FACTORS AFFECTING THE ADHESIVENESS OF HUMAN LEUCOCYTES AND PLATELETS IN VITRO*,‡

By JAMES E. GARVIN,§ M.D.

(From the Department of Biochemistry, Northwestern University Medical School, Chicago)

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Previous work in this laboratory (1) has shown that the polymorphonuclear neutrophils (PMN) from human blood adhere to a column of siliconized glass wool while the lymphocytes pass through in good yield. This paper describes further experiments undertaken to define some of the variables important in the adhesiveness of lymphocytes and platelets as well as the PMN, and to throw some light on the mechanisms involved. Whole blood rather than some simpler system was selected for study because of the artifacts involved even in such a simple process as centrifuging and resuspending human buffy coat in plasma (2), and because of its closer relevance to processes occurring *in vivo*; *e.g.*, the adherence of PMN to the walls of capillaries in areas of inflammation (3).

Materials and Methods

Preparation of Glassware.—All materials (except polyethylene) coming in contact with blood were treated to provide a relatively non-wetting surface. The columns, pipettes, syringes, etc., were treated with a 2 per cent solution of Dow Corning 200 fluid (obtained from Dow Corning Corp., Midland, Michigan) in chloroform and cured at 300°C for 30 minutes. The No. 20 stainless steel needles used for venipuncture were treated with monocote E (obtained from the Armour Laboratories, Kankakee, Illinois). The glass beads and glass wool were siliconized using Dow Corning No. 1208 vapor phase siliconing mixture (obtained from Dow Corning Corp.). The vapor of mixed chlorosilanes was produced by passing air through a bubbler containing the Dow Corning No. 1208. It was then passed through the actively agitated beads for exactly 1 minute, following which the beads were flushed with air for 5 minutes, washed exhaustively, cured at 110°C for 30 to 60 minutes, and finally washed exhaustively again. The glass beads used in these experiments were No. 100 superbrite glass beads (obtained from the Minnesota, Mining and Manufacturing Co., St. Paul). All glassware was washed only with 7 X detergent (obtained from Linbro Chemical Co., Inc., New Haven).

Column Preparation.—The columns were 16 cm in length by 1 cm in diameter with an

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§ Senior Research Fellow, United States Public Health Service.

outlet at the bottom of 0.3 cm. The glass wool mat, which was used to hold the beads in the column, was prepared from siliconized glass wool by matting a small portion of it so that when cut into a circular disc by a cork borer its weight was about 6 mg. The mat was carefully seated in the bottom of the column and the dry beads poured in and tapped down. The No. 100 beads were specified by the manufacturer as having a diameter of 0.02 cm, which gives a volume of 4.19×10^{-6} cm³ and a surface of 1.26×10^{-3} cm² for each bead. Since the density of the glass was 2.5 g./cm³, and 1 cm³ of packed beads was found to weigh 1.41 gm, it may be calculated that each centimeter of height of the columns contained about 106,000 beads having a glass surface area of 133 cm².

Regulation of Flow Rate.—The rate of blood flow through each column was regulated by a motor driven 10 ml. syringe. The cylinder of the syringe was controlled by an infusion-withdrawal apparatus (No. 600–900 obtained from the Harvard Apparatus Co., Dover, Massa-

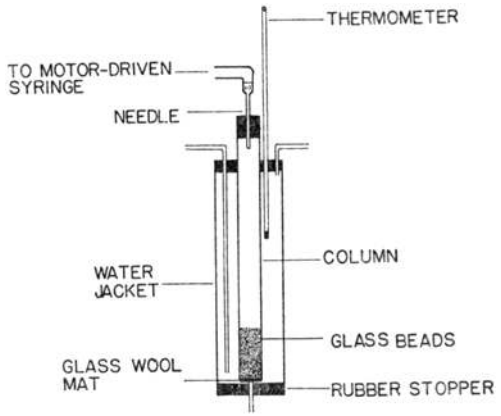

FIG. 1. Column used to measure adhesiveness of leukocytes and platelets. The internal diameter of the column was 1.0 cm.

chusetts). The syringe was connected to the top of the column by a piece of rubber tubing of 0.3 cm I.D., containing a T-tube. The opening of the T-tube to the ambient air was controlled by a pinch-cock. This arrangement made it possible to adjust the syringe without upsetting the column. In general we found that the blood tended to run somewhat faster than our usual rate (0.1 ml/minute) and that the syringe actually checked the flow. Nominal flow rates compared very closely with actual volumes delivered. Up to 6 columns (each with its own 10 ml. syringe) could be run simultaneously.

The average linear flow rates (in centimeters/minute) may be calculated from the nominal flow rate through the column by reference to the average cross-section of the column which is not occupied by beads. Since it was found that in a packed column about 56 per cent of the space is occupied by the glass beads, this open cross-section may be shown to be 0.345 cm². Hence the range of linear flow rates used in these experiments was from 0.29 cm/minute (nominal flow rate of 0.10 ml/minute) to 2.99 cm/minute (nominal flow rate of 1.03 ml/minute). The linear flow rate in peripheral capillaries of human subjects is given by Houssay (4) as being 3 to 4.8 cm/minute. Thus in most of these experiments the linear flow rate in the columns was about $\frac{1}{10}$ that *in vivo*.

Temperature Control.—The early experiments were conducted at room temperature which

ranged from 24–27°C. Temperature in the later experiments was controlled by a water jacket on each column as seen in Fig. 1.

Hematology.—Routine total white blood counts and differentials were carried out as described previously (1). Wet preparations were made by placing a drop of blood on a slide, adding a coverslip and rimming with vaseline. In the experiments on wet preparations at various temperatures a slide warmed or cooled by actively circulating water was used. This slide formed the cover of the chamber in which the water circulated. The platelet counts were made using the method of Brecher and Cronkite (5). Although smears of blood treated with 0.1 mg/ml of heparin (treated to remove phenol) showed clumping of platelets after 1 and 2 hours standing, clumping was rarely seen on the chambers during the counts. Furthermore there was no drop in platelet count over these same time intervals whether the temperature was 27°C or 37°C. These results differ from those of Copely and Robb (6) who observed a drop in platelet count with time in blood treated with heparin. We did not observe the extensive clumping of platelets on the chamber described by Hellem (7) in the presence of heparin. However, Hellem modified the platelet counting method of Nygaard (8) and employed citrate as the diluent, while the method of Brecher and Cronkite employs oxalate as the diluent.

Typical Experiment.—The blood was drawn with sterile precautions and after removing the needle from the syringe, the blood was gently squirted into a squat bottle containing 0.1 ml of heparin for every 10 ml of blood drawn. It was mixed for 1 minute by swirling and aliquots (usually about 5 ml) transferred to test tubes. Suitable additions were made and mixed with the bloods by gently forcing the mixtures up and down with polyethylene pipettes of large bore, and the tubes stoppered with paraffined corks. After incubation for 15 minutes at 37°C the bloods were mixed by inversion for 3 minutes and samples taken for total white counts, smears, and platelet counts. Using a serological pipette with a large outlet, 5 ml of blood mixture (called influent) was added to a column. After removing the small rubber stopper which had previously been placed in the outlet of the column, the top of the column was connected to a 10 ml syringe in the infusion-withdrawal apparatus. Working the syringe plunger by hand the blood was forced rapidly down the column until it just reached the glass wool. Now backing the plunger up slightly to prevent any flow out of the bottom of the mat, the plunger was attached to the infusion-withdrawal drive plate. After all the columns were thus prepared the infusion-withdrawal apparatus was started. The effluent bloods were collected as they emerged from the outlet tubes.

