Isolation and Properties of Human Blood-Group NN and Meconium-Vg Antigens*

Georg F. Springer, Yasushi Nagai,† and Herta Tegtmeyer

ABSTRACT: Blood-group antigens NN and Me-Vg were obtained as homogeneous substances from human red cells and meconium, respectively, and fully characterized chemically and biologically. For the first time a homogeneous erythrocyte membrane component of such high blood-group activity is described. Both substances induce anti-N specific antibodies in rabbits. They are also highly potent myxovirus receptors. The N and myxovirus specificities of the erythrocyte antigen are destroyed by sialidases and by proteases. Human N specificity is also destroyed by galactose oxidase. Specificities are carried by sialyl, sialylgalactopyranosyl, and β-galactopyranosyl structures. Both antigens are glycoproteins containing sialic acid, galactose, galactosamine, glucosamine, and mannose as the main carbohydrates. The Me-Vg antigen in addition contains a large amount of fucose. Components migrating on paper chromatograms like *N,O*-diacetylneuraminic acid have been identified in these antigens. This is the first account of these substances in products of human origin. The peptide part amounts to 44% in the NN antigen and to 13% in the Me-Vg antigen. Threonine and serine are prominent in both glycoproteins; the concentration of aromatic amino acids is low. Tryptophan and cystine are absent. Alkali degradation studies show that the threonine and serine are involved in the peptide–carbohydrate linkage; galactosamine and galactose are the carbohydrates destroyed by alkali in the NN antigen and glucosamine in addition in the antigen from meconium. Haptenic structures in which sialic acid and galactose predominate have been isolated by enzymatic and mild acid hydrolysis.

There are at least 14 human blood-group systems with over 60 blood-group antigens (*cf.* Race and Sanger, 1962; Wiener, 1963) whose mosaic structures are believed to be the products of one or more genes and their allelomorphs. Until recently chemical knowledge was confined to members of the two closely related ABH(O) and Lewis systems (Kabat, 1956; Morgan, 1960; Watkins, 1966). Most of this chemical information is based on studies, not of red cells, but of the abundant water-soluble substances in secretions which are similar in serological specificity to the ABH(O) and Lewis structures on human erythrocytes.

The first conclusive chemical evidence on the second human blood-group system to be discovered, the MN system (Landsteiner and Levine, 1928; *cf.* Wiener, 1963), was provided by Springer and Ansell (1958) and independently, shortly thereafter, by Mäkelä and Cantell (1958), who showed that strains of type A and B influenza viruses inactivated the M and N antigens of human erythrocytes. Since then glycoproteins of varying purity and with different degrees of blood-group M and N activity together with ABH(O) activity isolated from human erythrocyte stroma have been described (Baranowski *et al.*, 1959; Klenk and Uhlenbruck, 1960; Stalder and Springer, 1960; Kathan *et al.*, 1961; Nagai and Springer, 1962). Preparations specifically inhibiting the so-called anti-N reagent from *Vicia graminea* have been isolated from human meconium by Springer and Hotta (1963). In addition to their M and N specificities these substances inhibited hemagglutination by influenza viruses. More recently there have been reports on the isolation of blood-group specific dialyzable haptens from isolated M and N antigens (Nagai and Springer, 1962; Springer and Hotta, 1963; Hotta and Springer, 1965) and from erythrocyte stroma (Cook and Eylar, 1965).

The subject of the present paper is the isolation and definition of the chemical and biological properties of the human NN erythrocyte antigen (NN) which behaves as a single component in the systems tested and has a molecular weight of 595,000. Comparative studies on a homogeneous antigen (Me-Vg) from human meconium which was found to have a molecular weight of 520,000 and to neutralize the *V. graminea* reagent only are also reported. Some chemical data on the terminal structures responsible for the biological specificities are given. The analytical data for both substances which are also potent myxovirus inhibitors account

* From the Department of Immunochemistry Research, Evanston Hospital Association, and the Department of Microbiology, Northwestern University, Evanston, Illinois. *Received June 17, 1966.* This investigation has been supported by Grant No. AI-05681 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and part of the immunological work by Grant No. 63 G 119 from the American Heart Association. Preliminary accounts of the findings have been presented at the Annual Meetings of the American Society of Biological Chemists in 1962 and 1963. The Research Department is maintained by the Susan Rebecca Stone Fund for Immunochemistry.

† On leave from Fukushima Medical College, Fukushimashi, Japan.

for approximately 100% of the total of the macromolecules.

Materials and Methods

Preparation of Erythrocyte Stroma and Isolation of NN Antigen. Blood stored for 3–30 days at 2–6° in commercial 480-ml units containing 20% acid citrate dextrose N.I.H. formula B as anticoagulant was obtained from blood banks, and the MN, ABO, and Rh_0(D) blood types of each unit were determined with commercial typing sera (Ortho Pharmaceutical and Pentex) according to the instructions accompanying them. NN bloods were centrifuged for 40 min at 2000*g*, plasma and buffy coat discarded, and the packed red cells washed thrice with an equal volume of 0.85% aqueous NaCl. Erythrocytes from 10 units were combined and lysed in 10 volumes of distilled water at 5°. The pH was adjusted to 5.3 with acetic acid and phenol was added to a final concentration of 0.2%. The stroma settled after standing overnight at 5°, and the supernatant fluid was siphoned off, 10 volumes of water was added, and the pH was readjusted to pH 5.3. The sediment was resuspended in water as above and the entire procedure was repeated six times with phenol addition at every second water change. Subsequently the sediment was collected by centrifugation and stored at −20° until use. The yield was 30–40 g/10 units of blood.

The NN active material was prepared as shown in Figure 1. Stroma in 1.5% final concentration was extracted by the phenol–water procedure (*cf.* Westphal and Lüderitz, 1954). The following modifications were essential: (a) the aqueous phase contained 0.85% NaCl (Stalder and Springer, 1960) since little active material was obtained otherwise; (b) the extraction was carried out for 2 hr on a mechanical shaker at 23–25° (Springer and Hotta, 1964) since degradation, both biological and chemical, could be demonstrated after extraction at elevated temperatures (see Figure 1). Extraction of the phenol phase was repeated twice with one-half the original volume of water containing 0.43% NaCl. The activity resided in the aqueous phases and interphase which were combined and exhaustively dialyzed in the presence of toluene. The nondialyzable part was freed of precipitate and the soluble material was adjusted to 2% dry matter in 0.01 M phosphate buffer of pH 6.2 and purified by fractional centrifugation in a Lourdes AAC and a Beckman L preparative ultracentrifuge (3 hr/fraction). Material sedimenting between 32,700 and 96,600*g* was the most active while the sediment at 1250*g* and the supernatant after sedimentation at 150,900*g* had low activity only. The very active material was dissolved in water and fractionated with ethanol at 23–25°. Electrolytes facilitated precipitation, therefore sodium acetate was added in 0.1% final concentration throughout. The most active materials precipitated between 50 and 55% ethanol and were further purified on columns of 7% IONAGAR (Consolidated Laboratories) (Polson, 1961) which had been made 100 mesh with the aid of a sieve. The

1. *NN Erythrocyte Stroma*
   - suspend 1.5% w/v in 45% aqueous phenol containing 0.43% NaCl
   - shake at 23–25°; centrifuge at 2500*g*, 4°
   - remove aqueous layer; add 0.5 volume of 0.45% aqueous NaCl to phenol layer
   - reextract twice; combine aqueous layers and interphase; dialyze
2. *Crude NN Substance* (*ca.* 2% of 1.)
   - make 2% in PO_4 buffer (pH 6.2, 0.01 M)
   - centrifuge at 1250, 32,700, 96,600, and 150,900*g*
   - ethanol (containing 0.1% sodium acetate throughout) fractionation of 2% aqueous solution of 96,600*g* sediment
   - fraction precipitating between 50 and 55%
3. *Purified NN Substance* (12–13% of 2.)
   - 7% agar gel column (4 × 30 cm), 10% ethanol eluate, active material at 30–75 ml; reelute on 7% agar gel (2 × 30 cm); active material at 24–42 ml; adjust to 2% d.w. in PO_4 buffer at pH 6.2.
   - centrifuge: 1250, 32,700, and 96,600*g*; recentrifuge; 96,600*g* sediment on Sephadex G-200 column (2 × 30 cm) water eluate, active material between 40 and 49 ml, centrifuge, recentrifuge 96,000*g* sediment dialyze, electrodialyze
   - 96,000*g* sediment
4. *Highly Purified NN Substance* (1.5–2.0% of 2; activity 12–24 times that of 2.)

FIGURE 1: Outline of human erythrocyte NN antigen preparation.

eluate was again adjusted to 2% dry matter in pH 6.2 buffer. It was then centrifuged as shown in Figure 1. Material with the highest activity sedimented between 32,700 and 96,600*g*. All preparations obtained in this range showed only one peak in moving-boundary as well as paper electrophoresis; however, some were inhomogeneous in the ultracentrifuge. Therefore, minor components were removed by passing the precipitate, dissolved in water, through a Sephadex G-200 column. The peak area of the main fraction, found in the effluent between 40 and 49 ml, was recentrifuged and dialyzed. It behaved as one component in the ultracentrifuge and on electrophoresis between pH 1.5 and 11.0 (Bezkorovainy *et al.*, 1966). The NN antigen described here was assigned the lab no. Ca 825 and had been recovered from 150 units of blood (44 O, 74 A, 14 B, 18 AB; 121 were Rh positive and 29 Rh negative). These 150 units amounted to 15 lots which were combined at step 2 of Figure 1. The NN antigen was either used as such for chemical and physical analyses or after desalting by electrodialysis at 200 v through parchment membranes (Springer *et al.*, 1965).

Extraction and Purification of Meconium Antigen. Meconia were collected aseptically, as described previously (Springer and Horton, 1964), and stored at −20°. All meconia tested, regardless of the MN type

of the erythrocytes of the infant from which they originated, were found to possess significant activity with the *V. graminea* reagent only. Eleven blood-group AH(O) specific meconia were pooled and extracted twice with 25 volumes of distilled water by heating in a boiling water bath for 25 min and subsequent mechanical shaking at 23–25° for several hours after the addition of some toluene. The aqueous extracts were concentrated *in vacuo* and lyophilized after the removal of insoluble material by centrifugation at 3000*g*. This crude extract was made to 2% in water and fractionated with increasing ethanol concentrations in the presence of 0.1% sodium acetate (final concentration). Most of the active material precipitated at an ethanol concentration of 75–83%. This fraction was exhaustively dialyzed and precipitated with Na_2SO_4 at 23–25° as a 2% solution in water at 20, 25, 30, and 40% final concentration. This led to enrichment of the active material in the 30% precipitate which was dialyzed for 3 days against cold water. It was adjusted to 2% in water and submitted to fractional centrifugation. The most active material sedimented between 32,700 and 96,600*g*. Recentrifugation of this sediment under the same conditions yielded the most active material again at 96,600*g*. It was further purified on a Sephadex G-100 column (2 × 40 cm) by elution with water. The most active material appeared between 30 and 45 ml. It amounted to *ca.* 0.5% of the crude extract. After thorough dialysis and electrodialysis this material showed one sedimenting boundary in the ultracentrifuge and one peak on electrophoresis between pH 1.5 and 11.0 (Bezkorovainy *et al.*, 1966). This material was assigned the lab no. Ca 851 and is termed Me-Vg antigen.

Analytical Procedures. Total lipid was estimated after heating at 80° in a drying pistol. All solvents were ACS grade and were dried before use. The antigens were suspended in 70 volumes of methanol–chloroform (3:1, w/v) and heated for 48 hr at 56° in glass-stoppered, spring-fastened tubes. The extraction mixtures were renewed twice at regular intervals. Subsequently the residual material was extracted in the same way with ethanol–ether (1:1). During extraction the samples were shaken and the volume was adjusted for evaporated ether. The materials were dried *in vacuo* over KOH and paraffin. Total cholesterol was determined according to Zak *et al.* (1954).

