Specific Inhibition of Peptide-Chain Initiation by 2-(4-Methyl-2,6-dinitroanilino)-*N*-methylpropionamide†

Donald P. Weeks* and Robert Baxter

ABSTRACT: MDMP [2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide] is a potent inhibitor of mRNA-dependent amino acid incorporation in the *in vitro* wheat embryo amino acid incorporating system. At concentrations above 5×10^{-5} M, TMV-RNA-directed peptide synthesis is inhibited more than 95%. Amino acid incorporation by polyribosomes (obtained from germinating wheat embryos) is not affected by MDMP. Similarly, polyuridylic acid directed polyphenylalanine synthesis is not affected by MDMP. These data indicate that MDMP inhibits peptide-chain initiation and does not affect peptide chain elongation. More precise localization of the MDMP inhibition is obtained by studying the effect of the inhibitor on the initiation reactions involved with Met-tRNA binding to ribosomes. MDMP, *per se*, has no effect on the mRNA-dependent binding of Met-tRNA. It does, however, prevent the release of bound Met-tRNA by puromycin. Sedimentation analyses of the products of the Met-tRNA binding reaction show that radioactively labeled Met-tRNA is associated with 80S monoribosomes in control incubations, whereas label is found only with the 40S ribosomal initiation complex in MDMP-treated incubations. Thus, MDMP appears to prevent peptide chain initiation by specifically blocking the interaction of 60S ribosomal subunits with the 40S ribosomal subunit–mRNA–Met-tRNA complex. In parallel experiments, potassium fluoride is shown to inhibit chain initiation in a similar manner.

Much progress has been made in elucidating the scheme of protein chain initiation in eukaryotic organisms. The overall sequence of mRNA, initiator Met-tRNA and ribosomal subunit involvement has been clearly defined (Crystal *et al.*, 1971; Prichard *et al.*, 1971; Heywood and Thompson, 1971; Pragnell *et al.*, 1971; Hoerz and McCarty, 1971; Burgess and Mach, 1971; Weeks *et al.*, 1972) while the isolation and characterization of specific factors required for the constitutive reactions are well advanced (Crystal *et al.*, 1971; Heywood and Thompson, 1971; Seal *et al.*, 1972). Detailed anaylses of the individual partial reactions of initiation can be greatly facilitated by the availability of inhibitors with strict specificity, such as those which have been used to great advantage in delineating the partial reactions of procaryotic protein synthesis (Pestka, 1971). Presently, however, the number of inhibitors available which specifically interfere with known steps of eukaryotic peptide-chain initiation is quite limited [*i.e.*, aurintricarboxylic acid (Stewart *et al.*, 1971; Marcus *et al.*, 1970a), edeine (Obrig *et al.*, 1971), NaF (Hoerz and McCarty, 1971), and pactamycin (MacDonald and Goldberg, 1970; Seal and Marcus, 1972)]. We now report a new inhibitor of eukaryotic peptide-chain initiation, 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide (MDMP) (Figure 1[1]), which appears to block the coupling of the 40S ribosomal subunit mRNA–Met-tRNA initiation complex with the 60S ribosomal subunit and thus prevents formation of the 80S initiation complex.

We also confirm the observation of Hoerz and McCarthy (1971) that fluoride blocks initiation after the formation of the 40S ribosomal initiation complex. The inhibitions obtained with KF and MDMP are compared.

Materials and Methods

In Vitro Amino Acid Incorporating Systems. Conditions and requirements for the TMV-RNA-dependent and the polyribosome-dependent amino acid incorporating systems have previously been described (Weeks and Marcus, 1971). The preparation of ribosomes (Marcus *et al.*, 1968), polyribosomes (Weeks and Marcus, 1969), subunit-free S-100 (Weeks *et al.*, 1972), tRNA, tobacco mosaic virus (TMV) RNA, aminoacyl-tRNA (Marcus, 1970), and methionyl-tRNA (Seal *et al.*, 1972) have also been described.

In the experiments of Figure 2, either polyribosomes (100 μg) or TMV-RNA (10 μg) and ribosomes (5.0 A_{260} units) were added to an incubation mixture of 0.4-ml final volume containing: 0.08 ml of subunit-free S-100, 20 μg of tRNA, 45 mM KCl, 3.6 mM magnesium acetate, 1 mM ATP, 25 μM GTP, 8 mM creatine phosphate, 16 μg of creatine phosphate kinase, 25 mM Tris-acetate (pH 8.1), 2 mM dithiothreitol, and 0.125 μCi of [^{14}C]leucine. In the polyribosome assay the Mg^{2+} and K^{+} concentrations were raised to 5.0 and 70 mM, respectively (Weeks and Marcus, 1971). After incubation for 30 min at 30°, hot trichloroacetic acid precipitates were assayed for radioactivity (Marcus *et al.*, 1966). The order of addition to incubations described here was generally mRNA, MDMP, and ribosomes; variations of this sequence were found not to influence experimental results.

