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SEDIMENTATION BEHAVIOR OF ACTIVATED HUMAN GRANULOCYTES:
AGGREGATION AND VOLUME EFFECTS

by

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exposed to EAS. The altered gravity sedimentation behavior of endotoxin-activated granulocytes was abolished when calcium and magnesium were not present in the Ficoll density gradient. Granulocyte aggregation was inhibited by the absence of calcium and magnesium in the medium during granulocyte stimulation, whereas the changes in granulocyte shape and volume associated with granulocyte stimulation were not affected. The data indicate that the altered granulocyte sedimentation pattern in the presence of EAS and calcium and magnesium was produced by granulocyte aggregation and not by changes in granulocyte volume, or shape.

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ABSTRACT

Human peripheral blood granulocytes (PMN's) obtained from normal adults were studied by an analytical gravity sedimentation system. Exposure of PMN's to endotoxin-activated serum (EAS) in a Ficoll density gradient containing Hank's balanced salt solution with calcium and magnesium produced significantly different sedimentation patterns compared to the granulocytes exposed to normal serum under the same conditions. Experiments were performed to determine whether changes in granulocyte density, volume, shape, or aggregation were responsible for the sedimentation pattern of granulocytes exposed to EAS. The altered gravity sedimentation behavior of endotoxin-activated granulocytes was abolished when calcium and magnesium were not present in the Ficoll density gradient. Granulocyte aggregation was inhibited by the absence of calcium and magnesium in the medium during granulocyte stimulation, whereas the changes in granulocyte shape and volume associated with granulocyte stimulation were not affected. The data indicate that the altered granulocyte sedimentation pattern in the presence of EAS and calcium and magnesium was produced by granulocyte aggregation and not by changes in granulocyte volume, or shape.

INTRODUCTION

Activation of granulocytes by chemotactic factors is an area of intense research because of its relevance to the understanding of directed migration of cells, host defense mechanisms, and diagnosis of certain diseases. Granulocytes exposed to endotoxin-activated serum (EAS) in the presence of calcium and magnesium have been shown to have significantly different sedimentation behavior compared to granulocytes exposed to normal serum in the presence of calcium and magnesium (1). This finding is significant because gravity sedimentation of cells is a simple procedure to perform (2) and readily available for the study of granulocyte activation. It is important therefore to determine the physical changes that contribute to the alteration of the sedimentation characteristics of activated granulocytes. The explanation for the observed change in sedimentation of granulocytes treated with EAS is the subject of this report.

The sedimentation behavior of cells is dependent on Stoke's Law:

$$v = \frac{2gr^2\varrho_D}{9\eta k} \quad [1]$$

where v is the terminal velocity, g is the acceleration due to gravity, r is the particle radius, ϱ_D is the difference of the particle density from that of the support medium, η is the viscosity of the medium, and k is a factor depending on the shape of the particle.

Changes in cell shape and cell volume, and cell-to-cell aggregation affect sedimentation, and these changes have been shown to occur during granulocyte activation (3-6). Smith et al (4) have reported that in the presence of chemotactic factors granulocytes change from spherical cells to asymmetric cells with pseudopods. Granulocyte activation has been shown to increase the volume of the cells by about 10%, and granulocyte aggregation following stimulation with chemotactic factors has recently been implicated in several disease states (3,5,6).

In the study reported here, behavior of EAS-activated granulocytes in the presence and absence of calcium and magnesium was examined to explore the effects of granulocyte shape, size and aggregation on gravity sedimentation.

EXPERIMENTAL PROCEDURES

Human granulocytes were isolated from the peripheral blood of healthy donors by a process of dextran sedimentation followed by counterflow centrifugation (7). The final leukocyte preparations which contained $3.0-4.0 \times 10^6$ cells/ml were suspended in Hank's balanced salt solution (HBSS) in the presence or absence of calcium and magnesium. Gravity sedimentation at 37°C was performed in a shallow Ficoll density gradient (2.0% and 6.0% Ficoll 400) with HBSS both in the presence and absence of calcium and magnesium.

The sedimentation distribution of the granulocytes was followed optically as described previously (1). Briefly, a density gradient was formed in cylindrical quartz tubes and the cell sample was placed on top of the gradients. The tubes were scanned vertically by a light beam at regular time intervals and the light intensity was measured by a photometer (1). Each of the quartz tubes used in our study contained a uniform 2.4% concentration of endotoxin-activated serum (EAS), prepared as described previously (7), or control serum. In selected experiments the sedimentation columns were drained for analysis of the cells. Immediately after collection the cells from each of the 0.5 ml volumes were fixed in 0.1 ml of 7.5% glutaraldehyde, and were analyzed by phase microscopy for morphology and percentage of aggregation (4). Particle size was measured using a Coulter Model H4 Channelyzer equipped with a

Coulter Model ZB Counter. Arithmetic means and modes of volume distributions were measured between 100 and 1,000 cubic microns.

