DL-α-(Difluoromethyl)arginine: A Potent Enzyme-Activated Irreversible Inhibitor of Bacterial Arginine Decarboxylases†

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ABSTRACT: DL-α-(Difluoromethyl)arginine (RMI 71 897) is an irreversible inhibitor of both the biosynthetic and biodegradative arginine decarboxylases of *Escherichia coli* and of the biosynthetic arginine decarboxylases of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The K_i is close to 800 μM for the biosynthetic decarboxylase of *E. coli* and 140 μM for the biodegradative enzyme while the respective half-lives ($t_{1/2}$) calculated for an infinite concentration of inhibitor are 1.0 and 2.1 min. The inhibitor also blocks the arginine decarboxylase activity of *E. coli* and *Pseudomonas aeruginosa* in vivo, indicating that the compound is transported into the cell. DL-α-Methylarginine (RMI 71 669) was found to be a competitive inhibitor of both arginine decarboxylases from *E. coli*. These results suggest that it may be possible to use an arginine decarboxylase inhibitor in conjunction with known inhibitors of ornithine decarboxylase to block all putrescine biosynthesis in prokaryotic cells and thus to study the effects of such inhibition in these organisms.

All bacteria so far examined are able to synthesize putrescine, and a number of routes to this diamine have been observed (Tabor & Tabor, 1976; Pegg & Williams-Ashman, 1981). In *Escherichia coli*, these routes involve the decarboxylation of ornithine by ornithine decarboxylase, yielding putrescine directly, or the decarboxylation of arginine by arginine decarboxylase, producing agmatine, which is then converted into putrescine (Tabor & Tabor, 1976; Pegg & Williams-Ashman, 1981). *E. coli* has two distinct classes of ornithine and arginine decarboxylases which act either in biodegradative or in biosynthetic routes (Morris & Fillingame, 1974). The biodegradative decarboxylases are induced by growth at low pH in culture media enriched with amino acids (Gale, 1940), and their production seems to be primarily a defense mechanism against low environmental pH (Applebaum et al., 1975). The biosynthetic decarboxylases are responsible for the synthesis of putrescine during growth in neutral medium (Morris & Pardee, 1966; Morris & Koffron, 1969; Morris et al., 1970). All four enzymes are well characterized (Blethen et al., 1968; Wu & Morris, 1973a,b; Applebaum et al., 1975, 1977), and, as pyridoxal phosphate dependent enzymes, they appear to be ideal targets for enzyme-activated irreversible inhibitors of the types previously described for mammalian ornithine decarboxylases (Metcalf et al., 1978). The inhibition of ornithine decarboxylase and the consequent depletion of polyamines have been shown to significantly inhibit DNA synthesis and thereby block replication in mammalian cells (Mamont et al., 1976, 1978). No similar phenomena have thus far been reported in prokaryotic cells, because no specific inhibitor for arginine decarboxylase existed which could block this alternate route of putrescine formation in these organisms. However, mutants of *E. coli* that make neither ornithine nor arginine decarboxylase have been constructed. In the apparent absence of putrescine and spermidine, these grow at a reduced rate compared to their growth in the presence of polyamines (Hafner et al., 1979).

In this paper, we report studies on two such inhibitors which affect both the biosynthetic and biodegradative forms of arginine decarboxylase (EC 4.1.1.19). DL-α-(Difluoromethyl)arginine (RMI 71 897) is a novel specific enzyme-activated irreversible inhibitor of the two arginine decarboxylases while DL-α-methylarginine (RMI 71 669) acts as a competitive inhibitor against both enzymes.