Assays of the effects of inhibitors on initiation complex formation (Table II) are carried out in a two-step procedure similar to that described earlier by Marcus *et al.* (1970a). Initiation complex formation occurs during a preliminary incubation. This is followed by a second incubation in which the amount of [^{14}C]amino acid incorporation serves as a measure of the extent of complex formation during the preliminary incubation. The preliminary incubation is carried

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[1] Abbreviation used is: MDMP, [2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide.

out at 20° for 7 min and contains in 0.34-ml volume: 0.08 ml of subunit-free S-100, 10 μg of TMV-RNA, 5.0 A_{260} units of ribosomes, 20 μg of tRNA, 51 mM KCl, 1 mM ATP, 25 μM GTP, 8 mM creatine phosphate, 16 μg of creatine phosphate kinase, 25 mM Tris–acetate (pH 8.1), 2 mM dithiothreitol, 0.125 μCi of [^{14}C]leucine, and either 1.3 or 4.0 mM magnesium acetate. Owing to a kinetic lag in initiation complex formation (Marcus, 1970) and a very low rate of peptide-bond formation at 20°, little or no amino acid incorporation is observed during the preliminary incubation. After the preliminary incubation, aurintricarboxylic acid is added to a concentration of 40 μM and the concentration of Mg^{2+} is adjusted to 3.6 mM in all incubation mixtures. The second incubation is carried out at 30° for 8 min at which time amino acid incorporation is essentially complete. In incubation mixtures containing KF (in these experiments and others reported here), the concentration of KCl was lowered by 10 mM to compensate for the contribution of K^+ ion from KF. [The use of KF was required since at the high levels needed for inhibition (10 mM), Na ions *per se* are inhibitory to amino acid incorporating systems derived from plant tissues.]

Assays of polyuridylic (poly(U)) acid dependent polyphenylalanine synthesis were similar to those for amino acid incorporation in the TMV-RNA-dependent system described above but contained the following modifications: (1) [^{14}C]-phenylalanine was substituted for [^{14}C]leucine; (2) 10 μg of poly(U) replaced TMV-RNA; and (3) the Mg^{2+} and K^+ concentrations were raised to 7.6 and 60 mM, respectively.

Met-tRNA Binding to Ribosomes. The assay for Met-tRNA binding to ribosomes and the sucrose gradient analysis of this reaction have been described elsewhere (Weeks *et al.*, 1972). The reaction mixture in a total volume of 0.34 ml contained: 1.1 mM methionine, 20 mM Tris–acetate (pH 8.0), 1.1 mM ATP, 25 μM GTP, 10 μg of TMV-RNA, 3.6 mM $MgAc_2$, 2.6 mM dithiothreitol, 51 mM KCl, ribosomes (4.0 A_{260} units), 20 μg of [^{35}S] Met-tRNA (8500 cpm/μg), and initiation factors, C (step 1) and D (step 1) (Seal *et al.*, 1972). In incubations testing the effects of inhibitors on Met-tRNA binding, 0.1 mM MDMP and 10 mM KF were used. Following a 7-min incubation at 20°, puromycin (to 1.0 mM) was added to the appropriate tubes. After an additional 5 min in ice, the amount of Met-tRNA bound to ribosomes was determined by filtration through nitrocellulose filters (Marcus *et al.*, 1970b). The extent of Met-tRNA release by puromycin was determined by differences in the amounts of Met-tRNA retained on the nitrocellulose filters from incubations containing puromycin and control incubations that were treated identically except for the omission of puromycin.

