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TEMPERATURE EFFECTS ON SHAPE AND FUNCTION OF HUMAN GRANULOCYTES--ETC(U)
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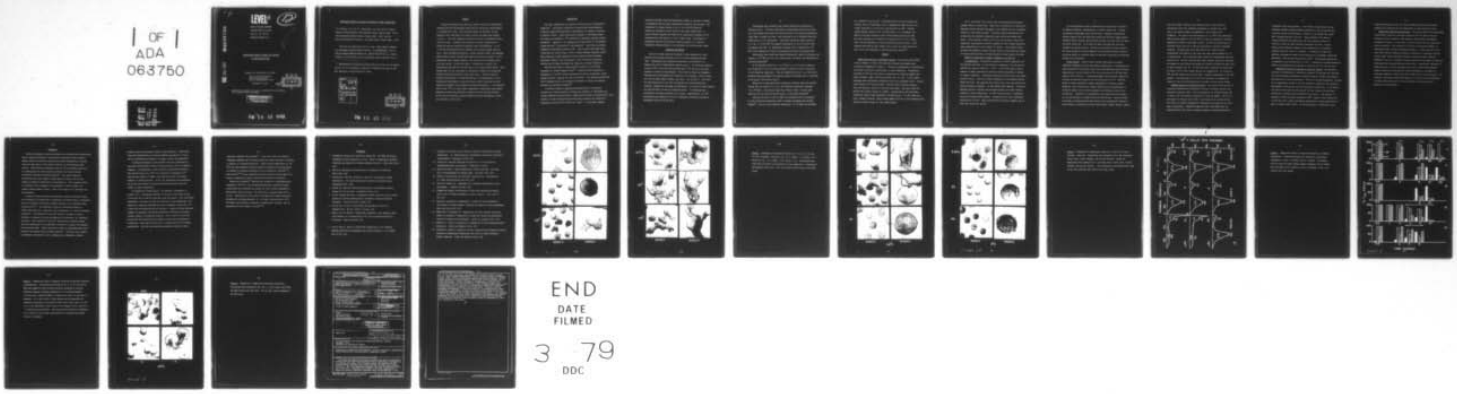

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TEMPERATURE EFFECTS ON SHAPE AND FUNCTION OF HUMAN GRANULOCYTES

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TEMPERATURE EFFECTS ON SHAPE AND FUNCTION OF HUMAN GRANULOCYTES

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SUMMARY

Scanning microscopic and functional studies were made of granulocytes isolated from CPD anticoagulated whole blood by counterflow centrifugation in a Beckman JE-6 rotor. The collection buffer was phosphate (20 mM) buffered saline (280 mOsM) with glucose (28 mM) and human serum albumin (1.2% w/v). The final suspension contained less than 2% mononuclear cells and 5% red cells. Incubation and fixation at various temperatures revealed two distinct temperature dependent shape transformations. At 22, 37, 40 and 45°C granulocytes were ameboid with extensive highly textured veils. These smoothed progressively, bullae and blebs formed, and membranes peeled finally leaving nonfunctional spheres with smooth surfaces. At 4°C, granulocytes were irregular spheres, less rugose but with numerous microvilli and nodules. Veiling was absent. Phagocytosis, initially low, progressively declined over 48 hours while cell surfaces became smooth. Some formed blebs, but all terminated as nonfunctional spheres with untextured surfaces containing occasional large single holes. Cellular stability estimated from changes in volume distributions, and phagocytosis by microfluorescence measurements of yeast and latex particle ingestion were also temperature dependent and paralleled the shape progressions. It is concluded that at body (37°C) or fever (40°C) temperatures, granulocytes have dynamic membranar surfaces characterized by extensive veiling and high function. At 4°C they are relatively inactive spheres devoid of pseudopodia or veils, yet functional at slow rates.

INTRODUCTION

The shape, metabolism, and function of blood cells are influenced by temperature. Particularly responsive are granulocytes whose membranes deform and undergo configurational transformation in response to chemical or physical stimuli. Human granulocytes suspended in autologous plasma were shown to be ameboid at 37°C consisting of spheroidal and multi-veiled extensions[1]. Reduction of the temperature of granulocytes to 4°C markedly reduced motility[2], phagocytosis[3], and metabolism[4]. Lysis also was greatly retarded for granulocytes stored at 4°C. Only 6 percent were lysed after 2 days while all cells stored at 22°C or higher underwent hypotonic lysis[3]. While elevation of body temperature during infection causes increased granulocyte numbers in the circulation, little is known of granulocyte function and the shape transformation induced by increased temperature.

Heat has been shown to induce extensive changes in cell surface morphology of cultured lymphoid cells[5,6]. Relatively short exposures to temperatures of 45-50°C caused intracellular structural alterations, active membrane movement, capping, and loss of microvilli. The associated inhibition of Na^+ dependent amino acid transport was thought to explain the observed membrane and cytological changes[7,8].

In previous studies we employed microfluorescence of fluorescein diacetate and ethidium bromide to estimate the stability of cytoplasmic and nuclear membranes of granulocytes at temperatures suitable for preservation[9]. Also, changes in volume distributions have been used to measure the integrity of granulocytes stored at 4°C and 22°C for 3 days[3]. In this paper scanning

electron microscopy identified morphologic changes in response to changes in temperature and two shape transformation sequences were observed. One progression of change occurred at 22, 37, 40, and 45°C which was similar but occurred at faster rates for the higher temperatures. A second different sequence was observed for granulocytes incubated at 4°C, characterized by the absence of veils or petal forms. Measurements of granulocyte stability and particle ingestion enabled us to identify morphologic features related to the functional and non-functional states.

MATERIALS AND METHODS

Fifty ml of whole blood was collected at room temperature by venepuncture into syringes containing 7.5 ml of citrate-phosphate-dextrose (CPD). Granulocytes were isolated by counterflow centrifugation in a Beckman JE-6 rotor as described by Lionetti et al.[9]. The blood was gradually cooled to 10°C in the rotor and the blood was at 10°C during the one hour required to isolate the granulocytes. The collection medium was phosphate-buffered saline (280 mOsm/kg H_2O) containing 0.02 M Na_2HPO_4, 0.028 M glucose, and 1.2% w/v human albumin (Armour, salt poor). The pH was 7.1. The rotor was sterilized prior to use with a 10% solution of sodium hypochlorite (Chlorox), flushed with the pump, and followed by 1.5 liters of sterile isotonic NaCl and 200-300 ml of sterile elutriation buffer. All solutions were sterilized by filtration through 0.22 u filters. Approximately 1.8×10^8 granulocytes were obtained in 8.0 ml of suspension containing less than 2% mononuclear cells and 5% red cells.

