[2] Sauer, K., and R. B. Park, *Biochim. Biophys. Acta*, **79**, 476 (1964).
[3] Sauer, K., and J. Biggins, *Biochim. Biophys. Acta*, in press.
[4] Park, R. B., and J. Biggins, *Science*, **144**, 1009 (1964).
[5] Sauer, K., and M. Calvin, *J. Mol. Biol.*, **4**, 451 (1962).
[6] Sauer, K., *Biophys. J.*, in press.
[7] Blout, E. R., and L. Stryer, these PROCEEDINGS, **45**, 1591 (1959).
[8] Stryer, L., and E. R. Blout, *J. Am. Chem. Soc.*, **83**, 1411 (1961).
[9] Tinoco, I., Jr., R. W. Woody, and D. F. Bradley, *J. Chem. Phys.*, **38**, 1317 (1963).
[10] Holcomb, D. N., and I. Tinoco, Jr., *Biopolymers*, in press.
[11] Park, R. B., and N. G. Pon, *J. Mol. Biol.*, **6**, 105 (1963).
[12] Anderson, A. F. H., and M. Calvin, *Nature*, **194**, 285 (1962).
[13] Sauer, K., and M. Calvin, *Biochim. Biophys. Acta*, **64**, 324 (1962).
[14] Ke, B., and R. M. Miller, *Naturwissenschaften*, **51**, 436 (1964).
[15] Katz, J. J., G. L. Closs, F. C. Pennington, M. R. Thomas, and H. H. Strain, *J. Am. Chem. Soc.*, **85**, 3801 (1963).
[16] Closs, G. L., J. J. Katz, F. C. Pennington, M. R. Thomas, and H. H. Strain, *J. Am. Chem. Soc.*, **85**, 3809 (1963).
[17] Anderson, A. F. H., and M. Calvin, *Arch. Biochem. Biophys.*, **107**, 251 (1964).
[18] Gouterman, M., *J. Mol. Spectr.*, **6**, 138 (1961).

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*THE PRESENCE OF SUBUNITS IN THE INHERITED GROUP-SPECIFIC PROTEIN OF HUMAN SERUM**

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The serum group-specific protein (Gc) was discovered by Hirschfeld during routine immunoelectrophoretic separation of normal human serum.[1] Variations in relative electrophoretic mobility of one of the precipitation lines in the α region enabled the classification of individual serums into three common phenotypes. Some serums contained a rapidly migrating component, others a slowly migrating component, while a third group was characterized by the presence of both components in approximately equal quantities.[1] Family studies have shown that the electrophoretic variations in these proteins are controlled by two codominant autosomal genes.[2] The most rapidly migrating component corresponded to the homozygote Gc 1-1, the more slowly migrating component to the homozygote Gc 2-2, while the intermediate heterozygous type corresponded to Gc 2-1. Conditions have already been defined which permit the resolution and recognition of the three phenotypes by starch gel electrophoresis.[3] The group-specific component migrates in the postalbumin region of the gel. A single rapidly migrating band was observed in individuals corresponding to the Gc 1-1 phenotype, and a more slowly migrating band corresponded to the Gc 2-2 phenotype. Serum of the heterozygous type Gc 2-1 revealed two bands of approximately equal intensity corresponding to the positions occupied by the Gc 1-1 and Gc 2-2 phenotypes.

Recent modification of the buffer system employed for starch gel electrophoresis has resulted in improved resolution and has disclosed heterogeneity in the protein bands characteristic of individuals homozygous for Gc 1-1 and 2-2.[4] Furthermore,

it has been shown that individuals heterozygous for Gc 2-1 possess three protein bands. Reports from other laboratories have also demonstrated heterogeneity in Gc 1-1 and Gc 2-1 immunoelectrophoretically[5] and by starch gel electrophoresis.[6] Ultracentrifugation analysis of purified native Gc 1-1 and Gc 2-2 revealed a molecular weight of 50,800 (±2900).[7] Amino acid analysis of Gc 1-1 and Gc 2-2 showed essentially the same amino acid composition.[7] The recent amino acid analysis of the Gc protein reported by Heimburger *et al.*[8] is in substantial agreement with the results obtained earlier from this laboratory.

