and A. Boparai, unpublished results.
(32) E. W. Sutherland, C. F. Cori, R. Haynes, and N. S. Olsen, *J. Biol. Chem.*, **180**, 825 (1949).
(33) R. H. Cornforth and G. Popják, *Methods Enzymol.*, **15**, 359 (1969).
(34) M. J. Kessler and J. M. Clark, *Anal. Biochem.*, **55**, 160 (1973).
(35) H. Rosenberg, *J. Chromatogr.*, **2**, 487 (1959).
(36) E. D. McCarthy and M. Calvin, *Tetrahedron*, **23**, 2609 (1967).

Inhibitors of Polyamine Biosynthesis. 4. Effects of α-Methyl-(±)-ornithine and Methylglyoxal Bis(guanylhydrazone) on Growth and Polyamine Content of L1210 Leukemic Cells of Mice

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L1210 leukemic cells of mice were incubated for a period of two generations in the presence of either α-methyl-(±)-ornithine, an inhibitor of ornithine decarboxylase, or methylglyoxal bis(guanylhydrazone), an inhibitor of *S*-adenosylmethionine decarboxylase. α-Methyl-(±)-ornithine produced a 50% decrease in spermidine levels, reduced putrescine to nondetectable levels, and caused a slight increase in spermine levels of the cells. However, DNA content of the cell suspension was not altered by α-methyl-(±)-ornithine. Thus putrescine and 50% of the cellular content of spermidine are not essential for DNA synthesis in these cells. Methylglyoxal bis(guanylhydrazone) produced a large increase in putrescine levels, the same decrease in spermidine levels as did α-methyl-(±)-ornithine, and approximately a 45% decrease in spermine levels. These changes were accompanied by a large decrease in the DNA content of the cell suspension. Since the two inhibitors caused a similar decrease in spermidine levels, it is unlikely that the inhibition of DNA synthesis by methylglyoxal bis(guanylhydrazone) is a result of a decrease in the cellular levels of spermidine. Rather, it seems likely that methylglyoxal bis(guanylhydrazone) inhibits DNA synthesis through a mechanism other than a decrease in polyamine levels.

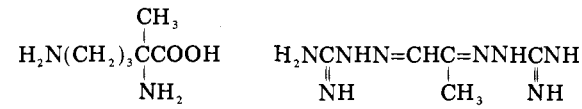
The conversion of many mammalian cells from a quiescent or a slowly growing state to a rapidly growing state is associated with an increase of polyamine levels in the cells.[1] This and the many effects in vitro of the polyamines on protein synthesis and on the synthesis and function of DNA and RNA suggest a causal relationship between the increase in polyamine levels and some aspects of cell growth.[1] One way to investigate the existence of such a relationship is to block the synthesis of polyamines and to determine if this inhibits any phase of cell growth.

The biosynthesis of polyamines in mammalian tissues proceeds through a number of enzymatic reactions. The decarboxylation of L-ornithine is catalyzed by ornithine decarboxylase, a pyridoxal phosphate requiring enzyme, to produce 1,4-butanediamine (putrescine).[2] *N*-(3-Aminopropyl)-1,4-butanediamine (spermidine) is formed from putrescine by two different enzymes, the putrescine-activated *S*-adenosyl-L-methionine decarboxylase and spermidine synthase.[3] The enzyme spermine synthase catalyzes the formation of *N*,*N*′-bis(3-aminopropyl)-1,4-butanediamine (spermine) from spermidine and decarboxylated *S*-adenosyl-L-methionine.

Several inhibitors of the enzymes involved in polyamine biosynthesis have been reported. Inhibitors of ornithine decarboxylase include α-methyl-(±)-ornithine[4,5] (α-MeOrn), (±)-5-amino-2-hydrazinopentanoic acid,[6] (±)-5-amino-2-hydrazino-2-methylpentanoic acid,[7] (±)-(*E*)-2,5-diamino-2-pentenoic acid,[8] and *N*-(5′-phosphopyridoxyl)ornithine.[9] Methylglyoxal bis(guanylhydrazone) (MGBG) is a potent competitive inhibitor of the putrescine-stimulated *S*-adenosyl-L-methionine decarboxylase in mammalian tissues and apparently acts by blocking the allosteric activating site for putrescine.[10] 1,1′-(Methylethanediylidenedinitrilo)bis(3-aminoguanidine), an analogue of MGBG, is also a potent irreversible inhibitor of mammalian and yeast *S*-adenosyl-L-methionine decarboxylase.[11]

