Human Haptoglobins: Estimation and Purification

By G. E. CONNELL and O. SMITHIES
Department of Biochemistry and Connaught Medical Research Laboratories, University of Toronto

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The presence in mammalian sera of haptoglobins, a group of proteins characterized by their ability to bind haemoglobin, was established by Polonovski & Jayle (1939). Smithies (1955) demonstrated by starch-gel electrophoresis that, in humans, three types of haptoglobins can be distinguished in different individuals. Genetic control of the haptoglobin types by a pair of autosomal alleles, Hp^1 and Hp^2, has been established (Smithies & Walker, 1956). The homozygote Hp^1/Hp^1 shows a single haptoglobin component on electrophoresis, whereas the other homozygous type Hp^2/Hp^2 shows many components. The heterozygote Hp^2/Hp^1 also shows several haptoglobin components, but these in general differ in their electrophoretic mobilities from those of either of the homozygotes. The differences between the three genetic types thus appear to be complex, and an investigation of haptoglobins will be of considerable interest to biochemists and geneticists.

Part I. Estimation of haptoglobin

Two methods of estimating haptoglobin have been described. Jayle (1951) uses a method depending on the difference between the peroxidase activity of haemoglobin and its complexes with haptoglobin. Ethyl hydrogen peroxide is the oxidizing substrate, and iodide is the electron donor; iodine liberated during the reaction is determined by titration with thiosulphate. Laurell & Nyman (1957) titrate haptoglobin with haemoglobin, using filter-paper electrophoresis to determine the end point at which unbound haemoglobin first appears. The method of estimating haptoglobin here described depends on the peroxidase activity of haemoglobin–haptoglobin complexes, as does the method of Jayle (1951). Hydrogen peroxide is used as the oxidizing substrate. Guaiacol is the hydrogen donor (cf. Maehly & Chance, 1954). The reaction conditions are chosen so that the peroxidase activity of free haemoglobin is essentially zero. The formation of tetraguaiacol during the reaction is followed spectrophotometrically. The method is calibrated against a haemoglobin standard so that the haptoglobin content of a solution can be expressed in terms of its haemoglobin-binding capacity (cf. Laurell & Nyman, 1957).

METHODS

Reagents for standard estimation procedure

Hydrogen peroxide. A solution of hydrogen peroxide, $0{\cdot}600 \pm 0{\cdot}005$ M, is prepared immediately before use by dilution (15- to 20-fold) of a stock solution. The dilute peroxide solution is discarded after 30 min. and if necessary a fresh dilution is prepared. The stock solution, approximately 30% (w/v) in hydrogen peroxide, is standardized at least once a week by titration with potassium permanganate (Schumb, Satterfield & Wentworth, 1955).

Guaiacol reagent. A buffered solution of guaiacol, $0{\cdot}030 \pm 0{\cdot}001$ M, is prepared as follows: 1·86 g. of guaiacol (analytical grade) and 50 ml. of M-acetic acid are added to 400 ml. of water. The pH is carefully adjusted to 4·0 with aqueous sodium hydroxide, with a pH meter standardized with 0·05 M-potassium acid phthalate (pH 3·97 at 20°). The volume is made up to 500 ml. with water. The guaiacol reagent is stable for several weeks when stored in the cold.

Methaemoglobin solution. Wet crystals of human oxyhaemoglobin, crystallized by the procedure of Drabkin (1950), are dissolved in water. The solution is made to contain approximately 0·2 m-equiv. of haemoglobin/l. (i.e. 3·3 g./l.) on the basis of Drabkin's extinction coefficients for oxyhaemoglobin. An equal volume of 0·4 mM-potassium ferricyanide is added to convert the oxyhaemoglobin completely into methaemoglobin. The methaemoglobin solution is then carefully diluted to $0{\cdot}050 \pm 0{\cdot}004$ mM. This concentration corresponds to the following extinction values calculated from Drabkin's data for methaemoglobin: 0·219 at 578 mμ; 0·218 at 562 mμ; 0·335 at 542 mμ. The solution is dispensed in small tubes and stored at −20°. It is stable for several months under these conditions.

Apparatus

A Beckman DU spectrophotometer is used to follow the peroxidase reaction. The temperature of the cell compartment is maintained at 30° with water circulating from a constant-temperature bath.

