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Homogeneity of Antibody-Producing Cells as Analysed by their Buoyant Density in Gradients of Ficoll

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Summary. Ficoll, a polymer of sucrose, has several advantages over proteins for preparation of density gradients used to study mammalian cells. In addition to its ease of preparation, Ficoll as a result of being an uncharged molecule, does not bind ions from solution and, therefore, does not affect the osmolality of the medium in which it is dissolved.

In studies on antibody-producing cells we found that the measurement of buoyant density in Ficoll at pH 7·2 gives an accurate measurement of cell density. In such gradients antibody-producing cells band in a single peak at a density of 1·070 g/cm^3. Two factors have a profound influence on the density profile of antibody-producing cells. Lowering the pH to 5·5 lowers their buoyant density to 1·062 g/cm^3. Creation of an inverse osmotic gradient varying from 294 m-osmolal at the top of the gradient to 286 m-osmolal at the bottom splits the single peak of antibody-producing cells into three distinct peaks. This effect of osmotic gradients could account for the multiple peaks observed when cells are separated on protein gradients.

INTRODUCTION

Heterogeneity of antibody-producing cells has been observed in several experimental situations. Morphological studies purport to show several distinct morphological types of cells synthesizing antibody (Harris, Hummeler and Harris, 1966; Cunningham, 1968). Recently, Haskill, Legge and Shortman (1969) analysed the buoyant density distribution of antibody-producing cells in the rat and found that these cells could be resolved into numerous sub-populations by flotation in gradients of bovine serum albumin (BSA). In contrast to these data, analysis of antibody-producing cells in the mouse by velocity sedimentation, which measures primarily cell volume, indicated that these cells are a homogeneous population (Phillips and Miller, 1970). Although there was some heterogeneity in sedimentation velocity this could almost entirely be explained in terms of cell volume changes associated with cell growth through the division cycle. If there were density heterogeneities of the magnitude reported by Haskill *et al.* (1969), the distributions of sedimentation velocity for antibody-producing cells should have been much broader than were observed and should have shown some fine structure.

To resolve this apparent discrepancy between the heterogeneity seen with density measurements and the homogeneity seen with velocity sedimentation, we have measured the density of antibody-producing cells in the mouse. In designing a method for measurement of the density of mouse cells, we attempted to avoid many of the pitfalls known to be

associated with separation of mammalian cells by flotation in density gradients (Leif, 1964; Shortman, 1968). Most of the previously reported density measurements have utilized gradients of BSA (Haskill *et al.*, 1969; Leif, 1964). The most disturbing feature of these reports has been their lack of reproducibility (Worton, McCulloch and Till, 1969). This problem could be caused by the nature of the gradient material. BSA is a natural product and its isolation may vary from one batch to another; variations in different preparations can generate different density profiles for the same population of cells (Shortman, 1968). In addition, because BSA is a charged molecule, it will bind ions and decrease the osmolality of the solution (Scatchard, Batchelder and Brown, 1946). Changes in osmolality are known to alter the density distribution of some classes of cells (Shortman, 1968; Legge and Shortman, 1968).

Some of these problems could be overcome if a synthetic, uncharged molecule were used to make density gradients. In this paper we described the use of Ficoll, a large polymer of sucrose, to generate density gradients. The data show that Ficoll gives an accurate, reproducible measurement of density, and that in the absence of osmotic gradients antibody-producing cells band as a single homogeneous peak.

MATERIALS AND METHODS

Animals

F_1 hybrids between C3H/HeOci and C57BL/6JOci (hereafter referred to as $C3B6F_1$) mice were used in these experiments. These mice were bred in the animal colony at the Ontario Cancer Institute. C3H/HeJ mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Fisher 344 rats (obtained from A. R. Schmidt Co., Wisconsin were used in some experiments. All animals were allowed free access to food and water.

Buffer

Maintenance of a constant tonicity and prevention of cell clumping dictated the choice of buffer material. To compare our data with that of Haskill *et al.* (1969), we chose to make our buffer 294 m-osmolal (Shortman, 1968); this choice precludes quantitative comparison of our results with published results of others which were carried out at different osmolality (Niewisch, Vogel and Matioli, 1967; Dicke, van Hooft and van Bekkum, 1968).

A major problem with Ficoll is its tendency to clump cells (Walder and Lunseth, 1963). Shortman (1968) noticed cell clumping with Dextran gradients and found the problem alleviated by using a dispersing agent, 2-naphthol-6, 8-disulfonic acid (dipotassium salt). With BSA the problem of clumping was overcome by lowering the pH to 5·1. He reported that this low pH did not noticeably affect the volume or viability of nucleated cells, but it caused marked swelling of red cells (Legge and Shortman, 1968). We find that the combination of the dispersing agent and a low pH (5·5) prevents clumping in Ficoll up to concentrations of 5×10^6 cells per ml. The composition of the buffer is as follows: 0·01M sucrose, 0·003 M KH_2PO_4, 0·1165 M NaCl, 0·015 M dispersing agent. When necessary, 294 m-osmolal Na_2HPO_4 was added to adjust the pH.

