References

Anthony, D. D., Zeszotek, E., and Goldthwait, D. A. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1026.
Berg, P. (1958), *J. Biol. Chem. 233*, 601.
Bremer, H., and Konrad, M. W. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 801.
Chamberlin, M., and Berg, P. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 81.
Deutscher, M. P., and Kornberg, A. (1969), *J. Biol. Chem. 244*, 3019.
diMauro, E., Snyder, L., Marino, P., Lamberti, A., Coppo, A., and Tocchini-Valentini, G. P. (1969), *Nature (London) 222*, 533.
Downey, K. M., and So, A. G. (1970), *Biochemistry 9*, 2520.
Goldberg, I. H., Rabinowitz, M., and Reich, E. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 226.
Jones, O. W., and Berg, P. (1966), *J. Mol. Biol. 22*, 199.
Khesin, R. B., Astaurova, O. B., Shemyakin, M. F., Kamzolova, S. G. and Manyakov, V. F. (1967), *Mol. Biol. 1*, 617.
Krakow, J. S., and Fronk, E. (1969), *J. Biol. Chem. 244*, 5988.
Lill, H., Lill, U., Sippl, A., and Hartmann, G. (1970), *in* Proceedings of the First Small Colloquium on RNA Polymerase and Transcription, Silvester, L., Ed., Amsterdam, North-Holland.
Maitra, U., and Hurwitz, J. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 815.
Niyogi, S. K., and Stevens, A. (1965), *J. Biol. Chem. 240*, 2593.
Richardson, J. P. (1969), *Progr. Nucleic Acid Res. Mol. Biol. 9*, 75.
Sentenac, A., Simon, E. J., and Fromageot, P. (1968), *Biochim. Biophys. Acta 161*, 299.
Sippel, A., and Hartmann, G. (1968), *Biochim. Biophys. Acta 157*, 218.
Stead, N. W., and Jones, O. W. (1967), *Biochim. Biophys. Acta 145*, 679.
Straat, P. A., and Ts'o, P. O. P. (1970), *Biochemistry 9*, 926.
Umezawa, H., Mizuno, S., Yamazaki, H., and Nitta, K. (1968), *J. Antibiot. 21*, 234.
Wehrli, W., Knusel, F., Schmid, K., and Staehlin, M. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 667.

Nicking Activity of an Endonuclease I–Transfer Ribonucleic Acid Complex of *Escherichia coli**

Werner Goebel† and Donald R. Helinski‡

ABSTRACT: An endonuclease I-tRNA complex, extracted from *Escherichia coli* JC411, but not present to any appreciable extent in an endonuclease I deficient *E. coli* mutant strain, was shown to catalyze in the presence of 0.5 M NaCl an average of a single nick per covalently closed circular DNA molecule of colicinogenic factor E_1 and the RFI form of ϕX-174.

A similar activity is shown by purified endonuclease I in the presence of *E. coli* tRNA and 0.5 M NaCl. *E. coli* tRNA forms a stable complex with endonuclease I that exhibits a sedimentation coefficient ($s^0_{20,w}$) of 6.0. Purified endonuclease I in the absence of tRNA exhibited an $s^0_{20,w}$ of 3.2 in sucrose gradient sedimentation velocity studies and characteristically degraded DNA to acid soluble oligonucleotides. The nicking activity of the endonuclease I–tRNA complex in the presence of 0.5 M NaCl requires Mg^{2+} and is inhibited by EDTA. With the RFI form of ϕX-174 the nick catalyzed by the endonuclease I–tRNA complex is not strand specific, but occurs with similar frequency in each of the complementary strands.

Endonuclease I of *Escherichia coli*, isolated and purified by Lehman *et al.* (1962), has been shown to catalyze the endonucleolytic cleavage of DNA to acid-soluble oligonucleotides of an average chain length of 7 nucleotides. Studies on the mechanism of degradation of DNA by this enzyme have revealed that the enzyme degrades DNA principally according to single hit kinetics (double strand scission, or chopping) (Cordomier and Bernardi, 1965; Studier, 1965). *Escherichia coli* endonuclease I has also been reported to be strongly inhibited by tRNA (Lehman *et al.*, 1962; Hurwitz *et al.*, 1965).

In this report the formation of a tRNA–endonuclease I complex that sediments more rapidly than unassociated endonuclease I in a sucrose gradient is described. The tRNA-associated endonuclease I exhibits limited endonuclease activity that occurs principally according to double hit kinetics (single strand scission, nicking reaction) in the presence of a high concentration of sodium chloride.

* From the Department of Biology, University of California, San Diego, La Jolla, California 92037. *Received June 10, 1970.* Supported by U. S. Public Health Service Research Grant AI-07194 and National Science Foundation Grant GB-11818.

† The present address of W. G. is Institut für Mikrobiologie and Molekularbiologie, Universitat Hohenheim, Hohenheim-Stuttgart, Germany.

‡ D. R. H. is a U. S. Public Health Service Research Career Development Awardee (No. K04-GM07821). To whom correspondence should be addressed.