Materials and Methods

Chemicals. L-[U-^{14}C]Arginine (300 mCi/mmol) was purchased from ICN (Irvine, CA). DL-[1-^{14}C]Arginine (20 mCi/mmol) was purchased from CEA–Research Products Intl. (Mt. Prospect, IL), and DL-[1-^{14}C]ornithine (58 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, England). All other reagents were of the highest grade commercially available and were used without further purification. DL-α-(Difluoromethyl)arginine (RMI 71 897), DL-α-methylarginine (RMI 71 669), and DL-α-(difluoromethyl)-ornithine (RMI 71 782) were synthesized at the Centre de Recherche Merrell International, Strasbourg, France (Bey et al., 1979).

Preparation of Enzyme Extracts. *E. coli* (MRC 59), *Pseudomonas aeruginosa* (ATCC 9027), and *Klebsiella pneumoniae* (ATCC 8045) were grown in Davis & Mingoli (1950) minimal media at 37 °C with vigorous aeration up to late log–early stationary phase. Cells were harvested by centrifugation, and washed once with phosphate-buffered saline, pH 7.2, and stored at −20 °C. About 1 g of frozen cells was resuspended in 4 mL of 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol and 0.1 mM EDTA and disrupted in a Branson 350 cell disrupter set at power setting 3.5 for 2 min. All sonications were done in a salt–ice bath. The cell debris was removed by centrifugation at 20 000*g* for 30 min. The supernatant solution, stored at −20 °C where enzyme activity was stable for several weeks, was used for measuring biosynthetic arginine and ornithine decarboxylases. Partially purified biodegradative arginine decarboxylase type II was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved in 2 mL of 10 mM potassium acetate–acid buffer, pH 5.25, containing 1 mM dithiothreitol and 0.1 mM EDTA.

Assay for Arginine Decarboxylase. Biosynthetic arginine decarboxylase was measured by a modification of the previously described method of Wu & Morris (1973a). The incubation mixture (1 mL) contained 100 mM Tris-HCl buffer, pH 8.25, 0.04 mM pyridoxal phosphate, 4 mM $MgSO_4$, 1 mM dithiothreitol, varying concentrations of L-arginine (0.1–0.5 mM), 0.17–1.0 μCi of L-[U-^{14}C]arginine, and the enzyme. The incubations were carried out for 20 min at 37 °C.

Biodegradative arginine decarboxylase was measured in a reaction mixture (1 mL) containing 100 mM potassium acetate

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buffer, pH 5.25, 0.04 7M pyridoxal phosphate, 1 mM dithiothreitol, 1 mM L-arginine, 0.17–1.0 μCi of L-[U-^{14}C]arginine, and the enzyme. The incubations were carried out for 15 min at 37 °C.

Most experiments were performed with L-[U-^{14}C]arginine as the substrate for arginine decarboxylase, but the release of $^{14}CO_2$ was also measured with DL-[1-^{14}C]arginine in all enzyme preparations to assure that we were in fact measuring release of the carboxyl group from arginine. The problem, however, with using racemic DL-arginine in the assay is that D-arginine is a potent inhibitor of arginine decarboxylase (K_i of 3.2 μM for the *Pseudomonas* enzyme; Rosenfeld & Roberts, 1976), and thus its presence in the assay mixture lowered the activity and changed the apparent K_m for arginine. L-[1-^{14}C]Arginine is not commercially available. The possibility that L-[1-^{14}C]arginine or DL-[1-^{14}C]arginine was converted to ornithine during the incubation and the $^{14}CO_2$ trapped would actually be released from this ornithine was ruled out by inhibiting all *Pseudomonas* ornithine decarboxylase activity in the preparation with 1.2 mM DL-α-(difluoromethyl)ornithine and then measuring the arginine decarboxylase activity. (*Pseudomonas aeruginosa* ornithine decarboxylase activity is effectively blocked by DL-α-(difluoromethyl)ornithine; Kallio et al., 1980.) The same specific activity of biosynthetic arginine decarboxylase was also observed after inhibition of ornithine decarboxylase in the other bacteria tested.

