ACTA CHEMICA SCANDINAVICA 3 (1949) 422—432

On the Turnover of Purines and Pyrimidines from Polynucleotides in the Rat Determined with N^{15}

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The study of the metabolic pathways of nucleic acids with the aid of isotopes has been seriously handicapped by the lack of convenient methods for the isolation of the different compounds of the nucleic acids. With the object of obviating this, especially with a view to isotope work, methods have been worked out at this laboratory for the preparation of the two nucleic acids (desoxyribonucleic acid = DNA, ribonucleic acid = PNA) from gram amounts of tissues [1], and for the preparation and separation of the purines from DNA, and the purines and pyrimidines from PNA [2-4] in mg amounts. Furthermore, a method has been elaborated for the degradation of adenine and guanine to the corresponding hydroxypurines [5].

By the employment of these methods, we were recently able to show that orotic acid containing N^{15} could be used by the rat for the synthesis of the PNA-pyrimidines in the liver [6]. In this experiment the N^{15} content of PNA-uridine was higher than that of PNA-cytidine. On the assumption, however, that the amino group of cytidine contained no isotope, the pyrimidine ring of cytidine would have had a higher isotope content than uridine. This was thought to be the case, but no evidence could be advanced, as at that time no method was available for the deamination of the small amounts of cytidine obtained in the experiment. The lack of a method was also the reason why the pyrimidines of DNA were not investigated.

The present investigation was carried out in order to decide these questions. For this purpose a method has been elaborated for the deamination of small amounts of cytidine. The separation of the resulting uridine from the non-deaminated cytidine was carried out by partition chromatography on starch. For the preparation of the pyrimidines from DNA the method of Vischer and Chargaff [7], hydrolysis with concentrated formic acid at 175° for 2 hours, was

employed. The experiments were carried out on nucleic acids obtained from the pooled kidneys, spleens and small intestines of the rats from the investigation of Arvidsson *et al.*[6]. The animals had received subcutanously six doses of 12.5 mg orotic acid/100 g of bodyweight (excess $N^{15} = 6.06$ %) at 12-hour intervals and had been killed 12 hours after the last injection.

Table 1. Purines and pyrimidines from polynucleotides from the mixed intestines, spleens and kidneys of two rats. These had received a total dose of 75 mg of orotic acid / 100 g of body weigth (excess $N^{15} = 6.06$ %) during 3 days. The value $\frac{E_{max.}}{\gamma N/ml}$ is a test for purity with respect to foreign nitrogen [2]. *For standard values see Reichard* [3] *and Table 4.*

	Excess N^{15}	$\frac{E_{max.}}{\gamma N/ml}$
PNA		
Cytidine	0.401	0.296
Cytidine ring	0.592	0.312
Amino group (calculated)	0.029	
Uridine	0.324	0.313
Adenine	0.004	0.179
Guanine	0.006	0.165
DNA		
Cytosine	0.091	0.227
Thymine	0.062	0.278
Adenine	0.005	0.182
Guanine	0.003	0.173

As can be seen from the table, the amino group of cytidine did not contain any significant amount of N^{15}. The N^{15} from orotic acid, furthermore, had entered the pyrimidines in DNA, though to a much smaller extent than in PNA. The purines contained no isotope.

The results of these experiments made it desirable to investigate the turnover rates of the nucleic acid pyrimidines with another precursor. Because of that, organs (kidney, spleen and small intestine) from a previous experiment of Hammarsten and co-workers [8], in which N^{15}-glycine had been used for the administration of isotope, were worked up for the pyrimidines from PNA and partly DNA. In this experiment the rats had received two subcutanous injections of 50 mg glycine/100 g of bodyweight (excess $N^{15} = 32$ %) at six-hour interval and had been killed six hours after the last injection.

In this case the isotope content of the deaminated cytidine is lower than that of the aminopyrimidine, indicating a relatively high turnover rate of the

Table 2. Pyrimidines from polynucleotides from the different organs of 28 rats. Each rat had received a total dose of 100 mg of glycine / 100 g of body weight during 12 hours.

