# CLIX. OBSERVATIONS ON SOME PROCESSES OF OXIDATION IN BLOOD-SERUM.

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The usual method for the estimation of the amount of oxygen combined as oxyhaemoglobin in a sample of blood consists in treating the fluid with potassium ferricyanide in faintly alkaline solution, when a complicated reaction occurs in which methaemoglobin is formed and an amount of oxygen equal to that originally present as oxyhaemoglobin is set free for measurement. But that the change which takes place when ferricyanide is added to so complicated a mixture as blood is completely expressed by this simple statement has long been questioned. Among the more recent work bearing on this point we may mention Conant and Fieser's [1924] demonstration of the dependance of the completeness of the reaction between potassium ferricyanide and oxyhaemoglobin on the degree of alkalinity of the medium in which that reaction takes place, and also the results of Smith, Dawson and Cohen [1920] which prove that in the van Slyke method of applying the ferricyanide reaction—a method in which the liberated oxygen is extracted at once from the reaction mixture by exposure to a vacuum—an appreciably greater amount of oxygen is liberated from a given quantity of blood than is obtained by the procedure of Henderson and Smith, in which the blood and ferricyanide are merely shaken together in a known volume of air and the amount of oxygen liberated is measured by the resulting increase in the percentage of oxygen in the gas phase. But apart from these and earlier investigations of the completeness of the reaction between ferricyanide and oxyhaemoglobin the observation has also been made that, when the oxygen has been set free in this way, if the shaking and exposure be continued, the liberated gas is slowly re-absorbed by the reaction mixture. This phenomenon is most marked with blood from anaemic patients, and with such blood it may proceed at such a rate as to render impossible any accurate estimation of the volume of oxygen originally liberated. The effect is so definite and of such magnitude that it seemed desirable to make an attempt to obtain some more precise knowledge as to the factors and substances involved when a mixture of blood and potassium ferricyanide absorbs oxygen under these conditions, and the results recorded in the present paper represent the information that we have collected.

For these experiments we used a compact form of Haldane's [1920, 1922] new blood-gas apparatus that is represented diagrammatically in Fig. 1. The main features are that the long rubber tubes of the original form of the apparatus are eliminated, and that the whole apparatus can be immersed in a thermostat bath through the glass sides of which the readings are taken. The zero line of the U-tube manometer is drawn on a small mirror which is arranged so that it is adjustable in a vertical direction in order to allow for slight variations in the level at which the manometric liquid (xylene) stands. The gas-measuring burette has a total volume of 0·2 cc. and is graduated in 0·002 cc. so that using mercury as confining fluid readings of gas volume correct to $\pm$ 0·0002 cc. can be obtained. This degree of accuracy is, however, far greater than that required for the purposes of this particular piece of work.

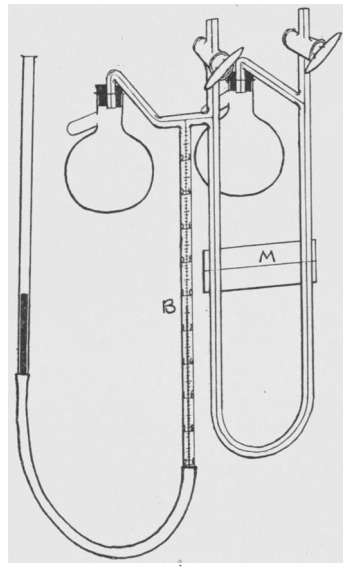


Fig. 1. Compact form of Haldane blood-gas apparatus. *B* is the gas-measuring burette containing mercury as confining fluid, and *M* the adjustable mirror carrying the zero line for the manometer U-tube.

The apparatus is most conveniently mounted on a plate of aluminium about $\frac{1}{8}$ in. thick with a suitable slot for receiving the mirror and its clamping screw.