In a typical experiment, 80 μL of the enzyme preparation was mixed with 80 μL of 300 mM Tris-HCl, pH 8.25, in the case of biosynthetic decarboxylase, and 300 mM potassium acetate, pH 5.25, in the case of biodegradative decarboxylase. The buffers contained 3 mM dithiothreitol and 0.16 mM pyridoxal phosphate. The biosynthetic enzyme buffer also had 12 mM $MgSO_4$. After a 5-min preincubation at 37 °C, 80 μL of DL-α-(difluoromethyl)arginine (DFMA) solution in water was added. At various times, 20-μL aliquots of this solution were transferred to the final assay mixture containing the substrate, resulting in a 50-fold dilution of the inhibitor. This transfer effectively stopped any further enzyme inhibition.

Assay for Biosynthetic Ornithine Decarboxylase. Ornithine decarboxylase activity was measured by the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine at 37 °C (Jänne & Williams-Ashman, 1973) in a reaction mixture (1 mL) containing 100 mM Tris-HCl buffer, pH 8.25 in the case of *E. coli* and pH 7.5 in the case of *Pseudomonas aeruginosa*, 0.04 mM pyridoxal phosphate, 1 mM dithiothreitol, 1 or 5 mM L-ornithine, 1.5–2.5 μCi of DL-[1-^{14}C]ornithine, and enzyme. The incubation was carried out for 20 min.

Definition of Enzyme Activity and Protein Estimation. In both the ornithine and arginine decarboxylase assays, the $^{14}CO_2$ produced was trapped and counted as described previously (Jänne & Williams-Ashman, 1973; McCann et al., 1975); reactions were linear and proportional to the amount of enzyme protein added. One unit of enzyme activity was defined as that amount catalyzing the release of 1 μmol of CO_2/h under the standard assay conditions. The specific enzyme activity was defined as activity units per milligram of protein. The protein concentrations were determined by the method of Böhlen et al. (1972) with crystalline bovine serum albumin used as a standard.

Results

Inhibition of Biosynthetic and Biodegradative Arginine Decarboxylase Activities by DL-α-(Difluoromethyl)arginine. The effects of the arginine analogue DL-α-(difluoromethyl)-arginine (DFMA) on the *E. coli* biosynthetic and biodegradative arginine decarboxylases were investigated. A rapid, time-dependent, and irreversible loss of both decarboxylase activities was seen after incubation with this compound. The inactivation process followed pseudo-first-order kinetics for at least two half-lives. The apparent dissociation constants, K_i, were determined (Table I) by plotting the half-lives of the enzyme activities as a function of the inhibitor concentration according to Kitz & Wilson (1962) as shown in Figure 1. The half-lives of the enzymes were calculated for a 10 μM concentration of DFMA and also for an infinite concentration of inhibitor (Table I). These results show that DFMA is slightly more effective against the biodegradative arginine decarboxylase than against the biosynthetic enzyme (Figure 1, Table I) but that both enzymes were very rapidly inhibited. As a control, DFMA at a 1 mM concentration had no detectable effect on either of the ornithine decarboxylases obtained from *E. coli*.

Table I: Kinetic Data for Irreversible Inhibition of *E. coli* Biosynthetic and Biodegradative Arginine Decarboxylases by DL-α-(Difluoromethyl)arginine

	biosynthetic arginine decarboxylase	biodegradative arginine decarboxylase
K_i (μM)	800	140
$t_{1/2}$ (min) at infinite DFMA concn	1.0	2.1
$t_{1/2}$ (min) at 10 μM DFMA	94	32
$t_{1/2}$ (min) at 100 μM DFMA	12	5
$t_{1/2}$ (min) at 100 μM DFMA + 1 mM arginine	64	9

