Separation of Cells Having Histochemically Demonstrable Glucose-6-Phosphatase from Suspensions of Hamster Kidney Cells in an Isokinetic Density Gradient of Ficoll in Tissue Culture Medium

Thomas G. Pretlow II, MD, Judith Jones and Sherron Dow

Suspensions of enzymatically disaggregated hamster kidney cells were separated primarily according to differences in diameter using velocity sedimentation in a previously described isokinetic density gradient and according to differences in density in an isopycnic density gradient. Cells which contained histochemically demonstrable glucose-6-phosphatase were thought to be cells from proximal tubules and constituted 46.5 ± 14.1% of the cells in the starting sample suspension of disaggregated kidney cells. The purest gradient fractions from experiments using velocity sedimentation contained 98.0 ± 0.6% cells which demonstrated glucose-6-phosphatase activity. More than 99.0% of these cells excluded trypan blue. Isopycnic sedimentation was not an effective means of purifying proximal tubule cells (Am J Pathol 74:275–286, 1974).

IT IS WIDELY RECOGNIZED that an understanding of the pathobiology of the kidney depends upon an understanding of renal physiology, biochemistry and cell biology.[1] In attempting to characterize the kidney biochemically, both in the normal and in the abnormal states, methods have been developed which permit the isolation of specific elements of the kidney for more specific investigation. Efforts to isolate specific elements of the kidney have included dissection of the renal cortex away from the medulla,[2–5] purification of glomeruli,[6–11] purification of tubules [10,12–14] and microdissection of several different components.[15,16] To our knowledge, there have been no reports of efforts to isolate single kinds of cells from kidneys. Wachstein and others [17–19]

From the Departments of Pathology and Engineering Biophysics, Schools of Medicine and Dentistry, University of Alabama in Birmingham, Birmingham, Ala.

Supported by Grants CA-12870, CA-13148 and DE-2670 from the National Institutes of Health and Grant ET-50 from the American Cancer Society; Dr. Pretlow is the recipient of Research Career Development Award K4-CA-70584-01 from the National Institutes of Health.

Accepted for publication October 8, 1973.

Address reprint requests to Dr. Thomas G. Pretlow, Department of Pathology, University of Alabama in Birmingham, The Medical Center, University Station, Birmingham, AL 35294.

have reported that the proximal tubule of most studied mammals (rat, mouse, rabbit, human, cat, dog and guinea pig) exhibits strong glucose-6-phosphatase activity and that other parts of the kidney do not. This suggests that glucose-6-phosphatase is a suitable marker for the identification of proximal tubule cells in suspension. In an investigation which we report here, after confirming the suitability of this enzyme as a marker for the proximal tubule in frozen sections of hamster kidney, we have developed a method for the purification of cells from the proximal tubule. After purification, purified cells from the proximal tubule were found to be viable. They have been maintained in culture for periods of 8 to 10 weeks.

Materials and Methods

Gradients and Centrifugation

Linear density gradients of sterile Ficoll (polysucrose, average molecular weight 400,000; Pharmacia Fine Chemicals, Piscataway, NJ) in Joklik's modification of minimum essential medium (Grand Island Biological Co, Grand Island, NY) were contained in 100-ml polycarbonate centrifuge tubes (Intenational Equipment Co tube 2806); centrifuged at 4.0 C in the MSE Mistral 6L centrifuge (VWR Scientific, San Francisco, Calif); constructed using the two-chambered gradient generator (Lido Glass, Stirling, NJ) which was described in an earlier report;[20] and collected using the gradient tapping cap (Halpro Inc, Rockville, Md) which was described previously.[20]

Velocity sedimentation was carried out using a previously described isokinetic gradient[21] which varied linearly from 2.7% w/w Ficoll at the sample-gradient interface, 13.7 cm from the center of revolution, to 5.5% w/w Ficoll at the gradient–cushion interface, 26.7 cm from the center of revolution. Isopycnic sedimentation was carried out in gradients which varied from 4.1% w/w Ficoll at the sample-gradient interface 14.2 cm from the center of revolution, to 43.0% w/w Ficoll at the gradient–cushion interface, 26.0 cm from the center of revolution. Isokinetic gradients were constructed on top of 5.5-ml cushions, and isopycnic gradients on top of 10-ml cushions of 45.0% w/w Ficoll. Solutions were sterilized by filtration (Millipore Corp, Bedford, Mass), except for Ficoll for isopycnic gradients, which is too viscous to be susceptible to sterilization by filtration and must be autoclaved as described previously.[20] The centrifuge speed was monitored continuously using an electronic stroboscope (General Radio, West Concord, Mass) during velocity sedimentation experiments.

