Ribonucleic Acid–Protein Cross-Linking within the Intact *Escherichia coli* Ribosome, Utilizing Ethylene Glycol Bis[3-(2-ketobutyraldehyde) ether], a Reversible, Bifunctional Reagent: Synthesis and Cross-Linking within 30S and 50S Subunits†

Linda A. Brewer,* Susan Goelz,‡ and Harry F. Noller

ABSTRACT: We have used the reversible, bifunctional reagent ethylene glycol bis[3-(2-ketobutyraldehyde) ether] to cross-link RNA to protein within intact ribosomal subunits from *Escherichia coli*. Here we describe the synthesis of this compound (termed bikethoxal) and demonstrate its ability to form covalent attachments between RNA and protein in the 5S RNA–L18 complex and within 30S and 50S ribosomal subunits. The reagent is a symmetrical dicarbonyl compound and reacts with guanine in single-stranded RNA and with arginine in protein. RNA–protein cross-links generated with this reagent are stable, as demonstrated by the comigration of ^{35}S-labeled ribosomal proteins with ribosomal RNA on neutrally buffered sodium dodecyl sulfate (SDS)–agarose gels. However, the cross-linked product is unstable in mildly basic conditions, allowing the identification of the linked macromolecules by conventional techniques. The reagent is potentially capable of cross-linking any combination of single-stranded RNA, single-stranded DNA, or protein; it should prove a useful probe of the RNA–protein proximities within the *E. coli* ribosome, since the SDS–agarose gel system we describe provides a rapid method of optimizing this RNA–protein cross-linking reaction.

The complexity and asymmetry of ribosomal structure require the development of novel techniques for its elucidation. A complete understanding of the mechanism of protein synthesis requires the development of methods for obtaining information about the sites of interaction between the RNA[1] and protein molecules of which the ribosome is composed. Although innovative techniques have been developed which have led to the localization of many proteins within the structure of the bacterial ribosome (Lake, 1980; Moore, 1980; Traut et al., 1980), an equivalent level of information and agreement concerning RNA conformation and RNA–protein interactions within the intact ribosome has been more difficult to obtain. Nevertheless, since the time when it was first suggested that most, if not all, of the ribosomal proteins would be found in domains containing RNA as well as protein (Kurland, 1974), a variety of techniques have been developed to probe these domains. Specifically, this was to obtain detailed topographical information concerning the spatial arrangement of the multitude of ribosomal proteins relative to their position near specific sequences in the three RNA chains of the intact ribosome (Zimmerman, 1980).

Until recently, cross-linking compounds which introduce a covalent bridge between the RNA and a protein were not extensively employed largely due to the paucity of suitable reagents (Ulmer et al., 1978; Zimmerman, 1980). In the last few years, however, a number of promising bifunctional reagents have been developed which irreversibly link RNA and protein [see Zimmerman (1980) for a review]. In conjunction with the elegant and painstaking techniques for cross-link site analysis worked out when direct ultraviolet irradiation of ribosomal subunits was used as a test system (Möller et al., 1978; Zwieb & Brimacombe, 1979; Maly et al., 1980), bifunctional reagents have (Wower et al., 1981) and are expected to continue to provide valuable information concerning the RNA sites of multiple simultaneous protein cross-links.

The search for reversible RNA–protein reagents, which would allow rapid analysis of cross-linked products by conventional techniques, has been more difficult. Although the reaction with formaldehyde is fully reversible, which allows rapid, positive identification of the proteins involved (Möller et al., 1977; Pon et al., 1977), the RNA–protein cross-linking reaction is too readily reversible, creating variability in the proteins observed to be cross-linked and difficulty in the isolation of intact ribonucleoprotein complexes (Ulmer et al., 1978; Zimmerman, 1980). A cleavable center in the bridge connecting cross-linked components is an advantage of butadiene dioxide, but an altered pattern of mobility for a few proteins and a loss of sharpness for others still occur (Bäumert et al., 1978; Sköld, 1981), perhaps as a result of the remaining portion of the reagent or the mild periodate cleavage step. In any event, it will be necessary to correlate cross-link site information gathered from a variety of bifunctional reagents with differing reaction specificity, length, flexibility, and chemical properties.

We have developed a bifunctional reagent, ethylene glycol bis[3-(2-ketobutyraldehyde) ether] which we term "bikethoxal", that has some advantages for investigating nucleic acid–protein topography in a complex macromolecule such as the ribosome. Preliminary reports of this work have been

† From the Department of Biological Sciences, Stanford University, Stanford, California 94305 (L.A.B.), and Thimann Laboratories, University of California, Santa Cruz, California 95064 (H.F.N. and S.G.). *Received January 31, 1983; revised manuscript received May 16, 1983.* This work is supported in part by Research Grants GM 17129 and RR 07135 from the U.S. Public Health Service to H.F.N. and from the University of California to L.A.B. L.A.B. was supported by A.A.U.W. and U.C. Fellowships.

* Address correspondence to this author at the Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305.

‡ Present address: Friedrich Miescher Institute, Basel, Switzerland.

