Studies on the Primary Structure of the Messenger RNA from Phage T4. Frequencies of the Mono-, Di-, and Trinucleotides Obtained from a Digest with Pancreatic Ribonuclease*

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The messenger RNA from bacteriophage T4 has been digested with pancreatic ribonuclease, and the resulting mono-, di-, and trinucleotides have been determined quantitatively by isotope dilution. The frequencies of the dinucleotides observed closely agree with the corresponding nearest neighbor frequencies in T4 DNA, providing further evidence that messenger RNA is complementary and, in addition, antiparallel to the template DNA strand. In contrast *Escherichia coli* ribosomal RNA and the RNA from phage f2 show little homology with *E. coli* DNA.

Studies on the secondary structure of messenger RNA from phage T4 (T4 m-RNA) have shown it to be quite similar to the structure of *Escherichia coli* ribosomal RNA (Bautz, 1963a,b). Yet the two RNA species are believed to serve two entirely different functions (Jacob and Monod, 1961). Direct experimental evidence supports this view, for it was found that upon separation of T4 m-RNA from the bulk of *E. coli* RNA (Bautz and Hall, 1962) the activity to stimulate amino acid incorporation into cell-free extracts is associated almost entirely with the T4 m-RNA (Bautz, 1962). Thus any attempt to understand the molecular basis of the function of messenger RNA calls for at least some knowledge about the distribution of the four nucleotides within the RNA molecules.

During the past few years methods have been developed allowing the fractionation and identification of oligonucleotides resulting from digests with pancreatic ribonuclease (Staehelin *et al.*, 1959) or with ribonuclease T1 (Rushizky and Sober, 1962). These methods have been used mainly for the characterization of oligonucleotides; however, quantitative analyses of the distribution of oligonucleotides are feasible and have been attempted in a few laboratories (Staehelin, 1961b; Berg *et al.*, 1962).

In this communication we report a procedure for the quantitative estimation of oligonucleotide frequencies resulting from a digest of isotopically labeled T4 m-RNA with pancreatic ribonuclease. A comparison of the dinucleotide frequencies with the corresponding nearest-neighbor frequencies in T4 DNA (Josse *et al.*, 1961) supports the existing evidence (Hall and Spiegelman, 1961; Weiss and Nakamoto, 1961) that phage-specific RNA is complementary in sequence to its homologous DNA. In analogy to the structure of DNA (Josse *et al.*, 1961), the sequence of messenger RNA was found to be antiparallel to the coding strand of DNA. *E. coli* ribosomal RNA and the autonomously replicating RNA from phage f2 show very few similarities with *E. coli* DNA.

Materials and Methods

DEAE-Cellulose.—Whatman powder DE 50, batch 542–550, capacity 1.0 meq/g, was used throughout. After removal of fines the powder was washed with 0.2 N NaOH until colorless, followed by repeated washings with water until *p*H 7.

Pancreatic ribonuclease A, lyophilized and phosphate free, was purchased from Worthington Biochemical Corp.

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Ribonuclease T1 was prepared from "Sanzyme R" powder (Sankyo Ltd., Tokyo) according to Takahashi (1961); its specificity had been checked by its ability to split GpU, ApGpC, and GpApC but not to degrade ApU and ApApC.

RNA.—High-molecular-weight yeast RNA was purchased from Worthington Biochemical Corp., Free hold, N. J.

Phage f2 RNA was provided by Dr. N. Zinder, Rockefeller Institute. *E. coli* ribosomal RNA was obtained by shaking a suspension of ribosomes prepared according to Nirenberg and Matthaei (1961) three times with phenol at 50°, followed by precipitation with ethanol. In spite of the phenol treatment some residual nuclease activity was encountered in one of several ribosomal RNA preparations which could be eliminated by precipitation in the cold with 10% trichloroacetic acid.