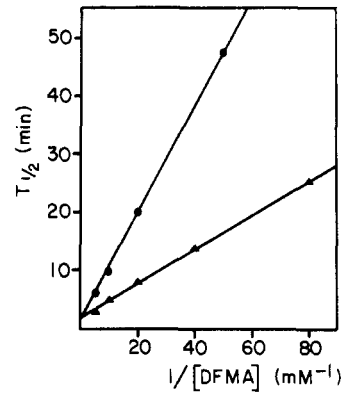

FIGURE 1: Dependence of the half-life of *E. coli* biosynthetic arginine decarboxylase (●) and biodegradative arginine decarboxylase (▲) upon the concentration of DL-α-(difluoromethyl)arginine. The rate of irreversible inhibition of both decarboxylases was followed by withdrawing samples for assays at different time points after mixing of enzyme and DL-α-(difluoromethyl)arginine. The half-lives of the enzyme activity at different concentrations of DFMA (0.0125–0.2 mM) were determined according to Kitz & Wilson (1962).

Evidence of the Enzyme-Activated Nature of the Inhibition of DL-α-(Difluoromethyl)arginine. The irreversible nature of the arginine decarboxylase inactivation by DFMA was indicated by a failure to restore more than 2–9% of enzyme activities by extensive dialysis (30 h at +4 °C against Tris-HCl, pH 8.25, or potassium acetate buffer, pH 5.25, containing 1 mM dithiothreitol and 0.1 mM EDTA). Thus, the inhibition is assumed to be due to a covalent bond formation between enzyme and inhibitor. Evidence of the involvement of the enzyme's active site in the inhibition by DFMA came from the protection against inactivation by the substrate L-arginine. Figure 2 shows the time-dependent inhibition of the arginine decarboxylases by 0.1 mM DFMA in the presence and absence of 1 mM L-arginine. The half-lives were clearly longer when the incubations were done in the presence of arginine (Table I). The presence of dithiothreitol in the preincubation medium

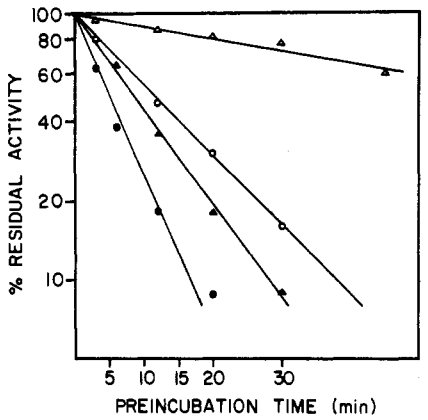

FIGURE 2: Time-dependent loss of biosynthetic and biodegradative arginine decarboxylase activity upon incubation with DL-α-(difluoromethyl)arginine and the protection against enzyme inactivation by the substrate, L-arginine. Biosynthetic arginine decarboxylase obtained from *E. coli* was preincubated with 0.1 mM DL-α-(difluoromethyl)arginine in the absence (○) or presence of 1 mM arginine (△) as was the biodegradative enzyme (●; +1 mM arginine, ▲). Aliquots of the preincubates were taken at the indicated times, and the residual enzymatic activity was measured as described under Materials and Methods.

and the absence of lag time before the onset of inhibition rule out the possibility of inhibition via an affinity-labeling mode by a diffusable alkylating species (Metcalf et al., 1978).

The enzyme-catalyzed decarboxylation of arginine by the biosynthetic arginine decarboxylase is highly temperature dependent in that extremely slow decarboxylation of arginine was observed at 13 °C. (When the 37 °C incubation is used as 100% enzyme activity, then there is only 8% of the activity present when the assay is done at 22 °C, 0.3% activity at 13 °C, and no activity at 0 °C.) In addition, the inhibition of arginine decarboxylase by DFMA was also highly temperature dependent so that at 13 °C no time-dependent inhibition was seen. This suggests that in order to produce the actual inhibitory species DFMA must have been turned over by the enzyme. All the results presented above suggest that DFMA inhibits arginine decarboxylase because it is accepted as a substrate by this enzyme and is decarboxylated, leading to the formation of an active Michael acceptor which irreversibly reacts with a nucleophilic residue of the enzyme.