	Spleen		Intestine		Kidney	
	Excess N^{15}	$\frac{E_{max.}}{\gamma N/ml}$	Excess N^{15}	$\frac{E_{max.}}{\gamma N/ml}$	Excess N^{15}	$\frac{E_{max.}}{\gamma N/ml}$
PNA						
Cytidine	0.145	0.275	0.285	0.279	0.087	0.290
Cytidine-ring	0.099	0.315	0.214	0.324	0.067	0.333
Amino group (calc.)	0.237		0.427		0.127	
Uridine	0.164	0.311	0.293	0.340	0.117	0.308
DNA						
Cytosine	0.060	0.219			0.028	0.209
Cytosine-ring	0.056	0.285				
Amino-group (calc.)	0.068					
Thymine	0.038	0.262			0.018	0.192 (?)
TCA insoluble	0.132				0.251	

amino group in cytidine. Uridine has the same (intestine) or a higher N^{15} content than the cytidine ring.

The purines from the PNA and DNA from intestine with N^{15} glycine as isotope precursor have been isolated previously and degraded to the corresponding hydroxypurines [5]. In order to get a more complete picture of some questions connected with the synthesis of purines in nucleic acids, adenine and guanine were now isolated from nucleic acids of spleen and kidney, too. The purines from the spleen were deaminated, but not those from the kidney, because of their very low N^{15} content. The results may be seen in Table 3, where the values for the intestine and regenerating liver from the previous experiment [5] are also given for comparison.

In the spleen the isotope content of the PNA-guanine is somewhat higher than that of adenine, though the relations are reversed for the corresponding hydroxypurines. This gives a high turnover rate for the aminogroup in guanine and a low for that of adenine. In DNA both hydroxypurines had a higher isotope content than the aminopurines. The results are in good agreement with those obtained from intestine. As for the kidney the isotope content there was rather low, but the difference between adenine and guanine for both PNA and DNA was significant. In both cases adenine had a higher turnover rate than guanine.

Table 3. Aminopurines and corresponding hydroxypurines from different organs from polynucleotides of the rat with N^{15}-glycine as a precursor. The experimental conditions were the same as in Table 3. The values for intestine and regenerating liver are taken from a previous publication [5] and are included for comparison.

	Spleen		*Kidney*		*Intestine*		*Regenerating liver*	
	Excess N^{15}	$\frac{E_{max.}}{\gamma N/ml}$	Excess N^{15}	$\frac{E_{max.}}{\gamma N/ml}$	Excess N^{15}	$\frac{E_{max.}}{\gamma N/ml}$	Excess N^{15}	$\frac{E_{max.}}{\gamma N/ml}$
PNA								
Adenine	0.285	0.181	0.065	0.162	0.46	0.171	0.43	0.178
Hypoxanthine	0.356	0.195			0.54	0.191	0.51	0.190
Amino group (calc.)	0.001				0.12		0.11	
Guanine	0.336	0.161	0.043	0.151	0.51	0.165	0.97	0.158
Xanthine	0.301	0.159			0.43	0.165	0.97	0.167
Amino group (calc.)	0.476				0.83		0.97	
DNA								
Adenine	0.165	0.187	0.054	0.178	0.27	0.169		
Hypoxanthine	0.205	0.192			0.33	0.192		
Amino group (calc.)	0.005				0.06			
Guanine	0.159	0.159	0.031	0.152	0.48	0.161		
Xanthine	0.196	0.158			0.59	0.169		
Amino group (calc.)	0.011				0.04			

EXPERIMENTAL

Preparation of purines and pyrimidine nucleosides from PNA. The preparation and separation of PNA and DNA were carried out according to Hammarsten [1]. The ribomononucleotides were isolated by precipitation with mercuric nitrate and decomposition with hydrogen sulfide as described previously[4]. If the precipitation with mercury was carried out at sufficiently low pH (not above 2), it was found that electrodialysis for the purification of the mononucleotides could usually be omitted.

It was found advantagous to separate the purines and pyrimidines before subjecting them to chromatography. This could be affected by splitting off the purines with acid and precipitating them with silver nitrate, according to Kerr and Seraidarian [9].

