A Pencillinase-Specific Ribonucleic Acid Component from *Bacillus cereus*. II. Partial Characterization of the Active Component*

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ABSTRACT: The active component, found in ribonucleic acid (RNA) extracts obtained from penicillinase constitutive *Bacillus cereus* 569/H membranes and causing an increased production of penicillinase in recipient cultures, was partially purified away from other RNA species by methylated serum albumin Kieselguhr (MAK) column chromatography and sucrose density gradient centrifugation to a specific activity (differential rate of enzyme synthesis per microgram of added RNA) of 100- and 800-fold greater than the crude RNA extract, respectively. The majority of this component eluted from MAK columns at a NaCl concentration between 0.6 and 0.7 M and had an estimated sedimentation value of 12–14 S on sucrose density gradient centrifugation. These partially purified fractions as well as their respective crude form were RNase sensitive whereas a number of 569/H membrane RNA extracts were found to be largely RNase and deoxyribonuclease (DNase) resistant but sensitive to combined nuclease treatment. Heating and rapid cooling converted these RNase-resistant active RNA preparations into sensitivity. The ionic strength of the buffer and possibly other factors of extraction and testing affected the nuclease sensitivity pattern of active 569/H RNA extracts. Such 569/H RNA extracts, centrifuged to density equilibrium in a mixed cesium salt solvent, were composed of at least two species, a major one of buoyant density of 1.619–1.621 g/cm^3 and a minor one of 1.548–1.554 g/cm^3. Comparative figures using the same solvent for 569/H DNA is 1.602–1.604 g/cm^3, while penicillinase protein is found at the meniscus. The lighter species was shown to be RNase sensitive and heat labile while the heavier species only became RNase sensitive after the RNA extract was heated at 100° for 10 min. The active RNA component examined in these centrifugation analyses survived extensive incubation with Pronase. The possible existence of the penicillinase-specific RNA component as a double-stranded RNA species, a RNA–DNA hybrid species, and as an RNA–protein complex was discussed.

A penicillinase[1]-specific component was demonstrated in RNA extracts obtained from protoplast membranes of *Bacillus cereus* 569/H (penicillinase constitutive) in the previous paper (Kirtikar and Duerksen, 1968). The recipient cell response to the added 569/H RNA, as shown by an increased differential rate of penicillinase activity production, was dependent on the added RNA concentration as well as the relative levels of inhibiting RNA species in the RNA extract. Serological identification of the penicillinase produced by recipient cells, whether homologous or heterologous species, as *B. cereus* 569/H enzyme demonstrates the degree of specificity of this active RNA component and along with the response of the system to various antibiotics suggests *de novo* penicillinase synthesis by the recipient cells. RNase sensitivity indicates the RNA nature of this penicillinase-specific fraction. This paper shall explore further the nuclease sensitivity of this active component and characterize it partially by MAK column chromatrography (Mandell and Hershey, 1960), sucrose density gradient (Britten and Roberts, 1960), and equilibrium density gradient centrifugation (Lozeron and Szybalski, 1966; Szybalski, 1968).

Materials and Methods

Previously Described Procedures. Procedures for RNA extraction from *B. cereus* 569/H except for the modifications described for some of the nuclease sensitivity experiments and the equilibrium density gradient centrifugation, testing of biological activity, growth of the donor and recipient cells, nucleic acid and protein estimations, and penicillinase assay have been described (Kirtikar and Duerksen, 1968). The bentonite

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[1] The trivial name, penicillinase, is used for this enzyme. The name suggested by the Commission on Enzymes of the International Union of Biochemistry is penicillin amidohydrolase (EC 3.5.2.6). Other abbreviations used are: DNase, deoxyribonuclease (deoxyribonucleate oligonucleotido-hydrolase (EC 3.1.4.5)); RNase, ribonuclease (polyribonucleotide 2-oligonucleotido-transferase (EC 2.7.7.16)); MAK, methylated serum albumin Kieselguhr; SLS, sodium lauryl sulfate; SSC, standard saline–citrate buffer; SPC, standard phosphate–citrate buffer.

TABLE I: Effect of Ionic Strength on Nuclease Sensitivity of an Active 569/H RNA Extract.[a]

RNA Treatment			Loss in RNA-Induced Rate
Prior to Nuclease Addn	Prior to Addn to Recipient Cells[b]	K_{RNA}[c]/K_{basal}[d]	$100 - [(RNA_t - B)/(RNA_c - B) \times 100]$ (%)
2× SSC	None	19.7	0
	Buffer	19.1	3
	RNase	11.3	45
	DNase	19.1	3
	RNase + DNase	3.8	85
1× SSC	None	11.2	0
	Buffer	6.9	42
	RNase	4.3	68
0.1× SSC	None	8.3	0
	Buffer	6.7	22
	RNase	2.66	77
0.01× SSC	None	4.98	0
	Buffer	5.28	−7
	RNase	1.53	87

[a] This 569/H RNA extract in 2× SSC when added to recipient cells gave a concentration of 510 μg/ml of culture. Dilution to the lower ionic strengths, hence, gave a concomitant reduction in final cultural RNA concentration. The extract also contained 0.5 μg of Folin-positive material (possibly trace phenol) and 0.2 μg of DNA/100 μg of RNA. [b] RNA extract aliquots, after the indicated additions, were incubated at 37° for 15 min prior to addition to recipient cultures. [c] Differential rate of penicillinase synthesis in recipient cultures after addition of 569/H RNA. [d] Differential rate of penicillinase synthesis in the uninduced culture ($K = 0.02$). B = basal.

addition in the extraction procedure was made only for those RNA extracts analyzed on MAK column chromatography and sucrose density gradient centrifugation. Often ethanol (two volumes of 95% ethanol at −20°) precipitation (16 hr) of RNA preparations brought to 0.1 M NaCl followed by a short (2–3 hr) dialysis was substituted for the extensive dialysis following phenol extraction. In some instances (nuclease sensitivity and equilibrium density gradient centrifugation studies) *B. cereus* 569 Sm^r, a streptomycin-resistant mutant of *B. cereus* 569, was used as recipient cultures with no noticeable difference in the results.

Nuclease Sensitivity. For the example presented in Table I, the RNA extraction from 569/H protoplast membranes was modified by treating the donor cells with chloramphenicol (20 μg/ml) for 1 hr prior to protoplast formation and by treating the washed protoplast membranes with sodium deoxycholate (0.02%) at room temperature for 5 min followed by centrifugation at 15,000*g* for 10 min. The supernatant was extracted for RNA. In all cases the ethanol-precipitated RNA was dissolved in and dialyzed against (2 hr) the desired buffer; usually standard saline citrate (SSC), 0.15 M NaCl–0.015 M sodium citrate (pH 7.0). Aliquots of the RNA preparations were incubated with RNase (100 μg/ml), DNase (100 μg/ml), or a combination of both for 15 min at 37°. Heat treatment of the indicated RNA aliquots prior to nuclease digestion was carried out at 70 and 100° for 10 min followed by rapid cooling in an ice bath.