Effect of DFMA on Arginine Decarboxylase Obtained from Other Bacterial Sources in Vitro. To determine if DFMA would affect other bacterial biosynthetic arginine decarboxylases, enzyme preparations were obtained from *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The rates of $^{14}CO_2$ release were clearly decreased in a time-dependent manner in both cases. The half-life of the *Pseudomonas* enzyme assayed in a manner identical with that described for the biosynthetic *E. coli* was 2 min as calculated at an infinite concentration of inhibitor. After the enzyme preparations from *Pseudomonas* and *Klebsiella* were incubated with 0.5 mM DFMA for 20 min, 6% and 17%, respectively, of the biosynthetic activities remained. Because of low enzyme activity in the *Klebsiella* preparation, the inhibitor was diluted only 20-fold.

Effect of DFMA on Biosynthetic Arginine Decarboxylase of E. coli and Pseudomonas aeruginosa in Vivo. In order to study the capability of DFMA to inhibit arginine decarboxylase activity in whole cells, bacteria were grown in Davis & Mingoli (1950) media in the presence or absence of 1 mM DFMA for 5 h with vigorous aeration at 37 °C. DFMA had no effect on the growth rate. When the cells were in a logarithmic phase of growth, they were harvested, and extracts were prepared for enzyme determination as described under Materials and Methods. As illustrated in Table II, biosynthetic arginine decarboxylase activity was inhibited 85% in *E. coli* cells grown in 1 mM DFMA and 94% in similarly grown *Pseudomonas aeruginosa* cells. Biosynthetic ornithine decarboxylase (ODC), which was used as a reference enzyme for overall protein synthesis, was not inhibited by DFMA, and, in fact, a slight stimulation of ODC activity was observed (Table II).

Table II: Effect of DL-α-(Difluoromethyl)arginine in Vivo on Biosynthetic Arginine and Ornithine Decarboxylases in Logarithmically Growing *E. coli* and *Pseudomonas aeruginosa* after 5-h Incubation

bacteria	DFMA (mM)	arginine decarboxylase activity [μmol h^{-1} (mg of protein)$^{-1}$]	ornithine decarboxylase activity [μmol h^{-1} (mg of protein)$^{-1}$]
E. coli	none	1.16	1.40
E. coli	1	0.18	1.68
P. aeruginosa	none	0.24	0.87
P. aeruginosa	1	0.01	1.04

Inhibition of Biosynthetic and Biodegradative Arginine Decarboxylase by DL-α-*Methylarginine.* It is known that several α-methyl amino acids are competitive inhibitors of their corresponding pyridoxal phosphate dependent amino acid decarboxylases (Bey, 1978). Both the *E. coli* biosynthetic and biodegradative arginine decarboxylases were inhibited in vitro by DL-α-methylarginine in a competitive manner with respect of the substrate, L-arginine (Figure 3A,B). The compound was clearly a more potent inhibitor of the biosynthetic enzyme, the reversal of what was seen when using DFMA as an inhibitor. The K_i obtained from a Lineweaver–Burk plot (Figure 3) was 0.27 mM for biosynthetic enzyme and 6 mM for biodegradative enzyme. The K_m values were 0.14 and 0.54 mM, respectively. The experiment was also repeated by using DL-[1-^{14}C]arginine as a substrate for the enzymes. While the same K_i values were obtained as above, because of the inhibitory effect of D-arginine, the apparent K_m values for arginine were slightly higher than those given above. DL-α-Methylarginine had no inhibitory effect on the *E. coli* biosynthetic ornithine decarboxylase.

