THE VALIDITY OF THE CENTRIFUGE METHOD FOR ESTIMATING AGGREGATE CELL VOLUME IN SUSPENSIONS OF THE EGG OF THE SEA-URCHIN, ARBACIA PUNCTULATA

HERBERT SHAPIRO[1]

(From the Physiological Laboratory, Princeton University, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

A problem which frequently arises in biological work is the determination of the total volume of the cells whose functional activities are being studied. This appears, for example, in measurements of oxygen consumption (Whitaker, 1933; Gerard and Rubinstein, 1934), where it may be desirable to give absolute, rather than relative figures. With free cells whose outline closely approximates that of a sphere, such as certain marine eggs, it is possible to estimate the total volume of material to a degree of precision depending upon the regularity of configuration of the particular species of cell, by measuring the diameter of the cells, and the concentration of cells with a suitable counting chamber. This procedure cannot be followed when cells are of irregular outline, for then the volume of the individual cell is not easily computed; one must then resort to a method such as centrifugalization. The problem then becomes one of ascertaining how closely the volume of the packed cells, as estimated from the length of the capillary occupied by the mass of material, approaches the true volume, and determining the complicating effect of extracellular structures such as enveloping membranes, or other layers of varying dimensions. A special case is considered here, viz., that of the egg of the Woods Hole sea-urchin, *Arbacia punctulata*. A justification for a detailed inquiry of a largely routine nature lies in the wide use of these cells for physiological work. Harvey (1932) has collocated the quantitative data pertaining to the various aspects of the chemical and physical properties of this cell. No validation of the centrifuge method has appeared, although numerous investigators have used it for this and other cells to determine egg volume. The *Arbacia* egg is good material for this study because, owing to its negligible departure from perfect sphericity of shape, its volume can be computed fairly precisely from a measurement of the egg diameter; and the material is obtainable in abundance. To consider the limits of error in the use of the centrifuge,

[1] National Research Council Fellow in the Biological Sciences.

under the conditions specified, for a measure of the aggregate volume of cell populations, the evidence will be given under these general headings: methods of measuring concentrations and volume (hemacytometer and centrifuge), distribution of egg sizes, the stacking of cells, extracellular structures, the general effects of centrifugalization on the packing of cells, and on their subsequent viability.

Relative Advantages of Counting and Centrifuging

In weighing the relative advantages of the two methods it is to be noted that the centrifuge method cannot be used where the volume of cells must be known before they are used, or where it is desired to follow the effect of experimentally imposed conditions upon embryological development after measurements have been made. The hemacytometer method, on the other hand, although slightly more laborious, and involving measurement of cell diameters and computation of their volumes, requires only small samples of material, and can thus be used without reference to considerations of quantity of material available.

Distribution of Cell Volumes

The measurement of the egg volume was the most precise datum available, and was computed, after determination of the diameter, from the formula,

$$V = 4/3\pi r^3.$$

Since volume is a cubic function of the radius, r,

$$dV/dr = 4\pi r^2,$$

and it may be noted that the difference in volume accruing from an error of 1 μ in the measurement of the diameter of a 74 μ egg is about 4 per cent; and that the radius is to be determined as accurately as possible.

A specially designed glass chamber, about 0.6 mm. deep, and with optically plane walls, was used to contain the eggs. After a suspension was pipetted into it, the open portion was covered over with a cover slip, and evaporation thus prevented. Egg diameters were measured with a filar ocular micrometer whose scale could be read to a fraction of a micron. Recalibrations of the ocular scale were frequent, to remain certain of its constancy. From each batch of eggs used, the diameters of about ten eggs were measured, and their volumes averaged, to secure a representative figure.

As regards the extent of dispersion of egg volumes, it can be said from inspection of Fig. 1 that the volumes of eggs from different

females vary considerably. It is not feasible, then, to assume a diameter of 74 μ (a figure commonly employed) when the variation is so great. The curve was drawn from data on the volume and diameter of eggs counted and measured throughout the summer of 1934. The mode occurred at slightly less than 72 μ diameter, corresponding to a volume of about 193,000 μ^3. The extremes encountered in these measurements were diameters of 64 and 81 μ. Whether the consider-