Doubly labeled T4 m-RNA was prepared as follows: *E. coli* cells were grown with aeration at 37° in a low-phosphate medium containing (mmoles/liter): Tris-HCl, 100; NH_4Cl, 20; $MgSO_4$, 1; glucose, 27; phosphate, 0.5; and $FeCl_3$, 0.005, until the culture reached an optical density at 660 mμ of 0.25. At this point ^{32}P was added at a concentration of 2–20 μc/ml of culture medium. When the cell suspension had reached an A_{660} of 0.40, T4 wild-type phages were added at a multiplicity of eight in the presence of 5 μg/ml DL-tryptophan. Three minutes after infection a 0.1-ml sample was diluted out into T4 antiserum, and subsequently tested for surviving bacteria. Five minutes after infection, [^3H]adenine (New England Nuclear Corp., specific activity 1 c/mmole) was added at a concentration of 1 μc/ml. Aeration was stopped after 3 more minutes and the culture was poured onto 0.4 volume of frozen buffer (Hall and Spiegelman, 1961).

The isolation of the RNA and the purification of the T4 m-RNA was performed as described elsewhere (Bautz, 1962).

Digestion of RNA and Chromatography of the Products.—Twenty mg of yeast RNA was incubated with ^{32}P + ^3H–labeled T4 m-RNA (the activity varied from 1×10^6 to 1.4×10^7 cpm for ^{32}P and from 3 to 6×10^5 cpm for ^3H in the individual experiments) in the presence of 2.0 mg of RNAase in 0.01 M Tris-HCl, *p*H 7.8. The total volume was 8 ml. The *p*H was kept above 7.5 by adding 0.05 N NaOH. After 30 minutes a few drops of toluene were added to prevent bacterial growth, and digestion was allowed to proceed for 12–14 hours. The digest was complete under these conditions as evidenced by the absence of 2′,3′-cyclic mononucleotides (Rushizky *et al.*, 1961). The toluene was extracted with ether, the residual ether was evaporated,

and the digest was loaded onto a column of DEAE-cellulose packed under pressure of 5 psi.

The elution of the oligonucleotides was first attempted using a nonlinear gradient of ammonium bicarbonate, *p*H 8.6, as described by Staehelin (1961a). However, owing to the type of mixing device used ("Rectangular Varigrad" of Buchler Instr., Fort Lee, N. J.), CO_2 was released rapidly from the vessel, resulting in a gradual increase in the *p*H of the effluent. Consequently, separation of the isomers of (ApGp)Cp and of (ApGp)-Up was not achieved.

The conditions were then modified as follows: A freshly made solution of 1 M ammonium bicarbonate was brought to *p*H 7.4 by bubbling CO_2 through, and was used without delay in a 6-chambered Varigrad of 300 ml each of 0.01, 0.3, 0.15, 0.4, 0.6, and 1.0 M ammonium bicarbonate. Elution was started at once. Column dimensions, 1 × 40 cm; flow rate, 0.5 ml/minute. The resulting profile (Fig. 1) can be obtained reproducibly as long as fresh bicarbonate is used for each run. The absorbancies at 260 and 290 mμ (A_{260} and A_{290}) were read on each fraction in a Zeiss spectrophotometer and 0.2 ml of each were counted simultaneously for ^3H and ^{32}P in a Packard Tri-Carb Model EX liquid scintillation spectrometer. The peak fractions were collected and evaporated at 40° *in vacuo*. Distilled water was added to the dry residue and evaporation was repeated several times until the ammonium bicarbonate had disappeared. The total dinucleotide fraction was pooled and rechromatographed on a DEAE-cellulose column (1 × 40 cm) with a linear gradient from 0.1 to 0.4 M ammonium bicarbonate, *p*H 8.6, the two vessels containing 400 ml each of buffer. The four dinucleotides were thus separated without overlap.

