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YEH, Alexander Kuo-Shi, 1940-
STUDIES ON HUMAN LEUKOCYTE NEURAMINIDASE.

The University of Oklahoma, Ph.D., 1973
Biochemistry

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

STUDIES ON HUMAN LEUKOCYTE NEURAMINIDASE

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1973

STUDIES ON HUMAN LEUKOCYTE NEURAMINIDASE

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ACKNOWLEDGEMENTS

I wish to acknowledge my heart-felt respect and thanks to Dr. Raoul Carubelli, my dissertation advisor and teacher, and a constant source of understanding and stimulating guidance.

I am also grateful to my former professors at West Texas State University and the professors at the University of Oklahoma Health Sciences Center for their teachings; to Mr. John F. Hall for his theoretical and practical lessons and expert advice in blood cell morphology; and to Dr. Russell T. Schultz of the Arthritic Out-Patient Clinic of Oklahoma University Hospital for his generous help in securing blood specimens from arthritic patients.

I would like to thank Dr. Tushar K. Chowdhury of the Department of Physiology and Biophysics, Dr. Albert M. Chandler, Dr. Jary S. Mayes and Dr. Martin J. Griffin of the Department of Biochemistry and Molecular Biology for their willingness to serve as members of my dissertation reading committee as well as for many valuable discussions during the performance of this research.

To the many investigators in the fields of hematology, enzymo-

logy and chemistry whose studies and discoveries have enriched my knowledge and helped my thinking, I would like to express my respect and appreciation.

I would like to thank Mrs. Opal Pratt and Mr. Bill Deal of the Blood Bank of Oklahoma University Hospital for their kindness and generous help in securing blood donors, to Mrs. Irene McMichael for her help in translating some words in German papers, to the fellow graduate student, Larry W. Schneider for helping with some statistical analysis.

For the conscientious typing of this dissertation after hours under the stress of last minute corrections and a pressing deadline, I would like to thank Miss Sharon Moline and Mrs. Fran Williams.

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STUDIES ON HUMAN LEUKOCYTE NEURAMINIDASE

CHAPTER I

INTRODUCTION

One of the criteria distinguishing viruses from bacteria before 1940 was that viruses were devoid of any intrinsic enzyme as part of their particle components. These views changed when investigations on the phenomenon of influenza virus-induced hemagglutination led to the discovery of neuraminidase.

Hirst (1) and McClelland and Hare (2) reported the agglutination of chicken erythrocytes when allantoic fluid from chick embryos, infected with influenza virus, were added to chicken blood. Hirst's assumption of the existence of a viral enzyme (3) was substantiated when it was shown that the hemagglutination caused by the indicator virus (4) can be inhibited by normal serum and by a number of biological substances of mucoid nature which lose their inhibitory property after pretreatment with the active virus or with a soluble enzyme present in the filtrates of Vibrio cholerae cultures (5). The nature of viral hemagglutination was further studied by Gottschalk (6) who observed that a low molecular weight compound was released from ovomucin concomittant with the loss of the inhibitory property. This small molecule was later identified as

N-acetylneuraminic acid (NANA) (7). The enzyme, originally believed to be at the surface of influenza virus particle and later found also in the cultures filtrates of Clostridium perfringens and Vibrio cholerae, was given the name "neuraminidase" and its action was defined by Gottschalk (8) as the hydrolytic cleavage of the glycosidic linkage joining the keto group of neuraminic acid to D-galactose or D-galactosamine and possibly to other sugars. Neuraminidase is now known to be present in myxoviruses and bacteria (9) as well as in mammalian and avian organs.

The presence of neuraminidase activity in mammalian plasma was first reported by Warren and Spearing (10) who found the enzyme both in bovine and in human preparations of Cohn fraction VI using fetuin as the substrate. Carubelli, Trucco and Caputto (11), using neuraminosyl trisaccharides as substrates (12), disclosed the presence of a soluble neuraminidase in the supernatant fraction of various rat organs. The enzyme levels were highest in the liver, brain and mammary gland. Shortly afterwards, Morgan and Laurell (13) reported neuraminidase activity in the whole homogenates of guinea pig, bovine and human brains, using endogenous substrates. The presence of a lysosomal mammalian neuraminidase was first described by Taha and Carubelli (14) who conducted studies on the intracellular distribution of this enzyme in rat liver and showed that the lysosomal fraction has the highest relative specific activity. The finding that neuraminidase belongs to the group of acid lysosomal hydrolases was confirmed by Mahadevan, Nduaguba and Tappel (15), who demonstrated neuraminidase activity in isolated rat liver and kidney lysosomes. Studies on the particulate neuraminidase of rat liver by Horvat and Touster (16) and by Aronson and De Duve (17), which established the structure-linked latency

of neuraminidase and its role in the digestion of glycoproteins, further supported the lysosomal nature of the particulate liver neuraminidase. However, Schengrund, Jensen and Rosenberg (18) have recently reported that a major fraction of the neuraminidase activity towards ganglioside substrate is localized in the plasma membrane of rat liver cells, and that only a minor portion of this enzyme activity was detectable in the lysosome-rich fraction.

Discrepancies between the properties of the neuraminidase present in the supernatant fraction of homogenates in isotonic KCl (11, 14) and the enzyme present in lysosomal fraction (15, 16, 17) were investigated in a rather detailed manner by Tulsiani and Carubelli (19). Although these differences had previously been attributed to allotopy (16), present experimental data indicate that in rat liver there are at least two different neuraminidases. These two neuraminidases differ in intracellular localization, in their pH optima and in their behavior in the presence of chlorides of various mono and divalent cations (19). On the intracellular localization of neuraminidase activity towards gangliosides, preliminary data of Tulsiani, Bhavanandan and Carubelli (20) were again consistent with the lysosomal nature of the particle-bound neuraminidase activity whether disialoganglioside or neuramin-lactose (NL) are used as substrates.

Experimental results from several sources now indicate that both the <u>particulate</u> and the <u>soluble</u> neuraminidases exist in various animal organs. The particulate neuraminidase has been found in rat mammary glands (21), in the brains of pigs (23), calves (13), rats (14), chicks (24) and human beings (25), and in the kidney of pigs (26). The soluble neuraminidase has also been detected in the brains of rats (27), pigs (28), and

human beings (25), in the kidney of rats (11) and rabbits (29). On the other hand, experiments with free cells such as HeLa cells (30) and Erlich ascites carcinoma cells (31) revealed the presence of a particulate neuraminidase but gave no evidence for a soluble neuraminidase in these cells.

Work on the neuraminidase activity in various blood cells is relatively recent. Gielen, Etzrodt and Uhlenbruck (32) have detected a particulate-bound neuraminidase in lysed bovine platelets and erythrocytes. Gielen, Schaper and Pink (33) reported the presence of neuraminidase activity in bovine leukocytes. More recently, Cabezas (34) scanned platelets of various animals and found that neuraminidase is present in all species investigated. Bosmann (35), working on the effect of surface sialic acid on platelet aggregation, found that human platelets contained low neuraminidase activity which could release sialic acid residues from the surface of intact platelets.

Neuraminidase activity has been found to increase in some pathological and experimental conditions such as in α-tocopherol deficiency (36) in the liver of tumor-bearing mice and rats (37) and in HeLa cells treated with hydrocortisone (30). More detailed studies on both particle-bound and soluble neuraminidases in the brain and liver of developing rats have been conducted by Carubelli and Tulsiani (27). It was found, in both organs, that the particulate neuraminidase is fully active at birth and the specific activity is fairly constant from a week before birth to about a month of age. The soluble neuraminidase activity, however, was very low in the fetus but increased markedly in late prenatal development in liver and in early postnatal development in brain. Age-related changes

of neuraminidase activities in the liver of developing chicks have been recently reported (38); again changes were observed only in the soluble enzyme. It is also of interest to mention that intracerebral innoculation of a non-neurotropic strain of influenza virus in experimental mice led to convulsion and that the death of the animal was concomittant with the peak of release of free neuraminic acid in the brain (39).

The biological role of neuraminidase rests on the biochemical and biological importance of neuraminic acid. Neuraminic acid is an acidic sugar with pKa $\simeq$ 2.6 (40). At physiological pH, its degree of dissociation is greater than 0.99. Whenever this sugar is present, it invariably occupies the non-reducing end of the corresponding glycoprotein or glycosphingolipid. In vitro studies have shown that with certain sialoglycoproteins the terminal NANA residue serve to prevent or retard their enzymatic breakdown (17). The negatively charged cell surface neuraminic acid has been implicated in intercellular repulsion. Loss of contact inhibition in neoplastic cells (41), decreased platelet aggregation upon incorporating NANA onto their surface (42), increased platelet aggregation by removing surface NANA (35) and the heavy aggregation of neuraminidase-treated peripheral myeloblasts in myeloblastic leukemia (43) are known in vitro examples of the role of NANA on cellular surface properties.

Human monocytes, when treated with neuraminidase, increase their phagocytic activity due to increased deformability. It has been suggested that this is caused by a lowering of the energy barrier for changes in membrane conformation (44, 45). On the other hand, recent studies indicate that the phagocytic activity of human polymorphonuclear leukocytes is not

affected by neuraminidase treatment (46).

Sialic acid has also been shown to act as a specific recognition site. The charged terminal neuraminic acid residue is known to be the essential component of human erythrocyte MN antigenic glycoprotein (47, 48). The homing property of rat lymphocytes can be altered by removing cell surface neuraminic acid residues (49). Neuraminic acid can also serve to block the penultimate D-galactose residue which is the recognition signal in the catabolic process of some serum glycoproteins and hormones. Removal of sialic acid from orosomucoid, fetuin, ceruloplasmin, haptoglobin, α_2-macroglobulin, thyroglobulin, human chorionic gonadotropin and follicle stimulating hormone led in each case to their rapid removal from circulation into liver parenchymal, but not into Kupffer cells (50). Tumor cells treated with neuraminidase showed enhanced immunogenicity and antigenicity (51, 52, 53). Heteroantigens of human lymphoid tissue cultured cells and of fresh human lymphocytes are unmasked after surface neuraminic acid is removed (54). Neuraminic acid content has been reported to increase in human solid tumor tissue (55), to remain unchanged in ascites hepatoma cells (56), and to decrease in the glycospingolipids of virus transformed fibroblasts (57). Human lymphocyte cultures treated with neuraminidase have been shown to respond with a marked increase in DNA-synthesis (58).

Human peripheral blood leukocytes are known to contain granules rich in acid and neutral proteolytic enzymes and various kinds of acid hydrolases (59) concerned with the digestion of heterologous and autologous materials. Their important role in the phagocytosis and digestion of incoming microorganisms as part of the body defense mechanisms is well established (60). However, discharge of their lysosomal contents has

been shown in various instances to be the cause of tissue injury. In immune-complex induced glomerulonephritis, neutrophiles have been seen infiltrating onto the basement membrane, releasing lysosomal enzymes to the surrounding tissues, and causing the dissolution of the membrane (61). In arteritis, neutrophiles are chemotactically attracted toward the target site, waging a series of attacks against the immune-complex, and resulting in local lesions of the blood vessel wall by the action of lysosomal enzymes (62). Chemotactically attracted polymorphonuclear leukocytes have been observed to be abundant in the synovial fluid of patients with rheumatoid arthritis and other forms of acute joint disease (63). Neutrophiles and mononuclear leukocytes of joint fluids have been observed to engulf immunoglobulin complexes in rheumatoid arthritis (63, 64).

It has been proposed (65) that among the initial events that start the cycle of inflammation and tissue destruction in rheumatoid arthritis, some as yet unidentified factor or factors alter normal IgG. The altered immunoglobulin then forms complexes with rheumatoid factors IgG_{RF} and IgM_{RF}. The large immune complex IgG-IgG_{RF} tends to fix complement, forming still larger complex (IgG-IgG_{RF})-C' which can further complex with IgM_{RF} (66). The high molecular weight immune complex precipitates on the membrane of the synovial cavity. As polymorphonuclear leukocytes are chemotactically attracted into the joint, phagocytosis of such complexes may lead to further inflammation by bringing about the release of lysosomal enzymes from the phagocytic cells (67).

The underlying mechanisms of the inflammation and tissue destruction observed in rheumatoid arthritis are quite complex (68, 69) and they involve a series of immune reactions plus complement activation. Although

the initiation event remains obscure, however, there is a possibility that it might be relatively simple. The heavy chains of human immunoglobulin G and immunoglobulin M are known to contain mannose, galactose, glucosamine, L-fucose and sialic acid (70, 71). The in vivo alteration of 7s IgG or the formation of IgG_{RF} and IgM_{RF} may result when the negatively charged sialic acid of the native immunoglobulins are cleaved off by neuraminidase. Alternatively, if desialization by itself does not directly lead to macroglobular aggregation, it might enhance or facilitate further deglycosylation or proteolytic digestion of the immunoglobulins by lysosomal enzymes and thus render the abnormal immunoglobulins foreign to the body's immune mechanisms. The neuraminidase activity may presumably be from the discharged lysosomal contents as the polymorphonuclear leukocytes are challenged by alteration in the surrounding tissues or by invading microorganisms. It has been proposed that the trigger for rheumatoid arthritis might be a physical injury, infection or a toxic chemical (65). All three are known factors which mobilize and activate polymorphonuclear leukocytes to violently engage in engulfing degraded substances, microorganisms and foreign chemicals; in the process, their lysosomal contents are often released.

It is possible that under certain conditions lysosomal neuraminidase may be depressed or missing as a result of inborn error of metabolism. This could lead to as yet unrecognized disease entities. For example, in Gaucher's disease, leukocyte glucocerebrosidase activity has been found to be 17% of that found in normal subjects (72); in Nieman-Pick disease, the white blood cell's sphingomyelinase activity is only 10% of that in control leukocytes (73); the leukocytes of patients with sulfatide lipidosis

(metachromatic leukodystrophy) have been found to contain only 10% of the arylsulfatase A activity found in normal individuals (74). Leukocyte β-glucuronidase activity has been known for a long time to be altered in various kinds of neoplastic diseases (75).

The goals of this study were to investigate neuraminidase activity in the mixed population of human peripheral blood leukocytes, in the fractionated lymphocytes and polymorphonuclear leukocytes. Detailed studies on the properties of leukocyte-neuraminidase and its subcellular distribution in lymphocytes and polymorphonuclears were to be carried out. Since, up to 1970, no studies concerning human peripheral leukocyte neuraminidase activity had been reported in the literature. In addition, the levels of neuraminidase activity were to be established in leukocytes from healthy adult human beings and possible alterations of this enzyme activity in disease were to be investigated.

CHAPTER II

MATERIALS AND METHODS

Materials

Chemicals and Solvents

All chemicals and solvents used for this study were of reagent grade. The potassium salts of neuramin-lactose (NL) and neuramin-lactose sulfate, isolated from rat mammary gland extracts and purified as described (12), were on hand in our laboratory. Ovine submaxillary glycoprotein was kindly provided by Dr. Alfred Gottschalk, Max Planck Institute for Virus Research, Tubingen, West Germany. Sialoglycopeptides were isolated from pronase digests of ovine submaxillary glycoprotein (76). Human and bovine erythrocyte mucoids prepared by phenol-saline extraction of the respective erythrocyte-ghosts were purified as described by Wintzer and Uhlenbruck (77). Crystalline synthetic NANA (Type IV), gangliosides from bovine brain (Type III), and Dextran (Type 200), were products of Sigma Company. Fetuin (B grade) from fetal calf serum, was purchased from Calbiochem. Dowex 1-X 8 (chloride form) 200-400 mesh was purchased from J.T. Baker Chemical Company.

Blood

Whole blood smear and differential counts of leukocytes were carried out using Wright's stain and a Leitz Dialux Research microscope. Total white counts were performed using disposable white count Unopettes, 1:250 dilution, of Becton-Dickinson Corporation, and a Bright-Line hemacytometer of American Optical Company. Small blood samples (10 ml) were drawn into heparinized Vacutainer tubes of Becton-Dickinson Corporation. Large blood specimens (1 pint) were drawn into JH-1 Blood-Pack of Fenwal Laboratories containing 2250 USP units of heparin.

The human blood, unless specifically stated, was freshly drawn from fasting, healthy adult donors.

The bovine blood was a gift from Wilson Certified Foods, Inc. The specimens were collected directly into plastic buckets containing anticoagulant or defibrinated by gently shaking with glass marbles.

Rat blood was drawn by heart puncture under ether anesthesia, using a heparinized syringe.

Instruments and Equipment

Spectrophotometric measurements were performed using a Beckman DU Spectrophotometer (Beckman Instruments Company), and a Cary Model 14 Recording Spectrophotometer (Applied Physics Corporation). Centrifugations at low temperatures were performed using an International Refrigerated Centrifuge Model PR-2, while an International Centrifuge Model V was utilized for centrifugations at room temperature. Ultracentrifugations were performed using either a Spinco Model L Ultracentrifuge or a Beckman L2-65 Ultracentrifuge, Beckman Instruments Company.

Methods

Analytical Methods

Neuraminidase (EC 3.2.1.18) assay. Neuraminidase activity was assayed by measuring the amount of NANA released from suitable substrates under the conditions of the assay.

Procedure. A typical incubation system contained 135 µl of an aqueous leukocyte suspension (ca. 10^7 cells), 15 µl of 1 M sodium acetate buffer pH 4.0, and 50 µl of substrate solution containing 200 nmoles of neuramin-lactose. The system was incubated in a water bath at 37° C for 3 hours. Water blank and control tubes, including enzyme without substrate and substrate without enzyme, were incubated concurrently. The enzymic reaction was stopped by adding 0.1 ml of periodate reagent which is the first step in the thiobarbituric acid method for the assay of free NANA.

Modified thiobarbituric acid method of Warren (78). Periodate oxidation of free NANA at room temperature in a strongly acidic medium, yields formyl-pyruvic acid. After reducing the excess periodate with arsenite, the formyl-pyruvic acid was condensed with thiobarbituric acid to yield a chromogen which had a maximum absorption at 549 nm.

Periodate reagent. This reagent is a 0.2 M solution of sodium metaperiodate in 9 M phosphoric acid.

Arsenite reagent. This reducing reagent consists of a 10% (w/v) solution of sodium arsenite in a solution 0.5 M in sodium sulfate and 0.1 N in sulfuric acid.

Thiobarbituric acid solution. This reagent is a 0.6% (w/v) solution of thiobarbituric acid in 0.5 M sodium sulfate.

Procedure. For the assay of NANA liberated by the enzymic reaction, 0.1 ml of periodate reagent was added to 0.2 ml of incubation mixture at the end of incubation time. The tubes were mixed and allowed to stand at room temperature for 20 minutes. At the end of periodate oxidation, 0.5 ml of arsenite reagent was added to consume the excess periodate reagent. The tubes were vortex-mixed twice to make sure that the liberated iodine was completely reduced. After adding 1.5 ml of thiobarbituric acid solution and mixing thoroughly, the tubes were placed in a boiling water bath for 15 minutes, cooled in ice water for 5 minutes. The chromogen was then extracted with 2 ml of cyclohexanone, the tubes were centrifuged for 3 minutes at 1500 rpm to separate the organic and aqueous phases. The upper layer (cyclohexanone) was transferred to a cuvette with a Pasteur pipette and the optical density was read at 549 nm. In this modified assay procedure the total volume of the reaction mixture was 2.3 ml instead of 4.3 ml and the chromogens were extracted into 2 ml of cyclohexanone instead of the 4.3 ml used in the original Warren procedure. These changes resulted in higher optical density readings and Beer's law was obeyed in the ranges of NANA concentration from 5 to 50 nmoles. The amount of free NANA can be determined from a standard curve. Approximate values were calculated from the equation: nmoles NANA = 43 x O.D. 549 nm.

