Actions of Aurintricarboxylate, Kasugamycin, and Pactamycin on *Escherichia coli* Polysomes†

Phang-Cheng Tai, Brian J. Wallace, and Bernard D. Davis*

ABSTRACT: The effects of several ribosomal inhibitors on a system initiating on viral RNA have been compared with their effects on purified *Escherichia coli* polysomes carrying out only chain elongation. With this more complete separation of the two processes we have confirmed previous reports that aurintricarboxylate specifically inhibits initiation at 10–20 μM but impairs chain elongation at higher concentrations. Kasugamycin is known to block initiation complex formation; but though it also has been reported to block chain elongation with poly(U) and poly(A), it was not inhibitory, up to high concentrations, with purified polysomes. This system also yielded unexpected results with pactamycin: this antibiotic has been reported to inhibit initiation specifically, in a poly(U) system including initiation factors and *N*-acetylphenylalanyl-tRNA; but with our systems the concentrations required to inhibit initiation or chain elongation overlap extensively. Hence pactamycin cannot be used as a selective inhibitor of initiation with *E. coli*, but kasugamycin can.

The use of viral RNA as a natural messenger for protein synthesis *in vitro* has been of great value in studying the mechanism of action of various antibiotics (see review by Pestka, 1971). However, it would be desirable to verify the assumption that the effects observed are the same as those occurring on cellular messenger, for viral and cellular mRNA may well differ in secondary structure and punctuation. Moreover, phage-RNA directed systems with cell extracts carry out both chain initiation and elongation and hence have not been ideal for distinguishing effects on these two processes. For this reason we have developed a method, described in the preceding paper (Tai *et al.*, 1973), for preparing endogenous or viral polysomes that lack initiation factors (IF): the ribosomes in these preparations are active in chain elongation but do not reinitiate after runoff.

Here we describe the application of such IF-free polysomes, compared with an initiation-dependent system, in analyzing the action of several ribosomal inhibitors. These studies have confirmed (and have extended to endogenous polysomes) the conclusion that aurintricarboxylate (Grollman and Stewart, 1968) and kasugamycin (Okuyama *et al.*, 1971) specifically inhibit initiation, at concentrations that do not inhibit chain elongation. The comparison between the two systems employed here thus seems reliable. Hence the contrasting result obtained with pactamycin is of particular interest: though this antibiotic can apparently inhibit initiation specifically with a crude reticulocyte system (Lodish *et al.*, 1971; Stewart-Blair *et al.*, 1971), or with a bacterial system employing poly(U) under conditions requiring IF (Cohen *et al.*, 1969a), we find that with natural messenger in a bacterial system it inhibits both chain elongation and initiation, in overlapping concentration ranges.

Materials and Methods

Preparation of IF-Free Polysomes. Polysomes were prepared by gel filtration on Sepharose 4B from lysates of *E. coli* strain MRE600, and also from a protein-synthesizing mixture containing an S30 extract of *E. coli* K12 and phage R17 RNA. All the preparations used were virtually free of IF, *i.e.*, with viral RNA added the post-runoff ribosomes incorporated less than 10% as much [^{14}C]valine as when supplemented with the optimal concentration of crude IF (Tai *et al.*, 1973).

In Vitro Protein Synthesis. The activity of IF-free polysomes was measured as described in the preceding paper (Tai *et al.*, 1973). Incubation was for 3 min, during which time the incorporation of [^{14}C]valine was linear; hence inhibition should reflect interference with the *rate* of chain elongation rather than with release of polypeptide. A longer incubation time (10 min) was used in some experiments and gave the same results as the shorter times.

Incorporation of [^{14}C]valine by NH_4Cl-washed ribosomes (plus crude IF and viral RNA) was used to estimate interference with chain initiation; the incubation time was longer (30 min) than for the IF-free polysomes. IF was prepared through the stage of $(NH_4)_2SO_4$ precipitation (Iwasaki *et al.*, 1968).

Ribosomal particles were analyzed in sucrose gradients as previously described (Tai *et al.*, 1973).

