Separation of Lipase-Positive Cells from Suspensions of Pancreas Cells in an Isokinetic Gradient of Ficoll in Tissue Culture Medium

John Blackmon, Annette Pitts and Thomas G. Pretlow II, MD

Lipase-positive cells were separated from suspensions of pancreas cells both by velocity sedimentation and by isopycnic sedimentation. Lipase-positive cells were 51.0 ± 9.6% of the disaggregated pancreas cells in the starting sample suspension. The purest gradient fractions from experiments using velocity sedimentation for cell separation contained a mean of 98.8 ± 0.6% lipase-positive cells. Cell separation using isopycnic centrifugation was less effective than cell separation using velocity sedimentation and resulted in a mean purity of only 89.8 ± 6.8% lipase-positive cells. Lipase positivity was assessed using the Gomori technic with the conditions for fixation slightly modified to be suitable for disaggregated cells (Am J Pathol 72:417–426, 1973).

IN BOTH *in vivo* and *in vitro* systems, the pancreas has often been selected for the study of cell differentiation, organ regeneration and control of protein synthesis.[1–4] The pancreas is particularly suitable for such studies because the pancreas exocrine cells produce several well-characterized enzymes,[1] the synthesis of which can be monitored during the course of acinar cell differentiation and regeneration. The synthesis of these proteins has provided an instructive system in which to study control of protein synthesis.[2,5] The availability of a chemically defined medium[6] for the organ culture of embryonic pancreas makes the pancreas a particularly advantageous system for the study of differentiation and macromolecular synthesis.

In an attempt to purify individual kinds of cells for study from embryonic pancreatic epithelium, Bernfield and Fell[7] reported that "Experiments with several density media revealed that solutions of Ficoll in an isotonic salt solution damaged cells least . . ." With

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 supported by Research Career Development Award K4-CA-70584-01 from the National Institutes of Health.

Accepted for publication May 2, 1973.

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

the subsequent development of a widely applicable method [8,9] for the separation of individual kinds of viable cells in gradients of Ficoll in tissue culture medium, it seemed useful to determine if pancreatic exocrine cells could be purified. We now report the results of our attempt to purify exocrine cells from disaggregated pancreas in gradients of Ficoll in tissue culture medium.

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 tissue culture medium (Grand Island Biological Co, Grand Island, NY) were constructed using the two-chambered gradient generator which was illustrated in an earlier report.[8] The gradients for both isopycnic and isokinetic gradient sedimentation were contained in 100-ml polycarbonate centrifuge tubes (International Equipment Co tube #2806). In experiments using both isopycnic and isokinetic centrifugation, centrifugation was carried out in the MSE Mistral 6L centrifuge (VWR Scientific, San Francisco, Calif). During isokinetic gradient centrifugation, the centrifuge speed was monitored continuously using an electronic stroboscope (General Radio, West Concord, Mass).

The gradients for isokinetic sedimentation were constructed as described previously [9] and 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). The isokinetic gradients were constructed on top of 5.5-ml cushions of 45.0% w/w Ficoll. Gradients for isopycnic sedimentation varied linearly 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 cm from the center of revolution). The isopycnic gradients were constructed on top of 10-ml cushions of 45.0% w/w Ficoll. Solutions of Ficoll in tissue culture medium for isokinetic gradients were sterilized by filtration (Millipore Corp, Bedford, Mass). Solutions of Ficoll to be used for the construction of isopycnic gradients are too viscous to be susceptible to sterilization by filtration and were sterilized in an autoclave as described in an earlier report.[8]

Pancreatic Cells for Starting Sample Suspension

Male 7- to 8-week-old, Golden Syrian hamsters were anesthetized using ether. The abdomens were entered and the pancreases were rapidly removed and placed in cold Eagle's minimum essential medium (Grand Island Biological Co, Grand Island, NY). The pancreases were minced to fragments of 2 mm in greatest dimension. The minced pancreases were then stirred gently on a magnetic stirrer in three successive 10-minute washes in minimum essential medium. After the last wash was discarded, the fragments of pancreas were stirred similarly in tissue culture medium containing 1 mg/ml pronase (EM Laboratories, Inc, Elmsford, NY) at room temperature. At 15-minute intervals, the tissue fragments were allowed to settle, the supernatant containing the suspended cells was decanted, and fresh pronase solution was placed in the flask with the tissue fragments. This process was continued until the tissue was exhaustively digested (approximately

11 to 13 digestions). The decanted cell suspensions were placed in an ice bath for 5 minutes immediately after being decanted from the tissue fragments; they were then centrifuged at 97*g* for 7½ minutes. The cell pellet was gently resuspended in 1 ml of tissue culture medium containing 10% fetal calf serum. The combined resuspended pellets were diluted such that the 7-ml starting sample suspensions which were layered over the gradients contained 19.0 to 26.6 $\times$ 10^6 cells for isopycnic separations and 32.2 to 45.7 $\times$ 10^6 cells for isokinetic separations.

