22.35. Found: C, 54.34, 54.21; H, 3.64, 3.73; N, 22.35, 22.48; dry ash, 0.40.

N-[α-(2-Amino-4-hydroxy-6-pteridyl)-*p*-anisoyl]-L-glutamic Acid (VIb).—To a mixture of 1.38 g. of sodium acetate and 1.80 g. of 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride was added 2.7 g. of crude diethyl *p*-(2-keto-3,3-diethoxypropoxy)-benzoyl-L-glutamate dissolved in 48 ml. of glacial acetic acid. The mixture was stirred under nitrogen and in the dark for 30 minutes at 25° and for one hour at 105–110°. The reaction mixture was cooled and centrifuged. The black solid was washed three times with water, dried and then washed with ethyl acetate. There was obtained 0.2 g. of material, $\lambda_{max}^{0.1\,N\ NaOH}$ 258 mμ, $E_{1\,cm.}^{1\%}$ 643; 366 mμ, $E_{1\,cm.}^{1\%}$ 178.

The acetic acid supernatant from the centrifuged product was concentrated and the black residue washed with water and ethyl acetate to give 1.37 g. of product, $\lambda_{max}^{0.1\,N\ NaOH}$ 258 mμ, $E_{1\,cm.}^{1\%}$ 674; 364 mμ, $E_{1\,cm.}^{1\%}$ 148. The combined products weighed 1.57 g. (60.8%).

A 0.5-g. sample of the crude pteridine was dissolved in 100 ml. of 0.1 *N* sodium hydroxide and diluted with 2 l. of water. To the solution was added 0.5 g. of calcium hydroxide and the mixture was stirred for 90 minutes, then heated to boiling and filtered. The solution was reheated to boiling and the *p*H adjusted to 3.0. A brown precipitate which settled on standing was collected, washed with water, and dried. There was obtained 0.24 g. of material $\lambda_{max}^{0.1\,N\ NaOH}$ 258 mμ, $E_{1\,cm.}^{1\%}$ 855; 364 mμ, $E_{1\,cm.}^{1\%}$ 185. Recrystallization from water containing a few drops of acetic acid gave a yellow solid which was collected by centrifugation. The solid was lyophilized and then dried for 5 hours at 100°.

Anal. Calcd. for $C_{19}H_{18}N_6O_7$: C, 51.58; H, 4.09; N, 18.99. Found: C, 51.08, 50.98; H, 4.25, 4.32; N, 18.67, 18.72; $\lambda_{max}^{0.1\,N\ NaOH}$ 259 mμ, $E_{1\,cm.}^{1\%}$ 889, 364 mμ, $E_{1\,cm.}^{1\%}$ 185.

α-(2,4-Diamino-6-pteridyl)-*p*-anisic Acid (VIc).—The *p*H of a mixture of 6.36 g. of 2,4,5,6-tetraminopyrimidine bisulfite[6] in 120 ml. of 65% ethanol was adjusted to 3.0 with dilute hydrochloric acid. A solution of 8.91 g. of IV in 40 ml. of 95% ethanol was added over a period of 20 minutes. The mixture was heated at 90° for 2 hours, then 3 ml. of concentrated hydrochloric acid added and heated for 15 minutes at 100°. The *p*H of the solution was adjusted to 4.5 with sodium hydroxide and the solution cooled to 2°. The yellow precipitate was collected by centrifugation and lyophilized to give 7.39 g. of product, $\lambda_{max}^{0.1\,N\ NaOH}$ 256 mμ, $E_{1\,cm.}^{1\%}$ 463; 364 mμ, $E_{1\,cm.}^{1\%}$ 130.

Saponification of 2.0 g. of the above material with 10% sodium hydroxide at room temperature gave 1.1 g. of crude VIc, $\lambda_{max}^{0.1\,N\ NaOH}$ 256 mμ, $E_{1\,cm.}^{1\%}$ 751; 364 mμ, $E_{1\,cm.}^{1\%}$ 200. Purification of this material was not attempted.

N-[α-(2,4-Diamino-4-hydroxy-6-pteridyl)-*p*-anisoyl]-L-glutamic Acid (VId).—In the manner described above 6.28 g. of IVa was treated with 2.96 g. of 2,4,5,6-tetraminopyrimidine bisulfite to give 3.95 g. of yellow solid, $\lambda_{max}^{0.1\,N\ NaOH}$ 256 mμ, $E_{1\,cm.}^{1\%}$ 559; 264 mμ, $E_{1\,cm.}^{1\%}$ 166. Saponification with 10% sodium hydroxide gave a yellow solid $\lambda_{max}^{0.1\,N\ NaOH}$ 257 mμ, $E_{1\,cm.}^{1\%}$ 528; 364 mμ, $E_{1\,cm.}^{1\%}$ 143. A mixture of crude VId and 250 mg. of calcium hydroxide in 75 ml. of 0.5 *N* sodium hydroxide was stirred for 1.5 hours at 25° and filtered. The *p*H was adjusted to 3.0 and the precipitate which formed collected and dried (40 mg.). Refrigeration of the mother liquors gave an additional 30 mg. of partially purified VId, $\lambda_{max}^{0.1\,N\ NaOH}$ 259 mμ, $E_{1\,cm.}^{1\%}$ 729; 368 mμ, $E_{1\,cm.}^{1\%}$ 167.