The apparent heterogeneity of the native Gc protein raised the possibility of the existence of polypeptide subunits in the Gc molecule. The present study was undertaken in an attempt to dissociate the Gc molecule and to obtain direct evidence for the presence of subunits. The recognition of more than one species of polypeptide chains comprising the Gc molecule would necessitate a new formulation of the number of genetic loci specifying the conformation of this protein.

Materials and Methods.—(a) *Purification of Gc:* Most of the studies were performed using a purified preparation of Gc 1-1; some experiments were also performed using a Gc 2-1 preparation. Isolation of Gc protein from plasma was carried out according to the following procedure. Plasma of known Gc type was dialyzed for 24 hr against barbital buffer (pH 8.6, ionic strength 0.1). A volume of 20 ml of the dialyzed plasma was centrifuged and separated on a starch block. Starch block electrophoresis was performed as described previously,[7] except that large blocks 51 × 55 cm were used for the first separation. Electrophoresis at 5°C and 3.6 volts/cm was continued until the albumin band had migrated 40 cm from the origin. (This separation was achieved in approximately 38 hr.) After electrophoresis the starch block was cut into half-inch segments and the protein eluted using suction. The Gc protein identified by starch gel electrophoresis extended from the cathodal edge of the albumin band to the anodal edge of the α_2 globulin peak. The tubes containing the Gc protein from the starch block were pooled and their contents concentrated by ultrafiltration[9] to a volume of 3 ml. After concentration, the proteins were separated by gel filtration on Sephadex G-100. A column 112 × 4 cm of Sephadex G-100, pH 8.0, was employed for gel filtration, utilizing a buffer containing 0.1 *M* Tris, 0.5 *M* NaCl, and 0.06 *M* HCl. The molecular weight difference between the large α_2 contaminating proteins and Gc allowed complete separation between Gc protein and these contaminants. When α_1-antitrypsin (mol. wt. 60,000) was present as a contaminant, it was necessary to perform a second starch block electrophoretic separation. The purity of the final preparation was judged by the appearance of a single precipitin arc following immunoelectrophoretic separation and development of precipitin lines with whole antihuman horse antiserum. In addition to revealing a single precipitin arc, such preparations revealed only the fast and slow bands characteristic of the Gc 1-1 phenotype on starch gel electrophoresis.[4] Contaminating proteins, if present, were quantitatively insufficient to appear as recognizable bands.

(b) *Starch gel electrophoresis:* Vertical starch gel electrophoresis was performed by a modification of the procedure described by Smithies.[3, 10] Electrophoresis was carried out for 5–6 hr at 15 volts/cm and 5°C in gels of 6-mm thickness. Two electric fans were placed at the sides of the gel, and the gels were air-cooled throughout electrophoresis. Each starch gel was prepared in a mixture of 450 ml of solution A and 50 ml of solution B. Solution A was composed of 6.29 gm of Tris (Sigma-121) and 1.6 gm of citric acid dissolved in 1 liter of distilled water. Solution B was the vessel buffer and was composed of 11.9 gm of boric acid and 1.2 gm of lithium hydroxide dissolved in 1 liter of distilled water.

(c) *Immunoelectrophoresis:* Immunoelectrophoresis was carried out in agar gel according to the microtechnique of Scheidegger as modified by Hirschfeld.[11] Specific anti-Gc serum was prepared in rabbits by immunization with purified Gc protein.

(d) *Agar gel diffusion:* The two-dimensional diffusion system on agar gel as developed by Ouchterlony[12] was employed.

(e) *Peptide patterns:* Tryptic digestion of 5–20 mg of heat-denatured Gc material was performed under a nitrogen barrier in a pH-stat for 1.5 hr. The reaction was carried out at 38°C,

pH 8.0, and with a 1% concentration of trypsin (Worthington, Cryst.). After tryptic hydrolysis was complete, the solution was centrifuged at 25,000 rpm for 1 hr. Aliquots were concentrated *in vacuo* on parafilm.

