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QUANTIFICATION OF COUNTERCURRENT DISTRIBUTION: FROM MOLECULAR PARTITION TO ANIMAL BEHAVIOR

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Summary. Countercurrent distribution is a very effective method for separating a variety of substances including molecules, cells, and animals on the basis of partition between two solvent or behavioral phases. Distribution of a substance in this multi-cycle procedure can be described in terms of its partition coefficient (K) or the fraction of the substance that moves to the mobile phase in each cycle (p). It is currently difficult to calculate the value of p or K directly from an experimental distribution. Such direct calculation will simplify comparison of various experimental populations for the properties which determine their distribution, such as the surface properties of cells or behavioral preferences of animals. This report describes, and provides a theoretical basis for, an extremely simple method to calculate p or K directly from an experimental countercurrent distribution.

The method of countercurrent distribution was introduced by Craig (1,2) to separate molecules on the basis of partition between two solvent phases. This method has since been adapted and used extensively (2-4) for separating ions, molecules, nucleic acids, proteins, chromosomes, membranes, cell organelles, viral particles, and cells; for studying interactions between macromolecules; and for distributing animals on the basis of behavioral partition. Countercurrent distribution is a multiple transfer method. Amount of a substance in different tubes after a number of transfers can be described by binomial distribution, equation 1 (1,5). The distribution depends upon the partition coefficient K of a substance between two phases, or a related parameter p (equation 1), which refers to the fraction of molecules or cells etc. in the mobile phase (2) or the strength of behavioral response in animals, for example the response of fruit flies to sensory stimuli (5). Countercurrent distribution is a very effective separation method due to an exponential relationship between the partition coefficient and the properties, for example the surface properties of cells or the behavioral response of animals, which determine it (2). Given the value of K or p, the distribution in an experiment can be computed using equation 1. This ability to calculate the results of the separation procedure, is an important advantage of the technique.

On the other hand, it is difficult to obtain the value of p directly from experimental distribution. As a result, comparisons between different populations of molecules (6,7), cells (8-

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10), flies (5,11) or other systems (12) are generally depicted by panels of distribution curves, with each curve showing a representative run for a particular population. If p must be specified, its approximate value is obtained by indirect methods. These include drawing a family of distribution curves with different p values and then selecting the curve that fits best with the experimental curve (11,13); determining the best fitting partition coefficient by non-linear regression analysis (14,15); and using the positions of the distribution maxima, or the contents of the adjacent tubes, to obtain an approximate value (13,14). A simple method for calculating p directly from experimental data will be extremely useful for describing the partition behavior of a substance, for estimating its purity or for quantitative comparisons between different systems. This report describes such a method.

Calculation of *p*. Countercurrent distribution separates experimental material, for example a membrane preparation or animals responding to a behavioral stimulus, on the basis of partition between two phases, for example two solvent phases or two behavioral phases. It is a multi-cycle procedure in which one phase is stationary and the other mobile. During each run, the material, e.g., a molecular species, a membrane preparation, viral particles or animals, distributes between the two phases, e.g., two solvent phases or two behavioral ones, on the basis of its partition coefficient between them. The final location of an individual ion, molecule or an animal depends upon the number of times it moves into the mobile phase during various runs. The overall distribution of a homogenous material can be calculated in terms of p and is given by the binomial distribution (16),

$$F_{(r)} = \frac{n!}{r!(n-r)!} p^r q^{n-r}, \tag{1}$$

where $F_{(r)}$ is the fraction of the total population appearing in the rth cavity, n is the number of cycles run, p is the fraction of the sample that partitions to the mobile phase during each run and $q = (1 - p)$.

Starting with a total number of N particles (molecules, viral particles or flies etc.), number of particles in the rth cavity, $N_{(r)}$, is given by

$$N_{(r)} = \frac{N n!}{r!(n-r)!} p^r q^{n-r}. \tag{2}$$

These $N_{(r)}$ particles partitioned to the mobile phase r times during n runs. For the discussion here, they will be said to have made r moves during n runs. The ratio between the total moves made by all the particles in all the cavities and the total moves theoretically possible for N particles during n runs is:

$$\frac{\sum_{r=0}^{n} \left\{ \frac{r N n!}{r!(n-r)!} p^r q^{n-r} \right\}}{nN}.$$

This expression simplifies to p itself, and can be used to calculate p from experimental distribution. Hence

$$p = \frac{\sum_{r=0}^{n} r F_{(r)}}{n} \quad . \tag{3}$$

We do not actually have to calculate the fractions as given in this equation. Instead of using fractions in the numerator, one can use whatever the original measured values are, e.g., number of flies, cell count, enzyme activity, titre of viral particles, concentration of metal ions etc., and include the total amount of substance in the denominator.

