and Scania (*7*)] for organic microfossils tends to substantiate our interpretation of the importance of environmental control in the occurrence of Silurian spores, although the absence of *any* organic microfossils from many rocks where we had predicted the occurrence of spores if environment of deposition rather than evolution was the critical factor can best be explained by postdepositional alteration (*30*). Abundant spores in our Llandovery and Ludlow age samples and in other latest Silurian age samples are known only from shallow-bottom or nearshore marine environments. Truly nonmarine Early Silurian environments have not yet yielded spores, but unmetamorphosed rocks of this age capable of providing spores have not been studied by us or reported in the literature.

The possibility of environmental control of Silurian spore occurrences related to water depth and shoreline proximity is further substantiated by the inverse relationship between abundant Silurian spores, both Early and Late, and marine organic microplankton (*9*). We find that abundant spores occur in rocks deposited either landward of those containing abundant and diversified marine microplankton or in rocks consistent with very shallow-bottom conditions. Strata conventionally accepted as marine or giving evidence of more offshore or deeper water environments yield abundant marine microfossils, including acritarchs, chitinozoans, or scolecodonts, but either no spores or rare spores. The results of our preliminary work suggest that these two groups of organic microfossils have occurrence patterns that are largely mutually exclusive and that may be correlated with marine versus nonmarine or shallow marine environments (*29*).

We suggest, therefore, that the known progressive increase in the frequency and taxonomic diversity of trilete spores that occurs from the Early Silurian to the Late Silurian–Early Devonian may be largely a function of biofacies correlated with geologic environment rather than a function of organic evolution as now concluded (*19, 22*). If the orderly evolutionary sequence that appears to be represented among Silurian spores is an environmental artifact and if the Silurian spores represent vascular plants, it may yet be found that Early Paleozoic vascular plants show a trend similar to the evolutionary history of many animal groups, which exhibit far greater diversity and morphological richness in their early rather than their later history. It remains for future work to determine whether or not plant spores will be recovered in sufficient abundance from nonmarine Early Silurian or older beds to warrant the conclusion that plants had already invaded the land.

JANE GRAY

Paleoecology Laboratory,
Museum of Natural History,
University of Oregon, Eugene 97403