Discussion

Bacterial arginine decarboxylases from *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* are irreversibly inhibited by the enzyme-activated α-difluoromethyl analogue of the enzyme substrate L-arginine. The mechanism of inhibition appears to be similar to that described by Metcalf et al. (1978) for the inactivation of mammalian ornithine decarboxylase by DL-α-(difluoromethyl)ornithine. DL-α-Methylarginine acts as a competitive inhibitor of these enzymes, again in a manner analogous to the effect of the DL-α-methyl analogue of ornithine on ornithine decarboxylase in mammalian cells (Mamont et al., 1976; McCann et al., 1977). Although it is not surprising that DFMA and α-methylarginine inhibit the arginine decarboxylases from these bacteria, one cannot automatically assume that this would be the case. Preliminary evidence has shown, for example, that (difluoromethyl)ornithine does not at all inhibit the *E. coli* or *Klebsiella* ornithine decarboxylases in vitro, but contrarily does irreversibly inactivate the ODC from *Pseudomonas* (Kallio et al., 1980).

Another significant finding (Table II) is that DFMA can inhibit the arginine decarboxylase activity in vivo in *E. coli* and *Pseudomonas aeruginosa*, indicating that the compound is transported into the cell from the medium. This is important

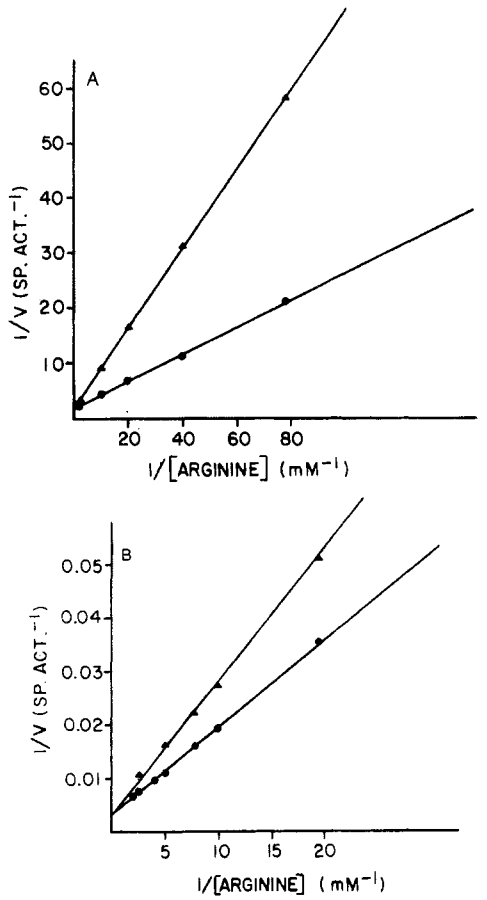

FIGURE 3: Effect of DL-α-methylarginine on biosynthetic and biodegradative arginine decarboxylase activity. Biosynthetic arginine decarboxylase activity (A) was measured at various concentrations of L-arginine in the absence (●) or in the presence of 0.5 mM α-methylarginine (▲). Crude extract obtained from *E. coli* (67 μg of protein/assay) was used as the source of the enzyme. Biodegradative arginine decarboxylase activity (B) was measured at various concentrations of L-arginine in the absence (●) or in the presence of 2.5 mM α-methylarginine (▲). Partially purified biodegradative arginine decarboxylase (3.5 μg of protein/assay) obtained from *E. coli* was used as the source of the enzyme.

when considering the possibility of using such an inhibitor as a means of enzymatically blocking polyamine biosynthesis in prokaryotic organisms. As these organisms have a dual pathway for synthesizing putrescine, via ornithine directly or via arginine decarboxylation to agmatine and then to putrescine, it would be necessary to simultaneously block both of these routes to inhibit putrescine biosynthesis. Such a block to putrescine biosynthesis in mammalian cells effected by ODC inhibition has been shown in a number of instances both in cell culture (Mamont et al., 1976, 1978) and in tumor cells in vivo (Prakash et al., 1980) to have a significant effect on DNA synthesis and ultimately on cell division. This manipulation may now be possible in bacterial cells and higher plants which have arginine decarboxylase in addition to ornithine decarboxylase. In fact, it has recently been shown (Hafner et al., 1979) that *E. coli* mutants which cannot synthesize putrescine at all via either the ornithine or the arginine routes show a 70% decrease in their overall growth rate as compared to wild-type cells. It will be interesting to see if the biochemical inhibition of putrescine synthesis has a similar or greater effect on the growth rate of *E. coli* and other bacterial species.

