of serum cholesterol, and that adding chromium (III) to this diet lowered cholesterol (*6*). Limited data from humans fed chromium indicated that chromium influenced the lowering of cholesterol concentrations, but the effect was inconsistent (*1*). We investigated the effects of dietary carbohydrates on hypercholesterolemia and a possible relationship of chromium and sucrose on serum cholesterol.

The study of hypercholesterolemia in the rat is facilitated by the addition of cholesterol and cholic acid to the diet. Such diets also containing sucrose or cooked potato starch as the carbohydrate (*7*) were fed to young adult male rats with no effort to control the chromium environment. Both sucrose and starch were fed because there is some evidence that a sucrose diet depletes body chromium stores (*8*) and causes higher concentrations of serum cholesterol (*7*). Chromic acetate was added to the drinking water of the experimental groups to supply 5 ppm of chromium (III) in the water. The same diets were fed to both the control and experimental animals, which were individually housed in raised galvanized cages with wire-mesh bottoms. Food and water were always available. All conditions were identical to those reported (*7*). The total chromium in the sucrose diet was 0.31 ppm and in the starch diet was 0.38 ppm, as determined by atomic absorption spectroscopy after the sample was subjected to dry ashing at 450°C. The cholesterol data were tested for significance by analysis of variance ($P \leq .05$). The standard deviations of the cholesterol values varied directly as the means; therefore, for statistical analysis, the cholesterol values were transformed to logarithms. The values presented were obtained by reconverting the data to their antilogs. The asymmetric distribution of the standard errors around their means results from the reconversion.

The serum cholesterol concentrations after an average of 35 days of feeding of the diets are shown in Table 1. The addition of chromium (5 ppm) to the drinking water significantly lowered the mean serum cholesterol. Chromium supplementation had the same effect when combined with either sucrose or starch. Chromium lowered the mean serum cholesterol in animals eating starch as well as those eating sucrose. Adding chromium to the drinking water of animals fed diets containing sucrose led to concentrations of serum cholesterol similar to the concentrations in the animals fed starch but not receiving chromium. Also the mean serum cholesterol of animals fed the starch diet is significantly lower than the mean of those fed the sucrose diet (*7*).

The mean weight gain and food consumption of each group during the first 28 days of the experiment before the animals were killed are shown in Table 2. The weight gain and food consumption of animals fed the starch-containing diets were slightly higher than those fed sucrose. The increased intake of the diet containing starch only gave a very minimum additional intake of total chromium, and it seems unlikely that this caused the significantly lower serum cholesterol in animals fed starch. These animals drank about 200 ml of water per week, and therefore the experimental groups received 4 to 5 mg of chromium (III) from the water during this 28-day period. The total amount of chromium received from the sucrose diet was 0.16 mg while the starch diet provided 0.24 mg over this same 28-day period.

The results of this experiment confirm and extend Schroeder's finding that chromium can lower serum cholesterol. Since chromium lowers the serum cholesterol of hypercholesterolemic rats housed in a normal laboratory environment whether they are eating starch or sucrose, we conclude that the elevation of cholesterol on sucrose diets cannot be due to chromium depletion alone and that chromium will lower the serum cholesterol concentration with any carbohydrate in the diet.

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Cell Sorting: Automated Separation of Mammalian Cells as a Function of Intracellular Fluorescence

Abstract. *A system for high-speed sorting of fluorescent cells was able to sort mouse spleen cells from Chinese hamster ovarian cells after development of fluorochromasia. Highly fluorescent fractions separated after similar treatment from mouse spleen cells immunized to sheep erythrocytes were enriched in antibody-producing cells by factors of 4 to 10.*

Separation of large numbers of functionally different cell types from the complex mixtures found in such organs as spleen, bone marrow, lymph nodes, liver, or kidney would be useful in biological and biochemical investigations. Direct fractionation methods, such as differential or isopycnic centrifugation, column fractionation, electrophoresis, and so forth, allow separation of large numbers of cells but produce only limited resolution of functionally different types. We are exploring a directed cell-sorting method where various parameters or criteria for separation can be used with a common sorting mechanism based on electrostatic deflection of charged droplets containing the cells. Here we report the use of intracellular fluorescence developed after exposure to the fluorogenic esterase substrate, fluorescein diacetate (FDA), as the parameter for directed sorting of cultured Chinese hamster ovarian (CHO) tumor cells from mouse spleen cells, and for concentration of antibody-producing hemolytic plaque-forming cells from spleens of mice sensitized to sheep red blood cells.