Standard estimation procedure

The three reagents are placed in a water bath at 30°. The solution to be tested is mixed with an equal volume of the methaemoglobin reagent. A portion (0·20 ml.) of the mixture is transferred to a cuvette of 1 cm. optical path and 2·75 ml. of the guaiacol reagent is added. The wavelength scale of the spectrophotometer is set at 470 mμ, the absorption maximum for tetraguaiacol, and the slit is opened to a convenient width (approximately 0·3 mm. with the DU spectrophotometer) so that the instrument can be operated at low sensitivity with the stable hydrogen lamp. The spectrophotometer is adjusted to give a reading of zero on the extinction scale with the cuvette containing the

sample in the optical path. The hydrogen peroxide reagent (0·05 ml.) is added from a polyethylene stirrer (a square of polyethylene sheet, 0·9 cm. × 0·9 cm., to which a polyethylene rod is attached centrally), which facilitates rapid addition of the hydrogen peroxide to the cuvette and thorough mixing. At the time of addition of peroxide a stopwatch is started. The extinction scale is set to successively higher values during the course of the peroxidase reaction, and the time at which the galvanometer needle passes zero is recorded for each extinction setting. The settings should be chosen so that, except for samples giving a very low peroxidase activity, the time between readings is of the order of 5–10 sec. A tape recorder is convenient for noting the times. Observations are continued until the rate slows appreciably.

The extinction readings ($\times 10^3$) are plotted against time (see Fig. 1). The slope in extinction units $\times 10^3$/sec. of the linear section of the progress curve is used as a measure of the peroxidase activity. The reaction shows a lag phase which is disregarded in calculating the slope. Samples giving a peroxidase activity of greater than 20 extinction units $\times 10^3$/sec. should be diluted and tested at a lower concentration. The haptoglobin content of the sample, in terms of its haemoglobin-binding capacity, is obtained from the measured peroxidase activity by reference to the calibration curve (Fig. 5).

RESULTS AND DISCUSSION

The method described above provides a rapid and accurate procedure for the estimation of haptoglobin in serum or in purified preparations. The reagents and specific reaction conditions for the standard estimation procedure were chosen on the basis of the following criteria.

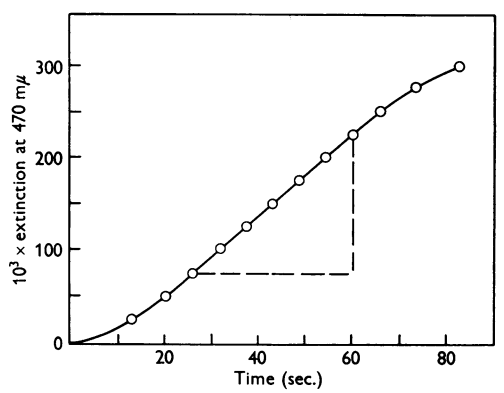


Fig. 1. A typical progress curve obtained during a haptoglobin estimation by the standard estimation procedure. The peroxidase reaction is followed by measuring the extinction at 470 mμ. The observed extinction ($\times 10^3$) is plotted against time (in sec.). The slope, in extinction units $\times 10^3$/sec., of the linear portion of the progress curve (indicated by the broken lines) is used as a measure of the peroxidase activity. The haptoglobin content of the test solution is obtained from this activity by reference to the calibration curve (Fig. 5).

Haemoglobin. Oxyhaemoglobin and methaemoglobin are closely similar in their peroxidase activities when combined with haptoglobin. However, methaemoglobin was selected for the standard estimation procedure because of its greater stability during storage. Although methaemoglobin prepared from crystalline oxyhaemoglobin has been used in the present work, methaemoglobin prepared directly from laked red cells would undoubtedly be satisfactory. The ferricyanide used in preparing the methaemoglobin does not interfere with the estimation of haptoglobin and it need not be removed.

Peroxide. Hydrogen peroxide and ethyl hydrogen peroxide are both satisfactory substrates for the haemoglobin–haptoglobin complex under the conditions described, but hydrogen peroxide is more convenient. The rate of the reaction is very sensitive to the concentration of peroxide (see Fig. 2). The concentration of hydrogen peroxide (0·01 M) for the standard estimation procedure was selected to give suitable reaction rates.

Guaiacol. Guaiacol was chosen as the reducing substrate because, at concentrations greater than 2·5 mM, it proved to be a powerful inhibitor of the peroxidase activity of free haemoglobin (Fig. 3). It also inhibits the activity of the haemoglobin–haptoglobin complexes, but to a much lesser extent. A guaiacol concentration of 27·5 mM in the reaction mixture permits advantageous use of this difference in inhibition.