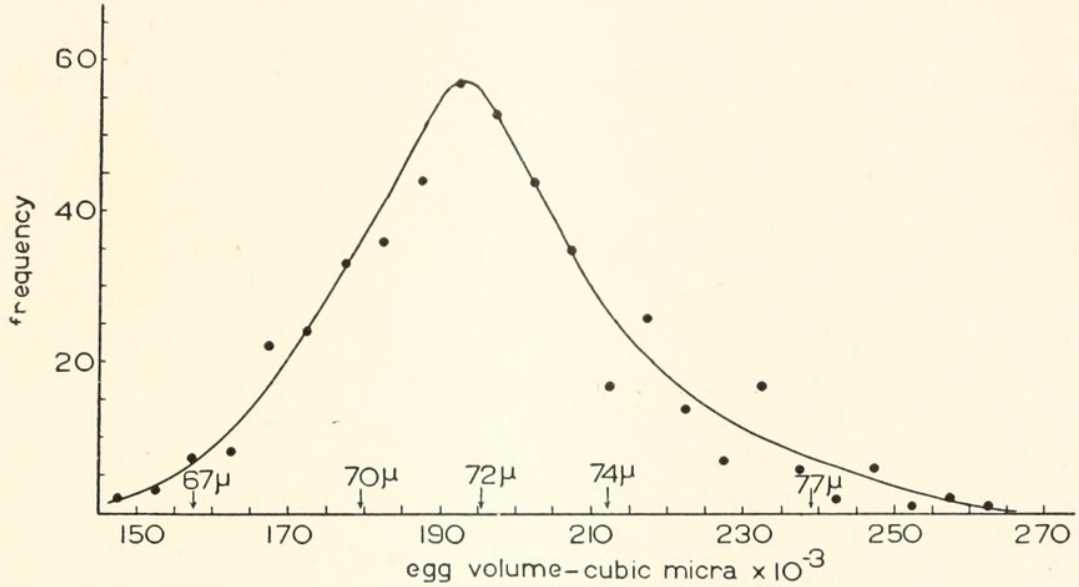

FIG. 1. Distribution of volumes of 467 eggs from 45 urchins selected at random during the season of 1934. The mode occurred at an egg diameter of slightly less than 72 micra.

ably wider distribution and different mode for *Arbacia* eggs reported by Glaser (1914) is intrinsically a seasonal effect and may obtain at other breeding periods, remains to be determined. Not only is the absolute diameter less variable for individual females, but the degree of dispersion of diameters of cells from one animal is approximately constant, regardless of the mode. To determine how individual urchins compare in the variation of egg volumes, 9 eggs were selected at random from each of 14 animals, and the cell volumes measured. The total variation in egg volume was similar to that of Fig. 1, but the coefficient of variation ($v = 100\sigma/M$, σ = standard deviation) was approximately the same for the eggs from each urchin; the average coefficient of variation was 5.4 per cent.

Theoretical Considerations: the Stacking of Cells

If rigid spheres be stacked with their centers in line with each other, they occupy 52.36 per cent of the volume of the container; when nested fully, 74 per cent. These values hold regardless of the absolute diameter of the spheres, provided they are all of the same size. Thus,

if *Arbacia* eggs were allowed to settle unhampered in a capillary tube, the volume read off should be 135.2 per cent of the true volume. They are prevented from actually nesting as close as this, under the influence of gravity alone, by the presence of the jelly, which envelops eggs freshly shed or removed from the ovaries. Owing to the presence of the jelly, whose average volume (from 15 cells) in one batch of freshly shed eggs was 1,123,000 μ^3 per egg, the eggs packed by gravity alone would occupy a volume equal to 9.9 times that of the eggs alone if the jelly envelope were to act as a rigid sphere. This ratio would be still higher (14.2 times) if we were to consider eggs which were taken after they had been allowed to remain in sea water for several hours, and whose jelly had consequently swollen to some degree.

Observations on the Jelly

Before they were counted, most of the eggs had large jelly envelopes; about 5 per cent had small coats of jelly. When centrifuged lightly (20 $\times$ gravity) in a test tube for one minute (to precipitate the eggs) the cells lose some of the outer looser layer of the jelly shell. In most of the batches of eggs used, even before handling, about 10 per cent of the eggs had little or no jelly. After pouring one batch of eggs back and forth between two tumblers, about nine times, about 50 per cent of the cells lost their jelly. Before sample drops were taken for counting, the eggs were thoroughly stirred in the tall Stender dish into which they had been poured originally. After about ten repeated resuspensions occasioned by withdrawal of samples for counting, about 50 per cent of the eggs were observed to have little or no jelly, and from the remainder the jelly was mostly sloughed off. The gradual reduction of the jelly envelope could be observed by examination of samples of the suspensions, between counts, in Chinese ink. To determine the amount of swelling which the jelly undergoes, the diameters of eggs and of jelly of cells freshly placed in a suspension of Chinese ink in sea water at 8:33 P.M. (urchin opened at 8:27 and eggs placed in dry Syracuse watch glass) were measured (8:35–8:44 P.M.). The average volume of the jelly of 15 cells was 1,123,400 μ^3 per cell. The same cells were measured again about two hours later (10:45–11:00 P.M.), and the average volume of the jelly was found to be 1,690,000 μ^3, or an increase, due to swelling, of about 50 per cent. When considered individually, the jelly of each cell, without exception, displayed the increase.

