long enough to attach ACh to tissue proteins. For these reasons we used a perfusion mixture of allyl alcohol and glutaraldehyde at *p*H 11, which allowed desacetylization of ACh and, simultaneously, transesterification. A bifunctional agent, glutaraldehyde allowed the newly formed hapten to be attached to tissue proteins.

Ten male rats (250 to 300 g) were perfused transcardially with glutaraldehyde (0.5*M*) and allyl alcohol (0.5*M*) in 0.1*M* cacodylate buffer (*p*H 11). The brains were removed, sectioned, and postfixed in the perfusion mixture without allyl alcohol. Sections (50 μm) were made with a Vibratome through the region of the medial septum. The sections were incubated at 4°C overnight with ACh antibody (1:1500), serum from unimmunized animals, or ACh antibody preadsorbed on choline–glutaryl–poly-L-lysine at the same dilution and were then processed for peroxidase-antiperoxidase immunocytochemistry. Figure 3 shows ACh-immunoreactive cells and fibers in the region of the medial septum. No positive staining was observed with the preadsorbed or pre-immune serum. Hence the perfusion mixture fulfilled our requirements in that (i) ACh was retained in the CNS tissue, (ii) the areas in which ACh was detected correspond to areas in which cholinergic neurons have been identified by other techniques (*1*, *2*), and (iii) as shown in Fig. 3 the ACh-immunoreactive cells maintained the morphological integrity of cholinergic neurons.

As a specific marker for cholinergic neurons in the CNS, the ACh antibody has several advantages over available immunocytochemical markers. Although antibodies to choline acetyltransferase are considered to be reliable markers for cholinergic neurons (*2*), preparation of the enzyme involves numerous purifications and specificity tests. Furthermore, application of the antiserum is restricted to species closely related to the one from which the enzyme was obtained. Such laborious procedures and restrictions are not encountered when raising ACh antibodies, since the antiserum is acquired after immunization with conjugates obtained through chemical reactions. We have successfully used the antibody to visualize cholinergic neurons in invertebrates (*10*).

The high specificity of the ACh antibody may enable the processes of cholinergic neurons to be visualized as well as the cell bodies. In a preliminary investigation fibers in the substantia nigra and striatum were stained with the antibody.

Recently we presented data (*11*) showing that the perfusion mixture can be used for the fixation of norepinephrine, dopamine, and serotonin in the rat CNS. This advantage of our procedure may enable interactions of the cholinergic system to be elucidated at both the light and electron microscopic levels with respect to other neurotransmitters. Unraveling these anatomic and functional relations may improve our understanding of neurological disorders such as Alzheimer's disease, parkinsonism, and Huntington's chorea, in which the role of ACh and other neurotransmitters is receiving more and more attention.

