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Separation and Subfractionation of Small Numbers of Cells ($\sim 10^6$) by Countercurrent Distribution in Dextran–Poly(ethylene Glycol) Aqueous-Phase Systems

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ABSTRACT

Separation and subfractionation of cells on the basis of subtle differences in surface properties by partitioning in dextran–poly(ethylene glycol) aqueous phase systems is an established method. We report here that the incorporation of fetal bovine serum into such systems permits countercurrent distribution of small quantities of cells ($\sim 10^6$). In the absence of serum such small quantities of cells are lost (probably by adherence) and cannot be recovered after countercurrent distribution.

Index Entries: Aqueous phase systems; cell partitioning; erythrocytes; cell separation; cell fractionation; dextran; poly(ethylene glycol); separation of, cells by countercurrent distribution; fractionation, of cells by countercurrent distribution; countercurrent distribution, separation of cells by.

INTRODUCTION

When aqueous solutions of dextran and of poly(ethylene glycol) are mixed above certain concentrations, they give rise to liquid, immiscible

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two-phase systems with a dextran-rich bottom and a poly(ethylene glycol)-rich top phase. Such systems can be buffered and made isotonic and are the basis for a highly useful, sensitive, and versatile method for cell separation by partitioning (*1,2*). Depending on polymer concentrations and ionic composition and concentration the phase systems have considerably different physical properties (*1*). Thus phase systems containing phosphate have an electrostatic potential difference between the phases (top phase positive) in which cell partitioning depends predominantly on surface charge-associated properties (*1,2*), though not necessarily the same charge as that measured by cell electrophoresis (*3*). Phases containing sodium chloride as the predominant salt have essentially no potential difference and, at lower polymer concentrations, give rise, at least in the case of red blood cells from different species, to cell partition coefficients that correlate extremely well with the ratio of membrane poly/monounsaturated fatty acids (i.e., a lipid parameter) (*1,2*). For detailed discussion of the phase systems and the surface properties reflected by cell partitioning see ref. *1*.

It is often desirable to separate or subfractionate relatively large quantities of cells. The capacity of the phases is great (*4*) and one can easily subject to countercurrent distribution on our thin-layer plates as much as 1 mL of packed red blood cells (i.e., about 10^{10} cells) for 120 transfers (or commensurately fewer cells for proportionately fewer transfers). In some experiments, in which only very few cells are available, it is essential to be able to separate or subfractionate small quantities of cells (e.g., about 10^6 cells). However, as one decreases the quantity of cells loaded on the countercurrent distribution apparatus one finds first a shift of the distribution curve to lower apparent partition ratios and as the quantity of cells loaded is further diminished one can no longer recover the cells. The latter phenomenon is most likely caused by an adherence of such small quantities of cells to the plates. We usually recover between 70 and 80% of cells when 10^9 cells are loaded. This amount diminishes to about 10 or 20% when 10^6 cells are used. Furthermore, when loading small quantities of cells, even the cells recovered do not give distribution curves comparable to those obtained with larger quantities of the same cell population or mixture.

We now report that the inclusion of fetal bovine serum in the phase systems resolves these problems. Small quantities of cells can be subjected to countercurrent distribution and recovered in approximately the same percentage as larger quantities of cells. In addition, the distribution curves give the same apparent partition ratios as do those obtained with larger quantities of cells (10^8–10^9). However, we do sometimes find a somewhat greater proportion of cells between the distribution peaks of two separated cell populations when small quantities ($\sim 10^6$) are subjected to countercurrent distribution that we do with larger quantities (see, for example, Fig. 1). The basis for this is not clear.