High-voltage electrophoresis followed by ascending chromatography was performed according to the method described by Ingram.[13] Three milligrams of Gc tryptic digest was applied in a volume of 10 μl of electrophoresis buffer to a piece of Whatman 3 MM paper, 25 cm wide and 90 cm long, with 7.5 × 12.5-cm tabs, in a small spot 30 cm from the anodal edge of the paper. High-voltage electrophoresis was carried out at pH 3.5 using a pyridine:acetic acid:water buffer (1:10:189 v/v, respectively). This step was performed in a Savant Lucite tank with coolant using 47 volts/cm for 1 hr. Ascending chromatography using pyridine:isoamyl alcohol:water solvent (35:35:30 v/v, respectively) was performed after the paper had been dried in an oven at 90°C for 10 min. The peptide patterns were developed by dipping the paper in a cadmium ninhydrin solution[14] consisting of cadmium acetate (100 mg), water (10 ml), acetic acid (5 ml), acetone (100 ml), and ninhydrin (1 gm). After remaining in a fume hood for 5 min, the paper was placed in a 90°C oven for 5 min. Peptide spots were pink, yellow, and orange depending upon their amino acid composition.

One-dimensional electrophoresis on a strip of Whatman 3 MM paper, 12.5 × 100 cm, was performed after applying 10 mg of concentrated digest contained in 40 μl of electrophoresis buffer in a vertical line 7.5 cm long which was located 35 cm from the anodal edge of the strip. Electrophoresis was performed for 2.5 hr with an initial voltage of 40 volts/cm. In most experiments the voltage did not fluctuate significantly during the electrophoretic separation. After electrophoresis the paper was dried and cut into 4–6 horizontal strips, each of which was subjected to a different specific stain.[15] On a single one-dimensional separation, it was possible to identify peptides having tyrosine, arginine, tryptophan, and histidine. Disulfide analyses were carried out by the procedure developed by Hartley.[16] After paper electrophoresis at pH 3.5, a guide strip was stained with ninhydrin and the remaining strip was placed in an atmosphere of performic acid overnight. After the paper had been dried *in vacuo* to remove the acid, it was sewed vertically along a new 25 × 110-cm paper and submitted to electrophoresis in a similar manner. Unaltered peptides would be arranged in a diagonal line, while each altered peptide arising from cleavage of a disulfide bond would appear as two new spots off the diagonal.

(f) *Gel filtration:* A 130 × 2.4-cm chromatography column was calibrated as described by Andrews[17] using Sephadex G-100 in 0.1 *M* Tris, 0.06 *M* HCl, 0.5 *M* NaCl buffer, pH 8.0 at 5°C. Fractions of 5.8 ml were collected and monitored at 280 mμ. Calibration of the column was achieved by determining elution volumes of 5 proteins with molecular weights varying from 13,700 to 82,000. Human transferrin (Grade C, Immunology, Inc.) has a molecular weight of *ca.* 82,000 and was eluted in 196 ml. The elution volume of human serum albumin (Cryst. Mann Research) which has a molecular weight of 69,000 was 225 ml. The molecular weight of chymotrypsinogen (3 × Cryst., Worthington) is 25,000; this protein was eluted in a volume of 330 ml. Soya bean trypsin inhibitor (3 × Cryst., Worthington) has a molecular weight of 21,500 and was eluted in 365-ml volume. The elution volume of ribonuclease (Cryst., Worthington) whose molecular weight is 13,700 was 382 ml, with small variations in different experiments. An inverse linear relationship was obtained between the elution volume and the logarithm of the molecular weights of the proteins used. Some gel filtration experiments were conducted in 0.6 *M* propionic acid.

(g) *Ultracentrifugation:* Ultracentrifugation analyses of native Gc 1-1 and Gc 2-2 preparations have been previously reported.[7] The molecular weight of reduced preparations of Gc was determined after the following steps. Two mg of purified Gc 1-1 in a volume of 2 ml was dialyzed against a solution of 6 *M* guanidinium chloride and 0.1 *M* mercaptoethanol at pH 8.5, for 12 hr at room temperature in 23/32 Visking casing. The dialysis sack containing the protein solution was transferred to a solution of 8 *M* urea and 0.1 *M* mercaptoethanol, pH 8.5, and was allowed to dialyze at 5°C for an additional 2 weeks in order to achieve equilibrium. The molecular weight was determined in 8 *M* urea and 0.1 *M* mercaptoethanol by the method developed by Yphantis.[18]

Results.—Purification of Gc: A comparison of the starch gel electrophoretic separation of a purified Gc 1-1 preparation and the serum of the same phenotype is illustrated in Figure 1. The heterogeneity of the Gc present in the whole serum also observed in the purified preparation, and is characterized by a fast- and

slow-moving band in the Gc region. Immunoelectrophoretic separation of purified Gc 1-1 and serum from an individual homozygous for Gc 1-1 is illustrated in Figure 2. A single precipitin arc in the Gc region was observed, indicating the absence of any contaminating protein which can react with antibodies against human serum.