Preliminary Purification of RNA. RNA was purified on several occasions by an initial passage over IR 120 resin-Sephadex G-25 columns by a modified procedure of Bautz and Hall (1962). The RNA precipitated from the usual Tris-KCl-Mg^{2+} buffer was suspended in and dialyzed against the suggested potassium acetate-Mg^{2+} buffer (pH 5). The early fractions (RNA peak) were pooled and the RNA was ethanol precipitated. Little loss in biological activity occurred provided Mg^{2+} ions at 0.01 M were present.

Chromatographic Separation of the RNA Species of 569/H RNA. Methylated serum albumin Keiselguhr (MAK) columns prepared according to the method of Mandell and Hershey (1960) were used. *B. cereus* 569/H membrane RNA was brought to a concentration of 0.3 M NaCl before adsorption to the column. Elution was achieved with a linear NaCl gradient of 0.3 M–1.5 M (200-ml reservoirs of each) at a rate of approximately 1 ml/min. Fractions (3 ml) were collected from the time of application of the sample. Absorbance at 260 and 280 mμ, RNA concentration, NaCl concentration (Mohr method), and biological activity determinations were made. At the concentrations of RNA used (3–5 mg) the columns were not overloaded. In some experiments, RNA was partially purified by passage through an IR 120 Sephadex

TABLE II: Effect of Temperature on Nuclease Sensitivity of an Active 569/H RNA Extract.[a]

RNA Treatment			Loss in RNA-Induced Rate
Prior to Nuclease Addn	Prior to Addn to Recipient Cells[b]	K_{RNA}[c]/K_{basal}[d]	100 − [(RNA$_t$ − B)/(RNA$_c$ − B) × 100] (%)
None (control)	None	4.2	0
	Buffer	5.7	−47
	RNase	3.41	21
	DNase	4.85	−20
	RNase + DNase	1.07	88
Heated 70°, 10 min, chilled	None	4.58	0
	Buffer	5.92	−38
	RNase	1.45	87
	DNase	5.74	−32
	RNase + DNase	<1	100
Heated 100°, 10 min, chilled	None	6.26	0
	Buffer	5.56	13
	RNase	1.2	97
	DNase	6.42	−3
	RNase + DNase	<1	100

[a] This 569/H RNA extract in SSC when added to recipient cells gave a concentration of 355 μg/ml of culture. The extract also contained 0.5 μg of Folin-positive material (possibly trace phenol) and 0.36 μg of DNA/100 μg of RNA. [b] RNA extract aliquots, after the indicated additions, were incubated at 37° for 15 min prior to addition to recipient cultures. [c] Differential rate of penicillinase synthesis in recipient cultures after addition of 569/H RNA. [d] Differential rate of penicillinase synthesis in the uninduced culture (0.0417). B = basal.

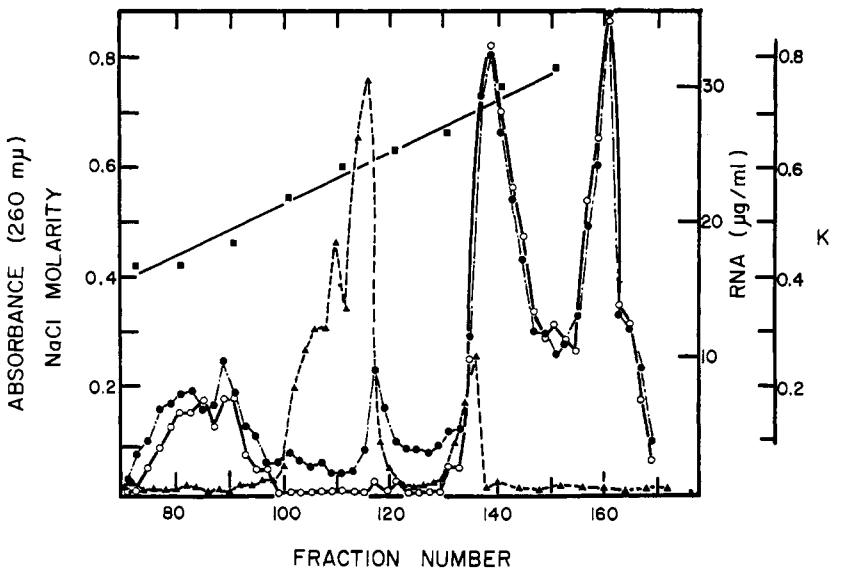


FIGURE 1: MAK column chromatography of *B. cereus* 569/H membrane RNA. Prior to fractionation, this RNA was partially purified on IR-120 resin G-25 Sephadex column (see Materials and Methods). The early fractions containing biologically active RNA were precipitated with ethanol and redissolved in TS2M buffer. This RNA (4.5 mg), brought to 0.3 M NaCl, was applied to a MAK column. Fractions (3 ml) were collected and assayed. (○—○) RNA; (●—●) $OD_{260\ m\mu}$; (■—■) NaCl concentration; (▲—▲) biological activity in recipient *B. cereus* 569 (expressed as *K* value of fraction-treated recipient culture). Approximately a 15-fold increase in the differential rate was obtained with the unfractionated RNA (K = 0.35) at 36-μg/ml concentration. The uninduced cells gave a K of 0.023. With penicillin at 0.3 and 10 units/ml the K values were 0.43 and 2.3, respectively.

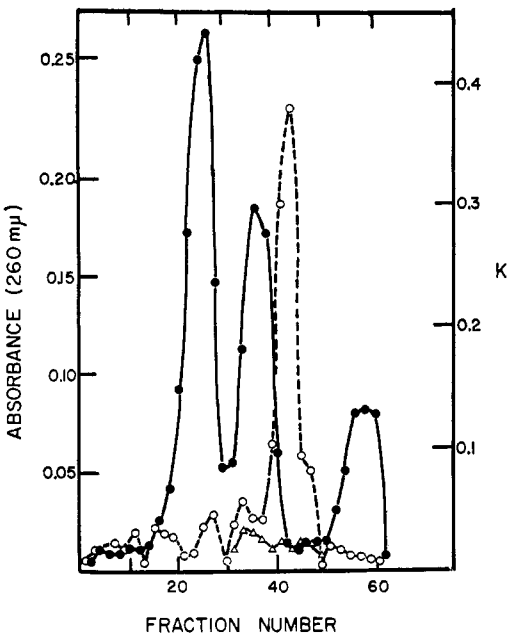


FIGURE 2: Sedimentation profile of *B. cereus* 569/H membrane RNA. The procedure for analysis of 1 ml (354 μg) of 569/H RNA is outlined in Materials and Methods. (●—●) $OD_{260\ m\mu}$; (○—○) differential rate of penicillinase synthesis (*K*); (△—△) *K* value of the corresponding cultures given parallel fractions after RNase treatment. Absorbancy peaks between fraction 19 and 28, 32 and 40, and 51 and 60 indicate, respectively, 23S rRNA, 16S rRNA, and 4S sRNA. Corresponding *K* values for uninduced, penicillin- (0.3 and 10 unit/ml) induced, and unfractionated RNA-treated recipient cells were 0.023, 0.43, 2.3, and 0.175, respectively.