References and Notes

1. F. Eckenstein and M. V. Sofroniew, *J. Neurosci.* **3**, 2286 (1983); A. I. Levey *et al.*, *Neuroscience* **9**, 9 (1983).
2. H. Kimura *et al.*, *Science* **208**, 1057 (1980); *J. Comp. Neurol.* **200**, 151 (1981); H. C. Fibiger, *Brain Res. Rev.* **4**, 327 (1982); M. M. Mesulam, *Neuroscience* **10**, 1183 (1983); K. Satoh, D. M. Armstrong, H. C. Fibiger, *Brain Res. Bull.* **11**, 693 (1983); C. R. Houser *et al.*, *Brain Res.* **266**, 97 (1983); B. H. Wainer *et al.*, *Neurochem. Int.* **6**, 163 (1984).
3. K. Landsteiner, *The Specificity of Serological Reactions* (Harvard Univ. Press, Cambridge, Mass., 1945); J. W. Goodman, in *The Antigens*, M. Sela, Ed. (Academic Press, New York, 1975), pp. 127–187.
4. S. Spector *et al.*, *J. Neurochem.* **30**, 685 (1978); K. Kawashima, M. Ishikawa, Mochizuki, *J. Pharmacol. Methods* **3**, 115 (1980).
5. L. J. Grota and G. M. Brown, *Endocrinology* **93**, 615 (1976); H. W. M. Steinbusch, A. A. J. Verhostad, H. W. F. Joosten, *Neuroscience* **3**, 811 (1978); M. Geffard *et al.*, *Brain Res.* **294**, 161 (1984); M. Geffard, P. Seguela, A. M. Heinrich-Rock, *Mol. Immunol.* **21**, 515 (1984); M. Geffard, P. Seguela, R. M. Buijs, *Neurosci. Lett.* **50**, 217 (1984); M. Geffard, J. Dulluc, A. M. Heinrich-Rock, *J. Neurochem.* **46**, 1221 (1985); P. Seguela *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1383 (1984).
6. The compound choline-glutaryl-polypeptide (ACh conjugate) was prepared as follows. First, 100 mg of bovine serum albumin (BSA), human serum albumin (HSA), or poly-L-lysine (PL) was dissolved in an alkaline solution (*p*H 9) and mixed with 100 mg of glutaric anhydride (GA) (Sigma). After dialysis against distilled water, the glutarylated BSA (GA-BSA), glutarylated HSA (GA-HSA), or glutarylated PL (GA-PL) was lyophilized. Then 30 mg of the lyophilized material was activated by ethyl chloroformate in anhydrous dimethylformamide containing 40 μl of triethylamine (Merck) for 5 minutes. Next, 1 ml of an aqueous solution containing 20 mg of choline hydrochloride (Fluka) 1 μl of [^{3}H]choline (specific activity, 80 Ci/mmol; New England Nuclear), and 40 μl of triethylamine was added to the activated GA-BSA, GA-HSA, or GA-PL. A 100-μl aliquot was counted for radioactivity, and, after dialysis against distilled water and centrifugation, another 100-μl aliquot was counted. The concentration of choline-GA was calculated from the radioactivity values and the volumes of the reaction medium before and after dialysis. The concentration of GA-BSA or of GA-HSA was determined for 1 ml of lyophilized immunogen after spectrophotometric analysis at 280 nm by taking into account the molar extinction coefficient of each protein after glutarylation. Molar coupling was calculated by dividing the concentration of choline-GA by that of the protein carrier. It was about 60.
7. J. L. Vaitukaitis *et al.*, *J. Clin. Endocrinol.* **33**, 988 (1971).
8. E. Engvall and P. Perlmann, *J. Immunol.* **109**, 129 (1972); J. E. Butler *et al.*, *Immunocytochemistry* **15**, 131 (1978).
9. Polystyrene well plates (Nunc) were coated with GA-BSA solution or choline-GA-BSA solution in 0.05*M* carbonate buffer (*p*H 9.6) at 4°C. After 16 hours they were filled with phosphate-buffered saline (PBS) containing 0.05 percent Tween 100 plus 1 percent bovine serum and incubated for 30 minutes at 37°C. The specific colored reaction measured from choline-GA-BSA enabled displacement curves to be established by competition. The antibody (1:5000) with or without the compounds was applied for 90 minutes at 37°C. Then the well plates were incubated for 45 minutes with goat antiserum to horseradish peroxidase–labeled rabbit antibody (1:2500) plus 1 percent bovine serum. Plates were washed with PBS and Tween 100 between each step. Peroxidase was assayed by incubation (10 minutes) with *o*-phenylenediamine dihydrochloride (10 mg/ml) in 0.1*M* citrate phosphate buffer (*p*H 5) containing 0.03 percent hydrogen peroxide. The reaction was stopped by adding 50 μl of 4*M* H_2SO_4 per well and the absorbance was read at 492 nm on a Multiskan Titertek. The blank values were subtracted from the experimental ones.
10. M. Geffard *et al.*, in preparation.
11. M. Geffard, *Soc. Neurosci. (Abstr.)* **130**, 17 (1984).

27 November 1984; accepted 11 March 1985

Ribonuclease P Catalysis Differs from Ribosomal RNA Self-Splicing

Abstract. *Two RNA-catalyzed reactions have been described, the* Tetrahymena *self-splicing ribosomal RNA and ribonuclease P. The* Tetrahymena *self-splicing reaction proceeds through a transesterification cascade that is dependent upon nucleophilic attacks by ribose 3′-OH groups. Periodate oxidation of the catalytic (or substrate) RNA, which destroys the nucleophilicity of RNA 3′ termini, did not inhibit ribonuclease P activity. Thus, catalysis by ribonuclease P differs from the self-splicing reaction.*

TERRY L. MARSH
NORMAN R. PACE*
Department of Biology and
Institute for Molecular and Cellular Biology, Indiana University, Bloomington 47405

*To whom correspondence should be addressed.

