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THE COMPUTER ANALYSIS OF VOLUME DISTRIBUTION CURVES

DEMONSTRATION OF TWO ERYTHROCYTE POPULATIONS OF DIFFERENT SIZE IN THE YOUNG GUINEA PIG AND ANALYSIS OF THE MECHANISM OF IMMUNE LYSIS OF CELLS BY ANTIBODY AND COMPLEMENT

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Guinea pig, rat and sheep erythrocytes were sized electrically using the hydrodynamic focusing technique. The experimental curves were approximated with a computer by linear and logarithmic normal distributions. Rat and guinea pig erythrocytes from adult animals were best approximated by one linear normal distribution and sheep erythrocytes by one logarithmic normal distribution. Two populations (I, II) of erythrocytes with different mean volume could be demonstrated in young guinea pigs by this analysis. Population I erythrocytes are small, have a lower electrophoretic mobility and are mainly present at birth. They are gradually replaced by the larger population II erythrocytes. Both types of erythrocytes are probably the result of separate differentiation pathways. The analysis of erythrocyte volume distribution curves during immune lysis by antibody and complement shows that intact and ghost erythrocytes are measured by electrical sizing. No volume changes were observed up to the EAC1-8 intermediate. After the addition of C9, a C9 dose-dependent part of the erythrocytes swell permanently to spheroids. The spheroid transformation is a temperature-dependent, all or nothing reaction which is independent of protein osmotic forces from the interior of the cell.

Electrical sizing is a convenient and fast method to obtain detailed information on the volume distributions of cells. The improvement of the original Coulter method[1] by the hydrodynamic focusing technique has significantly increased the volume resolution (15, 16, 20, 32–35) during the last few years. It has led to the observation of bimodal erythrocyte volume distribution curves during the ontogeny of erythropoiesis in the newborn and young rat, and several erythrocyte populations of different mean volume could be distinguished in these curves by graphical analysis (38). Later on, the volume distribution curves of the erythrocytes change gradually and become monomodal in adult animals (38, 39). The need for a more rapid and accurate method of routine analysis has prompted the development of a computer program which approximates the experimental volume distribution curves by one or several linear or logarithmic normal distributions. A second computer program visualizes the changes of volume distribution curves with time in the form of perspective plots. These plots give a better impression of the biological development of the different erythrocyte volume populations than do single analyzed volume distribution curves.

Two biologically important applications of the analysis are presented in this paper. One is the demonstration of two erythrocyte populations in the young guinea pig which apparently are the result of a differentiation process in the erythrocyte-forming tissues. The second is the analysis of volume distribution curves of erythrocytes during immune lysis by antibody and complement which shows that erythrocytes swell during the process of lysis and remain spheroids afterwards. The spheroid transformation is not caused by colloid osmotic forces but by an interference of complement with the structures of the membrane which are normally responsible for the maintenance of the biconcave form of the erythrocyte.

Both examples show that refined analysis can extract significant information from volume distribution curves of cells.

MATERIALS AND METHODS

Blood samples: Erythrocytes for sizing purposes were collected in the rat from the tail vein, in guinea pigs from the ear veins and in the sheep from the jugu-

[1] Coulter WH: US Patent No. 2656508, 1953.