G-25 column before application to the MAK column.

Sedimentation Analysis. For the analysis of 569/H membrane RNA extracted in the usual manner, a 24-ml linear sucrose density gradient (Britten and Roberts, 1960) ranging from 5 to 20% in TS2M buffer (pH 7.2) was prepared and equilibrated for at least 4 hr in the cold. A 569/H membrane RNA preparation (1 ml) (354 μg in Figure 2) was layered gradient wise with 1 ml of 4% sucrose on top of this gradient and the preparation was centrifuged for 11 hr at 24,000 rpm in the Spinco SW 25.1 swinging-bucket rotor in a Model L ultracentrifuge. Fractions (20 drop) (about 0.38 ml) were collected from the bottom of the tube by puncturing a hole with a 24-gauge syringe needle. Alternate fractions, diluted with 3 ml of TS2M, were examined for absorbance at 260 mμ while 0.5-ml aliquots of the remaining diluted (fraction plus 0.3 ml of TS2M) fractions were added to 10-ml recipient cultures (0.1 mg dry wt/ml) and penicillinase synthesis followed. Parallel fractions from a second gradient tube showing biological activity were tested for RNase sensitivity (Kirtikar and Duerksen, 1968).

For the analysis of a Pronase digest of lysed 569/H protoplasts, Pronase was added (500 μg/ml) to a lysed protoplast suspension in TS2M followed immediately by SLS addition. After a 30-min incubation at 37°, a 0.5-ml aliquot was applied to a 4.5-ml linear sucrose gradient (5–20%) and centrifuged in a Spinco SW 39L rotor at 35,000 rpm for 6 hr. Fractions (10 drop) (0.12 ml) were collected and after a fivefold dilution were analyzed as above.

Equilibrium Density Gradient Centrifugation. An additional step of Pronase digestion (Mills *et al.*, 1966) of the *B. cereus* 569/H membranes (after protoplast lysis and wash) was introduced into the usual procedure at the sodium lauryl sulfate incubation step just prior to the phenol extraction. The Pronase (California Corp. for Biochemical Research, Los Angeles) was autodigested (30 min, 37°) and added to membrane preparations at 1 mg/ml. The RNA obtained by phenol extraction of Pronase digested membranes, after ethanol precipitation, was solubilized in SPC buffer (0.15 M NaCl–0.10 M Na_2HPO_4–0.02 M sodium citrate, pH 7.1) and dialyzed for 2 hr *vs.* 100 volumes of the same buffer. Mixed CsCl–Cs_2SO_4 (optical grades) gradients (Lozeron and Szybalski, 1966) were prepared by adding eight volumes of CsCl (1.9 g/ml at 26°) solution to one volume of Cs_2SO_4 (2.0 g/ml at 26°) just prior to use. The density of solvents prepared in this manner was usually 1.7 g/cm^3. The solvent (3 ml) was added very slowly with stirring to 2 ml of RNA solution in buffer. The samples were equilibrated for at least 1 hr and centrifuged at 33,000 rpm in a SW 39L rotor in a Model L Spinco ultracentrifuge at 26° (temperature inside the chamber) for 57–60 hr. Dripping of 20 drops (approximately 0.25 ml)/fraction was carried out at 37°. *B. cereus* 569/H penicillinase (culture supernatant) and *B. cereus* 569/H DNA (extracted by a modified procedure of Marmur, 1961) were similarly analyzed. Purification of DNA was achieved with isopropyl alcohol precipitations and with omission of the RNase treatment. Thus, the DNA preparations were not entirely free of RNA. Nuclease and heating treatments of 569/H RNA extracts were carried out as indicated above. Densities were calculated for each gradient from immediate micropycnometer measurements (Szybalski, 1968) of undiluted fractions. For measurements of absorbance, RNA, DNA, protein, penicillinase, and biological activity, aliquots of fivefold-diluted fractions were used. A usual volume ratio of recipient culture to diluted fraction of 10:1 was used. The biological activity of the fractions is expressed here as the ratio of the RNA-induced, differential rate of penicillinase synthesis (*K*) of a recipient culture and the basal (uninduced) differential rate of penicillinase synthesis. A ratio of 1 would indicate no biological activity.

Results

Nuclease Sensitivities. As the number of RNA extractions performed increased, some variability in response of the biological activity to nuclease action became evident. For example, with some RNA preparations incubation with RNase considerably enhanced the biological activity while DNase had little effect. Incubation with both nucleases, however, eliminated the biological activity. Still other active extracts were only partially affected by either nuclease or a combination of the two. Tables I and II present an attempt to delineate some of the conditions affecting the response

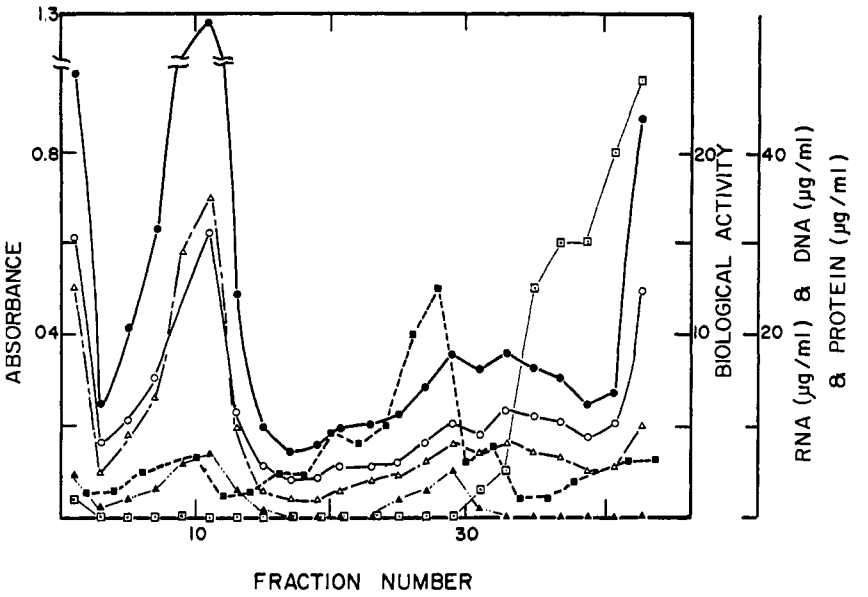


FIGURE 3: Sucrose density gradient sedimentation analysis of Pronase-digested lysed *B. cereus* 569/H protoplasts. The procedure is outlined in Materials and Methods. (●—●) $OD_{260\ m\mu}$; (○—○) $OD_{280\ m\mu}$; (△— - —△) RNA; (▲— ·· —▲) DNA; (⊡—⊡) protein; (■---■) biological activity.