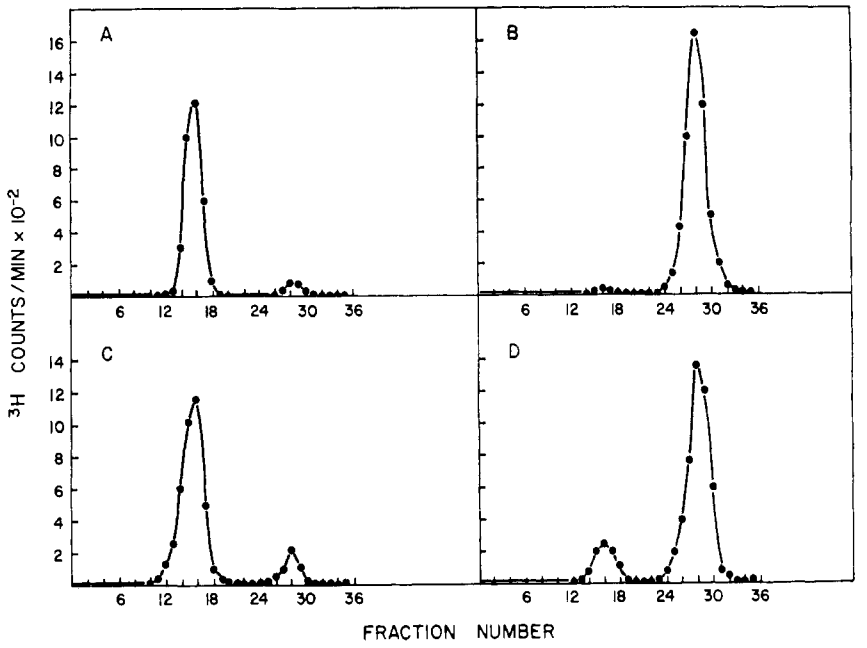

FIGURE 1: Nicking activity of a cleared Brij lysate: (A) ^{3}H-labeled *Col*E$_1$ DNA substrate (0.5 μg); (B) *Col*E$_1$ DNA incubated with cleared Brij lysate. The reaction mixture (0.30 ml total volume) contained: glycine buffer, pH 7.0, 25 μmoles; $MgCl_2$, 1.5 μmoles; NaCl, 150 μmoles; ^{3}H-labeled *Col*E$_1$ DNA, 1 μg; cleared lysate, 50 μg (protein). After 1 hr at 37°, the reaction was terminated by the addition of 10 μmoles of EDTA and 0.2-ml portions were removed for sedimentation analysis. (C) Reaction was carried out as described under B but 10 μmoles of EDTA was added to the reaction mixture at the start of the reaction and $MgCl_2$ was deleted. (D) Reaction was carried out as described under B except 10 μg of tRNA was added. All centrifugations were performed in a Spinco SW65 rotor for 90 min at 50,000 rpm (15°). The alkaline sucrose gradients (5–20%) contained 0.3 M NaOH, 1 M NaCl, 0.01 M Tris, pH 7.6, and 0.001 M EDTA. Fractions of 10 drops were collected.

Experimental Section

Bacterial Strains and Reagents. E. coli JC411 is a K12 strain that has been previously described (Clark and Margulies, 1965). The endonuclease I mutant, *E. coli* K12 strain 1100, was isolated by H. Hoffman-Berling. Lysozyme (B grade) and pancreatic RNase (A grade) were purchased from the California Corp. for Biochemical Research. *E. coli* tRNA was purchased from Miles Laboratories, Inc. DEAE-cellulose (DE52) was obtained from Whatman. The source of Brij 58 was Atlas Chemical Co.

DNA Preparations. ^{3}H-labeled *Col*E$_1$ DNA was prepared from Brij lysates of *E. coli* JC411 (*Col*E$_1$) by a procedure described previously (Clewell and Helinski, 1969). ^{14}C-labeled ϕX-174 RFI DNA was generously provided by Dr. M. Hayashi.

Bacterial Growth and Medium Conditions. Bacteria were grown at 37° to a logarithmic state (5 × 10^8 cells/ml) in a

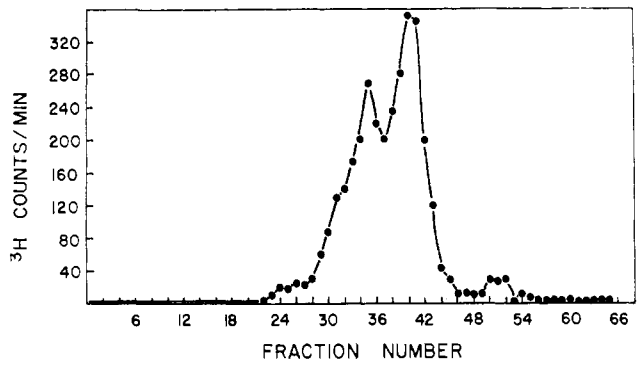

FIGURE 2: Separation of the DNA reaction product into circular and linear single strands. Supercoiled *Col*E$_1$ DNA (1 μg) was treated as described in Figure 1B. The reaction mixture (0.2 ml) was layered on top of an alkaline sucrose gradient (5–20%) and centrifuged at 57,000 rpm for 210 min (15°) in a Spinco SW65 rotor. Fractions 1–50 were 4-drop fractions. The remaining fractions contained 10 drops each.

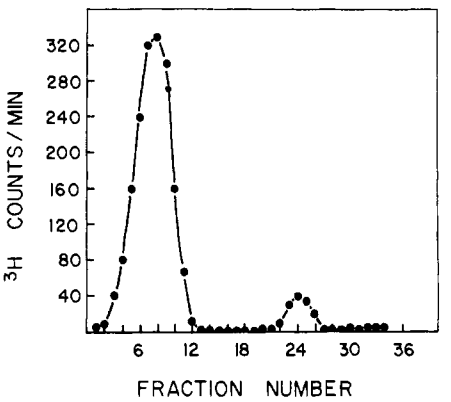

FIGURE 3: Effect of incubation of a cleared Brij lysate of an endonuclease I mutant with supercoiled *Col*E$_1$ DNA. Supercoiled *Col*E$_1$ DNA (1 μg) and 0.05 ml (45 μg of protein) of the cleared lysate of *E. coli* 1100 were incubated as described under Figure 1B. The alkaline sucrose gradient run was performed as described in Figure 1.

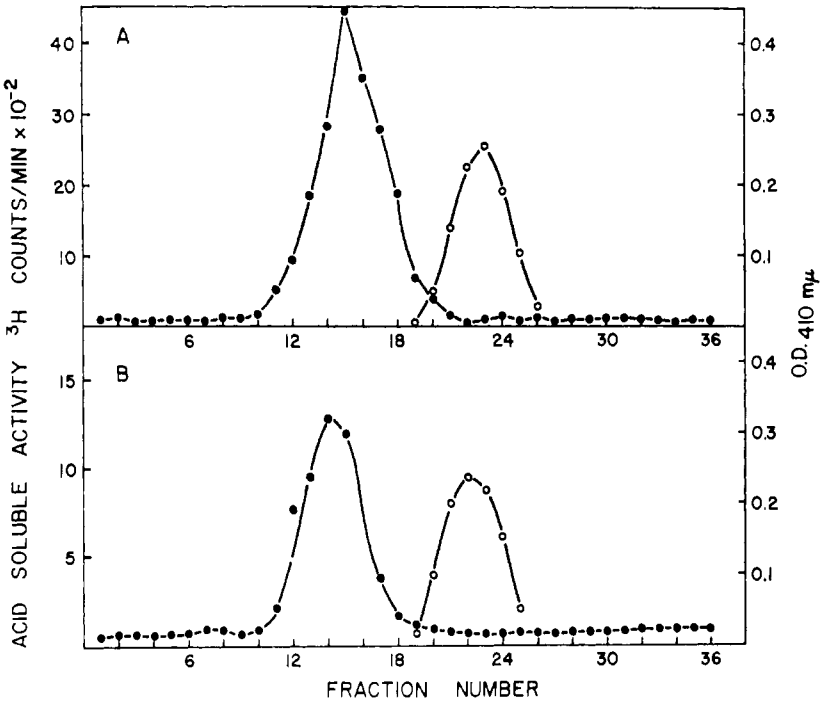

FIGURE 4: Sedimentation properties of unpurified endonuclease I. Cleared Brij lysate (0.2 ml; 0.4 mg of protein) and a preparation of endonuclease I (0.2 ml; 20 μg of protein) obtained by the procedure of Nossal and Heppel were layered on the top of a neutral 5 to 20% sucrose gradient containing 0.01 M phosphate buffer, pH 7.0. Purified human hemoglobin ($s^0_{20,w}$ of 4.5 S) was added as a reference. Centrifugation was carried out for 16 hr in an SW65 rotor at 50,000 rpm and 0°. Fractions 10 drop) were collected into 0.5 ml of glycine buffer, pH 7.0 (0.05 M): (A) cleared Brij lysate; (B) preparation obtained by procedure of Heppel and Nass; (●—●) acid solubilization activity in the presence of pancreatic RNase as described in the Experimental Section; (○—○) concentration of hemoglobin as measured by absorption.

phosphate buffered medium containing per liter: 1.0 g of NH_4Cl, 0.2 g of $MgSO_4$, 1.5 g of KH_2PO_4, 3.5 g of Na_2HPO_4, 5 g of Casamino acids (Difco), and 2.0 g of glucose. The medium was adjusted to pH 6.8.

