ing an inhibition of virus release from the membrane. In other systems, virus particles of low infectivity, such as MLV particles from interferon-treated TB cells (*3*) and VSV particles from interferon-treated L_B cells (*39*), were released. The low infectivity was related to the reduced amount of glycoprotein present in the released viruses and may have been attributable in part to a direct effect of interferon on the cell membrane. Therefore, it is possible that interferon and tunicamycin either markedly inhibit the release of membrane-associated viruses or cause an enhanced release of noninfectious virus particles lacking in glycoproteins. The failure of tunicamycin to enhance interferon's antiviral activity against EMC and mengo viruses (*40*), which have no membrane component, is consistent with both possibilities.

RADHA K. MAHESHWARI
Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, and *Department of Microbiology, School of Medicine and Dentistry, Georgetown University, Washington, D.C. 20007*

T. SREEVALSAN
Department of Microbiology, School of Medicine and Dentistry, Georgetown University

ROBERT H. SILVERMAN
Department of Pathology, Uniformed Services University of the Health Sciences

JOHN HAY
Department of Microbiology, Uniformed Services University of the Health Sciences

ROBERT M. FRIEDMAN
Department of Pathology, Uniformed Services University of the Health Sciences

References and Notes

1. R. M. Friedman, *Bacteriol. Rev.* **41**, 543 (1977).
2. W. E. Stewart II, *The Interferon System* (Springer-Verlag, New York, 1980).
3. P. K. Y. Wong, P. H. Yuen, R. MacLeod, E. H. Chang, M. W. Myers, R. M. Friedman, *Cell* **10**, 245 (1977).
4. P. M. Pitha, S. P. Staal, D. P. Bolognesi, T. P. Denny, W. P. Rowe, *Virology* **79**, 1 (1977).
5. R. K. Maheshwari and R. M. Friedman, *ibid.* **101**, 399 (1980).
6. J. Taylor-Papadimitriou, in *Interferon*, I. Gresser, Ed. (Academic Press, New York, 1980), vol. 2, p. 13.
7. I. Gresser, *Cancer Chemother. Rep.* **5**, 521 (1977).
8. H. M. Johnson, in *Interferon and Interferon Inducers*, D. A. Stringfellow, Ed. (Dekker, New York, 1980), p. 263.
9. R. M. Friedman, *Pharm. Ther.* **2**, 425 (1978).
10. E. H. Chang, F. T. Jay, R. M. Friedman, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1859 (1978).
11. E. Knight, Jr., and B. D. Korant, *Biochem. Biophys. Res. Commun.* **74**, 707 (1977).
12. L. D. Kohn, R. M. Friedman, J. M. Holmes, G. Lee, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3695 (1976).
13. C. Huet, I. Gresser, M. T. Bandu, P. Lindahl, *Proc. Soc. Exp. Biol. Med.* **147**, 52 (1974).
14. P. Lindahl, P. Leary, I. Gresser, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1859 (1973).
15. E. F. Grollman, G. Lee, S. Ramos, P. S. Lazo, R. Kaback, R. M. Friedman, L. D. Kohn, *Cancer Res.* **38**, 4172 (1978).
16. J. M. Weber and R. B. Stewart, *J. Gen. Virol.* **28**, 363 (1975).
17. R. M. Friedman and I. Pastan, *Biochem. Biophys. Res. Commun.* **36**, 735 (1969).
18. D. Brouty-Boye and M. G. Tovey, *Intervirology* **9**, 243 (1978).
19. M. Matsuyama, *Exp. Cell Res.* **124**, 253 (1979).
20. L. M. Pfeffer, E. Wang, F. R. Landsberger, I. Tamm, *Methods Enzymol.* **79**, 461 (1981).
21. A. Takatsuki, K. Arima, G. Tamura, *J. Antibiot.* **24**, 215 (1971).
22. D. K. Struck and W. J. Lennarz, in *Biochemistry of Glycoproteins and Proteoglycans*, W. J. Lennarz, Ed. (Plenum, New York, 1980), p. 35.
23. S. C. Kuo and J. O. Lampen, *Arch. Biochem. Biophys.* **172**, 574 (1976).
24. A. Takatsuki, K. Kohno, G. Tamura, *Agric. Biol. Chem.* **39**, 2089 (1975).
25. G. Tamura, T. Sasaki, M. Matsuhashi, A. Takatsuki, M. Yama Sakai, *ibid.* **40**, 447 (1976).
26. D. Duksin and P. Bornstein, *J. Biol. Chem.* **252**, 955 (Abstr.) (1977).
27. J. B. Ward, *FEBS Lett.* **78**, 151 (1977).
28. A. Frisch, H. Leukowitz, A. Loyter, *Biochem. Biophys. Res. Commun.* **72**, 138 (1976).
29. S. Hickman and S. Kornfeld, *J. Immunol.* **121**, 990 (1978).
30. A. Takatsuki, K. I. Shimizu, G. Tamura, *J. Antibiot.* **25**, 75 (1972).
31. D. Duksin and P. Bornstein, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3433 (1977).
32. R. K. Maheshwari, F. T. Jay, R. M. Friedman, *Science* **207**, 540 (1980).
33. R. K. Maheshwari, D. K. Banerjee, C. J. Waechter, K. Olden, R. M. Friedman, *Nature (London)* **287**, 454 (1980).
34. C. A. Ogburn, K. Berg, K. Paucker, *J. Immunol.* **111**, 1206 (1973).
35. C. Baglioni, *Cell* **17**, 255 (1979).
36. I. Kerr and R. E. Brown, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 256 (1978).
37. B. Lebleu, G. C. Sen, S. Shaila, B. Cabrer, P. Lengyel, *ibid.* **73**, 3107 (1976).
38. A. Billiau, H. Heremans, P. T. Allen, J. De-Maeyer-Guignard, P. DeSomer, *Virology* **73**, 537 (1976).
39. R. K. Maheshwari, A. E. Demsey, S. B. Mohanty, R. M. Friedman, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2284 (1980).
40. W. R. Fleischmann, personal communication.
41. T. Sreevalsan, E. Rozengurt, J. Taylor-Papadimitriou, J. Burchell, *J. Cell Physiol.* **104**, 1 (1980).
42. Supported by grant MV-130 from the American Cancer Society to R.K.M. We are grateful for the excellent technical assistance of E. White and B. Rani.