of active RNA extracts to nuclease action. Resistance to DNase digestion under varied experimental conditions was consistent, whereas sensitivity to RNase action depended more or less on pretreatment of the RNA extract and the ionic strength of the buffer as well as the presence or absence of certain ions. At comparatively high ionic strength (2× SSC) a 45% loss was observed while a combination of the two nucleases caused an 85% loss. Reduction in the ionic strength increased the loss due to RNase action. (At 0.01× SSC the RNA was at nonsaturating concentration.) Addition of Mg^{2+} ions at various concentrations, though protecting the activity of RNA extracts, did not markedly change the RNase sensitivity pattern for this RNA extract. The addition of chloramphenicol to the donor 569/H culture and introduction of the sodium deoxycholate step into the RNA extraction procedure would seem to be of little consequence since similar patterns of nuclease sensitivity have been observed for ethanol-precipitated RNA extracted by the usual method (*e.g.*, Table II). Enhancement of stimulatory activity of this RNA extract occurred on incubation with added buffer or DNase while partial sensitivity to RNase digestion was observed. Heating the RNA to 70 and 100° proportionately decreased the stimulatory effect of preincubation and DNase action and proportionately increased the sensitivity to RNase and combined nuclease action. It should be emphasized that not all RNA extracts showed initial resistance to RNase action and, in consequence, their nuclease sensitivity pattern varied from those presented here (see Discussion).

Fractionation of 569/H Membrane RNA on MAK Columns. The 569/H membrane RNA, after ethanol precipitation, passage through an IR 120-Sephadex G-25 column, and reprecipitation, was resolved into three expected, well-defined peaks upon chromatography on a MAK column (Figure 1): (a) 4 S at approximately 0.45 M NaCl, (b) 16 S at 0.7 M NaCl, and (c) 23 S at 0.8 M NaCl (Sueoka and Cheng, 1962). (The resolution of 16 and 23 S appears greater than is usually the case because of gradient difficulties.) The fractions between approximately 0.55 and 0.6 M NaCl (fractions 100–120) contained the major portion of the RNA component responsible for the biological activity of 569/H membrane RNA. Recipient cells treated with the peak fraction (116) synthesized penicillinase at a rate approximately one-third that of fully induced cells, giving an increase in specific activity ($K/\mu g$ of RNA) of approximately 100-fold over the initial RNA extract. A smaller peak of penicillinase synthesis stimulating activity is consistently noticed just slightly in advance of the 16S rRNA at a salt concentration of 0.68–0.7 M (fractions 130–133). On some of the MAK column purifications (not presented) small amounts of biologically active material eluted at a NaCl molarity just following the 23S rRNA. Only trace amounts of DNA were found in the active region in this run but subsequent MAK column chromatography demonstrated the usual presence of DNA in the biologically active fractions.

Sedimentation of 569/H Membrane RNA in Sucrose Gradients. The separation of RNA species of the 569/H membrane extract in a characteristic sedimentation pattern (Gros *et al.*, 1961) is shown by the ultraviolet absorbance profile in Figure 2. Presence of tRNA (4S) and the rRNA (16 and 23 S) in these RNA extracts would be expected since polysomes are firmly bound to these membranes (Schlessinger, 1963). The fractions representing the region of approximately 12–14 S gave a marked stimulation of the differential rate of penicillinase synthesis by the recipient cells. The active component in these fractions was RNase sensitive. The increased differential rate of enzyme synthesis brought

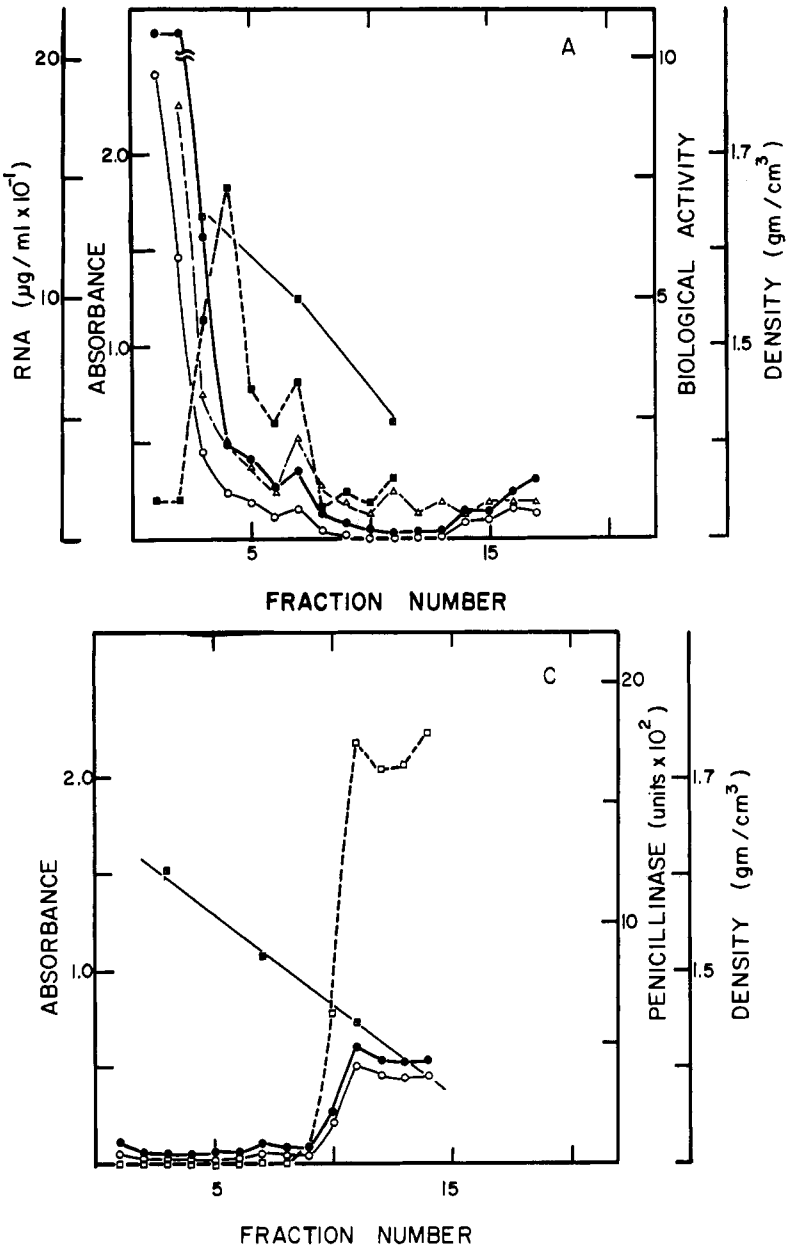
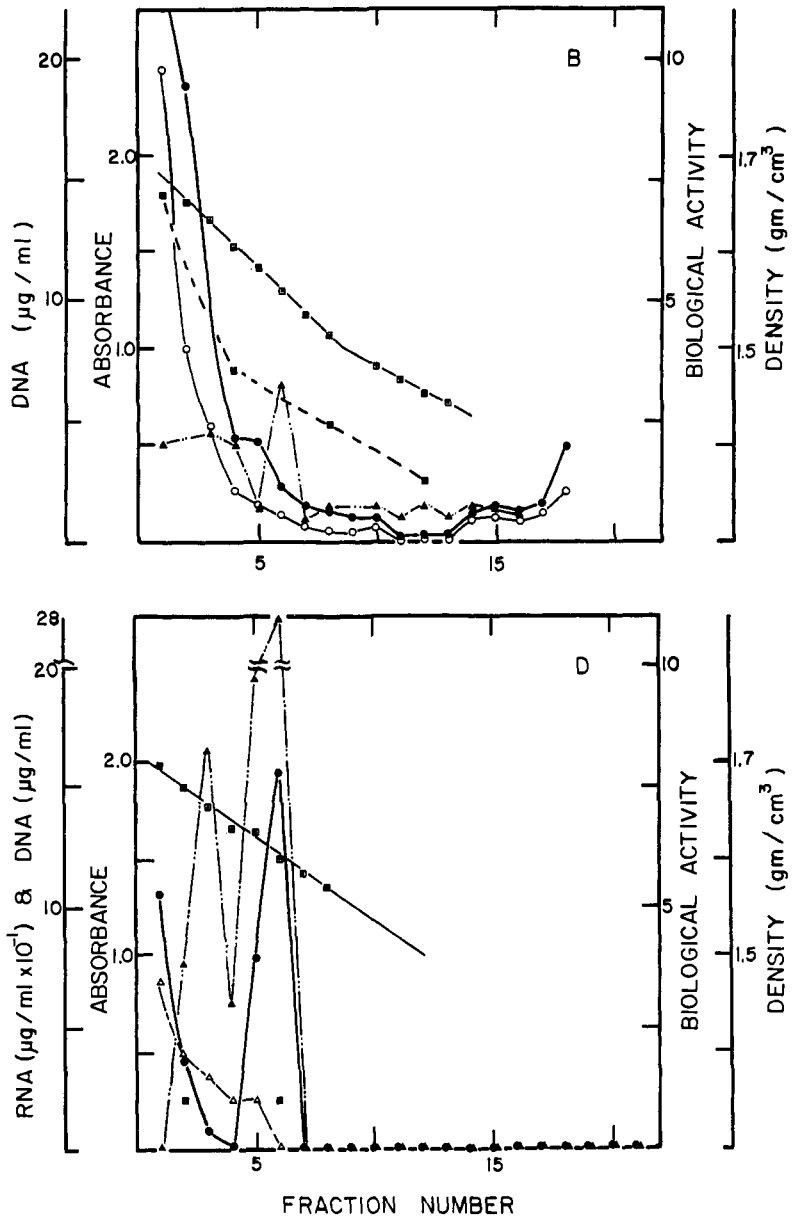


