Progesterone-Binding Globulin from the Serum of Pregnant Guinea Pigs, a Polydisperse Glycoprotein†

Robert M. Burton,‡ George B. Harding, Wael R. Aboul-Hosn, David T. MacLaughlin,§ and Ulrich Westphal*

ABSTRACT: Progesterone-binding globulin (PBG) was isolated from pooled serum of pregnant guinea pigs. The starting serum had a concentration of 1.2×10^{-5} M binding sites for progesterone with an association constant of 10^9 M^{-1}. The protein was purified by chromatographic methods to constant binding activity; it was homogeneous by the criterion of immunoelectrophoresis. The preparation appeared free of other serum proteins, but showed a wide distribution of molecular sizes in gel filtration and sodium dodecyl sulfate–polyacrylamide electrophoresis. The broad peak obtained on Sephadex G-200 consisted of proteins with molecular weights by sedimentation equilibrium between 70,000 and 120,000. Two arbitrary fractions, designated PBG I and PBG II, with molecular weights of 117,300 and 78,400, respectively, were characterized. Gel filtration studies gave the high Stokes' radii of 68 and 56 Å for PBG I and II, consistent with frictional ratios from sedimentation data of 1.8 and 1.6, respectively. An $s_{20,w}^0$ value of 4.7 S was obtained for purified but unfractionated PBG by schlieren methods. At lower concentrations, a strong concentration dependency was observed for PBG I and II resulting in higher extrapolated $s_{20,w}^0$ values. The absorptivities, $A_{1\ cm}^{1\%}$ at 279 nm, for PBG I and II were 4.4 and 5.0, respectively. Both PBG I and II could be further fractionated into arbitrary subfractions. PBG II thus gave active species with average molecular weights by the Yphantis method of 96,000, 75,000, and 67,000. A virtually continuous series of molecular sizes of PBG was demonstrated by three independent methods. The broad range of molecular sizes of active PBG, overlapping those of PBG I and II, was shown to be present in the unfractionated serum pool, as well as in the sera from individual pregnant animals. The amino acid compositions of PBG I and PBG II indicated identical polypeptide moieties. Tryptic peptide maps of PBG I and PBG II were indistinguishable. The polypeptide contents of PBG I and II are about 24 and 33%, respectively, with the remainder of the molecules essentially accounted for by carbohydrate, *i.e.*, 72 and 62%, respectively. No indications of subunit formation were obtained since treatment of PBG I or II with mercaptoethanol and urea or dodecyl sulfate did not alter their electrophoretic mobilities in polyacrylamide. PBG appears to be a polydisperse mixture of glycoprotein molecules having a common polypeptide core of approximately 27,000 molecular weight.

The serum protein responsible for specific binding of progesterone in the pregnant guinea pig is unique in several ways. It is not identical with the corticosteroid-binding globulin (Diamond *et al.*, 1969; Heap, 1969; Milgrom *et al.*, 1970), in contrast to observations in other species in which CBG[1] is the high-affinity serum binder for progesterone (Westphal, 1971; MacLaughlin *et al.*, 1972b). Secondly, the level of the progesterone-specific protein rises 100-fold or more during pregnancy (Diamond *et al.*, 1969), and thus reaches by far the highest concentration of any serum protein known to bind steroid hormones with high affinity (Westphal, 1971). Another distinctive property of this protein is its high carbohydrate content (MacLaughlin *et al.*, 1972a), exceeding that of other serum proteins capable of specific steroid binding. The progesterone-binding protein (PBG) migrates in the electric field as an α-globulin and therefore has been termed progesterone-binding globulin or PBG (Burton *et al.*, 1971).

PBG and CBG have been separated by chromatographic techniques (Burton *et al.*, 1971). Differences from and similarities with a progesterone receptor protein in the uterine cytosol of the pregnant guinea pig have been described (MacLaughlin *et al.*, 1972b). Isolation and characterization of progesterone-binding serum proteins from pregnant guinea pigs have been published from two laboratories (Allouch *et al.*, 1972; Milgrom *et al.*, 1973; Lea, 1973). In the present paper, we describe the isolation of PBG and studies of the polydispersity and other physicochemical properties not previously reported for this glycoprotein.

Experimental Procedure

Materials. Serum from guinea pigs near term of pregnancy was obtained from Wilson Animal Farms, Vincennes, Ind., and from Grand Island Biologicals, Grand Island, N. Y. The pregnant guinea pigs used as individual donors and New Zealand rabbits were obtained from S and S Research, LaGrange, Ky. Complete Freund adjuvant and Noble agar were products of Difco Laboratories, Detroit, Mich.

Radiolabeled steroids, *i.e.*, [4-^{14}C]progesterone (52.8 mCi/mmol), [1,2-^{3}H]cortisol (44 Ci/mmol), and [1,2-^{3}H]progesterone (33.5 Ci/mmol), were obtained from New England Nuclear. The radiochemical purity was checked periodically as described (Chader and Westphal, 1968; Chader *et al.*, 1972). Glass-redistilled deionized water and reagent grade chemicals

† From the Department of Biochemistry, University of Louisville School of Medicine, Health Sciences Center, Louisville, Kentucky 40201. *Received April 22, 1974.* This is communication No. 29 in the series Steroid–Protein Interactions; for the preceding paper see Harding et al. (1974). Our work was supported by a grant from the National Institute of Arthritis, Metabolism, and Digestive Diseases (AM-06369) and a research career award (U. W.) from the Division of General Medical Sciences (GM-K6-14, 138) of the U. S. Public Health Service. This paper is dedicated to Professor G. Ruhenstroth-Bauer on the occasion of his 60th birthday.

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[1] Abbreviations used are: CBG, corticosteroid-binding globulin or transcortin; PBG, progesterone-binding globulin.

were used throughout. All experiments were performed at 4° unless noted otherwise.

Fractionation Procedures. The pregnant guinea pig serum was dialyzed and either loaded with oversaturating amounts of [^{14}C]progesterone and [^{3}H]cortisol as described (Burton *et al.*, 1971), or [1,2-^{3}H]progesterone alone was added at a level of 0.644 μCi or 6.28 μg/ml of serum. Chromatography of 40–100 ml of steroid-labeled serum was performed on a short (10 × 10 cm) hydroxylapatite column equilibrated with 5 mM sodium phosphate (pH 6.8). Additional chromatographic purification utilized Sephadex G-200, DEAE-cellulose, hydroxylapatite, and finally three or four runs on Sephadex G-200. During this purification, lyophilization was routinely used to concentrate the active fractions. Radioactivity was measured in aliquots using a toluene–Triton X-100 scintillation solution (Kobayashi and Maudsley, 1969).

