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Properties and Activity of the Lipopolysaccharide–Receptor from Human Erythrocytes†

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ABSTRACT: We have isolated from human erythrocyte membranes a physicochemically homogeneous lipoglycoprotein with a molecular weight of 256,000. It is rich in *N*-acetylneuraminic acid, galactose, and hexosamines. The intact substance prevented attachment to erythrocytes of unheated and heated, smooth and rough lipopolysaccharide and protein–lipopolysaccharide of all gram-negative bacteria tested. It did not interact with other bacterial antigens and therefore is referred to as "lipopolysaccharide–receptor." The receptor physically and reversibly blocked those groupings on lipopolysaccharide which attach to red cells. Both citraconylation and dissociating polyacrylamide gel electrophoresis under standard conditions produced 1 large and 1 small fragment. Citraconylation-inactivated decitraconylation of the large fragment restored high activity but the small fragment remained inactive. Only the large fragment had a composition similar to the intact receptor. The light fragment had significantly less apolar amino acids and carbohydrate. Prolonged incubation of the small fragment in sodium dodecyl sulfate resulted in its further fragmentation. Circular dichroism spectra of the intact lipopolysaccharide–receptor showed α-helix, β-conformation and some flexible aperiodic conformation. Removal of lipid resulted in extensive disorganization. Receptor activity was destroyed by proteases; lipid and carbohydrate were not involved in the receptor's activity.

Endotoxin (=lipopolysaccharide, or O antigen) of gram-negative bacteria in minute quantities produces numerous noxious effects (Braude *et al.*, 1955; Microbial Toxins, V, 1971) for which attachment to host tissue is a prerequisite. Lipopolysaccharide has the ability to fix to human red cells *in vitro* (*cf.* Neter, 1956) and under extreme conditions *in vivo* (Boyden, 1953; Buxton, 1959; Springer and Horton, 1964). A receptor for lipopolysaccharide attachment has been isolated from the membrane of erythrocytes (Springer *et al.*, 1966b, 1973). It interacts with all lipopolysaccharide preparations tested and with the related Kunin antigen but not with other antigens of gram-negative or gram-positive bacteria and hereafter is referred to as lipopolysaccharide–receptor. Other compounds such as glycolipids, lipoproteins, and basic proteins also combine with lipopolysaccharide but are less active and, so far as investigated, nonspecific (Whang *et al.*, 1970).

Serological procedures indicated that lipopolysaccharide–receptor prevented lipopolysaccharide attachment to red cells by blocking sites on the lipopolysaccharide and not receptors on erythrocytes (Springer *et al.*, 1970). The lipopolysaccharide–receptor was obtained in apparently homogeneous form. It is a *N*-acetylneuraminic acid rich glycoprotein which also contains some lipid. Preliminary physicochemical analysis indicated the receptor to possess a molecular weight of about 230,000 (Springer *et al.*, 1973).

The present paper reports on detailed chemical, physicochemical, activity, and immunochemical analyses of the receptor and of fractions obtained from it by mild procedures.

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Materials and Methods

Preparation of Lipopolysaccharide–Receptor. Stroma was prepared from human erythrocytes of all blood groups as described previously (Springer *et al.*, 1966a). Red cells which had been stored at 4° for less than 1 month were used. Isolation of the lipopolysaccharide–receptor from the stroma was as described before (Springer *et al.*, 1973). Yield of highly purified receptor from erythrocyte stroma was about 0.4% on a weight basis.

Preparation of ^{32}P-Labeled Lipopolysaccharide. Escherichia coli O_{86} was grown on a fully defined glucose–salts medium devoid of blood group specific substances as described previously (Springer and Horton, 1964) except that ^{32}P, as $H_3{}^{32}PO_4$ 5 mCi/l. of medium, was added to the ordinary phosphate. [^{32}P]Lipopolysaccharide was isolated as described by Westphal and Lüderitz (1954) and protein–lipopolysaccharide as by Goebel *et al.* (1945). The material was purified by fractionation procedures employed earlier (Springer and Horton, 1964). Prior to use for coating of red cells, the lipopolysaccharide was heated in a boiling water bath in buffered saline pH 7.3–7.4 (see below) for 3 hr, cooled, and centrifuged at 2000*g* for 10 min to remove insoluble material. Alternatively "activation" of lipopolysaccharide was accomplished in a more physiological environment of 37° by incubation for 72–96 hr in the presence of 0.1% NaN_3.

Reagents. Reagent or equivalent grade chemicals were used throughout. Succinic and citraconic anhydrides, mercaptoethanol, and iodoacetic acid were purchased from Eastman Organic. Reagents used to prepare polyacrylamide gels were purchased from Bio-Rad Laboratories. Gel filtration media were obtained from Pharmacia Chemicals. Cytochrome *c* and ovalbumin were purchased from Sigma Chemical Corp., and myosin fragments and subunits were prepared in Dr. F. M. Booyse's laboratory by his procedure (Booyse *et al.*, 1972). Fibrinogen and catalase were obtained from Nutritional Biochemicals, and aldolase from Pharmacia. Bovine serum IgG was prepared as by Smith (1946). Phosphatidylethanolamine (24799) was from Pierce Chemical; bovine brain gangliosides (38B 8170, 79B 8100, 109B 8120) were from Sigma; acrolein (2037) was purchased from Eastman; neomycin sulfate was from Charles Pfizer & Co.; human hemoglobin, twice crystallized (M 1900, U 3177), was obtained from Mann Research as was dansyl chloride[1] 4213 and the standard dansylated amino acids as well as egg-white lysozyme three times crystallized L1087. Triethylamine was from Eastman, hydrazine 97%+ from Matheson Coleman and Bell. Urea was from Mallinckrodt Chemical; it was purified by passing through Amberlite MB3. Thin-layer chromatography plates, 20 × 20 cm, were precoated by E. Merck with a 0.25-mm layer of silica gel 60.

Antisera, Erythrocytes, and Solvent. Rabbit anti-*E. coli* O_{86} lipopolysaccharide sera were prepared and absorbed with human blood group O red cells to remove anti-human erythrocyte antibodies as previously described (Springer and Horton, 1969). Other blood grouping sera were purchased from Ortho Pharmaceutical or were gifts (*cf.* Springer *et al.*, 1972).

Sera against the lipopolysaccharide–receptor were prepared by immunizing New Zealand rabbits each weighing 2–3 kg, with four injections subcutaneous 2 mg each of alum-precipitated (Chase, 1967) lipopolysaccharide–receptor. Three rabbits received native receptor, three others receptor from which noncovalent lipid had been removed by chloroform–methanol (2:1) extraction (5 ml of extractant/7 mg of receptor) for 48 hr at 23–25°, and two additional rabbits were injected with lipopolysaccharide–receptor enzymatically desialized at 37° for 4 hr (*ca.* 90% *N*-acetylneuraminic acid removed). Injections were given at 3-day intervals, and blood specimens were obtained immediately prior to and 6–10 days after the last injection. The production of anti-lipopolysaccharide–receptor sera was also attempted in three White Leghorn chickens weighing 1–2 kg by three injections of lipopolysaccharide–receptor intravenously and intramuscularly and in two chickens by injection with desialized lipopolysaccharide–receptor at 1-day intervals. Receptor preparation, injection, lipopolysaccharide, and bleeding schedule were as described above.

Human blood group O erythrocytes were used in the coating and coating inhibition assays. All red cells were collected from healthy adult donors in the presence of acid–citrate–dextrose as anticoagulant and stored for less than 2 weeks at 1°. Buffered saline (0.1 M sodium chloride plus 0.05 M sodium phosphate (pH 7.3–7.4)) served as serum diluent and red cell suspending solution.