Preparation of spleen cells

Cells were suspended in 1 ml of buffered medium by gentle aspiration with a 1 ml syringe. Viability was assessed by measuring uptake of fluorescein diacetate (FDA) (Rotman and Papermaster, 1966).

Ficoll

Ficoll (Pharmacia, Uppsala) was dissolved in distilled water to give a 36 per cent solution (w/w). The solution was deionized using a mixed bed resin (AG-501-X8 (D), Bio-Rad) at a concentration of 100 g resin/500 g Ficoll. The solution was deionized twice, each time for 4 hours at 4° with continuous stirring. Filtration of the deionized mixture through gauze removed the resin; subsequent filtration through Millipore filters sterilized the Ficoll solution allowing it to be stored for long periods at 4°. As required, the Ficoll solution was then made up to be 294 m-osmolal with respect to the water present using the buffer described above. The pH was adjusted to the value desired by addition of 294 m-osmolal Na_2HPO_4. Water was added to allow for the osmolality of the Ficoll; for this purpose Ficoll was assumed to be a homogeneous molecule with a molecular weight of 400,000. This correction involved adding 7 ml of water to 3 liters of 36 per cent Ficoll in 294 m-osmolal buffer.

Density measurements

Measurement of refractive index provides a simple, rapid method for determining the density of unknown solutions. To prepare a standard curve for Ficoll of refractive index versus density, we made several solutions containing different concentrations of Ficoll. The density of these solutions was determined by weighing a large, known volume of solution (usually about 1 litre). Subsequent measurement of the refractive index (Abbey Refractometer, Bausch and Lomb) of each solution gave the desired standard curve. The density of fractions from each gradient was determined from this curve.

Gradients

Density gradients were prepared in the usual way using two chambers, one containing low density Ficoll and a mixing chamber containing initially high density material. A linear gradient was obtained by pumping low density material into the mixing chamber at half the rate that dense material was pumped out. The gradients were generated continuously over a 50-minute period. Cells were introduced into both chambers at equal concentrations, so that initially there was a uniform concentration of cells throughout the gradient. The advantages of loading cells in a way similar to this have been described by Shortman (1968).

Gradients were prepared in 30 ml cellulose nitrate tubes and centrifuged at 3800 ***g*** using an SW-25 rotor. Following Shortman's protocol, we allowed 1 hour for the cells to come to their equilibrium density. After centrifugation, 1 ml fractions were collected by upward displacement using dimethylphthalate (1·14 g/cm^3) as a displacement medium.

Cell counts

Total cells and nucleated cells in each fraction were enumerated using an electronic cell counter and pulse-height analyser as previously described (Miller and Phillips, 1969).

BSA solutions

BSA was prepared using a combination of the methods described by Shortman (1968) and by Worton *et al.* (1969). Briefly, BSA (Fraction V, Sigma) was dissolved in distilled water to give a concentration of 20 per cent (w/w). The solution was deionized as described above and then lyophilized. The deionized, powdered BSA was dissolved in buffer as described by Shortman (1968) to give solutions that were 294 m-osmolal on the assumption that the BSA molecules bound no salt.

Freezing point depression

To determine the osmolality of various solutions, we measured the freezing point of 5·0 ml samples of solution, monitoring temperature changes with a difference thermometer (Beckman Instruments). The correlation between freezing point depression and osmolality was determined with standard solutions of NaCl. Using the measured freezing point depression, the osmolality of experimental solutions was then read from this standard curve.

Measurement of cellular density using phthalate esters

The method used was essentially similar to that reported by Danon and Marikovsky (1964). Its major advantage is that density profiles can be determined without suspending the cells in dense media, thus avoiding possible osmotic effects of dense media on cells. Mouse blood, obtained from the retro-orbital sinus, was introduced directly into a microhaematocrit tube. This was followed by a mixture of dimethyl- and di-*n*-butyl-phthalate of known density. The tube was sealed and centrifuged and the proportion of cells above and below the phthalate mixture was estimated by measuring the length of the packed cell column on each side of the phthalate band. Only those cells denser than the mixture will sediment to the bottom of the tube. Although only one density at a time can be examined, many tubes can be processed simultaneously to give a complete density profile.

