Table 2. Effects of salicylanilides and dinitrophenol on housefly mitochondrial adenosine triphosphatase (ATPase). Each value is the average result from two experiments. Mitochondrial protein, 2.2 mg per flask.

Compound (molar conc.)	Oligomycin (2 μ per flask)	ATPase activity (P_i, μmole/30 min)
Control	−	3.83
	+	0.41
Dinitrophenol (10^{-4})	−	17.50
	+	0.69
IX (10^{-7})	−	17.25
	+	0.72
X (10^{-7})	−	17.25
	+	0.69
XI (10^{-7})	−	17.80
	+	0.75
XII (10^{-7})	−	17.80
	+	0.75
XIII (10^{-8})	+	12.05
	−	0.69
XIII (10^{-7})	−	18.34
	+	0.75

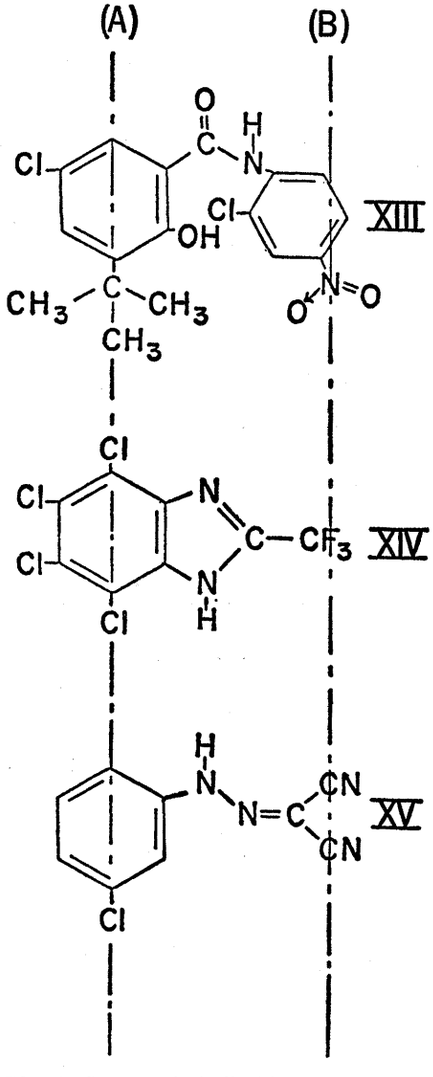

Fig. 1. Structural similarities among various uncouplers of oxidative phosphorylation. (A) Represents the plane of symmetry through the halogenated aromatic rings and (B) is the corresponding plane of symmetry through the electron withdrawing groups.

in many respects than the overall phosphorylating sequence.

Although the salicylanilides inhibit pyruvate oxidation in housefly mitochondria, they are categorized as uncoupling agents on the ground that adenosine triphosphatase, stimulated by the salicylanilides, is inhibited by oligomycin. Table 2 compares the salicylanilides with 2,4-dinitrophenol in effect on adenosine triphosphatase activity of housefly mitochondria in the presence and absence of oligomycin. In this capacity the salicylanilides are shown to be approximately 1,000 to 10,000 times more effective than dinitrophenol, which finding agrees favorably with the relative effectiveness of these compounds on the P_i–ATP exchange and oxidative phosphorylation.

Although the mode of action of these various uncouplers of oxidative phosphorylation is still obscure, they appear to possess certain common structural features. Inspection of molecular models reveals the presence of strong electron-withdrawing centers, such as NO_2, CN, or CF_3, located within a certain spatial distance from a halogenated aryl ring (Fig. 1). These common features and the greatly enhanced effect on oxidative phosphorylation and the related P_i–ATP exchange, resulting from the attachment of an additional bulky group such as naphthyl (II), biphenylyl (IV–XII), or *t*-butyl (XIII), suggest that inhibition results from preferential adsorption at an active site on the enzyme surface.

The close correspondence, in inhibition of the P_i–ATP exchange by the salicylanilide derivatives, between housefly and rat-liver mitochondria demonstrates the essential similarity of oxidative phosphorylation in vertebrates and invertebrates. These relatively simple and extremely active salicylanilides should prove to be useful tools for further investigation of this fundamental process.