Electrophoresis and Analysis of the Components.—The peaks were further separated by electrophoresis on Whatman 3MM paper in 0.05 M ammonium formate buffer, *p*H 3.5 (Markham and Smith 1952) for 2 hours at 40 v/cm or for 6 hours at 24 v/cm in a flat-plate apparatus (Savant Instruments, Inc., Hicksville, N. Y.), the lower voltage yielding a somewhat better separation of the components. In some cases it was preferred to purify GpGpCp and GpGpUp by descending paper chromatography with isobutyrate ammonia as solvent (Magasanik *et al.*, 1950). The spots were located on the paper with a short-wave ultraviolet lamp and, if labeled, by counting in a 4 π strip-counter (Atomic Accessories, Inc., Valley Stream, N. Y.) equipped with an integrating recorder. In this way the ratio of the separated components within a peak could be directly established. The spots were then eluted with distilled water and A_{260} was determined for each spot at neutral *p*H against paper blanks of equal size. Small portions of the eluted spots were counted in the scintillation counter to determine the ratio ^3H-^{32}P being identical with the ratio adenine-PO_4 in the oligonucleotides. The remainder of each spot was subjected to alkaline hydrolysis in the way described (*vide infra*), and the resulting mononucleotides were separated by paper electrophoresis. The base composition was determined by the ratio between the activities of the spots measured in the strip-counter. This procedure of measuring base ratios of oligonucleotides was found to be at least as accurate as the more tedious procedure involving elution of the spots and measuring the absorbance at 260 mμ.

The structures of the isomeric trinucleotides were established by digestion with ribonuclease T1 at an enzyme-substrate ratio of approximately 1:100 at *p*H 7.5, followed by electrophoresis and location of the resulting mono- and dinucleotides.

Alkaline Hydrolysis of Oligonucleotides with Barium Hydroxide (Loring *et al.*, 1951).—To 0.05 ml of a solution containing 0.2–0.5 μM of nucleotide, 0.1 ml of a 0.34 N solution of $Ba(OH)_2$ was added. The stoppered tube was incubated for 1 hour at 50° and the Ba $(OH)_2$ was then neutralized with H_2SO_4 (adjusted against the $Ba(OH)_2$ solution used). The sample was boiled for a few seconds and cooled. The $BaSO_4$ was removed by centrifugation, the supernatant was spotted on paper, and the resulting nucleotides were separated by electrophoresis as described. The recovery of the mononucleotides was 90% or better. This procedure was found to be useful for routine analysis because of the speed of hydrolysis; in addition the extremely low solubility product of Ba_2SO_4 yields the mononucleotides virtually salt free.

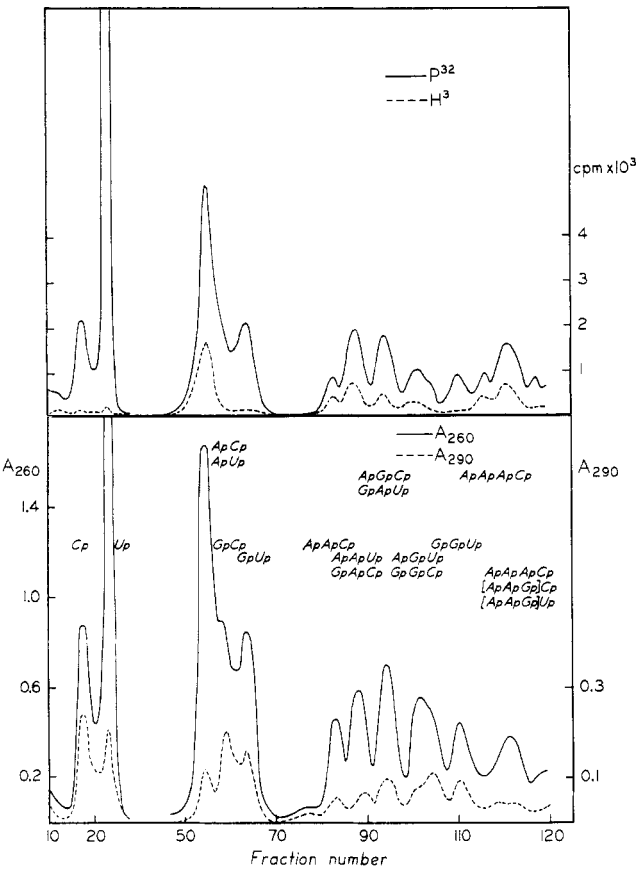

FIG. 1.—Chromatography of T4 m-RNA (total activity: 2.5 × 10^6 cpm ^{32}P, 3.0 × 10^5 cpm ^3H) digested with 2 mg of pancreatic ribonuclease in the presence of 20 mg of carrier yeast RNA at *p*H 7.8 in a volume of 8 ml; *p*H of starting buffer, 7.4. Fraction size, 6.6 ml. For details see Materials and Methods. Upper curve: elution profile of T4 m-RNA represented as cpm/ml of effluent. Lower curve: elution profile of carrier yeast RNA given as A_{260} and A_{290}.