Calculations.—In all cases the per cent recovery of particular formed elements was calculated as follows:

$$\frac{\text{No. of formed elements/mm}^3 \text{ of effluent blood}}{\text{No. of formed elements/mm}^3 \text{ of influent blood}} \times 100 = \text{Per cent recovery}$$

No. of a species of leukocyte/mm^3 was calculated as follows:

$$\text{Total WBC/mm}^3 \times \text{fraction of species in differential count} = \text{No. of species/mm}^3$$

Reagents.—The heparin was a sodium injection preparation (obtained from Lederle Laboratories Division, American Cyanamid Co., New York), and contained 1000 units of heparin (about 10 mg) per ml. It also contained 0.45 per cent phenol as a preservative and was used as supplied for all the room temperature experiments described in this paper. For all the experiments in which the temperature was controlled (see section Effect of Temperature on Adhesiveness of Leukocytes and Platelets to the end of the paper) this heparin preparation was processed to remove the phenol. One ml of the solution was taken to dryness in vacuum without heat, 1 ml of glass-distilled water was added back, and it was taken to dryness again.

This step was repeated and the vacuum left on for 30 minutes. By this time all odor of phenol had disappeared. The dry powder was then made back to the previous volume with glass-distilled water and used as the anticoagulant.

Krebs-Ringer phosphate was prepared as described by Krebs (9). The ethylenediaminetetraacetic acid disodium salt (EDTA) (obtained from Versenes Inc., Framingham, Massachusetts), the trisodium citrate, and the sodium oxalate were all reagent grade. These three anticoagulants were kept in a desiccator over $CaSO_4$ for at least 24 hours before being weighed out. The magnesium chloride and calcium chloride were both reagent grade. The final concentrations of all calcium chloride solutions were determined directly by titration with EDTA using calcein as an indicator (10). The concentrations of all magnesium chloride solutions were determined directly by analysis for chloride using the method of Volhard as modified by Koltoff and Sandell (11). The metabolic inhibitors used were as follows: sodium cyanide, analytical reagent, (Merck and Co., Rahway, New Jersey; 2,4-dinitrophenol, Eastman No. 102, (Eastman Chemical Co., Rochester, New York); iodoacetamide, (Nutritional Biochemicals, Cleveland, Ohio).

The Dowex A-1 chelating resin (obtained from the Dow Chemical Co., Midland, Michigan) was 50 to 100 mesh. It was cycled to the acid form and, after washing with excess 3.5 N NH_4OH, was heated at 80°C for 3 hours in 4N NH_4OH. It was then washed, converted to the acid form, and cycled back to the Na/K form with 2 N base such that the ratio of the equivalents of Na/K was 148/5. This was then washed slowly with 30 gallons of water. It was cycled from basic (still keeping the Na/K equivalents ratio of 148/5) to acid form two times, concluding in the basic form, and washed with 15 gallons of water. This last step was again repeated but with the change that a small column of the K/Na form was placed in the influent line of the wash water to remove the blue-green impurity found in our distilled water. Five ml aliquots of the effluent from this thoroughly washed resin still showed traces of white residue on drying, however the resin was used in this form.

Nucleic Acid Analysis.—The total nucleic acids were extracted from the column cuts by the hot perchloric acid method of Schneider (12). The relative amounts of these nucleic acids in each cut were determined by measuring the optical density of these extracts in 0.1 N perchloric acid at 265 mμ. This procedure was shown to give linear results with serial dilutions of buffy coat suspensions prepared by centrifugation.

Blood Donors.—Healthy male medical students were used as donors without regard to recency of food intake or time of day.

RESULTS AND DISCUSSION

Columns.—In preliminary experiments differences among various inert materials such as polystyrene, glass, siliconized glass, and paper were not striking. This suggested a common mechanism for the adherence of PMN on columns of these materials. On the other hand, when more reactive surfaces such as ion exchange resins, or ion exchange celluloses were used, more individuality in response was noted. These latter materials were therefore set aside temporarily as introducing complicating factors peculiar to each, and a standard column of siliconized glass beads was studied in detail.

Beads were preferred to glass wool because it was found very difficult to obtain uniform packing of the glass wool columns. Although smaller beads were satisfactory, No. 100 size was used for all experiments to minimize unnecessary turbulence. Fig. 1 shows the column with a water jacket. Similar columns

without a water jacket were used in the early experiments which were run at room temperature (24°–27°C).

Recoveries of Polymorphonuclear Neutrophils (PMN) and Lymphocytes in Successive Milliliters of Effluent Blood.—Fig. 2 shows the result of a single column run in which each successive milliliter of effluent blood was analyzed individually for PMN and lymphocytes. The removal of the PMN becomes

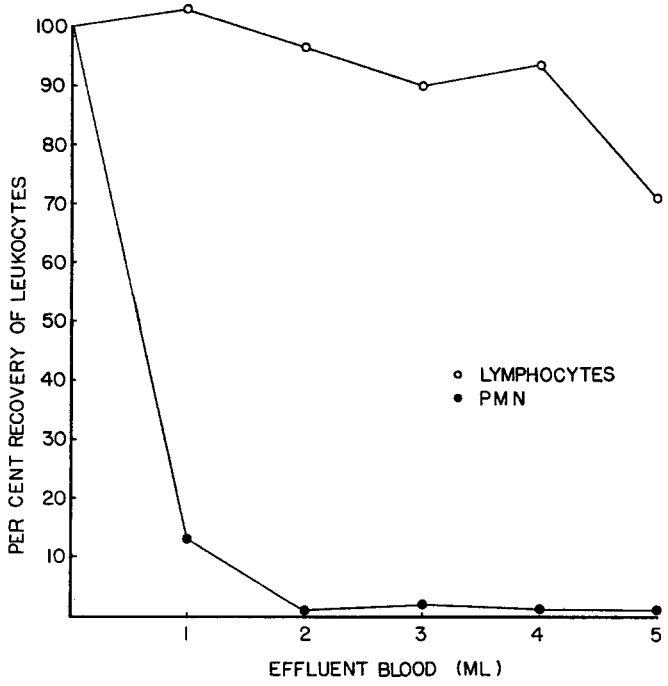

FIG. 2. Recoveries of polymorphonuclear neutrophils (PMN) and lymphocytes in successive milliliters of effluent blood. This is the result of a single column run at room temperature (24°C). The column was packed to a height of 4 cm with No. 100 siliconized glass beads, the flow rate was 0.10 ml/minute and the anticoagulant heparin (0.10 mg/ml). The blood was allowed to stand at room temperature for 1 hour before the column run began.

maximal after the 1st ml. has emerged from the column; subsequent 1 ml. portions show little additional change. There is a suggestion that the fraction of lymphocytes removed becomes higher as the run progresses, and some support for this appears in Table I. The reproducibility of the PMN and lymphocyte recoveries from one subject to another is shown in Table I, which summarizes the recoveries of both species in successive milliliters of effluent for 10 unselected normal subjects. The small standard deviations obtained on the PMN recoveries indicate a high degree of consistency, whereas the results

obtained from the lymphocyte recoveries show a larger variability. The high standard deviation shown for the lymphocytes in the 4th ml of effluent is largely the result of a single very high (153 per cent) recovery. The data are sufficiently consistent, however, to support the view that the effects shown reflect the influence of the variables in the system rather than those in the donors.