28 September 1982

Methoxyindoles and Photoreceptor Metabolism: Activation of Rod Shedding

Abstract. *Using an in vitro eye-cup preparation, we have evaluated a potential relationship between methoxyindole metabolism and photoreceptor disk shedding. Melatonin, 6-chloromelatonin, and 5-methoxytryptophol all activate rod disk shedding in culture. The effect is compound specific since serotonin and* N*-acetylserotonin are without effect, and it is similar to shedding in vivo because it is evoked by light and is quantitatively comparable to a normal intact animal response. The results suggest the involvement of 5-methoxyindoles in the control of rhythmic photoreceptor metabolism.*

The methoxyindoles, melatonin and 5-methoxytryptophol, are found in pineal tissue, where their synthesis and release occur in a circadian pattern with peak activity at night (*1*). The enzyme system for melatonin synthesis also exists in the retina (*2*) where the activity of serotonin *N*-acetyltransferase (NAT) (E.C. 2.3.1.5), a key enzyme controlling pineal melatonin rhythmicity (*1*), varies in a circadian pattern (*3*). The idea that rhythmic aspects of photoreceptor-pigment epithelial metabolism, such as disk shedding, may be controlled by melatonin or a related compound (*4*) is supported by observations that retinal NAT activity (*3*) and photoreceptor disk shedding (*5, 6*) are influenced similarly by alterations in the daily light-dark cycle.

We tested the hypothesis of a relationship between methoxyindoles and photoreceptor metabolism by evaluating the effects of such compounds on disk shedding in eye cups from *Xenopus laevis* (*7*). We based our approach on our previous observations that (i) light-evoked rod shedding occurs in culture provided that the medium-bicarbonate concentration is sufficiently high ($\geq$ 30 m*M*), (ii) that a low-bicarbonate medium (20 to 27.5 m*M*) does not support light-evoked shedding, and (iii) that colchicine activates light-evoked shedding in low-bicarbonate medium (*8*). Melatonin is similar to colchicine structurally and at high concentration has been reported to mimic some of its effects (*9, 10*). We therefore evaluated the effects of methoxyindoles by a procedure analogous to that used previously for colchicine (*8*).

