gested that adaptation of algae to low temperatures resulted from increased content of proteins—notably enzymes. Although this suggestion was later questioned (*8*), the possibility of increased protein synthesis being a means of responding to environmental stress remains. Although our studies of other regions (*3, 4*) have suggested such a role for protein synthesis, we have not observed such a phenomenon in the low temperatures of the Southern Ocean. Indeed, in such waters, the synthesis of lipid is the dominant physiological response of phytoplankton to stress.

The significance of this dramatic synthesis of lipid in the waters around Antarctica is unknown. At all stations, the populations were dominated by diatoms. Although Fogg (*9*) observed measured lipid synthesis when cultures of the diatom *Navicula* were nitrogen starved, there appears to be no evidence for nitrogen deficiency at the stations we studied since significant concentrations of nitrate were always observed (*10*). Mayzand and Martin (*11*) have proposed that organisms living in colder water with irregular or low food supply (for example, low light intensities for phytoplankton) might be expected to have larger lipid storage. The higher caloric content of lipid suggests that storage of high-energy products available for survival in dark periods could be of significance in the polar region; however, this is only one hypothesis. For example, Lee *et al.* (*12*) observed that phospholipids and galactolipids accounted for as much as 57 percent of the total lipid in diatoms. Such material can be important structural components [see the observations of Benson *et al.* (*13*) on chloroplast lipids]. Thus, the roles of lipids can be more than the simple one of storage products.

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Time: A New Parameter for Kinetic Measurements in Flow Cytometry

Abstract. *The measure of time was used as an additional parameter on an existing flow cytometer to study the kinetics of enzyme activities and cell-stain interactions. By correlating all fluorescent signals from single cells with time, the dynamics of a reaction can be followed for several minutes. This advanced application of flow cytometry is easily implemented and can be incorporated into any flow cytometer that has two-parameter analysis capability.*

Quantitative multiparameter flow cytometry is becoming widely used in basic research and clinical medicine (*1*). Flow cytometers presently in use range from the elegantly simple (*2*) to the highly complex (*3*), and several types of multiparameter flow instruments are commercially available (*4*). The growing number of applications of flow cytometry result from both instrumental innovations and new methodological approaches (*5*). Generally, flow cytometric measurements are made in an equilibrium state where all dynamic changes have gone to completion. Analysis of fluorescence from a fluorochrome such as mithramycin, which binds stoichiometrically to DNA, is carried out after the dye-binding reaction has reached equilibrium (*6*). Any fluctuation in the fluorescence intensity during such an analysis increases the coefficient of variation of the pulse height distribution and confounds the interpretation of the data (*7*). Several groups have recently made kinetic measurements, using flow systems (*8*). The increases in cellular fluorescence due to enzymatic conversion of a nonfluorescent substrate to a fluorescent product have been measured by sequential analyses at discrete time intervals after the addition of substrate. This technique provides quantifiable measurements of enzyme kinetics in whole, intact, and in certain cases viable cells. Because the fluorescence from large numbers of individual cells is measured, this method also can be used to detect population kinetic inhomogeneity within a single sample.

There are several important disadvantages associated with multiple discrete measurements in kinetic studies: (i) fast kinetic reactions will not be resolved because the rapidly changing fluorescent intensity will tend to smear the distribution, even over brief sampling time periods; (ii) heterogeneous kinetic populations will not be resolved unless the reactions are slow, and each subpopulation is quite distinct; and (iii) much of the statistical precision obtained by flow cytometric analysis is lost if data are collected for only a small fraction of the total time period.

We have incorporated time as one of the parameters recorded in a multiparameter flow cytometer. It is thus possible to analyze kinetic reactions continuously with each fluorescent event correlated to a specific time. Several examples are presented which illustrate the usefulness of this technique.