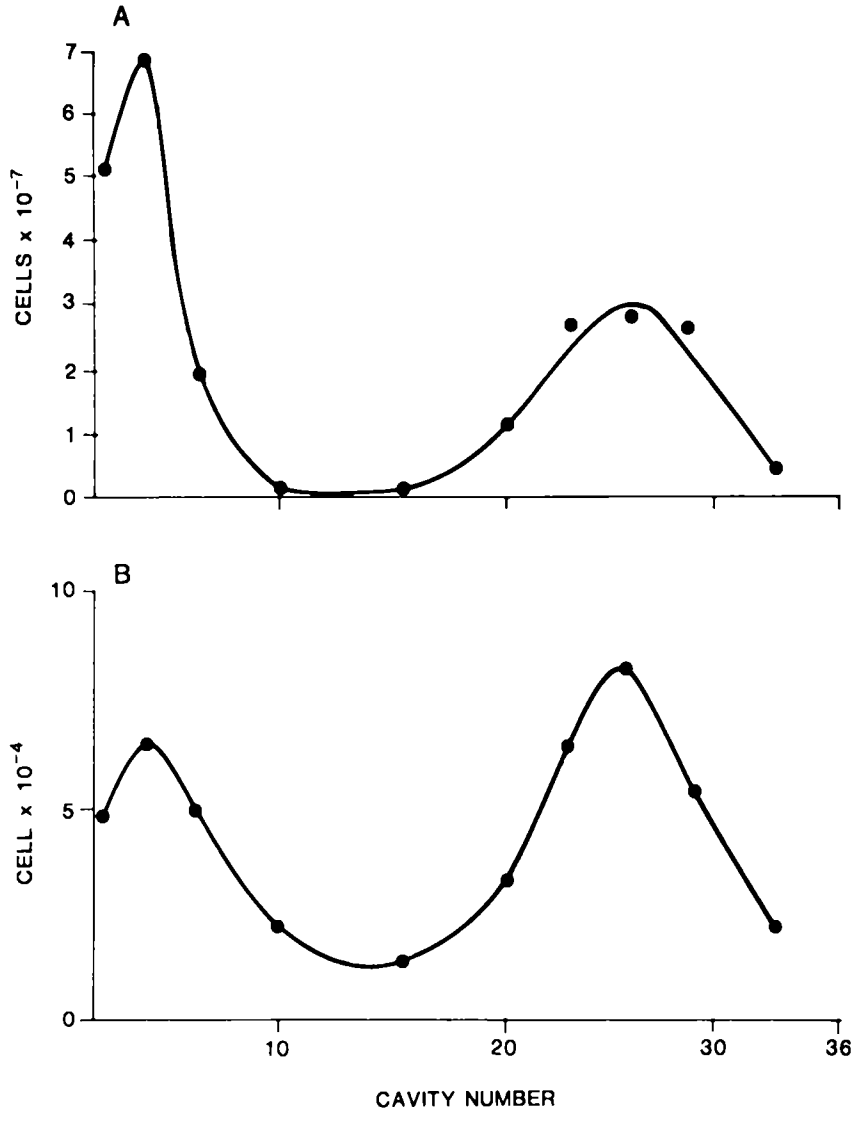

Fig. 1. Countercurrent distribution patterns of a 1:1 mixture of rat and rabbit red blood cells. It is known (*1*) that rat erythrocytes have a higher partition ratio than human red cells in a phase system with a high electrostatic potential difference between the phases. Two different cell quantities were examined: (A) 8×10^8 and (B) 1.9×10^6. Note that comparable apparent partition ratios are obtained for the two peaks independently of cell quantity loaded. Phase system 1 (see *Methods*), which measures charge-associated surface properties, was used. This system also contained heat-inactivated fetal bovine serum. In systems not incorporating fetal bovine serum small cell quantities ($\sim 10^6$) cannot be recovered from the countercurrent distribution plates. Conditions of countercurrent distribution : 36 transfers were completed using a 6-min settling time, 22-s shaking time. Temperature was 4–5°C. For additional details see text.

METHODS

Blood Collection

Rabbit blood was obtained from the ear marginal vein; rat blood, from male Sprague-Dawley rats, weighing between 300 and 450 g, by heart puncture; sheep blood was purchased from Mission Labs (Rosemead, CA). Rabbit and rat blood were collected in acid-citrate–dextrose (ACD) as anticoagulent. Sheep red blood cells were in Alsever's solution (1% 1). For the experiment in which rat young and old red cells were separated (see below) rat blood was collected in an Erlenmeyer flask containing a few glass beads and was defibrinated by gentle swirling.