FIGURE 4: Equilibrium density gradient centrifugation analysis. (A) *B. cereus* 569/H RNA extracted from a Pronase-treated protoplast membrane preparation. Preparation, of RNA, centrifugation, and analysis of fractions were carried out as outlined in Materials and Methods. (B) *B. cereus* 569/H membrane RNA was centrifuged in solvent 3 of Lozeron and Szybalski (1966). (C)

about by the peak fraction (43) was double that of the original RNA preparation, and the specific activity ($K/\mu g$ of added RNA) of this fraction was at least 800-fold greater than that for the crude extract. The recipient culture for this fraction apparently was not saturated with RNA.

Strong proteolytic enzymes had little effect on the active RNA component present in 569/H membrane preparations. Pronase digestion for at least 4 hr had no apparent effect on the activity of the subsequently isolated RNA. A decrease to bare visibility of the protein interphase of the phenol extraction takes place within 1 hr of Pronase digestion. In fact, noticeable separation of the active component from the bulk protoplast constituents by Pronase treatment of 569/H-lysed protoplasts is evident in 30 min by sucrose density gradient centrifugation analysis (Figure 3). The active component is found essentially free of measurable protein in a sedimentation position comparable to that of the active component in the analysis described in Figure 2. Treatment with Pronase of 569/H RNA extracts obtained in the usual fashion, followed by re-extraction, had little effect on the biological activity of these extracts.

Equilibrium Density Gradient Centrifugation Analysis. The mixed cesium salts solvent used in these centrifugation analyses was not inhibitory to penicillinase and at the diluted concentrations used only slightly inhibited the growth of the recipient cultures. The major portion of the 569/H RNA banded in the highest den-



B. cereus 569/H penicillinase. A culture supernatant was centrifuged and analyzed as described in A. (D) *B. cereus* 569/H DNA. DNA was prepared as outlined in Materials and Methods and centrifuged and analyzed as in A. The following symbols are consistent for Figures 4–6; (●—●) $OD_{260\ m\mu}$; (○—○) $OD_{280\ m\mu}$; (△— - —△) RNA; (▲—·—▲) DNA; (⊡—⊡) buoyant density; (□—□) penicillinase; (■···■) biological activity.

sity part (lowest in tube) of the gradient, ρ 1.66–1.67, with a trailing shoulder at ρ 1.621, and a small peak at ρ 1.548 (Figure 4A,D). When the proportion of the solvent was changed, most of the RNA was found at the bottom of the tube (Figure 4B) as well as most of the biologically active penicillinase RNA. The biologically active RNA component of these RNA extracts bands at two different densities (Figures 4A, 5B, and 6A), a major peak at ρ 1.621 coinciding with the RNA shoulder and flanked on either side by a DNA band, and a minor peak at 1.548 density coinciding with a minor RNA peak or shoulder. With a relatively crude 569/H DNA preparation, DNA (diphenylamine-reacting material) banded at two densities (Figure 4D), a major band at ρ 1.602 and a minor at ρ 1.655. These peak areas contained no biological activity. Analysis of 569/H culture supernatant showed that the penicillinase protein was found at the meniscus (Figure 4C).