Sucrose gradient sedimentation analysis of the products of the Met-tRNA binding reaction was performed as follows: (1) a Met-tRNA binding reaction identical with that described above [except for the substitution of 6 μg of STNV-RNA (the RNA of satellite tobacco necrosis virus) for TMV-RNA] was carried out; (2) MDMP or KF was added to control reaction mixtures *after* incubation in order to demonstrate that neither inhibitor caused dissociation of the 80S initiation complex once it was formed (the gradient patterns of such controls with either inhibitor were the same; the control treated with KF is shown in Figure 3A); and (3) incubated samples were layered on 7.5–25% linear sucrose gradients containing 40 mM KCl, 1 mM $MgAc_2$, 2 mM Tris–acetate (pH 7.6), and 3 mM mercaptoethanol. Following centrifugation in a SW 50.1 rotor for 80 min at 50,000 rpm, the gradients were scanned for absorbancy at 254 nm and divided into 20 fractions. Each fraction was analyzed for radioactivity retainable on nitrocellulose filters as in the Met-tRNA binding reaction described above.

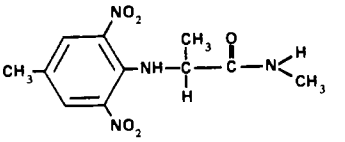

FIGURE 1: MDMP, 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide.

STNV-RNA was the generous gift of Dr. J. M. Clark, University of Illinois. Pactamycin was provided by Dr. G. S. Fonken of the Upjohn Co. MDMP was synthesized by Shell Research Limited, Sittingbourne, England.

Results

Inhibition of protein synthesis at the level of peptide-chain initiation or peptide-chain elongation can be clearly distinguished in the *in vitro* amino acid incorporating system derived from dry wheat embryos. Protein synthesis in this system using messenger-free 80S ribosomes and TMV-RNA as a mRNA source requires a factor-dependent peptide-chain initiation reaction (Seal *et al.*, 1972). However, amino acid incorporation by polyribosomes (obtained from germinating wheat embryos) is dependent only upon peptide-chain elongation (Marcus *et al.*, 1970). As seen in the data of Figure 2, the D isomer of MDMP inhibits amino acid incorporation in the TMV-RNA-dependent system by more than 95% at concentrations above 3×10^{-5} M. In contrast, amino acid incorporation by polyribosomes is not affected by MDMP. Hence MDMP appears to act exclusively at the level of peptide-chain initiation. Interestingly, the L isomer of MDMP is totally inactive in peptide synthesis inhibition (Figure 2) and has no effect on the level of inhibition obtained when mixed with a given concentration of the D isomer (unpublished data).

MDMP appears to affect only factor-dependent chain initiation since amino acid incorporation by polyuridylic acid [which attaches nonspecifically to the 80S ribosome (Falvey and Staehelin, 1970)] is not inhibited by MDMP (Table I). By comparison, aurintricarboxylic acid, which prevents mRNA attachment to ribosomes (Marcus *et al.*, 1970; Stewart *et al.*, 1971), completely blocks poly(U)-directed polyphenylalanine synthesis.

The degree of inhibition obtained with a given concentration of MDMP is dependent upon the Mg^{2+} concentration of the incubation medium. The inhibitory effect of MDMP gradually diminishes below 3.0 mM Mg^{2+} until at 1.3 mM Mg^{2+} the effect is essentially nil. The experiments of Table II illustrate this point and, more importantly, show that MDMP acts solely by preventing the formation of a functional initiation complex. In these assays advantage is taken of the ability to form an initiation complex during a preliminary incubation. The addition of aurintricarboxylic acid after the preliminary incubation prevents the formation of additional initiation complexes but does not interfere with amino acid incorporation by the initiation complex during a subsequent incubation (Marcus *et al.*, 1970a; Weeks *et al.*, 1972). Thus the extent of complex formation can be monitored by measuring the amount of amino acid incorporation in the second incubation. As noted above, MDMP does not interfere with peptide-chain initiation at 1.3 mM Mg^{2+} (Table II, line 3). On the other hand, aurintricarboxylic acid (in the *preliminary*