Kidney Cells for Starting Sample Suspension

Male 7- to 8-week-old, Golden Syrian hamsters were anesthetized with ether. The abdomens were entered, and the kidneys were perfused *in situ* with 250 ml of isotonic saline *via* the vena cava. Perfusion was carried out through a 22-gauge needle under a pressure of 120 cm of water at room temperature; kidneys were then excised, weighed and minced to fragments of 1 to 2 mm in greatest dimension. The fragments were gently agitated with a magnetic stirrer in three successive 10-minute changes of Joklik's medium containing 10% fetal calf serum (Grand Island Biological Co). The washing solutions were discarded, and the fragments were

similarly stirred in successive 50-ml, 20-minute changes of Joklik's medium containing 1 mg/ml pronase (EM Laboratories, Elmsford, NY) at room temperature. After being decanted from the tissue, the pronase solutions were cooled at 4.0 C and sedimented at 97*g* for 7½ minutes. The recovered cells were resuspended in five volumes of Joklik's medium with 10% fetal calf serum. After the tissue had been exhaustively digested, the first three 20-minute pronase digestions were found to contain many red blood cells and cells which were incapable of excluding trypan blue; the cells were discarded. The cells from the fourth through the thirteenth digestions were combined, filtered through a single layer of Nitex (Tobler, Ernst, and Traber, Elmsford, NY) having a pore diameter of 36 μ, sedimented at 97*g* for 7½ minutes, resuspended in 8 ml of Joklik's medium with 10% fetal calf serum, filtered again through a single layer of Nitex having a pore diameter of 36 μ and diluted such that the 7-ml starting sample suspensions which were layered over the gradients contained 29.6 to 30.8 $\times$ 10^6 cells for isokinetic cell separation experiments and 16.2 to 20.0 $\times$ 10^6 cells for isopycnic cell separation experiments.

Gradient Fractions

Gradients were collected in 4-ml fractions. All fractions from both isokinetic and isopycnic gradients were treated identically. Cell counts were performed using hemocytometer chambers. Refractive indices were measured on all gradient fractions in order to confirm the linearity of the gradients. Slides for microscopic examination were prepared in triplicate from all gradient fractions using the Cytocentrifuge (Shandon Southern Instruments, Inc, Sewickley, Pa); one series of slides was stained with Wright stain, one with the Wachstein[22] procedure for glucose-6-phosphatase and another with the Melnick[23] procedure for glucose-6-phosphatase. Differential cell counts were performed counting 200 cells from each fraction and 500 cells from the purest gradient fractions.

Results

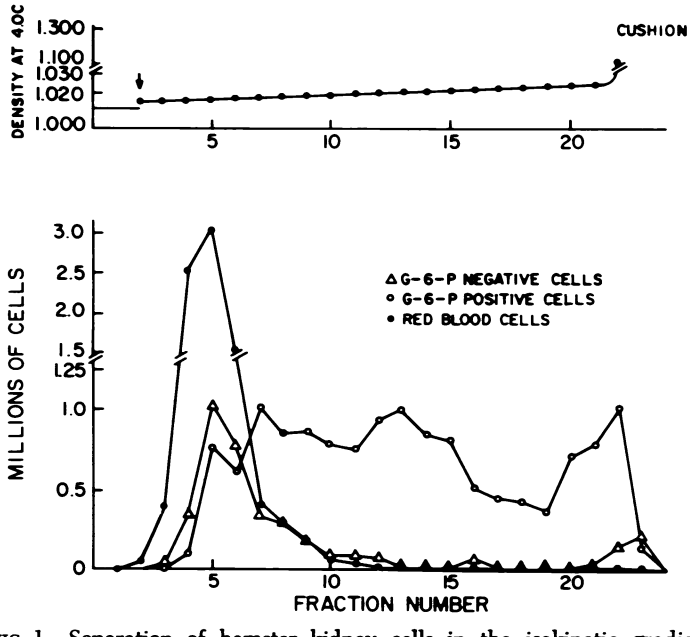
Sample Composition

We obtained an average of 21.1 $\times$ 10^6 cells/g of perfused hamster kidney. The standard deviation was $\pm$4.4 $\times$ 10^6; the maximum, 26.6$\times$ 10^6; and the minimum, 16.8 $\times$ 10^6 cells. These cells were 34.6 $\pm$ 14.7% red blood cells, 46.5 $\pm$ 14.1% cells which demonstrated glucose-6-phosphatase activity histochemically and 18.0 $\pm$ 5.4% cells which failed to demonstrate glucose-6-phosphatase activity. Significant differences between the two methods for the demonstration of glucose-6-phosphatase were not observed. More than 90% of the cells in the starting sample suspensions excluded trypan blue.

