The Specific Cleavage of Yeast Ribosomal Ribonucleic Acid with Nucleases*

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ABSTRACT: The fractionation by polyacrylamide gel electrophoresis of ribonucleic acid (RNA) species in the 4–15 S range is described; a relation between R_F in the gel and sedimentation coefficient has been determined. Yeast, as well as *Escherichia coli*, ribosomal RNA on mild digestion with endonucleases gives rise to essentially homogeneous intermediates, not more than about 12 in number. Only a few of these species can be resolved in the ultracentrifuge. Low concentrations of such components are in general present in ribosomal RNA prepared by the standard procedures. The presence of labile points (hot spots) in the polynucleotide chain is inferred, and it is suggested that these arise most probably from a tertiary structure of the ribosomal RNA, which makes particular parts of the chain available for nucleolytic attack.

The breakdown of ribosomal ribonucleic acid (RNA) into specific fragments has been observed on a number of occasions. Möller and Boedtker (1962) and later Midgeley (1965) noted for instance that under the action of what may now be identified with certainty as a ribosomal nuclease (Ohtaka and Uchida, 1963) RNA from the larger subunit of *Escherichia coli* ribosomes was degraded, presumably into two equally sized fragments, which sedimented with the RNA of the small subunit. The early course of degradation of mammalian ribosomal RNA with pancreatic ribonuclease was studied in the ultracentrifuge by Huppert and Pelmont (1962), who observed the formation of several well-defined sedimenting boundaries after mild treatment. This suggested the highly preferential cleavage of a small number of phosphodiester bonds, and in consequence the existence of what may reasonably be regarded as a type of subunit structure in the ribosomal RNA.

It has seemed of interest to investigate the possibilities of specific bond hydrolysis in the RNA from one of the more extensively studied species of ribosome over the widest possible range, to determine the number of labile phosphodiester bonds, and to investigate the size and nature of the products. In the ultracentrifuge the necessary resolution probably depends on the formation of hypersharp boundaries, and can certainly be achieved only in the high molecular weight range. There is consequently also little possibility of isolating separated components by this means. The severest limitation however is that only components present in substantial relative concentration can be detected in the ultracentrifuge.

For the purposes of a detailed investigation of the first stages of enzymic hydrolysis of ribosomal RNA we have adapted the technique of polyacrylamide gel electrophoresis. We have concerned ourselves primarily with the action of T1-ribonuclease on yeast ribosomal RNA.

Polyacrylamide electrophoresis was shown in an earlier publication (Richards and Gratzer, 1964) to afford high resolution and sensitivity for soluble ribonucleic acid (s-RNA) and other species in the same molecular weight range. For such fractionations 10% gels were found to be optimal, whereas in the present work the most useful working gel concentration was 5%. The performance of the gels has been evaluated, and methods for location and extraction of the RNA components have been evolved.

Experimental Section

Materials. Ribosomes from *Saccharomyces fragilis* in the exponential growth phase were prepared as described by Ohtaka and Uchida (1963), except only that higher ionic strength buffers were used (1 mM Tris, 2 mM magnesium chloride, 0.1 M potassium chloride, pH 7.2). The RNA was extracted with freshly distilled phenol, equilibrated with buffer, and three times precipitated with an equal volume of ethanol. *E. coli* ribosomes were prepared by the procedure of Tissières *et al.* (1959) and the RNA was extracted as just described. All preparations were treated with bentonite to remove endogenous nucleases, and in all the more recent experiments the ribosomes were purified by adsorption on a DEAE-cellulose column, as described in Clark and Marcker (1965). This was found to be a highly advantageous procedure in that the stability of the ribosomes was very greatly improved.

Ribonuclease-T1 was the purified product of the Sankyo Co. Ltd. Pancreatic ribonuclease was the three-times-recrystallized product of Worthington Biochemical Corp.