We used the Los Alamos Scientific Laboratory computer-based multiparameter cell sorter system (*9*) for all measurements. Time was measured with a linear voltage ramp generator. The ramp generator (Berkeley Nucleonics Corporation model LG-1) was modified to operate for long time periods by the addition of more capacitance (33 μF) to the timing circuit. This allows a sweep of 10 V (256 channels) in approximately 7 minutes. The output of the ramp generator was calibrated as a function of time and has an excellent linear behavior. For these studies, two analog signals are processed along with the time ramp. One of these is the fluorescence pulse whose time behavior is being measured, and the other is a light scatter signal. When a cell passes through the laser beam, the light scatter signal initiates a signal processor to peak sense and hold the light scatter and fluorescence signals as well as the voltage level present from the ramp generator. By using the light scattered by a cell to trigger data acquisition, every cell passing through the flow chamber is analyzed, even those with zero or very low fluorescence. The signals are digitized and transferred to a minicomputer (PDP-11/20), where they are stored on a magnetic disk as correlated raw event files (*9*). The initial start-up time delay in sample analysis is usually between 30 and 40 seconds. This delay could be re-

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duced by at least a factor of 2 if we were to incorporate shortened tube lengths and pressurized sample-substrate mixing. At the time the sample flow is established through the laser beam, the ramp generator and data acquisition run are both started. Counting rates are held below 10^3 cells per second.

The first example of fluorescence kinetics compares esterase (E.C. 3.1.1) activity in two cell lines, CHO and PYS, as assayed by measuring the fluorescence of fluorescein enzymatically liberated from the fluorogenic substrate fluorescein diacetate (FDA) (Eastman Kodak). The esterase activity for the CHO cells is significantly higher than for the PYS cells (Fig. 1). The rates are 7.4 and 1.5 channels per minute (*10*), respectively, and are apparently linear with time. The CHO cells have two populations whereas the PYS cells have three populations, one of which is very bright and off scale on the upper limit. The very dim cells on the lower limit in Fig. 1, A and B, probably represent cells with disrupted membranes which are unable to retain fluorescein (*11*). Classical enzyme measurements would not discriminate among these subpopulations but would instead give an averaged kinetic value for the total population. By sending the photomultiplier signal into two or more fluorescence amplifiers at different gains, one can follow heterogeneous populations with markedly different rates.

We also used this technique to study several aspects of cell-stain interactions. Unfixed mouse spleen cells were stained for DNA with propidium iodide in hypotonic citrate (*12*), and the reaction kinetics were measured. Nuclei from cells at stages G_1, S, and G_2 + M were stoichiometrically stained within 1.5 minutes and reached equilibrium after 7 minutes. For fixed (70 percent ethanol) mouse spleen cells, binding of the DNA-specific fluorochrome mithramycin (*6*) was at equilibrium at approximately 4 minutes. We studied the kinetics of dye leaching by resuspension of the mithramycin-stained cells in phosphate-buffered saline (PBS) (*13*). The fluorescence intensity decreased approximately 54 percent during the first 40 seconds and reached an equilibrium value of a 78 percent decrease (*14*) in approximately 10 minutes.

Although the sequential discrete measurement method is acceptable for studies of enzyme systems which have slow turnover rates and which are being followed for long periods of time, the continuous time technique is much more precise for fast reactions that are being observed for only a few minutes. Population inhomogeneity can be readily detected. In heterogeneous populations the addition of another parameter such as cell size or DNA content allows further discrimination in these kinetic measurements (*15*). Other applications in which time is used as a parameter in flow cytometry include analysis of the binding kinetics of fluorescent tags that are specific for cell membrane receptor sites (*16*) and examination of the uptake of fluorescent or fluorescence-quenching chemotherapeutic drugs such as adriamycin or its analogs (*17*).

Temporal studies of cells by flow cytometry need not be confined to fluorescence measurements but may be used with any measurable parameter such as the apparent decreases in cell volume as measured by the Coulter effect during fixation in ethanol (*14*). Indeed, with this technique, kinetic studies of changes in fluorescent intensity of solutions can be made in a flow cytometer either by electronically gating the measured d-c fluorescence levels for short intervals (for example, 10 μsec) or by chopping the laser beam, and processing the analog signals. For a computer-based system, one can use a computer clock to record the time for each event. In the present studies, we used the voltage ramp generator because it is an available technique for any flow cytometer system that has a hard-wired two-parameter analyzer, and, since it does not require software modification, it is immediately usable for any computer-based system. Since many of the flow cytometers presently in use are capable of two-parameter analysis, the instrumentation technique we have described is readily available for general application.