Our ordinary procedure has been to place 1 cc. of the blood or other material under investigation together with 2 cc. of dilute ammonia solution (4 cc. of a solution of d. = 0·880 diluted to 1 l.) or other alkaline solution in the reaction-bottle and to place a similar mixture containing 1 cc. of distilled water in place of the blood or serum on the control side. We used in the side-tube of each bottle 0·5 cc. of a saturated solution of potassium ferricyanide, and from the final colour of our reaction mixtures it was evident that this always constituted a great excess. The bottles having been fitted to the rest of the apparatus, the whole was lowered into the thermostat and when temperature equilibrium had been attained the taps were closed and the

ferricyanide tilted into the experimental material. The apparatus was shaken by hand for 2 minutes and then replaced in the bath for a further 3 minutes. By adjustment of the mercury reservoir tube the level of the xylene was brought to the zero line on the mirror and the reading of the gas volume then noted. This gave a starting-point for the observations of the subsequent absorption of oxygen, which was followed usually for about an hour by shaking the apparatus for 2 minutes and allowing it to rest in the bath for the following 3 minutes before each reading. By this procedure we, of course, systematically neglected the amount of oxygen absorption occurring during the first 5 minutes of each experiment; we did this in order to avoid occasional irregularities due to the mixing of the materials or to evolution of gas from small quantities of haemoglobin present, and all the curves we give are plotted from results consistently obtained in this way. But we have convinced ourselves that no appreciable difference is produced by the inclusion of these initial 5-minute intervals in the diagrams and that our conclusions are therefore in no way

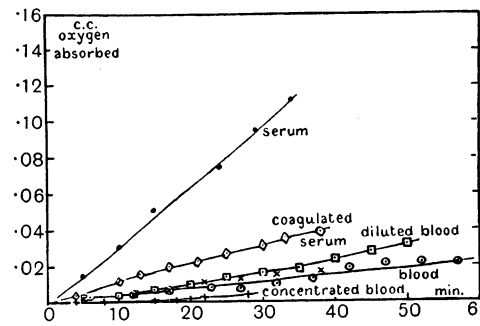


Fig. 2. Effect of variation of the concentration of corpuscles on the rate of absorption of oxygen by blood in the presence of ferricyanide. The points denoted by crosses were obtained in the presence of NaF. The oxidation curve for coagulated serum is also given.

affected either qualitatively or quantitatively thereby. All our experiments were carried out at room temperature, which rarely varied outside the limits of 15–18°, and all the gas volumes recorded in our diagrams have been reduced to N.T.P.

With this technique it was easy to show, in the first place, that the absorption of oxygen by a mixture of defibrinated blood and ferricyanide is due entirely to the serum and that the corpuscles play no part in it. For example in Fig. 2 we record the results of observations made on the blood of a male patient, S., who possessed a haemoglobin content of 60 % of normal and a red cell count of $3{\cdot}44 \times 10^6$ per mm.$^3$. It will be seen that doubling the concentration of the corpuscles by centrifuging almost completely abolishes the absorption of oxygen, while dilution of the blood with an equal volume of its own serum very considerably increases it. The rate at which the pure serum itself takes up oxygen in the presence of ferricyanide is very considerable, amounting to about 0·1 cc. per cc. of fluid in 30 mins. It is evident, then, that we are not here dealing with an absorption of oxygen by immature

red corpuscles such as might have been expected to occur in the blood of an anaemic patient, and that the oxidation is so much more marked in this than in normal blood simply because the anaemic blood contains the larger proportion of serum. As a matter of fact the rate of this oxygen absorption in the whole untreated blood even of this patient is such as might easily be overlooked in an ordinary oxygen determination, and in the blood of a normal person would be even less. With the serum from this same blood we convinced ourselves that absorption of oxygen does not occur in the absence of ferricyanide, obtaining a volume difference of only 0·0005 cc. during 25 minutes' shaking of the serum alone in air. We also showed that this oxidation is not dependent on a process of glycolysis inasmuch as the rate of absorption of oxygen by 1 cc. of this blood was totally unaffected by the addition of 0·2 cc. of 0·5 % sodium fluoride solution according to Evans' recommendation [1922] (see the points represented as crosses in Fig. 2). We further obtained evidence that we were not dealing with an enzyme reaction of any kind by observing the rate of absorption of oxygen by 1 cc. of serum that had been heated by holding the bottle in boiling water for 5 mins. before the experiment. Certainly in this case the rate of oxidation was much reduced (Fig. 2), but this is readily to be explained by the slow rate of diffusion and the difficulty of adequate shaking caused by the presence of large masses of coagulum.