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2 October 1967

Nucleotide Sequence of KB Cell 5S RNA

Abstract. *The nucleotide sequence of 5S RNA derived from KB carcinoma cell ribosomes has been determined. The molecule has a length of either 120 or 121 nucleotides with uridine at its 3′-terminus and guanylic acid at its 5′-terminus. If, in addition to Watson-Crick base-pairing, one accepts occasional base-pairing of guanylic acid to uridylic acid, long sequences of complementary nucleotides can be identified within the molecule. Two regions of the molecule contain sequences complementary to four or five bases in the pentanucleotide sequence guanylic acid, ribothymidylic acid, pseudouridylic acid, cytidylic acid, guanylic acid, which is common to most transfer RNA molecules. This is the first time the sequence of an animal-cell RNA has been determined.*

In many cell species, the ribosomes contain a low-molecular-weight ribonucleic acid (5*S* RNA) whose function is unknown (*1*, *2*). The introduction by Sanger *et al.* of an improved procedure for the separation and identification of P^{32}-labeled oligonucleotides by two-dimensional paper electrophoresis (*3*) has facilitated the study of the primary structure of 5*S* RNA and other low-molecular-weight homogeneous RNA species. Enzymatic digests of 5*S* RNA yield only a small number of oligonucleotides that are specific for the cell species of its origin and are present in quantities, which suggests that 5*S* RNA has a single nucleotide sequence, or a limited number of closely related sequences (*4*–*6*).

We used this oligonucleotide frac-

tionation procedure (i) to isolate and establish the sequence of all the oligonucleotides present in complete pancreatic and T1 ribonuclease digests of 5S RNA derived from KB cells (human epidermoid carcinoma line), and (ii) to identify the products of its partial digestion by ribonuclease T1 after their isolation and purification by column chromatography. As a result we were able to establish the entire nucleotide sequence of 5S RNA from KB cells.

Unlike previous studies (*7–10*) on the sequence of tRNA's (*11*), our work did not require large amounts of RNA, since the final identification of the sequences depended only on the radioactivity of the RNA and not on its optical density. Extensive procedures to purify the RNA were not needed since 5S RNA is homogeneous when separated from other RNA by methylated-albumin-kiesselguhr column chromatography. The 5S fraction contains less than 7 percent contaminating tRNA as judged by its content of pseudouridine, and its internal homogeneity is indicated by the fact that enzymatic digests of KB cell 5S RNA isolated by this method yield only a small number of specific oligonucleotides (*5*) which are present in molar amounts consistent with a single molecule, or a small number of very similar molecules. Three trinucleotides, however, are present in the pancreatic ribonuclease digest in traces (*5*), from 0.1 to 0.15 mole. These could be derived from another RNA contaminating the 5S RNA (*4*), or could result from oversplitting of large oligonucleotides of the 5S RNA itself.

The P^{32}-labeled KB cell 5S RNA was obtained as described (*5*). The RNA was completely digested by pancreatic and T1 ribonuclease, the oligonucleotides were isolated, and their sequences were determined by techniques similar to those of Sanger *et al.* (*3, 4*).

Figure 1 shows the radioautographs given by the two-dimensional paper-electrophoretic fractionation of pancreatic and T1 ribonuclease digests of KB cell 5S RNA. Because of the overlapping of oligonucleotides Nos. 9 and 10, and Nos. 53 and 56 in the fractionation of the T1 ribonuclease digest, simultaneous digestion of 5S RNA with this enzyme and alkaline phosphomonoesterose (PME) was carried out. Fractionation resulted in separation of the previously overlapping oligonucleotides (Fig. 2) and permitted their individual analysis.