In centrifuging in dilute suspensions in wide tubes, and in an isosmotic and isopycnotic medium, the jelly is drawn over toward the centripetal end of the egg. When a column of eggs packed in a capillary tube was examined after centrifuging, the centripetal end of the

column frequently showed a transparent region of varying length (1–1.5 mm.); this was taken to be jelly which had found its way to that region of the tube under the influence of centripetal force.

Comparison of Hemacytometers

Since the hemacytometer is an instrument originally designed for use with much smaller cells than *Arbacia* eggs and since, further, the diameter of the egg with its jelly usually greatly exceeds the depth of the 0.1-mm. slide, it was desirable to compare the latter instrument with one of twice its depth (0.2 mm.) and hence larger than the dimensions of the extracellular structures. For estimating the concentration of suspensions with hemacytometers, each batch of eggs was divided into three lots of the same concentration: *A*, for counting with hemacytometer of 0.1-mm. depth, *B*, for counting with 0.2-mm. hemacytometer, *C*, for centrifuging without having been subjected to preliminary counting, which entailed removal of the jelly in various degrees. As a matter of routine, some eggs were inseminated to determine their fertilizability. Only batches with a high percentage of fertilization (95 or more) were used. To remove large particles, the eggs were passed before counting and centrifuging through bolting silk, the size of whose mesh was approximately 175 μ on a side. In consequence of this treatment, about 10 per cent of the eggs were observed to have their jelly much reduced, or absent. The hemacytometers and cover slips were cleaned and dried thoroughly before each count was made. Before each sample was taken, the eggs were suspended uniformly, the suspension taken up quickly in a pipette, with the coverslip held in readiness by a pair of forceps, and a sample drop placed rapidly over the rulings, and the cover slid on immediately. The procedure usually resulted in a fairly even distribution of the cells. Care was taken to avoid irregularity in distribution of eggs in the hemacytometer. Only those drops were counted wherein the eggs appeared uniformly distributed after the cover glass was placed on the counting chamber. Counts on Lot *A* were made alternately with counts on Lot *B*. The 0.1 hemacytometer was ruled so as to contain four large squares (each 1 mm.^{2}) and the 0.2 instrument was ruled into 16 squares (1 mm.^{2}), hence more sample drops were counted, as a rule, with the shallower instrument. After counts were made by the above procedure, the concentration of Lot *A* was measured with the 0.2-mm. chamber, and commutatively, the 0.1-mm. chamber was used for Lot B. These latter measurements were fewer in number than those of the main series. The results, which are given in Table I, show for most counts, ap-

proximately a 12 per cent increase in the concentration when measured by the deeper slide. This is attributed to the influence of the jelly, and the action of the coverslip in tending to "squeeze out" eggs when placed over the drop on the shallower trough. In comparison with the centrifuge determinations, to be described presently, the results with hemacytometers were not found to be as reproducible. Averaging hemacytometer counts yielded a fair approximation to reproducible figures.

TABLE I

Comparison of concentration of egg suspensions as estimated by shallow hemacytometer (0.1 mm.) and by deeper hemacytometer (0.2 mm.).

Date	No. sample drops	Lot *A* 0.1 hcyt. *eggs per cc.*	No. sample drops	Lot *B* 0.2 hcyt. *eggs per cc.*	Difference in per cent *B/A*	No. sample drops	Count of Lot *A* with 0.2 hcyt.	No. sample drops	Count of Lot *B* with 0.1 hcyt.	Difference in per cent *A/B*
August 25	9	205,000				5	220,200			
29	9	391,500	6	394,600	+ 0.8	1	343,500	2	306,000	+12.2
30	11	192,000	5	231,500	+20.6	2	192,300	2	200,000	− 3.8
September 3 .	6	167,400	3	189,500	+13.2	2	251,800	1	158,000	+59.4
6 .	9	237,000	3	265,200	+11.9	1	286,500	3	260,000	+10.2
10 .	6	179,000	3	201,000	+12.3	2	251,000	4	210,300	+19.3
19 .	10	178,500	6	174,400	−2.35					

Centrifuge Tubes and Centrifuges

Centrifuge tubes of the type diagrammed in Fig. 2, with a capillary length which varied from 6 to 7 cm., were used. The tubes were sealed off at one end, to avoid possible loss of a slight amount of fluid at high centrifugal forces, as may occur in the use of tubes of conventional hematocrit design, with open ends. Inasmuch as sealing the tubes at one end in this way entails the formation of a meniscus, the closed portion of the capillary was blown out very slightly in an attempt to compensate. The error involved is about 0.5 mm., and in a column of 6 cm. this is a volumetric error of only a fraction of 1 per cent. Krueger (1930) placed a small drop of mercury in the bottom of the tube to secure a more nearly plane column end of cells. Hastings (1921) described the use of a graduated thermometer capillary as a hematocrit, designed by E. L. Scott. Ungraduated capillary tubes of bore 0.8 mm. and 1.6 mm. were used in the present experiments, and gave comparable results at 2,700 and 7,700 × gravity. The capillaries were calibrated for volume per unit length by filling with mercury to various lengths, and weighing precisely.