Immunization and assay for antibody-producing cells

Sheep erythrocytes (SRBC) were used as test antigen. They were washed three times with phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954) before use. For immunization, 4×10^8 SRBC were injected intravenously into mice and 2×10^9 SRBC intraperitoneally into rats. In some experiments studying cells making 7S-antibody, mice were given two injections of SRBC separated by a 4-week interval. The number of antibody-producing cells was enumerated using the direct plaque-forming cell (19S-PFC) assay of Jerne, Nordin and Henry (1963). Cells making 7S-antibody (7S-PFC) were developed with rabbit anti-mouse γ-globulin (Dresser and Wortis, 1965).

Cell viability in Ficoll

Tests of toxicity of Ficoll on antibody-producing cells were performed using spleen cell suspensions prepared from mice 4 days after immunization with sheep erythrocytes. The cells were suspended in 1 per cent BSA in PBS. Some of the suspension was placed in an ice-bath to serve as a control. Another sample was centrifuged, and aliquots were resuspended in 1 per cent BSA, 6 per cent Ficoll, or 36 per cent Ficoll. The conditions were identical to those used in the gradient. In particular, the buffer described above was used. Cells were incubated in these solutions for 3 hours at 4°, and then tested for PFC. The results are given in Table 1 and show no loss of viability under these conditions.

TABLE 1

SURVIVAL OF PFC IN FICOLL

Suspending medium	19S-PFC/10^6 spleen cells $\pm$ SE
Control (1 per cent BSA in PBS)	240 $\pm$ 23
Resuspend in:	
1 per cent BSA in PBS	230 $\pm$ 37
6 per cent Ficoll	230 $\pm$ 16
36 per cent Ficoll	240 $\pm$ 22

RESULTS

DISTRIBUTION OF MOUSE SPLEEN CELLS IN GRADIENTS OF FICOLL

The distribution of cells separated on a Ficoll gradient ranging from 10 to 29 per cent Ficoll at a pH of 5·5 is shown in Fig. 1. There are several points of interest in this figure. First, the density gradient is linear throughout the entire range of nucleated cells. Second, as expected, the distribution of nucleated cells is a broad distribution ranging in density from 1·050 to 1·096 g/cm^3. Third, the distribution of viable cells, as measured by uptake of FDA, shows that the majority of the damaged cells sediment in the dense regions of the gradient. This might be expected if cytoplasm leaks out of damaged cells, thereby increasing the contribution of the nucleus to the cell density. Fourth, the distribution of the erythrocytes shows a homogeneous population of cells at a mean density of 1·089 g/cm^3. This density of erythrocytes may be lower than the *in vivo* density since Legge and Shortman (1968) reported that erythrocytes swell at low pH.

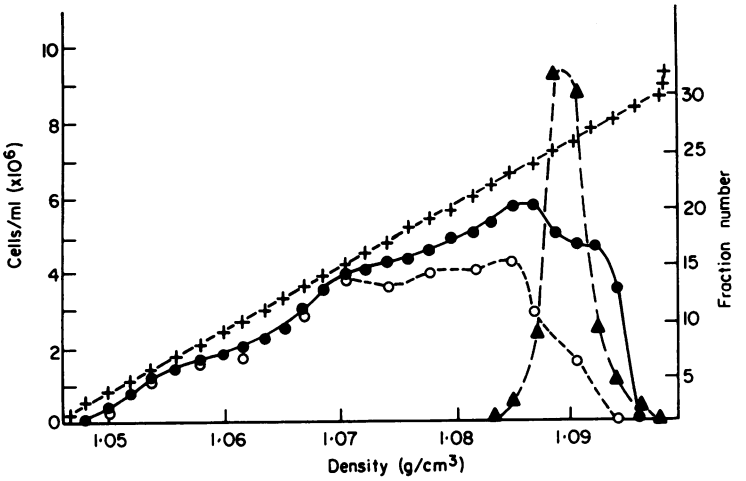

FIG. 1. Density separation profile for spleen cells from unimmunized C3B6F$_1$ mice in a linear Ficoll gradient at pH 5·5. The crosses show the density of each fraction. The other curves give the distribution of nucleated cells (●), viable cells (○), and erythrocytes (▲). Viable cells were assayed by their ability to concentrate FDA (Rotman and Papermaster, 1966). There was a 95 per cent recovery of nucleated and viable cells and an 83 per cent recovery of erythrocytes.

To test the reproducibility and resolution of separation on a Ficoll gradient, a rebanding experiment was performed. Spleen cells from an unimmunized mouse were separated as shown in Fig. 1. Two fractions from this separation were taken from different regions of the gradient, pooled, and re-separated on another gradient. The results of this experiment are shown in Fig. 2. Dashed lines are drawn through the two fractions that were pooled. The distribution of cells obtained on the second gradient shows that the cells re-band as narrow peaks in the same regions from which they were taken off the first gradient. The exact rebanding of the two fractions indicates that the separation procedure is highly reproducible; the position of each peak in the second gradient is within the density interval taken from the original gradient. The shoulder on the peak of dense cells presumably represents cells damaged during preparation of the second gradient (cf. Fig. 1). The narrow peaks observed in the second separation indicate a high degree of resolution; the width of each peak at half height is only 0·002 g/cm^3.