Live cells incubated with FDA accumulate fluorescein intracellularly since FDA can enter the cells and be hydrolyzed by intracellular esterases, but the fluorescein thus produced cannot readily leave through intact cell membranes. This phenomenon has been called "fluorochromasia" (*1*).

In these experiments a suspension of cells, after FDA treatment, passes through a small glass nozzle, forming a liquid stream. This stream is dark-field illuminated by an exciting beam of blue light from a mercury arc source, and the yellow-green fluores-

Table 1. Sorting of Chinese hamster ovarian (CHO) tumor cells from mouse spleen cells by fluorescence intensity, compared with sorting such cells by volume.

Cells	Cells exceeding threshold (percent)	
	Fluorescence* (cell sorter)	Volume† (electronic counter)
Mouse spleen	2	13
CHO	90	70
1 : 1.2 mixture of spleen and CHO tumor (before sorting)	47	48
Low-fluorescence fraction	2	23
High-fluorescence fraction	71	66

* These data were taken at the threshold used for detection. † The threshold was chosen to discriminate optimally between the signals generated by spleen cells and CHO tumor cells.

cence emitted by the cells is focused by a microscope on a photomultiplier tube through a yellow barrier filter. When a fluorescent cell generates a signal of designated amplitude at the photomultiplier a charging voltage pulse is applied to the stream. The stream breaks into droplets at the frequency of a low-power ultrasonic vibration applied to it, and those droplets formed while the charge is applied, including the one containing the fluorescent cell, remain charged after separation from the stream. These are deflected by an electric field between a pair of statically charged deflection plates and collected in a separate container from that collecting the undeflected droplets. The droplet-separating system is modified from that described by Sweet (*2*) and adapted by Fulwyler (*3*) for another cell-sorting system. Observation of fluorescence and other optical characteristics of cells in flow systems has previously been reported by Kamentsky *et al.* (*4*) and Van Dilla *et al.* (*5*).

The cell suspensions used were diluted to about 10^7 cells per milliliter, in Eagle's minimal essential medium containing 5 percent fetal calf serum. To develop fluorochromasia, 0.2 percent of a 0.5 percent solution of FDA in acetone was added and the samples were incubated at room temperature (23°C) for periods of 5 to 30 minutes (longer periods were found to be necessary for full development of fluorochromasia in small cells). The samples were then diluted in the same medium to a concentration of about 10^5 cells per milliliter and kept in the cold throughout the sorting operation. Flow rates were of the order of 1 ml/min.

In Table 1 we compare the efficiency of discrimination of mouse spleen cells from CHO tumor cells by volume and fluorescence intensity and show the efficiency of sorting by fluorescence intensity. Thresholds of volume and fluorescence intensity were chosen to best discriminate between the two cell types. The best fluorescence threshold was exceeded by the fluorescent signals given by 90 percent of the CHO tumor cells and by only 2 percent of the mouse spleen cells. The corresponding volume measurements were 70 percent and 13 percent respectively. A mixture of the two cell types was sorted into fractions containing cells exceeding and not exceeding the chosen threshold of fluorescence intensity. Analysis of the fluorescence intensity of the cells in the two fractions, presented in Table 1, showed efficient sorting. About 80 percent of the fluorochromatic signals were still detectable on a second passage through the instrument, indicating little cell damage. This was confirmed by a trypan blue dye exclusion test which showed that over 80 percent of the cells in both the initial sample and the deflected fraction were viable.

The poor correlation of volume with fluorescence intensity is also shown in Table 1. Although there is relatively good sorting of the small mouse spleen cells from the large CHO tumor cells in this experiment, the intensity of fluorochromasia is not a function of size alone. This has been demonstrated in several separations. For example, a suspension of spleen cells was sorted, deflecting only those cells giving signals above an arbitrary threshold (about 15 percent of the population). There was relative enrichment of the largest cells, but the concentration of even the smallest cells in the deflected sample (as measured by the Coulter counter) was actually two and one-half times as great as that in the control. Most of these small cells must have been among the most fluorescent 15 percent and thus more fluorescent than many larger ones, indicating increased esterase activity or different membrane characteristics than average cells.

