ON THE OXIDATION BY POTASSIUM FERRICYANIDE OF CERTAIN CONSTITUENTS OF THE SERUM IN ANEMIA

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In attempting to make measurements of the oxygen content of the blood of anemic rabbits by the method devised by Haldane (1898, 1920), it was found that after the liberation of the oxygen by the addition of potassium ferricyanide no steady end reading could be reached. The final solution began to absorb gas from the flask in quantities which, in a short time, might exceed the total volume of oxygen previously liberated. Such a difficulty has been described by Douglas (1910) also after studying the blood of anemic rabbits. More recently the reactions involved have been investigated by Parsons and Parsons (1927) and by Litarczek (1928).

Litarczek found that this reaction also occurred in the course of determinations made on normal blood though much less vigorously than in those made on anemic blood. He found, moreover, that it could be considerably intensified if the blood examined were taken from a normal individual shortly after a meal during which considerable quantities of fat had been consumed. Since the extraction of lipoids from the serum by ether greatly reduced the intensity of the reaction, Litarczek concluded that it depended upon the oxidation of fatty acids present in the blood, though he was unable to demonstrate that the lipoids so removed later exhibited any marked oxidative reaction in the presence of potassium ferricyanide.

Parsons and Parsons reached similar general conclusions, though they were able to show that, if the ether extraction of the serum
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were carried out by a method which excluded contact with air, the subsequent oxidation of the lipoid emulsion proceeded much more actively. They further undertook fractionation of the serum by the addition of varying concentrations of ammonium sulfate. After full saturation of the serum the filtrate was entirely inactive, while that obtained after one-third saturation exhibited almost as much activity as the original serum. In the former case the precipitate contained all the substances oxidizable under these conditions, and they interpret these results as being consistent with their view that the lipoids are the substances present in the serum which are responsible for the reaction.

Since the Haldane blood gas apparatus is widely used it is desirable to investigate any limitations in its applicability to the determination of blood gases. Such a study may result in a further insight into the changes in the blood after extensive bleeding, and so lay the basis for some modification of the original method which would render it more suitable for investigating the blood in anemia. This work was carried out with both these points in view, yet, though the reason for the systematic error now appears clearer, we were not able to develop any satisfactory modification of the method.

Methods

All estimations of the oxygen consumption of the blood, serum, or other solutions used, were made upon the respiration apparatus described by Warburg (1926). The quantities of the solutions used have been the same as those required for the Haldane blood gas apparatus (1920): 2 cc. of blood, serum, etc., 0.75 cc. of sodium carbonate (1 per cent) solution, and 0.25 cc. of a freshly prepared saturated solution of potassium ferricyanide. The potassium ferricyanide was placed in a side arm of the respiration flask and added to the solution in the main chamber after temperature equilibration at 37° had taken place.

EXPERIMENTAL

The participation of the red blood cells in this reaction, which might readily have been inferred from the observations of Morawitz (1909) upon the greatly heightened respiration of regenerating blood, has been excluded by Parsons and Parsons, and by Litarczek. The reaction is one which concerns some component of the serum
as can be seen from the illustrative experiment given in Table I, in which a comparison was made of the oxidation of whole blood, serum, and red cells from the same blood but resuspended in normal physiological salt solution.

**TABLE I**

Oxidation of Whole Blood, Serum, and Cells from Same Blood Resuspended in Normal Physiological Salt Solution

<table>
<thead>
<tr>
<th>Time</th>
<th>C.mm. oxygen consumed per cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole blood</td>
</tr>
<tr>
<td>15 min</td>
<td>24.5</td>
</tr>
<tr>
<td>30</td>
<td>40.0</td>
</tr>
<tr>
<td>45</td>
<td>56.1</td>
</tr>
<tr>
<td>60</td>
<td>72.8</td>
</tr>
<tr>
<td>75</td>
<td>89.4</td>
</tr>
</tbody>
</table>

The red cell count was 2.21 millions per c.mm. The reticulocyte count was 38 per cent. The temperature of the water bath was 37.5°.