Reagents. [^{14}C]Valine was obtained from New England Nuclear Corp., kasugamycin was a gift from Bristol Laboratories, and pactamycin (prepared by Upjohn Co.) was a gift from Dr. I. H. Goldberg.

Results

Inhibition of Polypeptide Synthesis by Aurintricarboxylate. Aurintricarboxylate at 10–20 μM, added at zero time, inhibited incorporation of [^{14}C]valine nearly completely with NH_4Cl-washed ribosomes plus phage R17 RNA (and IF), but these concentrations produced only 5–10% inhibition with IF-free endogenous or viral polysomes (Figure 1). At higher concentrations, however, aurintricarboxylate does inhibit chain elongation by an IF-free system (50% at 80 μM; Figure 1). It is clear that within a narrow concentration range aurintricarboxylate selectively inhibits an initiating system, while allowing already formed polysomes to continue translation of natural messenger at the normal rate.

Inhibition by Kasugamycin. Kasugamycin has been shown to strongly inhibit phage RNA-directed peptide synthesis in *E.*

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TABLE I: Effect of Kasugamycin on Amino Acid Incorporation by IF-Free Polysomes and NH_4Cl-Washed Ribosomes.[a]

Ksg concn (μM)	Per cent inhibition with		
	Endogenous polysomes	Viral polysomes	NH_4Cl-washed ribosomes
60	0	0	55
200	4	5	79
500	6	8	80
1000	8	7	85

[a] Reaction mixtures (see Methods), containing IF-free endogenous polysomes, R17 polysomes, or NH_4Cl-washed ribosomes (plus phage R17 RNA and IF), were incubated with various concentrations of kasugamycin for 10 min (polysomes) and 30 min (ribosomes). The Mg^{2+} concentration was 8 mM. Incorporation in control tubes without kasugamycin, taken as 100% activity, was 3220–5887 cpm for polysomes and 6000–9000 cpm for NH_4Cl-washed ribosomes; data shown are the averages of several separate experiments.

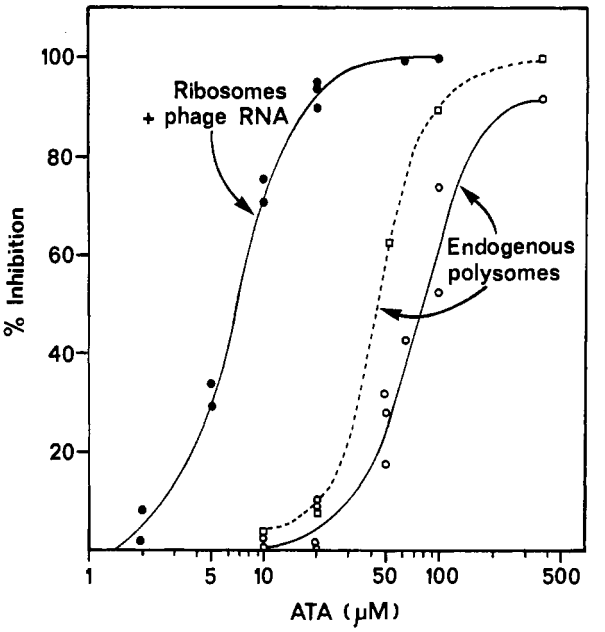

FIGURE 1: Inhibition of polypeptide synthesis by aurintricarboxylate. Reaction mixtures containing IF-free polysomes were incubated at 34° for 3 min (□) or 10 min (○) with various concentrations of aurintricarboxylate added. NH_4Cl-washed ribosomes (●) were incubated for 30 min with R17 RNA and IF. The incorporation in the control tubes without aurintricarboxylate (2123 and 5479 cpm for IF-free polysomes at 3 min and 10 min, respectively, and 8015 cpm for NH_4Cl-washed ribosomes) was taken as 100% activity.

coli (Sparling, 1970; Okuyama *et al.*, 1971). We have confirmed this finding with an S30 extract and with NH_4Cl-washed ribosomes (Figure 2 and Table I); a maximal effect was reached by 200 μM. In contrast, even at 1000 μM kasugamycin did not significantly reduce incorporation by IF-free endogenous or phage polysomes (Table I).

