35. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

4. THE 'LABILE IRON' IN BLOOD AND ITS INCREASE DURING CHOLEGLOBIN FORMATION[1]

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IT has been shown by Lemberg [1935] and Lemberg & Wyndham [1937] that the iron of a bile pigment-haematin compound (verdohaemochromogen) is more easily detached by acids than haemoglobin iron. If choleglobin is a bile pigment-haemoglobin it is to be expected that its formation during the coupled oxidation of haemoglobin and ascorbic acid will be accompanied by an increase in the 'labile iron'.

Barkan and his collaborators have shown in several papers that a small part of the iron in erythrocytes is split off more readily by acids than that of the remainder, and that 'leicht abspaltbares Bluteisen', for which we shall write in short ESI, accompanies oxyhaemoglobin through its purification and recrystallization. The initial values of ESI have, therefore, to be taken into account in the study of ESI in choleglobin solutions. According to Barkan & Schales [1937; 1938] the source of ESI is a bile pigment-haemoglobin ('pseudohaemoglobin') accompanying haemoglobin in the red cells, and inseparable from it on account of the identity of the protein parts (native globin). If this be correct, pseudohaemoglobin and choleglobin must be identical or very closely related. We have therefore reinvestigated the problem of the ESI in erythrocytes. While our experimental results agree with those of Barkan, except in one or two points, we arrive at different conclusions as to the explanation of these observations.

By this investigation we have also established the values of the initial ESI obtained from haemoglobin solutions in the presence of ascorbic acid in such concentrations as were used for the coupled oxidation. Thus we are enabled to measure the increase of ESI occurring during this reaction and to correlate it with the increase of choleglobin concentration, estimated spectrophotometrically with the method described in the preceding paper.

EXPERIMENTAL

Methods

Determination of ESI. Barkan incubates the diluted solution of haemoglobin with 0·4 % HCl for 16–24 hr., and then obtains by ultrafiltration a protein-free solution, in which he estimates the iron by a ferric thiocyanate method.

We found that our ultrafiltrates through Zsigmondy filters ('Cella finest' and 'Ultrafine') contained some protein breakdown products. These came out of solution at the *p*H used for the colorimetric estimation of iron as the ferrous $\alpha\alpha'$-dipyridyl complex in the method of Hill after Shorland & Wall [1936]. In

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order to use this method we prepared protein-free filtrates by trichloroacetic precipitation after incubation with HCl; blanks were, of course, carried out and the blank values subtracted. The results did not differ from those obtained with the Barkan method. A choleglobin solution, which according to the latter contained 10·25 % and in the presence of CO 11·3 % ESI, gave the values 11·0 and 10·25 % with our method. Barkan [1937] has also made use of trichloroacetic acid precipitation and confirmed earlier results of other workers that it gives the same results as the rather tedious ultrafiltration. He claims that in the presence of CO too low values of ESI in blood are occasionally found with the trichloroacetic acid method, so that the CO inhibition of the removal of ESI appears too high. The CO inhibitions that we observed in blood were, however, the same as those found by Barkan.

As a routine method we adopted the following procedure. To 1 ml. of the solution to be determined (blood diluted 10 times or choleglobin solution prepared from 0·7 % haemoglobin undiluted) was added 1 ml. of iron-free 0·8 % HCl; after mixing the solution was incubated overnight (16 hr.) at 37°. 1 ml. of 7 % trichloroacetic acid was then added, the mixture well shaken and the precipitate filtered off on a Whatman filter No. 42. The iron in an aliquot of the filtrate was then estimated by the method of Shorland & Wall.

If the acid treatment had to be carried out in CO or N_2, the solution was placed in the bottom part of a Thunberg tube and the HCl in the bent stopper. The CO was kept standing over alkaline pyrogallol solution, while the N_2 was freed from O_2 over heated copper asbestos.

Determination of total iron. A suitable aliquot of the solution was taken to dryness in a 6 in. × $\frac{5}{8}$ in. pyrex test tube. After ashing with 0·4 ml. conc. H_2SO_4 and 0·1 ml. perchloric acid (Merck) the solution was cooled and either made up to 10 ml. with glass-distilled water and analysed by the dipyridyl method; or after 2–3-fold dilution with water the solution was made alkaline with 5*N* NH_4OH containing ammonium thiolacetate. During this procedure it was cooled with ice water. After keeping for 5–10 min. with repeated shaking in order to keep the complex in the ferric state, the solution was compared colorimetrically with an iron standard treated in the same way. The two methods gave identical results.

