Jr., *Biochemistry* **3**, 1470 (1964); P. Hochstein, K. Nordenbrand, L. Ernster, *Biochem. Biophys. Res. Commun.* **14**, 323 (1964); W. D. Cash, M. Gardy, H. E. Carlson, E. A. Ekong, *J. Biol. Chem.* **241**, 1745 (1966); B. O. Christopherson, *Biochem J.* **106**, 515 (1968); E. D. Wills, *ibid.* **113**, 325, 333 (1969).
4. S. Orrenius, G. Dallner, L. Ernster, *Biochem. Biophys. Res. Commun.* **14**, 329 (1964); Z. A. Placer, L. L. Cushman, B. C. Johnson. *Anal. Biochem.* **16**, 359 (1966).
5. B. F. Trump, S. M. Duttera, W. L. Byrne, A. U. Arstila, *Proc. Nat. Acad. Sci. U.S.* **66**, 433 (1970).
6. K. S. Chio, U. Reiss, B. Fletcher, A. L. Tappel, *Science* **166**, 1535 (1969).
7. L. Napolitano, F. Lebaron, J. Scaletti, *J. Cell Biol.* **34**, 817 (1967).
8. E. D. Korn, *Fed. Proc.* **28**, 6 (1969); S. B. Fleischer, F. Fleischer, W. Stoeckenius, *J. Cell Biol.* **32**, 193 (1967); W. Stoeckenius and E. M. Engleman, *ibid.* **42**, 613 (1969).
9. E. A. Smuckler and M. Arcasoy, *Int. Rev. Exp. Pathol.* **7**, 305 (1969); B. F. Trump and A. U. Arstila, in *Principles of Pathobiology*, M. F. La Via and R. B. Hill Jr., Eds. (Oxford Univ. Press, New York, 1971), pp. 9–95; A. U. Arstila and M. A. Smith, *Fed. Proc.* **30**, 569 (1971).
10. M. Bassi, *Exp. Cell Res.* **20**, 313 (1960); E. S. Reynolds, *J. Cell Biol.* **19**, 139 (1963).
11. T. D. Gritzka and B. F. Trump. *Amer. J. Pathol.* **52**, 1225 (1968).
12. H. S. Utley, F. Bernheim, P. Hochstein, *Arch. Biochem. Biophys.* **118**, 29 (1967).
13. Supported by grant AM 10698 to B. F. Trump and grants from Sigrid Juselius Foundation and Finnish National Research Council for Medical Sciences to A.U.A.

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Opossum Fetuses Grown in Culture

Abstract. *Opossum fetuses explanted at limb bud stages have been successfully grown in culture for periods up to 20 hours. Blood circulation was maintained, and organogenesis continued at about the same rate as in vivo.*

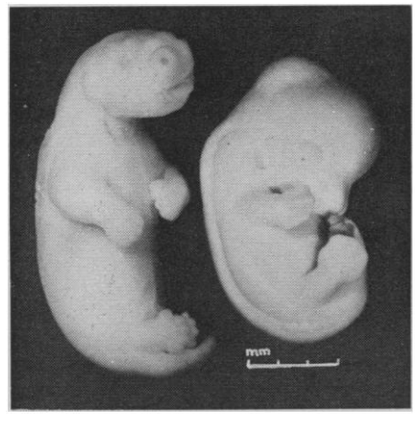
Despite the obvious advantages for observation and experiment of growing mammalian fetuses in culture, few successful methods have yet been devised. The main difficulty appears to be failure to provide any adequate substitute in culture for the allantoic placenta or to support the growth of this placenta if it is explanted with the fetus. Some workers have maintained the isolated fetus in conditions adequate for certain physiological studies (*1, 2*) but such "cultures" have not usually resulted in growth or differentiation.

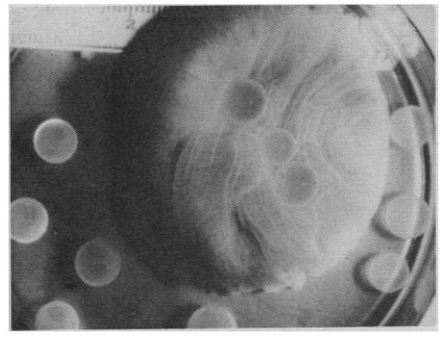
Only in rodents has there been much success in obtaining fetal development. The explanted rat fetus, for example, can now be grown for periods up to 2 or 3 days at any time between the primitive streak (8th gestation day) and 55-somite, limb bud (14th day) stages of development (*3*). Rodents are unusual among eutherian mammals in that during the early stages of organogenesis the respiratory and nutritional needs of the fetus are mediated entirely by a yolk-sac placenta; only later does the allantoic placenta become functional. The yolk-sac placenta is a relatively simple structure, and when explanted with the early fetus it grows and develops a blood circulation in a manner very similar to that in vivo. Hence the rodent fetus can grow and differentiate in culture until such time as the allantoic placenta becomes indispensable.