Content of easily removable water was assessed by drying at 110°. Drying to constant weight at 80° *in vacuo* was also done by Huffman Microanalytical Laboratories, prior to performing the following analyses: C and H (Pregl), N (Dumas), S (catalytic combustion with V_2O_5 addition to displace S from residue), P (Carius), $COCH_3$ (Kuhn–Roth) after hydrolysis with 25% H_2SO_4 for 4 hr at 100° or with 25% *p*-toluenesulfonic acid, CCH_3 as CH_3 (Kuhn–Roth), free COOH (Pregl), OCH_3 (Zeisel), and ash (with H_2SO_4). Total hexosamine (expressed as base) was determined by the method of Elson and Morgan (*cf.* Kabat, 1961) but hydrolysis was in 6 N HCl for 2 hr. Sialic acid was measured with the periodate–thiobarbituric acid assay (Warren, 1959) with and without hydrolysis, the direct Ehrlich and the resorcinol–HCl reactions (*cf.* Kabat, 1961). Methylpentose was determined as by Dische and Shettles (1951), and reducing values, expressed as glucose, according to Park and Johnson (1949). Total hexoses were determined together by the orcinol method of Winzler (1955) after correction for methylpentose, using a mannose–galactose (1:1) standard. All sugars were also determined singly after hydrolysis of the antigens (0.5 mg/ml) with 1 N HCl for 12 hr and separation on Whatman No. 1 or 3MM paper after three runs in solvent b (see below), elution with water, purification, and concentration (*cf.* Springer *et al.*, 1965). For individual determination of hexosamines there were the following modifications: antigen hydrolysis (10 mg/ml) was for 2 hr in 6 N HCl and solvent b_1 was used. All extinctions were measured in a Beckman DU spectrophotometer.

All quantitative amino acid analyses, except tryptophan, as well as additional determinations of glucosamine, galactosamine, and their ratios were performed by Dr. P. Weber and Mr. D. Grohlich in Professor R. Winzler's department. Analyses on "short" columns (0.9 × 50 cm) for hexosamines followed the procedures of Moore *et al.* (1958) and Kominz (1962); 0.975–1.000 mg was hydrolyzed in 1.0 ml of 3 N HCl for 6 hr at 115–118° under nitrogen in sealed tubes. After drying the hydrolysate was taken up in 1.0 ml of 0.7 M sodium citrate buffer, pH 5.28. Elution was in the same buffer, the flow rate 46 ml/hr at 25 psi. Quantitative amino acid determinations were carried out on microcolumns (0.9 × 140 cm) by hydrolyzing 0.490–0.510 mg of substance in 1.0 ml of 6 N HCl for 18 hr as above. The hydrolyzed material was dried *in vacuo*, taken up in 1.0 ml of 0.05 M sodium citrate buffer, pH 2.875, and analyzed as described by Piez and Morris (1960) in a modified, nine-chambered Technicon autograd analyzer. The buffer gradient was pH 2.85–5.00, 33.4 ml eluted/hr at *ca.* 140 psi. Further hexosamine and amino acid analyses were performed on a Beckman 120-C amino acid analyzer following the procedure of Spackman *et al.* (1958). For amino sugar determinations 1.0 or 2.0 mg of material was hydrolyzed under nitrogen with 4.0 ml of 3 N HCl for 3 hr at 100°. After drying in a rotary evaporator the material was dissolved in 1.5 or 2.5 ml of 0.2 N sodium citrate buffer, pH 2.20, containing 0.28 μM norleucine and 0.5 ml or 1.0 ml was added to the column. Amino acid determinations differed only in that hydrolysis was with 6 N HCl for 16 hr at 115°. Tryptophan was determined in this laboratory by the methods of Bencze and Schmid (1957) and Winkler (1934). Values given in the tables are those of one representative run of two or three consistent determinations.

The physicochemical methods employed have been described in more detail elsewhere (Bezkorovainy *et al.*, 1966), where also the appropriate references will be found. Briefly, sedimentation values were obtained in a Spinco Model E analytical ultracentrifuge at 59,780 rpm at 4° in a conventional cell. The solvent was 0.05 M cacodylate–0.1 M NaCl (pH 6.5). Antigen concentrations were determined after exhaustive dialysis

by drying measured volumes of both solvent and solution to constant weight at 70° *in vacuo*. Ultracentrifuge patterns were evaluated in an Excello microcomparator, corrected to standard conditions and extrapolated to zero concentration.

Diffusion measurements were carried out in a Spinco Model H electrophoresis apparatus in the same solvent as that used for the sedimentation experiments. Each "run" lasted for 6 days at 1°, employing a 2-ml microcell. The interference fringe pattern was used in calculating diffusion constants. Partial specific volumes for the two substances were calculated from their composition data by the method of Schachman *et al.* (1957); partial specific volumes used for the various sugars were the same as those arrived at previously (Bezkorovainy *et al.*, 1966).

Moving-boundary electrophoresis was performed interferometrically in an Antweiler apparatus. NN antigen and Me-Vg antigen were used as 2.0% solutions at pH 8.6 in 0.05 M barbital–0.05 M NaCl. The current density was 34 ma/cm. Horizontal zone electrophoresis was carried out on cellulose acetate at pH 8.6 in 0.05 M barbital and at pH 4.5 in 0.1 M acetate for 90 min at 26 v/cm. The strips were stained with Procion blue. Paper electrophoresis was performed as previously (Springer *et al.*, 1965) on Whatman No. 1 paper at 6–7 v/cm and 0.25–1.0 ma/cm for 6–12 hr. The following electrolytes were used: 0.05 M sodium borate (pH 9.2); 0.06 M sodium barbital (pH 8.6); 0.15 M sodium phosphate (pH 7.0); citrate·HCl (pH 3.0 and 1.5) both 0.1 N with respect to HCl added. Human serum and starch were included as controls. "Amido Black 10B," bromophenol blue, and sodium metaperiodate–potassium permanganate were used as staining reagents. Infrared and ultraviolet scans as well as viscosity determinations were carried out as described by Bezkorovainy *et al.* (1966). Ultrasonic treatment was performed on 0.5% solutions of the antigens in buffered saline. A Biosonic probe (Bronwill, Rochester, N. Y.) with needle tip probe BP-1-40 was used at 20 kcycles. In order to avoid overheating the experiments were performed in an ice bath and the samples were sonicated five times, 1 min each time, while allowing 1 min for cooling. Treated and untreated control samples were titrated in parallel.

Paper Chromatography, Preparative Electrophoresis, and Qualitative Color Reactions. Comparative and preparative chromatography was on Whatman No. 1, 3-MM, and 40 paper by the descending technique described previously (Springer *et al.*, 1965). The paper used for preparative separation was prewashed with acid and solvent and the desired compounds were rerun three to five times in the same solvent, for a period which gave optimal separation, isolated by elution, and purified as in earlier work, unless stated otherwise (for references unless given here see Springer *et al.*, 1964a, 1965). The following systems were used: (a) ethyl acetate–pyridine–water (10:4:3); (b) 1-butanol–pyridine–water (6:4:3), three runs of 20 hr each were always performed; (b_1) solvent b on $BaCl_2$-saturated paper (Heyworth *et al.*, 1961); (c) 1-butanol–propanol–0.1 N HCl (1:2:1) (Svennerholm and Svennerholm, 1958); (d) 1-butanol–ethanol–water (4:1:1) (Milks and Janes, 1956); (e) ethyl acetate–acetic acid–water (3:1:3) (*cf.* Gottschalk, 1960); (f) 90% aqueous phenol (v/w) containing 8-hydroxyquinoline (0.08%) in an atmosphere saturated with NH_3 in the presence of NaCN; (g) buffered phenol with buffer-washed filter paper at pH 12 (McFarren, 1951); (h) 1-butanol–acetic acid–water (4:1:1) (Reed, 1950). The developed chromatograms were stained for carbohydrates as previously (Springer *et al.*, 1964a, 1965). Ninhydrin in acetone (Toennies and Kolb, 1951) was used to stain amino acids and hexosamines, and periodate–thiobarbituric acid was the spray for detection of sialic acids (Warren, 1960).

Preparative paper electrophoresis at high voltage was performed with appropriate controls for 50 min using pyridine–acetic acid–water (10:4:86) as electrolyte (Springer *et al.*, 1965; Bezkorovainy *et al.*, 1966). The electropherograms were stained in the same way as the chromatograms.

Hydrolyses with Acids and Bases. Stability towards acids and bases was determined by heating a 1.0% solution of NN or Me-Vg antigen for 2 hr in a boiling water bath. The following buffers were used: (a–e) Mcilvaine Na_2HPO_4 (0.2 M), pH 2.2, pH 3.0, pH 4.0, pH 5.0, pH 6.0; (f,g) Soerensen 0.67 M; pH 7.0, pH 8.0; (h) Clark and Lubs 0.2 M Na-borate, pH 9.4; (i,j) glycine–NaOH 0.1 M, pH 10.0, pH 11.0. The compositions of buffers a–g, i, and j are described by Stauff and Jaenicke (1964) and h was prepared according to Latimer and Hildebrand (1953). After heating, the samples were dialyzed for 24 hr against 3 20-volume portions of water at 4°. Acid hydrolysis to study the composition of the macromolecules was carried out with 0.1–6 N HCl at 100° for the periods indicated in the Results Section or by extraction of a 2% antigen solution at pH 3.1 throughout for 1 hr in a boiling water bath (see Blix and Lindberg, 1960) followed by dialysis against 5 volumes of water at 4° for 3 hr and subsequently for 20 hr. Finally, soluble (see Painter, 1960) polyvinyltoluenesulfonic acid ion-exchange resin (Nagai and Springer, 1962) ET 181 (mol wt 400,000, Dow Chemical Co.) was used after purification, transformation into the H^+ cycle, dialysis, and concentration as recommended by the manufacturer. After precipitation with ethanol, it was freeze dried, dissolved in water to 0.04 N, transferred to carefully washed Visking dialysis casing (2.8-cm diameter) in 50-ml amounts (0.5 g of resin), and ~15 g of Amberlite IR-120 H^+ was added. The bag was then suspended in 800 ml of deionized water in a glass cylinder (diameter 7 cm, volume 1.2 l.), which was stoppered and fastened to an electrically driven rocking device (16 cycles/min) at ~15° below and above the horizontal. Heating tape (Briskeat) around the cylinder kept the water at 60°. Bent glass tubing inserted through a rubber stopper prevented increase in pressure and overflow. After heating for 1 hr, the water was discarded and replaced. The sample (50 ml, 2% in water) to be hydrolyzed was now added to the resin in the bag and the hydrolysis at pH 2.2–2.5 was begun. The water was changed after

1, 3, 5, and 8 hr. Each dialysate was dried *in vacuo* below 50°. Control experiments indicated <1% contamination from resin and dialysis casing.

IR-120 H^+ was removed by centrifugation as was the soluble resin after precipitation by 5% cetyldimethylbenzylammonium chloride at 23–25°. The separated resins were washed thoroughly with water, the aqueous layers were concentrated, and the residual antigen was precipitated with 10 volumes of 100% ethanol, washed three times with 100% ethanol, and dried as usual.

Enzymatic Hydrolyses. The following enzymatically active agents and enzymes were used: two strains of influenza virus type A: swine S_{15} and Melbourne; type B, the Lee strain. The viruses and bacterial sialidases were used as described by Springer and Ansell (1958). Crystalline *Vibrio cholerae* sialidase (=R.D.E.) (lot 10562, Behringwerke) was used most frequently, 1 mg of antigen was exposed to 1000 units of it for 24 hr (1 unit liberates 1 μg of *N*-acetylneuraminic acid at 37° in 15 min from acid α_1-glycoprotein); β-galactosidase from *Escherichia coli* (Wallenfels *et al.*, 1959) and *Diphococcus pneumoniae* (E. J. McGüire); coffee bean α-galactosidase was prepared in this laboratory (Springer *et al.*, 1964b); lysozyme (lot no. 1087, Mann Research Laboratories); galactose oxidase (Worthington, lot no. GAO 6544), β-*N*-acetylhexosaminidase from bovine spleen (Bhavanandan *et al.*, 1964); pronase (B grade, 45,000 proteolytic units/g; Calbiochem); papain and trypsin were both twice crystallized (Mann Research Lab). Enzyme activity was investigated under conditions specified by the supplier or as indicated in the Results Section. All incubations lasted from 24 to 80 hr; where necessary, activity was predetermined on standard substrates; all experiments included the following controls in the same final volume: (a) substrate alone, and, provided sufficient enzyme was available, (b) enzyme inactivated in a boiling water bath for 1 hr incubated alone as well as (c) with the substrate. After adding a few drops of toluene the samples were incubated at 37° and 23–25° for lysozyme; subsequently they were immersed in boiling water for 15–45 min. Particulate material was removed by centrifugation at ~100*g*. One aliquot of the supernatant was kept for investigation while two aliquots were dialyzed against 25 volumes of water which was changed three times. All samples were concentrated below 50° and dried *in vacuo*.

Deacetylation. This was carried out on a 0.1% solution of both antigens in 0.1 N NaOH for 3 hr at room temperature (Whiteside and Baker, 1960).

