ON THE DISTRIBUTION AND BIOLOGICAL SIGNIFICANCE OF THE NUCLEOSIDE PHOSPHOTRANSFERASES*

by

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Enzymes catalyzing the synthesis of nucleotides by transfer of phosphoric acid from organic phosphates to nucleosides have been studied recently in this laboratory[1,2]. This group of enzymes, for which the name nucleoside phosphotransferase has been proposed, is capable of phosphorylating nucleosides, but seems to be inactive toward alcohols and sugars[2]. The existence of such transfer enzymes in many cell types makes it possible to consider the phosphorylation of the corresponding nucleosides as one of the general pathways for the biosynthesis of nucleotides.

The present study constitutes an attempt to gauge the actual importance of the nucleoside phosphotransferases in cellular metabolism. The criteria on which a decision was to rest were the following. (a) Enzymes engaged in the biosynthesis of major constituents of living matter, such as the nucleic acids, should be distributed very widely. (b) Their activity should, in view of the important role in growth assigned to the nucleic acids, reflect the physiological state of the tissues. Accordingly, many animal and plant tissues were tested for nucleoside phosphotransferase activity and the distribution of the enzyme in several fractions of rat liver cells was determined. In addition, the changes in phosphotransferase activity were followed in several growth processes, such as the germination of wheat, the regeneration of liver and muscle, and during bacterial growth.

EXPERIMENTAL

Material. Sodium monophenylphosphate was prepared according to ASAKAWA[3]. Ribocytidine, ribouridine and 5′-riboinosinic acid were purchased from the Schwarz Laboratories (Mount Vernon, N.Y.).

Analytical methods. Phosphorus was determined by the method of KING[4], phenol by ultraviolet spectrophotometry[2], and protein by a modification of the biuret method[5]. The nucleotides were estimated by paper chromatography followed by spectrophotometry, as described previously[2].

Preparation of tissue extracts. All materials, except the bacteria and protozoa, were ground with ice-cold distilled water in a high-speed mincer for 10 minutes, and tissue fragments removed from the extracts by low-speed centrifugation (about $300 \times g$ for 10 minutes). The tissues were used fresh or kept frozen for no more than a few days before use. The latter procedure entailed no loss in the enzymic activities studied. Extracts also could be kept frozen for several days without loss in activity.

Enzyme assays. For the phosphatase assay, 0.05 ml of the suspension was added to 0.05 ml of substrate solution (0.2 *M* phenylphosphate, 0.2 *M* sodium acetate buffer, pH 5.2), the mixture

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was incubated at 30° for 30 minutes, 3 ml of 0.1 *M* NaOH were added, and the phenol liberated was determined, with a portion of the same suspension incubated with water instead of substrate serving as the blank. A unit of phosphatase activity was defined as the amount of enzyme necessary to liberate 1 micromole of phenol under the conditions of the test. For the phosphotransferase assay, 0.1 ml of the suspension diluted so as to contain about 6 units of phosphatase activity per ml, was added to 0.1 ml of substrate solution (0.08 *M* ribocytidine as acceptor, 0.4 *M* phenylphosphate or 0.08 *M* 5'-inosinic acid as donor, 0.2 *M* sodium acetate buffer, pH 5.2), the mixture was incubated for 20 hours at 30°, and the cytidylic acid and inorganic phosphate were determined. A unit of transferase activity was defined as the amount of enzyme necessary to form 1 micromole of nucleotide under the conditions of the test.

Cell fractionation. The procedure of SCHNEIDER AND HOGEBOOM[6] was used as basis for the fractionation. All operations were carried out in the cold. 3.0 g of fresh rat liver, which had been perfused successively with ice-cold 0.14 *M* NaCl and 0.25 *M* sucrose, were cut into small pieces and treated with 27 ml of 0.25 *M* sucrose in a glass tissue grinder for 5 minutes. The mixture was strained through gauze and centrifuged at 700 × *g* for 10 minutes. The sediment, washed by suspension in 8 ml of sucrose solution and centrifugation at 1,000 × *g* for 10 minutes, served as the nuclear fraction. The combined supernatants were centrifuged at 4,900 × *g* for 10 minutes, the sediment was suspended in 25 ml of sucrose and the suspension centrifuged at 6,000 × *g* for 10 minutes. The "poorly sedimentable layer"[7,8] was removed with the supernatant and the well packed pellet was used as the mitochondrial fraction. The microsomes were obtained by centrifugation of the combined supernatants, including the poorly sedimentable layer, at 75,000 × *g* for 60 minutes.