Resorcinol method for sialic acid (79). This method was used for the quantitative determination of total (i.e. bound and free) NANA.

Resorcinol reagent. Two hundred mg of crystalline resorcinol was dissolved in a mixture of 10 ml of distilled water and 80 ml of concentrated HCl. Then, 0.25 ml of 0.1 M $CuSO_4$ was added, and the volume was made up to 100 ml with distilled water. The reagent was prepared at least 4 hours prior to use.

Procedure. Sample aliquots containing about 50 to 100 nmoles of NANA in a final volume of 1 ml were mixed with 1 ml of resorcinol reagent. The tubes were heated in a boiling water bath for 15 minutes and cooled in ice water for 5 minutes. Two and five-tenths ml of isoamyl alcohol were added to the tubes which were then mixed and chilled again for 10 minutes in ice water. Following centrifugation for 10 minutes at 1500 rpm, the upper organic phase was decanted and its optical density at 580 nm was measured against a reagent blank. Standards containing 25 to 100 nmoles of NANA were analyzed concurrently.

Total NANA determination by thiobarbituric acid (TBA) method. Bound NANA was released by mild acid hydrolysis in 0.1 N H_2SO_4 at 80° C for 2 hours. The liberated NANA was then quantitated by the modified TBA method of Warren.

Protein determination (80). The method of Lowry, Rosebrough, Farr and Randall was used for the quantitative determination of proteins.

Folin phenol reagent. This reagent was purchased from Fisher Scientific Company.

Reagent A. This reagent was prepared by dissolving Na_2CO_3 (2% w/v) in 0.1 N NaOH.

Reagent B. This reagent was made by dissolving $CuSO_4 \cdot 5H_2O$ (0.5% w/v) in a 1% solution of sodium tartrate.

Reagent C. Reagents A and B were mixed in a 50:1 (v/v) ratio to yield reagent C which was freshly prepared shortly before use.

Procedure. Five to fifty µg of protein in 0.2 ml of water was introduced into test tubes. One ml of reagent C was added and the tubes were mixed and set at room temperature for 10 minutes. One-tenth ml of 2 N Folin reagent was added to each tube and the tube was rapidly vortex-mixed and set at room temperature for 30 minutes. The optical density was read at 600 nm against a blank prepared with distilled water. Known concentrations of crystalline bovine serum albumin were used as the standard.

Acid phosphatase (EC 3.1.3.2.) assay. Acid phosphatase was assayed using β-glycerophosphate as the substrate. The method of Lowry and Lopez (81) was used for the assay of the liberated inorganic phosphate.

Procedure. The incubation mixture contained, in a final volume of 1 ml, 0.1 ml of 1% (w/v) Triton X-100, 0.2 ml of 0.2 M sodium acetate buffer pH 5, 0.2 ml of 0.25 M β-glycerophosphate and enzyme preparation. Water blank, enzyme and substrate controls were run concurrently. A solution of inorganic phosphate (390.44 nmole KH_2PO_4/0.2 ml) was used as the standard. The tubes were incubated for 30 minutes at 37° C. At the end of the incubation, 0.3 ml of 10% trichloroacetic acid was added and the contents of the tubes were mixed and allowed to stand for 10 minutes at room temperature. Two and two-tenths ml of 0.1 M sodium acetate was added to each tube, the tubes were mixed and centrifuged at room temperature at 3000 x g for 20 minutes. One and five-tenths ml aliquots of the clear supernatant were transferred to clean test tubes, 0.3 ml of a freshly prepared reagent containing equal volumes of 1% ascorbic acid solution

and 1% ammonium molybdate in 0.1 N sulfuric acid solution were added, and the tube contents were immediately vortex-mixed. The tubes were allowed to stand for 10 minutes for color development and the optical density was read to 650 nm. The formation of phosphomolybdate complex and the solubility of the complex are highly pH-dependent. The procedure mentioned above gave a final pH of 3.5-3.9, which was within the pH range (2.8-4.7) recommended for this assay.

β-Glucuronidase (EC 3.2.1.31) assay. The method of Gianetto and De Duve (82) was used for the assay of β-glucuronidase. Phenolphthalein β-glucuronide was used as the substrate and the amount of phenolphthalein liberated was estimated by adjusting the pH to the basic range where phenolphthalein gave a red color with maximum absorption at 550 nm.

Procedure. The incubation mixture consisted of 0.2 ml of 0.75 M sodium acetate buffer pH 5.2, 0.2 ml of 1% Triton X-100 and 0.2 ml of 0.0125 M phenolphthalein B-glucuronide in a final volume of 2 ml. Water blank, enzyme and substrate controls and standard phenolphthalein solution (400 nmole/0.2 ml) were run together with the complete enzyme assay. The tubes were incubated at 37° C for 30 minutes and the reaction was stopped by adding 6 ml of glycine-carbonate buffer pH 10.4. After mixing, the tubes were centrifuged for 20 minutes at 3000 x g, and the optical density of the supernatant was read at 550 nm.

5'-Nucleotidase (EC 3.1.3.5) assay. 5'-Nucleotidase is widely accepted as a marker for plasma membranes (83, 84). Heppel and Hilmore (85) conducted thorough studies of this enzyme in membranes from bovine cells. Belfield and Goldberg (86) reported that alkaline phosphatase is also active toward 2'-,3'- and 5'-adenine mononucleotides under the

incubation conditions utilized for the assay of 5'-nucleotidase activity. However, when both β-glycerophosphate and adenosine 5'-monophosphate are present in the incubation mixture, alkaline phosphatase acts more selectively against β-glycerophosphate, leaving the bulk of the adenosine 5'-monophosphate unhydrolyzed. The procedure applied for the assay of 5'-nucleotidase activity is a combination of those of Heppel and Hilmore (85) and Belfield and Goldberg (86), in order to correct for the interference by alkaline phosphatase.

Procedure. The incubation mixture contained, in a final volume of 0.5 ml, 0.45 ml of enzyme preparation (ca. 0.1 mg to 0.5 mg of protein) and 0.05 ml of substrate in a solution of buffer and cofactors. For the control tubes, the glycine buffer (0.5 M pH 8.6) contained $MgCl_2$ (0.1 M) and β-glycerophosphate (1 M). For the experimental tubes, the above buffer also contained the substrate for 5'-nucleotidase at a 0.05 M concentration. The tubes were incubated at 37° C for 20 minutes and the reaction was stopped by adding 0.2 ml of 10% TCA. The tubes were mixed and allowed to stand at room temperature for 10 minutes. Two and three-tenths ml of 0.1 M sodium acetate was then added to each tube. The tubes were centrifuged at 3000 x g for 20 minutes. One and five-tenths ml aliquots were transferred to clean tubes and the orthophosphate was determined by the method of Lowry and Lopez described previously.

Leukocyte Isolation Methods

Isolation of human leukocytes. Leukocytes were isolated by several different method depending on the intended use of the isolated leukocytes.

a) Enzyme studies with mixed leukocytes (Figure 1). One pint of heparinized or citrate blood was centrifuged at 1,600 x g for 4 minutes at 20° C. The supernatant was decanted and utilized as a source of plasma and platelets required for another project. Leukocytes were then isolated from the sediment by a method based on the technique of Kampine and coworkers (72). The blood cells were suspended in 300 ml of a 1% solution of Na_2EDTA (ethylenediaminetetraacetic acid disodium salt) in 0.85% NaCl and the suspension was centrifuged at 400 x g for 10 minutes. The supernatant was decanted and discarded and the sediment was subjected to a second wash under identical conditions. These washings with Na_2EDTA-saline serve to eliminate most of the remaining platelets. The cells were then suspended in 300 ml of a solution containing 1.5% Dextran and 0.85% NaCl. The suspension was distributed in four 100 ml cylinders and allowed to sediment at room temperature. The leukocyte-rich supernatant was removed at 5 minute intervals over a period of 45 minutes. The final sediment was resuspended to the initial volume in dextran-saline solution and subjected to a second sedimentation under the same conditions. The leukocyte suspensions (slightly contaminated with erythrocytes) were pooled, placed in two 250 ml centrifuge flasks, and centrifuged for 10 minutes at 700 x g. The clear supernatant was discarded and the sedimented cells were then suspended in glass-distilled water (125 ml for each flask) in order to hemolyze contaminating erythrocytes. The suspension was stirred with a glass rod for 90 seconds, followed by quick addition of 12.5 ml of 9.35% NaCl solution to each flask, in order to restore isotonicity. The cell suspension was centrifuged for 6 minutes at 700 x g; the hemolysate and the layer of erythrocyte ghosts were care-

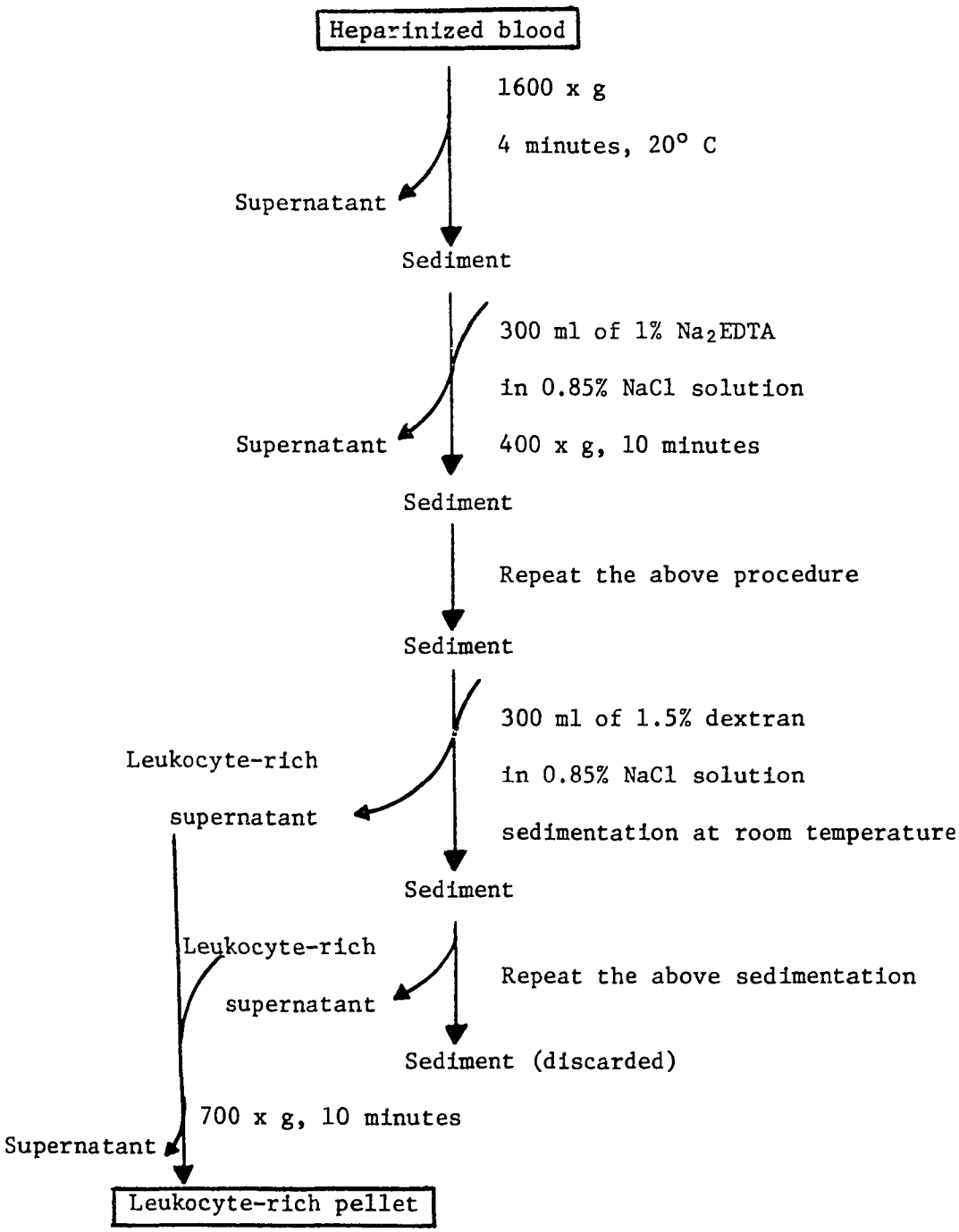


Figure 1. Procedure for the isolation of mixed leukocytes.

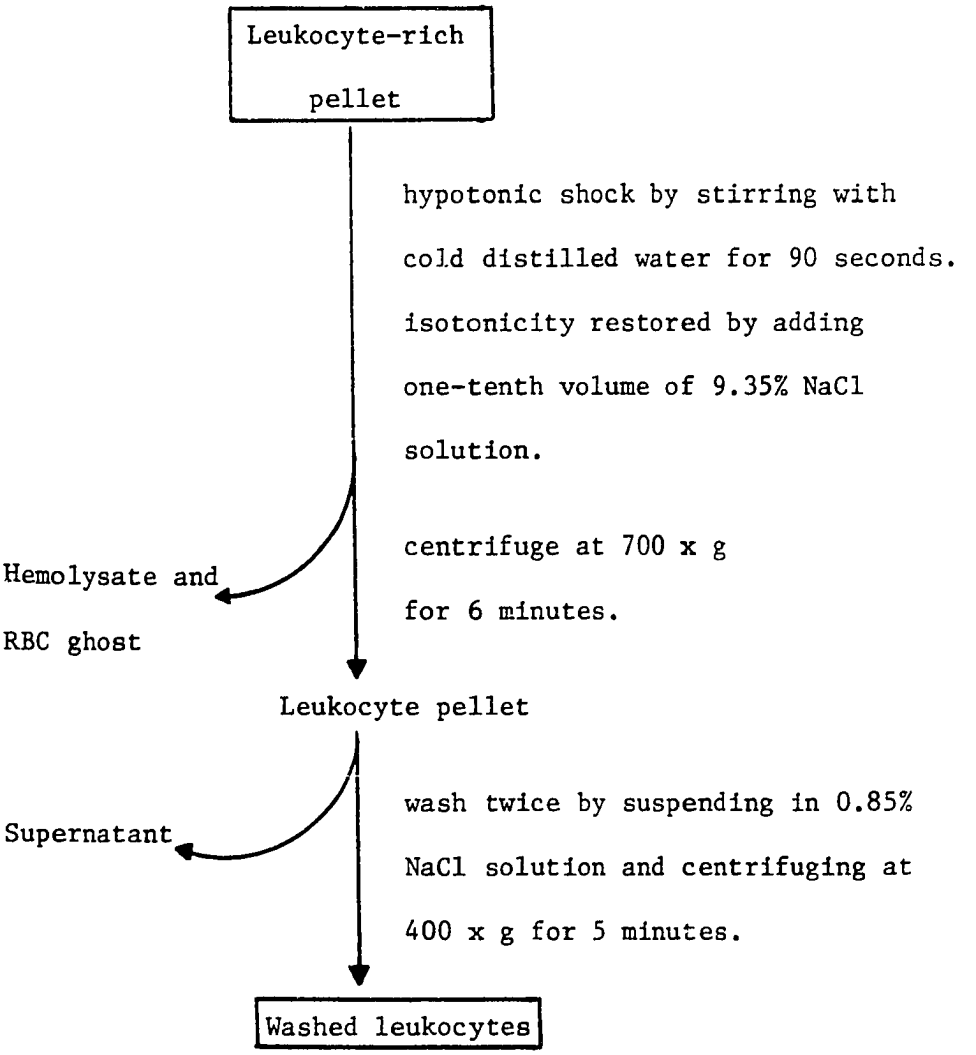


Figure 1 (continued). Procedure for the isolation of mixed leukocytes.

fully removed and discarded. The leukocytes from both flasks were then resuspended in 0.85% NaCl (total volume about 50 ml) and centrifuged at 400 x g for 5 minutes. After an additional wash with 0.85% NaCl following the same conditions, the leukocyte pellet was suspended in 7 ml of ice-cold, glass-distilled water, and the suspension was used for the various enzymic studies described below. In a typical experiment, the leukocyte count of the initial blood was 8,500 cells per µl, while the cell count of the suspension of purified leukocytes was 87,500 cells per µl. The overall recovery was about 15%.

b) Enzymic studies with isolated lymphocytes and polymorphonuclear leukocytes (PMN) (Figure 2). Five hundred ml of venous blood was drawn into a heparin-containing JH-1 Blood-Pack. A 200 ml portion was centrifuged at 1000 x g for 30 minutes in order to obtain the plasma which was decanted into an erlenmeyer flask, and stored at room temperature. This plasma was later warmed to 37° C and used for the elution of lymphocytes from the glass bead columns described below. The remaining 300 ml of blood was equally distributed into three 100 ml graduate cylinders, each containing 10 ml of 3% dextran in 0.85% NaCl. The blood was allowed to sediment at room temperature for 40 to 60 minutes. The supernatant was pipetted out at 5 to 10 minute intervals during the sedimentation with large-bore siliconized pipettes. The leukocyte-rich supernatant, in 50 ml polycarbonate centrifuge tubes, was centrifuged at 150 x g for 10 minutes at room temperature. The pellets were then resuspended in a total of 30 to 36 ml of the plasma and stored at room temperature in polycarbonate tubes sealed with Parafilm. The dextran-containing plasma was centrifuged at 1000 x g for 20 minutes and the clear supernatant (1.1 volume) was

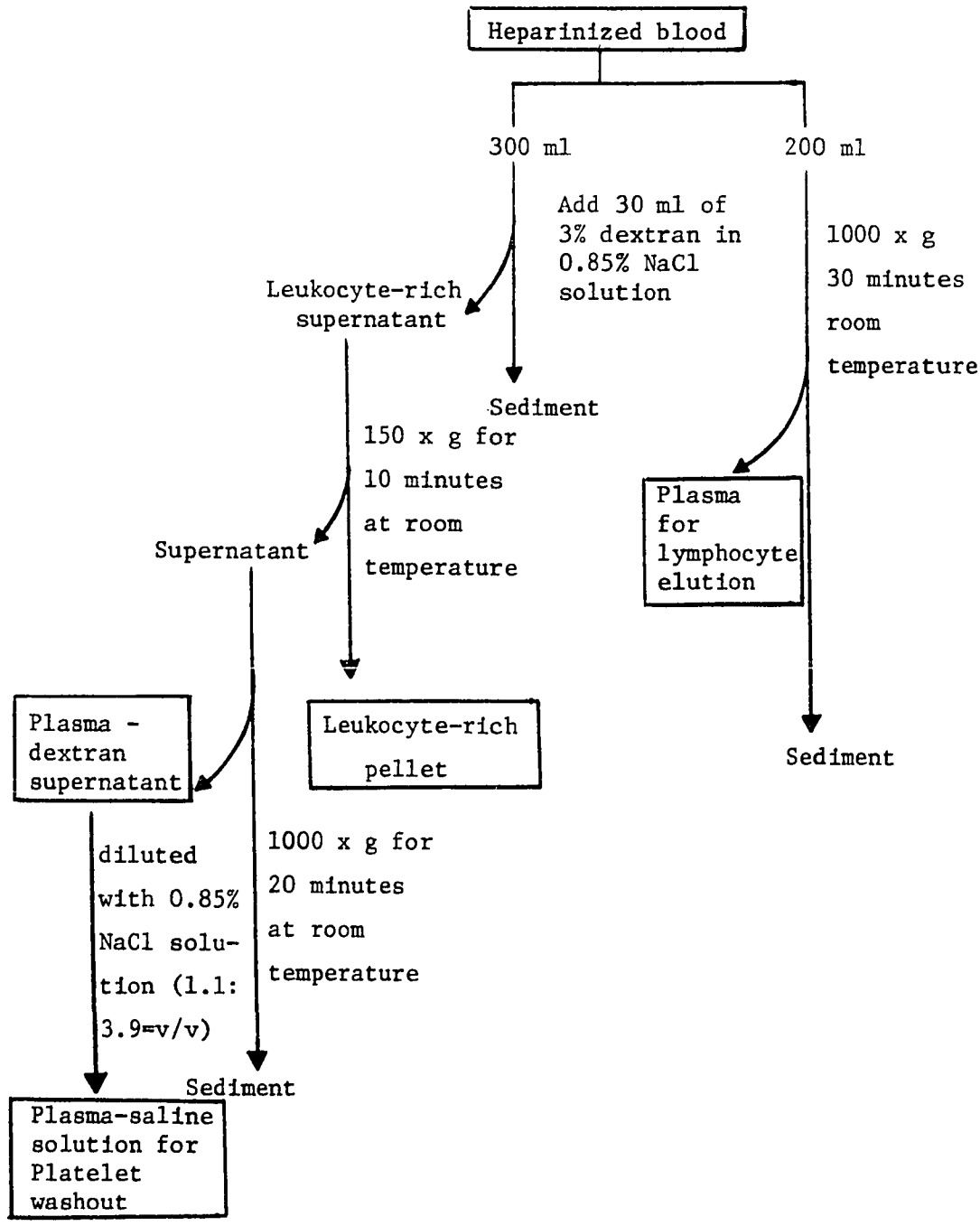


Figure 2. Procedure for the isolation of mixed leukocytes and plasma fractions utilized in the separation of lymphocytes and PMN by glass bead column.