In view of these findings it seemed desirable to examine the behavior in culture of embryos from a mammal that does not have a complex allantoic placenta and that relies almost entirely on a yolk sac. Such a mammal is the opossum *Didelphys marsupialis virginiana* (Kerr), a marsupial common in North America. The embryonic development of the opossum has been described in detail by McCrady (*4*). The gestation period is 12¾ days, and the young are born at a stage of development corresponding about to that of the mouse fetus of the same age (or rat fetus of 14 days) except that the forelimbs, lungs, pancreas, and a few other organs are precociously developed (Fig. 1). The allantois is present only as a simple sac that enlarges with urine during the last 3 days of gestation and probably has no placental function. But the yolk sac, which in rats is about 7 mm in diameter on the 13th day of gestation, develops in the opossum to over 20 mm in diameter, with folds closely adhering to those of the uterine endometrial surface.

Although the opossum breeds erratically in captivity and is much more difficult to maintain in self-perpetuating colonies than the common laboratory animals, a reliable method has been developed for obtaining embryos of predetermined age from recently captured opossums (*5*). The animals are trapped during the breeding season, at which time practically all the females either have young in the pouch or within a few days give birth to litters sired in the wild. If the young are removed from the pouch, a postlactational estrus follows a week later. This estrus is of exceptional intensity, and when the females are caged with males a high proportion of fertile matings can usually be achieved; the females that fail to mate at this estrus may do so at the end of the next cycle 28 days later. Because the young can be removed from the pouch at any desired time the method is very convenient for initiating timed pregnancies as required.

In the present study 12 females, newly trapped in Florida, were purchased from a dealer during February





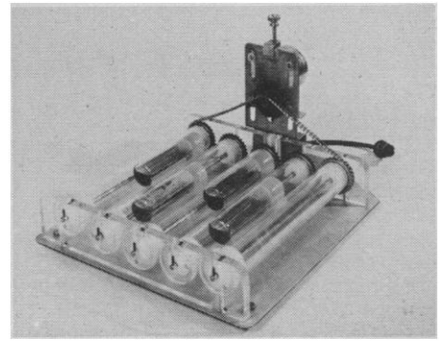


Fig. 1 (top left). Newborn opossum (left) and 14-day rat fetus (right) at same magnification. Forelimbs and parts of the head of the opossum are very advanced, but hindlimbs are only slightly more developed than in rat; tail is shorter. Fig. 2 (top right). Opened opossum uterus at 8 to 9 days of pregnancy. At this stage the embryos lie free in the uterine cavity. Fig. 3 (bottom right). Apparatus for rotating culture tubes.

and early March, and all were found to have pouch young (varying in age from 14 to 45 days) on arrival in the laboratory. At different times the pouch young were removed. Eight of the females were placed with males during postlactational estrus, and four were subsequently found to have embryos. Four of the females were placed with males during the second estrus following lactation, and three became pregnant. The animals were housed individually in rabbit cages (61 by 42 by 36 cm) or, for mating, were released into concrete-lined pits sunk into the ground, with wire mesh covers, and provided with bedding of dried grass. The pits were of two sizes; in the smaller, 8 feet long and 5 feet wide (244 by 152 cm), two or three females were placed with one male; in the larger, 17 feet by 5 feet, five females were placed with one or two males. The animals had free access to water and Gaines meal (General Foods Corp.).

