References and Notes

1. J. Barrowman and M. Craig, *Nature* **190**, 818 (1961); M. L. Craig and E. S. Russell, *Develop. Biol.* **10**, 191 (1964).
2. J. S. Kovach, P. A. Marks, E. S. Russell, H. J. Epler, *J. Mol. Biol.* **25**, 131 (1967).
3. E. S. Russell and S. E. Bernstein, in *Biology of the Laboratory Mouse* (McGraw-Hill, New York, 1966), p. 351; P. A. Marks and J. S. Kovach, *Curr. Top. Develop. Biol.* **1**, 213 (1966).
4. C. Baglioni and C. E. Sparks, *Develop. Biol.* **8**, 272 (1963); T. Borgese and J. F. Bertles, *Science* **148**, 509 (1965); V. D'Amelio and A. M. Salvo, *Acta Embryol. Morphol. Exp.* **4**, 250 (1961); R. C. Fraser, *Exp. Cell Biol.* **25**, 418 (1961); E. Kleihauer, I. O. Buss, C. P. Luck, P.G. Wright, *Nature* **207**, 424 (1965).
5. V. M. Ingram, *The Hemoglobins in Genetics and Evolution* (Columbia Univ. Press, New York, 1963); E. R. Huehns and E. M. Shooter, *J. Med. Gen.* **2**, 48 (1965).
6. J. L. Southard, H. G. Wolfe, E. S. Russell, *Nature* **208**, 1126 (1965).
7. T. H. J. Huisman, E. D. A. Martis, A. Dozy, *J. Lab. Clin. Med.* **52**, 312 (1958).
8. A. Rossi-Fanelli, E. Antonini, A. Caputo, *Biochim. Biophys. Acta* **30**, 608 (1958).
9. J. B. Clegg, M. A. Naughton, D. J. Weatherall, *Nature* **207**, 945 (1965).
10. R. A. Popp, *J. Biol. Chem.* **240**, 2863 (1965).
11. W. A. Schroeder, J. T. Cua, G. Matzuda, W. D. Feininger, *Biochim. Biophys. Acta* **63**, 352 (1962); W. DeWitt and V. M. Ingram, *Biochem. Biophys. Res. Comm.* **27**, 236 (1967).

12. These studies were supported in part by grants from PHS (GM-14552) and NSF (GB-4631). A.B. is a Leukemia Society scholar, and A.P. is supported by PHS grant TI-AM 5231 to Columbia University College of Physicians and Surgeons.

17 July 1967

Collagen-Derived Membrane: Corneal Implantation

Abstract. *Behavior of membranes derived from collagen was investigated in rabbit corneas. Disks of the membrane were placed between the lamellae of corneas which were then examined grossly, biomicroscopically, and histologically. The membranes remained clear, and almost no reaction or evidence of reabsorption was seen during an observation period of 6 months. These characteristics make the material potentially useful for heterotransplantation in the cornea.*

Synthetic polymers have attracted considerable attention in medicine, especially in ophthalmology. Only a few of these synthetics, however, have been found with the inertness and other physical properties needed for use in the eye. The search for new materials has overlooked biopolymers, largely because understanding of their molecular biology has been limited, and also because of such related problems as availability, plasticity, and tissue reaction.

New information about the collagen molecule has, however, permitted the development of a collagen-derived biopolymer with unique properties (*1–4*). This transparent material has a refractive index similar to that of the corneal stroma and is a polyelectrolyte which can be molded into any form desired. Binding heparin to it will produce a nonclotting surface. Pore aperture can be varied to selectively permit or deny passage of different sized molecules. Before any practical use can be made of a new biological substance, its behavior in vivo must be studied. This material was studied by means of its implantation into the cornea.

The preparation of the collagen biopolymer has been described elsewhere (*1, 4*). Collagen from calfskin was used exclusively, although any tissue rich in this protein is a possible source.

Enzyme-treated collagen was polymerized from solution into transparent membranes. The surface charge was unaltered, and the pore size in the membranes used was 30 to 50 Å. A similar membrane used for hemodialysis was shown to permit relatively free passage of water, gases, Na^+, and K^+ (*1*).

Adult albino rabbits (2 to 3 kg) were used throughout. The animals were anesthetized with sodium pentobarbital (30 mg/kg of body weight) injected intravenously. Tetracaine-HCl (0.5 percent) was used topically for additional anesthesia.

Collagen membranes were sterilized by exposure to dry heat at 120°C for 2 hours. Disks 5 mm in diameter were removed from the membrane with a trephine and placed intralamellarly into 28 rabbit corneas. Lids were left open, neosporin ointment was applied immediately after surgery, and some animals received antibiotics prophylactically.

