Quantitative Variations of Haptoglobins in a Caucasian Family*

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The inheritance of the common haptoglobin types is well understood. No satisfactory genetic explanation, however, has been advanced to explain quantitative variations in haptoglobin levels, such as those seen in well individuals with either anhapto-globinaemia or hypohaptoglobinaemia or the various 2-1 modified types (Sutton and Karp, 1964). Many environmental or host processes are known to influence the haptoglobin level. Haemolysis and hepatocellular failure are known to cause a decrease or absence of haptoglobin (Nyman, 1959). Increased haptoglobin levels have been described in a variety of disorders associated with inflammation or tissue necrosis (Nyman, 1959; Jayle and Moretti, 1962; Mathies, Schattenkirchner, and Liebrich, 1963; Robert, Mombelloni, and Crosti, 1961). Administered androgen increases the haptoglobin concentration, and the administration of diethylstilboestrol and ethinyloestradiol to healthy women decreases the haptoglobin level (Borglin and Nyman, 1961). Haptoglobin levels usually reach adult or near adult levels by the age of 1 year (Bergstrand, Czar, and Tarukoski, 1961). In Nyman's (1959) extensive study, haptoglobin levels were higher in males than in females and varied with the haptoglobin phenotypes. In 144 males the average levels were as follows: type 1-1, 144 mg./100 ml.; type 2-1, 116 mg./100 ml., and type 2-2, 88 mg./100 ml. In 84 normal women, type 1-1 averaged 115 mg./100 ml., type 2-1, 95 mg./100 ml., and type 2-2, 79 mg./100 ml. Thus, in general, the *Hp2^m* allele results in lower haptoglobin levels and is also generally associated with both anhapto-globinaemia

and various *Hp2-1M* subtypes. The terms 'anhapto-globinaemia' and 'Hp o' signify only the absence of haptoglobin by particular techniques and do not imply complete absence of haptoglobin.

Only a few family studies of haptoglobins using quantitative techniques have been carried out (Gottlieb, Ross, Greenberg, and Wisch, 1963a; Gottlieb, Wisch, and Ross, 1963b; Matsunaga, Murai, and Matsuda, 1962; Murray, Robinson, and Visnich, 1966). Anhapto-globinaemia has been reported only rarely in Caucasian families (Galatius-Jensen, 1958; Harris, Robson, and Siniscalco, 1958; Makela, Eriksson, and Lehtovaara, 1959; Gohler, 1964), but a recent report suggests that anhapto-globinaemia and hypohaptoglobinaemia may not be uncommon in Caucasians (Murray *et al.*, 1966). Sutton and Karp (1964) recently suggested that quantitative studies of three-generation pedigrees might be helpful in understanding anhapto-globinaemia. The present paper is a report of quantitative variation in the haptoglobin levels over four generations in a Caucasian family in which several members had low or absent haptoglobin levels. A Caucasian family of this size in which quantitative levels were studied has not been reported hitherto.

In addition, a modified benzidine staining method, which allows the preservation of the gels and stain with no appreciable change in intensity or clarity of the individual *Hp-Hb* bands for over a year, is described.

Patients

Certain members of the kindred described had skeletal malformations, and the haptoglobins and various blood groups were investigated for use as 'markers' in linkage studies. Six of the 39 members are deceased, and 25 were studied for *Hp* typing. Their ages are given in the Figure only if they are under 22. There was no evidence of significant disease in any family member at the time of study, except for II.2 who had basilar artery disease.

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Fig. Pedigree data. The haptoglobin phenotypes are shown directly below the pedigree symbols, e.g., I.2 is 2-2. III.13 was anhypohaptoglobinaemic on the first sample, but showed a weak Hp 2-1d pattern on the second sample. The quantitative levels in mg./100 ml. are shown in parentheses below the phenotype. Lastly, the age of the individual is given if 21 years of age or under. The predominance of low Hp levels in the younger members of the pedigree and the association of low levels with the Hp^s alleles is apparent. The qualitative and quantitative techniques correlate well except between the levels of 40 and 60 mg./100 ml. Of particular interest is the presence of border line values (under 60 mg./100 ml.) in all family members in direct line between the anhypohaptoglobinaemic individuals. I.2 died one year after the studies.

Laboratory Methods

Blood samples. Sera were separated within two hours after collection and frozen until used. Blood was also collected in tubes containing 1 ml. acid-citrate-dextrose solution for complete blood group studies to confirm inheritance.

Starch gel electrophoresis. All sera were run on vertical starch gel electrophoresis (Smithies, 1959a, b). Hydrolysed starch (S-676, Connaught Medical Research Laboratories, Toronto, Canada) was used in the concentration at which the migration distance of β globulin was approximately 2.5 times the migration distance of the slow α -2 globulin, namely 13.7 g./100 ml. borate buffer. The gels were run at room temperature (25-30°C.).

