SIGNIFICANCE OF RIBONUCLEOTIDE REDUCTION IN THE BIOSYNTHESIS OF THE DEOXYRIBOSE MOIETY OF REGENERATING RAT LIVER DEOXYRIBONUCLEIC ACID*

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In Escherichia coli (Bagatell et al., 1959) and Lactobacillus leichmannii (Manson, 1960) the exclusive biosynthetic pathway yielding deoxyribonucleotides involves reduction of the corresponding ribonucleotides. That this pathway operates at least to some extent in animal tissues is evident from the results of tracer experiments in various mammalian organs (Rose and Schweigert, 1953), tumor cells (Horecker et al., 1958) and chick embryos (Reichard, 1959). Racker (1952) originally postulated the participation of deoxyribose aldolase (E. C. 4. 1. 2. 4) in the formation of deoxyribonucleotides in vivo. Although the function of deoxyribose aldolase has not been definitely established, it has been regarded to be that of a degradative enzyme in the catabolism of deoxyribosyl compounds. However, results of experiments with regenerating rat liver slices and tumor cells were recently interpreted in favor of the participation of the aldolase in the biosynthesis of deoxyribonucleotides (Groth and Jiang, 1966).

In the present paper the extent to which deoxyribonucleotides arise by direct reduction of the corresponding ribonucleotides was investigated. Rats subjected to partial hepatectomy were injected simultaneously with inorganic ^{32}P and ^{14}C-cytidine. The liver nucleic acids were isolated; RNA was degraded to nucleoside 2',3'-monophosphates and DNA to nucleoside 5'-monophosphates. The ^{32}P and ^{14}C were measured simultaneously in a liquid scintillation counter. Assuming rapid equilibration of the injected ^{32}P with the

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nucleic acid percursors, and provided that the ^{14}C distribution between cytosine and pentose remained constant, an identical ratio of specific activities of $^{32}P:^{14}C$ in CMP and dCMP would constitute strong evidence for direct reduction as the sole source of deoxyribonucleotides. Such a result was actually obtained.

EXPERIMENTAL PROCEDURES

Two male albino rats, each weighing 130 g, were subjected to partial hepatectomy (Bergstrand et al., 1948). At 22, 25 and 28 hours after the operation each rat received intravenously in the tail an injection of 1.25 μc of ^{14}C-cytidine, specific activity 256 mc/mmole, uniformly labeled (Radiochemical Centre, Amersham, England), 1.9 μmoles of unlabeled cytidine, and 100 μc of carrier-free $NaH_2{}^{32}PO_4$ in a volume of 0.3 ml neutral saline solution. Three hours after the final injection the rats were sacrificed by decapitation, the livers (combined weight, 5.3 g) homogenized in 20 ml of solution which was 0.05 M with respect to both Tris-HCl buffer, pH 7.4 and Na_2HPO_4. The sodium nucleates were prepared and the RNA hydrolyzed with KOH (Hecht and Potter, 1956). After acid precipitation of the DNA, the solution of 2',3'-ribonucleotides was neutralized and diluted to 150 ml.

The DNA was suspended in a small volume of water and the pH adjusted to 7.5. After the addition of 0.1 mg pancreatic deoxyribonuclease (E.C. 3.1.4.5) (1 x crystallized, Sigma) and 0.4 ml each of 0.5 M $MgCl_2$ and 0.025 M $CaCl_2$ the volume was diluted to 4 ml and incubation carried out at room temperature for 2 hours. The pH was then raised to 9.5, 0.1 mg snake venom phosphodiesterase (E.C. 3.1.4.1) (B grade, Russel's viper, Cal. Biochem.) added and incubation conducted at 37° for 3 hours. The pH was then adjusted to 7, the hydrolysate heated at 100° for 5 minutes, clarified by centrifugation and diluted to 50 ml.

The nucleic acid hydrolysates were applied to separate columns of Dowex-1-formate X8, 200-400 mesh (length 10 cm, diameter 0.9 cm) and 10 ml fractions collected at 20 minute intervals. For separation of ribonucleotides the column was eluted in succession with 0.05 M formic acid, 0.25 M ammonium formate, pH 5 and 2.5 M formic acid; for separation of deoxyribonucleotides with 0.05 M, 0.3 M, 1.2 M and 2.5 M formic acid. The absorbance of each fraction was recorded at 280 and 260 mμ and small aliquots plated to confirm the coincidence of radioactivity and ultraviolet absorbance. The peak fractions were pooled, the UMP eluate passed through a short column of Dowex-50-H^+ to remove ammonium ion, and the solutions evaporated to dryness. The eight nucleotides, which were obtained in quantities of 3 to 11 μmoles,

were dissolved in a small volume of water and checked for purity by determination of absorbance ratios (Cohn, 1957).