Preparation of Cleared Brij Lysate. E. coli JC411 was grown in 1 l. of medium to a logarithmic state and the cells were harvested by centrifugation. The pellet (5–7 g, wet weight) was washed once with cold Tris-HCl buffer, pH 7.0 (0.01 M), and resuspended in 15 ml of a 20% sucrose solution in Tris-HCl buffer (pH 7.0, 0.01 M). Lysozyme (25 mg) was added and the mixture was incubated at room temperature for 15 min. Brij 58 then was added to a final concentration of 0.5%. Lysis of the cells occurred upon incubating the mixture for 5 to 10 min at room temperature. The viscous lysate was centrifuged for 15 min at 48,000*g* in the cold. Cell debris and more than 95% of the total DNA were removed during this step. The pellet was discarded and the clear supernatant was stored at 0°. This material is referred to as cleared Brij lysate.

The Purification of Endonuclease I. Enzyme extract used for the purification of endonuclease I was obtained from 100 g of frozen JC411 cells after grinding the cells with 150 g of alumina powder (Alcoa A305) in a mortar. The cell paste was extracted several times at 0° with a total of 400 ml of 0.01 M phosphate buffer, pH 8.0. The crude extract was centrifuged for 30 min at 10,000*g* in the cold. The pellet was discarded and the supernatant was cleared by centrifuging for 1–2 hr at 48,000*g*. The purification of the endonuclease I was carried out as described by Weissbach and Korn (1963). The two DEAE-cellulose chromatography steps were used, but not the hydroxylapatite chromatography step. The peak fractions of the two endonuclease I peaks obtained were pooled separately, concentrated by lyophilization, dialyzed against 0.05 M glycine buffer, pH 7.0, and stored at −20°.

DNA Acid Solubilization Activity. Chromosomal DNA of *E. coli*, labeled with [^3H]thymine and isolated by the phenol procedure followed by methylated albumin kieselguhr chromatography as previously described (DeWitt and Helinski, 1965), was used as the substrate for the measurement of acid solubilization activity. The incubation mixture contained 1.5 μmoles of $MgCl_2$, 25 μmoles of glycine buffer, pH 7.0, 2–3 μg of DNA (containing approximately 5×10^3 cpm), and a portion of the sample to be analyzed in a total volume of 0.3 ml. The reaction mixture was incubated for 30 min at 37° and then cooled to 0° and 0.1 ml of cold Salmon sperm DNA (500 μg/ml) was added. The acid-insoluble DNA was precipitated by adding 0.1 ml of perchloric acid (7%) and keeping the mixture for 5–10 min at 0°. The precipitate was centrifuged and 0.4 ml of the supernatant was placed in 10 ml of Bray's solution (Bray, 1960) for counting. Where indicated 5 μg of pancreatic RNase was added to the reaction mixture before incubation at 37°.

Counting of Radioisotope. Fractions obtained from the sucrose gradient centrifugations and containing radioisotope-labeled DNA were spotted or collected on 1-in. square filter papers and then immersed in succession in cold 10% trichloroacetic acid, ethanol, and ether, and counted in a Beckman liquid scintillation counter. Toluene containing 5 mg/ml of 2,5-diphenyloxazole was the fluor used for the counting of these samples.

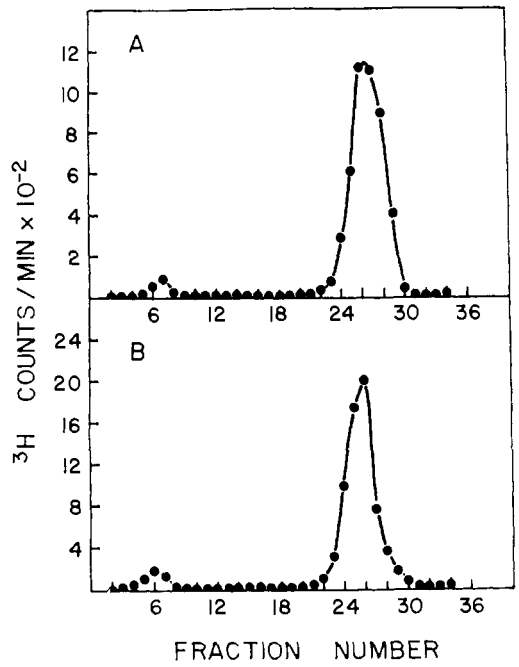

FIGURE 5: Nicking activity of a peak fraction from a sucrose gradient containing endonuclease I activity. Supercoiled *Col*E$_1$ DNA was incubated with 0.1 ml of fraction 15 from the sucrose gradient shown in Figure 4A. The reaction mixture contained in a total volume of 0.3 ml: glycine buffer, pH 7, 25 μmoles; $MgCl_2$, 1.5 μmoles; ^{3}H-labeled supercoiled *Col*E$_1$ DNA, 0.5 μg; 0.1 ml of enzyme solution (8 units, as judged from the acid solubilization activity in the presence of pancreatic RNase). The reaction was carried out as described in Figure 1: (A) no further addition; (B) 150 μmoles of NaCl and 6 μg of tRNA were added to the reaction mixture. Sedimentation analyses were performed on an alkaline sucrose gradient as described in Figure 1.

Results

Endonuclease Activity of a Cleared Brij Lysate of E. coli JC411. E. coli JC411 cells, grown to the log phase, were gently lysed by the lysozyme–Brij procedure. Incubation of the cleared lysate prepared by this procedure with tritium-labeled, covalently closed, *Col*E$_1$ DNA for 1 hr did not result in the formation of acid-soluble material. To determine whether the cleared lysate possessed limited endonuclease activity, the reaction mixture was centrifuged in an alkaline sucrose gradient. Under these centrifugation conditions, covalently closed, circular *Col*E$_1$ DNA sediments with a sedimentation coefficient of 63 S (Bazaral and Helinski, 1968), while the single strands of the denatured open circular, or linear, form of *Col*E$_1$ DNA sediment as 26S molecules. As shown in Figure 1, the cleared lysate in the presence of 0.5 M NaCl converted virtually all of the supercoiled *Col*E$_1$ DNA into a form that has the sedimentation properties in an alkaline sucrose gradient of the single strands of either the open circular or linear form of *Col*E$_1$ DNA. The conversion did not take place in the presence of 0.01 M EDTA (Figure 1C) and the addition of tRNA had little effect on this activity (Figure 1D).