Alkali Degradation. This was performed in a manner similar to that described by Anderson *et al.* (1965) and Kabat *et al.* (1965). Each antigen (4.50 mg) was incubated under nitrogen at 22–25° for 63 hr with 0.5 ml of 0.5 N NaOH, then the pH was adjusted to 6.0 with HCl, and the samples were dried *in vacuo* over KOH and P_2O_5. Controls contained antigens with NaCl in amounts stoichiometric to those of the proper samples after neutralization. Amino acids, galactosamine, and glucosamine of the alkaline-degraded and control samples were determined separately as described above by Dr. P. Weber in Professor R. Winzler's department in a Beckman-Spinco 120-C analyzer.

For analyses of hexosamine and hexose by paper chromatography and colorimetry, aliquots were hydrolyzed for 10 hr with 4.0 ml of 1 N HCl, and humin was removed. The vacuum-dried samples were taken up in water and aliquots were used for Elson–Morgan determinations. The remainder of each sample was deionized with Amberlite MB-3 resin, the resin was washed with copious amounts of water, and supernatants and washes were combined. Hexoses were determined by the Park–Johnson test after chromatography on Whatman No. 1 paper in solvent b. Known amounts of sugar, chromatographed as the samples proper, served as standards. All values found for the samples are expressed as per cent of the controls.

Antisera, Erythrocytes, and Solution. All antibodies were saline agglutinating ("complete") except the anti-s ("incomplete"). Human anti-N and anti-M sera were either obtained in this laboratory or were gifts. The human anti-N "Armstrong" (Gershowitz, 1963) proved to be most sensitive. All human anti-N sera were used after appropriate absorption by the donating laboratory and reabsorption in this one, since they all possessed additional antiblood-group antibodies. Some of the antisera, including those of other specificities, have been described previously (Springer and Ansell, 1958; Springer *et al.*, 1964a); the latter were monospecific for human red cell antigens as far as could be determined. Rabbit anti-N and anti-M sera, used as a routine in the assays, were purchased from Ortho Pharmaceutical and from Hyland Lab. Hemagglutinins were also extracted with 0.85% aqueous NaCl from the seeds of *V. graminea* (Ottensooser and Silberschmidt, 1953) which was grown under supervision of this laboratory.

O,MM, O,NN, and other human erythrocytes as well as those from four horses were obtained, stored, and used as described previously (Springer *et al.*, 1964a). NN erythrocytes from up to three different donors were tested in parallel. The diluent and the erythrocyte-suspending solution were aqueous 0.85% NaCl, containing 0.025 M phosphate buffer, pH 7.2 (buffered saline). The cells were used as a 0.5 or 1.0% suspension.

Blood-Group Hemagglutination Inhibition. The procedures employed for the saline-agglutinating antibodies have been described (*cf.* Springer *et al.*, 1964a). Special care was taken that the pH was always between 7.0 and 7.5. Hemagglutination inhibition with the blocking anti-s was performed in the same way, except that a standard antiglobulin test at 22–25° with a serum from Ortho Pharmaceutical was performed on the red cells after the ordinary incubation period. In some instances the procedure was scaled down and 0.02 ml of all reagents was used. A laboratory standard NN antigen was included in all assays. All active macromolecules were tested at least five times and the averages are given in Table I. Activities are expressed in terms of dilution of inhibitor before addition of

TABLE I: NN and Me-Vg Antigens: *in Vitro* Activity and Enzymatic Inactivation.

Antigen	Treatment	mg/ml Completely Inhibiting the Action on Human Erythrocytes of Four Hemagglutinating Doses of: Anti-N: Human (Armstrong)	Anti-N: Human (Konugres SN-6, Sturgeon 25543)	Anti-N: Rabbit (55,56,57)	*Vicia graminea*	Influenza Virus PR8
Highly purified NN,[a] Ca 825	None	0.01–0.02	0.6–2	0.1	0.003–0.005	0.003
	R.D.E.	*b*	*b*	*b*	0.003	0.425
	Trypsin	*b*	*b*	2.5	*b*	0.135
Me-Vg,[a] Ca 851	None	*b*	*b*	*b*	0.1	0.03
	R.D.E.	*b*	*b*	*b*	0.1	>1
	Trypsin	*b*	*b*	*b*	0.1	0.03

[a] Inactive with three human and four rabbit anti-M sera. [b] No inhibition of agglutination at 10 mg/ml or less.

serum and erythrocyte suspension.

Microprecipitin and Agar Gel Diffusion Tests. These were carried out with sterilized monospecific reagents as described by Springer *et al.* (1965). Crude and purified NN and Me-Vg antigens before and after treatment with R.D.E. were investigated. Anti-N and anti-M sera were of human and rabbit origin. Human anti-A sera and a horse antipneumococcus type XIV serum and homologous antigen were also included. The sera were clarified by centrifugation at 860*g*, 5° for 15 hr, and the extract from *V. graminea* for 2 hr at 13,000*g*. Negative controls consisted of serum or *Vicia* extract alone, and 1.0% antigen solution alone.

Immunizations. Male New Zealand albino and Dutch-colored rabbits weighing between 1.2 and 2.2 kg were injected subcutaneously and intravenously with 0.1% NN or Me-Vg antigens in 0.85% aqueous NaCl containing 0.4% phenol. Each animal received 2.0–2.5 mg of antigen over a 1-week period and was bled immediately before the first injection and 1–2 weeks after the last injection. A "booster" of 0.5–0.7 mg was given 10 days following the second bleeding; blood was again obtained 3–5 days later. The sera were separated by centrifugation, decomplemented at 56° for 25 min, diluted 1:2 or 1:4, and used unabsorbed or after absorption at 23–25° with one-half to one-eighth volume of washed human O,MM or as control O,NN erythrocytes for 30 min to 1 hr at 23–25° with frequent agitation; after erythrocyte removal pre- and postimmunization specimens were tested simultaneously.

Inhibition of Virus Hemagglutination. This procedure was carried out and the results were interpreted as described by the *U. S. Army Med. Dept.* (1945). All inhibitors were titrated in twofold geometrical dilutions in 0.25-ml volumes of buffered saline. To each tube four hemagglutinating units in 0.25 ml of heat-inactivated influenza viruses were added. After shaking, the tubes were incubated for 30 min at 23–25°. Then 0.5 ml of an 0.5% suspension of either human group O or chicken erythrocytes was added, and the samples were shaken again and read after 1-hr incubation. A standard was included in all tests. Active samples were tested at least three times and activities are expressed as in "Blood-Group Hemagglutination Inhibition."

Experimental and Results Section

General and Analytical Properties of Highly Purified NN and Me-Vg Substances. These antigens were obtained as light yellow powders. Weight loss at 110° (0.1 mm over P_2O_5) was 9–10% for the NN antigen and approximately 14% for the Me-Vg antigen. Constant weight was reached in less than 20 hr. At pH 1–12 the materials gave clear, slightly yellow solutions in concentrations up to 1%. While the Me-Vg antigen dissolved immediately, dissolution took up to several hours for the NN antigen, especially between pH 3.5 and 4.5. Electrodialysis resulted in a weight loss of 9.4% for the NN antigen and 8.8% for the Me-Vg antigen. Freshly electrodialyzed solutions had a pH of 3.45 and 4.00, respectively. The NN antigen had a reducing value of 1.5% and a maximal value of 33.5% was reached after 12-hr hydrolysis with 1 N HCl at 100°; the corresponding figures under the same optimal conditions for the Me-Vg substance were 2.5 and 55%. Both antigens behaved as a single component in the ultracentrifuge, on moving-boundary and zone electrophoresis. The molecular weights as determined from the sedimentation and diffusion constants were 595,000 for NN antigen Ca 825 and 520,000

TABLE II: Carbohydrate Constituents of Highly Purified NN and Me-Vg Antigens.

Structural Units	Quantitative Determination by[a]	NN antigen, Ca 825 %	NN antigen, Ca 825 Moles/Mole of Antigen	Me-Vg antigen, Ca 851 %	Me-Vg antigen, Ca 851 Moles/Mole of Antigen
Sialic acid	Warren	16.2	312	9.3	157
	Ehrlich	12.4	239	10.4	175
	Resorcinol	15.7	302	8.7	146
Total hexosamine	Elson–Morgan	9.8	325	23.5	682
Galactosamine	E. M.[b] after paper chromatography	5.7	189	6.7	194
	A. A. A.[c]	4.07	135	8:14	236
	A. A. A.[d]	4.56	153	6.82	198
Glucosamine	E. M.[b] after paper chromatography	6.3	209	15.5	450
	A. A. A.[c]	3.12	104	15.72	456
	A. A. A.[d]	3.07	102	14.34	416
Total hexose	Orcinol	15.0	495	28.0	808
Galactose	P. J.[e] after paper chromatography	11.1	367	20.3	586
Mannose	P. J. after paper chromatography	5.4	179	7.5	216
Glucose	P. J. after paper chromatography	0.3	10	1.7	49
Methylpentose	D. S.[f]	0.8	29	7.9	250
	D. S. after paper chromatography	0.7	25	8.4	266

[a] For description of procedures see text. [b] Elson–Morgan reaction. [c] Amino acid analyzer, short column. [d] Amino acid analyzer, Beckman 120-C. [e] Park–Johnson procedure. [f] Dische–Shettles method.

for the Me-Vg antigen Ca 851 (Bezkorovainy *et al.*, 1966).

Moving-boundary and zone electrophoresis showed only a single peak for the NN and the Me-Vg antigens at every pH tested (Bezkorovainy *et al.*, 1966). The infrared spectra were closely similar for both antigens and are in agreement with the chemical analytical data; they need no comment. Both antigens absorbed in the ultraviolet region between 255 and 282 mμ: the NN antigen showed an absorption peak between 260 and 277 mμ but the Me-Vg antigen a plateau. Both preparations were optically levorotatory. The relative viscosity of the Me-Vg antigen was considerably higher than that of the NN antigen. These physical properties are described in more detail by Bezkorovainy *et al.* (1966).

Lipid solvents extracted <5% by weight of Ca 825 and <1% of Ca 851. Virtually all activity remained in the insoluble residue. The total cholesterol content was <0.5% for either antigen. The NN antigen retained 2.47% and the Me-Vg antigen 0.72% ash. Analytical values (per cent) for the NN antigen were: C, 49.59; H, 6.53; N, 8.37; S, 0.15; $COCH_3$, 5.10; CCH_3, 5.60; free COOH, 2.79; OCH_3, 0.40; and P, <0.05; for the Me-Vg antigen: C, 46.19; H, 6.35; N, 4.60; S, 0.53; $COCH_3$, 9.30; CCH_3, 4.47; free COOH, 2.36; OCH_3, 0.35; and P, <0.05.

Immunology. The activities of the two substances in blood-group hemagglutination inhibition assays are listed in Table I. Full activity of the NN antigen was exhibited by 0.005 mg/ml with all human NN erythrocytes tested and extracts of 2 varieties of *V. graminea* seeds, 0.01 mg/ml with the human anti-N serum "Armstrong," and 0.1 mg/ml with all commercial and self-prepared rabbit anti-N sera. There were, however, significant variations with two other human anti-N sera, depending on the source of the human NN erythrocytes. The activity of NN antigen preparation Ca 825 as well as that of all others tested was much lower with these antisera and there was only a trace of activity with serum SN-6 and certain erythrocytes (Table I, column 4). All determinations with human anti-N described in this article were therefore carried out with the "Armstrong" serum.

The NN antigen isolated from a pool containing all blood-groups also inhibited anti-s at a concentration of 0.6 mg/ml, two of three anti-S at 2.5 mg/ml, anti-A_1 at 1 mg/ml, and anti-A_2 at 0.3 mg/ml. It was inactive with one anti-S serum. It did not possess M, M^g, B, K, P, $Rh_0(D)$, Lu^a, "infectious mononucleosis antigen" or H(O) activity, the latter measured with human and eel antisera. The Me-Vg antigen isolated from blood-group AH(O) meconium possessed A_1 (0.01 mg/ml), A_2 (0.003 mg/ml), and H(O) (0.1 mg/ml) activities in addition to *Vicia* specificity but no other, specifically no human and rabbit N, M, M^g, S, s, K, and Lu^a.

Closely similar activity of the antigens with the *V. graminea* extract was noted when horse instead of human erythrocytes were used. Human and rabbit anti-N after absorption with NN erythrocytes did not agglutinate horse erythrocytes.

