Observations on the Binding of Haemoglobin and Haematin by Serum Proteins in the Rabbit, Rat and Guinea Pig

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When haemoglobin is added to human plasma, it is bound through the globin portion of the molecule to an α_2 -globulin (Jayle, Boussier & Badin, 1952; Nyman, 1959). It has also been suggested that in human sera haemoglobin combines with albumin (Neale, Aber & Northam, 1958) and with a β_1 -globulin (Neale *et al.* 1958; Tombs, 1960), but these complexes could not be found by Allison & ap Rees (1957), Aber & Rowe (1960) or Nyman (1960). Liang (1957) investigated the haemoglobin-binding properties of various animal plasmas and claimed that haemoglobin was bound by an α_2 -globulin in the horse, dog, pig and duck, by albumin in the turtle, by fibrinogen in the ox, and by both an α_2 -globulin and albumin in the frog.

In humans, the reaction between alkaline haematin and plasma proteins differs from that between haemoglobin and plasma protein. Heilmeyer (1933) was the first to suggest that a reaction took place between alkaline haematin and plasma proteins; Fairley (1938, 1941) showed that the reaction was between haematin and serum albumin and he termed the complex methaemalbumin. On electrophoresis, methaemalbumin migrates at the same rate as uncombined albumin (Fišer-Herman & Davorin, 1953). Other complexes besides 'haematin-albumin' have been described, but there is not complete agreement as to their rate of migration on paper electrophoresis. Fišer-Herman & Davorin (1953) describe a haematin-protein complex migrating in the α_2 -globulin region; on the other hand, Neale *et al.* (1958), Aber & Rowe (1960) and Nyman (1960) have all described a haematin-protein complex travelling in the β -globulin. Moreover, Aber & Rowe (1960), using the technique of free-solution electrophoresis, also found haematin-protein complexes migrating in the α_2 - and γ -globulin regions.

The reaction between alkaline haematin and animal plasma proteins has also been investigated. Fairley (1941) found that a complex was formed between rabbit albumin and alkaline haematin, although this complex did not give the characteristic absorption spectrum of human and simian methaemalbumin. Fišer-Herman & Davorin (1953), using paper electrophoresis, found haematin-

protein complexes migrating in the albumin and in the α_2 -globulin region in monkey plasma (*Macacus rhesus*); in the cow and horse they found a complex migrating in the albumin region, in the pig a complex migrated in the α - and β -globulin region, and in the chicken there were complexes in the albumin and α -globulin region.

Further observations are presented here on the binding of both haemoglobin and alkaline haematin by plasma proteins obtained from the rabbit, rat and guinea pig. The investigations were made with the technique of electrophoresis on cellulose acetate paper.

METHODS

Electrophoresis. Electrophoresis was carried out on cellulose acetate paper supported horizontally in air, with a current of 0.4 mA/cm. width of paper. The buffers were (1) barbiturate (*I* 0.05, pH 8.6) made according to the directions of White, Beaven & Ellis (1956), and (2) sodium phosphate (*I* 0.15, pH 6.4).

Nomenclature of proteins. The protein bands revealed by paper electrophoresis have been named on the basis of comparison with those of human serum. In the rabbit, a band migrating at the same rate as the β_1 -globulin band of human serum has also been termed the β_1 -globulin band. In the rat and the guinea pig, there is a band migrating at the same rate as the β_2 -globulin of human serum and this has been termed the β_2 -globulin band in these animals. All bands between the albumin and β_1 -globulin have been termed α -globulins; in the rabbit and rat there are two bands, termed α_1 - and α_2 -globulin, and in the guinea pig three bands, termed α_1 -, α_2 - and α_3 -globulin.

Establishment of the combining capacities of protein. The method of Nyman (1959) was used. This consists in adding increasing amounts of haemoglobin to constant volumes of serum and determining by paper electrophoresis the concentration of haemoglobin which just saturates the haptoglobins. Haemoglobin was added to give concentrations rising by 10 mg./100 ml. The haemoglobin present in the original serum was also determined and added to the calculated haemoglobin concentration. The result was then expressed to the nearest 5 mg./100 ml. Haemoglobin was stained with dianisidine by the method of Owen, Silberman & Got (1958) and proteins were stained with Ponceau S.