KALAMAZOO, MICHIGAN

[CONTRIBUTION FROM THE MCARDLE MEMORIAL LABORATORY, THE MEDICAL SCHOOL, UNIVERSITY OF WISCONSIN]

Intramolecular Heterogeneity in Nucleic Acid Biosynthesis[1,2]

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Non-uniform labeling of the nucleotide phosphorus and pyrimidines has been found in partial ribonuclease digests of RNA from cell fractions of rat liver and Flexner–Jobling carcinoma following simultaneous administration of P^{32}, orotic acid-6-C^{14} and glycine-2-C^{14}. Uniform labeling of phosphorus, pyrimidines and purines from DNA degradations, and of RNA purines has been demonstrated. Such non-uniformity was not found with alkaline hydrolysates of RNA. The specific activities of the phosphorus of a given 2′- and 3′- or 5′-mononucleotide were approximately the same. Terminal phosphorus had a higher specific activity than average nucleotide phosphorus. The compositions of partial ribonuclease digests varied among the cell fractions.

There is now at hand abundant evidence from this Laboratory and many others that demonstrates intracellular heterogeneity in nucleic acid biosynthesis. When radiophosphorus is administered to animals, the ribonucleic acid (RNA) isolated from the nuclei of liver and other tissues has a higher specific activity than that of the cytoplasmic particles; the specific activity of the RNA from the soluble fraction is intermediate.[4–7] A similar phenomenon is observed in the RNA purines following administration of glycine-2-C^{14} [6] and in the RNA pyrimidines biosynthesized from orotic acid-6-C^{14}.[8]

It was felt that further information might be gained concerning the mechanisms of nucleic acid biosynthesis in normal and neoplastic tissues by an investigation of the intramolecular distribution of radioactivity in partial degradation products of nucleic acids. We were encouraged in this belief by the interesting results of Steinberg and Anfinsen[9] who found non-uniform labeling in various fractions derived from ovalbumin biosynthesized both *in vitro* and *in vivo* in the presence of labeled carbon dioxide or alanine. Furthermore, Bendich, *et al.*,[10] have isolated from each of several rat tissues two desoxyribonucleic acids (DNA) that differ in their physical and metabolic characteristics.

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(2) An abstract of part of this work appears in *Federation Proc.*, **12**, 247 (1953).

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(8) R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.*, **195**, 257 (1952), and in press.

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(10) A. Bendich, P. J. Russell and G. B. Brown, *ibid.*, **203**, 305 (1953).

A promising experimental approach to this problem was afforded by the studies of Zamenhof and Chargaff with DNA[11] and of Magasanik and Chargaff with RNA.[12] They carried out desoxyribonuclease and ribonuclease digestions of nucleic acid preparations in dialysis bags, and demonstrated the formation of dialyzable fragments and a non-dialyzable fraction, which they termed the "core." They showed also that the proportions of the purine and pyrimidine bases changed throughout the incubation. We have carried out this type of enzymatic degradation on nucleic acids isolated from the livers and tumors of rats bearing transplantable tumors after injection simultaneously with P^{32} inorganic phosphate, glycine-2-C^{14} and orotic acid-6-C^{14}.

Our knowledge of the structure of the ribonucleic acids and the determination of enzymatic specificities has been greatly enlarged by the recent work of Brown and Todd,[13] Markham and Smith,[14] and Cohn and Volkin.[15,16] It is now established that RNA consists of polynucleotide chains in which the individual nucleotides are connected by phosphodiester linkages between the 3′- and 5′-positions of the ribose moieties. It is not yet certain whether branching occurs in the polynucleotide molecules. Alkaline and ribonuclease hydrolyses result eventually in mixtures of the 2′- and 3′-mononucleotides (nucleoside-2′- or 3′-phosphates); snake venom diesterase degradation gives predominantly the 5′-mononucleotides (nucleoside-5′-phosphates). The phosphate in the terminal position of the polynucleotide chains can be removed by the action of phosphomonoesterase. It was thus possible for us to make a comparison of the specific activities of the terminal and average nucleotide phosphorus, and of the specific activities of the individual 2′- and 3′-mononucleotides and the 5′-mononucleotides from a single sample of RNA.