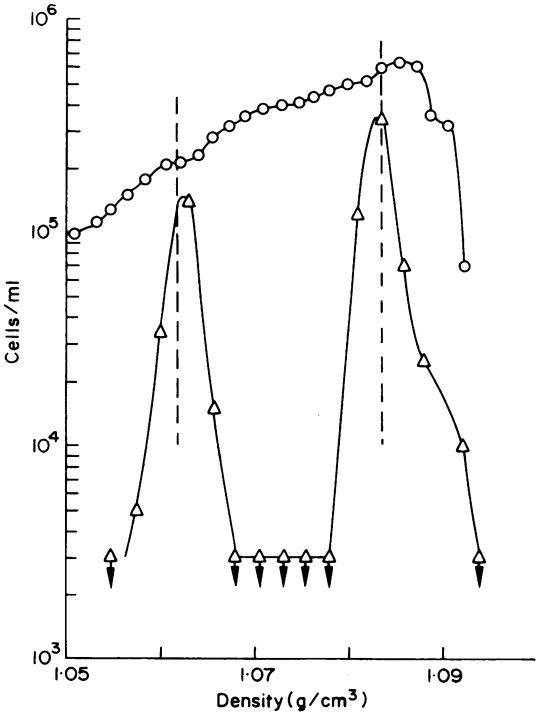

FIG. 2. Rebanding of nucleated spleen cells. Spleen cells from unimmunized mice were separated in Ficoll gradients (pH 5·5). The open circles show the nucleated cell profile in the first gradient (the recovery of nucleated cells loaded was 88 per cent). The dotted lines show the two fractions rebanded on a second gradient. The open triangles show the nucleated cell profile in the second gradient. The triangles with downward arrows indicate maximum values; no cells were observed in these fractions the limit for detection was 3000 cells/ml. The recovery of cells in the light density region was 94 percent; the recovery of cells in the dense region of the gradient was 85 per cent.

To determine the reliability of the density measurements in gradients of Ficoll, we decided to compare the values for cell densities determined in Ficoll gradients with those measured by an independent method. For the latter, we chose the method described by Danon and Marikovsky (1964) using phthalate esters. The comparison required using Ficoll gradients at pH 7·2, which in turn required lowering the concentration of mouse cells to prevent clumping at this pH. Because of the sensitivity of erythrocytes to various physiological stresses, we used C3H/HeJ erythrocytes to compare the two methods. The results of these two measurements are shown in Fig. 3. Because the measurements with phthalate esters give the cumulative distribution of cells, we have also plotted the results in Ficoll as a cumulative distribution. On the Ficoll gradient the median density of erythrocytes was 1·1010 g/cm^3. With the other method, the erythrocyte density was 1·1024 g/cm^3. The close agreement of these two independent methods for measuring density of mouse erythrocytes indicates that the Ficoll gradient gives a good estimate of the actual density of these cells. The slight difference in median density may be due to slight differences in osmolality between mouse serum and the Ficoll gradients. Mouse serum is 325 m-osmolal (Bernstein, 1966) while our Ficoll was prepared as 294 m-osmolal.

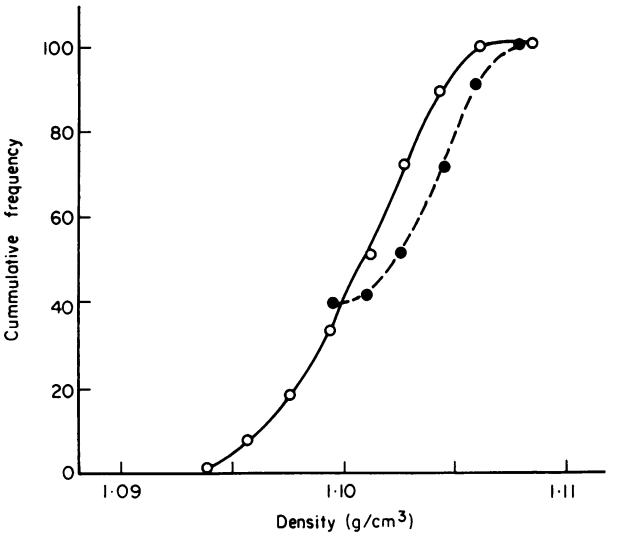

FIG. 3. Comparison of two methods of measuring the density of C3H erythrocytes. The open circles show the cumulative distribution obtained on a continuous Ficoll gradient, pH 7·2, and the closed circles show the distribution for red cells in serum determined by the method of Danon and Marikovsky (1964).