The blood ESI and its behaviour towards CO and reducing substances

Thirteen determinations of the ESI on seven samples of human blood exposed to air (ESI_{O_2}) gave results which varied from 3·6 to 7 % of the total iron, the mean being 5·35 % and the standard deviation ± 1·17 %. Thirteen determinations on the same batch of crystalline horse oxyhaemoglobin gave values ranging from 3·5 to 11 %, with a mean of 6·9 % and a standard deviation of ± 2·5 %. During the preparation of crystalline horse oxyhaemoglobin the ESI_{O_2} varied from 4·9 % in the cells to 5·4, 5·7 % in the first crystallization, 4·0, 3·5 % in the second and 8·5, 9·8 % in the third. Recrystallization thus did not alter the ESI_{O_2} significantly. These values agree with those found by Barkan and other observers.

Effect of carbon monoxide. We can also confirm that under CO much less iron is detached than in air. The ESI observed under CO (ESI_{CO}) is only about one-third of that observed when the splitting is carried out in air (ESI_{O_2}). The average amounts of ESI_{CO} as % of ESI_{O_2} in thirteen estimations on crystalline horse oxyhaemoglobin varied from 21 to 47 with a mean of 31 and a standard deviation of ± 5. Determinations on sheep blood came within this range. This would mean an inhibition of the detachment of iron by CO of 69 % on the average.

Effect of reducing substances and inert gases. Barkan & Berger [1928] have already found that H_2 has an effect similar to that of CO.

Table 1 shows the effect of evacuation and addition of reducing substances ($Na_2S_2O_4$ and ascorbic acid) on the detachment of iron. These experiments were carried out with both crystalline horse oxyhaemoglobin and laked sheep cells. No difference was observed, so they are given together. Evacuation and ascorbic acid in concentrations of 30 mg. per 100 ml. or more decrease the detachment of iron by acids to the same degree as CO or H_2. The amount split off in presence of $Na_2S_2O_4$ is a little larger, because the SO_2 developed on acidification of the solutions removes some iron from haemoglobin.

Table 1. *Influence of evacuation and reducing agents on blood ESI*

Conditions	ESI found in % of ESI_{O_2}	No. of experiments averaged
In vacuo	33	2
Added $Na_2S_2O_4$, 50 μg. per ml.	89	2
„ 100 „	51	5
„ 300 „	46	8
„ excess	53	4
Added ascorbic acid, 50 μg. per ml.	80	2
„ 100 „	80	6
„ 300 „	38	6
„ 1200 „	25	2

Barkan & Schales [1937] claim that by the addition of small amounts of $Na_2S_2O_4$ the inhibition of the iron detachment by CO can be further increased, and base important conclusions on this observation. While the theoretical side of the question will be discussed later, we point out here that the experiments of Barkan & Schales lend only slender support to this claim. The authors report three experiments in which the ESI_{CO} was further lowered by addition of 130–330 μg. $Na_2S_2O_4$ per ml. Examination of their figures reveals, however, that in two of these experiments the amount split under CO in the absence of $Na_2S_2O_4$ was abnormally high (ESI_{CO} still 75 % and 58 % of ESI_{O_2}, instead of the usual 31 %); and that ESI_{CO} in the presence of the reducer (25 % of ESI_{O_2}) was not significantly smaller than usually without reducer and still inside the range of the figures for ESI_{CO} in percentage of ESI_{O_2} (21–47 % in our experiments). It is very likely that the saturation of these solutions with CO was somehow incomplete and that the effect of the reducer was only to bring it to completion. In one experiment only ESI_{CO} was 33 % of ESI_{O_2} without reducer and was further decreased by the addition of $Na_2S_2O_4$ to 14 %. Occasional stray results do, however, occur and cannot be considered as sufficient evidence.

We have therefore repeated these experiments using various concentrations of $Na_2S_2O_4$ and also of ascorbic acid added to solutions of crystalline horse oxyhaemoglobin (Table 2).