Experiments with guaiacol at low concentrations, and with other hydrogen donors (e.g. leuco-Malachite green, *p*-toluidine), showed that human serum contains substances which inhibit the formation of the coloured products in the peroxidase reaction, presumably by competing with the added leuco dye. Guaiacol at 27·5 mM is sufficiently concentrated to overcome completely the inhibiting effect of serum. The other hydrogen donors tested are not sufficiently soluble to achieve this effect.

Effect of pH. Polonovski & Jayle (1939) observed that the pH optimum for the peroxidase activity of free haemoglobin is shifted to a more acid pH in the presence of haptoglobin. The pH optimum for free haemoglobin under the present conditions is 5·3, and the reaction rate diminishes rapidly as the solution is made more acidic (see Fig. 4). On the other hand, the peroxidase activity of the haemoglobin–haptoglobin complex increases on the acid side of pH 5·3, reaching its greatest value at pH 4·0. In still more acid solutions the rapid fading of tetraguaiacol prevents accurate measurement of the rate. Experiments with leuco-Malachite green as the hydrogen donor, instead of guaiacol, suggest that the true pH optimum for the complex is near pH 3·0. A pH of 4·0 was selected for the standard estimation procedure since at this pH the

peroxidase activity of free haemoglobin is negligible and the observable activity of its complexes with haptoglobin is nearly maximal.

These effects of pH on the peroxidase activity of haemoglobin and its complexes with haptoglobin suggest that haptoglobin protects haemoglobin from the denaturing effect of acid solutions. Thus, as the pH increases, the peroxidase activity is progressively less affected by the presence of haptoglobin.

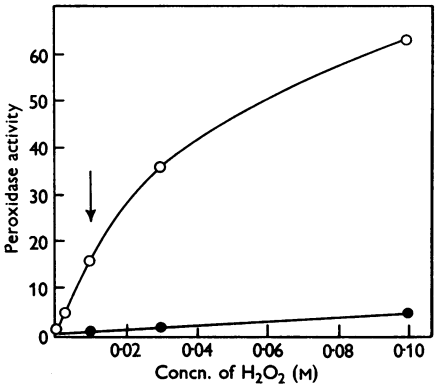


Fig. 2. Effect of hydrogen peroxide concentration on the peroxidase activity of methaemoglobin in the presence (○) and absence (●) of haptoglobin. Standard conditions were used for each determination except that the concentration of hydrogen peroxide was varied. The concentration of hydrogen peroxide selected for the standard estimation procedure is indicated by the arrow.

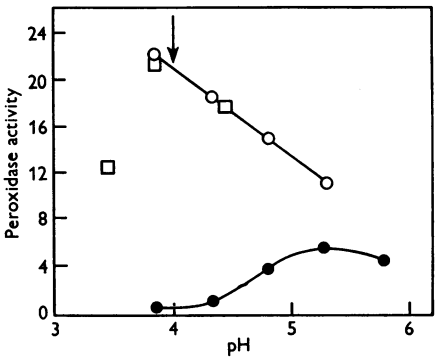


Fig. 4. Effect of pH on the peroxidase activity of methaemoglobin in the presence and absence of haptoglobin. ●, Methaemoglobin alone; ○, methaemoglobin plus haptoglobin, acetate-buffer solutions; □, methaemoglobin plus haptoglobin, lactate-buffer solutions. Standard conditions were used for each determination except that the pH and, where necessary, the buffering anion were varied. The pH selected for the standard estimation procedure is indicated by the arrow.

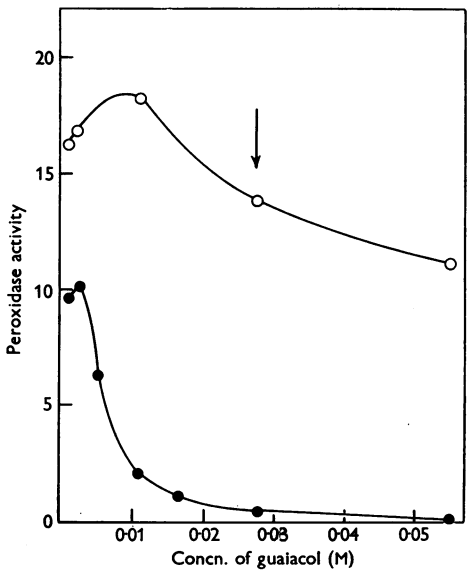


Fig. 3. Effect of guaiacol concentration on the peroxidase activity of methaemoglobin in the presence (○) and absence (●) of haptoglobin. Standard conditions were used for each determination except that the concentration of guaiacol was varied. The concentration of guaiacol selected for the standard estimation procedure is indicated by the arrow.