DENSITY DISTRIBUTION OF ANTIBODY-PRODUCING CELLS

To obtain a reasonable estimate of the *in vivo* density distribution of antibody-producing cells, separations were done at pH 7·2, using a low cell concentration (7×10^5 cells/ml) to prevent clumping of cells. Spleen cells taken from mice 4 days after a second immunization with SRBC were used as a source of antibody-producing cells. The density distributions of 19S-PFC and 7S-PFC were determined on a 10–29 per cent Ficoll gradient adjusted to pH 7·2. Fig. 4 gives the results of this determination. Note that the distribution of nucleated cells is different from that obtained at pH 5·5 (cf. Fig. 5); at the low pH there are more cells with low density which implies that cells swell slightly in an acidic medium. The 19S-PFC and 7S-PFC appear to be homogeneous populations of uniform density with 7S-PFC having slightly greater density than 19S-PFC. Our results disagree with those of Haskill *et al.* (1969) who found numerous distinct density peaks for 19S-PFC in spleens from immunized rats. Some of the differences could be due to differences in the experimental conditions as Haskill *et al.* (1969) measured the density of PFC in the rat using BSA gradients at pH 5·1. We considered three factors that could account for the observed discrepancy: pH differences, species differences and osmotic effects.

(*a*) *Effect of pH on density of PFC*

Spleen cell suspensions containing 19S-PFC and 7S-PFC were separated as above except that the gradient was prepared at pH 5·5. The results for this separation at low pH are shown in Fig. 5. Although the PFC distribution (bottom) is still homogeneous with only a single peak of activity, the activity is shifted to significantly lower density compared to pH 7·2. Thus, a change in pH does not affect the homogeneity observed on Ficoll gradients and cannot account for the heterogeneity seen on BSA gradients. The shift toward lower density at pH 5·5 is also reflected in the distributions of nucleated cells (top). Legge

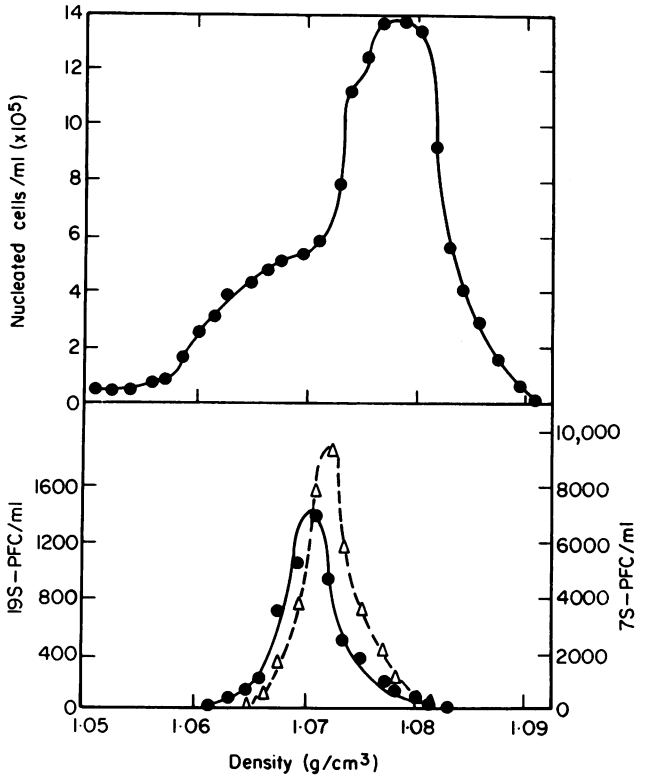

FIG. 4. Density profile of mouse PFC at pH 7·2. C3B6F$_1$ mice were immunized twice with SRBC. Four weeks separated the two immunizations, and the mice were killed 4 days after the last immunization. The spleen cells from these mice were then separated on a Ficoll gradient at pH 7·2. The upper half of the figure gives the distribution of nucleated cells. Because of their high density at this pH, the erythrocytes formed a pellet at the bottom of the gradient. The lower half of the figure shows the distribution of 19S-PFC (●) and 7S-PFC (△). The recovery of PFC activity was 98 per cent.

and Shortman (1968) previously reported that erythrocytes swell in acidic solutions, but they did not detect any swelling or shift in density for nucleated cells at pH 5·1. Perhaps the differences between this finding and our data are caused by differing effects on cell membranes of the materials used to prepare the density gradients.

