hours. The chloroform was removed by distillation and the oily residue was dissolved in 35 ml. of hot ethanol. The needles which formed upon cooling were removed by filtration and dried to produce 1.2 g. of 3,5,4'-tribromo-4-ethoxybiphenyl with melting point 100–101°. A mixture of this material with some made by ethylation of 3,5,4'-tribromo-4-hydroxybiphenyl[10] did not change the melting point. The yield was 33.0% of the theoretical.

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[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

The Preparation and Properties of Mammalian Ribonucleic Acids[1]

BY E. VOLKIN AND C. E. CARTER

A method, which is of general application to mammalian tissues, is described for preparing purified ribonucleic acids. The mammalian ribonucleic acids have been characterized with respect to mononucleotide analytical composition as well as nitrogen and phosphorus content. The extent of liberation of titratable phosphate groups by ribonuclease has been determined. Preliminary experiments with the analytical ultracentrifuge reveal that the nucleic acid preparations exist essentially as single sedimenting boundaries.

A method for the preparation of mammalian ribonucleic acids employing high concentrations of guanidine hydrochloride in the fractionation procedure is reported in this paper. Aside from the facility of preparation the method has the advantage of avoiding conditions of acidity and alkalinity which might degrade tissue ribonucleic acids and, by virtue of the protein denaturant action of guanidine salts, of minimizing the possibility for enzymatic degradation.

Ribonucleic acids prepared from yeast by alkaline extraction are reported to have a molecular weight of about 17,000[2,3] while a highly labile ribonucleic acid prepared from tobacco mosaic virus by heat denaturation had a molecular weight of about 300,000.[4] Relatively high molecular weight ribonucleic acids have been prepared from viruses by Markham, *et al.*,[5] and from bacterial surfaces by Stacey.[6] Pancreas ribonucleic acid has been prepared by Levene and Jorpes,[7] Hammarsten,[8] Kerr and Seraidarian,[9] and Allen and Bacher.[10] However, the particle size or homogeneity of these latter preparations was not assessed.

In view of the apparent dependence of the composition of ribonucleic acids upon the methods of preparation as well as the source[9,10] certain considerations appear necessary for a proper assessment of physical and chemical descriptions of ribonucleic acid preparations: (a) The preparative procedure and source, (b) a statement of the physical-chemical homogeneity of the ribonucleic acid, (c) a description of the degradation of the ribonucleic acid by ribonuclease, and (d) a statement of the mononucleotide composition of the ribonucleic acid. It is with reference to these considerations that mammalian ribonucleic acids prepared by the guanidine salt procedure hereinafter described have been studied.

Experimental and Results

Method of Preparation of Mammalian Tissue Ribonucleic Acid.—The method of isolation of ribonucleic acid from tissue homogenates consisted of (a) the removal of desoxyribonucleic acid as a nucleic acid–protein complex,[11] (b) the precipitation of the ribonucleic acid from a cold 2 *M* guanidine hydrochloride solution in which the large bulk of protein remains soluble, and (c) further purification of the ribonucleic acid by chloroform extraction[12] and alcohol precipitations.

The possibility of the occurrence of nuclease action on ribonucleic acid during the preliminary steps of the preparation can be obviated by immediately homogenizing the tissue in concentrated guanidine hydrochloride. The latter reagent is an effective protein denaturant. These procedures were found to be applicable to a number of mammalian tissues; details of the methods of preparation follow.

Fresh or frozen tissue was cut in small pieces and blended for 6 to 8 minutes with 3 volumes per gram tissue of a 0.15 *M* sodium chloride–0.02 *M* phosphate buffer, *p*H 6.8. A few drops of octyl alcohol were added to reduce foaming. The homogenate was then centrifuged at 3000 g. for 30 minutes. Essentially all the desoxyribonucleic acid was removed in the form of an insoluble nucleic acid–protein complex as described by Mirsky and Pollister.[11] All operations were carried out between 2 and 5°.