Velocity Sedimentation

Using the methods which have been described in detail previously,[20,21] it was determined that cells which demonstrated glucose-6-phosphatase activity could be separated from other kidney cells following centrifugation for 14 minutes at 4.0 C using a centrifugal force of 55*g*. In light

of the studies of Wachstein and others [17,19] using kidneys from several different mammals, and in light of frozen sections of hamster kidney which we stained for glucose-6-phosphatase using the same methods which we used in staining cell suspensions, we believe that the cells which exhibit histochemically detectable glucose-6-phosphatase are cells from proximal tubules. These cells were purest in fractions 13 through the gradient–cushion interface (Text-figure 1) and were generally distributed in two modal populations. One of these modal populations (Figure 1) was located in fraction 13 (±1 fraction) and consisted of primarily single cells. The cells in this modal location were 95.9 ± 3.6% cells which stained positively for glucose-6-phosphatase (Figure 1), 3.5 ± 3.1% cells which failed to exhibit glucose-6-phosphatase and 0.5 ± 0.3% red blood cells. The second modal population of cells which stained positively for glucose-6-phosphatase was located in fractions 20 through the gradient–cushion interface. In general, it is a dangerous practice to compare the sizes of cells in life based on their sizes when examined after fixation and staining on a smear or cytocentrifuge prep-



TEXT-FIG 1—Separation of hamster kidney cells in the isokinetic gradient. In this experiment, 30.1 × 10^6 hamster kidney cells were separated primarily according to differences in diameter in the isokinetic gradient using a centrifugal force of 55g for 14 minutes at 4.0 C. Cells which demonstrate glucose-6-phosphatase activity are widely separated from the other kinds of kidney cells. An *arrow* marks the sample–gradient interface on the density plot.

aration, since different cells shrink or spread out differently during drying, fixation and staining; however, in examining the cells from this second modal population in a hemocytometer chamber, they appeared to be comprised of some very large single cells, some dimers and some larger aggregates. The larger aggregates were located at the gradient–cushion interface. The purest fraction of cells which exhibited glucose-6-phosphatase in this modal population was generally located in the fraction immediately before the fraction which contained the gradient–cushion interface. The cells in this fraction were 98.0 ± 0.6% cells which stained positively for glucose-6-phosphatase, 1.9 ± 0.6% cells which lacked demonstrable glucose-6-phosphatase and 0.1 ± 0.1% red blood cells. It appears that the decreased purity which was found in the fraction which contained the gradient–cushion interface simply reflects the presence of aggregates which contained heterogeneous kinds of cells from the kidney. The modal population of cells which lacked histochemically demonstrable glucose-6-phosphatase was variably located in fraction 5 or fraction 6 (Figures 2 and 3).

Isopycnic Sedimentation

Isopycnic sedimentation was carried out using a centrifugal force of 800*g* for 90 minutes. Isopycnic centrifugation was not an effective means of purifying any of the identified cell types from the kidney. Following isopycnic sedimentation, red blood cells, cells which exhibited glucose-6-phosphatase activity and nucleated cells which failed to exhibit glucose-6-phosphatase activity were broadly distributed between densities of 1.05 and 1.12 g/ml. Interestingly, the red blood cells differ from red blood cells obtained from tissues which are disaggregated using collagenase [24] or lysozyme [25] and from red blood cells which have not been treated with any enzyme in that the pronase-treated red blood cells are much more heterogeneous with respect to density than red blood cells which have not been exposed to pronase. This effect of pronase has been observed in our laboratory previously [26] and probably represents selective injury of the red blood cell by pronase. In preliminary experiments with kidney and in the evaluation of methods for the disaggregation of other tissues, we have found that fewer red blood cells per gram of tissue are obtained using pronase than are obtained using trypsin, collagenase, lysozyme, etc.