Eye cups were prepared by surgical removal of cornea, iris, and lens in dim red light from eyes of postmetamorphic *X. laevis* that had been maintained in cyclic light (12 hours of light and 12 of darkness) at 25°C for at least 3 weeks before use. Disk shedding was evaluated as described previously (*7*) by counting the dense bodies (phagosomes) within pigment epithelium that were derived from the shed and internalized fragments of photoreceptor outer segments. Melatonin activated light-evoked disk shedding at a concentration of 0.5 m*M* (Fig. 1). The response occurred only in light and did not differ significantly from that

reported for colchicine (*8*). Melatonin was effective only when included in both dark preliminary incubation and light incubation periods (Fig. 1). In these experiments eye cups were incubated in dark before the beginning of light exposure or continued maintenance in darkness. Neither preliminary incubation only in 0.5 m*M* melatonin nor the addition of 0.5 m*M* melatonin at the time of light onset caused significant shedding. In this series of experiments melatonin at 1.0 m*M* had no effect (Fig. 1). This result is similar to our finding that colchicine's effect was diminished at higher concentration (*8*). The medium used for Fig. 1 can be made permissive for disk shedding without the addition of melatonin by raising the HCO_3^- concentration to 35 m*M* (*7*); under this condition, melatonin did not cause a further significant (α = .05) increase in disk shedding.

Melatonin also mimics darkness by activating shedding in eye cups from animals treated with constant light (Fig. 2). For these experiments, animals were kept in constant light for 4 days to block disk shedding, and eye cups were prepared in white light. As demonstrated previously (*7*, *8*), in vitro incubation for 3 hours in darkness or for 1 hour of darkness followed by 2 hours of light led to substantially more shedding than incubation in light alone did (Fig. 2). Although little shedding occurred in light alone, the addition of 0.5 m*M* melatonin increased shedding to a level comparable to that seen in 3-hour dark incubations. In contrast, 0.05 m*M* melatonin was ineffective.

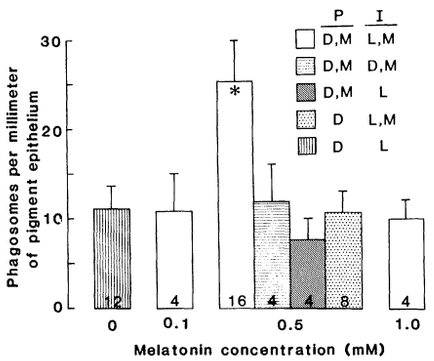

Fig. 1. Effects of melatonin on in vitro shedding. Means and standard errors are based on the number of eye cups indicated at the bottom of the columns. Asterisks signify values significantly different from controls (*$P < .025$) based on analysis of variance followed by Dunnett's *T* statistic (*18*). For all groups, preparation of eye cups in dim red light was followed by a preliminary incubation (*P*) in darkness (*D*) of 45 minutes, followed by incubation (*I*) in light (*L*) or *D* for 2.5 hours. Melatonin (*M*) was initially dissolved in dimethyl sulfoxide (DMSO), which was present in each incubation at 0.2 percent. The DMSO had no effect on disk shedding. As indicated by the key, melatonin was absent (bar with vertical stripes) or was present either continuously in light (white bar) or darkness (horizontal stripes), during the preincubation only (diagonal stripes) or during the light incubation only (stippled bars). The medium was composed of 82.5 m*M* NaCl, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 25 m*M* $NaHCO_3$, 5 m*M* glucose, and a mixture of amino acids (*7*). It was equilibrated with 95 percent O_2 and 5 percent CO_2 (final *p*H of 7.4 ± 0.1).

Table 1. Activation of rod disk shedding in light by 6-chloromelatonin and 5-methoxytryptophol. Dimethyl sulfoxide (DMSO) used to increase drug solubility was present at 0.2 percent. Statistical comparisons with control data were made with analyses of variance followed by Dunnett's *T* statistic. 5-Methoxytryptophol was soluble at 100 μM without DMSO; in a separate experiment without the solvent, it resulted in a twofold activation of shedding in light but not in darkness.

Treatment	Concentration (μM)	Eye cups (No.)	Phagosomes per millimeter of pigment epithelium
Control		12	10.2 ± 1.6
6-Chloromelatonin	0.1	4	8.9 ± 2.2
	1	4	13.1 ± 5.4
	10	8	28.2 ± 3.8*
	50	4	32.9 ± 5.9*
	100	8	22.8 ± 3.1†
	500	4	11.0 ± 2.9
Control		8	13.2 ± 2.0
5-Methoxytryptophol	1	4	18.3 ± 4.4
	10	4	32.4 ± 5.4‡
	100	8	34.8 ± 7.3*

*$P < .005$. †$P < .01$. ‡$P < .025$.