Analytical Gel Filtration. Stokes' radii of PBG components were estimated by chromatography on a 2 × 60 cm column of Sephadex G-200 (Andrews, 1970) which was equilibrated with 50 mM sodium phosphate (pH 7.4) at 4°, and calibrated for carbohydrate-free proteins (Westphal and Harding, 1973).

Gel Electrophoresis. The technique of Ornstein (1964) and Davis (1964) was applied, using 7% polyacrylamide gels and Tris-glycine buffer of pH 8.9. The proteins were stained according to Weber and Osborn (1969); duplicate gels were sliced and counted to assess the distribution of bound [^{3}H]progesterone (Chader *et al.*, 1972).

The low pH–urea system for polyacrylamide gel electrophoresis of Takayama *et al.* (1966) as modified by Zahler *et al.* (1970) was employed at room temperature. Preelectrophoresis was carried out for 3 hr at 5 mA/gel; 200-μg samples in 50 μl of the aqueous phenol–acetic acid–urea solution were applied to 100 × 6 mm columns of 10% polyacrylamide; electrophoresis then proceeded for 4–6 hr at 130–150 V with a constant current of 2.5 mA/tube.

Electrophoresis in the presence of 1% sodium dodecyl sulfate using 8 and 5% polyacrylamide gels was utilized for fractionation on the basis of molecular size (Weber and Osborn, 1969; Dunker and Rueckert, 1969). The gel systems were calibrated with essentially carbohydrate-free proteins (Chader *et al.*, 1972).

Ultracentrifugal Studies. Molecular weights were determined by the high-speed sedimentation equilibrium technique of Yphantis (1964) using a Beckman-Spinco Model E analytical ultracentrifuge equipped with sapphire cell windows and a 49-mm polarizing filter for work with Rayleigh interference optics. Protein solutions were dialyzed overnight at 4° against 0.1 M sodium chloride containing 1 mM sodium azide, and diluted with dialysate to 0.02 and 0.01% concentration. The centrifugations were carried out at 19,160 or 15,220 rpm at 5°; in all experiments equilibrium was attained after 18–22 hr as judged by fringe shifts of less than 10 μ over 1 hr or greater intervals. Weight average molecular weights were calculated for the entire cell, and point average molecular weights computed from four adjacent points (Yphantis, 1964).

Rayleigh optics were also used for sedimentation velocity studies at protein concentrations of 0.1, 0.05, and 0.03% and a speed of 47,660 rpm (Westphal *et al.*, 1974). A partial specific volume of 0.660 ml/g was calculated (Gibbons, 1972) from the amino acid and carbohydrate composition, and from an estimated sodium ion content.

Sucrose gradient centrifugations were performed using a 5–20% (w:v) gradient in 50 mM phosphate buffer (pH 7.4). This buffer gave the same results as the Tris-Cl–EDTA buffer used previously (MacLaughlin *et al.*, 1972b). Sedimentation proceeded for 18 hr at 4° in the Beckman L 3-50 ultracentrifuge equipped with an SW 50.1 swinging bucket rotor at 300,000*g* (maximum radius). The tubes were punctured and fractions of approximately 0.16 ml were collected by drop counting and analyzed for radioactivity. In view of slight variations in drop volume, the values were corrected to milliliters.

Immunochemical Studies. The steroid-binding fraction from the hydroxylapatite chromatography of pregnant guinea pig serum (Burton *et al.*, 1971) was employed as immunogen; mixtures of 0.2 ml of protein solution and 0.2 ml of Freund adjuvant were injected. Initially, two injections of 5 mg of protein each were made into the foot pads of three 4-lb male New Zealand rabbits, followed by four similar subcutaneous injections in the scapular region at weekly intervals. The same immunization schedule was followed using whole serum of pregnant guinea pigs (10 mg of protein for each injection). Blood was collected by cardiac puncture in the 5th, 6th, 7th, and 8th weeks after initial immunization.

Microimmunoelectrophoresis was carried out at 22° on various purified PBG preparations for 1 hr at 250 V using the LKB 6800 A apparatus. A 50 mM sodium barbital buffer of pH 8.6, containing 10 mM diethylbarbituric acid and 50 mM sodium acetate, was employed. Precipitin lines were stained with Amido-Schwarz after rinsing the gels with 0.2 M sodium chloride for 16 hr.

Carbohydrate Analysis. Standard colorimetric methods (Winzler, 1955) were employed for hexose, hexosamine, and fucose; sialic acid was determined according to Warren (1959). Because of limited quantities of purified PBG, reagent volumes were scaled down by a factor of 10 so that 20–50 μg of glycoprotein sufficed for each analysis utilizing micro cells. Standard curves obtained by the scaled-down methods were linear, passed through the origin and agreed with the curves constructed from the original method for each carbohydrate tested. As an additional control, samples of α_1-acid glycoprotein were analyzed together with PBG and gave carbohydrate values as reported (Jeanloz, 1972).

For qualitative determination of neutral sugars, PBG was hydrolyzed at 100° in 2 N HCl for 3 hr (Spiro, 1966). Thin-layer chromatography was performed on plates of silica gel G (Brinkmann Instruments, Inc., Westbury, N. Y.). The limit of detection for glucose was about 10% of total hexose.

Amino Acid Analyses. Samples of PBG I and II were hydrolyzed for 24 and 72 hr in 6 N HCl (Moore and Stein, 1963). The analyses were performed on a Durrum 600A analyzer by Biochemical Data Corporation (Dr. D. H. Strumeyer), Highland Park, N. J. Destruction of even "stable" amino acids occurred during the first 24 hr of hydrolysis; tryptophan, methionine, and cystine were not determined. Norleucine when added after hydrolysis was fully recovered.