To the solution of the four mononucleotides sulphuric acid was added to 0.1 *N*. The solution was kept at 100° for one hour in a water-bath. After

cooling and neutralisation with *N* NaOH to pH 2, the purines were precipitated with one-fifth by volume of *N* silver nitrate. The silver purines were washed, decomposed with HCl, and subjected to chromatography on a starch column, as described previously [2,5].

The mother liquor, after the precipitation of the silver purines, contained the pyrimidine nucleotides, ribose phosphoric acid, ribose and phosphoric acid. The nucleotides, and probably most of the ribose phosphoric acid and phosphoric acid, were precipitated as silver salts by silver nitrate at neutral pH. This procedure has been used by Kerr and Seraidarian [9] for the precipitation of the purine mononucleotides.

The supernatant from the silver purines was continuously neutralized with 0.1 *N* NaOH. A white to yellowish precipitate of the silver nucleotides was formed, which, after a further addition of alkali, turned brownish owing to the formation of silver oxide. The addition of alkali was stopped when the solution with the suspended precipitate had taken on a distinctly light-brown colour.

The silver salts were allowed to precipitate in the ice box for 48 hours, centrifuged, washed twice with 0.05 *N* silver nitrate, twice with alcohol, and twice with ether. The dry precipitate was suspended in water and decomposed with hydrogen sulphide.

The silver sulphide was centrifuged off, washed twice with 5 ml of hot water, and the washings were added to the supernatant. The resulting solution was neutralized with *N* NaOH to pH 4, the nucleotides were converted into the nucleosides with the aid of prostate phosphatase, and the nucleosides separated by starch chromatography. The details of these procedures have been described elsewhere [3,4].

Preparation of purines and pyrimidines from DNA. The DNA in Hammarsten's procedure [1] is precipitated with lanthanum. This must be removed before any further steps can be taken. For this purpose the lanthanum precipitate was finely suspended in 1 ml of molar potassium carbonate. 9 ml water were added, the solution was heated 5 minutes in a water-bath and centrifuged. The whole procedure was repeated twice with 0.5 ml of potassium carbonate and 4.5 ml of water. The combined supernatants, which contained the DNA, were neutralized with concentrated acetic acid to a slightly acid reaction and precipitated with 4 volumes of alcohol. The precipitate was centrifuged off and dried with alcohol and ether.

For the further preparation of the bases from DNA, two alternatives were tried out.

The first was the complete method of Vischer and Chargaff [7], in which the purines were precipitated as the hydrochlorides by dry HCl in methanol. After removal of the excess HCl the purines were then separated by chromato-

graphy on starch. The pyrimidines were obtained from the supernatant from the cristallisation of the purines by hydrolysis with concentrated formic acid at 175° for two hours, as described by Vischer and Chargaff. The dark-brown solution after the hydrolysis was diluted to 10 ml with water and centrifuged. The supernatant was evaporated several times *in vacuo*. Before the last evaporation, the solution was neutralized with 0.1 *N* NaOH. The residue after the last evaporation was dissolved in butanol water and run on a starch column.

By this method a fairly good yield (70—80 %) for the pyrimidines was obtained, as compared with the values of Vischer and Chargaff. As in their case, the yield of the purines however, was not very satisfactory. Higher yields of these could be obtained by a method following the same principles as for the isolation of the bases from PNA.

In that case 30—50 mg of the dry DNA, free from lanthanum, were dissolved in 10 ml 0.1 *N* sulphuric acid and hydrolyzed for 1 hour at 100°. In experiments which were not carried out on specially pure samples of DNA, some matter usually remained undissolved, probably from protein in the DNA. This was centrifuged off at the end of the hydrolysis. After neutralisation to pH 2, the purines were precipitated and treated as described for PNA. The solution left after the precipitation of the purines contained the pyrimidines in some indefinite form (»thymic acid»). They were precipitated with silver at neutral reaction, and the silver was removed by hydrogen sulfide, as in the case of the PNA pyrimidine mononucleotides.

The pyrimidines were then split off with formic acid and, after the removal of the acid, separated by chromatography.

