Our previous studies suggested that aryl hydrocarbon hydroxylase generates an active intermediate and is responsible for polycyclic hydrocarbon toxicity (*3*, *5–7*). Phenolic products of enzymatic hydroxylation are either of low or no carcinogenicity. Thus, the ultimate carcinogenic form is likely a reaction intermediate. In the case of DMBA this may be one of the hydroxymethyl derivatives which have been found to be carcinogenic (*16*). The carcinogenic forms may also be epoxides, carbonium ions, or radical cations (*17*). The mechanism of some microsomal aromatic hydrocarbon hydroxylations seems to involve an epoxide intermediate. Thus, naphthalene hydroxylation has been shown to proceed via naphthalene 1,2-epoxide (*18*). Epoxides may be metabolized to hydroxylated derivatives by an epoxide hydrase (*18*, *19*). We do not know whether the inhibitory effect of 7,8-benzoflavone is specific to DMBA tumorigenesis or whether this inhibitor is equally effective on tumorigenesis induced by other polycyclic hydrocarbons. This requires investigation.

Since 7,8-benzoflavone and 5,6-benzoflavone show biological affinity for the enzyme system as well as the induction receptor site for polycyclic hydrocarbons, it is possible that they have affinity for a hypothetical receptor site other than the enzyme at which the carcinogenic polycyclic hydrocarbons initiate tumorigenesis. Although this possibility is not eliminated, the data presented suggest that the tumorigenesis inhibitory activity of 7,8-benzoflavone is due to inhibition of the aryl hydrocarbon hydroxylase and that this enzyme system is responsible for activation of the administered DMBA to its carcinogenic form.

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Structure of RNA in Ribosomes

Abstract. *The 50S and 30S ribosomes and 23S and 16S RNA were hydrolyzed with ribonuclease A. The rate constants and number of fragments produced were determined for each reaction. The conformation of 23S RNA changes when the RNA is extracted from the ribosome. Specific regions of the RNA in 50S and 30S ribosomes are protected from hydrolysis by the ribosomal proteins.*

The role of ribosomal RNA (rRNA) in the structure and function of ribosomes has not been resolved. Optical rotatory dispersion and x-ray diffraction studies suggest that the average secondary structure is the same for protein-free rRNA and for rRNA in ribosomes (*1*, *2*). Most of the phosphate groups of rRNA, both protein-free and in ribosomes, bind dye molecules and magnesium ions, which suggests that the phosphates are available to the solvent (*1*, *3*). Ribonuclease hydrolyzes the RNA in ribosomes, which suggests that RNA may be a surface component of the ribosome (*4*). Examination of hydrolysis products of protein-free rRNA gives evidence that a definite tertiary structure exists for RNA in solution (*5*). Previous work has not shown differences in conformation for protein-free rRNA and RNA in ribosomes.

To determine whether specific regions of rRNA in ribosomes differ in conformation from the same regions of protein-free rRNA, we studied the initial stages of the ribonuclease-catalyzed hydrolysis reactions of 50*S* and 30*S* ribosomes and their 23*S* and 16*S* rRNA components by high resolution methods.

Ribosomes were extracted from *Escherichia coli* D10 (grown in tryptone medium) by standard methods (*6*). The 50*S* and 30*S* subunits were separated by zonal centrifugation in Beckman Ti14 rotor (*7*). Sucrose was removed with a diafiltration cell (Amicon). The 23*S* and 16*S* rRNA's were extracted from the 50*S* and 30*S* subunits, respectively, by the phenol–sodium dodecyl sulfate (SDS) method.

The hydrolysis reaction was carried out in a solution (50 μl) containing RNA (either free or in ribosomes, 0.5 mg/ml), magnesium acetate (0.005*M*), and tris(hydroxymethyl)aminomethane (0.005*M*, *p*H 7.4). Pancreatic ribonuclease (Worthington) was present at 0.01 μg/ml for 50*S*, 23*S*, and 16*S*, and at 0.4 μg/ml for 30*S*. The solutions were incubated at 0°C for the desired time. Addition of SDS to a concentration of 0.2 percent stopped hydrolysis and dissociated the ribosomal proteins from rRNA. Three small sucrose crystals were dissolved in each solution, which was then layered on a polyacrylamide gel. Electrophoresis resolved the undegraded RNA and the hydrolysis products (*8*).