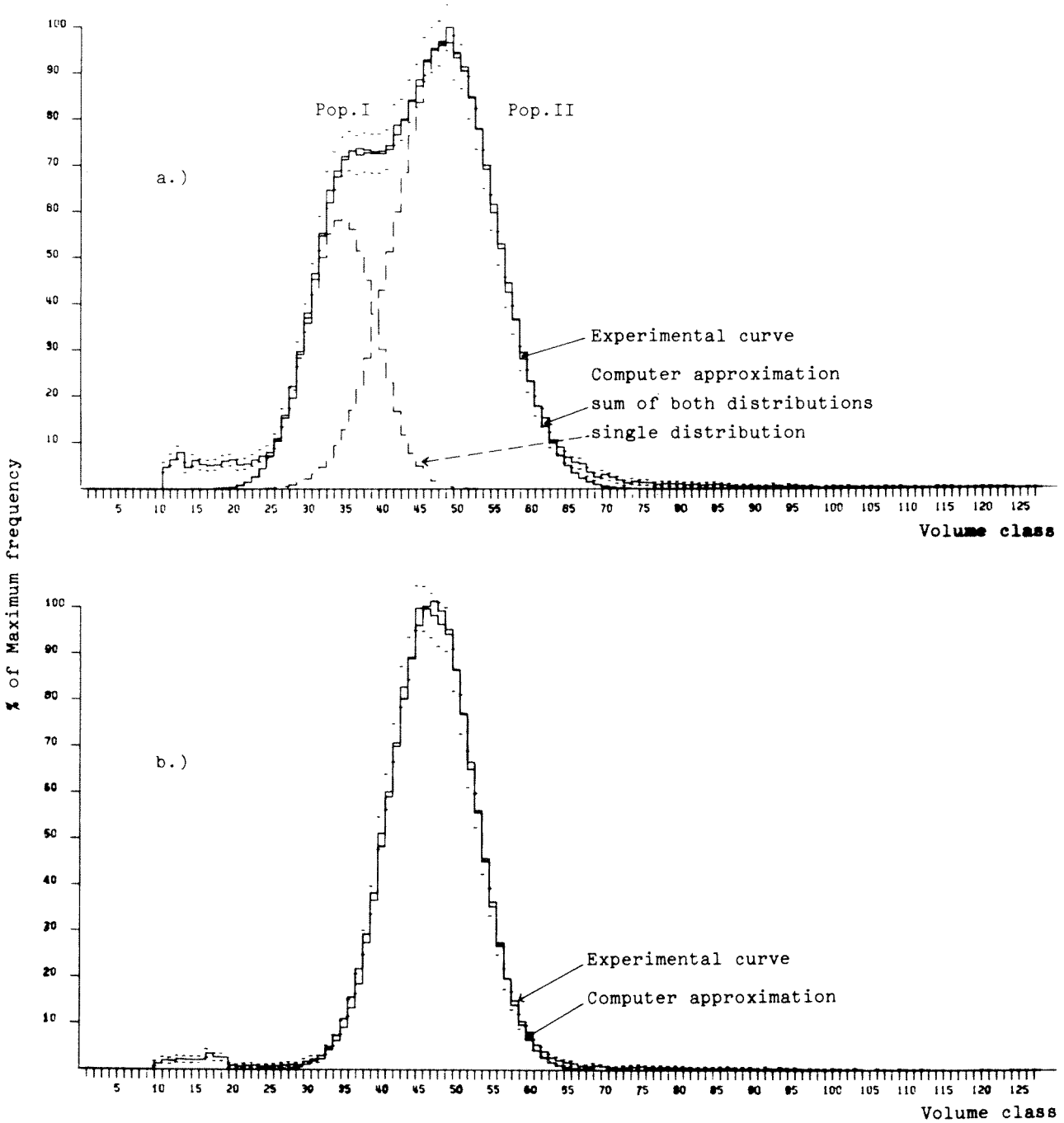

FIG. 4. Volume distribution curves of guinea pig erythrocytes of a 26-day-old animal (a) and of an adult guinea pig (b). The volume distribution curve of the younger animal is bimodal (a). The approximation by two linear normal distributions corresponding to population I and II erythrocytes (69.5 ± 9.4 μm^3; 97.6 ± 13.6 μm^3; CV(I) = 13.5%; CV(II) = 13.9%) fits the experimental curve well. Later, the population I erythrocytes disappear from the circulation and only population II erythrocytes can be demonstrated in the adult animal (b) (94.0 ± 11.2 μm^3; CV = 11.9%). Population II erythrocytes are well approximated by one linear normal distribution of volume. One volume class corresponds to 2.01 μm^3.

were sequentially attached to sheep erythrocytes by incubation. The volume distribution curves were measured at each step. Up to the EAC1-8 intermediate no measurable changes in the mean volume of the erythrocytes or in the shape of the curve were detected (Fig. 8a). On addition of C9, the cells lysed and the curve became bimodal (Fig. 8b), with one erythrocyte population having the volume of untreated sheep erythrocytes and one with a larger volume. The volume of large cells was similar to the volume obtained by maximal hypotonic swelling. Large cells were therefore spheroids, which is confirmed by the spheroid form of the erythrocytes in the orifice (Fig. 1b). Their mean volume was typically 30–35% higher than that of small erythrocytes. Both populations were well approximated by linear normal distribu-

tions. The number of large cells was proportionate to the C9 concentration. With small doses only a few large cells were formed (Fig. 9a), and a higher plateau of large cells (Fig. 9b) was reached with more C9.