A. J. BOUCOT

Department of Geology,
Oregon State University,
Corvallis 97331

References and Notes

1. O. Corna, *Geol. Zb. Geol. Carpathica* **21**, 183 (1970).
2. J. M. Schopf, in *Aspects of Palynology*, R. H. Tschudy and R. A. Scott, Eds. (Interscience, New York, 1969), chap. 10, pp. 163–192. Schopf reviews the status of previously described pre-Silurian sporelike bodies, some with weakly developed tetrad marks. He regards these as only dubious evidence for the presence of land plants in the early Paleozoic.
3. W. B. N. Berry and A. J. Boucot, *Geol. Soc. Amer. Spec. Pap. 102* (1970).
4. D. W. Fisher, *Bull. Amer. Ass. Petrol. Geol.* **38**, 1979 (1954).
5. J. Gray, in *Handbook of Paleontological Techniques*, B. Kummel and D. M. Raup, Eds. (Freeman, San Francisco, 1965), pp. 530–587.
6. D. W. Fisher, *Bull. Buffalo Soc. Natur. Sci.* **21**, 13 (1953); F. H. Cramer and M. d. C. R. D. de Cramer, *Can. J. Earth Sci.* **7**, 1077 (1970). Cramer and Cramer also report the presence of tetrads in the Power Glen Formation, which they describe as "tetragonal"—a configuration that we have not encountered among our specimens—and which they appear to regard as acritarchs. They refer to the tetrads as leiospheres "with non-characteristic features" but later as "miosporelike palynomorphs." Surprisingly, they also state, "Specimens in tetrads, as well as those which occur single, lack *clear* trilete laesurae." Tetragonal tetrads with the individual spores arranged in a single plane would not bear the triradiate marks and contact areas of spore specimens arrranged in the common tetrahedral tetrad arrangement.
7. J. Gray, unpublished data; S. Laufeld, J. Gray, A. J. Boucot, in preparation.
8. K. Faegri and J. Iversen, *Textbook of Pollen Analysis* (Hafner, New York, 1964).
9. J. Gray and A. J. Boucot, in *Abstracts* (Cordilleran Section meeting, Geological Society of America, Boulder, Colo., 1971), vol. 3, p. 127.
10. W. H. Lang, *Phil. Trans. Roy. Soc. London Ser. B Biol. Sci.* **227**, 245 (1937); D. Edwards, *Palaeontology* **13**, 451 (1970).
11. J. Obrhel, *Geologie* **11**, 83 (1962).
12. A. M. Ishchenko, *Geol. Zh. Akad. Nauk USSR* **29**, 101 (1969).
13. H. P. Banks, *J. Indian Bot. Soc.*, in press.
14. F. H. Cramer, *J. Paleontol.* **43**, 485 (1969).
15. ———, *Rev. Micropaléontol.* **11**, 61 (1968).
16. W. E. Evitt, personal communication.
17. A. B. Reaugh, in *Abstracts* (Southeastern Section meeting, Geological Society of America, Boulder, Colo., 1971), vol. 3, p. 343.
18. W. S. Hoffmeister, *Micropaleontology* (*N.Y.*) **5**, 331 (1959); J. L. Wray, in *Palynology in Oil Exploration* (Special Publication 11, American Association of Petroleum Geologists, Tulsa, Okla., 1964), pp. 90–96. The zonation for the Libyan spore occurrence of Hoffmeister is from unpublished information on associated graptolites provided by W. B. N. Berry.
19. J. B. Richardson and T. R. Lister, *Palaeontology* **12**, 201 (1969).
20. F. H. Cramer, *Notas Comun. Inst. Geol. Minero Espana* **85**, 71 (1966); *Bol. Inst. Geol. Minero Espana* **77**, 225 (1967).
21. S. Jardiné and L. Yapaudjian, *Rev. Inst. Fr. Pet.* **23**, 439 (1968).
22. W. G. Chaloner, *Biol. Rev.* (*Cambridge*) **45**, 353 (1970).
23. R. Castenholz, personal communication; W. Adey, personal communication; J. Wray, personal communication.
24. J. H. Johnson, in *Manual of Phycology*, G. M. Smith, Ed. (Chronica Botanica, Waltham, Mass., 1951), pp. 193–202.
25. K. M. Drew, *ibid.*, pp. 167–191.
26. F. M. Hueber, *Ann. Mo. Bot. Gard.* **48**, 125 (1961).
27. If Corna's Ordovician spores, cuticular material, and tracheids [see (*1*)] are generally accepted as bona fide, then the entire problem of the origin of vascular land plant characters is shifted back well before the Silurian.
28. K. Faegri and L. van der Pijl, *The Principles of Pollination Ecology* (Pergamon, New York, 1966); R. P. Wodehouse, *Pollen Grains* (McGraw-Hill, New York, 1935).
29. J. Gray and A. J. Boucot, in *Abstracts* (Annual meeting, Geological Society of America, Boulder, Colo., 1970), vol. 2, p. 560.
30. J. Gray, and A. J. Boucot, in preparation.
31. Fieldwork was partly supported by NSF grant 27350 to Oregon State University; NSF grant 4233 to the University of Oregon supported laboratory work in the Paleoecology Laboratory of the Museum of Natural History. We thank W. Kilgour, Sanborn, New York, for advice and assistance in collecting samples from western New York; D. Hoskins, Pennsylvania Geological Survey, for advice concerning eastern Pennsylvania; D. W. Fisher, New York Geological Survey, for advice concerning the New York Silurian; and C. Rexroad, Indiana Geological Survey, for help concerning material from Ohio and Kentucky. For critical comments on a preliminary version of the manuscript we thank W. Adey, H. P. Banks, W. E. Evitt, D. W. Fisher, F. M. Hueber, and J. Wray. M. A. Sherwood prepared the spore illustrations.

26 April 1971; revised 21 June 1971 ■

Homograft Rejection Delayed by Treatment of Donor Tissue in vitro with Antilymphocyte Serum

Abstract. *Treatment of rabbit corneal tissue in vitro with pooled rabbit serum delays the onset of corneal homograft rejection in the host. Addition of antilymphocyte serum results in a further significant delay in the onset of rejection. The mechanisms by which such treatment of donor tissues may modify the antigenic content of the material are discussed.*

The use of antilymphocyte serum (ALS) (*1, 2*), as well as other immunosuppressive agents (*2, 3*) in the treatment of donor animals to modify the homograft reaction is well documented in the literature. We now report the effect of treatment of corneal donor tissue with ALS in vitro prior to its use for penetrating keratoplasty.