The cells were mixed and suspended by gentle agitation. A measured sample was pipetted up, and the suspension of cells allowed to flow quickly from the pipette (to avoid settling) into the wide tubing spliced to the capillary. It is not necessary initially to fill the capillary before centrifuging for the air is displaced by the suspension during centrifuging, and the cells and the liquid fill the capillary in a continuous column. The capillary can be cleaned out easily by inserting into it a capillary pipette attached to a tap water suction pump, and immersing the mouth of the tube under water.

The length of the column was measured by placing the tube on a mirror (to avoid parallax) parallel to a steel rule graduated into millimeters. In this way the length of the column could be estimated to about 0.2 mm. The agreement between duplicate determinations of total egg volume by centrifuging was good, and, in the writer's hands, the centrifuge gave less variable results than the hemacytometer. The chief variable in readings of this nature seems to arise from the inhomo-

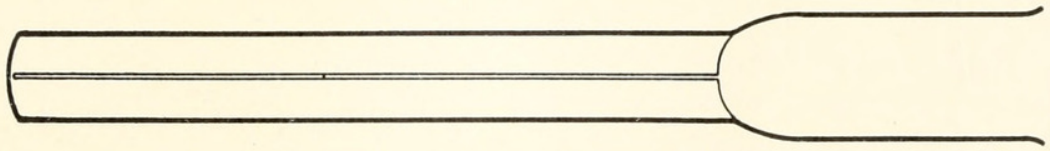

FIG. 2. Diagram of centrifuge tube, to show capillary and cup for receiving cell suspension. Used for centrifuging at 2,700 times gravity.

geneity in the distribution of the eggs in the suspension from which samples are pipetted. Seventeen duplicate determinations of volume by centrifuging at 2,700 × gravity showed an average agreement of 2.4 per cent, which was much better than duplicate determinations by successive hemacytometer counts. The centrifuge used was a type generally available (International, size 2, head 325); the centrifugal force attainable with this machine was 2,700 × gravity, and large tubes of any size could be accomodated. The centrifuge method may be used in two ways: (*a*) for ultimate packing; or (*b*) where a high speed centrifuge allowing nearly complete packing is not available, the introduction of a conversion factor, when the relative centrifugal force and duration of centrifuging are maintained constant.

General Effects of Centrifuging Cells

The first run was made on material whose measurements are given in Fig. 3. It is clear that for any given mass of cells the degree of packing of the cells depends upon two variables, viz., the duration of centrifuging, and the centrifugal force. Each of the component curves

becomes asymptotic to a given capillary volume at a particular centrifugal force. In this sense, perhaps, constant volumes are not obtained, but if centrifuged long enough, the volume is constant within the errors of measurement. At an early stage of packing, it is relatively easy, at low centrifugal forces, to compress the egg suspension to a smaller volume. At any specified centrifugal force a final degree of packing of cells is attained after a minimum amount of centrifuging. In this

