B.C. Further supporting evidence suggests a low sea level between 4000 and 4500 B.C. The Kruzensternian transgression proposed by Hopkins (*7*) appears to be in general agreement with these provisional events.

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## Oligoribonucleotides: Improvement in Chromatographic Separation

Abstract. *The separation of mixed oligoribonucleotides obtained from enzymatic digestion of soluble RNA into fractions containing mixed oligonucleotides of identical chain length is improved by absence of terminal phosphate. Each fraction may be separated into individual components by ion-exchange (Dowex) chromatography. Combination of these two chromatographic procedures yields maximum information on primary structure of the oligonucleotides.*

One of the problems attendant upon characterization of the linear nucleotide sequence of nucleic acids by digestion with specific enzymes is the separation of the resultant mixture of oligonucleotides into individual components. No chromatographic system is known which is capable of resolving all the possible fragments in a single operation. We report here a satisfactory procedure which depends upon preliminary separation of the oligonucleotide mixture on the basis of oligonucleotide length, followed by a chromatographic resolution of fractions containing mixed oligonucleotides of identical chain length (isopleths) into individual components.

Tomlinson and Tener (*1*) reported that pancreatic ribonuclease digests of soluble RNA could be fractionated on columns of DEAE cellulose (*2*) in 7.0*M* urea at *p*H 7.5 with a linear sodium acetate gradient. At this *p*H, and in the presence of concentrated urea, the major binding force is a function of the phosphate residues, and hence fractionation occurs on the basis of oligonucleotide length (number of phosphate residues per oligonucleotide). A similar technique was used by Salas *et al.* (*3*) to separate oligonucleotides of general form (Ap)*n*C utilizing DEAE cellulose and an exponential gradient of NaCl in 8.0*M* urea, 0.01*M* tris-HCl at *p*H 7.8.

Bartos, Sober, and Rushizky (*4*) confirmed these results for pancreatic ribonuclease digests of high-molecular-weight RNA; but they also reported that takadiastase ribonuclease $T_1$ digests did not give simple and unambiguous resolutions on DEAE cellulose. This was attributed to subfractionation of each isopleth according to the ratio of purine to pyrimidine. The same authors (*5*) further reported adequate resolution of a digest of high-molecular-weight RNA from bacteriophage in 7.0*M* urea, with DEAE-Sephadex as adsorbent.

Our attempts to apply this technique to the study of $T_1$ ribonuclease digests of serine sRNA from yeast gave the results shown in Fig. 1*a*. The resolution obtained is only partially satisfactory because of increasing overlaps with isopleths of increasing chain length. Various attempts to improve resolution by modification of the elution gradient were unsuccessful, but perfect resolution could be obtained by removal of terminal monoester phosphate from the oligonucleotide fragments. As expected, the dephosphorylated oligonucleotides are eluted at much lower concentrations of NaCl (Fig. 1*b*).

In accordance with the results of Tomlinson and Tener (*1*), fractionation occurs on the basis of the number of phosphate residues per oligonucleotide, and peaks I, II, III, IV of Fig. 1*b* correspond to mono-, di-, tri-, and tetra- . . . nucleotides. The low yields of peaks VI and VIII in Fig. 1*b* are due to the fact that hexa- and octanucleotides are absent from $T_1$ digests of serine sRNA from yeast.

Hydrolysis of phosphodiester bonds of RNA by $T_1$ ribonuclease proceeds in two steps: (i) formation of a cyclic 2′,3′-phosphodiester; and (ii) opening of the cyclic bond. In cases where this process ceases after step 1 (for example, methylated guanines, *5*), dephosphorylation by phosphomonoesterase is impossible. Therefore, oligonucleotides consisting of (*n*) nucleotides terminating in a cyclic phosphate are eluted in peak number (*n* + 1) owing to the presence of the terminal phosphate. With this restriction, which may be eliminated by exposure of oligonucleotide fragments to *p*H 2 to open the cyclic 2′,3′-phosphate (*3*) and by further treatment with *Escherichia coli* monoesterase at *p*H 7, the above technique is useful in elucidation of oligonucleotide structure.

Extrapolation from isoplethic origin (that is, oligonucleotides isolated from each *n*th isopleth should contain *n* component bases) serves as an internal check on adequacy of analytical procedures. Deviation in estimation of chain length from values calculated on

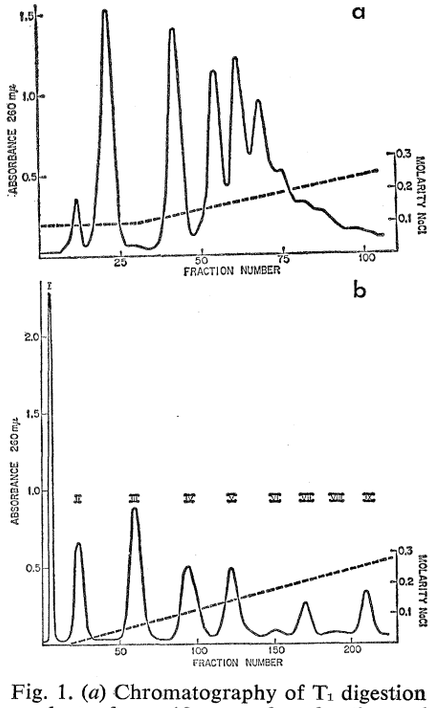


Fig. 1. (*a*) Chromatography of $T_1$ digestion products from 12 mg of unfractionated yeast sRNA on a column (0.7 by 96 cm) of DEAE-Sephadex A-25, coarse, in 7.0*M* urea, *p*H 8.4; 10-ml fractions. The pattern was unchanged at *p*H 7.6 to 8.4. (*b*) Chromatography of products of digestion with $T_1$ and phosphomonoesterase of 13 mg of partially purified serine sRNA; DEAE-sephadex column (0.7 by 98 cm) in 7.0*M* urea, *p*H 7.6; 9-me fractions.