Table 1 lists the code numbers and the corresponding sequence of the oligonucleotides shown in Figs. 1 and 2. The following procedures were used to establish their sequence (*3, 4*). The spots were eluted, their base composition was determined by alkaline hydrolysis, and, for the short oligonucleotides, their sequences were assigned according to the results of enzymatic digestion by pancreatic or T1 ribonuclease, micrococcal nuclease, or snake-venom phosphodiesterase in the case of dephosphorylated oligonucleotides. To establish the sequences of the longer oligonucleotides it was necessary to digest them partially with spleen or snake-venom phosphodiesterase and analyze the products by alkaline hydrolysis or by digestion with pancreatic ribonuclease. The longest oligonucleotide (No. 55) has 13 nucleotides; its sequence is based on a large number of separate analyses by alkaline hydrolysis and pancreatic ribonuclease digestion of the intact oligonucleotide and of the many digestion products resulting from partial degradation by spleen and snake-venom phosphodiesterase.

The molar yield of each oligonucleotide was calculated by measuring its radioactivity in a low-background gas-flow counter and dividing the result by the number of component bases multiplied by the amount of the radioactivity corresponding to 1 mole of phosphate in the particular fractionation. This last value was calculated by dividing the total recovered radioactivity by 120. The experimental molar yield obtained by this method for each oligonucleotide was compared with the corresponding theoretical molar yield (Table 1) based on the sequence which was ultimately determined for KB cell 5S RNA (Fig. 3). With the following exceptions, the experimental and theoretical molar yields are in close agreement.

The 5′-terminal oligonucleotides in both enzymatic digests, spots 1′ and 6′, were recovered in only half-molar amounts. However, when an alkaline hydrolyzate of KB cell 5S RNA, labeled with C^{14}-nucleoside precursors, was chromatographed on a Dowex-1-formate column (*1*), the only 3′,5′-diphosphate identified coincided with the pGp marker and was recovered in a yield of 0.9 mole. Another unusual feature is that in fractionations of both the pancreatic and T1 ribonuclease digests two spots are present, corresponding to the 5′-end groups. On alkaline hydrolysis both yield only pGp in the T1 ribonuclease digest, and only Up and pGp in the pancreatic ribonuclease digest. Whether this duplication is due to an artifact of the experimental procedures, or to an undetermined structural feature of the molecule at this point is not yet known.

In the T1 ribonuclease digest there are two other oligonucleotides each present in nearly half-molar amounts: spots 50-A and 50-B. Presumably they represent alternate forms of the 3′-end groups of the molecule since they both

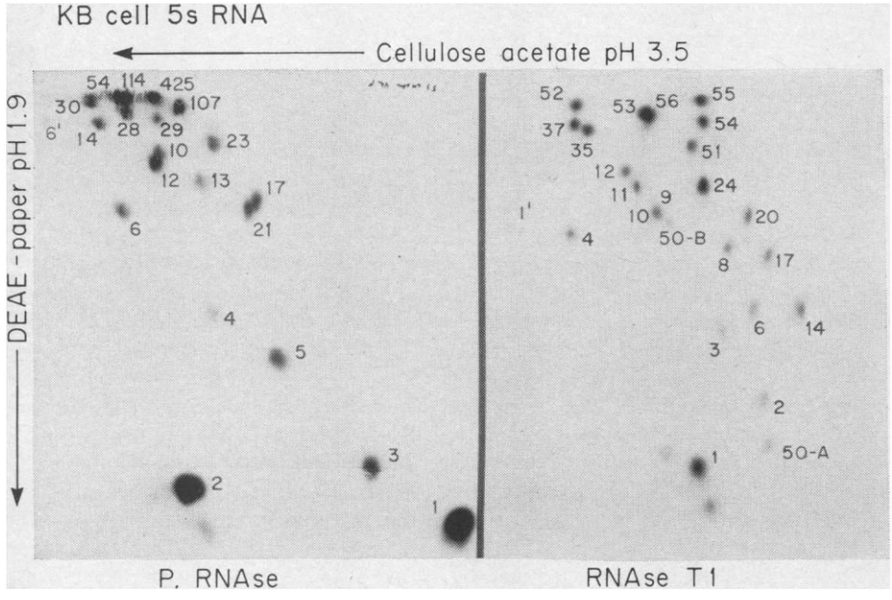

Fig. 1. Radioautographs given by two-dimensional paper-electrophoretic fractionation of pancreatic ribonuclease and ribonuclease T1 digests of P^{32}-labeled KB cell 5S RNA. The sequence of the oligonucleotides is given in Table 1.

contain a free 3′-hydroxyl group and have nearly identical sequences, differing by only one base.