Methods. ELECTROPHORESIS. Polyacrylamide gel elec-

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trophoresis was performed in a vertical system, as described by Davies (1964) using eight tubes of 6 mm i.d., and a constant-current power supply. In most respects the technique was as previously described (Richards *et al.*, 1965) except for the use of a 5% (w/v) gel, with no spacer gel. The monomer mixture (95% acrylamide, 5% *N,N'*-methylenebisacrylamide, supplied by British Drug Houses, Ltd.) was dissolved in Tris–HCl buffer (0.486 M Tris, pH 8.4), as previously described (Richards *et al.*, 1965), and poured into the electrophoresis tubes to a height of *ca.* 6 cm, after degassing and addition of 10% ammonium persulfate as initiator. Water was layered onto the surface of the solution with a fine pipet to protect it from atmospheric oxygen. Polymerization ensued in about 0.5 hr at room temperature. The samples (*ca.* 250 μg of RNA in up to 0.1 ml) were applied on disks of Whatman 3MM filter paper at the tops of the gels. Six or more such disks could be applied without loss of resolution. The samples were then covered in a slurry of washed sand in reservoir buffer to prevent back diffusion. The reservoir buffer was Tris–diethylbarbituric acid (0.0033 M Tris, 0.03 M diethylbarbituric acid, pH 7.03, free acid and free base being used to avoid the presence of ions of high mobility). Electrophoresis was carried out at a current of 5 ma/tube for about 40 min, bromphenol blue being used as a marker in one tube to show the progress of the solvent front.

After electrophoresis the gels were immediately removed from the tubes by rimming with a needle and expulsion with gentle water pressure if necessary. The RNA was fixed and stained by immersing the gels in a solution of 1% acridine orange and 2% lanthanum acetate in 15% acetic acid. After 24 hr the gels were washed and destained electrophoretically as previously described (Richards *et al.*, 1965). For densitometry, the gels were scanned with a Joyce-Loebel microdensitometer, using a blue filter.

EXTRACTION OF RNA FROM THE GELS. It is possible to recover RNA from excised zones, stained as described above. The gel slice is homogenized with 0.1 M sodium chloride solution in a Potter homogenizer; EDTA is added, and the extract is made neutral, the RNA–acridine orange complex being then brought into solution. The dye can be removed quantitatively from the RNA by extraction with phenol. This method, while making it possible to isolate RNA from the finest zones, involves its exposure to acetic acid and lanthanum ions for the prolonged period of the staining procedure, and a certain degree of damage is therefore most likely. For the purposes of molecular weight determination therefore, a milder, though less sensitive, procedure was evolved. A small proportion of acridine orange was added to the RNA before electrophoresis, and the most intense zones could then be distinguished by the fluorescence of the acridine orange under a Minerolit ultraviolet lamp. The zones were cut out and the RNA extracted by homogenization with 0.1 M sodium chloride solution as before. The RNA was precipitated with ethanol and redissolved in 0.2 M sodium chloride for sedimentation.

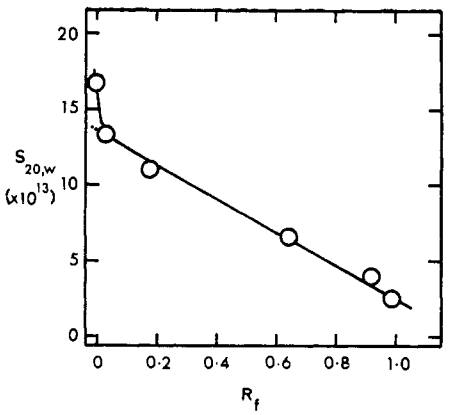

FIGURE 1: Calibration of 5% polyacrylamide gels. The plot shows the relation between sedimentation coefficient ($s_{20,w}$) and R_F of RNA species in the gel.