Several workers have used some of the compounds mentioned above to inhibit the biosynthesis of polyamines. Fillingame and Morris[12] used low concentrations of MGBG to block the increase in spermidine and spermine levels normally observed in bovine lymphocytes stimulated with concanavalin A. This treatment did not affect the synthesis or accumulation of RNA but it did decrease the incorporation of [^{3}H]thymidine into DNA.[13] The lowering of [^{3}H]thymidine incorporation induced by MGBG was confirmed by Otani et al.[14] in phytohemagglutinin stimulated cells from lymph nodes of guinea pigs. There are a few studies on the effects of inhibitors of ornithine decarboxylase on cell growth. Inoue et al.[15] reported a decrease in DNA synthesis in mouse parotid glands stimulated with isoproterenol after treating the mice with (±)-5-amino-2-hydrazinopentanoic acid. However, Harik et al.[16] observed no decrease in [^{3}H]thymidine incorporation into DNA of rat hepatoma cells after exposure to (±)-5-amino-2-hydrazinopentanoic acid.

To further investigate the effects of inhibitors of polyamine biosynthesis on cell growth, we carried out the studies presented in this report. We determined the effects of α-methyl-(±)-ornithine on cellular polyamine levels and cell growth of L1210 leukemic cells of mice grown in culture and compared these effects with those produced by methylglyoxal bis(guanylhydrazone).



α-MeOrn MGBG

Results and Discussion

The rate of growth of the L1210 leukemic cells in culture was determined by measuring the DNA content of the cell suspension because the number of cells and protein content of the cultures correlated linearly with the DNA content in the presence or absence of MGBG or α-MeOrn (results not presented). The control cells divided logarithmically during these experiments and the doubling time for the

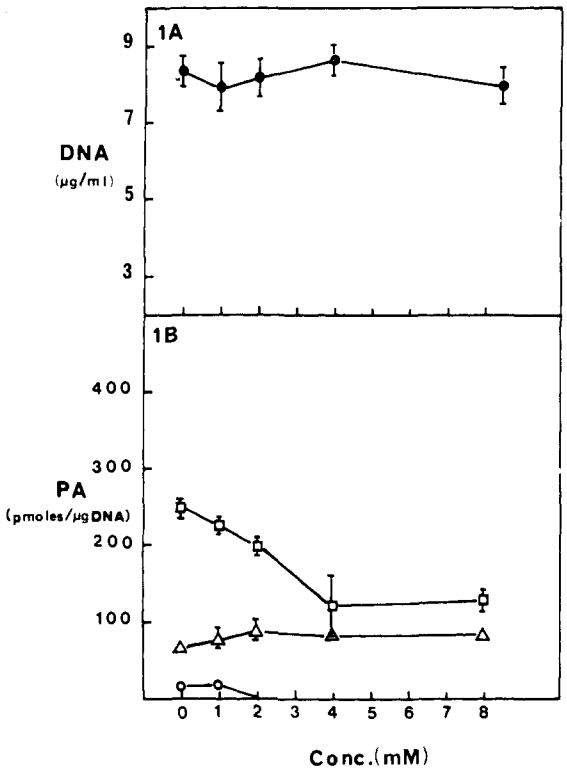


Figure 1. Effects of α-methyl-(±)-ornithine on the DNA concentration (1A) and polyamine levels (1B) of suspensions of L1210 leukemic cells of mice: DNA, concentrations of DNA in the cell suspension (μg/ml); PA, polyamine levels expressed as picomoles of the polyamine/μg of DNA in the cell suspension; Conc., concentration of α-methyl-(±)-ornithine (mM) in the cell suspension; ●, DNA; ○, putrescine; □, spermidine; and △, spermine. Each point represents the mean of determinations from three separate cultures. The vertical bars represent the ±SD. Where the SD is very small, the vertical bars are not shown.

