that this cell contains choline acetyltransferase at a concentration slightly greater than the highest activity reported for cholinergic mammalian axons (for example, ventral spinal roots) (*18*). However, there is evidence in one other species of *Aplysia* that the pigmented cell R2, which has an enzyme concentration comparable to that of L10, mediates an action which is not cholinergic. A branch from R2 to the giant cell in the left pleural ganglion produces a postsynaptic potential which was not blocked by curare and was not simulated by acetylcholine applied iontophoretically (*19*). Although these pharmacological data are not conclusive, they suggest that R2 may be a neuron which is not cholinergic, but which nonetheless has the capacity to synthesize acetylcholine. Alternatively, it is possible that R2 releases acetylcholine in addition to a noncholinergic transmitter.

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17 April 1968

Centrifugation of Mammalian Cells on Gradients: A New Rotor

Abstract. *A short-arm rotor increases separation of viable mammalian cells, from mixtures, by low-speed centrifugation; continuous Ficoll density gradients in tissue-culture media are used. We describe the theory and experimental demonstration of the superior separation achieved with this new rotor.*

Ability to isolate single species of cells from mammalian tissues would be widely useful because experimentation on specific cell types within whole tissues is complicated by the cells' intimate association with other cells differing markedly in structure and function. We are currently developing methods for resolution of disaggregated solid tumors into fractions containing purified suspensions of such things as viable malignant cells, fibroblasts, reticuloendothelial cells, and inflammatory cells.

Viable mammalian cells of predetermined diameters and densities may be sedimented at predictable rates (*1*) on density gradients of Ficoll (*2*) in tissue-culture medium; the computer-integrated differential sedimentation equation is used for determination of the optimal speed and duration of centrifugation. Anderson's low-speed A-XII zonal rotor is used.

We have now devised a similar system for the predictable separation of cells by gradient centrifugation; ordinary, parallel-walled polycarbonate centrifuge tubes of 100-ml capacity are used in the International Equipment Company centrifuge, model PR-2. This system has the advantage of easily maintained sterility, and permits multiple experiments in less time. Ehrlich ascites tumor cells and HeLa cells, separated from mixtures by this procedure, have been subsequently regrown in mice and in tissue culture, respectively. Rabbit thymocytes and Ehrlich ascites tumor cells also have been separated from mixtures in 98- to 100-percent purity.

We now report theoretical and experimental verification of the fact that greater separation of particles, sedimenting in solution in a centrifuge tube, can be achieved by movement of the centrifuge tube closer to the axis of revolution.

Consider a system consisting of two spherical particles, 1 and 2, sedimenting in a homogeneous solution of given density ρ_S and viscosity η, in which particle 2 sediments faster by virtue of its larger diameter a or greater density, or of both (*3*, *4*). After setting of the angular velocity during centrifugation (ω) constant, let both particles start sedimenting at a specific distance r_0 from the axis of revolution. Proceed for the time (t) that it takes particle 2 to travel distance l from r_0. During this time, particle 1 sediments to a position r_1 intermediate between r_0 and (r_0+l). The differential sedimentation equation states that the velocity of either particle is

$$\left(\frac{dr}{dt}\right)_i = C_i r$$

where

$$C_i = \frac{a^2_i(\rho_i - \rho_S)\,\omega^2}{18\eta}$$

Therefore

$$t = \frac{1}{C_2} \ln \frac{r_0 + l}{r_0} = \frac{1}{C_1} \ln \frac{r_1}{r_0}$$

and

$$r_1 = r_0 \left(1 + \frac{l}{r_0}\right)^{\alpha}$$

where

$$\alpha = C_1 / C_2$$

Distance S between the two particles after time t is therefore

$$S = r_0 + l - r_0 \left(1 + \frac{l}{r_0}\right)^{\alpha}$$

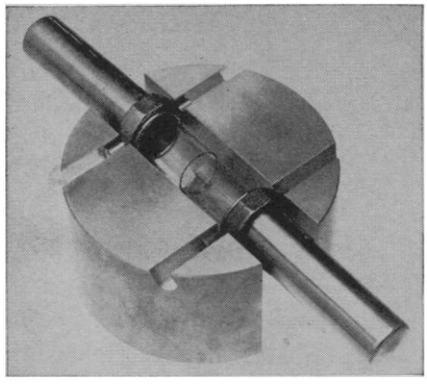

Fig. 1. Short-arm rotor with 100-ml polycarbonate centrifuge tubes in place.