Collection and Counting of Separated Cells

Following centrifugation, the gradients were collected in 4-ml fractions using the technic which was described and the gradient tapping cap which was illustrated previously.[8] Fraction 1 from both isopycnic and isokinetic gradients differed from the other fractions in that it consisted of the 7-ml starting sample volume initially layered over the gradients. Refractive indices were measured using aliquots from all gradient fractions to confirm the linearity of the gradients. Cell counts were done on all gradient fractions using hemocytometer chambers. Several slides for microscopic examination were made from each gradient fraction and stained in parallel using Wright stain and a modification of the Gomori technic for lipase as described by Thompson.[10] Differential cell counts were carried out using the slides stained for lipase activity; 200 cells from each gradient fraction and 500 cells from each of the purest gradient fractions were counted.

Staining

Slides were dried at room temperature for 10 to 15 minutes and placed for successive 24-hour periods in acetone at 4.0 C, acetone at room temperature and Bouin's fixative[10] at room temperature. Preliminary studies with a variety of fixatives at several different temperatures demonstrated that the conditions employed for fixation were critical, and that the described conditions resulted in good preservation of lipase activity.

After fixation, slides were washed in ten changes of distilled water at room temperature over the course of approximately 10 minutes and incubated for 64 hours at 37.0 C in substrate solution[11] containing Tween® 80 (polyoxyethylene sorbitan mono-oleate, Sigma Chemical Co, St. Louis, Mo), 0.5% calcium chloride and 15% glycerin in maleate buffer at pH 7.0 to 7.4. The only modification of Gomori's[11] substrate solution was the substitution of Tween 80 for Tween 40 or Tween 60 used by Gomori in his earlier work. This substitution was made because of later work by Gomori which demonstrated that the hydrolysis of Tween 80 is more specific for lipase in distinguishing esterase from lipase activity.[10,12]

After incubation in the above describe substrate for 64 hours, slides were washed in five changes of water over approximately 5 minutes at room temperature and placed in 2% lead nitrate in water for 15 minutes. Slides were then washed in repeated changes of distilled water at room temperature and placed in 1% ammonium sulfide for 1½ minutes. After washing in running water for 3 minutes, the slides were counterstained with hematoxylin for 1 minute, washed in water, dehydrated in graded alcohols, cleared with tetrachloroethylene and mounted in Permount (Fisher Scientific, Atlanta, Ga).