Effect of Time between Venipuncture and Column Run on Recovery of Polymorphonuclear Neutrophils (PMN) and Lymphocytes.—Many chemical changes have been shown to occur in shed blood (13, 14). It became important, therefore, to obtain information on the effect of the delay between drawing the blood and passing it through a column. Fig. 3 compares the results of column runs

TABLE I

*Recoveries of Polymorphonuclear Neutrophils (PMN) and Lymphocytes in Successive Milliliters of Effluent Blood**

Milliliter analyzed	Per cent recovered in effluent blood‡	
	Polymorphonuclear neutrophils (PMN)	Lymphocytes
1st	15 ± 10	86 ± 15
2nd	5 ± 4	79 ± 14
3rd	5 ± 5	69 ± 16
4th	4 ± 6	73 ± 38

* These experiments were conducted under the same conditions as described for the experiment shown in Fig. 2, except that the temperature varied from 23°–27°C.

‡ Average ± the standard deviation of the mean for 10 unselected, fed, adult males.

commenced immediately after the blood was drawn with the results obtained after 1 and 2 hours' delay. Except for the 1st ml of effluent blood, there does not appear to be much difference, but the recoveries from the 1st ml do suggest that if the column is run without delay the PMN are less adherent. These experiments, of course, do not reveal the effect of changes occurring in the first 10 minutes after the blood is drawn, and these early changes may be very important. A later series of experiments, also run in triplicate, using heparin from which the phenol had been removed gave essentially the same result.

Effect of Flow Rate and Column Length upon Recoveries of Polymorphonuclear Neutrophils (PMN) and Lymphocytes.—Fig. 4 A shows the relationship between per cent recovery of PMN and column length at four different flow rates. It is clear that at the higher flow rates there is a much greater retention of PMN on the longer columns. Thus, at 1 ml/minute the recovery of PMN drops from 90 to 25 per cent as the column length increases from 0.5 to 5 cm. At the lower flow rates the effect of column length is less noticeable, especially in columns 3

cm in length or greater. This suggests that at these slower flow rates, the PMN are chiefly retained on the upper 2 or 3 cm. of the column. These results are consistent with the hypothesis that retention on the column depends upon a PMN striking a glass bead under conditions permitting permanent adhesion.

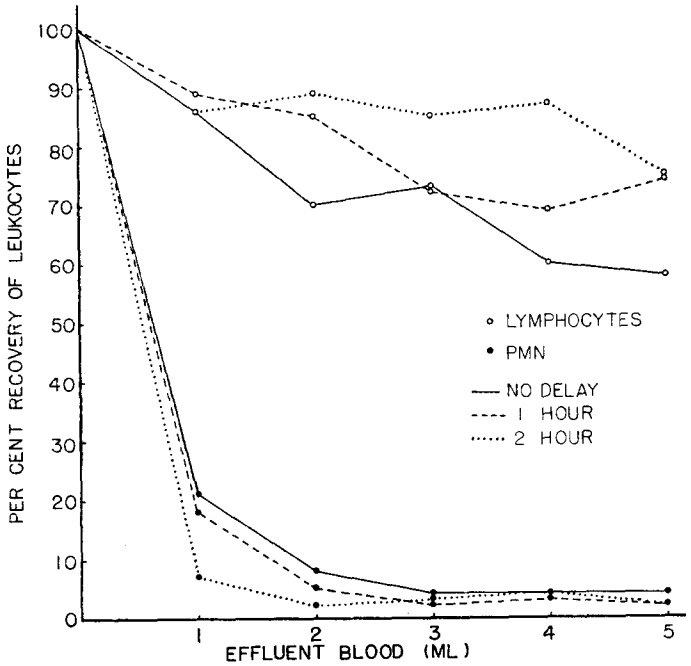

FIG. 3. Effect of time between venipuncture and column run on recovery of polymorphonuclear neutrophils (PMN) and lymphocytes. Blood samples from three separate donors were run with minimal delay and after standing 1 and 2 hours at room temperature (25°–27°C). Each point on the graph is an average obtained from three experiments. The columns were packed with No. 100 siliconized glass beads to a height of 2 cm, the flow rate was 0.2 ml/minute, and the anticoagulant was heparin 0.10 mg/ml. When the samples were run with minimal delay the time elapsing between the venipunctures and the beginning of actual flow through the columns was about 10 minutes. The columns were run at room temperature.

The longer column would provide the greater probability of a hit, while the slower flow rate would be less likely to sweep the PMN back into the stream. These average figures suggest furthermore that there are a few PMN of low adhesive character since they are not removed by even the longest columns.

Fig. 4 B shows a similar plot for lymphocytes. There does not appear to be any clear relationship between lymphocyte recoveries and flow rate or column length. The lack of correlation between the two sets of curves (those for the PMN recoveries and those for the lymphocyte recoveries) indicates a measure

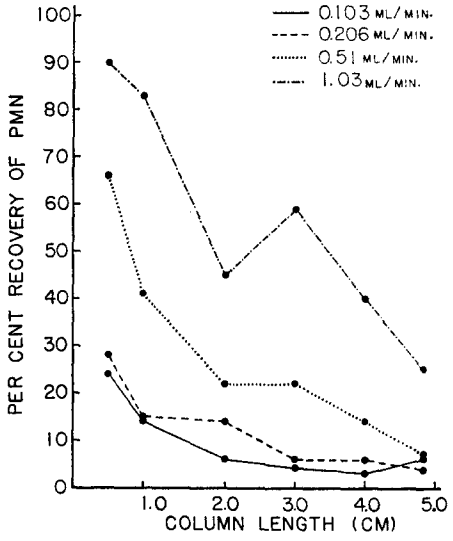

FIG. 4A

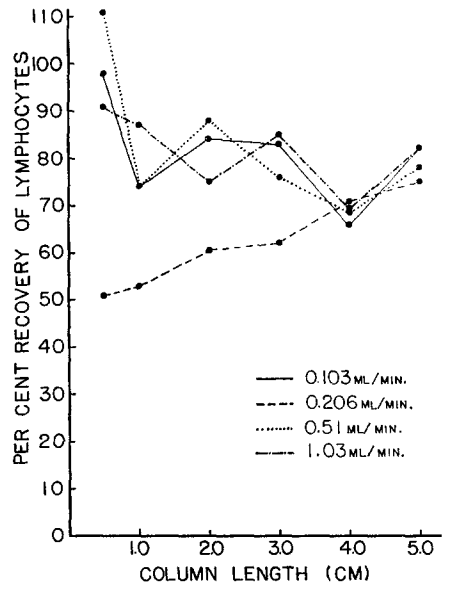

FIG. 4B

FIG. 4 A and 4 B. Effect of flow rate and column length upon recoveries of polymorphonuclear neutrophils (PMN) and lymphocytes. Each point on the graphs is an average obtained from three experiments. Twelve donors were used, blood from each being run at one flow rate through 6 columns of different length. The columns were run 3 at a time about 1 hour apart with the column lengths randomized between the first and second runs. The blood was allowed to stand 1 hour at room temperature before the first 3 columns were run. The columns were packed with No. 100 siliconized glass beads, the anticoagulant was heparin (0.1 mg/ml), and the columns were run at room temperature (23°–27°C). Six ml of blood was added to each column and the entire volume of effluent collected in one receiver. The recoveries were based on the comparison of the cells found in these effluents with the cells found in the original bloods.