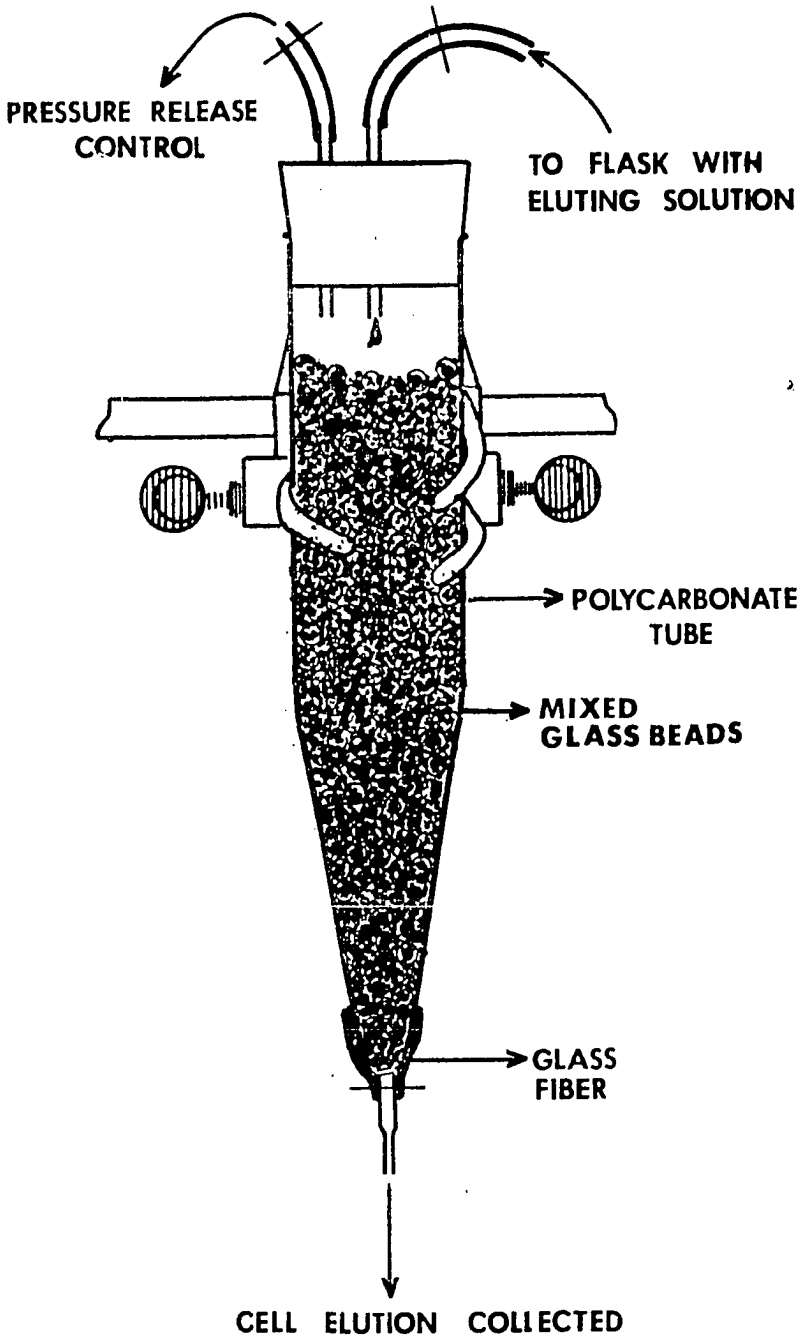


Figure 3. Picture showing the glass bead column used as described under Methods.

diluted with 0.85% NaCl (3.9 volume) in order to prepare the plasma-saline solution which was equilibrated to 37° C in a water bath and utilized for platelet wash-out from the glass bead columns.

The columns were fashioned from 50 ml polycarbonate conical centrifuge tubes as shown in Figure 3. In order to maintain the desired column flow rate and to have enough glass bead surface area, two sizes of glass beads were applied to each column. The large glass beads of Corning Glass Works are 3 mm in diameter, and the small glass beads of Matheson Scientific are 0.2 mm in diameter. Both glass beads were washed with cold nitric acid followed by rinsing with tap water and distilled water till near neutral pH. The columns, glass beads, glass fiber and pipettes were siliconized with 1% Silicad, rinsed with distilled water and dried at 37° C overnight.

The procedure (Figure 4) for the separation of lymphocytes and PMN using the glass bead column was adapted from that of Rabinowitz (87). Prior to packing, a thin-layer of glass fiber was placed at the tip of the column. Fifteen grams of the large-sized glass beads were first poured into the column after which 20 grams of the small-sized glass beads were also poured into the column. Finally, 30 grams of the large-sized glass beads were introduced into the column. The packed columns were then equilibrated in a 37° C constant temperature warm room before leukocyte suspensions were transferred into it. After introducing the leukocyte suspension, 4.2 to 5.0 x 10^8 cells in 12 ml of plasma, the two upper outlets were closed and the columns were kept at 37° C for 30 minutes. At the end of the incubation period, the internal pressure was released through the pressure release outlet. The clamp on the latter

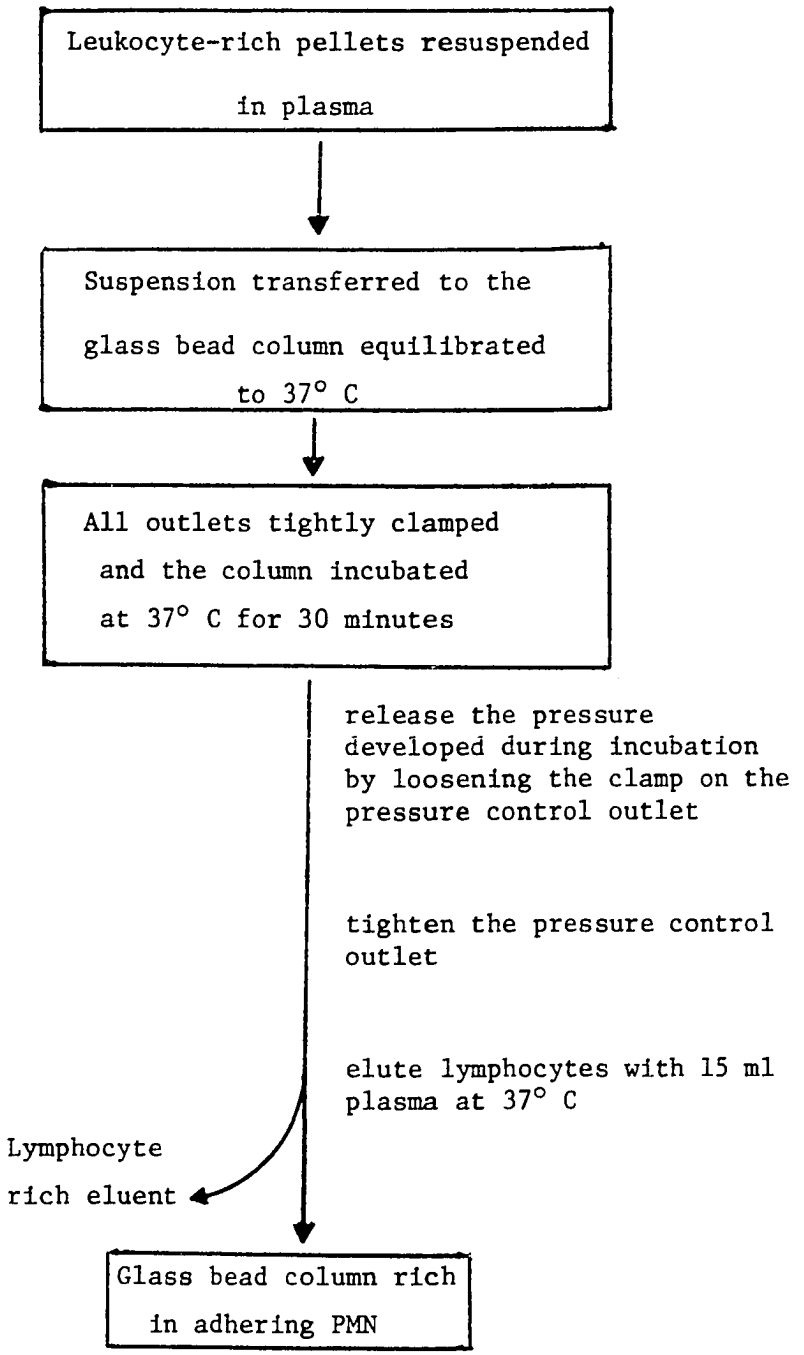


Figure 4. Procedure for the separation of lymphocytes and PMN on glass bead column.

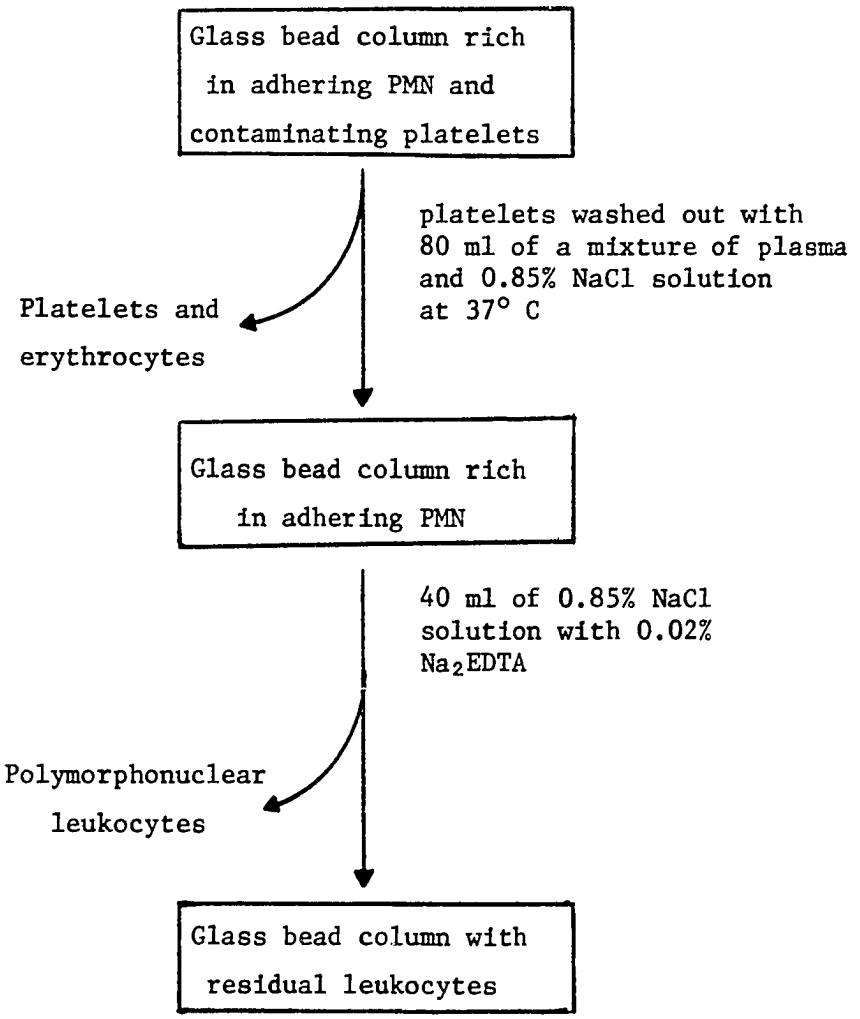


Figure 4 (continued). Procedure for the separation of lymphocytes and PMN on glass bead column.

was tightened, the other outlets were opened and the lymphocyte fraction was eluted with 15 ml of undiluted plasma. After the lymphocyte fraction was eluted from the column, platelets were washed out by eluting the column with 80 ml of a 0.85% NaCl solution containing 20% (v/v) of plasma and a very low concentration (ca. 0.5%) of dextran. The PMNs were then eluted with 40 ml of a 0.85% NaCl solution containing 0.02% disodium EDTA and a few drops of phenosulfonephthalein. The elution rate was about 1.5 ml/minute. The separated lymphocytes and PMN fractions were from 85% to 92% pure (Figure 5).

c) For the determination of leukocyte neuraminidase levels in individual blood samples. About 10 ml of blood was drawn into a heparinized vacutainer and the specimen was chilled in crushed ice. All subsequent steps were conducted at 0° to 4° C. The blood was immediately transferred to a 50 ml polycarbonate conical centrifuge tube containing 20 ml of a 1% solution of Na_2EDTA in 0.85% of NaCl. After thorough mixing by gentle stirring with a glass rod, the material was centrifuged for 5 minutes at 400 x g. The supernatant fraction was decanted and discarded. The erythrocytes were then hemolyzed by adding 40 ml of ice-cold, glass-distilled water to the sedimented blood cells. The hypotonic suspension was stirred for 90 seconds with a glass rod, and then 4 ml of ice-cold, 9.35% NaCl solution was added under continuous stirring in order to return the leukocytes to an isotonic environment. The suspension was then centrifuged for 10 minutes at 400 x g and the clear supernate, as well as the loosely packed layer of erythrocyte ghosts, were carefully removed with a Pasteur pipette and discarded. The leukocyte pellet was quantitatively transferred to a 12 ml conical centrifuge

5a. Mixed leukocytes in the leukocyte-rich supernatant of the heparinized blood sedimented in the presence of dextran (magnification 250x).

5b. Purified lymphocytes derived from 5a and eluted on glass bead column with undiluted plasma (magnification 450x).

5c. Aggregates of platelets, some erythrocytes and residual lymphocytes eluted with 20% plasma - 0.5% dextran in 0.85% NaCl solution (magnification 1000x).

5d. Purified PMN eluted on glass bead column with 0.02% Na_2EDTA in 0.85% NaCl solution (magnification 250x).

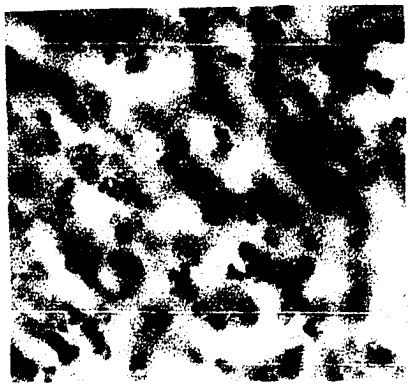


Figure 5a

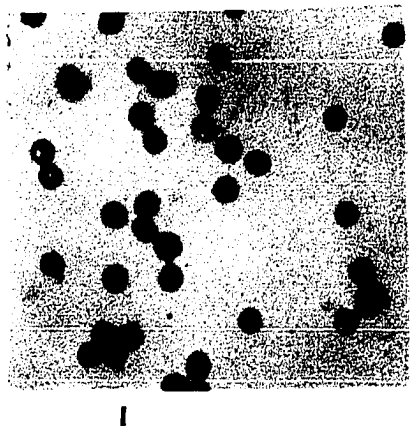


Figure 5b



Figure 5c



Figure 5d

tube with several small aliquots of 0.85% NaCl (totai volume 5 ml). The suspension was centrifuged for 4 minutes at 400 x g, and the supernate and buffy layer were decanted and discarded. The leukocytes were subjected to a second wash with isotonic saline and an aliquot of 20 µl of this cell suspension was taken and diluted 1:250 for cell count. The rest of the washed-cell suspension was centrifuged. The leukocyte pellet was then uniformly suspended in 0.5 ml of ice-cold, glass-distilled water.

Isolation of bovine leukocytes. Bovine blood leukocytes were obtained either by hypotonic shock of the whole blood or by following the procedure used by Gielen, Schaper and Pink (33) as described below. Fresh bovine citrated blood (120 ml of 0.25 M citrate buffer pH 6.2 to 1000 ml of bovine blood) was diluted 1:10 with a solution 0.7% in NaCl and 1% in Na_2EDTA. The blood suspension was then centrifuged at 700 x g for 15 minutes and the buffy coat was removed with a Pasteur pipette and saved. The sedimented blood cells were resuspended after adding a volume of 0.9% NaCl solution equal to the amount of supernant that was decanted. The suspension was subjected to this treatment for two more times. All leukocyte-rich buffy layers were combined and washed twice by resuspending in 0.85% NaCl and centrifuging at 700 x g for 10 minutes. The final leukocyte-rich pellet was exposed to hypotonic shock in 0.001 M Tris-HCl buffer, pH 7.0 in 1% Na_2EDTA for 20 minutes and centrifuged at 35,000 x g for 30 minutes. The hypotonic shock was repeated once more and the leukocyte pellets were suspended in 0.1 M sodium acetate buffer, pH 5.5, containing 1 mM $CaCl_2$ and dialyzed at 4° C for 16 hours against the same buffer. After the dialysis, the suspension was centrifuged at 65,000 x g for 25 minutes and the pellet, suspended in 0.1 M acetate buffer, pH 5.5, was homo-

genized with a Dounce homogenizer and the whole homogenate used as a source of bovine leukocyte neuraminidase.