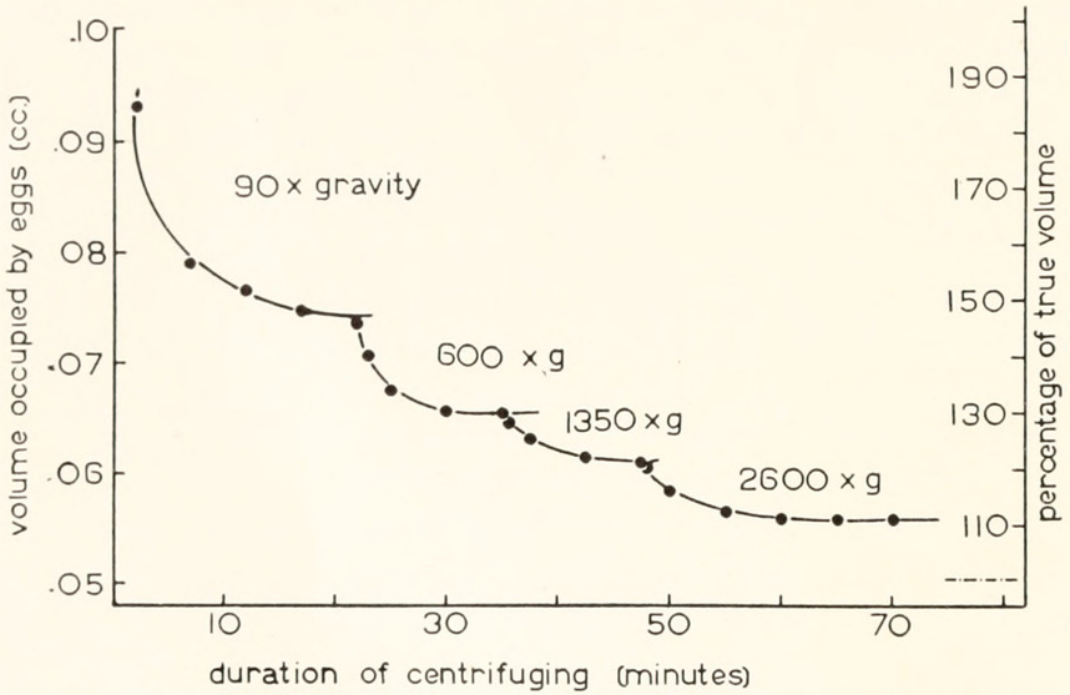

FIG. 3. Effect of duration of centrifuging, and centrifugal force on the volume occupied by a suspension of cells in a capillary. All the data were obtained from a single suspension of cells centrifuged in the same tube. The volumes were measured at various times after starting the centrifuge, and when an approximately constant value had been attained, the centrifugal force was raised. At any given centrifugal force the volume decreases rapidly at first and then approaches asymptotically a constant value. The dot-dash line near the bottom of Figs. 3 and 4 represents in each case the "true volume" as determined from hemacytometer measurements. It is to be emphasized that these curves were not intended to be used as a basis for standard conversion factors at centrifugal forces lower than 2,700 times gravity; they are presented largely to represent the nature of the changes which occur. Figs. 3, 4, and 5 were drawn from the same set of data.

particular run, the total "centrifuge volume" of eggs contained in 1 cc. of suspension (0.0559 cc.), as estimated from the volume of the capillary occupied by the packed eggs, was about 10.6 per cent higher than that computed from the hemacytometer (0.0506 cc.). The ratio of 1.8 to 1 of cell volume by centrifuge and hemacytometer, reported by Gerard and Rubinstein (1934), is understandable when it is noted that low centrifugal forces (400 and 750 times gravity) were used, and relatively short periods of centrifuging (1 to 10 minutes). The further analysis of

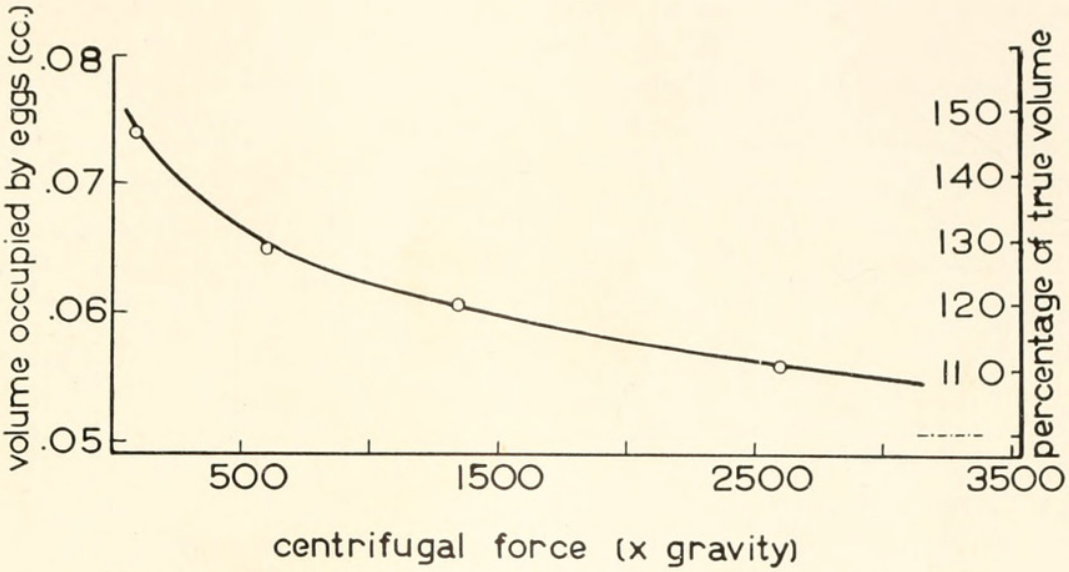

FIG. 4. Relation between centrifugal force and final (asymptotic) value.

this set of data is given in the remaining figures. In Fig. 4, the asymptotic values are plotted for each centrifugal force, and from Fig. 5 it appears that for progressively equal decrements of the excess volume, as read from the capillary length, it was required to increase the centrif-