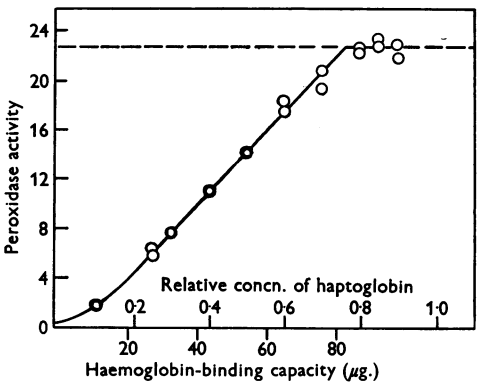


Fig. 5. Effect of increasing amounts of haptoglobin on the peroxidase activity of methaemoglobin measured by the standard estimation procedure. Coincident points are indicated by heavier circles. The haptoglobin solutions used in these experiments were obtained by diluting a solution of purified haptoglobin type 2-2. The upper scale on the abscissa indicates the relative concentration of haptoglobin. The haemoglobin-binding capacity of a given haptoglobin solution can be obtained from its peroxidase activity, measured by the standard estimation procedure, by reference to the lower scale on the abscissa (see text, Part I, Calibration of the method). The horizontal broken line indicates the peroxidase activity of 82·5 μg. of methaemoglobin when fully saturated with haptoglobin (see text).

Calibration of the method. A series of haptoglobin solutions of increasing dilution was prepared from a purified haptoglobin preparation (type 2-2), and the peroxidase activity was determined for each solution by the standard estimation procedure, but with a more accurately standardized haemoglobin solution ($0{\cdot}05 \pm 0{\cdot}0005$ mM) than is normally used. In Fig. 5 the observed activity is plotted against the relative concentration of haptoglobin. Curves identical within experimental error have been obtained with haptoglobins of the other two common types. The curve rises initially and then becomes linear. At high haptoglobin concentrations there is a point of inflexion and the curve becomes parallel to the abscissa. The maximum peroxidase activity obtained, corresponding to this horizontal part of the curve, was found to be independent of the type of haptoglobin used. A mean value of $22{\cdot}5 \pm 0{\cdot}55$ extinction units $\times 10^3$/sec. was obtained for ten samples of haptoglobin, including all three common types (see broken line in Fig. 5).

The meaning of the point of inflexion was established by preparing a similar curve, with only half the standard amount of methaemoglobin being used in the reaction mixture. This curve reached a plateau at approximately half the former maximum activity value. Thus at the point of inflexion all of the added methaemoglobin is present as haptoglobin complex, and further addition of free haptoglobin does not increase the peroxidase activity. This interpretation is confirmed by the results of a starch-gel electrophoresis experiment which showed that the solutions with activities corresponding to the plateau contained no free methaemoglobin.

Since $82{\cdot}5\ \mu$g. of methaemoglobin (0·1 ml. of 0·05 mM-solution) is used in the standard estimation procedure, the amount of haptoglobin corresponding to the inflexion point has a haemoglobin-binding capacity of $82{\cdot}5\ \mu$g. With this point of reference, the lower scale of the abscissa of Fig. 5 was laid out, expressing the amount of haptoglobin in the reaction mixture in terms of its haemoglobin-binding capacity. Thus in order to determine the haptoglobin content of a given solution the peroxidase activity of the solution is first measured by the standard estimation procedure. The haptoglobin content expressed in terms of a haemoglobin-binding capacity is then read from the curve in Fig. 5. Numerically the haemoglobin-binding capacity of the test solution (in μg.) is the same as the amount of haemoglobin (in mg.) which would be bound by 100 ml. of the same solution.

It seems desirable to express amounts of haptoglobin in terms of haemoglobin-binding equivalents because the function of haptoglobin is thought to be related to its ability to bind haemoglobin and to prevent the loss in the urine of haemoglobin liberated into the blood stream (Allison & ap Rees, 1957; Laurell & Nyman, 1957). Furthermore, since haemoglobin solutions of known concentration can be made accurately, haptoglobin determinations in different laboratories can be directly compared, provided that a calibration curve is prepared.

