowych w siewkach pszenicy

Streszczenie

Badano wbudowanie [^{14}C] glicyny, [^{14}C] mrówczanu sodu, [^{14}C] dwuwęglanu sodu, [^{14}C] karbamoilo-fosforanu, [^{14}C] octanu sodu i [8—^{14}C] adeniny w pochodne purynowe w odciętych siewkach pszenicy. Substraty te (poza octanem) były intensywnie wbudowywane w pochodne purynowe frakcji kwaso-rozpuszczalnej oraz znacznie słabiej do zasad purynowych frakcji kwaso-nierozpuszczalnej. Guanina posiadała znacznie niższe aktywności właściwe niż adenina.

Adenina, adenozyna, AMP i ADP miały zbliżone aktywności właściwe, nawet po krótkim okresie dokarmiania [1—^{14}C] glicyną.

Przemiana [1—^{14}C] glicyny do pochodnych purynowych była niemal całkowicie hamowana przez nadmiar adeniny.

Badania wskazują, że biosynteza puryn i jej hamowanie w roślinach wyższych jest podobna jak i w innych organizmach.