SEDIMENTATION. Sedimentation coefficients of the extracted RNA zones were measured with a Spinco Model E analytical ultracentrifuge, using ultraviolet-absorption optics. Weight-average sedimentation coefficients were determined from scans of the boundaries with a Joyce-Loebel microdensitometer. Partly digested ribosomal RNA samples were also examined at concentrations of 3 mg/ml, using the schlieren optical system. Digestion of ribosomal RNA: Aliquots of RNA solution (0.5 mg of RNA) in 0.01 M Tris–buffer, pH 7.4, were digested for 1 hr at 0° by addition of from 0.01 to 10 μg of nuclease in solution to each, the over-all volume being then *ca* 0.2 ml. At the required time the reaction was arrested by addition of 0.1 ml of a 1% suspension of bentonite (Fraenkel-Conrat *et al.*, 1961). After low-speed centrifugation, the RNA was precipitated with ethanol, and redissolved in 0.1 ml reservoir buffer for the electrophoresis. Digestion experiments were carried out also in the presence of 10 mM magnesium ions.

Results

Calibration of the Polyacrylamide Gels. Corresponding zones from seven of the eight electrophoresis tubes, located as described by the fluorescence of added acridine orange, were extracted, pooled, and the sedimentation coefficients of the RNA determined. The eighth gel was stained with acridine orange. The positions of the extracted zones were expressed as R_F values, *i.e.*, ratio of distance migrated by the zone to that of the solvent front. The distances of migration were measured on the stained gel, on which the solvent front could be distinguished as a fine orange line, concave toward the origin. Figure 1 shows a plot of R_F *vs.* sedimentation coefficient (corrected to $s_{20,w}$), which is seen to be linear over most of the range, resembling therefore the calibration previously set up (Richards *et al.*, 1965) for small RNA species migrating in 10% polyacrylamide gels. Boedtker (1960) has shown that the sedimentation co-

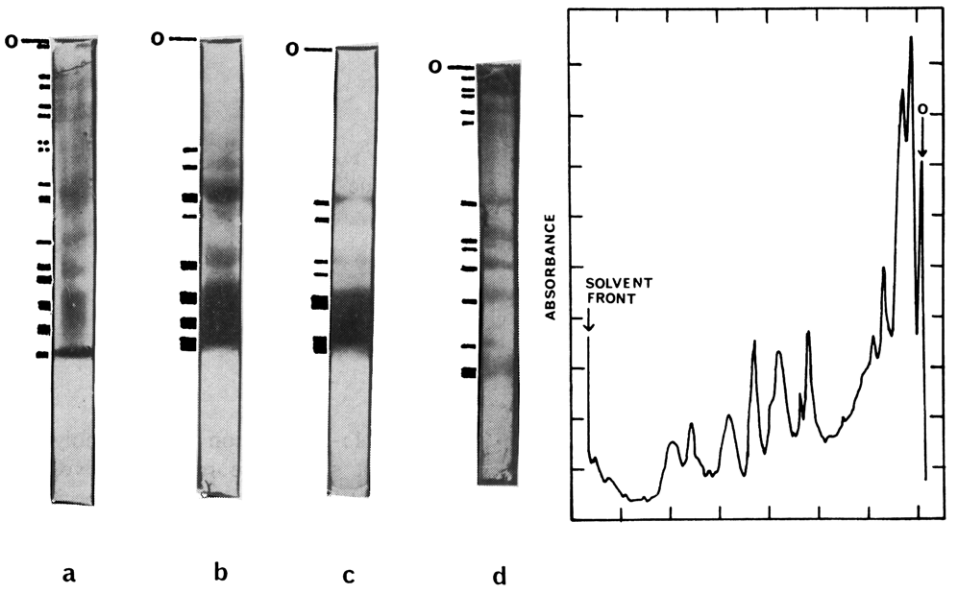

FIGURE 2: Polyacrplamide gel electrophoresis of partly digested yeast ribosomal RNA. The gels show typical stages in the mild enzymic digestion of the RNA: (a–c) with T1-ribonuclease; (d) with pancreatic ribonuclease. The origin is denoted by o. Note that the absolute migration distance (*i.e.*, the time of electrophoresis) is greater in (d) than in (a–c) which are from a single experiment. A densitometer trace of an electrophoretic pattern of a typical partial digest (pancreatic ribonuclease) is also shown.

efficients of viral RNA homologs follow the relation $s^0_{20,w} = 0.020 M_w^{0.50}$, which is obeyed with remarkable precision over a wide range of molecular weight. The molecular weight scale shown in Figure 3 is based on this relation. The linear portion of the plot is thus governed by the relations

$$s^0_{20,w} = 13.75 - 11.25 R_F$$

$$M_w = (6.88 - 5.63\ R_F)^2 \times 10^4$$

It is now possible to relate the RNA degradation products to one another.