Isolation of rat blood leukocytes. Ten ml of rat blood was added to a tube with 2 ml of a solution containing 14 mg of NaCl, 100 mg of dextran and 1 mg of sodium heparin. The tube was allowed to stand at room temperature for 45 minutes. The leukocyte-rich supernatant was removed with a Pasteur pipette and centrifuged for 10 minutes at 500 x g. The supernatant was discarded and the pellet was resuspended in 5 ml of ice-cold, glass-distilled water for 90 seconds followed by the addition of 5 ml of 1.7% NaCl solution. After centrifuging at 150 x g for 5 minutes, the hemoglobin-containing supernatant and erythrocyte ghosts were gently removed with a Pasteur pipette. The leukocyte pellets were then washed twice with 0.85% NaCl solution and finally suspended in ice-cold glass-distilled water to be used for enzyme assay.

Preparation of erythrocyte sialoglycoproteins. Human and bovine erythrocyte glycoproteins were extracted from erythrocyte stroma by the phenol-saline extraction method of Wintzer and Uhlenbruck (77). Purification was carried out by isoelectric focusing or by precipitation with cold ethanol.

Procedure. Human or bovine blood was diluted with 0.85% NaCl solution 1:2 (v/v), and centrifuged at 700 x g for 10 minutes. The supernatant and buffy coat were discarded and the sedimented blood cells were resuspended in 0.85% NaCl to the original volume and centrifuged under the same conditions to remove platelets and the leukocyte-rich buffy layer. This procedure was repeated 4 times. The final washed-erythrocyte sediment was suspended in 20 times its volume of cold glass-

distilled water and the suspension was acidified with glacial acetic acid (1 ml per 20 liter of erythrocyte suspension). The material was then allowed to stand at 4° C overnight. The hemoglobin-containing supernatant was carefully removed and the mucoid-rich bottom portion was centrifuged at 1000 x g for 20 minutes in 250 ml centrifuge flasks. The mucoid pellet was washed twice with cold distilled water and then suspended in 1% NaCl solution and stirred overnight at 4° C. The suspension was then mixed with an equal volume of 90% phenol. The temperature of the mixture was raised to about 65° C by constant stirring in an 80° C water bath. After reaching that temperature (about 20 to 30 minutes) the extraction mixture was set overnight in a 4° C cold room. After sedimenting in the cold room, the upper aqueous phase was transferred into dialyzing tubes and dialyzed for 72 hours against 4 changes of 1000 volumes of distilled water. With bovine mucoid preparations, the dialysate was rotary evaporated at 37° C and lyophilized. The crude products were dissolved in water and either precipitated with cold ethanol (65% final concentration) or dialyzed against 1% ampholine, pH 6.0, at 4° C overnight and then subjected to isoelectric focusing (77).

Preparation of human leukocyte subcellular fractions.

Procedure 1 (Figure 6). The lymphocytes and PMN eluted from the glass bead columns were subjected to hypotonic shock in order to eliminate contaminating erythrocytes by suspending them in 0.2% NaCi for 60 seconds followed by restoration of isotonicity. After centrifuging at 150 x g for 10 minutes, the ghosts and hemolysate were decanted and the cell pellets were suspended in ice-cold 0.34 M sucrose at a concentration of about 2 x 10^7 cells per ml of sucrose solution. The cell suspensions

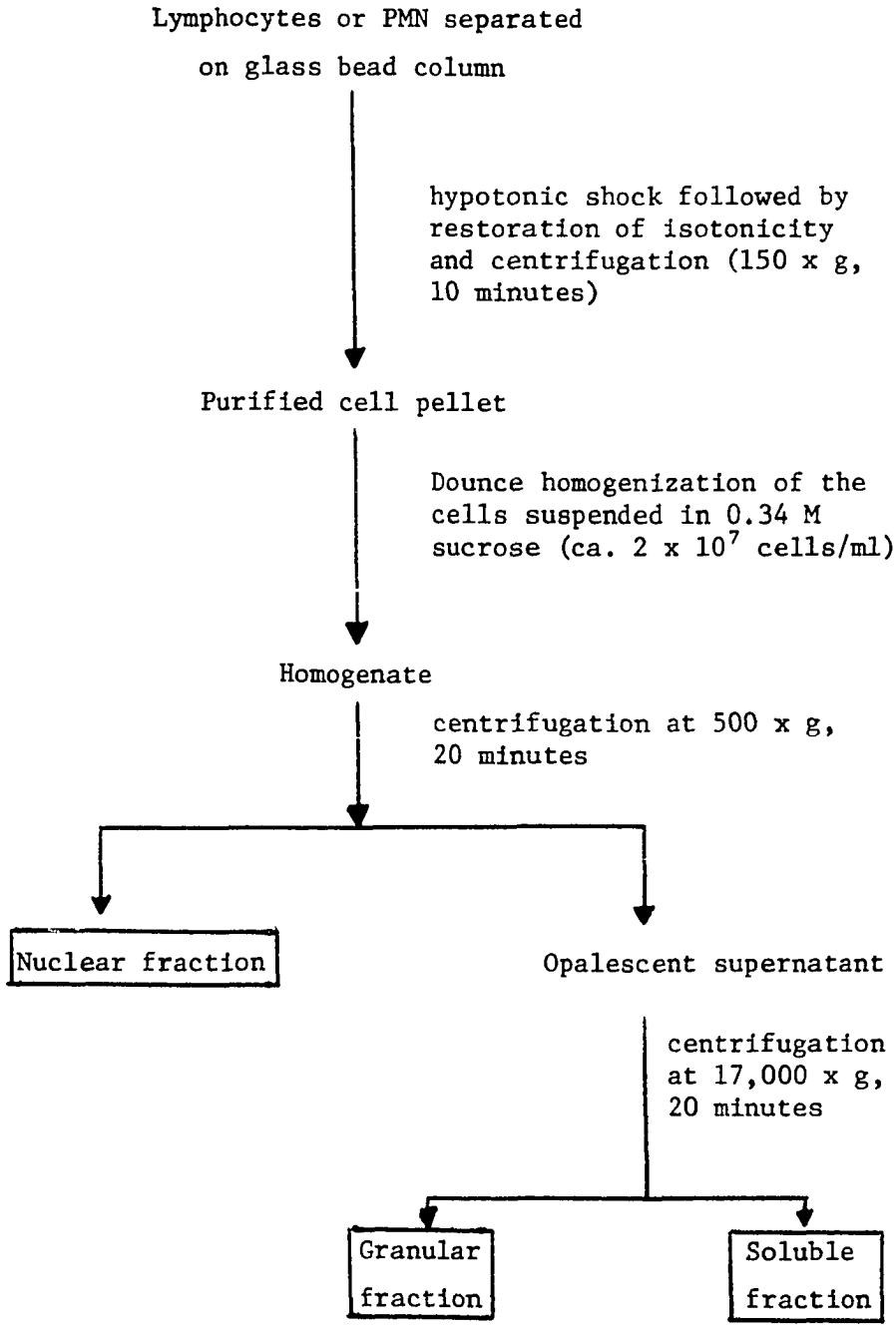


Figure 6. Procedure 1 for the preparation of subcellular fractions on isolated lymphocytes and PMN.

were homogenized with a Dounce homogenizer (30 to 40 strokes were required for complete homogenization).

The homogenates were diluted to 35 ml in a 50 ml centrifuge tube with the same solution and centrifuged at 500 x g for 20 minutes. The pellet, designated the nuclear fraction, was separated from the supernatant which was then centrifuged at 17,000 x g for 20 minutes. The supernatant, designated the soluble fraction, was decanted and the undisturbed pellet, designated the granular fraction, was suspended in cold, glass-distilled water.

Procedure 2 (Figure 7). The isolated lymphocytes and PMN were subjected to hypotonic shock as described in Procedure 1. The cells were then washed once with ice-cold 0.85% NaCl and centrifuged in a refrigerated centrifuge (4° C) at 150 x g for 10 minutes. The pellets were resuspended in about 10 ml of 0.34 M sucrose solution. The cells were then homogenized with a Dounce homogenizer. The homogenate was then quantitatively transferred to a 50 ml conical centrifuge tube and the final volume made to 35 ml with 0.34 M sucrose solution. A 3 ml aliquot was removed and saved. The rest of the homogenate was centrifuged at 500 x g for 20 minutes. The opalescent supernatant was transferred into a 38.5 ml thin-walled cellulose nitrate tube and the loosely-packed pellet was washed with 1 ml of 0.34 M sucrose and centrifuged again under the same conditions. The second supernatant was carefully removed with a Pasteur pipette and combined with the first supernatant; .he resulting pellet was designated the nuclear fraction. The supernatant was then ultra-centrifuged in a Beckman Type 30 rotor at 30,000 rpm for 1 hour after the maximum speed was reached. A clear supernatant and a pellet were obtained.

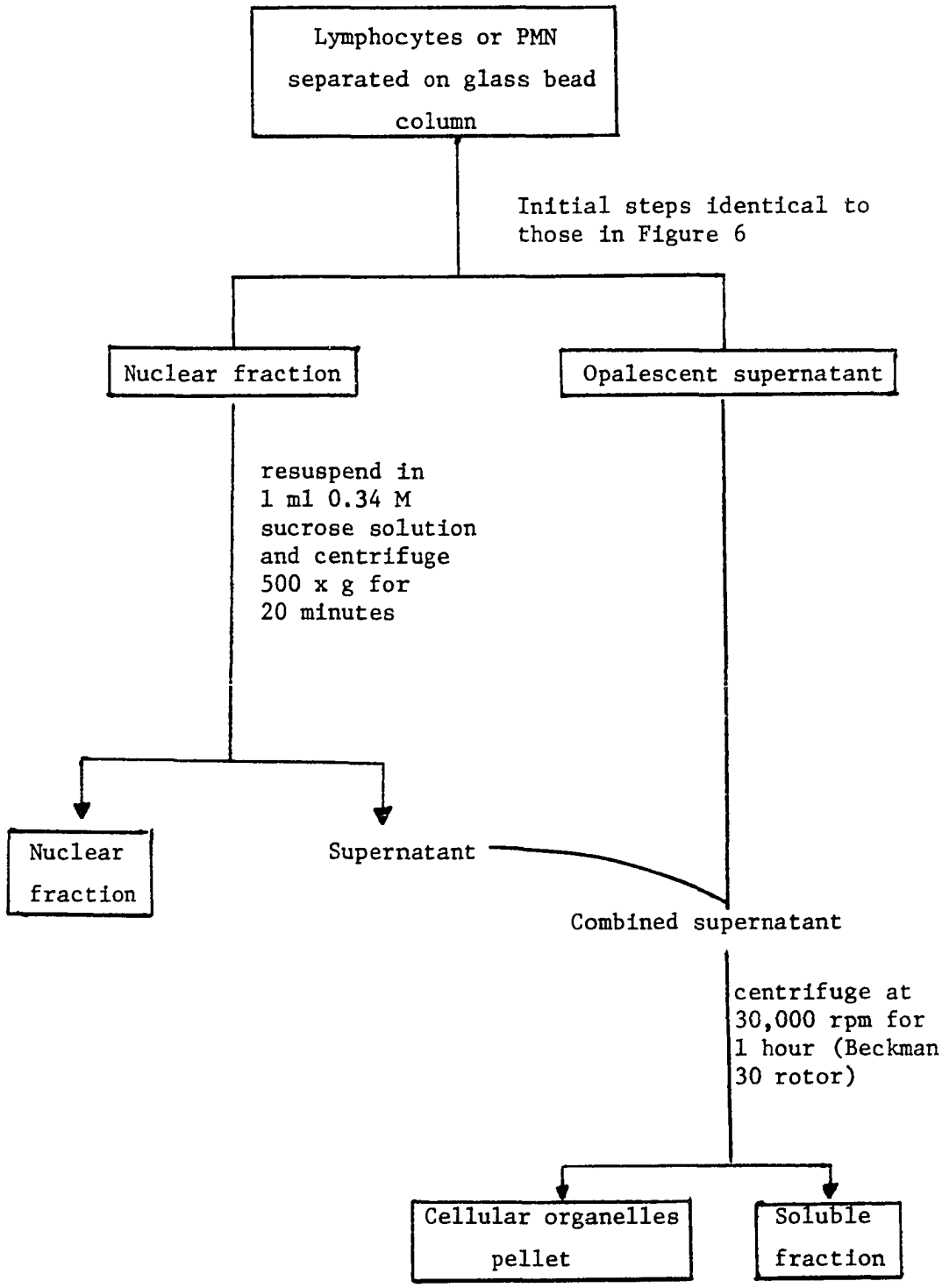


Figure 7. Procedure 2 for the preparation of granular pellets from isolated lymphocytes and PMN for sucrose density gradient centrifugation.

The uppermost portion of the pellet was a layer of fluffy, light-pink membranous material; beneath this layer, the color of the pellet changed from pale greenish-yellow to yellowish-green at the bottom of the tube.

Procedure 3 (Figure 8). The pellets obtained in the centrifugation of postnuclear supernatant (Procedure 2) at 30,000 rpm for 1 hour were suspended in 6 small aliquots of 0.34 M sucrose (total volume 6 ml) by gently stirring the contents o f the tube with the pestle of a Dounce homogenizer followed by vortex-mixing with the pestle loosely held inside the tube. The suspension was transferred to an ice-cold Dounce homogenizer. Since the leukocyte granules cling very tenaciously to stainless steel spatulas, their use must be avoided in the resuspension and transfer of the pellets. The suspensions, homogenized for 5 or 6 strokes, were layered on top of discontinuous sucrose density gradients (Figure 8). Fractionation of human polymorphonuclear leukocyte homogenate on continuous sucrose gradient have been reported by Folds, Welsh and Spitznagel (88). With the knowledge of the densities of the isopycnic bands, discontinuous sucrose density gradient systems (Figure 8) were established. For the fractionation of polymorphonuclear leukocyte organelles, the gradient consisted of 6 ml of the homogenate in 0.34 M (11.64% w/v) sucrose and 10 ml each of sucrose solutions, 0.88 M (30% w/v), 1.11 M (38% w/v) and 1.46 M (50% w/v). The lymphocyte homogenate, also 6 ml in 0.34 M sucrose solution, was layered on a gradient (Figure 8) consisting of 10 ml each of sucrose solutions, 0.88 M (30% w/v), 1.17 M (40% w/v) and 1.69 M (58% w/v). With both gradients, the isopycnic ultracentrifugation was carried out using a Beckman SW 27 rotor at

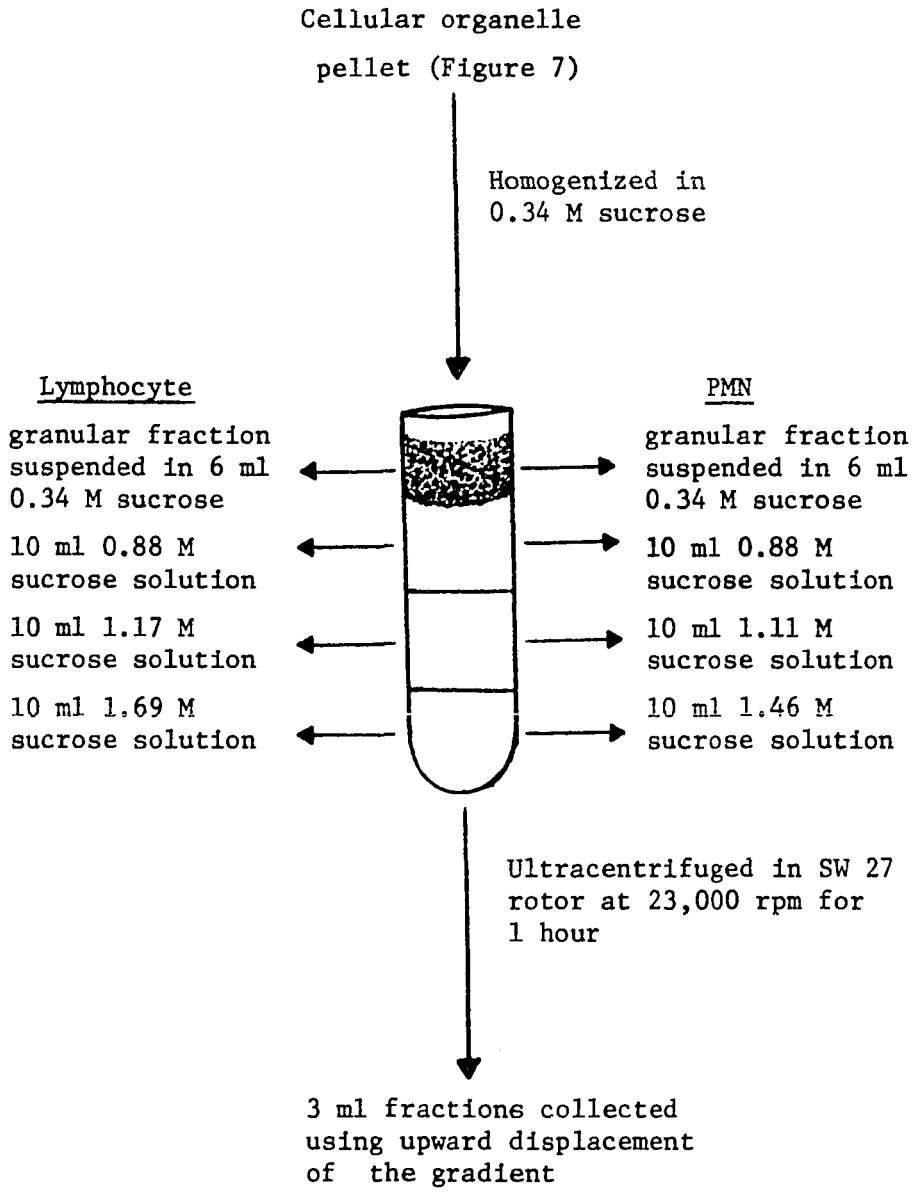


Figure 8. Discontinuous sucrose density gradient centrifugation utilized for the fractionation of the granular fraction of lymphocytes and PMN.

23,000 rpm for 1 hour. Successive 3 ml fractions were collected from the top. The gradient was displaced with 60% sucrose for PMN and 65% sucrose for lymphocyte gradient.

CHAPTER III

RESULTS

Isolation of Leukocytes and Separation of Lymphocytes from Polymorphonuclear Leukocytes

Isolation of Leukocytes

Human leukocytes. In the early stages of this study, the leukocyte-rich pellet was subjected to hypotonic shock in ice-cold, glass-distilled water for 90 seconds to hemolyze contaminating erythrocytes. Although normal leukocytes do not appear to be adversely affected by this treatment, leukocytes from patients with various diseases (allergy, herpes zoster, some forms of arthritis, etc.) were found to be more fragile; microscopic examination of leukocyte smears showed that a significant portion of leukocytes from these patients burst during the hypotonic shock. Since minor damage, not easily detected under the microscope, might also occur in normal leukocytes, we decided in later experiments to use 0.2% NaCl solution instead of glass-distilled water to lyse the contaminating erythrocytes.

Methods for the isolation of leukocytes from whole heparinized blood using dextran sedimentation (89) are, in general, effective and

simple to perform. However, it appears that many factors may influence the rate of erythrocyte rouleaux (adherence of erythrocytes along their concave surfaces, thus forming columns in rows like piles of coins) formation since aliquots of the same blood specimen placed in different graduated cylinders often gave quite different sedimentation rates. When mixing the blood with dextran-saline solution, the graduated cylinders must be inverted gently to avoid trapping air bubbles. Occasionally, larger amounts of dextran-saline solution were needed to hasten the sedimentation rate, or a second sedimentation was required to increase the leukocyte yields. In most cases, the method listed in Figure 2 gave about 55 to 65% of leukocyte recovery from whole blood. This percentage of recovery is comparable to that reported by Rabinowitz (87). In our initial studies using packed blood cells as starting material, the yields were considerably lower, running about 15%.