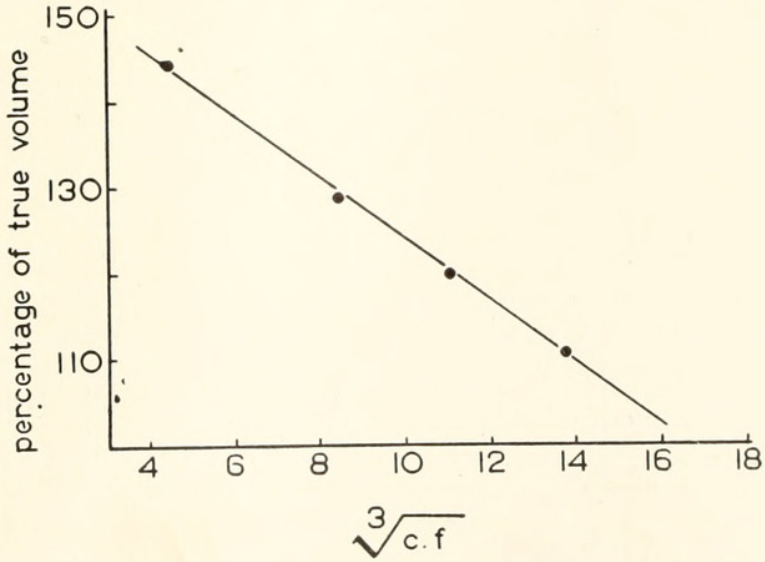

FIG. 5. To show the nature of the dependence of the asymptotic volume occupied by *Arbacia* eggs in a capillary, upon centrifugal force (C. F.).

ugal force, not in equally proportional increments, but rather in cubes of these increments. The expression for this rectilinear relation,

$$P = -3.68\ (C.F.)^{1/3} + 160,$$

where P is the percentage of the true volume, and $(C.F.)$ is the centrifugal force (relative to gravity), holds with good approximation for this

TABLE II

Comparison of total volume of unfertilized eggs as determined by hemacytometers, and by centrifuging in capillary tubes at 2,700 × gravity for 20 minutes. Below, data on fertilized eggs, using volume of unfertilized eggs as basis of comparison.

Date	Volume by hemacytometers	Volume by centrifuge (2,700 × g)	Difference
	cc.	*cc.*	*per cent*
August 25	.0766	.0816	+ 6.5
	.0506	.0537	+ 6.2
29	.0880	.0787	−10.5
	.0440	.0416	− 5.5
	.0440	.0404	− 8.1
	.0880	.0752	−14.6
	.0880	.0744	−15.5
30	.0434	.0424	− 2.4
	.0434	.0452	+ 4.1
	.0656	.0647	− 1.4
	.0434	.0436	+ 0.6
September 3	.0371	.0457	+23.2
	.0742	.0906	+22.1
	.0742	.0925	+24.5
6	.1068	.1242	+16.3
	.0534	.0624	+16.9
	.03204	.03687	+15.1
	.1068	.1238	+16.0
10	.1317	.1289	− 2.1
	.03512	.0373	+ 6.2
19	.0715	.0612	−14.4
	.1072	.0940	−12.3
	.1072	.09514	−11.3
*	.0867	.0940	+ 8.4
*	.0578	.0612	+ 6.0
*	.0867	.09514	+ 9.8
*20	.10632	.1042	− 2.0
*	.0709	.0680	− 4.0
*	.10632	.1028	− 3.4
*	.1122	.1204	+ 7.3
*	.1122	.1185	+ 5.6
	Fertilized Eggs		
August 29	.088	.0763	−13.3
	.088	.0794	− 9.7
30	.0651	.0739	+13.7
September 3	.03902	.04836	+23.8
	.07804	.1000	+28.2

* Volume determined in these cases by dilution method, not hemacytometer.

series of measurements, and affords a satisfactory conception of the nature of the dependence of the "centrifuge volume" upon the centrifugal force. For $P = 100$, i.e., the volume by centrifuge is equal to the true volume (or that calculated by hemacytometer), a centrifugal force of 4,330 × gravity is necessary, as calculated from this empirical formula, assuming that a linear extrapolation can be made. For other sets of data, the curve as a whole would be shifted slightly to the left or to the right, and the extrapolated value would change accordingly.

Results: Agreement between Hemacytometer and Centrifuge Volumes

In Table II, where the data comparing centrifuge and hemacytometer volumes are summarized, a minus sign before the differences indicates that the volume as estimated by centrifuge at the given centrifugal force was less than that as measured by hemacytometer, by the given percentage; and conversely, the plus signs indicate a greater volume by centrifuge as compared to hemacytometer. The values obtained from the two hemacytometers were averaged, and used as a standard basis for comparison with the volumes obtained by centrifuging. A negative value in the comparison of centrifuge with slide would seem to indicate that the slide estimate was too high, or may arise from differences in sampling due to inhomogeneity. The values in the table may be summarized by noting that of 29 determinations at 2,700 × gravity, 15 showed an average of 12 per cent greater volume than that estimated by hemacytometer, and 14 showed an average of 7.7 per cent less than that evaluated by the hemacytometers. Thus the two methods (hemacytometer, 0.1 and 0.2 mm. depths, and centrifuging at 2,700 × gravity for 20 minutes) agreed to within approximately 10 per cent, which may be taken as an average for all the experiments. A few experiments on fertilized eggs are also included in the table.