The method described can be applied equally well to serum and to purified preparations of haptoglobin. If a sample of purified haptoglobin is added to serum, the haptoglobin content of the mixture determined by the standard estimation procedure is equal, within experimental error, to the sum of the haptoglobin contents of the original serum and of the purified haptoglobin preparation. Thus the constituents of normal serum do not interfere with the estimation.

Part II. Purification of haptoglobin

Jayle, Boussier & Tonnelat (1956) and Boussier (1958) have described methods of purifying haptoglobins from serum by salt-fractionation and preparative electrophoresis. The present paper describes a procedure involving a single stage of adsorption and desorption by which haptoglobins of high purity uncombined with haemoglobin can be prepared from serum with 40–50 % yields.

METHODS

Dowex 2X-10 (200–400 mesh) anionic-exchange resin*, as the chloride, is used to adsorb the haptoglobins from dialysed serum at pH 4·2. The resin is set up in a column after the adsorption and is washed with water. The haptoglobins are eluted with 0·05 M-NaCl. All steps are carried out at room temperature unless otherwise stated.

Preparation of serum. Small samples of serum were prepared directly from clotted whole blood with precautions to avoid haemolysis. Larger samples were obtained from blood-bank acid–citrate–dextrose (A.C.D.) blood, only bottles in which the plasma appeared to be essentially free from haemolysis being used. The plasma was separated by centrifuging and 3 M-calcium chloride was added in amounts equivalent to the citrate present. The plasma was allowed to clot in centrifuge tubes overnight, and the serum was obtained by centrifuging at 25 000 *g* for 30 min. in a refrigerated centrifuge. Sufficient M-disodium citrate was added to combine with the Ca^{2+} added previously, and the serum was stored at $-20°$ until required.

Adsorption to resin. Sodium acetate buffer is prepared (0·2 M in acetic acid and 0·04 M in sodium hydroxide). The pH is carefully adjusted to 4·2, with a pH meter standardized with 0·05 M-potassium acid phthalate (pH 3·97 at 20°). Serum is mixed with 3 vol. of this buffer and the resulting solution is dialysed against 10 times its volume of water acidified to pH 4·2 with hydrochloric acid. The dialysis is carried out in the cold room with stirring, and the external

* *Note added in proof*: Dowex 2 is not now available in the fine-mesh grade. Dowex 1X-8 (200–400 mesh) appears to be an adequate substitute.

liquid is changed five times at 12 hr. intervals. Thorough dialysis is essential. The final pH should be between 4·2 and 4·7.

The resin is prepared by washing twice in a column with M-sodium hydroxide solution, water, M-hydrochloric acid and water. The last washing with water should be very thorough. One volume of packed resin in water with 0·4 vol. of dialysed serum and 0·6 vol. of water are mixed in a beaker, stirred for 15 min. and then poured into a column for washing and elution. The resin column is washed with water until the extinction of the effluent falls below 0·02 at 280 mμ. 0·05M-Sodium chloride solution is layered on to the top of the column and elution under gravity flow is carried out. A convenient pressure head is obtained when the height of the liquid above the top of the resin is approximately one-fifth of the length of the resin column. The appearance of the sodium chloride front is detected by a simple conductance tip on the outflow of the column (see Fig. 6). Collection of the effluent is commenced as soon as the conductivity begins to increase. The haptoglobin is obtained in the first 2–80 ml. of effluent with applied volumes of dialysed serum of 12–600 ml. Serial fractions are analysed for haptoglobin by the method described above, and for total protein by measuring the extinction at 280 mμ. The maximum concentration of haptoglobin obtained is between two and four times the initial concentration in the undiluted serum. Preparations have been carried out with as little as 1 ml. and as much as 150 ml. of undiluted serum. The yields and purity of the haptoglobin are independent of the amounts of serum used over this range. The resin can be regenerated by treatment with 2M-HCl at 100° for 24 hr. followed by exhaustive washing. Treatment with alkali is unnecessary.

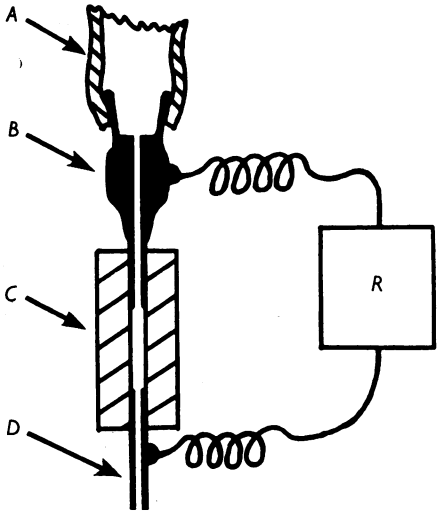


Fig. 6. A simple conductance tip for following the conductivity of column effluents. *A*, Rubber tube connected to outflow of column; *B*, 16-gauge hypodermic needle forming upper electrode of conductance cell; *C*, Perspex block; *D*, part of 16-gauge hypodermic needle forming lower electrode of conductance cell. Needle electrodes *B* and *D* are tightly fitted into a hole drilled through *C*. *R* is a simple resistance meter (model 630, Triplett Electrical Instrument Co., Bluffton, Ohio, U.S.A.).