Intact Ribosomal RNA. Intact ribosomal RNA barely enters the 5% gel. After electrophoresis the top of the gel is found always to be intensely stained, and a rather ill-defined zone is observed about 1 mm from the end, which almost certainly represents the RNA of the small subunit. However, according to the particular preparation, some RNA is always detected in the body of the gel. In all preparations there is a light polydisperse smear of RNA, and generally zones are also observed. These vary in number and intensity with the preparation and it has not so far proved possible to eliminate this material entirely in any experiments.

Washing with 0.5 M ammonium chloride (Spirin 1964) did not consistently improve the resulting pattern, but the use of a DEAE-cellulose column as described led to a substantial improvement. In some preparations when the DEAE-cellulose treatment was not used an extraordinary series of sharp zones, perhaps 15–20 in number, is observed, but the circumstances of cell growth or RNA preparation under which these appear have not yet been defined.

The integrity of the initial RNA preparations is satisfactorily demonstrated by the complete absence of new components when the RNA was examined after recovery from solution in pure formamide as described by Stanley and Bock (1965), and even after heating in formamide. It may be pointed out that the formamide procedure, allied with the gel electrophoresis, represents probably the most sensitive available method for detecting hidden breaks in the phosphodiester chain. The zones are all labile to pancreatic ribonuclease (boiled to destroy deoxyribonuclease activity) and must, therefore, certainly represent RNA species. The best preparations which have been observed show the rather faint zones indicated in Figure 3, although with rabbit reticulocyte RNA almost completely clear gels are consistently obtained (Gould, 1966). *E. coli* ribosomal RNA, including that from a ribonuclease-free strain (*MRE* 600), gives results rather similar to those with the yeast RNA.

The Course of Digestion of Ribosomal RNA with T1-Ribonuclease. The exposure of the ribosomal RNA to the lowest concentrations of T1-ribonuclease (up to 0.1 μg/ml) leads to the appearance of well-defined zones in the 5–12 S range, typical examples being shown in Figure 2a–c. Some of these zones are always present in the original RNA, to an extent varying, as has been described with the preparation. It is interesting to note (Figure 3) that on enzyme treatment these zones are intensified as judged both visually and by densitometry of the gels. At higher enzyme concentrations the slower