Physical Analyses. After freeze-drying, the substances were dried to constant weight at 23–25° at 10^{-1} to 10^{-2} mm over P_2O_5. Drying to constant weight at 60° for hydrazinolysis was done by us, and at 80° by Huffman Microanalytical Laboratories for determination of water loss, and before measurement of P (Ma and McKinley, 1953) and wet ash analyses. All other analyses were carried out on preparations dried to constant weight at 23–25°; results reported are uncorrected for residual moisture and ash.

Sedimentation and diffusion constants of the lipopolysaccharide–receptor and its fragments were determined as previously described (Bezkorovainy *et al.*, 1971) except that the ultracentrifugal speed was 52,000 rpm instead of 59,780 rpm. Viscosity was measured in an Ostwald viscometer at 27°. Intrinsic viscosity [η] was determined as previously (Bezkorovainy and Grohlich, 1967). The curve obtained was fitted to the points by the least-squares method using a minicomputer program. Molecular weights were computed from diffusion and intrinsic viscosity data (Schachman, 1957). From the molecular weight, sedimentation constant, and intrinsic viscosity data β values were calculated by the method of Scheraga and Mandelkern (1953) and partial specific volume $\bar{V}$ by the Svedberg equation. Absorbance measurements were done in a Zeiss PMQ II spectrophotometer and as described by Springer *et al.* (1965). Optical activity of 0.2% aqueous receptor solutions (0.05% for the small fragment) was determined in a Perkin-Elmer 141 digital readout polarimeter.

Circular dichroism was measured with a Durrum-JASCO Model CD-SP dichrograph improved by Sproul Scientific Instruments. The sensitivity scale setting was 2×10^{-5} or 5×10^{-5} dichroic optical density difference per 1 cm on the recorder chart (Jirgensons, 1973a,b). The residue molar ellipticities were calculated taking a mean residue weight of 145 for the lipopolysaccharide–receptor.

Gel electrophoresis was carried out in a dissociating buffer system in 0.1 M phosphate (pH 7.1) containing 1.0% sodium dodecyl sulfate, 2.5% 2-mercaptoethanol, and 5 M urea on 5% polyacrylamide according to Booyse *et al.* (1972). Preincubation of the receptor in 1.0% sodium dodecyl sulfate was either for 2 hr at 37° or under the same conditions followed by incubation at 23–25° overnight. Standards used were the light chains of bovine IgG, ovalbumin, cytochrome *c*, and

[1] Abbreviations used are: dansyl, Dans, 1-dimethylaminonaphthalene-5-sulfonyl; Fuc, L-fucose; Gal, D-galactose; GalN, D-galactosamine; Glc, D-glucose, GlcN, D-glucosamine; Man, D-mannose; U, unit.

myosin fragments with molecular weights of 110,000, 150,000, and 220,000, respectively. Electrophoresis on polyacrylamide gel, in a presumably nondissociating buffer system in the presence of 0.1% sodium dodecyl sulfate in 0.1 M phosphate buffer (pH 7.0), was done as previously (Bezkorovainy *et al.*, 1971) except that acrylamide (5%) and methylenebisacrylamide were used in one-half the recommended concentration. The standards were chymotrypsinogen, ovalbumin, aldolase, catalase, and fibrinogen. The apparatus used in both procedures was Canalco Model 1200 at a constant current of 2 mA/gel for 16 hr in the dissociating buffer and for 3–4 hr at 8 mA/gel in the nondissociating buffer. The gels were stained with either Coomassie Blue or Amido Black 10B.

Agar gel electrophoresis was done for 90 min at a potential difference of 20 V/cm on 3.25 × 4 in. glass slides coated with $^1/_{16}$ in. of 1.25% "Ionagar," Colab, in 0.1 M Veronal buffer at pH 8.6. After drying, the slides were stained as described above. Cellulose acetate electrophoresis was performed for 25–60 min on Sepraphore III strips (Gelman Instruments) with equipment and buffers at hydrogen ion concentrations from pH 3.1 to pH 9.0 as given earlier (Springer *et al.*, 1973) at 13.3 V/cm and 0.17–0.20 mA/cm at 23–25°. Human serum and RBY dye (Gelman) served as controls.

Chromatography and Colorimetric Carbohydrate Assays. Preparative column chromatographic separation of citroconylated lipopolysaccharide–receptor was carried out on Sephadex G-200 (2.5 cm × 90 cm) at 4°, the eluent was 0.1 M NaHCO3, and 2-ml fractions were collected at 30-min intervals.

For paper chromatographic analysis 0.05–0.1% receptor was hydrolyzed in 1 N HCl at 100° for 10 hr under nitrogen and the acid removed by repeated freeze-drying and addition of water. Qualitative paper chromatography on Whatman No. 1 paper with descending technique was as described previously (Springer *et al.*, 1965). The paper was prewashed with acetic acid and developing solvent. Glc, Gal, Man, and Fuc were included on the chromatograms as controls. The following solvent systems were used: (a) 1-butanol–pyridine–water (6:4:3), three runs of 20 hr each; (b) 1-butanol–ethanol–water (10:1:2); (c) 1-butanol–acetic–water (4:1:1). In quantitative assays, guide strips of the developed chromatograms were stained for carbohydrates with aniline oxalate as described previously (Springer *et al.*, 1965), Man was then eluted and determined with the Park–Johnson assay (1949). Man was also determined spectrophotometrically (Dische *et al.*, 1949). Gal was measured with galactose dehydrogenase obtained from Worthington Biochemical Corp., who also supplied the Glucostat reagent for the Glc assay and the appropriate instructions. Methylpentose was measured on the total hydrolysate as by Dische and Shettles (1951), total hexosamine as by Gatt and Berman (1966), and *N*-acetylneuraminic acid by the thiobarbituric acid (Warren, 1959) and resorcinol procedures (Svennerholm, 1958). GalN and GlcN were separated on the amino acid analyzer as the amino acids (see below) except that prior hydrolysis was for only 4 hr in 4 N HCl; the value of each amino sugar is expressed on the basis of the value obtained from total hexosamine determination.

Enzymatic Hydrolyses. The following enzymes were used (quantities/mg of receptor): insolubilized papain lot 5 10 U (Miles-Yeda) and lot 52C8520 (Sigma), crystalline mercuripapain lot M 398 (0.8 U) and twice crystallized trypsin lot P 1592 (500 μg) (Mann), β-galactosidase from *E. coli* lot BGC GA 20 U (Worthington), α-galactosidase lot 2 prepared from coffee beans in this laboratory (0.35 mg) (Springer *et al.*, 1964), and neuraminidase from *Vibrio cholerae* lot 372 H, (400 U) (Behring Diagnostics). The enzymes were used with controls as previously described (Springer *et al.*, 1966a) and in buffers specified by the supplier except that in which the neuraminidase was purchased was adjusted to pH 6.8 by addition of 0.05 M NaOH, and to the one for the β-galactosidase, 10^{-3} M Mg^{2+} was added (Professor K. Wallenfels, personal communication). α- and β-hexosaminidases which we found to be free of sialidase and protease activity were the gift of Dr. B. Weissmann. Bovine albumin was added to both hexosaminidases as suggested by Weissmann (1969 and personal communication). Because of the known lability of the receptor (Springer *et al.*, 1970) 0.05 M citric acid–sodium citrate buffer (pH 5.2) instead of one with optimal pH was used. A total of 0.051 U of α- and 0.291 U of β-hexosaminidases, respectively, were added over a 24-hr period. Incubation was for 72 hr at 37° except for the galactosidases and hexosaminidases where the incubation lasted for approximately 140 hr and one-half of the original quantity of enzyme was added after 70 hr. The experiments were terminated by exhaustive dialysis at 4° except that insolubilized papain was removed by centrifugation.