Treatment of 569/H RNA with DNase alone had no significant effect on the biologically active peaks (major at ρ 1.622 and minor at ρ 1.532 (Figure 5B)). Treatment with RNase alone or in combination with DNase caused the minor peak of biological activity to disappear (Figure 5A,C). There was a shift in the density of the major peak (ρ 1.615 in Figure 5A and ρ 1.613 in Figure 5C) and in the latter case seemingly coinciding with some diphenylamine-reacting material. The minor peak also appears to be heat labile as shown by its disappearance from the RNA extract heated to 100° (10 min) and rapidly cooled (Figure 6B). Here again a

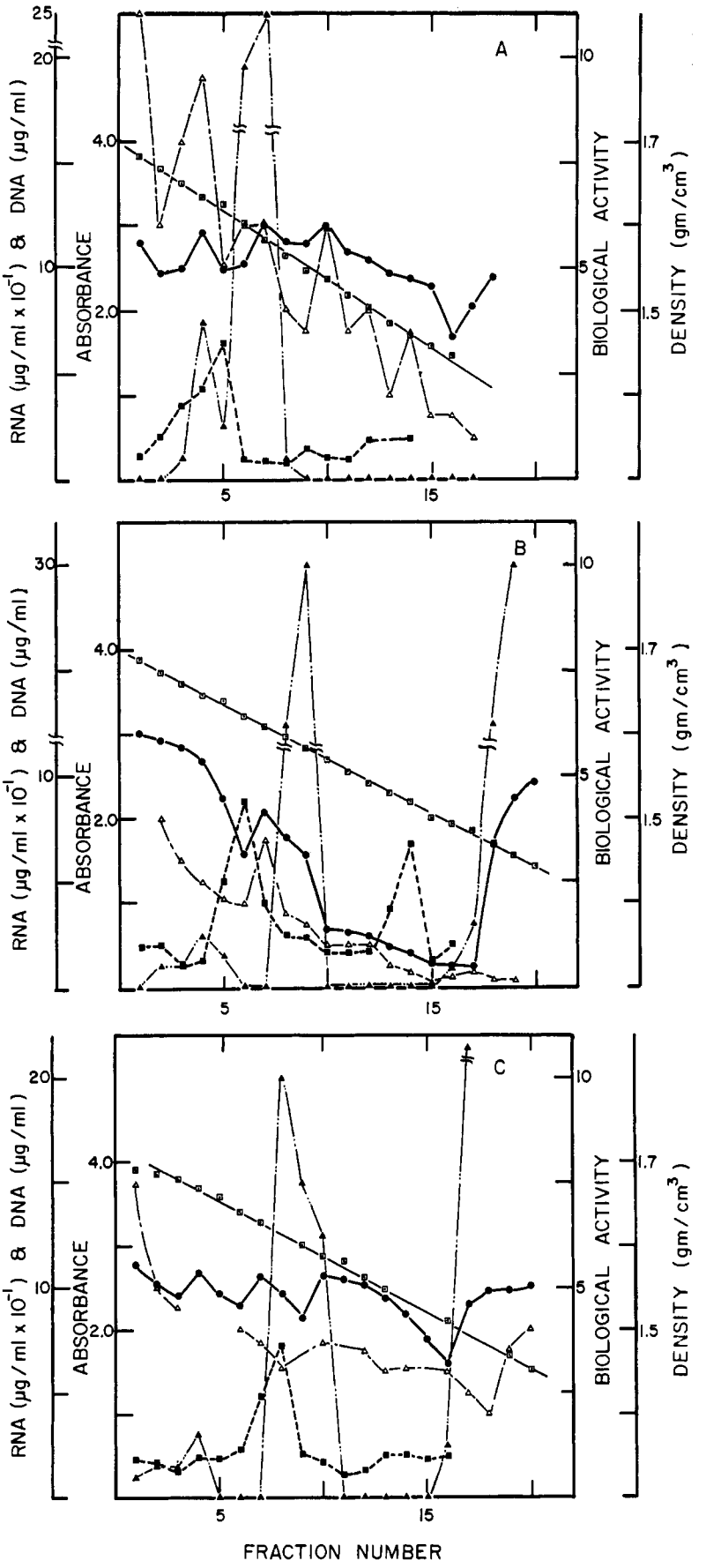


FIGURE 5: Equilibrium density gradient centrifugation analysis of *B. cereus* 569/H membrane RNA after nuclease treatment. (A) Post-RNase treatment. An aliquot of a 569/H RNA extract was incubated (37° for 30 min) with RNase at 100 μg/ml of RNA just prior to the centrifugation analysis. (B) Post-DNase treatment. A second aliquot of the same 569/H RNA extract was incubated similarly with DNase at 100 μg/ml of RNA. (C) Postcombined nuclease treatment. A third aliquot of the same 569/H RNA extract was incubated similarly with RNase and DNase each at 100 μg/ml.