Mild hydrolysis of the 50*S* ribosome produced two fragments of rRNA

(Fig. 1). In the profile of the RNA separated from untreated 50*S* particles (Fig. 1, curve a), there is a large band due to 23*S* rRNA, a small band called 50A, a smaller band which is 16*S* rRNA marker, and a small band called 50B. Small amounts of bands 50A and 50B are usually found in preparations of 50*S* ribosomes and appear to be due to hydrolysis by *E. coli* ribonucleases. Such preparations show normal activity for protein synthesis in vitro (*9*). Treatment of the ribosomes with pancreatic ribonuclease A (Fig. 1, curve b) caused a continuous diminution of the height of the 23*S* RNA band as a function of time and a concomitant increase of bands 50A and 50B. The band due to 16*S* RNA remained constant. The log of the molecular weight of RNA is a linear function of the migration distance (*8*). For gels calibrated with 23*S* and 16*S* RNA's (1.1×10^6 and 0.55×10^6 g/g-mole, respectively), the molecular weights of bands 50A and 50B were 660,000 and 410,000, respectively. The sum of these values is within 3 percent of the molecular weight of 23*S* RNA. We conclude that bands 50A and 50B are the two primary fragments produced by the first hydrolysis of 23*S* RNA in the 50*S* ribosome structure and that little of these fragments has been removed by subsequent hydrolysis.

Protein-free 23*S* RNA was hydrolyzed under conditions identical to those used for the 50*S* particles. Before 23*S* RNA was treated with ribonuclease, a large band due to 23*S* was seen at the left with several very small bands (due to some residual degradation) toward the right of 23*S* (Fig 1, curve c). Treatment of the 23*S* RNA with ribonuclease A caused a diminution of the 23*S* band and the appearance of six bands of lower molecular weight (Fig. 1, curve d). The molecular weights of these RNA fragments were determined as above. From left to right, the fragment designations with molecular weights are: 23C (910,000), 23D (800,000), 23A (660,000), 23B (410,000), 23E (290,000), and 23F (218,000). Fragments 23A and 23B appear to be identical to bands 50A and 50B, and the sum of the molecular weights of these two RNA fragments again equals the value for 23*S* RNA. The molecular weights of the remaining pairs of RNA fragments also follow this relation within 3 percent variation as follows: 23C + 23F = 23D + 23E = 23*S*. These relations suggest that the six fragments resulted from the hydrolysis of intact 23*S* RNA