It would seem that the jelly of the *Arbacia* egg (which, as in these experiments, has been exposed to sea water for approximately one to three hours) is easily displaced or squeezed out by the eggs in centrifuging, and hence does not exert a significant effect in these experiments, for wherever comparisons of Lots *A*, *B*, and *C* were made, where there was no partial reduction of jelly in *C* since it was not mixed and resuspended repeatedly for counting, the measurements of volume by centrifuging resulting from use of portions from each of the lots were identical within experimental error.

Dilution Method

By the use of a procedure suggested by Dr. A. K. Parpart, a check on the two methods described above was made by an independent dilution method. Small portions of egg suspension were diluted to

varying degrees in large volumes of sea water, placed in glass-stoppered bottles, and inverted several times to suspend the eggs uniformly. Then a capillary tube, slightly larger than 1 mm. in bore, and marked off at a length of about 9 cm. (total volume to mark, 0.168 cc.) was quickly immersed and withdrawn, and the total number of eggs contained in the capillary counted directly by examination with a binocular dissecting microscope. Knowing the dilution and the number of cells in the capillary, the original number of cells in the concentrated sample could be calculated. A protocol of some results obtained by this method, which was not studied as thoroughly as the hemacytometer method, is given below. The egg concentration estimated by this dilution method gave figures about 15 per cent lower than those estimated by the hemacytometer.

No. samples counted	Av. concentration by hcytr.	Dilution method	No. eggs per cc.	Centr. tube	$\frac{\text{Centr. vol.}}{\text{Hcyt. vol.}}$	$\frac{\text{Centr. vol.}}{\text{Dilution vol.}}$
10	176,500 cells/cc.	5 cc. : 140	141,520	B	$\frac{.06115}{.07148}=85.6\%$	$\frac{.06115}{.0578}=106\%$
		5 cc. : 500	148,570	D	$\frac{.0940}{.1072}=87.7\%$	$\frac{.0940}{.0867}=108.4\%$
		5 cc. : 1,000	138,700	C	$\frac{.09514}{.1072}=88.7\%$	$\frac{.09514}{.0867}=109.8\%$

In another series of determinations, the dilution and centrifuge methods only were compared, and gave very good agreement. The data are given below; all figures were obtained from measurements on a single suspension.

Comparison of Dilution and Centrifuge Method

No. samples counted	Dilution	Average concentration	Tube	$\frac{\text{Centrif. vol.}}{\text{Dilution vol.}}$
4	2 cc. : 140 cc. s.w.	185,000 cells/cc.	B	$\frac{.068}{.0709}=0.96$
8	2 cc. : 500 " "	184,700 " "	C	$\frac{.1042}{.1063}=0.98$
6	2 cc. : 1,000 " "	167,400 " "	D	$\frac{.1028}{.1063}=0.97$
7	6 cc. : 1,000 " "	177,700 " "		

Data on the Absolute Respiratory Rate of Unfertilized Arbacia Eggs

It may be of interest to note that measurements of the respiration (9/20/34) of eggs taken from urchins kept in laboratory aquaria for a period of about four weeks, with Warburg manometers, at 25.9° C., showed a Q_{O_2} (cu. mm. oxygen per 10 cu. mm. eggs per hour) of 1.03 for unfertilized eggs, and 3.04 for fertilized eggs (average of three determinations of each). The absolute volume of eggs was determined by combining the values obtained by centrifuge and dilution methods. Reduced to 21° C. by applying a Q_{10} of 4.1 for unfertilized eggs (Rubinstein and Gerard, 1934), the Q_{O_2} becomes at that temperature 0.52. More representative is the average of a series of measurements carried out from July to September, 1934, with Warburg and Fenn respirometers, at 25.9° C., and using the hemacytometer method for estimating egg volume (reported in detail elsewhere: Shapiro (1935)). The Q_{O_2} for unfertilized eggs was 1.5. When reduced to 21° C. this value becomes 0.75.