Novel biopolymer-catalyzed reactions involving the manipulation of nucleic acid phosphodiester bonds by catalytic RNA's have been reported. The first example, discovered by Cech and coworkers, is the "self-splicing" reaction of an intron-containing precursor of 26*S* ribosomal RNA (rRNA) from *Tetrahymena thermophila* (*1*). The 413-nucleotide intervening sequence is precisely excised from the mature rRNA through the action of the RNA itself, when guanosine is used as a cosubstrate. There is no requirement for a protein in this reaction.

The second example of an RNA-cata-

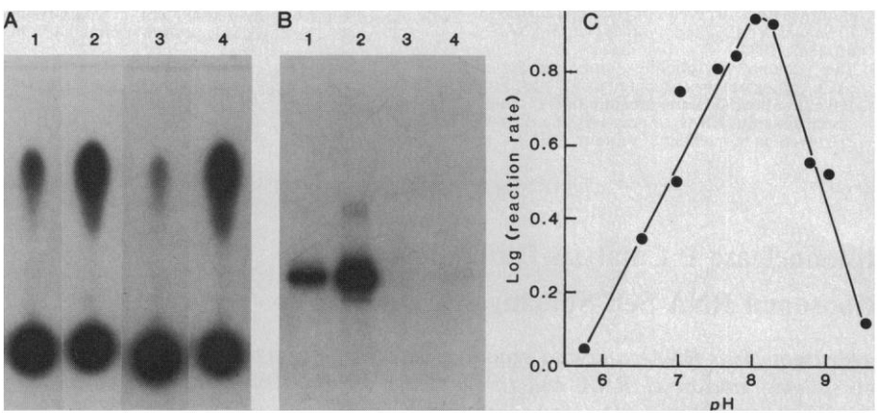
lyzed reaction is the processing of transfer RNA (tRNA) precursors by ribonuclease P (RNase P), which cleaves precursor-specific segments from the tRNA precursors to generate the mature 5′ terminus (*2*). The RNases P of *Escherichia coli* and *Bacillus subtilis* consist of single elements of protein (molecular weight of approximately 17,000) and RNA (approximately 400 nucleotides in length). Under physiological conditions, both the protein and RNA subunits are required for enzymatic action. However, in the presence of high monovalent and divalent cation concentrations with no cosubstrates, the RNase P RNA alone accurately cleaves precursor tRNA to generate a mature 5′ terminus (*2, 3*).

The *Tetrahymena* ribosomal RNA (rRNA) self-splicing reaction and the RNase P reaction both involve phosphodiester bond scissions. Intron excision in the self-splicing reaction is proposed to occur in two steps (*1*). First, the 3′-OH of the guanosine cosubstrate would initiate nucleophilic attack on the 5′ side of the intron sequence, which breaks the rRNA precursor chain and adds the guanosine cosubstrate to the intron by a 5′,3′-phosphodiester linkage. The 3′ intron-exon junction may then undergo nucleophilic attack by the newly formed 3′ end of the mature rRNA sequence. The result would be the release of the intron and joining of the mature rRNA sequences. The 3′ end of the excised intron subsequently attacks near the 5′ end of the intron, releasing a short oligomer and forming a circular RNA. Nucleophilic attack by a ribose 3′-OH occurs in each of these chain-breaking reactions: (i) by the guanosine cosubstrate, (ii) by the newly formed 3′ end of the rRNA resulting from scission of the 5′ intron boundary, and (iii) by the 3′ end of the excised intron during cyclization. Consistent with such a mechanism is the observation that modification of either the 2′- or the 3′-OH of the guanosine cosubstrate abolishes the reaction.