Granulocytes were incubated under sterile conditions as described by Contreras et al.[3]. Multiple collections of granulocytes from the sterilized rotor were pooled and divided into covered sterile 50 ml Corning polypropylene tubes under a laminar flow hood. Each contained 14 ml of buffered collection medium and 5.5×10^7 granulocytes. Tubes were maintained without agitation at 4, 22, 37, 40 and 45°C and sampled periodically for up to 48 hours under the laminar flow hood. At appropriate intervals two ml containing 8×10^6 PMN's were withdrawn and fixed for microscopy and tested as described below.

Granulocytes were counted and volume distributions made with dilutions containing 2×10^5 PMN's per ml with a Coulter Model ZH counter and Channelyzer as previously described[9].

At the specified intervals 1.3×10^6 PMN's in 0.3 ml of the incubation were fixed with 2.7 ml of 2% glutaraldehyde in Millonig's buffer maintained at the incubation temperature. They were dehydrated, dried, coated with gold-palladium and photographed with scanning electron microscopy (SEM) as described by Lin et al.[5] and Lionetti et al.[9].

Reactivity of granulocytes with fluorescein diacetate (FDA) and ethidium bromide (EB) was tested on 0.2 ml of the incubated suspensions containing 2.0×10^6 PMN's. Wet mounted slides were counted within 1.0 minute after admixture of cells with the FDA-EB mixture, as previously described[9].

Ingestion of latex or yeast by PMN's was estimated from the percentage of cells containing fluorescent latex[9] or zymosan pretreated with ethidium bromide[10]. One ml of the incubation containing 4.0×10^6 PMN's was withdrawn

as 2 aliquots of 0.5 ml each. Fluorescent latex (0.025 ml Fluolite-ICI, Finland) (875×10^6 particles, 0.1-0.3 u diameter) was added to one 0.5 ml aliquot resulting in a ratio of ca. 400 particles per PMN. Ethidium treated zymosan (0.025 ml, 360×10^6 particles, 1-1.5 u diameter) was added to the other yielding a ratio of 200 zymosan particles per PMN. Both were incubated for 30 minutes in autologous serum at 37°C, washed twice, and the percentage of cells containing particles counted. Latex ingestion was rated as high, medium, low or zero, and yeast ingestion as high, low or zero according to criteria previously defined[10].

RESULTS

Temperature effects on granulocyte surface. That granulocytes exhibit a host of shapes is evident from scanning microscopy photographs taken at different temperatures (Figure 1). These are representative of granulocytes fixed after 20 minutes of equilibration at each temperature. Granulocytes collected at 10°C and fixed while in suspension were primarily spheres with highly irregular rugose (wrinkled) surfaces (Fig. 1A, top). A small number displayed short multifolded, exoplasmic ruffles and cytoplasmic veils. Granulocytes brought to 4°C and then fixed, revealed totally different topology. They were spherical, devoid of veils and less rugose. Many short blebs and modules were evident (Figure 1A; middle). Granulocytes at 22°C and higher assumed the ameboid shape described by Lichtman et al.[1]. At 22°C these were polar, randomly oriented, tightly rugose spheres with one or more extended veils. (Figure 1A bottom, Figure 1B, top, middle bottom).

At 37, 40 and 45°C, the initial state was characterized by greatly extended leafy or folded veils. These were so extensive as to obscure the spherical portion and make them appear to be composed entirely of membrane (Figure 1B, top, middle, bottom). Functional studies demonstrated that granulocytes were maximally phagocytic where veiling was maximum (See below). Brief exposure of PMN's at 45°C for the 20 minutes required for temperature equilibration elicited a sequence of shape changes suggesting great susceptibility to heat (Figure 1B, bottom). Veils became greatly extended and commenced to smooth and peel while the spherical body reduced its highly textured wrinkled surface gradually forming nodules and blebs.

Effects of incubation on surface, membranes, and function of granulocytes. Granulocytes incubated at various temperatures for time periods sufficient to evoke changes in morphology, size and particle ingestion are shown in Figures 2, 3 and 4. At 22°C and higher a common sequence of shape transformation occurred related principally to changes in membrane properties. Cytoplasmic extensions gradually lost their highly puckered texture. Folds and wrinkles disappeared as the membranes became smooth and filmy. The spherical parts became less rugose with patches suggesting residues of membrane. On some spheres blebs appeared. Ultimately, membranes peeled or detached leaving smooth spheres with small membrane residues or slight indentations. At 40°C after two hours, a significant fraction had become smooth spheres. Progressively all cells transformed similarly for 21 hours. Many contained holes revealing a membrane and an inner core (Figure 2A, bottom).

At 4°C granulocytes slowly transformed into spheres with no evidence of membrane extension, transformation, or peeling (Figure 2B). Instead granulocytes transformed slowly over 48 hours. Membranes retracted and formed irregular villi and digitiform projections with ball shaped endings. Gradually these retracted leaving spheres covered with loose folds of lesser curvature (Figure 2B). By 48 hours all PMN's were spherical with many irregular but smooth contours. Microvilli had shortened to small nodules. The final observation revealed spheres with even smooth surfaces devoid of structures except for vesicular blebs in some cells consistent with membrane pinched off by exocytosis.

Volume studies. Volume studies revealed granulocytes to exhibit temperature dependent swelling and possible fragmentation. Distributions of sizes obtained with the Coulter cell counter and Channelyzer clearly showed granulocytes to be most stable at 4°C, to be less stable at 22°C and least stable at 40°C (Figure 3). Volume measurements from electronic signals are reliable for spherical particles such as granulocytes at 4°C. Distorted shapes at higher temperatures negated measurements of absolute volumes. Therefore, we have plotted only channel numbers, which are proportional to volume, the median value of which represents the midrange of sizes in the distribution. Instability of granulocytes was characterized by increases in size and decreases in number within each distribution. Previous studies on storage of granulocytes had shown swelling and fragmentation[3] characterized by shifts in distribution toward higher channel numbers (greater volume).