In considering the various constituents of serum that might be responsible for the formation of autoxidisable substances under the influence of potassium ferricyanide we first of all suspected that the effect might be due to sugar. But we found that, under the conditions of our experiment—that is, in faintly alkaline solution—1 cc. of 0·5 % glucose solution absorbed no appreciable amount of oxygen when shaken with air in the presence of ferricyanide, the actual change in volume observed at the end of an experiment lasting 41 mins. amounting to only 0·0008 cc.

We were left, therefore, with the alternative possibilities that the autoxidisable constituents arise either from the proteins of the serum, or from the lipins, or possibly from a colloidal complex of the two. We therefore attempted to fractionate the serum in various ways and to study the oxidisability of the separate fractions. We tried, for example, to extract the lipins by shaking with ether, and found that the residual aqueous fluid showed a much slower rate of oxygen absorption than the original serum, and that this reduction of oxidisability was the more marked the more efficient the ether extraction. When the ether extract was evaporated to dryness in a dish floating on warm water a residue was obtained that turned brownish during the last stages of the evaporation. When this residue was re-emulsified in some of the extracted serum no appreciable increase in the rate of oxidation was produced, undoubtedly because of oxidation of the lipins during the evaporation. When we repeated the experiment in such a way that the ether extraction and also the evaporation of the extract were carried out in an atmosphere of nitrogen, we found that when the lipin residue obtained was re-emulsified in the extracted

serum the rate of oxygen absorption was markedly increased. The result of this experiment is recorded in Fig. 3 in which it is to be noted that the curve labelled "extracted serum + lipins" was obtained with 1 cc. of extracted serum to which the lipins obtained from 1·5 cc. of the original serum had been added, so that in this experiment the effect of the addition of the lipins is exaggerated. In passing we would call attention to the particularly rapid rate of absorption of oxygen shown in this experiment by the mixture of untreated serum and ferricyanide, 0·24 cc. oxygen being taken up by 1 cc. serum in less than 50 mins. This is associated with the circumstance that the blood used in this experiment was obtained from a patient, F., exhibiting so marked a degree of lipaemia that the serum was opaque and milky with the excessive amount of fat it contained. We have confirmed these effects of the ether extraction and re-addition of lipins using serum containing only a normal content of these substances; we have found further that extraction with light

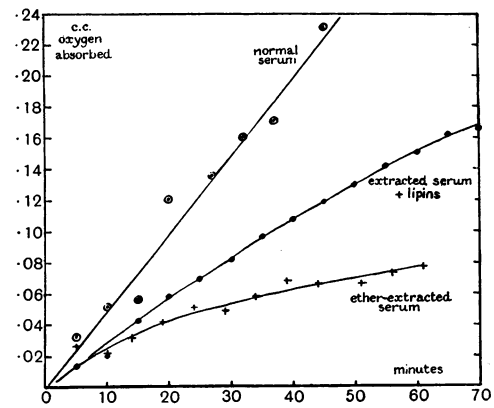


Fig. 3. Effect of ether extraction and re-addition of the lipins obtained by evaporation of the extract out of contact with air.

petroleum also reduces to a similar extent the rate at which the serum will absorb oxygen in the presence of ferricyanide. The fact that extraction with these lipin solvents reduces the oxidisability of the serum does not necessarily indicate that the lipins themselves are concerned in the effect, for, these solvents produce a certain amount of precipitation of the serum-proteins. This effect is not very marked in the case of ether, but with light petroleum emulsion formation is very troublesome, and vigorous shaking of the mixture of serum and petroleum results in an emulsion of the consistence of vaseline, which separates only imperfectly into petroleum and aqueous layers in the centrifuge, a compact mass of material giving protein reactions separating between the two main liquids. The reduction of the oxidisability of the serum described in these experiments might therefore well be due to an effect of the extracting fluid on the proteins, but the fact that the re-addition of the extracted lipins does in some measure increase the rate of oxidation of the extracted serum can be interpreted only as indicating a direct participation

of the lipins in the process of oxidation here under study. By re-addition of the extracted lipins, however, we have never succeeded in restoring the rate of oxidation to that shown by the original unextracted serum, but whether this incompleteness of the restoration of the oxidisability is due merely to incompleteness of mechanical dispersion we are at present not able to say.