Rat leukocytes. Heparinized rat blood gave consistently good sedimentation rates when treated with dextran-saline solution and no problems were encountered in the course of leukocyte isolation.

Bovine leukocytes. Bovine blood, subjected to the same dextran-saline treatment as described for human blood, gave practically no sedimentation upon standing up to 12 hours at room temperature or at 37° C. Various anticoagulants and different amounts and concentrations of dextran-saline solution were tried but none of the methods appreciably enhanced the sedimentation rate. In the course of these experiments, it was observed that when 1% Na_2EDTA in 0.7% NaCl was used as anticoagulant (approximate ratio - 2 volumes of blood to 1 volume of Na_2EDTA-saline solution) and the mixture was centrifuged at 1000 x g for 20

minutes, the upper layer of the sediment was very rich in lymphocytes. This lymphocyte-rich layer was carefully removed using a Pasteur pipette. However, since the underlying layer was richer in granulocytes, and there was no clear boundary, the composition of these leukocyte mixtures varied from one preparation to another.

Separation of Lymphocytes from PMN

Leukocytes were sedimented using 3% dextran in isotonic saline solution instead of 5% dextran in Hank's solution as utilized by Rabinowitz (87). The elution patterns from the glass bead column were similar to those reported by Rabinowitz.

The recovery of leukocytes from the glass bead column ranged from 55 to 65%. Heavy erythrocyte contamination should be avoided, since it tends to reduce lymphocyte recovery and affects the column flow rate causing some operational problems in the course of elution.

Properties of Human Leukocyte Neuraminidase

Unless specifically stated, all studies were conducted using aqueous suspensions of mixed human leukocytes prepared as described under Methods. Dounce homogenization or treatment with ultrasound (a 30 second burst delivered by the microprobe of a Bronson Bio-Sonicator at 70% efficiency) did not increase the enzyme activity of the suspension or of the supernatant fraction.

Kinetic Studies

Optimal pH. Aqueous suspensions of mixed human leukocytes showed optimal neuraminidase activity at pH 4.0 (Figure 9). As can be seen on

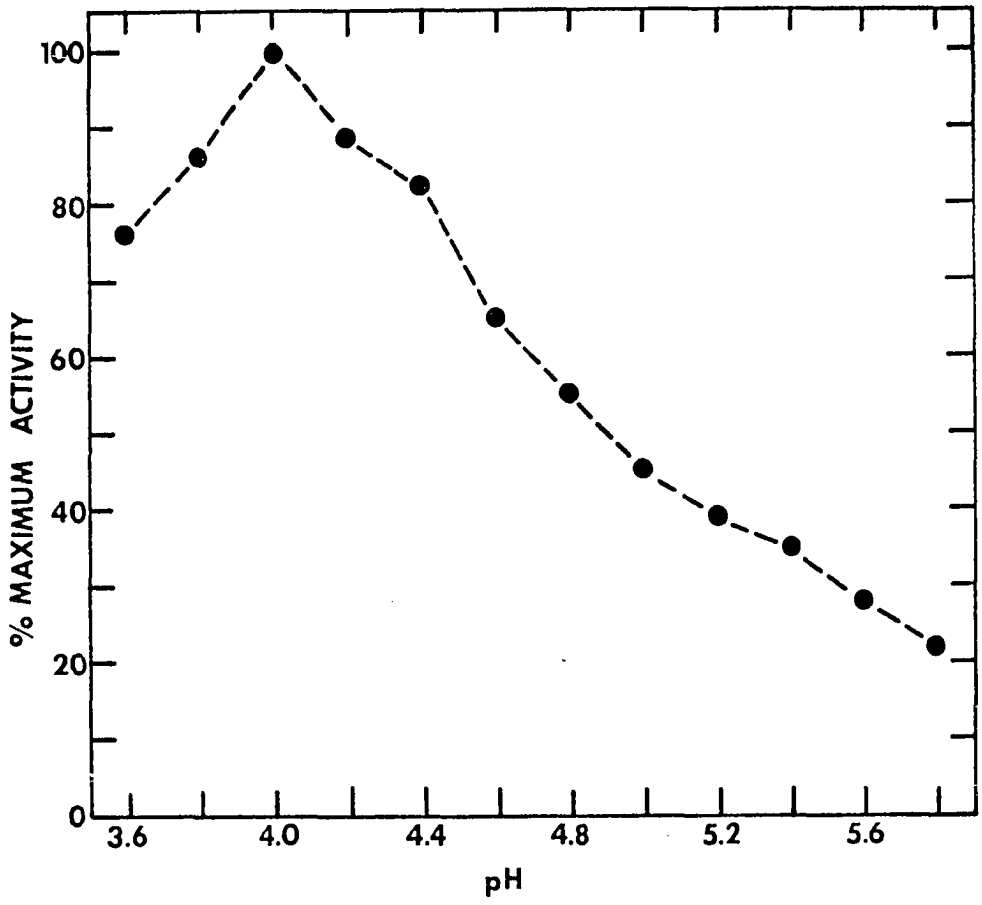


Figure 9. The pH versus activity curve of human leukocyte neuraminidase. The enzyme activity was measured with neuramin-lactose (200 nmoles) as the substrate. The enzyme preparations consisted of an aqueous suspension of human leukocytes (10^7 cells per tube) and sodium acetate - acetic buffers (75 mM final concentration).

the pH-activity curve (Figure 9), a small yet noticeable hump was always observed at pH 4.4. When leukocytes were homogenized in distilled water or in isotonic KCl and ultracentrifuged at 105,000 x g for 1 hour, only 15 to 20% of the total activity was detected in the supernatant fraction assayed at pH 4.0 and just traces of neuraminidase activity could be detected at pH 5.8.

Both bovine and rat leukocyte neuraminidases showed optimal activity at pH 4.0 using neuramin-lactose as substrate. Since Gielen, Schaper and Pink (33) assayed bovine leukocyte neuraminidase at pH 5.5 in the presence of 1 mM $CaCl_2$, the possible effect of $CaCl_2$ on the pH optimum was studied. However, our data indicated that the pH optimum of bovine leukocyte neuraminidase remained at 4.0 in the presence or in the absence of 1 mM $CaCl_2$, using NL or human or bovine erythrocyte membrane sialoglycoprotein as substrates. No significant amounts of enzyme-released sialic acid could be detected even after 12 hours of incubation at pH values ranging from 3.6 to 5.8. Both glycoproteins contained sialic acid which was effectively hydrolyzed by Clostridium perfringens neuraminidase. After a 3 hour incubation at pH 5.0 and 37° C, about 57% and 19% of the total sialic acid was released from human and bovine erythrocyte membrane sialoglycoprotein, respectively. Under the same conditions, 74% of the sialic acid from NL(2→3) was hydrolyzed by the bacterial enzyme.

Progress curves of human leukocyte neuraminidase. The rate of hydrolysis of NL(2→3) under the standard assay conditions is linear for at least 3 hours (Figure 10). A linear relationship was also observed between enzyme concentration (human leukocyte count from 5 to 15 million) and NANA released in a 3 hour incubation period (Figure 11).

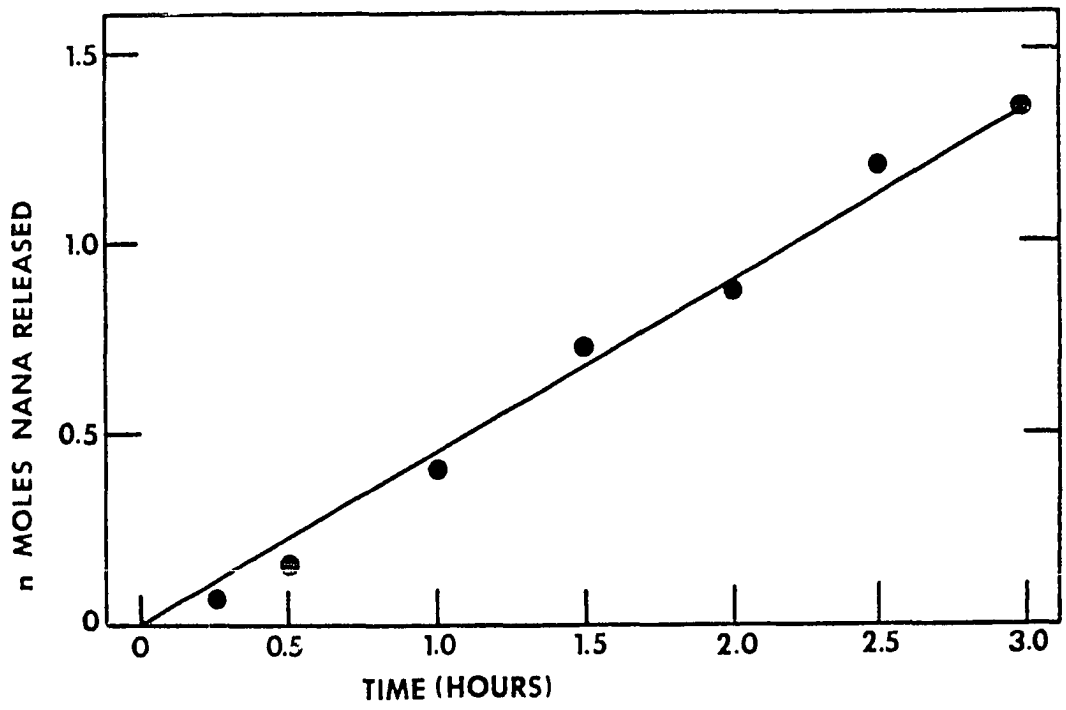


Figure 10. Progress curve showing the linear relationship between NANA released and length of incubation.

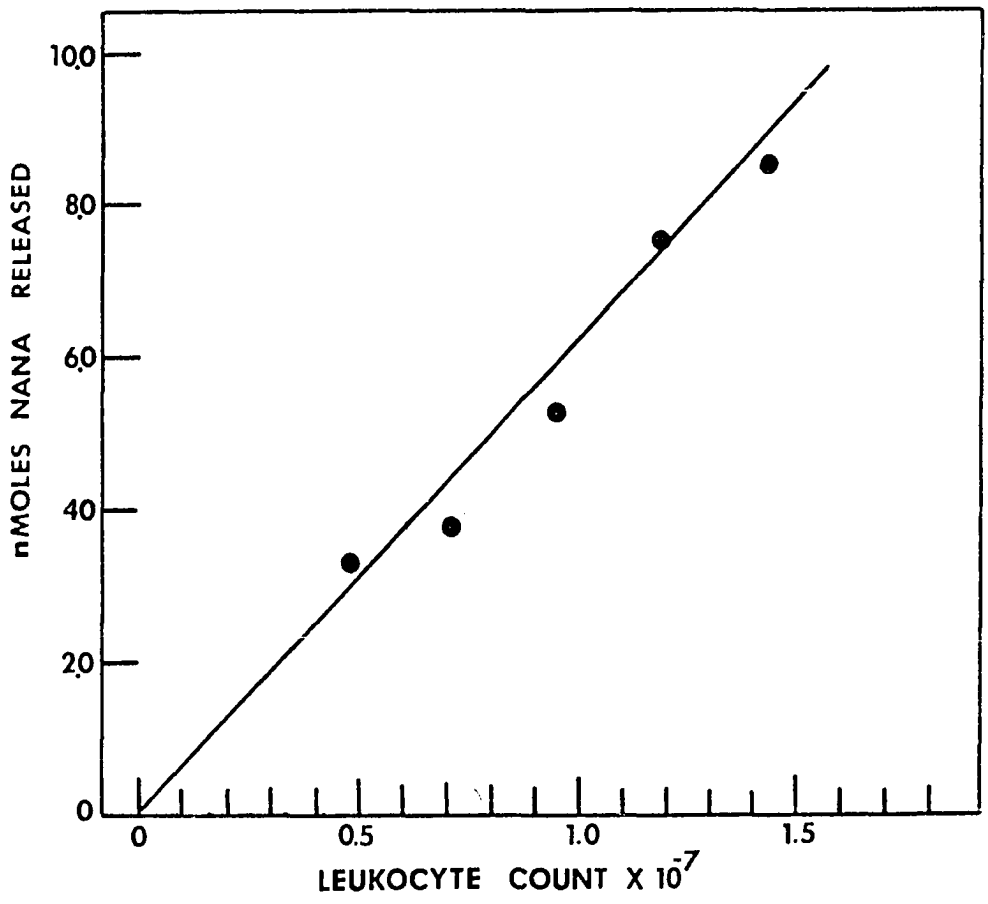


Figure 11. Linear dependency of NANA release on the concentration of human leukocyte neuraminidase in the incubation system.

Km for neuramin-lactose. The Km value for NL calculated from the [S]/V versus [S] plot (90) is 5.62×10^{-4} M (Figure 12), very similar to the values previously reported for the lysosomal neuraminidases of rat mammary glands (21) and Hela cells (30).

Enzyme stability. Storage of the leukocyte suspension for 24 hours at 4° C in the refrigerator resulted in a 10 to 15% loss of neuraminidase activity, while frozen storage for the same length of time resulted in about 8% loss of enzyme activity. At the end of 1 hour of preincubation at 37° C in distilled water, less than 60% of the initial activity could be detected. Freezing and thawing also proved to be deleterious for the neuraminidase of human leukocytes. Four cycles of freezing and thawing resulted in about 50% loss of activity; thereafter, the losses were less pronounced and at the end of 10 cycles of freezing and thawing some 40% of the initial activity was still present. Exposure to sucrose or incubation in the presence of sucrose at concentrations between 0.24 M and 1.14 M did not inhibit leukocyte neuraminidase activity.

Effect of divalent cations. The effect of several divalent cations, Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2} and Hg^{+2}, was studied by adding the respective chloride at concentrations between 1×10^{-3} M and 1×10^{-6} M. Except for Hg^{+2}, which caused about 25% inhibition at 1 mM concentration, these cations had little or no effect on the enzyme activity. A similar pattern was also observed with these divalent cations and rat leukocyte neuraminidase.

Effect of anions. The effect of LiCl, NaCl and KCl on leukocyte neuraminidase was studied at concentrations between 2×10^{-1} M and

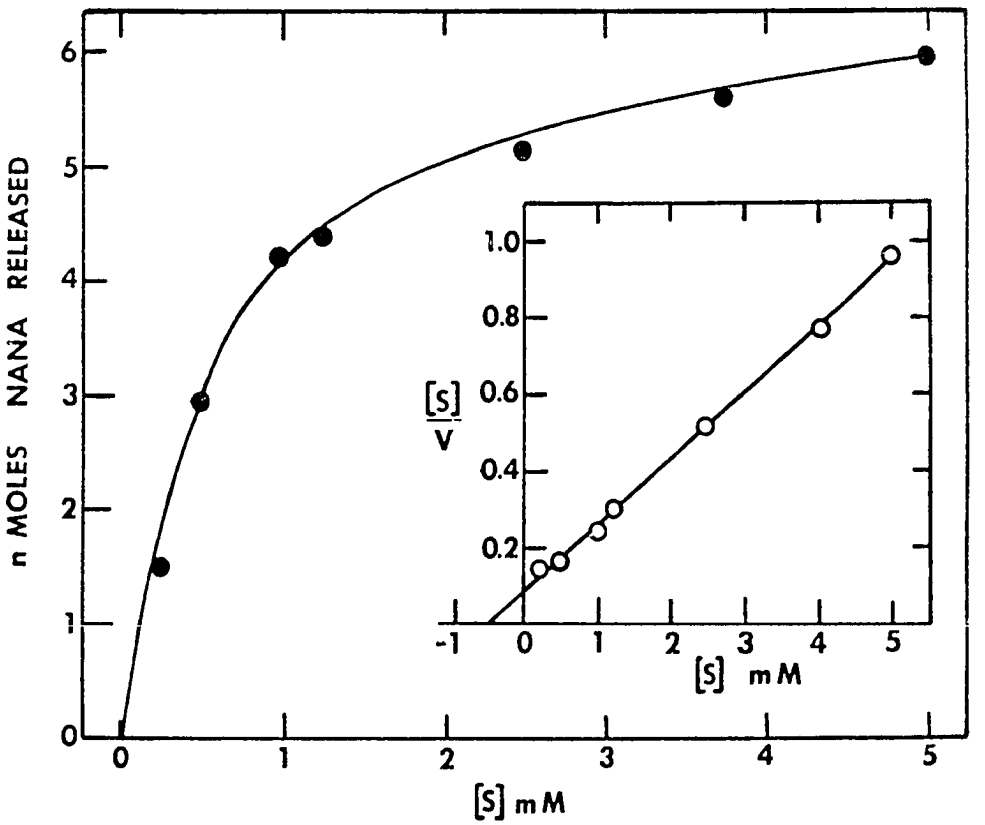


Figure 12. Effect of substrate concentration on the rate of hydrolysis. Assays conducted as described under Methods except for the concentration of neuramin-lactose which was varied as shown.

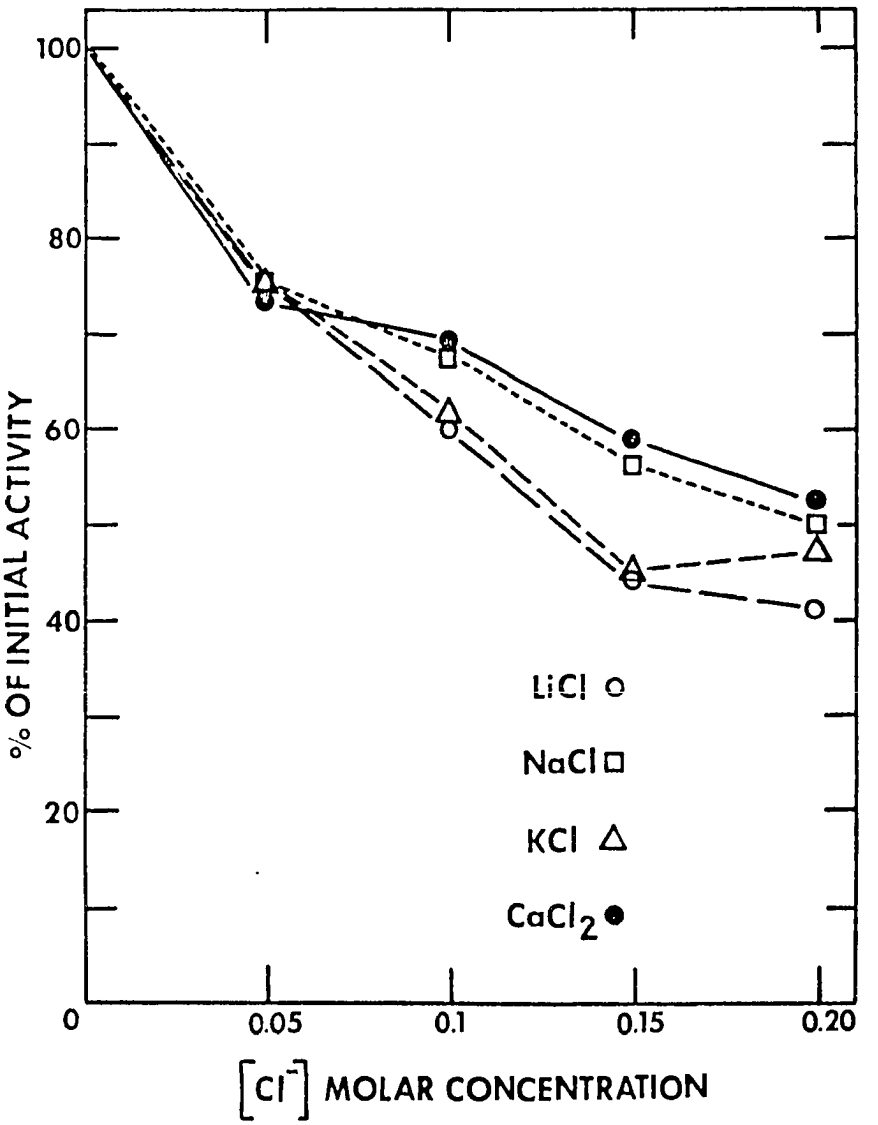


Figure 13. Effect of chloride concentration on human leukocyte neuraminidase. The salts were added to the enzyme system just prior to the start of the incubation period.

