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The Determination of Haptoglobins in Normal Human Serum

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Haptoglobins are proteins which combine with haemoglobin or certain derivatives of haemoglobin. Under certain conditions, combination of haptoglobin with haemoglobin or methaemoglobin enhances the peroxidase activities of the latter substances (Polonovski & Jayle, 1940; Guinaud, Tonnelat, Boussier & Jayle, 1956). This finding has been made the basis of methods for the determination of haptoglobins. Jayle (1951) estimated haptoglobins in serum by measuring the effect of the haemoglobin-haptoglobin complex on the reaction between ethyl hydroperoxide and iodide ions; liberated iodine was determined by titration with thiosulphate. More recently Connell & Smithies (1959) have utilized the reaction between guaiacol and hydrogen peroxide to determine the peroxidase activity of methaemoglobin-haptoglobin complex, measuring the rate of oxidation of guaiacol colorimetrically.

This paper presents observations on the use of the guaiacol-hydrogen peroxide reaction in the determination of haptoglobins and on the concentration of haptoglobins in normal human serum. A preliminary account of certain aspects of the work has already appeared (Owen, Better & Hoban, 1960).

MATERIALS

Guaiacol reagent. Standard reagent (Connell & Smithies, 1959) was prepared as described by Owen, Better & Hoban (1960). In experiments to test the effect of pH, similar reagents were prepared in which the guaiacol concentration was kept constant (3.72 g./l.) and the pH varied with appropriate additions of 0.1 M-sodium acetate (pH 3.2-5.0) or sodium phosphate (pH 6.0-7.0) buffers.

Hydrogen peroxide. This was 0.05 M as described by Owen *et al.* (1960).

Methaemoglobin solution. To 10 vol. of human erythrocytes, previously washed three times with 0.15 M-NaCl, were added 10 vol. of water and 1 vol. of peroxide-free ether. The mixture was shaken for 1 min. and centrifuged,

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and the clear haemolysate was pipetted off. The haemoglobin in the haemolysate was determined colorimetrically as oxyhaemoglobin with a calibration curve constructed from a standard haemoglobin solution supplied by Red Cross Transfusion Service, Sydney. The haemolysate was then diluted until the haemoglobin concentration was 1 g./100 ml. To 25 ml. of this solution was added 10 ml. of potassium ferrieyanide (100 mg./100 ml.) to convert the haemoglobin into methaemoglobin. After 10 min. the volume was made up to 450 ml. The concentration of methaemoglobin was then determined by measurement of the extinction of the solution in a spectrophotometer (Unicam SP. 400) at 500 $m\mu$, with a millimolar extinction coefficient (ϵ mm) of 38.2 (Allison & ap Rees, 1957). This coefficient is based upon haemoglobin; not upon haematin. The solution was then diluted until the concentration was exactly 50 mg./100 ml.

Sera. Normal blood samples were obtained from blood donors of European (Caucasian) descent. The samples were allowed to clot and serum was obtained in the usual way. Sera containing visible amounts of haemoglobin were discarded. Sera were normally diluted 1 in 5 with 0.15M-NaCl before assay.

METHODS

Assay of peroxidase activity. Unless otherwise stated, the calorimetric assay of haptoglobin was carried out as follows: 1 ml. of the diluted serum was mixed with 1 ml. of methaemoglobin solution; 5.0 ml. of guaiacol reagent was then placed in a test tube in a water bath at 25°. After 10 min., 0.1 ml. of the mixture of test solution and methaemoglobin was added. Immediately after, 1.0 ml. of 0.05M-hydrogen peroxide, previously warmed to 25°, was added and the contents of the tube were mixed. After 10 min. the tube was removed from the bath and the extinction of the solution measured in a spectrophotometer (Unicam SP. 1400) at 470 $m\mu$. With 10 mm. diameter matched test tubes the instrument was set to zero with water and readings were made within 1 min. With multiple tubes, timing was such that all tubes were treated similarly. A blank was performed with a mixture of equal volumes of test material and water. A calibration curve was prepared as described by Owen *et al.* (1960). Haptoglobin concentrations have been expressed in terms of bound methaemoglobin.