Many of the larger oligonucleotides, in the pancreatic ribonuclease digest more than in the T1 ribonuclease digest, are present in yields lower than those expected: from 0.6 to 0.8 mole. This was true not only in analyses of whole 5*S* RNA digests but also in analyses of purified partial-digestion fragments of KB cell 5*S* RNA. This suggests that the low recovery of these oligonucleotides is due to the methods of RNA digestion and fractionation rather than to heterogeneity of the nucleotide sequence (*4*).

The molar yields of the mononucleotides Cp and Up in the pancreatic ribonuclease digests were also lower than expected. This low yield may be due to the conditions of digestion which release variable quantities of 2′,3′-cyclic nucleoside monophosphates. A more reliable estimate of the total content of Cp and Up in the RNA can be obtained by determining the sum of these nucleotides in all the T1 ribonuclease digestion products (*4*).

Aside from the alternate 3′-end groups, quantitative analysis of the molar yields of oligonucleotides from both pancreatic and T1 ribonuclease digests shows no other clear evidence of heterogeneity in the sequence of KB cell 5*S* RNA.

Comparison of Table 1 to the list of oligonucleotides obtained from digests of 5*S* RNA from *Escherichia coli* (*4*) reveals that there are definite differences in the two cell species. The 3′-terminal nucleoside is uridine in both KB cell 5*S* RNA and *E. coli* 5*S* RNA; but the 5′-terminal nucleotide in the *E. coli* 5*S* RNA is uridylic acid, while in the KB cell 5*S* RNA it is guanylic acid. The sequences of most of the larger oligonucleotides also differ in the two cell species.

To arrange all the oligonucleotides obtained from digests of KB cell 5*S* RNA into one linear sequence, it was necessary to study a large number of partial-digestion fragments of the RNA. These were obtained by techniques similar to those used by Zachau *et al.* (*9*). The KB cell 5*S* RNA was digested with T1 ribonuclease at 0°C for 1 hour, in a solution of 0.2*M* tris-HCl, *p*H 7.5, containing no magnesium, and in the presence of a large excess of nonradioactive *E. coli* tRNA. The digests were extracted with phenol and applied to columns (200 by 0.6 cm) of micro-