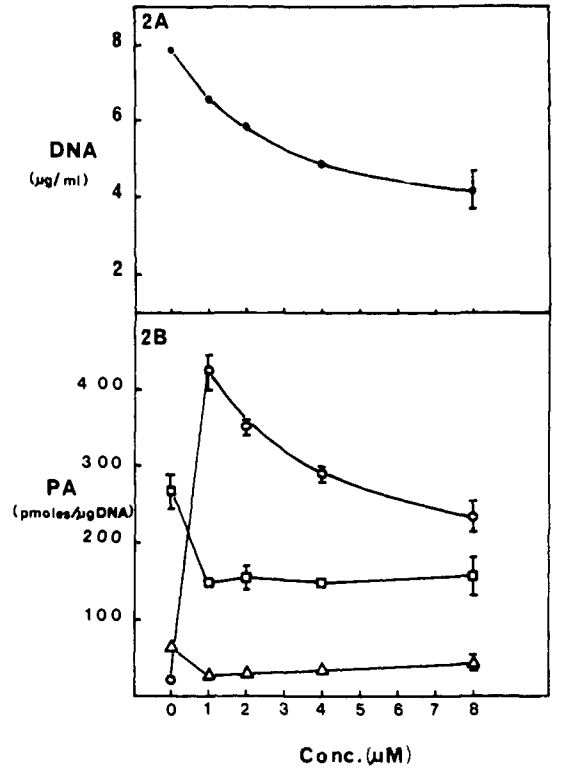


Figure 2. Effects of methylglyoxal bis(guanylhydrazone) on the DNA concentration (2A) and polyamine levels (2B) of suspensions of L1210 leukemic cells of mice: Conc., concentration of methylglyoxal bis(guanylhydrazone) (μM) in the cell suspension. See Figure 1 for explanation of symbols and other abbreviations.

untreated cells was about 0.56 day which is similar to the previously reported doubling time of 0.6 day.[17] Cell viability determined by the trypan blue dye-exclusion technique was greater than 95%. None of the concentrations of drugs tested caused an increase in the number of nonviable cells.

The polyamine concentrations were determined by a sensitive and specific method developed in our laboratory.[18] The perchloric acid extracts of the cells were treated with 5-(dimethylamino)-1-naphthalenesulfonyl chloride (dansyl chloride) and the dansyl derivatives of the polyamines were analyzed by high-pressure liquid chromatography on a microparticle bonded phase column with a fluorescence detector. The polyamine levels are reported as the ratios of the amount of polyamine (picomoles) to the amount of DNA (micrograms) in an aliquot of cells. This ratio reflects the cellular concentration of the amines and allows the comparison of the cellular levels of the amines in cell cultures with different numbers of cells.

Incubation of L1210 cells in the presence of relatively high concentrations of α-MeOrn (1–8 mM) produced a decrease in the cellular levels of putrescine and spermidine but a slight increase in the levels of spermine (Figure 1B). Concentrations of 2 mM or more of α-MeOrn decreased putrescine levels so that they were not detectable. Spermidine levels decreased from 258 ± 19 pmol/μg of DNA in the absence of the inhibitor to 122 ± 42 pmol/μg of DNA in the presence of 4 mM α-MeOrn. A further increase in the concentration of the inhibitor caused no greater decrease in spermidine levels. Although the increase in spermine produced by α-MeOrn was small, it occurred in all experiments in which α-MeOrn was added to the cell culture. It was necessary to ensure that this apparent rise in spermine levels was not due to a derivative of α-MeOrn with the same chromatographic mobility as spermine. Therefore, during the high-pressure liquid chromatographic analysis of selected samples, the column eluates corresponding to the fluorescent peak of the dansylspermine were collected. These fractions were concentrated and the residues were examined by two-dimensional TLC on silica gel plates. All the samples examined contained only one fluorescent component with a chromatographic mobility identical with that of dansylspermine.

The effects of α-MeOrn on the cellular polyamine levels of L1210 cells in culture were not accompanied by a decrease in the rate of growth of these cells since there was no decrease in the concentration of DNA in the cell culture (Figure 1A).

Addition of MGBG (1–8 μM) to L1210 cells in culture produced a concentration-dependent decrease in the number of cells as indicated by a decrease in the concentration of DNA in the cell suspension (Figure 2A). MGBG also altered the cellular levels of polyamines in the following way. The levels of putrescine were increased 16-fold at the lowest concentration of MGBG used (1 μM) and fell progressively with higher concentrations of this compound (Figure 2B). This finding confirms reports of other workers.[12,14] MGBG at a 1-μM concentration produced approximately a 50% decrease in the cellular levels of spermidine and spermine. The decrease in the levels of the amines was maximal at this low concentration of MGBG and did not decrease further to parallel the progressive decrease in the DNA content of the cell suspension observed at the higher concentrations of MGBG.