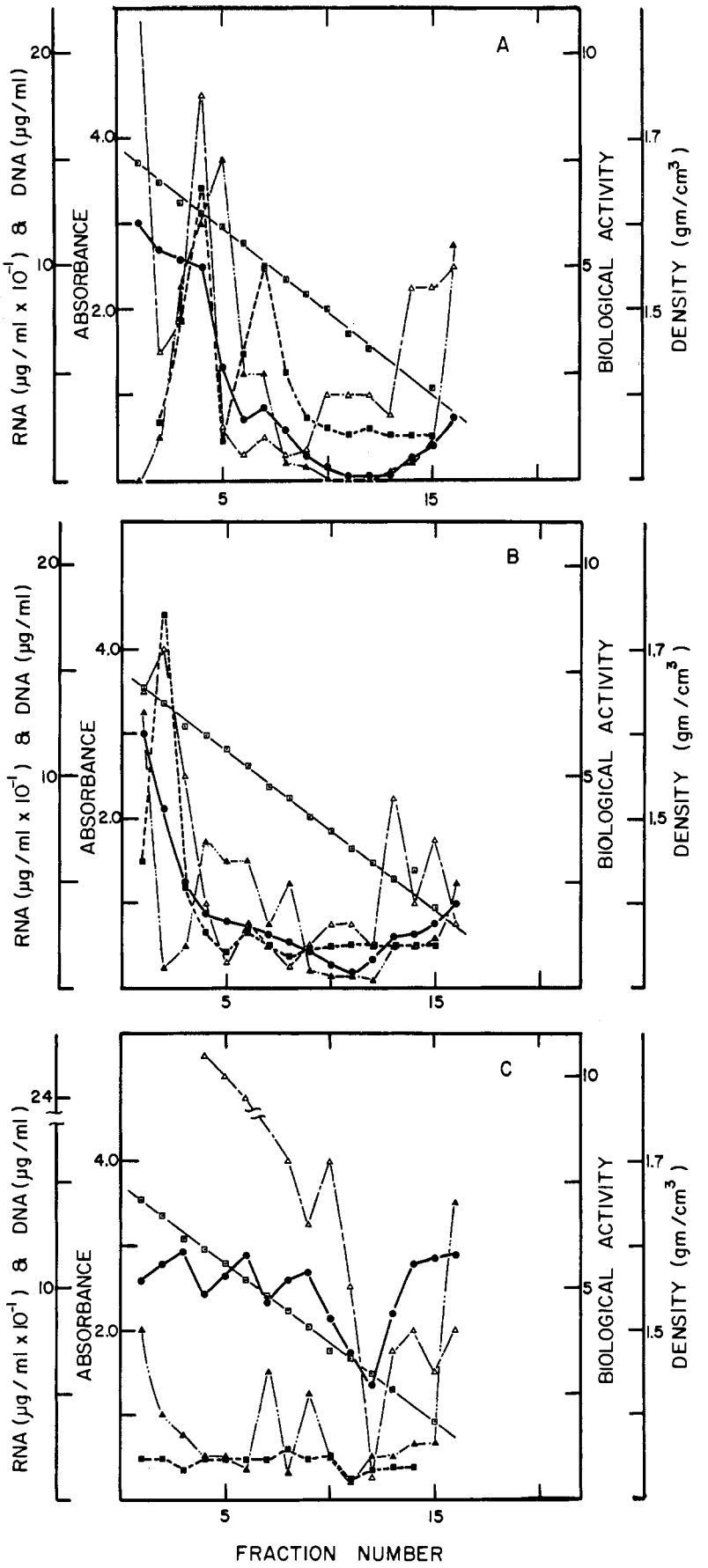


FIGURE 6: Equilibrium density gradient centrifugation analysis of *B. cereus* 569/H membrane RNA after heat and RNase treatment. (A) Untreated control RNA. An aliquot of a 569/H RNA extract was centrifuged and analyzed similarly to that of Figure 4A. (B) Postheat treatment. A second aliquot of the same 569/H RNA extract was heated at 100° for 10 min, rapidly cooled in an ice bath, and a portion of this treated aliquot immediately was centrifuged and analyzed. (C) Postheat and RNase treatment. The remaining portion of the heat-treated aliquot was incubated with RNase as in Figure 5A just prior to centrifugation.

shift in the density of the major peak (ρ 1.632) is observed. But, the peak disappears completely when this heated RNA extract was treated with RNase and analyzed (Figure 6C). The nuclease sensitivity pattern of these treated RNA preparations prior to centrifugation is similar to that presented in Table II.

Discussion

Fractionation of Active RNA Extracts. MAK column chromatography fractionates DNA and various RNA species based on size, base composition, and hydrogen-bond content (Mandell and Hershey, 1960; Kano-Sueoka and Spiegelman, 1962; Sueoka and Cheng, 1962). Thus, smaller RNA molecules elute at low salt concentrations and RNA species containing a greater AU content come off the column at a higher salt concentration. The amino acid composition and molecular weight of penicillinase (Datta and Richmond, 1966) suggest that penicillinase mRNA, containing more AU than GC, would be eluted off the column between 4S and 16S RNA. Thus, elution of the active component between 0.6 and 0.7 M NaCl is consistent with the column characteristics. Association with other nucleic acid species or the active component existing as a double-stranded or hybrid molecule, however, could be the determining factor of the elution position. RNase sensitivity of the active fractions as well as the elution of penicillinase prior to 0.3 M NaCl on a control column suggests again that the active component is not reversibly inactive penicillinase.

On the basis of Kurland's (1960) data for rRNA, the approximate molecular weight of the estimated 12–14S penicillinase-specific component is $3.56–4.27 \times 10^5$. Assuming a code ratio of three (Kurland, 1960; Guttman and Novick, 1963), this "messenger" could code a protein weighing 39,000–48,000 daltons. The molecular weight of 31,600 for penicillinase (Datta and Richmond, 1966) falls close to this calculated range. These calculations are only approximate and were made without any correction for possible association of this component with other RNA species, DNA, or its existence as a double-strand or hybrid molecule.

Buoyant Density Characteristics of the Active Component. The characterization of the penicillinase-specific RNA component of the 569/H RNA extracts by equilibrium density gradient centrifugation further indicates the complexity of the system suggested by the nuclease sensitivity patterns. The density patterns of 569/H penicillinase and 569/H DNA as compared to that of the active 569/H membrane RNA largely eliminate DNA or reversibly inactive penicillinase protein as candidates for the penicillinase-specific component found in 569/H RNA extracts. That an RNA species is indeed involved in the biological activity of 569/H RNA extracts is demonstrated by the density patterns of heat-treated and nuclease-treated 569/H RNA extracts. The distribution of the biologically active component consistently at two different densities indicates that the penicillinase-specific RNA component exists in these 569/H RNA extracts as at least two distinct species. The first and less dense, minor species is RNase sensitive and heat labile. The heat lability of this minor species is as yet unexplained but the apparent increase in the amount of biological activity of the more dense, major band suggests a fate for the lost minor species. This species possibly is a single-stranded RNA specific for penicillinase synthesis. The second and more dense, major species is RNase resistant unless heated and rapidly cooled whereupon it becomes RNase sensitive. It would appear, therefore, that this species is, at minimum, a double-stranded RNA, and possibly a hybrid RNA–DNA (some DNA is routinely found in these fractions) or a double-stranded RNA coupled with some DNA. The variability of the biological activity of 569/H extracts to nuclease action, the sensitivity of the active component of some 569/H extracts only to combined RNase and DNase action, and the apparent decrease and density shift of the more dense, major component upon combined nuclease treatment lend some significance to the presence of DNA.

Only the minor band (ρ 1.588) of RNA obtained from T_2 bacteriophage-infected macrophage cells generated antibody in spleen fragments leading to a reduction in plaque counts (Gottlieb *et al.*, 1967). This lighter band of apparent GC content of 54% as opposed to 41% for the corresponding DNA, disappeared upon Pronase treatment, and, therefore, the gradient displacement was probably due to a protein component and not a difference in base composition. Though our results show some similarities in the buoyant density profiles, the properties of the biologically active component(s) of 569/H membrane RNA are somewhat different than the suggested immunogenic RNA–protein complex. Firstly, Pronase treatment of the membranes prior to extraction eliminates neither the major nor the minor activity band. Furthermore, Pronase has no noticeable effect on the biological activity of 569/H RNA extracts. Secondly, sensitivity of the minor activity band to RNase tends to decrease the importance of a protein complexing with RNA as suggested by the lighter buoyant density. The loss of the minor activity band upon heating is accompanied by an apparent proportional increase in biological activity of the denser activity band. Thirdly, the buoyant density greater than the DNA of the major RNA activity band and the decrease of the buoyant density of the residual biological activity after combined nuclease treatment, decreases the possibility of protein association and suggests a nucleic acid association. Such a meaningful association is suggested further by the loss of the major activity band upon heating followed by RNase treatment.

