raise (Fig. 1 and Table 1) or lower (*12*) blood pressure, depending on the animal's starting blood pressure, affirms the hypothesis that the ability of tyrosine to accelerate catecholamine synthesis depends on the firing frequency of catecholamine-producing cells. In hypertensive rats, it seems likely that noradrenergic neurons in the brainstem fire frequently and that sympathetic outflow from more peripheral neurons is diminished (*12*); in hypotensive animals, this situation is probably reversed. If tyrosine exerts a similar state-dependent effect on blood pressure in humans, it may have advantages over catecholamine infusions in the treatment of shock.

LYDIA A. CONLAY
Laboratory of Neuroendocrine Regulation, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge 02139, and *Department of Anesthesia, Massachusetts General Hospital, Boston 02114*
TIMOTHY J. MAHER
Laboratory of Neuroendocrine Regulation, Massachusetts Institute of Technology, and *Massachusetts College of Pharmacy and Allied Health Sciences, Boston 02115*
RICHARD J. WURTMAN
Laboratory of Neuroendocrine Regulation, Massachusetts Institute of Technology

References and Notes

1. H. A. J. Struyker, G. M. W. Smeets, G. M. Brower, J. N. van Rossu, *Neuropharmacology* **13**, 837 (1974).
2. W. DeJong, P. Zandberg, B. Bohus, *Prog. Brain Res.* **42**, 285 (1975); W. DeJong, *Eur. J. Pharmacol.* **29**, 179 (1974); _____ and F. P. Nijkamp, *Br. J. Pharmacol.* **58**, 593 (1976).
3. J. P. Chalmers and R. J. Wurtman, *Circ. Res.* **28**, 480 (1971); J. P. Chalmers and J. L. Reid, *ibid.* **31**, 789 (1972).
4. M. J. Allwood, A. F. Cobbold, J. Ginsburg, *Br. Med. Bull.* **19**, 132 (1963).
5. R. J. Wurtman, F. Larin, S. Mostafapour, J. D. Fernstrom, *Science* **185**, 183 (1974); A. Carlsson and M. Lindqvist, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **303**, 157 (1978).
6. R. A. Alonzo, J. C. Agharanus, R. J. Wurtman, *J. Neural Transm.*, in press. Tyrosine caused a dose-dependent increase in urinary catecholamine excretion which was blocked by treatment with carbidopa; tyrosine also caused a fourfold increase in urinary dopa.
7. A. Alousi and N. Weiner, *J. Physiol. (London)* **56**, 1491 (1966); H. Thoenen, R. A. Mueller, J. Axelrod, *J. Pharmacol. Exp. Ther.* **169**, 249 (1969).
8. C. J. Gibson and R. J. Wurtman, *Life Sci.* **22**, 1399 (1978).
9. E. Melamed, F. Hefti, R. J. Wurtman, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4305 (1980).
10. M. C. Scally, I. Ulus, R. J. Wurtman, *J. Neural Transm.* **41**, 1 (1977).
11. A. F. Sved, J. D. Fernstrom, R. J. Wurtman, *Life Sci.* **25**, 1293 (1979).
12. _____, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3511 (1979); M. R. Bresnahan, P. Hatzinikolauo, H. R. Brunner, H. Gavras, *Am. J. Physiol.* **239**, H206 (1980).
13. N. A. Scott, R. A. DeSilva, B. Lown, R. J. Wurtman, *Science* **211**, 727 (1981).
14. Heparin (400 U/g) was then administered intraarterially.
15. Intraarterial blood pressure was recorded with a Grass model 70 polygraph and Statham transducers P23DC and P23AC.
16. Blood volume was estimated as 55 ml/kg [H. Donaldson, *The Rat* (Wistar Institute, Philadelphia, 1924)].
17. The other amino acids were L-tyrosine methylester HCl, L-tryptophan methylester HCl, L-valine, and L-leucine; all solutions were adjusted to *p*H 5.0.
18. One hour after adrenalectomy, blood pressure had not changed significantly ($P < .2$).
19. W. H. Oldendorf, *Am. J. Physiol.* **221**, 1629 (1971).
20. Supported in part by NIH grant AM-14228, NASA grant NGR-22-009-627, the Center for Brain Sciences, and the Metabolism Charitable Trust. L.A.C. is supported by National Research Training Award GM-07592.