All eyes were examined grossly, and most biomicroscopically, at frequent intervals for signs of reaction, such as perilimbal vascular dilation, corneal edema and vascularization, hyperemia of the iris, and cells and flare in the anterior chamber. Animals were killed at regular intervals, and the eyes were fixed in 10 percent neutral formalin. Histologic sections were made of corneas which were then stained with hematoxylin and eosin, Hale's colloidal iron, and periodic acid–Schiff reagent.

In 25 eyes, the only visible reaction after the implantation was a mild dilation of the perilimbal vessels lasting from the 1st to the 3rd day. There was no iris hyperemia, corneal edema, or vascularization. There was no reaction at the interface of cornea and membrane. Throughout the period of observation, the membranes maintained their transparency and original appearance. There were no corneal erosions and no extrusions of the membrane.

In three eyes, there was a violent reaction marked by corneal infiltration and vascularization which did not respond to antibiotics. After about 3 weeks, small, grey punctate lesions were seen on the surface of the membranes in all three cases. Microscopic examination showed that they were fungus.

Six animals were killed at weekly intervals, and six were killed at monthly intervals. The corneal stroma appeared normal in all tissue sections, with the exception of the stroma posterior to the membranes which seemed somewhat edematous after 4 months. At no time were more than a few cells seen near the membrane. These cells appeared to be macrophages and fibroblasts, with an occasional mononucleocyte. The membranes appeared unchanged during the 6-month observation period. No cellular invasion or repopulation was seen. Membranes in place for several months stained a pale blue with Hale's colloidal iron stain, thus suggesting the presence of host acid mucopolysaccharide in the membrane.

Collagen materials implanted into the cornea have always provoked a cellular response and are eventually reabsorbed. The behavior of plain catgut is well known (*5*). This familiar material, prepared from ovine and bovine intestinal collagen, is well tolerated but produces a much greater cellular response than collagen membranes do. Other collagen-rich implants, such as rat tail tendon (*6*), cartilage (*7*), and dissolved collagen fiber (*8*), have provoked tissue responses of varying degree, but always significantly greater than those caused by the collagen membranes used here.

Collagen placed in the cornea has inevitably been reabsorbed, but at a highly variable rate. Payrau polymerized collagen in the living rabbit cornea and found no evidence of it after 24 hours (*8*). Plain catgut is reabsorbed in a few days (*9*), and cartilage is resorbed over a period of months (*7*).

It is not definitely known why this collagen membrane when implanted

into the cornea should act differently from other materials rich in this protein. It is likely, however, that the explanation lies in some alteration of the collagen used in the membrane. Collagen is composed of a repeating "monomer," tropocollagen, which is itself composed of three chains of amino acids arranged in a coiled helix (*2*). Peptides of low molecular weight, termed telopeptides, extend from the body of tropocollagen (*4*). There is increasing evidence that these telopeptides determine species difference of collagen and play a determinative role in its molecular biology (*1, 3, 4*). The feature distinguishing the collagen membrane from other collagen-rich materials used in the cornea is that most of the telopeptides were removed during preparation of the membrane by enzymatic proteolysis (*10*). A further important point is the extensive introduction of intra- and intermolecular cross-links into the collagen membrane by heat or ultraviolet irradiation (*1*). It is known that cross-links stabilize the tropocollagen molecule and play an important role in determining the resistance to reabsorption (*11*).

MICHAEL W. DUNN, TOMIO NISHIHARA
KURT H. STENZEL
A. WHITLEY BRANWOOD
ALBERT L. RUBIN

Departments of Surgery (Ophthalmology) and Pathology and *Rogosin Laboratories, Department of Medicine, New York Hospital-Cornell Medical Center, New York*

References and Notes

1. T. Nishihara, A. L. Rubin, K. H. Stenzel, *Trans. Amer. Soc. Artif. Int. Organs* **13**, 243 (1967).
2. J. Gross, J. H. Highberger, F. O. Schmitt, *Proc. Nat. Acad. Sci. U.S.* **40**, 679 (1965).
3. A. L. Rubin, D. Pfahl, P. T. Speakman, P. F. Davison, F. O. Schmitt, *Science* **139**, 37 (1963); F. O. Schmitt, L. Levine, M. P. Drake, A. L. Rubin, D. Pfahl, P. F. Davison, *Proc. Nat. Acad. Sci. U.S.* **51**, 493 (1964); A. H. Kang, P. Bornstein, K. A. Piez, *Biochemistry* **6**, 788 (1967).
4. A. L. Rubin, M. P. Drake, P. F. Davison, D. Pfahl, P. T. Speakman, F. O Schmitt, *Biochemistry* **4**, 181 (1965).
5. W. H. Mead and A. Ochsner, *Surgery* **7**, 485 (1940); S. D. McPherson, Jr., *Amer. J. Ophthalmol.* **51**, 118 (1961).
6. T. Larmi, *Acta Ophthalmol. Suppl.* **9**, 63 (1961).
7. P. Payrau and C. H. Dohlman, *Ann. Oculist (Paris)* **197**, 375 (1964).
8. P. Payrau, G. Offret, J. P. Faura *et al.*, *Arch Ophthalmol. (Paris)* **23**, 129 (1963).
9. W. L. Hughes, L. P. Guy, H. H. Romaine, *Arch. Ophthalmol. N.Y.* **32**, 363 (1944).
10. M. P. Drake, P. F. Davison, S. Bump, F. O. Schmitt, *Biochemistry* **5**, 301 (1966).
11. I. Banga and J. Balo, *Biochem. Z.* **342**, 330 (1965).
12. Supported in part by grant HE 08736 from the U.S. Public Health Service and by the New York Heart Association. M.W.D. is a PHS postdoctoral fellow.