Electrophoretic analyses. Paper electrophoresis was carried out as described by Owen, Mackay & Got (1959). Starch-gel electrophoresis was carried out essentially as described by Smithies (1955). In determination of the haptoglobin type, haemoglobin was added to sera to give a final concentration of approx. 200 mg./100 ml. A dianisidine reagent (Owen, Silberman & Got, 1958) was used to locate zones containing the haemoglobin-haptoglobin complex after electrophoresis on paper or in starch gel.

RESULTS

Guaiacol reaction

The progress of the reaction under standard conditions and the effect of temperature are shown in Fig. 1. With the standard procedure and with diluted serum as a source of haptoglobin, the

extinction of the reaction mixture reached a maximum at 10 min. and then decreased. At a higher temperature (30°) a lower peak value was reached sooner and was sustained for a shorter period. At a lower temperature (20°) a higher peak value was reached later and was sustained for longer. Fading of the developed colour was accelerated if the tubes were exposed to diffuse bright daylight, but it was not abolished by placing the tubes in the dark.

The effect of adding more of the methaemoglobin-haptoglobin complex to the reaction mixture after the peak extinction had been reached is shown in Fig. 2. In this experiment, an additional amount of the complex equal to that present initially was added. The increment was considerably higher than the first peak.

The increase in sensitivity of the guaiacol reagent on aging is demonstrated in Fig. 3. The reagent was stored at room temperature (about 20°) in diffuse daylight. Storage at 2° decreased the aging effect but did not abolish it.

The effect of pH on the reaction is shown in Fig. 4. The optimum pH for colour development with the methaemoglobin-haptoglobin complex was about 4 whereas the optimum pH for colour development with methaemoglobin was about 6.

The method of using pooled normal serum to construct a calibration curve has been described by

Fig. 1. Effect of temperature on the reaction between guaiacol and hydrogen peroxide in the presence of methaemoglobin-haptoglobin complex. The standard procedure (see text) was used except that readings were made at intervals and various temperatures were employed. Serum contained 95 mg. of haptoglobin/100 ml. Colours were developed in the photometer cuvettes, which were removed from the water bath for 10 sec. for each reading. Room temperature was 23.5°. Δ , 20°; $\circ$, 25°; $\bullet$, 30°.

Owen *et al.* (1960), who give a typical curve. The curve covers the range 0–50 mg./100 ml. Usually sera were diluted 1 in 5 so that the range covered with sera was 0–250 mg./100 ml. If sera gave values within 10% of the peak value a fresh assay was set up by using a higher dilution. When sera diluted 1 in 5 gave values (uncorrected for dilution) below 10 mg./100 ml. they were reassayed without dilution. This uncovered an anomalous effect. Apparent haptoglobin values for such sera varied with the dilution (Table 1). Paper electrophoresis of these sera gave values in agreement with those obtained by the colorimetric procedure without dilution of serum. The dilution effect was only noticeable when sera with low haptoglobin contents were diluted before assay. No dilution effect was noticed when sera with high (i.e. > 40 mg./100 ml.) haptoglobin contents were diluted.

The reproducibility of the method was examined by assaying replicate samples of two sera. The results for the mean $\pm$ standard deviation and coefficient of variation respectively were: serum A (*n* 6) 145 ± 3.6 , 2.5%; serum B (*n* 12) 197 ± 4.1 , 2.1%.

Fig. 2. Destruction of the peroxidase activity of methaemoglobin-haptoglobin complex in the presence of guaiacol and hydrogen peroxide. Standard procedure (see text) was used. Serum contained 95 mg. of haptoglobin/100 ml. Colour was developed in the photometer cuvettes which were removed from the water bath for 10 sec. for each reading. Room temperature was 23.5°. At the time indicated by the arrow a quantity of methaemoglobin-haptoglobin complex, equal to that present initially, was added.

