Ferrihaemoglobinaemia * (Methaemoglobinaemia) in Man and the Horse

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(Received 26 September 1944)

Ferrihaemoglobin has been known for many years to occur in blood under a great variety of pathological and experimental conditions, but only recently has the suggestion been made that small amounts may also be present normally. The matter, however, is still a subject for controversy. Amundsen (1941) has observed in 40% of 82 normal human blood specimens that the carbon monoxide capacity of the fresh blood was less than the CO capacity after reduction with $\text{Na}_2\text{S}_2\text{O}_4$, by amounts which varied up to 2.5 vol. %. The discrepancy, it is suggested, may be due to the presence of ferrihaemoglobin. These figures are supported by those of Havemann, Jung & Issekutz (1939), in whose hands spectrophotometric methods have shown that in healthy persons up to 8% of the total blood pigment may be present as ferrihaemoglobin. On the other hand, Cox & Wendel (1942), using a method (Wendel, 1938) which is claimed to detect ferrihaemoglobin spectroscopically when its concentration exceeds 4% of the total pigment, deny the normal existence of ferrihaemoglobin in the blood of man and numerous other species. In the case of man, this is understandable, since only in a few individuals have other workers found the proportion to be above 4%.

This paper comprises the results of a gasometric investigation of normal human and horse blood, with a view to the provision of further evidence on the point at issue. Ferrohaemoglobin has been determined, by means of its oxygen capacity, in all the specimens of blood. 'Total haemoglobin' (a phrase used here to indicate the sum of ferrohaemoglobin and ferrihaemoglobin, but excluding carbon monoxide haemoglobin) has been determined, again in all the specimens, by a technique which is also based on the oxygen capacity of ferrohaemoglobin. This procedure, developed by various workers from the original of Conant & Fieser (1924-5), is believed to be sounder than any involving the use of CO, since oxygen is a more specific reagent for ferrohaemoglobin than is CO. The latter combines not only with ferrohaemoglobin and a number of haemochromo-

gens, but also with a series of compounds intermediate between these and the bile pigments (Lemberg, Legge & Lockwood, 1941). A significant difference between ferrohaemoglobin and 'total haemoglobin' may be regarded as being due to the presence of ferrihaemoglobin, especially as the technique for the determination of 'total haemoglobin' does not embrace carbon monoxide haemoglobin.

METHODS

Collection of blood

Since ferrihaemoglobin can act as a hydrogen acceptor in certain enzymic oxidations (Warburg, Kubowitz & Christian, 1930 *a, b*), all blood specimens have been drawn into flasks surrounded with ice and containing powdered potassium oxalate as anti-coagulant. The rapid cooling should inhibit enzymic reduction of ferrihaemoglobin almost completely.

Analytical methods

Ferrohaemoglobin determinations. These have been made by the standard oxygen capacity technique (Van Slyke & Neill, 1924).

Iron determinations. These have been made either colorimetrically with thioacetic acid and ammonia, or, more recently, by the more accurate titanous sulphate method of Ramsay (1944).

'Total haemoglobin'. This has been estimated by a modification of the procedure of Conant, Scott & Douglass (1928). It seems desirable to report the modified method in full, because it is somewhat simpler, and no less accurate than the original. The main advantages are that the determination can be carried out on a much smaller sample of blood, and entirely in the extraction chamber of the Van Slyke manometric apparatus. The principle remains unaltered: determination of oxygen capacity after reduction with a faintly alkaline titanium solution.

Reagents. (1) TiCl_3 : commercial 15-20% TiCl_3 diluted with an equal volume of *N*-HCl, and stored in the usual way under hydrogen. It is convenient to include a burette in the permanent set-up.

(2) Alkaline citrate: 0.1M-citric acid containing such a quantity of NaOH that, when 9 ml. are mixed with 1 ml. TiCl_3 , the pH of the mixture lies between 7 and 8. The quantity of NaOH required varies according to the acidity of different commercial preparations of TiCl_3 . Conant used a similar solution containing tartrate, borate and alkali, but the author prefers the citrate, which is easier to prepare and to use.

(3) 'Titanous citrate': a solution prepared not more than an hour or so before use (and preserved from air under

* In order to avoid confusion which might otherwise ensue, the terms ferrihaemoglobin and ferrohaemoglobin are used throughout this paper instead of methaemoglobin and haemoglobin (cf. Coryell, Stitt & Pauling, 1937).

liquid paraffin) by diluting 1 ml. TiCl_3 to 10 ml. with the alkaline citrate solution. The pH of such mixtures should be checked occasionally, but it is worth noting that round about pH 8 'titanous citrate' solutions change colour from mauve to greenish black. Solutions of ferro- and ferrihaemoglobin, moreover, do not seem to be denatured by alkali unless the pH exceeds 10 (Conn, Kistiakowsky & Roberts, 1940).