Estimation of the rate of turnover of the haemoglobin-haptoglobin complex. A rabbit whose total haemoglobin-binding capacity was estimated to be 40 mg. was injected with 60 mg. of haemoglobin. Plasma samples were obtained at frequent intervals over the following 24 hr. and

estimates of the haemoglobin-binding capacity in the presence of excess of haemoglobin made as follows. Two 20 μ l. volumes of serum were placed side by side on Whatman no. 1 paper, and electrophoresis was carried out with phosphate buffer. The site of the haptoglobin-haemoglobin complex was determined by staining one of the electrophoretograms, and the complex eluted from the other by the method of Dent (1947) with 2 ml. of phosphate buffer. The haemoglobin content of the complex was estimated with dianisidine: to the eluate was added 1 ml. of acetic acid, 1 ml. of 0.1% (w/v) dianisidine in 10% (v/v) acetic acid, and 1 ml. of H_2O_2 (10 vol.). The extinction was determined at 460 $m\mu$ after 30 min. A solution of known haemoglobin concentration was used as a standard. The method gave a linear relationship between haemoglobin concentration and extinction and was not affected by the presence of phosphate or barbiturate. The factor normally present in serum which inhibits the colour development was not present in the eluates. The total plasma haemoglobin was also determined, with benzidine, and the free haemoglobin content calculated from the difference between the total and the combined haemoglobin.

Alkaline haematin was prepared by the method of Gatterman (1941).

RESULTS

Rabbit. A haemoglobin-haptoglobin complex migrated in the same position as the α_2 -globulins (Fig. 1). No combination with albumin was detected with haemoglobin concentrations up to 400 mg./100 ml. The haemoglobin-combining capacities of the complex in the α_2 -globulin region in seven adult rabbits (lop-eared variety) were 10, 15, 25, 25, 35, 45 and 45 mg. (average 30 mg.)/100 ml. of serum. Haematin in low concentrations formed a complex which migrated in the α_2 -globulin region,

and in higher concentrations a complex was also formed which migrated with albumin. In one experiment, haemoglobin saturated the complex in the α_2 -region when the concentration reached 6 mM, whereas haematin saturated the complex at a concentration of 25 mM, that is, approximately four times the concentration required for saturation with haemoglobin. If just sufficient haemoglobin was added to serum to saturate the haptoglobin, then the further addition of haematin displaced the haemoglobin which appeared as free haemoglobin in the β -globulin region (Fig. 1).

The results of the experiment on the rate of turnover of the haemoglobin-haptoglobin complex in the rabbit are shown in Fig. 2. The initial half-time of removal of the complex was 40 min., and the free haemoglobin was also removed at approximately the same rate. Haptoglobin rapidly reappeared in the plasma and was raised above its original level 24 hr. later.

Rat. In sera obtained from Wistar rats, the haemoglobin-haptoglobin complex migrated with the α_2 -globulins. Six estimates of the haemoglobin-combining capacity gave values of 25, 30, 30, 30 and 50 mg./100 ml. of serum. On the addition of small quantities of haematin, a haematin-protein complex was found to migrate in the β_1 -globulin region: at higher concentrations, a complex was also found migrating with albumin (Fig. 3). All the rat sera examined gave some staining with dianisidine in the α_2 -globulin band, but

Fig. 1. Electrophoresis of rabbit sera in barbiturate buffer, pH 8.6. (a) Stained with Ponceau S; (b), (c), (d) and (e) stained with dianisidine. (a) Rabbit serum alone. (b) Rabbit serum with haemoglobin (final concentration, 100 mg./100 ml.). The free haemoglobin is in the β_2 -globulin position. (c) Rabbit serum with haematin (final concentration, 1 mg. and 3 mg./100 ml.). (d) Rabbit serum with haemoglobin in a concentration insufficient to saturate the haptoglobin. (e) The haemoglobin-serum mixture used for (d) with the addition of haematin (3 mg./100 ml.). The haemoglobin which was attached to the α_2 -globulin has been displaced by the haematin and is in the β_2 -globulin position. Hb, Haemoglobin; α_1 , α_2 , β_1 , β_2 and γ , corresponding globulins.

Fig. 2. Haptoglobin-haemoglobin (●) and free haemoglobin (○) in the serum of a rabbit after the injection of haemoglobin in excess of the haptoglobin-binding capacity.

since the addition of haematin did not appear to intensify the colour, it was considered that there was no haematin-protein complex migrating in the α_2 -globulin region.