References

Applebaum, D. M., Sabo, D. L., Fischer, E. H., & Morris, D. R. (1975) *Biochemistry 14*, 3675–3681.

Applebaum, D. M., Dunlap, J. C., & Morris, D. R. (1977) *Biochemistry 16*, 1580–1584.

Bey, P. (1978) in *Enzyme-Activated Irreversible Inhibitors* (Seiler, N., Jung, M. J., & Koch-Weser, J., Eds.) pp 27–41, Elsevier/North-Holland, New York.

Bey, P., Vevert, J. P., VanDorsellaer, V., & Kolb, M. (1979) *J. Org. Chem. 44*, 2732–2742.

Blethen, S. L., Boeker, E. A., & Snell, E. D. (1968) *J. Biol. Chem. 243*, 1671–1677.

Böhlen, P., Stein, S., Dairman, W., & Udenfriend, S. (1972) *Arch. Biochem. Biophys. 155*, 213–220.

Davis, B. D., & Mingoli, E. S. (1950) *J. Bacteriol. 60*, 17–28.

Gale, E. F. (1940) *Biochem. J. 34*, 392–413.

Hafner, E. W., Tabor, C. W., & Tabor, H. (1979) *J. Biol. Chem. 254*, 12419–12426.

Jänne, J., & Williams-Ashman, H. G. (1973) *J. Biol. Chem. 246*, 1725–1732.

Kallio, A., McCann, P. P., Bey, P., & Sjoerdsma, A. (1980) *J. Cell Biol. 87*, 319a.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.

Mamont, P. S., Böhlen, P., McCann, P. P., Bey, P., Schuber, F., & Tardif, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1626–1630.

Mamont, P. S., Duchesne, M. C., Grove, J., & Bey, P. (1978) *Biochem. Biophys. Res. Commun. 81*, 58–66.

McCann, P. P., Tardif, C., Mamont, P. S., & Schuber, F. (1975) *Biochem. Biophys. Res. Commun. 64*, 336–341.

McCann, P. P., Tardif, C., Duchesne, M. C., & Mamont, P. S. (1977) *Biochem. Biophys. Res. Commun. 76*, 893–899.

Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P. L., & Vevert, J. P. (1978) *J. Am. Chem. Soc. 100*, 2551–2553.

Morris, D. R., & Pardee, A. B. (1966) *J. Biol. Chem. 241*, 3129–3135.

Morris, D. R., & Koffron, K. L. (1969) *J. Biol. Chem. 244*, 6094–6099.

Morris, D. R., & Fillingame, R. H. (1974) *Annu. Rev. Biochem. 43*, 303–325.

Morris, D. R., Wu, W. H., Applebaum, D., & Koffron, K. L. (1970) *Ann. N.Y. Acad. Sci. 171*, 968–976.

Pegg, A. E., & Williams-Ashman, H. G. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R., & Marton, L. J., Eds.) pp 3–42, Marcel Dekker, New York.

Prakash, N. J., Schechter, P. J., Mamont, P. S., Grove, J., Koch-Weser, J., & Sjoerdsma, A. (1980) *Life Sci. 26*, 181–194.

Rosenfeld, H. J., & Roberts, J. (1976) *J. Bacteriol. 125*, 601–607.

Tabor, C. W., & Tabor, H. (1973) *Annu. Rev. Biochem. 45*, 285–306.

Wu, W. H., & Morris, D. R. (1973a) *J. Biol. Chem. 248*, 1687–1695.

Wu, W. H., & Morris, D. R. (1973b) *J. Biol. Chem. 248*, 1696–1699.