Because differences in composition of the ribonucleic acids from different cell fractions have been demonstrated,[17] we have attempted to use preparations as homogeneous as possible by studying only RNA samples derived from separated cell fractions.

Experimental

Source of Materials.—Female rats,[18] weighing 150–170 g. were used. The tumor-bearing animals carried 10-day-old multiple subcutaneous transplants of Flexner–Jobling carcinoma.

The glycine-2-C^{14} was purchased from Tracerlab, Inc., on allocation from the U. S. Atomic Energy Commission, and the orotic acid-6-C^{14} was synthesized in these laboratories.[19]

Crystalline ribonuclease and desoxyribonuclease were purchased from General Biochemicals, Inc., Chagrin Falls, Ohio. Phosphomonoesterase was prepared from hypertrophic human prostate glands by the method of Markham and Smith.[14b] Snake venom diesterase was purified as described by Hurst and Butler.[20] The first chromatographic peak from the water elution afforded the diesterase.

Radioactivity Determinations.—The P^{32} was counted in the liquid state in annulus counters (Radiation Counter Laboratories) or dipping counters (Tracerlab, Inc.). Under these conditions, C^{14}, if present in the samples, was not counted. Total phosphorus analyses were carried out as described by LePage.[21]

The chromatographically separated purine and pyrimidine bases were plated on aluminum discs from 0.05 *N* HCl, counted in internal, gas-flow, proportional counters[22] and corrected for self-absorption. They were then eluted from the discs with 0.1 *N* HCl and the quantities were determined by their ultraviolet absorption spectra, using the constants of Vischer and Chargaff.[23] All samples were counted sufficiently long to achieve at least 10% precision, and were determined at least in duplicate.

Nucleic Acid Isolations.—The cell fractionations were carried out on homogenates of perfused livers and of tumors in isotonic sucrose[24] with the modifications previously described.[6] The nuclei were washed with citric acid, and the mitochondrial fraction contained the "poorly sedimented layer."[25] The sodium nucleates were precipitated with ethanol from hot NaCl extracts of the acid-insoluble residue of individual cell fractions.[6]

Analytical data for sodium nucleates prepared by this method have been published.[6] It is worth noting that the "phosphoprotein" of high specific activity[7] is not extracted from the acid-insoluble precipitate by hot NaCl. Furthermore, all samples have been chromatographed on ion-exchange after hydrolysis, and radioactivity has *only* been found in those fractions corresponding to the mononucleotides.

Enzymatic Incubations.—The phosphomonoesterase incubation of microsomal RNA (7 mg.) was carried out at *p*H 5.3 and 37° for 36 hours with 2 mg. of the enzyme preparation. The nucleic acid and enzyme were precipitated with three volumes of cold ethanol, and after standing in the cold overnight the supernatant fluid from centrifugation was counted and analyzed for inorganic phosphate. No organic phosphate was detectable.

The diesterase incubation was performed on 10 mg. of microsomal RNA in 3 ml. of solution with 2 mg. of the enzyme preparation at 37° for 48 hours. The solution was maintained at *p*H 9 by frequent neutralizations during the course of the incubation. The mixture was placed directly on an ion-exchange column and chromatographed as described below.

The ribonuclease incubations of cytoplasmic RNA samples were carried out in dialysis bags (23/32 NoJax, Visking Corp., Chicago) at 37°. The samples had been previously dialyzed in the absence of enzyme against 0.2 *N* acetate buffer at *p*H 6 for 18 hours. Enzyme was then added to the bag[12] and the dialysis was carried out against buffer for 8 hours. The dialysate was removed, and dialysis against buffer was carried out for an additional 10 hours. The material remaining in the dialysis bag at the end of this time was arbitrarily designated as the core.

The nucleic acids from the nuclear fraction contained both DNA and RNA and were treated, after predialysis against buffer, by the following procedure. Ribonuclease was added and the dialysis was carried out as just described. The total DNA and the RNA core remained in the dialysis bag. The contents were made 0.1 *M* to NaOH and incubated at 37°.[26] The RNA core was hydrolyzed to mononucleotides which were dialyzable. Dialysis was continued until no more material was removed. The DNA, which remained non-dialyzable, was treated with desoxy-

(11) S. Zamenhof and E. Chargaff, *J. Biol. Chem.*, **187**, 1 (1950).

(12) B. Magasanik and E. Chargaff, *Biochim. Biophys. Acta*, **7**, 396 (1951).

(13) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952).

(14) (a) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952); (b) 558 (1952); (c) 565 (1952).

(15) W. E. Cohn, D. G. Doherty and E. Volkin, "Phosphorus Metabolism," Vol. 2, McElroy and Glass, ed., The Johns Hopkins Press, Baltimore, Md., 1952, p. 339.