The particulate fractions were suspended in water prior to enzyme studies; the final supernatant was used as such since the activities were found to be unaffected by 0.25 *M* sucrose. For the protein determination, 7% trichloroacetic acid was added to portions of each fraction and the resulting precipitates were dissolved in 0.1 *N* NaOH.

Germination of wheat. Spring wheat seeds were washed with water and kept in covered glass vessels on moist filter paper. Care was taken not to include moldy tissue.

Microbial and protozoal cultures. Flasks containing 300 ml of medium (0.8% nutrient broth, 1.3% proteose peptone, 3.3% glucose, 0.1% $NaHPO_4$) were inoculated with 15 ml portions of a 24 hour culture of *E. coli* in the same medium, and incubated at 37° with agitation. At various intervals, flasks were removed, the turbidity of the culture was determined by spectrophotometry at 650 mμ, and the bacteria were collected by centrifugation at 2,800 × *g* for 15 minutes and washed by suspension in water and centrifugation at 800 × *g* for the same period. The bacteria were kept at —15° for one day and extracted with water in the cold either by grinding with glass powder for 10 minutes or by vibration with small glass beads ("ballotini", 0.4 mm diameter[9]) for one hour. The latter procedure afforded a nearly complete cell breakage. The extracts were centrifuged at 800 × *g* for 20 minutes, and the supernatants used for enzyme assays.

Serratia marcescens was obtained from a 24 hour culture grown on nutrient broth and the extracts were made by vibration with "ballotini".

*Tetrahymena pyriformis** was grown for three days at 25° in the dark in 2% proteose peptone and 0.2% yeast extract. Prior to the final cultivation, the organisms were subjected to several transfers from 3-day old cultures in the same medium. The extraction was performed by grinding with glass powder.

Regenerating tissues. Four stages of regenerating liver were obtained from rats on the first, second, third, and fourth day after partial hepatectomy**. At the end of this period, the livers, originally reduced to one-third, had returned to almost normal size.

The specimens of regenerating rabbit muscle[10] were kindly supplied by Dr. G. C. GODMAN. Samples of rat hepatoma induced by azo-dye were obtained through the courtesy of Dr. J. A. MILLER, University of Wisconsin, and Dr. E. HIRSCHBERG of this University.

RESULTS AND DISCUSSION

1. *Distribution of the nucleoside phosphotransferases*

Nucleoside phosphotransferase activity was found in all the tissues tested, although the values found for the amount of enzyme present, as well as for the phosphorylation ratio (P.R.), *i.e.* the ratio of phosphate transferred to inorganic phosphate formed[2], covered a very wide range. In the animal organs, these values were quite constant for

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fresh tissues, but the plant materials showed considerable variations, probably owing to differences in the physiological state of the tissues.

The distribution studies were performed with two different donors, in order to differentiate between the three types of enzyme previously described[2]. As can be seen in Table I, a tentative grouping of the enzymes is possible which is based on the ratio of the transfer rates from phenylphosphate and 5'-inosinic acid and on the nucleotide isomers synthesized. The phosphotransferases occurring in plants apparently can be characterized by their ability to form only the 5'-nucleotides, while the enzymes present in animal tissues produce, in addition, a small amount of the 3'-isomers (5 to 10%). As regards the ratio of transfer rates (penultimate column in Table I), no essential differences seem to distinguish the bacteria from the higher plants and it is possible that the same type of enzyme occurs throughout the plant kingdom. Though the experimental material is much too exiguous for a decision, it would seem that in animals there occurs a continuous variation of the ratio of transfer rates with the stages of evolution, with the lowest forms (represented by *Tetrahymena*) having values close to those of plants.