Chemical Procedures. Succinylation and reduction alkylation procedures were those previously described (Bezkorovainy *et al.*, 1969; Bezkorovainy *et al.*, 1971; Bezkorovainy and Grohlich, 1972). Citraconylation and decitraconylation were based on previous descriptions (Nakagawa and Perlmann, 1972; Bezkorovainy *et al.*, 1972). Citraconylation was performed at 23–25° in an excess of citraconic anhydride at pH 8.0. After completion of the reaction, the unreacted materials were removed by dialysis which was not exhaustive, however, in order to prevent release of bound citraconyl groups and precipitation. Decitraconylation was in 0.1 M citric acid–sodium citrate buffer for 20 hr at pH 4.5 and 37° (10 mg of receptor/ml). Thereafter, the material was adjusted to pH 7.0 and exhaustively dialyzed against water. The number of free amino groups in the citraconylated and decitraconylated receptor was quantitated by the trinitrobenzenesulfonic acid procedure of Habeeb (1966) with apotransferrin as standard.

Amino acid analyses except Trp and Cys were performed on a Beckman 120 C automatic amino acid analyzer following the procedure of Spackman *et al.* (1958), employing UR 30 Dowex 50 resin in a column (90 × 0.6 cm) for neutral and acidic and Dowex PA35 in a 10 × 0.6 cm column for the basic amino acids. Hydrolyses for these analyses were as described before (Springer and Desai, 1971). Trp, Cys-SO_3H, and Met were determined as before (Springer and Desai, 1971). Acetyl was measured after hydrolysis in 2 N HCl in methanol for 4 hr at 100° in a sealed tube by the Ludowieg–Dorfman procedure (1960).

In the assessment of terminal amino acids standards, controls and lysozyme were included. NH_2-terminal amino acids were determined by the dansyl method as applied to proteins (*cf.* Gray, 1967). The dansylated, hydrolyzed, and dried material was extracted as described by Narita (1970). The hydrolyzed, dansylated receptor, the control lysozyme, and standard Dans amino acids were separated on tlc plates by two-dimensional chromatography with methyl acetate–isopropyl alcohol–ammonium hydroxide (9:7:4) followed by chloroform–methanol–acetic acid) (15:4:1); some one-dimensional runs were also performed in chloroform–benzyl alcohol–acetic acid (30:10:1) (*cf.* Seiler, 1970). Separation was observed by ultraviolet light. The plates were sprayed with triethylamine–isopropyl alcohol (1:4 v/v) and the areas containing Dans amino acids were scraped off, extracted with

methanol–ammonium hydroxide (specific gravity 0.91) (95:5, v/v), and determined fluorometrically as described by Seiler (1970) in a G.K. Turner fluorometer III. All readings were corrected for a blank, extracted from an area of the plate which contained no Dans amino acid. All values obtained for the samples proper were corrected for the losses which occurred with the authentic dansylated amino acid in question which had been run by itself and also was added to the dansylated macromolecule at the start of the hydrolysis. Recoveries of these internal standards were from 40 to 44% and in accordance with the literature (Gray, 1967). The values of determinations on the duplicate samples were closely similar throughout and arithmetic averages are given.

COOH-terminal amino acids were identified and quantitated after hydrazinolysis under strictly anhydrous conditions (Akabori *et al.*, 1952) following the procedure of Braun and Schroeder (1967). Amberlite CG-50(H^+) was added to the hydrazinolytic mixture and isolation of the COOH-terminal amino acids was by ion exchange chromatography, as described above, after the neutral and acidic amino acids had been separated on an Amberlite CG-50(H^+) column (1 × 3 cm) as described by Fraenkel-Conrat and Tsung (1967).

Lipid extraction was based on the procedure of Folch *et al.* (1957); 40–50 mg of lipopolysaccharide–receptor was suspended in predried chloroform–methanol 2:1 (v/v) in a glass-stoppered spring-fastened Pyrex tube and stirred magnetically for 24 hr at 23–25°. The phases were then separated by centrifugation, the residue reextracted twice, and the supernatants were pooled and washed twice with 0.74% NaCl. Thus the noncovalently bound lipids were recovered in the organic phase, freed of solvent by a stream of nitrogen, and dried to constant weight over paraffin, KOH, and P_2O_5 *in vacuo*. The residue was then hydrolyzed for separation of tightly bound lipid in a sealed tube at 100° for 2 hr in 1% acetic acid in a nitrogen atmosphere (Galanos *et al.*, 1971). After drying to constant weight, the material was extracted with chloroform–methanol as described above. All fractions were soluble in buffered saline to >350 μg/ml after they had been shaken mechanically overnight at 4°.

Red Cell Coating and Its Inhibition. The procedures have been described earlier (Springer *et al.*, 1970). Lipopolysaccharide and 10% red cell suspensions were incubated with agitation at 37° for 45 min. The red cells were sedimented, exhaustively washed, and suspended (0.5%) in buffered saline. The smallest quantity of lipopolysaccharide just producing maximal hemagglutination by homologous antiserum was defined as 1 coating U (Springer *et al.*, 1970). The inhibition assay differed from the coating assay in that the inhibitor was added to 1 coating U of lipopolysaccharide and incubated with shaking at 37° for 45 min before washed and packed red cells were added. The smallest quantity of receptor or receptor analog which inhibited 1 coating U of lipopolysaccharide >95% was defined as 1 inhibitor U (Springer *et al.*, 1970). These serological units did not strictly correspond with the more sensitive and accurate radiologic assays, but proved useful in comparative screening studies.

Hemagglutination and Hemagglutination Inhibition Tests. The method of measuring hemagglutination by antibodies (reciprocal titers) was the same as described previously as were the standards and controls, which were included in all tests, and also the definition of activities (Springer and Horton, 1964). The conditions of the assay, volume of reagents (0.05 ml), erythrocyte concentration (0.5%), and incubation temperature (23–25°) were constant throughout. In inhibition assays, four minimum agglutinating doses of serum were used. Agglutination end points were determined microscopically by at least two persons, one reading blind.

Inhibition of virus hemagglutination was determined by a microprocedure with Takatsi micro titrators and plastic U trays (Cooke Engineering Co.). All volumes were 25 μl; the PR8 strain of Type A and Md strain of Type B influenza virus donated by Dr. J. McGuire were employed under the conditions described previously (Springer *et al.*, 1969).

Measurement of ^{32}P Activity. Uptake or removal of ^{32}P-labeled lipopolysaccharide was measured on red cells as well as on the supernatant fluids and washes. The latter were placed on unlined planchets and dried at 80°, while red cell suspensions were sedimented, washed four times with 20 vol of buffered saline, hemolyzed, and assayed on planchets lined with lens tissue. Activity was measured with a gas-flow counter with automatic sample changer and printer (Nuclear-Chicago). A uranium mock-^{32}P standard from International Chemical and Nuclear was used for calibration of the counter. Decay was corrected for by inclusion of a ^{32}P-labeled lipopolysaccharide standard.