Under the conditions employed there was no significant inactivation for any of the blood-group specificities as a result of ultrasound treatment, even though human N activity sometimes decreased by one tube. N and *Vicia* activity of aqueous solutions were unchanged after 24 hr at 23–25° in water or autoclaving at 15 lb (120°) for 30 min, but after 24 hr at pH 4.15 at 23–25° human N activity showed a slight decrease. The influence of different hydrogen ion concentrations at 100° is depicted in Figure 2. The N activity of the antigens after heating 2 hr in a 100° water bath at pH 7.0 or in water was equal and taken as 100%. Such heating did not alter activity of the NN substance measured with rabbit and *Vicia* reagents; however, human N activity decreased by 90–95%. Between pH 2.2 and 9.0 the activity determined with *Vicia* did not decrease significantly; in fact, it increased four- to eightfold between pH 4 and 5. N activities with human and rabbit antisera remained virtually unchanged between pH 5.0 and 7.5; outside of this range they decreased steeply. At alkaline pH the Me-Vg antigen showed the same stability of its *V. graminea* receptor as did the NN antigen, but it was inactivated in the acid range at pH 3.0. ET 181 resin under continuous dialysis inactivated the N specificities of the residual antigens during the first hour but left the *Vicia* specificity intact up to at least 10-hr hydrolysis. Hydrolysis with 0.1 N HCl for 1.5 hr, however, destroyed this specificity also.

Under the conditions employed for deacetylation the blood-group activity of the NN antigen decreased by approximately 90% as measured with human and *Vicia* reagent, but there was hardly any inactivation with rabbit anti-N. No change in the *Vicia* activity of the Me-Vg antigen was observed.

Hemagglutination Inhibition by Model Compounds. Slight (1.2 mg/ml) but strictly specific inhibiting activity of the *V. graminea* reagent was shown by cow colostrum mucoprotein preparation 311. Its activity did not increase following mild acid hydrolysis. Cow colostrum mucopolysaccharide Na-116 and glycopeptides isolated from it were inactive. The *Vicia* reagent was also inhibited (0.6–1.2 mg/ml) by a sponge (*Grantia sp.*) surface glycopeptide, which was inactive with anti-M, -N, -Rh_0(D) serum of human and rabbit origin, but inhibited anti-A, -B and -H(O) sera. None of the naturally occurring amino acids, over 30 peptides, and over 30 mono- and oligosaccharides, including the component sugars of the antigens, showed any *in vitro* activity when measured with any of the anti-N reagents and the *Vicia* extract provided they were tested at about pH 7. This included gangliosides I–IV, dog brain mucolipid A and C, *N*-glycolyl- and *N*-acetylneuraminic acid, one *N,O*-diacetylneuraminic acid preparation, colominic acid, and oligosaccharides from human milk and human chorionic gonadotropin. Cytolipin H, ribonucleic acid, deoxyribonucleic acid, and the purine and pyrimidine bases were inactive. Corn phytoglycolipid and some phospholipids acted unspecifically in that they weakly inhibited some anti-N and numerous other antibodies to about the same extent (2.5–10 mg/ml).

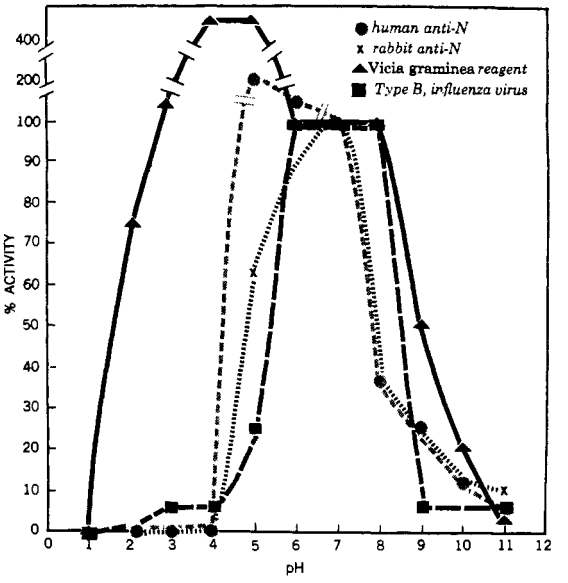

FIGURE 2: Stability of blood-group and myxovirus receptor activity of NN antigen toward acids and bases at 100°. Activity at pH 7.0 = 100%.

Immunoprecipitation. In agar gel diffusion the two antigens reacted visibly only with the *Vicia* extract. One band only was observed for each antigen at concentrations of 1.5–7 mg with 0.25–0.5 ml of standard plant extract but not at lower antigen levels. The bands given by the two antigens fused. Prior R.D.E. treatment did not change the reactivity. The antigens did not precipitate with anti-A or antipneumococcus type XIV horse serum either before or after R.D.E. treatment; the latter serum gave strong precipitates with as little as 2.5 μg of Pneumococcus type XIV antigen.

In the microprecipitin test only Me-Vg antigen reacted with *Vicia* extract. However, after treatment with R.D.E. both antigens precipitated in the narrow range of 10–5 mg/ml of antigen concentrations. There was no precipitation with human anti-M, -N, and -A nor with several rabbit anti-N sera. Me-Vg antigen at concentrations of 10–0.1 mg/ml precipitated antipneumococcus type XIV serum whether the antigen had been pretreated with R.D.E. or not. NN antigen precipitated in this serum only after prior exposure to R.D.E. at concentrations of 10–1 mg/ml. The homologous antigen precipitated this serum at 1 μg/ml.

Immunization. Both NN and Me-Vg substances were immunogenic in rabbits, but only the NN antigen produced anti-N antibodies with regularity. All three rabbits who received NN antigen showed a 16-fold increase in anti-N specific antibody. Acid-desialized NN substance evoked an 8–16-fold increase in anti-N titer in three of six rabbits. Of 17 rabbits immunized with the Me-Vg antigen in its native or desialized form, only one injected with intact antigen showed a clear N specific response from <1:1 to 1:16. None of five uninoculated control rabbits showed an increase in anti-N titer. NN substance inhibited antisera produced with either NN antigen or desialized NN antigen to the same extent as commercial rabbit anti-N sera (Table I) but only the sera produced with acid-desialized NN antigen were in addition uniformly inhibited by acid- and R.D.E.-desialized NN substance; of the three rabbit antisera against ordinary NN substance one was weakly inhibited by acid-desialized NN antigen and none by the R.D.E.-desialized substance. Ordinary and desialized Me-Vg antigens did not inhibit these sera. The antiserum which resulted from immunization with Me-Vg antigen was completely inhibited by 1 mg/ml of Me-Vg as well as NN antigen. MM antigen and other blood-group antigens did not inhibit any of the absorbed antisera.

Inhibition of Influenza Virus Hemagglutination. Highly purified NN antigen preparations were extraordinarily powerful *in vitro* inhibitors of influenza virus hemagglutination (Table I). Me-Vg antigen Ca 851 possessed 10–25% of the activity of the NN substance when tested with the A type PR 8 and Melbourne virus strains. When determined with the Maryland 1/59-Type B_1 strain, both showed lower activity but the Me-Vg antigen was found to be four times as active as the NN substance. Activities against a given strain were closely similar when measured with either human blood-group O or chicken erythrocytes. Myxovirus inhibitory activity of the NN antigen resisted boiling between pH 5 and 8 and that of the Me-Vg antigen between pH 5 and 10, but rapidly declined under more acid or more alkaline conditions. Both antigens retained their myxovirus receptor activity under the conditions used for deacetylation.

Action of Enzymes on Blood-Group and Antiviral Activities. The activities of the highly purified NN macromolecule measured with human serum "Armstrong" rabbit anti-N and with influenza viruses were destroyed to the same extent by all sialidases and by all proteases investigated. The receptor detected by rabbit anti-N was destroyed last. Activity determined with *Vicia* reagent was resistant to the sialidases tested (Table I) but destroyed by proteases. One R.D.E. treatment released >90% of the total sialic acid from the NN antigen and 70–80% of that of the Me-Vg substance in dialyzable form. After trypsin action on NN antigen preparations none or only insignificant amounts of the total sialic acid were found in the dialysate, none of it free. Pronase rendered much sialic acid positive material dialyzable (see below). Treatment of NN antigen and acid-desialized NN antigen with galactose oxidase (2:1 substrate:enzyme ratio, w/w) or *D. pneumoniae* β-galactosidase (0.5 U/mg of substrate) destroyed the *Vicia* specificity of the NN antigen to >95%. Remarkably, galactose oxidase reduced activity as measured with human anti-N >90% and that determined with rabbit anti-N by 50–75% even though this enzyme did not attack sialic acid and did not contain sialidase as also determined in this laboratory. It did not decrease the myxovirus receptor activity. Partially purified preparations generally were not completely inactivated by proteases or galactose oxidase. α-Galactosidase, *E. coli* β-galactosidase, β-hexosaminidase, and lysozyme were without effect on any of the receptors tested. The *Vicia* receptor of the Me-Vg antigen was not affected by any of the enzymes used; its myxovirus receptor activity was destroyed by R.D.E. but not by trypsin.

Chemical Properties. Paper chromatography of acid hydrolysates showed that the predominant carbohydrates shared by both antigens had chromatographic and staining properties of galactose, *N*-acetylneuraminic acid, *N*-acetylgalactosamine, *N*-acetylglucosamine, and mannose. In addition, much fucose was released from the Me-Vg antigen. Among the components set free at pH 3.1 those positive in the Warren test predominated but there were also small amounts of *N*-acetylhexosamine, fucose, serine, and hexosamine or alanine. Between 75 and 95% of the total sialic acid was released from both antigens by this procedure. When developed on Whatman No. 1 or 40 papers in solvent b up to five Warren-positive spots, two migrating faster and two slower than *N*-acetylneuraminic acid, were obtained from both antigens. The major fraction migrated like *N*-acetylneuraminic acid. In some cases one of the spots from the Me-Vg antigen migrating slower than *N*-acetylneuraminic acid disappeared upon R.D.E. treatment but a spot migrating like *N,O*-diacetylneuraminic in solvents a and b on Whatman No. 1 and 40 papers remained. It was always present in hydrolysates at pH 3.1 of the Me-Vg antigen but could only occasionally be demonstrated in hydrolysates of the NN antigen. Hydrolysates of the NN antigen uniformly showed a component on Whatman No. 1 paper with an R_{NANA} of approximately 1.45 and $R_{N,O\text{-diacetyl-NA}}$ 0.74 in solvent a; the values were 1.40 and 0.80 in solvent b. This component was not always demonstrable in hydrolysates of the Me-Vg antigen.

In some instances sialic acid from both antigens crystallized upon drying the dialysates of the 0–3-hr hydrolysates obtained with ET 181 resin. It was recrystallized by dissolving it in a small amount of water, and adding of ice-cold methanol and ether. The crystals decomposed between 183 and 186°, migrated in solvents a and b like authentic *N*-acetylneuraminic acid, and were quantitatively accounted for as *N*-acetylneuraminic acid in the Warren reaction.

Hydrolysis with 0.1 N HCl for 1.5 hr released, in addition to sialic acid(s), some of all component sugars from both antigens. The predominant spots migrated on paper in solvents b and d like glucosamine, galactose,

TABLE III: Amino Acid Analysis of NN and Me-Vg Antigens.

Amino Acid	NN Antigen, Ca 825			Me-Vg Antigen, Ca 851		
	a	*b*	*c*	*a*	*b*	*c*
Aspartic acid	2.93	131	6.3	0.92	36	6.3
Threonine	4.26	213	10.3	2.99	131	22.8
Serine	3.58	203	9.8	1.34	66	11.5
Glutamic acid	4.21	170	8.2	1.04	37	6.4
Proline	2.93	151	7.3	1.85	83	14.4
Glycine	1.46	116	5.6	0.55	38	6.6
Alanine	2.21	148	7.2	0.72	42	7.3
Valine	3.07	156	7.5	0.57	25	4.3
Isoleucine	2.52	114	5.5	0.37	15	2.6
Leucine	4.07	185	8.9	0.72	29	5.0
Tyrosine	1.78	58	2.8	0.15	4	0.7
Phenylalanine	1.85	67	3.3	0.33	10	1.7
Lysine	2.31	94	4.5	0.35	13	2.3
Histidine	2.08	80	3.9	0.25	8	1.4
Arginine	2.54	87	4.2	0.35	10	1.7
Methionine[d]	2.46	98	4.7	0.92[e]	28[e]	4.9[e]
Sum	44.26	2071		13.42	575	
Tryptophan	0.00			0.01		
NH_4^+	0.77	255		0.77	222	
Methionine sulfoxide				0.06		

[a] g of amino acid/100 g of antigen. [b] Moles of amino acid/moles of antigen (nearest integer). [c] Moles of amino acid/100 moles of total amino acid. [d] Values obtained by deducting galactosamine from sum of methionine and galactosamine (see text). [e] Includes value derived from methionine sulfoxide (see text).

and *N*-acetylglucosamine. The Me-Vg antigen in addition released much fucose. This was followed, after additional hydrolysis for 5 hr with 1 N HCl, by the appearance of galactosamine in large amounts, some mannose, and traces of glucose. Additional small amounts of these sugars were liberated when a 10-hr hydrolysis with 3 N HCl followed. There was no indication of the presence of mannosamine in either antigen with any solvent including b_1.

