STUDIES ON THE NUCLEOSIDE PHOSPHOTRANSFERASE OF CARROT

III. ON THE SYNTHETIC ABILITY OF THE TRANSFER ENZYME

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(Received September 4th, 1959)

SUMMARY

The purified nucleoside phosphotransferase of carrot, which has been deprived of most of the contaminating phosphomonoesterase, is a versatile enzyme permitting the conversion to the 5′ nucleotides of unusual nucleosides, such as the ribosides of purine, 2,6-diaminopurine, and 5-bromouracil, and the glucoside or arabinoside of adenine. It is, moreover, able to use cyclic or 2′ nucleotides as phosphate acceptors, as shown by the production of adenosine 2′,5′-diphosphate and the cyclic adenosine (2′,3′), 5′-diphosphate. The latter is hydrolyzed by alkali to the 2′,5′- and 3′,5′-diphosphates of adenosine.

INTRODUCTION

The purification of the nucleoside phosphotransferase of plant tissues—an enzyme catalyzing the formation of 5′ nucleotides through the transfer of organically bound phosphoric acid to the 5′ position of deoxyribo- or ribonucleosides[1]—has recently been described in detail[2,3]. This has made possible a brief investigation of the synthesizing potential of this enzyme. We study here the ability of several unusual nucleosides, and even of nucleotides, to act as phosphate acceptors. Some of the findings have been already mentioned in a preliminary note[4].

EXPERIMENTAL

Materials

A nucleoside phosphotransferase preparation of carrot, purified (step VI in a previous publication[2]) and deprived of phosphomonoesterase by treatment with Celite[3], was used in all experiments.

The following nucleosides were kindly given us by Dr. G. B. BROWN of the Sloan-Kettering Institute, New York: 9-β-D-ribofuranosylpurine (nebularine)[5,6]; 9-α-L-

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arabofuranosyladenine[7]; 9-β-D-glucopyranosyladenine[8,9], and 2,6-diamino-9-β-D-ribofuranosylpurine[10]. All other nucleosides and nucleotides were commercial products.

Procedures

The previously described standard method for the assay of enzymic activity was employed, with sodium monophenylphosphate (100 μmoles/ml) as the donor and a nucleoside or nucleotide (20 μmoles/ml) as the acceptor in 0.1 *M* acetate buffer of pH 5.2 (protected with 0.001 % of ethyl mercurithiosalicylate)[2]. In addition to the procedures previously applied for the isolation and estimation of the products[2,3] a mixture of 79 vol. saturated ammonium sulfate, 19 vol. water, and 2 vol. isopropanol[11] was used for the chromatographic separation of the 2′- and 3′-adenylic acids and of the 2′,5′- and 3′,5′-diphosphates of adenosine.

RESULTS AND DISCUSSION

Some unusual nucleosides as acceptors

The versatility of the purified nucleoside phosphotransferase of carrot is exemplified in Table I which gives a qualitative survey of the production of the 5′-phosphates of several unusual nucleosides. Before being subjected to the action of the enzyme, the acceptors were all shown to be homogeneous chromatographically, and the appearance of a second adsorption zone served as indication of phosphate transfer. Purine riboside and adenine arabinoside were phosphorylated within 2 h of incubation; the other acceptors required longer periods.

TABLE I

PHOSPHATE TRANSFER TO SEVERAL NUCLEOSIDES*

Nucleoside as acceptor	*Relative chromatographic position of nucleotide***	*Absorbance ratios*					
		Nucleoside			*Nucleotide*		
		$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$	$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$
9-β-D-ribofuranosylpurine	0.47	0.74	0.26	0.06	0.74	0.32	0.15
9-α-L-arabofuranosyladenine	0.62	0.84	0.24	0.045	0.84	0.23	0.035
9-β-D-glucopyranosyladenine	0.52	0.85	0.19	0.017	0.86	0.15	0.025
2,6-diamino-9-β-D-ribofuranosylpurine	0.52	1.14	0.79	1.00	1.10	0.81	1.00
5-bromo-3-β-D-ribofuranosyluracil	0.47	0.50	1.83	1.45	0.47	1.91	1.41

* Incubation at 30° under standard conditions (100 μmoles phenylphosphate, 20 μmoles acceptor per ml of 0.1 *M* acetate buffer of pH 5.2). After chromatography in the isobutyric acid–ammonium isobutyrate solvent, the remaining nucleoside and the newly formed nucleotide were eluted with 0.01 *N* HCl and the eluates read in the spectrophotometer.