Peptide Mapping. Five-milligram samples of PBG I and PBG II were incubated overnight at 37° in an 8 M urea solution containing 0.5 M Tris-Cl (pH 8.5) and 0.2 M mercaptoethanol (Burton and Deutsch, 1970), and were then allowed to react with 30 mg of iodoacetamide for 10 min at room temperature. After exhaustive dialysis against water and lyophilization, 0.5 ml of a suspension was added containing 0.1 M ammonium bicarbonate (pH 7.8), 10 mM calcium chloride, 1 mM sodium azide, and 0.1 mg of L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin (Worthington Biochemicals, Freehold, N. J.). The samples were digested for 48 hr at 37°, lyophilized and relyophilized, and suspended in 40 μl of 0.1% phenol red, and 20 μl was applied to Whatman 3MM paper for two-dimensional chromatography and electrophoresis essentially according to Bennett (1967). The top phase of a butanol–acetic

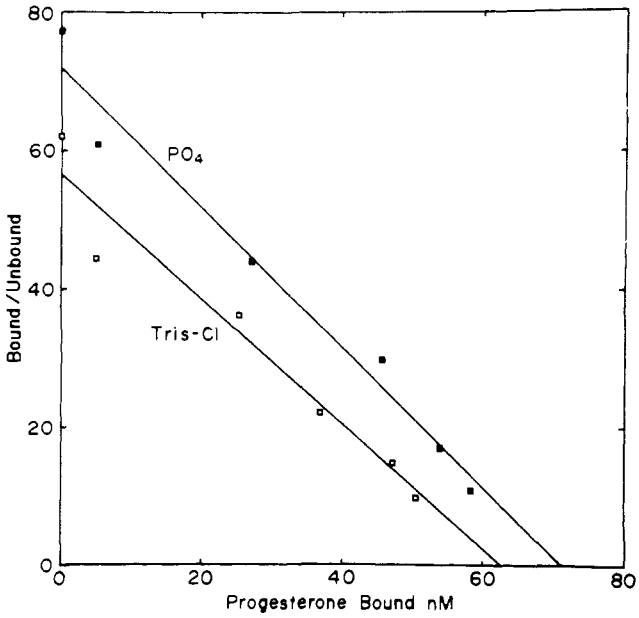

FIGURE 1: Analysis of binding of progesterone to PBG in pregnant guinea pig serum. The serum was diluted 1:200 with 0.01 M Tris-Cl buffer (pH 7.4), containing 0.15 M sodium chloride (open squares) or with 50 mM sodium phosphate (pH 7.4) (filled squares). At this high dilution, the binding parameters are not significantly affected by the presence of albumin. Equilibrium dialysis was performed at 4° for 44 hr; inside volume, 1 ml; outside volume, 15 ml.

acid–water mixture (4:1:5, v/v) was employed in descending chromatography (20 hr); the pH 3.7 pyridine–acetate buffer system was used for the subsequent paper electrophoretic separation at 60 V/cm for 1 hr. The dried paper was stained with cadmium–ninhydrin reagent (Dreyer and Bynum, 1967).

Results and Discussion

Binding Parameters of Starting Material. A Scatchard plot for binding of progesterone to PBG in the serum used is shown in Figure 1. The concentration of high-affinity binding sites in the undiluted serum is approximately 1.2×10^{-5} M, corresponding to 1.0–1.4 mg of PBG/ml, based on one binding site per molecule and molecular weights between 78,000 and 117,000. The apparent association constant for the binding of one progesterone molecule to PBG is about 1×10^{9} M^{-1}, reproduced in a number of experiments. The values obtained in the two buffers are not considered significantly different.

Purification of PBG from Serum of Pregnant Guinea Pigs. A highly enriched PBG fraction was obtained by successive chromatography on hydroxylapatite and Sephadex G-200 (see Figures 4 and 5 in Burton *et al.*, 1971). Further purification by DEAE-cellulose chromatography at pH 8.6 resulted in additional separation of protein-bound progesterone from other proteins including CBG. The purification of PBG to a constant ratio of bound [^{3}H]progesterone to protein absorbance was obtained by a second fractionation on hydroxylapatite; it was followed by three to four G-200 filtrations. The resulting PBG preparation appeared homogeneous in sedimentation velocity ultracentrifugation (Figure 14 in Burton and Westphal, 1972).

PBG I and PBG II. After the last gel filtration (Figure 2A), the earlier half and the later half of the eluate were designated PBG I and II, respectively. Upon rechromatography of these arbitrary fractions of active PBG (Figure 2B,C), the elution volumes for PBG I and PBG II remained nearly the same as before. The yield of purified PBG from 89 ml of serum was 46 mg at the last Sephadex G-200 step before separation into

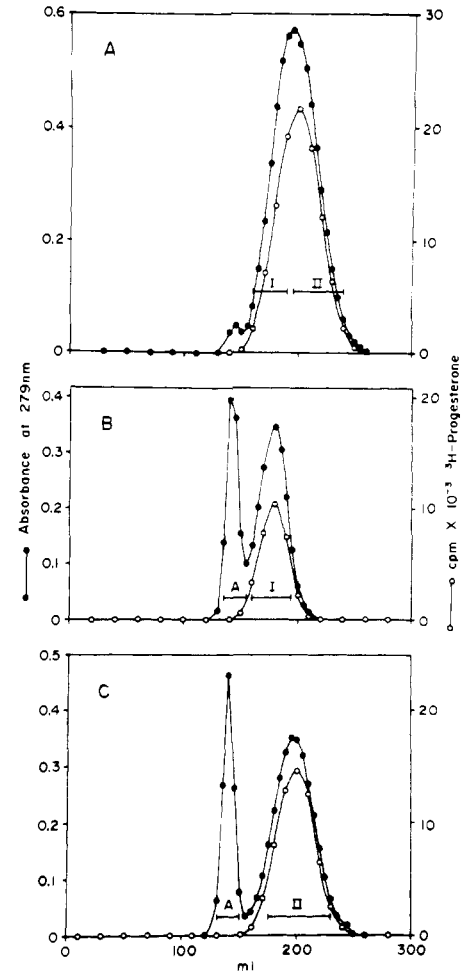

FIGURE 2: (A) Sephadex G-200 chromatography of PBG which had been purified to constant specific activity. (B and C) Rechromatography of PBG I (B) and PBG II (C) on the same Sephadex G-200 column. The lyophilized protein, obtained from 89 ml of radioprogesterone and radiocortisol-labeled serum, in 4.0 ml of 0.2 M ammonium bicarbonate (pH 7.8) containing 1 mM sodium azide, was applied to a 2.5 × 90 cm column of Sephadex G-200 equilibrated with that buffer. The flow rate was about 20 ml/hr. One-tenth-milliliter aliquots of the fractions were counted. The brackets indicate pooling for further analysis. With each active fraction, a small amount of nonbinding protein aggregate eluting near the void volume was observed. The height of this peak "A" (this figure, B and C) does not correctly indicate protein concentration because it results from light scattering by the aggregated protein as also observed with CBG (Chader *et al.*, 1972).

PBG I and II, which were obtained in amounts of 18 and 21 mg, respectively. The overall yield thus was about 44%.

Polyacrylamide gel electrophoresis of PBG I at acidic pH in 4 M urea showed migration as a single band. Reduction with β-mercaptoethanol and alkylation with iodoacetamide in the presence of 8 M urea did not produce any component of significantly greater mobility than that of the untreated protein. Similar results were obtained with PBG II. This suggests that PBG does not consist of disulfide-linked subunits.