The curves showed skewing to the right and loss of area within the distribution while cell fragments accumulated, as evident from a new peak at low channel numbers corresponding to the volumes of the fragments[3]. The initial size distributions (4, 22, 40°C) were symmetrical. Median channel numbers at 4°C and 22°C of 62 and 61 respectively indicated populations of PMN's of equivalent volumes. At 40°C the median number 64 indicates larger cells due to swelling and possibly veiling or psuedopod formation. No mononuclear cells were present but a small red cell contamination was observed in the lower size range (median numbers 14, 12, 13 respectively). The volume data shows clearly that granulocytes were most stable at 4°C. No shift occurred which would indicate swelling or shrinking. The numbers of cells within the populations likewise revealed that small loss of cells occurred, (Ca. 3%). On the other hand, incubation for longer than 20 hours at 22°C or 40°C caused swelling and fragmentation. By 24 hours at 40°C and 22°C almost total loss occurred. Fragments accumulated in the lower channels. The magnitudes of cell loss were calculable by comparing the number of cells in the PMN population at anytime with the initial number.

Membrane stability by microfluorescence. The stability and function of granulocyte membranes was determined by studies of esterase activity with fluorescein diacetate (FDA), from the accumulation of ethidium bromide (EB) in nuclei, and from studies of ingestion of latex and zymosan. The sum of percentages of esterase active PMN's, those whose nuclei reacted with EB, plus PMN's lost through fragmentation (Channelyzer) accounted for all cells under investigation. Assumptions made were that viable PMN's were all esterase positive and intact cytoplasmic membranes allowed fluorescein to

accumulate within the cytoplasm. It was further assumed intact nuclear membranes excluded ethidium bromide preventing its reaction with DNA and resulting intense red fluorecence. Finally, it was assumed that viable PMN's ingested particles maximally. These parameters were shown to change predictably with temperature and with incubation (Figure 4). Initially, nearly all PMN's produced fluorescein from FDA. A slight decline with increasing temperature was accompanied by an increase in ethidium reactivity from 1 to 5 percent. On incubation, nuclear permeability increased as evident from EB uptake while cytoplasmic fluorescein diminished. This was particularly seen at 45°C. These changes accelerated as temperature was increased from 4 to 45°C. Fragmentation followed maximum EB uptake as determined from percentages of remaining cells in the size plots.

Temperature effects on ingestion of latex and zymosan. The phagocytic functions of granulocytes as measured by ingestion of particles was consistent with the fluorescein accumulation, ethidium exclusion, volumes, and stability data observed at various temperatures. PMN's were maximally phagocytic initially at all temperatures (Figure 4) and lost phagocytic capability with incubation. Considerable variation was found with increasing temperatures. At 4°C despite high total overall activity the capacity for high ingestion of both yeast (dotted bar) and latex (cross hatched bar) was minimal. High capacity for ingestion of yeast and latex increased to 40°C. However, these indices fell at 45°C even after a short (20 minutes) equilibration, showing sensitivity to heat in excess of 40°C (105°F). In the late stages of incubation at each

temperature phagocytic cells lost high and medium capacities for particle ingestion and displayed only low capabilities to ingest yeast and latex.

Temperature inactivated granulocytes. The surface features of granulocytes inactivated by heat over a four hour period at 45°C are shown in Figure 5. Progressive loss of rugosity, bleb formation, high polarity and membrane stripping is evident. At four hours, veils were absent and the granulocytes were all spherical. The existence of residues of cytoplasmic membrane is revealed by slight rugosity and membrane patches. At this time most of the cells (80%) were yet capable of producing fluorescein in cytoptasm, and only a small number (20%) accumulated ethidium, reflecting nuclear membranes permeable to the dye. Phagocytic function decreased as the number of yeast and zymosan particles internalized were clearly less. Nevertheless, adherence of latex and yeast to the cell periphery was readily visible and measureable. Complete ingestion of particles was limited presumably by the absence of sufficient membrane to engulf more than a small fraction of the particles. In contrast, granulocytes brought alternately to 4°C from 37°C could repeatedly (6 times) extend and retract veils and increase and decrease particle ingestion (to be published).

DISCUSSION

Whereas transmission electron microscopy has elucidated much intracellular detail, scanning techniques of granulocytes in suspension have revealed a dynamic surface and cellular topology which differs substantially from that seen with light optics of wet mounted, settled, or stained spread preparations. Granulocytes in autologous plasma at 37°C were ameboid consisting of rugose spheres with highly convoluted surfaces from which extended multiveiled, pleated and leaflike membranes[1]. The ameboid shape was inhibited and the cells assumed a rugose spherical shape at 4°C or when exposed to metabolic inhibititors or cytochalasin B. Such a dynamic surface is consistent with the capacity of granulocytes to deform, spread, and locomote towards chemical stimuli. Data of this paper are in agreement with these findings.

By means of phase contrast and transmission electron microscopy, Bessis and colleagues have learned much of temperature and aging effects on leukocytes. Veils on neutrophils were shown to undergo retraction in wet mounted slides subjected to 4°C[11]. The granulocyte surface as described by Bessis is covered with microvilli, projections, and invaginations in a state of continual agitation[12]. Cold (between 0°C and 10°C) caused an increase in cellular viscosity, a decrease in motion and withdrawal of projections, all completely reversible. Heat (between 45°C and 55°C) induced major disruptive changes. They were liquifaction of the cytoplasm, contraction of nucleus and cytoplasm and generalized edema. Bessis described the death of polymorphonuclear granulocytes on wet mounted slides in phase contrast[12]. There were gross changes in pseudopods, retraction of veils, fragmentation of membranes, Brownian

movement within the cytoplasm, sphering, and plasmolysis. Additionally there were manifestations of increased membrane permeability to water, such as vacuolization and edema of cytoplasm, nucleus and organelles[12].

In scanning microscopy, surviving PMN's from long incubations became bald, smooth spheres, devoid of surface texture, veils, or membrane fragments. Of significance is that spherical granulocytes obtained by heat inactivation at 40 or 45°C or by retraction of membrane at 4°C retained some capacity to adhere, and within the limits of available membrane, to ingest particles. At 4°C, all granulocytes were phagocytic but did not have avidity for particles (particularly yeast) that was evident at higher temperatures.