** With the R_F of the corresponding nucleoside taken as 1.00. Uridine 5′-phosphate had, under these conditions, a relative R_F value of 0.50 with respect to uridine.

Adenylic acids as acceptors

The nucleoside phosphotransferases of plants have been shown to transfer phosphate specifically to the 5′ position of the acceptor[1,12]. The extensive purification of the transfer enzyme of carrot, resulting in the removal of most of the phosphomonoesterase activity[2,3], made possible the study of the behavior of nucleotides as

phosphate acceptors. The two isomeric adenylic acids having a free 5′-hydroxyl, *i.e.*, the 2′- and the 3′-phosphates of adenosine, were investigated.

The results are summarized in Table II. 2′-adenylic acid functions as acceptor (Expt. 1), more than 10% of the nucleotide being converted to adenosine 2′,5′-diphosphate; at the same time a small amount of adenosine, produced by the dephosphorylation of the acceptor, gives rise to some 5′-adenylic acid by phosphate transfer. 3′-adenylic acid behaves differently (Expt. 2): it does not accept phosphate directly—adenosine 3′,5′-diphosphate could not be detected—but, being split more rapidly than the 2′ isomer, it is, *via* adenosine, converted to 5′-adenylic acid in a significant yield (15%). In the absence of phenylphosphate as the donor (Expt. 3), the mixture of 2′- and 3′-adenylic acids produces both 5′-adenylic acid and adenosine 2′,5′-diphosphate; the 3′ nucleotide acts as the phosphate donor and is, at the same time, the source of the adenosine that is converted to the 5′ isomer. It will be noticed that, had the specificity of the enzyme been unknown, the conversion of 3′- to 5′-adenylic acid could have been interpreted as evidence of intramolecular phosphate migration. In these experiments adenosine diphosphate was readily separable from the other products in the isobutyrate solvent system, as it moved much more slowly than the adenylic acids.

TABLE II

ADENYLIC ACIDS AS PHOSPHATE ACCEPTORS*

Expt. No.	*Initial composition of assay mixture (per ml)*	*Products (μmoles/ml) formed during incubation (h)*				
			3	*6*	*24*	*56*
1	100 μg enzyme 100 μmoles phenylphosphate 20 μmoles adenosine 2′-phosphate	Adenosine Adenosine 5′-phosphate Adenosine 2′,5′-diphosphate	0.2 0 0.5	 0 1.2	0.6 0.4 2.2	1.4 0.4 2.2
2	100 μg enzyme 100 μmoles phenylphosphate 20 μmoles adenosine 3′-phosphate	Adenosine Adenosine 5′-phosphate Adenosine 2′,5′-diphosphate	1.1 0.3 0	1.7 0.5 0	3.3 2.3 0	6.4 3.0 0
3	100 μg enzyme 20 μmoles adenosine 2′-phosphate 20 μmoles adenosine 3′-phosphate	Adenosine Adenosine 5′-phosphate Adenosine 2′,5′-diphosphate	0.7 0.1 0	1.5 0.3 0.1	3.8 1.1 0.5	10.8 2.8 0.9

* The incubation was carried out at 30° and pH 5.2. At the beginning of the experiments none of the compounds listed as products was present in the assay mixtures. The products were separated on Whatman No. 1 filter paper in the isobutyric acid–ammonium isobutyrate solvent; the eluates in 0.01 *N* HCl were estimated spectrophotometrically.

Cyclic nucleotides as acceptors

When the cyclic nucleotides adenosine (2′,3′)-phosphate, uridine (2′,3′)-phosphate or cytidine (2′,3′)-phosphate were incubated with the enzyme and phenylphosphate, a new, more slowly moving product appeared on the chromatograms in the isobutyrate solvent. It had retained the spectral characteristics of the parent compound and represented the 5′-phosphate derivative of the cyclic nucleotide. The case of cyclic adenylic acid was investigated in more detail. The cyclic adenosine (2′,3′), 5′-diphosphate also offered a way to the preparation of adenosine 3′,5′-diphosphate which, as pointed out above, cannot be prepared by direct phosphate transfer to the 3′ nucleotide.