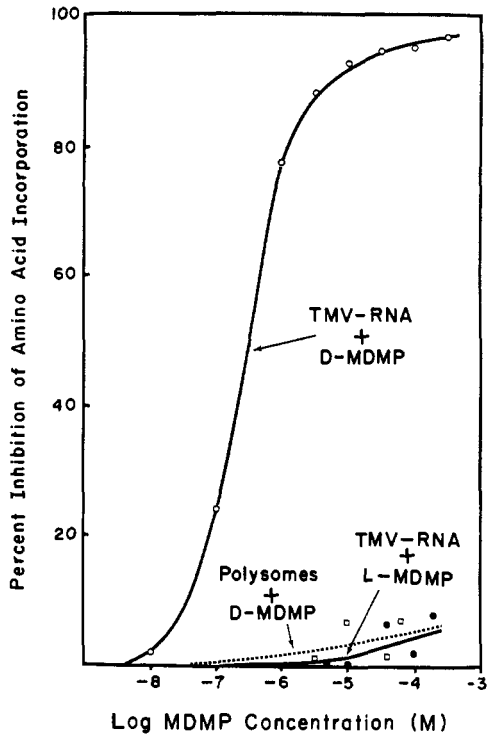

FIGURE 2: Effect of MDMP on TMV-RNA-dependent and polyribosome-dependent amino acid incorporation. The TMV-RNA-dependent amino acid incorporation system was incubated with increasing concentrations of D-MDMP (○) or L-MDMP (●). The polyribosome system was likewise treated with D-MDMP (□). Amino acid incorporation by control incubations (*i.e.*, without inhibitor) was 20,473 cpm for the TMV-RNAs ystem and 5660 cpm for the polyribosome system. The conditions of incubation are detailed in Materials and Methods.

TABLE II: Inhibitory Effect of MDMP and KF on Initiation Complex Formation at Different Mg^{2+} Concentrations.[a]

Preliminary Incubation		Amino Acid Incorporation Incubation (with ATA)		[^{14}C]-Amino Acid Incorporation
Mg^{2+} Concn (mM)	Inhibitor Treatment	Inhibitor Treatment	Cpm	% Inhibn
1.3			895	
1.3	ATA		29	97
1.3	MDMP		801	11
1.3	KF		278	69
4.0			2126	
4.0	MDMP		176	92
4.0		MDMP	2082	2
4.0			1984	
4.0	KF		584	72
4.0		KF	1202	39

[a] Following initiation complex formation in the preliminary incubation, aurintricarboxylic acid (ATA) (40 μM) was added to prevent further initiation. The extent of complex formation was then measured by assaying the ability to support amino acid incorporation in a subsequent incubation. The Mg^{2+} concentration in the second incubation was adjusted to 3.6 mM in all cases (see Materials and Methods for details of incubation). Concentrations of inhibitors used were 0.1 mM MDMP 10 mM KF, and 0.04 mM ATA.

incubation) completely blocks initiation complex formation and KF inhibits approximately 70%. By raising the Mg^{2+} concentration in the preliminary incubation to 4.0 mM, over 90% inhibition of initiation complex formation can be obtained with MDMP. It should be noted that MDMP added after the preliminary incubation has no effect on amino acid incorporation. Potassium fluoride again inhibits initiation by about 70%. In contrast to MDMP, however, KF appears to also exert an inhibitory effect on peptide chain elongation. At lower concentrations (*i.e.*, 1 mM), KF inhibits neither peptide chain initiation nor chain elongation (unpublished results).

More precise localization of the inhibitory effect of MDMP within the multistep peptide-chain initiation process was achieved in experiments studying the reactions involved with mRNA-dependent binding of Met-tRNA to ribosomes. The data of Table III show that MDMP and KF have very little effect on the initial binding of Met-tRNA to ribosomes. However, the Met-tRNA bound in the presence of MDMP or KF is not releasable from the ribosome by reaction with puromycin. Previous investigations (Weeks *et al.*, 1972) have shown that Met-tRNA is initially bound to the 40S ribosomal subunit in a 40S subunit–mRNA–Met-tRNA complex. This complex subsequently joins with a 60S ribosomal subunit to form an 80S initiation complex. Since reactivity with puromycin requires that Met-tRNA be bound to the "P" site of the large sunbunit (Thach and Thach, 1971; Tanaka *et al.*, 1971), *i.e.*, requires the formation of the 80S complex, experiments were performed to determine if the lack of puromycin reactivity of Met-tRNA in the presence of MDMP could be due to interference with the formation of a stable 80S initiation complex. Indeed, the experiments of Figure 3 clearly show that formation of an 80S initiation complex is blocked by both MDMP and KF. The peak of Met-tRNA radioactivity in the MDMP and KF gradients corresponds precisely to that previously shown for the 40S ribosomal subunit–mRNA–Met-tRNA complex (Weeks *et al.*, 1972). Another inhibitor of peptide-chain initiation which blocks puromycin release but does not interfere with Met-tRNA binding is pactamycin (Seal and Marcus, 1972). In contrast to MDMP and KF, this inhibitor, as indicated by Seal and Marcus, does not interfere with the formation of an 80S initiation complex

TABLE I: Effect of MDMP on Polyuridylic Acid Directed Synthesis of Polyphenylalanine.[a]

Conditions	[^{14}C]Phenylalanine Incorporation	
	Cpm	% Inhibn
Control	46,508	
+MDMP (100 μM)	43,221	7
+ATA (30 μM)	53	99

[a] The conditions of the incubation are described in Materials and Methods.