The effect of kasugamycin on the initiating system varies with Mg^{2+} concentration (Figure 2a): 200 μM kasugamycin produced 90% inhibition at 6 mM Mg^{2+} and only 65% at 12 mM. Because this kasugamycin concentration might no longer be saturating at elevated Mg^{2+} 600 μM kasugamycin was also tested: its effect at various Mg^{2+} concentrations was the same as that observed with 200 μM. Figure 2 further shows that the ribosome concentration had little influence and the endogenous polysomes remained unresponsive to kasugamycin over a broad range of Mg^{2+} concentration.

Inhibition by Pactamycin. As Figure 3 shows, pactamycin above 1 μM strongly inhibited peptide synthesis with IF-free endogenous and phage polysomes; at 10 μM the inhibition was almost complete. As with a reticulocyte system (Felicetti *et al.*, 1966), this inhibition is not due to an effect on amino acid activation, for the tRNA's in the S100 fraction were charged with [^{14}C]amino acids equally well in the presence or absence of the antibiotic (results not shown). The inhibition was accompanied by stabilization of polysomes: during 10 min of incubation in a reaction mixture the polysomes decreased by 85% in the absence of pactamycin but only by 30% when 30 μM pactamycin was present (Figure 4). Variation in Mg^{2+} concentration had little effect on the partial inhibition exerted by 3 μM pactamycin (Figure 5).

Figure 3 also shows that an initiating system (programmed with phage RNA) was only moderately more sensitive to pactamycin: the curve for inhibition *vs.* concentration overlapped substantially with that obtained with IF-free polysomes, 50% inhibition being produced by 1 and 3 μM, respectively. Pactamycin therefore cannot be considered a selective inhibitor of initiation with bacterial ribosomes.

Because of the unexpected sensitivity of chain elongation on IF-free polysomes to pactamycin it seemed advisable to test whether pactamycin fails to block the puromycin reaction with this system as with certain other systems (Cundliffe and McQuillen, 1967; Cohen *et al.*, 1969a). In the presence of 30 μM pactamycin, just as in its absence, the addition of puromycin released more than 80% of the nascent peptide (which sedimented above the ribosomes in a sucrose gradient; results not shown); it also released the ribosomes from the polysomes (Figure 4c). In contrast, sparsomycin, a known inhibitor of peptidyl transfer, prevented ribosome release by puromycin (Figure 4d).

Discussion

To shed light on the actions of aurintricarboxylate, kasugamycin, and pactamycin, we have compared their effects on two systems: IF-free polysomes, which can carry out peptide chain elongation but not initiation, and an initiating system programmed by phage R17 RNA, which carries out both processes. The results have confirmed earlier conclusions concerning aurintricarboxylate and kasugamycin, derived from systems that did not separate initiation and elongation as sharply. Pactamycin, however, yielded unexpected results.

(*i*) *Aurintricarboxylate.* It has previously been shown that aurintricarboxylate, at 10 μM, completely inhibits peptide synthesis in an initiating system and prevents attachment of viral messenger to ribosomes but has less effect on peptide synthesis when added after initiation. Moreover, at this concentration it only slightly inhibited endogenous incorporation by an S30 extract, though at 30 μM inhibition was 50% (Grollman and Stewart, 1968; Stewart *et al.*, 1971). However, S30 extracts are not entirely satisfactory for assessing inhibition of chain elongation, for the activity is low and may depend to an unknown extent on nonspecific incorporation and reinitiation. The present studies with IF-free polysomes show unequivocally that in a narrow concentration range (10–20 μM) aurin-