(b) Density of PFC in the rat

To determine whether or not heterogeneity of 19S-PFC is a unique feature of rats, we examined the density profile of 19S-PFC from spleens of Fischer rats, immunized 3 days earlier with SRBC, on a Ficoll gradient at pH 5·5. The distribution that we obtained is shown in Fig. 6. The arrows in this figure give the location of the major PFC peaks in BSA gradients at pH 5·1 observed by Haskill *et al.* (1969). Our data indicate that 19S-PFC in the rat are a homogeneous population with most of the PFC in a single peak at a density of 1·057 g/cm^3. In addition to the increased homogeneity in gradients of Ficoll, we find the PFC distribution shifted to lower density in comparison to the results with BSA gradients. As mentioned above, the density shift of nucleated cells at low pH may be a characteristic of our system.

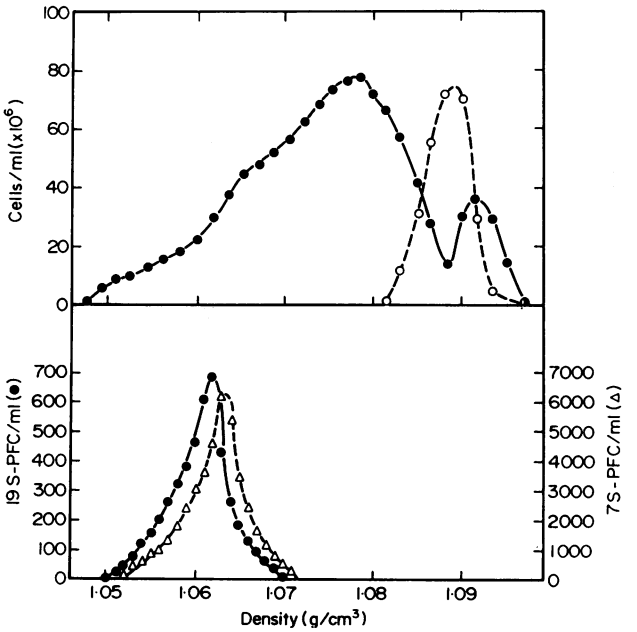

FIG. 5. Density profile of mouse PFC at pH 5·5. C3B6F$_1$ mice were given two injections of SRBC separated by 4 weeks. 4 days after the last injection, the mice were killed and their spleen cells separated on a Ficoll gradient at pH 5·5. The upper half of the figure shows the distributions of nucleated cells (●) and erythrocytes (○). The lower half of the figure shows the distribution of 19S-PFC (●) and 7S-PFC (△) in the same gradients. There was 100 per cent recovery of all cells in this experiment.

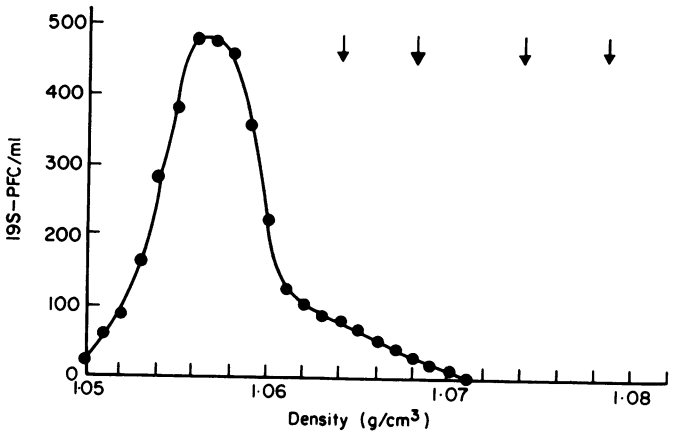

FIG. 6. Density profile of 19S-PFC from rats. Fischer 344 rats (20 weeks old) were immunized with 2×10^9 SRBC. Three days later their spleens were removed, and the cells separated on a Ficoll gradient at pH 5·5. The PFC activity from this gradient is shown in the above figure. The largest arrow gives the location of the major PFC peak observed by Haskill *et al.* (1969) on BSA gradients; the other arrows show the position of the minor populations observed by these workers.

(c) *Effects of osmolality on density profiles of* 19*S-PFC*

BSA is a charged macromolecule known to bind ions from solution (Scatchard *et al.*, 1946). This property of BSA could affect the osmolality of BSA gradients. The greater concentrations of BSA in the dense regions of the gradient would cause binding of more ions than in the lighter regions, thereby creating an osmotic gradient with the dense regions being more hypotonic than the light regions. We therefore examined the possibility that such osmotic gradients could account for some of the differences between our data and those reported by others.