The derivative of S with respect to l gives the separation occurring between the particles per unit distance traveled by particle 2:

$$\frac{dS}{dl} = 1 - \alpha \left(\frac{r_0}{r_0 + l}\right)^{1-\alpha}$$

Now, as $r_0 \rightarrow 0$ (that is, as the centrifuge tube containing particles 1 and 2 is moved closer to the axis of revolution), the separation between the particles, per unit distance traveled by particle 2, increases as limit 1 is approached. Thus is demonstrated the theoretical reason for moving the centrifuge tube closer to the axis of revolution for better separations.

So we constructed a rotor that could provide the desired short initial distance from the axis of revolution (Fig. 1). It was designed to hold a 100-ml polycarbonate tube (IEC tube 2806) and to be used in the model PR-2 centrifuge. The trunion-ring grooves are elevated above the top of the rotor shaft so that in the horizontal position the tops of the centrifuge tubes are 0.45 cm from the center of revolution. This rotor is 7 cm in diameter and made of naval brass; it weighs 13.6 kg—slightly less than the largest conventional PR-2 centrifuge rotor filled to capacity with water. The high mass was chosen for slow deceleration, and minimal swirling of the gradients during deceleration.

The new rotor was calibrated with two populations of styrene divinylbenzene spheres (*5*) with diameter ranges of 6 to 14 and 12 to 35 μ, respectively (approximately the range of sizes observed in mammalian cells). The finer of the two populations exhibited a broad modal diameter in the range from 6 to 11 μ; the coarser, a modal diameter between 26 and 33 μ. These particles are spherical and constant in density and diameter, and lack significant tendency to form aggregates under our experimental conditions.

Identical, 10-cm, linear density gradients, 2.5 to 16 percent (by weight) of Ficoll in water, were constructed in centrifuge tubes. One of two identical 2.5-ml portions of a water-suspended mixture of latex particles of the two sizes (in concentrations previously determined to be suitable for photography) was layered over each gradient. One of the resultant gradients, with the overlying sample of suspension of latex particles, was centrifuged at 400 rev/min and 4°C with the short-arm rotor. The other gradient was centrifuged in the same manner with the conventional long-arm rotor (IEC 269). The initial radius for the gradient on the short-arm rotor was 2.5 cm; on the long-arm rotor, 11.1 cm. The duration of centrifugation was calculated in each case (by means of the computer-integrated sedimentation equation) so that peak separations by the two rotors could be easily compared.

Figure 2 shows both greater distance between peaks and greater resolution of the heterogeneous particle diameters within each peak when the short-arm rotor was used. Wall effect is a more important consideration when the centrifuge tube is moved closer to the center of rotation. Sector-shaped centrifuge tubes have been designed (*6*) for conventional rotors to overcome wall-effect problems and would be advantageous for use with the short-arm rotor.

We call attention to improvement of separation by shortening of the initial radius in gradient centrifugation; this principle is applicable to any system of particles or macromolecules having different sedimentation coefficients. Use of the computer-integrated sedimentation equation, in conjunction with a short-arm rotor, provides the experimenter with a highly efficient method for purifying mixtures of viable cells on density gradients composed of Ficoll in tissue-culture media.

