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THE DISTRIBUTION OF ^{32}P IN THE MONONUCLEOTIDES OF NORMAL AND REGENERATING LIVER RIBONUCLEIC ACIDS

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By Elliot Volkin and Charles E. Carter

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THE DISTRIBUTION OF ^{32}P IN THE MONONUCLEOTIDES OF NORMAL AND REGENERATING LIVER RIBONUCLEIC ACIDS

By Elliot Volkin and Charles E. Carter

Investigations of the turnover of nucleic acid phosphorus generally employ crude nucleic acid fractions and assume homogeneity of distribution of isotopic phosphorus within the complex nucleic acid molecule.[1] The work reported in this paper deals with the ^{32}P turnover in liver ribonucleic acid purified by the guanidine salt method.[2] The specific activities of the component monoribonucleotides were determined following mild alkaline degradation of the parent nucleic acid and separation of the mononucleotides on the anion-exchange resin, Dowex-1.[3]

Experimental

One rabbit was injected intraplurally with 900 microcuries of inorganic ^{32}P and after a period of three hours the liver (about 100 g.) removed for the preparation of ribonucleic acid. Three adult rats each received 1 millicurie of inorganic ^{32}P intraplurally and one hour later were sacrificed. From the pooled rat livers (about 30 g. each) ribonucleic acid was isolated. Ribonucleic acid was prepared from the pooled regenerating liver tissue (about 20 g. each) of 4 rats, each of which forty-eight hours previously had been subjected to subtotal hepatectomy. Conditions with regard to the amount of isotope injected and the time of sacrificing were the same as in the experiment with normal rats.

Preparation of Ribonucleic Acid. -- The method of preparing ribonucleic acid from mammalian tissue[2] involves a preliminary precipitation of the desoxyribonucleic acid as a protein complex which is removed with the large

cytoplasmic particles by centrifugation.[4] The supernatant solution containing the small granule and soluble portion of the cytoplasm is made 4 molar with guanidine hydrochloride which results in the precipitation of a gelatinous ribonucleic acid-protein complex. The ribonucleic acid is purified by extraction of the complex with chloroform-octyl alcohol, a process which denatures and precipitates the protein.[5] Multiple repetition of this process plus repeated alcohol precipitations of the nucleic acid result in a pure, protein-free ribonucleic acid. Material so prepared sedimented in the analytical ultracentrifuge as a single, symmetrical component. The phosphorus content of such preparations was consistently between 8.8 and 9.1%.

Separation of Mononucleotides. -- Liver ribonucleic acid was hydrolyzed in 0.5 N NaOH at 37° for seventeen hours, using 20 mg. of nucleic acid per ml. of alkali. These conditions for alkaline hydrolysis, which are milder than those employed for analytical determinations of ribo- and desoxyribonucleic acids,[6] were found to convert about 20-40% of the adenylic acid moiety to adenosine. When pure adenylic acid, either the "A" or "B" isomer,[3,7] were employed under similar conditions, no adenosine was formed, indicating that the phosphorus of adenylic acid is more labile in the internucleotide linkage. (A detailed study of the kinetics and products of the alkaline degradation of ribonucleic acids will appear elsewhere.) However, approximately 90% of nucleic acid phosphorus, employing these conditions, was recovered as mononucleotide and this adequately met the purpose of the investigation.

For analysis, the alkaline hydrolysate of the nucleic acid was partially neutralized by adding 1/2 volume of 0.5 N HCl and diluted with water to 100 ml., then put on the resin column (Dowex-1, bed size 8 cm. x 0.72 cm.^{2}) which had previously been converted to the chloride form, and washed with 200 ml. of water, 20 ml. of 0.05 M NaCl and 10 ml. of 0.01 N HCl. The effluent collected

during this preliminary treatment contains material not held by the anion exchanger, i.e., some purine and pyrimidine bases and adenosine.[3] Under the above conditions the material not bound to the column was found to contain almost exclusively adenosine. This was determined by collecting the break-through and preliminary wash, concentrating by vacuum distillation and submitting the concentrate to paper chromatography.[7] Also, the spectrum of the effluent collected to this point showed a ratio of optical density at 280 mμ/260 mμ of 0.15 - 0.19 at neutral pH, which is characteristic of adenosine. Since the amount of material which did not remain on the column was less than 5-10% of the total hydrolysate, this fraction was neglected in determinations of specific activity of the phosphorus of the mononucleotides. This fraction, however, which is almost exclusively adenosine, was included in the estimation of adenylic acid content of the nucleic acid. Since the break-through fraction has not been rigorously analyzed for small amounts of other base or riboside derivatives of nucleic acid this omission may introduce an error of 1-3% for each mononucleotide constituent.