As suggested by Lichtman et al.[1], the membrane is apparently retracted at 4°C. Nevertheless function is retained in 50 percent or more of the cells for at least 26 hours when veils were absent. Heat inactivated granulocytes, initially very avid for particles at high temperatures, lost capacity for ingestion coincident with loss of membrane (Figures 2A, 4, 5) as seen from the morphology data of this paper. Membrane stripped spherical residues of temperature inactivated granulocytes nevertheless retained a residual capacity for phagocytic function. After 2 hours at 40 and 45°C function was best characterized as adherence rather than internalization of particles. This might be due to one or a combination of the following possibilities. Heat may have inactivated complement proteins in serum

required to opsonize the particles[13]. It may also reflect the residual cytoplasmic membrane still stretched around the nucleus deficient in quantity or capacity to incorporate particles. Finally extreme temperatures (4, 40, 45°C) may have disrupted thermally sensitive cytoskeletal structures which are required to maintain morphology as well as phagocytic functions[6,14]. Other studies[13,15] using cytoskeletal structure sensitive drugs support the notion of temperature induced structural and functional alterations.

Roberts and Steigbigel[16] observed that incubation of polymorphonuclear leukocytes at 40°C for 1 hour increased bactericidal capacity and phagocytosis which they suggested may account for fever induced enhancement of bactericidal capacity in vivo. Our observation, in vitro suggest this to be true. Additionally our data shows that temperature induced enhancement processes are also time dependent, i.e. at higher temperatures (40, 45°C) and longer time, significant depression of phagocytosis occurred. This is consistent with the findings of others[17,18].

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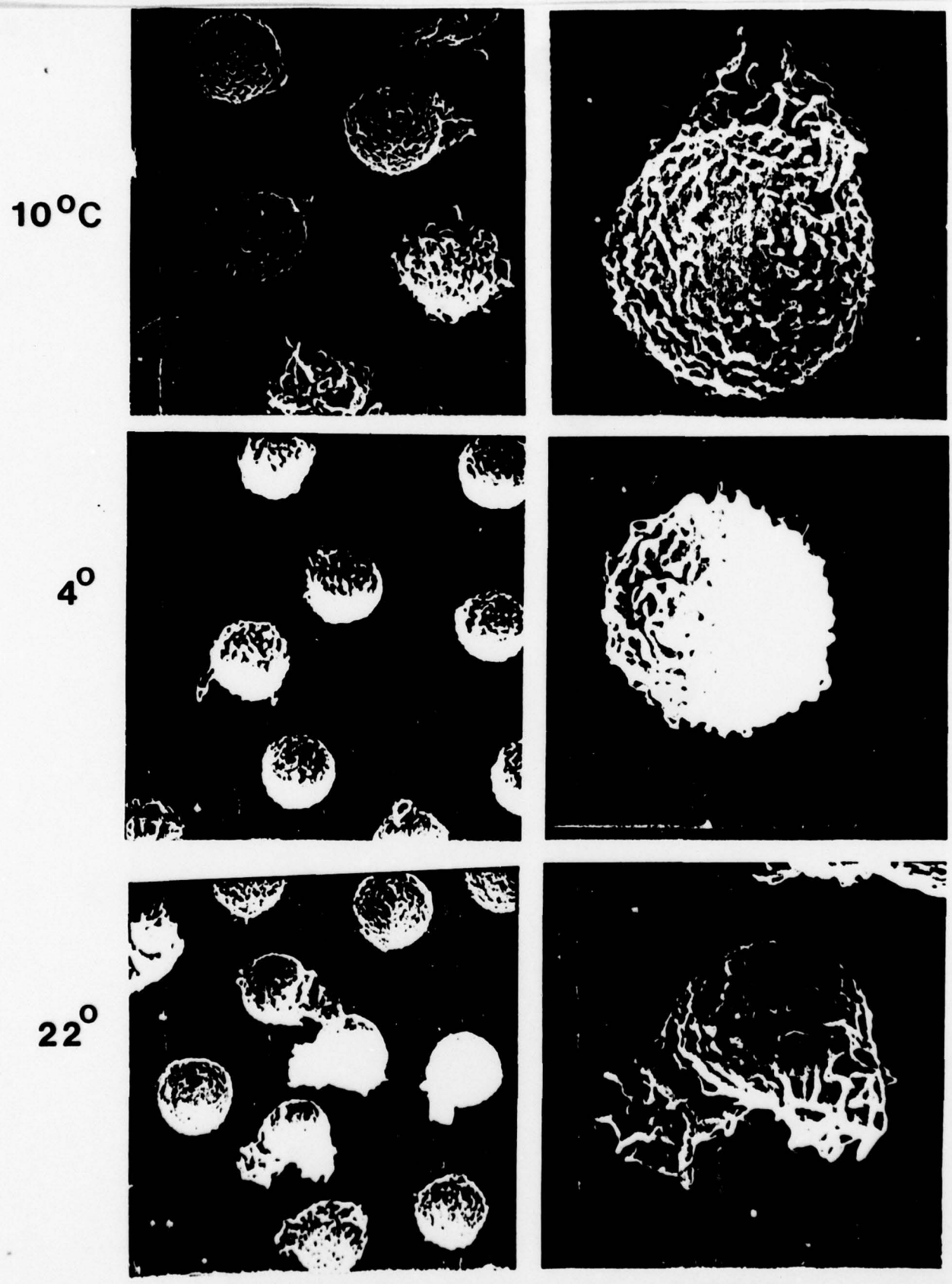