8 November 1980; revised 16 December 1980

Cadmium-113 Nuclear Magnetic Resonance Studies of Bovine Insulin: Two-Zinc Insulin Hexamer Specifically Binds Calcium

Abstract. *By use of cadmium-113 nuclear magnetic resonance spectroscopy, a specific calcium ion binding site has been identified in the bovine two-zinc insulin hexamer. This site is composed of six glutamyl carboxylate groups clustered in the center of the hexamer, and is distinct from the normal zinc ion binding sites.*

We present here results of ^{113}Cd nuclear magnetic resonance (NMR) experiments which provide evidence that Ca^{2+} binds strongly to a specific site in the central core of the bovine two-zinc insulin hexamer. The Ca^{2+} site is composed of six glutamyl carboxylate groups (Glu B-13) clustered in the center of the hexamer, and is distinct from the normal Zn^{2+} binding sites (*1*). X-ray crystallographic studies of porcine zinc insulin by Blundell *et al.* (*2*) showed that the hexamer is torus-shaped (~ 50 Å in diameter by 35 Å high). The two Zn^{2+} ions per hexamer are located 17 Å apart on the threefold symmetry axis, which traverses the central cavity. Each Zn^{2+} ion is coordinated to three imidazolyl nitrogen atoms (from His B-10) and three water molecules (see Fig. 1). The crystallographically identified Zn^{2+} sites (*2*) are designated site I, and the Ca^{2+} site proposed here is designated site II (Fig. 1).

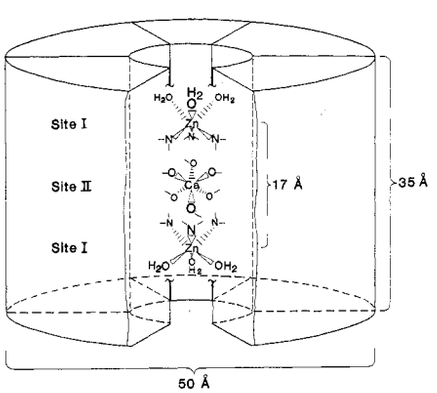

Fig. 1. Three-dimensional structural representation of the zinc insulin hexamer, showing the proposed Ca^{2+} binding site (site II). Ligation about Zn^{2+} ions (3 His, 3 H_2O, site I) is based on the x-ray crystal structure (*1*). The experiments represented in Fig. 2 are consistent with a single site for Ca^{2+}, but do not exclude exchange among several identical sites (that is, sites composed by pairing the carboxylates). The insulin monomer facing the viewer has been cut away to show the positions and liganding of the metal ion sites.

Synthesized in the beta cells of the pancreatic islets of Langerhans, insulin is stored in secretory granules. Under the electron microscope, immature granules appear to contain insulin in an amorphous state. In mature granules, insulin forms a dense array of 50-Å units, with packing similar to that found in the crystalline hexamer (*3*). Because of the high content of zinc in pancreatic islets and the requirement for divalent metals in forming rhombohedral crystals, it has been proposed that the two-zinc insulin hexamer, $(Zn^{2+})_2(Ins)_6$, is the storage form of insulin (*2*).

Substitution of $^{113}Cd^{2+}$ ions for naturally occurring divalent metal ions and direct observation of the ^{113}Cd NMR signal has proved useful in the study of metalloproteins (*4–7*). Cadmium-113 chemical shifts depend strongly on the number and type of ligands coordinated to the metal ion.

The ^{113}Cd NMR spectrum of ^{113}Cd-substituted bovine insulin hexamer at *p*H 8.0 (Fig. 2A) exhibits two resonances of relative area 2:1. Peak I has a chemical shift of 165 ppm and peak II occurs at −36 ppm. When $^{113}Cd^{2+}$ is added in small increments from an initial ratio of 0.3 equivalent per hexamer to a final ratio of 3.0, the resulting ^{113}Cd NMR spectra (not shown) have the same chemical shifts and 2:1 relative area. When this species, which is designated $(^{113}Cd^{2+})_3(Ins)_6$, was titrated from *p*H 8.0 to *p*H 10.4 by gradual addition of NaOH, peak I underwent a downfield shift, which leveled off at 201 ppm. Peak II was unaffected. Least-squares fitting of peak I chemical shifts versus *p*H to a sigmoidal curve gave an apparent pK_a value of 8.7. We propose that this *p*H dependence is caused by conversion of metal-bound water to hydroxide ion (*8*). Gradual addition of NaCl produced a

0036-8075/81/0501-0560$00.50/0

downfield shift of peak I, which reached 177 ppm at 1.0*M* NaCl, without affecting peak II.