TABLE I

DISTRIBUTION OF THE NUCLEOSIDE PHOSPHOTRANSFERASES IN PLANT AND ANIMAL TISSUES*

Tissue	*Phosphatase activity*	*Phosphotransferase*				*Relative transfer rates***	*Nucleotide isomers formed*
		Phenylphosphate as donor		*5'-Inosinic acid as donor*			
		Activity	*P.R.*	*Activity*	*P.R.*		
Wheat seed***	68	15	0.008	9	0.046	0.6	5'
Potato tuber	106	67	0.028	134	0.306	2.0	5'
Potato sprout	102	64	0.020	197	0.330	3.1	5'
Pea seed	152	4	0.001	3	0.014	0.8	5'
Pea seedling	136	14	0.003	14	0.026	1.0	5'
Spinach leaves	250	65	0.010	37	0.039	0.6	5'
Carrot root	38	62	0.146	39	0.460	0.6	5'
Serratia marcescens			0.120		0.705	3.4	5'
E. coli			0.023		0.268	0.8	5'
Tetrahymena pyriformis	514	1,500	0.069	1,800	0.206	1.2	5', 3'
Frog liver	570	99	0.018	37	0.050	0.37	5', 3'
Chicken liver	193	112	0.028	38	0.049	0.34	5', 3'
Rat liver	230	508	0.098	50	0.057	0.10	5', 3'
Rabbit skeletal muscle	12	6	0.022	0.8	0.015	0.13	5', 3'
Calf thymus	200	64	0.032	10	0.052	0.15	5', 3'
Calf liver	70	67	0.043	7	0.013	0.10	5', 3'
Human spleen	284	202	0.036	16	0.021	0.08	5', 3'

* Activities expressed as units per g of wet weight.
** Ratio of phosphotransferase activities with 5'-inosinic acid and phenylphosphate as the respective donors.
*** First day germination.

The bacterial systems present a peculiarity caused by the presence of a strong cytidine deaminase. This enzyme rapidly converts all the cytidine present in the substrate to uridine; and for this reason only uridylic acid is found as the product of transferase action.

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As is evident from Table II, the same type of phosphotransferase occurs in all organs of the rat; but certain tissues, *viz.*, spleen, liver and kidney, have a very high activity, while the activity of others, such as muscle and brain, is quite low.

TABLE II

DISTRIBUTION OF NUCLEOTIDE PHOSPHOTRANSFERASE IN RAT ORGANS*

Organ	*Phosphatase activity*	*Phosphotransferase*				*Relative transfer rates***
		Phenylphosphate as donor		*5'-Inosinic acid as donor*		
		Activity	*P.R.*	*Activity*	*P.R.*	
Liver	230	508	0.098	50	0.057	0.10
Spleen	590	640	0.077	56	0.056	0.09
Kidney	208	392	0.092	32	0.042	0.08
Testes	100	100	0.079	7	0.026	0.07
Brain	60	57	0.051	7	0.039	0.12
Lung	134	80	0.044	9	0.037	0.11
Small intestine	92	96	0.071	17	0.047	0.18
Heart	52	32	0.049	5	0.018	0.15
Skeletal muscle	24	8	0.022	0.8	0.010	0.10
Prostate	72	73	0.048	9	0.017	0.12

* Activities expressed as units per g of wet weight.
** Ratio of phosphotransferase activities with 5'-inosinic acid and phenylphosphate as the respective donors.

The results of the fractionation experiments with hepatic cell components (Table III) indicate a preferential localization of the phosphotransferase in the cytoplasmic particles. The specific activity of the nuclear fraction, as given here, is probably too low, since no attempt was made completely to remove the contaminating unbroken cells.

TABLE III

DISTRIBUTION OF PHOSPHOTRANSFERASE IN RAT LIVER CELL FRACTIONS

Fraction	*Protein % of total*	*Phosphatase*		*Phosphotransferase***		
		Activity % of total	*Specific activity**	*Activity % of total*	*Specific activity**	*P.R.*
Total	100	100	1	100	1	0.096
Nuclei	21	12	0.6	10	0.5	0.106
Mitochondria	13	19	1.5	22	1.7	0.131
Microsomes	28	42	1.5	49	1.7	0.123
Supernatant	31	28	0.9	13	0.4	0.042
Recovery	93	101		94		

* In terms of protein, as determined by the biuret method.
** With sodium phenylphosphate as the donor.

2. *Behavior of the nucleoside phosphotransferases during growth*

The changes in phosphotransferase activity were followed by measuring both the activity of the tissues (activity per g of wet tissue) and the P.R. values. The determination of the latter offered a convenient means of comparing the change in the enzyme under investigation with that of the acid phosphatase.

