who reported values ranging from 156 to 643 in the blood from random patients (ages not stated).

Summary. Ascorbic acid and the sum of ascorbic, dehydroascorbic and diketogulonic acids in the mast cell fraction (about 92% mast cells) from peritoneal washings of normal rats, and in mouse ascites tumor mast cells (about 98% mast cells), were determined per 10^6 cells and per μg protein-nitrogen. Ascorbic acid (551 mμg/10^6 cells) comprised about 87% of the sum of the acids in the normal cells and about 98% (271 mμg/10^6 cells) of the sum in the tumor cells. The concentrations of ascorbic acid in the normal and tumor cells were 13 and 10 mμg/μg protein-nitrogen, respectively. The ascorbic acid per 10^6 cells in the rat macrophage cell fraction (96% macrophages) was 38% of that in the mast cell fraction, while the ascorbic acid per μg protein-nitrogen in the former was 1⅓ times that in the latter.

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Pathogenesis of Acute Inflammation. VI. Influence of Osmolarity and Certain Metabolic Antagonists Upon Phagocytosis and Adhesiveness by Leucocytes Recovered from Man.* (30098)

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A clear understanding of the process responsible for the adhesion of polymorphonuclear granulocytes (PMNG) to vascular endothelium during acute inflammation has not been obtained. It is recognized that this event represents a prime step in the mobiliza-

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tion of blood borne phagocytic cells for defense of the host against a variety of noxious agents. Stemming from the results of experiments concerned with the *in vitro* adhesiveness of PMNG recovered from peritoneal exudate of rabbits(1) and from the venous blood of healthy human donors(2), it seemed that an enzymatic mechanism, probably proteolytic in nature, might be responsible at least in part for initiating this event even though confirmatory evidence from experiments with intact subjects was lacking.

The studies reported here deal with the same *in vitro* experimental model(1,2) and in particular define in detail certain conditions that influence the phagocytic and adhesive properties of PMNG recovered from venous blood of healthy human donors. It was found that inhibition of both functions of white blood cells was accomplished only when high concentrations of the metabolic antagonists were used and that hyperosmolarity of the test solutions was responsible for most of the observed effect.

Materials and methods. Glassware and solutions. Glass apparatus and equipment used for handling blood cells and bacterial cultures were siliconed, washed to eliminate contaminating chemicals, then baked for 2 hours at 170°C to destroy bacterial pyrogens(1). Chemicals to be studied were added directly to serum or were dissolved in either deionized distilled water or pyrogen free isotonic saline (Baxter Laboratories) before being added to the autologous serum samples.

Donors. Healthy, male, medical students served as professional donors and most studies were performed using blood from a select few. Intercurrent infection or other illness eliminated donors from use for at least 6 weeks or until recovery was judged complete.

Reagents. The following chemicals were used for experiments: Epsilon amino caproic acid (EACA) (Aldrich Chemical Co., Milwaukee, Wisc.), p-tosyl-L-arginine methyl-ester hydrochloride (TAME) (free base), diisopropyl fluro-phosphate (DFP) (Mann Research Laboratories, Inc., New York), ethylene diamine tetra acetate (EDTA) (J. T. Baker Chemical Co., Phillipsburg, N. J.), dinitrophenol (DNP) (Eastman Kodak, Rochester, N. Y.), quinidine HCl (Brewer and Co., Inc., Worcester, Mass.), L-ornithine hydrochloride, L-lysine hydrochloride, L-citrulline (Nutritional Biochemicals Corp., Cleveland, O.), glycine (Merck and Co., Inc., Rahway, N. J.), and 2-pyridinealdoxime methochloride (PAM) (Campbell Pharmaceuticals, Inc., New York).

Technique for harvesting leucocytes. Sera for resuspending autologous white blood cells were harvested prior to experimentation and stored aseptically at either 4°C or frozen at −18°C until required. Frozen samples were thawed at 25°C and no effort was made to inactivate complement. Whenever possible, the exact amount of a chemical to be studied was added to 2.0 ml serum. Otherwise, concentrated solutions of these compounds, always in a volume of 0.1 ml, were added to 2.0 ml serum samples in order to insure constant dilution ratios.

Venous blood was obtained in syringes containing EDTA for anticoagulation according to previously described methods(3). The technique for obtaining a preparation of PMNG by adding a small amount of clinical dextran to reduce contamination by platelets and erythrocytes has also been noted(3). The button of PMNG harvested from the platelet containing plasma was then washed twice in 5.0 ml Na/K saline with centrifugation performed at 800 rpm. Following the second wash, the white blood cell centrifugate from 8.0 ml whole blood was resuspended in 1.0 ml autologous serum. A white cell count on this mixture revealed approximately 1×10^7 PMNG per ml.