It is evident from this experiment that the red blood cells are not greatly concerned in the reaction, indeed probably a large part of the oxygen consumption in the flask containing the cell suspension took place in the small quantity of serum still adherent to the cells. That less oxygen was consumed by the whole blood than by the serum alone was probably due to the smaller quantities of serum present and also to the lower effective concentration of potassium ferricyanide, part of which had reacted with the hemoglobin present. Both of these factors also probably contribute to the intensification of the reaction when anemic blood rather than normal blood is examined.

**Constituent of Serum Responsible for Reaction**

The serum in anemia exhibits two important changes: a relative lipemia, and a considerable increase in the purine nitrogen. The former was first described by Boggs and Morris (1909) in the blood of rabbits rendered anemic by bleeding, and has since been somewhat extensively studied, notably by Bloor (1921, 1925). An increase in the concentration of the serum purine bodies has been described by Krafka (1929) in a dog recovering from an experi-
mental anemia, and by Riddle (1930) in patients recovering from pernicious anemia as a result of liver treatment.

In order to determine whether the change in the purine concentration might play a part in the enhanced oxidation, the serum from an anemic rabbit was dialyzed inside a collodion sac against an equal volume of normal physiological sodium chloride solution. Since the purine bodies are small readily diffusible substances it was expected that, were they the substances concerned, the oxidation reaction of the solutions on the two sides of the membrane would rapidly attain the same value. The following tabular matter gives the results obtained.

<table>
<thead>
<tr>
<th>Duration of dialysis</th>
<th>C.mm. oxygen consumed per cc.</th>
<th>Sugar concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Original serum</td>
<td>Inside</td>
</tr>
<tr>
<td>hrs.</td>
<td>18</td>
<td>202.0</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>139.2</td>
</tr>
</tbody>
</table>

The sugar determinations were made by the method of Hagedorn and Jensen. The duration of each experimental determination was 75 minutes.

This experiment shows that the rate of diffusion of the oxidizable material, even through a fairly permeable collodion sac, is slow, and that when equilibrium has been attained for the serum sugar the activity of the serum inside the membrane is still nearly twice that of the solution outside. Further evidence against the participation of the purine bodies was the absence of any absorption of oxygen by the urine of an anemic animal upon the addition of potassium ferricyanide.

Oxidation of Serum in Relation to Lipoids

Fishberg and Fishberg (1928) have studied the lipemia which occurs in rabbits after repeated bleeding. They found that the total fat content of the blood may increase 8 or 9 times, figures which are in good agreement with those of Boggs and Morris. Since it is well known that the unsaturated fatty acids are more readily oxidizable in vitro than saturated ones, it seemed possible that they might be the constituents involved in this reaction. Were this so the oxidizability of the serum should bear some relation to the degree of iodine absorption by the fats which it con-
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tains. This possibility was explored by comparing the oxygen consumption of the serum when potassium ferricyanide was added to it with the iodine absorption of the fats extracted by the method devised by Bloor, Pelkan, and Allen (1922). The oxygen consumption of the serum was determined as before by the Barcroft-Warburg apparatus, and the iodine absorption by the method of Gibson and Howard (1923) upon the extracted serum fats. Several rabbits were used, both in their normal condition and after the production of a severe grade of anemia by successive bleedings. The results will be seen in Table II.