For measurements of volume and aggregation, the granulocytes were incubated at 37°C in plastic test tubes (Falcon 2005) under conditions similar to those used in the sedimentation experiment except that the Ficoll gradient was omitted. In one set of experiments, the test tubes were inverted twice before each sampling to assure uniform mixing of cells. In a second set, sampling was obtained from a site 1.5 cm above the bottom of the tube without inversion in order to eliminate granulocyte adherence to the sides of the test tube. The samples were analyzed for granulocyte counts and the volume distribution of granulocytes was measured using a Coulter Model H4 Channelyzer.

RESULTS AND DISCUSSION

The data in this report confirmed our previous results that the sedimentation of EAS human granulocytes in the presence of calcium and magnesium has a positive skewness toward higher velocity sedimentation while granulocytes exposed to normal serum in the presence of calcium and magnesium sedimented in a symmetrical distribution (Figure 1). While the sedimentation FIG.1 patterns of the control and EAS-treated granulocytes were markedly different, the mean sedimentation rate was similar (Figure 1). During two experiments the sedimentation column was drained and the fractions containing the sedimenting cells were analyzed. The total number of particles in each fraction closely resembled the original light absorption pattern. As seen in Figure 2, the modal particle size was similar in all of FIG.2 the fractions suggesting that the predominant particle in each fraction had a similar size. The modal particle volume represents the most frequently occurring cell volume and mean particle volume represents the average cell volume in the sizing interval of the Coulter Channelyzer. The average mode of $350 \pm 4 \mu^3$ for the granulocytes treated with EAS in the presence of calcium and magnesium was significantly greater ($p < 0.001$) than the average mode for unstimulated granulocytes, 320 ± 22 ($n = 19$), suggesting that the major portion of the granulocytes in each fraction was activated. This was supported by morphologic data (see Table 1) which showed that only 8-17% of the cells in each fraction remained in their original

spherical shape and the remainder of the cells were asymmetric in shape, or aggregated. Mean particle size showed a progressive increase in the direction of sedimentation, increasing 20% over the 13 fractions. The increase in mean particle size may reflect an increased percentage of granulocyte aggregates. Phase microscopy revealed an increase in aggregates from 1% in Fraction 1 (the trailing edge) to 22% in Fraction 13 (the leading edge) (Table 1).

An experiment was designed to separate granulocyte volume and shape changes from granulocyte aggregation. Granulocytes were treated with normal serum in HBSS with calcium and magnesium and with EAS in HBSS in the presence and absence of calcium and magnesium. Granulocytes treated with EAS both in the presence and absence of calcium and magnesium showed a rapid increase in their volume (Figure 3).

By phase microscopy nearly all the granulocytes were spherical in shape prior to treatment with EAS. However, following EAS stimulation the granulocytes rapidly developed an asymmetric shape. The change in granulocyte morphology following treatment with EAS was independent of the presence of calcium and magnesium in the medium (Figure 3). At one and one-half hours $89 \pm 3\%$ of the granulocytes treated with EAS and calcium and magnesium exhibited an asymmetric shape while $92 \pm 5\%$ of the granulocytes treated with EAS without calcium and magnesium had an asymmetric shape. However, the loss of granulocytes during incubation, indicating granulocyte

aggregation and/or granulocyte adherence to the surface of the test tubes, was significantly greater for the cells treated with EAS in the presence of calcium and magnesium than in either normal serum containing calcium and magnesium, or EAS without calcium and magnesium (Figure 4).

To demonstrate that granulocyte aggregates were produced, additional experiments were performed. Test tubes containing granulocytes treated with EAS in the presence and absence of calcium and magnesium and treated with normal serum containing calcium and magnesium were allowed to settle. The tubes were not inverted so that adherence was minimized. The granulocytes were collected at a distance 1.5 cm from the bottom of the tube throughout a 90-minute period of sedimentation. Granulocytes treated with EAS in the presence and absence of calcium and magnesium had a similar increase in volume (Figure 5). Granulocytes treated with EAS in the presence of calcium and magnesium had fewer cells at the sampling site of the test tube - which was kept undisturbed during sedimentation - (Table 2) and volume distribution showed that doublets occurred (Figure 5). Larger aggregates were also demonstrated by use of a larger sizing range in the Coulter Model H4 Channelyzer. Granulocytes treated with EAS in the absence of calcium and magnesium exhibited an increased volume, absence of aggregation and a slower rate of settling in the test tube (Table 2).