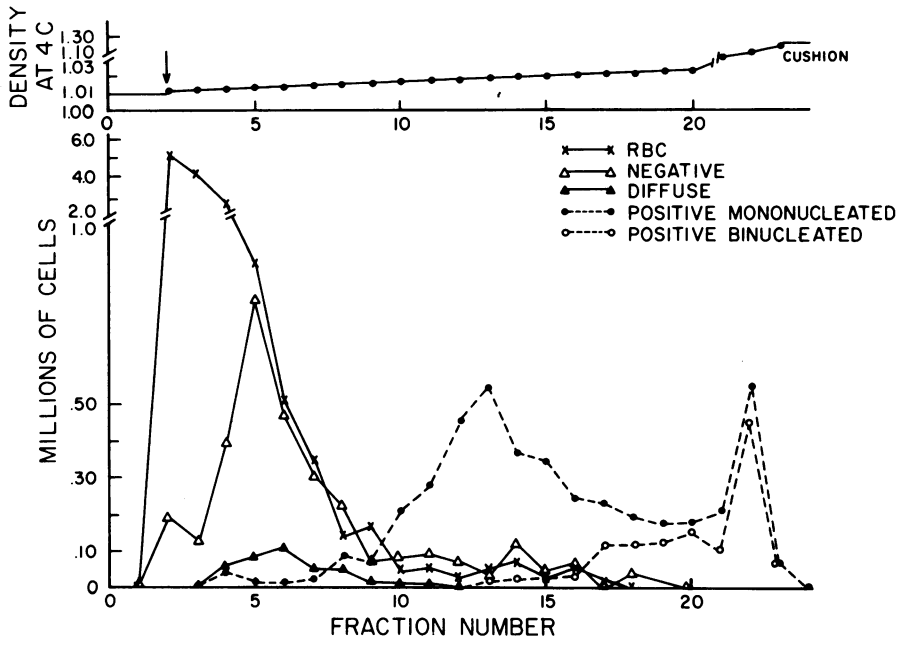
Results

Sample Composition

From a total of nine groups of hamsters (5 hamsters/group) which were used in cell separation experiments, an average of 26.9 $\times$ 10^6 pancreas cells/group (of 5 hamsters) was obtained. The standard deviation was $\pm$ 9.2 $\times$ 10^6; the maximum, 45.7 $\times$ 10^6; and the minumum, 19.0 $\times$ 10^6 cells. The starting sample suspension contained 35.2 $\pm$ 11.4% red blood cells, 2.7 $\pm$ 1.4% mast cells, 11.1 $\pm$ 2.9% lipase-negative mononucleated cells, 29.0 $\pm$ 7.3% lipase-positive mononucleated cells and 21.9 $\pm$ 5.7% lipase-positive binucleated cells. A photomicrograph of the cells in the starting sample suspension stained for lipase activity is shown in Figure 1. Most of the cells which stained with the described procedure for lipase exhibited the lipase activity as discrete dark yellowish-brown cytoplasmic granules. These cells are the cells which are, in the context of this report, termed lipase-positive. In addition, 2.7 $\pm$ 1.4% of the cells in the starting sample suspension stained diffusely a pale pink during the "lipase" procedure. These cells which demonstrated a "diffuse" pink staining when stained for lipase activity are thought to be mast cells, since their modal location in the isokinetic gradient coincided with the location of mast cells as demonstrated by Wright stained preparations from the same gradient fractions.

Velocity Sedimentation in the Isokinetic Gradient

Using the methods which were described previously,[8,9] it was determined that the purest preparations of lipase-positive cells could be obtained following sedimentation in the isokinetic gradient at 4.0 C for 13 minutes using a centrifugal force of 24.3*g* measured at the sample-gradient interface 13.7 cm from the center of revolution. Using these conditions, lipase-positive cells (Figure 2) were obtained with a mean purity of 98.8 $\pm$ 0.6% from repeated experiments. The most highly purified lipase-positive cells were obtained in fractions containing the modal population of binucleated lipase-positive cells (fractions 16 to 20 from the isokinetic gradient). The distributions of all cell types in the isokinetic gradients are shown in Text-figure 1. Separation of cells in the isokinetic gradient occurs primarily because of differences in cell diameters; the effective purification of lipases-positive cells is accomplished primarily because they are larger than the other cells from the disaggregated pancreas. Similarly, the modal populations of mononucleated and binucleated lipase-positive cells are separated because the modal di-



TEXT-FIG 1—Separation of pancreas cells using velocity sedimentation in the isokinetic gradient. In this experiment 37.2 × 10^6 pancreas cells were layered over an isokinetic gradient and centrifuged with a centrifugal force of 24.3*g* for 13 minutes at 4.0 C. Cells are separated in the isokinetic gradient primarily because of differences in diameter. Lipase-positive cells are widely separated from lipase-negative cells and cells (mast cells) which exhibit a diffuse pale pink staining when stained for lipase activity. The modal populations of mononucleated and binucleated lipase-positive cells are separated. An *Arrow* marks the sample-gradient interface on the density plot.

ameter of the binucleated cells is larger than the modal diameter of the mononucleated cells.