In view of the evidence that exists in favour of the view that the lipins of the serum are present in the form of colloidal complexes with the proteins, we carried out also an experiment on the separation of the proteins and lipins of the serum by the method described by Hardy and Gardiner [1910]. Serum was run into some six times its volume of alcohol previously cooled to $-8°$; the precipitated proteins were filtered off at a temperature not rising above $0°$, then freed from alcohol by thorough washing with ether and finally dried in the vacuum desiccator. One half of this material was dissolved in a volume of water equal to half that of the serum originally taken, and the oxidation of this solution in the presence of ferricyanide was observed. The lipins were recovered from the combined filtrates by evaporation *in vacuo*. The residue was emulsified by shaking in a volume of distilled water

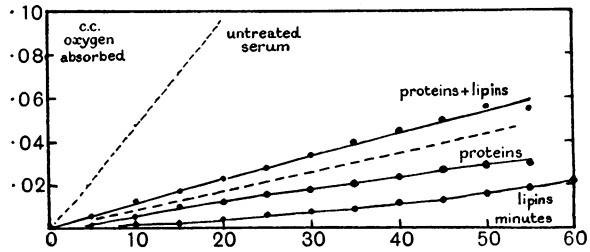


Fig. 4. Rate of oxidation in the presence of ferricyanide of the proteins and lipins separated from serum by ice-cold alcohol. The unlabelled dotted line represents the arithmetical sum of the curves for lipins and proteins respectively.

equal to that of the original serum, the process being conducted in an atmosphere of hydrogen. One half of the resulting emulsion was used for an oxidation experiment, while in the other the remaining half of the protein precipitate was dissolved by shaking in hydrogen to yield a "resynthesised" serum whose oxidisability was also studied. The results of this experiment are shown in Fig. 4 from which it will be seen that both the protein and the lipin fractions take up oxygen slowly in the presence of ferricyanide, and that the mixture of the two, in the proportions and concentrations in which they originally existed in the serum, absorbs oxygen at a rate hardly greater than that to be expected from the arithmetical summation of the effects due to the two constituents separately. From this it would seem that both proteins and lipins are concerned in the process of oxidation and that neither exerts an appreciable accelerating influence on the oxidation of the other. If a lipin-protein complex is re-formed under the conditions of our experiments it does not show any appreciably greater susceptibility to oxidation than is possessed by its separate lipin and protein constituents. It will be noticed, however

how far we were in this experiment from achieving a re-synthesis of the original serum by a re-union of its separated constituents: it is evident that the process of separation had produced deep-seated and irreversible changes of the colloidal structure of the serum and consequent reduction in the rate of oxidation—an effect apparently analogous to the diminution of the rate of oxygen absorption observed by Warburg [1914] to follow successive destruction of structure in nucleated red blood cells.

Our next observations are concerned with the fractionation of the serum by means of ammonium sulphate. A first experiment showed that when serum is saturated with this salt the oxidisable substances are completely precipitated—the filtrate absorbing no oxygen at all when treated with ferricyanide in the presence of ammonia according to our usual procedure.