Recovery

Following separation in the isokinetic gradient, 79.2 ± 2.4% of the cells which stained positively for glucose-6-phosphatase, 52.8 ± 5.8% of

the cells which failed to demonstrate glucose-6-phosphatase and 72.7 ± 1.6% of the red blood cells which had been layered over the gradients were recovered from the gradients. After isopycnic centrifugation, 12.9 ± 8.4% of the cells which exhibited glucose-6-phosphatase, 24.8 ± 23.9% of the cells which lacked demonstrable glucose-6-phosphatase and 19.8 ± 2.8% of the red blood cells which had been layered over the gradients were recovered from the gradients. Undoubtedly, the wall effect artifact [27] accounts for some of the attrition of cells in both isopycnic and velocity cell separation. The unusually and disproportionately great loss of cells during isopycnic centrifugation probably reflects the deleterious effect of the much higher centrifugal force and longer duration of centrifugation which are required for isopycnic cell centrifugation. Isopycnic centrifugation is carried out at 800*g* for 90 minutes; isokinetic centrifugation, at 55*g* for 14 minutes.

Viability

Following purification in the isokinetic gradient, more than 99% of the purified cells from proximal tubules (fractions 13 through the gradient–cushion interface) excluded trypan blue. In experiments which will be reported in detail in a later report, these cells have been maintained in tissue culture for 8 to 10 weeks.

Discussion

The availability of pure viable cells from the proximal tubule should facilitate the specific biochemical and physiologic characterization of the proximal tubule and its function. The existing methodology has limited the biochemical characterization of components of the kidney to studies of those components which have been purified previously, *ie*, cortex as dissected from the medulla,[2–5] glomeruli,[6–11] tubules [10,12–14] and microdissected component of the kidney.[15,16] While often lacking the precision of more quantitative biochemical technics, ultrastructural and histochemical studies [28–31] have made it possible for our knowledge to be extended to the level of specific kinds of cells. Similarly, our knowledge of the pathogenesis of renal diseases at the cellular level has been derived in large measure from the use of light and electron microscopy.[32–38] Increasingly, our knowledge of the abnormal kidney is being advanced by the use of biochemical technics [39–40] in conjunction with light and electron microscopic studies. Virchow [41] stated that man's understanding of disease is dependent upon "Physical and chemical investigation of . . . disturbances, either active or passive, of large or small groups of living units, whose functional capacity is altered in

accordance with the state of their molecular composition and is thus dependent on physical and chemical changes of their contents." Man's realization of Virchow's goals will be facilitated as methods are developed for the isolation and chemical characterization of these "living units."

The separation of single kinds of cells from the kidney suggests numerous possibilities for future investigations. The role of renin in the pathogenesis of nephrogenic hypertension [42,43] is well established; the control of renin synthesis and release could be further elucidated using purified cells *in vitro* in an environment which is free of the many unknown or partially known variables *in vivo*. Jacobson's [44] discovery of the role of the kidney in the production of erythropoietin has had a major impact on the study of hemopoiesis. If one could purify cells capable of the production of erythropoietin *in vitro*, one might not only have the opportunity to study the control of erythropoietin synthesis but also be able to produce an important hormone with possible therapeutic utility. The successful culture of proximal tubule cells suggests the possibility of confirming Oberling's [45] ultrastructural observation that renal cell carcinomas arise from tubular epithelium. If cultured proximal tubule cells could be transformed *in vitro*, one might establish the histologic type of the resultant tumor following heterotransplantation to the anterior chamber of the eye.[46,47]

In conclusion, we hope that the method which is reported here for the separation of viable cells from the proximal tubule will be useful in the investigation of normal and abnormal cells from the proximal tubule.

References

1. Trump BF, Valigorsky JM, Dees JH, Mergner WJ, Kim KM, Jones RT, Pendergrass RE, Garbus J, Cowley RA: Cellular change in human disease: a new method of pathological analysis. Hum Pathol 4:89–109, 1973
2. Heidrich H-G, Kinne R, Kinne-Saffran E, Hannig K: The polarity of the proximal tubule cell in rat kidney: different surface charges for the brush-border microvilli and plasma membranes from the basal infoldings. J Cell Biol 54:232–245, 1972
3. Chesney RW, Sacktor B, Rowen R: The binding of D-glucose to the isolated luminal membrane of the renal proximal tubule. J Biol Chem 248:2182–2191, 1973
4. Berger SJ, Sacktor B: Isolattion and biochemical characterization of brush borders from rabbit kidney. J Cell Biol 47:637–645, 1970
5. Bondi EE, Devlin TM, Ch'ih JJ: Distribution of two mitochondrial populations in rabbit kidney cortex and medulla. Biochem Biophys Res Commun 47:574–580, 1972
6. Greenspon SA, Krakower CA: Direct evidence for the antigenicity of the