To the supernatant solution enough solid guanidine hydrochloride was added, with rapid stirring, to make the solution 2 molar with respect to guanidine hydrochloride. The solution was placed in a 38° bath and allowed to stand at this temperature for 30 minutes, then chilled at 0° for 1 hour. Under these conditions most of the protein of the tissue extract remained soluble, while a gelatinous precipitate formed which contained ribonucleic acid and a small amount of protein. The precipitate was washed twice with a cold solution of 2 *M* guanidine hydrochloride in *p*H 6.8 saline–phosphate buffer. By this process any desoxyribonucleoprotein which remained soluble in the high guanidine concentration was removed by the washing process. To remove the contaminating protein the precipitate was then suspended in 2 *M* guanidine hydrochloride (one volume per gram of original tissue) and extracted with chloroform–octyl alcohol (5:1).[12] The suspension of nucleic acid in guanidine hydrochloride was added to an equal volume of the chloroform:octyl alcohol mixture, warmed to 40°, then shaken mechanically for 30 minutes. The mixture was centrifuged and the upper aqueous layer containing the nucleic acid removed. The extraction of the aqueous solution at 40° was repeated twice with fresh chloroform:octyl alcohol. Extractions in the cold, or in saline or water solutions, resulted in incomplete separation of the nucleic acid from protein. Nucleic acid was precipitated in the cold from the guanidine solution by adjusting the acidity to *p*H 4.2–4.5 with acetic acid and adding two volumes of cold ethanol. The white, flocculent ribonucleic acid precipitate was cen-

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(1) Work performed under Contract Number W-7405-Eng-26 for the Atomic Energy Commission.

(2) H. L. Loring, *J. Biol. Chem.*, **128**, LXI (1939).

(3) M. Kunitz, *J. Gen. Physiol.*, **24**, 15 (1940).

(4) S. S. Cohen and W. M. Stanley, *J. Biol. Chem.*, **144**, 589 (1942).

(5) R. Markham, E. F. Mathews and K. M. Smith, *Nature*, **162**, 88 (1948).

(6) M. Stacey, *Symposia of the Soc. for Exp. Biol.*, No. 1, 86 (1948).

(7) P. A. Levene and E. Jorpes, *J. Biol. Chem.*, **86**, 389 (1930).

(8) E. Hammarsten, *ibid.*, **43**, 243 (1920).

(9) S. E. Kerr and K. Seraidarian, *ibid.*, **180**, 1203 (1949).

(10) J. E. Bacher and F. W. Allen, *ibid.*, **183**, 641 (1950).

(11) A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.*, **30**, 117 (1946).

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TABLE I

ANALYTICAL COMPOSITION, RIBONUCLEASE HYDROLYSIS AND SEDIMENTATION CONSTANTS OF MAMMALIAN RIBONUCLEIC ACIDS

RNA source	Nitrogen–phosphorus composition % P	% N	N/P	Ion-exchange analysis: Units recovered, %	Molar ratios: Cytidylic	Adenylic	Uridylic	Guanylic	Purine/pyrimidine ratio	Ribonuclease action acid equivalents per mole phosphorus	Sedimentation constants ($S_{20,w}$)
Rabbit liver 1				94	1.70	1.01	1.00	2.03	1.13		2.31, 5.50[a]
Rabbit liver 2	9.19	16.1	1.74	92	1.69	1.05	1.00	2.02	1.12	0.41	
Rat liver				95	2.21	1.17	1.00	2.14	1.03		
Regenerating rat liver	8.70	15.6	1.79	97	1.97	1.08	1.00	2.05	1.05	.38	
Mouse liver	9.18	16.1	1.74	96	1.49	1.16	1.00	1.88	1.22	.41	
Mouse hepatoma				98	1.87	1.21	1.00	1.87	1.07		
Calf liver 1				96	1.77	1.19	1.00	2.13	1.20		2.23
Calf liver 2	9.34	16.3	1.73	93	1.84	1.15	1.00	2.15	1.16	.40	
Calf spleen	8.79	15.6	1.77	99	2.05	1.16	1.00	2.28	1.13	.39	2.09
Calf thymus	9.10	17.2	1.89	96	2.13	1.53	1.00	3.64	1.65	.26	2.17
Calf pancreas	9.30	17.42	1.87	100	1.77	1.05	1.00	3.62	1.69	.25	2.68

[a] $S_{20,w}$ *p*H 4.8, all other values obtained at *p*H 6.8 (*cf.* text).

trifuged and washed twice with cold 70% alcohol. The precipitate was then dissolved in water, carefully adjusted to *p*H 6.8 with dilute sodium hydroxide and any insoluble material (denatured protein) centrifuged off. The ribonucleic acid was purified by adding enough 1 *M* sodium chloride to bring the final concentration to 0.05 *M* sodium chloride and precipitating the sodium ribonucleate with 2 volumes of cold ethanol. The product was washed twice with cold 70% ethanol.

