ON THE ACTION OF CERTAIN OXIDISING AGENTS UPON BLOOD-PIGMENT¹

BY J. A. MACWILLIAM, M.D., *Professor of Physiology in the University of Aberdeen.*

From the Physiological Laboratory, University of Aberdeen

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While the destructive effects of chlorates on red blood corpuscles and the conversion of the haemoglobin into methaemoglobin have long been known the further effects have not been much studied. Formation of haematin, ultimate disappearance of absorption bands, and conversion of the blood into a dark solid mass have been stated to occur.²

JELLYING OF BLOOD AND COLOUR CHANGES

The jellying effect of chlorate on blood is a remarkable one, and occurs as one of the results of the chlorate influence being prolonged much beyond the phase of methaemoglobin formation. It can be easily produced by mixing blood with crystals or strong solutions of sodium (*e.g.*, 10 per cent.) potassium or ammonium chlorate in amounts sufficient to give a strength of 2 to 5 per cent. chlorate in the mixture. When crystals are used the fluid must, of course, be stirred until solution is complete; otherwise there may be excessive percentages in certain portions of the fluid—with disturbing effects. With blood that has not been much diluted the jelly formed is very firm, coherent and elastic, and very difficult to reduce to a state of fine subdivision. Its formation is much accelerated by acidulation of the blood and by warmth (30° to 40° C.). When no acidulation is used the jelly

1. The main facts in this paper were communicated to the Physiological Society at the Oxford meeting in July, 1904, when illustrative specimens were shewn. Publication was delayed in the hope of carrying the investigation further in certain directions.

2. A list of papers is given at the end of the article on Chlorates in Cushny's Textbook of Pharmacology. See especially von Mering's paper in the *Berlin klin. Wochenschr.*, No. 44, 686 (1883).

is at first always dark red in colour when seen in mass—probably in twenty-four to forty-eight hours after the chlorate has been added, at room temperature. At a later stage, much hastened by acidulation and warmth, the red colour gives place to a deep green, as seen by reflected light, though thin slices viewed by transmitted light show a brownish-red tint. At a still later stage under the influence of decided acidulation and warmth the green colour may gradually change to a yellowish tint.

Sometimes the jelly contracts to a certain extent and squeezes out a clear fluid or serum. This fluid is rich in protein, etc., but gives no appreciable iron reaction with ammonium sulphide, ferrocyanide, etc., even after prolonged application of various methods for revealing 'masked' iron; the iron of the haemoglobin has evidently been retained in the jelly or clot. The squeezing out of fluid is very variable as regards its occurrence and amount; very often there is none.

When blood is diluted beyond a certain point jellying under the influence of chlorate fails and is replaced by a coloured precipitate varying in coarseness and tendency to coherence. The results are essentially similar whether the blood has been laked by dilution with water, etc., or diluted with isotonic salt solution. Ox blood which had been kept for many years behaved like fresh blood, and the results got with the blood of different animals (ox, horse, sheep, rabbit, rat, etc.), and with human blood, were essentially similar.

The influence of the reaction of the blood on these changes is very marked. Addition of alkaline salts (sodium carbonate, etc.) retards chlorate action very markedly, while acidulation accelerates the changes very strikingly. Samples of blood which were unusually alkaline shewed considerable resistance to the chlorate influence; the effect of slight acidulation of portions of the same blood was very notable. Hence the plan of administering alkalis in cases of chlorate poisoning has obviously a sound basis. Acidulation may be done by adding dilute acids or acid salts like acid sodium phosphate. With different samples of ox blood I have often used half to three volumes of 0.2 per cent. HCl, or about one volume of 0.5 per cent.

HNO₃. To make the mixture faintly acid to litmus paper varying amounts of acid are required, care being taken to guard against the acidity being sufficient to cause the formation of acid haematin, etc.; this was checked by the use of the spectroscope. Amounts of acid insufficient to make the mixture react acid to litmus are able to favour chlorate action markedly.

The relations of the development of the colour changes to the jellying vary considerably. With acidulation and warmth the green phase may be arrived at very early, indeed almost as soon as the jellying, while with an alkaline reaction in the cool the jelly may remain red for many days. Similarly, when the blood is diluted so much that a flocculent precipitate occurs instead of jelly formation the particles may become green at a very early stage in presence of pronounced acidulation.