[1] Abbreviations: bikethoxal, ethylene glycol bis[3-(2-ketobutyraldehyde) ether]; SDS, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt; Na_4EDTA, (ethylenedinitrilo)-tetraacetic acid tetrasodium salt; NMR, nuclear magnetic resonance; IR, infrared; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; RNA, ribonucleic acid; T_1 RNase, ribonuclease T_1 (EC 3.1.27.3); Gdn·HCl, guanidine hydrochloride.

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presented (Brewer & Goelz, 1979; Brewer, 1980). Like other dicarbonyl compounds, bikethoxal is expected to form stable covalent bonds; however, these bond are labile in mildly basic solutions in the absence of borate ion (Litt, 1969). This reversibility greatly simplifies the identification of cross-linked products. Further, the analysis of the sites of protein cross-linking within the RNA chain will be greatly facilitated since the sequences of most of the sites of kethoxal (a monofunctional dicarbonyl reagent of similar molecular specificity) modification are known (Noller, 1974; Chapman & Noller, 1977; Herr & Noller, 1978; Hogan & Noller, 1978).

We report here the synthesis and characterization of a novel, bifunctional, cross-linking reagent capable of covalently, but reversibly, joining protein to RNA within the *Escherichia coli* ribosome. The capability of this reagent to produce cross-links between any combination of single-stranded RNA, single-stranded DNA, and protein under physiological conditions is discussed, as well as the advantages of a reagent fully reversible under mild conditions. Furthermore, cross-linking between RNA and protein in the 5S RNA–L18 complex and within both ribosomal subunits is demonstrated.

Experimental Procedures

Synthesis of Bikethoxal. A solution of selenium dioxide (1.1 g, 10 mmol; Matheson Coleman & Bell) dissolved in 1,2-ethanediol (0.655 mL, 12 mmol) was added dropwise with continuous stirring into 2.0 mL (20.7 mmol) of 2-butenal (trans) (Eastman) which had been heated to 80 °C under a nitrogen atmosphere. This reaction mixture was held under nitrogen at 80 °C for 5 h (Rabjohn, 1949; Tiffany et al., 1957), cooled to room temperature, and centrifuged (300*g*, 15 min) to remove precipitated selenium. The compound ethylene glycol bis[3-(2-ketobutyraldehyde) ether] was stored at −20 °C.

Spectra of the compounds were obtained on a Perkin-Elmer 237B (IR), a JEOL JNM-FX60 (NMR), or a Cary 14 (UV).

Preparation of 5S RNA, Ribosomal Protein L18, and L18–5S RNA Complexes. ^{32}P-Labeled and unlabeled 5S RNA were prepared in the A form (Noller & Garrett, 1979). Ribosomal protein L18 was prepared as described by Hindennach et al. (1971). 5S RNA–L18 complexes were prepared essentially according to the procedure of Garrett & Noller (1979). However, approximately 10^5 cpm of ^{32}P-labeled 5S RNA was used in each reaction. Also, a 10-fold (rather than a 4–5-fold) molar excess of L18 was used to form the complexes.

Cross-Linking of L18–5S RNA Complexes with Bikethoxal. The 5S RNA–L18 complexes or 5S RNA was reacted with 3.5 mg/mL (0.015 M) ethylene glycol bis[3-(2-ketobutyraldehyde) ether] in the modification buffer used to form the complexes (Garrett & Noller, 1979). All reactions were incubated for 1 h at 33 °C with gentle mixing. Following the reaction, the solution was adjusted to 20 mM borate to stabilize the RNA–protein cross-link. Excess reagent was removed by dialysis for 28 h at 6 °C into a buffer containing 5 mM Hepes, pH 7.0, 20 mM borate, and 0.1 mM Na_4EDTA. Samples were frozen and lyophilized to decrease the volume to 0.05 mL; 0.5 μg of T_1 RNase [Sankyo; 20:1 (w/w) RNA–RNase] in 1 μL of a buffer containing 0.01 M Tris-HCl, pH 7.0, and 0.001 M Na_4EDTA was added to each sample.

Isolation of 5S Oligonucleotides Covalently Bound to L18. To dissociate noncovalently bound macromolecules, samples were adjusted to 6 M guanidine hydrochloride (Sigma) and incubated for 20 min at room temperature. Samples were then applied directly to a Sephadex G-50 (Sigma) column (11 cm × 0.3 cm i.d.) that had been equilibrated with 0.02 M sodium acetate and 7 M urea at 6 °C. Fractions were analyzed for radioactivity. Samples were eluted with the same buffer (6 °C), and fractions were alalyzed for radioactivity. The peak of radioactivity found in the excluded volume, fractions 9–17, was pooled. These ^{32}P-labeled oligonucleotides found to co-chromatograph with L18 were dialyzed at 6 °C into a 10 mM acetic acid buffer adjusted to pH 5.0 with pyridine and then lyophilized.