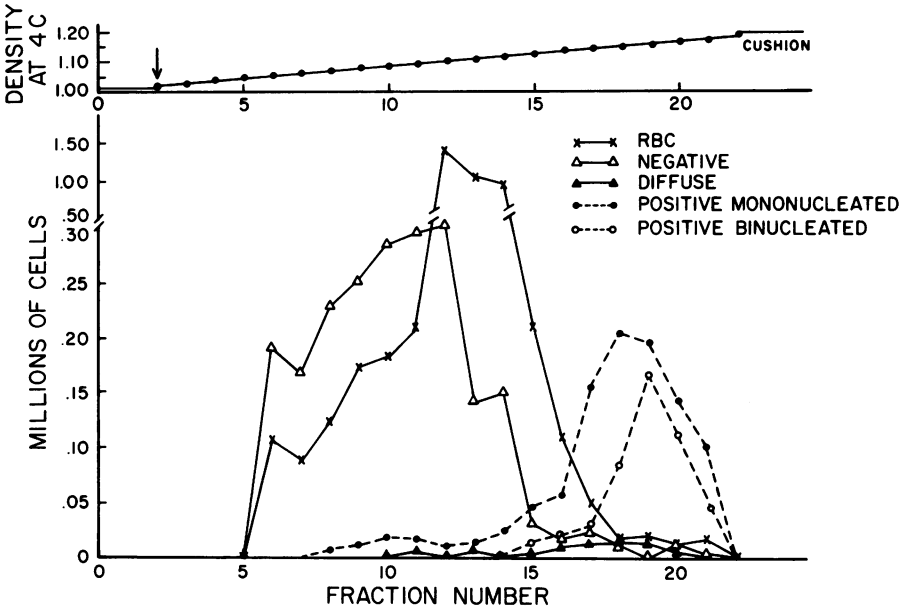
Isopycnic Sedimentation

Following sedimentation in the described isopycnic gradient using a centrifugal force of 850*g* measured at the sample-gradient interface 14.2 cm from the center of revolution, lipase-positive cells were not as well purified as those in the isokinetic gradient. In the purest fractions, lipase-positive cells were obtained with a mean purity of 89.8 ± 6.8%. In contrast to the consistently high degree of purification achieved using the isokinetic gradient, both the locations of the cells and their relative purities varied considerably following isopycnic sedimentation. The densities of the modal populations of the various cell types varied as much as 0.03 g/ml from experiment to experiment; despite this variation, the mononucleated and binucleated

lipase-positive cells invariably exhibited similar densities in the context of any partciular experiment. The lipase-positive cells were always found in fractions having densities between 1.11 and 1.17 g/ml. Cells lacking in lipase activity were found in fractions having densities between 1.06 and 1.11 g/ml. In all experiments, the modal populations of lipase-positive cells were separated from cells which lacked demonstrable lipase activity (Text-figure 2); however, there was considerable overlap of the two populations at their upper and lower limits of density, and the purity of the lipase-positive cells was always inferior to that achieved using sedimentation in the isokinetic gradient.

Discussion

The availability of pure pancreas exocrine cells will facilitate studies of the regulation of protein synthesis and cell differentiation using pancreatic exocrine cells. Since a chemically defined medium for the organ culture of embryonic pancreas has already been developed,[6]



TEXT-FIG 2—Separation of pancreas cells using isopycnic centrifugation. In this experiment, 19.0 × 10^6 pancreas cells were layered over the density gradient and centrifuged with a centrifugal force of 850*g* for 90 minutes. The modal populations of lipase-positive cells were resolved from the modal populations of other kinds of cells but were not as pure as lipase-positive cells obtained by cell separation in the isokinetic gradient. Binucleated and mononucleated lipase-positive cells show similar density distributions. An *arrow* marks the sample-gradient interface.

it will be important to determine if purified pancreas exocrine cells can be cultured in this defined medium. The culture of purified exocrine cells in a chemically defined medium would offer very precisely defined experimental conditions for the study of cell differentiation, the control of protein synthesis, the response of cells to ethionine-induced regeneration,[13] etc.

While the purest cells were obtained using centrifugation in the isokinetic gradient, it is interesting to note that, in contrast to most other kinds of mammalian cells which we have examined,[14] pancreas exocrine cells could be considerably purified using isopycnic centrifugation. The pancreatic exocrine cell is more dense than most mammalian cells. The cardiac myocyte and the mast cell are the only cells other than pancreas exocrine cells which we have obtained in good purity using isopycnic centrifugation. These three cell types differ from most other mammalian cells in that they are more dense than the mammalian erythrocyte. One might speculate that these very dense cells derive their high densities from the high level of cytoplasmic protein which they have in common. As measured using isopycnic centrifugation, it is interesting to note that the mononucleated and binucleated pancreatic exocrine cells exhibit very similar density distributions.