Guinea pig. When haemoglobin was added to guinea-pig serum, a haemoglobin-haptoglobin was first seen to migrate with albumin (Fig. 4). When the haemoglobin concentration was raised further, some of the sera showed a dianisidine-staining band in the β -globulin region. Although free haemoglobin also migrates in this region, this probably does not represent free haemoglobin as, on raising the haemoglobin concentration still further, another complex appears which migrates in the α_3 -globulin region. When electrophoresis was carried out in phosphate buffer (pH 6.4) no staining appeared in the β -globulin region. It is possible that the complex migrating in the β -globulin region at pH 8.6 might migrate at pH 6.4 either with free haemoglobin or with the complex in the α_3 -

globulin region and thus be obscured. With phosphate buffer the binding capacities in the albumin region were 30, 50 and 50 mg./100 ml. of serum, and those of the complex travelling with the α_3 -globulins was 20, 20 and 40 mg./100 ml. of serum in the three sera examined.

When haematin was first added to sera, a complex was formed which ran with albumin. Raising the concentration of haematin gave next a complex migrating in the β -globulin region; finally a complex appeared in the α_3 -globulin region. In one serum a further complex was found in the α_1 -globulin region.

When haemoglobin is added to human sera, haematin can be produced (Fairley & Bromfield, 1937). It is thus possible that the dianisidine-staining complexes seen after addition of haemoglobin to guinea-pig sera are really haematin-protein complexes. However, the complex migrating in the albumin region after addition of haemoglobin was slightly slower than the complex produced by the addition of haematin. Similarly, the haemoglobin complex in the α_3 -globulin region ran slightly slower than the haematin complex. This suggests that haemoglobin and haematin form two distinct complexes. The complexes seen in the β -globulin region after addition of haemoglobin and haematin both ran at the same speed; there is thus the possibility that haemoglobin could be partially broken down to give a haematin-protein complex migrating in this region.

Fig. 3. Electrophoresis of rat serum in barbiturate buffer, pH 8.6. (a) Serum stained with Ponceau S. (b) Serum with the addition of haemoglobin (final concentration, 50 mg./100 ml.). (c) Serum alone. Some haemoglobin is present owing to haemolysis before separation of the serum. (d) The same serum as used in (c) with the addition of haematin (3.0 mg./100 ml.). (b), (c) and (d) were stained with dianisidine. For abbreviations see Fig. 1.

Fig. 4. Electrophoresis of guinea-pig serum in barbiturate buffer, pH 8.6. (a) Serum alone, stained with Ponceau S. (b) Serum with the addition of haemoglobin. (c) Serum with the addition of haematin (final concentration, 3 mg./100 ml.). (b) and (c) were stained with dianisidine. For abbreviations see Fig. 1.

DISCUSSION

The observations made by Fišer-Herman & Davorin (1953) and Liang (1957) have shown that there is considerable variation between animals and man in the ability of their plasma proteins to bind haemoglobin and haematin. The findings presented here supplement these observations. Of the sera obtained from the rabbit, rat and guinea pig, only that of the rat shows properties similar to human sera. As in human sera, the haemoglobin-haptoglobin complex of the rat migrates with the slow α -globulins only, and a haematin-protein complex migrates in the β -globulin and albumin regions. Aber & Rowe (1960) found in human sera that there was a tighter binding between the haematin and protein of the complex migrating in the β -globulin region than between the haematin and protein migrating in the albumin region; similarly, in the rat, the addition of haematin forms a complex in the β -globulin region before forming a complex in the albumin region, suggesting a tighter binding with the former than with the latter.

In the rabbit, a haematin-protein complex has been found which migrates with albumin. This supports the finding of Fairley (1941), who demonstrated a combination between albumin and

haematin by ultracentrifugal analysis. The haemoglobin-haptoglobin complex in the rabbit migrates in the α_2 -region, as in humans. In humans the linkage in the haemoglobin-haptoglobin complex is between the globin and haptoglobin (Nyman, 1959) whereas in the rabbit there are two observations which suggest that the linkage may be through the haem portion of the molecule. First, the protein appears to bind four times as many haematin as haemoglobin molecules, suggesting that each binding site can take either four free haematin molecules or four haematin incorporated in a haemoglobin molecule. Secondly, the addition of haematin to a serum containing the haptoglobin-haemoglobin complex in the α_2 -globulin region always resulted in the appearance of a dianisidine-staining compound in the position where free haemoglobin migrates, suggesting that free haematin displaces haemoglobin from the binding sites on the protein, and thus that the haemoglobin binds through haematin and not through globin. A further difference between human and rabbit haptoglobin is that rabbit haptoglobin is replaced within a few hours after its removal as haemoglobin-haptoglobin complex, whereas human haptoglobin takes several days to be replaced (Laurell & Nyman, 1957).