5×10^{-2} M. More than 50% inhibition was observed at 2×10^{-1} M concentration of these salts. When the incubation mixture contained $CaCl_2$ at the same molar concentrations of chloride ion, similar degrees of inactivation were observed (Figure 13). These experiments indicate that the inhibitory effect of alkali chlorides is not due to the monovalent cation, but might either be due to a specific effect of the chloride anion interfering with the enzyme substrate binding or to a non-specific effect because of the increased electrolyte concentration in the incubation system, since high ionic concentrations may change the rate constants by changing the activity coefficients of enzyme macromolecule, substrate and ionic species of the buffer. When various halides at a final concentration of 0.15 M were added to the incubation system it was observed that the extent of the inhibition increased from 32% with NaCl to 49% with NaBr and to 79% with NaI. When citrate buffer at a final concentration of 75 mM was used neuraminidase activity was only 58% of that with acetate buffer.

Effects of detergents. Low concentrations of Triton X-100 caused strong inhibition of leukocyte neuraminidase. At 0.05% (v/v) of Triton X-100 only 54% of the initial enzyme activity was detected; higher concentrations of detergent caused a proportionally smaller loss of activity (Figure 14). Loss of neuraminidase activity was also observed upon addition of sodium deoxycholate. About 80% of the initial activity was recovered in the presence of 0.1% (w/v) of deoxycholate; higher concentrations of this bile salt failed to cause any further loss of activity (Figure 14).

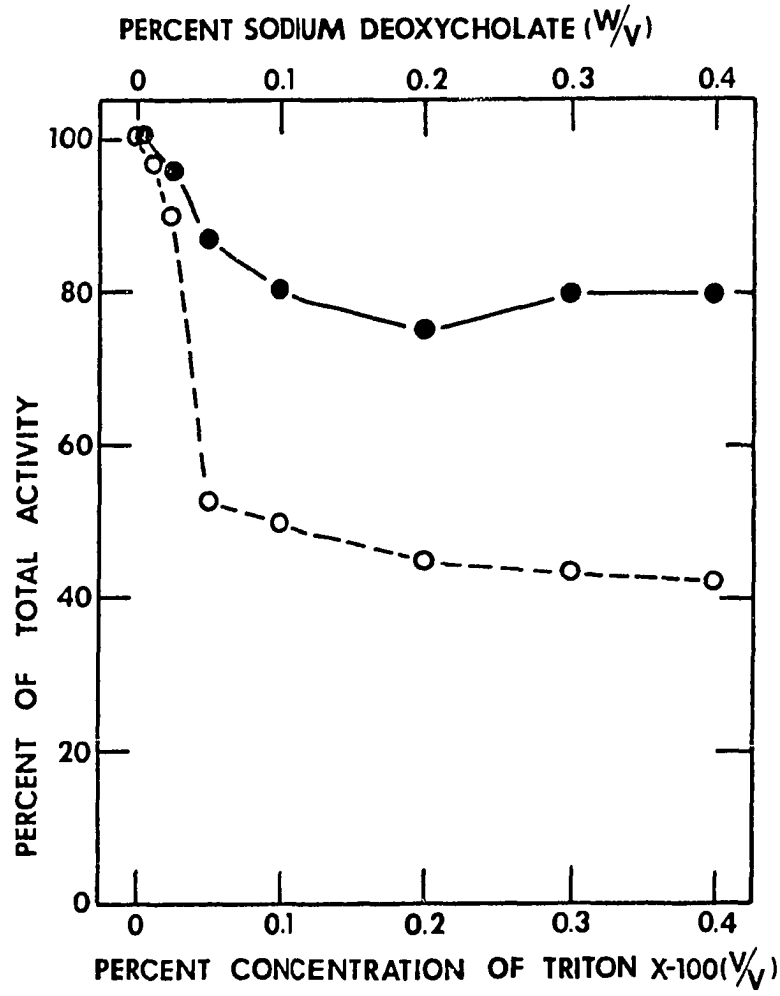


Figure 14. Effect of Triton X-100 (O···O) and of deoxycholate (●—●) on human leukocyte neuraminidase. Increasing concentrations of these compounds were added to the enzyme preparation and the mixtures were kept in an ice bath for 15 minutes prior to the neuraminidase assay.

Substrate Specificity

The rate of hydrolysis of ten different substrates by the neuraminidase of human leukocytes is shown in Table 1. Neuramin-lactose sulfate was hydrolyzed at a higher rate than NL(2→3) (Figure 15). Mixed bovine-brain gangliosides, NL(2→6) and ovine submaxillary glycoprotein were comparatively poor substrates. However, the sialoglycopeptides from pronase digests of the salivary glycoprotein were hydrolyzed at a very fast rate. Little or no sialic acid was released when fetuin, urinary sialoglycoprotein or erythrocyte membrane sialoglycoproteins were used as substrates.

Levels of Leukocyte Neuraminidase Activity in Normal and Diseased Subjects

Levels of Neuraminidase Activity in Mixed Leukocytes

Peripheral blood leukocyte neuraminidase activity was measured in a limited number of healthy adult males and females. The mean specific activity (Table 2), expressed as nmoles NANA/3 hour/10^6 cells, for 11 young male adults was 0.65 with a standard deviation of $\pm$ 0.21, the wide range of activities from 0.38 to 1.01 is reflected in the large standard deviation. For 5 young female subjects, the mean was 0.44 with a standard deviation of $\pm$ 0.07. Statistical evaluation of the levels of leukocyte neuraminidase activity in arthritic patients gave no correlation between the enzyme activity and age, sex or treatment of the patients.

Levels of Neuraminidase Activity in Lymphocytes and PMN

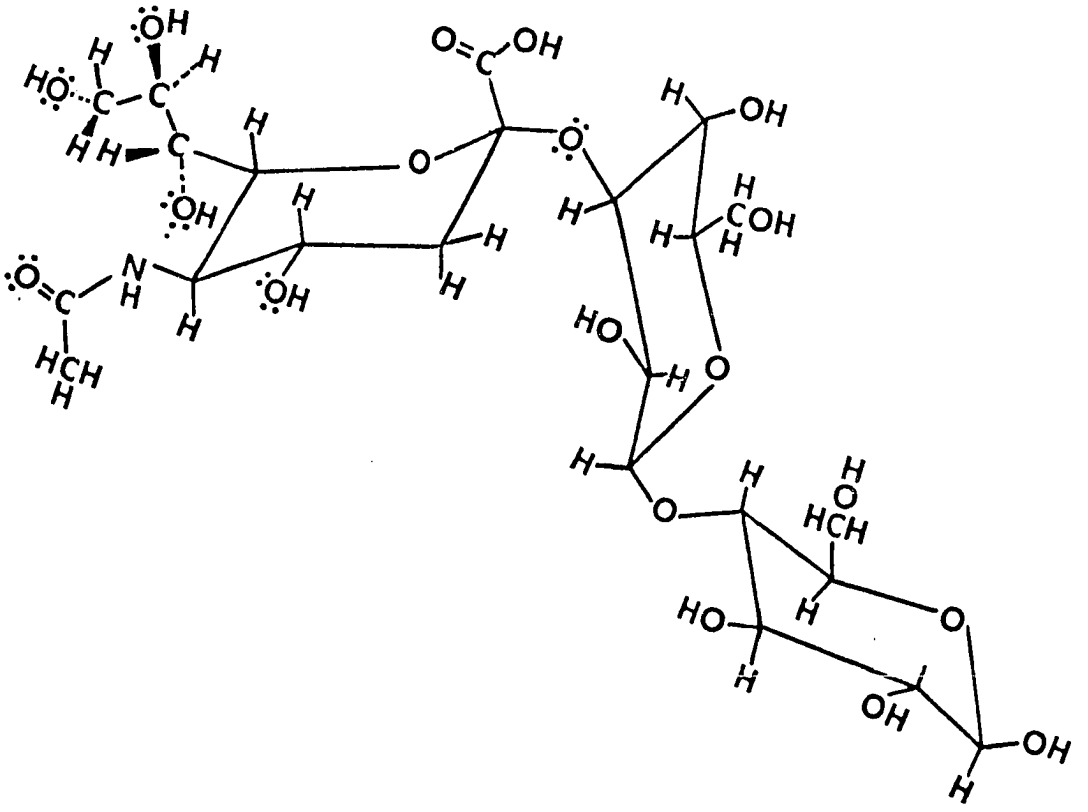


Figure 15. Molecular structure of the trisaccharide, N-Acetyl-neuraminosyllactose [α-D-N-acetylneuraminosyl-(2→3)β-D-galactopyranosyl-(1→4)-D-glucopyranose].

TABLE 1

HYDROLYSIS OF VARIOUS SUBSTRATES BY THE NEURAMINIDASE OF HUMAN LEUKOCYTES

Substrates	*Specific Activity
Neuramin-lactose (2→3)	0.49
Neuramin-lactose (2→6)	0.20
Neuramin-lactose sulfate	1.39
Sialoglycopeptides from OSM	1.28
Ovine submaxillary glycoprotein	0.22
Bovine brain gangliosides	0.05
Human erythrocyte membrane sialoglycoprotein	trace
Bovine erythrocyte membrane sialoglycoprotein	trace
Fetuin	trace
Tamm and Horsfall urinary sialoglycoprotein	trace

* nmoles NANA released/10^6 cells /3 hours.

TABLE 2

NEURAMINIDASE ACTIVITY IN LEUKOCYTES
OF NORMAL HUMAN BEINGS

Male Donors			Female Donors		
No.	Age years	Specific Activity [a]	No.	Age years	Specific Activity [a]
1	21	0.45	1	23	0.48
2	21	1.01	2	24	0.38
3	22	0.38	3	26	0.48
4	22	0.51	4	26	0.52
5	22	0.78	5	26	0.36
6	22	0.98			
7	23	0.41			
8	23	0.58			
9	23	0.62			
10	23	0.74			
11	25	0.65			
Mean SD:		$0.65^{b\pm}0.21$	Mean SD:		$0.44^{b\pm}0.07$

[a] nmoles NANA released from NL per 10^6 of cells in 3 hours incubation period.

[b] the difference in the means from male and female donors were statistically insignificant.

The neuraminidase activities in lymphocytes and PMN were determined in male subjects only. Similar specific activities, expressed as nmoles NANA released/3 hours/10^6 cells, were found for lymphocytes 0.62 ± 0.13 (mean $\pm$ S.D., n = 4) and for PMN, 0.50 ± 0.07. The specific activities, when expressed as nmoles NANA released/3 hours/mg protein, were 6.72 ± 1.52 (mean $\pm$ S.D. n = 4) for lymphocytes and 4.80 ± 0.87 for PMN.

Subcellular Distribution of Leukocyte Neuraminidase Activity

The intracellular localization of neuraminidase activity was investigated in human lymphocytes and granulocytes isolated by glass bead columns as described under Methods. The cells were suspended in 0.34 M sucrose and homogenized with Dounce homogenizer; the completeness of homogenization was ascertained by phase contrast microscopy. The homogenates were then fractionated by two different procedures.

By the first procedure (Figure 6) of differential centrifugation nuclear fraction (500 x g for 20 minutes), granular fraction (17,000 x g for 20 minutes) and the soluble fraction were isolated. Neuraminidase activity as well as the lysosomal marker enzymes, acid β-glycerophosphatase and β-glucuronidase activities were measured in each fraction. The average of the results obtained from three experiments (Table 3) showed that 16.30% of the total lymphocyte protein was recovered in the granular fraction while 13.18% and 62.96% were recovered in the nuclear and soluble fractions, respectively. With granulocytes, 21.82% of total protein was found in the granular fraction, while 13.02% and 61.10% were found in the nuclear and soluble fractions, respectively. Practically all the neuraminidase activity was found in the granular fractions

TABLE 3

PROTEIN DISTRIBUTION IN SUBCELLULAR FRACTIONS ISOLATED FROM HUMAN LYMPHOCYTES AND POLYMORPHONUCLEAR LEUKOCYTES

Cell Fractions [a]	Percent Recoveries [b]	
	Lymphocytes	PMN
Nuclear fraction	13.18±1.96	13.02±4.05
Granular fraction	16.30±0.91	21.82±3.09
Soluble fraction	62.96±2.00	61.10±1.88

[a] Fractions prepared by subcellular fractionation procedure 1 (Fig. 6).

[b] The results show the Mean ± SD from three different experiments.

TABLE 4

DISTRIBUTION OF ENZYME ACTIVITIES IN LYMPHOCYTE AND POLYMORPHONUCLEAR LEUKOCYTE SUBCELLULAR FRACTIONS

Cell Fractions [a]	Percent of the Activity Present in Whole Homogenates [b]					
	β-Glucuronidase		Acid-β-glycerophosphatase		Neuraminidase	
	Lymphocytes	PMN	Lymphocytes	PMN	Lymphocytes	PMN
Nuclear Fraction	3.76±1.61	3.50±1.25	5.00±0.50	6.56±3.85	3.98±1.76	5.92±2.16
Granular Fraction	81.32±3.00	81.07±6.73	70.85±4.05	72.12±2.77	105.73±10.19	98.43±9.50
Soluble Fraction	9.26±2.50	7.83±3.51	14.67±3.36	10.74±5.00	0	0

[a] Fractions prepared by subcellular fractionation procedure 1 (Fig. 6).

[b] The results show the mean ± S.D. from three different experiments.

with both lymphocytes and PMN (Table 4). The other two hydrolases showed some extragranular activity, but the bulk (70 to 80%) was recovered in the granular fraction (Table 4). The Relative Specific Activity Profiles (Figure 16) show similar patterns for neuraminidase and for the two lysosomal marker enzymes.

Although these findings indicate that leukocyte neuraminidase is possibly a lysosomal hydrolase, one must recognize that the granular fraction obtained by the isolation procedure (Figure 6) is quite heterogeneous. Preliminary experiments also showed that the postnuclear pellets, sedimented at 17,000 x g for 20 minutes, contained large amounts of 5'-nucleotidase activity, a marker enzyme for plasma membrane. In view of some recent reports suggesting the presence of a neuraminidase active on sialoglycolipids of plasma membrane from rat liver (18) and rat brain synaptosomes (91), further fractionation of the particulate fraction was imperative in order to pinpoint with some accuracy the intracellular localization of leukocyte neuraminidase.

For these experiments, the postnuclear supernatant of homogenates in 0.34 M sucrose was ultracentrifuged for 1 hour at 78,500 x g. The clear supernatant was saved and the sediment, resuspended in 0.34 M sucrose solution, was layered on top of the discontinuous sucrose gradient and centrifuged as described in Figure 8. After centrifugation, the PMN granular fraction gave three clearly visible bands. The protein distribution profile (Figure 17) showed the three bands with peaks at fractions 3, 6 and 9 and a pink-colored membranuous sediment at the bottom of the tube. The profiles of the distribution of enzyme activities (Figure 18) revealed that, although there is some overlap, 5'-nucleotidase

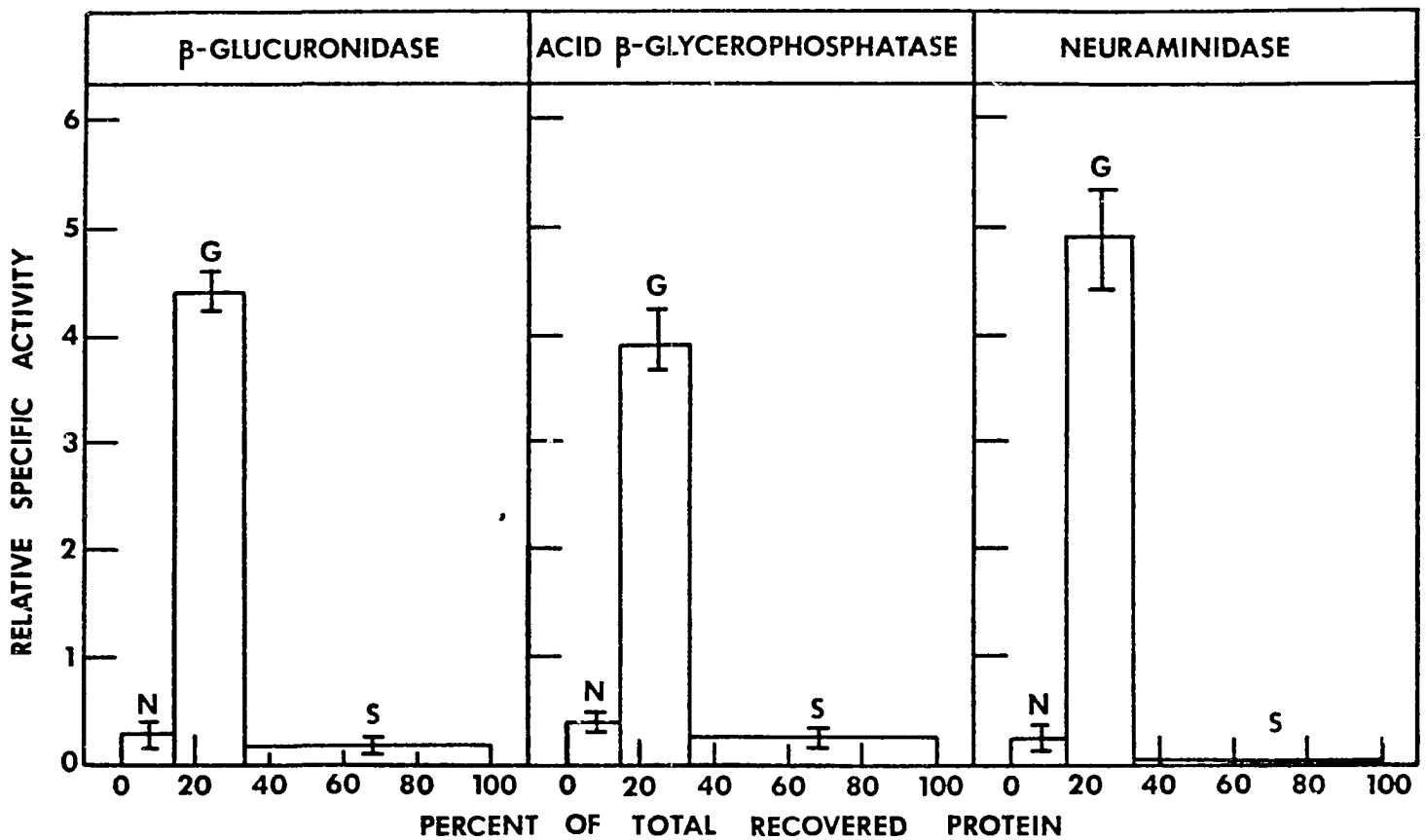


Figure 16a. Relative Specific Activity

$$\left(\frac{\%\text{ of total recovered enzyme activity}}{\%\text{ of total recovered protein}}\right)$$

of β-glucuronidase, acid β-glycerophosphatase and neuraminidase in human lymphocyte nuclear fraction (N), granular fraction (G) and soluble fraction (S) prepared by subcellular fractionation procedure 1 (Figure 6).