Bernfield and Fell[7] reported separation of two cellular components from disaggregated embryonic pancreatic epithelium. Following their separation, they were able to culture the separated cells and observe the synthesis of zymogen. While these authors made no attempt to evaluate the purity of separated cells using histochemical criteria, they noted that the observed amylase activity which they assayed in their gradient fractions was not monodisperse.

In conclusion, it is hoped that the described technic for the purification of pancreatic exocrine cells will provide a useful means of studying protein synthesis and differentiation *in vitro* in a single kind of cell independent of the other cell types, unknown hormonal stimuli, nutritional variations and nervous stimuli which are present *in vivo*.

References

1. Rutter WJ, Wessells NK, Grobstein C: Control of specific synthesis in the developing pancreas. Natl Cancer Inst Monogr 13:51–65, 1964
2. Jamieson JD, Palade GE: Synthesis, intracellular transport, and discharge of secretory proteins in stimulated pancreatic exocrine cells. J Cell Biol 50: 135–158, 1971
3. Mizoguchi M, Parsa I, Marsh W, Fitzgerald P: Pancreas acinar cell differen-

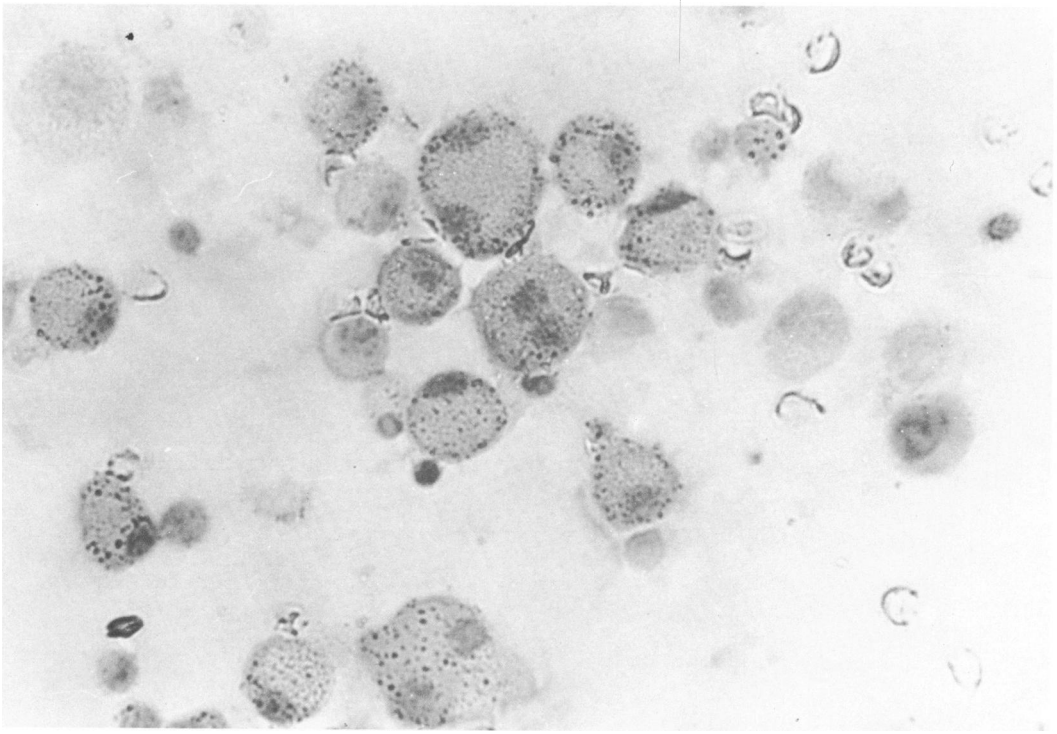
tiation. VII. Autoradiographic demonstration of intracellular radioactivity with S-adenosyl-L-(C^3H_3) methionine. Am J Pathol 69:309–330, 1972