We first measured the osmolality of BSA solutions using freezing point depression. Shortman (1968) observed precipitation of highly concentrated BSA solutions near the freezing point. However, we found this problem could be overcome by preparing the BSA solutions at a pH of 5·5 instead of 5·1. With the exception of the pH change and a different method of de-ionizing the BSA, we attempted to follow the protocols described by Shortman for the preparation of BSA. The freezing point depression of solutions containing various concentrations of BSA was measured and the osmolality calculated from this measurement. The data for these experiments are shown in Table 2, and show that in our hands BSA gradients of 15–35 per cent (w/w) BSA would also be osmotic gradients varying from 288 to 276 m-osmolal, respectively. These values may be compared with the values of 284 and 262, respectively, calculated from the theory of Scatchard *et al.* (1946) for ionic binding by BSA. It is important to note that BSA is more effectively deionized with ion-exchange resins than by dialysis. Therefore, the BSA used in these experiments might be expected to generate more of an osmotic gradient than the dialysed BSA used by Haskill *et al.* (1969).

TABLE 2

OSMOLALITY OF BSA SOLUTIONS

BSA conc. (w/w)	Salt	Calculated osmolality (m-osmolal)	Expected freezing point depression*	Observed freezing point depression†	Measured osmolality (m-osmolal)‡
0	NaCl	260	−0·484°	−0·483°±0·006°	260±7
0	NaCl	270	−0·503°	−0·503°±0·004°	270±4
0	NaCl	280	−0·522°	−0·525°±0·004°	281±4
0	NaCl	294	−0·548°	−0·548°±0·006°	294±6
0	Buffer No. 1§	294	−0·548°	−0·548°±0·002°	294±2
15 per cent	Buffer No. 2¶	294	−0·548°	−0·536°±0·003°	288±3
25 per cent	Buffer No. 2	294	−0·548°	−0·527°±0·004°	283±4
35 per cent	Buffer No. 2	294	−0·548°	−0·515°±0·002°	276±2

*Calculated from the expected osmolality as follows: $\Delta T_f = K_f m_s$ where ΔT_f is the freezing point depression, K_f the cryoscopic constant for water and m_s the molality of solute. For water K_f has a value of 1·862° kg/mole.

† These values are the average and standard errors from three measurements of freezing point depression.

‡ These values can be read from a standard curve made from the saline solutions or calculated from theory. The standard errors were calculated using the errors in the freezing point measurements.

§ Buffer used for Ficoll gradients.

¶ Buffer described by Shortman (1968).

Unfortunately, we were unable to measure the freezing point depression of our Ficoll solutions because they solidified near their freezing points. However, if osmotic gradients generate some of the peaks observed in BSA gradients, we should be able to duplicate this phenomenon by superimposing an osmotic gradient on a density gradient of Ficoll. Accordingly, Ficoll gradients were prepared in which there was a linear osmotic gradient ranging from 294 m-osmolal at the top to 286 m-osmolal at the bottom. A spleen cell suspension from mice immunized 4 days previously with SRBC was separated on three of

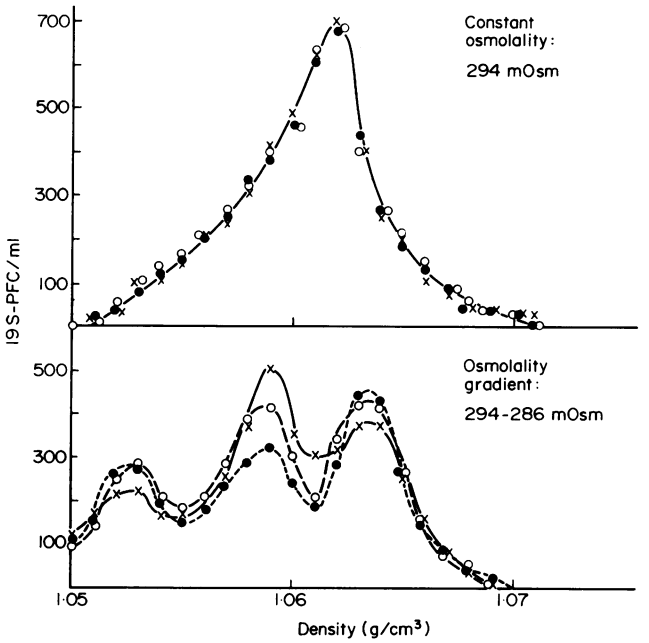

FIG. 7. Effect of osmolality on the density profile of 19S-PFC. C3B6F$_1$ mice were immunized twice with SRBC. Four days after the last immunization their spleen cells were separated on a normal Ficoll gradient that was 294 m-osmolal throughout (a), or on a Ficoll gradient which also contained an osmotic gradient (b). The osmotic gradient varied from 294 m-osmolal at the top (density 1·045 g/cm^3) to 286 m-osmolal at the bottom (density 1·072 g/cm^3). The same suspension was fractionated on three gradients with constant osmolality and on three density gradients with an osmolality gradient. The different symbols refer to 19S-PFC obtained from the different gradients.