Under certain conditions the blood, especially when diluted to a certain extent, may, after the addition of chlorate, present the appearance of a deep green fluid with little or no jelly or precipitate. Such conditions are (1) when the reaction is too alkaline or insufficiently acid, (2) when the percentage of chlorate is insufficient, and (3) when the temperature is too low, the reaction and amount of chlorate present being such as to require warmth for complete precipitation. The addition of acid (*e.g.*, acetic) in such cases usually leads to precipitation or jellying immediately or in a short time; if much acid is added, the development of the yellow phase is brought on. Alcohol also causes precipitation in the green fluid, the coloured substance being thrown down among others.

The effects obtained with bromates were quite similar to those following the use of chlorates. Iodates on the other hand, have not the same action; a red precipitate is thrown down with properties differing from those got with chlorates and bromates, the blood not being acidulated.

Similar results are obtained when washed red blood corpuscles are used instead of blood.

Solutions of the stromata were prepared by Halliburton's¹

1. *Journ. of Physiology*, Vol. X, p. 532.

modification of Wooldridge's method, and these were tried with chlorate in the same way as blood, but no jellying or precipitation followed. On the other hand the solution of haemoglobin left after removal of the stromata gave the characteristic results with chlorate—very rapidly indeed on account of the previous acidulation with acid sodium sulphate used to precipitate the stromata.

Blood plasma and serum were found to give negative effects with chlorate, whether acidulated or not; acid sodium phosphate was used to acidulate. Even when kept in a warm chamber for considerable periods this was the case; except where the plasma or serum had been contaminated with some haemoglobin—leading to precipitation by the chlorates—the serum pigment remained apparently unchanged, at least for long periods. Similarly negative results were obtained with egg-albumin.

EFFECTS ON SOLUTIONS OF HAEMOGLOBIN

From the foregoing observations it is evident that striking effects of chlorates on the blood are essentially effects upon the haemoglobin, and not upon the ordinary proteins. This conclusion was confirmed by the characteristic effects got with solutions of haemoglobin crystals and of methaemoglobin; the former were prepared by different methods, commonly by some of the modifications of Hoppe-Seyler's method. The same phenomena as regards colour changes, jellying, etc., were readily observed. Samples of haemoglobin obtained from Grüber, Merck and others were also used. Solutions of CO-haemoglobin were found to behave in much the same way. As a rule no acidulation was found to be necessary with the haemoglobin solutions; the chlorate or bromate was simply added to the usual amount. Cautious acidulation accelerates the changes.

Haematin solutions on the other hand quite fail to give these results; if blood is treated in such a way as to break up its haemoglobin into haematin and globin, treatment with chlorate in the usual way does not produce the effects seen when unchanged blood or haemoglobin is dealt with.

SEPARATION AND PROPERTIES OF THE MODIFIED HAEMOGLOBIN

From haemoglobin solutions as well as from blood the product was separated in conditions of greater or less purity. When blood was used the most successful results were obtained when considerable dilution was employed, *e.g.*, ox blood diluted with sixteen volumes of water and cautiously acidulated with 0.2 per cent. HCl, or 0.1 to 0.5 per cent. HNO₃, chlorate or bromate being then added to the amount of 2 per cent. Methaemoglobin was rapidly formed and soon a flocculent precipitate appeared—very marked in less than half an hour. Centrifuged and subjected to repeated and thorough washing first with normal saline and then with distilled water. Boiling was tried with one portion, and helped the separation of the precipitated matter without causing any evident change of colour.

In other cases the blood was diluted only three or four times with very weak acid and after addition of chlorate or bromate kept constantly stirred to prevent jellying, a coarse flaky precipitate (green) being got in an hour or two; this was washed thoroughly.

The substance obtained in the dry state is a dark powder (looking somewhat like haematin) with metallic lustre.

Microscopically it presents the appearance of yellow or brownish-yellow polygonal plates, varying much in size and form. With the micro-spectroscope no absorption bands are visible. Slices of the coloured jelly in the moist state shew similarly negative appearances with the spectroscope. The methaemoglobin spectrum resulting from the addition of chlorate to the blood gradually disappears as a result of more intense and prolonged influence.