glomeruli in the production of nephrotoxic serums. Arch Pathol 49:291–297, 1950

7. Kaplan BS, Drummond KN: Glucose metabolism by isolated rat glomeruli in hyperproteinemic proteinuria. Lab Invest 27:590–593, 1972
8. Misra RP: Isolation of glomeruli from mammalian kidneys by graded sieving. Am J Clin Pathol 58:135–139, 1972
9. Quadracci LJ, Striker GE: Growth and maintenance of glomerular cells *in vitro*. Proc Soc Exp Biol Med 135:947–950, 1970
10. Cade-Treyer D: Isolation of pure fractions of viable calf kidney tubules and glomeruli: *In vitro* culture, immunochemical and esterase zymogram analysis. Ann Inst Pasteur (Paris) 122:263–282, 1972
11. Burlington H, Cronkite EP, Reincke U, Zanjani ED: Erythropoietin production in cultures of goat renal glomeruli. Proc Natl Acad Sci USA 69:3547–3550, 1972
12. Burg MB, Orloff J: Oxygen consumption and active transport in separated renal tubules. Am J Physiol 203:327–330, 1962
13. Dawson AG: Preparation and some properties of a suspension of fragmented tubules from rat kidney. Biochem J 130:525–532, 1972
14. Hillman RE, Rosenberg LE: Amino acid transport by isolated mammalian renal tubules. III. Binding of L-proline by proximal tubule membranes. Biochim Biophys Acta 211:318–326, 1970
15. Mattenheimer H, Pollak VE, Muehrcke RC: Quantitative enzyme patterns in the nephron of the healthy human kidney. Nephron 7:144–154, 1970
16. Oliver J: Nephrons and Kidneys. New York, Harper and Row Publishers, Inc, 1968
17. Wachstein M: Histochemical staining reactions of the normally functioning and abnormal kidney. J Histochem Cytochem 3:246–270, 1955
18. Wachstein M, Bradshaw M: Histochemical localization of enzyme activity in the kidneys of three mammalian species during their postnatal development. J Histochem Cytochem 13:44–56, 1965
19. Jacobson NO, Jorgensen F, Thomsen AC: On the localization of some phosphatases in three different segments of the proximal tubules in the rat kidney. J Histochem Cytochem 15:456–469, 1967
20. Pretlow TG, Boone CW: Separation of mammalian cells using programmed gradient sedimentation. Exp Mol Pathol 11:139–152, 1969
21. Pretlow TG: Estimation of experimental conditions that permit cell separations by velocity sedimentation on isokinetic gradients of Ficoll in tissue culture medium. Anal Biochem 41:248–255, 1971
22. Wachstein M, Meisel E: Histochemistry of hepatic phosphatases at a physiologic pH: with special reference to the demonstration of bile canaliculi. Am J Clin Pathol 27:13–23, 1957
23. Melnick PJ: Histochemical enzymology of leukemic cells, Pathology of Leukemia. Edited by GD Amromin. New York, Harper and Row, Publishers, Inc, 1968, pp 136–137
24. Pretlow TG II, Glick MR, Reddy WJ: Separation of beating cardiac myocytes from suspensions of heart cells. Am J Pathol 67:215–226, 1972
25. Pretlow TG, Williams EE: Separation of hepatocytes from suspensions of mouse liver cells using programmed gradient sedimentation in gradients of Ficoll in tissue culture medium. Anal Biochem 55:114–122, 1973