The nucleotide compositions of T4 m-RNA, phage f2 RNA, and the commercial yeast RNA were determined under the conditions employed previously for *E. coli* ribosomal RNA and for T4 m-RNA (Bautz and Hall, 1962). For the calculation of nucleotide compositions the following molar extinction coefficients for the four mononucleotides were used: cytidine-2′,3′-monophosphate, 7.6 × 10^3; uridine-2′,3′-monophosphate, 10.0 × 10^3; adenosine-2′,3′-monophosphate, 15.0 × 10^3; guanosine-2′,3′-monophosphate, 11.4 × 10^3.

Quantitation of Carrier and Labeled Nucleotides.—The total A_{260} of each of the one mono-, one di-, and five trinucleotide peaks was determined. After removal of salt, the specific activity as ^{32}P counts was determined.

TABLE I
PAPER ELECTROPHORESIS OF TRINUCLEOTIDES IN 0.05 M AMMONIUM FORMATE, pH 3.5

Nucleotide	R_{Up}
Up	1.0
ApApCp	0.4
ApApUp	0.7
GpApCp	0.5
ApGpCp	0.5
GpApUp	0.85
ApGpUp	0.8
GpGpCp	0.55
GpGpUp	1.05

From the specific activity the total amount of ^{32}P present in the peaks before evaporation was calculated. The relative amounts of the further fractionated components in each peak were determined by A_{260} readings and by ^{32}P counts. The radioactivity provides a direct measure of the amount of nucleotides recovered; the relative amounts of carrier nucleotides within a peak were determined as μmoles using the extinction coefficients given by Stanley (1963).

CALCULATION OF OLIGONUCLEOTIDE FREQUENCIES. — (a) Random distribution: These calculations are based on the nucleotide composition determined for the individual RNA species. *Example:* Frequency of the dinucleotide ApCp present in T4 m-RNA after an exhaustive digest with pancreatic ribonuclease. Frequency as mole % phosphate = mole % Ap × mole % Cp × (mole % Cp + Up) × 2 = 0.313 × 0.177 × (0.177 + 0.302) × 2 = 0.053 = 5.3% of total nucleotide. All other frequencies have been calculated accordingly. The sum of the mono-, di-, and trinucleotides in T4 m-RNA thus computed is 0.654, or 65.4% of total nucleotide.

(b) Distribution of nucleotides found: In one of the three analyses on T4 m-RNA, all peaks including the ones containing tetranucleotides and the higher oligonucleotides were freed from salt (the presence of substantial amounts of salt in the later fractions results in some quenching of ^{32}P counts), and the total radioactivity in each peak was determined. With 95% of the counts recovered, 66.1% of these were in the fractions containing the mono-, di-, and trinucleotides, a value which agrees closely with the 65.4% expected from a random distribution of nucleotides. It was then assumed for all three analyses that the sum of the mono-, di-, and trinucleotides recovered was 65.4% of total nucleotide. For T4 m-RNA, the amount of each component is thus expressed as the fraction of the mono-, di-, and trinucleotides recovered multiplied by 0.654. The oligonucleotide frequencies in *E. coli* ribosomal RNA and in phage f2 RNA have been computed analogously.