The chemical shift of peak I is consistent with Cd^{2+} bound to a mixture of nitrogen and oxygen ligands. This fact, plus the responsiveness to *p*H and Cl^-, lead us to assign peak I to Cd^{2+} bound to the crystallographically identified zinc sites (site I in Fig. 1). Because of the upfield chemical shift of peak II, the relative peak area of 2:1, and the insensitivity to *p*H and Cl^-, we suspected that peak II involves coordination of Cd^{2+} through oxygen ligands to a single site. Each Ca^{2+} site in parvalbumin (two sites) and concanavalin A (one site) involves hexacoordination through oxygen ligands, and when occupied by $^{113}Cd^{2+}$ gives resonances at −94, −98, and −132 ppm (*6, 7*).

In the insulin x-ray crystal structure (*2*), six carboxylate groups from Glu B-13 residues converge at the center of the two-zinc hexamer. Blundell *et al.* (*2*) mention a crystalline hexamer containing 3 equivalents of Cd^{2+}. Patterson and Fourier maps indicated that this $(Cd^{2+})_3(Ins)_6$ is isomorphous with $(Zn^{2+})_2(Ins)_6$. Two of the Cd^{2+} ions occupy the normal (His B-10) zinc binding sites, and the third Cd^{2+} is located "in the region of the B-13 glutamate residues" in the center of the molecule (*2*). The UO_2^{2+} and Pb^{2+} ions have also been found to bind in this region (*9, 10*). Therefore we propose that peak II in Fig. 2A arises from Cd^{2+} coordinated to the Glu B-13 residues (site II in Fig. 1).

Noting that Ca^{2+} binding sites in metalloproteins such as parvalbumin (*6*), concanavalin A (*7*), and thermolysin (*11*) consist primarily of carboxylate ligands, and that Cd^{2+} and Ca^{2+} are very similar in Pauling ionic radii (0.97 Å), we hypothesized that site II might be a specific Ca^{2+} binding site in the native insulin hexamer. As shown in Fig. 2B, addition of Ca^{2+} to $(^{113}Cd^{2+})_3(Ins)_6$ causes a decrease in the relative area of peak II. The concentrations and types of metal ions given in the legend to Fig. 2 refer to the predominant occupancy of sites I and II, and not necessarily to the total composition of ions in the sample. The first equivalent of Ca^{2+} gave a ratio of 6:1 for peaks I and II (data not shown), and the second equivalent (Fig. 2B) a ratio of 10:1. Equilibrium was fully reached in less than the spectral acquisition time of 8.5 hours. The displaced $^{113}Cd^{2+}$ does not give rise to a detectable resonance. The most likely explanation is that due to intermediate exchange rates among a number of weak binding sites on insulin (*2, 12, 13*) or buffer molecules, the resonance for the displaced $^{113}Cd^{2+}$ is too broad for detection. These experiments indicate that Ca^{2+} and Cd^{2+} have comparable affinities for site II, supporting the idea that insulin is a Ca^{2+} binding protein. On the other hand, site I strongly prefers Cd^{2+} over Ca^{2+}.

To test the divalent metal ion specificities of sites I and II, we added 2 equivalents of Zn^{2+} to $(^{113}Cd^{2+})_3(Ins)_6$ and observed the complete disappearance of peak I (Fig. 2C). Again, no resonance was observed for the displaced $^{113}Cd^{2+}$. Thus, site I strongly prefers Zn^{2+} over Cd^{2+}, and site II strongly prefers Cd^{2+} over Zn^{2+}. With ionic radius 0.7 Å, Zn^{2+} is probably too small for effective coordination to site II. The low affinity of Ca^{2+} for nitrogen ligands probably accounts for its inability to compete with Zn^{2+} and Cd^{2+} for site I.

The uniqueness of the Ca^{2+} site proposed in Fig. 1 raises mechanistic questions concerning the assembly of the hexamer and the accessibility of site II in preformed hexamers. We prepared two hexamer samples, each containing exchange-inert Co^{3+} at the His B-10 sites (site I in Fig. 1) by oxidation of the corresponding Co^{2+} derivatives. Binding of Co^{2+} to insulin has been extensively investigated (*2, 13*). Solution studies show Co^{2+} forms a $(Co^{2+})_2(Ins)_6$ species in which Co^{2+} clearly is tightly bound to site I in place of Zn^{2+}. The first sample was oxidized to Co^{3+} after the addition of 1 equivalent of $^{113}Cd^{2+}$. The ^{113}Cd NMR spectrum (Fig. 2D) shows only the presence of peak II. Addition of excess Ca^{2+} caused no decrease in the signal-to-noise ratio of peak II, even after 48 hours. The second sample was oxidized to Co^{3+} before the addition of 1 equivalent of $^{113}Cd^{2+}$. No ^{113}Cd resonance was observed even 48 hours after addition, indicating that substitution at site II must occur either before assembly of the hexamer or by removal of the "capping" metal ion or ions.