Peptide patterns: A typical peptide pattern obtained for a Gc 2-1 preparation is shown in Figure 3. In all experiments conducted, 23 to 27 peptide spots were clearly visible. A preparation of Gc 1-1 yielded a similar pattern both in the number and approximate position of the peptide spots obtained. If the peptide bonds of all the lysine and arginine residues in the molecule were available for tryptic hydrolysis, 54 peptides would be expected following digestion with this enzyme. One-dimensional electrophoresis of Gc 1-1 revealed approximately 18 peptide bands. Following electrophoresis, the paper was cut longitudinally into five half-inch strips and stained for the presence of specific amino acids (Fig. 4). Five peptide bands showed the presence of histidine, compared to the 6–7 residues found by amino acid analysis. Seven arginine-containing peptides were found on the one-dimensional strips compared to 13–14 residues in the amino acid analysis. Tyrosine stains were positive in 7 peptides compared to 15–16 tyrosine residues in the amino acid analysis. Since 19–23 one-half-cystine residues had been found in the amino acid analysis, the possibility existed that the native Gc molecule might contain 10–12 disulfide bonds. However, the results obtained from disulfide analyses of purified Gc 1-1 and Gc 2-1 preparations suggested the presence of only 5 or 6 peptides containing di-

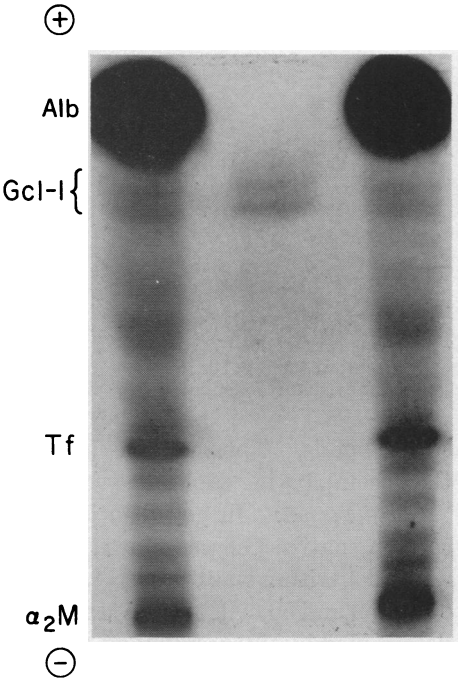

FIG. 1.—Starch gel electrophoresis of whole serum from Gc 1-1 phenotype (right and left patterns) and purified Gc 1-1 (center). The positions of the major protein bands are indicated: albumin (*Alb*), the two protein bands in the Gc region (*Gc*), transferrin (*Tf*), and the α_2 macroglobulin ($\alpha_2 M$).

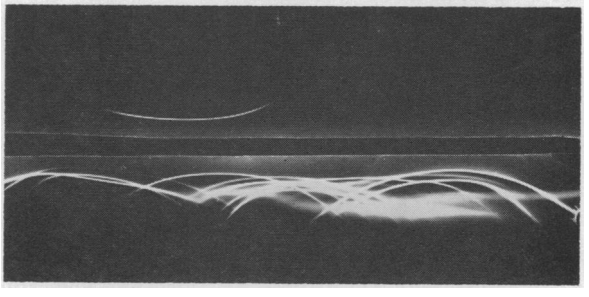

FIG. 2.—Immunoelectrophoretic analysis of a purified Gc 1-1 preparation (4 mg/ml) (*above*) and serum of type Gc 1-1 (*below*). Antiserum: Horse antiserum 306, Institut Pasteur, Paris.