Horse antiserum to rabbit lympho-

Table 1. Effect of in vitro treatment of donor corneal grafts on date of onset of homograft rejection.

Treatment	Animals (No.)	Mean onset of reaction (days)	Significance
HARLS (5 percent) in rabbit serum	15*	19.5	$P < .01$†
Rabbit serum (100 percent)	10	14.9	$P < .01$†
Horse serum (5 percent) in rabbit serum	10	14.2	$P < .01$†
Direct transplantation	7	9.7	

* Excluding three animals that have remained clear for more than 180 days. † The delay in rejection between the 5 percent HARLS group and the serum groups is significant at $P < .05$.

cytes (HARLS) was prepared as previously described (*4*). It had agglutinating titers of 1 : 4000 for lymphocytes and 1 : 16 for red cells. Albino rabbits weighing 3.0 to 5.0 kg were used. After enucleation the entire cornea and a rim of sclerae were removed from the donor eye. The donor tissue was then placed in one of three solutions at 4°C or transplanted directly. The solutions were 100 percent pooled rabbit serum in which complement had been inactivated, 5 percent HARLS in rabbit serum, and 5 percent horse serum in rabbit serum. After being soaked for 24 hours at 4°C, the donor tissue was placed epithelial side down in a paraffin block with a fashioned trough. A 6-mm corneal button was trephined, and with the host anesthetized with pentobarbital a penetrating keratoplasty was performed with a continuous 8-0 black silk suture. Clean, but not sterile technique was used. The animals received daily topical applications of atropine ointment and antibiotic drops until the suture was removed on the 10th day after the operation. On the 14th day all the corneal grafts were successful and skin grafts were then performed between the same pairs of donors and recipients in order to achieve a uniformly high rate of corneal graft rejection (*2, 4, 5*). The skin grafts were placed subcutaneously in the ventral abdominal wall. The animals were examined daily from the day of skin grafting for evidence of corneal homograft rejection. Because of their deep location, the skin grafts were not directly observable, but the presence of the graft and the inflammatory response of the surrounding tissue verified its continued presence. Soaked corneas from each group were frozen in liquid freon, suspended in liquid nitrogen, sectioned, and stained with fluorescein-labeled rabbit antiserum to horse γ-globulin (*6*).

The results reported here are those observed during the 6 months after skin transplantation (Table 1). The mean onset of corneal graft rejection was 9.7 days in the group in which tissue was transplanted directly (control group). It was prolonged to 14.9 days when the corneal buttons were first soaked in rabbit serum ($P < .01$) and to 14.2 days when 5 percent horse serum in rabbit serum was used ($P < .01$). Soaking the tissue in 5 percent HARLS in rabbit serum delayed the mean onset of rejection to 19.5 days in those that rejected the graft. This delay is significant when compared to that for the control group ($P < .01$) and when compared to that for the group treated with rabbit serum ($P < .05$). Furthermore, three animals in the HARLS group retained clear grafts for 6 months whereas none in any of the other groups did. These results are suggestive but not conclusive ($.05 < P < .10$).

Studies with fluorescein-labeled antiserum to horse γ-globulin failed to demonstrate binding of this protein to the donor tissue. As observed with the light microscope the structure of the cornea appeared normal, with all cell layers present and intact.

All immunosuppressive agents, including ALS (*7*), used to prevent homograft reaction in cases of organ transplantation have serious side effects associated with their use. Recently, we reported (*2*) the efficacy of treatment of donor animals in modifying corneal homograft rejection and suggested that organs from patients treated with ALS could serve as treated donor material. Extending this work we have treated the donor material in vitro and significantly delayed the onset of rejection without exposing the donor animal to any immunosuppressive therapy. Soaking of the donor material in pooled serum somewhat delayed the onset of rejection, and adding 5 percent ALS to the soaking solution resulted in grafts that remained clear for an additional period of time.

The intriguing question which remains is, "Why does previous treatment of donor tissue in vivo or in vitro modify the homograft reaction?" It has been postulated by Guttman and Lindquist (*3*) that the effectiveness of the immunosuppressive agent is dependent upon the destruction of nonparenchymal cells of hematopoietic origin, which are the stimuli to the effector mechanisms of rejection. In this work donors of renal allografts were treated with a variety of immunosuppressive agents (cyclophosphamide, methotrexate, and procarbazine), and a significant reduction in the amount of kidney transplant rejection compared to the known control series was noted. We do not believe that this mechanism is operative in our experiments because the avascular cornea contains few nonparenchymal cells.