Nuclease Sensitivity and Some Speculation on the Nature of the Active Component. Isolated mRNA shows considerable sensitivity to RNase while the RNA of the ribosomes, and importantly the mRNA found in polysomes (Warner *et al.*, 1963), or in a double-stranded or DNA hybrid form is relatively resistant. The RNA extracts examined in the preceding paper as well as the active fractions obtained from MAK column chromatography and sucrose density centrifugation were inactivated by RNase action. Varying degrees of RNase sensitivity to complete resistance as

well as, on some occasions, enhancement of the biological activity of a number of the 569/H RNA preparations suggested that complicated factors were involved in the response of the biological activity of 569/H RNA extracts to nucleases. The ionic strength of the buffer as well as the presence of certain ions are some of the factors effecting the relative resistance of these RNA preparations to RNase; a result obtained by Bishop (1966) for a double-stranded (replicative form) phage RNA. He also found that digestion of total nucleic acid with DNase and RNase prior to MAK column fractionation resulted in a substantial loss of this double-stranded RNA. Possibly, in our case, the growth parameters of the donor culture and especially some of the conditions of RNA extractions would have an effect on the nuclease sensitivity of these RNA preparations. Of course, heating would be expected to and does eliminate RNase resistance. The pattern of nuclease sensitivities (inactivation by RNase plus DNase or heat plus RNase) as well as the cesium salts equilibrium density gradient centrifugation does definitely suggest that the biologically active fraction does exist, in large measure, as a double-stranded or hybrid nucleic acid molecule in these 569/H RNA preparations. The active fraction was soluble in 10% NaCl, a characteristic exhibited by several double-stranded nucleic acids (Bishop, 1966). The elution of the active 569/H RNA component along with some DNA from MAK columns at a NaCl molarity (0.6–0.7 M) similar to that where Evans (1964; Spizizen *et al.*, 1966) found the RNA component for sulfanilamide resistance that was more or less sensitive to either DNase or RNase and where DNA–RNA hybrids and double-strand RNA species apparently elute (Cherry, 1964) gives additional support to the above suggestion.

The function of a double-stranded RNA as well as a two- to three-stranded form in the replication of RNA coliphage has been suggested (Bishop, 1966; Erikson and Franklin, 1966). Demonstration of DNA–RNA hybrids have been reported for an *in vitro* system containing ϕX 174 DNA (Hayaski *et al.*, 1965) and for infected cells (Hayashi and Hayashi, 1966). But the general *in vivo* significance of such hybrids in genetic transcription (Evans, 1964; Spiegelman and Haruna, 1966; Spizizen *et al.*, 1966) should be implicated only indirectly since some of the natural DNA–RNA complexes may be artifacts of high ionic conditions of isolation and demonstration (Konrad and Stent, 1964) and since the demonstration of the natural occurrence of DNA–RNA hybrids has relied heavily on their resistance to RNase action. The RNase resistance of rapidly labeled RNA can be markedly increased by artificial hybridization with its complementary DNA region (Hayashi *et al.*, 1965) and the RNA in both the artificial and the natural DNA–RNA hybrids can be made susceptible to RNase by breaking the hybrid with heat (Hayashi and Hayashi, 1966). It seems somewhat tenuous, therefore, to definitively state that such species are directly involved in the production of penicillinase in the recipient cultures. It has been suggested (possibly prematurely), based on the semistable heterozygous nature of the transformed cells, that the capability of the introduced *Pneumococcus* sulfanilimide-resistant RNA fraction (DNA–RNA hybrid?) resides within the cell in a cytoplasmic state capable of limited replication by cellular polymerases (Evans, 1964; Spizizen *et al.*, 1966). It is our suspicion that the hybrid or double-stranded nature is an isolation artifact. These speculations on the structural nature of the active species do not distract from the essential role of a RNA species specific for penicillinase synthesis in the recipient cells. This characterization of the active RNA species offers supporting evidence for our suggestion of *de novo* penicillinase synthesis in recipient cultures receiving *B. cereus* 569/H RNA extracts.

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Biosynthesis of Long-Chain Hydrocarbons. II. Studies on the Biosynthetic Pathway in Tobacco*

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ABSTRACT: Two classes of long-chain hydrocarbons (C_{25}–C_{35}), branched (mainly iso and anteiso) and normal, occur in tobacco in almost equal proportions. The biosynthetic pathway for hydrocarbons has been studied through measurements of the incorporation of radioactive fatty acid substrates into the branched-chain hydrocarbons by the excised leaf. Acetate is the best precursor and its incorporation into the alkane fraction is independent of light. Carboxyl-labeled butyrate, valerate, caproate, and caprylate are all incorporated to the extent of 0.01–0.05% of the added activity into long-chain hydrocarbons. The amount incorporated into branched hydrocarbons was 40–90% of that incorporated into normal hydrocarbons. Furthermore, about 80% of the radioactivity of the branched-chain hydrocarbons isolated from the experiment with [8-^{14}C]caprylate is found in the methyl carbon. These results, together with previous findings that the terminal branched portions of the branched-chain hydrocarbons are derived from the amino acids valine, leucine, and isoleucine, suggest that the hydrocarbons very likely are produced from precursors resulting from a condensation of two long-chain fatty acids, at least one being a normal fatty acid. This mechanism is consistent with the observed relative distributions of long-chain fatty acids and of hydrocarbons in tobacco.

The wide occurrence of long-chain hydrocarbons (C_{25}–C_{35}) in the plant kingdom, particularly in higher plants, is a well-known fact. They occur mostly in the cuticle waxes of leaves and stems. More recently similar hydrocarbons have been found in the animal kingdom, wool wax (Downing *et al.*, 1960), beeswax (Downing *et al.*, 1961), and even in beef brain (Nicholas and Bombaugh, 1965). Consequently the occurrence of long-chain hydrocarbons is of wide generality in nature and the mechanisms of their biosynthesis are of general interest.

Biosynthesis of long-chain hydrocarbons has not been studied to any significant extent; the incorporation of acetate into long-chain hydrocarbons has been reported (Matsuda, 1962; Kolattukudy (1965) and very recently Kolattukudy (1966) has presented evidence showing that *n*-nonacosane, the only major hydrocarbon in *Brassica oleracea*, may be produced by elongation of the common fatty acids.

The present investigation deals with the biosynthetic mechanism of the long-chain hydrocarbons which occur most commonly in higher plants, *viz.*, C_{25}–C_{35}. Studies on the precursors of branched portions of iso and anteiso hydrocarbons have been published in the first paper (Kaneda, 1967). This paper, dealing with the biosynthesis of branched-chain hydrocarbons in tobacco, presents evidence supporting the "condensation mechanism" for the hydrocarbon synthesis as contrasted with the elongation mechanism referred to in the previous paragraph.

Experimental Procedure

Tobacco Plants. Nicotiana tabacum var. "yellow gold" (a "Bright" type) (4–5-months old) (Kaneda, 1967), was used throughout the present work. Unless specified, leaves approximately half-way up the stem of the plant, weighing 10–20 g (1.0–2.0 g dry wt) and with dimensions of about 20 × 30 cm, were excised at a position 1–2 cm from the junction with the stem and used immediately for experiments.

Chemicals. Standard hydrocarbons (branched and normal series) were the same preparations synthesized previously (Kaneda, 1967). Three additional branched-chain hydrocarbons, 3,22-dimethyltetracosane, 3,24-di-

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