DISCUSSION

Approximation of erythrocyte volume distribution curves by mathematical functions: The question of the shape of erythrocyte volume distribution curves has been controversial for some time. Mono- (2, 16, 29, 42), bi- (3, 21) and trimodal (19) distribution curves have been proposed for erythrocytes from adult human and animal individuals. The better understanding of the electrical sizing process (6, 14, 16, 20, 26, 34) and the use of the hydrodynamic focusing technique (14, 15, 20, 32, 33–35) have shown that the volume distributions of erythrocytes from the adult rat, mouse, guinea pig, rabbit, sheep and man are monomodal (2, 16, 29, 35, 37–42). The large amount of data from the volume distribution curves, which are obtained with multichannel analyzers, makes it desirable to approximate the experimental curves by mathematical functions. They reduce the data into a few descriptive parameters which facilitate the comparison of the curves. The volume distribution curves of rat (Fig. 2) and guinea pig (Fig. 4b) erythrocytes of adult animals were therefore approximated by a computer with Gaussian normal distributions. They were better fitted by linear (Fig. 2a) than by logarithmic (Fig. 2b) distributions. Both functions approximate well the central part of the curve. However, the logarithmic function rises too steeply in the lower left part of the curve to give a good approximation (Fig. 2b). The advantage of the linear approximation, besides the better fits, is that the parameters for the mathematical description are simpler. An exception to the linear approximation are freshly drawn sheep erythrocytes. They are better approximated by a logarithmic distribution (Fig. 3a). The shape of the experimental curve does change, however, when the erythrocytes are stored *in vitro* at 0°C. *In vitro* aged sheep erythrocytes, as they are used for complement titrations, are consistently better approximated by linear than by logarithmic distributions (Fig. 8a). The reason for this could be that certain erythrocytes lyse during storage or that they change their form or elasticity during aging (18).

TABLE I

Hematologic Parameters of the Young Guinea Pig

Days after Birth	Blood Erythrocyte Concentration	Reticulocyte Concentration (‰ of Erythrocyte Concentration)	No. of Experiments
	$\times 10^6/mm^3$		
0	3.40 ± 0.38[a]	26 ± 9	2
3	3.45 ± 0.42	30 ± 4	4
11	2.13 ± 0.51	40 ± 13	4
17	3.37 ± 0.45	53 ± 13	4
26	3.45 ± 0.30	24 ± 6	4
33	3.39 ± 0.32	23 ± 5	3
38	3.08 ± 0.30	19 ± 4	2
45	3.58 ± 0.11	18 ± 2	2
53	3.48 ± 0.28	13 ± 3	2

[a] ± S.E.

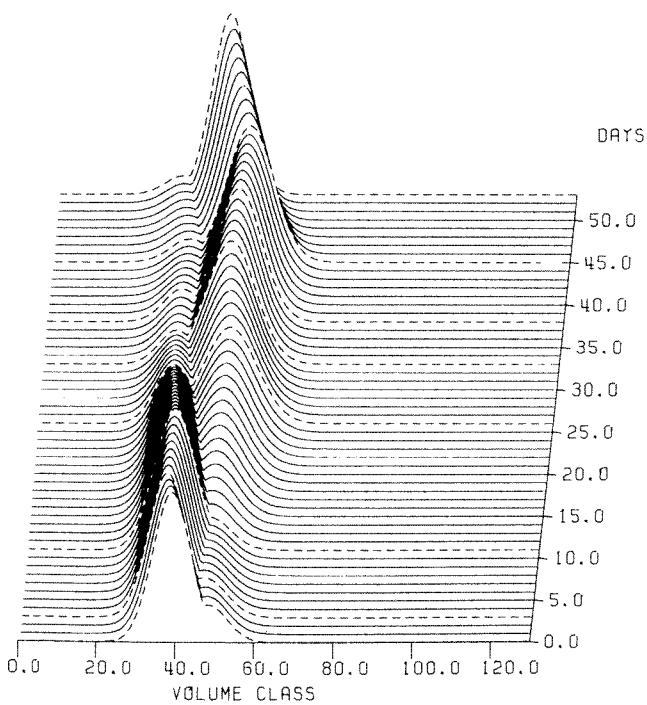

FIG. 5. Changes of the volume distribution curves of guinea pig erythrocytes during the postnatal development. The volume distribution curves of the same guinea pig were measured repeatedly between birth and 53 days of age. They are represented by the dotted lines in the perspective plot. The volume is plotted on the x-axis, the relative frequencies on the y-axis and the time after birth on the z-axis. The solid line curves are calculated by linear interpopulation to increase the 3-dimensional impression of the plot. The guinea pig enters life with population I erythrocytes which are gradually replaced by population II erythrocytes. Population I erythrocytes disappear from the circulation around day 67.