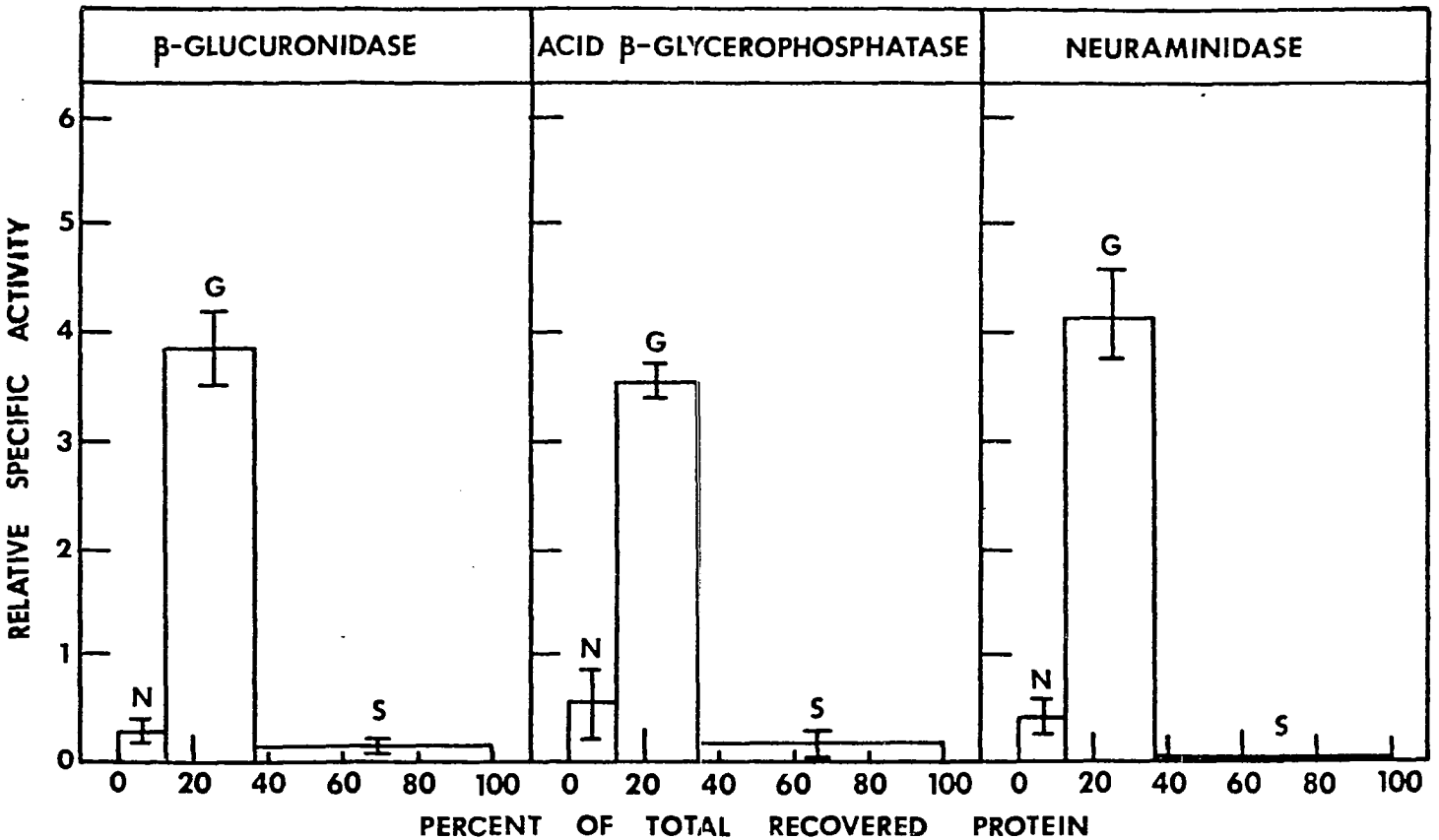


Figure 16b. Relative Specific Activity

$\left(\frac{\%\text{ of total recovered enzyme activity}}{\%\text{ of total recovered protein}}\right)$ of β-glucuronidase, acid β-glycerophosphatase and neuraminidase in human polymorphonuclear leukocyte nuclear fraction (N), granular fraction (G) and soluble fraction (S) prepared by subcellular fractionation procedure 1 (Figure 6).

activity is concentrated in the lighter half of the gradient while β-glucuronidase, acid β-glycerophosphatase and neuraminidase activity appeared concentrated in the heavier half of the gradient. Some differences were observed in the patterns of distribution of β-glucuronidase and acid β-glycerophosphatase activities. This is not unexpected, since β-glucuronidase has been found not only in lysosomes but also in endoplasmic reticulum (92). The pattern of the distribution of neuraminidase activity is, to a large extent, similar to that of acid β-glycerophosphatase except for fraction 1, where neuraminidase has a relative specific activity of about 1.6, suggesting the presence of either some solubilized neuraminidase or a very light membrane fraction enriched in neuraminidase. The distributions of these four enzymes point out that most of the PMN neuraminidase behaves as a lysosomal enzyme. However, the possibility of a minor fraction, or a different form, of neuraminidase localized in the plasma membrane can not be ruled out.

When the fractionation system utilized for granulocytes was tried on lymphocytes, a rather large portion of the material sedimented to the bottom of the gradient as a yellowish-green pellet, rich in lysosomal enzymes. Since Weissmann, Douglas, Hirschhorn and Brittinger (93) reported that practically all hydrolytic activities from human lymphocyte postnuclear supernatant were recovered in a discontinuous sucrose-density gradient at sucrose concentrations between 1.4 M and 1.7 M, a different gradient was used for lymphocytes. For these experiments, the granular fraction, resuspended in 6 ml of 0.34 M sucrose was layered in top of a density-gradient composed of three layers of 10 ml each of 0.88, 1.17 and 1.69 M sucrose solutions, respectively (Figure 8).

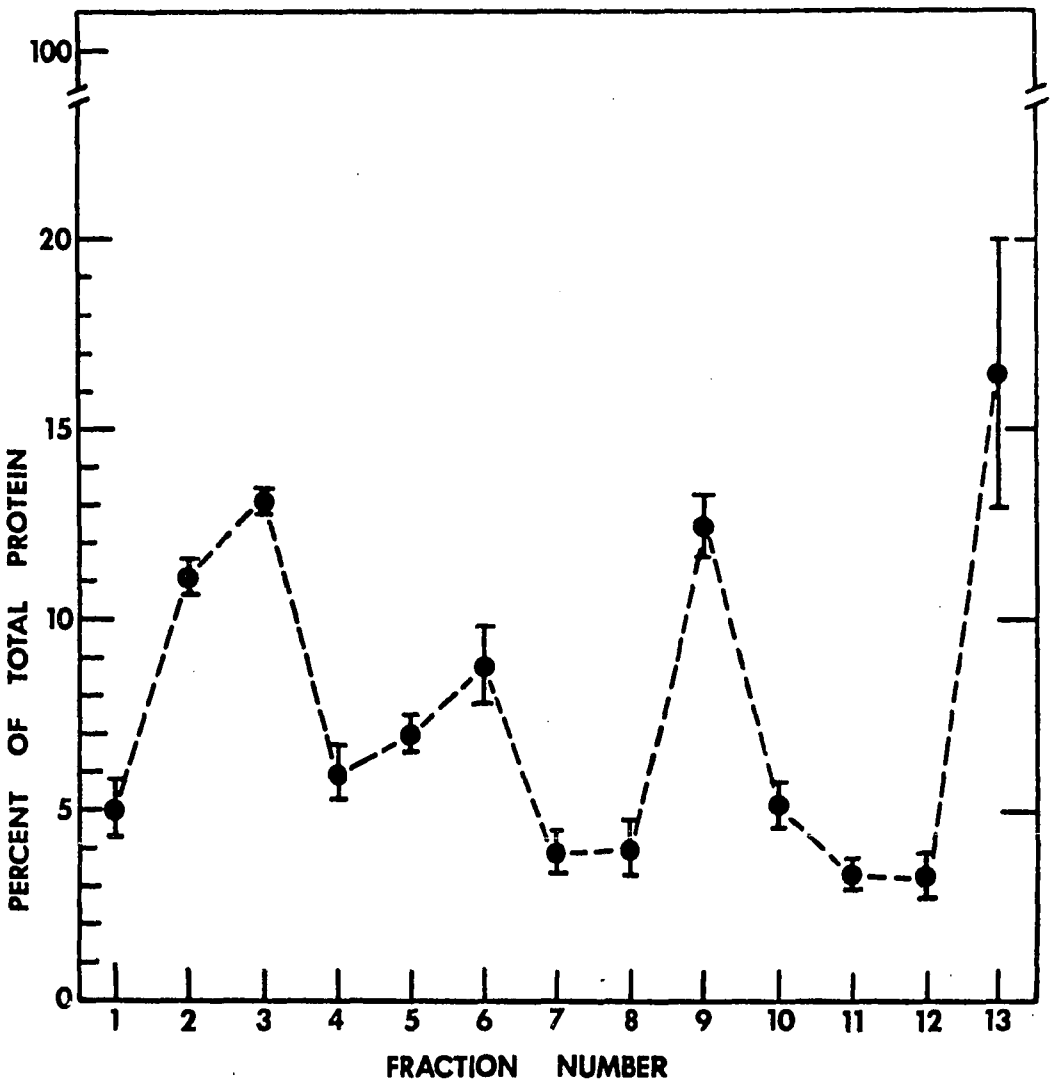


Figure 17. Protein distribution profile of the postnuclear particulate fraction of PMN leukocytes in the discontinuous sucrose gradient (Figure 8). The 3 ml fractions were collected by upward displacement with heavy sucrose. The density increases from left to right. The results represent mean ± S.D. of three separate experiments.

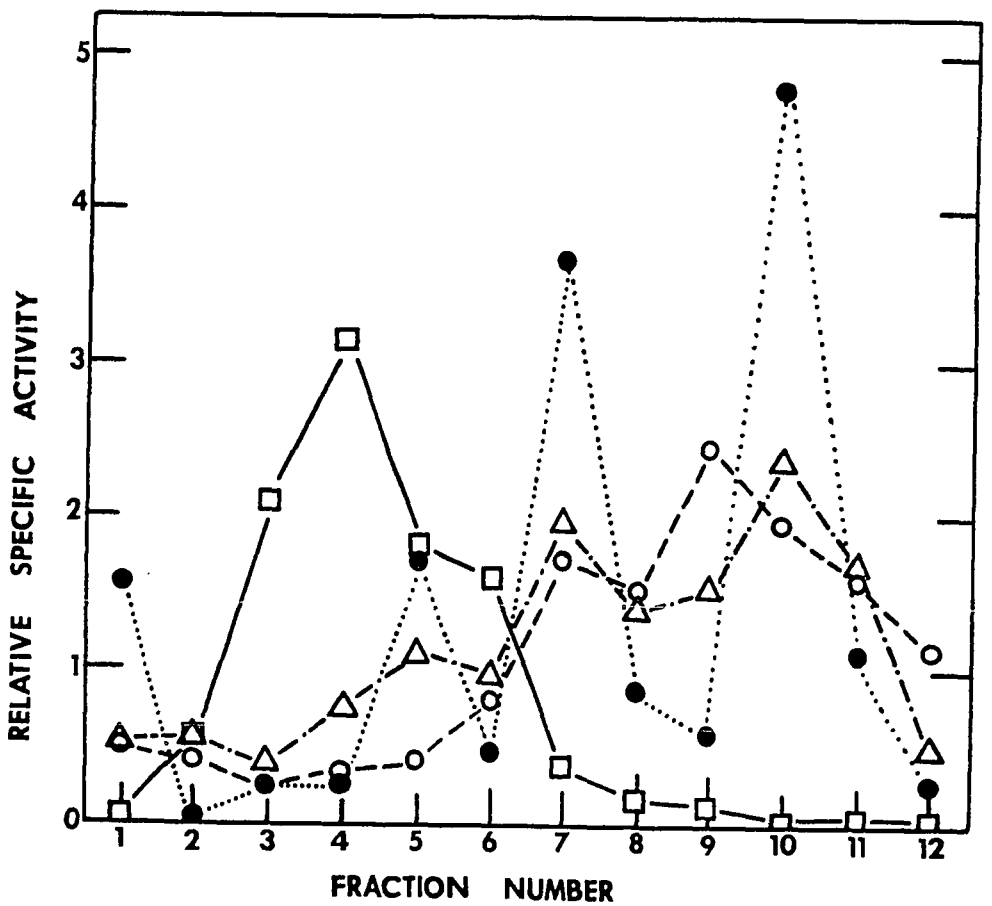


Figure 18. Relative Specific Activity profiles of 5'-Nucleotidase (□—□), β-glucuronidase (○--○), acid β-glycerophosphatase (△··△) and neuraminidase (●·····●) of human polymorphonuclear leukocyte post-nuclear particulate fraction (Figure 7) ultracentrifuged on the discontinuous sucrose gradient (Figure 8).

The protein distribution profiles from two experiments (Figure 19) were very similar. Since different gradients were utilized, a direct comparison with the profiles of PMN is not possible. However, 5'-nucleotidase was again confined to the lighter half of the gradient where the activities of the other hydrolases were lowest. Similarly, β-glucuronidase and acid β-glycerophosphatase and neuramindase were enriched in the heavier half of the gradient. It should also be noted that the first fraction again, has a higher relative specific activity for neuraminidase compared to other acid hydrolases (Figure 20).

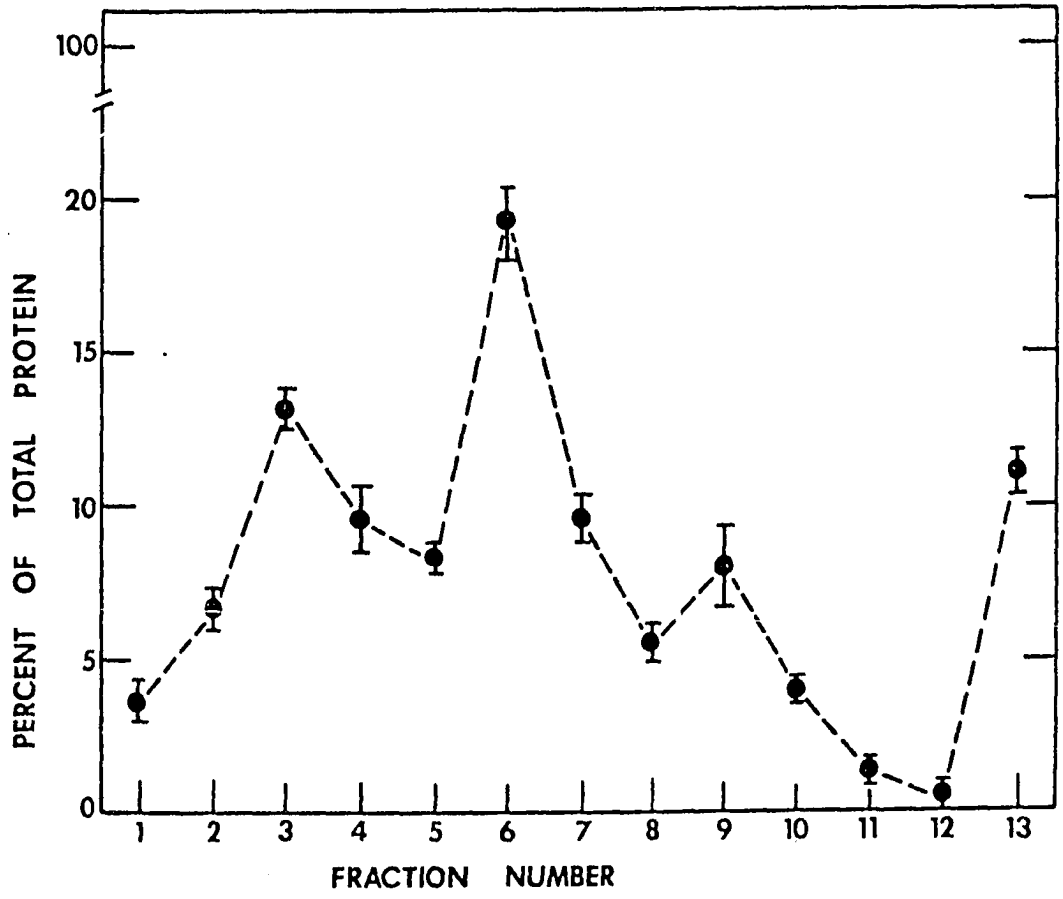


Figure 19. Protein distribution profile of the postnuclear particulate fraction (Figure 7) of lymphocytes in the discontinuous sucrose gradient (Figure 8). The 3 ml fractions were collected by upward displacement with heavy sucrose. The density increases from left to right. The results represent the mean and the range of the values obtained in two separate experiments.

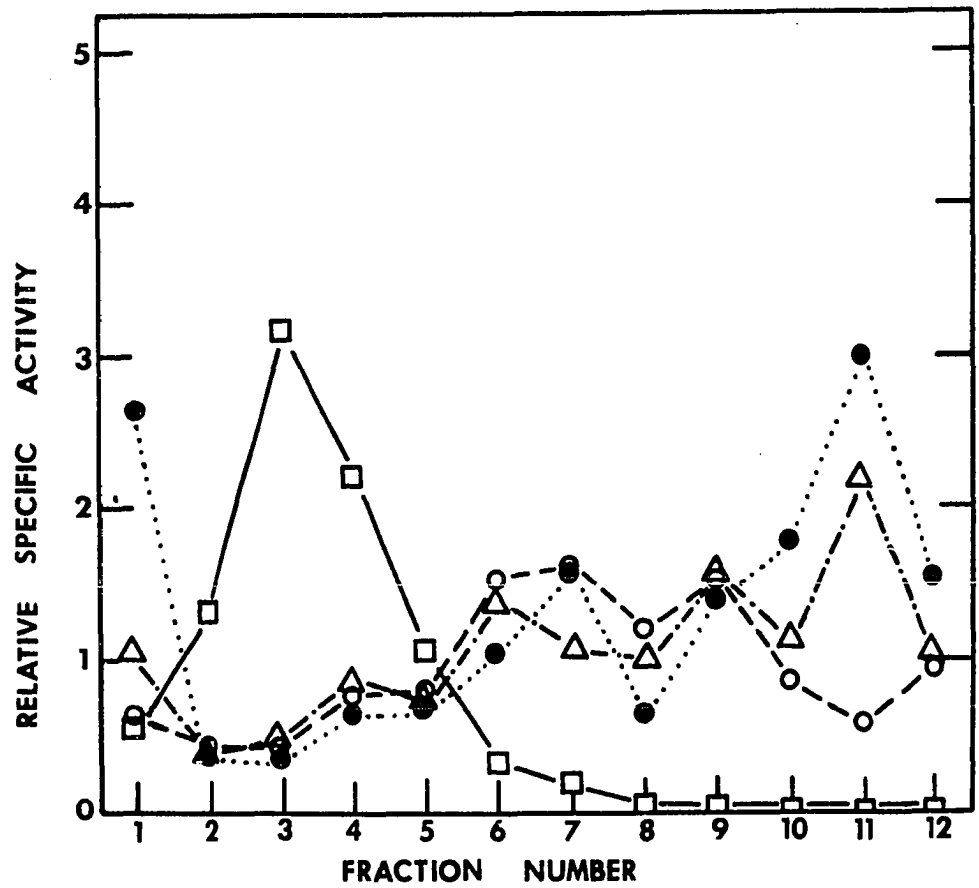


Figure 20. Relative Specific Activity profiles of 5'-nucleotidase (□—□), ß-glucuronidase (○--○), acid ß-glycerophosphatase (△···△) and neuraminidase (●····●) of human lymphocyte postnuclear particulate fraction (Figure 7) ultracentrifuged on discontinuous sucrose gradient (Figure 8).