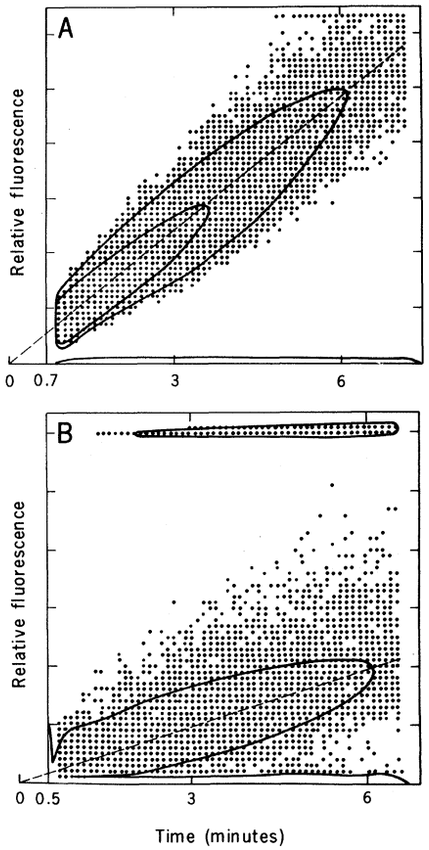

Fig. 1. The enzymatic liberation of fluorescein from the fluorogenic substrate FDA is shown as two-parameter contour distributions for two cell types, (A) CHO and (B) PYS. Chinese hamster (line CHO) cells, originally obtained from Dr. T. T. Puck, were grown in Spinner culture maintained in F-10 medium (Gibco, Grand Island, New York) supplemented with 15 percent fetal calf serum (Biocell, Carson, California). The isolation of parietal yolk sac (PYS) cells from an embryonal carcinoma cell line originally isolated from the solid teratocarcinoma OTT 6050 has been described in (*18*). The cells were maintained in medium 199 (Gibco) supplemented with 15 percent newborn calf serum (Biocell). Harvested cells were pelleted from media and resuspended in PBS, *p*H 7.2, at concentrations of approximately 10^5 cells per milliliter. Immediately before flow cytometric analysis, FDA was added to single-cell suspensions to a final concentration of 2.5 μg/ml for assay of esterase activity (*11*). The lower level threshold for the dots is 40 cells in (A) and 10 cells in (B); contours are at 60 and 100 cells in (A) and 20 cells in (B). The PYS sample was run at twice the gain setting on the fluorescence amplifier as the CHO sample. The dashed lines passing through the origin show approximate slopes for these distributions. Both (A) and (B) have subpopulations with very low or zero fluorescence intensities. The population in the PYS sample that is off scale on the upper limit accounts for 4 percent of the total cells. When observed at lower fluorescence gain settings (data not shown), this population increases in fluorescence intensity very rapidly. Computer reprocessing of the raw event data with discrete time windows yields fluorescence distributions that are equivalent to the distributions obtained by sequential discrete measurements (*8*).

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Aboriginal Indian Residence Patterns Preserved in Censuses and Allotments

Abstract. *Early reservation annuity censuses and allotment ledgers, analyzed in concert, allow identification of sociologically significant subdivisions of Native American tribes. Using this method, Southern Cheyenne* manhao *or "bands" can be located on the allotment map of 1892 as discrete clusters of individuals known by name, age, and sex. Measurement of linear distances among individual allotments of family members enables us to quantify jural rules of postmarital residence and confirms in a test case that the descendents of the bands at the Sand Creek Massacre in fact resided matrilocally.*

The Dawes Act of 1887 required Native Americans to select small individually owned allotments of land from the larger areas of tribal reservations. In selecting allotments, Indian people such as the Cheyennes of the American Plains exhibited patterns of choice which preserve for modern analysis their aboriginal social structure. By 1892, the Southern Cheyennes had selected 2132 allotments of approximately 160 acres each spread over 4 million acres in western Oklahoma (*1*). My first hypothesis was that the contiguity and spacing observed in the pattern of selected allotments represent the relationships then existing not only within extended families but also among kin-based political units called *manhao* in the native language. These units, which have been imprecisely described as "bands" or "clans" in English, consisted of approximately 200 to 800 persons each.