Benzidine stain. The sliced gels were stained in the standard manner with amido black 10B (Smithies, 1959a). The other half of the gel was stained with a modified benzidine procedure originally designed for thin-layer starch gel electrophoresis (Baur, 1963). The modified procedure, in contrast to the usual benzidine stain, allowed the preservation of the stained gel for a much longer period. The stain solution consisted of 0.2% aqueous benzidine dihydrochloride (Fisher Scientific Co.), 0.2% aqueous sodium nitroprusside (Fisher Scientific Co.), and 1% glacial acetic acid. Just before use, 0.2 ml. 30% hydrogen peroxide for every 100 ml. stain solution was added. The sliced gel was immersed in this mixture for several minutes allowing the Hb to stain blue. After draining, 3% aqueous

sodium pyrophosphate (reagent grade) was added for 3-5 minutes. The gel was next immersed in absolute methanol for 30 minutes. The original method called for 4 minutes in methanol. Finally, distilled water was added directly to the methanol producing a 10-15 to 1 ratio of water/methanol. This was changed from the method of Baur (1963), in which the gel was washed with distilled water for 15 minutes, and finally immersed in a glycerol/acetic acid/sodium nitrate solution to make the gels white and firm with blue stained Hp-Hb bands. The modification described produced a less friable gel with greater contrast between the Hp-Hb bands and the ivory background. It was excellent for observing qualitative and gross quantitative differences in samples. The stained gels were then wrapped in plastic to prevent dehydration and stored in the cold. There was no detectable difference upon inspection of the gels one year later.

Quantitative paper electrophoresis. Serum haptoglobin quantitation was performed as described by Nyman (1959). Normal adult values have ranged from 26 to 260 mg./100 ml. in various studies (Nyman, 1959; Shinton, Richardson, and Williams, 1963; Jayle and Moretti, 1962; Murray *et al.*, 1966), but no definitive criteria for normal levels have emerged.

Results

Four children had extremely low or absent haptoglobins (Table). III.11 was anhypohaptoglobinaemic on two occasions by the usual starch gel technique and by the quantitative determination of the second

sample. III.13 showed no haptoglobin staining by either benzidine or amido black staining on the first sample, but the first three of the usual Hp 2-1 bands were faintly present on benzidine staining on the second sample four months later. This is comparable to Sutton and Karp's (1964) Hp 2-1d phenotype and will be called 2-1d throughout this communication. Amido black staining was absent in the haptoglobin region but was otherwise normal. No haptoglobin was found by the quantitative technique on the second sample. IV.1 was anhapto-globinaemic on one occasion, but a second sample could not be obtained and there was insufficient serum for quantitation. III.12 showed a weak Hp2-1 pattern on both occasions and a quantitative level of 15 mg./100 ml. All of the family members in direct line between the two sibships, where low levels of haptoglobin occurred, had low normal values in the 46-57 mg./100 ml. range. In each case when it was measured, however (II.1, II.11, III.1), their spouses were well within the normal range.

TABLE

REPEAT HAPTOGLOBIN FINDINGS IN SEVERELY AFFECTED SUBJECTS

Subject	Sample 1	Sample 2 (4 months later)	
	Gel Phenotype	Gel Phenotype	Quantitative Level
III.11	o	o	o
III.12	Hp 2-1 (weak)	Hp 2-1 (weak)	15 mg./100 ml.
III.13	o	Hp 2-1 (weak)	o
IV.1	o	—	—

The results of the quantitative and qualitative studies are presented in the Fig. Both techniques revealed low or absent levels when the quantitative levels were less than 40 mg./100 ml., and were also in good agreement when the levels were over 60 mg./100 ml. Between these levels (46-57 mg./100 ml.), however, the techniques were in poor agreement. The reasons for these discrepancies are not clear, but presumably they reflect the semi-quantitative nature of both techniques and emphasize the need for a better quantitative technique. At present it seems best to employ both techniques, but the technical limitations add significantly to the difficulties in arriving at a satisfactory hypothesis for the haptoglobins.

Because of the presence of atypical or weak Hp patterns, the three sibs, III.11, III.12, and III.13 had complete haemograms done at the drawing of the second sample. Packed cell volume estimates, reticulocyte counts, platelet counts, white blood cell

counts, and differentials were normal. Blood group studies were consistent with paternity as shown in Fig. 1.