Microprecipitin and Agar Gel Diffusion Tests. Native and enzymatically desialized lipopolysaccharide–receptor preparations were employed with the following anti-reagents: rabbit and chicken anti-lipopolysaccharide–receptor sera, anti-pneumococcus Type XIV horse serum No. 635, the albumin gland extract of *Helix pomatia*, donated by Professor G. Uhlenbruck, as well as extracts from the seeds of *Vicia graminea*, *Arachis hypogaea*, *Dolichos biflorus*, and *Lotus tetragonolobus*. The seeds were either grown under the supervision of this laboratory or obtained commercially and extracted by standard procedures (Springer *et al.*, 1972) but at eightfold seed concentrations. The anti-reagents were centrifuged at *ca.* 17,500*g* at 4° for 4 hr immediately before use. Microprecipitin tests were carried out as previously at 1° in capillaries of 0.5–0.9-mm diameter and 90-mm length (Springer *et al.*, 1966a).

The two-dimensional double-diffusion Ouchterlony procedure was performed at 4° on agar gel immuno plates No. 085-072, pattern B (Hyland Labs.). The reagents were used in a 5–7-μl volume; the anti-reagent was in the center well. Both types of precipitin tests were observed for about 1 month.

Results

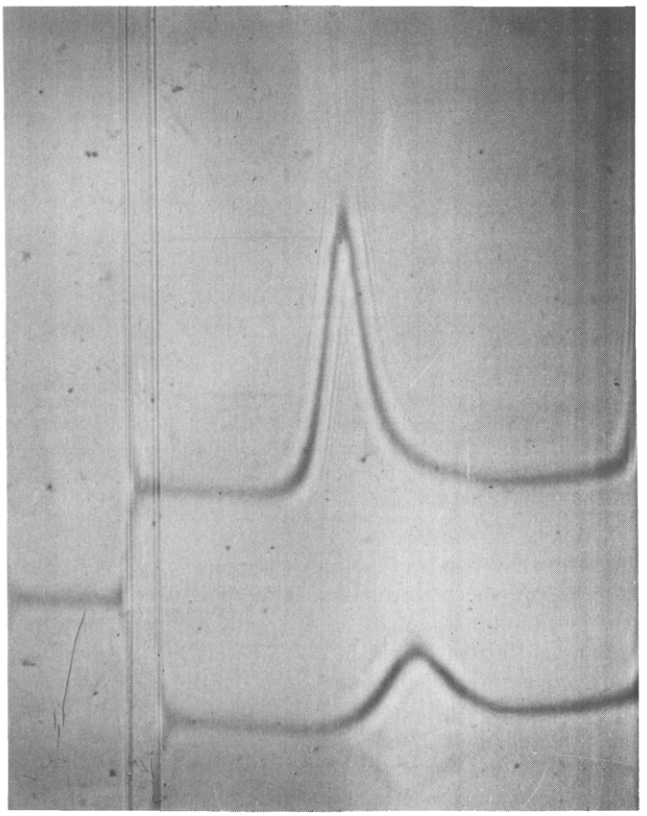
General and Physicochemical Properties of Lipopolysaccharide–Receptor. The highly purified receptor was a white powder readily and immediately soluble in water or buffered saline up to 6% at 1°; the dry receptor retained full activity at 23–25° for several years and in physiological solutions for at least 1 week at 4° and for 72 hr at 37°. It lost about 40% of its activity upon incubation at 56° for 6 hr at pH 7.0 and >80% below pH 6.0 and above pH 8.0. Heating in a boiling water bath or autoclaving at 121° destroyed all receptor activity in <15 min. Preincubation with lipopolysaccharide did not protect it from inactivation. The receptor lost 2.8–5.6% water on drying at 23–25° and 8.48% at 80°; it contained 0.49% ash and <0.02% P. The average receptor quantity just giving >95% inhibition of coating was 25 μg/ml for 23 lipopolysaccharides or protein–lipopolysaccharides of smooth as well as rough gram-negative bacteria whether the bacterial antigens had been activated at pH 7.4 by heating or for prolonged periods at 37°. Only the fixation of lipopolysaccharides was prevented (Springer *et al.*, 1970).

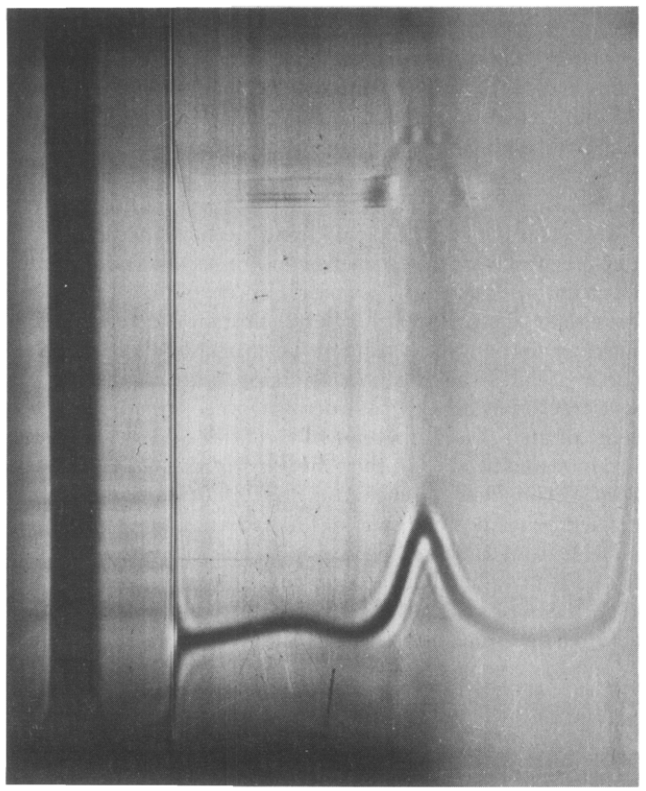
The receptor possessed no human blood group A_1, B, M, or $Rh_0(D)$ activity when tested at 10 mg/ml. It possessed traces of A_2 activity giving inhibition at 2.5–5.0 mg/ml under

standard conditions; it had similar activities when measured with human or eel anti-human blood group H(O) sera and with rabbit but not with human anti-N sera. The receptor was nonpyrogenic at concentrations of at least 1 mg/kg body weight (U. S. Pharmacopoeia XVIII, 886, 1970).

Physical Properties. The lipopolysaccharide–receptor was electrophoretically homogeneous and migrated with a negative charge between pH 4.0 and pH 9.0 on cellulose acetate strips and on agar plates, at pH 8.6 it migrated like α_1-globulin; its isoelectric point was close to pH 3.0. The receptor also showed a single band after electrophoresis in polyacrylamide gel using the nondissociating buffer. It appeared to be homogeneous in the ultracentrifuge (Figure 1a). The physical parameters determined for the intact lipopolysaccharide–receptor are listed in Table I. The receptor is either highly asymmetric or highly hydrated as indicated by the frictional ratio f/f_0. The reduced viscosity values of the receptor were 17.4, 16.4, 16.2, 16.6, and 16.7 ml/g at receptor concentrations of 9.8, 8.2, 5.9, 3.7, and 1.8 mg/ml. An intrinsic viscosity value of 16.3 ml/g was calculated from these data. The molecular weight of the receptor determined by sedimentation, diffusion, and intrinsic viscosity was 256,000. From this figure and the sedimentation constant of 6.5 S a partial specific volume of 0.759 ml/g was calculated. Polyacrylamide gel electrophoresis in the nondissociating buffer gave a closely similar molecular weight; 250,000–260,000 was found for three different receptor preparations.