These transfer experiments with cyclic adenylic acid (barium salt) as the acceptor are shown in Table III. After incubation at 30° in 0.1 *M* acetate buffer (pH 5.2) for 24 h, portions were applied as stripes on Whatman No. 1 filter paper sheets and subjected to descending chromatography overnight in the isobutyric acid–ammonium isobutyrate solvent. The papers were dried in a vacuum over P_2O_5 and the slowest adsorption zone, which represented the diphosphate produced by the enzyme, was eluted with borate buffer of pH 7.0. Samples of the eluates served for spectroscopy, determination of inorganic phosphate, and for the determination of the ratio of organic phosphate to adenosine after hydrolysis with conc. $HClO_4$. It will be seen that phenylphosphate and 5′-adenylic acid were about equally effective as phosphate donors, whereas 5′-uridylic acid was less active.

TABLE III

CYCLIC ADENYLIC ACID AS PHOSPHATE ACCEPTOR*

Expt. No.	*Transfer enzyme µg/ml*	*Donor*	*µmoles/ml*	*Adenosine (2′,3′)-phosphate as acceptor µmoles/ml*	*Adenosine (2′,3′), 5′-diphosphate produced*				
					Yield µmole/ml	*Absorbance ratios*			*Molar ratio org. P/adenosine*
						$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$	
1	100	Phenylphosphate	100	20	2.1	0.82	0.18	0.02	1.9
2	100	5′-Adenylic acid	20	20	2.0	0.81	0.21	0.04	2.2
3	100	5′-Uridylic acid	20	20	1.2	0.79	0.20	0.04	1.9

* Incubation at 30° and pH 5.2 for 24 h. See text for experimental details.

The cyclic diphosphate is hydrolyzed by alkali to a mixture of the two isomeric adenosine diphosphates, *viz.*, 2′,5′- and 3′,5′-diphosphate. A preparation of the cyclic compound, adenosine (2′,3′), 5′-diphosphate, which migrated as a single component in both the isobutyrate and the ammonium sulfate–isopropanol solvent systems (see below), was dissolved in water (1 mg/ml), cooled in an ice-bath, and made 0.5 *N* with respect to NaOH. After 30 min, the mixture was neutralized and subjected to paper chromatography in the $(NH_4)_2SO_4$–isopropanol solvent, with untreated cyclic adenosine diphosphate and adenosine 2′,5′-diphosphate, produced by enzymic phosphate transfer, as described before, as reference compounds. The treatment with alkali brought about the disappearance of the cyclic diphosphate; it produced two new, faster-moving compounds of which one coincided in position with authentic adenosine 2′,5′-diphosphate. Since both products, which had typical adenosine spectra, contained two phosphate groups per adenosine, the slower-moving new compound must have been adenosine 3′,5′-diphosphate. The results are summarized in Table IV.

Additional remarks

The purified nucleoside phosphotransferase of plant tissues permits, as was pointed out before[2], the preparation of many otherwise inaccessible 5′ nucleotides. This is exemplified by the description, in this paper, of the enzymic phosphorylation of several unusual nucleosides. It is also useful for the preparation of deoxyribonucleotides. The remarkable versatility of the transfer enzyme is likewise demonstrated by its ability to use nucleotides as acceptors. Derivatives that are normally

difficult to obtain, such as the 2′,5′- and 3′,5′-diphosphates of adenosine—components of coenzyme II (TPN) and coenzyme A, respectively—can be prepared with relative ease.

TABLE IV

HYDROLYSIS OF CYCLIC ADENOSINE (2′,3′),5′-DIPHOSPHATE*

Compound	Relative R_F before hydrolysis**	Hydrolysis products					
		Relative R_F**	Yield, % of starting material	Absorbance ratios			Molar ratio org. P/adenosine
				$\frac{A_{230}}{A_{260}}$	$\frac{A_{240}}{A_{260}}$	$\frac{A_{265}}{A_{260}}$	
Adenosine (2′,3′),5′-diphosphate	0.82	—	—	***	***	***	***
Adenosine 2′,5′-diphosphate	—	1.70	34	0.85	0.20	0.02	2.1
Adenosine 3′,5′-diphosphate	—	1.30	66	0.82	0.22	0.03	1.8

* In 0.5 N NaOH for 30 min at 4°. See text for experimental details.

** Relative R_F values, with the R_F of 5′-adenylic acid taken as 1.0, in the ammonium sulfate–isopropanol–water solvent. Other relative R_F values, in the same solvent, were: cyclic adenosine (2′,3′)-phosphate 0.30; 3′-adenylic acid 0.50; 2′-adenylic acid 1.0. Adenosine 2′,5′-diphosphate produced, as described above, by enzymic phosphate transfer to 2′-adenylic acid, had a relative R_F of 1.67.

*** See Table III for data.

ACKNOWLEDGEMENT

This work was supported by a research grant from the National Science Foundation.

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