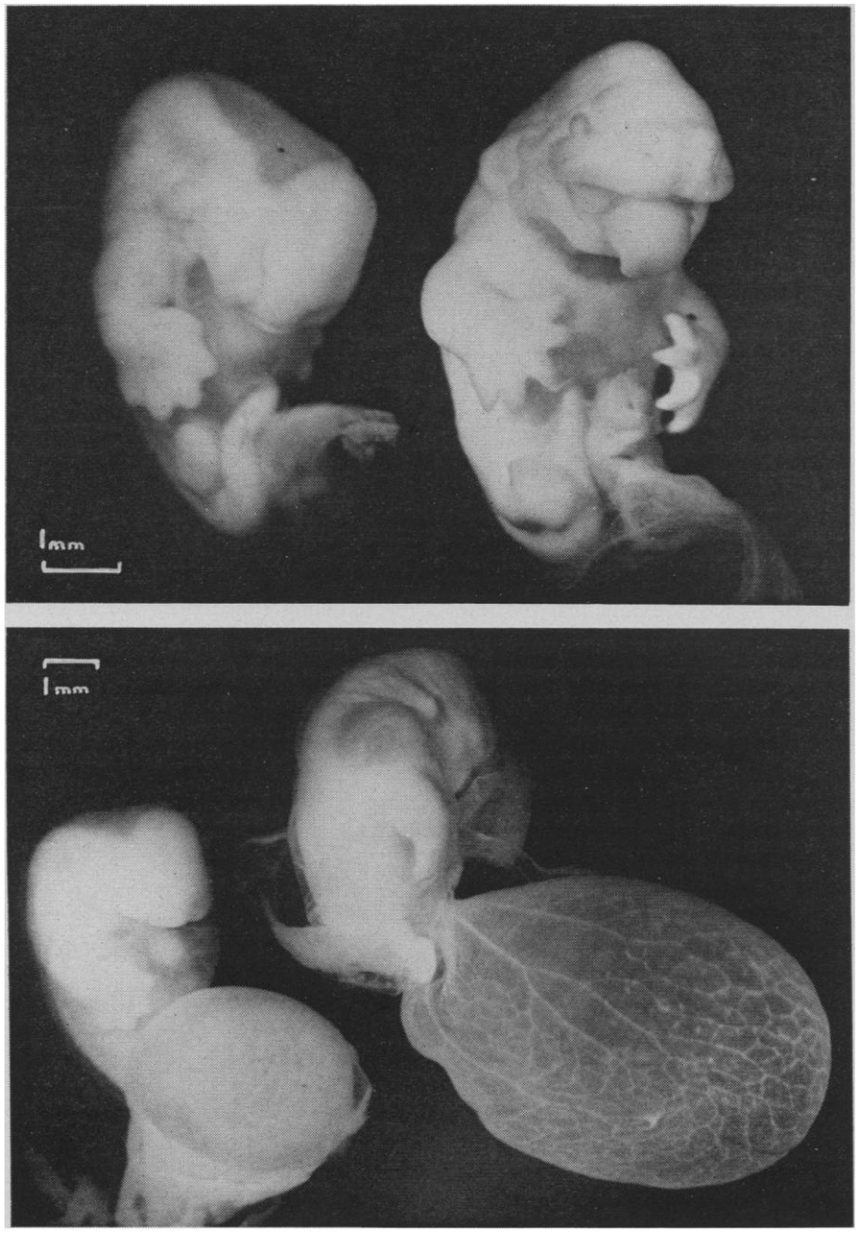


Fig. 4. Opossum embryos from the same litter, all explanted at 11 days' gestation. Left, as explanted (McCrady stage 31–32); right, after 20 hours in culture (McCrady stage 33). (Top) Dissected free of all membranes to show growth of the fetus. Among the externally visible developments in culture are the digits on the forefoot, the change of the hind limb from bud to "paddle" stage, and the formation of the eyelid folds. Also, and characteristic of stage 33, the head has become raised from the chest with mouth open and tongue protruding. The forelimb has lengthened from 2.3 to 3.1 mm, the hind limb from 1.0 to 1.5 mm; and the total weight has increased from 26 to 39 mg (all measurements made after fixation). (Bottom) Dissected free of the yolk sac but photographed to show the growth of the allantois in culture. Although this membrane does not form a complex placenta in the opossum, toward the end of gestation it enlarges and fills with urine.

It is not at present possible to detect, by palpation or any other nonsurgical method, the stage of gestation of a pregnant opossum (or indeed to ascertain even that the animal is pregnant). It is therefore important to know the time of mating. The present study gives some indication of the accuracy with which this can be predicted from the time the pouch young are removed. The four successful matings during the first postlactational estrus all occurred 4 to 6 days after removal of the young; the three during the second estrus were more dispersed and occurred 30 to 35 days after removal of the young [Mizell *et al.* (*5*) give further data on predicting time of mating].