these gradients and on three normal, isotonic gradients at pH 5·5. The data for this experiment are shown in Fig. 7. Fig. 7(a) shows the distribution in normal gradients and again demonstrates the reproducibility of the separation; the PFC peak in these separations was at a density of 1·062 g/cm^3 as in the experiment shown in Fig. 5. Fig. 7 (b) gives the distribution of PFC in the osmotic gradient and shows that the osmotic gradient generated three peaks of PFC. Although the proportion of cells in each peak was variable, the positions of the peaks were highly reproducible in the three gradients. Thus, osmotic effects may account for at least one of the differences between our results and those reported by Haskill *et al.* (1969); osmotic gradients can generate multiple sub-populations within an otherwise homogeneous population of cells. However, osmotic effects do not explain the markedly different densities in the two reports. The presence of a slight osmotic gradient shifted the cells within the normal range, not outside of it. Again this difference may be related to the different pH at which the experiments were performed and/or to the different materials used to make the gradients.

DISCUSSION

Two important conclusions can be drawn from the above experiments. First, separation of cells on Ficoll gradients at pH 7·2 gives a good estimate of the actual *in vivo* density of a cell. Second, introduction of slight osmotic gradients can cause a marked alteration in the density profile of an otherwise homogeneous population of cells.

There are several reasons for postulating that density separations in Ficoll are indeed separations on the basis of true cell density. Several of the controls are given in this paper. The best argument is that it is unlikely that the rebanding and reproducibility would be satisfactory if the gradients themselves were affecting the density of the cells. The results obtained for mouse erythrocytes agree well with the value obtained with the other method used.

Another test is to use the measured cell density to calculate the sedimentation velocity of antibody-producing cells. Under previously defined conditions, the sedimentation velocity of 19S-PFC at 4° and pH 7·2 was found to be 4·7±0·2 mm/hour (Phillips and Miller, 1970). Theoretically, the sedimentation velocity should be given by the expression,

$$s = \frac{2(\rho_c - \rho_0)\boldsymbol{g}\, r^2}{9\eta},$$

where s is the sedimentation velocity, ρ_c the density of the cells, ρ_0 the density of the supporting medium, η the viscosity of the supporting medium, $\boldsymbol{g}$ the acceleration due to gravity and r the radius of the cell. We have shown that spleen cells sedimenting at 4·7 mm/hour have an average radius of 4·3 μ (Miller and Phillips, 1969). Using the measured density for 19S-PFC at pH 7·2 of 1·070 g/cm^3 (Fig. 4) and values of 1·006 g/cm^3 for ρ_0 and 0·0157 poise for η (Miller and Phillips, 1969), we calculate 19S-PFC should sediment at 4·6 mm/hour. The excellent agreement between the observed and calculated sedimentation velocities for 19S-PFC provides additional evidence that our measured density is correct.

The implication of our data is that antibody-producing cells in the spleen of a mouse near the time of the peak response are a homogeneous population of cells. Our previous data on sedimentation velocity of antibody-producing cells (Phillips and Miller, 1970) and the present data on density measurements support this conclusion. We suggest that the dispersion observed by Haskill *et al.* (1969) in the rat is a manifestation of slight osmotic changes in their BSA gradients and does not represent a true dispersion of cellular densities. Although morphological studies of antibody-producing cells also have shown some polymorphism (Harris *et al.* 1966; Cunningham, 1968) the reliability of morphological assessment is questionable because of the unknown effects of fixatives on cell morphology and because of the subjectivity of the assay. Quantitative, reproducible physical parameters, such as size and density show little heterogeneity, at least among spleen cells synthesizing antibody against sheep red cell antigens.

Recognition of the importance of osmolality may lead to a new method for separating cells. When the osmolality can be varied in a measurable and reproducible fashion, as it can using agents such as Ficoll which are non-ionic, it should be possible to fractionate cells on the basis of their susceptibility to osmolality changes. Presumably, *in vivo*, most classes of cells are characterized by a single unique density. However, it might be possible to distinguish physiologically different sub-populations within a particular class by looking at the susceptibility of that population to changes in osmotic pressure. If some of the peaks reported by Haskill *et al.* (1969) were generated by osmotic gradients, their data support the idea that cells in different physiological states have differing susceptibilities to osmotic shock. For example, they showed that cells susceptible to vinblastine are confined to only a small portion of the gradient, as are the antibody-producing cells that find their way into the circulation. These observations are consistent with our hypothesis that cells in different physiological states can be separated on osmotic gradients.

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