Because of the high concentration (0.5 m*M*) of melatonin needed to elicit a response, we evaluated the effects of 6-chloromelatonin, whose half-life in blood and potency as an inhibitor of gonadal function in rats are greater than those of melatonin (*11*). The 6-chloromelatonin also activated shedding in a dose-related manner (Table 1), but was effective at 1/50 the concentration (10 μM) of melatonin. Like colchicine (*8*) and melatonin, it was less effective at high concentration (0.5 m*M*). Its greater potency suggests that it may be more stable than melatonin under our culture conditions or that it may interact more effectively with melatonin receptors.

As a test of specificity, we examined the effects of serotonin, *N*-acetylserotonin, and 5-methoxytryptophol, compounds normally found in pineal and retinal tissue. Neither serotonin nor *N*-acetylserotonin promoted significant (α = .05) shedding at concentrations of 0.1 and 0.5 m*M*. However, 5-methoxytryptophol was a potent activator of shedding with an effective concentration for the full response at 1/50 the required concentration of melatonin (Table 1). This result, along with the observation that 5-methoxytryptophol is synthesized in the retina (*12*) through a pathway with different components than that for melatonin (*13*), is consistent with a role for the compound as a physiological effector of photoreceptor metabolism.

The similarity of the effects of methoxyindoles and colchicine suggests that they may activate shedding through a similar mechanism. Melatonin at high concentration affects events presumed to be mediated by microtubules and may act by binding to tubulin (*9*). However, it is questionable whether melatonin interacts with tubulin as colchicine does (*10*). Without further information, colchicine in our system could equally well be thought to mimic melatonin through effects on melatonin receptors. Understanding the nature of both drug effects may ultimately provide insight into the normal mechanisms of methoxyindole action or, conversely, into the nature of colchicine side effects unrelated to microtubules.

The demonstration of melatonin- and 5-methoxytryptophol-activated disk shedding in vitro represents the first direct evidence of a relationship between retinal methoxyindole metabolism and photoreceptor disk shedding. In the earliest study of photoperiod-related shedding in rats, such a relationship was suggested because both phenomena oc-

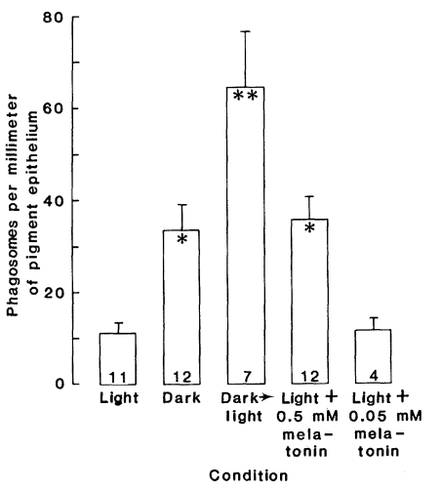

Fig. 2. Eye cups were prepared in room light from animals maintained in constant light for 4 days and placed in medium identical to that in Fig. 1 except that $NaHCO_3$ was raised to 35 m*M* (equilibrated with 95 percent O_2 and 5 percent CO_2; *p*H 7.7). The increase in $NaHCO_3$ made the medium permissive for dark-induction of shedding (see bars "Dark " and "Dark → light") (*7*). Before fixation, eye cups were maintained for 3 hours under conditions indicated on the abscissa. "Dark → light" signifies darkness for 1 hour followed by 2 hours of light. Asterisks signify values significantly different from controls (*$P < .025$; **$P < .005$).

curred rhythmically and because reserpine, a drug known to block the rhythm of melatonin synthesis in the pineal gland, also blocked disk shedding (*5*). It is now known, however, that an intact pineal is not necessary for rhythmic disk shedding in rats or for light-evoked shedding in frogs (*14*). In addition, many experiments including studies of in vitro disk shedding (*7, 15*) strongly suggest that the principal features of the shedding control system are intrinsic to the eye (*16*). A role for methoxyindoles in the regulation of shedding remains tenable, however, because such compounds are actively synthesized within the retina (*2, 3, 17*).

Note added in proof: Subsequent to the acceptance of this report for publication we found that melatonin in a medium containing ascorbic acid, but lacking DMSO, significantly activated shedding at concentrations of 0.05 to 0.5 μ*M*.