Development of Eggs after Centrifugal Compression in a Capillary

After centrifuging, unfertilized eggs were removed from the capillary and examined microscopically. They were found to be well stratified in the usual manner, drawn out into cylinders, with corners somewhat truncated, a configuration lending itself to more intimate packing of the cells. One batch of cells which had been centrifuged 20 minutes at 2,700 × gravity was left in the capillary 24 minutes in the packed condition, and then removed and inseminated. They retained their elongated form after fertilization, and proceeded to cleave and develop. The pigment remained at one pole of the cell. This was demonstrable repeatedly and indicates that cells can easily survive these experimentally induced conditions. The toughness of the fertilization membrane prevents eggs from elongating, when centrifuged after insemination. The fertilized eggs, after packing, were poorly stratified and in some cases practically unstratified, and polygonal in outline, and retained the fertilization membrane, which usually enveloped the cell closely. They tended to resume the spherical configuration when replaced in sea water after centrifuging. On several occasions it was found that cells fertilized and then packed in a capillary by centrifuging at 7,700 × gravity for 20 minutes would upon removal from the tube proceed to develop as far as free-swimming plutei. Whether water is actually abstracted from the cell as a result of centrifugal compression, is an open question. R. S. Lillie has observed (1918) that fertilized cells, which have shrunken in hypertonic sea water faster than resting egg cells, appear denser than unfertilized

eggs exposed for the same period of time in that they sink faster to the bottom of the container.

Related Experiments at Higher Centrifugal Force

Numerous experiments were made on living eggs swollen and shrunken, and dead cells of various volumes fixed in formalin and centrifuged at 7,700 × gravity to determine volume. The technique involved the use of large volumes of fluids and subsequent decantation of the supernatant fluid, following light centrifuging, in order to get the cells into the small tubes used, which entailed their addition in several small portions, with centrifuging between additions. This procedure was necessary because of the short length of the slots in the high speed centrifuge head, and the use of a long capillary column led to the sacrifice of the wide tube at the upper end, which might receive all the cells at once. The cells were added in small quantities, and the supernatant fluid removed after each preliminary centrifuging to make way for the rest of the cells. A slight (but indeterminate) loss of cells occurred owing to the adhesion of some of the material to the walls of the large test tubes in which they were kept during swelling or shrinking. Thus it was difficult for these reasons to demonstrate the adequacy of the centrifuge method for such cells. Data for normal, living unfertilized and fertilized eggs centrifuged for 20 minutes at 7,700 × gravity were also obtained, and may be summarized by stating that of 15 determinations, 6 showed an average volume 10.6 per cent less and 9 an average volume 12.8 per cent greater than that estimated by hemacytometers. It is of interest to add that when eggs are placed in successive small lots in these small tubes, they appear rhythmically stratified, i.e. each lot shows a light brown layer at the centripetal end.

Summary

Observations of the aggregate volume of *Arbacia* eggs, when centrifuged in capillary tubes, as a function of centrifugal force, and of duration of centrifuging were made. Data on the comparisons of such total cell volume contained in suspensions of *Arbacia* eggs as evaluated by three methods (counting in hemacytometers, centrifuging at 2,700 and 7,700 × gravity, and direct counts of diluted suspensions) are given. It is submitted that the centrifuge method is reliable, to within approximately 10 per cent, for estimation of the total volume of cells in a suspension of unfertilized eggs of *Arbacia punctulata* in sea water provided that it be used with the necessary force and duration for sufficient packing of the cells.

Cells so centrifuged are in the living state, and remain viable, for

upon removal from the capillary, they will proceed to cleave and to undergo considerable embryological development.

LITERATURE CITED

GERARD, R. W., AND B. B. RUBINSTEIN, 1934. A Note on the Respiration of Arbacia eggs. *Jour. Gen. Physiol.*, **17**: 375.

GLASER, O., 1914. The Change in Volume of Arbacia and Asterias Eggs at Fertilization. *Biol. Bull.*, **26**: 84.

HARVEY, E. N., 1932. Physical and Chemical Constants of the Egg of the Sea-urchin, Arbacia punctulata. *Biol. Bull.*, **62**: 141.

HASTINGS, A. B., 1921. The Physiology of Fatigue. Physico-chemical manifestations of fatigue in the blood. *Public Health Bull.* 117, p. 16.

KRUEGER, A. P., 1930. A Method for the Quantitative Estimation of Bacteria in Suspensions. *Jour. Gen. Physiol.*, **13**: 553.

LILLIE, R. S., 1918. The Increase of Permeability to Water in Fertilized Sea-urchin Eggs and the Influence of Cyanide and Anaesthetics upon this Change. *Am. Jour. Physiol.*, **45**: 406.

RUBINSTEIN, B. B., AND R. W. GERARD, 1934. Fertilization and the Temperature Coefficients of Oxygen Consumption in Eggs of Arbacia punctulata. *Jour. Gen. Physiol.*, **17**: 677.

SHAPIRO, H., 1935. The Respiration of Fragments Obtained by Centrifuging the Egg of the Sea-urchin, Arbacia punctulata. *Jour. Cell. Comp. Physiol.* **6**: 101.

WHITAKER, D. M., 1933. On the Rate of Oxygen Consumption by Fertilized and Unfertilized Eggs. IV. Chaetopterus and Arbacia punctulata. *Jour. Gen. Physiol.*, **16**: 475.