Finally, to test the role of granulocyte aggregation and

that of granulocyte size and shape, gravity sedimentation was performed using granulocytes treated with EAS in the absence of calcium and magnesium. The positive skewness exhibited by granulocytes exposed to EAS in the presence of calcium and magnesium was completely abolished when granulocytes were exposed to EAS in the absence of calcium and magnesium (Figure 6). In fact, the modal sedimentation rate of granulocytes exposed to EAS without calcium and magnesium, $1.69 \pm 0.25 \times 10^{-4}$ cm/sec, was slower than that of the granulocytes exposed to normal serum containing calcium and magnesium, $1.97 \pm 0.47 \times 10^{-4}$ cm/sec. A decreased sedimentation rate for granulocytes treated with EAS in the absence of calcium and magnesium is consistent with Stoke's Law which can be rewritten as follows:

$$v = \frac{2gr^2\rho_D}{9\eta k} = \frac{g}{6\pi\eta k} \cdot \frac{\text{"mass"}}{r} \quad [2]$$

The sedimentation velocity of a particle decreases as its radius increases, providing that its mass remains constant. The frictional coefficient ($6\pi\eta k$) increases for an asymmetrical mass and it is least for a perfect sphere (8). As granulocytes are activated, they undergo a shape change from a sphere to an asymmetric cell. The assymetry of the granulocytes increases the frictional coefficient and in turn it decreases the sedimentation rate.

The modal sedimentation rate of granulocytes treated with EAS in the presence of calcium and magnesium, $1.86 \pm 0.31 \times 10^{-4}$ cm/sec, was also slower than granulocytes treated with normal

serum containing calcium and magnesium. However, because of the positive skewness, the mean peak sedimentation rate was 4.5% faster for granulocytes treated with EAS in the presence of calcium and magnesium than in granulocytes treated with normal serum containing calcium and magnesium. The presence of granulocyte aggregates with their greater sedimentation rate can account for the positive skewness of the peak sedimentation rate as compared to the sedimentation mode.

The presence of single granulocytes in the higher sedimentation velocity fractions can be explained by the dissociation of the aggregates into single cells after they had migrated to that position as granulocyte doublets, triplets, quadruplets, etc. Reversible aggregation of granulocytes treated with EAS in the presence of calcium and magnesium has been noted by other investigators (3).

Our results suggest that aggregation is the mechanism for the altered granulocyte sedimentation pattern in the presence of EAS containing calcium and magnesium. Granulocyte size and shape changes induced by treatment with EAS were not affected by the absence of calcium and magnesium in the support medium while granulocyte aggregation was inhibited. When granulocyte aggregation was inhibited by treatment of granulocytes with EAS in the absence of calcium and magnesium, the sedimentation pattern was unchanged compared to granulocytes treated with normal serum containing calcium and magnesium. The slower

rate of granulocyte sedimentation in the absence of granulocyte aggregation was due to the increased granulocyte volume and the asymmetric cell shape consistent with Stoke's Law.

Recent studies suggest that in vivo granulocyte aggregation may occur in certain diseases (6,9). Gravity sedimentation offers an alternative and novel approach to the study of in vitro granulocyte aggregation and its regulation.

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The opinions or assertions contained herein are those of the authors, and are not to be construed as official or reflecting the views of the Navy Department or Naval Service at large.

REFERENCES

1. Catsimpoolas, N., Kurtz, S.R., Skrabut, E.M., Griffith, A.L. and Valeri, C.R.: (1979) Science 205, 936.
2. Miller, R.G. and Phillips, R.A.: (1969) J. Cell Physiol. 73, 191.
3. O'Flaherty, J.T., Kreutzer, D.L. and Ward, P.A.: (1978) Am. J. Pathol. 90, 537.
4. Smith, C.W., Hollers, J.C., Patrick, R.A. and Hassett, C.: (1979) J. Clin. Invest. 63, 221.
5. Hammerschmidt, D.E., Harris, P., Wayland, J.H. and Jacob, H.S.: (1978) Blood 52, Supp. 125.
6. Jacob, H.S., Craddock, P.R., Hammerschmidt, D.E. and Moldow, C.F.: (1980) New Engl. J. Med. 302, 789.
7. Lionetti, F.J., Hunt, S.M., Lin, P.S., Kurtz, S.R., and Valeri, C.R.: (1977) Transfusion 17, 465.
8. Van Holde, K.E.: Physical Biochemistry (Prentice Hall, New Jersey, 1971) p. 79.
9. Craddock, P.R., Hammerschmidt, D.E., White, J.G. and Jacob, H.S.: (1976) Blood 48, Supp. 961.