JOSEPH C. BESHARSE
DAIGA A. DUNIS
Departments of Anatomy and
Ophthalmology, Emory University School of Medicine,
Atlanta, Georgia 30322

References and Notes

1. J. Axelrod, *Science* **184**, 1341 (1974); D. C. Klein, in *The Neurosciences: Third Study Program*, F. O. Schmitt and F. G. Worden, Eds. (MIT Press, Cambridge, Mass., 1974), pp. 509–515.
2. W. B. Quay, *Life Sci.* **4**, 983 (1965); D. P. Cardinali and J. M. Rosner, *Endocrinology* **89**, 301 (1971); W. A. Gern and C. L. Ralph, *Science* **204**, 183 (1979).
3. S. Binkley, K. B. Reilly, M. Hryschchyshyn, *J. Comp. Physiol.* **139**, 103 (1980); H. E. Hamm and M. Menaker, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4998 (1980).
4. For a review, see J. C. Besharse, *Prog. Retinal Res.* **1**, 81 (1982).
5. M. M. LaVail, *Science* **194**, 1071 (1976).
6. J. C. Besharse, J. G. Hollyfield, M. E. Rayborn, *J. Cell Biol.* **75**, 507 (1977); A. I. Goldman, P. S. Tierstein, P. J. O'Brien, *Invest. Ophthalmol. Vision Sci.* **19**, 1257 (1980).
7. J. C. Besharse, R. O. Terrill, D. A. Dunis, *Invest. Ophthalmol. Vision Sci.* **19**, 1512 (1980); J. C. Besharse, D. A. Dunis, B. Burnside, *J. Gen. Physiol.* **79**, 775 (1982); J. C. Besharse and D. A. Dunis, *Exp. Eye Res.*, in press.
8. J. C. Besharse and D. A. Dunis, in *The Structure of the Eye*, J. G. Hollyfield, Ed. (Elsevier/North-Holland, New York, 1982), pp. 85–96. Both colchicine (1 m*M*) and Colcemid (0.25 m*M*) activated rod disk shedding when eye cups were incubated in darkness with the drug and then exposed to light.
9. S. Banerjee, V. Kerr, M. Winston, J. K. Kelleher, L. Margulis, *J. Protozool.* **19**, 108 (1972); S. Banerjee and L. Margulis, *Exp. Cell Res.* **78**, 314 (1973); D. P. Cardinali and F. Freire, *Mol. Cell. Endocrinol.* **2**, 317 (1975); R. S. Piezzi and J. C. Cavicchia, *Anat. Rec.* **200**, 115 (1981).
10. Although it has been reported that melatonin competes for colchicine binding sites in a crude preparation of brain tubulin [M. Winston, E. Johnson, J. K. Kelleher, S. Banerjee, L. Margulis, *Cytobios* **9**, 237 (1974)], subsequent analysis suggests that melatonin neither prevents in vitro polymerization of tubulin nor blocks colchicine binding [M. Poffenbarger and G. M. Fuller, *Exp. Cell Res.* **103**, 135 (1976)].
11. M. E. Flaugh, T. A. Crowell, J. A. Clemens, B. D. Sawyer, *J. Med. Chem.* **22**, 63 (1979).
12. P. Pévet, M. G. M. Balemans, W. C. Legerstee, B. Vivien-Roels, *J. Neural Transm.* **49**, 229 (1980).
13. R. J. Wurtman and Y. Ozaki, *ibid.* **13** (Suppl.), 59 (1978).
14. M. M. LaVail and P. A. Ward, *Invest. Ophthalmol. Vision Sci.* **17**, 1189 (1978); M. Tamai, P. Tierstein, A. Goldman, P. O'Brien, G. Chader, *ibid.*, p. 558; J. R. Currie, J. G. Hollyfield, M. E. Rayborn, *Vision Res.* **18**, 995 (1978); S. F. Basinger and J. G. Hollyfield, *Neurochemistry* **1**, 81 (1980).
15. J. Flannery and S. K. Fisher, *Invest. Ophthalmol. Vision Sci.* **18**, 638 (1979).
16. P. S. Tierstein, A. I. Goldman, P. J. O'Brien, *ibid.* **19**, 1268 (1980); J. G. Hollyfield and S. F. Basinger, *Nature (London)* **274**, 794 (1978).
17. P. C. Baker, W. B. Quay, and J. Axelrod [*Life Sci.* **4**, 1981 (1965)] demonstrated high ocular hydroxyindole-*O*-methyltransferase (E.C. 2.1.1.4) activity in *X. laevis*. We have found that NAT activity in *X. laevis* retinas shows a sixfold increase over daytime values at night. The NAT rhythm persists in constant darkness and is suppressed in constant light (M. Iuvone and J. C. Besharse, *Brain Res.*, in press). Disk shedding in *X. laevis* is affected in a similar fashion in altered lighting regimes (*6*).
18. B. J. Winer, *Statistical Principles in Experimental Design* (McGraw-Hill, San Francisco, 1962).
19. We thank D. M. Forestner for technical assistance, J. Corley for preparing the manuscript, C. Hartzell and M. Iuvone for critical comments, and M. E. Flaugh of Lilly Research Laboratories for the gift of 6-chloromelatonin. Supported by NIH grant EY02414. J.C.B. was supported by NIH research career development award EY0169.