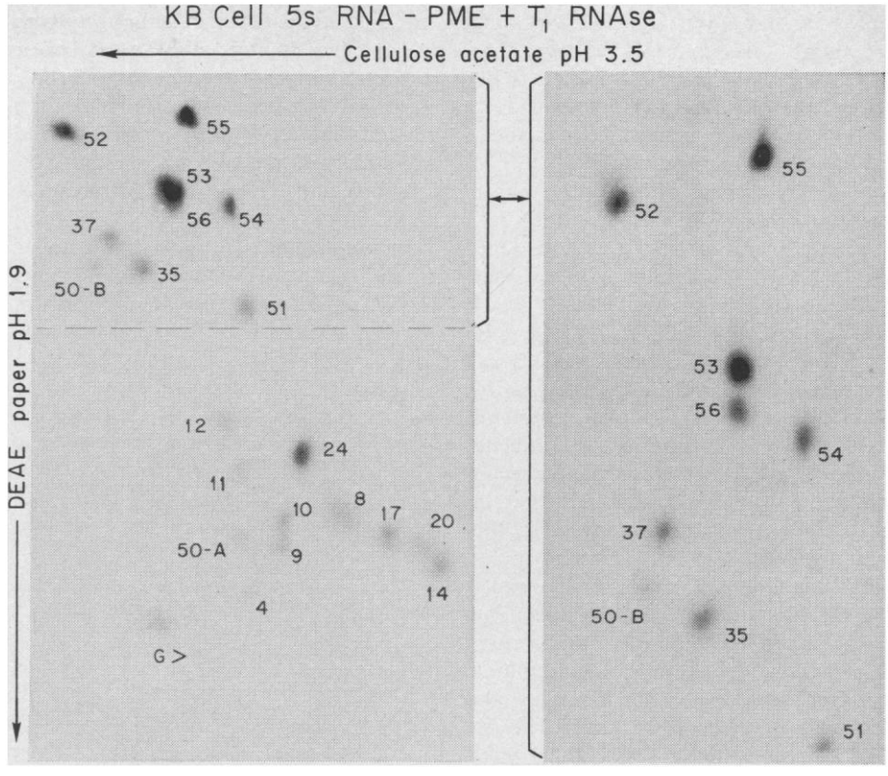

Fig. 2. Radioautographs given by two-dimensional fractionation of P^{32}-labeled KB cell 5*S* RNA digested simultaneously with T1 ribonuclease and alkaline phosphomonoesterase (PME). In the fractionation result shown on the right, the second-dimension electrophoresis was continued for 12 hours instead of the usual 3½ hours. G > represents cyclic guanosine 2′,3′-phosphate. The oligonucleotides are numbered as in the T1 ribonuclease digest of Fig. 1, and their sequence is that given in Table 1, without the terminal 3′-phosphate.

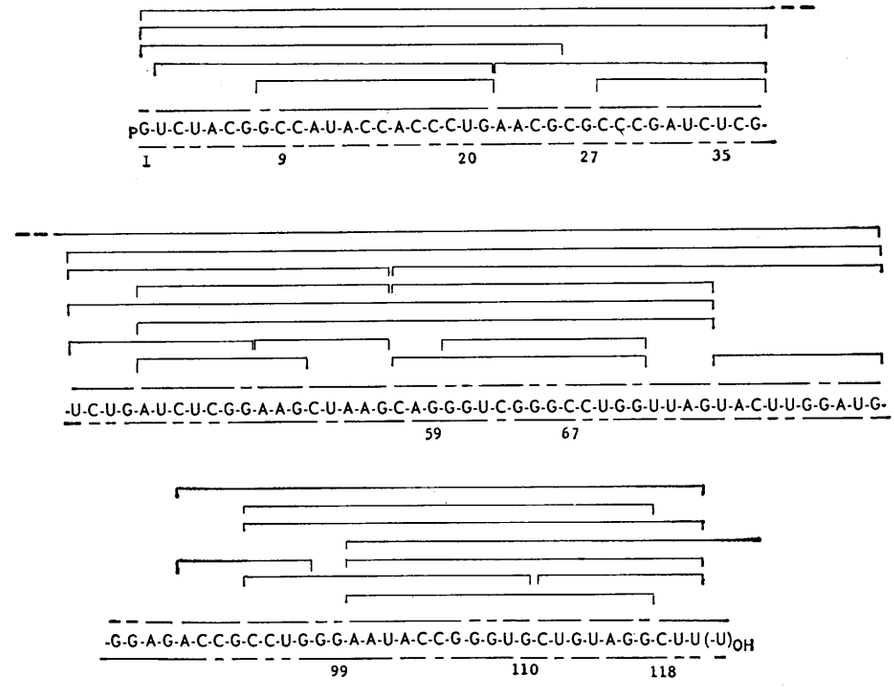

Fig. 3. Sequence of KB cell 5*S* RNA. The lines that overscore the sequence indicate the ribonuclease T1 digestion products, and the lines that underscore the sequence indicate the products of pancreatic ribonuclease digestion. The brackets indicate the composition in oligonucleotides of various T1 ribonuclease partial-digestion fragments, determined by their complete digestion by pancreatic or T1 ribonuclease.