Biological implications of the Ca^{2+} binding site are not clear. Calcium is believed to be involved in the storage of insulin in the pancreas, the release of insulin into the bloodstream, and the action of insulin at target cells (*14–18*). There is evidence that Ca^{2+} is taken up by rhombohedral zinc insulin crystals (*19*). Pancreatic storage granules contain high concentrations of Ca^{2+} as well as Zn^{2+}, and $^{45}Ca^{2+}$ accumulated within the granules is not readily exchangeable (*20*). Despite the presence of normal amounts of Zn^{2+}, depletion of Ca^{2+} impedes granule maturation in cultured mouse islets (*19*). Finally, experiments

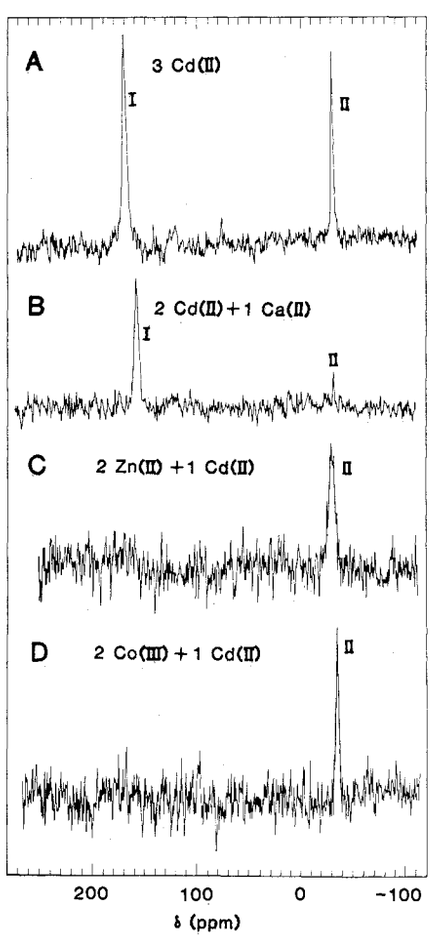

Fig. 2. Cadmium-113 Fourier transform NMR spectra of aqueous ^{113}Cd insulin derivatives in H_2O at 25°C and *p*H 8.0. Chemical shifts are relative to external 0.2*M* $CdSO_4$ in H_2O-D_2O (1:1). The number and type of metal ions given refer to the predominant occupancy of sites I and II, and not necessarily the composition of ions in the sample. (A) Spectrum of 2.6 m*M* insulin hexamer plus 7.7 m*M* $^{113}CdSO_4$. Line widths of peaks I and II are 70 and 20 Hz (total acquisition time, 12 hours). (B) Spectrum of 2.9 m*M* insulin hexamer after addition of 2 equivalents of Ca^{2+} per hexamer to a sample originally containing 3 equivalents of bound $^{113}Cd^{2+}$ (Fig. 2A). One Ca^{2+} equivalent was added before the addition of $^{113}Cd^{2+}$ and one after (total acquisition time, 8.5 hours). (C) Spectrum of 2.9 m*M* insulin hexamer after addition of 2 equivalents of Zn^{2+} to a sample originally containing 3 equivalents of bound $^{113}Cd^{2+}$ (total acquisition time, 4.5 hours). (D) Spectrum of 2.7 m*M* insulin hexamer containing 1 equivalent of Cd^{2+} and 2 equivalents of Co^{3+} per hexamer (total acquisition time, 5 hours). The sample was prepared in dilute H_2O_2 and was concentrated and washed by ultrafiltration, using a Diaflow UM-10 membrane (Amicon Corp.). Amino acid analysis of Co^{3+} peptides generated by proteolytic cleavage of $(Co^{3+})_2(In)_6$ have shown that Co^{3+} occupies the normal Zn^{2+} site. The oxidation conditions used do not alter the amino acid composition of insulin (*22*). Reagent preparation and spectral conditions are described in (*23*).

indicate that only a small fraction of the $^{65}Zn^{2+}$ taken up by cultured rat islets is associated with the storage granules (*21*). These findings, in addition to the results presented in this report, point to a more important role for Ca^{2+} in the storage of insulin than was previously supposed.