Preparation of Isotopically Labeled Rat Young Red Blood Cells

Rat young red blood cells were obtained by the centrifugation method of Murphy (*5*). Approximately 7–10% from the top of the centrifuged cell column was collected representing a cell subpopulation of younger mean age [as verified previously by isotopic (*6*) and enzyme marker (*7*) analysis]. White blood cells were removed from the young red cell preparation by the method of Beutler (*8*). Hemocytometer counts indicated the depletion of white cells to be more than 95%. Young red blood cells were then isotopically labeled with [^{51}Cr] chromate as previously described in detail (*6*) using 150 μCi [^{51}Cr] chromate to label the 0.35 mL of cells.

Preparation of Cell Mixtures for Countercurrent Distribution

(I) Labeled young cells and unlabeled, unfractionated rat red cells (approximately 0.75 mL) from the same rat from which the young cells were prepared were each washed five times with ten times the cell volume of phosphate-buffered saline, pH 7.0 (PBS). The two cell preparations were then suspended in 10 mL of PBS and cells in an aliquot of each were counted electronically using a Celloscope (Particle Data, Chicago, IL) fitted with a 76 μm orifice tube and operating on the Coulter principle. Aliquots of each cell suspension corresponding to the same number of cells ($\sim 10^8$) were pipeted into a test tube containing 2 mL of PBS and gently mixed. The mixture was centrifuged, the supernatant solution discarded and the cells resuspended in 4 mL of top phase of the system to be used in countercurrent distribution (see below).

(II) In somewhat analogous fashion red blood cells (all unlabeled) from rabbit, rat, and sheep were washed five times as above with PBS. Cells were suspended in 10 mL of PBS and aliquots of cells counted. Mixtures were prepared that contained equal numbers ($\sim 10^9$) of rat red cells + rabbit red cells or rat red cells + sheep red cells. Each of these cell mixtures was suspended in 4 mL of top phase of the system in which countercurrent distribution was to be carried out (see below).

Additional dilutions of aliquots of the suspensions were made using the respective top phase as diluent. For actual quantities loaded in the experiments see captions to Figs. 1–3.

Dextran–Poly(ethylene Glycol) Aqueous Phases

Two different two-polymer aqueous phases having different physical properties, prepared as described by Walter (*1*), were used. Their compositions were as follows: system 1 contained 5% (w/w) dextran T500, lot no. 11648 (Pharmacia Fine Chemicals, Piscataway, NJ), 4% (w/w) poly(ethylene glycol) 6000 ("Carbowax 6000", recently renamed "8000", Union Carbide, NY), 0.09*M* sodium phosphate buffer, pH 6.8 (made of equimolar quantities of mono- and dibasic sodium phosphates), 0.023*M* sodium chloride and 5% (w/w) heat-inactivated fetal bovine serum (Irvine Scientific Co., Irvine, CA); system 2 had 4.9% (w/w) dextran, 3.4% (w/w) poly(ethylene glycol), 0.01*M* sodium phosphate buffer, pH 6.8, 0.14*M* sodium chloride, and 5% (w/w) head-inactivated fetal bovine serum. The phases were put through a 0.45 μm filter (Nalgene Labware, Rochester, NY) to remove any suspended materials (originating in the fetal serum). System 1 has a high electrostatic potential difference between the phases, top phase positive (9); system 2 has virtually no potential difference between the phases. System 1 has a higher interfacial tension than system 2 (*1*). The significance of these physical properties have been discussed previously (*1,2*) in relation to surface properties of cells reflected in such systems by partitioning. Phase system 1 is charge-sensitive while phase system 2 is noncharge-sensitive. Phase systems were equilibrated at 4–5°C in a separatory funnel. Top and bottom phases were then separated and used in the countercurrent distribution experiments described below.

Countercurrent Distribution of Red Blood Cell Mixtures

We use a thin-layer countercurrent distribution apparatus with circular Plexiglas plates having 120 concentric cavities (10). The bottom plate cavities have a capacity of 0.7 mL. In the experiment depicted in Fig. 1 the separation of rat and rabbit red blood cells in phase system 1 was studied as a function of quantity of cell mixture loaded. Two load mixes (A and B) were prepared having different cell concentrations in top phase of system 1 (see caption to Fig. 1). To load the countercurrent plates all cavities received 0.5 mL of bottom phase of system 1. Cavities 0–2 and 60–62 each received 0.7 mL of one of the two "load mixes" (A or B). All other cavities received 0.7 mL of top phase. Plates were loaded in this manner to give a stationary interface (see ref. *11* for full discussion). Countercurrent distribution was carried out on our automatic unit: 22 s shaking time, 6 min settling time, 36 transfers, at 4–5°C. By loading cells in the manner indicated we could carry out this comparative experiment