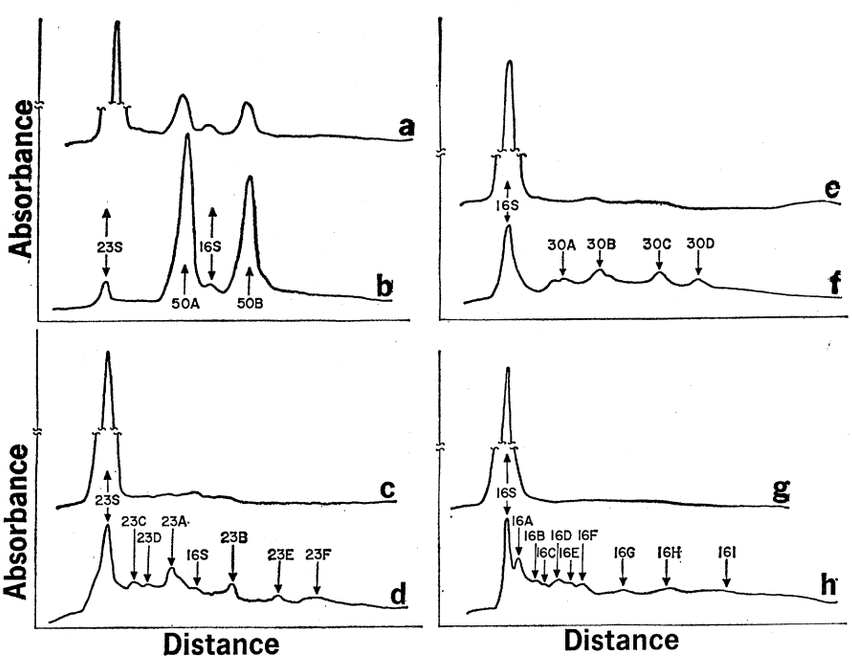

Fig. 1. Polyacrylamide gel electrophoresis of RNA fragments. (a) 50*S* ribosomes, no ribonuclease, 3 percent gel; (b) 50*S* ribosomes, plus ribonuclease for 40 minutes, 3 percent gel; (c) 23*S* rRNA, no ribonuclease, 3 percent gel; (d) 23*S* rRNA, plus ribonuclease for 40 minutes, 3 percent gel; (e) 30*S* ribosomes, no ribonuclease, 4 percent gel; (f) 30*S* ribosomes, plus ribonuclease for 20 minutes, 4 percent gel; (g) 16*S* RNA, no ribonuclease, 4.4 percent gel; (h) 16*S* RNA, plus ribonuclease for 27 minutes, 4.4 percent gel.

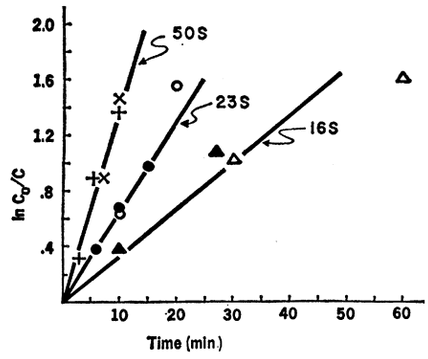

Fig. 2. The kinetics of depletion of intact RNA by hydrolysis with pancreatic ribonuclease A. Duplicate runs are shown as follows: X, +, 50*S* ribosomes; ○, ● 235 rRNA; △, ▲ 16*S* rRNA. All were treated with 0.01 μg ribonuclease A per milliliter.

at three sites by three separate parallel reactions, each giving two RNA fragments. For the 50*S* ribosome, there is one site for hydrolysis and two fragments are produced. We conclude that either two of the three sites available in 23*S* RNA are sterically protected by protein in the 50*S* particle or that the interactions of proteins with 23*S* RNA in the ribosome changes the tertiary or secondary structures of the RNA to eliminate these sites (*10*).

The concentration (C) of nonhydrolyzed 50*S* ribosomes in the reaction mixture is proportional to the absorbance (height) of the 23*S* band in the gel scan. The natural logarithm of C_0/C was plotted against time, resulting in a straight line (Fig. 2, line labeled 50*S*) as expected for pseudo first-order kinetics at constant enzyme concentration. The slope of this line gives the pseudo first-order rate constant (k'). For 50*S*, k' equals 13.8×10^{-2} min^{-1}.

The rate of hydrolysis of the 23*S* rRNA was similarly analyzed (Fig. 2, line labeled 23*S*). The linear relation obtained again confirms pseudo first-order kinetics with k' of 23*S* equal to 6.5×10^{-2} min^{-1}. This k' is the sum of rate constants for three separate, parallel reactions of the 23*S* RNA. No product inhibition is observed for the reactions of either 23*S* RNA or the 50*S* ribosome.

Because the reactions for both protein-free 23*S* RNA and 50*S* ribosomes were run at the same enzyme concentration, the ratio of the second-order rate constants (k50*S*/k23*S*) is equal to the ratio of the pseudo first-order rate constants (k'50*S*/k'23*S*) and is about 2. Since fragments 50A and 50B are identical to 23A and 23B in elec-

trophoretic mobility, we conclude that hydrolysis occurs at about the same point in the sequence of nucleotides for both protein-free 23*S* RNA and 23*S* RNA in the 50*S* ribosome. The rate constant for the hydrolysis of this site in the 50*S* ribosome is over twice the rate for the site in protein-free 23*S* RNA. Therefore this region of RNA has a different conformation in the 50*S* ribosome than in the protein-free 23*S* RNA.