(4) 0.5% $\text{K}_3\text{Fe}(\text{CN})_6$ containing 0.1% octanol and either 0.3% white saponin or 0.25% Lissapol C. (a long-chain sulphonic acid detergent kindly supplied by Imperial Chemical Industries Ltd.).

(5) NaOH and $\text{Na}_2\text{S}_2\text{O}_4$ for the absorption of CO_2 and oxygen (Peters & Van Slyke, 1932).

Procedure. This description should be intelligible to anyone who is familiar with the manipulation of the Van Slyke manometric apparatus. 1 ml. blood or pigment solution is admitted to the extraction chamber from a rubber-tipped 1 ml. pipette, and followed by 1 drop octanol. The chamber is evacuated, and shaken gently for 3 min., in order to remove oxygen, which might otherwise interfere with the subsequent reduction. The vacuum is released, the liberated gases are expelled, and the extraction is repeated. If much ferrohaemoglobin is present, the colour of the solution at this point suggests that most of the oxygen has been removed. On the other hand, solutions containing a very high proportion of ferrihaemoglobin contain little oxygen, and the preliminary extraction may be omitted. Experiment has shown that small proportions of carbon monoxide haemoglobin remain unaffected by this extraction of gases.

A few ml. water are poured into the cup, and 0.25 ml. 'titanous citrate' is run into the chamber from a rubber-tipped, subdivided 1 ml. pipette. The stopcock is washed through by running in 0.25 ml. water, and the chamber is again evacuated. It is shaken gently for 5 min., during which period ferrihaemoglobin is reduced to ferrohaemoglobin. After the reduction the vacuum is partially released by the admission of mercury, and air is then allowed to fill the extraction chamber at atmospheric pressure. The apparatus is next shaken briskly for 5 min., to ensure the complete oxygenation of all ferrohaemoglobin. At the same time the excess titanium is oxidized to the inert titanate state, a process which occurs very rapidly in the presence of air. When oxygenation is complete, all gases are expelled from the chamber and the cock between the chamber and the cup is closed. 3 ml. $\text{K}_3\text{Fe}(\text{CN})_6$ solution (previously freed from dissolved oxygen by extraction in the Van Slyke apparatus and stored, if necessary, under liquid paraffin for a few hours) are run into the cup, and 2 ml. are admitted to the extraction chamber. The cock is then sealed with mercury. The chamber now contains 3.5 ml. fluid, and the remainder of the analysis is precisely similar to the usual oxygen determination. When the calculation is made, however, there is a small difference. This arises from the fact that to each 1 ml. of pigment solution there has been added 0.25 ml. titanous citrate and 0.25 ml. water before equilibration with oxygen. This means that the 'c' correction must be larger than that used in the straightforward oxygen determination, because it must include oxygen dissolved physically in the additional 0.5 ml. solution. It is necessary to determine the 'c' correction accurately, using 0.25 ml. titanous citrate, 0.25 ml. water (these being equilibrated with air by mixing and shaking in the extraction chamber) and 3 ml. air-free $\text{K}_3\text{Fe}(\text{CN})_6$. Variations in temperature should theoretically cause fluctuation of the

correction, but in practice this is so slight as to be negligible.

The method has been tested on ferrihaemoglobin solutions prepared from crystallized horse oxyhaemoglobin by oxidation with nitrite or ferricyanide, followed by dialysis against distilled water. If concentrated solutions are required, precipitation must be prevented by dialysis against 0.1M- Na_2HPO_4 or 0.1M- $\text{Na}_2\text{B}_4\text{O}_7$. All dialyses have been done at about 2°. There is no apparent difference in behaviour between preparations made with ferricyanide and those made with nitrite. No independent accurate method of determining ferrihaemoglobin has been available, so that it has been rather difficult to check the accuracy of the results, but the oxygen capacity after reduction has been compared with the protein-iron content (iron precipitated by trichloroacetic acid).

RESULTS

All figures quoted here have, where necessary, been corrected for dissolved oxygen in accordance with the recommendations of Sendroy, Dillon & Van Slyke (1934).

Table 1 gives the results of the determination of total haemoglobin in 'pure' ferrihaemoglobin solutions, and a comparison of the oxygen capacities found with the protein-iron contents of the solutions. The analytical results show clearly that the procedure described is satisfactory for the determination of ferrihaemoglobin.