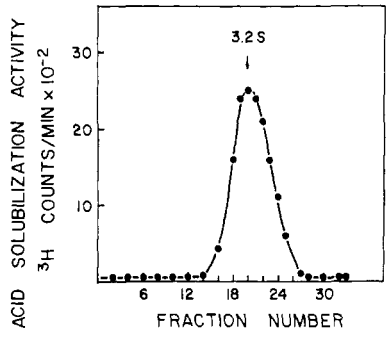

FIGURE 6: Sedimentation properties of endonuclease I purified by DEAE-cellulose chromatography. The combined peak fractions (0.2 ml; 30 μg of protein) of endonuclease activity from the second DEAE-cellulose column was layered on a 5–20% sucrose gradient and centrifuged as described in Figure 4. Nuclease activity was determined as described in the Experimental Section. Pancreatic RNase was not added to the reaction mixture.

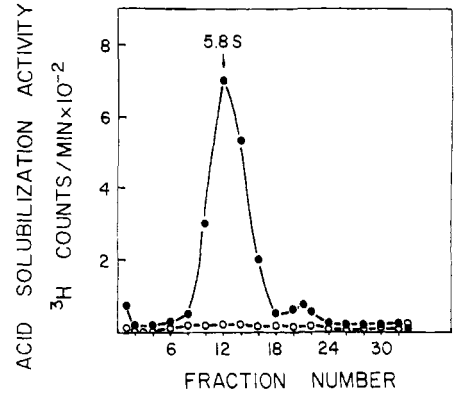

FIGURE 7: Sedimentation properties of endonuclease I purified by DEAE-cellulose chromatography and mixed with tRNA. The combined endonuclease I containing fractions described in Figure 6 were mixed with a solution of tRNA (0.2 ml; 120 μg) at 0°; centrifugation conditions were as described in Figure 4. Endonuclease activity was determined as described in the Experimental Section in the presence (●—●) and absence (○—○) of pancreatic RNase.

Centrifugation of the DNA product of the activity of the cleared Brij lysate for a prolonged period in an alkaline sucrose gradient yielded results which indicated that the supercoiled *Col*E$_1$ DNA was converted mostly into an open circular DNA form containing a single nick in one of the two DNA strands. Vinograd and Lebowitz (1966) have demonstrated that the single strand circular and linear components of an open circular duplex DNA molecule can be separated on an alkaline sucrose gradient. Figure 2 shows that the conversion product of the action of the cleared Brij lysate similarly can be separated into two components that sediment as predicted for the circular and linear single strands, suggesting that the conversion involved predominately a nicking (single strand scission) rather than a chopping (double strand scission) mechanism. This result also indicated that essentially a single nick occurred during the reaction since little slower sedimenting, or lower molecular weight, single stranded DNA was observed.

A cleared Brij lysate prepared from the mutant *E. coli* 1100 strain that has been shown to lack most of its endonuclease I activity exhibited essentially no nicking activity under the reaction conditions that resulted in the virtually complete

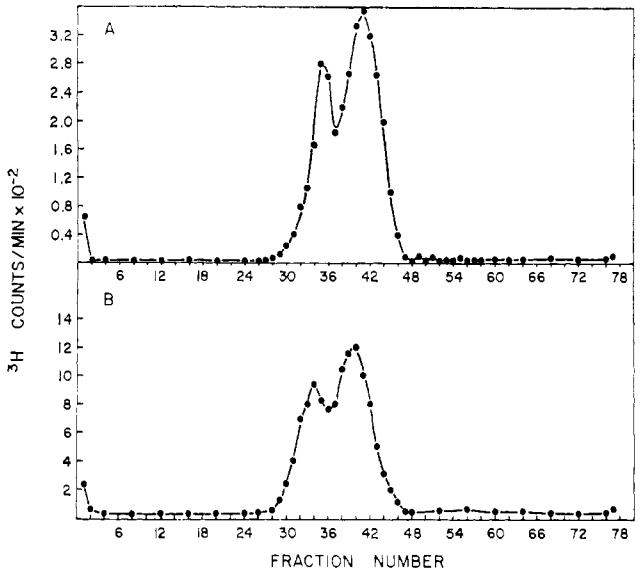

FIGURE 8: Nicking activity of purified endonuclease I in the presence of tRNA and 0.5 M NaCl. The reaction mixture contained in a total volume of 0.3 ml: glycine buffer, pH 7.0, 25 μmoles; $MgCl_2$, 1.5 μmoles; NaCl, 150 μmoles; tRNA, 6 μg; ^{3}H-labeled supercoiled *Col*E_1 DNA, 1 μg; and endonuclease I from peak I (A) and peak II (B) of a second DEAE-cellulose column (5 μg of protein in each case). After 2 hr at 37° the reactions were terminated by the addition of 10 μmoles of EDTA. The nicked circular *Col*E_1 DNA was separated into its circular and linear strands as described in Figure 2 except that the first 70 fractions were 4 drop and the remaining 6 contained 10 drops. Essentially 100% of the counts in the assay mixture were recovered on the gradient.

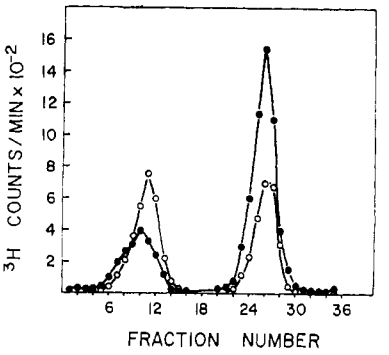

FIGURE 9: Endonculease I activity after 1 hr of incubation of the reaction mixture. ^{3}H-labeled supercoiled *Col*E_1 DNA (1 μg) was incubated in the reaction mixture described in Figure 8A for 1 hr at 37°. At the end of that time 10 μg of ^{14}C-labeled ϕX-174 RFI DNA was added to the reaction mixture and the mixture was incubated for an additional hour. A 0.2-ml portion of the reaction mixture then was run on an alkaline sucrose gradient for 120 min at 50,000 rpm in an SW65 rotor. Fractions (10 drop) were collected: (●—●) *Col*E_1; (○—○) ϕX-174 RFI.

conversion of the supercoiled form to the slower sedimenting form in the presence of the cleared Brij lysate of the *E. coli* JC411 strain (Figure 3). The JC411 strain possesses normal levels of endonuclease I. Because of this result and the data to be reported in other sections, the nicking activity of the cleared Brij lysate of JC411 is ascribed to endonuclease I.