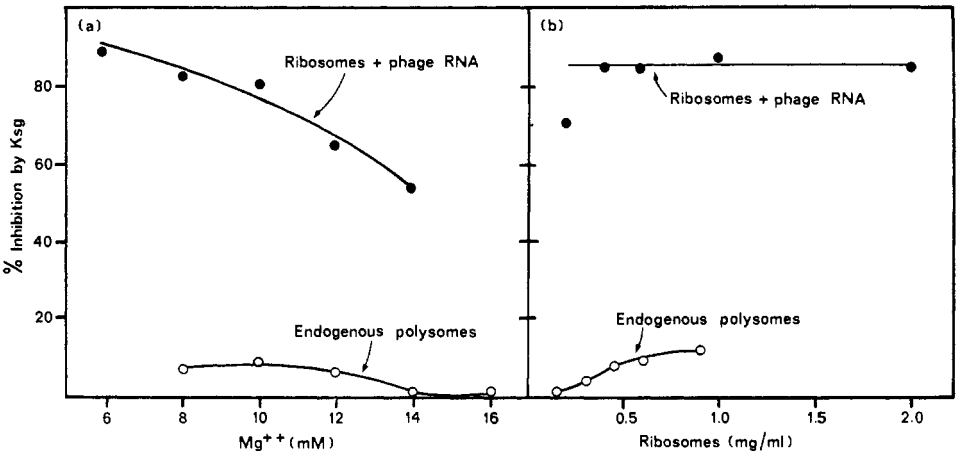

FIGURE 2: Effects of Mg^{2+} and ribosome concentration on the inhibition of peptide synthesis by kasugamycin. Reaction mixtures, containing either IF-free endogenous polysomes (○) or preincubated S30 extract ("ribosomes") with viral RNA added (●), were incubated with 200 μM kasugamycin at 34° for 10 and 30 min, respectively. In Figure 2a the ribosome concentration was 0.5 mg/ml; in Figure 2b the Mg^{2+} concentration was 8 mM.

tricarboxylate specifically inhibits initiation (Figure 1), as originally proposed. Moderately higher concentrations also inhibit chain elongation on endogenous messenger, which is consistent with evidence that aurintricarboxylate can inhibit various reactions in peptide chain elongation (Miller and Weissbach, 1970; Siegelman and Apirion, 1971a,b).

(ii) Kasugamycin. We have found that kasugamycin inhibits an initiation-dependent system, but even at concentrations far above those required for maximal effect in this system it does not affect the amount or rate of chain elongation on IF-free polysomes (Table I). Kasugamycin thus acts selectively over a broad concentration range (200–1000 μM); hence it may be more reliable than aurintricarboxylate in experiments where such selectivity is required, *e.g.*, in helping to define other antibiotic actions. However, it should be noted that inhibition of *E. coli* ribosomes by saturating concentrations of kasugamycin is not quite complete, even at low Mg^{2+} concentrations, and the extent of inhibition decreases with increasing Mg^{2+} (Figure 2). The selective action of kasugamycin in an initiating system may be accounted for by its observed ability to block formation of a 30S initiation complex (Okuyama *et al.*, 1971).

In contrast to the results obtained with purified polysomes, kasugamycin has been found to inhibit incorporation of preformed complexes with poly(U) or poly(A) (Tanaka *et al.*, 1966). This greater sensitivity of the poly(U) system is un-