The lipopolysaccharide–receptor dissociated upon either succinylation or citraconylation. The latter procedure resulted maximally in 46% citraconylation of the potentially available amino groups. An ultracentrifugal pattern of the most extensively citraconylated material is shown in Figure





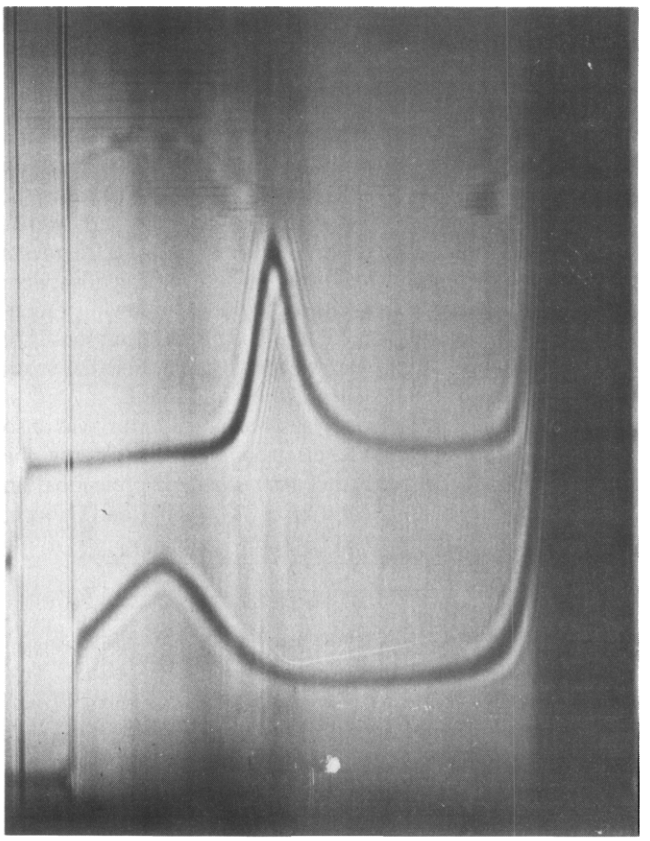


FIGURE 1: (a) Ultracentrifugation of lipopolysaccharide–receptor in 0.1 M $NaHCO_3$ (upper left). Upper pattern, 10 mg/ml; lower pattern, 5 mg/ml. Photo was taken 75 min after reaching speed, at a bar angle of 60°. (b) Citraconylated lipopolysaccharide–receptor, 10 mg/ml in 5.1 M $NaHCO_3$ (above). Photo was taken 72 min after reaching speed, at a bar angle of 60°. (c) Citraconylated lipopolysaccharide–receptor after separation of the heavy component A (upper pattern) and the light component C (lower pattern) on Sephadex G-200 column (lower left). Both were at concentrations of 10 mg/ml in 0.1 M $NaHCO_3$. Photo was taken 68 min after reaching speed, at a bar angle of 60°.

TABLE I: Physical Properties of Lipopolysaccharide–Receptor and Component Fragments.

Parameter	Lipopoly-saccharide–Receptor	Large Com-ponent[a]	Small Com-ponent[a]
$s^0_{20,w}$	6.5	6.5	1.5
$D^0_{20,w}$	2.5	2.8	5.3
$\bar{V}$ (ml/g)	0.759	0.716[b]	0.74[b]
$[\eta]$ (ml/g)	16.3		
dS/dC	1.6×10^{-4}		
Mol wt (sed, diff, $[\eta]$)	256,000	202,000[d]	28,000[d]
Mol wt (polyacrylamide)[c]	255,000	95,000	31,500
f/f_0	2.1		
$[\alpha]^{29}_D$	−26.5°	−21.5°[d]	+10.0°[d]
$A^{1\%}_{276\ nm}$	7.280	6.575[d]	5.800[d]

[a] After citraconylation and separation on Sephadex G-200. [b] Calculated, disregarding lipid. [c] After polyacrylamide gel electrophoresis, intact receptor in nondissociating, fragments in dissociating buffer 2-hr sodium dodecyl sulfate preincubation only; arithmetic average of 2–3 experiments. [d] Decitraconylated.

1b. The sample consisted now of a large and a small fragment. These were separated on Sephadex G-200 as shown in Figure 2, where it can also be seen that fraction C absorbed significantly only at 230 nm. The two major fractions were analyzed in the ultracentrifuge. Figure 1c shows that separation of the fragments was complete. In different experiments the apparently homogeneous small fragment amounted to 10–20% of the homogeneous large fragment. Both fragments could be completely decitraconylated. Similar fractions were obtained on succinylation with or without following reduction and alkylation of the receptor. The physical properties of the large fragment, molecular weight 202,000, and the small one, molecular weight 28,000, are given in Table I.

Dissociation of the lipopolysaccharide–receptor was also achieved on polyacrylamide electrophoresis employing the appropriate buffer; it separated after standard preincubation in sodium dodecyl sulfate for 2 hr at 37° (Weber and Osborn, 1969) into two bands of molecular weights 95,000 and 30,000. If, however, the preincubation with sodium dodecyl sulfate was in addition overnight, the receptor separated into three additional, smaller bands. Molecular weights measured from the leading edges of the bands in a typical experiment were 95,000, 30,000, 20,000, 10,000, and 7000, respectively, with a known error of ±10% (Weber and Osborn, 1969). The component of molecular weight 20,000 may consist of aggregates of fractions with molecular weights 10,000 and 7000. This view is supported by acrylamide gel electrophoresis in dissociating buffer of the ultracentrifugally homogeneous small fragment of the citraconylation experiments. After its decitraconylation and prolonged sodium dodecyl sulfate incubation it also showed components of molecular weights of 30,000, 20,000, 10,000, and 7000. The decitroconylated large fragment, on the other hand, revealed the same components as the native receptor upon electrophoresis in the dissociating buffer.

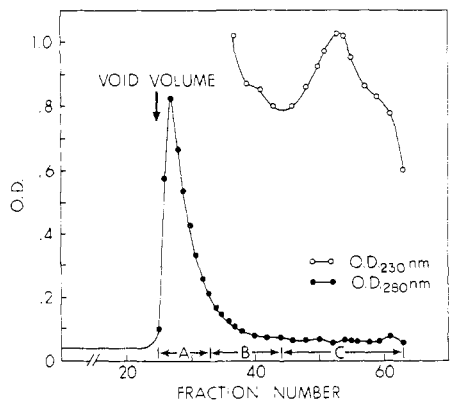


FIGURE 2: Separation of the large and small components of the lipopolysaccharide–receptor after its citraconylation on Sephadex G-200 with 0.1 M $NaHCO_3$; 43 mg of receptor were applied, the recoveries were 32.3, 2.2, and 6.5 mg in fractions A, B, and C, respectively. Fractions B and C were pooled according to readings at 230 nm, see inset. Fractions of 2 ml were collected.

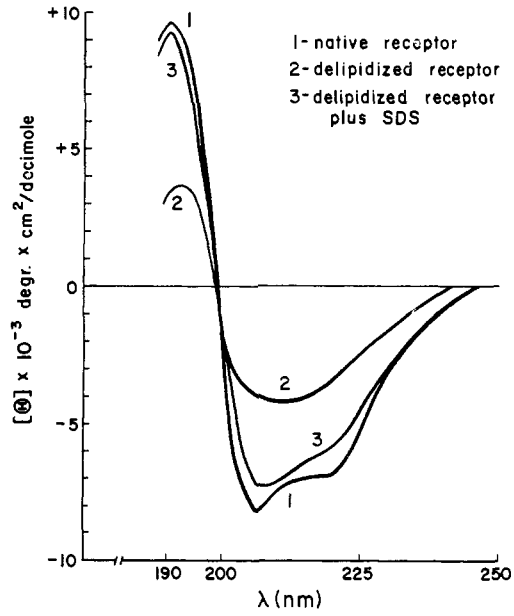


FIGURE 3: Circular dichroism of lipopolysaccharide–receptor. Curve 1, native lipoglycoprotein 0.01% in aqueous solution. Curve 2, delipidated glycoprotein 0.01% in aqueous solution, pH 6.3. Curve 3, 0.0068% delipidated material in 0.005 M sodium dodecyl sulfate (pH 6.8).