The phosphotransferase activity increases very markedly during the germination of

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wheat, as can be seen in Table IV. The P.R. values show a similar increase, and this is a consequence of the fact that the acid phosphatase activity rises to a much smaller extent. Table IV also shows that most of the phosphotransferase is present in the growing shoot, while the phosphatase activity is about evenly distributed between the shoot and the remnant of the grain.

TABLE IV

CHANGES IN NUCLEOSIDE PHOSPHOTRANSFERASE ACTIVITY DURING GERMINATION OF WHEAT*

Germination, number of days	*Phosphatase activity*	*Phosphotransferase*			
		Phenylphosphate as donor		*5'-Inosinic acid as donor*	
		Activity	*P.R.*	*Activity*	*P.R.*
0	(106)	(20)	0.007	(14)	0.045
1	68	15	0.008	9	0.046
2	108	84	0.029	68	0.152
3	162	213	0.054	187	0.298
4	154	461	0.124	425	0.543
6 (cotyledon)	238	34	0.006	18	0.029
6 (shoot)	290	945	0.184	1,055	0.623
10 (shoot)	520	1,270	0.257		

* Activities expressed as units per g of wet weight; values in parentheses as units per g of dry weight.

In the case of rat liver, changes in the physiological state do not seem to produce any alterations in the phosphotransferase content (see Table V). Both the transferase and the phosphatase activities remain unchanged in the course of regeneration, and also in hepatoma. But it will be noticed that even normal liver is characterized by a very high phosphotransferase activity. This is in contrast to the experiments with rabbit muscle, in which transferase activity and phosphorylation ratio, low in the normal tissue, increased markedly during regeneration.

TABLE V

BEHAVIOR OF PHOSPHOTRANSFERASE DURING PHYSIOLOGICAL CHANGES IN LIVER AND MUSCLE*

Organ	*Phosphatase activity*	*Phosphotransferase*	
		Activity	*P.R.*
Rat liver, normal	230	508	0.098
Rat liver, regenerating**	244	424	0.093
Rat hepatoma	282	402	0.107
Rabbit muscle, normal	12	6	0.022
Rabbit muscle, regenerating***	16	31	0.090

* Activities expressed as units per g of wet weight.
** Average of the values for 1st, 2nd, 3rd, and 4th day after the operation.
*** Seventh day after the operation.

During the growth of *E. coli*, the phosphotransferase activity also shows significant changes (see Fig. 1). Only the variations of the P.R. values are recorded here, but the changes in transferase content are similar, as the phosphatase activity remains un-

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changed throughout the whole growth cycle. In these experiments, uridine was used as the acceptor instead of cytidine, in order to avoid the interfering action of the cytidine deaminase. The P.R. increases sharply in the phase of rapid growth and drops steeply as the rate of growth decreases. The close connection between the phosphotransferase activity and the rate of growth is illustrated by the similarity of the two curves. It may be noted that the P.R. increases already during the lag phase. This may indicate that the non-dividing cells undergo physiological changes which make them ready for growth.