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(17) D. Elson and E. Chargaff, ref. 15, p. 339.

(18) Obtained from the Holtzmann Rat Co., Madison, Wis.

(19) C. Heidelberger and R. B. Hurlbert, THIS JOURNAL, **72**, 4704 (1950).

(20) R. O. Hurst and G. C. Butler, *J. Biol. Chem.*, **193**, 91 (1951).

(21) G. A. LePage, *Methods in Medical Research*, **1**, 345 (1948).

(22) We gratefully acknowledge the skilled technical assistance of Mrs. Edith Walestad and Mrs. Dorothy McManus in these measurements.

(23) E. Vischer and E. Chargaff, *J. Biol. Chem.*, **176**, 703 (1948).

(24) W. C. Schneider and G. H. Hogeboom, *ibid.*, **183**, 123 (1950).

(25) V. R. Potter, R. O. Recknagel and R. B. Hurlbert, *Federation Proc.*, **10**, 646 (1951).

(26) Under these conditions no dialyzable fragments were obtained from purified DNA preparations.

ribonuclease[11] at 37°, and dialysis was carried out for 36 hours. The material remaining in the bag was arbitrarily designated as the DNA core.

Chemical Hydrolyses.—The ribonucleic acids dialysates and cores were hydrolyzed in 0.1 *N* NaOH at 37° for 24 hours, which converted them into a mixture of the 2′- and 3′-nucleotides. Under the conditions of RNA hydrolysis with *N* NaOH at 37° some conversion of cytidylic into uridylic acid occurs. However under the present conditions, with 0.1 *N* NaOH at 37°, this conversion does not take place to any measurable extent and the sodium nucleates are quantitatively hydrolyzed to mononucleotides.[6,8] The separated purine nucleotides were hydrolyzed with 1 *N* HCl at 100° for 1 hr., diluted to a concentration of 0.1 *N* in HCl, and placed on 6 × 20 mm. columns of Dowex-50. The $P^{32}O_4$ was washed through with 25 ml. of water and the purines were eluted with 6 *N* HCl, evaporated and plated.

The separated pyrimidine nucleotides were hydrolyzed with 9 *N* perchloric acid 1 hour at 100°. They were then neutralized with KOH, cooled, and the $KClO_4$ was removed by centrifugation. The samples were diluted to 15 ml. and placed on 1 × 20 cm. columns of Dowex-1-formate. The pyrimidines were eluted with water, evaporated and plated. The $P^{32}O_4$ remained on the column.

In experiment 3, the individual RNA dialysates and cores were hydrolyzed with 1 *N* HCl at 100° for 1 hour and the DNA dialysates and cores were similarly treated for 20 minutes to give pyrimidine-2′- and 3′-mononucleotides, purines and inorganic phosphate.

Chromatographic Separations. Procedure A.—The products of alkaline hydrolysis were neutralized and placed on 1 × 20 cm. columns of Dowex-1-formate. They were eluted with formic acid continuously increasing from 0–4 *M* in a mixing apparatus.[27] Five-ml. fractions were collected automatically and sharp separation of the nucleotides was obtained.[6]

The incubation mixture from the diesterase degradation was placed on a Dowex-1-formate column and eluted with water until all ultraviolet-absorbing nucleotides were removed. The columns were then eluted with increasing concentrations of formic acid as described above. The fact that the diesterase treatment gives only 5′-nucleotides and no 3′-nucleotides was further confirmed by ion-exchange chromatography on Dowex-1-formate eluted with increasing concentrations of ammonium formate at *p*H 5, which gives good separation of the 3′- and 5′-mononucleotides.[28]

Procedure B.—This method was developed to utilize hand-operated columns for large numbers of samples. The mixture of pyrimidine nucleotides, purines and inorganic phosphorus resulting from 1 *N* HCl hydrolysis was neutralized and placed on 3 × 5 cm. columns of Dowex-50 (H^+ form). The columns were washed with water, and the inorganic "purine" phosphate (derived from purine-3′-nucleotides) and uridylic acid were obtained in the effluent. Cytidylic acid was eluted with 2 *N* HCl, and purified by rechromatography. Adenine and guanine were eluted with 6 *N* HCl, dried *in vacuo*, and placed on 5 × 30 mm. Dowex-50 columns. The guanine was eluted with 2.5 *N* HCl, the adenine with 4 *N* HCl. The mixture of inorganic phosphorus and uridylic acid was placed on 3 × 5 cm. columns of charcoal (Nuchar B) and washed with water. The inorganic phosphorus was found in the effluent, the uridylic acid was eluted with 10% pyridine.

The "pyrimidine" phosphorus was counted with the liquid counters, and the pyrimidine-C^{14} content was determined in the internal counters by means of differential absorbers after the P^{32} was allowed to decay.