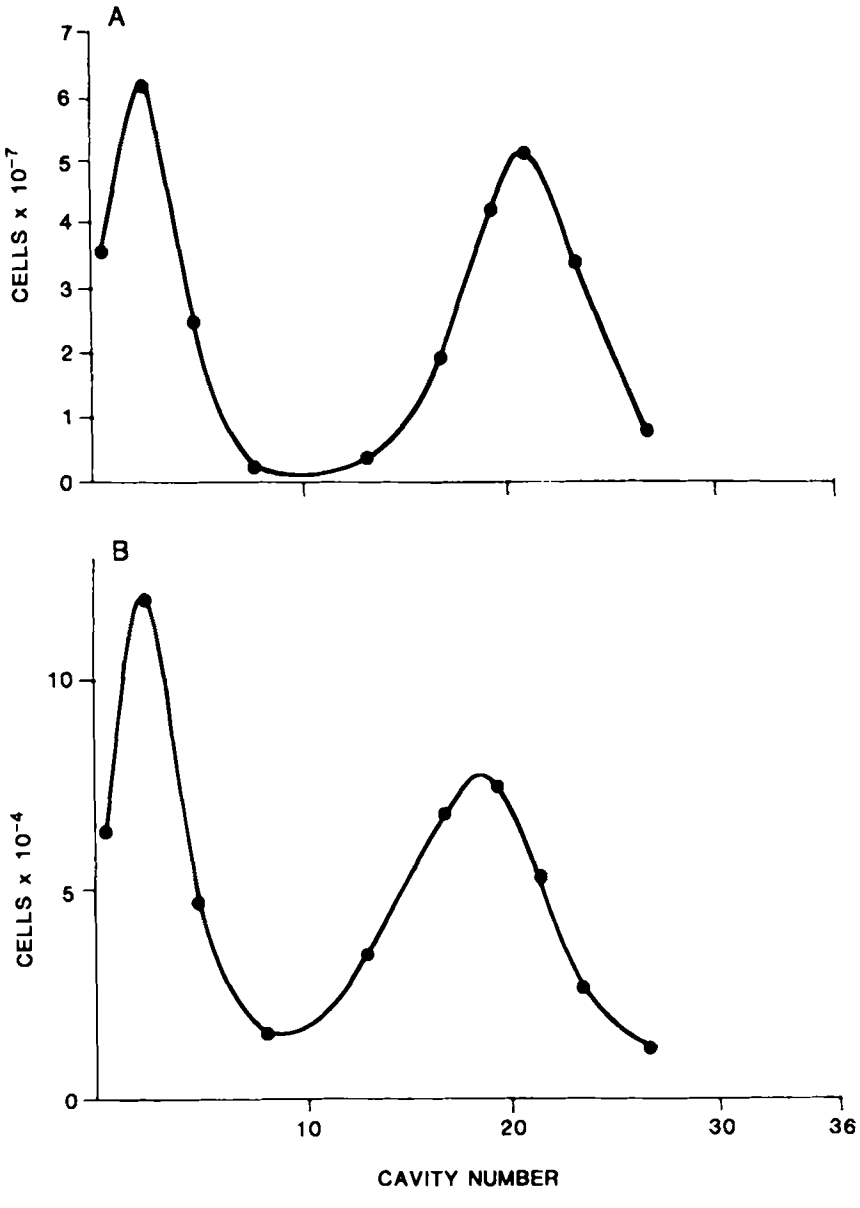

Fig. 2. Countercurrent distribution patterns of a 1:1 mixture of sheep and rat red blood cells. It is known (*1*) that rat erythrocytes have a higher partition ratio than sheep red cells in phase systems without an electrostatic potential difference between the phases and close to the critical point (i.e., low polymer concentrations). Two different cell quantities were examined: (A) 9.4×10^8; (B) 1.85×10^6. Note again that comparable distributions are obtained in the two experiments independently of cell quantity loaded. Phase system 2 (see *Methods*), which is noncharge-sensitive, was used. This system also contained heat-inactivated fetal bovine serum. In systems not incorporating fetal bovine serum small cell quantities ($\sim 10^6$) cannot be recovered. Conditions as in Fig. 1 except that a 7-min settling time was used. See text for additional details.