Preparation of Ribosomal Subunits. Radioactively labeled ribosomal subunits were prepared by growing *E. coli* MRE600 on low phosphate medium (Garen & Levinthal, 1960), supplemented with 20 mCi of carrier-free [^{32}P]orthophosphate (ICN), or on low sulfate medium (Sun et al., 1974), supplemented with 5 mCi of carrier-free [^{35}S]sulfate (ICN). Labeled cells were opened by the lysozyme freeze–thaw method of Ron et al. (1966). Ribosomes and ribosomal subunits were isolated essentially according to the method of Staehelin et al. (1969), except that the buffer in which the 70S ribosomal pellet was resuspended and the subunits isolated by sucrose density gradient centrifugation contained 1 mM $MgCl_2$. Ribosomal subunits were stored at −80 °C in a buffer containing 20 mM Tris-HCl, pH 7.5, 60 mM NH_4Cl, 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. Unlabeled subunits were prepared in parallel with radioactive subunits.

Cross-Linking Reaction Conditions. For determining the extent of RNA–protein cross-linking with varying bikethoxal concentrations, ^{35}S-labeled 50S ribosomal subunits (1.5×10^4 cpm) and unlabeled 50S subunits (to total 40 A_{260} units) were incubated in a buffer containing 0.04 M $KHCO_3$, pH 8.5, and 0.01 M $MgCl_2$. Bikethoxal (in this buffer) was added to various final concentrations. Reaction mixtures were incubated for 1 h at 37 °C, before the addition of 1 M potassium cacodylate to make a final concentration of 0.04 M and pH 7.0. Incubation was continued for 1 h at 37 °C. Following the reaction, excess reagent was removed by precipitation of cross-linked subunits with 0.66 volume of ethanol (−20 °C). Ribosomal pellets were resuspended in distilled water, immediately frozen (−80 °C), and lyophilized.

As a control, ^{35}S-labeled 50S ribosomal subunits were treated in the same manner as in the cross-linking reactions, except that bikethoxal was omitted. Also, ^{35}S-labeled 30S subunits were incubated under the same conditions at a 3.5 mg/mL bikethoxal concentration.

Reaction conditions for ^{35}S- or ^{32}P-labeled 30S subunits in the sedimentation experiments were essentially the same as described above. The bikethoxal concentration was 3.5 mg/mL, and incubation times are noted in the figure legends. For the experiments in which SDS–agarose gels were the method of assessing the extent of RNA–protein cross-linking, ^{35}S- or ^{32}P-labeled 30S or 50S subunits were incubated in a reaction buffer containing 0.04 M potassium cacodylate, pH 7.0, and 0.01 M magnesium chloride. Variations in pH, magnesium ion concentration, incubation time and temperature, preincubation of the reagent, or the addition of other components to the buffer are as noted in the figures and figure legends.

RNA–Protein Cross-Links Isolated by Urea–Sepharose 4B Chromatography. Lyophilized cross-linked or control 50S ribosomal subunits were dissociated by a 20-min, room temperature, incubation in 0.05 mL of a buffer containing 6 M guanidine hydrochloride, 0.02 M potassium cacodylate, and 0.02 M potassium borate, pH 7.0. Dissociated 30S subunit samples were applied directly to a Sepharose 4B column (9.0 cm × 0.3 cm i.d.) that had been equilibrated in 0.02 M potassium cacodylate, pH 7.0, 0.02 M potassium borate, 0.5 M sodium acetate, and 8 M urea, at 4 °C. The samples were

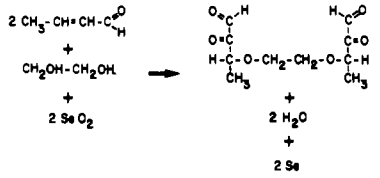

FIGURE 1: Reaction scheme for the synthesis of bikethoxal.

eluted with the same buffer (4 °C). Fractions were analyzed for radioactivity and for absorbance at 260 nm.

RNA–Protein Cross-Links Isolated by SDS–Agarose Gel Electrophoresis. Cross-linked and control ^{35}S- and ^{32}P-labeled subunits in 0.020–0.025 mL were applied to 2% agarose (Sigma, type II) gels containing 0.5% SDS in the Tris–borate–EDTA buffer system described by Peacock & Dingman (1968); 100-mL gels (20 × 20 × 0.3 cm) were poured and assembled for horizontal electrophoresis in an apparatus similar to that described by Shinnick et al. (1975). For best resolution, electrophoresis was at 80 mA, constant current for 1.5 h. Gels were air-dried and autoradiographed (Sommer & Traut, 1976). Densitometric analysis of autoradiograms were made with a Quick Scan Jr. TCL (Helena Laboratories).

Sedimentation Analysis of Cross-Linked 30S Subunits. Cross-linked or control ^{35}S- or ^{32}P-labeled 30S ribosomal subunits in 0.105 mL were layered on top of 3.4-mL 5–20% sucrose gradients in 10 mM Tris-HCl (pH 7.5), 30 mM ammonium chloride, 6 mM 2-mercaptoethanol, and 1 mM magnesium chloride and centrifuged at 6 °C for 1.75 h at 42000 rpm (International B-60 Ultracentrifuge, SB405 rotor).