In the second method the tissue was immediately homogenized with 3 volumes per gram tissue of cold 2.5 *M* guanidine hydrochloride solution. The rest of the procedure followed that of the first method, except that the ribonucleic acid–protein complex was washed at least three times with cold 2 *M* guanidine hydrochloride to ensure complete removal of any contaminating desoxyribonucleic acid. Excess foaming, which occurred during the blending in the presence of guanidine hydrochloride, was alleviated by adding a few drops of octyl alcohol after the solution had warmed a few minutes in the 38° bath.

Duplicate liver ribonucleic acid preparations made by the two methods yielded essentially identical analytical compositions (see table), indicating that in liver little or no enzymatic hydrolysis occurred in the first procedure.

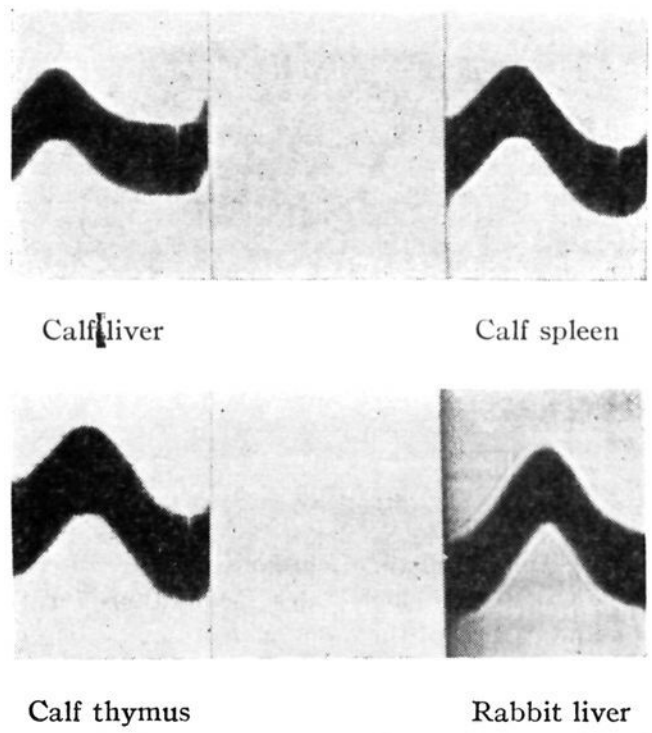
The mammalian ribonucleic acids readily dissolved in water to give clear, colorless solutions. Preparations to be stored were lyophilized from water solutions. Concentrations as high as 20 mg. per ml. failed to give a reaction with diphenylamine reagent, indicating that all the nucleic acid was of the ribose type. Similar concentrations gave negative biuret tests. The yield of ribonucleic acids varied from 20 to 30% of the total tissue ribonucleic acid.

Nitrogen–Phosphorus Analysis of Mammalian Ribonucleic Acids.—Nitrogen content was determined by the semimicro Kjeldahl method while phosphorus was analyzed by the method of Fiske and SubbaRow. Determinations were carried out on carefully weighed duplicate samples of lyophilized ribonucleic acid preparations. The result of the analyses and the corresponding nitrogen–phosphorus (N/P) ratios are recorded in the table. It can be seen that, with the exception of calf pancreas and thymus ribonucleic acid, this ratio lies between 1.73 and 1.79, the values of the latter preparations being considerably higher.

Sedimentation in the Analytical Ultracentrifuge.—The degree of molecular homogeneity of the ribonucleic acid preparations was estimated in the analytical ultracentrifuge (Spinco Model E). The centrifugations were carried out in 0.2 *M* sodium chloride–0.05 *M* phosphate buffer *p*H 6.8, while one preparation (rabbit liver 1) was also analyzed in *p*H 4.8 0.2 *M* sodium acetate buffer. Further details of the analyses are given in the text of Fig. 1.

Figure 1, which shows the sedimentation velocity photographs of some ribonucleic acid preparations, reveals that the preparations move as essentially single boundaries.

The sedimentation constants ($S_{20,w}$) calculated for these preparations are shown in the table. These values do not vary greatly for ribonucleic acid preparations from different sources. It should be noted that the $S_{20,w}$ value for rabbit liver ribonucleic acid at *p*H 4.8 is almost double that at *p*H 6.8, probably as a result of aggregation.