FIGURE 1A

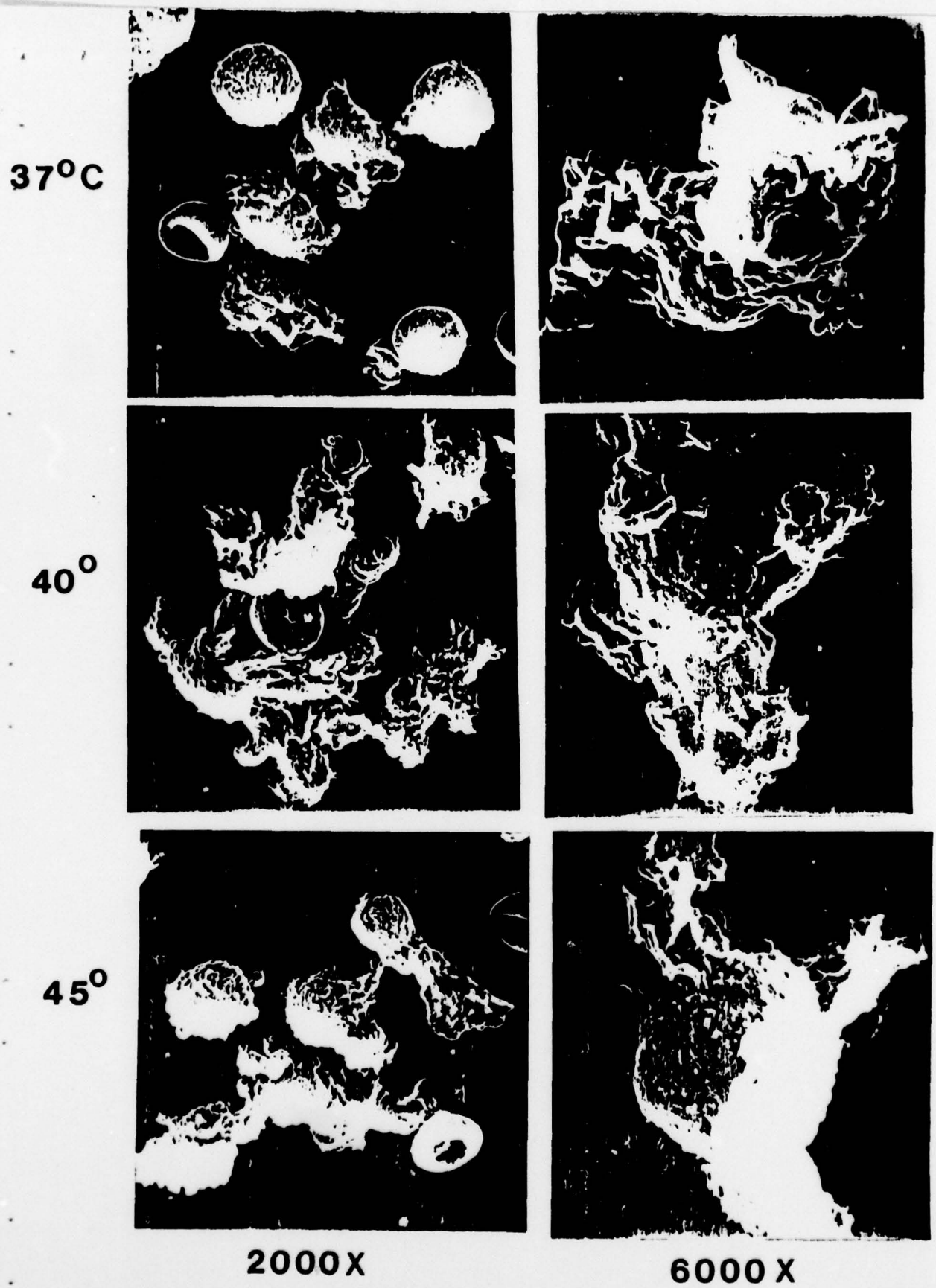
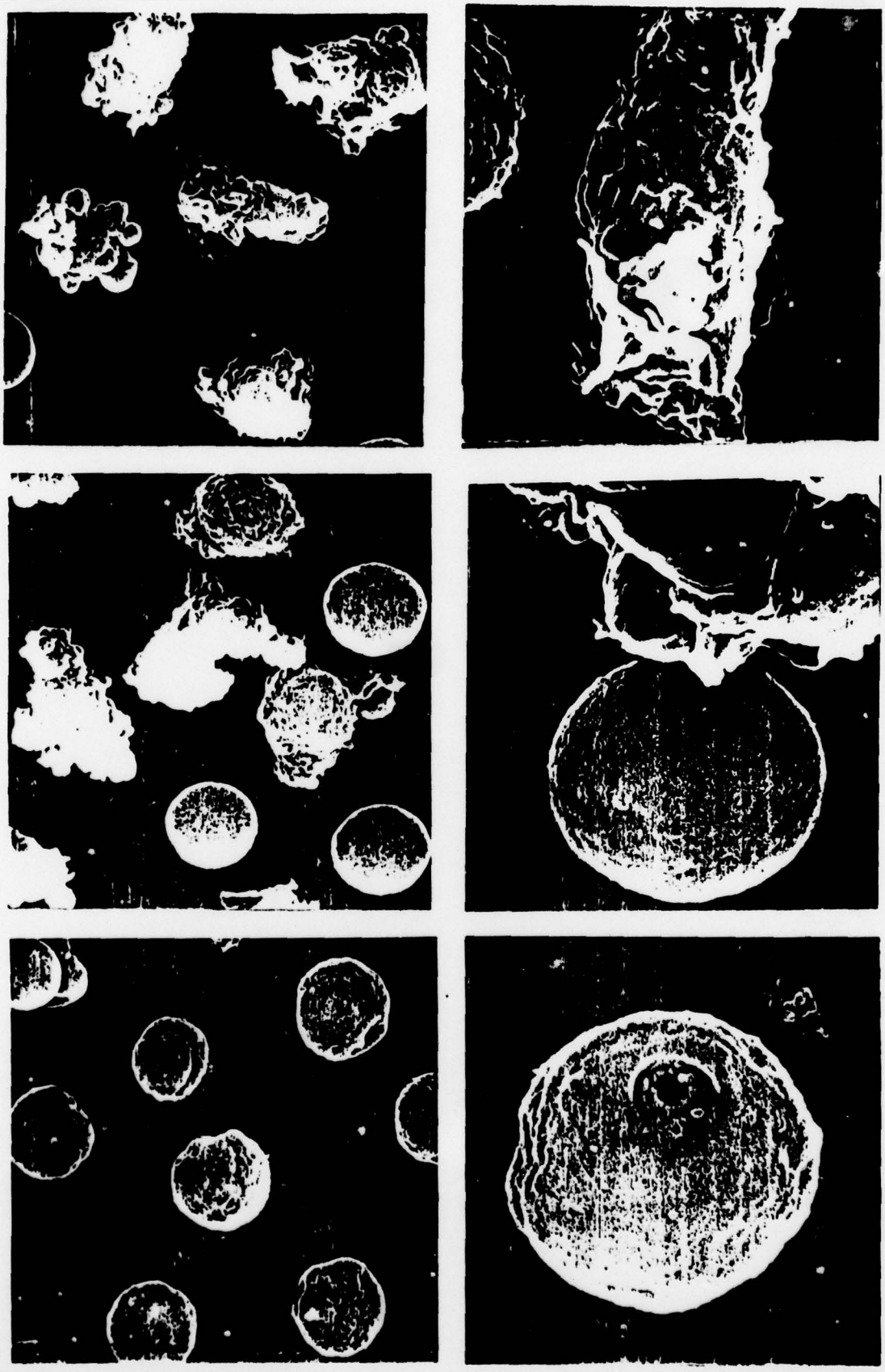