Both antigens, after hydrolysis with 1 N HCl for 5 hr, released ninhydrin-positive substances migrating in solvents f–h like aspartic acid, glutamic acid, serine, glycine, threonine, alanine, histidine, valine, lysine, phenylalanine, proline, leucine, and methionine as well as hexosamines. Hydrolysis with 3 N HCl for 10 hr yielded more of the same substances from both antigens plus arginine.

Based on these findings quantitative measurements were carried out, the results of which are listed in Tables II and III. The sum of all components including those listed under "general properties" accounts for the entirety of both macromolecules. Sugar determinations in each case consisted of more than one procedure per sugar and were generally in good agreement with one another, except that the hexosamine values for the NN antigen by the Elson–Morgan method were uniformly higher than on the amino acid analyzer determined by two different methods. Each carbohydrate value represents the average of at least two or a typical value of three determinations. Galactose, sialic acid, galactosamine, glucosamine, and mannose are the predominant carbohydrates of the NN antigen. The Me-Vg antigen, in addition, contains much fucose which occurs only in trace amounts in the NN substance.

The molar ratio sialic acid:total hexosamine:galactose is close to 1:1:1 in the NN antigen. In the Me-Vg antigen this approaches 1:4:5. As will be seen from Table II the glucosamine:galactosamine ratio is different for the two antigens; for the NN substance it is approximately 1:1 by Elson–Morgan determinations on paper chromatographic eluates and 0.75:1 on the amino acid analyzer; it is 2:1 for the Me-Vg antigen. The galactose:mannose ratio for the NN antigen is 2:1 and close to 3:1 for the Me-Vg substance. The most abundant monosaccharide, on a molar basis, in both antigens is galactose. Comparing the percentages of carbohydrates of both antigens, it is evident that sialic acid is the only carbohydrate present in higher concentration in the NN antigen.

Threonine is the predominant amino acid in both antigens (Table III). This is followed by serine, leucine, and glutamic acid in the NN substance. There is more proline than serine in the Me-Vg substance, and alanine

is the fourth most common amino acid. In the NN antigen the sum of acid amino acids amounts to about three-quarters and that of the basic amino acids to two-thirds of that of the hydroxyamino acids. In the Me-Vg antigen these figures are one-third and one-sixth.

Methionine did not separate from galactosamine on the column described by Piez and Morris (1960), but on the short column by Moore *et al.* (1958) glactosamine alone was determined since methionine was destroyed. The methionine value was therefore calculated from the glucosamine:galactosamine ratio given by the antigens in these two different amino acid analyzing systems, with the assumption that the hexosamine ratio was the same and any difference between the ratio on the microcolumn and that on the short column was due to methionine. The aromatic amino acids, with the exception of proline, were present in the lowest concentration in both antigens; there was virtually no tryptophan. Cystine was not demonstrable. Good agreement was found for both antigens between the N values obtained by the Dumas method (8.37% for the NN substance and 4.60% for the Me-Vg substance) and those arrived at by calculation from the amino acid and amino sugar composition (about 8.5% for the NN and 4.5% for the Me-Vg antigen. CH_3 values were calculated based on monoacetylation of the hexosamines and sialic acid. They were somewhat lower than those actually found; calculated for NN antigen: acetyl 1.4%; leucine, valine, isoleucine, threonine, alanine, and fucose in this descending order account for 3.3% giving a total of 4.7 against 5.60% found. The corresponding calculated CH_3 value for the Me-Vg antigen is 3.7%; of this 2.1% is for acetyl and 1.6% for fucose, threonine, leucine, valine, alanine, and isoleucine in this sequence; 4.47% CH_3 was found. Calculated acetyl was 4.1% for the NN antigen, while 4.62, 5.57% were the figures found. Acetyl calculated for the Me-Vg antigen was 5.9%; 9.49, 9.41, and 9.01% were the values of the determinations. Based on the hydrolytic studies the free carboxyl appears to be largely due to the sialic acid, which according to calculation accounts for 2.4% COOH for NN and 1.5% for Me-Vg. This agrees with the analytical data. The methoxyl values are due to the SCH_3 of the methionine; the calculated values of 0.5 and 0.2% for the NN and Me-Vg antigens, respectively, are in agreement with the direct determinations. Similarly, the small percentage of S in both antigens is from methionine.

Alkaline Degradation. The most striking results of alkali degradation are depicted in Table IV, which shows that galactosamine, galactose, threonine, and serine were the components destroyed in both antigens. Results obtained by the Elson–Morgan reaction and on the amino acid analyzer were in agreement. In the Me-Vg antigen glucosamine also decreased by 30%. Mannose remained unaffected in both antigens, and there was no demonstrable change in fucose and sialic acid content. Tyrosine was decreased while glycine increased by *ca.* 20% in the NN antigen. No other amino acid was significantly affected in either antigen.

TABLE IV: Components of NN and Me-Vg Antigens Destroyed by 0.5 N NaOH (23°, 63 hr).[a]

Component	Quantitative Determination by	% Destruction: NN Antigen, Ca 825	% Destruction: Me-Vg Antigen, Ca 851
Hexosamine, total	Elson–Morgan	48	43
Galactosamine	A. A. A.[b]	80–90	61
Glucosamine	A. A. A.[b]	0	30
Galactose	P. J.[c] after chromatography	66	~30
Threonine	A. A. A.[b]	48	46
Serine	A. A. A.[b]	39	34
Tyrosine	A. A. A.[b]	56	0

[a] For procedures and unaffected components see text. [b] Amino acid analyzer Beckman, 120-C. [c] Park–Johnson procedure.

Isolation of Sialic Acid Containing N-Specific Haptens. These were obtained from the "crude" or "purified" NN (see Figure 1) and Me-Vg antigens by hydrolysis with (1) HCl at pH 3.1, or (2) ET 181 resin at pH 2.2–2.5, or (3) pronase. Both the residual antigens and the dialysates were studied serologically but only the latter were subjected to preliminary chemical investigation. Inhibition given by the most active haptenic structures obtained by the three methods was between 0.6 and 1.2 mg/ml with human anti-N and *Vicia* reagent. The hapten activity of *Vicia* active substances was somewhat higher when horse instead of human erythrocytes were used. There was never more than a trace of activity with rabbit anti-N serum. Where indicated, dialysates were pooled and cleaned with IR-120 H^+ and Darco-G 60 as described previously (Springer *et al.*, 1964a). The composition was determined by the Elson–Morgan and Warren procedures, and for components other than hexosamine and sialic acid preliminary evidence was obtained by paper chromatography in solvents a, b, and g both unhydrolyzed, and after hydrolysis for 45 min at 80° in 0.1 N HCl, after 8 hr with 1 N HCl at 100°, and after 24 hr with 6 N HCl. (1) Upon hydrolysis at pH 3.1 almost 20% of the NN and Me-Vg antigens (w/w) became dialyzable. The material was separated (*ca.* 30 hr for each of three runs) on Whatman No. 40 paper sheets in solvent b, eluted with water, and centrifuged at 4°, and the supernatant fluids were lyophilized. About one-quarter of the fractions obtained showed activity. One fraction inhibited human and rabbit anti-N while all other active ones inhibited either only *Vicia* or *Vicia* and human anti-N. Those inhomogeneous fractions containing material with chromatographic properties like *N,O*-diacetylneuraminic acid showed weak human N

activity. Some chemical properties of these Warren-positive substances from both antigens have been described above. The majority of hydrolysates of fractions with human N activity showed galactose amounting to approximately one-third of the sialic acid. Sometimes fucose was also found in hydrolysates from NN antigen; it occurred generally in those of meconium substance. Some inactive fractions had the same over-all composition, even though fractions consisting solely of sialic acid and *N*-acetylhexosamine were only found among the inactive material. None of the fractions contained any amino acids in significant amount.

(2) ET 181 hydrolysis released up to 25% (w/w) of the NN antigen in dialyzable form. Approximately 20% of the total sialic acid was liberated from the NN antigen during the first hour, 60% by 3 hr, and >90% by 8-hr hydrolysis. From the Me-Vg antigen more sialic acid was released during the first 3 hr; one-third of the antigen (weight) and >90% of the total sialic acid became dialyzable. The residual sialic acid could usually be removed from both antigens by R.D.E. treatment. Material with chromatographic properties of oligosaccharides was found in all fractions from both antigens besides of all the component sugars. Free sialic acid, galactose, and *N*-acetylhexosamine appeared first in addition to oligosaccharide fractions which migrated slower than sialic acid. With the ninhydrin reagent spots like threonine, serine, glycine, glutamic acid, some aspartic acid, and hexosamine and frequently also valine were found at all stages of ET 181 hydrolysis. Usually two-thirds of the dialyzable material from the NN antigen showed hapten activity, but in some instances the yield of active material was much lower. From the Me-Vg antigen approximately 8%, released during the first 5 hr, possessed both human and *Vicia* activity; another 5% released later had *Vicia* activity only.

High-voltage electrophoresis of serologically active hydrolysates from NN antigen yielded three fractions with human N activity, two of which migrated to the anode while one remained at the point of application. The slower of the two anodically migrating fractions was the most active and largest. It had mobility M_{NANA} 0.66. Chromatography of this material on Whatman No. 1 paper in solvent a showed it to consist of three components of which the fastest contained the activity. Elution and rechromatography of this latter component in solvent a showed one major spot, R_{NANA} 0.70, and a minor one with R_{NANA} 1.40 and $R_{N,O\text{-diacetyl-NA}}$ 0.8 in solvent b; both were Warren positive. Colorimetric and paper chromatographic analysis of the major component after hydrolysis indicated a ratio of 2:1 sialic acid:galactose. From the Me-Vg antigen hydrolysate only one N-active fraction was isolated. It had the same electrophoretic properties as the most active hapten material from the NN antigen and on chromatography revealed three components.

Other dialysates of ET 181 treated NN and meconium antigens were separated on Sephadex G-15 columns (0.8 × 80 cm) by elution with water and collection of 1-ml fractions; peaks were pooled and cleaned with Amberlite IR-120 H^+ and Darco-G 60 charcoal as described previously (Springer *et al.*, 1964a). More than 90% of one weakly human N-active dialysate from NN substance was eluted in one chromatographically homogeneous peak which on hydrolysis yielded NANA and galactose (*ca.* 2:1). A 3-hr ET 181 hydrolysate of meconium substance gave two major chromatographically homogeneous peaks; the first containing 28% of the starting material eluted between 15 and 19 ml; the homogeneous eluate possessed no human N specificity and showed sialic acid, glucosamine, galactose, and fucose (2:1:1:1) on hydrolysis. The second fraction (11% yield) eluted between 21 and 23 ml; it inhibited human anti-N at 0.6 mg/ml and *Vicia* at 5 mg/ml. On hydrolysis sialic acid, galactose, and fucose were found. None of these fractions yielded amino acids. Elution of 45 mg of a Me-Vg fraction obtained between 5- and 8-hr hydrolysis from a Sephadex G-50 column gave one active fraction at 8 and 9 ml in 0.4% yield which inhibited both human anti-N and the *Vicia* reagent at 1.2 mg/ml. It was not investigated chemically.

(3) Pronase digestion of NN antigen (substrate: enzyme ratio 20:1, w/w, 0.05 M Na-Veronal buffer, final pH 7.2, plus traces of Ca^{2+}; 37°, 65-hr, toluene added) rendered approximately 35% of the substrate dialyzable; of this 23.5 mg was placed on a Sephadex G-15 column (0.8 × 80 cm) and eluted with water and 1-ml fractions were collected; a major Warren- and ninhydrin-positive peak, amounting to approximately 25% of the applied sample, eluted from 17 to 24 ml. It possessed human N activity (0.6 mg/ml), and traces of activity with rabbit and *Vicia* reagents. Elson–Morgan and Warren reactions and chromatography in solvent b after hydrolysis for 45 min at 80° in 0.1, 5 hr in 1, and 16 hr in 6 N HCl showed that it contained one sialic acid (terminal):two galactose:one galactosamine: one serine:one glycine:one threonine:one aspartic acid: one glutamic acid:one lysine:one leucine:one valine. It contained no free amino acids or carbohydrates.