Calf liver Calf spleen

Calf thymus Rabbit liver

Fig. 1.—Analytical ultracentrifuge photographs of some mammalian ribonucleic acid preparations: The ribonucleic acids were analyzed at concentrations between 15 and 20 mg. per ml. in 0.2 *M* sodium chloride, 0.05 *M* phosphate buffer, *p*H 6.8, except for the rabbit liver ribonucleic acid which was run in *p*H 4.8 0.2 *M* sodium acetate buffer. The photographs shown were taken approximately one and a half hours after maximum centrifugal speed was reached, except the picture of rabbit liver ribonucleic acid which was taken after one hour. The mean ultracentrifugal force was 254,500 g. and the inclined bar in the Philpot–Svensson optical system was at 65° angle. Rotor temperature was maintained between 25 and 28°. For calculation of sedimentation constants (see table), 5 pictures were taken at 16-minute intervals.

The Nucleotide Composition of Mammalian Ribonucleic Acids.—For analysis the ribonucleic acid was converted to mononucleotides by dissolving in 0.5 *N* sodium hydroxide

at a concentration of 15 to 20 mg. per ml. and maintained at 37° for 17 hours. The digest was then diluted with water to 0.02 *N* sodium hydroxide and run through the anion-exchange resin (Dowex-1, bed size 6 cm. by 0.72 sq. cm., which had previously been converted to the chloride form). Excess hydroxyl ion was removed from the resin by allowing 0.01 *M* ammonium chloride to pass through the column until the *p*H of the effluent reached neutrality. No purine and pyrimidine bases and ribosides resulted under the conditions of alkaline hydrolysis employed. The elution of mononucleotides from the column was effected with increasing concentrations of hydrochloric acid according to the procedure described by Cohn.[13] Quantitative determination of mononucleotides in the effluent fractions was carried out by ultraviolet spectrophotometry, based on the following molar extinction coefficients at 260 mμ in 0.01 *N* hydrochloric acid; cytidylic 12,750; adenylic 13,900; uridylic 9,930; and guanylic acid 11,800.

Figure 2 illustrates the elution of rat liver ribonucleic acid mononucleotides from the anion-exchange column and is typical of all such runs. In all nucleotides derived from the mammalian ribonucleic acids which we have examined, adenylic and guanylic acids were present as isomers (*cf.*[13,14]). The relative proportion of each of the purine nucleotide isomers was constant for the several preparations, approximately 40% of the total nucleotide being accounted for by the "A" form. Under the conditions employed in these determinations isomeric pyrimidine nucleotides[15] were not separated.

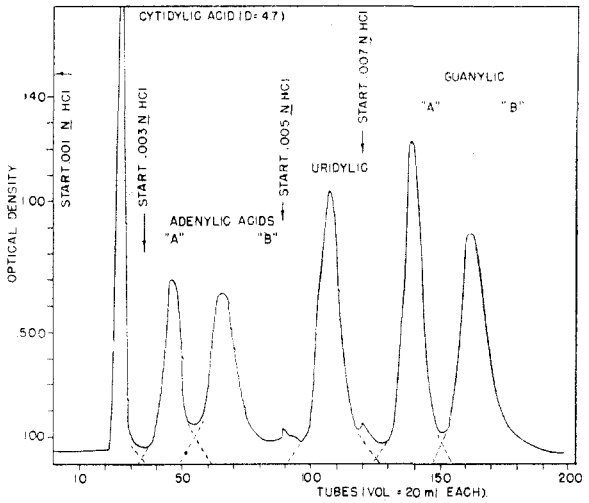


Fig. 2.—Anion-exchange chromatography of rat liver ribonucleic acid mononucleotides: conditions for the adsorption and elution of the mononucleotides are described in the text.

The nucleotide compositions of eleven ribonucleic acid preparations from various sources are shown in the table. For comparative purposes, nucleotide compositions are expressed as molar ratios, with that nucleotide of lowest quantity (uridylic acid) assigned unity. Total recovery of mononucleotides is expressed as the per cent. of the total 260 mμ absorption units[18] contained in the nucleic acid alkaline digest. There are differences in ribonucleic acid mononucleotide composition for the same tissue from different species and, likewise, the ribonucleic acid of various tissues from one species differ in composition. Of the pyrimidine nucleotides cytidylic acid predominates over uridylic acid, while of the purines, guanylic acid is always in excess of adenylic acid. In this respect, the extremely high guanylic acid contents of calf thymus and pancreas are noteworthy. The purine/pyrimidine ratios are between 1.0 and 1.2 in all ribonucleic acids except those of pancreas and thymus, where the high guanylic acid content increases this ratio to 1.7.