TABLE 1

THE PERCENT OF AGGREGATED CELLS, ASYMMETRIC CELLS, AND SPHERICAL CELLS AFTER 3.5 HOURS OF SEDIMENTATION AT 37°C THROUGH THE FICOLL GRADIENT CONTAINING 2.4% EAS IN HBSS WITH CALCIUM AND MAGNESIUM

<u>FRACTION #1</u> (Trailing Edge)	<u>% AGGREGATED CELLS</u>	<u>% ASYMMETRIC CELLS</u>	<u>% SPHERICAL CELLS</u>
1	1.0	85.0	14.0
2	4.5	78.5	17.0
3	5.0	81.0	14.0
4	6.0	84.0	10.0
5	7.5	84.5	8.0
6	7.0	83.5	9.5
7	10.5	75.0	14.5
8	13.0	76.0	11.0
9	11.5	72.0	16.5
10	12.0	73.5	14.5
11	21.0	66.0	13.0
12	20.0	68.0	12.0
13	22.0	64.0	14.0

(Leading Edge)

The sum of aggregated cells, asymmetric cells, and spherical cells was 100% for each fraction.

TABLE 2

GRANULOCYTE COUNTS IN UNDISTURBED TEST TUBES INCUBATED AT 37°C (n = 3)*

<u>Sedimentation Time (min)</u>	<u>Normal Serum with Calcium and Magnesium (C)</u>	<u>EAS with Calcium and Magnesium (T1)</u>	<u>EAS without Calcium and Magnesium (T2)</u>
0	100.0	100.0	100.0
10	77.0 ± 8.3	72.6 ± 7.2	81.1 ± 8.6
20	71.9 ± 4.3	62.3 ± 4.9	76.2 ± 10.5
30	63.5 ± 11.2	51.9 ± 11.5	75.2 ± 2.4
45	57.9 ± 3.3	42.9 ± 5.8	68.7 ± 2.7
60	49.7 ± 2.4	38.1 ± 6.8	56.8 ± 5.4
90	36.0 ± 2.9	24.6 ± 4.3	37.4 ± 7.4

*Percent cell counts found at 1.5 cm from bottom of the test tube as compared to the number of cells at the same position at the beginning of test.

FIGURE 1

Granulocyte gravity sedimentation at 37°C in the presence of 2.4% endotoxin activated serum (T1) or 2.4% normal serum (C) in a Ficoll gradient made with Hank's balanced salt solution containing calcium and magnesium.