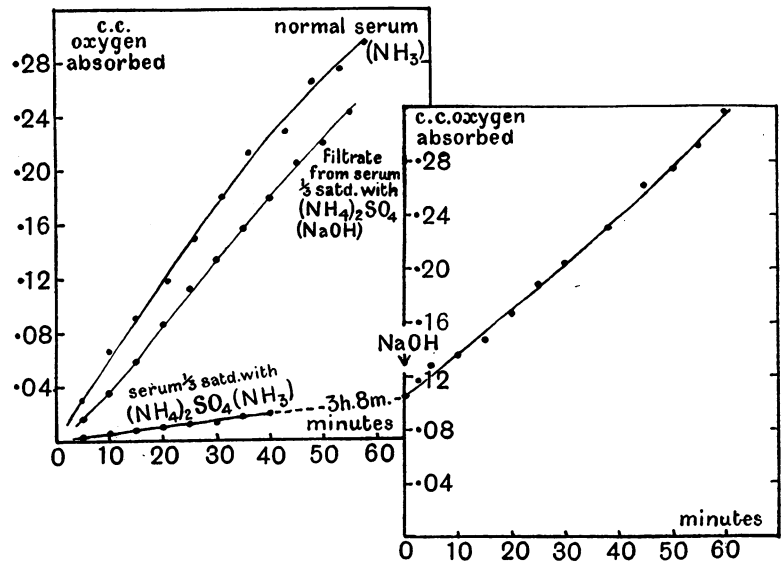


Fig. 5. Effect of one-third saturation with ammonium sulphate and subsequent addition of NaOH. The alkali present during the oxygen absorption is mentioned in brackets at the end of the description of each curve.

But the precipitated proteins were also practically devoid of oxidisable properties when treated in this way. This result suggested that possibly the large amount of ammonium sulphate present in these experiments had depressed the ionisation of the ammonia used as alkali to such an extent that the final mixture was far less alkaline than in our previous experiments, and that therefore the oxidising potential of the ferricyanide was so much reduced that it was incapable of giving rise to the autoxidisable substances whose behaviour we are here studying. It is possible also that the rate of absorption of oxygen by these autoxidisable substances themselves might have been reduced by a reduction in the alkalinity of the medium. We therefore proceeded to the experiment whose results are recorded in Fig. 5. In this case

we compared the rate of absorption of oxygen by untreated serum in the presence of ammonia with that shown by serum mixed with one-third its volume of saturated ammonium sulphate solution. In this mixture the rate of oxygen absorption was very slow until 1 cc. of approx. *N* NaOH solution was added at the point indicated. The result of the increased alkalinity was shown in an immediate increase in the rate of oxidation to a value not far different from that of the untreated serum. Evidently, then, the depressant effect of the ammonium sulphate is to be ascribed to the reduction it produces of the alkalinity of the reacting mixture. In Fig. 5 we include also the results of an experiment on the *filtrate* from serum one-third saturated with ammonium sulphate, and examined in the presence of 1 cc. *N* NaOH instead of ammonia. It is evident that one-third saturation with ammonium sulphate does not precipitate any appreciable amount of the substance or substances responsible for the oxidations we are studying—a result confirmed by experiments described below.

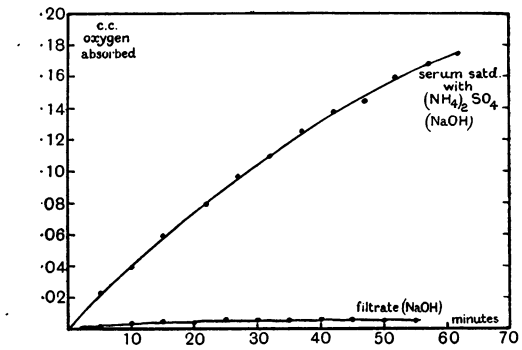


Fig. 6. Precipitation of the oxidisable materials by full saturation of the serum with ammonium sulphate.

On repeating now the experiment with full saturation with ammonium sulphate and using 1 cc. *N* NaOH as alkali it was easy to show that this procedure precipitated completely the oxidisable substances, for the filtrate from serum saturated with ammonium sulphate showed no oxygen uptake even in strongly alkaline solution, while the unfiltered mixture of serum and ammonium sulphate under the same conditions absorbed oxygen at a rate commensurate with that observed with the untreated serum (Fig. 6). The oxidisable material was therefore contained in the precipitate. A further series of observations illustrating the successive precipitation of the active material by ammonium sulphate is recorded in Fig. 7, from which it will be seen that, as mentioned above, one-third saturation with the salt removes only a small portion of the oxidisable substances while half saturation brings down a much larger fraction. In all these experiments we used such an amount of each of the various filtrates as corresponded to 1 cc. of the original serum, and added ammonium sulphate solution to the reaction mixtures in

such amounts that the concentration of this substance, together with the amount of NaOH and the total volume of the liquid under test, was the same for all experiments of any one series, so that they are strictly comparable *inter se*. As we were able to confirm the results of Fig. 7 on another sample of serum we may state definitely that the oxidisable substances are precipitated completely by full saturation with ammonium sulphate, partially by half saturation and hardly at all by one-third saturation with ammonium sulphate.