The latter procedure gave very good yields for the purines but considerably lower yields for the pyrimidines. The cause of this was that part of the pyrimidines was precipitated together with the purines with silver at acid pH. These pyrimidines, in the form of »thymic acid», were carried together with the purines to the purine chromatography. They showed, however, very slight solubility in alkaline butanol-water (the chromatographic medium for the purines). Therefore they were left as an insoluble residue when the purines were dissolved for chromatography. This residue may be dried, and hydrolyzed with formic acid, and the pyrimidines separated by chromatography, which procedure increased the yield.

Chromatographic separation of pyrimidines. Fig. 1 shows a chromatogram of the pyrimidine fraction from DNA. In order to ascertain the identity of the different compounds, a mixture of the three pyrimidines, cytosine, uracil and thymine, was subjected to chromatography under identical conditions. The result is shown by Fig. 2. By calculating the *R*-values [10], determining the

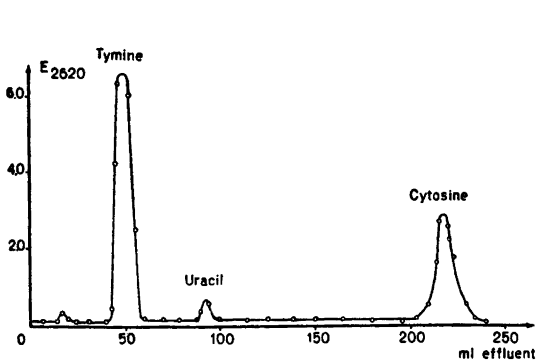

Fig. 1. Chromatographic separation of the DNA-pyrimidine fraction from 30 mg of DNA. Length of column: 205 mm, diameter: 22 mm. The peak in front of the thymine is an artefact and does not show the typical pyrimidine light absorption.

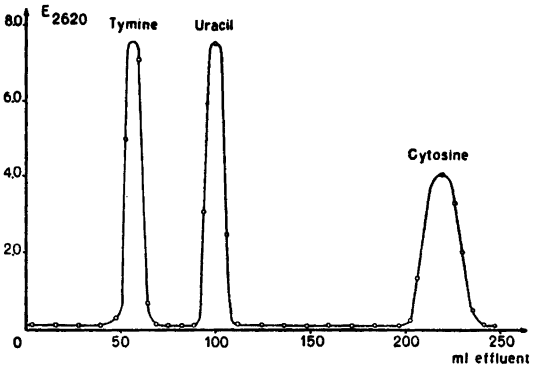

Fig. 2. Chromatographic separation of 2.2 mg thymine, 2.5 mg uracil and 2.3 mg cytosine. Length of column: 220 mm, diameter: 22 mm. Yield for each pyrimidine 90—100 %.

typical absorption curve in the ultra violet, and the constant $\frac{E_{\text{max.}}}{\gamma\text{N/ml}}$ [2], the identity of the pyrimidines was established. The results are summarized in Table 4.

Table 4. Characteristic values for the compounds obtained after chromatography of the hydrolysis products from DNA, as compared with those for standard pyrimidines. All light absorption values are determined in N HCl.

	R-value	$E_{\text{max.}}$ (Å)	$\frac{E_{\text{max.}}}{\gamma\text{N/ml}}$	$\frac{E_{\text{max.}}}{E2480}$
Products from DNA				
First compound	1.55	2650	0.265	1.83
Second compound	0.84	2590		1.30
Third compound	0.36	2750	0.215	3.72
Standard pyrimidines				
Thymine	1.48	2650	0.270	1.73
Uracil	0.83	2590	0.283	1.33
Cytosine	0.38	2750	0.229	3.48

The finding of uracil, even if in rather small amounts, seems to be rather surprising. Uracil is not supposed to be present in DNA. The presence of PNA in the DNA was highly improbable, as the experiment was carried out with a pure specimen of DNA, prepared according to Hammarsten [1]. The