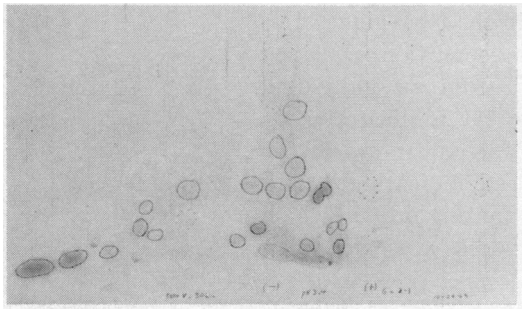

FIG. 3.—Peptide pattern of a tryptic digest of Gc 2-1. High-voltage electrophoresis was carried out for 1 hr at pH 3.5 with 77 volts/cm, and in a buffer of pyridine:acetic acid:water (1:10:189 v/v, respectively). Ascending chromatography in pyridine:isoamyl alcohol:water (35:35:30) was performed for 8 hr.

sulfide bonds. The peptide evidence and results of specific stains are summarized in the accompanying table.

Gel filtration: In gel filtration experiments using a calibrated Sephadex G-100 column, native Gc was eluted in a volume corresponding to a molecular weight whose logarithm was between 4.6960 and 4.7040, corresponding to molecular weights of 49,660 and 50,690. These results are in close agreement with the molecular weight determined by ultracentrifugal analyses of native Gc 1-1 and Gc 2-2, where a value of 50,800 was obtained.

After treatment with solutions of 6M guanidinium chloride and 8 *M* urea in the presence of 0.1 *M* mercaptoethanol, the elution volume of Gc was increased significantly, indicating a decrease in molecular weight. The elution curve following gel filtration of native Gc is superimposed over the elution of a reduced preparation of Gc in Figure 5. The data in Figure 5 were obtained from two gel filtration experiments. The elution volume of reduced Gc was 275 ml, compared to 230 ml elution volume of native Gc. Elution volumes cited here corresponded to the maximum optical density in each chromatographically separated peak. As is evident from Figure 5, the reduced preparation also contained Gc material which was eluted in

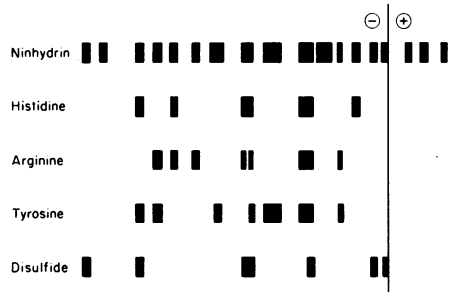

FIG. 4.—One-dimensional paper electrophoresis of a tryptic digest of Gc at pH 3.5, using 40 volts/cm for 2.5 hr. The color reactions specific for various amino acids were carried out as described in ref. 15.

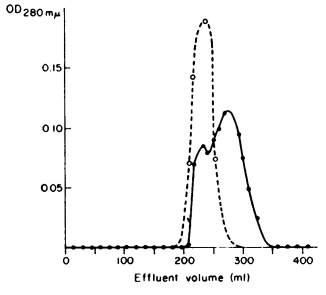

FIG. 5.—Gel filtration of native Gc (interrupted lines) and reduced Gc. The elution volume of reduced Gc was increased significantly, indicating a decrease in molecular weight. The reduced preparation also contained native Gc material.

TABLE 1

CHARACTERISTICS OF THE TRYPTIC PEPTIDES OF Gc

Amino acid analysis	Number of residues*	Tryptic Peptides	
		Expected	Observed
Lysine and Arginine	53	54	23–27
Histidine	7	7	5
Tyrosine	16	16	7
Arginine	14	14	7
Half-cystine†	19–23	10–12‡	6

* Based on 51,000 molecular weight values for Gc 1-1 and Gc 2-2.
† Based on a value range obtained from two analyses of hydrolysates prepared from native Gc 1-1 and Gc 2-2.
‡ Assuming that 19–23 half-cystines are covalently linked by 10–12 disulfide bridges.