Demonstration of two erythrocyte popula-

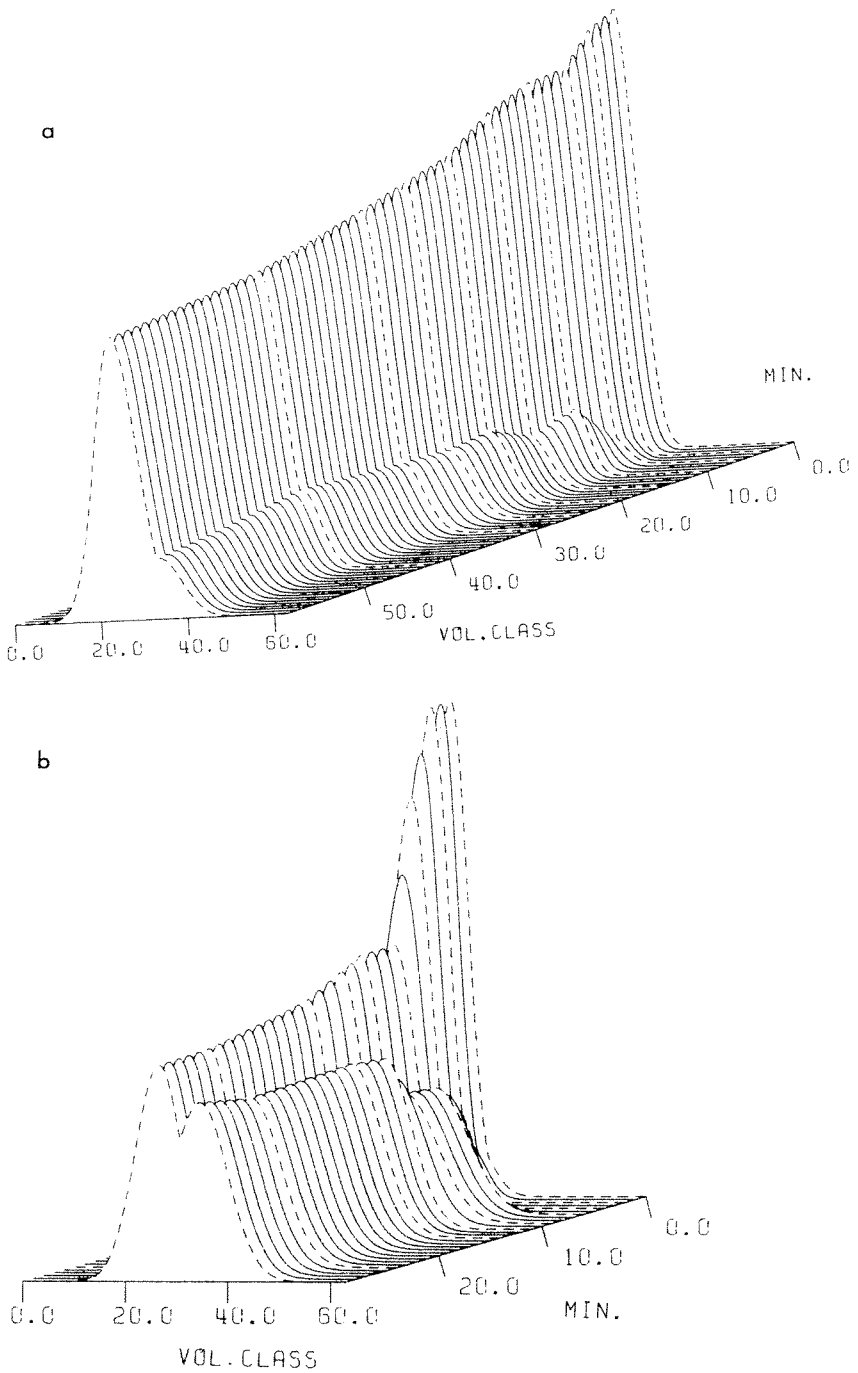

FIG. 9. C9 dose dependence of large cell formation. EAC1-8hu cells were incubated with 2 (a) or 5 (b) CH50 doses of C9hu at 30°C. Lysis was complete in 70 and 30 min, respectively. The formation of large cells is C9 dose dependent. It begins approximately 5 min after the start of the reaction and soon reaches a constant level, which increases with higher doses of complement C9.

ume population which is characteristic for the adult animal. In humans, indications for several erythrocyte volume populations have been obtained during embryonal life (37) and are under investigation after birth.