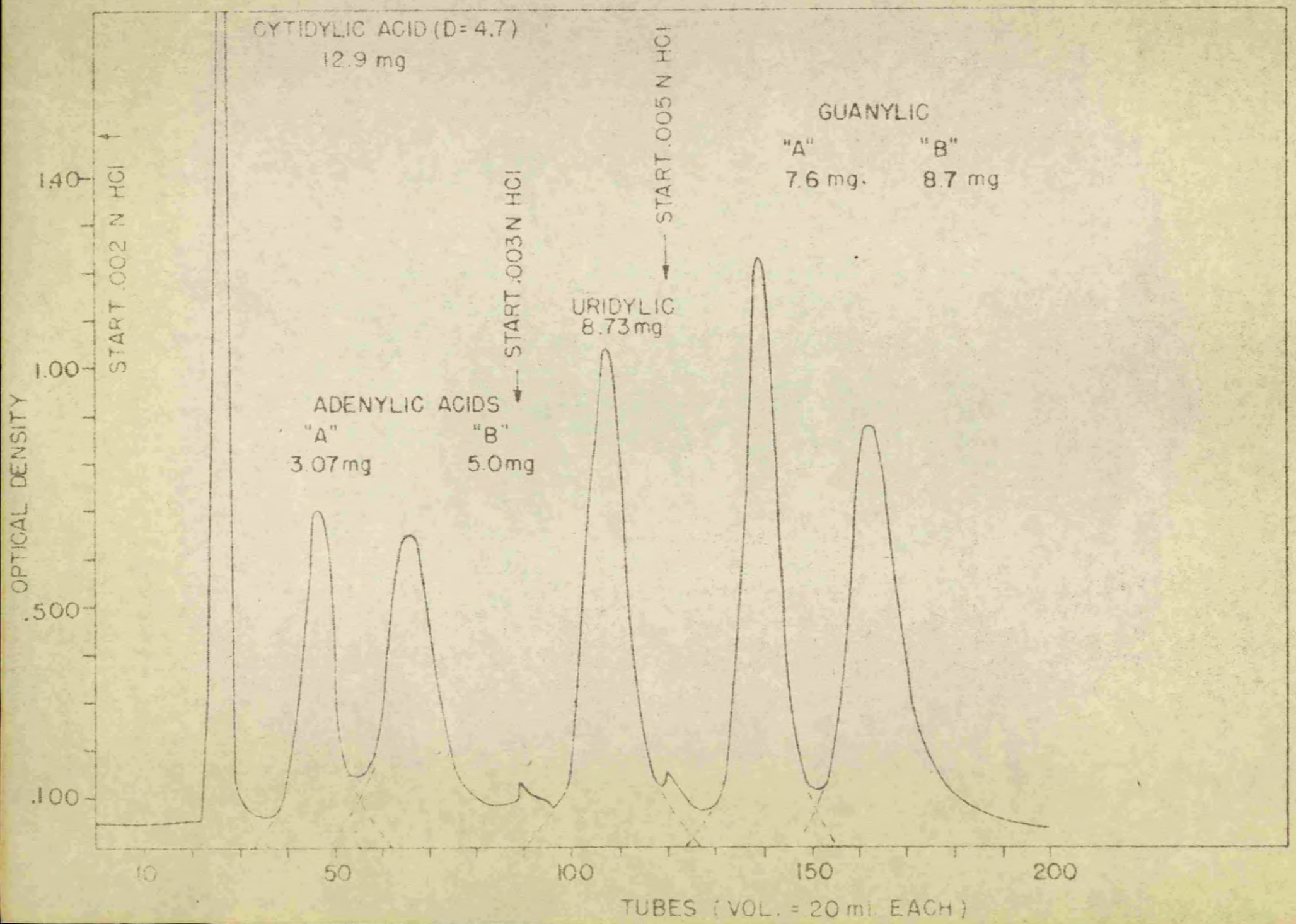
The elution of mononucleotides from the anion-exchange column by increasing concentrations of HCl in the wash (see Fig. 1) follows the procedure described by Cohn.[3] The isomeric purine nucleotides obtained by this procedure are believed to be adenosine 2' and 3' phosphate and guanosine 2' and 3' phosphate, although rigorous proof by synthesis has not as yet been obtained. Quantitative determination of mononucleotides in the effluent fractions by ultraviolet spectrophotometry were based on the following molar extinction coefficients of the nucleotides in 0.01 N HCl: adenylic acid, 13,400 (260 mμ), guanylic acid, 12,000 (260 mμ), cytidylic acid, 12,800 (278 mμ),[8] and uridylic acid, 9,890 (262 mμ).[8]