Serum for culturing was obtained from male and female opossums, including those providing the embryos, with the animals under Penthrane (methoxyflurane, Abbott) anesthesia. The abdomen was opened, the dorsal aorta was exposed by dissection, and up to 60 ml of blood was withdrawn into a plastic, disposable syringe with a No. 16 needle and transferred to centrifuge tubes. After the blood had clotted it was centrifuged and the serum was decanted. Clotting was slow, and it was best to leave the blood for 18 hours or more before centrifuging; otherwise clots tended to develop in the decanted serum.

The uterus from each female was transferred to a dish, usually containing Tyrode or Hanks saline, and carefully opened by cutting longitudinally through the wall with fine scissors. The younger embryos with their membranes lie freely in the uterine cavity (Fig. 2) and can easily be tipped or flushed out, but the yolk sacs of the older embryos adhere to the endometrium and to each other and have to be pulled away from the uterus [see Padycula and Taylor (*6*) for a recent study of endometrial differentiation in the pregnant opossum]. The number of embryos in each pregnant female varied from 12 to 24.

The nutrient medium used was tissue culture medium 199 or Ham's F10, with 20 percent serum. The embryos were explanted and the medium pre-

pared aseptically, and antibiotics were added (50 units of penicillin and 50 μg of streptomycin per milliliter). The culture chambers were glass specimen tubes (30 by 100 mm or 25 by 75 mm) with silicone rubber stoppers. Half the capacity of each tube was filled with the nutrient medium and the other half with either 5 percent CO_2 and 95 percent O_2 or 5 percent CO_2 in air, renewed every 6 to 8 hours. The tubes were laid horizontally on rollers and continuously rotated at 30 to 40 revolutions per minute during incubation (Fig. 3) at 37°C. Rotation keeps the explanted embryos gently swirling around in the medium and improves oxygenation of the medium by continuously exposing a fresh layer to the gas phase; with rat embryos the method gives much more growth than can be obtained in static cultures (*7*).

The best results were obtained from four litters explanted at 10 to 11 days' gestation. At 10 days the embryo has about 28 somites, the heart has just begun to beat, and the forelimbs are flattening to the paddle shape; but the hind limb buds and allantois have scarcely begun to extend from the body surface. The yolk-sac diameter is about 17 mm; a vascular area covers a third to a half its surface. At this stage the yolk sac lightly adheres to the endometrium but can be pulled away easily and transferred undamaged to the culture vessel. By 11 days the vascular area of the yolk sac adheres more firmly to the endometrium and the avascular areas of adjacent yolk sacs are fused. The yolk sacs at this stage can be pulled away in a mass from the endometrium but are damaged and collapse when separated from one another. However, it is possible to keep the vascular area intact, (about 25 mm in diameter), and this can be transferred to the culture vessel with the embryo.

Table 1 summarizes the culture conditions and results of the 33 embryos explanted into 14 roller tubes at 10 to 11 days' gestation. Survival was assessed by the length of time that a good blood circulation could be seen in the yolk-sac blood vessels. Medium 199 was used in most of the cultures, but Ham's F10 gave similar results. In two cultures the medium was augmented with uterine fluid, and probably some yolk-sac fluid, from the same uterus as the embryos; but this did not have any beneficial effect. However, the results were better when the atmosphere contained 95 percent oxygen instead of air, as shown particularly by the proportion of embryos maintaining a blood circulation for 12 hours or more.

Table 1. Survival of embryos from four litters explanted at 10 to 11 days of gestation and incubated in tissue culture medium 199 or Ham's F10 with 20 percent fetal calf serum (FC) or opossum serum (O).

Age of embryos (days)	Tube No.	Medium	Serum	O_2 (%)	Number of embryos			
					Total	Blood circulation maintained		
						6 hr	12 hr	18 hr
11	1	199	FC	20	3			
11	2	F10	O	20	2	1		
11	3	199	O	95	3	1	1	
10	4	199	O	20	3	2	1	
10	5	199*	O	20	3	1		
10	6	199	O	95	3	3		
11	7	199	O	20	2			
11	8	199	O	95	2	2	2	2
11	9	199	O	95	2	2	2	
11	10	199*	O	95	2	1	1	
11	11	F10	O	95	2	2		
11	12	F10	O	95	2	2	2	
11	13	199	O	95	2	1		
11	14	199	O	95	2	2		

* Culture also contained "uterine washings."

The embryos continued to grow and differentiate in culture; development was conspicuous when good blood circulation was maintained in the yolk sac. The rate of differentiation appeared to be about the same as in vivo. The longest-lived embryos explanted at 10 days developed from McCrady stage 29 to stage 30–31, and those explanted at 11 days developed from stage 31–32 to stage 33 (Fig. 4). The embryos explanted at 11 days commonly showed hemorrhage during the culture period, probably resulting from injury at explantation.