Rabbit liver and calf liver ribonucleic acids prepared by homogenizing prior to the addition of guanidine hydrochloride (no. 1) and by homogenizing in the presence of guanidine hydrochloride (no. 2) have analytical compositions which agree within 5% for each nucleotide component.

The Action of Crystalline Ribonuclease on Mammalian Ribonucleic Acid.—Fifty-mg. samples of ribonucleic acid were dissolved in 5 cc. of water and the solution adjusted to *p*H 7.2 with dilute sodium hydroxide. Then 1 ml. of a 5-mg.-per-ml. solution of crystalline ribonuclease (Armour), dissolved in 0.1 *M* phosphate buffer *p*H 7.2, was added to the nucleic acid solution under stirring. As the reaction progressed the solution was maintained at *p*H 7.2 by the addition of 0.05 *N* sodium hydroxide from a 1.0-ml. buret. The temperature of the digest was maintained between 25 and 27°. The hydrolysis was essentially complete in 2 hours, but all values recorded in the table are for 6-hour periods.

The extent of hydrolysis of mammalian ribonucleic acids by ribonuclease is shown in the table, expressed as equivalents of alkali used per mole of nucleic acid phosphorus. With the exception of calf pancreas and thymus, it is evident that about 0.4 of the total secondary phosphate groups in these nucleic acids are released by the enzyme ribonuclease.

Discussion

The fact that these preparations include only 20 to 30% of the tissue total ribonucleic acid, and appear to exist as single molecular species, probably indicates that the products represent only one of several ribonucleic acids contained in the cell. Lacking a biological test for activity it is doubtful whether the term "native ribonucleic acid" applied to mammalian ribonucleic acid is very useful. A test for the integrity of primary internucleotide linkages might be the extent of liberation of acid groups by ribonuclease acting on ribonucleic acids prepared from a given tissue. However, the intramolecular order of nucleotides may modify such a value since different preparative procedures may cleave the ribonucleic acid molecule in different regions resulting is some preparations which, though rich in enzymatically susceptible pyrimidine internucleotide linkages, represent small degraded particles of the original molecule. It is of interest that those mammalian ribonucleic acids whose pyrimidine nucleotide content is about 50% of the total yield 0.4 equivalent of acid groups per mole of phosphorus by ribonuclease action; whereas, calf thymus and pancreas ribonucleic acids which contain only about 38% pyrimidine nucleotide yield a corresponding value of 0.25, similar to that for yeast.[16]

The preparation of pancreas ribonucleic acid described by Bacher and Allen[10] was specifically designed to prevent ribonuclease degradation during isolation procedures; it resulted in material having a ratio of labile phosphate to total phosphate of 48.5%, indicating a purine to pyrimidine nucleotide ratio of close to unity. Since the pancreas and thymus used for the ribonucleic acid preparations in the studies described herein were not immediately homogenized in guanidine hydrochloride after slaughtering, it is possible that nuclease degradation accounts for the high purine/pyrimidine nucleotide ratios reported in this paper, and by others.[7,8,9] In the case of rabbit liver, however, immediate homogenization of the tissue in guanidine hydrochloride or addition of the guanidine salt after preliminary homogenization in saline and removal of the desoxy-

(13) W. E. Cohn, THIS JOURNAL, **72**, 1471 (1950).
(14) C. E. Carter, *ibid.*, **72**, 1466 (1950).
(15) W. E. Cohn, *ibid.*, **72**, 2811 (1950).
(16) F. W. Allen and J. J. Eiler, *J. Biol. Chem.*, **137**, 757 (1941).

ribonucleoprotein did not influence the purine/pyrimidine nucleotide ratio.

Analyses for phosphorus and nitrogen in the ribonucleic acids prepared by the guanidine salt method yielded N/P ratios which compared favorably with the values of 1.74 to 1.77 calculated from mononucleotide analyses of ribonucleic acids (other than thymus and pancreas, the latter two having a ratio of 1.86). The postulate of a statistical tetranucleotide does not coincide with the results of mononucleotide analysis of mammalian ribonucleic acids by ion-exchange chromatography, in agreement with previously reported analyses.[17] The pig liver ribonucleic acid analyzed by a chromatographic method[17] had molar proportions of cytidylic, adenylic, uridylic and guanylic acids of 2.1, 1.4, 1 and 2.4, respectively, values in fair agreement with those reported here for liver ribonucleic acids from other species. The sedimentation constants shown in the table reveal no striking differences among those preparations analyzed. It should be noted that the sedimentation constant of rabbit liver ribonucleic acid almost doubles as the *p*H is lowered from 6.8 to 4.8. This probably indicates an increased state of aggregation in more acid solution. Data concerning the physical-chemical homogeneity of mammalian ribonucleic acids prepared by other techniques[7,8,9,10] is available only for the non-dialyzable residue of ribonuclease-treated pancreas ribonucleic acid.[18]