<u>Specific Activity Determinations</u>. -- Specific activity determinations were

Figure 1

Nucleotides derived from rabbit liver

Molar Ratio			Percent Composition	
Cyt	=	4.0	Cyt	29
Ad "A"	=	.88	Ad	17.1
"B"	=	1.44	Ur	20
Ur	=	2.70	Guan	33.3
Gu "A"	=	2.10		
"B"	=	2.41		


CYTIDYLIC ACID (D= 4.7)
12.9 mg
START .002 N HCl
START .003 N HCl
START .005 N HCl
ADENYLIC ACIDS
"A"
3.07mg
"B"
5.0mg
URIDYLIC
8.73mg
GUANYLIC
"A"
7.6 mg.
"B"
8.7 mg
OPTICAL DENSITY
1.40
1.00
.500
.100
10
50
100
150
200
TUBES (VOL. = 20 ml. EACH)

based on spectrophotometric determinations of the nucleotides which were then converted to equivalent phosphorus contents. These values were occasionally checked with direct determinations of organic phosphorus and were found in agreement. The ^{32}P content was measured by counting aliquots of solutions slowly dried on porcelain capsules. For specific activity determinations, the tubes collected corresponding to the peak of each nucleotide fraction were employed (see Fig. 1). Four to six determinations for each nucleotide peak were made.

Results and Discussion

The figure demonstrates a typical elution diagram of the mononucleotides derived from rabbit liver ribonucleic acid. In the experiment illustrated the mononucleotides plus adenosine represent 96% of the ribonucleic acid phosphorus.

The table summarizes the results obtained for the percentage composition and specific activities of the mononucleotides derived from rabbit, normal rat and regenerating rat liver ribonucleic acids. As shown in the table, these analyses reveal a relatively high percentage of cytidylic and guanylic acids in rabbit and rat liver ribonucleic acids. Although Levene[9] proposed a tetranucleotide structure (equimolar proportion of the four bases) of yeast ribonucleic acid, he recognized that this generalization was inapplicable to ribonucleic acid isolated from mammalian pancreas, which contained a comparatively high guanylic acid content. We have found that, in general, mammalian ribonucleic acids have relatively high guanylic and cytidylic acid contents. While regenerating rat liver ribonucleic acid, when compared with that from the normal rat, exhibits an almost identical mononucleotide composition, rabbit and rat liver ribonucleic acids were found to differ slightly in their uridylic and adenylic acid contents. The nucleotide composition of rat liver ribonucleic acid was similar to that

TABLE I

ANALYTICAL COMPOSITION AND SPECIFIC ACTIVITIES OF MONONUCLEOTIDES DERIVED FROM RABBIT, NORMAL RAT AND REGENERATING RAT LIVER RIBONUCLEIC ACIDS

Mononucleotide	(A) Rabbit			(B) Normal Rat			(C) Regenerating Rat		
	Weight % composition	Moles x 10^5 per 50 mg. nucleic acid	S.A.[1,2]	Weight % composition	Moles x 10^5 per 50 mg. nucleic acid	S.A.[1,2]	Weight % composition	Moles x 10^5 per 50 mg. nucleic acid	S.A.[1,2]
Cytidylic	26.5	4.15	10.2 ± 0.5	29.0	4.50	4.29 ± 0.09	28.6	4.42	12.1 ± 0.6
Adenylic "A"	6.3		9.8 ± 0.5	6.3		6.37 ± 0.12	7.0		19.3 ± 0.3
Adenylic "B"	10.3		10.7 ± 0.4	10.2		6.38 ± 0.10	8.7		18.9 ± 0.8
(+Adenosine as Adenylic)[3]	5.3			9.6			11.3		
Total Adenylic	21.9	3.17		26.1	3.76		27.0	3.89	
Uridylic	18.0	2.81	10.3 ± 0.7	13.2	2.06	4.38 ± 0.21	14.5	2.24	13.8 ± 0.3
Guanylic "A"	15.6		10.1 ± 0.3	14.7		4.10 ± 0.20	13.2		13.5 ± 0.2
Guanylic "B"	17.9		9.8 ± 0.7	16.8		4.12 ± 0.11	16.6		13.8 ± 0.3
Total Guanylic	33.5	4.70		31.5	4.36		29.8	4.13	

[1] S.A. = Specific activity in terms of counts/minute/γ-phosphorus. Values are averages of four to six determinations each.