The adenine and guanine analyses reported in Table I were obtained by paper chromatography with the butanol–ammonia solvent system of Chargaff, *et al.*,[29] following hydrolysis with HCl. The papers were eluted, and the ultraviolet absorption was measured.[23]

Dosage.—In each experiment four rats were used, and the organs were pooled. The labeled precursors were administered by intraperitoneal injection:

(27) H. Busch, R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.*, **196**, 717 (1952).

(28) We are greatly indebted to Dr. R. B. Hurlbert for instruction in this procedure, R. B. Hurlbert, H. Schmitz, A. Brumm and V. R. Potter, *ibid.*, in press.

(29) E. Chargaff, R. Lipshitz, C. Green and M. E. Hodes, *ibid.*, **192**, 223 (1951).

Experiment 1:—P^{32}, 270 μc./100 g. sacrificed in 40 min.
Experiment 2:—P^{32}, 100 μc./100 g.; glycine-2-C^{14}, 12 μc./100 g.; orotic acid-6-C^{14}; 1 μc./100 g. sacrificed in 4 hours.
Experiment 3:—P^{32}, 33 μc./100 g.; glycine-2-C^{14}, 33 μc./100 g.; orotic acid-6-C^{14}, 2 μc./100 g. sacrificed in 4 hr.

Results and Discussion

The data in Table I clearly show that in addition to differences in composition among the ribonucleic acids of each cellular component[17] there are marked variations in their nucleotide sequences. It is striking that in the nuclear and supernatant RNA during the course of ribonuclease hydrolysis and dialysis the guanine is liberated much more rapidly than the adenine, whereas in the particulate mitochondria and microsomes the reverse is true. Because both situations obtain, it cannot be argued that such an effect is due to a difference in permeability of the dialysis membrane to the two bases, but can most reasonably be explained on the basis of differences in the nucleotide sequences among the cell fractions. Thus it appears that the structures of the ribonucleic acids of the particulate elements of the cell differ markedly from those of the nuclei and the soluble fraction, at least in rat liver. It is of interest in this connection that we have shown[6] that nucleic acid purine biosynthesis, following administration of glycine-2-C^{14}, takes place most rapidly in the nuclear fraction, with the supernatant intermediate, and the particulates the slowest. It is not yet possible to discern the exact relationship between nucleic acid structure and the mechanism of its biosynthesis, yet it seems self-evident that a close correlation must exist. It is the aim of the work reported here to gather data relating biosynthesis and structure, and it is to be hoped that some of the results that cannot yet be interpreted may eventually be clearly understood when our knowledge of nucleic acid structure is extended to include the nucleotide sequences.

TABLE I

RIBONUCLEASE HYDROLYSIS OF LIVER NUCLEIC ACIDS

Hours	Accumulated % of total material in dialysate: Total P	Adenine	Guanine
	Nuclei		
2	34	0.8	32
6	51	35	40
24	70	92	80
	Mitchondria		
2	60	77	0.9
6	85	88	40
24	87	92	81
	Microsomes		
2	34	52	34
6	44	76	42
24	75	78	69
	Supernatant		
2	71	0.2	52
6	82	1.1	63
24	85	78	78

The results given in Table I also support the contention that ribonucleic acids exist as complex mixtures,[14,15] and indicate that much research, both physical and chemical, needs to be devoted

to the question of structural heterogeneity of ribonucleic acid.

In initiating the isotopic experiments it appeared to us that, since the phosphorus atoms are held in diester linkages between the 3'- and 5'-carbons of the ribose of adjacent nucleotides, interpretation of results of partial enzymatic degradations would rest upon a knowledge of the comparison of the specific activities of the phosphorus of the 2'- and 3'- and of the 5'-mononucleotides. Accordingly, rats were sacrified 40 minutes after administration of P^{32}, and the microsomal RNA was isolated. This fraction was selected because it contains the largest amount of RNA in the cell of any single fraction. One aliquot was subjected to alkaline hydrolysis, and the specific activities of the separated 2'- and 3'-nucleotides were determined. No difference in specific activity between the 2'- and the 3'-nucleotides was observed, which is to be expected since they both result from a cyclic diester intermediate.[13,14a] Another aliquot was degraded with diesterase to give the 5'-mononucleotides, whose specific activities were determined. Finally, a third aliquot was treated with phosphomonoesterase, and the specific activity of the inorganic phosphorus was measured. The results in Table II show that there is no marked difference in the specific activities of the 5'- as compared with the 2'- and 3'-nucleotides. It is felt that the small differences observed are experimentally significant but not sufficiently large to be conducive to interpretation.