Results

Synthesis of Bikethoxal. The reaction between 2-butenal (trans) and 1,2-ethanediol to form ethylene glycol bis[3-(2-ketobutyraldehyde) ether] is outlined in Figure 1. The oxidation by treatment with selenium dioxide (Tiffany et al., 1957) and condensation were achieved in good yield. NMR and mass spectra of the product were both consistent with the formation of bikethoxal. The IR spectra were complex, and the appearance of certain absorbance maxima not present in spectra of the reactants was characteristic of the synthesis of this reagent. The 1100-cm^{-1} peak was consistent with the formation of an ether linkage and not present in spectra of either reactant. The 1638-cm^{-1} peak (C=C stretch) of one of the reactants, 2-butenal (trans), was not found in bikethoxal. Further, the 1700-cm^{-1} peak (C=O stretch) characteristic of kethoxal was found in the IR spectra of bikethoxal but neither of the reactants.

Bikethoxal was soluble in aqueous solution and was stable for at least 6 years when stored at −20 °C. However, the selenium precipitate which formed during storage in solution, while negligible quantitatively, appeared to interfere with the reaction of bikethoxal and RNA. Therefore, this precipitate was removed by centrifugation before using solutions of the reagent.

Specificity of Bikethoxal. Bikethoxal, like other 1,2-dicarbonyl compounds, reacts selectively with guanine in RNA (Shapiro & Hachmann, 1966; Shapiro et al., 1969) and with the guanidino function of arginine and lysine in protein (Glass & Pelzig, 1978). The structures of the mono- and diadducts, as well as those of complexes stabilized by borate, are summarized by Expert-Bezançon & Hayes (1980). Although the products of these addition reactions are relatively stable in acidic solutions and their stability can be increased with borate, the guanosine adduct (Shapiro & Hachmann, 1966) and the major arginine adduct under the conditions of these experiments (Glass & Pelzig, 1978) are unstable and decompose into

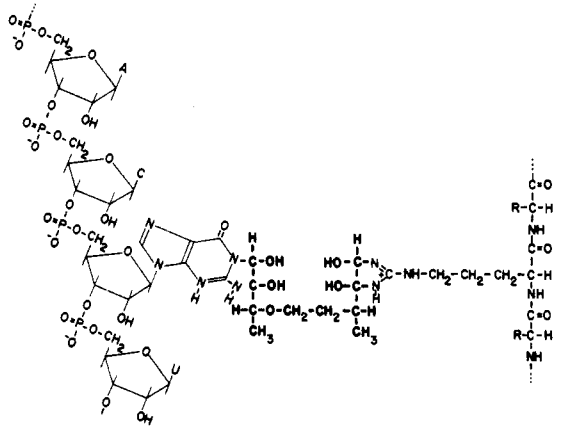

FIGURE 2: Cross-linking reaction scheme between RNA and protein with bikethoxal. The reaction of glyoxal derivatives such as bikethoxal, ethylene glycol bis[3-(2-ketobutyraldehyde) ether] (drawn in bold letters), with guanine (in the RNA chain, drawn schematically on the left) and arginine (in the protein, drawn schematically on the right) has been studied in detail (Shapiro & Hachmann, 1966; Takahashi, 1968; Shapiro et al., 1969; Glass & Pelzig, 1978).

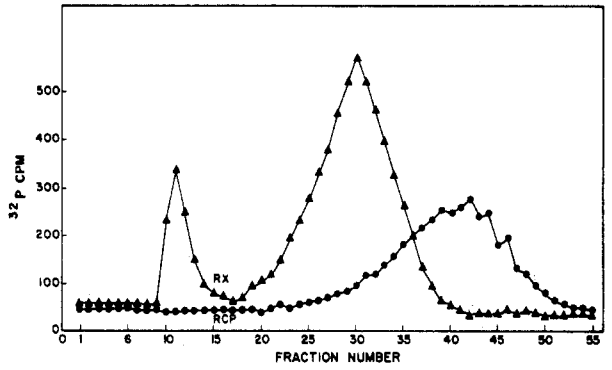

FIGURE 3: Separation of 5S RNA oligonucleotides cross-linked to ribosomal protein L18. ^{32}P-Labeled 5S RNA–L18 complexes were cross-linked with bikethoxal (▲) or incubated in the absence of bikethoxal (●). The complexed RNA was digested with T_1 RNase. After dissociation by incubation with Gdn·HCl, T_1 oligonucleotides cross-linked to L18 were separated from unbound sequences by urea–Sephadex G-50 chromatography.

their components in mildly alkaline solutions in the abscence of borate.

The results of experiments in which bikethoxal was reacted with guanosine or arginine were consistent with those finding using other 1,2-dicarbonyl compounds (glyoxal derivatives). The only exception was that since bikethoxal is a symmetrical bifunctional reagent, products likely to be the result of addition to both ends of the reagent were formed below pH 8.0 or following 8 h or more of reaction at 37 °C (results not shown).

A schematic representation of the structure of an RNA–protein cross-link formed by bikethoxal is shown in Figure 2. The bridge length of the reagent was calculated to be between 8 and 9 Å.