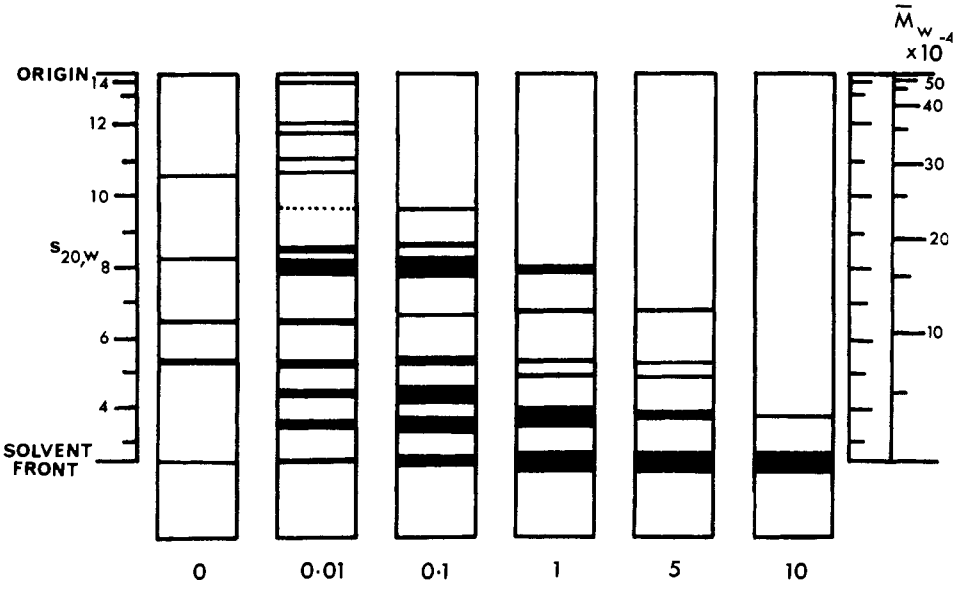

FIGURE 3: The course of T1-ribonuclease digestion of yeast ribosomal RNA. The figures are the amounts in milligrams of enzyme added to 0.5 mg of RNA samples all of the same concentration (10 mg/ml final). The scale shows the sedimentation coefficients corresponding to R_F values, taken from the data of Figure 1. The molecular weight scale is based on the relation between $s_{20,w}$ and $\bar{M}_w$ given by Boedtker (1960).

migrating zones begin to diminish in intensity and the faster moving zones in general become more intense. At an enzyme concentration of 50 μg/ml, under the standard conditions of treatment, only one intense zone remains, which migrates with the solvent front.

The patterns do not change significantly with the presence or absence of magnesium ions. Eight major zones are usually to be seen. There is also evidence however of the generation of components near the origin, which are difficult to distinguish because their distance of migration is so small. It is possible to draw up schemes according to which the degradation process could occur within the molecular weight range with which we are concerned. None, however, can at present be regarded as unique in accounting for the observed electrophoretic patterns. It is difficult at this stage to define the degradation process more precisely, since the background of nonspecific degraded material present from the outset vitiates attempts at accurate densitometric estimation of RNA concentrations in the zones. Attempts at quantitative evaluation of the various digestion stages, both by densitometry and dye extraction as described, show that the intermediate zones (between the top of the gel and the solvent front) contain of the order of 30% of the total RNA in the intermediate stages of enzyme attack (Figure 2a and d). In the earlier stages, a preponderant proportion of the RNA remains at the top of the gel, and in the later, it appears at the solvent front. There seems little doubt that a substantial part at any rate of the RNA passes through the intermediate stages represented by the zones but the difficulties which have been mentioned preclude more quantitative interpretation at this stage.

DIGESTION WITH PANCREATIC RIBONUCLEASES. The action of pancreatic ribonuclease on ribosomal RNA has been less fully investigated. Seven major zones in the main part of the gel are observed, but the pattern of zones formed very close to the origin is also noteworthy (Figure 2d).

DIGESTION WITH ENDOGENOUS RIBOSOMAL NUCLEASE. It is known that yeast ribosomes possess a nuclease, active at room temperature, the presence of which leads to spontaneous degradation of the usual ribosomal RNA preparations (Ohtaka and Uchida, 1963). Accordingly, solutions of ribosomal RNA in the usual buffer system were allowed to stand at 20° and at 37° for intervals up to 18 hr, and then examined on the polyacrylamide gels. Some zones were observed, but the definition and reproducibility were poor by comparison with the patterns produced by the controlled action of added nucleases.

DIGESTION OF RIBOSOMAL RNA FROM *E. coli*. Mild treatment of this RNA with pancreatic ribonucleases was also found to give rise to a pattern of electrophoretic zones. This has not yet been investigated in more detail. The initial material shows evidence of small components before degradation at least equal in proportion to those of yeast RNA.

Sedimentation of Partially Degraded Ribosomal RNA. A number of partly digested samples, corresponding to the range encompassed in Figure 3, were examined in the analytical ultracentrifuge, using the schlieren optical system, and a speed of 50,740 rpm. More than four boundaries could never be observed with any certainty, and it was clear that the resolution attainable in the ultracentrifuge was too limited for a useful comparison with the gel electrophoresis results to be made.