Accepting the existence of different erythrocyte populations, it is of interest to know more about their origin and the principles that regulate their appearance. Two possibilities will be discussed here. One is the transformation of erythrocytes which are already in the blood into erythrocytes of another volume, and the second is the direct formation of new erythrocyte populations by the hemopoietic tissues. The transformation hypothesis was examined in the sheep by radiolabeling the erythrocytes of a newborn animal with ^{51}chromium. The label is limited to one cohort and does not appear in subsequently produced erythrocytes,[3] thus

[3] Valet G., Franz G., Lauf PK: The demonstration of several erythrocyte volume populations in the young sheep by electrical sizing and their association

making the transformation hypothesis unlikely as a general explanation for the formation of different erythrocyte populations. The alternative is that the erythrocyte populations are produced directly by the hemopoietic tissues.[59] Iron incorporation into the hemoglobin of new erythrocytes or a temporary increase of the reticulocyte concentration would be indicative for this mechanism. [59]Iron incorporation is, in fact, demonstrable in the sheep,[3] and the reticulocytes (Table I, Fig. 6) increase in the young guinea pig from day 11 on to a peak at day 17 after birth, when population II erythrocytes appear in larger quantities in the blood. Both parameters therefore show that the new erythrocyte populations are directly produced by the hemopoietic tissues. The interesting question is whether the erythrocyte populations are the result of a changing differentiation mechanism during the ontogeny of erythropoiesis. This problem cannot be approached with the volume parameter alone because the production of erythrocytes with a different mean volume could be caused simply by nutritive influences during growth without any change in the differentiation process. Changes of the differentiation mechanism would be more likely if other volume-independent parameters would also differ. Such differences do in fact exist. The mean electrophoretic mobility (1) of the population I erythrocytes in the guinea pig is significantly lower (Table II) than that of population II erythrocytes in the adult animal. It changes at the same time as the population II erythrocytes increase (Fig. 6). The volume and electrophoretic data together suggest that the erythrocyte populations I and II are qualitatively different from one another. The data are therefore compatible with the hypothesis that the erythrocyte populations in the blood are the result and the indicator of several distinct differentiation pathways during the ontogeny of erythropoiesis. This is further supported by measurements of the volume distribution curves of rat liver, spleen and bone marrow cells and of blood leukocytes during the hemopoietic development, where characteristic changes in the mean volume and height of the several volume peaks in such curves are observed (7). These measurements demonstrate that, in synchrony to the appearance of the blood erythrocyte volume populations, the volume patterns of the cells of different hemopoietic tissues change. The mechanism which triggers the formation of the erythrocyte populations is not yet known. The analysis of the volume distribution curves will, however, certainly be of great value in the further characterization of the underlying mechanisms.

Analysis of volume distribution curves of erythrocytes during immune lysis: Another application of the analysis of volume distribution curves is the mechanism of immune lysis of cells and bacteria by antibody and complement. This mechanism is only poorly understood at present, and it is unclear if it occurs by physicochemical interaction of the complement components (9, 22) or by an enzymatic attack of the membrane (9, 12, 22). It is known that erythrocytes swell during the process of lysis (5, 17). Electrical sizing seemed promising for a better understanding of the swelling mechanism because single cells can be measured by this method, in contrast to the radiolabeling (5) and osmotic (17) techniques. An important question in this respect was, however, if cells are measurable by electrical sizing during and after lysis. To determine this, the cell concentration in a lytic experiment was electrically counted with a Coulter counter at a constant threshold setting (Fig. 7). There was no measurable change of the erythrocyte concentration during and after lysis, although 100% of the cells were lysed at the end of the experiment, as determined by the hemoglobin in the supernatant (Fig. 7). The answer to the above question is, therefore, that intact and ghost erythrocytes are measured by electrical sizing, since both methods use the same measuring principle. To explain the measurability of erythrocyte ghosts, the cell membrane has either to remain impermeable for the electrical current during lysis or the lesions have to reseal after a short time. Indications for the first possibility are that the lesions do not clearly penetrate the whole membrane as open channels (28). Flux measurements show, in addition, that the substance exchange through the membrane during lysis is lower, as predicted by free diffusion in the case of open channels (17). The cell membrane could therefore still have a sufficiently high resistance which allows electrical sizing, even though vari-

with the genetically determined high (HK) and low (LK) potassium and the L, M antigen cell polymorphism. In preparation.