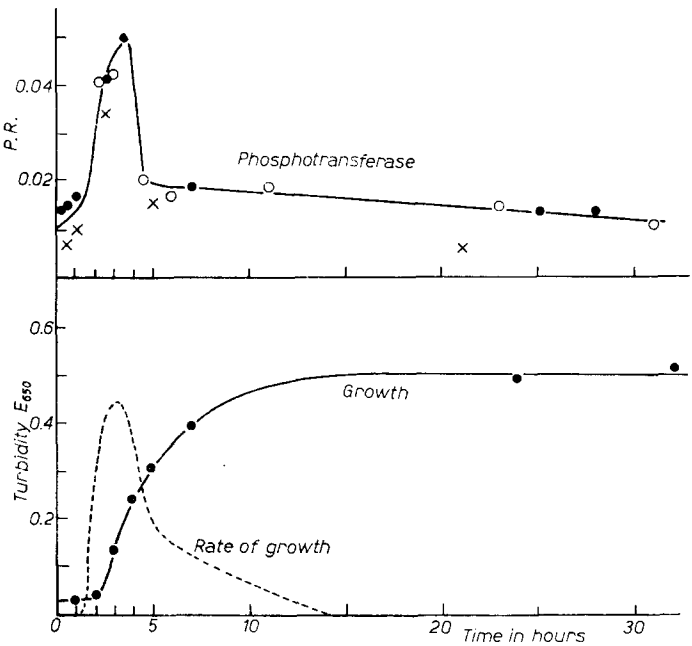

Fig. 1. Activity of nucleoside phosphotransferase during growth of *E. coli*. × Extraction by grinding with glass powder. ○ ● Extraction by vibration with "ballotini". (Two separate experiments.) The ordinate in the lower part of the figure does not apply to the curve for the rate of growth which was computed from the growth curve.

3. *Interpretations*

The wide distribution of the nucleoside phosphotransferases, their occurrence in higher plants, bacteria, protozoa and vertebrates, strongly suggests that this enzymic activity is one of the attributes of growing cells. As was pointed out in a previous paper[2], however, differences in the specificity of the transfer reaction catalyzed by extracts from different organisms demonstrate the existence of several transferase types. The plant phosphotransferases differ from those of animals by the nucleotide isomers formed; but this distinction may not have great physiological significance, as the 5'-nucleotides are, respectively, the only and the major isomers produced in both instances. Differences in the effectiveness of various phosphate donors may be of greater importance. In lower organisms, bacteria as well as protozoa, phenylphosphate and 5'-inosinic acid are equally effective donors (Table I). This is essentially also true of higher plants in which the considerable fluctuation in the relative rates of transfer from these two donors may be due to extraneous factors (*e.g.*, phosphatase action on the donor or the newly formed nu-

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cleotide) rather than to differences in specificity. That the progressive decrease, in animals, in the relative efficiency of the transfer from 5′-nucleotides may have evolutionary significance, has been pointed out above.

All mammals studied up to now appear to contain the same type of phosphotransferase which is identical with the rat liver enzyme described previously[2]. The specificity characteristics of this "mammalian enzyme" remained the same in the various rat organs examined here; no tissue specificity of the nucleoside phosphotransferases has been observed, with one exception. While the enzyme present in human spleen conforms to the general pattern, the human prostate phosphotransferase shows unusual properties: its activity is extremely high and its specificity unusual; large amounts of the 2′- and 3′-nucleotides are formed; the deoxyribosides are much better acceptors than the corresponding ribosides[2]. No enzyme of similar properties has been found in any other tissue, not even in the prostate of the rat. One could ask whether this type of enzyme is perhaps limited to hypertrophic glands, the material serving as the source of our preparations.

The characteristics of the mammalian nucleoside phosphotransferases seem to remain unchanged in the different tissues, but the enzyme contents of these tissues vary considerably (Table II). Although the parallelism is not complete—the moderate activity of the small intestine is surprising—it does seem that organs engaged in active cell formation are rich in nucleoside phosphotransferase. On the whole, it is inviting to assume that the uneven distribution of the transfer enzymes parallels, or reflects, differences in the anabolic activities of the several organs, especially as concerns their efficiency in synthesizing nucleic acids and proteins. A comparison of the data assembled in Table II with the corresponding rates of turnover of total nucleic acid phosphorus might contribute to an evaluation of the importance of the nucleoside phosphotransferases for the formation of nucleotides in different organs.

As concerns the location of nucleoside phosphotransferase within the cell, the major part appears to reside in the particulate fractions of the cytoplasm (Table III). Studies on the incorporation of radioactive phosphorus into rat liver cell fractions have, on the other hand, shown that the most rapid incorporation into nucleic acid takes place in the nucleus[11]. It is not inconceivable that nucleotides are formed in the cytoplasm by the phosphate transfer under discussion here and assembled as polynucleotides in the nucleus.

The biological significance of nucleoside phosphotransferases is, perhaps, best illustrated by the studies with growing systems (Tables IV, V, and Fig. 1). The most marked increase in activity is observed in germinating wheat; but bacteria and, to a lesser extent, regenerating muscle provide additional evidence. It could, of course, be objected that many enzymes increase with the onset of germination. An inspection of Table IV will, however, show that the increase in phosphotransferase by far surpasses the rise in acid phosphatase. Moreover, the latter occurs in both the endosperm and the shoot, whereas the rise in nucleoside phosphotransferase is confined to the growing shoot.