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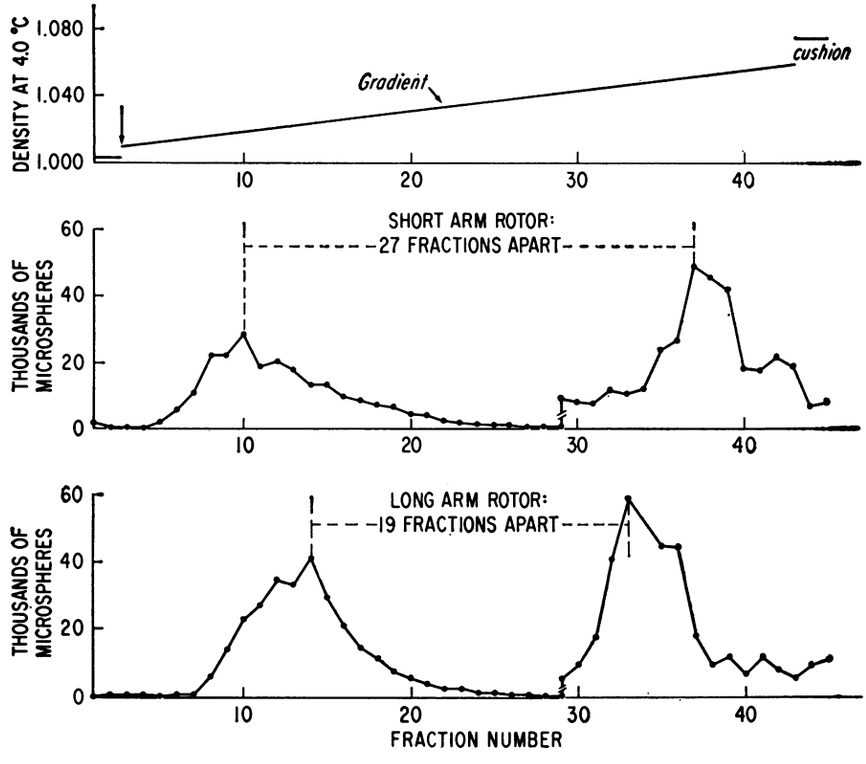

Fig. 2. With identical gradients and samples, the short-arm rotor improves separation between peaks, and resolution of the heterogeneous particles within peaks. The arrow indicates the sample-gradient interface. Centrifugation was carried out for 60 minutes with the long-arm rotor and 138 minutes with the short-arm rotor. The microscope count for fractions 29 to 45 is plotted × 10 to facilitate comparison of peak locations.

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17 July 1968

Oncogenicity by Methyl Methanesulfonate in Male RF Mice

Abstract. *The incidences of lung tumors and thymic lymphomas were increased in young adult male RF mice receiving 30 milligrams of methyl methanesulfonate per kilogram of body weight daily in the drinking water throughout life. Differences in oncogenicity between treatment with methyl methanesulfonate and with dimethylnitrosamine or diethylnitrosamine suggest a qualitative difference between the site (or sites) of alkylation by methyl methanesulfonate and by dimethylnitrosamine or diethylnitrosamine within the nucleic acids.*

The mutagenic and carcinogenic action of alkylating agents has been partly attributed to alkylation of nucleic acids, which results in an altered cellular genome (*1, 2*). Diethylnitrosamine (DEN) and dimethylnitrosamine (DMN), potent carcinogens in various animal species (*3, 4*), alkylate nucleic acids, especially at the 7-position of guanine (*5*). Methyl methanesulfonate (MMS) is reportedly as effective as DMN in alkylating DNA and RNA (*2*); it also methylates at the 7-position of guanine in bacteriophages (*6*). No reports, however, incriminate MMS as a carcinogen; repeated doses have been given to male rats for study of the effect on fertility (*7, 8*), but even after prolonged treatment no tumors were observed (*9*). We now report demonstration of an oncogenic effect by MMS on lung and thymus in mice.