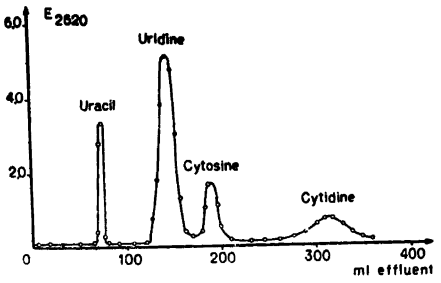

Fig. 3. Chromatographic separation of the products obtained after 48 hours hydrolysis at 100° with 0.4 N HCl of 8.3 mg of cytidine. Length of column: 185 mm, diameter: 22 mm. The peaks correspond to 0.7 mg of uracil, 4.1 mg of uridine, 0.5 mg of cytosine and 1.1 mg of cytidine.

explanation seemed to be that the uracil was formed during the hydrolysis from cytosine. This became still more probable when a model experiment with pure cytidylic acid, after hydrolysis with formic acid at 175° for two hours, gave rise to some uracil (5—10 % uracil of the cytosine formed).

Deamination of cytidine and cytosine. Various possibilities exist for the splitting off of the amino group of cytidine. One method outlined by Loring and Ploeser [11], consists in refluxing cytidine with 0.4 *N* sulphuric acid for 48 hours. This method has been tried out, though hydrochloric acid was substituted for sulphuric acid.

8.3 mg cytidine were dissolved in 5 ml 0.4 *N* HCl and refluxed on a sand-bath for 48 hours. The HCl was removed by repeated evaporation in vacuo. The residue was dissolved in 2 + 1 + 1 ml butanol-water and put on top of a starch column 22 mm in diameter and 185 mm in length. The result of the experiment is shown in Fig. 3.

Very little of the cytidine was left unaltered, but most of it had been converted into uridine. A considerable part of the nucleosides, however, had been converted into the free pyrimidines, and the method seemed to be unsuitable for the deamination of cytidine. The deamination of the free cytosine to uracil, however, has been carried out in this way.

Cytidine has been converted into uridine by treatment with an aqueous solution of pyridine (1 part pyridine + 3 parts water). No free pyrimidines

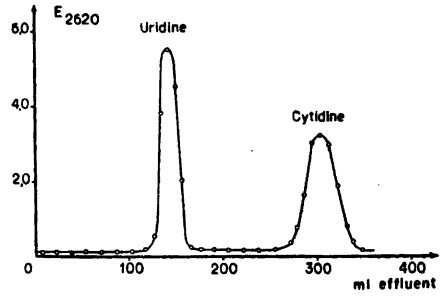

Fig. 4. Chromatographic separation of the products obtained after 5 hours hydrolysis at 160° with aqueous pyridine of 10.1 mg of cytidine. Length of column: 185 mm, diameter 22 mm. The peaks correspond to 4.5 mg of uridine and 5.1 mg of cytidine.

were formed in this way. 10.1 mg cytidine were treated with 1 ml of pyridine-water in a sealed tube at 160° for 5 hours. The pyridine was removed by four evaporations *in vacuo*; the residue was dissolved in butanol-water and subjected to chromatography (fig. 4).

As can be seen, about half the amount of cytidine has been deaminated and no free pyrimidines have been formed.

Deamination of aminopurines. This was carried out as described in a previous publication [5].

DISCUSSION

The experiment with *orotic acid* as a precursor for the pyrimidines of nucleic acids in the rat clearly indicates two things. Firstly, as might have been expected, the N^{15} of orotic acid only enters the ring of PNA-cytosine, and the amino nitrogen of this base is derived elsewhere. Secondly orotic acid may act, not only as a precursor for the pyrimidines of PNA, but also for the pyrimidines of DNA.

As regards the first finding, the results show that, with orotic acid as a precursor, the cytosine ring is rebuilt at a quicker rate than the uracil in PNA. This is very obvious in the present investigation, in which the PNA was derived from the combined intestines, spleens and kidneys. The cytosine ring contained about double the amount of N^{15} as compared with the uracil. This holds true also for the previous experiment with liver [6], if one assumes that liver does not differ from the other organs and thus does not use orotic acid nitrogen for the amino group of cytosine. On that assumption, the difference between the calculated cytosine ring (1.308 % excess N^{15}) and uracil (1.133 %) for liver is not as large as for the organs in the present experiment. The obvious conclusion from these data is that the synthesis of cytosine in these experiments was not preceded by a synthesis of uracil.