Demonstration of RNA–Protein Cross-Linking in the 5S RNA–L18 Complex. ^{32}P-Labeled 5S RNA (A form) was incubated with ribosomal protein L18 under saturation binding conditions. These 5S RNA–L18 complexes, as well as samples of 5S RNA alone, were then incubated with or withvout bikethoxal. Following exhaustive digestion of the 5S RNA with T_1 RNase, ^{32}P-labeled oligonucleotides covalently bound to L18 were separated from unbound oligonucleotides by urea–Sephadex G-50 chromatography (Figure 3). First of all, when ^{35}S-labeled 50S subunits were dissociated in Gdn·HCl, all of the ^{35}S-labeled protein migrated in the excluded volume on urea–Sephadex G-50 columns (data not shown). Since L18

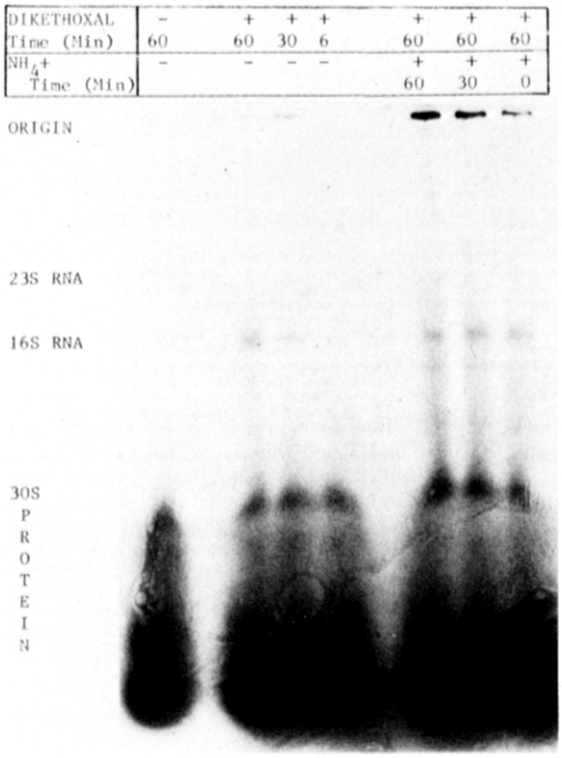

FIGURE 4: Cross-linking of ^{35}S-labeled 30S protein to 16S RNA with bikethoxal. ^{35}S-Labeled 30S ribosomal subunits were incubated with or without (control) bikethoxal at 37 °C for the periods indicated. Ammonium acetate (to a final concentration of 0.2 M) was added to the reaction and was present for the periods indicated. The subunits were dissociated with SDS, and the RNA and protein components were separated by SDS–agarose gel electrophoresis. This is an autoradiogram of the dried gel.

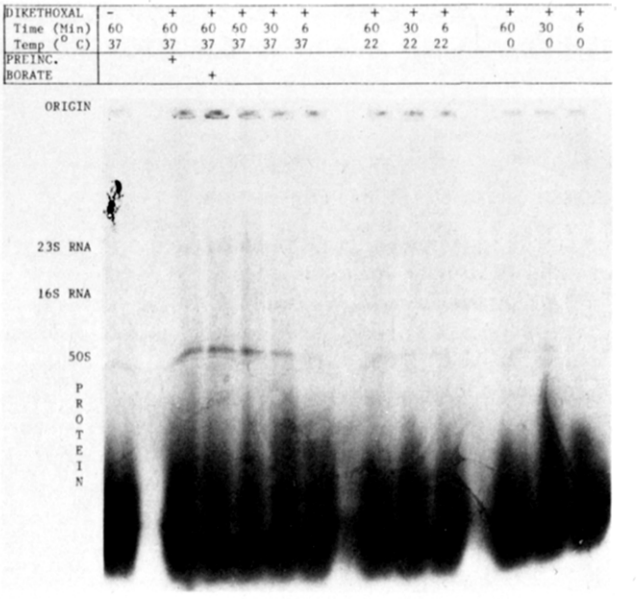

FIGURE 5: Cross-linking of ^{35}S-labeled 50S protein to 23S RNA with bikethoxal. ^{35}S-Labeled 50S ribosomal subunits were incubated with or without (control) bikethoxal. Samples were incubated at various temperatures and for varying periods of time (as indicated). There was one sample to which bikethoxal preincubated in reaction buffer for 1 h at 37 °C was added and another to which borate (to a final concentration of 20 mM) was added after cross-linking. The subunits were dissociated with SDS and the RNA and protein components separated by SDS–agarose gel electrophoresis. This is an autoradiogram of the dried gel.

is a 50S subunit protein, it migrated in the excluded volume. Next, if 5S RNA alone was digested with T_1 RNase, the ^{32}P-labeled oligonucleotides were not found in the excluded volume, but migrated more slowly. Similarly, when ^{32}P-labeled 5S RNA–L18 complexes were treated with T_1 RNase, the ^{32}P-labeled oligonucleotides migrated more slowly than L18. However, if ^{32}P-labeled 5S RNA–L18 complexes were treated with bikethoxal, a significant proportion of the ^{32}P-labeled oligonucleotides migrated in the excluded volume in the same position as L18.