Immunoelectrophoresis of PBG I and II resulted in a single precipitin band for each preparation (see Figure 15 in Burton and Westphal, 1972). PBG migrated as an α-globulin. A single arc was also obtained with the antiserum against whole serum. Purity of our active PBG preparations was further substantiated in conventional gel electrophoresis by the absence of the sharp well-stained bands given by most serum proteins; instead, a broad zone was obtained (see below Figure 6, gels 6 and 7). Thus, sedimentation data, gel electrophoresis, and immunoe-

lectrophoresis are consistent with the absence of contaminating proteins. Electrofocusing gave a single binding species with a p*I* of 2.8 (Harding *et al.*, 1974).

Determination of progesterone binding to PBG I by equilibrium dialysis and evaluation by the Scatchard procedure (Westphal, 1969) gave an apparent association constant of $K = 2.9 \times 10^9$ M^{-1} with $n = 0.8$ binding site per molecule. The n value was calculated using the molecular weight of 117,000, *i.e.*, the value for the smallest species determined in the Yphantis method (see next paragraph). The number of binding sites, therefore, is a minimum estimate; we assume one binding site for progesterone per molecule. The affinity constant for PBG II was approximately the same (unpublished results by S. D. Stroupe and G. B. Harding).

Sedimentation Equilibrium Studies. For evaluation of the polydisperse behavior of PBG by high-speed equilibrium ultracentrifugation, the two fractions, PBG I and II, were used. Markedly different molecular weights were found; the concentration *vs.* radius plot (Yphantis, 1964) for PBG I gave a weight average molecular weight of 117,300, calculated from the slope obtained in five such plots from experiments at 0.02 and 0.01% concentration. The molecular weight for PBG II from similar determinations was 78,400.

Sedimentation Velocity Studies. Extrapolation to zero concentration of $s_{20,w}$ values obtained for purified but unfractionated PBG resulted in $s_{20,w}^0 = 4.7$ S. Sucrose gradient centrifugation also gave (MacLaughlin *et al.*, 1972b) approximately 4.7 S for a PBG sample prepared by hydroxylapatite chromatography of pregnant guinea pig serum. Both PBG I and II at lower concentrations exhibit a strong concentration dependence of the sedimentation coefficients; approximate extrapolated values for $s_{20,w}^0$ were 6.1 and 5.2 S.

Frictional ratios (f/f_0) of 1.8 and 1.6 for PBG I and PBG II, respectively, were calculated from $s_{20,w}^0$ and from the molecular weights obtained by sedimentation equilibrium (Tanford, 1961). The concentration dependence of the sedimentation coefficients for PBG I and II at low concentrations is more pronounced than that seen for simple globular proteins (Tanford, 1961). Similarly, the frictional ratios of PBG I and II which are intermediate between those of globular (1.05–1.4) and fibrous proteins (2.3–3.5) may reflect extensive hydration and/or shape asymmetry (Schachman, 1959). Milgrom *et al.* (1973) reported $s_{20,w}^0 = 4.5$ S and a value of $f/f_0 = 1.685$ for their progesterone-binding protein, and Lea (1973) observed $s_{20,w}^0 = 4.5$ S and $f/f_0 = 1.74$.

Analytical Gel Filtration. Estimation of the molecular size of PBG I and PBG II by Sephadex G-200 filtration on the calibrated column gave Stokes' radii of 68 and 56 Å which would correspond to molecular weights, for compact globular proteins lacking carbohydrate, of 480,000 and 280,000, respectively. These data would again indicate very large hydration volumes resulting from the unusually high carbohydrate content of PBG (see below). Anomalously high values have been found for other glycoproteins (Andrews, 1970; Allen and Majerus, 1972; Chader *et al.*, 1972). Stokes' radii of progesterone-binding protein, determined by gel filtration, were reported to be 59 Å (Lea, 1973) and 47 Å (Milgrom *et al.*, 1973).

Absorptivity. The absorptivities at the maximum, $A_{1\text{ cm}}^{1\%}$ at 279 nm, were 4.4 and 5.0 for PBG I and II, respectively; the absorptivities at the minimum (254 nm) were 3.4 and 3.7. A_{280}/A_{260} for PBG I and II were 1.20 and 1.29, respectively. A value of 4.9 for $A_{1\text{ cm}}^{1\%}$ at 280 nm has been reported by Milgrom *et al.* (1973), whereas Lea (1973) found 7.3.

Size Heterogeneity of Active PBG in Serum. The observation of widely differing molecular sizes of PBG raises the question of their artifactual formation during purification or storage. Figure 3 shows the presence in unfractionated serum of active PBG having a broad range of sedimentation rates that overlap those of PBG I and PBG II. The sedimentation coefficients calculated (Martin and Ames, 1961) from the position of a bovine serum albumin marker (not shown) are 5.0 and 4.1 for PBG I and PBG II, respectively.

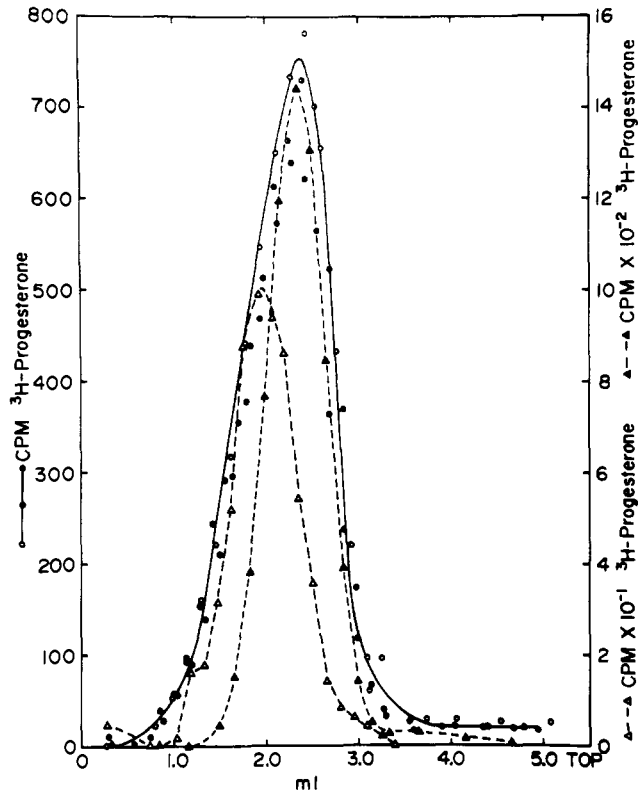

FIGURE 3: Sucrose gradient centrifugation of [^{3}H]progesterone-labeled, pooled serum of pregnant guinea pigs (circles), and of PBG I (open triangles) and PBG II (filled triangles). The material (0.2 ml of 1:1 diluted serum, with 25% of progesterone-binding sites of PBG labeled; or 0.2 ml containing 30 μg of purified PBG I or PBG II labeled with radioprogesterone) was layered on the 5-ml sucrose gradient. The three circular symbols indicate triplicate results obtained with the unfractionated serum.