TABLE II

SPECIFIC ACTIVITY OF P^{32} FROM MICROSOMAL RNA (40 MINUTES), C.P.M./μG. P (COR.)

Terminal P		2.5
Av. nucleotide P		1.4
	2',3'-P	5'-P
Adenylic	2.3	2.4
Guanylic	1.0	0.72
Cytidylic	1.1	1.3
Uridylic	1.7	1.5

It appears very likely that free mononucleotides are intermediates in nucleic acid biosynthesis[6,8,30–33] but it is not yet established whether the 3'- or 5'-nucleotides are on the biosynthetic pathway. This point is now under investigation. However, since their specific activities are similar in the polynucleotide (at least in the liver microsomal RNA studied here) even at as short a time as 40 minutes, interpretation of specific activity data of nucleotides obtained by partial ribonuclease degradations will not be complicated by considerations of whether the phosphorus was incorporated in a 3'- or a 5'-nucleotide.

On the other hand, there is a marked difference between the specific activity of the "terminal" phosphorus and the average nucleotide phosphorus. The term "terminal" phosphorus must be qualified because it represented about 30% of the total nucleic acid phosphorus, a figure that appears to be too high.[14c] Regardless of whether it is high because of depolymerization of the nucleic acid, or because of impurities in the enzyme preparation, the fact that its specific activity is almost twice that of the average nucleotide phosphorus demonstrates conclusively that there is intramolecular heterogeneity in nucleic acid biosynthesis. It is of some interest that whereas the specific activity of the adenylic acid P approximates that of the terminal P it is probably not a terminal nucleotide.[14,15] The mechanistic implications of such heterogeneity are not clear on the basis of experiments using P^{32} alone, so the work was extended to include other labeled nucleic acid precursors as well.

Table III gives the results of an experiment in which P^{32}, glycine-2-C^{14} and orotic acid-6-C^{14} were administered simultaneously to rats. The animals were sacrified after four hours and the microsomal RNA was isolated. The preparation was incubated with ribonuclease as described in the experimental section, and 8- and 18-hour dialysates and a core were obtained. It should be pointed out that the dialysis was stopped somewhat before it had gone to completion in order to assure enough material to carry out the necessary isolations. It will be noted that the dialysates are rich in substances that give pyrimidine mononucleotides on hydrolysis, and the core is rich in purine components, especially guanine. This is in agreement with the observations of Magasanik and Chargaff,[12] and arises as a consequence of the specificity of ribonuclease,[14,15] which is unable to split internucleotide phosphodiester bonds, in which the phosphorus is attached to the 3'-position of a purine nucleotide. It is also of interest that the cores from our RNA preparations are dialyzable up to 90% in the presence of 2 *M* sodium chloride, in agreement with the findings of Markham and Smith[14c] with yeast and virus ribonucleic acids. Thus these "cores" are probably not "structural" in the sense of being a continuous backbone in the middle of the molecule, but are rather polynucleotide units, dispersed throughout the molecule. that contain linkages resistant to enzymatic hydrolysis. That these "core" units are not highly polymerized is indicated by the fact that they dialyze in strong salt solutions.

TABLE III

SPECIFIC ACTIVITIES OF P^{32} AND C^{14} FROM MICROSOMAL RNA, C.P.M. $\times$ 10/μG. OF P OR BASE

	8 hours			18 hours			Core		
	%	P^{32}	C^{14}	%	P^{32}	C^{14}	%	P^{32}	C^{14}
Adenylic	38	84	1.1	26	97	0.84	36	68	0.35
Guanylic	28	53	0.34	21	58	0.28	51	61	0.35
Cytidylic	65	70	53	13	74	47	22	60	31
Uridylic	60	80	122	20	77	98	20	61	71

It will be noted from Table III that the specific activities of the 3'-phosphates in both dialysis fractions are essentially the same or slightly higher in the 8-hour samples. However, the specific activities of the P^{32} in the nucleotides of the core are significantly lower. Therefore, regardless of the true nature of the core, an intramolecular heterogeneity or non-uniform labeling within the poly-

(30) M. E. Balis, D. H. Levin, G. B. Brown, G. B. Elion, H. Vanderwerff and G. H. Hitchings, *J. Biol. Chem.*, **200**, 1 (1953).

(31) H. Schmitz, R. B. Hurlbert and V. R. Potter, *ibid.*, in press.

(32) M. Edmonds and G. A. LePage, *Federation Proc.*, **12**, 199 (1953).

(33) G. A. LePage, *Cancer Research*, **13**, 178 (1953).

nucleotide chains has been demonstrated. The same situation obtains in the pyrimidine bases. The specific activity of the C^{14} in the core is distinctly lower than in the dialyzable fractions. The situation is not as clear in the case of the purines. Although the adenine in the core has a lower specific activity than in the dialyzable fractions, no such difference is found with guanine. The explanation of this observation is not yet clear. Nevertheless, it can be said with certainty that there is non-uniform labeling in the phosphorus of all nucleotides except possibly in guanylic acid and in the pyrimidine bases within the RNA molecule.