Decreases in the total pool of polyamine nitrogen do not seem to be important in controlling cell growth as α-MeOrn (4 mM) produced a 30% decrease in total polyamine nitrogen with no decrease in DNA synthesis. Furthermore, in the presence of the highest concentration of MGBG (8 μM) the total amount of nitrogen in the polyamine pool (1.06 ± 0.10 nequiv of N/μg of DNA) was similar to that in the untreated cells (1.08 ± 0.05 nequiv of N/μg of DNA) although there was a large decrease in the DNA content of the cell suspensions.

A number of conclusions can be reached from the above results. It appears that at least 50% of the cellular spermidine and virtually all of the putrescine are unnecessary for growth of L1210 leukemia cells in culture. It also seems that the inhibition of growth of the L1210 cells produced by MGBG is not necessarily related to the effects of this compound on polyamine biosynthesis because the inhibition of cell growth did not parallel the effects of this compound on spermidine or spermine synthesis.

Our results do not exclude a causal relationship between cellular levels of polyamines and cell growth because the amines were not eliminated even with high concentrations of the inhibitors. The levels of spermidine with both α-MeOrn and MGBG and the levels of spermine with MGBG reached a minimum of about 50% of control levels and were not lowered further by higher concentrations of the inhibitors. The residual polyamines may be sufficient to support growth of L1210 cells in culture in the presence of α-MeOrn, and the inhibition of cell growth by MGBG in the presence of the remaining amines may be through a mechanism other than inhibition of the biosynthesis of polyamines.

The results presented here support those of Harik et al.[6] They reported that 5-amino-2-hydrazinopentanoic acid depressed putrescine levels in rat hepatoma cells and in liver remnants of partially hepatectomized rats but produced no effect on [^{3}H]thymidine incorporation into DNA of the rat hepatoma cells or on the incorporation of [^{14}C]orotic acid into RNA of the cells of the liver remnants. However, the levels of spermidine and spermine were not determined in this study, and it is not known if the inhibitor depressed these amines. In contrast, recently, Mamont et al.[19] reported that [^{3}H]thymidine incorporation into DNA of rat hepatoma cells and the number of cells in a culture were both decreased by incubation with α-methyl-(±)-ornithine. The difference between the results reported by Mamont et al. and those reported here could be attributed to differences between the cells used. For instance, the inhibitor produced a greater decrease in cellular spermidine levels in the rat hepatoma cells than in the L1210 cells.

Several workers have reported a correlation between an increase in the ratio of spermidine to spermine and increased growth.[20,21] In the present studies, such a correlation was not observed (Figure 3). The ratio of spermidine to spermine was greatly decreased by α-MeOrn which did not decrease cell growth and was affected only very slightly by MGBG which did inhibit cell growth.

Fillingame et al.[12,13] and Otani et al.[14] were able to reverse the effects of MGBG on DNA synthesis by adding spermidine to the cell culture. This reversal was assumed to be evidence that intracellular spermidine levels lowered by MGBG were being restored to normal levels. The reversal of the effects of MGBG on cell growth by adding spermidine or spermine was not attempted in the present studies as these amines have several effects on cells which would make the results difficult to interpret. Field et al.[22]