The occurrence of polydispersity in native serum PBG enveloping that of PBG I and PBG II was confirmed by polyacrylamide electrophoresis (Figure 4). This technique also demonstrates the wide zone of distribution of high-affinity binders for progesterone in the unfractionated serum, and within this zone, the more limited spread observed for the purified fractions PBG I and PBG II. The relative gel electrophoretic mobilities of PBG I and II are readily explained by their size relationship alone. Charge differences are less likely since sialic acid content and amino acid composition (see below, Tables I and II) do not account for such differences. This correlation of size to electrophoretic mobility is maintained in experiments with and without sodium dodecyl sulfate (see below, Figure 6).

The broad distribution of PBG seen on acrylamide electrophoresis of the pooled serum from pregnant guinea pigs was also observed when the fresh serum of a single animal was analyzed (Figure 4). Similarly, six individual sera from pregnant guinea pigs were evaluated by sucrose gradient centrifugation (not shown). In all cases, the broad distribution of active PBG was indistinguishable from that seen for the serum pool (Figure 3). The above results clearly exclude the possibility of PBG polydispersity arising as an artifact of pooling, storage, or puri-

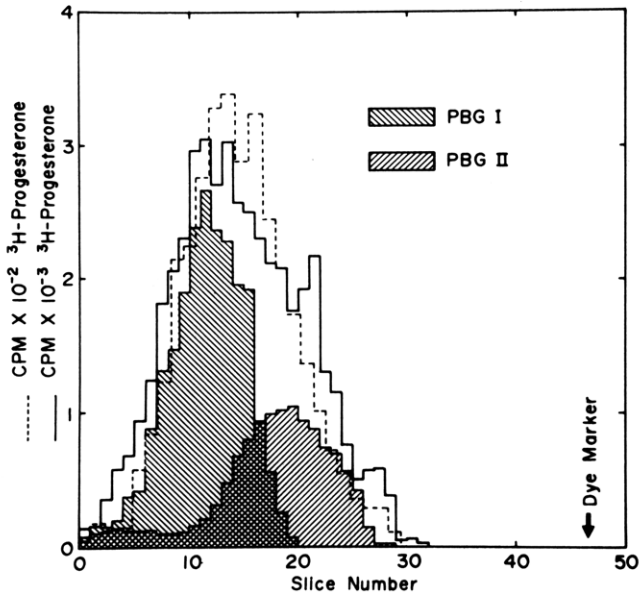

FIGURE 4: Polyacrylamide electrophoresis of PBG in pregnant guinea pig serum. Solid line, open area, pooled serum; broken line, serum from single animal; shaded areas, PBG I or PBG II. Labeling with [^{3}H]progesterone was done as described for Figure 3. The serum of the single animal, containing about 10% of the radioactivity added to the pooled serum sample, was run in a separate experiment and the results were adjusted by reference to the dye marker.

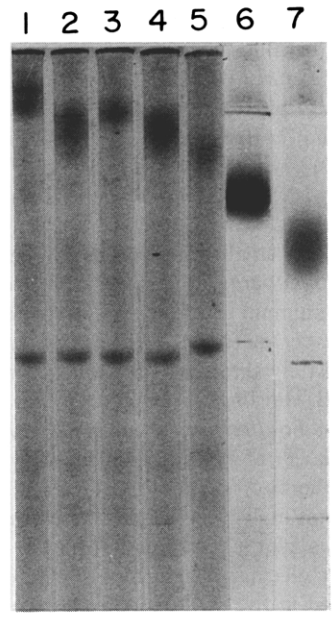

FIGURE 6: Polyacrylamide gel electrophoresis of PBG fractions in the presence of sodium dodecyl sulfate (gels 1–5) according to Weber and Osborn (1969). Samples of 27–33 μg of PBG and 2.5 μg of chymotrypsinogen were applied in 8% gels: origin, top; anode, bottom; gel 1, PBG I; 2, PBG II; 3, 4, and 5, PBG IIa, IIb, and IIc, respectively; gels 6 and 7 show electrophoresis of PBG I and PBG II, respectively, according to Ornstein (1964) and Davis (1964).

fication procedures. Rather, individual animals appear to possess a mixture of PBG molecules of varied size and, implicitly, of varied composition.

PBG Subfractions. The heterogeneity of PBG II was investigated by further gel filtration (Figure 5). The fractions indicated by the brackets (PBG IIa, IIb, IIc) were separately analyzed. Their migration rates in sodium dodecyl sulfate–polyacrylamide gel electrophoresis in comparison with those of PBG I and II are seen in Figure 6. It is evident that the subfractions of PBG II (gels 3–5) differ in mobility such that the early eluates have the lower electrophoretic mobility. The difference in migration rates between PBG I and PBG II in the presence of sodium dodecyl sulfate (gels 1 and 2) is also seen in the absence of sodium dodecyl sulfate (gels 6 and 7). This again suggests that the observed differences in gel electrophoretic migration rates result predominantly from differences in size.

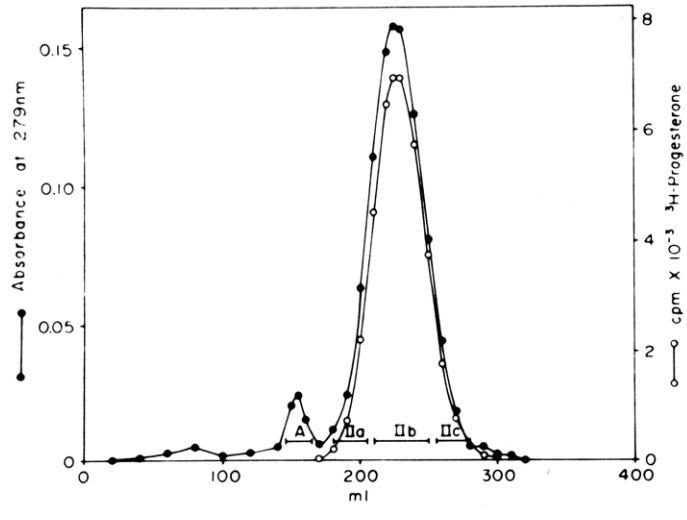

FIGURE 5: Sephadex G-200 chromatography of PBG II (Figure 2C). See Figure 2 for experimental conditions.