Table 2. *Influence of reducing agents on blood ESI in the presence of CO*

Conditions	ESI_{CO} in % of ESI_{O_2}
Without reducer, CO	28
Added $Na_2S_2O_4$, 130 μg. per ml.	14; 31
„ 300 „	31
Added ascorbic acid, 50 μg. per ml.	32
„ 120 „	15; 29
„ 300 „	28
„ 1200 „	24

Again a decrease of the iron split off was only observed in two experiments out of eight, and in each case repetition of the same experiment failed to confirm the low value. The decrease cannot therefore be considered as significant.

Experiments on the nature of the compound from which the blood ESI is derived

In the first paper of this series [Lemberg, Legge & Lockwood, 1939] we have already mentioned that CO does not decrease the amount of ESI liberated from choleglobin solutions, and have adduced this as evidence against the identity of choleglobin and the substance in blood which is the source of the ESI. The choleglobin solutions contain, however, ascorbic acid in concentrations between 50 and 100 mg. per 100 ml. and from Tables 1 and 2 it is evident that the blood ESI would also not be decreased much further by CO in the presence of ascorbic acid in this concentration. After removal of ascorbic acid from choleglobin solutions by prolonged dialysis CO has still no effect on the yield of ESI, but during dialysis oxycholeglobin, if present, may be transformed into ferric choleglobin and therefore not react with CO. It cannot be absolutely excluded, therefore, that CO may inhibit the removal of iron from an oxycholeglobin with reversibly bound O_2, the existence of which is not proved. It will be seen from the discussion below, that this point is no longer of importance, since we believe that the CO inhibition of the detachment of ESI in the blood has to be explained in a totally different way.

We have also briefly described spectroscopic observations which made us doubt the occurrence of choleglobin in red cells. They are given here in greater detail. When a strong solution of oxyhaemoglobin or of laked erythrocytes is treated with $Na_2S_2O_4$ in presence of air, the band of choleglobin is observed, and that of cholehaemochromogen when the haemochromogen is formed by pyridine or alkali and $Na_2S_2O_4$ without precautions. When, however, reduced haemoglobin is formed by evacuation or when the reductions with $Na_2S_2O_4$ are carried out in a medium free from O_2, no trace of these bands can be observed spectroscopically. In the first-mentioned experiments choleglobin is, therefore, an artefact, doubtless formed from haemoglobin by the action of traces of hydrogen peroxide, which is well known to arise by the action of O_2 on $Na_2S_2O_4$ solutions. While these experiments show once more the ease with which choleglobin arises from haemoglobin, they do not offer any evidence for its occurrence in red cells. In the absence of O_2 another absorption band was observed in solutions of reduced haemoglobin and in denatured globin and pyridine haemochromogens, lying at about 660 mμ. This band disappeared on oxygenation and reappeared on reduction. It was not observed in solutions of CO-haemoglobin or in laked blood treated with coal gas with or without addition of $Na_2S_2O_4$. This compound may be a source of some of the blood ESI.

From the spectrophotometric curves of CO-haemochromogen prepared from laked blood or crystalline oxyhaemoglobin (Fig. 1 of Part 3) we can deduce that choleglobin iron cannot form as much as 5% of the total iron. There is only a very slight hump of the absorption curve at 630 mμ indicating less than 1% of choleglobin. Even if pure CO-protohaemochromogen had zero absorption at 630 mμ, which from the form of the absorption curve is very unlikely, the choleglobin content could not be higher than 3·9%.

We have already briefly reported that the treatment of laked erythrocytes and of horse oxyhaemoglobin with acid yields, indeed, some bile pigment, and quantitative estimations will be given in the succeeding paper. While this proves the occurrence of some bile pigment haemoglobin in red cells, the bile pigment

yield was much lower than that expected from a bile pigment haemoglobin content of 5% which ought to be present if all ESI were derived from it.

We have therefore investigated whether some of the ESI may not originate from haemoglobin. The arguments which Barkan brings forward against this assumption will be discussed below, while here we present a few experiments. We have tried whether methaemoglobin synthesized in a way which excludes the presence of bile pigment-iron compounds, does not yield any ESI. Pure crystalline haemin should not, according to the way it is prepared, contain any bile pigment haematin; we have further made sure of this by extracting its ether-pyridine solution repeatedly with 20% HCl. After washing with water the precipitated haemin was dissolved in $N/10$ ammonia and coupled at once with the optimal amount of a native horse globin solution, the iron content of which was known. The alkaline haematin solution must not be allowed to stand, since we have observed that it is not very stable and after a few hours a band may be found at about 630 mμ after addition of native globin and $Na_2S_2O_4$. The synthesized methaemoglobin yielded even slightly more ESI (6·5–9·6%) than oxyhaemoglobin from red cells (Table 3). Inhibition by CO was not found, nor was it expected since the iron is in the ferric state. The experiment shows that the ESI of the blood may, at least partly, be derived from haemoglobin.