On the other hand, Guttman *et al.* (*1*) have suggested that ALS may act on the organ to be transplanted by coating or binding available antigenic sites. It is also possible that the treated donor tissue acts as a repository of ALS which is slowly released and continually bathes the regional lymph nodes preventing recognition of the foreign material (*8*). The failure to demonstrate the binding of HARLS in the donor tissue by the fluorescent antibody technique does not negate the possibility of the binding of smaller quantities of γ-globulin than are detectable by this technique. But it would seem unlikely, under these circumstances, that the donor button could act as a repository of ALS which would continuously bathe the regional lymph nodes.

Another possibility is that soaking of the tissue prior to skin grafting may allow soluble antigens, if present, to diffuse into the media. Earlier work by Kuwahara (*9*) on a lamellar heterograft system demonstrated that soaking of the donor button in recipient serum reduced the apparent antigenicity of the graft. Stocker *et al.* (*10*), on the basis of work by Kuwahara, have used donor material stored in autologous serum for human transplantation, and they express their clinical impression that the results achieved with this material were more favorable than ordinarily could have been expected in this particular group of cases.

As determined with the light microscope, the donor material appeared completely intact. Kobayashi (*11*) has recently reported the results of his ultrastructural investigation of rabbit corneas soaked in dialyzed rabbit serum. He concludes that morphologically cor-

neas soaked in serum for 72 hours are the most satisfactory for penetrating keratoplasty. The exact mechanism by which our soaking regimen decreases the apparent antigenicity of the donor material is not clear, and our results suggest that more than one mechanism may be operable.

The demonstration of the effectiveness of the treatment of corneal buttons with ALS in modifying the homograft reaction should be immediately applicable in man in those cases where graft rejection is expected to occur, that is, in patients who have already had graft reaction, highly vascularized corneal tissue, and so forth.

RONALD M. BURDE
STEPHEN R. WALTMAN
JUAN H. BERRIOS
Department of Ophthalmology, Washington University School of Medicine, St. Louis, Missouri 63110

References and Notes

1. R. D. Guttman, C. B. Carpenter, R. R. Lindquist, J. P. Merrill, *Lancet* **1967-I**, 248 (1967).
2. R. E. Lernor, R. M. Burde, S. R. Waltman, *Am. J. Ophthalmol.* **69**, 453 (1970).
3. R. D. Guttman and R. R. Lindquist, *Transplantation* **8**, 490 (1969).
4. S. R. Waltman, H. W. Faulkner, R. M. Burde, *Invest. Ophthalmol.* **8**, 196 (1969).
5. A. E. Maumenee, *Am. J. Ophthalmol.* **34** (Part 2), 142 (1951).
6. The rabbit antiserum to horse γ-globulin was obtained from Pentex Biochemicals, Kankakee, Illinois.
7. N. Kashiwagi, C. O. Brantigan, L. Brettschneider, C. G. Groth, T. E. Starzl, *Ann. Intern. Med.* **68**, 275 (1968).
8. P. Málek, J. Kolc, V. Hasková, V. Zástava, *Transplantation* **8**, 333 (1969); B. G. Grobbelaar, P. Brain, A. J. Rogers, *Lancet* **1970-I**, 1120 (1970).
9. Y. Kuwahara, *Jap. J. Ophthalmol.* **5**, 243 (1961).
10. F. W. Stocker, D. Levenson, N. Georgiade, *Arch. Ophthalmol.* **70**, 554 (1963).
11. S. Kobayashi, *Acta Soc. Ophthalmol. Jap.* **68**, 952 (1964).
12. We thank Dr. Reimut Wette, Director of Biostatistics, Washington University School of Medicine, for his help with the statistical evaluation. Supported in part by PHS grants EY-00004 and EY-00016 and by a grant-in-aid G-421 from Fight for Sight, Inc.