Haptoglobins in normal human serum

Sera were obtained from 152 Caucasian blood donors and the haptoglobin contents determined by the standard procedure. The genetic haptoglobin type was determined by starch-gel electro-

Fig. 3. Effect of age of guaiacol reagent on the reaction between guaiacol and hydrogen peroxide in the presence of methaemoglobin and of methaemoglobin-haptoglobin complex. Standard procedure (see text) was used. The guaiacol reagent had been stored at room temperature (about 20°) for: ○, 1 day; ●, 12 days; ■, 20 days. In each case the reaction was carried out with methaemoglobin alone (haptoglobin, absent), with a mixture of methaemoglobin and methaemoglobin-haptoglobin complex (haptoglobin, 21 mg./100 ml.) and with methaemoglobin-haptoglobin complex alone (haptoglobin, 50 mg./100 ml.).

Fig. 4. Effect of pH on the reaction between guaiacol and hydrogen peroxide in the presence of methaemoglobin (●) and methaemoglobin-haptoglobin complex (○) respectively. Serum contained 95 mg. of haptoglobin/100 ml. Standard procedure was used except that serum was not diluted, i.e. all methaemoglobin was bound to haptoglobin.

Table 1. *Effect of dilution on the apparent haptoglobin content of sera with low haptoglobin contents*

Haptoglobin concentrations are expressed in terms of bound methaemoglobin. Serum A, normal; serum B, from patient with pernicious anaemia. The standard procedure (see text) was employed except that various dilutions of serum were assayed.

Dilution factor	Serum A		Serum B	
	$E_{470\text{ m}\mu}$	Apparent haptoglobin concn. (mg./100 ml.)	$E_{470\text{ m}\mu}$	Apparent haptoglobin concn. (mg./100 ml.)
1	0.087	4	0.143	9
5	0.083	20	0.094	23
10	0.074	32	0.086	37
20	0.065	42	0.074	54
(Blank)	0.045	—	0.051	—

Table 2. *Haptoglobins in normal human serum*

Results are expressed as mg. of bound haemoglobin or methaemoglobin/100 ml.

Author	Method	n	Hapto-globin type*	Haptoglobin concn.		
				Mean	S.D.	Actual range
Present	Peroxidase-guaiacol	152†	All	93	40	4-220
		21	1.1	104	34	21-155
		80	2.1	102	37	28-220
		49	2.2	72	38	14-193
Nyman (1959)	Peroxidase-iodide (Jayle, 1951)	277	All	110	41	20-250
		58	1.1	136	30	50-220
		130	2.1	108	37	30-220
		86	2.2	82	34	20-180
Latham & Worley (1959)	Paper electrophoresis	10	All	128	—	98-171
Latham & Jensen (1959)	Paper electrophoresis	28	All	102	30	68-170
Owen, Mackay & Got (1959)	Paper electrophoresis	50	All	84	—	30-200
Allison (1958)	Starch-gel electrophoresis	—	1.1	120	26	—
		—	2.1	120	26	—
		—	2.2	100	20	—
Jayle & Vallin (1952)	Peroxidase-iodide	1000	All	74†	31	—
Brissaud (1941)	Peroxidase-iodide	—	All	69†	—	26-111

* Nomenclature of Smithies (1955).

† On the assumption that 1 Haptoglobin Index unit corresponds to a haemoglobin-binding capacity of 85 mg./100 ml. (Jayle, 1951).

‡ Includes two sera in which very low haptoglobin concentrations precluded typing.

phoresis. The results are shown in Table 2. The haptoglobin contents of the sera ranged from 4 to 220 mg./100 ml. Two sera contained so little haptoglobin (4 and 11 mg./100 ml.) that it was not possible to determine the haptoglobin type.