granular DEAE-cellulose, which were eluted by linear gradients of NaCl in 7*M* urea and 0.02*M* tris-HCl, *p*H 7.5. Each individual peak of radioactivity obtained from this first column was chromatographed again on either (i) a column (200 by 0.45 cm) of DEAE-Sephadex that was eluted by a linear gradient of NaCl in 7*M* urea, *p*H 3.5, or (ii) on a heated (55°C) column (200 by 0.6 cm) of DEAE-cellulose that was eluted by linear gradients of NaCl in 7*M* urea and 0.02*M* tris-HCl, *p*H 7.5. Some of the earlier peaks from the first column were also fractionated by two-dimensional paper electrophoresis in the same fashion as the complete RNA digests. These partial-digestion fragments were then analyzed, and in most instances they proved to be homogeneous segments of the molecule, containing 1 mole, or multiples thereof, of specific oligonucleotides. The oligonucleotide composition of each partial-digestion fragment was determined by digesting portions of it completely with pancreatic or T1 ribonuclease. The complete digests were then fractionated by a one- or two-dimensional paper-electrophoresis procedure and compared to a similar fractionation of a whole 5*S* RNA digest to identify the oligonucleotides present. The sequences of the oligonucleotides were not determined individually in each partial-digestion fragment. Each oligonucleotide was given the previously determined sequence of the oligonucleotide occurring in the corresponding position of a similar fractionation of a whole 5*S* RNA digest. In doubtful cases, however, the identification of the oligonucleotide was confirmed by alkaline hydrolysis or by appropriate enzymatic digestion.

Over 35 different partial-digestion fragments were analyzed. All the oligonucleotides can be grouped into three large partial-digestion fragments which correspond to three general regions of the molecule (Fig. 3). Together the three fragments contain all of the oligonucleotides listed in Table 1 except for No. 425 of the pancreatic ribonuclease digest. Smaller partial-digestion fragments permitted the arrangement of the oligonucleotides into a single possible logical linear sequence within each of the three large fragments or regions of the molecule. In most cases the order of the oligonucleotides in the smaller fragments could be established by complete digestion of portions of the fragment by both pancreatic and T1 ribonuclease. The results of both digestions are usually compatible with only one logical linear sequence. Longer fragments, overlapping these short fragments of known sequence, lengthen the sequence by progressive addition of oligonucleotides to the short fragments and order all the oligonucleotides into only one possible sequence within each of the three regions.

In two regions (residues 57 through 75 and residues 100 through 117) there was increased resistance to splitting by T1 ribonuclease, with the result that no short partial-digestion fragments were obtained after the primary partial digestion. This allowed alternate arrangements of the oligonucleotides in these regions. To obtain the necessary short fragments, we subjected the fragments from the resistant regions to partial digestion for a second time with T1 ribonuclease at 0°C for 45 minutes, under the same conditions as in the primary partial digestion. The digest was then extracted with phenol; the extract was placed on a column of DEAE-Sephadex and eluted with a linear NaCl gradient in 7*M* urea, *p*H 3.5 This procedure yielded shorter partial-digestion fragments which established the sequence of the doubtful areas.

Table 1. Sequence and molar yield of the oligonucleotides derived from pancreatic and T1 ribonuclease digestion of KB cell 5*S* RNA. The code numbers (spot No.) of the oligonucleotides correspond to those of Figs. 1 and 2. The sequence is given from the 5′-end (left) to the 3′-end (right) of the oligonucleotide. Each molar experimental yield (exp.) was determined as described in the text, and is listed opposite the molar theoretical yield (theor.) derived from the sequence ultimately determined for KB cell 5*S* RNA. The hyphens indicate phosphate groups.

Spot No.	Sequence	Moles (No.)	
		Exp.	Theor.
Pancreatic ribonuclease digestion products			
1	C-	13.2	17
2	U-	10.1	13–14
3	A-C-	4.8	5
4	A-U-	1.3	1
5	G-C-	4.2	4
6	G-U-	2.2	2
10	A-G-U-	1.1	1
12	G-A-U-	1.9	2
13	G-G-C-	0.94	1
14	G-G-U-	1.15	1
17	A-A-G-C-	1.03	1
21	G-A-A-C-	0.95	1
23	A-G-G-C-	0.73	1
28	G-G-A-U-	1.07	1
29	G-G-G-C-	0.76	1
30	G-G-G-U-	1.01	1
54	A-G-G-G-U-	0.59	1
107	G-G-A-A-G-C-	0.97	1
114	G-G-G-A-A-U-	0.8	1
425	G-G-G-A-G-A-C-	0.57	1
6′	pG-U-	0.5	1
T1 Ribonuclease digestion products			
1	G-	13.8	15
2	C-G-	1.27	1
3	A-G-	1.27	1
4	U-G-	1.08	1
6	C-A-G	0.89	1
8	A-A-G-	0.95	1
9	U-C-G-	0.99	1
10	C-U-G-	0.99	1
11	U-A-G-	1.0	1
12	A-U-G-	1.0	1
14	C-C-C-G-	1.02	1
17	A-C-C-G-	0.87	1
20	A-A-C-G-	0.85	1
24	C-C-U-G-	2.12	2
35	U-C-U-G-	1.19	1
37	U-U-A-G-	0.89	1
51	C-U-A-A-G-	0.86	1
52	U-A-C-U-U-G-	0.99	1
53	A-U-C-U-C-G-	1.84	2
56	U-C-U-A-C-G-	0.85	1
54	A-A-U-A-C-C-G-	0.8	1
55	C-C-A-U-A-C-C-–A-C-C-C-U-G-	0.58	1
1′	pG-	0.5	1
50-A	C-U-U_{OH}	0.65	0.5
50-B	C-U-U-U_{OH}	0.47	0.5