RESULTS

Table 1 summarizes the results of a haptoglobin preparation from the serum of an individual of haptoglobin type 2-1. In this preparation the yield of purified haptoglobin from the column amounted to 48 % of the haptoglobin present in the starting material. The total recovery (column washings and purified fractions) was 103 % of the haptoglobin of the starting material, so that no inactivation occurred during the purification procedure. The highest specific activity obtained in any fraction of this preparation was 42·7 mg. of haemoglobin-binding capacity/100 ml./$E_{280\,m\mu}$. In several preparations the specific activity of the richest fraction averaged 46·0. Preliminary studies in the ultracentrifuge and by electrophoresis in starch

Table 1. *A typical preparation of purified haptoglobin*

A.C.D. serum (150 ml.), haptoglobin type 2-1, was subjected to the purification procedure described under Methods. Fractions (10 ml.; 1–8) from the column were analysed for haptoglobin content by the standard estimation procedure, and for total protein by measuring the extinction at 280 mμ. Fractions 3 and 4, marked with asterisks, contained so much haptoglobin that their haptoglobin contents were determined on fivefold diluted samples; the figures in the corresponding rows of the table are, however, presented as if the dilutions had not been made. Hb, haemoglobin.

Fraction	$E_{280\,m\mu}$	Slope of progress curve (10^3 E/sec.)	Activity (mg. of Hb-binding capacity/100 ml.)	Specific activity (activity/E)	Total Hb-binding capacity (mg./fraction)
Starting material (diluted dialysed serum)	13·5	3·57	18·1	1·34	108·6
Column washings	4·0	0·57	2·9	0·72	58·0
1	0·02	0	0	—	0
2	1·68	19·3	71·7	42·7	7·2
3*	4·77	49·0	199	41·7	19·9
4*	3·42	29·7	134	39·1	13·4
5	1·76	19·6	72·5	41·2	7·3
6	0·94	8·9	36·7	39·0	3·7
7	0·57	3·7	18·7	33·0	1·9
8	0·33	1·6	8·8	26·4	0·9

gels, of typical preparations of purified type 1-1 haptoglobin, suggest that the best preparations obtained are approximately 80 % pure.

Purified haptoglobins were prepared from sera of the three common haptoglobin types, Hp 1-1, Hp 2-1 and Hp 2-2, and from serum of a fourth type, Hp 2-1 (Mod.), which is rarely seen in whites, but occurs comparatively frequently in negroes (approximately one in ten). This fourth type is referred to as Hp 2-1 (Mod.) because it appears to be a modified form of haptoglobin type 2-1; the rates of migration in starch gel of the individual type 2-1 (Mod.) haptoglobins are the same as those of the type 2-1 haptoglobins, but the relative concentrations of the individual components are different. A photograph of the results of a vertical starch-gel-electrophoresis experiment (Smithies, 1959) with these four preparations is presented in Plate 2, which shows the portion of the gel from the sample slots to the fastest migrating haptoglobin. All the components which are clearly visible in the photograph have been identified as haptoglobins, by showing that they will combine with added haemoglobin. When complexed with haemoglobin the individual haptoglobin components migrate in starch gel less rapidly than in the absence of haemoglobin, but the general appearance of the patterns for the four genetic types is otherwise unchanged.