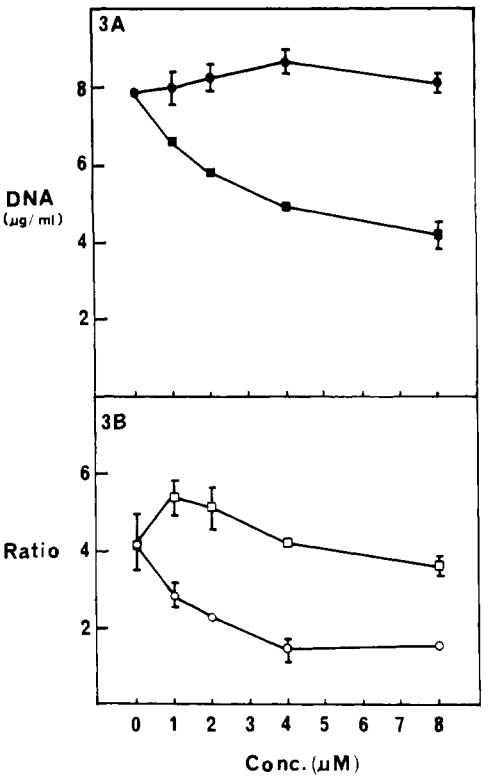


Figure 3. Comparison of the effects of α-methyl-(±)-ornithine and methylglyoxal bis(guanylhydrazone) on the concentration of DNA (3A) and on the ratio of spermidine to spermine (3B) in a suspension of L1210 leukemic cells of mice: DNA, concentrations of DNA in the cell suspension (μg/ml); Ratio, the ratio of spermidine to spermine in the cell suspension; Conc., concentration of α-methyl-(±)-ornithine (mM) or methylglyoxal bis(guanylhydrazone) (μM) in the cell suspension; ●, DNA concentration, and ○, ratio of spermidine to spermine in cell suspensions treated with α-methyl-(±)-ornithine; □, DNA concentration, and □, ratio of spermidine to spermine in cell suspensions treated with methylglyoxal bis(guanylhydrazone). Each point represents the mean of determinations from three separate cultures. The vertical bars represent the ±SD. Where the SD is very small, the vertical bars are not shown.

and Dave et al.[23] reported that spermidine and MGBG compete for uptake into several types of cells including L1210 leukemic cells of mice. High levels of spermidine added to the cell culture would be expected to decrease the effects of MGBG on cell growth by preventing its uptake into cells. Furthermore, addition of spermidine and spermine to cultured cells decreased the activity of ornithine decarboxylase in 3T3 cells,[24] in rat hepatoma cells,[25] and in human lymphocytes stimulated with phytohemagglutinin.[26] We likewise observed a decrease in ornithine decarboxylase activity of L1210 cells incubated with spermidine for short times. After 2 h of incubation of freshly diluted cells with 0.1 mM spermidine, the enzyme activity was 20% of control. Incubation of L1210 cells in the presence of 0.2 mM spermidine for 25 h resulted in a decrease of the cellular putrescine level so that it was not detectable.

Conclusions

α-Methyl-(±)-ornithine produced inhibition of polyamine biosynthesis in L1210 leukemic cells in culture. This inhibition of polyamine biosynthesis was not accompanied by inhibition of growth of these cells indicating that a large portion of the polyamines is not essential for cellular growth. These results, however, do not exclude a causal relationship between cellular levels of polyamines and cell growth since of the three amines studied only putrescine

was virtually eliminated. Spermidine levels were decreased to about 55% of basal levels and spermine was increased slightly. These residual levels could be sufficient to support cell growth. A comparison of the effects of α-MeOrn and MGBG on cellular polyamine levels and cellular growth suggests that MGBG produces its effect on cell growth through mechanisms other than inhibition of polyamine synthesis and underscores the need for a continued effort to produce potent and specific blockers of polyamine biosynthesis and especially inhibitors of spermidine biosynthesis.

Experimental Section

α-Methyl-(±)-ornithine monohydrochloride monohydrate was synthesized in our laboratory.[4] Methylglyoxal bis(guanylhydrazone) was obtained from Aldrich Chemicals (Milwaukee, Wis.). Fischer's medium for leukemic cells of mice (modified, 10×), horse serum, penicillin G sodium (U.S.P.), and streptomycin sulfate (U.S.P.) were obtained from Grand Island Biological Co. (Grand Island, N.Y.). Spermidine trihydrochloride, pyridoxal phosphate, and DNA from calf thymus were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cell Culture. L1210 leukemic cells were maintained in culture in Fischer's medium for leukemic cells of mice containing 10% horse serum, 13.4 mM sodium bicarbonate, 50 units/ml of penicillin G, and 50 μg/ml of streptomycin sulfate. When the cells reached a density of 10^6 cells/ml, 1 ml of cell suspension was transferred to 50 ml of fresh medium. The culture flasks were capped tightly and maintained at 37 °C. For experiments in which the effects of α-MeOrn or MGBG on polyamine and DNA levels were determined, the cells were diluted in fresh medium to a density of approximately 0.2×10^6 cells/ml. Aliquots of 4.4 ml of the suspension were placed in 15 × 100 mm sterile, disposable glass test tubes. Appropriate volumes of α-MeOrn, MGBG spermidine, or water (for control) were added to the cells. The solutions had been very carefully adjusted to the same pH as the Fischer's medium and sterilized by passing through a millipore filter. The tubes were capped tightly with a Teflon stopper and incubated at 37 °C for 20–24 h. All the above procedures were performed in a sterile transfer hood. To terminate the experiments, the cells were resuspended and two aliquots of 2 ml each were removed from each tube and placed in 12 × 75 mm test tubes. The tubes were centrifuged at 1000g for 5 min at room temperature. The supernatant solution was discarded and the cells in one tube were used for the determination of polyamines and those in the second tube for the determination of DNA.