the same position as native Gc. This material probably represented some molecules of Gc which were resistant to the denaturing and reducing agents employed in these experiments. On a calibrated Sephadex G-100 column, the elution volume of reduced Gc preparations was somewhat less than expected for a protein of 25,000 molecular weight and suggested a molecular weight slightly larger than 35,000, and thus approximately 10,000 greater than that found by ultracentrifugation. It is noteworthy in this connection that anomalous elution patterns have been observed during gel filtration for a number of proteins. Lipase and ribonuclease have both been observed to behave anomalously during gel filtration by Gelotte[19] who attributed their unusual behavior either to interaction between solute and gel or to a structural change in the protein molecule. Anomalous behavior of lysozyme, hemoglobin, and ovomucoid has been reported in thin layer chromatography using cross-linked dextran.[20] Thus, it is clear that although a detectable alteration in molecular weight of reduced Gc has been demonstrated by gel filtration, this method cannot be used to calculate with precision the exact molecular weight of the subunits. Attempts to calculate the molecular weight of the subunits of Gc by the method of gel filtration would yield a molecular weight substantially higher than that found by ultracentrifugation. It seems quite likely, however, that modification of the method of gel filtration will result in a molecular weight determination more closely in agreement with that obtained from the ultracentrifugal analysis.

Ultracentrifugation: The weight average molecular weight of native Gc 1-1 and Gc 2-2 was reported previously and found to be 50,800 (±2900).[7] However, a striking difference in molecular weight was found for the Gc protein following dialysis against 6 *M* guanidinium chloride and 8 *M* urea in the presence of 0.1 *M* mercaptoethanol. Assuming the partial specific volume to be 0.73, the weight average molecular weight of reduced protein was observed to be 25,000 (±5000). The molecular weight was thus reduced to one half the value of native Gc indicating the presence of two subunits of closely similar, if not identical, molecular weight.

Immunological studies: The possibility that the subunits of the Gc molecule might retain some or all of its native antigenic determinants was studied using the technique of Ouchterlony double diffusion in gel. A rather weak antigen antibody reaction was obtained which fused with the antigen antibody precipitate of native Gc (Fig. 6). The native Gc precipitin line spurred over the line formed by the subunits. It is apparent that under the conditions employed, the subunits have retained some of their antigenic determinants. Further experiments will be required to exclude the possibility that the native Gc molecule contains more antigenic determinants than are present in its subunits.

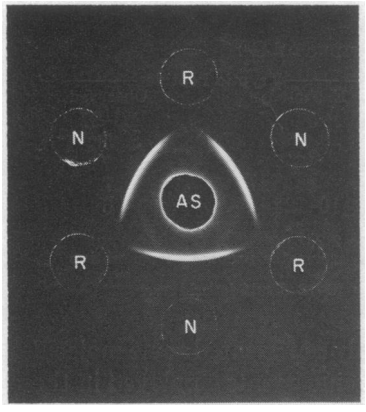

FIG. 6.—Two-dimensional diffusion test on agar gel demonstrated an antigen-antibody reaction of reduced (*R*) Gc which fused with the antigen-antibody reaction of native Gc (*N*). The native Gc precipitin line can be seen to spur over the line formed by the subunits. (*N* contains 2 μl of 4 mg/ml, *R* contains 5 μl of 2 mg/ml, *AS* contains 20 μl of rabbit specific anti-Gc serum).

Discussion.—The results reported in this study provide experimental evidence for the existence of subunits in the serum group-specific protein. The presence of two polypeptide chains was first suggested by the finding of one half the expected number of tryptic peptides after subjecting the protein to peptide analysis. This evidence was strengthened by the results obtained using specific stains for individual amino acids. One half the number predicted by amino acid analyses was found for tyrosine, arginine, and disulfides on one-dimensional electrophoresis. It was of some interest that the number of peptides containing histidine was slightly higher than expected, 5 rather than 3. The unexpected increase in the number of histidine-containing peptides may be explained from the results obtained in preliminary studies of the tryptic digests of purified fast and purified slow bands of Gc 1-1. In these studies, there is a difference in the migration of a histidine-containing peptide which migrates more rapidly toward the anode at pH 3.5 in the fast Gc band than a corresponding histidine peptide does in the slow Gc band. Therefore, in mixtures of fast and slow bands, such as the preparation studied here, it is possible that each of the two histidine peptides is visualized.

A decrease in the molecular weight of the Gc protein after reduction was indicated by gel filtration, and ultracentrifugation analyses confirmed the presence of two subunits each having a molecular weight of 25,000. The presence of polypeptide subunits in the Gc molecule explains the observed heterogeneity of the three Gc phenotypes.