Similar subfractionation of PBG I and sodium dodecyl sulfate gel electrophoresis of the subfractions in 5% polyacrylamide gel (not shown) gave analogous results: the earliest eluting fraction showed the slowest migration. Reduction with mercaptoethanol, at standard or more vigorous conditions (5% mercaptoethanol, 2 hr at 37°, or 20 min at 100°), did not result in the appearance of faster migrating components in the sodium dodecyl sulfate–polyacrylamide electrophoresis. Again, these findings indicate the absence of noncovalently bound or disulfide-bonded subunits.

Apparent molecular weights for PBG I and II were estimated by evaluation of the relative mobilities in sodium dodecyl sulfate gel electrophoresis. Calibration curves were prepared for 8% gels (Chader *et al.*, 1972) and for 5% gels (Westphal and Harding, 1973) using carbohydrate-free proteins. PBG I and PBG II showed wide protein bands indicating apparent molecular weights of approximately 100,000–250,000. As will be seen below, PBG is a glycoprotein with an unusually high carbohydrate content. It is well known that glycoproteins give anomalously high Stokes' radii in sodium dodecyl sulfate–acrylamide electrophoresis (Schubert, 1970; Segrest and Jackson, 1972; Westphal *et al.*, 1974). Nevertheless, the Stokes' radii of the subfractions clearly indicated intermediate molecular weights (PBG Ia, 2.0–2.5 $\times$ 10^5; Ib, 1.5–2.0 $\times$ 10^5; IIa, 1.5 $\times$ 10^5; IIb, 1.0–1.4 $\times$ 10^5; IIc, 1.0 $\times$ 10^5), thus confirming by an independent method the polydispersity of PBG.

Sedimentation equilibrium studies also revealed the weight differences of the PBG II subfractions. Weight average molecular weights of 96,000, 75,000, and 67,000 were calculated for PBG IIa, IIb, and IIc, respectively. It should be reiterated that these values are minimal estimates for arbitrary fractions of active PBG, and represent examples from a polydisperse series. The positive concentration dependence of the point average molecular weights of PBG IIa and IIb indicates further polydispersity within the subfractions (Figure 7). For additional evidence, PBG IIb was filtered over Sephadex G-200 and aliquots

TABLE I: Composition of PBGI and II.

Constituent	PBG I Mol Wt 117,300 %	PBG I Mol Wt 117,300 g/mol	PBG II Mol Wt 78,400 %	PBG II Mol Wt 78,400 g/mol
Polypeptide[a]	23.6	27,700	32.8	25,700
Hexose	27.7	32,500	24.5	19,200
N-Acetylhexosamine	31.3	36,700	25.0	19,600
Fucose	0.8	900	0.7	500
Sialic acid[b]	12.7	14,900	12.2	9,600
Total carbohydrate	(72.5)	(85,000)	(62.4)	(48,900)
Ash[c]	1.8	2,100	2.3	1,800
Total	97.9	114,800	97.5	76,400

[a] Unpublished results from amino acid and nitrogen analyses by S. D. Stroupe. [b] Reported as *N*-acetylneuraminic acid. [c] After 24 hr at 700°; unpublished results by S. D. Stroupe.

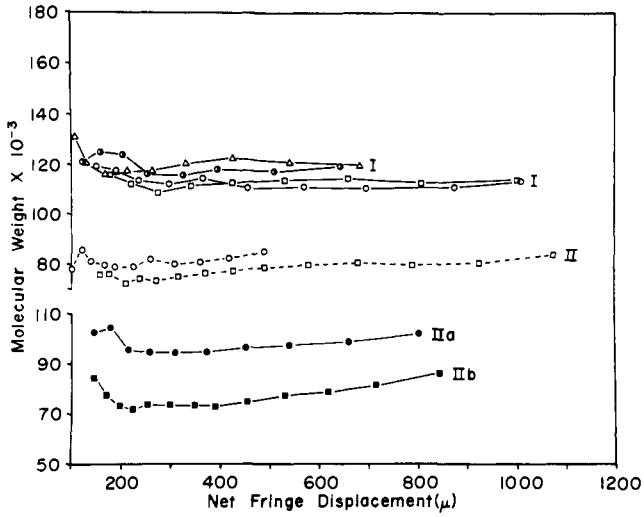

FIGURE 7: Point average molecular weights of PBG fractions as a function of protein concentration (net fringe displacement). (△, ◑) PBG I. 0.01%; (○, □) PBG I, 0.02%; (○) PBG II, 0.01%; (□) PBG II, 0.02%; the concentration of the other fractions was 0.02%. The speed was 19,160 rpm. All fractions were in 0.1 M sodium chloride–1 mM sodium azide at 5°.

of each eluate fraction were subjected to gel electrophoresis. A nearly continuous gradient of mobilities was observed in agreement with the polydispersity seen in Figure 7.

Polypeptide and Carbohydrate Composition. The results of carbohydrate analyses and estimates of polypeptide content from amino acid analyses are given in Table I. Carbohydrate is the major constituent. The molecules making up PBG I have an average of almost 24% polypeptide with nearly all of the remainder accounted for by carbohydrate, whereas the PBG II fraction contains about 33% polypeptide and a correspondingly lower amount of carbohydrate. These data suggest that the size–carbohydrate relationship is similar to that of other polydisperse glycoproteins.

The carbohydrate values of 62–72% (Table I) appear to be the highest reported for serum proteins. Qualitative analysis showed the presence of galactose and mannose and the absence of glucose (unpublished results by S. D. Stroupe). The relative abundance of hexosamine, galactose, and mannose, as well as the absence of glucose, argue against any contamination from carbohydrate-containing adsorbents. Similarly, "bleeding" from Sephadex columns as a source of the high carbohydrate values was ruled out by purifying PBG on polyacrylamide columns without the use of Sephadex; identical hexose values were obtained. Milgrom *et al.* (1973) reported a lower carbohydrate content of 48.7% for their preparation; Lea (1973) determined 42.0%.

Amino Acid Composition and Peptide Mapping of PBG I and PBG II. Results of amino acid analyses are presented in Table II. The data for PBG I and PBG II are very similar, and are consistent with the existence of a common polypeptide chain in PBG molecules of different size. The same conclusion is drawn from tryptic peptide maps for reduced and alkylated PBG I and II presented in Figure 8. On both maps, the same 21 major spots, identical in position and color for PBG I and PBG II, were observed. This result agrees with the content of 18–19 lysine plus arginine residues (Table II) calculated for PBG I (10.7 Lys and 8.5 Arg) and for PBG II (10.6 Lys and 7.5 Arg). The diffuse spot that has not moved chromatographically, but migrates in the electric field, is presumed to be glycopeptide. The finding of identical peptide maps is interpreted as indicating that the different amounts of carbohydrate present in PBG I and PBG II are attached to the same amino acid residues in a common polypeptide core; this is in accordance with present concepts of microheterogeneity in glycoproteins (Spiro, 1970).