Determination of Polyamines.[18] A 300-μl aliquot of 0.3 N perchloric acid was added to the cells to extract the polyamines. The tubes were centrifuged and the supernatant was stored at −20 °C until analysis. A 100-μl aliquot of the supernatant was transferred into a disposable glass test tube (16 × 125 mm) and diluted with 100 μl of water. Standard solutions for the calibration curve were prepared by mixing 100 μl of one of five standard solutions of polyamines, which contained various concentrations of putrescine, spermidine, and spermine in water, with 100 μl of 0.3 N perchloric acid. The following were added to each tube: 100 μl of a saturated solution (room temperature) of sodium carbonate, 20 μl of a 0.2 mM solution of propane-1,3-diamine dihydrochloride (internal standard), and 600 μl of a solution of 7 mg/ml of dansyl chloride in acetone. The tubes were stoppered with plastic caps and shaken gently overnight at room temperature in the dark. The tubes were immersed in a water bath at 40 °C and the liquid in each was evaporated in a stream of nitrogen. The residue in each tube was mixed vigorously with 0.5 ml of water and 5 ml of benzene for 20 s with a vortex mixer. The tubes were centrifuged at 1000g for 2 min and the benzene layer was transferred to a 12-ml conical centrifuge tube. The tubes were immersed in a water bath at 40 °C and the benzene was evaporated in a stream of nitrogen. The residue (dansylpolyamines) was stored at −20 °C for no more than 4 days.

High-pressure liquid chromatography was performed on a system composed of a U6K injector, two 6000 solvent delivery systems, a 660 solvent programmer (Waters Associates, Inc., Milford, Mass.), a LDC Model 1209 fluoromonitor (Laboratory Data Control, Inc., Riviera Beach, Fla.), and a two channel strip chart recorder (Honeywell, Fort Washington, Pa.). The separation of the dansylpolyamines was carried out on a Micropak CN-10 column (25 × 2.5 mm, Varian, Palo Alto, Calif.) with a solvent composed of cyclohexane–2-propanol (49:1) as solvent A and cyclohexane-methylene chloride–2-propanol (21:3:1) as solvent B. The residues of the crude dansyl derivatives obtained above were dissolved in 50 μl of methylene chloride and 5–10 μl of the solution was injected into the system. The sample was eluted in the isocratic mode with solvent A for 5 min and then with a programmed solvent gradient elution using the concave gradient curve number 7. The gradient changed from 100% of solvent A to 100% of solvent B in 15 min at a flow rate of 3 ml/min. Each sample was eluted in a total of 22 min and then the column was allowed to reequilibrate with solvent A for 3 min before a second sample was injected.

For selected samples, the column eluate corresponding to the fluorescent peak for dansylspermine was collected and concentrated. The residues were examined by two-dimensional TLC on silica gel GF plates (20 × 20 cm, 250 μ, Analtech, Inc., Newark, Del.) with a solvent of chloroform–triethylamine (12.5:1) in one dimension and ethyl acetate–cyclohexane (1:1) in a second dimension.

The areas of the chromatographic peaks were estimated by the triangulation method. The ratios of the areas of the peaks for each of the amines to that of the internal standard were calculated. The correlation curves were obtained by regression analysis to determine the line of best fit for the data points. The smallest detectable amounts of the amines are approximately 40 pmol of spermidine and spermine.

Determination of DNA. The cells in the second tube were washed twice with a basic salt solution buffered with Tris–HCl (pH 7.4) which had been chilled in ice.[27] The DNA was precipitated by the addition of 0.5 ml of 5% trichloroacetic acid and the tubes were centrifuged. The supernatant solutions were discarded and a 0.3-ml aliquot of 5% trichloroacetic acid was added to the pellet in each tube. The DNA was hydrolyzed by placing the tubes in a boiling water bath for 15 min. A 0.1-ml aliquot of the hydrolysate was analyzed for DNA by complexing with diphenylamine using the method of Schneider.[28] Calf thymus DNA was used as a standard.