Optical rotations of the lipopolysaccharide–receptor and the large fragment were negative and similar, that of the small fragment was positive. The specific absorption decreased with decreasing molecular size of the substance tested. Circular dichroic spectra in the far-ultraviolet are shown in Figure 3, curve 1, which indicates that the lipopolysaccharide–receptor is a macromolecule of considerable order which consists of some pleated sheet (β), about 15% α helix, and some unspecified loop and bend conformation (Crawford *et al.*, 1973; Lewis *et al.*, 1973). The presence of α helix and pleated sheet is indicated by the positive band at 190–195 nm and by the sloping plateau from 215 to 225 nm. Sialic acid may also contribute to the positive band at 190–195 nm (Jirgensons and Springer, 1968; Kabat *et al.*, 1969). If 110 is taken as the mean amino acid residue weight, a residue molar ellipticity of −5100 at 222 nm is compatible with 12–15% of α helix. Only a faint negative band was obtained in the near-ultraviolet at 265–285 nm.

Removal of lipid resulted in significant loss of conformational order (Figure 3, curve 2). The weak bands at 190–195

TABLE II: Amino Acids of the Lipopolysaccharide–Receptor and Its Citraconylated Components.

Amino Acid	Lipopolysaccharide–Receptor % of Receptor	Lipopolysaccharide–Receptor Mol/mol	Large Component[a] % of Component	Large Component[a] Mol/mol	Small Component[a] % of Component	Small Component[a] Mol/mol
Lysine	3.59	63[b]	2.67	37	3.30	7
Histidine	2.83	46	2.01	26	2.01	4
Arginine	3.62	53	2.96	34	2.48	4
Aspartic acid	5.78	111	3.49	53	4.71	10
Threonine	4.53	98	3.58	61	3.01	7
Serine	4.51	108	3.29	63	2.93	7
Glutamic acid	7.35	128	5.52	76	7.43	14
Proline	3.68	82	2.58	45	1.96	5
Glycine	2.20	75	1.42	38	1.54	6
½-Cystine	trace		0.0		0.0	
Alanine	2.92	84	2.08	47	2.30	7
Valine	3.96	86	3.31	57	2.82	7
Methionine[c]	1.35	20	1.00	14	0.35	1
Isoleucine	3.03	60	2.72	42	1.74	4
Leucine	5.02	98	4.11	63	3.91	8
Phenylalanine	3.41	53	1.59	23	1.83	3
Tyrosine	2.12	31	1.92	18	1.72	3
Tryptophan	1.18	15	1.01	10	0.64	1
Total	61.07	1211	45.26	707	44.38	98

[a] After decitraconylation. [b] Nearest integer. [c] As sulfoxide.

nm and at 205–220 nm indicated some remaining order. The conformation was recovered almost completely upon addition of sodium dodecyl sulfate (Figure 3, curve 3). Sodium dodecyl sulfate had virtually no effect on the spectrum of the native lipopolysaccharide–receptor.

Chemical Composition of Receptor and Receptor Fragments. The composition of the lipopolysaccharide–receptor and of its fragments are depicted in Tables II and III. Averages of two analyses each are given. The component fragments originated from different receptor lots than the intact receptor listed there. The peptide part of the intact lipopolysaccharide–receptor amounted to 61%, with monoaminodicarboxylic acids and hydroxyamino acids predominating. There was scarcity of Trp, aromatic, and sulfur-containing amino acids. *S*-Cystine was present only in traces.

N-Acetylneuraminic acid predominated by a factor of about 1.5 (molar basis) over the next common sugar Gal. There were approximately 12% more GalN then GlcN. The acetyl content of 288 mol/mol of receptor was in good agreement with the theoretical value of 255 mol/mol of receptor assuming monoacetylation of all *N*-acetylneuraminic acid and hexosamines. The receptor contained nearly 10% phosphorus-free, noncovalently bound lipid (Table III) whose removal did not affect the activity of the residual receptor; the extracted lipid had <10% activity of the receptor. No significant quantity of lipid was released upon hydrolysis.

Quantitation of NH_2-terminal amino acids gave 0.443 mol of Leu/mol of receptor and 0.123 mol of Ala; based on the peptide part of the receptor only (see Table III) the figures were 0.727 and 0.201, respectively. In repeated analyses NH_2-terminal Ala amounted to between 21 and 27.5% of Leu, a figure in reasonable agreement with the proportion of small fragment to large fragment obtained on citraconylation (see above).

Repeated COOH analyses uniformly gave a number of amino acids among which Ser (2.0), Leu (1.0), Gly (1.0), Ala (0.9), and Val (0.9) predominated; the figures in parentheses are the recoveries of mol of amino acid/mol of lipopolysaccharide–receptor. The control lysozyme analyses gave a recovery of at least 40% of the theoretical for the NH_2 terminus and 80% for the COOH terminus; in all instances only 1 amino acid was found on each terminus.

TABLE III: Composition of Citraconylated Lipopolysaccharide–Receptor and Its Components.

Structural Unit	Lipopolysaccharide–Receptor % of Receptor	Lipopolysaccharide–Receptor Mol/mol	Large Component[a] % of Component	Large Component[a] Mol/mol	Small Component[a] % of Component	Small Component[a] Mol/mol
Carbohydrates						
N-Acetylneuraminic acid	16.32[b]	135[c]	16.22	106	9.17	8
Galactose	6.14	88	6.11	69	3.03	5
Mannose	1.46	20	1.34	15		
Glucose	0.11	1				
Fucose	0.92	15	0.78	10	0.92	2
Glucosamine[d]	4.78	57	3.97	45	2.27	4
Galactosamine[d]	5.38	63	6.00	68	3.49	5
Total hexosamine	10.10	144	10.03	115	5.76	9
Acetyl[e]	2.58	153				
Total carbohydrate	35.11		34.42			
Total peptide	61.07					
Lipids						
Noncovalent	9.61					
Covalent	<0.33					
	108.7					

[a] Decitraconylated. [b] Warren procedure, 16.67% by resorcinol procedure. [c] Nearest integer. [d] Corrected for hexosamine loss during hydrolysis. [e] Exclusive of the acetyl of *N*-acetylneuraminic acid.

TABLE IV: Inhibition of *E. coli* O_{86} [^{32}P]Lipopolysaccharide Fixation to Human Erythrocytes by Lipopolysaccharide–Receptor Before and After Chemical Treatment.

Receptor Added 5 U	% Inhibition of Lipopolysaccharide Uptake 15 U
Intact	80.5
Delipidized	75.0
Desialized	87.8
α- or β-hexosaminidase treated	80
α- or β-galactosidase treated	80
Papain or trypsin treated	<25
Acrolein treated	<10
Large fragment citraconylated	17.6[a]
Large fragment decitraconylated	54.8
Small fragment decitraconylated	<5

[a] 46% citraconylation.

A comparison of the amino acid composition of the receptor fragments resulting from citraconylation with the original lipopolysaccharide–receptor (Table II) showed that they were generally similar, on a percentage basis, to the original material. The fragments, especially the small one, contained less Asp, His, Thr, Ser, Val, Ile, Leu, Phe, and, the small one only, less Pro and Trp than the native receptor. Table III shows the closely similar carbohydrate composition of the native receptor and the large fragment. The small fragment, however, had less carbohydrate.