Activity of Endonuclease I Purified by Sucrose Gradient Centrifugation. The cleared lysate of the JC411 strain was fractionated by sucrose gradient centrifugation. Nuclease activity as measured by the acid solubilization of ^{3}H-labeled supercoiled *Col*E_1 DNA in the absence of pancreatic RNase was not detected in any of the fractions of the gradient. When the acid solubilizing activity of each fraction was tested in the presence of pancreatic RNase, a homogenous peak of endonuclease activity was observed at a position corresponding to approximately 6 S (Figure 4A). Endonuclease I activity released from the cells by the procedure of Nossal and Heppel (1966) and fractionated by sucrose gradient centrifugation gave an identical peak of endonuclease activity when the fractions were assayed in the presence of pancreatic RNase (Figure 4B).

The peak fraction (fraction 15) of the sucrose gradient described in Figure 4A also was examined for nicking activity. As shown in Figure 5, this material in the absence of pancreatic RNase catalyzed the rapid conversion of the supercoiled *Col*E_1 DNA to the slower sedimenting form of *Col*E_1 DNA in the presence, or absence, of tRNA. The results of strand separation of the product of this reaction by prolonged centrifugation in an alkaline sucrose gradient indicated that the product in this case was once again the open circular DNA form of the *Col*E_1 factor.

Nicking Activity of Purified Endonuclease I. The further purification of endonuclease I was carried out by chromatography on a DEAE-cellulose column essentially as described by Weissbach and Korn (1963). The material containing

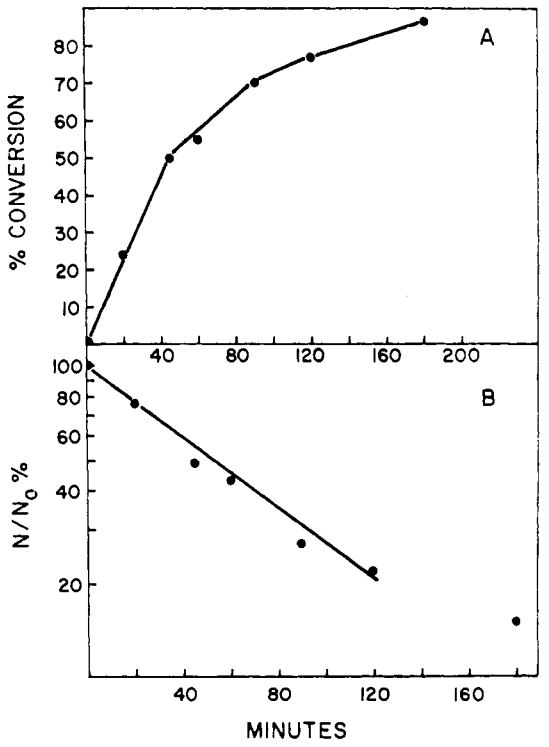

FIGURE 10: Kinetics of the nicking activity of endonuclease I in the presence of tRNA and 0.5 M NaCl. (A) The reaction conditions are described in Figure 8A. Samples were removed at the times indicated. Nicking activity was measured by centrifugation on an alkaline sucrose gradient as described in Figure 9. (B) N/N_0 refers to the fraction of the amount of supercoiled *Col*E_1 DNA initially present that is nicked in a given time period.

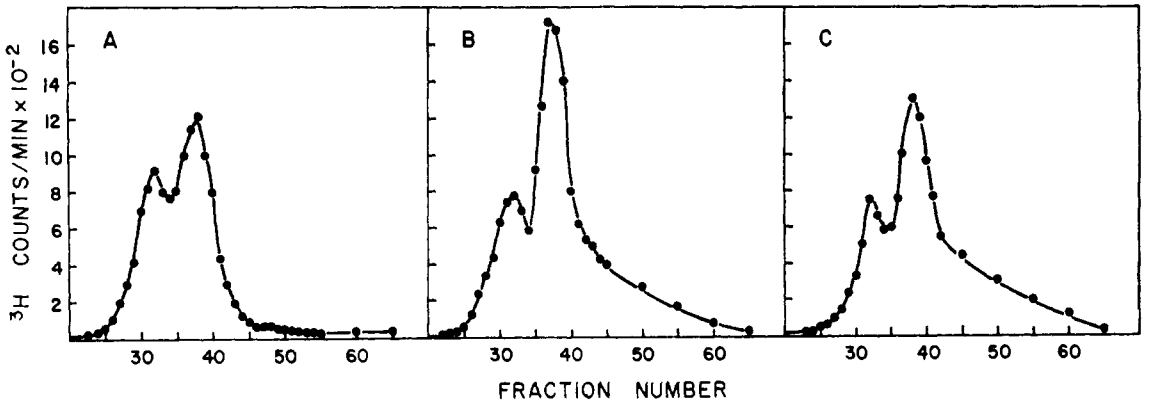

FIGURE 11: Activity of endonuclease I in the presence of tRNA and 0.5 M NaCl during longer incubation times. ^{3}H-labeled supercoiled *Col*E$_1$ DNA (1 μg) was incubated under the reaction conditions described in Figure 8A for (A) 2 hr, (B) 4 hr, and (C) 6 hr. Analysis of the conversion product was carried out by alkaline sucrose gradient centrifugation as described in Figure 2.

endonuclease I activity (tRNA inhibitable endonuclease activity), eluting in two peaks, was pooled and rechromatographed on a second DEAE-cellulose column. Two peaks of activity again were obtained. Material from each peak was combined and centrifuged in a sucrose gradient. A single sedimenting peak of endonuclease activity with a sedimentation coefficient of 3.2 S was observed in the absence of pancreatic RNase (Figure 6). As shown in Figure 7, the addition of tRNA to the combined fractions from the second DEAE-cellulose chromatography resulted in an increase in the sedimentation coefficient of the material exhibiting endonuclease I activity to a value approximating that observed for the endonuclease I of the cleared Brij lysate, or the enzyme released from the cells by the Nossal and Heppel procedure (Figure 4). Detection of acid solubilizing endonuclease activity in this faster sedimenting peak required the addition of pancreatic RNase to the assay medium.