Guinea-pig sera differ from human sera in that the complex first formed on the addition of both haemoglobin and haematin migrates with the albumin. There is also the possibility that a haemoglobin-haptoglobin complex migrates in the β -globulin region, although, as discussed above, the dianisidine-staining material in this region could be a haematin-protein complex.

In connexion with the finding that haematin forms complexes migrating in the α -globulin region in the rabbit and guinea pig, Aber & Rowe (1960) found a complex migrating with the α -globulins and another with the γ -globulins in humans. This was only seen if the sera were examined by free electrophoresis; they could not be demonstrated on filter-paper electrophoresis, as the haematin was preferentially absorbed by the paper.

The results presented here on the binding of haemoglobin in the rabbit and rat do not agree with those of Liang (1957), who did not find any binding of haemoglobin by the proteins of plasma from rabbits and guinea pigs in Hong Kong. Murray & Connell (1960) also examined rabbit sera and found that they had an average binding capacity of only 10 mg./100 ml. of serum, which is distinctly lower than that of the rabbits examined in the present work. It is possible that there are considerable variations between different strains of rabbits and guinea pigs.

SUMMARY

1. The complexes formed by the addition of haemoglobin and haematin to sera obtained from the rabbit, rat and guinea pig have been investigated by paper electrophoresis.

2. In the rabbit, a haemoglobin-haptoglobin complex migrates with the α_2 -globulins and a haematin-protein complex migrates with albumin and with the α_2 -globulins.

3. In the rat, a haemoglobin-haptoglobin complex migrates with the α_2 -globulins and a haematin-protein complex migrates with albumin and with the β_1 -globulins.

4. In the guinea pig, haemoglobin-haptoglobin complexes migrate with albumin and α_3 -globulin; in some sera, a complex probably also migrates with the β -globulins. Haematin-protein complexes migrate with albumin, α_3 -globulin and β -globulin.

5. In the rabbit, the haemoglobin-haptoglobin complex was removed from the plasma with a half-time of 40 min.

REFERENCES

- Aber, G. M. & Rowe, D. S. (1960). *Brit. J. Haematol.* **6**, 160.
- Allison, A. C. & ap Rees, W. (1957). *Brit. med. J.* **ii**, 1137.
- Dent, C. E. (1947). *Biochem. J.* **41**, 240.
- Fairley, N. H. (1938). *Nature, Lond.*, **142**, 1156.
- Fairley, N. H. (1941). *Quart. J. Med. (New Ser.)*, **10**, 115.
- Fairley, N. H. & Bromfield, R. J. (1937). *Trans. R. Soc. trop. Med. Hyg.* **28**, 307.
- Fiser-Herman, M. & Davorin, P. (1953). *Biochem. Z.* **324**, 96.
- Gatterman, L. (1941). *Laboratory Methods of Organic Chemistry*, p. 407. London: Macmillan and Co. Ltd.
- Heilmeyer, L. (1933). *Medizinische Spektrophotometrie, Jena*, p. 117.
- Jayle, M. F., Boussier, G. & Badin, J. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 1063.
- Laurell, C. B. & Nyman, M. (1957). *Blood*, **12**, 493.
- Liang, C. C. (1957). *Biochem. J.* **66**, 552.
- Murray, R. K. & Connell, G. E. (1960). *Nature, Lond.*, **186**, 86.
- Neale, F. C., Aber, G. M. & Northam, B. E. (1958). *J. clin. Path.* **11**, 206.
- Nyman, M. (1959). *Scand. J. clin. lab. Invest.* **11**, suppl. 39.
- Nyman, M. (1960). *Scand. J. clin. lab. Invest.* **12**, 121.
- Owen, J. A., Silberman, H. J. & Got, C. (1958). *Nature, Lond.*, **182**, 1373.
- Tombs, M. P. (1960). *Nature, Lond.*, **186**, 1055.
- White, J. C., Beaven, G. H. & Ellis, M. (1956). *Ciba Foundation Symp., Paper Electrophoresis*, p. 43.