TABLE III: Effect of MDMP and KF on the Binding of Met-tRNA and Its Release by Puromycin.[a]

Conditions	[35S]Met-tRNA Bound to Ribosomes (cpm)			% Inhibn of Met-tRNA Binding	% Inhibn of Puromycin Release
	−Puromycin	+Puromycin	Δ		
Control	2059	1198	861		
+MDMP (0.1 mM)	1981	1863	118	3	86
+KF (10 mM)	1993	1849	144	3	83

[a] Met-tRNA binding and puromycin release were carried out as described in Materials and Methods. After the Met-tRNA binding incubation with or without MDMP or KF, puromycin (1.0 mM) was added for 5 min at 0° prior to filtration through nitrocellulose. Control incubations were handled identically except for the omission of puromycin.

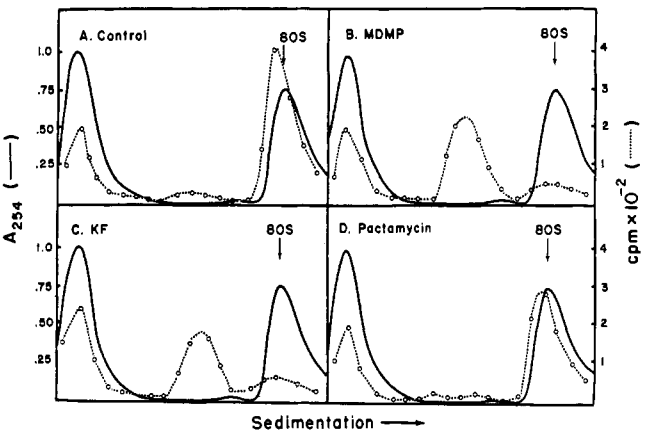

FIGURE 3: Sucrose gradient analysis of the products of the Met-tRNA binding reaction. [35S]Met-tRNA binding was carried out in the presence of either: (A) no inhibitor, (B) 0.1 mM MDMP, (C) 10 mM KF, or (D) 0.001 mM pactamycin. Details of the Met-tRNA binding reaction and sucrose gradient analysis are given in Materials and Methods.

formation (Figure 3D). Thus, MDMP and KF appear to block a specific interaction of the 40S subunit–mRNA–Met-tRNA complex with the 60S subunit. The apparent loss of radioactively labeled Met-tRNA from the 40S initiation complex during the extended period required for centrifugation, fractionation, and filtration of the sucrose gradients may well be related to the observed instability of tRNA bound to the "A" site relative to that bound into the "P" site (Erbe *et al.*, 1969).

Discussion

The results reported here clearly establish MDMP as a specific inhibitor of peptide-chain initiation. It appears that MDMP acts by preventing the interaction of the 40S ribosomal subunit–mRNA–Met-tRNA complex with the 60S ribosomal subunit to form the stable 80S ribosome initiation complex that is essential for peptide synthesis. The specificity of this inhibitor should prove valuable to future probes of the complex reactions involved in peptide-chain initiation. Indeed, the present observations *per se* provide additional confirmation of the sequential role of ribosomal subunits in eukaryotic peptide-chain initiation recently demonstrated by Weeks *et al.* (1972). These investigators have shown that isolated 40S ribosomal subunits incubated with TMV-RNA, initiation factors, ATP and GTP bind the initiator species of tRNA, Met-tRNA$_i$, to form 40S subunit–mRNA–Met-tRNA complexes. These complexes were shown to couple with 60S subunits to form 80S initiation complexes functional in protein synthesis. The same sequence of reactions is implied from the present data using 40S ribosomal subunits derived directly from the equilibrium dissociation of 80S ribosomes during incubation. Supplied with mRNA and initiation factors these subunits can bind Met-tRNA in the presence of MDMP. They are, however, effectively prevented from participating in the next step of initiation, the attachment of the 60S ribosomal subunit. This feature of MDMP inhibition offers the opportunity to "trap" the 40S initiation complex to study the requirements for its formation and to examine certain of its features. For instance, it may be possible to ascertain whether Met-tRNA is bound into the acceptor site of the 40S subunit (and thus requires translocation for entry into the peptidyl site of the 60S subunit) or to another site, such as the "pre-P" site proposed by Thach (1971). Also, investigations of conformation changes (Schreier and Noll, 1971; Chaung and Simpson, 1971) which might occur in the 40S subunit during initiation complex formation would be greatly facilitated by the use of MDMP.