RESULTS

The elution profile in Figure 1 shows that the mono- and dinucleotides appear largely unresolved. However, as the pH of the effluent gradually increases due to the release of CO_2 the trinucleotides are eluted in five distinct peaks. All three unresolved pairs differ greatly in electrophoretic mobility (Table I) and can thus be separated with ease by paper electrophoresis. The two pairs of isomers are separable under these conditions. Moreover, the first of the tetranucleotides is eluted only after GpGpUp. Since the two mono- and the four dinucleotides can be separated quantitatively by one-dimensional paper electrophoresis or by rechromatography on DEAE-cellulose at pH 8.6, this procedure turned out to provide the quickest analysis of frequencies of up to the trinucleotides.

Table II shows the results of three different analyses on T4 m-RNA. The values obtained for a given nucleotide agree well with each other in most cases. The agreement also holds for the frequencies determined on the carrier yeast RNA (Table II), which in turn agree roughly with the data reported by Staehelin (1961b) on high-molecular-weight yeast RNA. The values marked with a [b] are inaccurate because of accidental losses and have therefore been left out in computing the mean values. A comparison of the error limits given in Table II shows that the accuracy of an analysis done by radioisotope counting is of the same order as the measurement of absorbancies on the carrier nucleotides. In addition, the simultaneous analysis of labeled and cold oligonucleotides precludes the occurrence of a number of possible errors without detection.

Table III shows the nucleotide distributions of three different RNA species, including a heterogeneous sample of messenger RNA synthesized on its DNA template represented by T4 m-RNA, an RNA species assumed to be a "nonmessenger" represented by *E. coli* ribosomal RNA, and phage f2 RNA as an autonomously replicating and homogeneous messenger species.

The distribution of the di- and trinucleotides in T4 m-RNA shows small but nevertheless marked deviations from the calculated random distribution. The deviations of the dinucleotide frequencies are explained by the fact that complementary sequences in T4 DNA are not exactly random either (Josse *et al.*, 1961), their deviations being opposite or parallel to the ones observed in RNA, depending whether one assumed m-RNA to be read parallel or antiparallel to the coding strand of DNA (Table IV). The agreement observed clearly favors an antiparallel mechanism of RNA synthesis already established for *in vitro* DNA synthesis (Josse *et al.*, 1961). Using the products of two nearest-neighbor frequencies as a rough approximation of trinucleotide frequencies in T4 DNA, and comparing them with the corresponding values for T4 m-RNA, it becomes apparent that the agreement observed for the dinucleotides also holds for the trinucleotides except for GpGpUp. Such a comparison immediately explains the observed bias between the pairs of isomers of GpApCp and ApGpCp, and of GpApUp and ApGpUp. For the isomers of (ApGp)Cp the same bias is observed in *E. coli* ribosomal RNA and in yeast RNA (previously noted by Staehelin, 1961b). The bias for the isomers of (ApGp)Up, however, is not paralleled by any of the other RNA species. Furthermore, T4 m-RNA differs from the other two species listed in its ApApCp and ApApUp content, whereas *E. coli* ribosomal RNA deviates most strongly in its ApUp content. The same deviation has been observed by Stanley (1963) on the 16 S ribosomal RNA component of *E. coli*. Phage f2 RNA seems to be somewhat related to *E. coli* ribosomal RNA; it does not, however, show an abnormal value for ApUp. It is of interest to note that both *E. coli* ribosomal RNA and phage f2 RNA show few similarities with *E. coli* DNA (Table IV).

DISCUSSION

The RNA synthesized after infection with phage T4 provides the most suitable material for the present studies, since it seems to be entirely composed of messenger molecules. The fact that up to 95% of this RNA can form complexes with denatured DNA (L. Bautz, unpublished data) and that none of it can be classified as either s-RNA or ribosomal RNA supports this concept. The procedure employed for the isolation

TABLE II

MONO-, DI-, AND TRINUCLEOTIDE FREQUENCIES IN T4 M-RNA AND YEAST RNA[a]