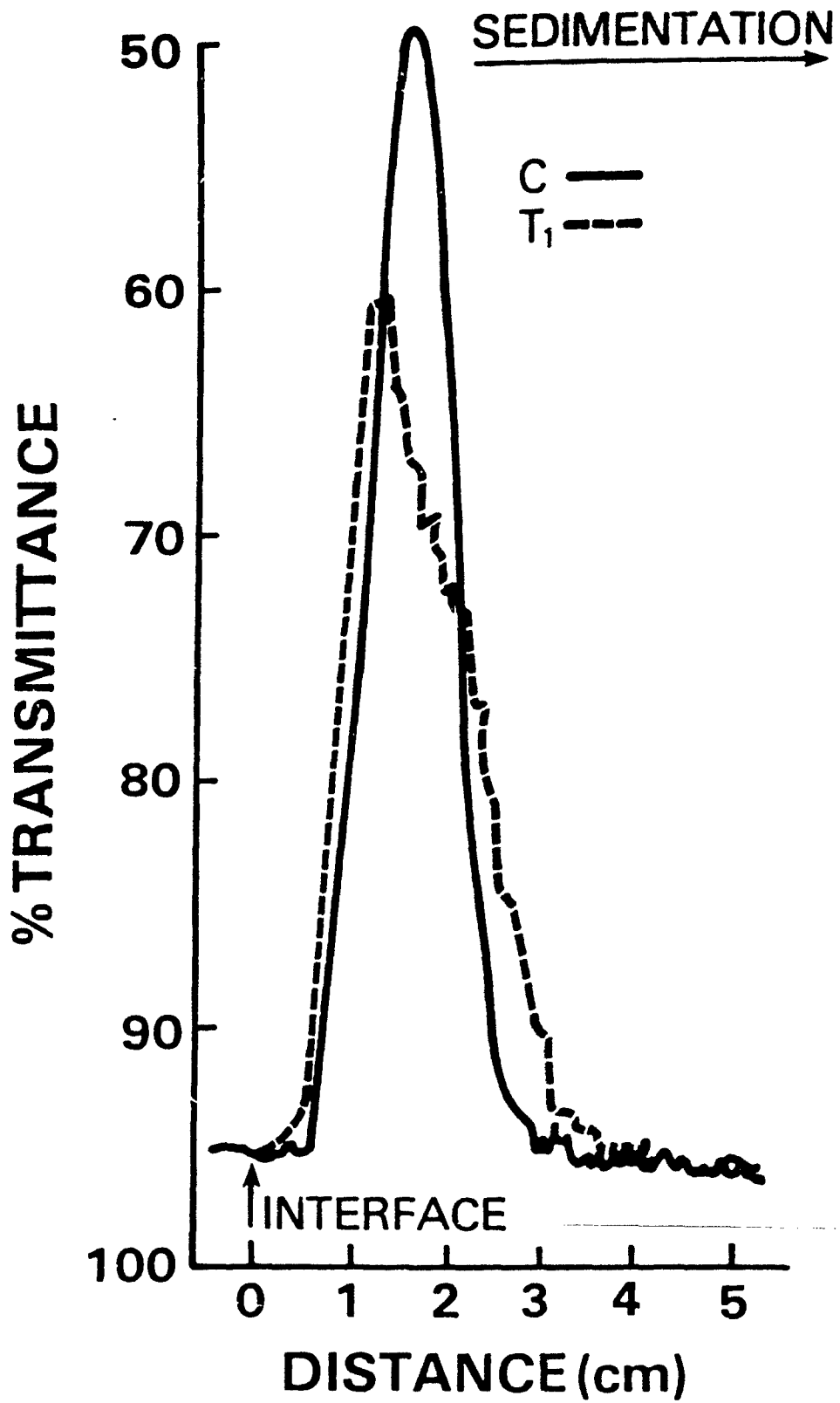
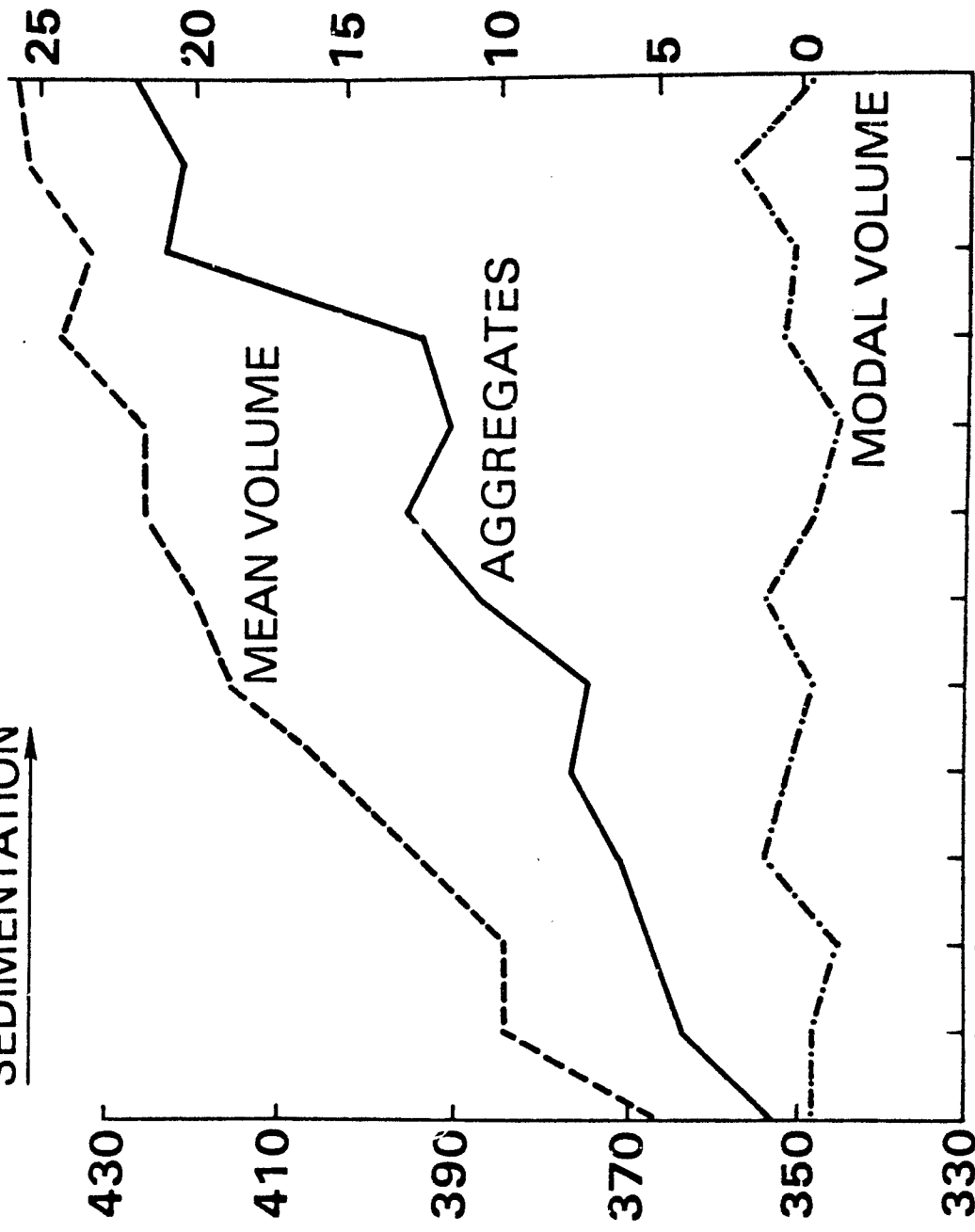