FIGURE 1E

Figure 1. Morphology of granulocytes stored at 4, 10, 22, 37, 40, and 45 C for 20 minutes. Figure 1A: top - 10 C, middle - 4 C, bottom - 22 C. Figure 1B: top - 37 C, middle - 40 C, bottom - 45 C. Granulocytes were fixed after 20 minutes of equlibration at each temperature. Granulocytes were magnified 2000 times in the left column and 6000 times in the right column.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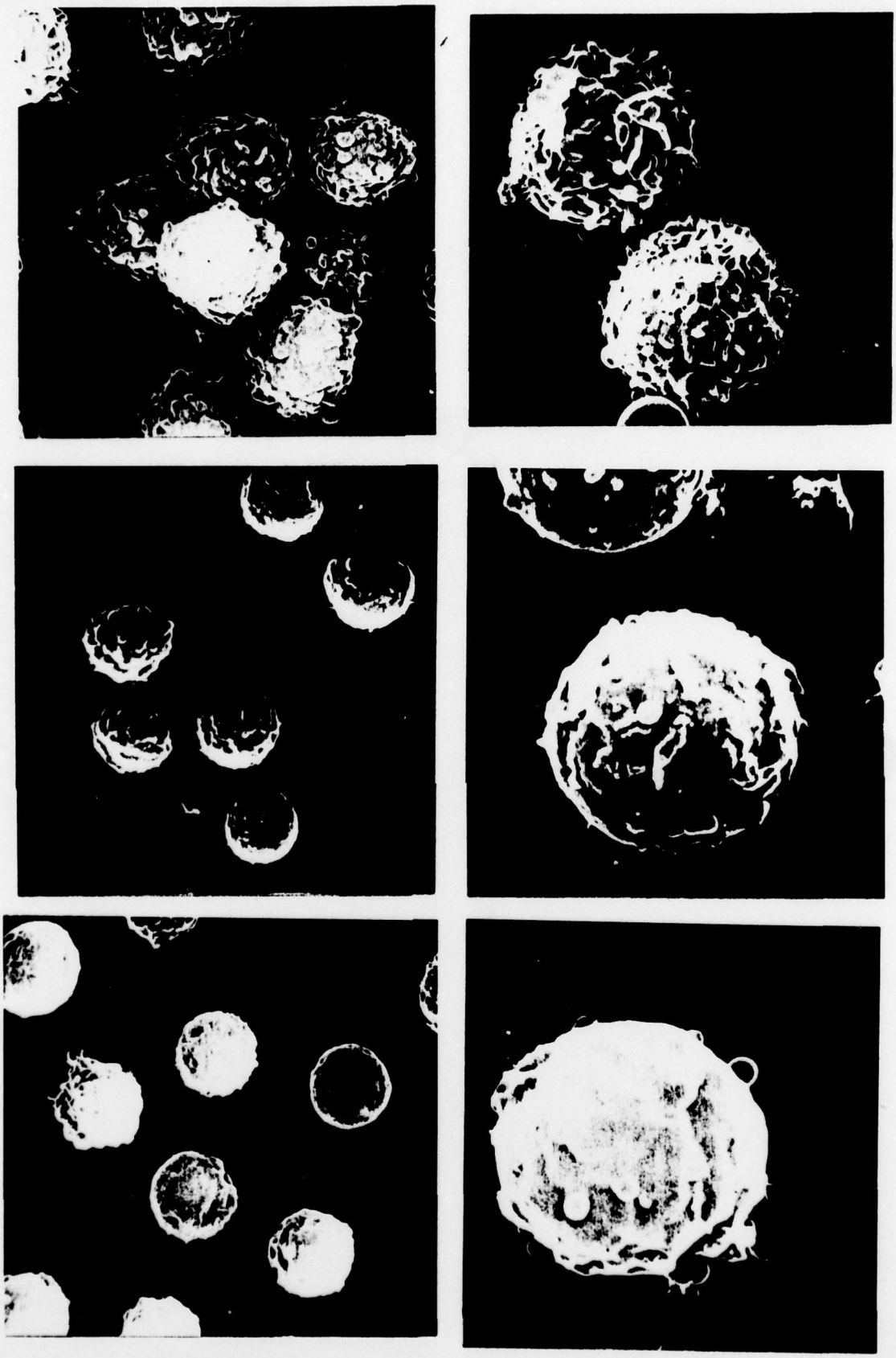
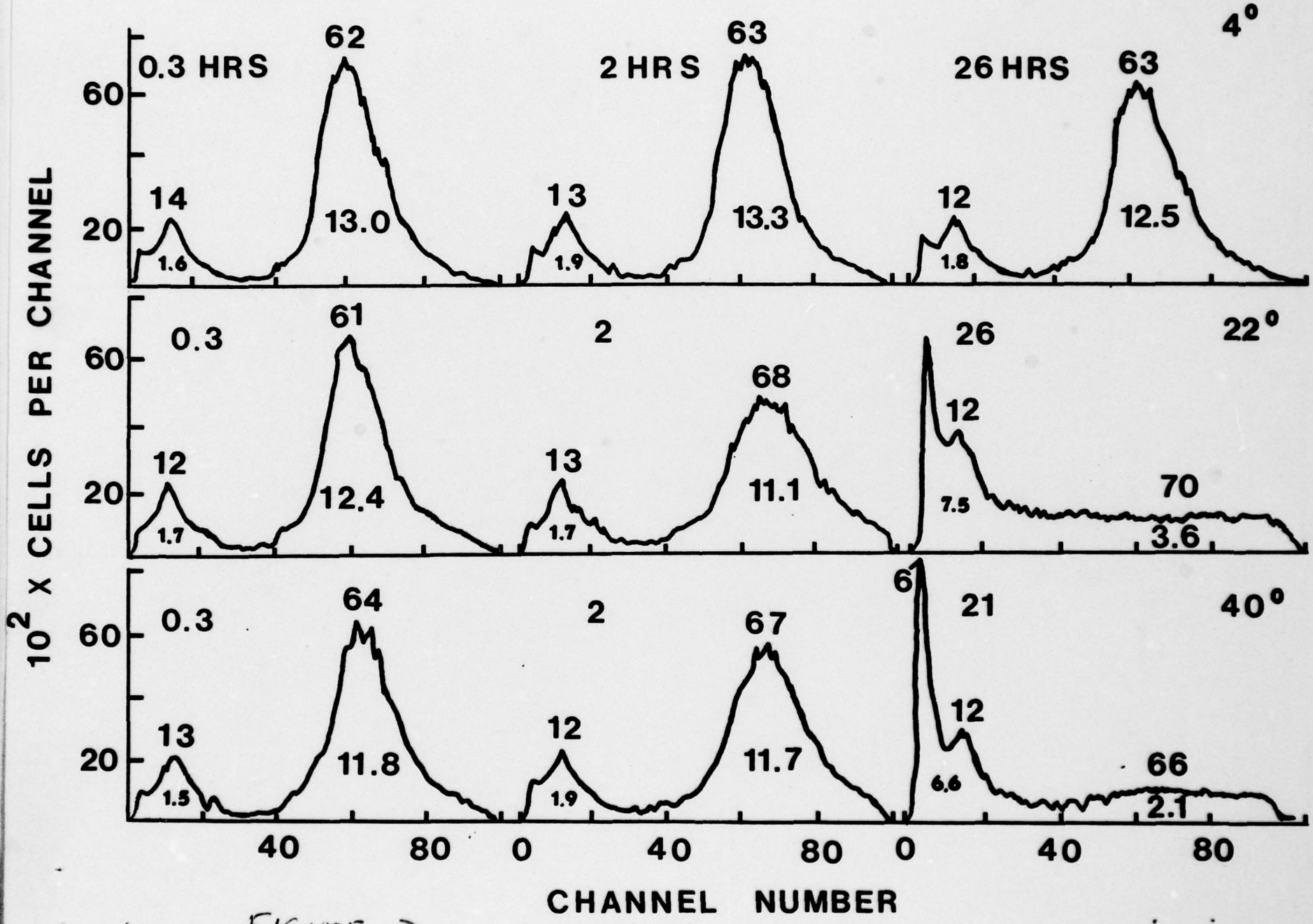