Discussion

Aronson and McCarthy (1961) first noted that degradation of *E. coli* ribosomal RNA by heat, or by (presumably) endogenous nuclease, leads to the appearance of intermediates of 13.1, 8.8, and 4.4 S. Möller and Boedtker (1962) observed that the RNA from the large subunit of *E. coli* ribosomes appeared to undergo specific fission into two halves on mild treatment, and Midgeley (1965) has since shown that this is brought about by endogenous nuclease. Huppert and Pelmont (1962) showed that under the right circumstances the action of pancreatic ribonuclease on ribosomal RNA from ascites cells could generate five components, detectable in the ultracentrifuge. It is not of course possible to determine from the boundary profiles whether these components are monodisperse, or represent polydisperse populations. As the work of Huppert and Pelmont also showed, any greater number of intermediates could no longer be resolved in the ultracentrifuge, especially in the slower sedimenting range where the boundaries are broader.

These limitations are not present in the gel electrophoresis technique. It is necessary at this point to stress that the less prominent zones observed in the polyacrylamide gels represent components present in too low concentrations, to be observed in the ultracentrifuge. Certainly the components revealed in gel electrophoresis of the initial ribosomal RNA preparations cannot be perceived in the ultracentrifuge, even at high total concentrations and with schlieren optics. It is of interest that Bachvaroff and McMaster (1964) and Tsanev (1965) have recently also found evidence of unexpected minor components in ribosomal RNA preparations, by electrophoresis in 1.25% agar gel. (The basis of the separation in this medium is not clear. It is known (Gordon and Reichard, 1951; Gordon *et al.*, 1950) that such agar gels have no important molecular sieving function, at least for deoxyribonucleic acid (DNA) and proteins of molecular weights below about 10^6.)

We are now led to the question of what proportion of the RNA in the degradation experiments is contained in the intermediate electrophoretic zones. In the work on the degradation of reticulocyte ribosomal RNA (Gould, 1966) it appears clear that all, or nearly all, of the RNA passes through some or all of the intermediate stages represented by the zones in the 5–12 S region. In the yeast RNA the same conclusion is not at this stage justified. The overlay of randomly degraded material makes it at least possible that the RNA accumulating at the solvent front is in part degraded by random hydrolysis. Until initial preparations are obtained which are free of degraded material the question cannot be answered with any certainty.

It is important to point out that all the zones, as well as the background material, consist of RNA and not DNA, acid polysaccharides, or acridine orange binding proteins: more extensive treatment with pancreatic or T1-ribonucleases leads to clear gels, showing no trace of acridine orange staining.

The most significant conclusion which emerges from the results is the existence of labile points (hot spots) in the polynucleotide chain. The number of such points may in principle be deduced from the number of zones generated. Thus if there are n hot spots, the number of all the components which can result, Z (including the undegraded species), is given by

$$Z = \tfrac{1}{2}(n + 2)(n + 1)$$

(This number could of course be reduced in the event of degeneracy.) Thus for three hot spots, ten zones would be generated from one chain, including the original material, which does not migrate in the gel, whereas for four hot spots there would be fifteen zones from one chain. If these points are spaced in fairly even manner along the chain, it may be supposed that some of the species which can be formed will still be very large, and will not move far enough from the origin to be unambiguously resolved. The results, therefore, should be altogether consistent with the presence of from two to four hot spots in each of the two kinds of polynucleotide chain in the ribosome. As digestion proceeds, so each hot spot will be attacked, and the large fragments will give place to the smaller ones (as in Figure 3). Thereafter less labile bonds in the chain will be hydrolyzed, and as the secondary structure is broken down, so more bonds yet will become available for cleavage.

It may be noted that the existence of unpaired regions in ribosomal RNA is to be inferred from many of its properties (Petermann, 1964). Many lines of evidence (see for instance Timasheff *et al.*, 1961; Spirin, 1964; Spencer and Poole, 1965) indicate that the molecule in fact consists of rather short helical regions joined by single-stranded segments. It would be specious to seek to explain the present results in terms of the preferential lability of all these random portions of the chain *per se:* on such a basis, an order of magnitude more zones would be produced than is compatible with the patterns which have been shown.