Bacterial culture. A rough strain pneumococcus handled as described elsewhere was used for all experiments(1).

Bacterium-leucocyte mixtures. White blood cells were suspended in autologous serum containing appropriate concentrations of compounds to be studied and subjected to rotational mixing at 37°C for 30 minutes. Spontaneous loose clumping by cells during incubational contact with test compounds was minimal and viability was well preserved as determined by supravital stains with trypan blue. A washed centrifugate of the 4-hour

pneumococcus culture was then added to the leucocyte suspension to give a pneumococcus-PMNG ratio of 20 to 1. There then followed rotational mixing of combinations at 9 rpm with estimates of phagocytosis and clumping of cells into microscopic and macroscopic aggregates at 30 minutes as described before (1). The osmolarity of preparations was determined at the conclusion of each experiment by a Fiske model G osmometer. Hydrogen ion concentration of preparations both before and after rotational mixing was measured with a Beckman Model G pH meter. Viability of PMNG after mixing was assessed with trypan blue supravital stains. A better assay for viability was found, however, when PMNG were separated from serum containing the test compound and resuspended in normal autologous serum containing pneumococcus. In this instance brisk phagocytosis and adhesion of cells indicated that the inhibition was reversible and that injury was negligible.

Results. 1. *Influence of hydrogen ion concentration upon phagocytosis and clumping by human leucocytes.* The pH of the reconstituted PMNG-serum mixture with pneumococcus added was found to vary between 7.85 and 7.95 in a long series of experiments. By using certain buffers(1) the initial pH of this combination could be varied over a wide range from a low of 4.0 to a high of 10.0; but after 30 minutes rotational mixing, there was always a tendency to drift toward neutrality. It was soon apparent that human PMNG reacted to the pH of the suspending medium much as did rabbit cells(1), *i.e.*, both ingestion of bacteria and cellular aggregation were almost completely blocked below a pH of 5.10. At pH levels of 5.50 to 5.75 phagocytosis began to appear and was associated with moderate cellular clumping; and above pH 6.0, both reactions were brisk without any evidence of inhibition. With each increased increment of pH to a level of 9.0, phagocytosis and clumping by PMNG was active; however, above this point there was a sharp reduction in both functions which seemed to correlate well with cellular death.

2. *Influence of dinitrophenol (DNP) upon phagocytosis and clumping by human leucocytes.* It had been noted previously(2) that exposure of human PMNG to 0.4×10^{-1} M DNP in serum for 30 minutes inhibited almost completely both phenomena; whereas, 0.4×10^{-3} M had little inhibitory effect on either. Measurement of the osmolarity of 0.4×10^{-1} M DNP in serum yielded a value of 305 milliosmoles as compared to either serum alone, 300 milliosmoles, or serum with 0.1 ml saline, 297 milliosmoles (Table I). Of obvious importance on the other hand was the fact that the pH of 0.4×10^{-1} M DNP in serum was 5.5; well within inhibitory ranges as just noted in the preceding section. Non-inhibitory, less concentrated solutions of DNP were found to have pH ranges within more physiologic ranges.

3. *Influence of osmolarity of proteolytic enzyme inhibitors upon phagocytosis and clumping by human leucocytes.* As described earlier(2), several metabolic antagonists were found to block both phagocytosis and clumping by PMNG harvested from our human donors. One of these, EACA, an agent capable of blocking proteolysis, was active in concentrations of 0.5 M and higher. From these results, a number of compounds of similar configuration were also examined for inhibitory activity in this system and those exhibiting a substantial effect have been listed in Table I. A number of structurally related compounds were subjected to the same screening procedures but since none was found to exhibit antagonistic behavior, they have not been enumerated. These results were interpreted at first as supporting evidence for a specific but rather broad blockade of proteolytic enzyme activity(1,2). Subsequently, however, it was found that each inhibited only at very high osmolarities (Table I) although the pH ranges were within acceptable, non-inhibitory ranges. Of the structurally similar compounds, L-ornithine hydrochloride, L-lysine hydrochloride, L-citrulline, and glycine were also inhibitory but only at very high concentrations.

In view of the known depressant influence of hypertonic solutions upon the function of phagocytes(4,5,6) it became essential to determine whether or not similar concentrations of other substances would also block these

TABLE I. Characteristics of Substances Capable of Preventing the Phagocytic and Clumping Reactions of Human Leucocytes *in vitro*.