Two litters of embryos were explanted at 8 to 9 days' gestation. In the first the embryos all had about 8 to 10 somites, with yolk sacs about 8 mm in diameter and surrounded by the shell membranes. They were incubated in roller tubes with medium 199 containing 20 percent opossum serum and the same gas phases used with the older embryos. Within 1 hour the yolk sacs were collapsing away from the shell membranes, and after 6 hours all embryos were dead. It was thought possible that the embryos were damaged by the alkaline *p*H of the medium before it became equilibrated with the CO_2, particularly as measurements of *p*H of the uterine fluid in some pregnant opossums gave values of 6.5 to 7.0. This was further suggested by the results from the second litter: the yolk sacs of the embryos placed in medium mixed with (pregnant) uterine fluid or pre-equilibrated with 5 percent CO_2 remained expanded; the others did not. (The uterine fluid may have had other beneficial effects in addition to lowering *p*H, but the numbers of embryos are too small to be sure of this.) The two best embryos had beating hearts after 18 hours in culture; but the yolk sacs did not expand beyond the size at explantation, and there was little evidence of embryonic growth.

In this preliminary study the numbers of embryos used were fairly small and the culture apparatus was very simple. Only a few variations in culture conditions could be tried. Nevertheless the older fetuses survived and developed well for up to 20 hours, and there seems little doubt that with further work this period could be extended. Already the most advanced stage attained in culture (McCrady stage 33) is within 24 hours of the end of normal gestation, suggesting the possibility of rearing opossums from cultured fetuses; it is known that newborn young can be placed by hand in the pouch to attach to the nipples and continue development (*5*). As a source of embryos for studying fetal development in culture, and perhaps for following the postnatal results of experiments on the fetus, the opossum has considerable promise.

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References and Notes

1. D. P. Alexander, H. G. Britton, N. M. Cohen, D. A. Nixon, in *Foetal Autonomy*, G. E. W. Wolstenholme and M. O'Connor, Eds. (Churchill, London, 1969), p. 95.
2. W. M. Zapol, T. Kolobow, J. E. Pierce, G. G. Vurek, R. L. Bowman, *Science* **166**, 617 (1969).
3. D. A. T. New, in *Methods in Mammalian Embryology*, J. C. Daniel, Ed. (Freeman, San Francisco, 1971), p. 305; D. A. T. New and P. T. Coppola, *J. Reprod. Fertil.* **21**, 109 (1970); D. A. T. New and J. C. Daniel, *Nature* **223**, 515 (1969).
4. E. McCrady, Jr., *The Embryology of the Opossum*, American Anatomical Memoir No. 16 (Wistar Institute, Philadelphia, 1938).
5. M. Mizell, R. A. Warriner III, D. E. Ramsey, R. Spencer, in preparati → M. Mizell and J. J. Isaacs, *Am. Zool.* **10**, 141 (1970).
6. H. A. Padycula and J. M. Taylor, *Anat. Rec.* **169**, 394 (1971).
7. D. A. T. New and P. T. Coppola, in preparation.

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Prostaglandin E_1 in Platelet Harvesting: An in vitro Study

Abstract. *Prostaglandin E_1 (10^{-8} to 10^{-7} molar) is effective in improving the preparation of human platelet concentrates from plasma rich in platelets and from whole blood. A procedure has been developed for the use by blood banks, on a trial basis.*

The demand for platelet concentrates has increased steadily in view of their usefulness in treating hemorrhage due to thrombocytopenia, and their relation to chemotherapy and radiation and immunosuppressive therapy. Platelets are in short supply, however, because they can be stored only for a short time and because the cost, in time and materials, necessary to prepare them in therapeutic quantity is high. Usually, after centrifugal separation, platelets are suspended in one-tenth volume of autologous plasma. Until recently, this procedure was performed at low temperatures; however, the cold reduces the viability of platelets (*1*). This suggests that preparation and storage of platelet concentrates (PC) at room temperature will improve both the harvesting of the platelets and the maintenance of their function in vivo.

During the high-speed centrifugation of platelet-rich plasma (PRP), massive aggregates form because of the release of cell components or the exposure of sticky sites. Prostaglandin E_1 (PGE_1) is one of the most potent inhibitors of platelet aggregation known (*2*). It inhibits the rounding of platelets induced by adenosine diphosphate (ADP). This rounding is associated with a stickiness which then results in the aggregation of platelets when they come in contact with one another (*3*). The effect of PGE_1 on the aggregation and shape of platelets during their collection and storage was studied to provide a basis for the trials described by Becker *et al.* and Valeri *et al.* (*4*).