We have compared the RNase P RNA cleavage of tRNA precursors to the rRNA self-splicing reactions as to the requirement for ribose 3′-OH. The RNase P reaction does not require a ribose-containing cosubstrate and, if analogous to self-splicing, would involve attack of the precursor tRNA by the 3′ end of the RNase P RNA. This would release the precursor segment and result in the formation of a transient conjugate between the RNase P RNA and the tRNA through a 5′, 3′-phosphodiester linkage. This intermediate conjugate would then dissociate to yield mature tRNA and regenerated RNase P RNA.

Treatment with periodate oxidizes the 3′-terminal hydroxyl groups of RNA. The resulting dialdehyde is inactive as a nucleophile and cannot participate in phosphodiester bond formation, as would be required if RNase P RNA were to act in a manner analogous to the *Tetrahymena* self-splicing reaction. We treated the *B. subtilis* RNase P RNA with periodate and tested the modified RNA for activity under ionic conditions optimal for catalysis by RNA alone (Fig. 1A). There was no significant difference in activity between the native and oxidized forms at two concentrations of RNase P RNA. At an RNA concentration of 12 ng/ml, the activity of the oxidized form was 80 percent of that observed with the native form, reflecting different recoveries during the steps subsequent to oxidation.

In order to examine the efficiency of oxidation, we tested the ability of the periodate-treated RNase P RNA to act as a substrate in an RNA ligase condensation with [5′-^{32}P]cytidine-3′,5′-bisphosphate (pCp). Successful oxidation would abolish addition of pCp to the RNA 3′-terminus as a phosphodiester bond cannot be formed. The oxidized



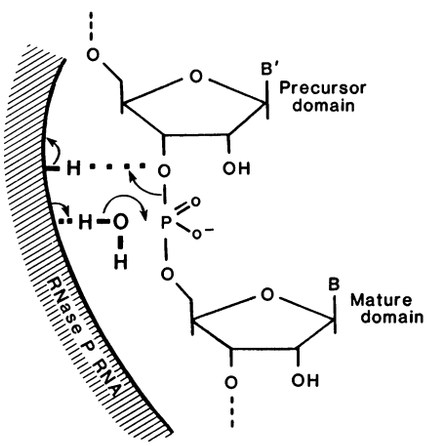


Fig. 1 (left). RNase P assays and RNA ligase substrate assays of native and periodate-oxidized RNase P RNA, and *p*H dependence of RNase P. The isolation of RNase P RNA from *B. subtilis* and the construction of the semisynthetic substrate were as described (*2*). Periodate oxidation was carried out as described (*7*). (A) RNase P activity of native and oxidized RNA. The assay was performed as described (*2*), with the following modifications. RNA was tested for activity in 50 m*M* tris-HCl, *p*H 8.0, 1.2*M* NH_4Cl, 0.25*M* $MgCl_2$. The *p*H optimum was determined in 50 m*M* tris-HCl or 50 m*M* Hepes, 0.6*M* NH_4Cl, 0.15*M* $MgCl_2$, 0.05 percent NP40. The reaction was stopped by a tenfold dilution with cold 3 m*M* EDTA, and a 10-μl sample was then spotted onto a thin-layer polyethyleneimine chromatography plate (Brinkmann) and developed with 1.0*M* formic acid that had been adjusted to *p*H 4.3 with pyridine. The RNase P reaction is linear with time and amount of RNase P RNA added (*2*). RNase P activities of native (lanes 1 and 2) and oxidized (lanes 3 and 4) RNA were determined with 12 ng (lanes 1 and 3) and 60 ng (lanes 2 and 4) of RNase P RNA per reaction. Incubation was for 10 minutes at 37°C. (B) The capacity of RNase P RNA to act as an acceptor in an RNA ligase reaction was tested as follows. Approximately 250 ng of RNase P RNA (native or oxidized) was incubated at 4°C with 0.2 mCi [5′-^{32}P]cytidine bisphosphate and 3.0 U of RNA ligase (NEN) for 16 hours. The reaction volume was 19 μl and ionic conditions were as described (*7*). The acceptor capacity and integrity of RNase P RNA were determined by polyacrylamide gel electrophoresis (6 percent acrylamide, 7*M* urea, 50 m*M* tris, 50 m*M* boric acid, 1.0 m*M* EDTA), autoradiography, and silver staining (*8*). RNase P RNA ligase acceptor activity was tested with 12 ng (lanes 1 and 3) and 60 ng (lanes 2 and 4) of native (lanes 1 and 2) and oxidized (lanes 3 and 4) RNase P RNA per reaction. (C) The *p*H dependence of the RNA reaction was determined with 10 ng of RNase P RNA and 1 to 2 ng of substrate. Incubation was at 37°C and samples were removed at 2, 4, 8, and 15 minutes. The amounts of substrate and product were determined by cutting out the appropriate spots (identified by autoradiography) from the PEI plates and scintillation counting. The logarithm of the linear reaction rate between 8 and 15 minutes is plotted as a function of *p*H. Hepes buffer was used from *p*H 5.0 to 7.0 and tris buffer from *p*H 7.0 to 9.5. Fig. 2 (right). A scheme for hydrolysis of tRNA precursors by RNase P RNA. The hatched boundary indicates the RNase P–RNA surface. B and B′ are bases. The phosphodiester bond connecting the precursor and mature domains of the precursor tRNA substrate is depicted.