Effects of Spermidine on Ornithine Decarboxylase Activity. L1210 leukemic cells were suspended at a concentration of 10^6 cells/ml in fresh medium. The suspension was incubated at 37 °C for 1 h and then spermidine was added to a final concentration of 0.1 mM. Aliquots (10 ml) of the cell suspension were removed immediately after the addition of spermidine (control), after 1 h, and after 2 h. These aliquots were centrifuged, the supernatant solutions were discarded, and 1.2-ml aliquots of a solution of the following composition were added to the cells: Na_2HPO_4, 15.4 mM; KH_2PO_4, 9.6 mM (pH 7.0); dithiothreitol, 5 mM; and pyridoxal phosphate, 0.2 mM. The cell suspensions were frozen and thawed three times to lyse the cells. The tubes were next centrifuged at 1000g for 30 min. A 1.0-ml portion of the resulting supernatant solution was removed for analysis of the activity of ornithine decarboxylase. The reaction was initiated by adding 0.6 μCi of [1-^{14}C]ornithine to the solution and was carried out as described previously.[2]

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References and Notes

(1) U. Bachrach, "Function of Naturally Occurring Polyamines," Academic Press, New York, N.Y., 1973, pp 47–95.
(2) A. E. Pegg, *Ann. N.Y. Acad. Sci.*, 171, 977 (1970).
(3) A Raina, J. Jänne, P. Hannonen, E. Höltta, and J. Ahonen in "Polyamines in Normal and Neoplastic Growth", D. H. Russell, Ed., Raven Press, New York, N.Y., 1973, p 167.
(4) M. M. Abdel-Monem, N. E. Newton, and C. E. Weeks, *J. Med. Chem.*, 17, 447 (1974).
(5) J. J. Ellington and I. L. Honigberg, *J. Org. Chem.*, 39, 104 (1974).
(6) S. I. Harik and S. H. Snyder, *Biochim. Biophys. Acta*, 327, 501 (1973).

(7) M. M. Abdel-Monem, N. E. Newton, and C. E. Weeks, *J. Med. Chem.*, 18, 945 (1975).
(8) N. Relyea and R. R. Rando, *Biochem. Biophys. Res. Commun.*, 67, 392 (1975).
(9) J. S. Heller, E. S. Canellakis, D. L. Bussolotti, and J. K. Coward, *Biochim. Biophys. Acta*, **403**, 197 (1975).
(10) H. G. Williams-Ashman and A. Schenone, *Biochem. Biophys. Res. Commun.*, **46**, 288 (1972).
(11) A. E. Pegg and C. Conover, *Biochem. Biophys. Res. Commun.*, **69**, 766 (1976).
(12) R. H. Fillingame and D. R. Morris, *Biochemistry*, 12, 4479 (1973).
(13) R. H. Fillingame, C. M. Jorstad, and D. R. Morris, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 4042 (1975).
(14) S. Otani, Y. Mizoguchi, I. Matsui, and S. Morisawa, *Mol. Biol. Rep.*, 1, 431 (1974).
(15) H. Inoue, Y. Kato, M. Takigawa, K. Adachi, and Y. Takeda, *J. Biochem.*, 77, 879 (1975).
(16) S. I. Harik, M. D. Hollenberg, and S. H. Snyder, *Nature (London)*, **249**, 250 (1974).
(17) G. J. Dixon, E. A. Dulmadge, and F. M. Schabel, Jr., *Cancer Chemother. Rep.*, **50**, 247 (1966).
(18) N. E. Newton, K. Ohno, and M. M. Abdel-Monem, *J. Chromatogr.*, **124**, 277 (1976).
(19) P. S. Mamont, P. Böhlen, P. P. McCann, P. Bey, F. Schuber, and C. Tardif, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1626 (1976).
(20) D. H. Russell, *Cancer Res.*, **32**, 2459 (1972).
(21) O. Heby, L. J. Marton, C. B. Wilson, and H. M. Martinez, *FEBS Lett.*, **50**, 1 (1975).
(22) M. Field, J. B. Block, V. T. Oliverio, and D. P. Rall, *Cancer Res.*, **24**, 1939 (1964).
(23) C. Dave and L. Caballes, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **32**, 736 (1973).
(24) J. L. Clark and J. L. Fuller, *Biochemistry*, 14, 4403 (1975).
(25) T. C. Theoharides and Z. N. Canellakis, *Nature (London)*, **255**, 733 (1975).
(26) J. E. Kay and K. J. Lindsay, *Biochem. J.*, **132**, 791 (1973).
(27) C. B. Favour in "Immunological Methods", J. F. Ackroyd, Ed., F. A. Davis Co., Philadelphia, Pa., 1964, p 195.
(28) W. C. Schneider, *Methods Enzymol.*, **3**, 680 (1957).