Table 3. *ESI in synthesized methaemoglobin*

The iron contained in the native globin solution (0·50 μg. per ml.) has been subtracted from the ESI values found.

	Total iron	ESI_{O_2}	ESI_{CO}	ESI_{N_2}
μg. per ml.	7·7	0·71; 0·56	0·74	0·50

Finally we have investigated the claims of Barkan & Berger [1930] that the CO-inhibition of the detachment of iron in mixtures of CO-haemoglobin and oxyhaemoglobin is much larger than it ought to be, if it were proportional to the saturation of haemoglobin with CO, and that the CO inhibition persists even after the reconversion of CO-haemoglobin into oxyhaemoglobin. This, indeed, would prove that the CO causing the inhibition of the iron splitting is bound to a compound different from haemoglobin. The experiments of these authors are, however, open to criticism. They claim to get the same results whether they add a known amount of CO dissolved in water to a solution of oxyhaemoglobin, or mix solutions of oxyhaemoglobin and CO-haemoglobin (saturated with CO) of about 1·5% haemoglobin concentration (blood diluted ten times) in the required proportions. This cannot be correct, since 100 ml. of 1·5% CO-haemoglobin solution contain 2 ml. of CO bound to haemoglobin, but at 15° at least 2·5 ml. CO in physical solution, and probably more, since this figure is based on the solubility in water, and that in protein solution is likely to be higher. The CO-haemoglobin content in the mixtures was not determined, and it is obvious that the mixtures must have contained a much greater proportion of CO-haemoglobin than Barkan & Berger assumed from the proportion in which the solutions had been mixed. There is, therefore, no evidence that the presumptive disproportionality between the degree of CO-inhibition of the iron splitting and the CO-haemoglobin concentration really exists. We can also not confirm the second claim. While the authors still find a considerable CO-inhibition of the iron detachment after 48 hr. of vigorous aeration of a CO-haemoglobin solution, we found that after 24 hr. aeration the ESI had returned to its original value.

The increase of ESI during choleglobin formation

During the coupled oxidation of haemoglobin and ascorbic acid the ESI of the solution rises simultaneously with the increase in absorption at 630 mμ due to choleglobin. In a series of experiments we have measured the ESI of choleglobin solutions and have compared the increase in ESI with the choleglobin concentration estimated spectrophotometrically as described in Part 3.

The choleglobin solutions contained 50–100 mg. ascorbic acid per 100 ml. From the results reported above it can be deduced that haemoglobin solutions containing ascorbic acid in this concentration yield ESI of about 1·5 % of the total iron. This value was therefore subtracted from the ESI found in our choleglobin solutions. The values thus obtained are given as ΔESI in Table 4. The procedure of subtracting the initial ESI is justified, even if the ESI or part of it were attributable to choleglobin, since the average absorption of CO-protohaemochromogen at 630 mμ has also been subtracted from the absorption values at 630 mμ for the estimation of the choleglobin content.

The values of choleglobin (including cholehaemochromogen in the 80 min. experiments at 37° in Table 4 are in percentage of initial haemoglobin and those of ΔESI in percentage of total iron, while in the bottom row (green pigment) the first figure means cholehaemochromogen in percentage of total haemochromogen (proto + chole) and the second figure ESI in percentage of total iron.