7 August 1967

Vertebrate Regeneration System: Culture in vitro

Abstract. *With standard tissue-culture techniques and media, various components of the lizard tail regenerate have been maintained in culture for 8 months. Differentiation of two cell types, melanophores and striated muscle, has been obtained. Myoblast proliferation and fusion can be selectively controlled by altering the culture medium.*

The details of tail regeneration in a number of lizard species have been described (*1, 2*). As in other regeneration systems, the developmental capacities of these regeneration cells are in dispute. In addition, the various interactions involved in the initiation of the regenerate, as well as those occurring during its differentiation, have not been adequately resolved. These basic aspects of vertebrate regeneration have proven difficult to clarify with available techniques in vivo. Studies of regeneration in vitro have been hampered by the general paucity of techniques for the culture of cells from lower vertebrates. Although considerable progress along these lines has been made in recent years (*3*), all reported attempts to culture the components of a regenerate have achieved, at best, maintenance and limited outgrowth (*4*). We describe techniques for the culture in vitro of various components of the tail regenerate in the lizard *Anolis carolinensis*.

The portion of a tail bearing a regenerate of known age was first swabbed with ether to remove fungal spores. The regenerate was then removed and placed in Hanks's salt solution containing antibiotics (*5*). The regenerate was cut into circular segments approximately 1 to 2 mm thick and placed in dissociation medium, consisting of growth medium (GM-I) containing 2.0 mg of collagenase per milliliter (*5*). The *p*H was maintained at 7.2 with a bicarbonate buffer and gassing with a mixture of 95 percent air and 5 percent CO_2. Dissociation was carried out at 26°C for various lengths of time (2 to 8 hours) depending on the component to be isolated for culture. Following this preliminary loosening of the components, the tissue slices were carefully dissected with the aid of tungsten needles to yield the material for culture (that is, wound epithelium, promuscle aggregates, procartilage aggregate, or general mesenchymal cells of the regenerate). The isolated components were then placed in fresh dissociation medium, gassed, and dissociated with occasional pipetting for an additional 2 to 4 hours until suspensions of single cells were obtained. The fully dissociated cells were filtered through several layers of sterile lens paper to remove cell clumps, spun down in a clinical centrifuge, and then resuspended in a known volume of growth medium. The cells were counted in a hemocytometer, diluted to appropriate concentrations, and plated in either 60-mm Falcon plastic petri dishes or in Falcon 30-ml flasks.

During the initial phases of this study, pooled cultures of dissociated cells obtained from early regenerates were used to screen various media. Although several commercially available media and various combinations of added biologicals were tested for their ability to support growth of the cells, two formulations were found to support growth for long periods of time and in some cases additionally support differentiation of the cultured cells. Growth Medium I (GM-I) consisted of Coon's (*6*) modification of Ham's F-10 medium containing 5 percent chick embryo extract, 10 percent fetal calf or horse serum, 1 percent GIBCO antibiotic mixture (*5*), and 0.07 percent bicarbonate. Growth Medium II (GM-II) contained Eagle's basal medium with glutamine and the same concentrations of embryo extract, fetal calf or horse serum, antibiotic, and bicarbonate. Although both media supported long-term proliferation of the lizard cells, a higher plating efficiency and more rapid growth was consistently obtained with GM-I. However, GM-I failed to support differentiation of striated muscle, whereas GM-II did support muscle formation. All cultures were incubated at 26°C in sealed chambers gassed with 95 percent air and 5 percent CO_2 and maintained at 100 percent humidity. Every 4th day half of the medium in each dish was exchanged for fresh medium.

Presumptive muscle cells, obtained from 3- to 6-mm regenerates, were plated at a concentration of 2×10^3 to 3×10^3 cells per dish. In all cases the