Demonstration of RNA–Protein Cross-Linking within 30S and 50S Ribosomal Subunits. When 30S ribosomal subunits were treated with bikethoxal, protein was cross-linked to 16S RNA. Cross-linking was demonstrated by the comigration of ^{35}S-labeled protein with 16S RNA on SDS–agarose gels. Figure 4 is an autoradiogram of a dried SDS–agarose gel. ^{35}S-Labeled 30S ribosomal subunits were incubated with or without bikethoxal. Subunits were dissociated with SDS and the RNA and protein separated by rapid electrophoresis. In the left-hand section of the gel it can be observed that without cross-linking no ^{35}S-labeled protein comigrates with 16S RNA. However, when bikethoxal is added, increasing the incubation period increases the amount of ^{35}S-labeled protein migrating in the position of 16S RNA.

Similarly, when 50S subunits were treated with bikethoxal, protein was cross-linked to 23S RNA. Figure 5 shows ^{35}S-labeled protein co-migrating with 23S RNA when ^{35}S-labeled 50S subunits were treated with bikethoxal. Further, increasing the time and temperature of incubation with the reagent increased the amount of cross-linking to 23S RNA.

The effect of various buffer components on the cross-linking reaction was tested with these gels. There was some concern that Tris would react with bikethoxal, forming a Schiff's base. However, the presence of 20 mM Tris-HCl had no observable effect upon the extent of the cross-linking reaction (data not shown). Similarly, 5 mM Mops did not interfere with cross-linking. Figure 4 shows that the presence of 0.2 M ammonium acetate did not reduce the extent of the cross-linking reaction. That is, the amount of ^{35}S-labeled protein comigrating with RNA after 1 h of incubation with bikethoxal was the same although ammonium ion had been added at various times during the reaction. Further, since ^{35}S-labeled bands appeared between 16S RNA and 30S protein with increasing time of reaction in the presence of ammonium ion, the formation of protein–protein cross-links may have been favored.

Other ions and conditions were also tested. The addition of borate following the reaction did not significantly increase the stability of the RNA–protein cross-link under these reaction and electrophoretic conditions (Figure 5). Also, the presence of magnesium ion between 1 and 20 mM had no observable effect. However, at magnesium concentrations below 1 mM the extent of the reaction appeared to increase slightly, perhaps due to unfolding of the subunit (data not shown). Furthermore, preincubation of the cross-linking reagent did not increase or decrease the extent of the cross-linking reaction as can also be seen in Figure 5.

The pH optimum of the RNA–protein cross-linking reaction was determined. The relative extent of cross-linking was quantified by densitometric analysis of cross-linked bands on autoradiograms of these gels. Figure 6 shows that RNA–protein cross-liking of bikethoxal under these conditions was maximal below pH 7.0. However, limited solubility of ribosomes at pH 6.0 restricted the range of analysis.

A separate method was used to quantify the extent of cross-linking. The proportion of protein cross-linked to RNA with bikethoxal in ^{35}S-labeled ribosomal subunits was deter-

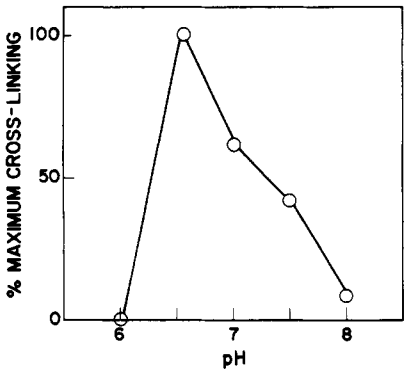

FIGURE 6: pH dependence of cross-linking with bikethoxal. Reaction was at 37 °C for 2 h. The yield of cross-linked protein was determined by the proportion of radioactive protein which comigrated with RNA on SDS–agarose gels (as described under Experimental Procedures).

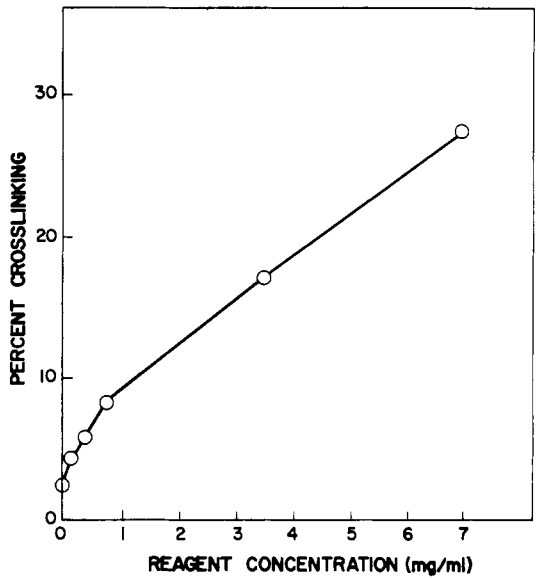

FIGURE 7: Dependence of the extent of RNA–protein cross-linking of 50S subunits on bikethoxal concentration. The reaction was at 37 °C for 1 h at pH 8.5 and then for 1 h at pH 7.0. The yield of cross-linked proteins was determined by the proportion of radioactive protein which comigrated with RNA on urea–Sepharose 4B columns (as described under Experimental Procedures).

mined by analyzing the proportion of radioactivity comigrating with RNA on urea–Sepharose 4B columns. It was found that 15% of the 50S protein was cross-linked to RNA at 3.5 mg/mL bikethoxal (the standard reagent concentration used in these experiments). Under this same set of conditions about twice as much protein was cross-linked to RNA in 30S subunits (data not shown). The dependence of the proportion of protein cross-linked to RNA upon reagent concentration is shown in Figure 7. The amount of protein cross-linked to RNA increased with increasing bikethoxal concentration. Although the rate of increase decelerated at higher concentrations, the reaction had not reached saturation when up to 25% of the protein was cross-linked at a 7.0 mg/mL bikethoxal concentration.