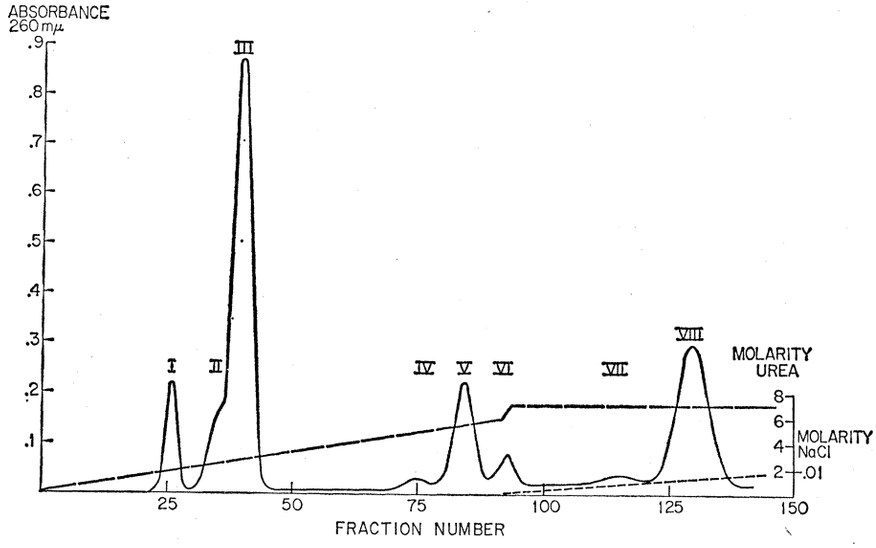


Fig. 2. Chromatography of 98 optical-density (260 mμ) units of dinucleotides derived from pancreatic ribonuclease digest of partially purified serine sRNA. The volume of the first gradient was 1450 ml of 0 to 7.0*M* urea, *p*H 3.3 (meter reading), adjusted with HCl. The second gradient was 1000 ml of 0 to 0.01*M* NaCl in 7.0*M* urea, *p*H 3.3 (adjusted with HCl). Fractions were 11 ml. Peak I, ApC; peak II, GpMeC; peak III, GpC; peak IV, Ap; peak V, ApU; peak VI, unidentified; peak VII, GpΨ; peak VIII, GpU. Recovery more than 90 percent.

the basis of isopleth separation may be due to (i) the presence of bases which have escaped detection either because of intrinsic spectroscopic properties (for example, dihydrouridine) or because of chemical lability; or (ii) the inadequacy of resolution of individual isoplethic components. The first alternative is illustrated by the data of Holley *et al.* (*7*) as follows. The trinucleotide diHUpApG that appears in the trinucleotide fraction would, after hydrolysis, yield only two components (A and G) when examined as usual for ultraviolet absorption at 260 mμ. The second alternative is illustrated by the original report of Dutting and Zachau (*8*) that the $T_1$ digest of yeast serine sRNA contains a hexanucleotide but no heptanucleotides. This result appears to differ from the results shown in Fig. 1*b*.

However, upon further investigation, Melchers, Dutting, and Zachau (*9*) subsequently amended their original interpretation and now suggest that hexanucleotide (Up,Up,ApApC)G (*8*) is absent, but that a heptanucleotide (Cp,ApApCp,Up,Up)G is present in the $T_1$ digest of serine sRNA. As noted, we were able to conclude directly from the data of Fig. 1*b* that yeast serine sRNA did not yield a hexanucleotide upon $T_1$ digestion, but rather a heptanucleotide, the composition of which, determined independently, agrees with that now proposed by Melchers, Dutting, and Zachau (*9*).

Resolution of a mixture of oligonucleotides of equal chain length can be obtained by use of chromatography on Dowex 1 × 2 $Cl^-$ at *p*H 3.3 with linearly increasing gradients of urea or NaCl. The limiting concentration of NaCl increases with increasing chain length of the fragments from 0.01*M* for dinucleotides to 0.31*M* for decanucleotides. As in the case of mononucleotides (*10*), the resolving power of Dowex is much greater than that of DEAE, which has been used by other investigators (*11*) to separate isoplethic oligonucleotides into their components. This general procedure is equally useful for $T_1$ or pancreatic ribonuclease digests. An example of the degree of resolution possible is shown in Fig. 2.

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## Spin Label Studies in Chlamydomonas

Abstract. *When spin label is added to* Chlamydomonas *the organism is apparently unaffected, but the paramagnetic resonance signal of the spin label decreases. Irradiation with visible light greatly accelerates this decrease, which is partially reversible. If the cells are grown in the presence of the spin label and washed well, no spin label signal is detectable. However, such cells can no longer catalyze the destruction of added spin label in the light. This finding suggests that the molecule is bound to a specific site, which undergoes a change in conformation with illumination.*

The technique of labeling biological molecules with a compound which is a permanent free radical has recently been introduced (*1*). The presence of such a molecule, called a "spin label," can be detected by means of electron paramagnetic resonance (EPR) spectroscopy. The compounds consist of a nitroxide radical with a pair of methyl groups on each side. The molecule available to us (Fig. 1) is notably stable in aqueous solution. The nitroxide radicals have a sharp, strong, three-line paramagnetic resonance spectrum. Binding of the spin label to another molecule in a way which affects its rotational freedom is evidenced by a change in the EPR spectrum. However, even in the absence of steric effects, binding may still be demonstrated.

Having studied for some years the naturally occurring paramagnetic resonance spectra induced by light in photosynthetic systems, in the belief that these