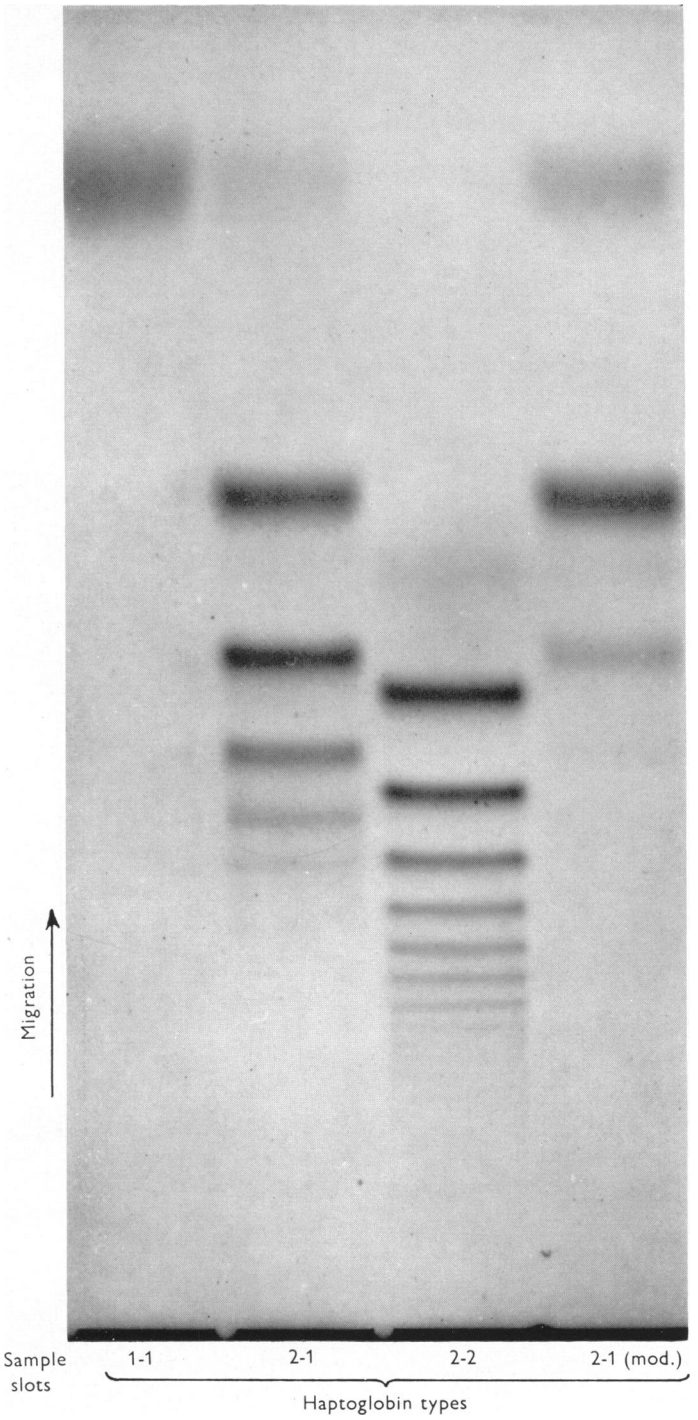
DISCUSSION

The absorption of haptoglobin at pH 4·2 by the positively charged Dowex 2 resin suggests that haptoglobin is a protein with a low isoelectric point. Most serum proteins at this pH have a net positive charge. The method for the preparation thus appears to depend on the selection of a pH at which haptoglobin is the chief serum protein with a net negative charge. This hypothesis receives confirmation from the observation that haptoglobin of progressively greater purity was obtained as the pH of adsorption was decreased from 5·5 to 4·3. Adsorptions below pH 3·9 yielded no haptoglobin. Rabbit haptoglobin has been successfully prepared by the procedure here applied to human haptoglobins, which suggests that haptoglobins in species other than man may be acidic proteins.

Haptoglobins purified by the present procedure are of value for further investigation of their properties only in so far as the haptoglobins are undamaged by the purification. Evidence bearing on this question is provided by the experiment illustrated in Table 1, in which the haptoglobin content of the serum was completely accounted for in the column washings and the purified fractions. No significant loss of haemoglobin-binding capacity occurred. Further evidence is provided by the results illustrated in Plate 2, which show that there are at least seven components in purified haptoglobin of type 2-1 and more than 12 in purified haptoglobin of type 2-2. The number of components seen in these preparations is thus closely similar to the number which can be demonstrated in whole sera of the same genetic type and of equivalent haptoglobin content (cf. Smithies, 1959, serum samples 1 and 2). Furthermore the behaviour in starch-gel electrophoresis of the purified haptoglobins of the several genetic types, both with respect to the mobility and the relative amounts of the different components, is the same as the behaviour of the respective haptoglobins in the sera from which they were obtained. This is particularly well illustrated by the preparation of haptoglobin type 2-1 (Mod.) illustrated in Plate 2. The principal difference between this type and haptoglobin type 2-1 is in the relative amounts of protein in the different haptoglobin zones. The relative proportions which were observed in the original serum of this type were completely preserved in the purified preparation. All the purified haptoglobins retain their ability to bind haemoglobin.