Sera from a further 800 Caucasian donors were examined qualitatively for the presence of haptoglobin by means of paper electrophoresis. Haemoglobin was added to the sera before electrophoresis to give a concentration of 200 mg./100 ml. of serum and the stained electrophoretic strips were examined for peroxidase activity in the α_2 -globulin zone. All sera showed some peroxidase activity in this position, though in some the activity was slight. In some sera, a peroxidase area was also

noted in the albumin zone. This was presumably due to the presence of methaemalbumin and was found whenever sera were kept with haemoglobin in excess of the haptoglobin-haemoglobin-binding capacity. Thus methaemalbumin was detected in sera which contained excess of haemoglobin and which had stood at 2° for 12 hr. or at room temperature for shorter periods.

DISCUSSION

The complex nature of the overall reaction between guaiacol, hydrogen peroxide and the methaemoglobin-haptoglobin complex is illustrated in Figs. 1-3. The cessation of colour de-

velopment within minutes is presumably due to destruction of the peroxidase activity of the methaemoglobin-haptoglobin complex, for addition of more complex allows the reaction to proceed again for a while. Inactivation of other peroxidases by hydrogen peroxide is well recognized (Lemberg & Legge, 1949).

The immediate oxidation product of guaiacol is tetraguaiacol. However, this substance is not stable (Maehly, 1954). Reference to Fig. 1 indicates that fading is less at 20° than at higher temperatures, which suggests that this temperature (20°), or lower, would be more suitable for the measurement of haptoglobins. However, in Melbourne the room temperature is frequently higher than 20° and, to avoid the need for a water bath maintained below room temperature, a temperature of 25° was chosen for the standard procedure.

The increased sensitivity of the guaiacol reagent on aging or after some colour has been developed are possibly different expressions of the same phenomenon. Certainly aged guaiacol develops a slight brown tinge. It seems possible that an oxidation product of guaiacol, or of some impurity present, increases the rate of oxidation of guaiacol by hydrogen peroxide under the influence of peroxidase. Whatever the nature of the phenomenon allowance has to be made for it, and for precise work a calibration curve should be constructed each time the method is used.

We cannot at present offer a certain explanation for the dilution anomaly noted with sera containing very low haptoglobin levels though it may possibly be due to the presence of a β -globulin with weak haemoglobin-binding capacity (Neale, Aber & Northam, 1958; Nyman, 1959). We believe, however, that the values obtained for such sera by the colorimetric procedure without prior dilution of the serum are correct, since they agree with the results obtained by the electrophoretic method. Accordingly it is our practice to assay sera diluted initially 1 in 5. Any serum which has an apparent haptoglobin content of less than 50 mg./100 ml. is then reassayed without dilution.

The method described here for the estimation of haptoglobins is simpler than that devised by Connell & Smithies (1959), which requires a temperature-controlled spectrophotometer. Hommes (1959) has described a method involving isolation of the haemoglobin-haptoglobin complex by paper electrophoresis and estimation of its peroxidase activity by a relatively simple method. However, the electrophoresis requires several hours as does the electrophoresis method of Latham & Worley (1959). Jayle's (1951) method is simple and precise but involves the use of ethyl hydroperoxide, a potentially dangerous reagent.

The coefficient of variation of results obtained

with the present method is slightly less than that found by Nyman (1959) using the method of Jayle (1951), i.e. 2.6.

The present method gives values for haptoglobin essentially in agreement with those obtained by other methods (Table 2). Moreover, our findings confirm earlier reports (Allison, 1958; Nyman, 1959) that the serum haptoglobin level, in terms of haemoglobin-binding capacity, depends on the genetic haptoglobin type. The dependence of the mean haemoglobin-binding capacity of sera on the haptoglobin type could be due to differences in the haemoglobin-binding capacity (mg./mg.) of the different molecular species of haptoglobin or to different amounts of haptoglobin. It has been suggested (Allison, 1959) that haptoglobin type 1.1 consists of monomeric forms whereas haptoglobin type 2.2 consists mostly of polymeric forms. If this is correct it is conceivable that, with type 2.2 haptoglobin, steric hindrance would decrease the haemoglobin-binding capacity relative to that of type 1.1 haptoglobin.