Discriminating between *manhao*, and *notxestozeo* or "military societies," also sometimes called bands, has been a classic problem in Cheyenne ethnography, and both the basis of organization of the *manhao* and the identities of their members have been widely debated (*2*). Although members of extended families are listed together by name, age, sex, and relationship on official allotment and annuity ledgers, descriptions of the larger units have been preserved only equivocally, in oral tradition and ethnographic accounts (*3*). Official rosters do not recognize these larger units, although they can be discovered from the rosters by a cybernetic control of this data, along lines of inquiry suggested by modern Cheyenne informants (*4*).

According to informants, and also according to historical accounts, extended families within the same *manhao* usually took their annuities together and also chose adjoining land allotments (*5*). If we analyze annuity lists, then, the consecutive ledger numbers assigned to individuals within a *manhao* should be closer than ledger numbers between members of different *manhao*. The locations of land allotments should also be instructive, since we learn from plotting family allotments on a map that members of the extended family took allotments together. Members of a *manhao* also can be predicted to have taken allotments together, as a group of extended families clustered on the allotment map.

A discrimination of hypothesized *manhao* from the allotment data is complicated, however, by the fact that some bands apparently took groups of allotments adjoining the allotments of other bands, with no space between them on a map of allotments (Fig. 1). But this problem can be solved by looking back at the annuity lists and subjecting them to an analysis in concert. In particular, I analyzed names appearing on at least two of the official annuity censuses of 1888, 1891, and 1895. The censuses were analyzed two at a time, with each individual identified by a pair of ledger numbers, each from a different ledger. Then these numbers were plotted as abscissa and ordinate for each person on Cartesian coordinates. On the resulting scattergram, not only did members of the same extended family appear as a small cluster of points, but clusters of families, the hypothesized *manhao*, began to appear. The individual land allotments of people in the same large group found on the map confirmed that these people all took their allotments together. By plotting each discovered "*manhao*" as a discrete unit, it was possible to identify the boundaries between groups which were contiguous on the map (Fig. 1) and to confirm that smaller groups of allotments were in fact single-band or single-*manhao* units. When an outline map of allotments arranged as bands (hypothesized *manhao* units) was shown to Southern Cheyenne informants in April 1979, they confirmed that these units were indeed the traditional *manhao*, and they supplied provisional names for the groups.

Although each Cheyenne band lived within the boundaries of the allotments taken by members of the band, an individual usually did not live on his or her personal allotment, according to government documents and modern informants (*6*). Nevertheless, the distribution of personal allotments strongly suggests that members of families, lineages, and bands took their land in patterns that symbolized their consanguineal, affinal, and political relationships to one another. When we assume that map distances can be taken as symbolic parameters of social distance, "kin closeness" can be quantified in miles and puts Cheyenne kinship studies on a firm data base. It is then possible to enter the classic debate concerning whether Cheyenne "bands" in the late 19th century were matrilocal, bilateral, or shifting from matrilocal to patrilocal forms as part of a general Plains emphasis on the importance of agnatic structures (*3*, *7*).

One problem of particular interest has been whether the Cheyenne camp attacked during the Sand Creek Massacre in 1864 was composed of *manhao* organized on matrilocal principles, in contrast to the patrilocal "Dog Soldiers" who had withdrawn to the northeast (*8*). The Sand Creek camps allegedly honored the matrilocal jural rule whereby "The people in each camp all belonged to the same clan" (*9*). It is now possible to test the actual matrilocality of these bands because of control of this early official data, and because of the recent collection by fieldworkers of genealogies of

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