Table 1. *Analysis of 'pure' ferrihaemoglobin solutions*

Reagent*	Total haemoglobin (vol. %)	Fe (mg./100 ml.)	ml. O_2 /55.84 mg. Fe
$\text{K}_3\text{Fe}(\text{CN})_6$	4.48, 4.57, 4.62 4.52, 4.51, 4.61	11.4	22.3
NaNO_2	8.41, 8.51	21.7	21.8
NaNO_2	9.79, 9.74	24.0	22.7

* Reagent used for preparation of ferrihaemoglobin.

Ferrohaemoglobin and total haemoglobin have been determined in samples of blood from 38 normal human beings, all from males except no. 16, which was from a female. Table 2 gives the results of these analyses, with the differences between ferrohaemoglobin and total haemoglobin calculated as vol. O_2 % and also as percentages of the total haemoglobin present. The accuracy of the analyses does not warrant the calculation of the percentages more closely than to the nearest 0.5%. In Table 3 are given the results of similar analyses made on 17 samples of blood from apparently normal horses. All determinations, except five marked with an asterisk in the tables, were made at least in duplicate, and some of the more surprising figures in Table 3 were confirmed by triplicate or even quadruplicate analysis.

Table 2. *Ferrohaemoglobin and total haemoglobin content of human blood*

Subject	Ferro-haemoglobin (vol. %)	Total haemoglobin (vol. %)	Difference	
			Vol. %	% of total haemoglobin
1	16.86	17.06	0.20	1
2	19.55	20.93	1.48	7
3	20.05	20.52	0.47	2.5
4	20.92	21.17	0.25	1
5	20.20	21.04	0.84	4
6	20.37	21.03	0.66	3
7	19.73	20.62*	0.89	4.5
8	19.40	19.96	0.56	3
9	20.49	20.37	-0.12	-0.5
10	20.81	21.39	0.58	2.5
11	19.62	20.25	0.63	3
12	21.37	21.62	0.35	1.5
13	20.74	21.55	0.81	3.5
14	19.68	19.68	0.00	0
15	19.89	20.06	0.17	1
16	15.42	15.94	0.52	3
17	19.99	20.28	0.29	1.5
18	19.78	19.85	0.07	0.5
19	21.03	21.06	0.03	0
20	20.11	20.45*	0.34	1.5
21	21.93	21.85	-0.08	-0.5
22	20.80	20.80*	0.00	0
23	20.53	20.56	0.03	0
24	20.17	20.33	0.16	1
25	21.10	21.13	0.03	0
26	20.40	20.93	0.53	2.5
27	20.62	21.33	0.71	3.5
28	20.26	20.22	-0.04	0
29	19.30	19.64	0.34	1.5
30	19.52	20.62	1.10	5
31	19.49	19.46	-0.03	0
32	21.85	22.07	0.22	1
33	19.13	19.70	0.57	3
34	20.07	21.32*	1.25	6
35	19.08	19.88	0.80	4
36	20.36	20.94	0.58	3
37	21.80	21.88	0.08	0.5
38	20.42	20.63	0.21	1

* These determinations were not made in duplicate.

Table 3. *Ferrohaemoglobin and total haemoglobin content of horse blood*

Horse	Ferro-haemoglobin (vol. %)	Total haemoglobin (vol. %)	Difference	
			Vol. %	% of total haemoglobin
1	14.34	14.80	0.46	3
2	14.73	19.82	5.09	25.5
3	19.76	20.36	0.60	3
4	17.56	21.35	3.79	17.5
5	17.00	18.54	1.54	8
6	14.81	15.55	0.74	4.5
7	14.76	15.90	1.14	7
8	16.94	17.12*	0.18	1
9	17.99	19.37	1.38	7
10	16.19	17.14	0.95	5.5
11	17.45	18.56	1.11	6
12	14.75	15.18	0.43	3
13	15.74	15.76	0.02	0
14	16.40	17.21	0.81	4.5
15	15.09	17.28	2.19	12.5
16	16.14	18.06	1.92	10.5
17	16.87	16.96	0.09	0.5

* This determination was not made in duplicate.

DISCUSSION

The standard error of a single oxygen determination, either before or after reduction with 'titanous citrate', is about 0.06 vol. %. It has therefore seemed statistically sound to conclude that differences between ferrohaemoglobin and total haemoglobin of not less than 1.5 %, or about 0.3 vol. %, may be considered real. It is believed that there are no chemical or manipulative grounds to invalidate this conclusion, and, in any case, many of the differences, particularly in the equine analyses, are so much greater than this limit that there can be no question of their reality. The few negative differences recorded in Table 2 are well within the limits of error, and may therefore safely be ignored.