Table 4. *ESI from choleglobin*

Conditions of experiment	Time of incubation min.	Choleglobin	ΔESI	Ratio ΔESI : choleglobin
25°, *p*H 8·5	90	28·3	23·9	0·84
	120	42·3	28·4	0·67
37°, *p*H 8·5	15	17·9	11·7	0·65
	30	33·3	19·5	0·59
	80	45·5	23·8	0·52
37°, *p*H 7·2	30	14·1	12·2	0·86
	80	34·6	21·5	0·62
Green pigment	—	77·3	51	0·66
				Average 0·66

The increase of ESI under various conditions is evidently proportional to the amount of choleglobin formed, but the yield of ESI is only 66 % of that expected theoretically from the amount of choleglobin. The reason for this discrepancy is that a part of the cholehaematin remains unsplit and is still contained as such in the precipitate of denatured protein. If this precipitate is carefully washed free from trichloroacetic acid and redissolved in *N*/10 NaOH, a spectrophotometric determination of the absorption at 630 mμ of the CO-haemochromogen shows that 31 % of the cholehaematin has remained unsplit in this precipitate. It is possible that this observation means that choleglobin is not homogeneous and consists of two compounds, one easily split by acids and one that is not easily split, but the incomplete splitting cannot be considered as definite evidence against uniformity. We have observed similar incomplete splittings with verdohaemochromogen [Lemberg & Wyndham, 1937].

CO does not inhibit the detachment of iron from choleglobin solutions. In a few experiments a slight inhibition by CO (10–20 %) was found, but this is within the experimental range of error and in other experiments ESI_{CO} was found higher than ESI_{O_2}; usually both were equal (e.g. ESI_{O_2} 14·8 μg. per ml.,

ESI_{CO} 15·8, ESI_{CO} in presence of $Na_2S_2O_4$ 15·6). Even after the ascorbic acid had been removed by prolonged dialysis CO had no effect (ESI_{O_2} 18·9 % of the total iron, ESI_{CO} 19·4 %, ESI_{CO} in presence of $Na_2S_2O_4$ 18·4 %). A solution of cyanmetacholeglobin (cf. Part 2) yielded 8·9 % ESI_{O_2}, 7·3 % ESI_{CO} and in presence of $Na_2S_2O_4$ without CO 9·6 %, with CO 10·0 % ESI, all in percentage of total iron. All these differences lie within the range of experimental error.

Discussion

From their observation that CO decreases the yield of labile iron from laked erythrocytes or oxyhaemoglobin solutions to about one-third of its value, Barkan & Schales have concluded that the ESI arises from a bile pigment-haemoglobin (pseudohaemoglobin). They assume that the iron detached by acid splitting in air, but not in CO, i.e. $ESI_{O_2}-ESI_{CO}$ (about 65 % of the ESI and 3·5 % of the total iron), is derived from a bile pigment-oxyhaemoglobin (EO_2), EO_2 being easily split by acids in contradistinction to the CO compound (ECO). The iron that is detached even in the presence of CO (ESI_{CO}), about 35 % of ESI and 1·5 % of the total iron, they believe to be the ferric form of the same bile pigment haemoglobin (E′), since they observed that it can be partially converted into the CO-inhibitable form by reduction with $Na_2S_2O_4$.

In our experiments described above we have been able to confirm the greater part of Barkan's experiments with the exception of the presumptive conversion of E′ into ECO by reduction and CO, for which we do not find conclusive evidence, and their observation that the CO inhibition of the detachment of iron occurs under conditions in which haemoglobin is no longer combined with CO.

We have shown that a small yield of biliverdin can indeed be obtained from erythrocytes and haemoglobin solutions by treatment with acids, and this lends support to the view that at least part of the ESI is attributable to a bile pigment-haemoglobin. The quantitative experiments described in Part 5 indicate, however, that the concentration of this bile pigment-haemoglobin in blood or haemoglobin preparations is too small to account for more than a small part of the ESI.

There are in fact, considerable difficulties in accepting Barkan's deductions about the nature of the ESI. The effect of CO on ESI is not specific, and Barkan explains the decreasing effect of inert gases on the yield of ESI by the assumption that only EO_2 (oxypseudohaemoglobin) and E′ (pseudomethaemoglobin) undergo splitting, while the iron in E (reduced pseudohaemoglobin) as well as in ECO (carboxypseudohaemoglobin) is resistant to acid. For chemical reasons this does not appear likely. Experience with haemoglobin derivatives shows that CO-haemoglobin and reduced haemoglobin are much more readily split than oxy-haemoglobin or methaemoglobin. It is not impossible that the properties of pseudohaemoglobin derivatives are exactly opposite to those of haemoglobin derivatives. Experience with known bile pigment-haematin compounds, e.g. verdohaemochromogen, shows that the ferric and ferrous forms are both split by acid, and that CO, while combining, does not prevent the splitting (cf. Part 5). Choleglobin appears to behave in the same manner.