[2] The specific activities of the undegraded nucleic acids from rabbit, normal rat and regenerating rat livers were 10.3, 4.68 and 14.4, respectively. The respective nucleic acid specific activities calculated from the sum of the mean values of the nucleotide specific activities were 10.15, 4.77 and 14.7.

[3] Calculated from material not bound to column in initial break-through (see text).

found by Chargaff et al.[10] for pig liver, although the adenylic acid content of the latter was somewhat lower. As was the case with all ribonucleic acids which have been similarly analyzed in this laboratory, two isomeric adenylic and two isomeric guanylic acids were found.[3,7] The relative proportions of the isomers is also typical, about 60% of the total in each case being accounted for by the second or "B" peak.

It is apparent from the table that in the rabbit, three hours after injection of ^{32}P, essentially uniform labeling of the mononucleotides of the liver ribonucleic acid had occurred. The experimental conditions were therefore altered in order to determine if an initial inhomogeneous incorporation of isotope took place in the liver ribonucleic acid. For this purpose, a shorter period of time, one hour, was allowed to elapse between injection of the isotope and sacrificing the animal, and to achieve sufficiently high specific activities in this length of time a smaller animal, the rat, was employed. It can be seen from the table that, under these conditions, in both normal and regenerating rat liver ribonucleic acid the adenylic acid isomers, which were equally labeled, had specific activities about 50% higher than the other mononucleotides. The specific activities of the latter were essentially the same. Since the specific activity of the undegraded nucleic acid phosphorus was almost identical with the mean value of the total mononucleotide specific activity (see footnote 2 of table), it is assumed that the phosphorus represented by the adenosine component did not have a significantly higher specific activity than that determined for adenylic acid. Regenerating rat liver ribonucleic acid, when compared with the normal, exhibits almost identical mononucleotide composition and the same pattern of isotope distribution among the nucleotides. However, in the case of the rapidly growing tissue the specific activities of the nucleotide phosphorus are about three times the value of the normal, in agreement with previous studies on nucleic acid turnover in regenerating liver.[11]

Although the experiments with normal and regenerating rat liver ribonucleic acids demonstrate higher phosphorus specific activities of the derived adenylic acids than the other mononucleotides, it cannot be assumed that the entire adenylic acid moiety undergoes a comparably higher metabolic turnover. Also, since nucleotides are bound in doubly esterified phosphate linkage in the nucleic acid molecule, it is possible that alkaline cleavage might result in either one of two phosphorus atoms being associated with adenosine in the derived nucleotide. The phosphorus of the derived adenylic acid thus may have been associated with the metabolism of the adjoining riboside rather than the adenosine moiety itself. The data presented here does indicate, however, that an inhomogeneous distribution of ^{32}P occurs in liver ribonucleic acids.

Barnum[12] has recently reported that nucleolar ribonucleic acid had a higher specific activity following ^{32}P administration than the nucleic acid of other fractions. It must be emphasized that the ribonucleic acids isolated in this study were derived from the small granule and soluble portions of the liver cell cytoplasm. This preparation contains the major part of the cell ribonucleic acid,[13] but does not include nucleolar or mitochondrial ribonucleic acid. The mononucleotide composition and specific activity distribution in ribonucleic acid contained in the latter portions of the cell may be quite different from those reported here.

Summary

One hour after injection of inorganic ^{32}P into the rat, the adenylic acid moiety of the ribonucleic acid had about 50% higher specific activity than the other mononucleotides, the specific activities of the latter being comparable. The isomeric adenylic acids were equally labeled.

The mononucleotide composition and specific activity of regenerating rat

liver ribonucleic acid followed the same pattern as the normal, although specific activities of the mononucleotides were about three times higher than their normal counterparts.

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