The results of both pancreatic and T1 ribonuclease digestion of the individual bracketed fragments, and the various overlaps between the different brackets, establish the complete sequence of the molecule—a single logical linear sequence—with the following exception. The fragments obtained allow two alternate positions for the dinucleotide CpGp placed in positions 26 and 27. By the data shown, it could also be located in positions 36 and 37, at the far right-hand end of the large fragment encompassing residues 1 to 37. This alternate position was disproved by the following experiment. The large fragment of the region was treated with 0.1*N* HCl to open the cyclic terminal 2′,3′-phosphate bonds, neutralized, and then treated with PME, which splits off the terminal phosphate. The digest was extracted with phenol and then completely digested with T1 ribonuclease and fractionated by two-dimensional paper electrophoresis. The dinucleotide CpGp was not dephosphorylated, and therefore it must be placed internally within the fragment. That 75 to 80 percent of oligonucleotide No. 53 was dephosphorylated is confirmation of its position at the end of the fragment.

Once the sequence of each of the three large regions was established, it was possible to arrange them in relation to one another. One contained the 5′-end group, another had the 3′-end group, and the third had no end group. One very large partial-digestion fragment contained all of the 5′-end and central regions, and provided an overlap confirming the arrangement of these two large regions of the molecule. There is no fragment which overlaps the central and 3′-end regions. The point which joins these two regions of the molecule must be very susceptible to the action of T1 ribonuclease. The missing overlap,

however, is given by the oligonucleotide GpGpGpApGpApCp (No. 425) derived from complete digestion of the RNA by pancreatic ribonuclease. It is the only oligonucleotide from Table 1 not already accounted for in the partial-digestion fragments obtained.

Since it is the only pancreatic ribonuclease product ending in . . . GpApCp, it must overlap with the oligonucleotide ApCpCpGp which is the only T1 ribonuclease digestion product beginning in ApCp . . . and is present at the far left of the 3′-end region of the RNA. In summary, the results shown in Fig. 3 indicate the complete linear sequence of KB cell 5*S* RNA.

The presence of regions in the molecule that show marked and reproducible variability in susceptibility to digestion by T1 ribonuclease, as noted above, suggests that the molecule has a specific secondary structure. If, in addition to classic Watson-Crick base-pairing of nucleotides, one proposes occasional Gp-to-Up base-pairing, as assumed in the cloverleaf model for tRNA (*7, 8*), it is possible to construct different models of the possible secondary structure of KB cell 5*S* RNA. The molecule contains two sets of complementary sequences, nine nucleotides in length. Residues 1 through 9 (Fig. 3) can form base pairs with residues 118 through 110, thus joining the 5′-end to the 3′-end of the molecule as in the cloverleaf model for tRNA (*7, 8*); and residues 27 through 35 can form base pairs with residues 67 through 59. There are a number of additional complementary sequences containing from 3 to 6 nucleotides. They can be arranged in different patterns. Selection of a final model of the secondary structure of KB cell 5*S* RNA will depend on the detailed comparison of its sequence to that of 5*S* RNA in other cell species, such as that of *E. coli* 5*S* RNA (*12*). In *E. coli* 5*S* RNA there is also the possibility for base pairing of the 5′-end of the RNA with its 3′-end for a length of 10 nucleotides

In KB cell 5*S* RNA there are two sequences, one of seven nucleotides and another of six nucleotides, which occur twice in the molecule: the sequence GpApUpCpUpCpGp (residues 31 to 37 and 41 to 47) and the sequence GpCpCpUpGpGp (residues 66 to 71 and 93 to 98). In *E. coli* 5*S* RNA there are sequences of ten and eight nucleotides which also occur twice (*12*). However, in *E. coli* 5*S* RNA these sequences are repeated in different halves of the molecule, while in 5*S* RNA from KB cell they are repeated in the same half of the sequence.