CHAPTER IV

DISCUSSION

Isolation of Leukocytes

Skoog and Beck (89) were the first investigators to use dextran to enhance erythrocyte sedimentation in the isolation of human blood leukocytes. Although the underlying mechanism of erythrocyte rouleaux formation is not yet fully understood, the authors observed that the increase in erythrocyte sedimentation rate is highly dependent on both the molecular weight and the concentration of the dextran.

In several of our trial experiments, it was observed that the erythrocyte sedimentation rate is actually retarded to a variable degree by diluting the blood with different amounts of 0.85% NaCl or with sucrose solutions of various concentrations. These observations suggest that erythrocytes will not sediment simply by decreasing the blood viscosity, but rather that the initial erythrocyte agglomeration in the form of rouleaux is essential for these cells to sediment. Both sucrose and dextran are polyhydroxyl, hydrogen-bond forming compounds; however, they differ in composition, structure and, perhaps most significantly, in their molecular dimension.

It would appear that in the course of rouleaux formation, dextran molecules serve as bridges between erythrocytes. This can be achieved either by specific interactions between dextran and certain saccharides of the erythrocyte membrane or by non-specific interactions such as hydrogen bonding. Since inter-erythrocyte repulsion forces are affected by the intercellular distances, one might expect that the stability of the bridged erythrocytes would be considerably less with the small-sized sucrose molecule than with dextran.

The fact that dextran enhances human and rat erythrocyte sedimentation rates while being completely ineffective towards bovine erythrocytes seems to support the possibility of specific interactions between these polysaccharides and receptor groups on the erythrocyte surface. On the other hand, the possible effect of differences in the plasma glycoproteins or in the orientation of the receptor groups on the bovine erythrocyte membrane, which might prevent non-specific interactions, can not be ruled out.

Properties of Human Leukocyte Neuraminidase

Human leukocyte neuraminidase is tightly bound to the particulate fraction of the cell. Unlike the other two hydrolases, β-glucuronidase and acid β-glycerophosphatase, no neuraminidase activity was recovered in the non-sedimentable fractions of sucrose homogenates (Table 4). Dounce homogenization or ultrasonic treatment failed to solubilize this enzyme.

Using NL as substrate, pH optimum of 4.0 was found with preparations of mixed leukocytes, isolated lymphocyte and PMN and with their respective granular fractions. Since the pH-activity curve (Figure 9)

always shows a hump at pH 4.4, it is quite possible that at least two isoenzymes might eventually be found. The optimal pH of leukocyte neuraminidase is similar to that observed in the particulate neuraminidase of liver (19), brain (27) and mammary glands (21) of rats, in the liver of developing chicks (38) and in Hela cells (30). A soluble neuraminidase with a pH optimum of 5.8, such as the one present in the cytosol of various rat organs (11, 19-22) could not be detected. Since human plasma contains a soluble neuraminidase (10) with an optimal pH of 5.5, it is tempting to speculate that the latter, although extracellular, might fulfill the biological functions performed by the cytosol neuraminidase in solid tissues.

In recent experiments with rat peritoneal polymorphonuclear neutrophiles, Jensen and Bainton (94) showed that upon phagocytosing neutral red-stained inactive yeast, degranulation of the neutrophiles occurred with a concomitant drop of intracellular pH to near 3.5 to 4.5 within 7 to 15 minutes. These findings seem to support the view that the in vitro optimal pH of 4.0 of human leukocyte neuraminidase may also be the in vivo optimal pH for this enzyme.

Since it had been reported that bovine leukocytes exhibited neuraminidase activity against bovine erythrocyte membrane sialoglycoprotein at pH 5.5 in the presence of 1 mM $CaCl_2$ (33), extensive comparative studies were conducted following the published procedures for the leukocyte isolation. Using the same sialoglycoprotein as substrate, little or no neuraminidase activity could be detected. Instead, it was found that the bovine leukocyte neuraminidase activity also has an optimal pH of 4.0. Calcium chloride, at a final concentration of 1 mM, did not

alter the optimal pH or exert inhibitory or activation effects on bovine leukocyte neuraminidase activity toward NL, human erythrocyte membrane sialoglycoprotein or bovine erythrocyte membrane sialoglycoprotein. In a more recent publication from the same research group (95), it was stated that Ca^{+2} has no effect on either human or bovine leukocyte neuraminidase and, in the same paper, all the neuraminidase activity determinations were performed at pH 4.3. Unfortunately, no explanations are offered for the discrepancies in optimal pH and Ca^{+2} requirement between their present and initial publications (95, 33).

Both Triton X-100 and sodium deoxycholate inhibit human leukocyte neuraminidase. This is in contrast to their action on acid β-glycerophosphatase and β-glucuronidase of PMN and lymphocytes, which possess a structure-bound latency typical of lysosomal enzymes (93, 96). Whether this represents a specific inhibition of the bound enzyme or of the solubilized neuramindase is not clear at this time.

Gielen and Schaper (95) reported that the poor release of sialic acid from leukocyte endogenous substrates was significantly increased if Triton X-100 in a final 0.1% concentration was added to the incubation system or when the leukocyte-homogenates were pretreated with trypsin or pronase to solubilize the particle-bound neuraminidase. In our laboratory, the activity of human leukocyte neuramindase against endogenous substrates was tested at pH values ranging from 3.6 to 5.8. After 3 and 12 hours of incubation, the thiobarbituric acid reaction produced a strong chromogen color. When measured at 549 nm, the optical density was highest for the tube incubated at pH 5.6. However, the spectra of all incubation tubes revealed maximum absorption at 532 nm, and not even

a shoulder could be detected at 549 nm. It would appear that under these experimental conditions employed, instead of a highly active neuraminidase activity against endogenous substrate, there is actually a very active production of malondialdehyde (97) in the course of the incubation. The presence of an active neuraminidase in the incubation systems was ascertained using NL as exogenous substrate. In view of the results described above, more detailed studies, such as fractionation of the incubation mixture by ion exchange chromatography prior to the thiobarbituric acid assay and a careful examination of the spectra of the resulting chromogens, will be necessary in order to clarify this point.

Under the assay conditions described under Methods, the kinetic behavior of human leukocyte neuraminidase follows the pattern predicted by the classical Michaelis-Menten equation. The Km was detemined to be 5.62×10^{-4} M (Figure 12). Since of the initial 1 mM NL concentration in the incubation system less than 10% of the substrate was hydrolyzed at the end of the incubation, one can assume that the effect of pH on the enzyme reaction rate (Figure 9) was measured at conditions of near zero-order kinetics.

It was observed that at equimolar concentrations of chloride anion, LiCl, NaCl, KCl as well as $CaCl_2$ exerted about the same extent of inhibition (Figure 13). The possibility of non-specific inhibition due to increased ionic strength is unlikely since at equimolar concentrations of NaCl, NaBr and NaI, the inhibitory effects were markedly different. If the inhibitory effects of NaCl, NaBr and NaI were non-specific, such large, differing degrees of inhibition should not be expected since the anions Cl^-, Br^- and I^- at similar ionic strength would be expected to

have about the same activity coefficients (98).

EDTA has been reported to inhibit viral (99), bacterial (100) and mammalian (10, 16) neuraminidases. In addition to the strong chelating property of Na_2EDTA, which might decrease the activity of certain cations thus indirectly inhibiting the enzyme activity, one must also consider that such inhibition may be due to the direct effect of the EDTA anion on some critical site of the enzyme. The present results indicate that non-chelating anions Cl^{-1}, Br^{-1} and I^{-1} as well as the weak chelator, citrate anion, all exhibit different degrees of inhibition, suggesting a specific anionic inhibitory effect at or near the active site of human leukocyte neuraminidase. The potency of such an anionic inhibitory effect seems to be more dependent on the size (and hence the polarizability of the halid anions) than on the basicity of the halide anions concerned since the inhibitory effects are on the same order of the ionic radii ($I^{-1} > Br^{-1} > Cl^{-1}$), and polarizability ($I^- >> Br^- > Cl^-$) (101), while the order of basicity of the anions concerned (102) is in the opposite direction (i.e. $Cl^- > Br^- > I^-$).

Role of the Functional Groups of Neuramin-Lactose in the Formation of the Enzyme-Substrate Complex

The mechanism of action and the specificity of neuraminidase have been tested by various investigators using a number of substrates. Synthetic substrates with unmodified glycon (N-Acetylneuraminosyl group) such as α-methyl ketoside (103), α-benzyl and α-phenyl ketosides (104) of NANA have been reported to be hydrolyzed by bacterial neuramindase, while all β-anomers investigated are not attacked. Modification of the

carboxyl group of the glycon by esterification (103) and reduction (105) of the α-phenyl ketosides or conversion of the carboxyl group to the amide form of the α-benzyl ketoside (105) have been reported to yield the products which are not susceptible to bacterial neuraminidases. All these experimental data indicate that the α-anomeric configuration and a free carboxyl group are essential for the neuraminosyl group to be enzymatically hydrolyzed.

The modifications of the carboxyl group described above, resulting in elimination of the negative charge, can also bring about secondary effects. These include alteration of the molecular volume, change of the molecular hydrophilicity or introduction of new hydrogen-bonding groups. All these factors play some role in enzyme action and hence must be carefully evaluated.

Another way to probe for the role of the functional groups involved in the formation of an enzyme-substrate complex would be to study the effect of various salts on the enzyme activity. The pronounced inhibitory effect of the Hg^{+2}, for example, could be due either to the blocking of the dissociated carboxyl group of NL by the weak Lewis acid Hg^{+2}, or to blocking of carboxyl groups or thio groups on the enzyme molecule. The various degrees of inhibition observed with different anions strongly suggests the possible involvement of the carboxyl group of NL in the formation of enzyme-substrate complex. The sharp decrease in enzyme activity at pH values higher that 4.0 suggests the possible presence of certain basic amino acids in the active site of neuraminidase, most possibly histidine. Since the side chain of histidine has a pKa of 6.0, at pH 4.0, the protonated histidine side chain may exert a coulombic

attractive force toward the dissociated carboxyl group on the substrate molecule (pKa for sialic acid ≈ 2.5). The positive charge on the side chain could be shielded by anions and prevented from exerting electrostatic interaction or charge transfer complex with the substrate. The results of the present studies using various salts support the postulation that the negative charge of the carboxyl group on the substrate is essential for the NANA α-ketosides to be hydrolyzable by neuraminidase (104, 105) and provides an explanation for the inhibitory effects of anion.

In addition to the free carboxyl group, a contribution of hydrogen-bonding forces in the formation of the transition-state complex is almost certain when one considers the availability of the C_5-acetamido group and the hydroxyl groups at C_4, C_7, C_8 and C_9 of the substrate molecule. Decreased enzyme activity at pH values lower than 4.0 may be due, at least in part, to the decreased availibility of the potential hydrogen-bonding groups on the substrate and enzyme molecules.

Neuraminidase Activity Levels in Leukocytes of Normal and Diseased Human Subjects

In view of the limited number of determinations performed so far, no attempt was made to investigate possible statistical differences between the levels of neuraminidase activity in normal male and female donors. It is interesting to note that, with normal male adults, the levels of neuraminidase activity in mixed leukocytes cover a fairly wide range of values. Both isolated lymphocytes and PMN were found to contain similar specific activities per million cells or per mg of protein, with lymphocytes showing slightly higher values than those of PMN. This

finding is in conflict with Gielen and Schaper's remark (95) that the major neuraminidase activity of leukocytes stays in granulocytes and monocytes. It is impossible to discuss this discrepancy any further at this time since these authors have not published the experimental data on which they based the above comments.

Statistical evaluation of the leukocyte neuraminidase activity in arthritic patients gave no correlation between neuraminidase levels and age, sex or treatment of the patients. Additional studies with more suitable patients (i.e. same treatment, similar severity of symptoms, sex, age, etc.) as well as studies with experimental animal models would be desirable in order to gain additional information on the possible role of leukocyte neuraminidase in the conceptual model for arthritis outlined in the Introduction.

From the results of the present studies, it seems clear that intracellular distribution of human leukocyte neuramindase parallels fairly closely that of lysosomal enzymes. The subfractionation of the postnuclear particulate fraction of both lymphocytes and PMN by discontinuous density gradient ultracentrifugation afforded the opportunity for a detailed study of the distribution patterns of plasma membrane and lysosomal marker enzymes. The distribution profiles strongly indicate that lysosomes are the intracellular sites of storage for most, if not all, of human leukocyte neuraminidase. The small amount of neuraminidase activity found in fractions rich in the plasma membrane marker enzyme could be due to insufficient resolution of these fractions with the methods used in this study; on the other hand, the possibility that a small amount of neuraminidase might be present in the plasma membrane deserves further

exploration. If the latter case were true, it would be of interest to see if it is the same enzyme or a different isoenzyme with the possibility of a different metabolic role. The presence of neuraminidase and lysosomal marker enzyme activities in the light density fractions of the gradient also suggests possible involvement of the Golgi apparatus. Nyquist and Mollenhauer (106) have recently shown the existence of acid phosphatase activity in the Golgi apparatus of rat testis. This is not too surprising since lysosomes originate from Golgi apparatus.

Heterogeneity of the Isolated Lymphocytes and Polymorphonuclear Leukocytes

It should be noted that the lymphocytes and PMN isolated for these studies do not represent two homogeneous populations of cells with well-defined biological functions. Humoral immune response and cell mediated immunity are known to involve functionally different lymphocytes (107). In light of the definite relationship between cellular functions and their biochemical makeup, it would appear probable that neuraminidase activity might not be equally distributed in B and T lymphocytes (107). Heterogeneity of 5'-nucleotidase activity in lymphocytes of chronic lymphocytic leukemia (108) and diminished acid phosphatase activity in lymphocytes of chronic lymphocytic leukemia (109), have recently been reported. The possibility that neuraminidase might not be uniformly distributed in the polymorphonuclear neutrophiles, basophiles and eosinophiles should also be given careful consideration since this point could be of great importance when studying alterations of neuraminidase activity under various pathological conditions.

Closing Remarks

Finally, one may ask, what is the importance of the finding that human leukocytes contain neuraminidase activity? Naturally, this question will be answered gradually in the years to come as additional research gives us a clearer insight into the multiple facets of this problem. However, a brief review of our current knowledge in the field of leukocyte lysosomal enzymes will shed some light on possible areas of biochemistry which may be affected by these findings. Hirschhorn and Hirschhorn (110) have published experimental evidence which suggests that labilization of lymphocyte lysosomes might be accompanied by widespread gene activation. Tchorzewski, et al. (111, 112) have reported that an enzyme, non-proteolytic in nature, present in the extract of guinea pig PMN lysosomes can stimulate in vitro lymphocyte blastic transformation. By analogy with bacterial neuraminidase which is known to stimulate lymphocyte division in vitro (58), the possible involvement of lysosomal neuraminidase in these processes appears as a reasonable and likely possibility.

Considering the unique properties and the topochemical localization of sialic acid which make it responsible for most of the negative charges on the surface of mammalian cells, one would be inclined to speculate that leukocyte neuraminidase might play a role in the regulation of the electrical charge of the membranes of circulating cells, endothelial cells of blood vessels and of the plasma membranes of leukocytes themselves. Changes in the electrical charge of the membranes would lead to a new state of dipole orientation. Thus, through the action of neuraminidase on their plasma membrane or nuclear membrane constituents, leuko-

cytes could not only enjoy the ability for cellular conformational changes, but also control the communications between its nuclear and extranuclear components. Among the large number of biological phenomena affected by removal of sialic acids we can mention, among others, permeability of the cell membranes, the immunogenic properties of glycoproteins and the regulation of the half-life of plasma glycoproteins. These points should suffice to give an overview of the possible far reaching implications of future studies along these lines.

CHAPTER 5

SUMMARY

Mixed leukocytes isolated from peripheral human blood contain neuraminidase activity. The enzyme, which is wholly recovered in the sedimentable fraction of leukocyte-homogenates, exhibits maximum activity at pH 4.0 and has a Km of 5.62×10^{-4} M with neuramin-lactose as the substrate. Hg^{+2} at 1 mM concentration is inhibitory, while other divalent cations (Ca^{+2}, Mg^{+2}, Cu^{+2} and Zn^{+2}) under identical experimental conditions did not significantly affect the enzyme activity. Chlorides of alkali metals at, or near, isotonic concentrations were inhibitory. Similar inhibition was obtained with $CaCl_2$ at the same molar concentration of chloride ion relative to the concentration of alkali chloride. Using sodium halides (NaCl, NaBr, NaI), a direct correlation between inhibitory power and the size of the anion was established. Detergents such as Triton X-100 and sodium deoxycholate were inhibitory even at low concentrations. Higher hydrolysis rates were observed with low molecular weight substrates (milk trisaccharides and sialoglycopeptides) than with gangliosides and glycoproteins. A method for the isolation of leukocytes from small samples of blood, and a sensitive modification of the thiobarbituric acid assay were developed. This allowed the determination of the normal levels of leukocyte neura-

minidase activity in normal human donors of both sexes. This method has also been utilized in initial studies, now in progress, for the determination of neuraminidase activity in leukocytes from human patients affected with various diseases. Initial studies of the neuraminidase activity present in leukocytes from bovine and rat leukocytes have also been performed.

Studies conducted with isolated human lymphocytes and PMN leukocytes showed that the enzyme is present in both cells. The properties of their enzymes are identical to those described above for mixed leukocytes.

No evidence for a soluble neuraminidase could be detected in human leukocytes. The distribution of neuraminidase and several marker enzymes was investigated in particulate subcellular fractions obtained by ultracentrifugation of leukocyte homogenates in a discontinuous sucrose gradient. With both lymphocytes and PMN leukocytes, most of the neuraminidase activity follows a distribution pattern similar to that of the acid lysosomal hydrolases. The possibility of some neuraminidase activity bound to plasma membranes can neither be affirmed nor ruled out at this time.

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