Sixty-three 11-weeks-old male mice from a noninbred subline of the RF/Un strain were given MMS (*8*) for life in drinking water at 20 mg/100 ml. Fresh drinking solutions were prepared daily, and the water consumption per cage (initially eight mice) was measured daily. Mice were weighed weekly, and the intake of MMS was calculated in milligrams per kilogram of body weight. Groups each of four mice were killed and necropsied after 3, 6, 9, and 12 months of treatment; the remainder were necropsied at death or killed when in a moribund condition, and tissues from all major organs were examined histologically. A group of 162 untreated RF male mice, handled similarly, served as controls.

Of the 47 treated mice allowed to die, 70.2 percent had primary pulmonary tumors, compared to 38.9 percent of 162 controls (Table 1). The mean age at death of mice with lung tumors was slightly less in the treated group than in the control group: 19.7 versus 22.0 months. In the 28 mice examined histologically, the incidence was 82 percent, suggesting that the overall incidence would have been even higher if lung tissue from all mice had been examined microscopically. The tumors were adenomas morphologically indistinguishable from those observed either of spontaneous origin or after treatment with DEN (*3*) or DMN (*4*).

Thymic lymphomas developed in 14.9 percent of the treated mice and in 3.7 percent of the controls (Table 1); these neoplasms originated in the thymus, infiltrated locally into the lungs and heart, and then spread throughout the body. The disease process appeared identical in treated and untreated mice, with mean ages at death of 17.3 and 15.4 months, respectively. Myeloid leukemia was not observed in the mice treated with MMS but was diagnosed in 4.3 percent of the controls. Other types of leukemias and lymphomas were predominantly reticulum-cell sarcomas in both treated and control mice, the overall incidences being 44.7 and 60.5 percent, respectively; the incidences were not different when adjusted for differences in survival time. Other pathological changes in the treated mice did not differ from those in the controls.

Mice receiving MMS showed no gain in weight, in contrast with the controls whose mean weight increased from 35 g at 11 weeks of age to 43 g at 43 weeks. Although the calculated daily dose levels of MMS were from five to 30 times the sublethal levels of DEN and DMN, respectively (*3, 4*), the mean survival time of treated mice (18.4 months) was not greatly different from that of the controls (20.5 months). On the basis of the mean survival time, the mean cumulative dose for all treated mice was 13,800 mg/kg—approximately 30 mg/kg daily.

Alkylation occurs primarily at the 7-position of guanine in both RNA and DNA after administration of MMS (*10*), as after DMN or DEN (*1, 2, 5*). The fact that levels of methylated guanine in rat kidney after administration of MMS are comparable to those following nitrosomethylurea or DMN (*11*) suggests that the precise location or grouping of the alkylation, along the nucleic acid chain, may be important. The significance of alkylation of proteins or of bases other than guanine in the oncogenic process is unknown; however, the 1- and 3-positions of the imidazole ring of histidine amino acid residue are principal sites of reaction with DMN (*12*), whereas MMS reacts with the sulfhydryl group of cysteine, with no detected reaction with the histidine side chain (*13*).

If one measures by induction of lung tumors, MMS is slightly more effective than DEN (*3*) but less so than DMN (*4*). While DEN and DMN fail to induce leukemia (*3, 4*), MMS is leukemogenic, resembling x-rays (*14, 15*) and nitrogen mustard (HN2), triethylene melamine, and myleran (*14–16*), all of which induce thymic lymphomas. The fact that MMS fails to reduce the latent period for lung tumors and thymic lymphomas suggests that carcinogen-screening programs require observation periods sufficiently long for detection of late-occurring as well as early tumors.

Table 1. Observed incidences (percentages) of neoplasms in 47 male RF mice treated daily with methyl methanesulfonate (MMS) at 30 mg/kg in the drinking water; controls numbered 162. "Other" leukemias were primarily reticulum-cell sarcomas.

Item	Treatment	
	MMS	None
Tumor sites		
Lung	70.2	38.9
Liver	4.2	3.7
Stomach	2.1	0
Leukemias		
Thymic lymphoma	14.9	3.7
Myeloid	0	4.3
Other	44.7	60.5

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