Finally, we note that the Zn^{2+} binding site (site I in Fig. 1) is not present in insulin from certain species (for instance, guinea pig and coypu); however, the carboxylate groups (either Glu or Asp) (*1*) at B-13 which form site II are conserved in all insulin species sequenced to date (*2*).

J. L. SUDMEIER
S. J. BELL
Department of Chemistry, University of California, Riverside 92521
M. C. STORM
M. F. DUNN
Department of Biochemistry, University of California, Riverside

References and Notes

1. Abbreviations: Glu, glutamic acid; His, histidine; Asp, aspartic acid; B-13 and B-10, B-chain positions 13 and 10, respectively.
2. T. Blundell, G. Dodson, D. Hodgkin, D. Mercola, *Adv. Protein Chem.* **26**, 279 (1972).
3. M. H. Greider, S. L. Howell, P. E. Lacy, *J. Cell Biol.* **41**, 162 (1969).
4. I. M. Armitage, R. T. Pajer, A. J. M. S. Uiterkamp, J. F. Chlebowski, J. E. Coleman, *J. Am. Chem. Soc.* **98**, 5710 (1976).
5. J. L. Sudmeier and S. J. Bell, *ibid.* **99**, 4499 (1977).
6. T. Drakenberg and B. Lindmann, *FEBS Lett.* **92**, 346 (1978).
7. D. B. Bailey, P. D. Ellis, J. A. Fee, *Biochemistry* **19**, 591 (1980).
8. M. F. Dunn, *Struct. Bonding (Berlin)* **23**, 61 (1975).
9. M. J. Adams *et al.*, *Nature (London)* **224**, 491 (1969).
10. D. C. Hodgkin *et al.*, in *Insulin Action*, I. B. Fritz, Ed. (Academic Press, New York, 1972), p. 1.
11. P. M. Coleman, J. N. Jansonius, B. W. Matthews, *J. Mol. Biol.* **70**, 701 (1972).
12. J. M. Summerell, A. Osmond, G. H. Smith, *Biochem. J.* **95**, 31 (1965).
13. L. W. Cunningham, R. L. Fischer, C. S. Vestling, *J. Am. Chem. Soc.* **77**, 5703 (1955).
14. M. P. Czech, *Annu. Rev. Biochem.* **46**, 359 (1977).
15. T. Clausen, in *Biochemistry of Membrane Transport*, G. Semenza and E. Carafoli, Eds. (Springer, Berlin, 1977), p. 481.
16. E. Gylfe and B. Hellman, *Biochim. Biophys. Acta* **538**, 249 (1978).
17. H. A. Pershadsingh and J. M. McDonald, *Nature (London)* **281**, 495 (1979).
18. E. G. Siegel, C. B. Wollheim, G. W. G. Sharp, *FEBS Lett.* **109**, 213 (1980).
19. S. L. Howell, M. Tyhurst, H. Duvefelt, A. Andersson, C. Hellerstrom, *Cell Tissue Res.* **188**, 107 (1978).
20. S. L. Howell, W. Montague, M. Tyhurst, *J. Cell Sci.* **19**, 395 (1975).
21. G. M. Grodsky, private communication.
22. M. C. Storm and M. F. Dunn, in preparation.
23. Bovine insulin was purchased from Sigma, and divalent metal ions were removed by Chelex-100 ion-exchange resin as previously described [M. F. Dunn, S. E. Pattison, M. C. Storm, E. Quiel, *Biochemistry* **19**, 718 (1980)]. Concentrated metal ion–free insulin solutions were prepared by dissolving lyophilized insulin in twice-distilled water. The *p*H was raised to 10.5 with NaOH to effect complete solution and was then carefully lowered to 8.00 with dilute $HClO_4$. Cadmium-113 oxide (isotopic purity, 96 percent; Oak Ridge National Laboratory) was dissolved in H_2SO_4 and neutralized with NaOH to *p*H 6.0. Stock solutions of $CoCl_2$ and $Zn(NO_3)_2$ were purchased from Alfa Products as the atomic absorption standards. The NMR spectra were obtained at ~ 19.97 MHz, using a modified multinuclear Bruker WH90D-18 (18-inch, 2.11-T magnet) with quadrature phase detection for all nuclei. The probe has a homemade insert designed for sample tubes of 15-mm outer diameter (containing 5 ml of solution) and an external D_2O field/frequency lock. The sweep width was 15 kHz, acquisition time was 0.27 second (4K real data points), and exponential multiplication producing line broadening of 10 Hz was employed. The 90° pulse was 12.5 μsec for a pulse power of 25 watts, and a flip angle of 30° was employed. No proton decoupling was used.