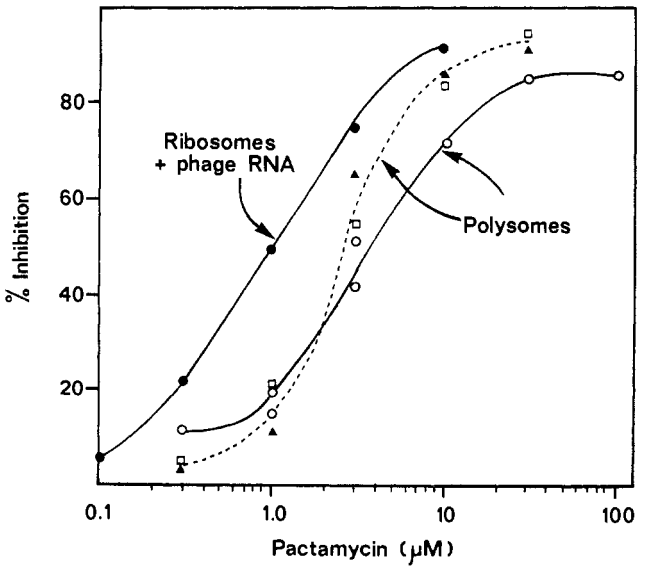

FIGURE 3: Inhibition of polypeptide synthesis by pactamycin. Incorporation in the absence of pactamycin, taken as 100%, was 1663 or 3499 cpm for IF-free endogenous polysomes incubated at 34° for 3 min (□) or 10 min (○), respectively; 2655 cpm for IF-free R17 polysomes incubated for 10 min (▲), and 6894 cpm for NH_4Cl-washed ribosomes with R17 RNA and crude IF incubated for 30 min (●).

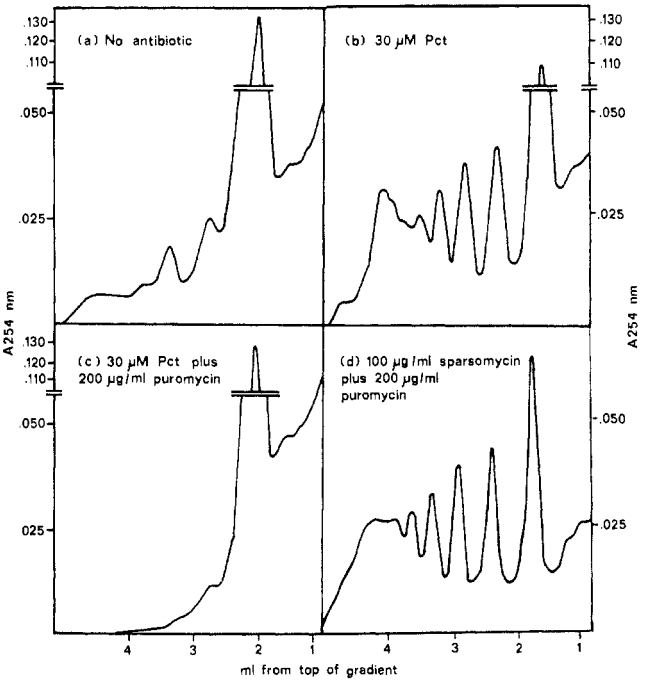

FIGURE 4: Stabilizing effect of pactamycin on *E. coli* polysomes. Reaction mixtures for protein synthesis with IF-free polysomes were as described in Methods. Antibiotics were added as indicated and the mixtures were incubated at 34° for 10 min, chilled, and layered onto 4.0 ml of 10–30% sucrose gradients with an 0.8-ml cushion of 45% sucrose. The gradients were centrifuged in an SW 50.1 rotor at 45,000 rpm for 50 min and analyzed in an Isco gradient analyzer.

usual, for with many antibiotics the inhibition with synthetic messengers is less marked than that with natural messengers (reviewed by Pestka, 1971). It is of interest that kasugamycin appears to inhibit binding of aminoacyl-tRNA in the poly(U) system (Tanaka *et al.*, 1966; Okuyama *et al.*, 1971), though as we have seen it does not block chain elongation on natural mRNA.

(*iii*) *Pactamycin.* In an IF-dependent system with *N*-acetylphenylalanyl-tRNA, phenylalanine, *E. coli* ribosomes, and poly(U), pactamycin inhibited formation of initiation complexes; however, it did not inhibit incorporation after initiation with this system, though it was noted to have some effect on an S30 extract (Cohen *et al.*, 1969a; Goldberg *et al.*, 1973). We have found that with IF-free polysomes, including viral polysomes, 10 μM pactamycin inhibits chain elongation almost completely (Figure 3). Moreover, inhibition of this system was essentially independent of Mg^{2+} concentration (Figure 5), though the inhibition of polyphenylalanine synthesis (apparently at initiation), and of *N*-acetylphenylalanyl-tRNA binding, was apparently restricted to Mg^{2+} concentrations below 6 mM (Cohen *et al.*, 1969a,b).