In order to confirm and extend these results, an experiment was carried out in tumor-bearing animals and measurements were made of the DNA and the RNA of nuclei, mitochondria, microsomes and supernatant fractions in liver and Flexner-Jobling carcinoma. A comparison of liver and tumor with respect to nucleic acid biosynthesis has been under investigation in this Laboratory for some time.[6,34-36] In this experiment the livers of tumor-bearing animals were used. However, we have shown[6] that the only significant difference in nucleic acid biosynthesis between the livers of normal and tumor-bearing animals is found in an enhancement of the incorporation of labeled precursors into DNA nucleotides. This effect is correlated with an increased mitotic rate in the livers of tumor-bearing animals. In such livers, there were no detectable differences in RNA biosynthesis as compared with those from normal animals.[6]

The experiment was conducted as described in the experimental section. It should be noted that the P^{32} specific activities were obtained as purine-P and pyrimidine-P, instead of in the separated nucleotides. Dialysis was carried out for 18 hours.

The results obtained with the DNA of liver and tumor are given in Table IV. Although small variations of the individual specific activities have been found between dialysate and core in both tissues, there appear to be no major differences. Thus, with respect to partial desoxyribonuclease degradation in both liver and tumor, there is no significant non-uniform labeling in the DNA, at least at the time interval studied here, as contrasted with the findings with RNA. It appears from this criterion that there is intramolecular homogeneity in DNA synthesis, which is additional support for our conclusions[6] that DNA is synthesized from various precursors at the same rate. Although Bendich, *et al.*,[10] have shown in many tissues that there are at least two DNA's that differ with respect to solubility and metabolic rates, they have found only one in liver, and have reported no examinations of the DNA of tumors. Therefore, there is no contradiction between the present finding and theirs. Moreover, it is possible that the two DNA's obtained from a tissue, even though of different specific activities, might show uniform labeling in partial enzymatic degradations of this sort. However, the relationship of these two aspects of DNA biosynthesis and structure will have to await further experimentation.

TABLE IV

SPECIFIC ACTIVITIES OF P^{32} AND C^{14} FROM DNA, C.P.M./μG.

	Purine-P	Pyrimidine-P	Thymine	Cytosine	Adenine	Guanine
Liver						
Dialysate	1.2	1.4	0.39	2.2	0.21	0.34
65% core	1.6	1.9	0.39	3.2	0.23	0.59
Tumor						
Dialysate	11		1.1	4.8	4.8	11
6% core	12		1.2	5.5	5.4	13

The data for the RNA of the various cell fractions in liver and tumor are given in Table V. In the case of liver it is evident that both the purine and pyrimidine phosphorus of the dialysate have a higher specific activity than the core in all cell fractions, which confirms and extends the results presented in Table III. In the case of uracil and cytosine the dialysate has a higher specific activity than the core in the nuclei, mitochondria and microsomes. However, in the supernatant there is uniform labeling throughout the molecule.

TABLE V

SPECIFIC ACTIVITIES OF P^{32} AND C^{14} FROM LIVER RNA, C.P.M./μG.

	Purine-P	Pyrimidine-P	Uracil	Cytosine	Adenine	Guanine
Nuclei						
Dialysate	11.1	18	27	19	2.7	3.3
18% core	8.5	12	9.1	16	2.8	2.2
Mitochondria						
Dialysate	3.6	4.1	6.8	6.3	0.52	0.80
22% core	1.7	3.5	5.3	4.7	0.47	1.0
Microsomes						
Dialysate	6.2	5.7	11	5.1	0.69	1.1
16% core	5.4	3.3	6.0	2.7	0.95	1.1
Supernatant						
Dialysate	4.4	13	9.9	9.5	1.8	2.7
18% core	1.1	5.8	11	9.9	1.9	4.9
Tumor RNA						
Nuclei						
Dialysate	20		2.0	0.5	19	20
22% core	13		1.6	1.0	21	23
Mitochondria						
Dialysate	5.7		0.6	0.7	4.7	23
12% core	2.7		1.2	1.5	5.5	13
Microsomes						
Dialysate	18		1.0	0.8	10	16
18% core	9.3		1.7	1.3	10	18
Supernatant						
Dialysate	14		5.0	1.5	7.8	21
22% core	5.9		6.0	2.6	5.9	31

The specific activities of the adenine and guanine of the dialysate and core show small variations in both directions, but no systematic trend is discernible. Hence we may conclude that the labeling of the RNA purines tends to be uniform, at least as revealed by this type of enzymatic partial degradation.