FIGURE 2B

Figure 2. *Morphology of granulocytes stored at 4 C and 40 C for up to* 48 hours. Figure 2A: Granulocytes were incubated at 40 C and fixed after 1 hour (top), 2 hours (middle), and 21 hours (bottom). Figure 2B: Granulocytes were incubated at 4 C and fixed after 2 hours (top), 26 hours (middle), and 48 hours (bottom). The granulocytes were magnified 2000 times in the left column and 6000 times in the right column.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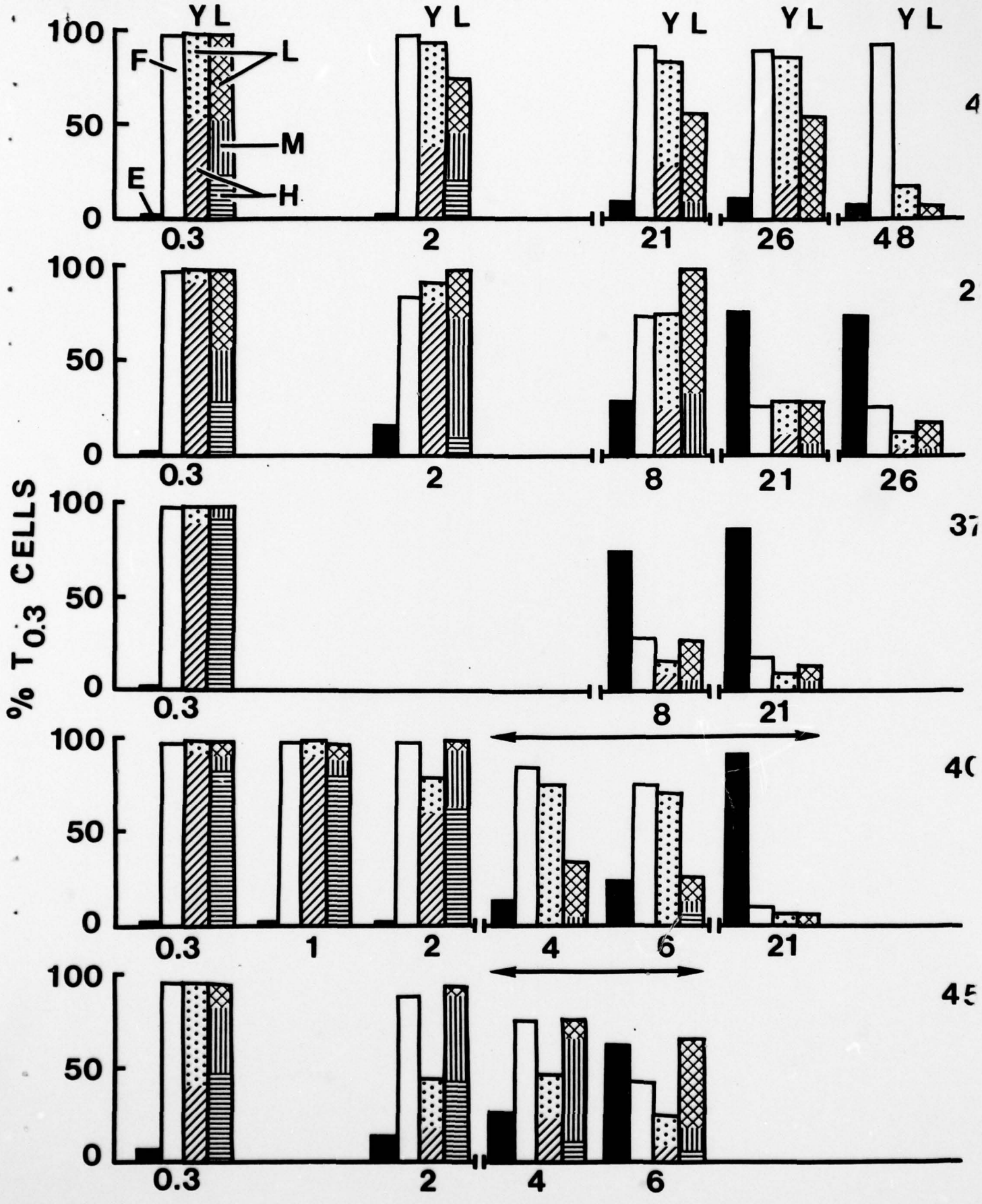
10² X CELLS PER CHANNEL

FIGURE 3

Figure 3. Temperature effects on volume distributions of incubated granulocytes. Volume distributions are plotted for granulocytes incubated at 4 (top), 22 (middle) and 40 C (bottom). The numbers over the peaks are median channel numbers (midrange) and the numbers under the peaks are the numbers of cells in thousands in the 0.1 ml aliquots that were counted.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TIME (HOURS)

FIGURE 4

Figure 4. Temperature effects on membrane integrity and particle ingestion by granulocytes. Incubations were carried out at 4, 22, 37, 40 and 45 C. They were sampled at time intervals previously estimated to cause demonstrable changes in membrane permeability to fluoresceindiacetate (F, white bars), ethidium bromide (E, black bars), yeast (Y) and latex (L) ingestion. H, M, and L refer to high, medium, and low capacities for ingestion of particles, the criteria of which were: Yeast, high is ≥ 5 and L is < 5 per granulocyte; Latex, high is > 30, medium is 5-15, and low is < 5 particles per granulocyte. The arrows define intervals of incubation at 40 C and 45 C during which internalization of particles was reduced in favor of adherance.