The mechanism of inhibition of chain elongation by pactamycin involves stabilization of the polysomes (Figure 4), as has also been recently observed by Goldberg *et al.* (1973) with *E. coli* ribosomes translating viral RNA. Moreover, 800 μM pactamycin prevented ribosomal subunit exchange in extracts of *E. coli* (Kaempfer and Meselson, 1969). This antibiotic has also been found to stabilize polysomes in reticulocyte systems when added at concentrations high enough to inhibit chain elongation (Lodish *et al.*, 1971; Stewart-Blair *et al.*, 1971). It does not inhibit the puromycin reaction and hence does not block translocation or peptidyl transfer (Figure 4; see also Cundliffe and McQuillen, 1967; Cohen *et al.*, 1969a), and in contrast to fusidic acid (Modolell and Davis, 1969) it does not block release of the ribosomes from puromycin-treated polysomes. These results suggest, by exclusion, interference with recognition, but more direct evidence is required.

An initiation-dependent viral system was only *ca.* three times more sensitive to pactamycin than were the polysomes (Figure 3). Hence, pactamycin cannot be used to inhibit initiation selectively with bacterial ribosomes and natural messenger. With extracts of rabbit reticulocytes the discrimination seems somewhat better: initiation was totally blocked by a pactamycin concentration that only moderately slowed chain elongation (Lodis *et al.*, 1971; Stewart-Blair *et al.*, 1971).

The extra sensitivity of initiating systems may be due to an effect on formation of initiating complexes, but it is also possible that pactamycin has an increased chance of affecting subsequent chain elongation when it interacts with a nonpolysomal rather than a polysomal ribosome. In this connection it may be noted that pactamycin has greater affinity for free than for complexed reticulocyte ribosomes, from reticulocytes (Macdonald and Goldberg, 1970), or from *E. coli* (Goldberg *et al.*, 1973).

In the present work IF-free viral polysomes responded to aurintricarboxylate, kasugamycin, and pactamycin just like IF-free endogenous polysomes. This result supports the assumption that viral RNA is a reliable model for cellular messenger in studying antibiotic action, despite probable differences in secondary structure and punctuation. In contrast, the poly(U) system with acetylphenylalanyl-tRNA, even though dependent on IF, does not seem to be a reliable model.

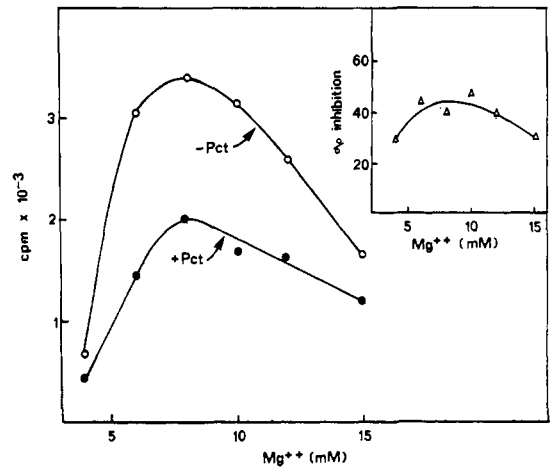

FIGURE 5: Effect of Mg^{2+} concentration on the inhibition of polypeptide synthesis by pactamycin. Reaction mixtures containing IF-free polysomes, adjusted to various Mg^{2+} concentrations, were incubated at 34° for 10 min either with (●) or without (○) 3 μM pactamycin; the per cent inhibition (△) at various Mg^{2+} concentrations is given in the inset.

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Interaction of Pyruvate with Pyruvate Carboxylase and Pyruvate Kinase as Studied by Paramagnetic Effects on ^{13}C Relaxation Rates†