Discussion

Several hypotheses have recently been suggested to explain the genetics of hypohaptoglobinaemia and anhapto-globinaemia (Gottlieb *et al.*, 1963b; Parker and Bearn, 1963, 1965; Gohler, 1964, Sutton and Karp, 1964). None of these has proved completely satisfactory. The predominance of low levels of haptoglobin in the younger age-groups and quantitative variations have been particularly difficult to explain on a purely genetic basis. The possibility of environmental effects has been discussed by most authors but not considered central to the explanations offered.

The present family raises several additional points which even the broad hypothesis of Parker and Bearn (1963, 1965) does not explain. These include the presence of two sibs with very low levels of Hp (III.11, III.12), one of whom has a Hp 2-1 pattern rather than Hp 2-1M, and the change from Hp o to Hp 2-1d in III.13. In addition, this hypothesis requires each parent of an Hp o individual to have at least one mutant control gene. The presence of normal Hp levels not only in a Hp 1-1 but a Hp 2-2 parent (II.11 and III.1) is against this hypothesis. Conversely, each of the family members in direct line between the Hp o patients shows a low normal value (46-57 mg./100 ml.). These observations suggest that the genetic basis of both low and absent haptoglobin levels is the presence of a single mutant gene. Similar results have been obtained in other large families where quantitative studies have been done (Gottlieb *et al.*, 1963b). It is of particular interest that in each of the hypohaptoglobinaemic families reported by Murray *et al.* (1966), one of the parents had levels in the same 46-57 mg./100 ml. range or lower, though only two were considered low by their criteria (less than 30 mg./100 ml.).

Although Sutton and Karp's hypothesis (1964) is more useful in understanding the present family, certain limitations warrant comment. The genetic evidence presented in their paper forced a pooling of phenotypes Hp 2-1a and Hp 2-1b as normal, and Hp 2-1c and Hp 2-1d as alternative expressions of the *Hp 2^{cd}* gene. Whether these genotypes will remain discrete is not clear. Either additional genetic evidence or a change from Hp 2-1cd to Hp 2-1e in the same subject would necessitate further alteration in the hypothesis. There are few available data to test this possibility, since sequential studies are infrequently reported. Sutton and Karp were forced to conclude that the genetic basis for

anhaptoglobinaemia was obscure, and offered no genetic reason why some $Hp\ 2^m/Hp\ 1$ genotypes should be expressed as $Hp\ 0$. Again III.12 is not explained.

Several modifications in emphasis can be made to Sutton and Karp's hypothesis, which extend the hypothesis, and which can be tested. It is postulated that there is an $Hp\ 2^m$ allele, or more likely a series of alleles as suggested by Sutton and Karp (1964), which lead to lower levels of $Hp\ 2$ product. The rise to adult levels is delayed in the presence of these alleles, there is an increased sensitivity to certain environmental effects or agents which lower Hp levels, and the maximum Hp level in the presence of one $Hp\ 2^m$ allele is 46–57 mg./100ml. A subject with a $Hp\ 2^{cd}/Hp\ 1$ genotype might be observed as $Hp\ 0$ at age 2, $Hp\ 2-1d$ at age 8 with a level of 15 mg./100 ml., as $Hp\ 2-1$ or $Hp\ 2-1d$ at age 40 with a weak gel pattern and a quantitative level of 25–57 mg./100 ml. An appropriate environmental stimulus would return him to $Hp\ 0$ at any time. Sequential testing of subjects with $Hp\ 2-1M$ and $Hp\ 0$ phenotypes could provide confirmation of this hypothesis in a few years. It would not be necessary to observe the full range of possibilities in a single person. The change from $Hp\ 0$ to $Hp\ 2-1d$ in III.13, as well as similar earlier reports (Galatius-Jensen, 1958; Sutton, Neal, Livingstone, Binson, Kunstadter, and Trombley, 1959; Blumberg and Gentile, 1961) provide support for this hypothesis. This hypothesis is essentially that of Sutton and Karp (1964), with emphasis on the wide range of the age and environmental effects. The various types of low or absent haptoglobins in the present family appear best explained by the presence of a single $Hp\ 2^{cd}$ gene, with differences in phenotypic and quantitative levels resulting from age and environmental effects.

In view of the many known host and environmental effects on Hp levels summarized in the introduction, it seems more productive to observe these families carefully rather than to create new hypotheses at this time.

Summary

Quantitative levels of haptoglobin were determined over 4 generations in a Caucasian family in which several members had low or absent haptoglobins. A satisfactory genetic explanation of the hypohaptoglobinaemic members of the present and other similar families has not evolved. It appears very likely that a significant portion both of the

quantitative and phenotypic variation described is due to interaction of the environment and age with the various $Hb\ 2^m$ alleles. Serial studies in these families over a period of years will be necessary to resolve these questions.

A modified staining technique is reported which produces well-stained gels for over a year.

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