4. Vinijchaikul K, Fitzpatrick PJ: Pancreas acinar cell regeneration. XII. Protein metabolism as determined by ^{3}H-leucine autoradiography. Am J Pathol 66:407–477, 1972
5. Berg NB, Young RW: Sulfate metabolism in pancreatic acinar cells. J Cell Biol 50:469–483, 1971
6. Parsa I, Marsh WH, Fitzgerald PJ: Chemically defined medium for organ culture: differentiation of rat pancreas anlage. Exp Cell Res 59:171–175, 1970
7. Bernfield M, Fell PE: Separation of the proliferating and differentiating cell populations of cultured embryonic pancreatic epithelium. Proc Natl Acad Sci USA 58:2227–2234, 1967
8. Pretlow TG, Boone CW: Separation of mammalian cells using programmed gradient sedimentation. Exp Mol Pathol 11:139–152, 1969
9. Pretlow TG: Estimation of experimental conditions which permit cell separations by velocity sedimentation on isokinetic gradients of Ficoll in tissue culture medium. Anal Biochem 41:248–255, 1971
10. Thompson SW: Selected Histochemical and Histological Methods. Springfield, Ill, Charles C Thomas Publisher, 1966
11. Gomori G: Distribution of lipase in the tissues under normal and under pathologic conditions. Arch Pathol 41:121–129, 1946
12. Barka T, Anderson PJ: Histochemistry. New York, Harper and Row, Publishers, Inc, 1963
13. Fitzgerald PJ, Vinijchaikul K, Carol B, Rosenstock L: Pancreas acinar cell regeneration. III. DNA synthesis of pancreas nuclei as indicated by thymidine-^{3}H autoradiography. Am J Pathol 52:1039–1066, 1968
14. Pretlow TG: Problems connected with the separation of different kinds of cells. Int Rev Exp Pathol 14: (In press)

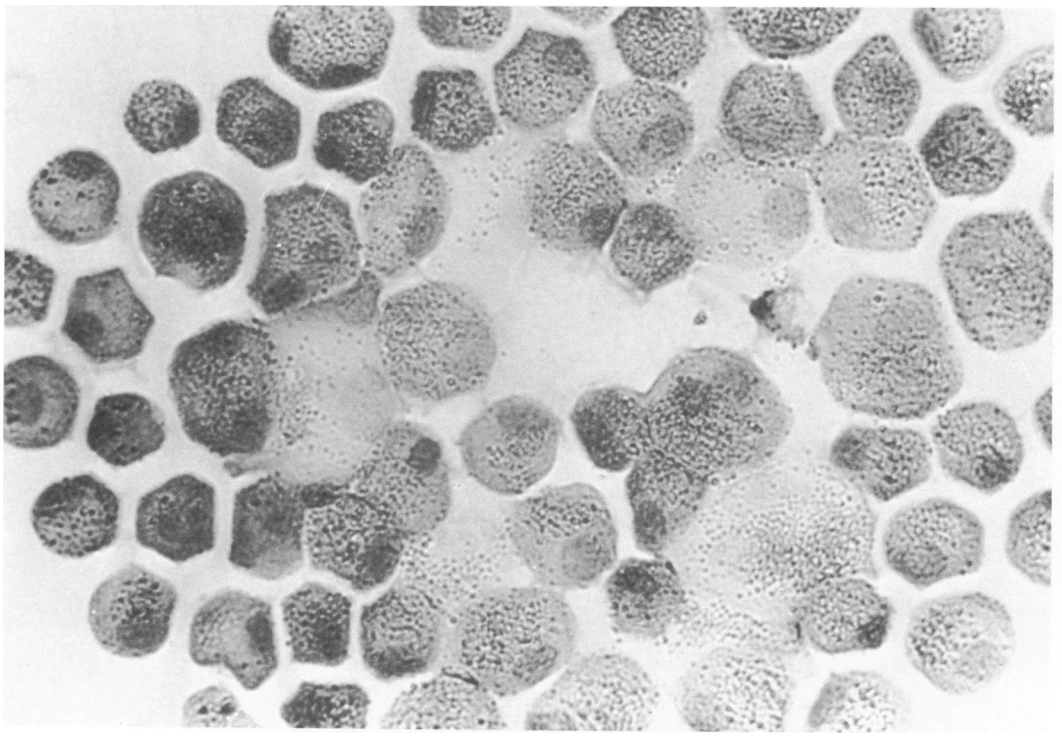
Acknowledgments

We thank Miss Margaret Scalise and Mr. Choice Jennings for their expert technical assistance.

Mr. Blackmon is a medical student year-out fellow in pathology.



1



2

Fig 1—Starting sample suspension of pancreas cells (Gomori stain for lipase, × 500).
Fig 2—Cells from fraction 19 of the isokinetic gradient. This fraction contains purified lipase-positive cells (Gomori stain for lipase, × 500).

[*End of Article*]