Synthesis of [1-Sarcosine,8-*O*-methylserine]angiotensin II and 1-Substituted Analogues of [8-Threonine]angiotensin II as Antagonists of Angiotensin II[1]

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[1-*N*-methylisoleucine,8-threonine]- (I), [1-dimethylglycine,8-threonine]- (II), [1-guanidineacetic acid,8-threonine]- (III), des-1-aspartic acid-[8-threonine]- (IV), and [1-sarcosine,8-*O*-methylserine]angiotensin II (V) were synthesized by Merrifield's solid-phase procedure to study the effect of (a) substituents in position 1 on the antagonistic activity of [1-sarcosine,8-threonine]angiotensin II, and (b) a change in size and branching in position 8 of [1-sarcosine,-8-*O*-methylthreonine]angiotensin II. The analogues I–V caused an initial rise in blood pressure (30 min of infusion, 250 ng/kg/min in vagotomized ganglion-blocked rats) of 8.05, 11.7, 3.50, 4.5, and 11.16 mmHg. The pA_2 values (rabbit aortic strips) obtained were 7.68, 7.53, 7.23, 7.53, and 9.66, and the dose ratios (in vagotomized ganglion-blocked rats infused at 250 ng/kg/min) obtained were 2.37, 4.49, 1.02, 1.47, and 24.04, respectively. The results obtained indicate that (a) the nature of the substituent in position 1 has an important influence on the biological activity of these peptides, and (b) the potency of antagonists I–IV (all less potent antagonists than [1-sarcosine,8-threonine]angiotensin II) is very much influenced by the length and branching of the side chain in position 8. The in vivo antagonistic activity of [1-sarcosine,8-*O*-methylthreonine]angiotensin II is reduced considerably by shortening the chain length by one carbon atom as is in V.

We have previously reported that all known antagonists of the pressor action of angiotensin II, e.g., [Sar[1],Ala[8]]- and [Sar[1],Ile[8]]angiotensin II, cause an initial agonist activity which is equal to 1–2% of that of the parent hormone.[2,3] In continuation with our earlier work,[3] the present investigation is an attempt to find potent antagonists of the pressor and myotropic activity of angiotensin II having no or low agonist activity. Comparative infusion studies in rats with several analogues indicate that [Sar[1],Thr[8]] angiotensin II[4] shows the lowest agonist to antagonist ratio,[5] while [Sar[1],Thr(Me)[8]]angiotensin II is the most potent antagonist thus far synthesized.[3] In order to study the effect of substituents in the one position on the antagonistic activity of [Sar[1],Thr[8]]angiotensin II, we report the synthesis of some analogues in which position 1 has been replaced with a hydrogen atom, *N*-methylisoleucine, dimethylglycine, or guanidineacetic acid. Similarly, [Sar[1],Ser(Me)[8]]angiotensin II has been synthesized to study the effect of a change in size and branching in an ether-linked side chain in position 8.

All the analogues reported in this paper have been synthesized by the solid-phase procedure.[6] Determinations of the pressor activity[7] of the analogues as compared to angiotensin II (expressed as percent) and the comparative antagonistic activity [2] (expressed as dose ratio) have been carried out on vagotomized ganglion-blocked rats, as are the infusion studies[2] to determine the initial pressor activity (expressed as mmHg). Inhibition of contractile activity of angiotensin II has been studied on isolated spirally cut rabbit aortic strips (expressed as pA_2 values).[4,8,9]

Results and Discussion

Comparative initial agonist properties (bolus injection in rats) (Table I) indicate that the replacement of position 1 in [Sar[1],Thr[8]]angiotensin II with a hydrogen atom, guanidineacetic acid, dimethylglycine, or *N*-methylisoleucine reduces the initial pressor activity. Similar results