26. Blackmon J, Pitts A, Pretlow TG: Separation of lipase-positive cells from suspensions of pancreas cells in an isokinetic gradient of Ficoll in tissue culture medium. Am J Pathol 72:417–426, 1973
27. Anderson NG: Studies on isolated cell components. VIII. High resolution gradient differential centrifugation. Exp Cell Res 9:446–459, 1955
28. Sternberg WH, Farber E, Dunlap CE: Histochemical localization of specific oxidative enzymes. II. Localization of diphosphopyridine nucleotide and triphosphopyridine nucleotide diaphorases and the succindehydrogenase system in the kidney. J Histochem Cytochem 4:266–283, 1956
29. Longley JB, Fisher ER: Alkaline phosphatase and the periodic acid Schiff reaction in the proximal tubule of the vertebrate kidney: a study in segmental differentiation. Anat Rec 120:1–21, 1954
30. Beard ME, Novikoff AB: Distribution of peroxisomes (microbodies) in the nephron of the rat: a cytochemical study. J Cell Biol 42:501–518, 1969
31. Longley JB: Histochemistry of the kidney, The Kidney, Vol I. Edited by C Rouiller, AF Muller. New York, Academic Press, Inc, 1969, pp 157–259
32. Simon GT, Chatelanat F: Ultrastructure of the normal and pathological glomerulus.[31] pp 261–349
33. McCluskey RT, Vassalli P: Experimental glomerular diseases.[31] Vol II, pp 83–198
34. Morrison AB, Gardner KD Jr: The effect of potassium deficiency on the reabsorption of protein in the renal tubule of the rat. J Exp Med 118:479–488, 1963
35. Cotran RS: Experimental pyelonephritis.[31] Vol II, pp 269–361
36. Choie DD, Richter GW: Cell proliferation in rat kidney induced by lead acetate and effects of uninephrectomy on the proliferation. Am J Pathol 66: 265–276, 1972
37. Elsas LJ, Hayslett JP, Spargo BH, Durant JL, Rosenberg LE: Wilson's disease with reversible renal tubular dysfunction: correlation with proximal tubular ultrastructure. Ann Intern Med 75:427–433, 1971
38. Shimamura T, Morrison AB: Vascular permeability of the renal medullary vessels in the mouse and rat. Am J Pathol 71:155–166, 1973
39. Wilson H, Spargo B, Getz GS: Changes in kidney medullary phospholipid metabolism in the potassium-deficient rat: part I. Am J Pathol 71:295–314, 1973
40. Wilson H, Spargo B, Getz GS: Changes in kidney medullary phospholipid metabolism in the potassium-depleted rat: part II. Am J Pathol 71: 315–330, 1973
41. Virchow R: Cellular pathology, Disease, Life, and Man: Selected Essays. (Translated from the original German published in Virchows Archiv 8:1, 1885, by LJ Rather) Stanford, Calif, Stanford University Press, 1958, p 100
42. Goldblatt H, Katz YJ, Lewis HA, Richardson E: Studies on experimental hypertension. XX. The bioassay of renin. J Exp Med 77:309–313, 1943
43. Helmer OM, Page IH: Purification and some properties of renin. J Biol Chem 127:757–763, 1939
44. Jacobson LO, Goldwasser E, Fried W, Plzak L: Role of the kidney in erythropoiesis. Nature (Lond) 179:633–634, 1957
45. Oberling C, Riviere M, Haguenau F: Ultrastructure des epithéliomas a cellules claires du rein (hypernephromes ou tumeurs de Grawitz) et son

implication pour l'histogenèse de ces tumeurs. Bull Assoc Franc Cancer 46: 356–381, 1959

46. Greene HSN: The significance of the heterologous transplantability of human cancer. Cancer 5:24–44, 1952
47. Greene HSN: The heterologous transplantation of human testicular tumors. Cancer Res 14:516–518, 1954