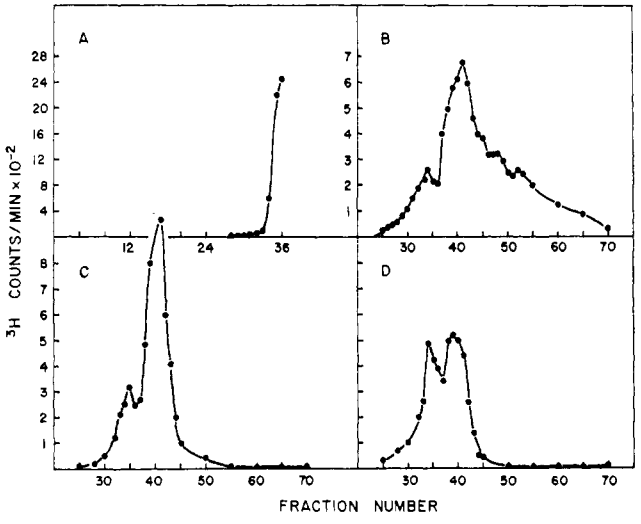

FIGURE 12: The effect of salt concentration and tRNA on the activity of endonuclease I. The reaction mixture contained in a total volume of 0.3 ml: glycine buffer, pH 7.0, 25 μmoles; $MgCl_2$, 1.5 μmoles; ^{3}H-labeled supercoiled *Col*E$_1$ DNA, 1 μg; and endonuclease I from combined peaks I and II from the second DEAE-cellulose column, 5 μg (protein). After 2 hr at 37° the reaction was terminated by the addition of 10 μmoles of EDTA. The reaction product was analyzed by alkaline sucrose gradient centrifugation as described in Figure 2: (A) no further additions; (B) NaCl added at a final concentration of 0.5 M; (C) 6 μg of tRNA added; (D) 6 μg of tRNA and 0.5 M NaCl (final concentration) were added.

The nicking activity of endonuclease I obtained from the second DEAE-cellulose column was determined by incubating supercoiled *Col*E$_1$ DNA with material from each of the two chromatographic peaks of endonuclease activity in the presence of tRNA and 0.5 M NaCl. As shown in Figure 8, the endonuclease I material in either peak from the DEAE-cellulose column was capable of converting the supercoiled *Col*E$_1$ DNA to a slower sedimenting form that could be resolved into circular and linear single strands in an alkaline sucrose gradient. Thus, under these conditions purified endonuclease I exhibits predominately nicking activity. Unlike tRNA relatively high concentrations of *E. coli* ribosomal RNA did not alter the sedimentation coefficient of the purified endonuclease I or promote the nicking activity of the purified enzyme.

To test whether the catalysis of approximately a single nick in the covalently closed *Col*E$_1$ DNA by endonuclease I is due to a limited amount of the enzyme, 10 μg of the supercoiled or RFI form of the DNA of bacteriophage ϕX-174 was added to a reaction mixture containing 1 μg of supercoiled *Col*E$_1$ DNA plus tRNA and 0.5 M NaCl for a 1-hr incubation period. As shown in Figure 9, sufficient enzyme activity is present in the reaction mixture after the 1 hr of incubation to catalyze the conversion of 50% of the added ϕX-174 RFI DNA to a slower sedimenting form. Alkaline sucrose gradient analysis of this slower sedimenting form of ϕX-174 DNA indicated that the conversion once again involved a nick to form II, or the open circular form of ϕX-174 RF DNA (see Figure 14).

Kinetics of Nicking Activity of Purified Endonuclease I. The extent of conversion with time of supercoiled *Col*E$_1$ DNA to the open circular form was followed by removing at various times samples of a reaction mixture containing supercoiled *Col*E$_1$ DNA and purified endonuclease I in the presence of tRNA plus 0.5 M NaCl and analyzing the samples in alkaline sucrose gradients. As shown in Figure 10, the extent of conversion of supercoiled *Col*E$_1$ DNA to the slower sedimenting denatured DNA in the alkaline gradient increases with time and when the logarithm of the fraction of super-

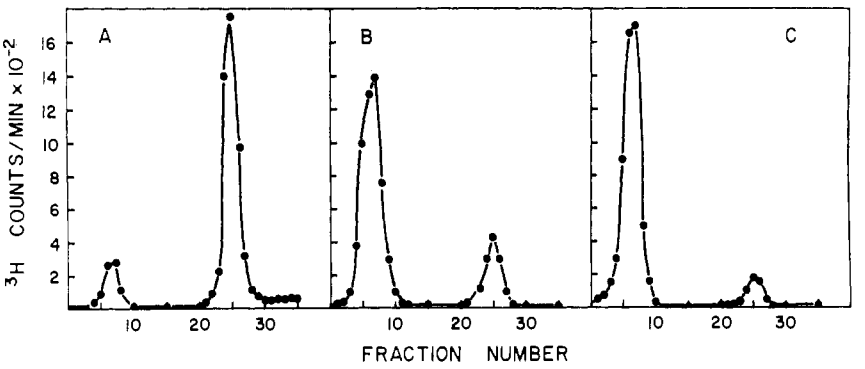

FIGURE 13: Requirement for Mg^{2+} for the nicking activity of endonuclease I. (A) The reaction conditions are described in Figure 8a; (B) Mg^{2+} was deleted from the reaction mixture; (C) Mg^{2+} was deleted and 0.025 M EDTA (final concentration) was added to the reaction mixture. Alkaline sucrose gradient centrifugation was performed as described in Figure 9.

coiled DNA remaining is plotted against time, a straight line relationship, as expected for first-order kinetics, is observed. Similar kinetics are observed when supercoiled *Col*E$_1$ DNA is relaxed with pancreatic DNase (Bazaral and Helinski, 1968). As indicated in Figure 11, alkaline sucrose gradient analysis of the product of the reaction at various times of incubation revealed that there was essentially a single nick in the circular *Col*E$_1$ DNA molecules at the end of 2 hr of incubation while at the end of 4 and 6 hr of incubation fewer circular single strands and smaller fragments of the linear single strands were recovered. Thus, on prolonged incubation additional nicks and possibly double strand cleavages occurred.

Effect of NaCl Concentration on Endonuclease I Activity. The requirement of both 0.5 M NaCl and tRNA for the catalysis by purified endonuclease I of approximately a single nick in the supercoiled *Col*E$_1$ DNA molecule during a 30-min incubation period is shown in Figure 12. In the absence of tRNA and under conditions of low salt concentration (Figure 12A), purified endonuclease I degraded supercoiled *Col*E$_1$ DNA to acid-soluble fragments as previously described (Lehman *et al.*, 1962). In the presence of 0.5 M NaCl without tRNA single-strand cleavages occur as indicated by the recovery of circular single strands (Figure 12B). Under these conditions multiple nicks or double-strand cleavages also occur as indicated by the recovery of a significantly lower amount of circular strands and a substantial amount of lower molecular weight linear single strands (Figure 12B). In the presence of tRNA and a low salt concentration, the nicking activity occurs; however, the substantial reduction of circular single strands and the increase in linear single strands without the production of a substantial amount of lower molecular weight linear strands indicate double strand cleavages in addition to the nicking activity of endonuclease I under these conditions (Figure 12C). Finally, in the presence of 0.5 M NaCl and tRNA (Figure 12D) the reaction appears to proceed largely by the single strand cleavage mechanism with the catalysis of approximately a single nick per molecule.