ous substances are exchanged through the membrane during lysis (5, 17). Concerning the second mechanism, there is a question whether the resealing of temporarily current-permeable lesions would be compatible with the experimental data. The following calculation will show that this is the case. The erythrocyte concentration in the assay is 4.7×10^7 cells/ml. The lysis between 15 and 25 min after the start of the experiment is 50%, as shown by the hemoglobin in the supernatant, and is approximately linear (Fig. 7). This means that 2.35×10^7 cells/ml are lysed in 10 min or 3.9×10^4 cells/ml in 1 sec. Knowing that the electrically counted cell concentration of 4.7×10^7/ cells/ml does not measurably change, and assuming that only a consistent decrease of the cell concentration by more than 2% would be detectable with the Coulter counter, a maximum of 9.4×10^5 cells/ml could continuously escape monitoring by electrical counting because they are temporarily permeable for the electrical current. The concentration of these cells is stationary during the time of maximal lysis between 15 and 25 min. The disappearance rate of 3.9×10^4 cells/ml $\times$ sec multiplied by the mean lifetime of the state of permeability is then equal to the stationary concentration of the 9.4×10^5 cells/ml which are temporarily permeable for the electrical current. The maximal mean lifetime or permeability is obtained by dividing 9.4×10^5 cells/ml by 3.9×10^4 cells/ml $\times$ sec. It is 24 sec. Very similar times of 15–25 sec have been determined during hypotonic lysis of erythrocytes (27). Both mechanisms, impermeability and resealing, are therefore compatible with the data from electrical sizing.

The analysis of the volume distribution curves of erythrocytes at different intermediate states of the complement reaction shows that the erythrocytes swell only when all nine components are present on the cell surface (Fig. 8b). The swollen erythrocytes have the volume of maximally swollen erythrocytes in hypotonic solutions. They are spheroids, as is shown by photographing them in the orifice (Fig. 1b), and their mean volume is 30–35% higher than that of normal sheep erythrocytes. Since the mean volume of these large cells is always constant, and since no population with an intermediate mean volume between small and large cells is observed (Fig. 8b), the spheroid transformation of the biconcave erythrocyte is an all or nothing phenomenon. The number of swollen erythrocytes is complement dose dependent (Fig. 9a and b), and additional experiments show (40) that the large cells in lytic experiments are erythrocyte ghosts. Besides the large ghosts, small ghosts are consistently present (Figs. 8b, 9a and 9b) which have the same volume as intact erythrocytes. These cells cannot be intact erythrocytes because lysis is 100% at the end of all experiments, as determined by the hemoglobin in the supernatant. The question is whether these cells ever swelled prior to lysis or if they lysed while being small. The latter possibility would be in contradiction to the colloid osmotic concept of lysis (5, 17). Considering the maximal lytic velocity of 3.9×10^4 cells/ml $\times$ sec (experiment of Fig. 7), as compared to the electrically measured cell concentration of 4.7×10^7 cells/ml, only 0.083% of all cells lyse/sec. If the time for swelling by colloid osmotic forces and returning to the original volume after lysis is in the order of a few seconds, those cells would not become apparent in the volume distribution curves because their overall concentration is too small. The electrical sizing studies therefore do not exclude the possibility of a transient swelling of those erythrocytes which are small ghosts after lysis. The permanent swelling of the large erythrocyte ghosts (Figs. 8b, 9a and 9b) is, however, not explained by colloid osmotic forces from the intracellular protein because the cells have lost their hemoglobin during lysis. This swelling is caused by a temperature-dependent influence of complement on the membrane and can also be demonstrated when erythrocyte ghosts are washed free of soluble protein prior to the incubation with complement (40). A possible sequence of the events during lysis is: first, that the cells swell with high-complement doses by interference of the complement components with the membrane structures responsible for biconcavity or with low-complement doses by protein osmotic forces; second, that the membrane ruptures due to colloid osmotic forces and third, that the ghosts with many lytic sites remain spheroids and those with few lytic sites on their membrane return to their original volume.

The important advantage of the analysis of volume distribution curves during the immune lysis is that the dynamic processes of the lytic mechanism can be investigated for large numbers of individual erythrocytes in a short time.

The static electron microscopic (10) and the overall radiolabeling techniques (8) are more limited in this respect. Electrical sizing should therefore be of value in further studies on the mechanism of immune lysis.

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