24. Supported by NSF grant CHE-7811548, NSF grant BNS76-20218-01, PHS grant 1R01 GM25877, American Diabetes Association grant 40660, and a grant from the UCR Committee on Research. The UCR Bruker WH90D-18 multinuclear Fourier transform NMR spectrometer was provided by NIH Biomedical Sciences grant 5S05RR 07010-09 and NSF grant MPS75-06138. We are grateful to G. G. Dodson for helpful discussions and for sharing his unpublished data with us.

18 November 1980

Evidence for Endomycorrhizae in Pennsylvanian Age Plant Fossils

Abstract. *Fossil fungal chlamydospores from the tissues of a number of Pennsylvanian age fossil plants are described. Features such as size, shape, wall organization, and the structure of the subtending hyphal stalk suggest affinities with the modern endomycorrhizal fungus* Glomus.

Paleobiological studies suggest that the fungi are an ancient group that may have been among the earliest life forms to have evolved. Filamentous fragments that may represent hyphal remains and possible fungal spores were identified from Precambrian sediments (*1*). Despite the uncertainties in accurately identifying these Precambrian remains, unquestionable fungi representing all of the major divisions are known from the Paleozoic to the Recent (*2*).

Of the Paleozoic fungi that have been described (*3*), several different forms were identified in the petrified plant material that accumulated during the formation of the Carboniferous coal swamps. Although fungi are known to have oc-

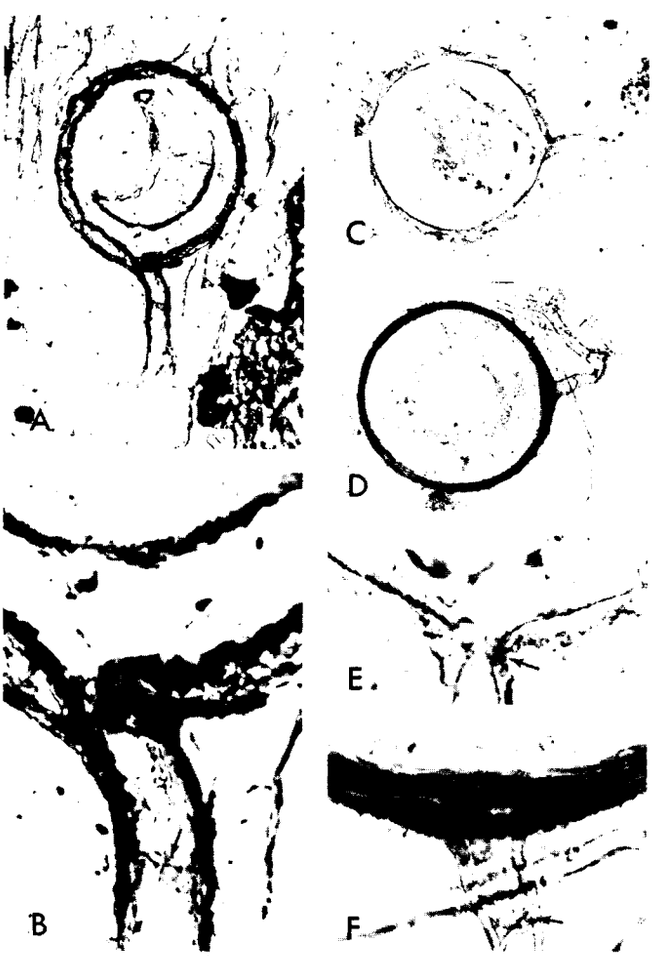

Fig. 1. Endomycorrhizal chlamydospores. (A) Fossil chlamydospores showing the stalk and multilayered wall (× 160). (B) Hyphal attachment to fossil chlamydospore showing curved occlusion in the stalk (arrow) (× 740). (C) Fossil chlamydospore with simple hyphal attachment (× 160). (D) *Glomus mosseae* chlamydospore (× 160). (E) Simple hyphal attachment of fossil chlamydospore showing thickening of wall (arrow) at spore base (× 740). (F) *Glomus mosseae* chlamydospore with curved septum in the stalk (× 740).

0036-8075/81/0501-0562$00.50/0