From experiments studying the effects of NaF on protein synthesis in reticulocytes, Hoerz and McCarty (1971) proposed that NaF blocked peptide-chain initiation at a point after 40S ribosomal attachment to mRNA and before subsequent 60S coupling. Recent work by Obrig *et al.* (1971) shows that edeine, a specific inhibitor of ribosome attachment to mRNA, only partially blocks peptide synthesis by ribosomes from NaF-treated cells—the incomplete inhibition being explained by the presence of 40S subunits attached to mRNA. Because of the obvious similarity between the mode of action proposed for NaF and that shown here for MDMP, we tested the influence of KF on peptide-chain initiation in the *in vitro* wheat embryo system. These experiments have provided direct *in vitro* confirmation that fluoride does not interfere with the formation of the 40S ribosomal subunit–mRNA–Met-tRNA initiation complex but does prevent its coupling to the 60S subunit. Since MDMP and KF are grossly different molecules it should prove both interesting and informative to determine if they affect identical molecules and partial reactions involved with 80S initiation complex formation. For the moment MDMP would appear to have certain advantages in studies where either inhibitor might ostensibly be used. First, the interaction of D-MDMP with its target molecule must be quite specific since even its enantiomorph apparently does not bind to the same site (*i.e.*, the L isomer offers no competitive inhibition to the action of the D isomer). Secondly, the concentration at which maximal inhibition is attained with MDMP (Figure 2) is approximately 1000-fold less than that required for maximal inhibition with KF. Indeed, the very high concentrations of KF needed for inhibition (both *in vivo* and *in vitro*) and high degree of nonspecific binding of the small negatively charged fluoride ion may greatly hamper attempts at the molecular definition of

KF's mode of action. In addition, the apparent inhibitory effect of KF on peptide-chain elongation and incomplete inhibition of chain initiation (Table II) would seem to make it a less attractive reagent, in general, for studying the mechanisms of protein biosynthesis.

Since the participation of the peptidyl site of the large subunit is a requisite for puromycin release of Met-tRNA (Thach and Thach, 1971), the prevention of 60S subunit coupling to the 40S ribosome initiation complex becomes the obvious reason for the prevention by MDMP and KF of the puromycin reaction with Met-tRNA (Table III). Yet mere coupling of the initiation complex with a 60S subunit is not sufficient to ensure puromycin reactivity. Seal and Marcus (1972) have noted that while pactamycin prevents puromycin release of Met-tRNA, it does not prevent the formation of an 80S ribosome initiation complex (see Figure 3D). Taken together, the observations concerning inhibition by MDMP, KF, and pactamycin indicate that in addition to a number of partial reactions which must precede the initial binding of Met-tRNA, the initiation process must also contain a complex of steps required for the eventual participation of Met-tRNA in peptide bond formation.

MDMP is a potent herbicide (Yates, 1968). Its primary mode of action *in vivo* appears to be at the level of protein synthesis (Baxter and Weeks, 1971). The pattern of polyribosome decay following administration of MDMP to germinating wheat embryos (manuscript in preparation) is consistent with the selective inhibition of peptide chain initiation. Interestingly, the concentration curve for protein synthesis inhibition *in vivo* (Baxter and Weeks, 1971) is almost coincident with that obtained *in vitro* (Figure 2). Another point which arises when evaluating *in vitro* results in terms of *in vivo* implications is that the degree of inhibition obtained *in vitro* is highly dependent on the Mg^{2+} concentration of the incubation mixture, *i.e.*, complete inhibition requires the effective Mg^{2+} concentration to be above 2.2 mM Mg^{2+} (3.0 mM Mg^{2+} minus 0.8 mM Mg^{2+} chelated by 1.0 mM ATP). Thus, since nearly 100% inhibition of protein synthesis can be achieved *in vivo* with MDMP, the intracellular Mg^{2+} concentration in the vicinity of polyribosomes, *vida infra*, would appear to be at least 2.2 mM.

Initiation of peptide synthesis in the *in vitro* rabbit reticulocyte system appears to be selectively blocked by MDMP (L. Gedamu, personal communication). However, the inhibitory effect of MDMP may be restricted to eukaryotic organism since neither growth, nor protein synthesis, in bacteria appear to be affected by the compound (manuscript in preparation).

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