RNase P RNA did not serve as an RNA ligase substrate. The oxidized RNA bonds contained less than 2 percent of the [^{32}P]pCp incorporated into the unmodified RNA, as determined by scintillation counting of gel slices and remained intact, as judged by silver staining (*4, 5*). Incubation of oxidized RNase P RNA in the high salt buffer of the RNase P assay had no effect on its later ability to serve as an RNA ligase substrate, demonstrating that the oxidized terminus is stable under the unusual ionic conditions of the assay. Furthermore, periodate-oxidized precursor tRNA could serve equally well as substrate for either native or oxidized RNase P RNA (*5*). Thus, the 3′ termini of neither the RNase P RNA nor its substrate are required for accurate processing. The mechanism of the RNase P reaction is different from those that occur during intron excision from *Tetrahymena* rRNA.

Since the 3′-OH of the RNase P RNA is not the initiating nucleophile in the RNase P processing reaction, it is likely that a hydroxyl group from water serves that role. The RNase P reaction had a significant dependence on hydroxide concentration (Fig. 1C) with an optimum at *p*H 8 to 8.5. The *p*H at the half-maximum reaction rate does not correlate with any of the reported *p*K values for nucleic acid ionizable groups, consistent with dependence on hydroxide concentration. Above an approximate *p*H of 9, all activity was lost, probably because of denaturation of the catalyst or substrate RNA's due to deprotonation of U-N3 (at *p*H 9 to 9.5).

Reactions involving hydroxide attack on phosphate esters are commonly catalyzed by proteins. The amino acid side chains may provide proton exchange mechanics to activate a water molecule positioned to attack the esterified phosphorus (Fig. 2). We envisage that the precursor tRNA substrate binds to the surface of the RNase P RNA in a manner analogous to the binding of a substrate to a protein surface, and that nucleic acid groups manipulate the reactants. A hydrated Mg^{+2}-coordinate complex offers attractive geometry for the reaction (*6*), but the actual catalysis must derive from the RNase P RNA. The proton donor-acceptor roles might be provided by nucleoside base tautomeric transitions, nonterminal 2-OH groups, or, conceivably, internucleotide phosphates with high *p*K values. The delineation of the active site, the identification of chemically important groups, and the roles of high concentrations of mono- and divalent cations required for the RNA reaction remain to be investigated.

References and Notes

1. K. Kruger, P. J. Grabowski, A. J. Zaug, J. Sands, D. E. Gottschling, T. R. Cech, *Cell* **31**, 147 (1982).
2. C. Guerrier-Takada, K. Gardiner, T. Marsh, N. Pace, S. Altman, *ibid.* **35**, 849 (1983).
3. C. Guerrier-Takada and S. Altman, *Science* **223**, 285 (1984).
4. B. R. Oakley, D. R. Kirsch, N. R. Morris, *Anal. Biochem.* **105**, 361 (1980).
5. T. L. Marsh and N. R. Pace, unpublished data.
6. K. Haydock and L. C. Allen, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
7. S. H. Leppla, B. Bjoraker, R. M. Bock, *Methods Enzymol.* **12**, 236 (1968). Briefly, approximately 1.0 to 1.5 μg of RNase P RNA in H_2O was mixed with a 100-fold molar excess of sodium metaperiodate. A subcatalytic amount of 3′-labeled RNase P RNA was added to all reactions, or to control reactions run in parallel, as a means of assessing recovery. The periodate was added under darkroom safety lights and the reaction mixture was incubated for 1 hour at room temperature in the dark. Then the 15-μl reaction mixture was diluted 1:10 with H_2O, sodium acetate (*p*H 5.4) was added to a final concentration of 0.2*M*, and the RNA was precipitated with three volumes of ethanol. The RNase P RNA was centrifuged (Eppendorf), dried briefly under vacuum, and resuspended in 15 μl of H_2O. The resuspended RNA was divided into two portions; one was tested for RNase P processing activity and the other for effectiveness as a substrate in an RNA ligase reaction.
8. D. A. Stahl *et al.*, *Nucleic Acids Res.* **9**, 6129 (1981).