Nucleotide	T4 m-RNA Found I	T4 m-RNA Found II	T4 m-RNA Found III	T4 m-RNA Mean	T4 m-RNA Error Limit	Yeast RNA Found I	Yeast RNA Found II	Yeast RNA Found III	Yeast RNA Mean	Yeast RNA Error Limit
Cp	8.4	7.7	7.8	8.0	0.4	10.2	9.2	10.4	9.9	0.5
Up	14.2	14.9	14.9	14.7	0.4	11.1	12.4	12.1	11.9	0.7
ApCp	4.9	4.4	4.5	4.6	0.2	4.1	4.3	3.9	4.1	0.2
ApUp	9.3	7.4[b]	9.2	9.3	0.1	5.8	5.0[b]	5.5	5.7	0.2
GpCp	3.8	4.1	3.9	3.9	0.2	4.6	4.3	4.9	4.6	0.3
GpUp	5.6	5.1	4.9	5.2	0.4	5.1	5.1	4.6	4.9	0.3
ApApCp	2.2	2.9	2.7	2.6	0.4	2.8	2.8	2.9	2.8	0.1
ApApUp	4.5	4.7	4.5	4.6	0.2	2.5	2.7	2.6	2.6	0.1
GpApCp	1.5	1.6	1.7	1.6	0.1	1.9	1.7	2.1	1.9	0.2
ApGpCp	2.0	2.2	2.1	2.1	0.1	2.4	2.4	2.5	2.4	0.1
GpApUp	3.4	3.6	3.6	3.5	0.1	2.4	2.6	2.4	2.5	0.1
ApGpUp	2.6	3.0	2.6	2.7	0.2	2.9	3.0	2.8	2.9	0.1
GpGpCp	0.9	1.5	1.4	1.3	0.3	2.8	2.6	2.4	2.6	0.2
GpGpUp	2.4	2.3	1.9	2.2	0.3	3.1	3.5	2.8	3.1	0.4

[a] Given in mole % of total nucleotide. [b] Value omitted from calculation of mean (see text).

TABLE III

MONO-, DI-, AND TRINUCLEOTIDE FREQUENCIES IN T4 M-RNA, *E. coli* RIBOSOMAL RNA, AND PHAGE f2 RNA

Nucleotide	T4 m-RNA Random[a]	T4 m-RNA Found	*E. coli* Ribosomal RNA Random[b]	*E. coli* Ribosomal RNA Found	Phage f2 RNA Random[c]	Phage f2 RNA Found[d]
Cp	8.5	8.0	9.7	10.0	12.1	12.7
Up	14.5	14.7	8.8	8.5	11.4	11.5
ApCp	5.3	4.6	4.8	4.5	5.6	5.8
ApUp	9.0$_5$	9.3	4.3	3.1	5.2	5.3
GpCp	3.5	3.9	6.3	6.6	6.9	6.9
GpUp	6.0	5.2	5.7	5.2	6.5	5.5
ApApCp	2.5	2.6	1.8	2.9	1.9	2.9
ApApUp	4.2$_5$	4.6	1.6	1.6	1.8	1.6
GpApCp	1.6$_5$	1.6	2.3	2.3	2.4	2.2
ApGpCp	1.6$_5$	2.1	2.3	3.0	2.4	2.4
GpApUp	2.8	3.5	2.1	2.1	2.2	2.0
ApGpUp	2.8	2.7	2.1	2.2	2.2	2.0
GpGpCp	1.1	1.3	3.1	2.7	3.0	3.6
GpGpUp	1.9	2.2	2.8	2.8	2.8	2.3

[a] The base ratio of T4 m-RNA was found to be cytosine, 17.7%; uracil, 30.2%; adenine, 31.3%; guanine, 20.7%. [b] The base ratio of *E. coli* ribosomal RNA used was cytosine, 22.5%; uracil, 20.5%; adenine, 24.6%; guanine, 32.4% (Bautz and Hall, 1962). [c] The base ratio of phage f2 RNA was found to be cytosine, 25.0%; uracil, 23.5%; adenine, 22.9%; guanine, 28.6%. [d] These data represent the mean values of two independent determinations.

of T4 m-RNA involving adsorption on homologous DNA already implies sequence complementarity between T4 DNA and T4 m-RNA. However, the structure of native DNA-RNA hybrid molecules has eluded detailed analysis, because homogeneous samples are not available as yet; consequently a double-stranded nature of DNA-RNA hybrids could only be inferred from their close resemblance with native DNA (Hall and Spiegelman, 1961; Geiduschek *et al.*, 1961). The homology between RNA and DNA found here on the oligonucleotide level lends further support to the structural concept of DNA-RNA hybrids without necessarily proving complete sequence homology on the polynucleotide level.