Discussion

The human NN erythrocyte antigen and the novel antigen (Me-Vg) from human meconium are fully described here for the first time. Both substances behaved as single components in the agar gel diffusion assay described here and no inhomogeneity was found by physical tests (Bezkorovainy *et al.*, 1966). Quantitative chemical analyses account for approximately 100% of both antigens. Both are glycoproteins and in addition to being blood-group substances are potent influenza virus receptors. Sialic acid and galactose are involved in blood-group N specificity as determined with both human and rabbit anti-N sera; the myxovirus receptor activity is also dependent on the presence of sialic acid; the *Vicia* specificity of the NN antigen depends apparently on a β-galactopyranosyl group (see below). The NN antigen may serve as a model compound not only in blood-group serology, human bio-

chemical genetics and virology but in a number of other fields since it is the first physically homogeneous cell surface receptor to be described which exhibits blood-group hemagglutination-inhibiting activity equal to that of blood-group ABH(O) glycoproteins from fluids when tested with their respective antibodies containing the same amount of agglutinating units. Lipid extractants removed <5% material (weight basis) with only 1–5% of the activity/unit weight of the insoluble material; it was not investigated chemically, although the activity was reproducible. Thus, some N-active erythrocyte material is probably bound to lipid and chemically different from the antigen described here. The isolation of blood-group N-active substances from human erythrocyte stroma with lipid solvents (Stalder and Springer, 1960, 1962) supports this conclusion. The Me-Vg antigen is characterized biologically by its reactivity with *V. graminea* agglutinins and influenza viruses but it fails to inhibit human and rabbit anti-N sera and thus shares only some serological properties with the NN antigen. It contains virtually no lipid. Neither substance had a chorionic gonadotropin effect as determined by the method of Edgren (1956); this was considered in view of the ability of influenza viruses to destroy this hormone (*cf.* Brossmer and Walter, 1958).

Both antigens possess a similar qualitative composition, but there are quantitative differences. On a molar basis galactose is the predominant sugar in both. In the NN antigen this is followed by sialic acid, then the two hexosamines and mannose in approximately equal concentration. In contrast, glucosamine is the second most abundant sugar in the Me-Vg antigen, followed by fucose and then galactosamine and sialic acid in similar concentrations. Glucose occurred only at the end of a vigorous hydrolysis; it is questionable if it is a building stone of the two macromolecules. According to results in the amino acid analyzer (six different determinations, two listed in Table II), galactosamine predominates over glucosamine in the NN antigen; this unusual finding was not made for the ABH(O) blood-group glycoproteins (Kabat *et al.*, 1965). Separate determination of these two hexosamines by the Elson–Morgan reaction after chromatography and elution gave a considerably higher value than on the amino acid analyzer and indicated a ratio of 1:1. The determination of total hexosamine by the Elson–Morgan reaction lies halfway between the two other values. Such a discrepancy for values obtained by different methods was not observed for the Me-Vg antigen (Table II).

The most common amino acid (molar basis) in both antigens is threonine. Threonine and serine amount to 416 moles or *ca.* 20% of the total amino acid content of the NN antigen and 197 moles or 35% of that of the Me-Vg antigen (Table III). The proportion of threonine to serine is about 1:1 in the former antigen and 2:1 in the latter. Glutamic and aspartic acids are found in both macromolecules in a proportion of about 1:1 and in the NN antigen amount to two-thirds of threonine and serine. The three basic amino acids were present in low and nearly equal concentration in antigen Ca 851; they were also found in equal proportions in antigen Ca 825 where their total amounted to almost as much as the acidic amino acids. Both antigens contain considerable amounts of proline, leucine, alanine, and valine, and both have little tyrosine, phenylalanine, and histidine and lack cystine and tryptophan.

The elements determined analytically are accounted for by the different components and their functional groups. Generally there is good quantitative agreement. One discrepancy was the excess of acetyl found which was about 1.6 times as much in the Me-Vg antigen and 1.2 times in the NN antigen as that calculated on the basis of monoacetylation of neuraminic acid and hexosamine. The observed acetyl value of 9.30% for the Me-Vg antigen would allow for diacetylation of all sialic acid. Paper chromatography indicates the presence of at least one *N,O*-diacetylated neuraminic acid in both antigens; its concentration is higher in the Me-Vg antigen. The occurrence of *N,O*-diacetylneuraminic acid in man has not previously been described. In the NN antigen only very little of the second carboxyl group of glutamic and aspartic acids could be free since it would add 2.3 to the 2.4% COOH calculated to be due to the sialic acid; the total free carboxyl found was 2.79%. The theoretically free carboxyl groups of the Me-Vg antigen which are not attributable to sialic acid amount to only 0.63%.

The studies with alkali under nitrogen indicate a β-carbonyl elimination involving galactosamine, galactose, and threonine (*cf.* Ballou, 1954; Anderson *et al.*, 1965; Kabat *et al.*, 1965); in addition, glucosamine is involved in the Me-Vg antigen (Table IV). Based on the experiments reported, there is no indication for a significant contribution of any other component. In the NN antigen the hydroxyamino acids could accommodate all hexosamine (galactosamine plus glucosamine) or all galactose but not both. The destruction of galactosamine in the alkali-treated NN antigen could be calculated only approximately from the peak eluted from the column. Thus 85% destruction corresponds to the loss of 130 moles of galactosamine/mole of NN substance. Approximately 240 moles of galactose was destroyed. This compares to the destruction of *ca.* 100 moles of threonine and *ca.* 80 of serine. Obviously not all carbohydrates destroyed are linked to an amino acid. The experiments permit no decision as to which sugar is predominantly involved in such linkages and which is destroyed as a result of a peeling degradation of the liberated oligosaccharide from the reducing end (*cf.* Kabat *et al.*, 1965). The sugars destroyed are likely to be located less distant from the terminal nonreducing end groups of the macromolecule than those of the Me-Vg antigen; it is reasonable to assume the presence of at least two different carbohydrate chains. According to the chemical and immunochemical results of these studies, the terminal sugar unit in one instance is sialic acid and in the other galactose, unless one postulates branched structures. Even if the chains are all alike the chain

length will probably not exceed four to six sugar units based on the molar proportions of the sugars and since sialic acid is the most peripheral sugar. The relative shortness of the chains is also compatible with the moderate excess of 2:1 of carbohydrate destruction over that of hydroxyamino acids, thus making it unlikely that a peeling reaction is responsible for the destruction of >60% of the sugars. If there is only one sialic acid at the terminus of each chain carrying this sugar, then there would be approximately 300 such chains/mole of NN antigen Ca 825; there are indications, however, that some chains carry 2 sialic acids. The rather extensive destruction of tyrosine is noteworthy but its significance is not clear since there are only a total of 58 moles of tyrosine in the NN molecule. The increase in glycine in the NN antigen may result from the degradation of the hydroxyamino acids in a β-elimination reaction (Adams, 1965).

In the Me-Vg antigen there is not enough serine and threonine to bind all the hexosamine or all the galactose but enough to bind all the galactosamine. However, approximately 40% of the galactosamine was unaffected by alkali and, assuming the elimination reaction to be complete, not involved in the sugar–peptide linkages. Only 60 moles of threonine and 22 moles of serine are destroyed by alkali/mole of antigen *vs.* approximately 120 moles of galactosamine, 175 moles of galactose, and 135 moles of glucosamine, *i.e.*, over five times as many carbohydrates as hydroxyamino acids have been destroyed. Therefore, extensive carbohydrate destruction by a peeling reaction must have occurred. These results and the much higher total carbohydrate to amino acid ratio make it necessary to assume the presence of longer carbohydrate chains in the Me-Vg antigen than in the NN antigen, even if one considers that the serological and enzymatic results clearly indicate a number of different carbohydrate chains; that bearing the *Vicia* specificity does not appear to be the predominant one.

There is a remarkable resemblance in the amino acid composition of the antigens described here with the human blood-group ABH(O) and Le^a glycoproteins isolated from secretions (Carsten and Kabat, 1956; Pusztai and Morgan, 1963). However, in the NN antigen Ca 825, threonine, serine, and proline amount to a smaller percentage of the peptide moiety, the basic amino acids are present in about the same proportion as in the ABO substances, while the leucines and aspartic and glutamic acids form a considerably larger part of the peptide moiety in the NN antigen. The quantitative composition of the Me-Vg antigen peptide moiety is more closely similar to that of the ABO glycoproteins.

The data reported here for preparation Ca 825 give the average composition of the NN antigen in 150 donors. Individual variations undoubtedly occur. An earlier preparation (Springer, 1965) of comparable purity obtained from a pool of 60 donors had considerably more sialic acid (24%) and much less methionine (0.63%); otherwise there was close agreement in amino acid composition of both antigen preparations. Amino acid and carbohydrate values resembling those reported here were found by Kathan and Winzler (1963) for a myxovirus receptor with mol wt 31,000 isolated from human red cells. The NN antigens prepared in this laboratory uniformly had approximately 50% more lysine, alanine, leucine, and proline, and significantly less hexosamine. The myxovirus receptor extracted with hot phenol under alkaline conditions may be the deaggregated form of the antigen described here whose molecular weight is approximately 20 times larger (*cf.* Bezkorovainy *et al.*, 1966). This assumption is supported by the similar biological properties of both materials. The myxovirus receptor isolated by Kathan *et al.* (1961, 1963) contained up to 5% of the blood-group activity of the NN antigen described here depending on the antiserum used. The NN antigen in turn is an exceedingly potent myxovirus receptor. There is no close agreement between the amino acid values of NN antigen Ca 825 and those obtained by Uhlenbruck and Weber (1964) on a physically undefined NN erythrocyte antigen preparation; notable is the discrepancy in threonine, serine, proline, and glycine content. Comparable studies of an additional homogeneous meconium antigen are not available; previous data (Springer, 1965) were obtained with inhomogeneous material from the same pool as the present preparation.

The high galactosamine:glucosamine ratio of the NN antigen appears to be unique. The remarkable proportion of approximately 1:1:1 for sialic acid:hexosamine:galactose obtained for the NN glycoprotein does not necessarily indicate a linear order of these molecules on the same chain. It has been shown here that in the NN and Me-Vg antigens a substantial part of the sialic acid is linked to galactose and some possibly to a second sialic acid. Only small amounts of sialic acid and hexosamine containing structures (more from the Me-Vg antigens) were obtained from both antigens in the present study, possibly due to the method of isolation.

The most striking results of enzymatic hydrolyses not reported previously for either crude or isolated antigen preparations or intact red cells (*cf.* Springer and Ansell, 1958; Springer, 1965) are those obtained with galactose oxidase and β-galactosidase. Surprisingly, the former enzyme destroyed NN specificity determined with human anti-N sera and decreased N activity determined with rabbit anti-N sera. Similar observations were made for the human MM antigen and its corresponding antisera and the M-like antigen on sheep erythrocyte glycoprotein (G. F. Springer, H. J. Callahan, M. Nikiforuk, and E. T. Wang, in preparation). It is known that galactose oxidase oxidizes both galactose and galactosamine (Avigad *et al.*, 1962). It is therefore probable that this enzyme oxidizes either a subterminal or branched galactose or galactosamine, which in either case is involved in the N specificity. From the hapten studies this sugar is more likely to be galactose.

The *Vicia* specificity of the NN antigen is inactivated by galactose oxidase, as shown by Krüpe and Uhlenbruck (1964) and here. It has now been demonstrated

for the first time that galactose in β-glycopyranosidic linkage is most likely involved in *Vicia* specificity of the NN antigen since one of the two β-galactosidases tested inactivated the *Vicia* receptor by >95%. None of the other glycosidases including the β-hexosaminidase had any effect on the *Vicia* activity.

The other results of enzymatic hydrolyses of the isolated NN antigen are in general agreement with earlier observations on intact red cells. Inactivations frequently were less complete with crude antigen preparations. The only discrepancy was the reported destruction of the *V. graminea* receptor by crude R.D.E. on the red cell (Springer and Ansell, 1958). Reinvestigation with crystalline R.D.E. showed that the "*Vicia*" receptor was resistant to sialidase action both on the red cell surface or in isolated form. The *Vicia* receptor of the Me-Vg antigen was not affected by any of the enzymes tested. This may possibly be due to lack of accessibility of the receptor to the enzyme.

Successive sialidase and mild acid hydrolysis indicate that >95% of the sialic acid is in the periphery of both macromolecules, linked either terminally or subterminally to another sialic acid. From both antigens one to two sialic acid containing oligosaccharides were released at pH 3.1.

Care must be exerted in interpretation of paper chromatographic results. While the spots obtained on Whatman No. 1 paper were reproducible under all conditions employed and distinct from all controls used, it was found that one of the Warren-positive spots with an R_{NANA} of *ca.* 0.66 in either solvent a or b on Whatman No. 40 paper (three runs) arose from synthetic *N*-acetylneuraminic acid when it was mixed with the same buffer as present in commercial R.D.E. The buffer in which the R.D.E. was dissolved consisted of 0.05 M sodium acetate, 0.9% NaCl, and 0.1% $CaCl_2$.