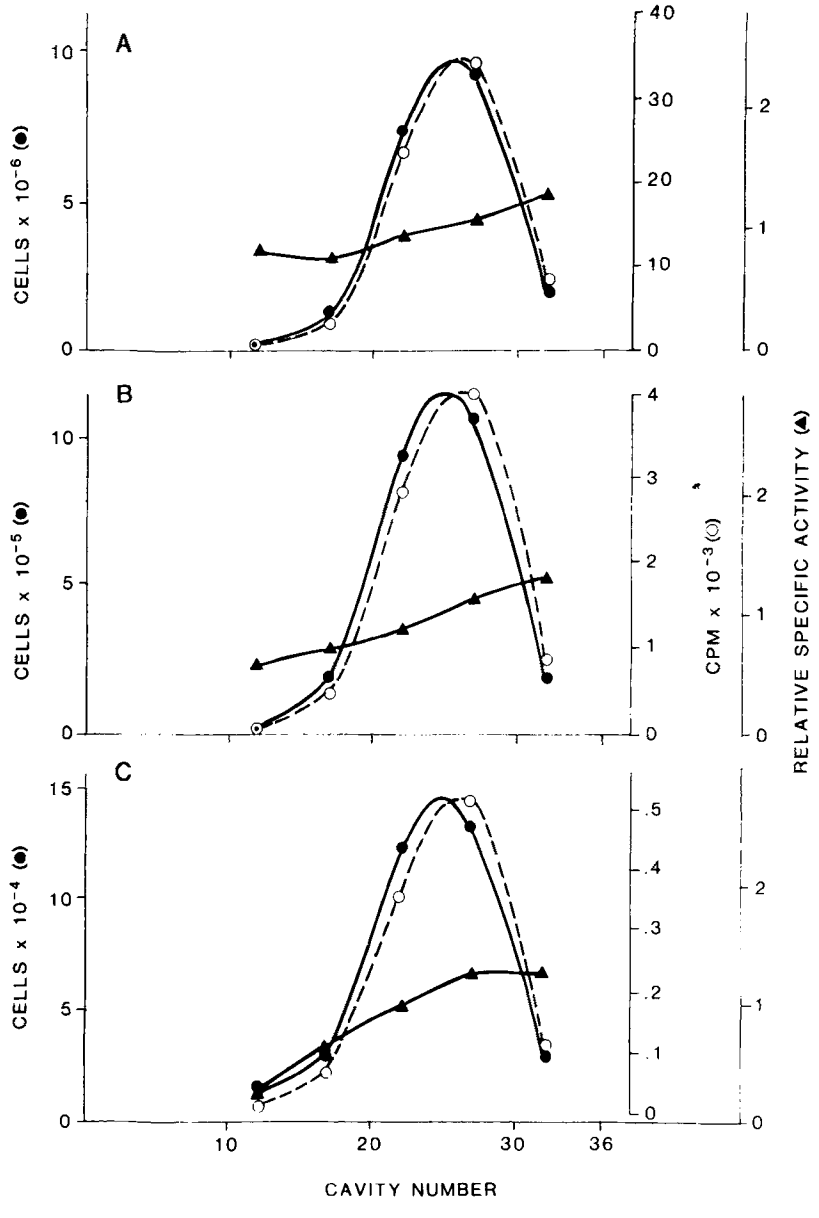

Fig. 3. Countercurrent distribution patterns of a 1:1 mixture of isotopically [^{51}Cr]-labeled rat younger red blood cells and unlabeled, unfractionated rat erythrocytes. Younger cells were obtained by the centrifugal method of Murphy (*5, see* text). It is known that young red cells have a higher mean partition ratio than unfractionated cells (2) in a phase system with a high electrostatic potential difference between the phases. Three different cell quantities were examined: (A) 0.98×10^8; (B) 1.2×10^7; and (C) 1.7×10^6: ●, shows the distribtion curve of the entire cell mixture in terms of cell counts; ○, depicts the distribution of the younger labeled cells in cpm; ▲, presents the relative specific activity through the distribution (a measure of the extent of the separation). Note that comparable distributions and resolutions are obtained in the three experiments independently of cell quantity loaded. Phase system 1 (*see* Methods), which measures charge-associated surface properties, was used. This system also contained heat-inactivated fetal bovine serum. In systems not incorporating fetal bovine serum small cell quantities ($\sim 10^6$) cannot be recovered. Conditions as in Fig. 1. See text for discussion.

on the two quantities of cell mixtures simultaneously and without overlap.