Oligosaccharide variations have been implicated in explaining the dispersity of glycoproteins (Spiro, 1970). The size and charge heterogeneity of porcine ribonuclease have been shown to be conferred by carbohydrate associated with the enzyme (Reinhold *et al.*, 1968; Jackson and Hirs, 1970). Heterogeneity in size and in gel electrophoretic mobility has been reported for the human serum glycoprotein testosterone–estradiol binding globulin (Rosner *et al.*, 1969); sialic acid differences have been implicated (Van Baelen *et al.*, 1969). The glycoproteins yeast invertase (Neumann and Lampen, 1967) and human granulocyte vitamin B_{12} binding protein (Allen and Majerus, 1972) exhibit broad distributions on gel electrophoresis, resembling the patterns obtained with PBG in the present study.

Comparison with Published Results. The progesterone binding protein preparations obtained by Milgrom *et al.* (1973) and

TABLE II: Amino Acid Composition[a] of PBG I and PBG II.

	PBG I	PBG II
Asp	10.4	9.7
Thr	5.8	5.7
Ser	8.3	8.9
Glu	13.2	14.5
Pro	4.6	4.8
Gly	4.3	5.2
Ala	4.3	4.7
Val	5.3	5.2
Ile	3.7	3.4
Leu	10.1	9.8
Tyr	2.4	2.2
Phe	8.3	7.2
His	1.8	1.7
Lys	5.0	5.3
Arg	4.8	4.6

[a] The results are given as average values from 24- and 72-hr hydrolyses, in grams per 100 g of polypeptide.

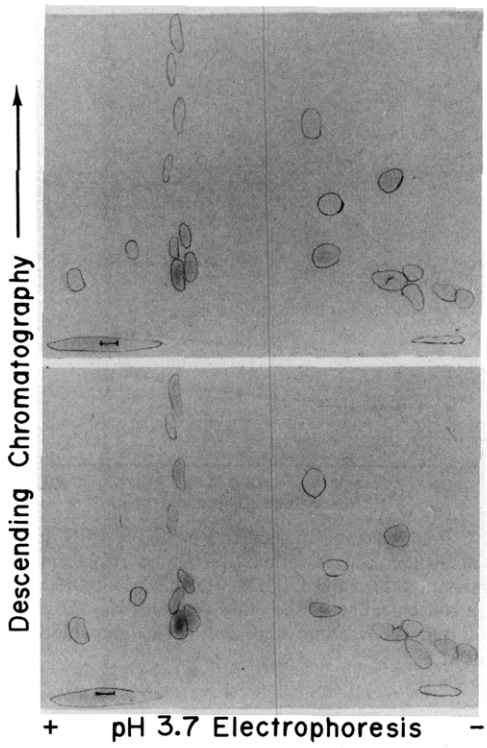

FIGURE 8: Tryptic peptide maps of PBG I (upper half of figure) and PBG II (lower half). In either fingerprint the area of application is indicated by a bracket at lower left. For details see Experimental Procedure.

by Lea (1973) have molecular weights by sedimentation equilibrium of 76,000 and 82,800, respectively. The purification method of Milgrom *et al.* resulted in a selection of about 20% of the original binding activity accumulated in a final Sephadex G-200 peak from which an unspecified number of central fractions were taken for the studies reported. Lea utilized 19.8 mg of purified protein or about 13% of the total from 150 ml of serum containing about 150 mg of progesterone-binding protein (1.2×10^{-5} M binding sites, 82,800 molecular weight).

The progesterone-binding proteins obtained in the two laboratories appeared homogeneous, and similar in size to our PBG II; however, they constitute but a small fraction of the total polydisperse glycoprotein. We have obtained similar species in our subfractionation studies. In contrast to this approach, our objective was to investigate the entire size range of PBG molecules that is demonstrable by various methods in unfractionated sera.

The anomalous behavior of glycoproteins in gel permeation, observed for PBG in our laboratory, has also been described by Lea (1973). Milgrom *et al.* (1973) found molecular weights for the progesterone-binding protein of 79,000 by sodium dodecyl sulfate–polyacrylamide electrophoresis, *vs.* 76,000 by equilibrium sedimentation. The absence of the carbohydrate effect in this result is unexplained. The type of PBG heterogeneity described by Lea (four peaks in DEAE-Sephadex chromatography) has not been seen in our studies. Aggregation by noncovalent interactions or by disulfide formation has been ruled out as an explanation for the occurrence of PBG I. Lea (1973) suggests heterogeneity in the carbohydrate content of PBG as a possible contributor to the inhomogeneity. Our results support this assumption.

Although the amino acid analyses reported from three laboratories show a general similarity, none of them is in satisfactory agreement with either of the other two. The destruction of amino acids during hydrolysis observed in our laboratory, and the relatively high range of error given by Milgrom *et al.* (1973), point to the difficulties of obtaining reproducible results. No explanation is apparent for the reported differences in total polypeptide and carbohydrate content. The possibility that the strains of guinea pigs utilized by the different investigators produce variant glycoproteins has to be considered. We have applied the purification procedure of Milgrom *et al.* (1973), which includes preliminary heating at 60° and ammonium sulfate fractionation, to the pregnant guinea pig serum used in our laboratory. We obtained the same type of polydispersity and similarly high carbohydrate values as those described in this paper.

References

Allen, R. H., and Majerus, P. W. (1972), *J. Biol. Chem. 247,* 7702.

Allouch, P., Baulieu, E. E., and Milgrom, E. (1972), *C. R. Acad. Sci. 275,* 1707.

Andrews, P. (1970), *Methods Biochem. Anal. 18,* 1.

Bennett, J. C. (1967), *Methods Enzymol. 11,* 330.

Burton, R. M., and Deutsch, H. F. (1970), *Immunochemistry 7,* 145.

Burton, R. M., Harding, G. B., Rust, N., and Westphal, U. (1971), *Steroids 17,* 1.

Burton, R. M., and Westphal, U. (1972), *Metabolism 21,* 253.

Chader, G. J., Rust, N., Burton, R. M., and Westphal, U. (1972), *J. Biol. Chem. 247,* 6581.