In DNA, too, cytosine has the highest turnover rate of the two pyrimidines. In this case the material was not sufficient for deamination, but there is no reason to assume that the amino group contains any isotope. On the assumption that all of the N^{15} is located in the cytosine ring, this would have about double the isotope content of thymine. When comparing the pyrimidines of DNA with those of PNA, one notes that the latter contain about five times as much N^{15} as the DNA pyrimidines. This finding is somewhat surprising, in view of the results obtained with glycine-N^{15} as a precursor. There the turnover ratio PNA/DNA varies between 1 and 2 for the three organs, intestine, spleen and kidney. The same results [12] were obtained with P^{32}. At first sight it would seem that the rat is able to use orotic acid to a lesser degree for the

synthesis of DNA pyrimidines than glycine as compared with the synthesis of PNA pyrimidines.

With glycine as a precursor, the turnover rates for the cytosine ring and uracil from PNA are reversed as compared with orotic acid. Now uridine has a significant higher isotope content than deaminated cytidine. The results show, furthermore, that cytidine, conformably with guanine, has an »active» amino group. The calculated isotope content of this is invariably higher than that of the ring. In DNA the low N^{15} content makes it impossible to interpret the results with certainty. At least in the case of spleen it seems certain that the turnover rate of cytosine is higher than that of thymine.

Another question which can be answered from the results of the present investigation is that of the existence or non-existence of uracil in PNA. It has at times been questioned whether uracil exists at all as a natural building stone of PNA or whether it has not its origin from deamination of cytosine during the preparation of the nucleic acid or during the preparation of the pyrimidines from PNA. The results with both N^{15}-orotic acid and N^{15}-glycine clearly indicate a difference in turnover rates for deaminated cytosine and uracil from PNA. This should afford fairly definite proof of the primary existence of uracil in PNA.

The results obtained in respect of the purines from spleen and kidney confirm the earlier finding for intestine [5]. In the spleen the higher isotope content of PNA-guanine as compared with PNA-adenine is reversed after deamination. In the kidney PNA-adenine has a higher isotope content than guanine. These findings can be easily fitted into the theory of Brown *et al.* [13] that adenine is a precursor for PNA-guanine.

In DNA the difference between the two purine rings is not significant for the spleen, while the turnover rate of adenine is higher than that of guanine for the kidney.

SUMMARY

A method is described for the preparation of cytosine and thymine from 30—50 mg desoxyribo nucleic acid by the use of hydrolysis with formic acid according to Vischer and Chargaff [7] and partition chromatography on starch.

The conversion of cytidine into uridine with the help of 0.4 *N* acid and aqueous pyridine is investigated, and the products are separated by chromatography on starch.

It is shown that orotic acid can act as a precursor, not only for PNA pyrimidines, but also for DNA pyrimidines. In the pyrimidines from PNA the cytosine ring is shown to have a higher turnover rate than uracil.

The results obtained with orotic acid as a precursor for pyrimidines are compared with those obtained with glycine as a precursor.

Some supplementary experiments are made with the purines from the spleen and kidney of the rat with N^{15}-glycine, with the object of investigating the possibility of using adenine as a precursor for guanine.

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Received March 20, 1949.

Short Communications

Pyrimidine Nucleosides as Precursors of Ribonucleic Acid (PNA) Pyrimidines

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It has been shown with the aid of N^{15} that the free pyrimidines uracil, thymine [1] and cytosine [2] cannot be utilized as precursors for polynucleotides by the rat. This does, however, not necessarily mean that these bases when bound as nucleosides or nucleotides cannot enter polynucleotides. The finding of Loring and Pierce [3] that pyrimidine ribonucleosides and nucleotides are from 10 to 60 times as active as free pyrimidines for a pyrimidine deficient strain of *Neurospora* strongly suggests the necessity of trying tracer marked nucleosides or nucleotides as precursors for polynucleotides.

Because of that we have grown yeast on a medium in which the nitrogen source of