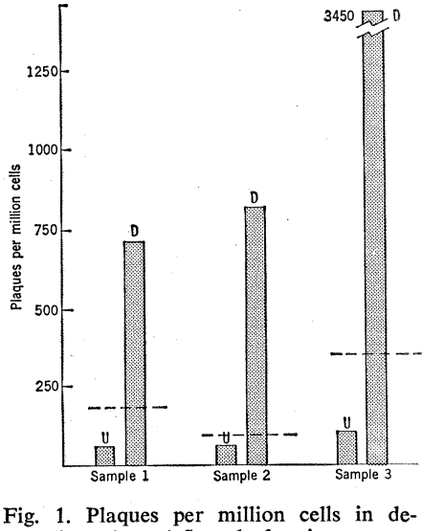

Fig. 1. Plaques per million cells in deflected and undeflected fractions, compared to original. *U*, Undeflected fraction; *D*, deflected fraction; ---, original. Ratio of *D* to *U* is 14 for sample 1, 16 for sample 2, and 34 for sample 3.

In a search for other biological activity associated with high fluorochromatic ability, cell samples from spleens of mice immunized with sheep erythrocytes were prepared at times from 4 to 9 days after injection and run through the instrument, which deflected the most fluorescent 8 to 10 percent of the cells. The enrichment of highly fluorescent cells in the deflected sample, and their depletion in the undeflected sample, were confirmed on a rapid cell spectrophotometer similar to that described by Kamentsky *et al.* (*4, 6*). The ability of the cells to form hemolytic complement dependent plaques was then assayed by using a modification of the Jerne hemolytic plaquing technique (*7*). Results are shown in Fig. 1. All deflected fractions contained much higher proportions of plaque-forming cells than the original samples, while the undeflected samples were correspondingly depleted. Apparent recovery of plaque-forming cells in the two sorted fractions varied from 60 to 110 percent of the number in the unseparated control. While there were more than ten times as many cells in the undeflected samples as in the deflected samples, the former always contained fewer of the plaque-formers than the latter. Since on the 4th day after immunization most of the plaque-forming cells produce 19*S* antibody while on the 9th day most produce 7*S*, cells producing antibody of both types were enriched in the deflected sample.

Although the present version of the sorter has only two sorting channels, sequential passes allow multiple fractions to be obtained. Simple modifications will permit a simultaneous collec-

tion of several fractions. Use of fluorochromasia from FDA as the sorting parameter in this cell sorter may have other applications in selecting functionally different groups of cells. The possibility of substituting other fluorogenic substrates for FDA should be considered as well as the use of other fluorescent dyes and of fluorescent antibody techniques.

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Chloroplast Replication and Growth in Tobacco

Abstract. *Tobacco etioplasts replicate at least twice during light-induced development. Their size is doubled over the same period. The two processes do not show the same kinetics. 5-Fluorodeoxyuridine inhibits chlorophyll synthesis during the normal course of "greening" by inhibiting chloroplast growth. This compound also inhibits cytokinin-induced chloroplast replication.*

Chloroplasts in higher plants and algae contain their own DNA (*1*) and complete protein-synthesizing machinery (*2*), but very little is known about the function of this DNA in chloroplast development. To study the effect of inhibitors of DNA synthesis on the development of etioplasts into chloroplasts, we used the inhibitor 5-fluorodeoxyuridine (FUDR), which inhibits DNA synthesis in bacteria and tobacco (*3, 4*) primarily by inhibiting thymidylate synthetase (*5*). The effect is reversible with thymidine.