If the above statistical standard be accepted, it then appears that in 21 human cases out of 38, and 14 equine cases out of 17, a significant difference has been found between ferrohaemoglobin and total haemoglobin. Bearing in mind the basis of the analytical methods employed, this is *prima facie* evidence for the presence, in certain cases, of ferrihaemoglobin, but conclusive proof is lacking. The figures are supported by the similar ones of Ammundsen (1941), which were obtained by a less specific method, and both sets bear out the contention of Havemann *et al.* (1939) that human blood may contain ferrihaemoglobin. There remain the contrary findings of Cox & Wendel (1942), which are difficult to reconcile with those of the other workers. It is true that in none of his samples has the present author been able to demonstrate the ferrihaemoglobin absorption band at about 630 m μ , but it is now generally admitted that spectroscopic examination for this band is far from being a sensitive test for ferrihaemoglobin in the presence of much oxyhaemoglobin (cf. Campbell & Morgan, 1939). Furthermore, it has not so far been possible in this laboratory to make the procedure of Wendel (1938) nearly as sensitive as that author claims.

One of the most striking observations that has been made in these experiments is that there is a marked quantitative difference between man and the horse. The percentage of horse bloods showing a significant difference between ferrohaemoglobin and total haemoglobin (about 80 %) is greater than that of human bloods (55 %). Moreover, the average difference in such samples between ferrohaemoglobin and total haemoglobin is very much greater (horse 1.58 vol. %, human 0.68 vol. %). Further work on the differences is therefore much easier in the case of horse bloods, and it is mainly as a result of additional work on that species that additional evidence (described below) has been accumulated.

Iron determinations have been made on many of the blood specimens analyzed, and in every case the amount of oxygen bound by 55.84 mg. Fe

approaches the theoretical value of 22.4 ml. much more closely after reduction with Ti than before. In Table 4 are given some characteristic figures. These are consistent with the hypothesis that some of the iron in horse blood is present in the ferric state, and that it does not combine with oxygen until it has been reduced to the ferrous condition. Similar results have been obtained with human blood, although they are hardly conclusive, since the initial differences between ferrohaemoglobin and total haemoglobin are so much smaller.

Table 4. *Comparison of oxygen capacity of horse blood with its iron content*

Horse	ml. O ₂ bound by 55.84 mg. Fe	
	Before reduction	After reduction
2	16.5	22.1
3	21.2	21.9
4	17.6	21.4
14	21.3	22.6
15	19.4	22.2
16	19.5	21.9

It has, moreover, been shown by Campbell & Morgan (1939) and by Cox & Wendel (1942) that ferrihaemoglobin formed in blood experimentally or during sulphonamide therapy may disappear spontaneously when the blood is shed. During the present work several opportunities have occurred for checking the oxygen capacity of horse blood for hours and even days after withdrawal. The value has been found to increase until it approaches closely that obtained from the fresh blood after reduction with 'titanous citrate'. For example, horse blood no. 15 showed originally an oxygen capacity of 15.09 vol. % (mean of three estimations made between 30 and 75 min. after withdrawal), while, after 30 hr. at 2°, the value had risen to 17.24 vol. %. The total haemoglobin corresponded initially to an oxygen capacity of 17.28 vol. %, and remained virtually unchanged. Although this experiment was done at the temperature of the refrigerator, it has been found that increasing the

temperature up to 15, 25 or 37° has no apparent effect on the increase in oxygen capacity. Neither has the addition of glucose to the blood any influence, a finding which seems related to that of Cox & Wendel (1942) in their study of experimental ferrihaemoglobinaemia. Similar but less striking results have again been obtained with human blood.

Hence, in spite of the absence of direct spectroscopic evidence, it is difficult to escape the conclusion that most samples of horse blood, and a fair proportion of human blood samples, behave as if they contain a certain amount of ferrihaemoglobin. Hardly more than traces have been found in human blood, but horse blood sometimes contains amounts of the order of 20 % of the total haemoglobin present. In all cases, however, the ferrihaemoglobin disappears when the blood is kept for some hours or a day or two.

SUMMARY

1. A modification is described of the Conant-Scott-Douglass technique for the determination of total haemoglobin (ferrohaemoglobin plus ferrihaemoglobin, but excluding carbon monoxide haemoglobin).

2. In 21 out of 38 normal human bloods the total haemoglobin has been found to exceed the ferrohaemoglobin by 1.5 % or more.

3. In 14 out of 17 normal horse bloods the total haemoglobin has been found to exceed the ferrohaemoglobin by more than 1.5 %. In most cases the differences are far greater than those found in human blood.

4. Circumstantial evidence is presented which tends to confirm the hypothesis that the differences are due to the presence of ferrihaemoglobin.

The author is indebted to Prof. Henry Dryerre for encouragement and advice, and to Mr J. W. Burgess, M.R.C.V.S., for assistance in obtaining samples of horse blood. A part of this work was reported in an oral communication to the Physiological Society at Edinburgh in July 1943.

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