Seeing that from the results of the ether extraction experiments we concluded that the lipins of the serum are to a large extent concerned in the oxidations we are considering, we were naturally led to compare the behaviour of lecithin emulsions towards ammonium sulphate with that of the serum itself. It is easy to show that a 1 % emulsion in distilled water of Merck's lecithin (prepared from eggs) is rapidly and completely precipitated by saturation with ammonium sulphate, less rapidly but still as completely by half saturation and that even one-third saturation with the salt will throw out the

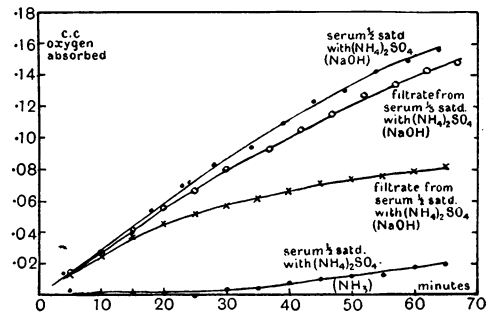


Fig. 7. Progressive precipitation of the oxidisable materials by increasing concentrations of ammonium sulphate.

whole of the lipin in the space of 2 to 3 hours. As the liquids from which the results of Fig. 7 were obtained were allowed to stand overnight before filtering, it might have been expected that the precipitation of the lipins would have been complete even in the case of the serum only one-third saturated with ammonium sulphate. But it is also easy to show that the presence of proteins very much modifies the conditions of precipitation of lecithin by salts. For example, if a mixture of 1 cc. of a 1 % emulsion of lecithin lightly stained with Sudan III and 1 cc. of 1 % gelatin solution be added to 10 cc. *N*/100 NaOH and the solution be then one-third saturated with ammonium sulphate by the addition of 6 cc. of the saturated solution, it is evident from the red colour remaining in the liquid that the bulky precipitate that is produced does not contain the whole of the lipin, although, in the absence of the protein, lecithin itself is completely precipitated under these conditions. In this alkaline solution the protein exerts a protective action on the lecithin. If we can assume that similar relationships obtain between the proteins and the lipins of the serum we can at once account for our observations.

These results have a bearing on the general question of the relationship of lipins to proteins in serum. As Chick demonstrated [1914], the euglobulin of serum is a complex of pseudoglobulin and phospholipin and is precipitated by one-third saturation of the serum with ammonium sulphate. That this procedure does not render the serum lipin-free is evidently due to the fact that the serum-proteins exert a protective action on the lipins similar to that which we have observed to be exerted on lecithin by gelatin: we can thus account for Chick's observation that after precipitation of the euglobulin from serum by one-third saturation with ammonium sulphate there remains sufficient lipin in the filtrate to combine slowly with pseudoglobulin to form a further quantity of euglobulin.

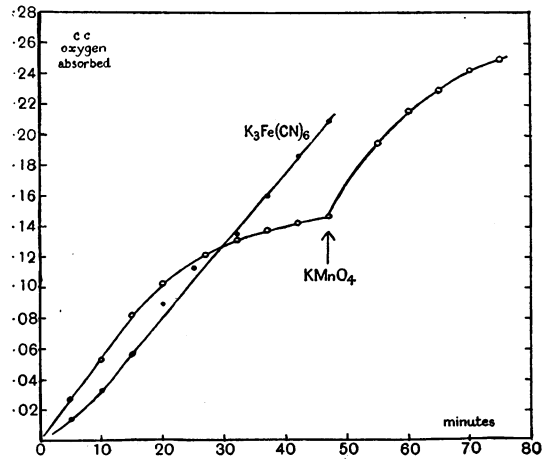


Fig. 8. Effect of substitution of potassium permanganate for ferricyanide: at the arrow a second portion of permanganate was added to the serum.