Action of Enzymes and Chemicals on the Lipopolysaccharide–Receptor. Table IV lists the activities of highly purified lipopolysaccharide–receptor untreated as well as after various chemical treatments. Removal of >96% of the receptor's lipid, exhaustive enzyme treatment with RDE, α- or β-hexosaminidase, and α- or β-galactosidase had no significant effect on the receptor's activity, even though repeated treatment with RDE released >99% of the receptor's *N*-acetylneuraminic acid and α-galactosidase 3.42% of its Gal. None of the other glycosidases released a significant amount of carbohydrate. The proteases papain and trypsin inactivated the receptor >85%. Papain released *ca.* 25% dialyzable inactive products.

Because of the blocking effect of acrolein on the red cell sites to which lipopolysaccharide attaches (Springer *et al.*, 1966b) its effect on the isolated receptor was determined by incubation under standard conditions of 20 U of receptor with 40 U of acrolein. This was followed by exhaustive dialysis. This treatment led to >90% loss of the receptor's activity (Table IV). Crotonaldehyde and D-glyceraldehyde had a similar effect on the receptor while none of these aldehydes blocked lipopolysaccharide.

Maximal citraconylation decreased the activity of the large fragment by nearly 80%. Decitraconylation largely reversed this effect (Table IV); the small fragment did not regain any activity.

Mode of Action of Lipopolysaccharide–Receptor. We have shown previously that the lipopolysaccharide–receptor interacted only with lipopolysaccharide and not with the red cell surface (Springer *et al.*, 1973). We investigated, therefore, if the receptor irreversibly blocked the combining sites on the lipopolysaccharide molecule. Figure 4 demonstrates that this was not the case. While rather small quantities of lipopolysaccharide–receptor bound lipopolysaccharide quite firmly, there was nevertheless an endotoxin transfer from the receptor to red cells. Thus from a complex which had been formed by preincubation of 10 μg of lipopolysaccharide with 35 μg of receptor/ml, 18% was transferred to red cells after 1 hr; the transfer increased to approximately 40% after 5 hr. The transfer of lipopolysaccharide from hemoglobin, which possesses 10% of the inhibitory activity of the lipopolysaccharide–receptor (Springer *et al.*, 1970), to red cells was much faster even though nearly 15 times more of this protein than of receptor were employed. In both instances the transfer reached a plateau by the 6th hr of incubation. No lipopolysaccharide dissociated from the receptor on incubation of the complex in buffered saline only. Similar results were obtained when bacterial protein–lipopolysaccharide was used instead of lipopolysaccharide or when lipopolysaccharide had been activated at pH 7.4 at 37° for 72–96 hr.

Table V shows that transfer of lipopolysaccharide already fixed to red cells also took place if the red cells were incubated with receptor. Such a transfer was also noted for some receptor analogs such as gangliosides and phosphatidylethanolamine provided they were present in large quantity. Transfer did not occur to seeming analogs such as neomycin and acrolein.

Table VI lists activities of the lipopolysaccharide–receptor which give significant biological and chemical information but which appear not directly related to that activity which forms the main subject of this study. The first three columns of figures show that native, desialized as well as delipidized receptor are immunogenic in rabbits. All three rabbits immunized with native receptor as well as those which received delipidized receptor formed precipitating antibodies against it. Only 1 of the 2 rabbits immunized with desialized receptor responded with antibody formation. Native, desialized and

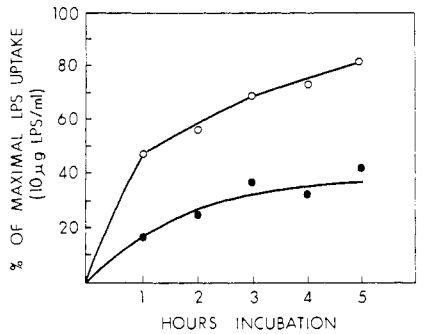


FIGURE 4: Transfer of *E. coli* O_{86} [^{32}P]lipopolysaccharide (10 μg/ml) from inhibitor to red cells: lipopolysaccharide–receptor, 35 μg/ml (●); human hemoglobin, 500 μg/ml (○).

TABLE V: Removal by Inhibitors of [^{32}P]Lipopolysaccharide (10 μg/ml) After Its Fixation to Red Cells.

Inhibitor	μg/ml	% [^{32}P]-Lipopolysaccharide Removal 1.5 hr
Lipopolysaccharide–receptor	625	22.4
Ganglioside	2,000	23.3
Phosphatidylethanolamine	2,500	21.5
Neomycin	3,000	2.3
Acrolein	10,000	2.3

TABLE VI: Serological and Influenza Virus Inhibiting Activities of the Native and Chemically Altered Lipopolysaccharide–Receptor.[a]

Receptor	Precipitation with Anti-Reagent						Influenza Virus Inhibiting Activity	
	Rabbit Anti-Lipopolysaccharide–Receptor			Anti Pneumococcus XIV	*Arachis hypogaea*	*Helix pomatia*	Strain	
	Native	Desialized	Delipidized	β-Gal[b]	β-Gal	α-GalNAc	A/PR8	B/Md
Native	0.02[c]	0.02	<0.01	>5[c]	>5	>5	0.05[d]	0.01
Desialized	0.02	0.01	0.02	1.25	0.01	0.01	>5	>5
Delipidized	0.1	0.04	0.01					
Citraconylated large fragment	0.01	0.01						
Decitraconylated small fragment	>5							

[a] Average of >3 experiments throughout. [b] Immunodominant complementary structure. [c] Smallest quantity (mg/ml) giving definite precipitation. [d] Smallest amount (mg/ml) completely inhibiting agglutination of human blood group O erythrocytes by four agglutinating doses of virus.

also delipidized receptor were of similar activity as antigens as well as precipitinogen except that the delipidized receptor was a somewhat less efficient precipitinogen. The 46% citraconylated large fragment precipitated to the same extent as native lipopolysaccharide–receptor. Agar gel diffusion studies with rabbit anti-receptor sera paralleled the microprecipitin assays; only 1 line resulted in each instance.

The native receptor did not precipitate with any of the heterologous anti-reagents employed while the desialized lipopolysaccharide–receptor precipitated extensively with *Helix pomatia* and *Arachis hypogaea* extracts, there was also slight precipitation with horse anti-pneumococcus Type XIV serum. These findings indicate the presence of subterminal β-galactosyl and possibly α-*N*-acetylgalactosaminoyl groupings. There was no precipitation, however, with *Vicia graminea* extract (β-D-galactosyl specificity), *Dolichos biflorus* extract (α-*N*-acetyl-D-galactosaminosyl specificity), and *Lotus tetragonolobus* extract (α-L-fucosyl specificity) before or after desialization.

The moderate antiviral activities depicted in the last two columns of Table VI are in keeping with earlier observations on glycoproteins of the size and sialic acid content of the lipopolysaccharide–receptor (Springer *et al.*, 1969). Our observation that papain treatment led to >90% inactivation of myxovirus inhibitory activity of the receptor as measured with the PR/8 virus, but was virtually without effect toward the B/Md virus inhibitory activity, likewise agreed with the earlier findings.

Discussion

Lipopolysaccharides of gram-negative bacteria are among the most potent toxins known (Microbial Toxins, V, 1971). As for any toxin or drug the attachment of lipopolysaccharide to host tissue components is a prerequisite to their action. Yet while much is known of the nature of endotoxins (Milner *et al.*, 1971; Lüderitz *et al.*, 1973) our knowledge as to the mode of action of these substances is woefully inadequate with the exception of the demonstration of the interaction of lipopolysaccharide with complement; here, however, antibody appears to be a prerequisite (Gilbert and Braude, 1962). Furthermore, activation of the complement system is only one of the many incitements of multiple effector systems (*cf.* Gewurz *et al.*, 1971).