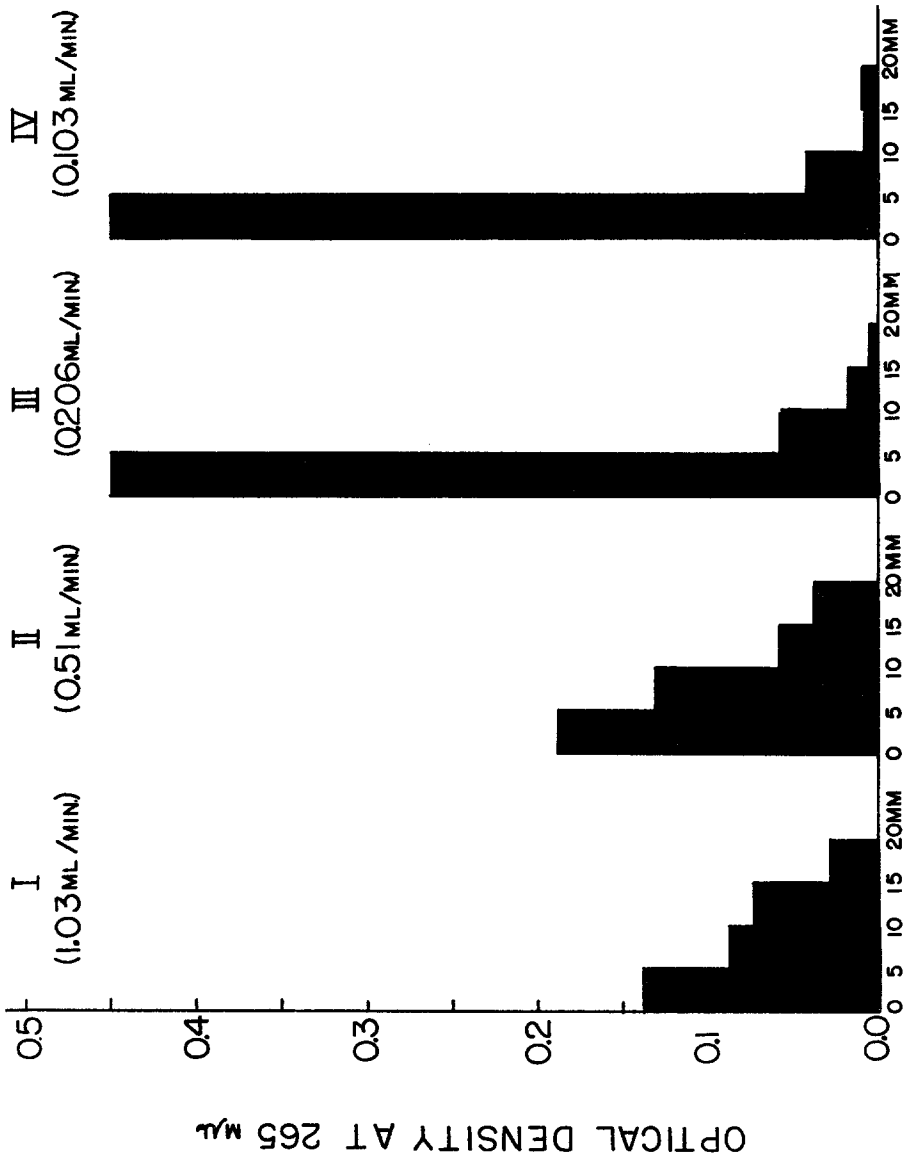

FIG. 5. Relationship between flow rate and position of the leukocytes and platelets retained on the glass bead columns. Twenty ml of blood was obtained from a single donor, heparinized (0.1 mg/ml) and allowed to stand at room temperature for 1 hour. It was then divided into four equal portions each of which was added to a column packed to a height of 2 cm with No. 100 siliconized glass beads. After the blood had passed through (0.1 ml/minute), two 1 ml washes of Krebs-Ringer phosphate were gently added, each being passed through also at the 0.1 ml/minute rate. The columns were then as white as before the run. They were cut off just above the level of the packing and extruded 5 mm at a time. These cuts were extracted for total nucleic acids by the method of Schneider (12). The optical densities of these extracts at 265 mu were then determined. Fig. 5 shows data from a single experiment, but 4 other similar experiments were done with essentially the same result.

of independence between the degree of adhesiveness of the two cellular species. However, since in almost all circumstances some members of both species were retained on the column, the prior adherence of one cannot be ruled out as a requirement for the adherence of the other.

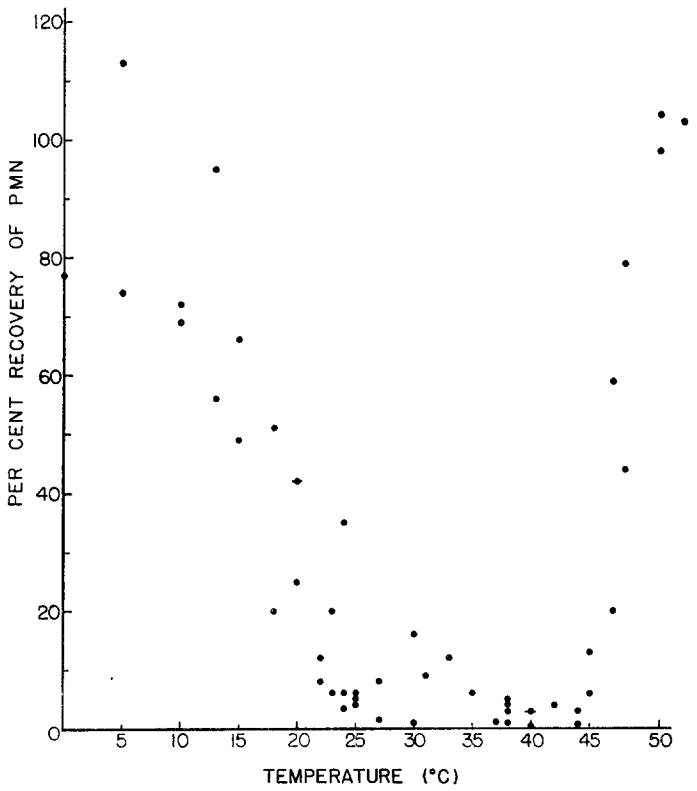

FIG. 6. Effect of temperature on the recovery of polymorphonuclear neutrophils (PMN). Each point represents one column run, in which 5 ml of heparinized blood (0.10 mg/ml) was allowed to stand 1 hour at room temperature and then run through 4 cm of No. 100 siliconized glass beads, at 0.10 ml/minute. Four columns, each at a different temperature, were run simultaneously using blood from one donor. A spread of at least 10°C was maintained in each set to minimize individual differences in donors. Thirteen separate donors were used making a total of 52 separate column runs.