9. We thank B. Pace and C. Reich for assistance. Supported by NIH research grant GM34527 (N.P.R.).

6 December 1984; accepted 29 March 1985

Preparation of Bispecific Antibodies by Chemical Recombination of Monoclonal Immunoglobulin G_1 Fragments

Abstract. *Preparation of bispecific antibodies by the chemical reassociation of monovalent fragments derived from monoclonal mouse immunoglobulin G_1 is inefficient because of side reactions during reoxidation of the multiple disulfide bonds linking the heavy chains. These side reactions can be avoided by using specific dithiol complexing agents such as arsenite and effecting disulfide formation with a thiol activating agent such as 5,5′-dithiobis(2-nitrobenzoic acid). In this way bispecific antibodies were obtained in high yield and free of monospecific contaminants from monoclonal mouse immunoglobulin G_1 fragments. The bispecific antibodies were used as agents for the selective immobilization of enzymes.*

MAUREEN BRENNAN
Department of Fine Structure, Boston Biomedical Research Institute, Boston, Massachusetts 02114
PETER F. DAVISON
Department of Fine Structure, Boston Biomedical Research Institute, and *Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts 02115*
HENRY PAULUS
Department of Metabolic Regulation, Boston Biomedical Research Institute, and *Department of Biological Chemistry, Harvard Medical School*

Bispecific antibodies, hybrid immunoglobulins with two different antigen-binding sites, have been prepared from polyclonal rabbit immunoglobulins (*1-3*). They have many potential uses, ranging from immunodiagnostic procedures to targeted delivery of drugs. Nevertheless, the applications of bispecific antibodies would be considerably enhanced if they could be derived from monoclonal antibodies. The preparation of bispecific monoclonal antibodies by fusion of antibody-producing cells was recently described by Milstein and Cuello (*4*). Although their procedure produces a mixture of hybrid antibodies with various assortments of chains, which must be fractionated to yield the desired bispecific molecules, they suggested that it is superior to a chemical reconstitution approach on account of certain technical problems (*4*). These problems include the need to dissociate immunoglobulins into half-molecules without damaging the antigen-binding sites and to reform three disulfide bonds linking the heavy chains, characteristic of mouse immunoglobulins, without allowing interfering side reactions, such as formation of disulfide bonds within chains. Nevertheless, it has been possible to obtain hybrid monoclonal antibodies in low yields (*5*). We report here a chemical procedure for preparing bispecific antibody fragments from monoclonal mouse immunoglobulin G_1 (IgG_1), a procedure that avoids these problems and generates the desired bispecific reagent in high yield as the only product, obviating the need for further purification.

To dissociate reduced immunoglobulin half-molecules under mild conditions, we used the method of Nisonoff and Mandy (*1*), who removed the Fc′ portion of rabbit IgG by limited pepsin hydrolysis to yield $F(ab')_2$. The cleavage of monoclonal mouse IgG_1 with pepsin to yield $F(ab')_2$ proceeded readily, provided that the reaction was carried out at a slightly lower *p*H than that with rabbit IgG (*6, 7*). About 80 percent of the monoclonal IgG_1's were converted to $F(ab')_2$ with a good yield (75 to 95 percent) by incubation with pepsin (2 percent by weight) in 0.1*M* sodium acetate (*p*H 4.2) for 18 hours at 37°C. On reduc-