FIGURE 2

Size and percent aggregation of granulocytes after 3.5 hours of sedimentation at 37°C through the Ficoll gradient containing 2.4% EAS in Hank's balanced salt solution with calcium and magnesium.

SEDIMENTATION →

% AGGREGATED PARTICLES



PARTICLE VOLUME (μ^3)

(TRAILING EDGE) (LEADING EDGE)

FRACTION NUMBER

FIGURE 3

Percent of original granulocyte volume as a function of incubation time at 37°C after addition of EAS (T₁ or T₂) or control serum (C) in a test tube: C = 2.4% normal serum with calcium and magnesium in the support medium; T₁ = 2.4% EAS with calcium and magnesium in the support medium; and T₂ = 2.4% EAS without calcium and magnesium in the support medium.

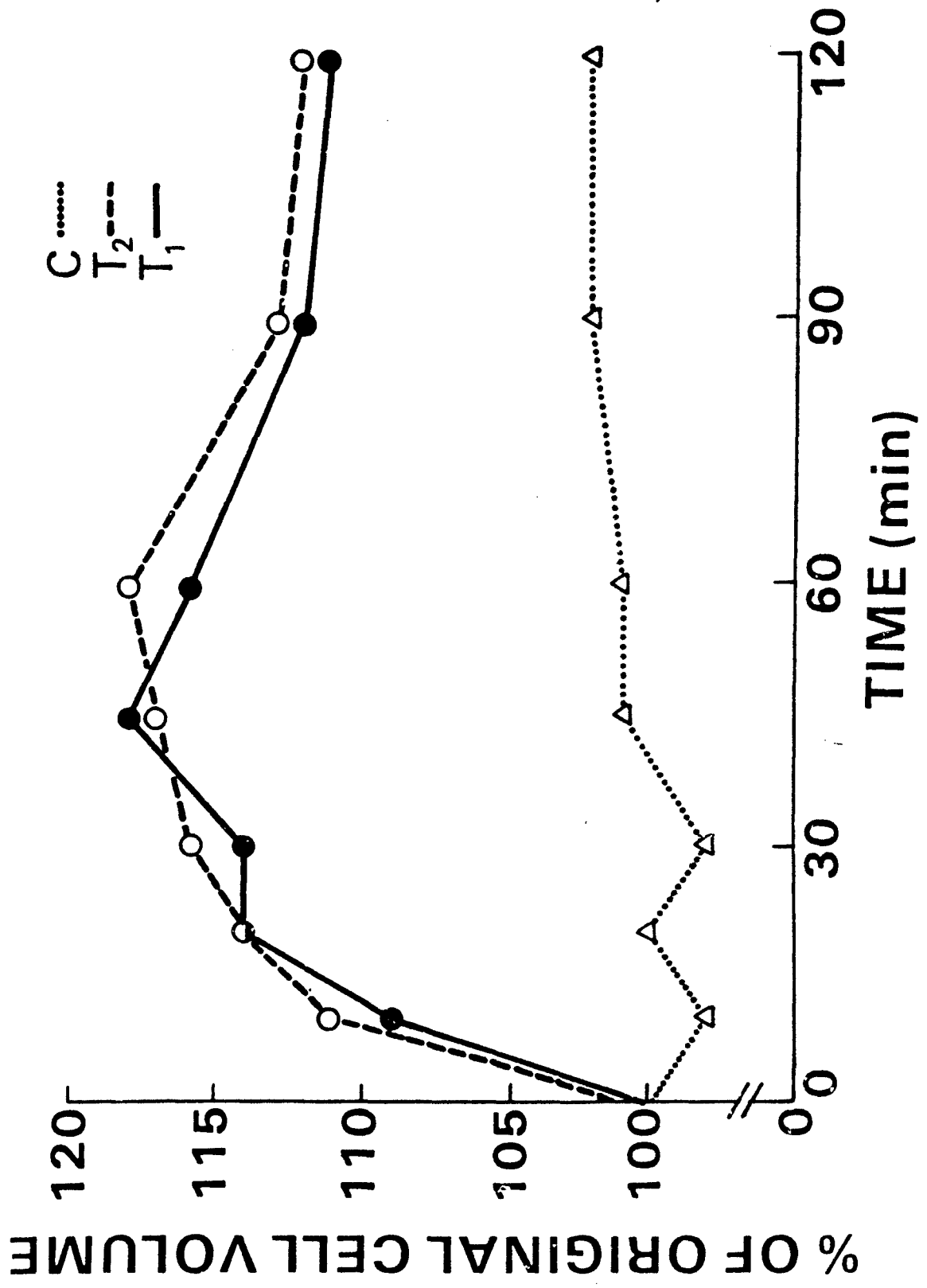