Speculation of the occurrence of subunits in the fast and slow bands of Gc 1-1 and Gc 2-2 has led to at least two possible interpretations in the light of the present study. There is a possibility that a single polypeptide chain is common to both the fast and slow bands with the second polypeptide chain being qualitatively different in each. Alternatively, one of the two bands, either the fast or the slow band, is a dimer composed of two identical subunits and the other band has one of the same subunits associated with a different polypeptide chain. The first assumption has an attractive structural similarity to the results obtained from studies on human hemoglobin in which hemoglobins A, A_2, and F are formulated by $\alpha_2 \beta_2$, $\alpha_2 \delta_2$, and $\alpha_2 \gamma_2$, respectively. It is clear that further resolution of the polypeptide structure of Gc will require the isolation and characterization of the fast and slow protein bands of Gc.

According to current genetic concepts, the information dictating the primary amino acid sequence of each polypeptide chain resides in one gene. Thus, the results of these studies suggest that the genetic control of the structure of the Gc molecule requires the activity of at least two different structural genes. It follows that structural mutations may be expected to occur affecting the different polypep-

tide chains comprising the fast and slow protein bands of Gc 1-1 and Gc 2-2.

Summary.—Evidence for the existence of subunits in the inherited group-specific protein of human serum is presented. The possibility of subunits within the Gc molecule was first raised by the electrophoretic heterogeneity observed in purified Gc 1-1 and Gc 2-2 in starch gel electrophoresis. The finding of Gc peptide patterns containing one half the expected number of tryptic peptides strongly supports this possibility. Reduction of the Gc molecule resulted in a decrease in the molecular weight of the Gc protein as judged by gel filtration. Analytical ultracentrifugation analyses of the reduced Gc protein confirmed the presence of subunits, each having a molecular weight of 25,000. The results presented here suggest that the primary structure of the Gc molecule is controlled by more than one structural gene.

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[1] Hirschfeld, J., B. Jonsson, and M. Rasmuson, *Nature*, **185**, 931 (1960).

[2] Cleve, H., and A. G. Bearn, in *Progress in Medical Genetics*, ed. A. G. Steinberg and A. G. Bearn (New York: Grune and Stratton, 1962), vol. 2, p. 64.

[3] Parker, W. C., H. Cleve, and A. G. Bearn, *Am. J. Human Genet.*, **15**, 353 (1963).

[4] Bearn, A. G., F. D. Kitchin, and B. H. Bowman, *J. Exptl. Med.*, **120**, 83 (1964).

[5] Reinskou, T. A., *Acta Pathol. Microbiol. Scand.*, **59**, 526 (1963).

[6] Akfors, K.-E., and L. Beckman, *Acta Genet. Statist. Med.*, **13**, 231 (1963).

[7] Cleve, H., J. H. Prunier, and A. G. Bearn, *J. Exptl. Med.*, **118**, 711 (1963).

[8] Heimburger, N., K. Heide, H. Haupt, and H. E. Schultze, *Clin. Chim. Acta*, **10**, 293 (1964).

[9] Everall, P. H., *J. Exptl. Med. Lab. Technol.*, **15**, 209 (1958).

[10] Smithies, O., *Biochem. J.*, **71**, 585 (1959).

[11] Hirschfeld, J., *Acta Pathol. Microbiol. Scand.*, **47**, 160 (1959).

[12] Ouchterlony, O., *Acta Pathol. Microbiol. Scand.*, **26**, 507 (1949).

[13] Ingram, V. M., *Biochim. Biophys. Acta*, **28**, 539 (1958).

[14] Heilmann, J., J. Barrollier, and E. Watzke, *Z. Physiol. Chem.*, **309**, 219 (1957).

[15] Bailey, J. L., *Techniques in Protein Chemistry* (Amsterdam: Elsevier Publishing Co., 1962), pp. 18–24.

[16] Brown, J. R., and B. S. Hartley, *Biochem. J.*, **89**, 59 (1963).

[17] Andrews, P., *Biochem. J.*, **91**, 222 (1964).

[18] Yphantis, D. A., *Biochemistry*, **3**, 295 (1964).

[19] Gelotte, B., *Acta Chem. Scand.*, **18**, 1283 (1964).

[20] Morris, C. J. O. R., *J. Chromatog.*, **16**, 167 (1964).