(34) G. A. LePage and C. Heidelberger, *J. Biol. Chem.*, **188**, 593 (1951).

(35) C. Heidelberger and G. A. LePage, *Proc. Soc. Exptl. Biol. Med.*, **76**, 464 (1951).

(36) E. P. Tyner, C. Heidelberger and G. A. LePage, *Cancer Research*, **12**, 158 (1952).

In the Flexner–Jobling carcinoma the specific activity of the purine P^{32} is higher in the dialysate than in the core in all RNA's studied. Unfortunately owing to the decay of the P^{32}, comparable data on the pyrimidine phosphorus are not available. In all cases except one, the uracil and cytosine of the core have a higher specific activity than those of the dialysate, in contrast to the findings with liver. In the tumor, as in liver, there is no systematic trend in the purines and it may be concluded once again that there is uniform labeling in the purines.

It would be anticipated that the intramolecular heterogeneity that has been repeatedly demonstrated is revealed as a consequence of the ribonuclease specificity and that a comparable experiment with alkaline hydrolysis and concomitant dialysis, in which the alkali would be expected to attack the internucleotide bonds at random, would show uniform labeling. A number of such comparisons have been carried out with completely consistent results. A typical comparison is made in Table VI for the case of liver RNA uracil and shows as expected that there is uniform labeling in the alkaline hydrolysis of all cell fractions, whereas there is non-uniform labeling in all the enzymatic degradations.

TABLE VI

SPECIFIC ACTIVITY OF C^{14} IN LIVER RNA URACIL, C.P.M./μG.

	% core	Enzymatic hydrolysis	% core	Alkaline hydrolysis
Nuclei				
Dialysate		27		22
Core	18	9.1	25	19
Mitochondria				
Dialysate		6.8		4.2
Core	22	5.3	34	4.7
Microsomes				
Dialysate	16	10.6	33	8.2
Core		6.0		8.5
Supernatant				
Dialysate		9.9		9.2
Core	18	10.8	33	10.0

In comparing the results of the partial degradation of the RNA fractions from liver and tumor, similar effects are observed with the phosphorus and purines in both cases. However, in the liver the pyrimidines of the dialysate have a higher specific activity than those of the core, whereas in the tumor the reverse is true. This may be due to the increased rate of nucleic acid synthesis that has been found in these tumors as compared with liver,[6,34–36] but proof of this point must await further experimentation at different time intervals after injection of the labeled precursors.

The fact that uniform labeling is found with the purines and non-uniform labeling with the pyrimidines is somewhat surprising and cannot be interpreted successfully at this time. It may reflect the fact that orotic acid is a more proximal precursor than glycine or that purine and pyrimidine nucleotides are grouped differently in the nucleotide sequence. It might also indicate that some pyrimidine transribosidation takes place.[37] In any event, regardless of these or other possible explanations, considerably more research must be done, particularly with respect to determinations of nucleotide sequence, before these phenomena are fully understood.

The explanation for the difference in behavior between the phosphorus and purines is likewise not at hand, although it has been shown[6] that the rates of incorporation of P^{32} and glycine-2-C^{14} into RNA nucleotides are different.

It has been inferred[5] that, because of the high initial specific activity of the nuclear RNA as compared with the cytoplasmic RNA's after administration of P^{32}, the ribonucleic acid is synthesized in the nucleus and then passes into the cytoplasmic fractions. Recent calculations based on kinetic data[38] do not support this concept, but rather, that there is a precursor common to all the cell fractions. The fact that the various cell fractions show non-uniform labeling to different extents, particularly in the pyrimidines from the supernatant RNA in liver, suggests that if indeed the RNA's of various cell fractions are interconvertible there must be considerable alteration of the nucleotide sequence during the process.

The implications of non-uniform labeling resulting from the biosynthesis of macro-molecules have been thoroughly discussed by Steinberg and Anfinsen.[4] As a result of their demonstration of non-uniform labeling of alanine and other amino acid residues in the ovalbumin molecule, they concluded that the protein could not have been synthesized by an instantaneous template mechanism, but rather by a stepwise mechanism. It appears from the present study that the RNA polynucleotide molecules may also be formed by a stepwise process, but definitive interpretation is rendered impossible at present because of the complexity and lack of homogeneity of the ribonucleic acids and because of the broad generality of the degradative procedures here used. Work is now in progress to measure the specific activities of well-characterized di- and tri-nucleotides obtained during the course of ribonuclease degradation,[14,15] which should provide a more definitive understanding of this aspect of nucleic acid biosynthesis.

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(37) I. A. Rose and B. S. Schweigert, *J. Biol. Chem.*, **202**, 635 (1953).

(38) Private communication from Dr. C. P. Barnum.