Relationship between Flow Rate and Position of the Leukocytes and Platelets Retained on the Glass Bead Column.—Direct microscopic examination of the different levels of the glass bead columns by means of stained smears showed that most of the leukocytes and platelets were retained in the upper portions. Using columns 2 cm high an attempt was made to quantitate this localization

of formed elements and relate it to flow rate. The nucleic acid concentration in any cut was used as an index of the concentration of leukocytes and platelets at that level. Although the platelets make a smaller contribution to the nucleic acid concentration than the leukocytes, they must be included since they have been shown to contain considerable amounts of ribonucleoprotein (15). Contamination by red cells was minimized by gently washing the column with a Krebs-Ringer phosphate solution after the blood had been passed through. This wash was found to contain from 4 to 8 per cent of the leukocytes originally present in the 5 ml of blood added to the column.

The results of a typical experiment are shown in Fig. 5, in which the optical density at 265 mμ is used as the index of the relative numbers of the formed elements at each level. The figure shows that the expectations suggested by the earlier flow rate studies are borne out, namely that at the more rapid flow rates the retained components are spread down the column, whereas at the slower flow rates they remain near the top. Indeed, if one assumes that at a flow rate of 0.10 ml/min. most of the formed elements are retained on the upper 0.5 cm of the columns, it can be shown that they would have more than sufficient surface to occupy the entire area provided by the beads. Thus, assuming that 2.5×10^7 leukocytes adhere to a column during the passage of 5 ml of blood, and that the average diameter of these cells is about 15×10^{-4} cm (16), it can be calculated that they will occupy 44 cm^2 of surface. Similarly, assuming an average diameter for platelets of 2×10^{-4} cm (16) and a total number of 1.25×10^8/5 ml of blood, it is found that they also will occupy 44 cm^2. Since the top 0.5 cm of the column has a total area of only 67 $cm,^2$ it seems likely on this basis that there was some overlapping.

The microscopic examinations showed that the retained elements were in about the same proportions at the various levels, and that they were often intermixed. The PMN were sometimes single and sometimes in clusters; the platelets were usually single and, surprisingly, seldom in clusters. Strands of a material staining purple with Wright's stain were scattered through the smears, some with adjacent or attached clusters of PMN. Most of the cells and platelets were attached to the slides, rather than to the beads. It was not possible to determine whether this latter observation was an artifact of making the slides.

Effect of Temperature on Adhesiveness of Leukocytes and Platelets.—Fig. 6 shows that when blood is passed through the glass bead columns the temperature profoundly affects the adhesiveness of the PMN. The adhesiveness is greatest in the range 25°–44°C amounting to the removal of about 95 per cent of the PMN. Above and below this temperature range the adhesiveness falls off—more sharply as one goes to higher temperatures. At 52°C the PMN appear quantitatively in the effluent bloods. On the low temperature side (0°–5°C) recovery of PMN is about 75 per cent. Fig. 7 shows the effect of

temperature on the recovery of lymphocytes and platelets. The platelet data are clear cut in that there was almost no recovery of platelets above 5 per cent regardless of the temperature. The lymphocyte recoveries appear on the other hand to parallel the PMN but at a much higher level with a low plateau of about 50 per cent recovery in the 25–44°C range.

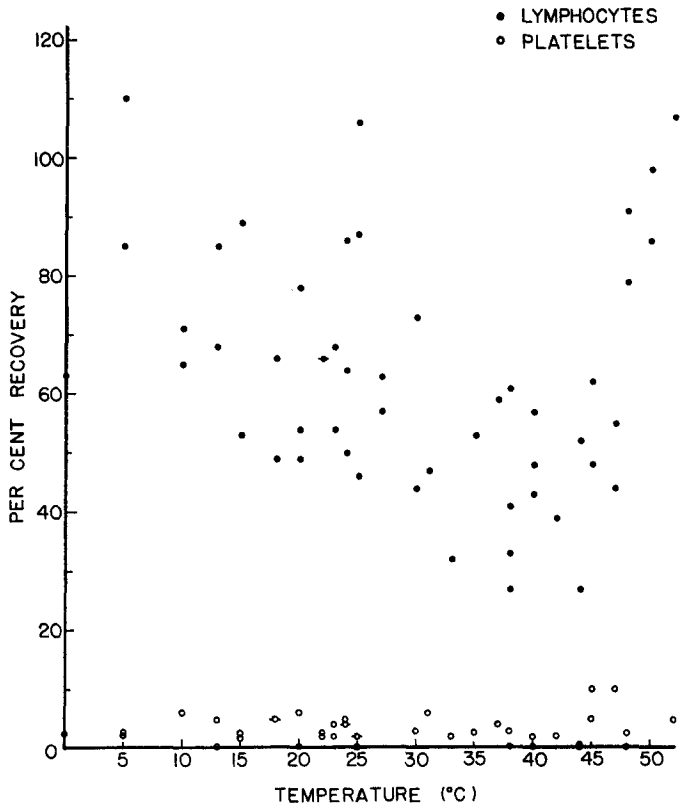

FIG. 7. Effect of temperature on the recovery of lymphocytes and platelets. These data are from the same experiments described under Fig. 6.

Direct microscopic observation of the ameboid movement of the PMN at different temperatures was carried out to supplement the column data. A striking parallelism between adhesiveness and ameboid movement was found. Thus maximum ameboid movement was noted in the range 30°–43°C. As the temperature was lowered below 30°C, there was a gradual loss of ability of the PMN to migrate. At 27°C a noticeable slowing was seen. This became marked by 23°C, and by 15°C almost no movement was observed. Movement was restored by raising the temperature. At the high end of the scale the cut-off

was much more rapid. At about 44°C a slowing of some PMN was seen, and by about 48°C all the cells were dead. Baudisch (17), studying granulocytes by a microscopic technique, also found a maximum of adhesiveness between 25° and 44°C.

The qualitative and quantitative association of ameboid movement with adhesiveness suggests some connection between the two processes but it may be that both depend upon the energy metabolism of the cell. The platelet adhesiveness appears to be independent of the temperature and suggests a

TABLE II

Effect of Various Anticoagulants on the Adhesiveness of Leukocytes and Platelets

Anticoagulant*	Per cent recovery‡ from glass bead columns§		
	Polymorphonuclear neutrophils (PMN)	Lymphocytes	Platelets
Heparin	4 ± 3	90 ± 29	7 ± 6
Heparin + EDTA	97 ± 7	77 ± 6	76 ± 22
Heparin + citrate	89 ± 34	89 ± 28	8 ± 3
Heparin + oxalate	94 ± 24	99 ± 11	7 ± 5

* Twenty ml. of blood was heparinized (0.10 mg/ml) and immediately 4.51 ml aliquots were combined with additions as follows: for heparin alone, 0.49 ml of 0.85 per cent NaCl; for heparin plus ethylenediaminetetraacetic acid disodium salt (EDTA), 0.49 ml of 102.7×10^{-3} M EDTA; for heparin plus citrate, 0.68 ml of 77×10^{-3} M sodium citrate; for heparin plus oxalate, 0.49 ml of 102.7×10^{-3} M sodium oxalate. These additions give final concentrations of EDTA, citrate, and oxalate in the influent bloods of 10×10^{-3} M. After the tubes stood at room temperature for 10 minutes the columns were started.

‡ Average ± maximum deviation from the mean obtained from three separate experiments.

§ No. 100 siliconized beads were packed to a height of 4 cm, the flow rate was 0.10 ml/minute, and the temperature of the columns was 37°C.

mechanism different from that of the PMN. The fact that most platelets adhered below 15°C while the PMN did not, shows that the prior adherence of the platelets is not a sufficient condition for the adherence of the PMN.