Chader, G. J., and Westphal, U. (1968), *J. Biol. Chem. 243,* 928.

Davis, B. J., (1964), *Ann. N. Y. Acad. Sci. 121,* 404.

Diamond, M., Rust, N., and Westphal, U. (1969), *Endocrinology 84,* 1143.

Dreyer, W. J., and Bynum, E. (1967), *Methods Enzymol. 11,* 32.

Dunker, A. K., and Rueckert, R. R. (1969), *J. Biol. Chem. 244,* 5074.

Gibbons, R. A. (1972), *in* Glycoproteins, Gottschalk, A., Ed., 2nd ed., Part A, Amsterdam, Elsevier, p 76.

Harding, G. B., Burton, R. M., Stroupe, S. D., and Westphal, U. (1974), *Life Sci.14,* No. 12 (in press).

Heap, R. B. (1969), *J. Endocrinol. 13,* 546.

Jackson, R. L., and Hirs, C. H. W. (1970), *J. Biol. Chem. 245,* 624.

Jeanloz, R. W. (1972), *in* Glycoproteins, Gottschalk, A., Ed., 2nd ed., Part A, Amsterdam, Elsevier, p 565.

Kobayashi, Y., and Maudsley, D. V. (1969), *Methods Biochem. Anal. 17,* 55.

Lea, O. A. (1973), *Biochim. Biophys. Acta 317,* 351.

MacLaughlin, D. T., Burton, R. M., Aboul-Hosn, W. A., and Westphal, U. (1972a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 430.

MacLaughlin, D. T., Harding, G. B., and Westphal, U. (1972b), *Amer. J. Anat. 135,* 179.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.

Milgrom, E., Allouch, P., Atger, M., and Baulieu, E. E. (1973), *J. Biol. Chem. 248,* 1106.

Milgrom, E., Atger, M., and Baulieu, E. E. (1970), *Nature (London) 228,* 1205.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.

Neumann, N. P., and Lampen, J. O. (1967), *Biochemistry 6,* 468.

Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121,* 321.

Reinhold, V. N., Dunne, F. T., Wriston, J. C., Schwarz, M.,

Sarda, L., and Hirs, C. H. W. (1968), *J. Biol. Chem. 243,* 6482.
Rosner, W., Christy, N. P., and Kelly, W. G. (1969), *Biochemistry 8,* 3100.
Schachman, H. K. (1959), The Ultracentrifuge in Biochemistry, New York, N. Y., Academic Press.
Schubert, D. (1970), *J. Mol. Biol. 51,* 287.
Segrest, J. P., and Jackson, R. L. (1972), *Methods Enzymol. 28,* 54.
Spiro, R. G. (1966), *Methods Enzymol. 8,* 3.
Spiro, R. G. (1970), *Annu. Rev. Biochem. 39,* 599.
Takayama, K., MacLennan, D. M., Tzagoloff, A., and Stoner, C. D. (1966), *Arch. Biochem. Biophys. 114,* 223.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley.
Van Baelen, H., Heyns, W., and DeMoor, P. (1969), *Ann. Endocrinol. 30,* 199.
Warren, L. (1959), *J. Biol. Chem. 234,* 1971.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Westphal, U. (1969), *Methods Enzymol. 15,* 761.
Westphal, U. (1971), Steroid-Protein Interactions, New York, N. Y., Springer-Verlag New York.
Westphal, U., Burton, R. M., and Harding, G. B. (1974), *Methods Enzymol. 36A* (in press).
Westphal, U., and Harding, G. B. (1973), *Biochim. Biophys. Acta 310,* 518.
Winzler, R. J. (1955), *Methods Biochem. Anal. 2,* 279.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.
Zahler, W. L., Fleischer, B., and Fleischer, S. (1970), *Biochim. Biophys. Acta 203,* 283.

Quantitative Interaction of *Ricinus communis* Agglutinin and Concanavalin A with Influenza and Vesicular Stomatitis Viruses and Virus-Infected Normal and Polyoma-Transformed Cells†

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ABSTRACT: The interactions of concanavalin A (Con A) and *Ricinus communis* agglutinin (RCA_I) with purified influenza and vesicular stomatitis virus (VSV) and virus-infected cells were examined using ^{125}I-labeled lectin binding and ferritin-lectin labeling. In quantitative binding experiments the number of Con A and RCA_I receptors per influenza virion was greater than for VSV, and there was a close correlation between the number of influenza lectin receptors and viral "spikes." Electron microscopic localization of ferritin–Con A and –RCA_I on purified influenza and VSV virus particles suggested that the viral spikes bind these lectins. Using ferritin–Con A labeling to influenza virus, Klein and Adams (*J. Virol. 10,* 833 (1972)) came to similar conclusions. When BHK cells were infected with influenza or VSV, there was an increase in Con A agglutinability within 30–120 min after infection (as reported by Rott *et al., Z. Naturforsch. B 27,* 227 (1972)), although the number of Con A-binding sites remained constant during the course of infection. RCA_I agglutinability was also enhanced, but the number of RCA_I-binding sites increased approximately sixfold during influenza infection; 75% of this increase was prevented by protein synthesis inhibitors and was probably due to viral component appearance and/or modification of the host cell surface. The remainder of the increase appeared during the early stages of infection, and it was not prevented by protein synthesis inhibitors, suggesting that this increase was due to modification of existing cell membrane components. Polyoma-transformed BHK cells were examined during influenza infection, and although the cells remained highly agglutinable at all times, the increase in RCA_I receptors per cell during infection was much lower than the corresponding increase in BHK cells.

Animal cells that have been transformed to the neoplastic state by a variety of agents usually agglutinate with plant lectins more readily than their normal counterparts (reviews: Lis and Sharon, 1973; Terner and Burger, 1973; Nicolson, 1974a,b). The increased lectin-mediated agglutinability of transformed cells has generally not correlated with increased numbers of lectin-binding sites after transformation (Arndt-Jovin and Berg, 1971; Cline and Livingston, 1971; Ozanne and Sambrook, 1971; Inbar *et al.,* 1971; Nicolson, 1973a; Nicolson and Lacorbiere, 1973), although in one laboratory this finding has been questioned (Noonan and Burger, 1973). It has been proposed on the basis of lectin receptor localization experiments that the increased agglutinability may be due, in part, to the disposition of the lectin-binding sites on the cell surface, an aggregation of lectin receptors being more likely to lead to cell agglutination than a dispersed distribution (Nicolson, 1971; Martinez-Palomo *et al.,* 1972; Nicolson, 1973b; Rosenblith *et al.,* 1973). However, other factors are also important in determining cell agglutinability such as cell zeta potential, cell re-

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