(17) E. Chargaff, B. Magasanik, R. Donigen and E. Vischer, THIS JOURNAL, **71**, 1513 (1949).

(18) J. E. Bacher and F. W. Allen, *J. Biol. Chem.*, **183**, 641 (1950).

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[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

The Incorporation of Isotopic Phosphate in the Mononucleotides of Liver Nucleic Acids[1]

BY E. VOLKIN AND C. E. CARTER

Investigations have been carried out on the *in vivo* incorporation of isotopic phosphate into the component mononucleotides of liver ribonucleic acid (RNA) and desoxyribonucleic acid (DNA). Mononucleotides were isolated by anion-exchange chromatography from hydrolysates of highly purified nucleic acids. This work reveals that a considerable variation in distribution of the isotope occurs among the nucleotides of DNA, thymidylic acid containing the highest activity. The distribution among the nucleotides in rabbit liver ribonucleic acid is essentially homogeneous, while rat liver and mouse liver ribonucleic acids exhibit a heterogeneous distribution of activity among the mononucleotides. These results, in conjunction with those of other workers, suggest that the evaluation of studies on nucleic acid turnover rates may be considered with regard to the metabolic turnover of the substituent components of nucleic acids, as well as the nature of the isotopically labeled precursor.

Introduction

The metabolic turnover of nucleic acid phosphorus is generally studied on nucleic acid fractions of tissues with the assumption of homogeneous distribution of isotopic phosphorus among the substituent nucleotides. A study of the specific activity of the mononucleotides derived from RNA and DNA isolated from mammalian liver following *in vivo* assimilation of inorganic isotopic phosphate is reported in this paper.

The results of this investigation which are summarized in the table show an essentially homogeneous distribution of isotopic phosphate among the nucleotides of rabbit liver RNA isolated from an animal sacrificed 180 minutes following injection of the isotope. In order to obtain high nucleic-acid activities in shorter incubation periods smaller animals, rats and mice, were employed. Under the conditions described in the table (20-min. incubation period) isolated ribonucleotides are unequally labeled with isotopic phosphorus. In all cases each pair of the isomeric purine nucleotides is found to be equally active[2a,b]; adenylic acid exhibits the highest activity and, in the case of mouse liver, guanylic acid has a significantly lower specific activity than other mononucleotides. A striking variation of distribution of isotopic phosphorus is found among the desoxyribonucleotides derived from mouse and rat liver desoxyribonucleic acids. In both species thymidylic acid exhibits the highest specific activity. In the mouse, desoxyadenylic acid closely approximates the activity of thymidylic acid while in the rat it is about half the thymidylic acid value in both the normal and regenerating livers. A further species difference is found in the distribution of activity in the purine desoxyribonucleotides; in the rat, essentially equal activities are found, whereas in the mouse the activity of desoxyadenylic acid is about twice that of desoxyguanylic acid. In experiments employing isotopic formate, species differences in the distribution of activity among the nucleotides of chick and rat nucleic acids have also been found.[3]

The experiments with normal and regenerating rat liver were not performed under comparable conditions so that the absolute specific activities are not applicable. However, it may be seen that the relative values show no significant differences in the nucleotide distribution of phosphate in the two groups.

A satisfactory degradation of mouse hepatoma DNA to mononucleotides was not achieved, consequently, a comparison of this category with normal liver is not available. The ribonucleotides from the two tissues exhibited no significant differences of activity under the conditions employed.

The foregoing data do not impair the value of experiments which utilize the incorporation of isotopic phosphate into nucleic acids to assess the metabolic activities of these compounds under various conditions. However, there is considerable evi-

(1) Work performed under Contract #W-7405-eng-26 for the Atomic Energy Commission.

(2) (a) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950); (b) W. E. Cohn, *ibid.*, **72**, 1471 (1950).

(3) J. R. Totter, E. Volkin and C. E. Carter, *ibid.*, **73**, 1521 (1951).