Effect of Various Anticoagulants on the Adhesiveness of Leukocytes and Platelets.—A comparison of the effects of four anticoagulants upon the adhesiveness of PMN, lymphocytes, and platelets is shown in Table II. When heparin alone was used the PMN and platelets were both largely retained on the columns, whereas the lymphocytes were recovered in good yield. However, when ethylenediaminetetraacetic acid (EDTA) plus heparin was used the adhesiveness of all three formed elements was blocked. Heparin plus citrate or heparin plus oxalate had similar effects, permitting both lymphocytes and PMN to pass through the columns but causing retention of most of the platelets. Similar experiments, also in triplicate, carried out at room temperature gave essen-

tially the same results. Since the three anticoagulants which permit passage of the PMN all act by removing divalent cations, an essential role for divalent cation(s) in the adhesiveness of the PMN is indicated.

The adhesiveness of the platelets also appears to be dependent upon divalent cation(s), although the concentration(s) required by the platelets seems to be much lower than that required by the PMN. This is readily seen from a comparison of the stability constants of EDTA and citrate for magnesium and calcium ions. The logs, of these stability constants are as follows: EDTA-Mg, 8.6; EDTA-Ca, 10.5 (18); Citrate-Mg, 3.2; Citrate-Ca, 3.2 (19). Thus it can be argued that although citrate binds these two divalent cations strongly enough to block the adhesiveness of the PMN, enough are left to permit the adhesiveness of the platelets. On the other hand, the much greater stability of the binding with EDTA effectively blocks the adhesiveness of both PMN and platelets. This difference in divalent cation dependence suggests that PMN adhere to the columns by a mechanism different from that of the platelets. The results on the adhesiveness of platelets are in accord with the findings of Zucker *et al.* (20) who, working with platelet-rich plasma, found more satisfactory resuspension of platelets after centrifugation when EDTA was the anticoagulant than when citrate was the anticoagulant. Also Hellem (21), studying adhesiveness of platelets in whole blood, found 35 per cent adhesive platelets when 10×10^{-3} M citrate was the anticoagulant and only 2 per cent adhesive platelets when 4×10^{-3} M EDTA was the anticoagulant.

Microscopic examination of wet preparations of influent bloods containing all additions was carried out. The slide preparations were incubated at 37°C for 10 minutes and then examined under oil immersion at room temperature. Active ameboid movement of the PMN was observed in the presence of heparin alone. The other influents were less active but slow change of shape with pseudopod formation occurred in all of them. The effluents from the columns containing the chelating agents were also examined in wet preparations. The PMN in all cases were changing shape rather actively and streaming of the granules could be seen. The cells, however, were only able to move within their own axes. In the EDTA effluent it was particularly clear that the cells, although able to change shape, could not adhere to the glass. These PMN in the EDTA effluents became more active with time, so that after incubation at 30°C for 1 hour after the effluent was collected, their activity was dramatic. They were able to move through the rouleaux of red cells by forming pseudopods which they used almost like legs. Definite movement from place to place was thereby achieved without adherence to the slide. The PMN in the citrate likewise became more active with time, but they seemed to be able to get some purchase (about 50 per cent of them) on the glass of the slide and move about. The oxalate preparations were usually actively moving but not as able to move from place to place as those containing citrate. It seems likely from these

observations that in the PMN there is a definite separation of the adhesive function from the capacity of the cells to change shape.

Effect of Divalent Cations on the Adhesiveness of Polymorphonuclear Neutrophils (PMN), Lymphocytes, and Platelets in Blood Treated with a Chelating Ion Exchange Resin.—Dowex A-1 chelating resin was used to remove the divalent cations from whole blood without introducing the uncertainties due to a dissolved reagent. Blood was passed through this resin and then physiological amounts of calcium and magnesium ions were added back separately and together.

(Forty ml of blood was drawn, heparinized (0.10 mg/ml and immediately cooled to 27°C in a water bath. Five ml was placed aside at room temperature, and the balance passed through a column of Dowex A-1 resin 2.8 cm in diameter by 4 cm in height at a rate of 1.23 ml/minute. Four experiments showed that under these conditions recovery of leukocytes averaged 88 per cent and recovery of platelets averaged 76 per cent. Aliquots of 4.5 ml from the effluent of this column were then placed in each of 4 test tubes and 4.5 ml of the heparinized blood which had not been through the resin was added to a fifth tube. Additions were then made as follows: to the heparin tube (this was the aliquot not passed through the resin column), 0.50 ml of Na/K saline; to the A-1 resin tube, 0.50 ml of Na/K saline; to the A-1 resin plus $CaCl_2$ tube, 0.50 ml of Na/K saline which was 14.9×10^{-3} M in $CaCl_2$; to the A-1 resin plus $MgCl_2$ tube, 0.50 ml of Na/K saline which was 5.95×10^{-3} M in $MgCl_2$; to the A-1 resin plus $CaCl_2$ plus $MgCl_2$ tube, 0.5 ml of Na/K saline which was 14.9×10^{-3} M in $CaCl_2$ and 5.95×10^{-3} M in $MgCl_2$. These additions are such that physiological concentrations of calcium (2.5×10^{-3} M) and magnesium (1.0×10^{-3} M) ions would be expected in the expanded plasmas of these influent bloods if one assumes that the hematocrit value was 45 per cent and that all the ions were contributed by the additions. Three pilot experiments showed that the calcium ion concentration in plasma after passage through a Dowex A-1 resin under these conditions was below 0.10×10^{-3} M.)

The effect of these procedures on the adhesiveness of the PMN, lymphocytes and platelets is shown in Table III. Similar experiments also carried out in triplicate, but at room temperature, gave essentially the same results. Apparently both calcium and magnesium ions are necessary for the full adhesiveness of the PMN. Indeed, the calcium ion alone had the paradoxical effect of increasing the recovery of the PMN while when combined with the magnesium ion it produced the greatest reestablishment of adhesiveness. Magnesium ions alone were fairly effective in restoring adhesiveness. As usual the lymphocytes did not show striking differences in the various systems. The data show clearly that only platelets from blood treated with the A-1 resin alone passed through the glass bead columns. Platelets from all other influents were largely retained. This result indicates that the adhesiveness of the platelets although dependent upon either calcium or magnesium ion is not as selective in this regard as the adhesiveness of the PMN. Zucker *et al.* (20) also found that the adhesiveness of platelets could be maintained by either calcium or magnesium ions, although it was their opinion that the naturally effective ion was magnesium. The fact

that when calcium ions alone were added to the blood treated with A-1 resin, the platelet recoveries were low (17 per cent) and the PMN recoveries were high (76 per cent), again shows that retention of platelets is not a sufficient condition for the retention of PMN.

Effect of Some Metabolic Inhibitors on the Adhesiveness of Polymorphonuclear Neutrophils (PMN) Lymphocytes and Platelets.—The relationship of adhesiveness to metabolic sources of energy was explored with several inhibitors.