While the results presented in this paper provide strong evidence of an active participation of the nucleoside phosphotransferases in nucleotide synthesis and represent the first instance of a *general* mechanism for the production of these important cell constituents, the extent to which nucleosides are available as direct precursors of nucleotides in the cell is not yet known. For that matter, it does not appear sensible to expect a single answer to the question of the origin of nucleotides in tissues. It is obvious that there exist several potential mechanisms[12–17] whose respective preponderance may

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depend, in any given case, upon the availability of the enzyme, the donor or the acceptor. It is, moreover, probable that different nucleotides are sometimes formed by different mechanisms; for instance, the enzymes catalyzing the condensation of free nitrogenous bases with a ribose-5-phosphate derivative seem to operate only in the cases of adenine, hypoxanthine and orotic acid[12–16], while the kinase concerned with the phosphorylation of nucleosides by ATP is specific for adenosine and 2-aminoadenosine[17]. It may be assumed that different synthetic mechanisms can operate simultaneously and that additional processes are brought into play during physiological states that are accompanied by an increased demand for nucleotides.

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SUMMARY

Nucleoside phosphotransferases were found in a large variety of plant and animal tissues. In plants and bacteria, only the 5′-nucleotides are formed, while in animals small amounts of the 3′-isomers are also produced. The rates of phosphate transfer from phenylphosphate and 5′-inosinic acid are of the same order of magnitude in plants, bacteria and protozoa, but in the mammals transfer from the nucleotide is much slower. The enzyme is unevenly distributed in the organs of the rat, with the highest activities in liver, spleen and kidney, and the lowest in muscle. The bulk of the enzymic activity is localized in the cytoplasmic particles. Considerable increases in the activity of the enzyme occur during germination of wheat, regeneration of rabbit muscle, and bacterial growth. The results point to an active role of the nucleoside phosphotransferases in nucleotide biosynthesis.

RÉSUMÉ

La nucléoside phosphotransférase a été décelée dans une grande variété de tissus végétaux et animaux. Chez les plantes et les bactéries, seuls les nucléotides-5′ sont formés, tandis que chez les animaux une petite quantité des isomères-3′ est aussi produite. Les vitesses de transfert de phosphate à partir du phenylphosphate et de l'inosine-5′-phosphate sont du même ordre de grandeur chez les plantes, les bactéries et les protozoaires, mais chez les mammifères le transfert à partir du nucléotide est beaucoup plus lent. L'enzyme est inégalement distribué dans les organes du rat; les activités les plus élevées se trouvent dans le foie, la rate et le rein, et la plus basse dans le muscle. La plus grande partie de l'activité enzymatique est localisée dans les particules du cytoplasme. Des accroissements considérables de l'activité de l'enzyme accompagnent la germination du blé, la régénération du muscle de lapin et la croissance bactérienne. D'après les résultats obtenus, la nucléoside phosphotransférase pourrait jouer un rôle important dans la biosynthèse des nucléotides.

ZUSAMMENFASSUNG

Nukleosidphosphotransferasen finden sich in einer grossen Anzahl von pflanzlichen und tierischen Geweben. Während in Pflanzen und Bakterien nur die 5′-Nukleotide gebildet werden, erzeugen die Enzyme aus Tiergeweben auch kleine Mengen der 3′-Isomeren. Die Geschwindigkeiten der Phosphatübertragung sind in Pflanzen, Bakterien und Protozoen von derselben Grössenordnung für Phenylphosphat und 5′-Inosinsäure als Donatoren; in Säugetieren geht jedoch die Übertragung vom Nukleotid viel langsamer vor sich. Die Verteilung des Enzyms in Organen der Ratte ist sehr verschieden: die höchste Aktivität wird in der Leber, Milz und Niere gefunden, die niedrigste im Muskel. Die Hauptmenge des Enzyms findet sich in der Partikelfraktion des Zytoplasmas. In keimendem Weizen, regenerierendem Kaninchenmuskel und während des Wachstums von Bakterien wird ein sehr beträchtlicher Anstieg der Enzymaktivität beobachtet. Die Ergebnisse deuten auf eine wichtige Rolle der Nukleosidphosphotransferasen in der Biosynthese von Nukleotiden.

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