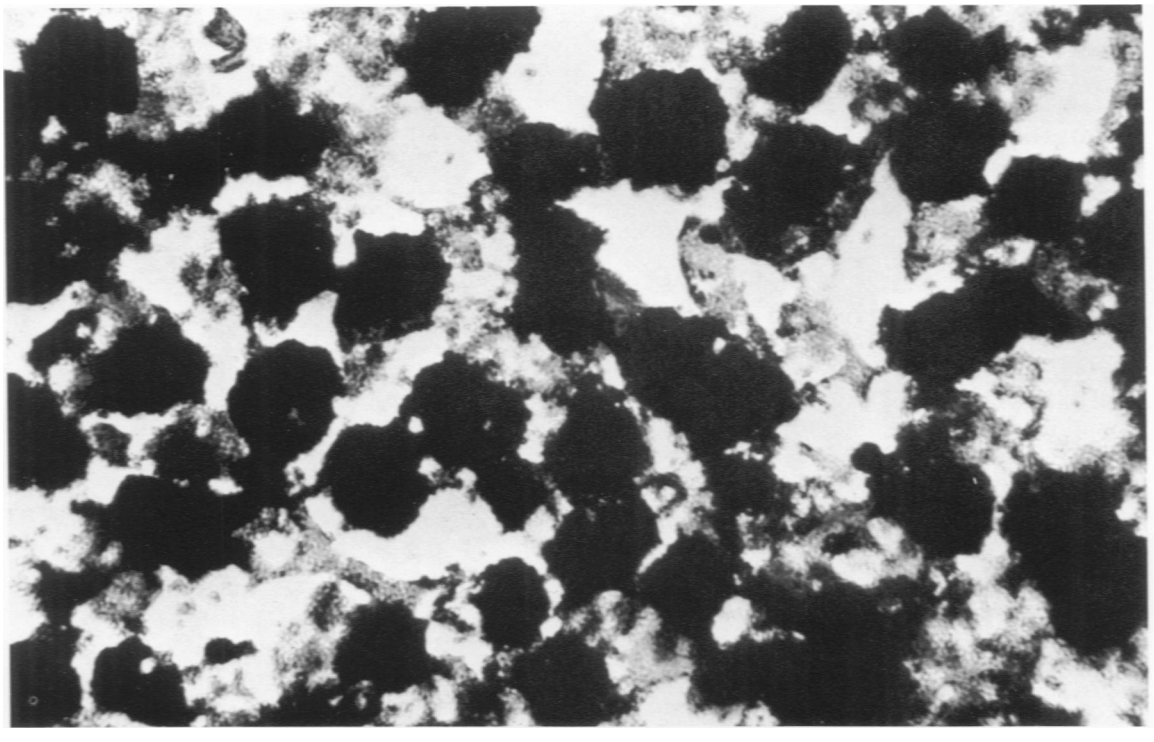
Acknowledgments

We thank Mrs. Annette Pitts and Mr. Henry Brown for their expert technical assistance.