TABLE II
Oxidation of Serum in Relation to Iodine Absorption of Serum Lipoids

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Red cells</th>
<th>O₂ consumed per 100 cc. serum*</th>
<th>I₅ absorbed per 100 cc. serum</th>
<th>Ratio (c)/(d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>(b)</td>
<td>c.mm.</td>
<td>mg.</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>4.98</td>
<td>14,200</td>
<td>133</td>
<td>107</td>
</tr>
<tr>
<td>15</td>
<td>2.81</td>
<td>23,300</td>
<td>178</td>
<td>131</td>
</tr>
<tr>
<td>21</td>
<td>3.57</td>
<td>34,700</td>
<td>253</td>
<td>138</td>
</tr>
<tr>
<td>34</td>
<td>2.68</td>
<td>34,900</td>
<td>241</td>
<td>145</td>
</tr>
<tr>
<td>21</td>
<td>2.11</td>
<td>38,700</td>
<td>360</td>
<td>108</td>
</tr>
<tr>
<td>21</td>
<td>4.39</td>
<td>43,600</td>
<td>345</td>
<td>126</td>
</tr>
<tr>
<td>21</td>
<td>2.73</td>
<td>44,400</td>
<td>323</td>
<td>137</td>
</tr>
<tr>
<td>23</td>
<td>2.73</td>
<td>44,500</td>
<td>310</td>
<td>143</td>
</tr>
<tr>
<td>23</td>
<td>2.73</td>
<td>45,200</td>
<td>290</td>
<td>156</td>
</tr>
<tr>
<td>21</td>
<td>2.73</td>
<td>46,200</td>
<td>350</td>
<td>132</td>
</tr>
</tbody>
</table>

* The duration of the oxygen consumption determinations was 4 hours.

It can be seen from Table II that the oxygen consumption of an anemic rabbit’s serum after the addition of an alkaline solution of potassium ferricyanide, is considerably greater than that of a normal fasted animal, and that coincident with this rise is an approximately similar rise in the iodine absorption value of the serum fats. The ratio between the two is to be found in the last column.

Should the unsaturated fatty acids be the constituents of the
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serum which are oxidized it might be expected that after oxidation they would no longer be able to absorb as much oxygen as before. This was tested experimentally. At the same time as the oxygen consumption of the specimen of serum from an anemic rabbit was measured in the Barcroft-Warburg apparatus, 7.5 cc. of a mixture of serum, sodium carbonate, and potassium ferricyanide, in the same proportions as in the respiration flask were shaken in a larger flask at the same rate in the same water bath. The only difference between the two was that one flask was larger and contained more than twice as much of the solution as the other. At the end of 4 hours the oxygen consumption of the serum was determined, and the iodine absorption of the fatty acids remaining in the mixture in the larger flask was estimated. This iodine absorption was compared with that of the fatty acids present in the original serum. The results will be found in Table III.

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>Influence of Oxidation of Serum on Iodine Absorption of Serum Lipoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen consumed per 100 cc. serum (4 hrs.)</td>
<td>Iodine absorption by serum fats</td>
</tr>
<tr>
<td>cc.</td>
<td>mg.</td>
</tr>
<tr>
<td>34.7</td>
<td>49.6</td>
</tr>
<tr>
<td>46.2</td>
<td>66.1</td>
</tr>
<tr>
<td>44.4</td>
<td>63.5</td>
</tr>
</tbody>
</table>

It is clear from these determinations that after oxidation of the serum mixture has taken place, the capacity of the serum fats for taking up iodine is definitely reduced. Further it can easily be calculated that about 10 atoms of oxygen have been taken up for every 3 atoms of iodine which might have been absorbed. It is evident therefore that the oxidation has not resulted entirely in the production of the corresponding hydroxy acids, as Lewkowitsch (1909) has described as the principal product of the oxidation of oleic acid by potassium dichromate. The oxidation of the serum fats must have progressed considerably further.
Form of Oxygen Consumption Curve for Serum

The consumption of oxygen by the serum takes place very rapidly at first, but the rate decreases progressively. This is well shown in Chart I, which indicates the oxygen consumption, both for normal and anemic serum for a period of 20 hours. The decrement in the rate does not follow any simple logarithmic rule, a result which might be expected from the probability that a number of unsaturated fatty acids are simultaneously oxidized, some of them more readily than others.