The lability of the hot spots is evidently very great indeed compared with that of the overwhelming majority of bonds in the chain. Their nature is obscure, and one may obviously consider the two alternatives of single labile bonds, or short labile regions. The apparent (though not yet certainly established) basic similarity of the cleavage process with pancreatic and T1-ribonucleases, which have quite different specificities, is an argument in favor of the latter alternative. The presence of one covalent bond of unusual type and high hydrolytic sensitivity in the phosphodiester chain of 23 S *E. coli* ribosomal RNA has been postulated (Midgeley, 1965). Alternatively one may conceive of the possibility that the ribosomal RNA chains have a definite tertiary structure, which makes limited regions sterically favorable for enzymic attack. An argument in favor of such a scheme is our failure to observe any electrophoretic zones of the type which have been described when limited hydrolysis is performed with 0.3 N alkali. In these experiments the RNA is exposed to the

alkali for periods of time varying between those too short for any degradation to be detected to those leading to extensive degradation to small fragments (Spencer and Poole, 1965). The absence of specific cleavage with alkali (which, it may be noted, also has a specificity lower than, but qualitatively similar to, that of pancreatic ribonuclease (Michelson, 1963)) suggests that the elimination of secondary and tertiary structure at the high pH annihilates the greater lability of the hot spots.

Further investigations along these lines are in progress. Attempts are being made to isolate the intermediates of T1-ribonuclease digestion from the acrylamide gels and to examine their nature and properties. The products of nuclease attack on whole ribosomes are also being studied.

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References

Aronson, A. I., and McCarthy, B. J. (1961), *Biophys. J. 1*, 215.

Bachvaroff, A., and McMaster, R. P. B. (1964), *Science 143*, 1177.

Boedtker, H. (1960), *J. Mol. Biol. 2*, 171.

Clark, B. F. C., and Marcker, K. A. (1965), *Nature 207*, 1038.

Davies, B. J. (1964), *Ann N. Y. Acad. Sci. 121*, 404.

Fraenkel-Conrat, H., Singer, B., and Tsugita, A. (1961), *Virology 14*, 54.

Gordon, A. H., Keil, B., Šebešta, K., Knessl, O., and Šorm, F. (1950), *Collection Czech. Chem. Commun. 15*, 1.

Gordon, A. H., and Reichard, P. (1951), *Biochem. J. 95*, 232.

Gould, H. (1966), *Biochemistry 5*, 1103.

Huppert, J., and Pelmont, J. (1962), *Arch. Biochem. 98*, 214.

Michelson, A. M. (1963), The Chemistry of Nucleosides and Nucleotides, New York, N. Y., Academic, p 340.

Midgeley, J. E. (1965), *Biochim. Biophys. Acta 95*, 232.

Möller, W., and Boedtker, H. (1962), Acides Ribonucléiques et Polyphosphates, C.N.R.S. International Colloquium, Strasbourg, 1961, p 99.

Ohtaka, Y., and Uchida, K. (1963), *Biochim. Biophys. Acta 76*, 94.

Petermann, M. L. (1964), The Physical and Chemical Properties of Ribosomes, Amsterdam, Elsevier.

Richards, E. G., Coll, J. A., and Gratzer, W. B. (1965), *Anal. Biochem. 12*, 452.

Richards, E. G., and Gratzer, W. B. (1964), *Nature 204*, 878.

Spencer, M., and Poole, F. (1965), *J. Mol. Biol. 11*, 314.

Spirin, A. S. (1964), *Struktur und Funktion des genetischen Materials*, Berlin, Akademie Verlag, p 163.

Stanley, W. M., and Bock, R. M. (1965), *Biochemistry 4*, 1302.

Timasheff, S. N., Witz, J., and Luzzati, V. (1961), *Biophys. J. 1*, 525.

Tissières, A., Watson, J. D., Schlesinger, D., and Hollingworth, B. R. (1959), *J. Mol. Biol. 1*, 221.

Tsanev, R. (1965), *Biochim. Biophys. Acta 103*, 374.