22 April 1971; revised 10 June 1971 ■

Calcium Requirement for Melanophore-Stimulating Hormone Action on Melanophores

Abstract. *The calcium ion is specifically required for the action of melanophore-stimulating hormone on melanosome dispersion within lizard* (Anolis carolinensis) *melanophores in vitro. The response to this hormone is directly related to the concentration of the Ca^{2+} ion. Lithium, choline, rubidium, and cesium will replace the sodium and potassium of Ringer solution if Ca^{2+} is present. Calcium ions are not required for melanosome dispersion itself, since theophylline or dibutyryl cyclic adenosine monophosphate reversibly darkens lizard skins in the absence of calcium.*

Melanophore-stimulating hormone (MSH) initiates the movement (dispersion) of melanosomes from a perinuclear (aggregated) position out into the dendritic processes of vertebrate melanophores (*1*). This leads to a rapid darkening of the skin of most poikilotherms and may precede and be prerequisite for melanin synthesis (*2*). A number of in vitro studies have been directed toward understanding the possible ionic requirements for MSH action. It has been reported that there is an "absolute" requirement for the sodium ion in the action of MSH on frog (*Rana pipiens*) melanophores (*3*). Not only was the calcium ion reported not to be required for melanophore responses to MSH, but, indeed, the response was said to be enhanced in a Ca^{2+}-free medium (*3, 3a*). Others (*4*) have suggested that melanosome dispersion within frog melanophores results from an increase in the intracellular level of calcium ions and a simultaneous decrease in intracellular potassium ions. Fingerman has implicated a role for Ca^{2+} ions in the hormonal control of crustacean chromatophores (*5*). Horowitz (*6*) showed that melanosome aggregation within melanophores of the lizard *Anolis carolinensis* was facilitated by the removal of Ca^{2+} ions from Ringer solution. Novales (*3, 3a*), on the other hand, reported that there is a sodium requirement for MSH action on melanophores of this lizard.

In the present report we demonstrate a specific Ca^{2+} ion requirement for MSH action on melanophores of the lizard *Anolis carolinensis*. We also show that neither sodium nor potassium ions are necessary for MSH action and, in addition, that the Ca^{2+} ion requirement is for an early event in MSH action, since this divalent cation is not necessary for melanosome movements per se. The in vitro response of the integumental melanophores of *Anolis* to MSH or other agents under varying ionic conditions was studied by photometric reflectance methods as described originally for frog skin (*1*) and modified slightly for *Anolis* skin (*7*). Melanosome dispersion in response to MSH results in a change from a bright green to a dark brown color and this change can be measured as a decrease in reflectance from the outer (epidermal) surface of isolated skins. Conversely, melanosome reaggregation resulting in a lightening of skins is correlated with an increase in reflectance.

As reported previously (*3, 3a*), MSH darkens *Anolis* skins immersed in Ringer (NaCl, 111 m*M*; $NaHCO_3$, 2 m*M*; KCl, 2 m*M*; $CaCl_2$, 1 m*M*) solution (Fig. 1). Skins residing solely in sodium chloride (120 m*M*) or in an equivalent amount of NaCl plus KCl (2 m*M*) are almost totally unresponsive to MSH. The addition of the Ca^{2+} ion (1 m*M*) to the NaCl solution permits a response equal to that of the Ringer control group of skins (Fig. 1). The degree of darkening of lizard skins to MSH is clearly directly related to the concentration of the Ca^{2+} ion present (Fig. 2).

These results indicated that either the Ca^{2+} ion is needed directly for MSH action or else its role is permissive to the action of the Na^+ ion. Other monovalent cations were substituted for sodium to determine whether the Na^+ ion is specifically required in addition to the Ca^{2+} ion for MSH action. Melanophore-stimulating hormone failed to darken skins in isotonic solutions (120 m*M*) of either lithium or choline chloride. The presence of Na^+ (1 m*M*) or K^+ (1 m*M*) ions in combination with either Li^+ or choline ions failed to reestablish a response of the melanophores to MSH. There was, however, a near

Table 1. Response (change in reflectance) of *Anolis* skins to MSH (6×10^{-9} g/ml), theophylline (10 m*M*), or dibutyryl cyclic AMP (DcAMP, 10 m*M*), in the presence or absence of calcium ion (1 m*M*). Each value represents the maximal mean percent reflectance change, ± standard error, of the skins comprising each experimental group. (Number of skins in parentheses.)

NaCl, EDTA (5 m*M*), MSH (6)	0 ± 0.67
NaCl, Ca^{2+}, MSH (6)	39 ± 3.69
NaCl, EDTA (5 m*M*), theophylline (6)	58 ± 2.31
NaCl, Ca^{2+}, theophylline (6)	58 ± 3.58
NaCl, EDTA (5 m*M*), DcAMP (8)	49 ± 2.54
NaCl, Ca^{2+}, DcAMP (8)	52 ± 1.44