A number of serologically active structures of small molecular size have been obtained by mild procedures. The fragments inhibited human anti-N (Armstrong) and *Vicia* reagents and occasionally, but to a lesser degree, rabbit anti-N. It is surprising that it was possible to prepare from the meconium antigen, which possessed no human N specificity, fractions which inhibited apparently specifically human anti-N. These active structures are not accessible to the human antibody in the macromolecule.

Only preliminary information has been obtained on the chemistry of the haptenic structures and the homogeneity of none of them has been rigidly proven. A blood-group N specific hapten containing two sialic acids and one galactose but no other carbohydrate has been isolated from an ET 181 hydrolysate of the NN antigen. Galactose was the predominant sugar in the blood-group N specific fragment isolated from the digest. The other components involved in the carbohydrate–peptide linkage (Table IV) were present in the fragment in the proportion 1:1:1. Acid hydrolysis at pH 3.1 also gave haptens which either contained only galactose in addition to sialic acid (in the proportion of 1:2 or 1:1) or some fucose or *N*-acetylhexosamine as further components.

Romanowska (1961) obtained a sialylhexosamine chromogen and a compound which contained galactose and sialic acid in the proportions 1:1 by mild alkaline hydrolysis of M and N substances. It was not reported if these materials were blood-group active. Cook and Eylar (1965) state that they prepared an N-active fraction with approximately 1.75 moles each of hexosamine and sialic acid per mole of galactose. Kathan and Winzler (1963) assumed that in their virus inhibitor all sialic acid was linked to hexosamine and that the hexosamine was largely unacetylated. Both antigens described here show an excess of acetyl over the theory for monoacetylated sialic acid and monoacetylated hexosamine. Similarly, in the blood-group ABH(O) glycoproteins an excess of acetyl over that accounting for monoacetylated hexosamine was generally found (*cf.* Kabat, 1956).

The chemistry of the two macromolecules described is viewed here in relation to their blood-group specific structures. While the present studies with isolated haptens implicate sialic acid–galactose structures in N specificity as measured with human anti-N reagent (Armstrong) no statement beyond this can yet be made. Free sialic acid, *N,O*-diacetylneuraminic acid, and D-galactose, and several of its derivatives were inactive.The structure detected by rabbit anti-N is probably larger than that responsible for human N specificity as indicated by the latter's much greater heat lability and susceptibility to galactose oxidase as well as the ability of desialized NN antigen to induce anti-N antibodies in rabbits, even though the latter may be predominantly *Vicia* specific. The *Vicia* specificity is not dependent on sialic acid (Nagai and Springer, 1961; Lisowska, 1963); in fact its removal under mild acid conditions increases the *Vicia* activity of the NN antigen (Figure 2). Attempts to determine the structure responsible for *Vicia* specificity by inhibition studies with known sugars failed. It is compatible with the preliminary hapten investigations and the findings with β-galactosidase and galactose oxidase that removal of sialic acid leads to an increase in *Vicia* and pneumococcus type XIV specificity, both of which seem to depend on a β-galactosyl unit.

It appears possible that both human N and *Vicia* specific structures have relatively small immunodeterminant groups not exceeding three to four carbohydrates in the former and two in the latter case. Sensitive immunochemical indicators of activity and specificity were the hemagglutination and hemagglutination-inhibition assays. The NN substance regularly induced anti-N antibodies. In rabbits, remarkably, acid-desialized NN antigen which had lost its *in vitro* reactivity with human and rabbit anti-N nevertheless remained a good antigen in induction of anti-N antibodies, which could be inhibited by both ordinary and acid-desialized NN antigens. The Me-Vg substance is poor in its ability to produce anti-N antibodies. Precipitin tests with anti-N sera were negative throughout; both antigens precipitated with *Vicia* extract, however. In agar a so-called "reaction

of identity" was given between the two chemically and physically different NN and Me-Vg macromolecules pointing once more to the necessity of caution in interpretation of immunochemical reactions (*cf.* Springer *et al.*, 1965).

Crude preparations of NN erythrocyte antigen contain the S and s antigens (Stalder and Springer, 1962). These activities could also be shown in the present homogeneous antigen; they may therefore be carried on the same molecule, supporting the well-known close genetical relationship of these antigens with the MN system (*cf.* Race and Sanger, 1962). Although the NN antigen had been isolated from a pool of all blood groups the only other specificities found were A_2 and weak A_1 in this and all other NN antigen preparations originating from different pools. The absence of Lu^a activity is noteworthy, since the antigens of the Lutheran system are the only other human erythrocyte antigens known to be inactivated by influenza viruses (Springer and Ansell, 1958).

The serological activity of NN antigen Ca 825 varied by approximately 1 decadic logarithm from one class of reagent to the other in increasing order from rabbit-, to human- (Armstrong) anti-N, to *Vicia* reagent. There was no significant change in this activity when different NN erythrocytes were used with different *V. graminea* extracts or various rabbit anti-N sera. It is, therefore, noteworthy that extreme variations in activity of the NN substance were found with the human anti-N sera. These variations were influenced, except for the serum Armstiong, by the origin of the NN erythrocytes, an indication that each of the tested human sera reacts with a somewhat different area on the erythrocyte antigen, and furthermore that the NN antigens of various individuals differ in their surface structure. It is probable, because of the faint N activity with some of the human anti-N sera tested, that even the relatively gentle isolation procedures employed in this study have not yielded the native NN antigen. The lability of the specificity determined with human anti-N sera is also demonstrated by the uniform loss of >75% of activity determined with the Armstrong serum when the glycoprotein was extracted from red cell stroma at 65° instead of at 25°, by the >90% loss of activity of a preparation isolated at 25° on boiling for 2 hr between pH 5.5 and 7.0, and finally by the slight decrease in human N specificity upon sonication in contrast to complete resistance of rabbit N and *V. graminea* specificity under the conditions employed. The lability of human N specificity contrasts with the relative stability of the human ABO substances (Kabat, 1956; Morgan, 1960) and may be an indication that an *O*-acetylated sialic acid contributes to human N specificity as determined with some sera. It is noteworthy that the two human sera which were inhibited by the smallest amounts of NN antigen also had the highest anti-N titers. From the standpoint of biochemical genetics these findings provide further evidence for the mosaic structure of blood-group antigens in general (Wiener *et al.*, 1964) since N specificity is recognized by antibodies which react with different chemical structures on the same macromolecule.

It has become evident throughout this work that the so-called anti-N reagent from *Vicia* interacts with a chemical structure not closely related to the receptors for rabbit and human anti-N. Wiener and Moor-Jankowski (1963) have described a rabbit anti-N serum which reacted with human and ape red cells like *V. graminea* extract. The similarity of this serum to *Vicia* extract was confirmed in this laboratory; however, it was not identical with *Vicia* extract since desialized NN antigen possessed only about 10% of the inhibiting activity of the intact antigen and the Me-Vg substance failed to inhibit this rabbit antiserum.

It has been assumed that the erythrocyte structures detected by the *Vicia* reagent are those of the N factor (Levine *et al.*, 1955; Allen *et al.*, 1960) and by others that *Vicia* receptor may be akin to the human H(O) blood-group substance in the ABH(O) system (Lisowska 1963; Uhlenbruck and Krüpe, 1963). Results obtained in this laboratory (Nagai and Springer, 1962; Springer and Hotta, 1964; Callahan and Springer, 1966) and those reported by Wiener and Moor-Jankowski (1963) with blood typing of chimpanzees can be interpreted as indicating that the *Vicia* receptor represents either a precursor substance of both the N and M antigen or that it is a heterophile antigen, in a way analogous to the receptor on the ABH(O) and Le^a substances which cross-reacts with antipneumococcus type XIV antiserum; it would not necessarily have to lay in the direct biosynthetic pathway of the N or M erythrocyte antigens. Significantly, human erythrocytes possessing solely the *Vicia* structure but no N or M specificity have not been found, in contrast to human red cells having only H(O) activity. That much of the *Vicia* receptor is ordinarily masked in human N substance and even more in the M substance is shown by the increase in activity of both antigens to an equal level by hydrolysis between pH 4 and 5, following which the *Vicia* activity of the NN antigen increased four- to eightfold and that of the MM antigen 12-24-fold. Full inhibition was given by 1 μg/ml of both. R.D.E. treatment increased the *Vicia* receptor activity of the NN antigen almost twofold, whereas that of the MM antigen was increased four-to eightfold, bringing the activity to the level of the "native" NN antigen. Similar to the *Vicia* receptor, the pneumococcus type XIV structure was found to be more accessible in some blood-groups (Le^a and H(O)) than in others (A and B) and also here, mild acid hydrolysis increased the cross-reactivity (Kabat, 1965; Morgan, 1960). Favoring the views presented here on the relation of *Vicia* specificity to human and rabbit N is the finding of equally "*Vicia*-active" glycoproteins in all meconia of infants of all blood types (MM, MN, and NN) and in glycoproteins isolated from horse, cow, and sheep (*cf.* Callahan and Springer, 1966).

M-specific glycoprotein fractions extracted in this laboratory with phenol at 65° from M^uM^u erythrocytes of three donors (L. Tracy, E. Brown, and one from blood furnished by Dr. R. Rosenfield) which are thought

to contain no N specificity (Allen *et al.*, 1960) inhibited agglutination of horse erythrocytes by *Vicia* agglutinins, and two of the glycoproteins (L. T. and Dr. Rosenfield's donor) inhibited the action of the *Vicia* agglutinin on human red cells in addition, a property considered to be characteristic for N substance (*cf.* Stalder and Springer, 1962). Complete inhibition was given by 0.6–1.2 mg/ml of partially purified M^uM^u preparations and amounted to 25–50% of that of corresponding crude glycoproteins from ordinary human MM erythrocytes. Human anti-N (Armstrong) was also inhibited by these two preparations, but not that of rabbit (one test only). The three M^uM^u antigen preparations possessed no S or s activity. These results indicate that the differences in N activity and *Vicia* receptor activity between MM red cells possessing antigen U and those lacking it are quantitative rather than qualitative.

Highly purified MM antigen preparations obtained by extraction at 23–25° did not inhibit any of a number of rabbit and human anti-N sera, while some of the preparations which were extracted at 65° did inhibit rabbit anti-N, although weakly (Stalder and Springer, 1962). Low-degree human N specificity, as measured with Armstrong serum only, also occurred *de novo* in some preparations upon mild acid hydrolysis (Nagai and Springer, 1962). N-active haptenic structures were isolated from MM antigen (Hotta and Springer, 1965). In contrast, no M activity could be produced from NN antigen. Clearly, the MM antigen contains N-specific structures. *Vicia* activity was present in all highly purified preparations of human MM substance. This varies from observations by Lisowska (1963) who found this activity absent in four of her six preparations.

N activity determined with human and rabbit antisera could not be separated from the virus-inhibitory properties. Both activities appear to be located on the same macromolecule. They were by far the highest in that NN antigen preparation (Ca 825) which had the largest molecular size of a number of preparations tested. Treatment with R.D.E. from *V. cholerae* or influenza virus neuraminidase or acid hydrolysis between pH 4 and 6 destroyed simultaneously both the blood-group activity measured with human and animal sera and the myxovirus receptor activity. *Vicia* activity on the other hand is not closely related to the myxovirus receptor properties since sialic acid is not involved in its specificity.

In a more general way, the original observation on the NN and MM antigens on intact red cells (Springer and Ansell, 1958) seems to have been the first one in which a blood-group specific structure has been shown to possess a second, biologically unrelated receptor function. The studies since then indicate that the virus inhibitory structures are part of the N-specific region reactive with human and rabbit anti-N. In contrast, the *V. graminea* receptor site is distinct from the virus receptor area. Chemical studies have now made likely the involvement of sialylgalactopyranosyl structures in blood-group N specificity and of a β-galactopyranosyl grouping in *Vicia* specificity.

The studies discussed here describe two biologically active glycoproteins and are a first step in the elucidation of the biochemical genetics of the second human blood-group system. They also point to the possibility that genetical schemes of erythrocyte blood-group systems in general (*cf.* Race, 1965) may be elaborated more completely once isolated homogeneous red cell antigens are available.