The dried substance is very stable and insoluble in water and most reagents, *e.g.*, alcohol, ether, chloroform, benzol, glycerine, etc. Also in acids like HCl and H₂SO₄, in solutions of alkaline salts and in weak solutions of caustic alkalis, while it dissolves after a time in strong solutions of NaOH (20 per cent., etc.) and KOH. A certain amount of solution takes place after many hours in weak alkalis in the warm chamber. The freshly precipitated moist substance is more soluble in dilute alkalis. Boiling causes no obvious effect upon the substance;

its colour—whether red or green—remains unchanged. When a solution has been made with strong alkali, boiling with lead acetate gives a negative result as regards reduction.

In 0.2 per cent. HCl the solution swells up but does not dissolve even with the aid of warmth.

A solution in strong alkali shows no definite absorption bands, and when treated with ammonium sulphide shows only a very faint haemochromogen spectrum.

Treatment of the substance with H_2SO_4 does not give anything resembling haematoporphyrin, or indeed any definite bands.

QUESTION OF FORMATION OF HALOGEN COMPOUND

In view of the results that have been obtained by Mulder¹ and various more recent workers on the combination of proteins with halogens, and especially those described by Hopkins and Pinkus² and by Kurajeff³ (combination of haemoglobin with iodine), the question suggests itself as to whether there is any combination of such a kind here. Examination of the chlorate and bromate products by Carius' method does not lend countenance to this suggestion, the halogen found being apparently of the nature of an impurity.

The red, green and yellow products obtained by the action of chlorate and bromate seem to be oxidation products of haemoglobin.

RETENTION AND LIBERATION OF THE IRON

The substance retains all the iron of the haemoglobin from which it was formed, and the iron is in very firm combination. There is no reaction with ferrocyanide and HCl, with Macallum's⁴ haematoxylin solution, nor with ammonium sulphide even when kept for long periods in the warm chamber. This is the case if the chlorate action in the preparation of the substance from haemoglobin solution or blood is not carried too far, in the presence of too much acid, etc.

1. *Journ. f. pr. Chem.*, Vol. XLIV, s. 487.

2. *Ber. d. deutsch chem. Gesellsch.*, Vol. XXXI, s. 1311 (1898).

3. *Zeitsch. f. physiol. Chemie*, Vol. XXXI, s. 527 (1901).

4. *Journ. of Physiology*, Vol. XXII, p. 25.

The presence of Fe in the solution can be demonstrated in the usual way by incineration, etc., or by setting free the iron by prolonged treatment with acid alcohol (Bunge's fluid, etc.) and warmth, or more easily by chlorate or bromate plus *strong* acidification and warmth. By using 10 per cent. solution of sodium chlorate with $\frac{1}{10}$ to $\frac{1}{5}$ volume of saturated salicyl-sulphonic acid in the warm chamber the whole of the Fe is usually removed in a day or two; as soon as it is set free the ferric salt strikes a red colour with the salicyl-sulphonic acid and the progress of the extraction is readily gauged by the depth of the coloration. When the Fe has been completely removed in solution an orange-yellow residue is left.

A similar method serves for the extraction of Fe from ordinary haemoglobin or from blood. A weaker solution suffices to extract a good deal of the Fe even at room temperature, and with such a solution (*e.g.*, containing 5 per cent. salicyl-sulphonic acid) testing with ferrocyanide can be readily done (without HCl), and with ammonium sulphide after neutralisation.

The iron of haematin may also be extracted by treatment with chlorate whether treated as (1) haematin in the solid state, HCl, etc., being used for strong acidulation, or (2) haematin dissolved in glacial acetic acid or salicyl-sulphonic acid alcohol. Heat accelerates the extraction. When the Fe is completely removed a yellow substance is left which is not ordinary haemato-porphyrin.

As regards the possibility of demonstrating the iron *in situ* in the modified haemoglobin or in ordinary haemoglobin by means of ammonium sulphide, ferrocyanide, etc., the difficulty is that when the Fe is set free it is readily dissolved out. In order to prevent this I sought a solution which should liberate the combined Fe but not dissolve it out, and obtained such by dissolving salicyl-sulphonic acid in ether to saturation. Treatment with this fluid liberated the Fe but did not remove it, and it was possible to stain it *in situ* in the crystals and in blood corpuscles. But in the latter case the injurious effects of prolonged action of the fluid constitutes a serious drawback as regards histological applications. In the case of the modified haemoglobin got by means of chlorate or bromate the substance is

so resistant that it was found quite possible to obtain successful preparations shewing the iron *in situ* by the black coloration with ammonium sulphide and with haematoxylin, and the blue with ferrocyanide.