Our failure to find any normal serum completely lacking in haptoglobin is of some interest in view of the reports (Sutton, Neel, Binson & Zuelzer, 1956; Allison, Blumberg & ap Rees, 1958; Harris, Robson & Siniscalco, 1958; Kirk, Lai & Hogben, 1960) that a small proportion of Caucasians do not have any serum haptoglobin. However, these authors all used starch-gel electrophoresis whereas in our experience starch-gel electrophoresis, with the filter-paper method of sample insertion (sample volume, 0.02 ml.), constitutes a less sensitive method of detecting haptoglobin than paper electrophoresis. Thus in our hands with (pathological) sera containing less than 10 mg. of haptoglobin/100 ml. and with added haemoglobin it is usually not possible to demonstrate haptoglobin (peroxidase-active) zones after electrophoresis in starch gel, although haptoglobin in concentrations as low as 2 mg./100 ml. is easily detected on paper electrophoresis. Our findings, therefore, together with those listed in Table 2 and other data (Galatius-Jensen, 1958; Moulec & Fine, 1959), indicate that complete ahaptoglobinaemia is rare in Caucasians if indeed it occurs at all.

Assuming that the serum haptoglobin types 1.1 and 2.2 represent homozygous alleles and that type 2.1 is a heterozygous form (Smithies & Walker, 1955; Kirk *et al.* 1960) the gene frequencies in our normal material are: Hp1 gene 40.7%, Hp2 gene 59.3%. In 150 persons this would give an expected approximate distribution of haptoglobin types as follows: type 1.1, 24; type 2.1, 80; and type 2.2, 54. These values do not differ significantly (χ^2 , 1.6; $P > 0.5$) from the observed values (Table 2). The greater incidence of the Hp2 gene in

Caucasians has been reported previously (for references, see Kirk *et al.* 1960).

Note added in proof. Since submitting this paper, we have observed that both caeruloplasmin and a globulin-haem complex (see Nyman, 1960) react with the dianisidine reagent used to detect the haptoglobin-haemoglobin complex after paper electrophoresis. Although caeruloplasmin migrates clearly ahead of the haptoglobin-haemoglobin complex on paper electrophoresis at pH 6.5, the globulin-haem complex and the haptoglobin-haemoglobin complex do not separate under these conditions. Accordingly the finding of a dianisidine reactive area in the α_2 -globulin zone (pH 6.5) does not necessarily indicate the presence of the haptoglobin-haemoglobin complex. Since some of the 800 normal Caucasian sera examined by paper electrophoresis were found to contain methaemalbumin, it is possible that they also contained the globulin-haem complex. Consequently our findings do not necessarily exclude a haptoglobinaemia in these sera.

SUMMARY

1. The peroxidase activities of methaemoglobin and of methaemoglobin-haptoglobin complex on the reaction between guaiacol and hydrogen peroxide have been examined and a method for the determination of haptoglobins in serum was devised.

2. The method has been used to determine the serum haptoglobin concentration in normal human serum. A mean value (as bound methaemoglobin) of 93 mg./100 ml. (s.d. 40) was obtained. The mean serum-haptoglobin concentration varied according to the genetic haptoglobin type.

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The Glycolytic Enzymes of Guinea-Pig Lung in Experimental Bagassosis

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In a previous study of experimental bagassosis Singh, Mitra, Venkitasubramanian & Viswanathan (1960) showed that in this condition there was an appreciable change in the activity of various res-

piratory enzymes and in the Q_{O_2} of guinea-pig lung. Also, V. N. Singh, T. A. Venkitasubramanian & R. Viswanathan (unpublished work) found a remarkable alteration in the chemical composition of