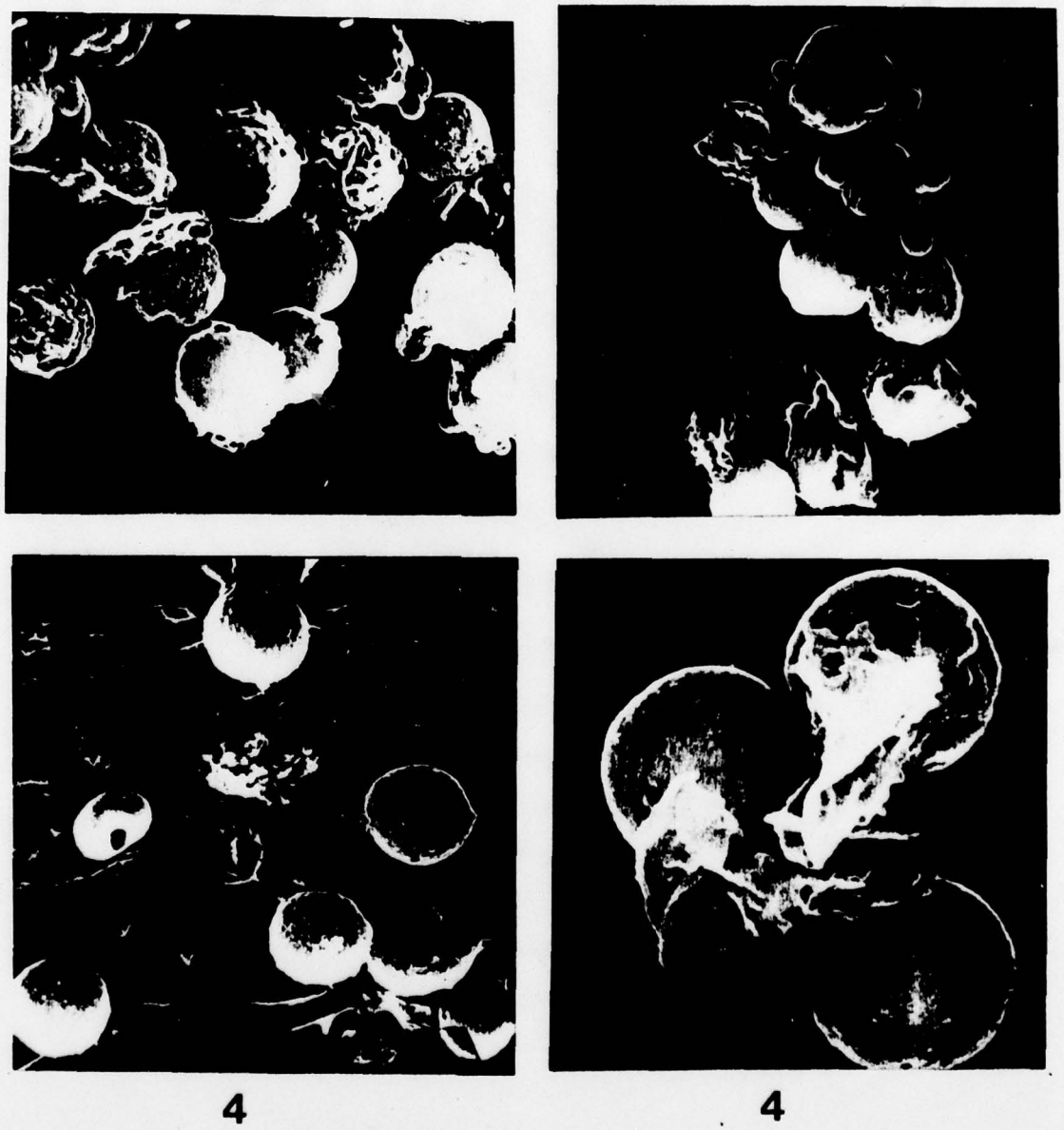

FIGURE 5

Figure 5. Morphology of temperature inactivated granulocytes. Granulocytes were incubated at 45 C for 1, 2 and 4 hours, then fixed. The magnification was 2000 times. For the lower right photograph it was 6000 times.

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granulocytes, counterflow centrifugation, volume distributions, phagocytosis, freeze preservation, microfluorimetry, temperature, shape.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number):
Scanning microscopic and functional studies were made of granulocytes isolated from CPD anticoagulated whole blood by counterflow centrifugation in a Beckman JE-6 rotor. The collection buffer was phosphate (20 mM) buffered saline (280 mOsM) with glucose (28 mM) and human serum albumin (1.2% w/v). The final suspension contained less than 2% mononuclear cells and 5% red cells. Incubation and fixation at various temperatures revealed two distinct temperature dependent shape transformations. At 22,

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37, 40 and 45°C granulocytes were ameboid with extensive highly textured veils. These smoothed progressively, bullae and blebs formed, and membranes peeled finally leaving nonfunctional spheres with smooth surfaces. At 4°C, granulocytes were irregular spheres, less rugose but with numerous microvilli and nodules. Veiling was absent. Phagocytosis, initially low, progressively declined over 48 hours while cell surfaces became smooth. Some formed blebs, but all terminated as nonfunctional spheres with untextured surfaces containing occasional large single holes. Cellular stability estimated from changes in volume distributions, and phagocytosis by microfluorescence measurements of yeast and latex particle ingestion were also temperature dependent and paralleled the shape progressions. It is concluded that at body (37°C) or fever (40°C) temperatures, granulocytes have dynamic membranar surfaces characterized by extensive veiling and high function. At 4°C they are relatively inactive spheres devoid of pseudopodia or veils, yet functional at slow rates.