FIGURE 4

Percent of original cell count as a function of incubation time at 37°C after addition of EAS (T₁ or T₂) or control serum (C):

C = 2.4% normal serum with calcium and magnesium in the support medium; T₁ = 2.4% EAS with calcium and magnesium in the support medium; and T₂ = 2.4% EAS without calcium and magnesium in the support medium.

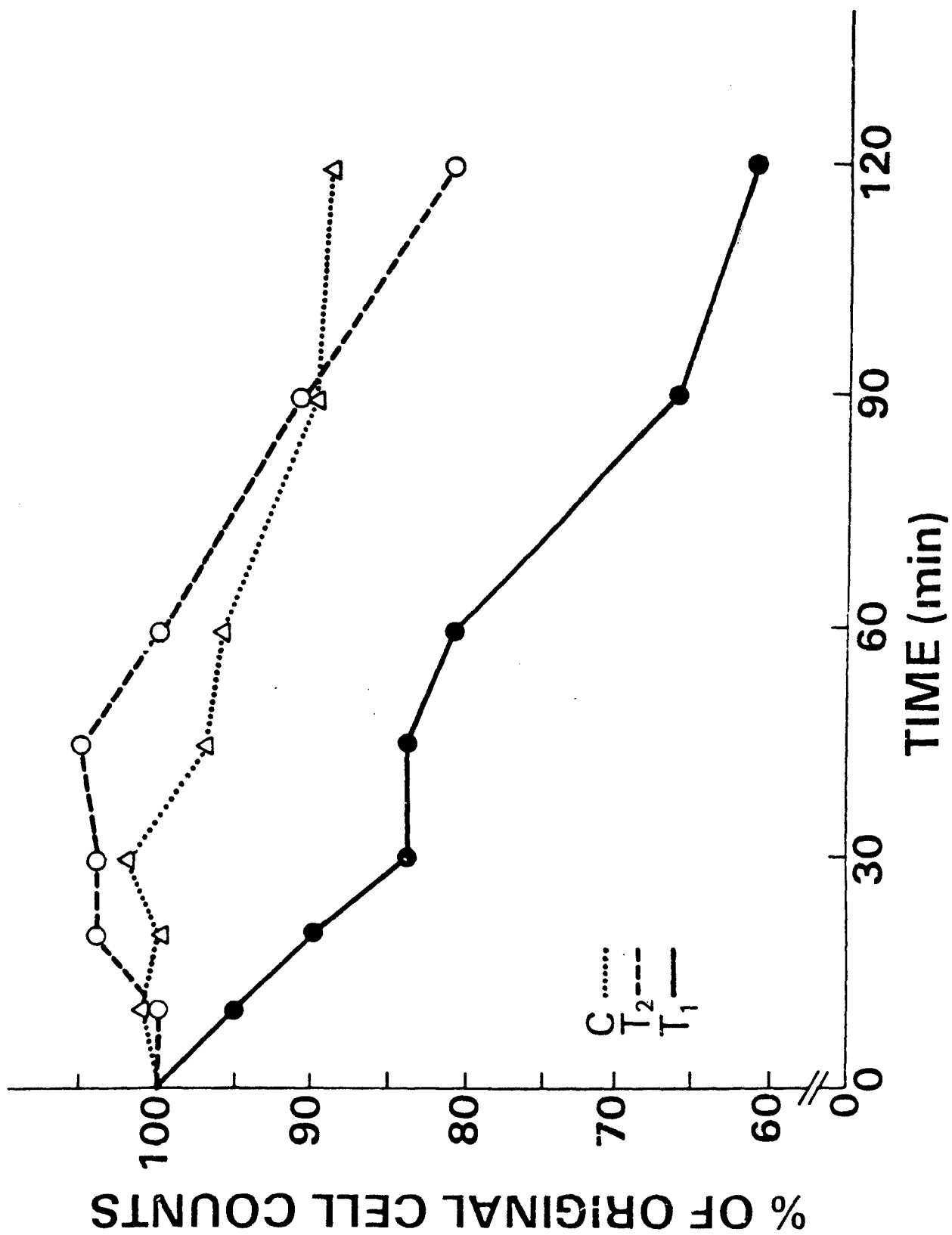


FIGURE 5

The volume distribution of granulocytes incubated undisturbed in test tubes at 37°C in normal serum containing calcium and magnesium (C) and in EAS in the presence (T₁) and absence (T₂) of calcium and magnesium. The samples were collected at a position 1.5 cm from the bottom of the test tube.