Requirement of Mg^{2+} for Nicking Activity of Endonuclease I. As shown in Figure 13, the nicking activity of endonuclease I is substantially reduced when Mg^{2+} was deleted from the reaction mixture. In the absence of Mg^{2+} and in the presence of 0.025 M EDTA, virtually no nicking activity occurs (Figure 13C).

Test of Strand Specificity of Nicking Activity. To obtain some indication of the strand specificity of the single-strand cleavage catalyzed by endonuclease I under the limited reaction conditions, the reaction was carried out with the RFI form of ϕX-174 DNA as the substrate. As shown in Figure 14A, ϕX-174 RFI DNA is converted by endonuclease I in the presence of tRNA and 0.5 M NaCl into a product that can be separated, approximately equally, into circular and linear single strands. Since the two complementary strands of ϕX-174 RF DNA can be separated by centrifugation to equilibrium on an alkaline CsCl gradient, it was possible to test whether the circular and linear strands of the reaction product each were a mixture of the individual ϕX-174 strands, or repre-

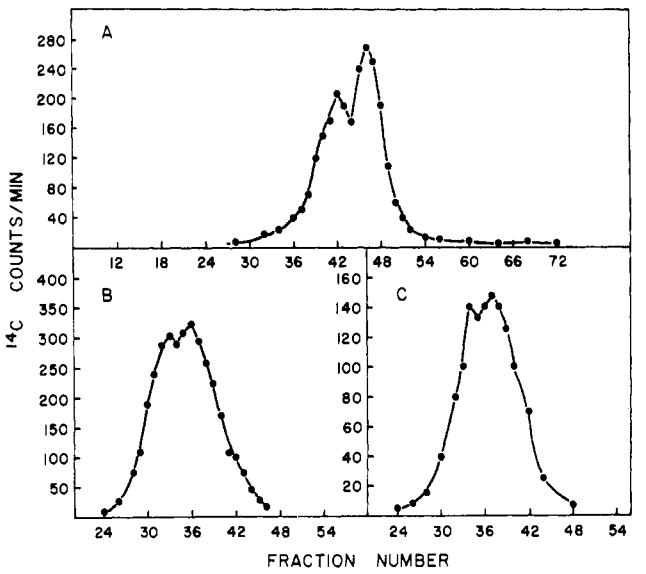

FIGURE 14: Test of strand specificity of nicking activity against ϕX-174 RFI DNA. (A) ϕX-174 RFI DNA (5 μg) was converted into the RFII form under the reaction conditions described in Figure 8a. The circular and linear single strands were separated by alkaline sucrose gradient as described in Figure 8; (B) fractions 36–41 of A were pooled and centrifuged in an alkaline sucrose gradient that contained 2.190 g of CsCl, 0.1 ml of 0.1 M EDTA, and 0.7 ml of 0.4 M phosphate buffer (pH 12.5) in a total volume of 1.8 ml. Centrifugation was performed in a Spinco SW65 rotor at 18° for 40 hr at 38,000 rpm as described by Siegel and Hayashi (1967). Fractions of 3 drops were collected. (C) Fractions 48–51 of A were pooled and centrifuged as described in B.

sented specifically one or the other individual strand. As shown in Figure 14B,C, both the circular and the linear strands of the RFII DNA produced by endonuclease I action consisted of approximately equal amounts of the heavy and light strands of ϕX-174 RF DNA that can be resolved by alkaline CsCl centrifugation. The same conclusion was arrived at from studies on the abilities of the circular and linear strands derived from the endonuclease I nicking of ϕX-174 RF DNA to hybridize with strand specific messenger RNA. In this case both the circular and linear strands hybridized with equal efficiency with ϕX-174 strand specific messenger RNA.

Discussion

Improvements in the ease and sensitivity of techniques for measuring endonuclease activity have permitted the identification of a number of endonucleases in bacteria. In *E. coli* three distinguishable endonucleases have been identified and characterized. Endonuclease I, complexed with RNA, can be released from the periplasmic space (between the cell membrane and the cell wall) of this organism (Cordomier and Bernardi, 1965). The complexed form of endonuclease I has virtually no activity in catalyzing the double strand cleavage of native DNA, but this activity is expressed after treatment with pancreatic RNase, or purification by procedures that result in the removal of the RNA (Lehman *et al.*, 1962). Endonuclease II of *E. coli* catalyzes a limited number of single strand cleavages in nonalkylated native DNA and both double strand and single strand cleavages in alkylated double stranded DNA (Friedberg and Goldthwait, 1969). In contrast to endonuclease I, endonuclease II activity does not require the addition of Mg^{2+} and is not inhibited by tRNA. Endonuclease III of *E. coli* catalyzes the double strand cleavage at specific sites of unmodified DNA and requires *S*-adenosylmethionine and ATP for its activity (Meselson and Yuan, 1968; Roulland-Dussoix and Boyer, 1969).

Several properties of the endonuclease–tRNA complex in this report indicate that the nuclease in the complexed state is endonuclease I. The nicking activity cannot be detected in a cleared Brij lysate of a mutant *E. coli* strain deficient in endonuclease I. Secondly, purified endonuclease I has been shown to be inhibited by tRNA and the release of this inhibition can be achieved by treatment with pancreatic RNase. Thirdly, the nicking activity of the endonuclease–tRNA complex requires Mg^{2+}, is inhibited by Versene, and does not require *S*-adenosylmethionine and ATP. Finally, the nicking activity is released from the periplasmic region under conditions known to release endonuclease I from this region of *E. coli*.

The effect of tRNA in shifting the mechanism of endonuclease activity of endonuclease I from a single hit to a double hit process has some analogy with the observed effect of divalent metal ions on the mechanism of DNase I (pancreatic DNase) degradation of DNA (Melgar and Goldthwait, 1968). The double hit kinetics of DNase I in the presence of Mg^{2+} is converted into single hit kinetics when Mg^{2+} is substituted by Mn^{2+}, Ca^{2+}, or Co^{2+}. These observations emphasize the importance of the environment with respect to the mechanism, and possibly specificity, of endonuclease activity. Protein inhibitors of endonuclease activity have also been reported (Dabrowska *et al.*, 1949; Lesca and Paoletti, 1969) and may play important physiological roles in modulating endonuclease activity.

The conversion of covalently closed circular DNA to open circular DNA by the endonuclease I–tRNA complex in the presence of high concentrations of salt showed first-order kinetics not unlike that exhibited by pancreatic DNase. However, the activity of the endonuclease I–tRNA complex differs from DNase I activity in at least one important respect. Virtually all of covalently closed circular DNA can be converted by the enzyme complex in the presence of 0.5 M sodium chloride into an open circular form with essentially one nick per molecule, while in the case of DNase I there are more than four average nicks per molecule under conditions of complete conversion of supercoiled DNA into the open circular form (Vinograd and Lebowitz, 1966). This may suggest a limited number of preferred sites on the DNA molecule that can be attacked by the endonuclease I–tRNA complex in the presence of high concentrations of salt. It is clear that at least for ϕX-174 RFI DNA, the enzyme complex does not show strand specificity. In addition the enzyme complex does not show substrate specificity in that it is capable of nicking the supercoiled DNA form of the *Col*E_1 DNA, RFI DNA of ϕX-174, and SV40 DNA (unpublished observation).