The comparison between the dinucleotide frequencies observed for T4 m-RNA and the corresponding nearest neighbor frequencies for enzymatically synthesized T4 DNA (Josse *et al.*, 1961) indicates that the RNA is synthesized in the direction antiparallel to the template DNA strand. Since the trinucleotide frequencies in T4 DNA were computed from the two composite nearest-neighbor frequencies, they represent only a rough approximation and are therefore not expected to compare as well with the homologous sequences in T4 m-RNA as do the dinucleotides. The agreement is nevertheless strong enough to suggest that the homology extends to sequences longer than dinucleotides. The nearest-neighbor frequencies measured by Weiss and Nakamoto (1961) on enzymatically synthesized T2 RNA are also in agreement with the corresponding frequencies in T2 DNA. Although, unlike *in vivo* RNA synthesis, the RNA polymerase does not seem to be able to discriminate between the two DNA strands *in vitro*, the structural similarities between the native and the enzymatically synthesized RNA leave little doubt that both are made by a common mechanism. These arguments can be extended to the enzyme system of the DNA polymerase; the agreement between our data and the data of Josse *et al.* (1961) support the view that the mechanisms of *in vitro* and *in vivo* DNA synthesis are identical.

One of the reasons for attempting the present analysis was to see whether a nonsense triplet could be detected by a strong deviation of its frequency from randomness, for it can be argued that, whenever a nonsense triplet was "in phase" for reading (Crick *et al.*, 1961), it should have been eliminated (by a mutational change in the complementary DNA triplet) through evolution. Assuming a triplet code, a trinucleotide should be "in phase" one out of three times; therefore the frequency

TABLE IV

COMPARISON OF NEAREST-NEIGHBOR FREQUENCIES BETWEEN T4 m-RNA AND T4 DNA; AND *E. coli* RIBOSOMAL RNA, PHAGE f2 RNA, AND *E. coli* DNA

Nucleotide	T4 m-RNA (found/-random)	T4 DNA[a] (found/random)		*E. coli* Ribosomal RNA (found/-random)	Phage f2 RNA (found/-random)	*E. coli* DNA[a] (found/-random)
		Antiparallel	Parallel			Antiparallel
ApCp	0.87	0.90	1.09	0.94	1.04	0.87
ApUp	1.03	1.04	0.90	0.72	1.02	1.08
GpCp	1.11	1.13	0.84	1.05	1.00	1.34
GpUp	0.87	0.84	1.05	0.91	0.85	0.87
ApApCp	1.04	0.98	1.17	1.61	1.52	1.04
ApApUp	1.08	1.12	0.99	1.00	0.89	1.28
GpApCp	0.97	0.91	1.05	1.00	0.92	0.78
ApGpCp	1.27	1.08	0.86	1.30	1.00	1.17
GpApUp	1.25	1.06	0.87	1.00	0.91	0.96
ApGpUp	0.96	0.82	1.07	1.05	0.90	0.76
GpGpCp	1.18	1.12	0.84	0.88	1.19	1.21
GpGpUp	1.15	0.84	1.05	1.00	0.82	0.79

[a] Data from Josse *et al.* (1961).

of a nonsense triplet should be reduced by one-third, assuming a perfectly random distribution of code words. A digest by pancreatic ribonuclease offers only a limited sample, namely, eight out of the sixty-four possible triplets. Among these none deviates in frequency enough to become a suspect of being "nonsense," since all the trinucleotides are present to more than 90% of theory. So far the results support the strong evidence from *in vitro* studies (Jones and Nirenberg, 1962; Wahba *et al.*, 1963) that very few if any nonsense code words exist. An additional analysis of nine more trinucleotide frequencies to bring the total number of trinucleotides investigated to seventeen is feasible (Bartos *et al.*, 1963) and should serve to elaborate this problem further.

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