EFFECTS OF PEPTIC AND TRYPTIC DIGESTION

Digestion with peptic fluid gave results strikingly different from those obtained with ordinary haemoglobin or methaemoglobin. Instead of there being a splitting of the pigment into the protein component and acid haematin which becomes precipitated, the substance in this case gradually dissolves forming a yellow solution in which proteoses and, later, peptone can be recognised. No haematin appears, and in the earlier stages there is little or none of the iron liberated; though with prolonged digestion an extensive setting free of iron occurs and some yellowish deposit may form in the fluid. The rapidity of digestion varies much with the exact condition of the substance tested; in the moist state and well subdivided (*e.g.*, a recently-formed flocculent precipitate) it is very rapidly dissolved (two to three minutes, etc.); the dried product on the other hand is much more slowly acted upon. No absorption bands are visible in the yellow solution. The digestibility of the substance is very notable in view of its generally insoluble and resistant character. Whether this material can be turned to useful account as a source of 'organic' iron for therapeutic or nutritive purposes remains to be seen.

When a neutralised digest taken at an early stage is dialysed it may be found that a certain amount of combined iron passes through into the dialysate and can be demonstrated there by methods for revealing the presence of 'masked' or 'organic' iron, *i.e.*, there is a certain amount of iron combined in the form of a diffusible compound. But if peptic digestion is at all prolonged the iron gradually becomes liberated. If treatment with the chlorate has been carried on too long, or too decided acidulation has been used, iron seems to be set free at an earlier stage of digestion.

Pancreatic fluid is similarly effective in digesting the substance

in question ; alkaline haematin is not formed as in the digestion of ordinary haemoglobin. With tryptic digestion the liberation of iron seems to be slower than with peptic fluid.

Treatment of blood or haemoglobin with a soluble persulphate (*e.g.* 2 to 3 per cent. sodium persulphate) also gives a modification of haemoglobin which is completely digested by peptic and pancreatic fluids without splitting off haematin. In the case of blood the modified haemoglobin is mixed with other blood proteins also coagulated by the reagent.

SOME APPLICATIONS OF THE CHLORATE AND BROMATE ACTION UPON HAEMOGLOBIN

In virtue of the above described action in coagulating haemoglobin while not similarly affecting ordinary proteins, chlorates and bromates may in certain cases be successfully used to separate haemoglobin from other proteins, etc., a suitable degree of slight acidification being provided by the use of acid sodium phosphate, etc., as in the case of serum, etc. The same may be done in urine containing haemoglobin (using 5 per cent. of the chlorate at room temperature for a day or two) with proper acidulation, but here certain constituents often interfere with the separation of the haemoglobin. The chlorate causes no precipitation in normal urine.

When it is desired to liberate and demonstrate the iron of the haemoglobin, *e.g.*, by the use of chlorate and strong acidulation with salicyl-sulphonic acid as already described, complications arise from the fact that certain urinary constituents (uric acid, kreatinin, etc.) discharge the red colour struck by the liberated iron with the salicyl-sulphonic acid—unless the amount of iron be large ; ammonium sulphide and ferrocyanide may likewise fail to demonstrate the presence of appreciable amounts of iron. Interfering constituents are also present in muscle extract, but not to any marked extent in serum or plasma.

In the absence of such disturbing agents it is very easy to liberate and demonstrate the iron of haemoglobin, etc. For class purposes two or three drops of blood may be mixed with sodium chlorate

solution (5 to 10 per cent.) and acidulated with salicyl-sulphonic acid to the amount of 2 to 5 per cent. The tube is left to stand for twenty-four hours, and the liberated iron shews by pink coloration of the fluid, which can also be tested by ferrocyanide and (after neutralisation with ammonia) by ammonium sulphide.

The pigments of serum, bile and urine are not precipitated or decolourised by treatment with chlorate (after acidulation with acid sodium phosphate) over periods vastly longer than are necessary to change haemoglobin in the way described.

Chlorate and bromate may be used as fixing agents for haemoglobin for certain histological purposes, etc., *e.g.*, in the case of the natural injection of organs and parts with blood.