Blood was drawn from healthy volunteers into blood bags containing an anticoagulant: acid, citrate, and dextrose (ACD). Sterile polystyrene test tubes (Falcon) were used for studies on centrifugation and storage. Platelet-rich plasma was prepared by centrifugation (375*g*, 15 minutes) and portions of it (3 ml, *p*H 7.1) were transferred into the plastic test tubes. Prostaglandin E_1 (*5*) (0 to 60 ng/ml) was added and mixed well. After centrifugation at room temperature (1500*g*, 15 minutes) the shape of the resultant pellet was examined. The platelets which were originally centrifuged without PGE_1 or at low concentrations of PGE_1 ($\leqq$ 2 ng/ml) spread out in a thin film over the bottom of the test tube. However, platelets mixed with greater concentrations of PGE_1 ($\geqq$ 5 ng/ml) lay in a smooth, clearly defined round pellet (Table 1). Plasma supernatant (2.5 ml) was then removed by suction, and the platelets were suspended in the remaining plasma by gentle pumping with a plastic pipette.

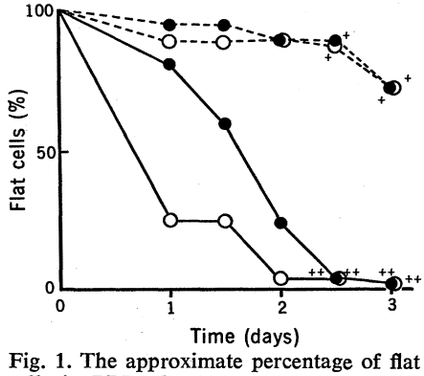

Fig. 1. The approximate percentage of flat cells in PRP after storage at room temperature (estimated with phase contrast microscopy); +, microscopic aggregates of less than ten cells; ++, macroscopic aggregates; open circles, at *p*H 6.5, closed circles, at *p*H 7.1; solid line, control (no PGE_1 added); dotted line, experimental [with addition of PGE_1 (60 ng/ml)].

The control sample (no PGE_1 added) showed visible aggregates. Low concentrations of PGE_1 ($\leqq$ 5 ng/ml) did not prevent microscopic aggregation, but higher concentrations of PGE_1 produced a smooth suspension of platelets. Microscopically, the shape of the free cells was flat in concentrations of PGE_1 greater than or equal to 10 ng/ml. These PC were then stored for 18 hours at room temperature (22°C). During storage, a gradual sedimentation of platelets occurred; however, when the PC's were shaken, a dispersion was observed in those with high PGE_1 concentrations ($\geqq$ 10 ng/ml). Since PGE_1 accelerates the dispersion of platelet clumps induced by ADP (*2*), we also studied the effects of adding PGE_1 immediately before suspension. When this method was used and the PC's were shaken, control samples showed only slight dispersion, even with 60 ng of PGE_1 per milliliter of PRP. Further aggregation during overnight storage was prevented, however. Acidic PRP (brought to *p*H 6.5 with excess ACD solution before centrifugation) showed far less aggregation when resuspended, even without PGE_1, although sphering of individual cells occurred rapidly.

The change in shape of platelets in PRP at *p*H 7.1 and *p*H 6.5 (made with excess ACD solution) was studied in sterile plastic test tubes with PGE_1 (60 ng/ml) or without PGE_1. Test tubes were kept in the vertical position at room temperature (22°C) for 0 to 3 days. Individual test tubes were inverted gently before examination to suspend the platelet sediment. Control samples (those without the addition of PGE_1) yielded massive aggregates at both *p*H values after 2 to 3 days of storage and subsequent suspension. PGE_1 again prevented the macroscopic aggregation and made suspension possible; however, a few fine aggregates were observed microscopically (Fig. 1). After 1 to 2 days of storage at acidic or neutral *p*H, most platelets became spherical when subsequently suspended. Acidic conditions were more destructive to cells than were neutral *p*H environments. The PGE_1 (60 ng per milliliter of PRP) slowed the course of cell sphering during storage at acidic or neutral *p*H. After storage for 3 days, more than 60 percent of the free cells were flat at both *p*H values, in spite of microscopic aggregation.

Cold-induced sphering of platelets is reversible during the first few hours (*6*); at low temperature, platelet cells may become sticky and, consequently,