24 September 1982

Collagen Formation by the Hepatocyte in Primary Monolayer Culture and in vivo

Abstract. *Immunohistochemical techniques were used to confirm biochemical evidence that parenchymal cells isolated from adult rat liver and maintained in nonreplicating monolayer culture for 2 days synthesized type IV basement membrane collagen. On continued incubation in serum-free medium, the hepatocytes also synthesized the interstitial collagens, types I and III. Consistent with these results in culture, type IV collagen was localized to the hepatocytes in slices of pathologic rat liver. Hence collagen formation is a previously unrecognized function of the hepatocyte that may be important in the pathogenesis of liver fibrosis or cirrhosis.*

A fundamental, but as yet unanswered, question about the pathogenesis of liver fibrosis is which type of cell in the liver is responsible for production of collagen, the major protein component of extracellular matrix (*1*). Although it is widely assumed that in the liver, as in most other tissues, collagen is elaborated exclusively by fibroblasts (*2*), or possibly by resting fibroblast precursors (*3*), we have provided evidence that the liver parenchymal cell also synthesizes collagen (*4–6*).

We have shown that the hepatocyte contains a substantial portion of total rat liver prolyl hydroxylase, a key enzyme

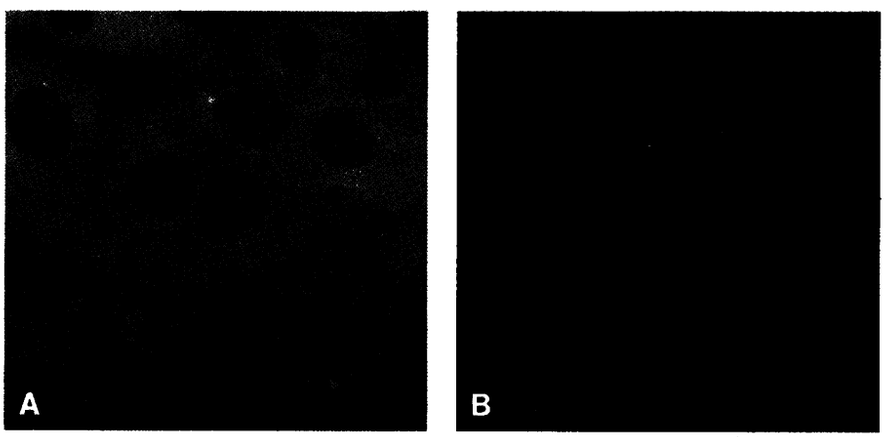

Fig. 1. (A) The presence of type IV collagen in 8-day-old rat hepatocytes in primary monolayer cultures was identified by indirect immunofluorescence. The cultures were reacted with rabbit antibodies to type IV collagen and then with fluorescein isothiocyanate–conjugated goat antibody to rabbit immunoglobulin (*9*). Exposure of parallel cultures to antibodies against type I and type III collagen (*17*) also resulted in positive staining (not shown). (B) The same cells stained with antibodies to albumin, then with rhodamine-conjugated immunoglobulin against the first antibodies. Hepatocyte cultures were prepared from livers of male Sprague-Dawley rats by perfusion in situ with 0.03 percent crude bacterial collagenase (type I, Sigma) (*5*). The hepatocytes were suspended in culture medium and plated at a density of 3.5×10^6 cells in 60-mm plastic culture dishes (*5*). Highly purified, undenatured collagens were used as antigens in the rabbits to prepare the specific antibodies to collagen types I, III, and IV (*9, 17*). The collagens were prepared by a series of salt fractionations followed by selective chromatography on DEAE-cellulose (*11*). Contaminating antibodies reacting to other types of collagens were removed by cross absorption and coupling the absorbed antiserums to immobilized purified antigen, washing, and then recovering type-specific antibodies by elution (*17*).