21 ml of blood was drawn, heparinized (0.10 mg/ml) and allowed to stand for 30 minutes at room temperature. Five ml aliquots were then transferred to each of 4 test tubes and

TABLE III

Effect of Divalent Cations on the Adhesiveness of Polymorphonuclear Neutrophils, (PMN), Lymphocytes, and Platelets in Blood Treated with a Chelating Ion Exchange Resin

System	Per cent recovery* from glass bead columns‡		
	Polymorphonuclear leukocytes	Lymphocytes	Platelets
Heparin	3 ± 3	66 ± 21	6 ± 2
A-1 resin	55 ± 16	73 ± 12	88 ± 47
A-1 resin + $CaCl_2$	76 ± 16	103 ± 24	17 ± 7
A-1 resin, + $MgCl_2$	29 ± 19	94 ± 27	18 ± 14
A-1 resin, + $CaCl_2$ + $MgCl_2$	14 ± 9	67 ± 19	12 ± 10

Additions were calculated to give the following concentrations in the expanded plasmas of the influent bloods: $CaCl_2$, 2.5×10^{-3} M; $MgCl_2$, 1.0×10^{-3} M.

* Average ± maximum deviation from the mean for three separate experiments using blood from different donors.

‡ No. 100 siliconized beads were packed to a height of 4 cm., the flow rate was 0.10 ml/minute, and the temperature of the columns was 37°C.

additions made as follows: to the control tube, 0.5 ml of Na/K saline; to the NaCN tube, 0.5 ml of Na/K saline which was 44×10^{-3} M in NaCN; to the dinitrophenol tube, 0.5 ml of Na/K saline which was 4.4×10^{-3} M in dinitrophenol; to the iodoacetamide tube, 0.5 ml of Na/K saline which was 44×10^{-3} M in iodoacetamide. The contents of each tube were mixed using a polyethylene pipette with a large opening, stoppered with a paraffined cork, incubated at 37°C for 30 minutes, and finally added to the columns.

Table IV compares the effects of three inhibitors, sodium cyanide, 2,4-dinitrophenol and iodoacetamide on the adhesiveness of the PMN, lymphocytes, and platelets. Iodoacetamide inhibited the adhesiveness of all three components, whereas the other inhibitors were without effect on any component. Sodium cyanide was further tested in triplicate experiments at 0, 4, 8, and 12×10^{-3} M. No effect on the adhesiveness of any component was noted. Another series of experiments using dinitrophenol at a concentration of 8×10^{-3} M likewise showed no effect of this inhibitor. It is concluded that the

adhesiveness of human PMN, lymphocytes, and platelets is dependent upon intact sulfhydryl groups and independent of both oxidative phosphorylation and oxidative pathways linked to cytochrome oxidase.

The study of factors affecting phagocytosis in several laboratories show an interesting parallelism with the experiments reported here on adhesiveness of PMN. Straus and Stetson (2), found that dinitrophenol was without effect on the phagocytosis of polystyrene latex particles by human PMN, whereas iodoacetate inhibited it. Similarly Sbarra and Karnovsky (22) working with guinea pig leukocytes showed that phagocytosis was independent of both cyanide and dinitrophenol but considerably inhibited by iodoacetate. The

TABLE IV

Effect of Some Metabolic Inhibitors on the Adhesiveness of Polymorphonuclear Neutrophils (PMN), Lymphocytes, and Platelets

Inhibitor*	Per cent recovery‡ from glass bead column§		
	Polymorphonuclear	Lymphocytes	Platelets
None	1 ± 1	71 ± 13	7 ± 4
NaCN	3 ± 3	76 ± 17	7 ± 5
DNP	2 ± 1	78 ± 30	5 ± 1
Iodoacetamide	103 ± 5	110 ± 36	67 ± 16

* The concentrations of the inhibitors in influent bloods were: NaCN, 4.0×10^{-3} M; 2,4-dinitrophenol, 4.0×10^{-4} M; iodoacetamide, 4.0×10^{-3} M.

‡ Average ± maximum deviation from the mean for three separate experiments using blood from different donors.

§ No. 100 siliconized beads were packed to a height of 4 cm, the flow rate was 0.10 ml/minute, and the temperature of the columns 37°C.

simplest explanation of these data would propose that both adhesiveness and phagocytosis depend upon an active process in the cell membrane which derives its energy from glycolysis. The experiments of Fisher and Ginsberg (23) with guinea pig leukocytes showed both depressed lactate production and depressed phagocytosis when the leukocytes were infected with influenza virus. This result is consistent with the above conclusion regarding glycolysis but it is possible that some other process is also involved. For example, in the studies of Philipson and Choppin (24) on the attachment of enteroviruses to erythrocytes they concluded that a sulfhydryl inhibitor prevented such an attachment by occupying the free —SH groups on the surface of the virus. Similar free —SH groups on the surface of the PMN might be necessary for adhesiveness and phagocytosis.

Effect of Concentration of Iodoacetamide on the Adhesiveness of Polymorphonuclear Neutrophils, Lymphocytes, and Platelets.—The effect of the concentra-

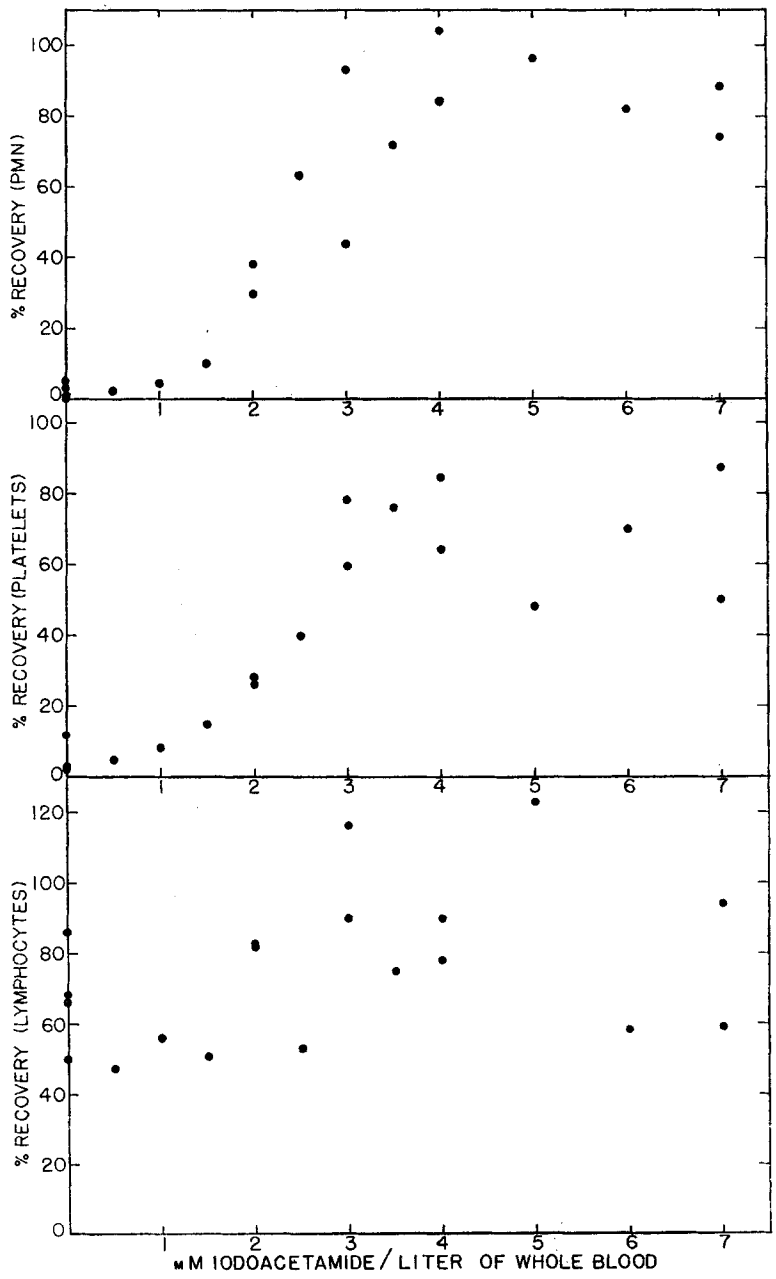

FIG. 8. Effect of concentration of iodoacetamide on the adhesiveness of polymorphonuclear neutrophils (PMN), lymphocytes, and platelets. The conditions of these experiments were the same as those described in Table IV.