Acknowledgments

The authors are obliged to Professor R. J. Winzler, Dr. P. Weber, and Mr. D. Grohlich for quantitative amino acid analyses, hexosamine ratios, and advice, to Professor S. Roseman for suggestions, to Dr. K. Hotta, Mrs. M. Nikiforuk, Mrs. K. Roberson, and Mrs. E. T. Wang for various preparations and assays. We thank the Directors of the Blood Banks of the Philadelphia General Hospital, The Hospital of the University of Pennsylvania, and the American Red Cross in Philadelphia for a continuous supply of blood. Allantoic fluid containing influenza viruses was given by Dr. B. Rubin and Dr. J. Sagin who measured inhibition of hemagglutination by influenza viruses in many instances and to whom we are also indebted for the chorionic gonadotropin assays. We are grateful to Professor E. Klenk for *N,O*-diacetylneuraminic acid, to Professor M. Heidelberger for the pneumococcus S XIV antigen, to Dr. H. Gershowitz for a large supply of human anti-N serum, Dr. T. J. Greenwalt, Dr. N. G. Maxwell, Dr. Ph. Sturgeon, Dr. A. S. Wiener, Dr. F. H. Allen, and Mr. F. W. Marquart for anti-M and anti-N sera, and to Dr. A. Konugres for erythrocytes carrying the M^g antigen and for anti-S, anti-s, and anti-K sera. We thank Professor K. Wallenfels for *E. coli* β-galactosidase, Professor S. Roseman and Dr. E. J. McGuire for *Clostridium perfringens* neuraminidase and *D. pneumoniae* β-galactosidase, Professor R. Kuhn for brain gangliosides, mucolipids, and numerous milk and colostrum oligosaccharides and glycoproteins, and Dr. R. Kathan for myxovirus receptor preparations. We thank Professor E. A. Kabat, Professor R. J. Winzler, and Mr. H. J. Callahan for evaluation of the manuscript. Professor F. Ottensooser gave the original two lots of *V. graminea* seeds, Professor M. M. Rapport cytolipid H, Professor H. E. Carter corn phytoglycolipids, and Dr. A. P. MacLennan the sponge glycolipid. Miss J. L. Hendry donated the antipneumococcus type XIV horse serum.

References

Adams, J. B. (1965), *Biochem. J. 94*, 368.

Allen, F. H., Jr., Corcoran, P. A., and Ellis, F. R. (1960), *Vox Sanguinis 5*, 224.

Anderson, B., Hoffman, P., and Meyer, K. (1965), *J. Biol. Chem. 240*, 156.

Avigad, G., Amaral, D., Asensio, C., and Horecker, B. L. (1962), *J. Biol. Chem. 237*, 2736.

Ballou, C. E. (1954), *Advan. Carbohydrate Chem. 9*, 91.

Baranowski, T., Lisowska, E., Morawiecki, A., Romanowska, E., and Strozecka, K. (1959), *Arch. Immunol. Terapii Dosw. 7*, 15.
Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.
Bezkorovainy, A., Springer, G. F., and Hotta, K. (1966), *Biochim. Biophys. Acta. 115*, 501.
Bhavanandan, V. P., Buddecke, E., Carubelli, R., and Gottschalk, A. (1964), *Biochem. Biophys. Res. Commun. 16*, 353.
Blix, G., and Lindberg, E. (1960), *Acta Chem. Scand. 14*, 1809.
Brossmer, R., and Walter, K. (1958), *Klin. Wochschr. 36*, 925.
Callahan, H. J., and Springer, G. F. (1966), *Federation Proc. 25*, 1387.
Carsten, M. E., and Kabat, E. A. (1956), *J. Am. Chem. Soc. 78*, 308.
Cook, G. M. W., and Eylar, E. H. (1965), *Biochim. Biophys. Acta 101*, 57.
Dische, Z., and Shettles, L. (1951), *J. Biol. Chem. 192*, 579.
Edgren, R. A. (1956), *Proc. Soc. Exptl. Biol. Med. 92*, 569.
Gershowitz, H. (1963), *Proc. 2nd Intern. Congr. Human Genet. Rome II*, 833.
Gottschalk, A. (1960), The Chemistry and Biology of Sialic Acids and Related Substances, Cambridge University, England, p 62.
Heyworth, R., Perkins, H. R., and Walker, P. G. (1961), *Nature 190*, 261.
Hotta, K., and Springer, G. F. (1965), *Proc. Xth Intern. Congr. Soc. Blood Transfusion*, Stockholm, 1964, Basel-New York, p 505.
Kabat, E. A. (1956), Blood Group Substances, New York, N. Y., Academic.
Kabat, E. A. (1961), *Exptl. Immunochem.*, 505, 511, 535.
Kabat, E. A., Bassett, E. W., Pryzwansky, K., Lloyd, K. O., Kaplan, M. E., and Layug, E. J. (1965), *Biochemistry 4*, 1632.
Kathan, R. H., and Winzler, R. J. (1963), *J. Biol. Chem. 238*, 21.
Kathan, R. H., Winzler, R. J., and Johnson, C. A. (1961), *J. Exptl. Med. 113*, 37.
Klenk, E., und Uhlenbruck, G. (1960), *Z. Physiol. Chem. 319*, 151.
Kominz, D. R. (1962), *J. Chromatog. 9*, 253.
Krüpe, M., and Uhlenbruck, G. (1964), *Z. Immfrsch. 126*, 408.
Landsteiner, K., and Levine, P. (1928), *J. Exptl. Med. 48*, 731.
Latimer, W. M., and Hildebrand, J. H. (1953), Reference Book of Inorganic Chemistry, 3rd ed, New York, N. Y., MacMillan, p 564.
Levine, P., Ottensooser, F., Celano, M. J., and Pollitzer, W. (1955), *Am. J. Phys. Anthropol. 13*, 29.
Lisowska, E. (1963), *Nature 198*, 865.
Mäkelä, O., and Cantell, K. (1958), *Ann. Med. Exptl. Biol. Fenniae (Helsinki) 36*, 366.
McFarren, E. F. (1951), *Anal. Chem. 23*, 168.
Milks, J. E., and Janes, R. H. (1956), *Anal. Chem. 28*, 846.
Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.
Morgan, W. T. J. (1960), *Proc. Roy. Soc. (London), B, 151* 308.
Nagai, Y., and Springer, G. F. (1962), *Federation Proc. 21*, 67d.
Ottensooser, F., and Silberschmidt, K. (1953), *Nature 172*, 914.
Painter, T. J. (1960), *Chem. Ind. (London)*, 1214.
Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 181*, 149.
Piez, K. A., and Morris, L. (1960), *Anal. Biochem. 1*, 187.
Polson, A. (1961), *Biochim. Biophys. Acta 50*, 565.
Pusztai, A., and Morgan, W. T. J. (1963), *Biochem. J. 88*, 546.
Race, R. R. (1965), *Ann. N. Y. Acad. Sci. 127*, 2, 884.
Race, R. R., and Sanger, R. (1962), Blood Groups in Man, 4th ed, Oxford, Blackwell Scientific Publications.
Reed, L. J. (1950), *J. Biol. Chem. 183*, 451.
Romanowska, E. (1961), *Nature, 191*, 1408.
Schachman, H., Colowick, S. P., and Kaplan, M. O. (1957), *Methods Enzymol. 4*, 32.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Springer, G. F. (1965), Mosbach Colloquium 15th Annual Meeting, German Society of Physiological Chemistry, Berlin, Heidelberg, New York, Springer-Verlag, Chapter 7.
Springer, G. F., and Ansell, N. J. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 182.
Springer, G. F., Desai, P., and Kolecki, B. (1964a), *Biochemistry 3*, 1076.
Springer, G. F., and Horton, R. E. (1964), *J. Gen. Physiol. 47*, 1229.
Springer, G. F., and Hotta, K. (1963), *Federation Proc. 22*, 2261.
Springer, G. F., and Hotta, K. (1964), *6th Intern. Congr. Biochem., N. Y. 11*, 183.
Springer, G. F., Nichols, J. H., and Callahan, H. J. (1964b), *Science 146*, 946.
Springer, G. F., Takahashi, T., Desai, P. R., and Kolecki, B. J. (1965), *Biochemistry 4*, 2099.
Stalder, K., and Springer, G. F. (1960), *Federation Proc. 191*, 7.
Stalder, K., and Springer, G. F. (1962), *Proc. 8th Congr. Europ. Soc. Haematol.*, Basel-New York, p 489.
Stauff, J., and Jaenicke, R. (1964), *in* Biochemische Taschenbuch II, Rauen, H. M., Ed., Berlin, Springer, pp 90–94.
Svennerholm, E., and Svennerholm, L. (1958), *Nature 181*, 1154.
Toennies, G. J., and Kolb, J. (1951), *Anal. Chem. 23*, 823.
Uhlenbruck, G., and Krüpe, M. (1963), *Z. Immfrsch. 124*, 342.
Uhlenbruck, G., and Weber, P. (1964), *Z. Immfrsch. 126*, 325.

U. S. Army Bull. Med. Dept. (1945), *6*, 777.
Wallenfels, K., Zarnitz, M. L., Laule, G., Bender, H., and Keser, M. (1959), *Biochem. Z. 331*, 459.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Warren, L. (1960), *Nature 186*, 237.
Watkins, W. M. (1966), *Science 152*, 172.
Westphal, O., and Lüderitz, O. (1954), *Angew. Chem. 66*, 407.
Whiteside, R. E., and Baker, E. E. (1960), *J. Immunol. 84*, 221.
Wiener, A. S. (1963), *Transfusion 3*, 173.
Wiener, A. S., and Moor-Jankowski, J. (1963), *Science 142*, 67.
Wiener, A. S., Moor-Jankowski, J., and Gordon, E. B. (1964), *J. Immunol. 92*, 391.
Winkler, S. (1934), *Z. Physiol. Chem. 228*, 50.
Winzler, R. (1955), *Methods Biochem. Analy. 2*, 290.
Zak, B., Dickenman, R. C., White, E. G., Burnett, H., and Cherney, P. J. (1954), *Am. J. Clin. Pathol. 24*, 1307.

The Interaction of Polypeptides and Proteins with Apocarboxypeptidase A*

Thomas L. Coombs and Bert L. Vallee

ABSTRACT: A gel filtration technique has been employed to characterize the binding of protein and polypeptide substrates to apocarboxypeptidase A-δ and to compare their behavior with that of dipeptides. Tyrosyl residues of the enzyme facilitate the binding both of di- and polypeptides. The introduction of increasing proportions of charged lysyl or glutamic acid residues into a tyrosyl copolymer, however, reduces and eventually abolishes binding. Both polypeptides and proteins bind more firmly to apocarboxypeptidase than dipeptides. Acetylation of the apoenzyme with *N*-acetylimidazole, while destroying dipeptide binding, does not abolish the formation of polypeptide or protein complexes. These findings suggest that the macromolecules bind to groups on the surface of the apoenzyme, in addition to those concerned with dipeptide binding, resulting in greater stability of the apoenzyme with polypeptide and protein substrates. Further, acetylation both of the substrate and of the apoenzyme suggests that these additional groups are neither free tyrosyl nor lysyl residues but may be hydrophobic in nature. The poly-L-lysyl-L-tyrosyl copolymer employed was not hydrolyzed, even though it binds to the apoenzyme and presumably to the metalloenzyme, in accord with previous observations with certain dipeptide substrates. These observations also suggest the existence of binding sites on the surface of the enzyme in addition to those employed for hydrolysis of dipeptides. The characterization of such polyamino acid– and protein–enzyme complexes is continuing.

The specificity characteristics and mode of action of carboxypeptidase A have been elucidated largely through kinetic studies of the hydrolysis of synthetic dipeptides and their analogs (Bergmann and Fruton, 1942; Neurath and Schwert, 1950; Smith, 1951; Neurath, 1960). Under physiological conditions, the enzyme is also likely to act on polypeptides and proteins, but technical problems have discouraged the kinetic study of their hydrolysis. However, where comparisons have been possible, significant differences in the kinetics of hydrolysis of dipeptides and of substrates with higher molecular weight have already been demonstrated (Green and Stahmann, 1952; Neurath *et al.*, 1954; Harris and Knight, 1955; Davie, 1956; Davie *et al.*, 1959; Miller, 1964; Katchalski *et al.*, 1964; Lehrer *et al.*, 1965; Schechter and Berger, 1965; Schechter *et al.*, 1965; Slobin and Carpenter, 1963a,b, 1966).

Equilibrium methods employing gel filtration and isotope exchange have contributed further insight into the mechanism of dipeptide hydrolysis. The reversible binding of $^{65}Zn^{2+}$ to metal-free apocarboxypeptidase has permitted a separation of the initial, substrate binding step from the subsequent catalytic event (Coleman and Vallee, 1962a,b, 1964). Peptide substrates bind to apocarboxypeptidase and prevent the otherwise instantaneous restoration of zinc carboxypeptidase; peptides which are not hydrolyzed do not prevent the restoration of the metalloenzyme and presumably do not bind. Acetylation of the "free" tyrosyl residues of carboxypeptidase abolishes the capacity of apocar-

* From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts. *Received June 30, 1966.* This work was supported by Grant-in-Aid HE-07297 from the National Institutes of Health of the Department of Health, Education and Welfare.