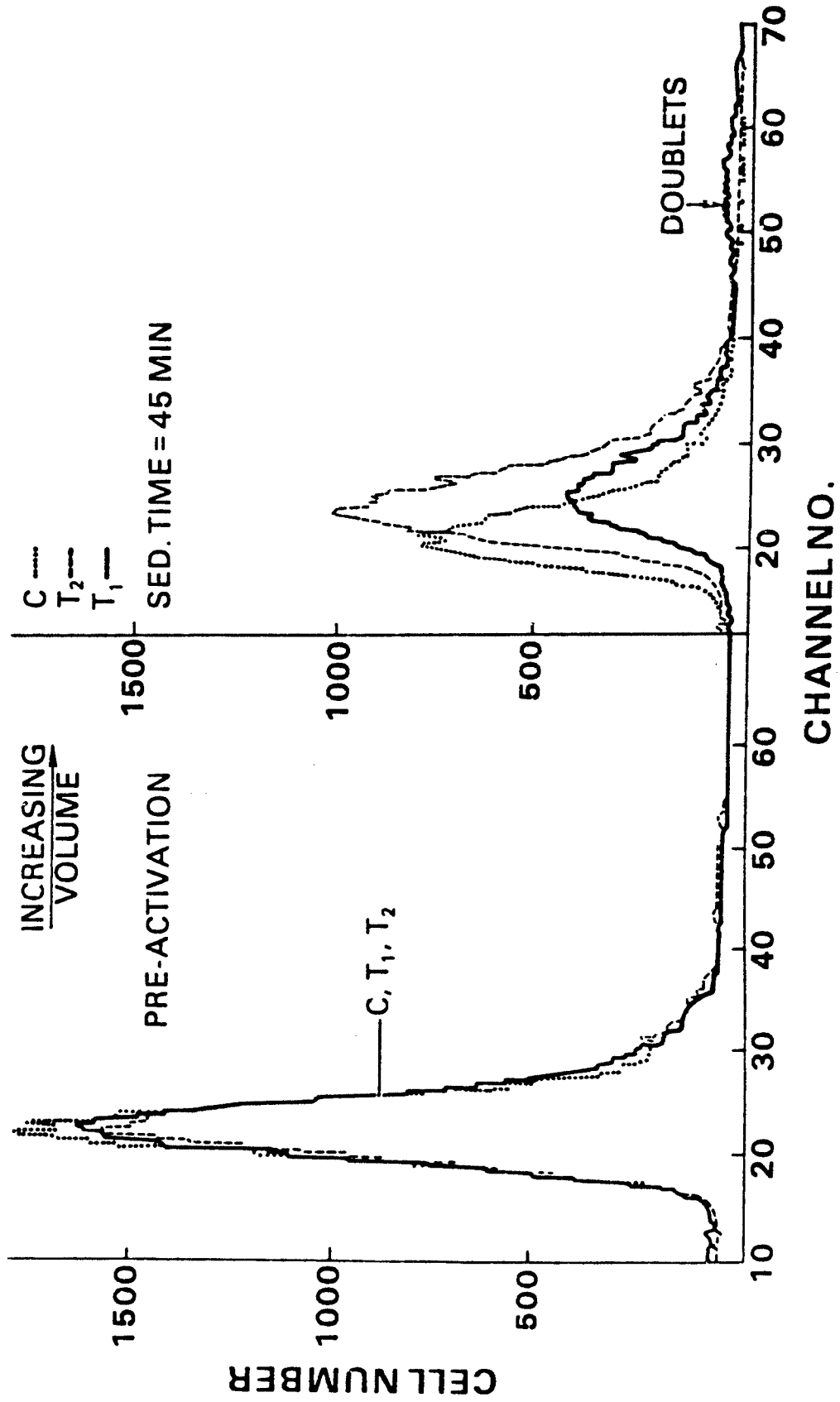


FIGURE 6

Granulocyte gravity sedimentation at 37°C in the presence (T₁ and T₂) and absence (C) of 2.4% EAS in the Ficoll gradient, C and T₁ in the presence of calcium and magnesium, and T₂ in the absence of calcium and magnesium.