The reaction still continued at a reduced intensity after 20 hours so that complete oxidation was not attained, nor is it possible to extrapolate from the data to discover when this point would be reached. The curve probably represents a summation of the different oxidation curves for fatty acids ranging from oleic acid on the one hand, to those with several unsaturated linkages, such as arachidic acid described by Hartley (1909) on the other. Further the rate of oxidation may be influenced by the occurrence
of autocatalytic reactions such as will be described below in the
oxidation of oleic acid.

Attempts to Inhibit the Serum Oxidation Reaction

Several efforts were made to inhibit, or at least reduce, the
intensity of the reaction. All were unsuccessful. The addition
of potassium cyanide to a final concentration of 0.005 M, and the
addition of phenylurethane up to saturation in the sodium carbonate
used, both of which Warburg had found to be inhibitors of both
biological oxidation systems and his charcoal model, were without
effect upon this reaction. The reason may have been that these
substances are only effective in inhibiting oxidation reactions
catalyzed by colloidal particles upon which they can be adsorbed.
On the other hand, as Kuhn and Meyer (1929) have suggested,
it is possible that potassium cyanide is without effect upon the
oxidation of fats, inhibiting only that of amino acids and carbo-
hydrates.

Oxidation of Oleic Acid by Potassium Ferricyanide

Since oleic acid is one of the principal unsaturated fatty acids
in the serum, experiments were carried out to examine its oxidiz-
ability by potassium ferricyanide under conditions similar to those
used for the serum. Two specimens of oleic acid were used, one
with an iodine number of 66, and specific gravity of 0.892 at 20°,
and another with one of 88 and specific gravity of 0.894 (the theo-
retical iodine number is 91). The oleic acid was dissolved in 1
per cent sodium carbonate solution in concentrations of about
0.4 gm. per cent. 2 cc. of this solution, together with 0.75 cc.
more of 1 per cent sodium carbonate solution, were placed in the
flask of a Barcroft-Warburg apparatus and after temperature
equilibrium at 37° had been reached, 0.25 cc. of a saturated solution
of potassium ferricyanide at the same temperature was added. The
oxygen consumption of the final mixture was measured. A con-
trol measurement was made in which the potassium ferricyanide
was omitted. Table IV gives the oxygen consumptions in the
experimental and control flasks.

Chart II shows the course of the reaction. From this it can be
seen that the reaction does not proceed uniformly, but when charted
shows a sigmoid curve. Such variations in the speed of progress
of the oxidation might be due to the products of the reaction affecting its further course. In order to obtain more definite evi-

TABLE IV

Table: Oxygen Consumption of Sodium Oleate Solutions in Presence of Potassium Ferricyanide

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>C.mm. oxygen consumed per cc. oleate solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With K₄Fe(CN)₆</td>
</tr>
<tr>
<td>1</td>
<td>156.5</td>
</tr>
<tr>
<td>2</td>
<td>180.5</td>
</tr>
<tr>
<td>3</td>
<td>114.0</td>
</tr>
<tr>
<td>4</td>
<td>179.2</td>
</tr>
</tbody>
</table>

The experiment lasted 210 minutes.

The experiment lasted 210 minutes.

CHART II. Curves showing the oxidation of oleic acid in the presence of sodium carbonate and potassium ferricyanide.
0.75 cc. from the experimental and control flasks of the previous experiment were added to fresh oleic acid and the oxidation of the two solutions again measured after the addition of potassium ferricyanide. In this way the fresh oleic acid solution was seeded with any possible autocatalytic products developed in the previous reaction. The results of this experiment are given in Chart III.