Etiolated leaves of *Nicotiana tabacum* L. var. Maryland Mammoth were obtained by cutting stems of mature, greenhouse-grown plants into 20-cm sections which were rinsed in 4.0 percent hypochlorite (commercial bleach with detergent) solution and placed in the dark in flasks containing a nutrient solution. Etiolated leaves of lateral shoots were harvested after 2 weeks, soaked in water containing a trace of laboratory detergent for 15 to 20 minutes, agitated in a 2.0 percent hypochlorite solution for 2 minutes, rinsed in sterile water, and placed in petri dishes on a layer of sterile filter paper. Disks (2 mm in diameter) from several leaves were placed together in each dish to randomize variation. All manipulations were performed under a dim green light. The dishes were kept in the dark for 2 hours before exposure to light; longer periods of preliminary treatment in the dark did not affect the result. The dishes were kept at 25°C in a light intensity of 30 lu/mm^2 provided by Sylvania cool white VHO fluorescent tubes with neutral density filters.

The standard culture medium (S+S) contained Murashige and Skoog salts (*6*), 0.8 percent agar, and 0.5 percent sucrose. Other components were added as indicated. Thymidine, uridine, and FUDR were sterilized by filtration before addition to the medium.

Chlorophyll was determined according to the method of Arnon (*7*). Chloroplasts and etioplasts in cells were counted and measured in sections from fixed tissue. The leaf disks were embedded in paraffin by standard methods, sectioned at 40 μm, and stained in safranin and then fast green. Plastids were counted by moving the plane of focus through whole cells. Chloroplast size was determined by measuring the greatest dimension with an ocular scale. The number of cells per leaf disk was determined by hemacytometer counts of cells separated in 5 percent chromic acid, and cell sizes were measured on photographs of the microscopic slides. Observations were made with a Zeiss photomicroscope equipped with a ×100 phase-contrast objective.

Disks were exposed to ^{3}H-thymidine (250 μc/ml, specific activity 11.7 c/mmole) for 2 hours, fixed by freezing, and embedded in paraffin (*8*). Sections were cut at 6 μm, treated with either deoxyribonuclease (Worthington, ribonuclease-free; 1 mg/ml in 0.1M acetate buffer, pH 5, and 0.005M $MgSO_4$) or with plain buffer, covered with Ilford L4 emulsion by the liquid-emulsion method (*9*), exposed for 8 to 10 days, and developed with Kodak D19 developer.

Material was fixed for electron microscopy in glutaraldehyde and OsO_4 as described (*10*). The tissue was embedded in Epon (*11*) and sectioned with a diamond knife. Sections were mounted on uncoated grids, then stained with lead citrate (*12*), and viewed in a Zeiss 9A electron microscope.

The etiolated tobacco leaves are not completely uniform, so the absolute values for chlorophyll content vary between experiments although the pattern remains the same. The graphs and tables, therefore, give representative data rather than average values.

Chlorophyll synthesis in the tobacco leaf disks is inhibited about 50 to 60 percent by FUDR ($10^{-4}M$) after 4 days in the light. This is reversed by thymidine at the same concentration but not by uridine (Table 1). 5-Fluorodeoxyuridine has little effect on fresh weight and none on cell size and cell number per leaf disk. The course of chlorophyll synthesis (Fig. 1) in the control shows the normal pattern of a lag phase followed by a rapid increase in chlorophyll content (*13*). During the lag phase there is no visible effect of FUDR, but the following rapid synthesis of chlorophyll is largely inhibited by FUDR. A reduction in chlorophyll content of the whole tissue could be the result of abnormal chloroplasts, fewer chloroplasts, or smaller chloroplasts.

Since chloroplasts from FUDR-treated

Table 1. Inhibition of chlorophyll synthesis by $10^{-4}M$ FUDR in etiolated leaf disks exposed to light for 4 days and reversal of this inhibition.

Additions to S+S medium	Chlorophyll (μg/g, fresh weight)
None	375.4
FUDR	220.5
Thymidine	414.4
Uridine	446.0
FUDR and thymidine	366.6
FUDR and uridine	228.6

Table 2. Effect of FUDR on kinetin-induced chloroplast replication. Etiolated disks cultured on S+S and kinetin and S+S, kinetin, and FUDR in the dark for 7 days and exposed to light for 7 days.

Additions to S+S medium	Chloroplasts (No./cell)	Chloroplast size (μm)
Kinetin	135	4.6
FUDR and kinetin (0.5 mg/liter)	50	2.5