The formation of an endonuclease–tRNA complex is not unique to endonuclease I. A tRNA inhibitable endonuclease of *Proteus mirabilis* also forms a stable complex with tRNA (Goebel and Helinski, 1969). In this case the kinetics of endonuclease activity is not significantly altered, but the presence of tRNA limits the activity of the endonuclease to the formation of DNA fragments of an average molecular weight of 5.5×10^5. A well-defined complex of tRNA and endonuclease has also been found for spleen acid DNase (Bernardi, 1964).

A physiological role for the endonuclease I–tRNA complex has no support at this time. It is of interest that endonuclease I after release from the periplasmic region is complexed with RNA. A vital role of the endonuclease I–tRNA complex for growth of cells is argued against, however, by the finding of normal growth in the case of an endonuclease I deficient mutant strain. Nevertheless, it is tempting to speculate on the potential role of the endonuclease I–tRNA complex in the initiation, or a subsequent step, of DNA replication, or in recombination. Finally, it should be noted that Lehman (1967) had previously suggested the possibility of low levels of nicking activity of endonuclease I in the presence of tRNA as demonstrated in this report.

Acknowledgments

We are indebted to Mrs. Susan Sheaffer and Mrs. Sally Jo Divis for their invaluable technical assistance.

References

Bazaral, M., and Helinski, D. R. (1968), *Biochemistry 7*, 3513.

Bernardi, G. (1964), *Biochem. Biophys. Res. Commun. 17*, 573.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Clark, A. J., and Margulies, A. D. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 451.

Clewell, D. B., and Helinski, D. R. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1159.

Cordomier, C., and Bernardi, G. (1965), *Biochem. Biophys. Res. Commun. 20*, 555.
Dabrowska, W., Cooper, E. J., and Laskowski, M., Sr. (1949), *J. Biol. Chem. 177*, 991.
DeWitt, W., and Helinski, D. R. (1965), *J. Mol. Biol. 13*, 692.
Friedberg, E. C., and Goldthwait, D. A. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 934.
Goebel, W., and Helinski, D. R. (1969), *Bacteriol. Proc.* 46.
Hurwitz, J., Becker, A., Gefter, M. L., and Gold, M. (1967), *J. Cell. Physiol. 70, Suppl. 1*, 181.
Lehman, I. R. (1967), *Annu. Rev. Biochem. 36*, 645.
Lehman, I. R., Roussos, G. G., and Pratt, E. A. (1962), *J. Biol. Chem. 237*, 819.
Lesca, P., and Paoletti, C. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 913.
Melgar, E., and Goldthwait, D. A. (1968), *J. Biol. Chem. 243*, 4409.
Meselson, M., and Yuan, R. (1968), *Nature (London) 217*, 1110.
Nossal, N. G., and Heppel, L. A. (1966), *J. Biol. Chem. 241*, 3055.
Roulland-Doussoix, D., and Boyer, H. W. (1969), *Biochim. Biophys. Acta 195*, 219.
Siegel, J. E. D., and Hayashi, M. (1967), *J. Mol. Biol. 27*, 443.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.
Vinograd, J., and Lebowitz, J. (1966), *J. Gen. Physiol. 49*, 103.
Weissbach, A., and Korn, D. (1963), *J. Biol. Chem. 238*, 3383.

Further Studies on the Fatty Acid Specificity of Rat Liver Sterol–Ester Hydrolase*

Hans J. Goller† and Demetrios S. Sgoutas‡

ABSTRACT: The specificity of rat liver cholesterol ester hydrolase activity (EC 3.1.1.13) was further investigated. Cholesterol esters of unsaturated fatty acids differing in the proximal or terminal position of the double bond were synthesized and their rate of hydrolysis indicated that a proximal portion of 9 carbon atoms for the fatty acid constituent is a requirement for an optimal hydrolysis of the corresponding cholesterol ester.

In addition, the enzymatic activity was shown to depend upon the chain length of the fatty acid moiety. When a series of saturated fatty acid cholesterol esters were studied, the activity increased from hexanoic to decanoic and then decreased gradually through the eicosanoic acid cholesterol ester. These observations are discussed in terms of a close matching of specially shaped acyl chains to a specially shaped complementary surface in the enzyme active site.

The most active hydrolytic enzyme in rat liver for cholesterol esters was found in the particle free cytoplasm of the cell (Deykin and Goodman, 1962; Swell *et al.*, 1964) and evidence indicated that it acted specifically on cholesterol esters of both common and uncommon fatty acids (Sgoutas, 1968).

Recently, the cholesterol esters of the 16 positional isomers of *cis*-octadecenoic acid were synthesized and their rate of hydrolysis with rat liver cholesterol ester hydrolase was studied (Goller *et al.*, 1970). It was clearly shown that cholesteryl *cis*-9-octadecenoate was hydrolyzed at the highest rate suggesting that this ester is the preferred substrate. Cholesteryl *cis*-9-octadecenoate, however, has the double bond in a symmetric position within the acyl chain and it does not by itself specify whether the proximal or the terminal portion governs the enzymatic selectivity.

Since it seemed desirable to us to emplore this parameter, we determined the enzymatic activity against cholesterol esters of unsaturated fatty acids with different chain lengths which had equal proximal but different terminal portions and different proximal but equal terminal portions. A dependence of the enzymatic activity upon the chain length of the acyl moiety was anticipated and since it was desirable to explore this parameter too, a homologous series of saturated fatty acid cholesterol esters were included as substrates. The results are reported in this communication.

It is hoped that relations between reactivity and structure of the cholesterol ester with regard to its acyl moiety might give useful information in predicting some features about the topography of the active site, the mode of binding, and the catalytic process.

Experimental Procedure

Microanalyses were performed by Clark Microanalytical Laboratories, Urbana, Ill. Instrumentation used in this study has previously been described (Sgoutas *et al.*, 1969). All solvents were reagent grade and were distilled before use.

Materials. Octadecanoic, hexadecanoic, tetradecanoic, 9-

* From The Burnsides Research Laboratory, University of Illinois, Urbana, Illinois 61801. *Received July 14, 1970.* This work was supported by Grant HE 10779-04 from the National Institutes of Health, U. S. Public Health Service.

† Visiting Scientist, supported by Grant HE 5368, U. S. Public Health Service, National Institutes of Health.

‡ To whom correspondence should be addressed at the Department of Pathology, School of Medicine, Emory University, Atlanta, Ga. 30322.