We should mention lastly that we made a few experiments to determine the effect of substituting other oxidising agents for the ferricyanide used in all the experiments so far described. As is seen in Fig. 8, for example, potassium permanganate in dilute ammonia solution acts even more powerfully in promoting the oxidation than ferricyanide, but the effect gradually passes off owing to the fairly rapid reduction of the permanganate by the proteins and other oxidisable constituents of the serum. On adding a further quantity of permanganate the rate of oxygen uptake is once more increased. So far as we are aware there are no experiments on the oxidation of lecithin in the presence of potassium permanganate with which we can compare this result obtained with serum. With potassium dichromate, which from the observations of Thunberg [1916] is known to accelerate the oxidation of ordinary lecithin, we obtained no acceleration of the oxidation of serum comparable with that produced by ferricyanide and permanganate, and this no matter whether we worked in an alkaline medium (1 cc. *N* NaOH + 1 cc. saturated ammonium sulphate being added to 1 cc. of the serum) or in an acid medium,

in which case 2 cc. approx. 0·1 *N* acetic acid were added to 1 cc. of serum (Fig. 9). Using ferric chloride also we found no appreciable absorption of oxygen to take place in the acid medium, but when the alkaline mixture was used the rate of oxygen uptake after a short initial delay due probably to the difficulty of efficient shaking of the mixture before the bulky precipitate of ferric hydroxide had shrunken together, was no less rapid than in the presence of ferricyanide (Fig. 9). This result again is just the opposite of that obtained by Warburg and Meyerhof [1913] with emulsions of ordinary lecithin, for they found that iron was a more efficient catalyst of the oxidation in acid solution than in neutral or alkaline.

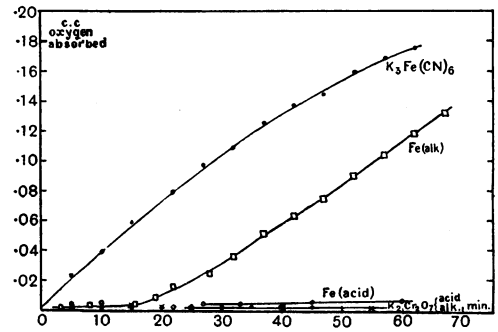


Fig. 9. Substitution of ferric salts and potassium dichromate for ferricyanide in the oxidation of serum.

It is natural to compare the results of this investigation with those obtained by Harris [1926] in his work on the oxidation of blood-plasma exposed to light. He found that when exposed to light 1 cc. plasma absorbs oxygen at the rate of about 0·024 cc. per hour, and ascribed this effect to the tyrosine and tryptophan present in the molecules of the plasma-proteins. But as this rate of oxidation is of the order of one-tenth of that which we have observed in the serum in the presence of ferricyanide, and, further, as Harris found that the addition of ferricyanide produces a marked degree of protection of the plasma against oxidation under the conditions of his experiments, it is evident that the processes of oxidation that occur when plasma is exposed to light can account for only a small fraction of the total oxidation that we have observed in the serum in the presence of ferricyanide.

In conclusion we would add that we do not claim any measure of completeness for the data and explanations here presented, but we have thought it worth while to publish in this form the results we have obtained so far on account not only of their practical bearing on the technique of blood-gas analysis, but also of their relation to the general study of biological oxidation systems.

SUMMARY.

In the presence of certain oxidising agents such as potassium ferricyanide and potassium permanganate human blood-serum absorbs oxygen at the rate of about 0·25 cc. per cc. per hour when shaken with air at room temperature. The same effect is observed in the presence of ferric salts in alkaline solution, but not with ferric salts in acid solution nor with dichromate at any reaction. Extraction with ether reduces the rate at which the serum will absorb oxygen in this way, but this can be restored in some measure by the re-addition of the extracted material provided that this is recovered by evaporation of the ether extract in an oxygen-free atmosphere. This suggests that the substances primarily responsible for the effect are lipins and this explanation fits in with the further observations that we have made on serum fractionated by means of ammonium sulphate, when the conditions determining the precipitation of lipins by this salt in the presence of proteins are taken into account.

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