In most models of the secondary structure of KB cell 5*S* RNA, the sequences GpApApUp (residues 99 to 102 and UpGpApApCp (residues 20 to 24) are not involved in base pairing. These sequences are complementary in an "antiparallel" fashion to four or five bases in the pentanucleotide sequence GpTpψpCpCp which is common to most tRNA's and is tentalively located in the loop of the limb of the molecule nearest its 3′-end (*7, 13*). This association may be fortuitous or it may be related to the function of 5*S* RNA in the ribosome. It is of interest that *E. coli* 5*S* RNA contains the sequences GpApApCp (residues 107 to 110) and CpGpApApCp (residues 43 to 47) which are also complementary to four or five bases in the pentanucleotide sequence GpTpψpCpGp of tRNA (*12*).

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11. Abbreviations used: tRNA, transfer ribonucleic acid; PME, alkaline phosphomonoesterase; DEAE, diethylaminoethyl; tris-HCl, tris (hydroxymethyl)aminomethane hydrochloride; T, ribothymidine; Ψ, pseudouridine; A, adenosine; C, cytidine; G, guanosine; U, uridine; the subscript OH is used to indicate the presence of a 3′-hydroxyl group; p, on the left of A, C, and the like, indicates a 5′-phosphate; and on the right, a 3′-phosphate. In Fig. 3 and Table 1, hyphens are used instead of p.
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Photoreactivation in vivo of Pyrimidine Dimers in Paramecium DNA

Abstract. *Cells of* Paramecium aurelia *labeled with tritiated thymidine were irradiated with ultraviolet light and then were either exposed to photoreactivating light or kept in the dark as controls. In the controls, the level of thymine-containing pyrimidine dimers did not change, but in cells exposed to photoreactivating light such dimers were destroyed. This is the first demonstration in a eukaryote of in vivo photoreactivation of thymine-containing pyrimidine dimers.*

Ultraviolet light (2200 to 3000 Å) causes both death and mutation in microorganisms and higher organisms. A large body of evidence indicates that cyclobutane-type pyrimidine dimers in DNA of irradiated organisms are important in the lethal and mutagenic effects of ultraviolet light (*1, 2*). The most convenient method for evaluating the biological role of these dimers is by means of photoreactivation—reversal of the ultraviolet-induced damage by irradiation with light of longer wavelengths. The photoreactivating ability of an organism may be observed in two ways: (i) biological photoreactivation, and (ii) the destruction of ultraviolet-induced pyrimidine dimers in vitro (*3*) and in vivo (*4*). In microorganisms the concurrent presence or absence of these two phenomena permits evaluation of the role of dimers in the lethal and mutagenic effects of ultraviolet light; however, such an evaluation has not been possible for organisms higher than bacteria. Although dimers have been found in the DNA of eukaryotes as a result of irradiation with ultraviolet light (*5*), there have been no demonstrations of in vivo destruction of dimers in eukaryotes. We report the first such case: the in vivo photoreactivation of thymine-containing pyrimidine dimers in the DNA of *Paramecium aurelia.*

The DNA of paramecia (syngen 7, stock 57) was labeled specifically (*6*) by letting the animals feed overnight on cells of *Escherichia coli* 15T$^-$ that had been grown in medium containing ^{3}H-methyl thymidine with high specific activity. The paramecia were irradiated in Dryl's (*7*) solution, which is transparent to ultraviolet light. A germicidal lamp, emitting mainly at 2537 Å, supplied the ultraviolet light exposure of 4000 erg mm^{-2} at a rate of 55 ergs