tion of iodoacetamide on the adhesiveness of PMN, lymphocytes, and platelets is shown in Fig. 8. Both PMN and platelets show a beginning effect at about 1.5×10^{-3} M which reaches 80 to 90 per cent inhibition of adhesiveness at 4×10^{-3} M. On the other hand, the adhesiveness of the lymphocytes shows no clear cut dependence upon the concentration of iodoacetamide. Microscopic observations on the motility of the PMN as a function of iodoacetamide concentration were carried out in wet preparations at room temperature. A close parallel between loss of the ability to change shape by pseudopod formation and loss of adhesiveness was demonstrated.

Since both PMN (25), and platelets (26) are known to have actively glycolyzing systems, the data are consistent with the hypothesis that the iodoacetamide acts on the adhesiveness by blocking glycolysis. However, the data are equally consistent with any hypothesis which ascribes the adhesiveness of the two formed elements to sulfhydryl-dependent mechanisms with similar sensitivities to iodoacetamide.

The effect of 5×10^{-3} M iodoacetamide on the clotting time of blood on the columns was also studied.

Five ml of freshly drawn blood was mixed gently with iodoacetamide in 0.5 ml of Na/K saline in the absence of heparin and quickly added to a standard 4 cm column. The column was then run at 0.10 ml/minute and 37°C in the usual manner. Two control columns from the same blood sample were run simultaneously. One contained blood plus the Na/K saline but without either heparin or iodoacetamide; the other contained only K/Na saline plus heparin. The time of clotting was determined by noting when the effluent drops stopped appearing.

In four separate experiments, using blood from different donors, the blood containing the iodoacetamide clotted in an average time of 7 ± 2.4 minutes (average $\pm$ standard deviation) while the blood containing only the Na/K saline clotted in an average time of 8 ± 1 minutes. In all instances a firm clot formed in each column, except of course, in the columns containing heparin. On dissection the clots in the columns containing iodoacetamide seemed the more easily disrupted. The significance of these results is discussed below.

GENERAL DISCUSSION

In a system as complex as shed whole blood containing an anticoagulant interpretation of the roles of the various components is necessarily limited at this time. However, some tentative conclusions are offered regarding the adhesiveness of the PMN and these will be discussed below. Platelet adhesiveness and lymphocyte adhesiveness will be mentioned only in passing in this discussion.

It appears improbable that all degrees of PMN adhesiveness depend upon prior adherence of platelets at the same site. This statement is made in the face of the finding in the present study that adherence of PMN was never observed under conditions in which platelets did not also adhere. It is based

on the microscopic observation of the migration of PMN on a glass slide. They can be seen to attach, flatten out, and move along the surface of the slide. It does not seem possible that they are always underlaid by platelets or platelet residues. However, a requirement for the prior adherence of the platelets on adjacent sites remains a possibility. Soluble platelet factors released after adherence of the platelets could then diffuse the short distance to the PMN and affect their adhesiveness. Indeed, soluble factors released even without adherence of the platelets may be essential. The influence of such factors will not be determined easily, since preparation of plasma free of "platelet activity" is very difficult (27, 28). Finally, other plasma factors not of platelet origin may also be involved.

Another possibility for the mechanism of the adherence of the PMN is that they attach to small strands of fibrin which have formed on the beads. According to this hypothesis there is just enough trauma to the platelets at the surface of the beads to produce an incipient clot. This view is supported by the finding of the amorphous strands of material on the stained smears from the column packing. On the other hand, it would be expected that adding back calcium ions to blood lacking it, would accelerate this process. However, the experiments with the blood treated with A-1 chelating resin show that the addition of calcium ions actually decreased the adhesiveness of the PMN while permitting the platelets to adhere strongly. It is possible, of course, that the adhesiveness of the PMN requires preconditions both on the adsorbant and on the PMN. Thus the development of the fibrin strands on the beads could be calcium-dependent, while the adhesive properties of the surface of the PMN could be magnesium-dependent.

The finding that a concentration of iodoacetamide (5×10^{-3} M) which has only a small effect on the clotting time almost completely inhibited the adhesiveness of the PMN also bears on this question. If small fibrin strands were alone responsible for the adherence of the PMN we would not expect inhibition of adhesiveness by iodoacetamide. Thus we have further support for an active process on the part of the PMN, although the data do not exclude some preconditions on the adsorbant.

Authors concerned with the *in vivo* adhesiveness of PMN to the endothelial wall also have attempted to assess the role of fibrin. Zweifach (29, 30) and also Wood (3) have advanced the theory that there is some gelatinous precipitate or fibrin formed over the injured endothelium to which the passing PMN adhere. Other authors, more recently, have opposed this view. Allison *et al.* (31) observing inflammation in the ear chamber of the hypofibrinogenemic rabbit found no decrease in stickiness of the PMN. Similarly Marchesi and Florey (32) present electron micrographs of developing inflammation which show cross-sections through endothelial cells and their adherent PMN. No morphological evidence of fibrin between the two apposed surfaces was demon-

strated. It appears possible that both views are correct and that the differences reported depend upon type of injury and animal. But whatever the changes on the endothelial surface ultimately prove to be, it is strongly suggested by the present *in vitro* work that the PMN also plays an active role in the process of adhesive contact.

SUMMARY

Some factors affecting the retention of human polymorphonuclear neutrophils (PMN), lymphocytes, and platelets on a siliconized glass bead column were explored.

PMN were more effectively retained when the flow rates were slow and the columns long. They were found largely on the upper portions of the columns except with rapid flow rates when they spread down the columns. PMN retention on the columns was greatest in the range 30°–43°C. Both magnesium and calcium ions were required for full adhesiveness; calcium ions alone were unable to restore adhesiveness to PMN from blood which had been treated with a chelating resin to remove divalent cations. The adhesiveness of the PMN was independent of cyanide and dinitrophenol, but was almost completely eliminated by iodoacetamide. Under all the conditions mentioned above in which adhesiveness was lost there was a concurrent loss of the usual ability of the PMN to migrate, but at least in the presence of EDTA, a capacity to change shape by pseudopod formation remained.

Lymphocytes were retained on the columns to a much lesser extent than the PMN under all conditions and, within limits, this retention was not related to either flow rate or column length. Maximum lymphocyte retention occurred in the range 30°–43°C. No dependence of lymphocyte adhesiveness was shown for divalent cations, cyanide, dinitrophenol, or iodoacetamide, but such dependence is not excluded by the data obtained.

Platelets were largely retained by the glass bead columns under most conditions and this was unrelated to temperature in the range 0°–50°C. Their adhesiveness was found to require either magnesium or calcium ions and to be blocked by iodoacetamide.

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