Inhibitory substance	Concentration in serum	Phagocytosis of pneumococcus	Clumping PMNG*	Osmolarity of mixture after rotation for 30 min	Hydrogen ion concentration of mixture after rotation for 30 min	Viability after rotational mixing by Trypan blue staining
	(moles/liter)	(%)		(milliosmoles/liter)	(pH)	(%)
Serum control		95	4 plus	300	8.3	95
Serum 0.9 ml plus saline 0.1 ml		95	4 plus	297	8.15	95
EDTA	1%	25	0	318	7.6	95
EACA	.5	0	0	853	8.25	95
L-ornithine hydrochloride	.25	0	0	717	7.75	95
L-lysine hydrochloride	.25	0	0	731	8.3	95
L-citrulline	.5	0	0	793	8.3	95
Glycine	.5	0	0	770	7.95	95
DNP	$.4 \times 10^{-1}$	0	0	305	5.5	90
DFP	$.4 \times 10^{-1}$	0	0	513	5.8	0
TAME	1.0×10^{-2}	95	4 plus	300	7.8	95

* 4 plus = 75-90% of PMNG clumped.

cellular activities. As an example of many compounds examined, glucose in concentrations of 600 milliosmoles and greater always blocked both phagocytosis and clumping by PMNG. It was thus concluded that the proteolytic enzyme antagonists studied were not capable of blocking either reaction; or to be more specific, none seemed to have blocking capability when used in physiologic concentrations.

4. *Influence of di-isopropyl flurophosphate upon phagocytosis and clumping by human leucocytes.* Based upon rather preliminary data, we also reported(2) that DFP in concentrations of 0.4×10^{-1} M blocked these functions of PMNG without compromising cellular viability; whereas, 0.4×10^{-2} M failed to exhibit the effect. This was so whether DFP was suspended in propylene glycol(7), isopropyl alcohol(8) or even in isotonic saline. In more recent studies, DFP concentrations of 0.4×10^{-1} M produced a brownish precipitate in serum and killed PMNG that were subsequently added. A check of the hydrogen ion concentration in these mixtures revealed values in the 5.8 range and an osmolarity of 513 milliosmoles.

Another compound, TAME, reputed to impair the permeability changes of acute injury(9) and to block the effect of DFP (10), was examined in this system. Exposure of PMNG to 1.0×10^{-2} M TAME for as long as 30 minutes failed to prevent either phagocytosis or the clumping reaction and had no detrimental influence upon cellular viability. Nevertheless, combinations of 1.0×10^{-2} M TAME with 0.4×10^{-2} DFP were injurious to the PMNG so that neither phagocytosis nor clumping developed. Apparently this last result did not relate to either an extreme of pH (6.5) or high osmolarity (337 milliosmoles). Although the toxic effects of DFP and TAME were not explained, other antagonists to DFP such as PAM were tested but not a single one influenced the action of DFP or for that matter showed other independent inhibitory activity.

5. *Influence of quinidine upon phagocytic and clumping activity of human leucocytes.* Spector and Willoughby noted that a number of compounds were capable of broad anti-inflammatory activity in laboratory animals injured by heat and of these, quinidine sulfate also inhibited the formation of a cellular exudate at the locus of damage(11). Since it was thought that the margination of leucocytes was inhibited by this chemical, it was of interest to test quinidine within our system. Accordingly, PMNG were incubated in serial 2-fold dilutions of quinidine hydrochloride for 30 minutes before rough strain

pneumococcus was added. Phagocytosis and clumping were vigorous in concentrations of quinidine ranging from 12 mg through 192 mg per ml but at 384 mg per ml and greater, phagocytosis was inhibited almost completely. Even at this high concentration some spontaneous clumping by PMNG was found and it should be pointed out that viability was approximately 75% that of control values. At even higher levels, that is 768 mg per ml quinidine hydrochloride and greater, viability of PMNG was very low and leucocytes failed either to ingest the bacterium or to aggregate. It is important to realize that these levels exceed by many times acceptable blood concentrations sought for in clinical practice.

Discussion. In the light of these findings, we conclude that there is no reasonable evidence to suggest that a proteolytic enzyme system instigates either the phagocytosis or the aggregation of blood borne phagocytic cells. It would indeed appear that our initial evidence with EACA was entirely a hyperosmolar effect, quite nonspecific, and probably unrelated to the structural configuration of the compound. Other substances (Table I) known to act either as analogues or as metabolic antagonists with respect to protein breakdown have also been found devoid of action in our system unless employed at excessive concentrations.