Hydrolysis of the 30*S* ribosome gave four RNA fragments with designations and molecular weights as follows (Fig. 1, curves e and f): 30A (210,000), 30B (130,000), 30C (50,000), and 30D (28,000). Hydrolysis of the protein-free 16*S* RNA gives nine fragments: 16A (450,000), 16B (320,000), 16C (290,000), 16D (225,000), 16E (195,000), 16F (160,000), 16G (120,000), 16H (53,000), and 16I (18,000). The presence of these bands was confirmed by staining gels with methylene blue. Because we lacked adequate low-molecular-weight markers, these values are not of sufficient accuracy to exclude the possibility that 30A = 16D, 30B = 16F or 16G, 30C = 16H, and 30D = 16I. However, fragments 16A, 16B, and 16C are not present in the fragments from 30*S*. This shows that sites of hydrolysis exist in protein-free 16*S* RNA which do not exist in the 30*S* ribosome. In the 30*S* ribosome these RNA sites are either sterically shielded by the ribosomal protein, or the interaction of proteins with 16*S* RNA changes the conformation of the RNA to eliminate these sites. We found no obvious relations between the molecular weights of fragments from the 30*S* ribosome or the 16*S* RNA. The 30*S* ribosome and 16*S* RNA also resulted in more fragments, respectively, than 50*S* and 23*S*. The hydrolysis reactions of 30*S* and 16*S* are probably more complicated than those of 50*S* and 23*S*.

The kinetics of degradation of 30*S* ribosomes and 16*S* RNA was examined by plotting ln C_0/C against time. For 16*S* RNA, initial points fit a straight line confirming pseudo first-order kinetics with k' (16*S*) equal to 3.3×10^{-2} min^{-1} (Fig. 2, line labeled 16*S*). The 30*S* ribosomes required much higher concentrations of ribonuclease to produce significant hydrolysis at 0°C than did 16*S* rRNA. The data for 30*S* gave curved lines which became asymptotic to a constant ordinate value suggesting enzyme inhibition. We estimate that k'(30*S*) is about 1/20 of k'(16*S*). Also, k'(23*S*)/k'(16*S*) is about 2, which suggests that the conformations of 23*S* and 16*S* RNA's differ.

Our results are consistent with those of Cahn *et al.* (*9*) who also observed fragments 50A and 50B from 50*S* ribosomes. They showed that 50*S* ribosomes containing these fragments have little or no activity in a cell-free amino acid incorporation system. Our rate constants indicate that the region of 23*S* RNA which is hydrolyzed in the 50*S* ribosome has a special conformation. This region is much more accessible to ribonuclease than is the same region in deproteinized RNA. In view of the inactivation observed by Cahn *et al.*, this region of RNA may have an important role in protein synthesis.

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Polymeric Structure of Spruce Lignin

Abstract. *From the Flory-Stockmayer theory and molecular weights of successive fractions of lignin obtained by extraction of spruce wood with a mixture of water, dioxane, and hydrochloric acid and by sulfonation, the average length of the primary chains was estimated to be 18 phenylpropane units. From data on the acid-catalyzed cleavage of model compounds in various solvents the degrees of cross-linking in four extraction processes were calculated. The average degree of cross-linking of intact lignin was found to be 0.277; that is, 5 out of every 18 phenylpropane units bear cross-linking benzyl ether groups. Of the five, three are also etherified at their phenolic ends (B groups), and two are not (X groups).*

In the sulfite pulping of wood, the only reactions of lignin positively known to have a bearing on the mechanism of delignification occur at the benzyl positions of its constituent phenylpropane units (*1*). We have accumulated experimental evidence that in other processes of delignification in acidified solvents, both aqueous and nonaqueous, the significant delignifying reactions also occur at the benzyl positions (*2*). In one key instance—delignification of softwoods by means of acidified 2,2-dialkoxypropane (*3*)—only reactions at the benzyl positions occur with sufficient speed and to an adequate degree to account for the experimental results (*4*). We have now examined the implications of these results in terms of currently accepted theories of polymer cross-linking (*5*) and have found that the extraction of lignin from wood can be treated as the depolymerization of a three-dimensional gel composed of relatively short linear (or slightly branched) polymer chains joined by frequent cross-linking benzyl ether groups.

These conclusions are distinct from those of Szabo and Goring (*6*), who assumed that delignification is the reverse of polymerizing a mixture of di- and trifunctional monomers. They further assumed that the lignins of the cell wall and the middle lamella have somewhat different structures and, hence, different patterns of delignification. By modifying the appropriate well-known mathematics (*5*), Szabo and Goring were able to generate theoretical curves of lignin concentration in secondary wall and middle lamella versus the total