It can be seen from Chart III that the course of the reaction in the two flasks is quite different. In the flask seeded from the original flask, in which no oxidation had previously taken place, the form of the curve reduplicates the original sigmoid, while in the other flask to which products of oxidation from the previous experiment had been added the curve is of paraboloid appearance. It would seem that while in the former it took time for the formation of products capable of affecting the subsequent course of the reaction, in the latter they were added, already formed, in the seeded solution.
The total quantity of oxygen taken up by the oleic acid during the period of observation is very much less than that taken up by the serum fats having the same capacity for the absorption of iodine. Only 1 atom of oxygen is used for every 2 or 3 potential iodine atoms, which is roughly the reverse of that for the serum fats. Consequently the oleic acid present in the serum is probably in only a minor measure responsible for the oxidation reaction with potassium ferricyanide, and other more unsaturated fatty acids account for the greater part of it.

DISCUSSION

The serum lipoids both of normal and anemic dogs have been extensively studied by Bloor (1923). In both he found that large proportions of the fatty acids present were unsaturated and probably largely oleic and linoleic acids with small quantities of even more unsaturated ones such as linolenic acid. In the serum in anemia however the proportion of linoleic to oleic acid was considerably increased, and at the same time the quantity of fatty acids in the serum was also somewhat raised. The rise he observed was, however, not very great, possibly because the dogs he examined were in a condition of chronic anemia in which, following Bloor's suggestion it is highly probable that extension of the erythropoietic tissue to the available marrow space had already taken place, leaving practically no further marrow fat to be removed by the blood. Horiuchi (1920) in examining the blood of rabbits in anemia found that daily hemorrhages induced a lipemia which attained a maximum in the course of a few days and then subsided to about its original value, even though bleeding was still kept up. In this case the lipemia appeared coincidently with the extension of the bone marrow, falling away after this had taken place and little further fat remained to be replaced. Unfortunately Horiuchi makes no mention of the degree of unsaturation of the lipoids at the height of the lipemia, though Boggs and Morris state that the iodine absorption of the serum fats is definitely elevated at this time.

The rise in the oxidizability of the serum in the presence of ferricyanide appears concurrently with the increase in concentration of the unsaturated fatty acids. That potassium ferricyanide is able to oxidize unsaturated fatty acids is definitely
shown by its oxidation of oleic acid dissolved in sodium carbonate solution. That it would also be able to oxidize linoleic and linolenic acids and any more unsaturated ones seems very probable. Kuhn and Meyer (1929) were able to catalyze the oxidation of oleic, linoleic, and linolenic acids with hemin; the more unsaturated the fatty acid, the more rapid was the subsequent oxidation. Warburg (1914) was able to catalyze the oxidation of linolenic acid by iron (ferrous ammonium sulfate) in acid solution, though oleic acid remained unoxidized under such conditions. Just as for the serum fats described in this paper, Warburg found that the oxidation of the linolenic acid was accompanied by a decrease in the iodine absorption values of the solution. A similar decrease was described by Meyerhof (1923) for the oxidation of linolenic acid by cysteine and by thioglycolic acid.

It seems therefore that potassium ferricyanide has an oxidation potential sufficient not only to convert the reduced hemoglobin to methemoglobin (Conant, 1923), but also to induce oxidations of other serum constituents which involve the taking up of free oxygen. Where these substances are present in any quantity, as in anemia, the Haldane method for blood oxygen determinations will be unsatisfactory and recourse must be made to Van Slyke's modification in which the ferricyanide-serum mixtures present a much smaller surface of contact with the oxygen which in turn is at a low partial pressure. In this way the opportunity for subsequent oxidation is greatly minimized.

**SUMMARY**

1. The constituents of the serum of rabbits made anemic by hemorrhage which interfere with the use of the Haldane blood gas apparatus for blood oxygen determinations appear to be unsaturated fatty acids.

2. The progress of the anemia is accompanied by an increase in the iodine absorption value of the serum fats and by an almost quantitatively similar increase in the oxidizability of the serum by potassium ferricyanide.

3. After oxidation of the serum by potassium ferricyanide the iodine absorption values of the serum fats fall.

4. The oxidation of an unsaturated fatty acid, oleic acid, under similar conditions is described and its relation to the oxidation of the serum constituents discussed.
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