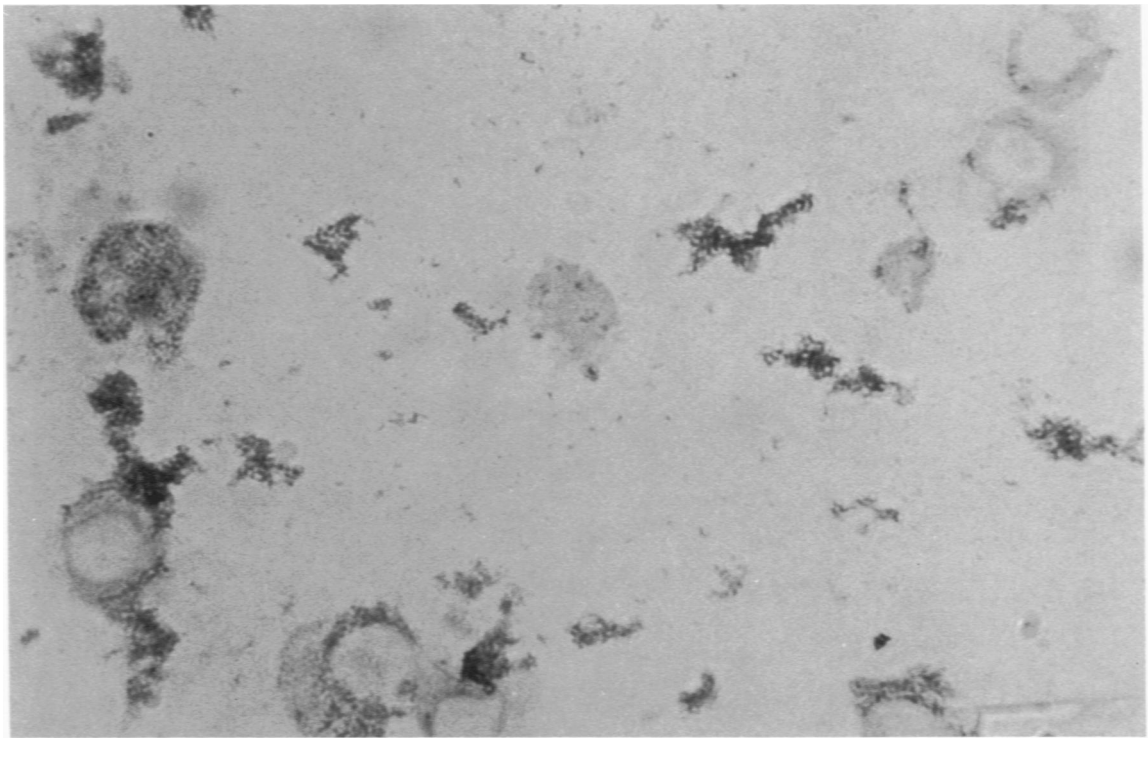
Legends for Figures

Fig 1—Cells from fraction 14 of the isokinetic gradient. Cells which demonstrate glucose-6-phosphatase histochemically are highly purified in fraction 13 through the gradient–cushion interface (Melnick stain for glucose-6-phosphatase, × 250).

Fig 2—Cells from fraction 6 of the isokinetic gradient. These cells demonstrate little or no glucose-6-phosphatase activity and are visible because of the phloxine counterstain. Red blood cells are not stained (Melnick stain for glucose-6-phosphatase, × 250).



1



2

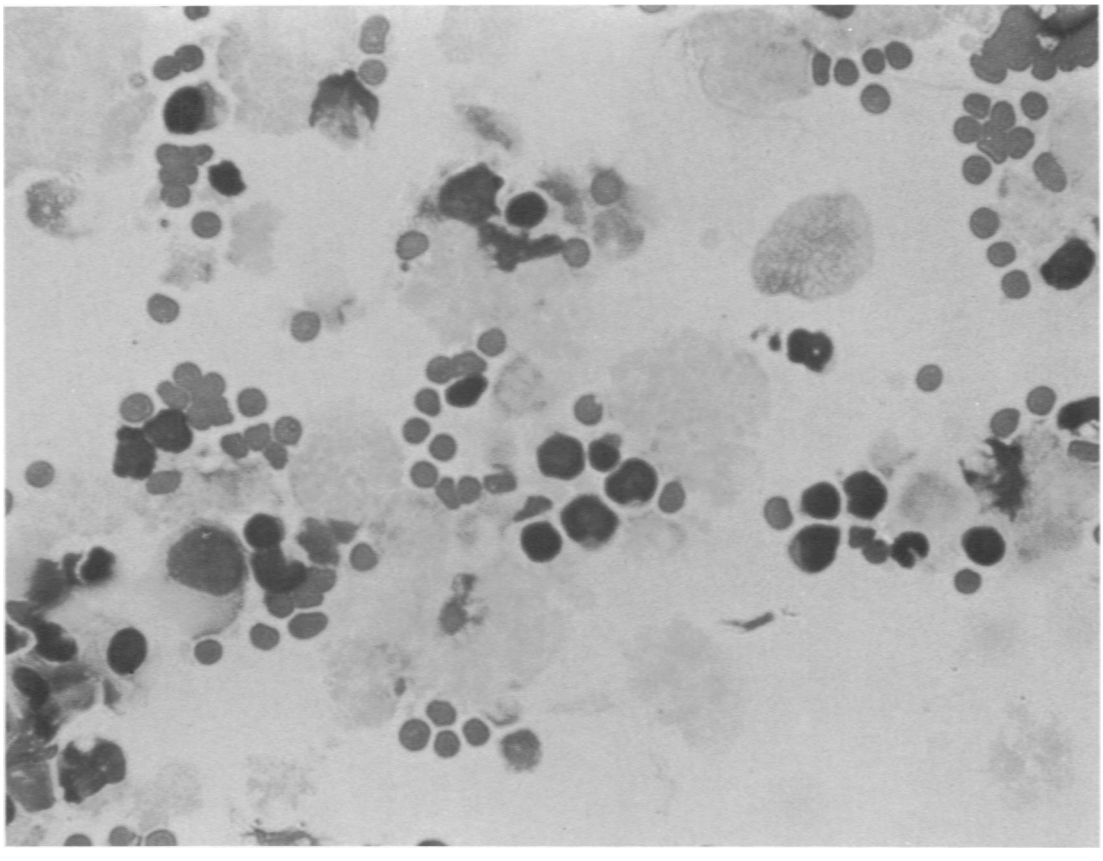


Fig 3—Cells from fraction 6 of the isokinetic gradient. Wright stain was used on all gradient fractions in order to determine the number of red blood cells present (Wright stain, × 250).