At the standard bikethoxal concentration (3.5 mg/mL), the effect of cross-linking on ribosome conformation was studied by sedimentation on sucrose gradients. Figure 8 shows that the sedimentation rate of 30S subunits does not appear to be significantly effected by cross-linking since the peaks of cross-linked and control subunits were coincident. The appearance of additional, small, faster sedimenting peaks was reduced, however, by decreasing the subunit concentration and reaction time.

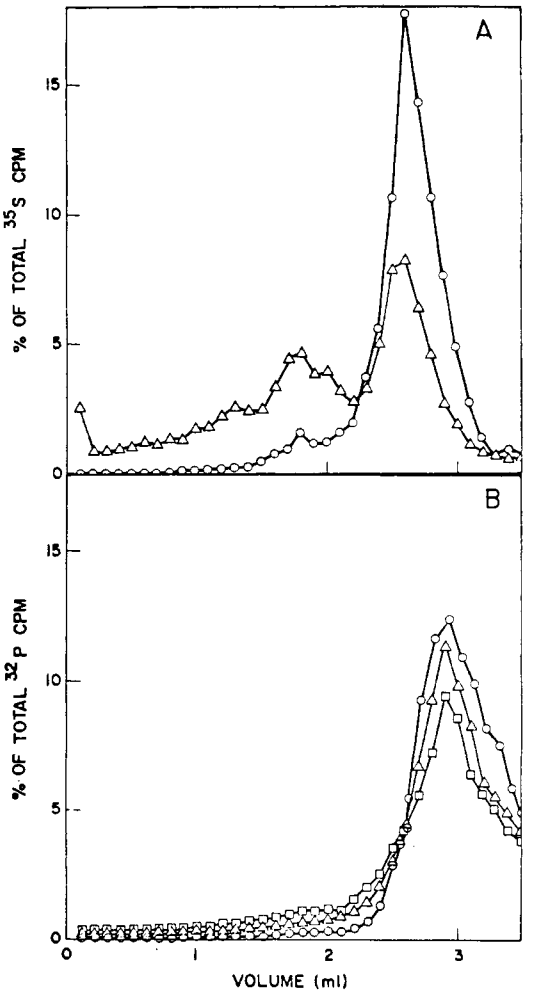

FIGURE 8: Sedimentation of 30S subunits cross-linked with bikethoxal. ^{35}S-Labeled or ^{32}P-labeled 30S ribosomal subunits were sedimented on 5–20% sucrose gradients as described under Experimental Procedures. Cross-linking with bikethoxal was 3.5 mg/mL at 37 °C. (A) ^{35}S-Labeled 30S subunits (0.5 A_{260} unit/mL) incubated 2 h at pH 8.0 and then 2 h at pH 7.0 with (△) or without (○) bikethoxal (3.5 mg/mL). (B) ^{32}P-Labeled 30S subunits incubated 0.5 h at pH 8.0 and then 1 h at pH 7.0 with 3.5 mg/mL bikethoxal [(□) 0.1 A_{260} unit/mL 30S or (△) 0.2 A_{260} unit/mL 30S] or without bikethoxal [(○) 0.1 A_{260} unit/mL 30S, control].

Discussion

Cross-linking of RNA to protein in the 5S RNA–L18 complex occurs when the ribonucleoprotein complexes are treated with bikethoxal. This is demonstrated by the appearance of ^{32}P-labeled T_1 oligonucleotides (derived from the 5S RNA), comigrating with L18 during Sephadex G-50 chromatography under denaturing conditions, following incubation with the bifunctional reagent. This technique provides a rapid positive screening procedure for putative RNA–protein reagents. Further, since the 5S RNA sequences protected from nuclease digestion (Douthwaite et al., 1979) or from modification by kethoxal (Garrett & Noller, 1979) by L18 are known, it is a fertile system for the comparison of these data with those from RNA–protein cross-linking studies.

Cross-linking of 30S protein to 16S RNA or of 50S protein to 23S RNA within intact 30S or 50S ribosomal subunits from *E. coli* occurs when subunits are treated with bikethoxal. This is demonstrated by the appearance of ^{35}S-labeled protein comigrating with RNA on SDS–agarose gels after incubation with the reagent. The amount of protein found in the cross-linked band increases with increasing time and temperature

in the presence of the reagent. Thus, it is unlikely that the protein comigrating with RNA after treatment with bikethoxal is a result of the reagent interfering with the dissociation of the subunit by SDS. Further, dissociation of the subunit by Gdn·HCl and separation of RNA from protein by chromatography on Sepharose 4B in the presence of urea and high salt gave the same results. That is, following incubation with bikethoxal ^{35}S-labeled protein cochromatographed with RNA.