Figure 2 depicts an experiment set up in a manner analogous to that just described but with rat + sheep red cells (instead of rat + rabbit) and using phase system 2. All other conditions were as in Fig. 1 except that the settling time was 7 min. (The closer a system is to the critical point, the longer the required settling time, 1.)

In Fig. 3 the subfractionation of rat red cells based on cell age (i.e., young cells vs rest of cell population) was tested as a function of quantity of cells loaded (A, B, or C as indicated in caption). Rat [^{51}Cr]-labeled young red cells mixed with unlabeled, unfractionated cells (see above) were loaded in a manner similar to that in Fig. 1 except that four instead of three cavities were loaded with cells (cavities 0–3, 40–43, 80–83). See caption to Fig. 3 for cell quantities used in these countercurrent distribution experiments. Countercurrent conditions were as indicated above for Fig. 1.

At the end of a run cavities were emptied directly into centrifuge tubes and 1 mL of PBS added to each tube to change the two-phase system into a single homogeneous suspending medium. Tubes were pooled by twos or threes, then centrifuged in the cold at 1200*g* for 10 min. The supernatant solutions were discarded and the cells suspended (using a Pasteur pipet) in 2 mL of PBS. Cell counts were obtained, electronically as above, on aliquots. In the case of isotopically labeled cells, an aliquot of the cell suspension was counted on a Beckman scintillation well-counter on the "[^{51}Cr]" setting.

Presentation of Data

Distribution curves are given in terms of number of cells in the different cavities. In the experiment using isotopes (Fig. 3) the distribution of the labeled cell population is shown in counts per minute (cpm). In addition, for Fig. 3, a relative specific activity is presented which is defined as:

$$\frac{\text{cpm/cell in a given cavity}}{\text{cpm/cell in the original mixed cell population prior to countercurrent distribution}}$$

RESULTS AND DISCUSSION

Small quantities of cells (i.e., 10^6) cannot be separated or subfractionated by countercurrent distribution in two-polymer aqueous phase systems because they are lost (probably by adherence) and cannot be recovered from the plates. The incorporation of heat-inactivated fetal bovine serum (or, possibly, other proteins) into aqueous phase systems resolves this problem.

The inclusion of FBS appears to have no effect on the partition ratio of cells when compared to the partition ratio of large quantities of cells in systems of similar composition but omitting FBS. Phases, containing FBS, permit separation of 10^6 cells not only in charge-sensitive phases but also non-charge-sensitive systems. Finally, demonstration of the presence of cells with different surface properties in a "single" cell population (e.g., rat red blood cells of different cell ages) is equally feasible with 10^6 cells as with higher cell numbers.

These experiments are presented in the figures. Figure 1 shows the separation, using two cell quantities in the load mixes, of rat and rabbit red blood cells based on charge-associated properties. Figure 2 depicts, in an analogous manner, the separation of sheep and rat red blood cells in a noncharge-sensitive phase system. Figure 3, in which a mixture of isotopically labeled rat young red blood cells and unlabeled, unfractionated rat red blood cells is examined (using three cell quantities), shows that the young cells have a higher partition ratio {in line with previously published findings (1), which have also established that the [^{51}Cr]-label *per se* has no effect on the cells' partition ratio (6)}.

FBS (heat-inactivated) has previously been incorporated into some phase systems, but for another purpose. Lymphocytes (*12*) monocytes (*13*), or cultured tumor cells (*14*) fractionated by countercurrent distribution were thought to benefit from the presence of FBS in maintaining their viability. In the experiments cited it was shown that viable cells were recovered after countercurrent distribution that could be cultured, stimulated with mitogens, were able to phagocytose or had metastatic potential. Based on these results it appears likely that the addition of FBS to phase systems will not constitute an impediment in most studies contemplated on cells separated or subfractionated by countercurrent distribution.

ACKNOWLEDGMENT

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