Along somewhat different lines and in an attempt to implicate the cholinesterase system as suggested by Becker and Miles(10), we were unable to show that DFP or its analogues were active unless used in lethal concentrations. Quinidine hydrochloride, an alkaloid with anti-inflammatory capability reputed, at least in part, to be due to anticholinesterase properties(11) was found to be quite ineffective in blocking our system.

So far then, there is evidence that drastic alteration of the cell surface by exposure to trypsin(1,2) will block both phagocytosis and cellular adhesion. This indicates only that the cell membrane, believed to consist of a lipid-carbohydrate-protein moiety, has been sterically altered in such a way as to prevent the joining together of chemical bonds required for membrane attachment attending phagocytosis and cellular adhesion. The fact that both calcium and magnesium, either singly or in combination, are required for the reaction(3,12) continues to suggest that enzymes may either regulate or drive the system but the nature of the reaction is as yet unknown.

A hitherto uninvestigated aspect of this problem concerns the role of carboxyl groups within the cell membrane as possible bonding sites for cellular adhesion. Bangham has suggested from studies of membrane surface charge that the negative zeta potential exhibited by PMNG in an electrophoretic chamber regardless of the basic pH of the suspending medium indicates a structure rich in carboxyl groups(13). Bangham suggested that these terminal blinding sites represented prime points for cellular binding and adhesion(13). We are now concerned with this possibility.

These studies also serve to emphasize difficulties that may arise from unsuspected physiologic abnormalities in an experimental system. The importance of osmolarity should be emphasized in this regard and rigid control must always be sought. Likewise, the possible effect of pH on a delicate system such as this must be considered with care as our experience with DNP served to emphasize.

Summary. A detailed analysis of conditions that prevailed within an *in vitro* system used for the study of the phagocytosis and adhesiveness of human polymorphonuclear neutrophilic granulocytes emphasized the critical role exerted by osmolarity and hydrogen ion concentration on the test system. It was found that neither proteolytic enzyme antagonists such as epsilon amino caproic acid nor metabolic antagonists such as dinitrophenol interfered with leucocytic function when used in subtoxic concentrations. The membrane alteration responsible for phagocytosis and clumping has yet to be described although evidence suggests it to be enzymatically controlled.

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Lack of Thymic Effect on Wound Healing.* (30099)

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Much evidence has been presented that the thymus plays an important role in antibody formation, transplantation immunity and delayed hypersensitivity in a variety of species of mammals. This influence has been related to the primacy of fetal and neonatal thymic function for lymphoid development and consequent immunologic competence. Little information is available concerning thymic and lymphocytic function in situations of questionable or non-immunologic nature, such as the inflammatory response. Inflammation following subcutaneous injection of turpentine was observed to be comparable in neonatally thymectomized and intact rats(1). However, mice thymectomized at birth were protected against the lethality of lymphocytic choriomeningitis virus administered one month later. Although virus multiplication was not inhibited, the inflammatory response was suppressed indicating the importance of tissue hyperactivity and the lymphocytic infiltrate in production of symptomatology(2).

This information prompted us to explore the effect of neonatal thymectomy on another model of inflammation and repair, notably wound healing.

Methods. Newborn Sprague-Dawley rats were randomized within 12 hours after birth so that one-half of each litter was thymectomized and the remainder utilized as controls. Thymectomy was accomplished through a short longitudinal sternal incision with a wire snare. Clean, but not aseptic precautions were enforced. All animals were placed in individual cages after weaning and maintained with rat chow supplemented with canned dog food, cabbage and apples *ad lib.* A 4 cm surgical incision of the skin of the lower back was made with a scalpel and the wound closed by 4 evenly distributed interrupted silk sutures. A uniform, circular defect was simultaneously produced in the dorsolateral aspect of the thorax with a 6 mm dermal punch in all animals 61 $\pm$ 1-72 $\pm$ 6 days of age (Table I). Groups were sacrificed at 2, 5, 8, 14 and 21 days following wounding.

Wound contraction was estimated by daily planimetry of the skin defect on the thorax. Tensile strength was determined from uniform transverse strips of areas between the sutures measuring 1 cm in width and 2 cm on each side of the wound. One end was suspended from a ring stand and weights and/or paper cup attached to the other. Water was slowly delivered into the cup until wound disruption occurred. Tensile strength was based on the average of 2 determinations on each wound. (Differences in readings ranged from 12-25 ml water.)

Other portions of the surgical wound and skin defect were fixed in 10% neutral formalin and absolute alcohol, dehydrated and imbedded in paraffin in the usual manner. Sec-

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