As an example, if 10 transfers are carried out for separation of red blood cells, and the observed concentrations in tubes *0* through *10* are (Hemoglobin absorbance units) 0.0, 0.0, 0.0, 0.0, 0.0, 0.2, 0.8, 5.6, 6.1, 4.0 and 0.0, then

$$p = (5 * 0.2 + 6 * 0.8 + 7 * 5.6 + 8 * 6.1 + 9 * 4.0) / (10 * 16.7) = 0.78.$$

Similarly, if we offer 5 behavioral trials ($n = 5$) each to 152 flies, and the number of flies in tubes *0* through *5* are respectively 0, 0, 5, 15, 41, and 91, then

$$p = (0 * 0 + 1 * 0 + 2 * 5 + 3 * 15 + 4 * 41 + 5 * 91) / (5 * 152) = 0.89.$$

Table 1. An illustration of p values calculated for data from literature. Data points were estimated from figures in the original sources. Standard deviation could not be calculated as the sources gave only a single representative curve for each experiment due to limitations of the graphical representation. 'Calculated p' refers to the value calculated by the method discussed here (equation 3). 'Reported p' refers to the value reported in the original reference. Fig. No. refers to figures in the original reference. n is the number of transfers (equation 1). Details on experiments are given in original references.

Sr. No.	Experiment	n	Calculated p	Reported p	(Ref.), Fig. No.
1	Response of taste-blind flies (*Drosophila* mutant *gusB*) to quinine sulphate	5	0.49	0.50± 0.05[a]	(11), 4
2	Fractionation of red blood cells from rat	50	0.77	0.67[b]	(18), 2
3	Fractionation of membranes enriched for nicotinic acetylcholine receptor	19	0.73	c	(19), 6A
4	Fractionation of Human CO-Hemoglobin	59	0.65	0.65[d]	(15), 2A
5	Fractionation of Human Carbonic Anhydrase II	59	0.43	0.40[d]	(15), 2B
6	Fractionation of chondroitin-6-sulfate	17	0.86	c	(20), 2C

[a]The original source estimated p by comparing experimental curves with theoretical curves for different values of p.

[b]Partition coefficient was determined in the original reference by single tube partition and reported as fraction of total cells found in the top phase. For the purpose of comparison here, p was calculated from the partition coefficient by equation (4).

[c]The original sources compared the data graphically without estimating p.

[d]The original source estimated K by assuming various values for it and determining the best fit by non-linear regression analysis. For the purpose of comparison here, p was calculated from K by equation (4).

Table 1 gives p-values calculated from data taken from literature. As in the above treatment, p in these calculations represents the proportion of flies that avoid quinine sulfate, or the fraction of enzyme that partitions to the top mobile phase. It is related to the partition coefficient K, in a simple manner (4), by the equation

$$K = \frac{p}{1-p} \quad . \tag{4}$$

The value of p, being a measure of probability, ranges from 0 to 1. On the other hand, K being a ratio of the substance in two phases, can assume any positive value. In some experiments, the results can be discussed more meaningfully in terms of p, e.g., as an index of flies' response to light, gravity or an olfactory substance. In other cases, partition coefficient may be a more relevant parameter, as e.g., for partition of cells in two phases. If required, K can be calculated from p using equation 4.

Discussion. Ability to calculate p directly from the distribution profile of a homogenous material allows a direct comparison of data. For example it can help in comparing normal flies with mutants (5), studying whether a human gene can rescue a fly mutant (17), or in resolving an interaction between macromolecules (14,15). In many cases when data have to be represented as panels of distribution curves showing representative runs (10,11), a tabular form of p values can represent data from multiple experiments along with statistical information. Experimental p values can be used to assess the purity of a material by comparing it with p from a pure substance. In general, partition coefficient obtained in a multi-cyle procedure will be more accurate than the value obtained from a single-step partition as is currently done in many cases.

It should be noted that in many cases, data are better represented in a graphical form, for example when one is dealing with a mixture of substances or is working with an impure material and needs to present the distribution profile. Therefore ability to calculate p directly from data does not replace, but complements, the graphical representation of countercurrent distribution. It must also be kept in mind that p values obtained from different experiments can be compared, as in the case of graphic curves, only if the experiments have been conducted under similar conditions, e.g., similar phase systems, similar ways of presenting behavioral stimuli, or similar time periods for settling of phases or presentation of stimuli.

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