The present paper reports in detail on the chemical, physicochemical, and some biological properties of a lipoglycoprotein which we termed lipopolysaccharide–receptor (Springer *et al.*, 1970) and which we have now obtained in homogeneous form as judged by various physical criteria. As shown in Figure 4 and Table V the interaction of the receptor with lipopolysaccharide is reversible. An equilibrium of distribution of lipopolysaccharide between receptor and red cells establishes itself depending on the quantity of the three components, red cells, lipopolysaccharide, and receptor, present.

Although the receptor is a lipoglycoprotein neither lipid nor carbohydrate appears to be involved in its activity. This is indicated by our removal of >99% *N*-acetylneuraminic acid without any decrease of the receptor's activity; likewise all other glycosidases were without effect on activity. The majority of the glycosidases did not even release the carbohydrate for which they were specific, which indicates that these structures were not in an accessible location of the macromolecule. All of the lipid could be removed, yet >90% of the inhibitory activity persisted and the lipid itself possessed no inhibitory activity. Furthermore, the small decitraconylated fragment was inactive. It may be assumed that because of the low carbohydrate and amino acid content it was lipid rich (Table III).

It is most likely that the peptide part plays a decisive role in the inhibitory activity of the receptor. This was indicated by the receptor's heat lability and sensitivity toward changes in hydrogen ion concentration at 56°, its inactivation by aldehydes which are known to form condensation products with the amino acids in proteins (French and Edsall, 1945), as well as its susceptibility to proteases. The citraconylation experiments seem to implicate Lys and more specifically its ε-amino groups (Nakagawa and Perlmann, 1972), but it cannot be decided if this effect is due to the alteration of the Lys residues or the result of a conformational change of the receptor molecule due to introduction of negative charges with resulting hydrogen bond disruption. Citraconylation did not interfere with the precipitability of the large receptor fragment with antibody.

Polyacrylamide gel electrophoresis after extensive preincubation in 1% sodium dodecyl sulfate in the dissociating buffer system revealed a more complex quaternary structure than did citraconylation; five components were obtained (excluding the one with molecular weight 20,000 which may be a

reaggregation product) in agreement with the five predominant amino acids of the COOH termini after the apparently harsh hydrazinolysis. The two NH_2 termini would correspond to the two fragments resulting from citraconylation or conventional polyacrylamide electrophoresis in a dissociating buffer system (Weber and Osborn, 1969). Future studies will have to determine the relations of the subunits to one another.

The high affinity of the lipopolysaccharide–receptor to endotoxin is remarkable, because both macromolecules possess a strong negative charge. The receptor, as shown in this paper, possesses the negative charge on account of its high *N*-acetylneuraminic acid content and lipopolysaccharide predominantly due to its phosphoric acid radicals (Lüderitz *et al.*, 1973). Removal of virtually all the *N*-acetylneuraminic acid had a slightly, but probably insignificant, activating effect. It is equally surprising that the receptor's lipid had no effect on its lipopolysaccharide-binding activity and by itself possessed no activity.

Strong evidence has accumulated that the lipid A part of lipopolysaccharide is responsible for its attachment to tissue components (*cf.* Lüderitz *et al.*, 1973). Possibly lipopolysaccharide interacts with those areas of the receptor of human erythrocytes which carry hydrophobic amino acids, of which the peptide part of the receptor possesses *ca.* 40%. The inactive small fragment resulting from citraconylation had significantly less hydrophobic amino acids than either the active intact receptor or its active large fragment.

The structures on the isolated receptor and those on the red cell surface which interact with lipopolysaccharide appear to be the same in both as shown by the inactivating effect of acrolein on the receptor groups in both locations and by the lack of any effect on either by neuraminidase (Springer *et al.*, 1966b, 1973). We have proven that the attachment of lipopolysaccharide to red cells is reversible as is also its fixation to the receptor; the process seems to obey the law of mass action.

The lipopolysaccharide–receptors on other cells may differ in nature and there may be more than one kind of lipopolysaccharide–receptor. Nevertheless, we have isolated for the first time a cell surface macromolecule which specifically prevents lipopolysaccharide fixation to red cells, whether the lipopolysaccharide is from smooth or rough cultures and present as lipopolysaccharide or bacterial protein–lipopolysaccharide complex. Our isolation of a cell-bound receptor substance which interacts with the endotoxins of gram-negative bacteria has practical clinical implications for endotoxic shock. This receptor appears to be a hitherto unrecognized major erythrocyte membrane component. Its recognition not only furthers the understanding of membrane structure but also of the mode of attachment of toxic substances to cells and tissue components.

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Equine Luteinizing Hormone and Its Subunits. Isolation and Physicochemical Properties†

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ABSTRACT: Equine luteinizing hormone (LH) was dissociated into subunits by incubation with 8 M urea. These subunits were separated by chromatography on DEAE-Sephadex A-50 and isolated by gel filtration on Sephadex G-100. The subunits, α and β, proved dissimilar as evidenced by disc electrophoresis, molecular weights, amino acid, and carbohydrate contents. The molecular weight of the α subunit was 12,500 while that of the β subunit was 23,000. The α subunit was rich in threonine, glutamic acid, and half-cystine residues, whereas the β subunit showed high amounts of proline, alanine, and half-cystine. The α subunit was shown to be similar in amino acid content to human chorionic gonadotropin-α and human LH-α while the β subunits of these hormones showed differences. The carbohydrate analyses showed that the α subunit was high in mannose whereas galactose was the predominant sugar in the β. The hexosamines were distributed fairly equally between the two subunits. Sialic acid was found in nearly a two to one ratio between the β and α subunits. The β subunit contained 9.47% sialic acid, whereas α had only 4.97%. The electrophoretic patterns of the subunits differed although both indicated heterogeneity. The α subunit exhibited three distinct bands. The β subunit migrated much further indicating a greater negativity and appeared as a broader more diffuse zone than the α. The individual subunits exhibited very low biological activity. Upon recombination, the activity was increased significantly, showing a recovery of 50% of the original LH activity.

Luteinizing hormone (LH)[1] from several species has been shown to consist of two nonidentical subunits. These species are ovine (Liu *et al.*, 1972a,b), bovine (Reichert *et al.*, 1969), porcine (Hennen *et al.*, 1971) and human (Rathnam and Saxena, 1971; Bishop and Ryan, 1973). The hormones HCG (Swaminathan and Bahl, 1970), FSH (Saxena and Rathnam, 1971; Papkoff and Ekblad, 1970), PMSG (Gospodarowicz, 1972), and TSH (Pierce and Liao, 1970; Pierce, 1971) have also been shown to consist of subunits. The subunits of these gonadotropins have been compared and the α subunits were found to be similar. The β subunits have been shown to differ and possess the biological specificity of the hormone.

Equine LH, which has been previously characterized in this laboratory (Braselton and McShan, 1970; Landefeld *et al.*, 1972), is shown in this study to consist of two dissimilar subunits. These subunits have been characterized as to their physicochemical properties and these properties were compared to those of the intact hormone. A comparison of equine LH subunits is also made with human LH and HCG subunits. The similarities and differences are examined and studied in this report.

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[1] Abbreviations used are: LH, luteinizing hormone; HCG, human chorionic gonadotropin; FSH, follicle-stimulating hormone; PMSG, pregnant mare serum gonadotropin; TSH, thyroid-stimulating hormone.