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ABSTRACT: The interaction of pyruvate-*1*-^{13}C and pyruvate-*2*-^{13}C with Mn(II), Mn(II)–pyruvate carboxylase, and Mn(II)–pyruvate kinase has been examined by measurements of ^{13}C longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the enriched carbon atoms. For calibration purposes, the interaction of ionic Mn(II) with pyruvate was studied initially. At 15.18 MHz, the increase in $1/T_1$ of the carboxyl carbon of pyruvate which results from interaction with ionic Mn(II) is approximately twofold greater than that observed for the carbonyl carbon. Interaction with Mn(II) also increases $1/T_2$ of the carboxyl carbon of pyruvate but has little effect on $1/T_2$ of the carbonyl carbon. Similar data are obtained when the relaxation rates of these carbon atoms are measured at 25.14 MHz. Calculation of distances between Mn(II) and the carboxyl carbon and the carbonyl carbon and the methyl protons of pyruvate from $1/T_1$ supports a structure for the binary Mn(II)–pyruvate complex in which pyruvate is coordinated as a monodentate carboxyl ligand. The hyperfine coupling constants calculated from $1/T_2$ and $1/T_1$ are also consistent with this structure. In contrast with ionic Mn(II), the bound Mn(II) of pyruvate carboxylase causes a threefold greater increase in $1/T_1$ of the carbonyl carbon of pyruvate as compared with that of the carboxyl carbon. Similarly, interaction with the bound Mn(II) causes a greater increase in $1/T_2$ for the carbonyl carbon of pyruvate as compared with that observed for the carboxyl carbon. These effects may be attributed to interaction of pyruvate with the bound Mn(II) since only minimal effects are observed on the relaxation rates of these carbon atoms in the presence of Mg(II)–pyruvate carboxylase. In the active quaternary pyruvate kinase–Mn(II)–phosphate–pyruvate complex the bound Mn(II) exerts approximately equal effects on the relaxation rates of the carboxyl and carbonyl carbon atoms of pyruvate. Hence, these data indicate that enzyme-bound pyruvate is oriented differently with respect to the metal ion as compared with the Mn(II)–pyruvate complex. The frequency dependence observed for $1/T_1$ of the carboxyl and carbonyl carbon atoms of pyruvate in the pyruvate carboxylase– and pyruvate kinase–pyruvate complexes permits estimation of the correlation times for the Mn(II)–pyruvate dipolar interaction as $\sim 5 \times 10^{-9}$ sec. Using these correlation times, the distances between Mn(II) and the carbon atoms of pyruvate on both enzymes (7.1–8.5 Å) calculated from $1/T_{1p}$ are consistent with complexes in which pyruvate is in the second coordination sphere of the bound Mn(II). On pyruvate kinase an inner sphere phosphate anion is suggested and is supported by the effect of the bound Mn(II) on the $1/T_1$ of the phosphate phosphorus in the quaternary complex.

The interactions of substrates and substrate analogs with Mn(II)–enzyme complexes have previously been studied by measurement of the ^{19}F (Mildvan *et al.*, 1967), ^{1}H (Mildvan and Scrutton, 1967), and ^{31}P (Nowak and Mildvan, 1972) relaxation rates. Data obtained in these studies have provided both structural and kinetic information on many enzyme–metal–substrate complexes as has been summarized in recent reviews (Mildvan and Cohn, 1970; Mildvan, 1970). The structure of the pyruvate complex of the Mn(II) metalloenzyme, pyruvate carboxylase, has previously been investigated by measurements of the relaxation rates of the protons of water (Mildvan *et al.*, 1966), and the methyl protons of pyruvate (Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1970). The results were interpreted as consistent with an enzyme–metal–pyruvate bridge complex in which the carbonyl group of pyruvate was directly coordinated by the enzyme-bound cation. While monodentate coordination of the carbonyl group of pyruvate by the enzyme-bound metal would be most effective in facilitating carboxyl transfer to pyruvate, other structures involving carboxyl coordination, bidentate coordination, or second sphere complexes could not be rigorously excluded by the data (Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1970). Similarly, previous studies of the relaxation rates of analogs of phosphoenolpyruvate in ternary complexes with Mn(II)–pyruvate kinase have indicated that these substrate analogs are coordinated to the enzyme-bound Mn(II) through the phosphoryl group (Mildvan *et al.*, 1967; Nowak and Mildvan, 1972). However, the orientation of pyruvate on this enzyme has not been investigated.

The present studies with pyruvate samples, which were enriched with ^{13}C in the carbonyl and carboxyl groups, were

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