The amount of protein cross-linked to RNA also increased with increasing bikethoxal concentration. However, the slope of the curve describing the dependence of the percent of protein cross-linked upon reagent concentration decreases at higher reagent concentrations. Also, an analysis of the time dependences of the initial incubation showed that after 1 h at 37 °C there was no significant increase in the amount of protein cross-linked. These results are in contrast to those obtained with the cross-linking reagents bis(2-chloroethyl)amine (Ulmer et al., 1978) or butadiene dioxide (Bäumert et al., 1978). With these RNA–protein cross-linkers the reaction tended to accelerate with increasing reagent concentration or incubation time, respectively. This suggests a cooperative unfolding of the ribosome. The results described here are similar to those obtained with (*p*-azidophenyl)acetic imido ester (Rinke et al., 1980) and with the time dependence of the reaction with (4-azidophenyl)glyoxal (Politz et al., 1981).

The proportion of protein cross-linked to RNA in 30S subunits was twice that found cross-linked to RNA in 50S subunits. This result was obtained by Rinke et al. (1980) with (*p*-azidophenyl)acetic imido ester at the same concentration. Since the length of the bridge formed between RNA and protein by both reagents is small (8–9 Å), the proportion of protein found cross-linked to RNA is unlikely to be an artifact introduced by linking protein which is actually quite distant from RNA in the ribosomal subunit. This is supported by the finding that cross-linking with a shorter version of the (*p*-azidophenyl)acetic imido ester does not lead to a more selective cross-linking reaction, but merely to a lower yield of the same products (Rinke et al., 1980).

SDS–agarose gel electrophoresis has potential to serve as a rapid method for searching for intersubunit cross-links and did provide a method for demonstrating intrasubunit cross-linking and for optimizing the reaction conditions. For example, increasing the reaction temperature to 37 °C or an incubation at pH 7.0 increased the extent of RNA–protein cross-linking. [Although the addition of 20 mM borate at pH 7.0 was not required to stabilize the cross-link under electrophoretic conditions, its presence would be expected to favor the reversible adduct of the reagent with arginine (Glass & Pelzig, 1978). Further, by analogy with other dicarbonyl reagents it is expected that manipulation of the pH and borate concentration in the reaction buffer would allow optimization of RNA–protein, protein–protein, or RNA–RNA functions of the reagent (Takahashi, 1968; Litt, 1969; Cheung & Fonda, 1979).] The components of "activation buffers" for ribosomal subunits (Zamir et al., 1971) did not interfere with the cross-linking reaction. However, ^{35}S-labeled bands appeared between RNA and protein on the gel with increasing time with ammonium acetate in the reaction buffer. This suggests that protein–protein cross-linking was favored by high ammonium ion concentrations.

Topographical artifacts introduced by cross-linking a large proportion of ribosomal protein to RNA cannot be ruled out, as with any chemical-modifying reagent. Nevertheless, in this context it should be noted that no detectable effect could be observed on the conformation of 30S subunits as a result of cross-linking with bikethoxal, as judged by sedimentation on sucrose gradients. However, small peaks of radioactivity sedimenting more rapidly than 30S subunits were observed on gradients with cross-linked subunits. It is likely that these effects were due to intersubunit cross-linking of 30S–30S dimers or multimers present at the 10 mM Mg^{2+} concentration in the reaction buffer. "Intersubunit cross-linking" was minimized by decreasing the subunit concentration.

In conclusion, these studies indicate that bikethoxal is a promising bifunctional reagent for RNA–protein cross-linking in the *E. coli* ribosome. Since it is capable of covalently, but reversibly, joining protein to RNA in both 30S and 50S ribosomal subunits without requiring the use of organic solvents, it may prove a good general reagent. Like other dicarbonyl reagents, bikethoxal is relatively specific. It should react with arginyl (and possibly lysyl) residues in protein (Takahashi, 1968; Glass & Pelzig, 1978) and with guanylic acid residues present in single-stranded regions of RNA (Litt & Hancock, 1967; Litt, 1969; Noller, 1974). Such specificity would be expected to minimize the effect of the reagent on ribosome conformation. Further, since the sites of kethoxal modification of *E. coli* ribosomal RNAs are known (Noller, 1974; Chapman & Noller, 1977; Hogan & Noller, 1978; Herr & Noller, 1978), the task of identifying the components of individual ribonucleoprotein complexes should be considerably simplified. Also, a stable but reversible bifunctional reagent provides the opportunity to identify the precise point of cross-linkage between RNA and protein, as well as to characterize the involved macromolecules rapidly and precisely. Furthermore, since bikethoxal is also theoretically capable of modifying guanylic acid residues in single-stranded regions of DNA, it would be possible to cross-link any combination of single-stranded DNA, single-stranded RNA, or protein. As a consequence, bikethoxal may also be a useful reagent for studying the tertiary structure of large RNA molecules, of small nuclear ribonucleoproteins, of the DNA replication fork, of chromatin, or of the portions of mononucleosomes proximal to the cleaved ends of the DNA helix.

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Registry No. Bikethoxal, 84031-85-6; selenium dioxide, 7446-08-4; 1,2-ethanediol, 107-21-1; (*E*)-2-butenal, 123-73-9; guanine, 73-40-5; arginine, 74-79-3.

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