Franglen & Gosselin (1958) described a simple experiment in which a quininoid dye was separated into two components by starch-gel electrophoresis. They established that these two components were in dynamic equilibrium, since either component separated again into two bands when it was isolated from the gel and subjected to a second electrophoresis. It is thus of great importance to establish that the large number of haptoglobin zones are not artifacts of the starch-gel electrophoretic method and that they represent 'real' protein components.

Two approaches to this problem were employed. Serum of haptoglobin type 2-2 was subjected to starch-gel electrophoresis. The regions of the gel containing the main haptoglobin zones were cut out, frozen, thawed and the proteins were obtained in solution by centrifuging (cf. Smithies, 1955). These haptoglobin solutions were kept at 37° for 20 hr., and were subjected to a second electrophoresis alongside the original serum. The individual haptoglobins retained their original electrophoretic characteristics. Storage for several days at 5° of individual haemoglobin–haptoglobin complexes similarly isolated from the gels was without effect on their electrophoretic properties. Further strong evidence for the reality of the many haptoglobin zones in the purified preparations was obtained by observing the behaviour of the preparations during starch-gel electrophoresis under a variety of conditions. The characteristic starch-gel patterns for the three common haptoglobin types were obtained at pH 9·0 in the presence of 8 M-urea, at pH 8·9 in ethylenediaminetetra-acetic acid buffer (0·01 M) and at pH 8·5 with barbiturate buffer of the ionic strength usual for filter-paper electrophoresis.



A photograph of the results of a starch-gel-electrophoresis experiment with four preparations of purified haptoglobin of the genetic types indicated. The portion of gel from the sample slots to the fastest-migrating haptoglobin is shown. The gel was stained with Amido-Black 10B.

Similar patterns, but with mobilities of reversed sign, were obtained at pH 2·1 and 1·7 in dilute hydrochloric acid gels. These results strongly suggest that the many haptoglobin zones demonstrated under the usual conditions of starch-gel electrophoresis, in whole sera and in the purified preparations, are not artifacts but represent distinct protein components.

SUMMARY

1. A procedure for the rapid, accurate estimation of haptoglobin in serum and purified preparations is described, based on the peroxidase activity of haemoglobin in its complexes with haptoglobin.

2. Hydrogen peroxide, guaiacol and methaemoglobin reagents are used, under well-defined conditions which reduce the peroxidase activity of free haemoglobin essentially to zero. The reaction is followed spectrophotometrically.

3. The method is calibrated so that the haptoglobin content of a solution can be expressed in terms of its haemoglobin-binding capacity.

4. Haptoglobin has been prepared in high purity from 1 to 150 ml. of serum in approximately 50% yield, by bulk-adsorption from dialysed serum at pH 4·2 with Dowex 2 X-10 anionic-exchange resin in the chloride form, followed by washing in a column and elution with 0·05 M-sodium chloride solution.

5. Purified haptoglobins from sera of the three common haptoglobin types and from serum of the modified type, Hp 2-1 (Mod.), have been examined by starch-gel electrophoresis.

6. Evidence is presented that the haptoglobins obtained are undamaged by the purification, and that the many individual haptoglobin components demonstrated in the several genetic types are not artifacts.

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The Genetic Control of Transferrins in Humans

By O. SMITHIES and O. HILLER
Connaught Medical Research Laboratories, University of Toronto

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Inherited differences in the serum proteins of normal individuals which are apparently unassociated with red-cell blood groups have been recently described. Smithies (1955) and Smithies & Walker (1956) reported evidence for the genetic control of serum haptoglobins; Grubb (1956) and Grubb & Laurell (1956) showed differences in the serum γ-globulins of different persons and demonstrated the genetic control of these differences. This paper describes the experimental procedures used and the family studies made to establish the existence of a third genetic system involving the serum proteins of normal individuals.

Smithies (1957) observed in the sera of two New York negroes (out of 50 studied) and five Australian aborigines (out of 23 studied) the presence of an additional β-globulin (D), not previously described. Family studies, reported by Horsfall & Smithies (1958), indicated that the presence or absence of β-globulins C and D in the serum was under genetic control. Three phenotypes were observed in the Australian aborigines corresponding to the possible combinations of two alleles at a single autosomal (i.e. not sex-linked) locus. In searching for the occurrence of β-globulin D in the sera of Canadian whites, Smithies (1958) found five