Simultaneous estimates of ^{32}P and ^{14}C were made with a Packard liquid scintillation counter type 3003.

The two nucleotides containing significant levels of ^{14}C, CMP and dCMP, were converted to the corresponding nucleosides by treatment with acid phosphatase (E. C. 3.1.3.2). Ten mg of enzyme (wheat germ, Sigma) were added to about 3 μmoles of each nucleotide in 2 ml volumes of 0.05 M acetate buffer, pH 4.6. After incubation for 3 hours at 37° the hydrolysate was neutralized and heated at 100° for 5 minutes, diluted to 10 ml and passed through a column of Dowex-1-formate X8, 200-400 mesh (length 5 cm, diameter 0.9 cm) in order to remove phosphate compounds. The nucleoside solutions were concentrated to a small volume and further purification performed by preparative chromatography on Whatman 3 MM paper with ethanol-5M ammonium acetate, pH 9.5-water (1:10:4) as solvent, using ultraviolet absorption for detection. The isolated nucleosides were characterized by their ultraviolet absorbance spectra (Cohn, 1957) and by paper chromatography (Reichard, 1958). The specific activities were determined on aliquots with a planchet counter.

Finally, 0.5 μmole aliquots of the cytidine and deoxycytidine, together with a specimen of the cytidine used for injection, were cleaved in hot formic acid (Reichard, 1957). After removal of the formic acid the preparations were purified by electrophoresis in 4 per cent formic acid (300 v, 12 ma, 2 hours) on Whatman 3 MM paper.

All values obtained by planchet counting were multiplied by the factor 1.24 to make them correspond to those found in the liquid scintillation device.

RESULTS AND DISCUSSION

After simultaneous injection of ^{14}C-cytidine and $NaH_2{}^{32}PO_4$ labeled nucleotides were isolated from regenerating rat liver RNA and DNA (Table I). Inasmuch as the biosynthesis of nucleic acids occurs from 5'-nucleoside triphosphates, the enzymic degradation of DNA assured that the phosphate remained attached to the original position. But in the case of RNA, which was degraded with alkali, the phosphate which appeared in the 2',3'-position of CMP was derived from a neighbouring nucleotide. Since all four ribonucleotides were found to have similar specific activities with respect of ^{32}P it is apparent that rapid equilibration had taken place and the source of the phosphate in 2',3'-CMP is hence immaterial so far as the interpretation of this experiment is concerned. As can be seen in Table I, the ratio of $^{32}P:{}^{14}C$ in CMP and dCMP is almost identical, thus indicating that direct ribonucleotide reduction is the

Table I. Distribution of ^{32}P and ^{14}C in the Nucleotides of Regenerating Rat Liver RNA and DNA after injection of $NaH_2{}^{32}PO_4$ and ^{14}C-Cytidine.

	Specific Activities (Counts/Minute/μMole)						
	Nucleotide			Nucleoside	Base	Pentose[2]	Base:Pentose
	^{32}P[1]	^{14}C	$^{32}P:^{14}C$	^{14}C	^{14}C	^{14}C	
CMP	13,170	5,860	2.24	5,770	2,470	3,300	0.75
AMP	16,600	nil	--	--	--	--	--
UMP	16,500	290	--	--	--	--	--
GMP	12,800	nil	--	--	--	--	--
dCMP	5,100	2,220	2.29	2,250	990	1,260	0.78
dAMP	9,750	nil	--	--	--	--	--
dTMP	8,650	200	--	--	--	--	--
dGMP	6,880	nil	--	--	--	--	--
Injected cytidine[3]	--	--	--	17,800	7,850	9,950	0.79

(1) Values confirmed by recounting after one half-life.
(2) Values obtained by difference.
(3) Aliquot diluted with unlabeled cytidine.

exclusive biosynthetic pathway in regenerating rat liver. The fact that the ^{14}C specific activity ratio of cytosine:pentose remained essentially constant in going from CMP to dCMP confirms once more the observation by Rose and Schweigert, (1953) that the bond between these moieties remains intact during the reduction process.

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