Barkan & Berger [1928] describe an experiment, in which they acidified the haemoglobin under hydrogen and then aerated the solution. The amount of ESI found under these conditions was not larger than without aeration (equal to ESI_{CO}). In order to explain this result the authors are forced to make the unlikely assumption that E does not unite with O_2 in acid solution. In the presence of acid, E must be split to pseudohaem and no acid haem has been observed that is not readily autoxidizable to acid haematin, whether in the class

of porphyrin-haematins or bile pigment-haematins. The final product obtained under the conditions of the experiment ought to be, therefore, the same as from EO_2 and E′, and the ESI found ought to be equal to ESI_{O_2}, not to ESI_{CO}.

Several authors [Taylor & Coryell, 1938; Ammundsen, 1939] have recently confirmed earlier observations of Klumpp [1935] that a difference of about 5% exists between the O_2 capacity of blood and its CO capacity in the presence of $Na_2S_2O_4$. This indicates the presence of ferric haemoglobin or ferric bile pigment-haemoglobin in normal blood. From magnetochemical observations Taylor & Coryell also conclude that the substance combining with CO in the presence of $Na_2S_2O_4$ but not combining with O_2 is a ferric compound. Barkan & Schales [1939] mention the results of Ammundsen as being explained by the presence of 'pseudohaemoglobin', but this is evidently only partly true. While E+E′ form 5% of the total blood iron, only E′ (1·5%) combines with CO in presence of $Na_2S_2O_4$ and not with O_2, E (3·5%) would combine with both and thus not contribute to the difference of the gas capacities. The existence of this difference, therefore, supports only the assumption that E′ is the ferric form of a bile pigment-haemoglobin, and again E can explain only a fraction of the difference found between the O_2 and CO capacities.

The whole problem is evidently far from being satisfactorily solved, and we offer, therefore, an alternative explanation which, while not fully satisfactory in all respects, appears to us more likely and in better agreement with the observed facts. We shall discuss separately the CO-inhibitable part of the ESI ($ESI_{O_2}-ESI_{CO}$, attributed by Barkan to EO_2) and the CO non-inhibitable part (ESI_{CO}, attributed by Barkan to E), since we do not believe that there is evidence for a chemical relationship of the two forms. There is no evidence that the effect of CO on the blood ESI is specific and that CO acts in a way different from any other inert gas. The experiments indicate solely that presence of O_2 is necessary for setting free of the iron from E. The simplest explanation appears to us to be that the CO-inhibitable iron fraction ($ESI_{O_2}-ESI_{CO}$) is formed from oxyhaemoglobin by an oxidation process during the action of acid on haemoglobin. Recently several authors have observed that the O_2 set free from oxyhaemoglobin by denaturation has strongly oxidative power. Holden [1936; 1937] and Rawlinson [1939] have shown that the globin part of oxyhaemoglobin undergoes oxidation during the denaturation. Lemberg & Legge [1938] found that the O_2 set free from oxyhaemoglobin by acids oxidizes ascorbic acid. In Part 5 it will be shown that under the same circumstances biliverdin is oxidized to biliviolin. Engel [1939] has recently observed that some plasma bilirubin is oxidized during the precipitation of oxyhaemoglobin by alcohol, and that it can be protected against this oxidation by ascorbic acid. In the same way ascorbic acid protects biliverdin from being oxidized during the acid denaturation of oxyhaemoglobin. It is only necessary to assume that the oxidative effect can be turned against the prosthetic group of the haemoglobin molecule as well as towards the globin part. It has already been suggested by Lintzel & Radeff [1928] that ESI is derived from haemoglobin and that the prosthetic group is partially destroyed by the action of acid; the authors' claim is based on their observation that the colour of the acid haematin derived from oxyhaemoglobin is several per cent less in intensity than from the same amount of carboxyhaemoglobin. Barkan & Berger [1930] have accumulated material against this hypothesis, but their arguments are not fully convincing, although Lintzel & Radeff's observation may be explained by a different colloidal state of the 'acid haematins' derived from oxyhaemoglobin and CO-haemoglobin. Some of Barkan's arguments, e.g. the fact that the detachment of ESI is irreversible though incomplete, and the lack of formation

or porphyrin, do not speak against our hypothesis. His experiments with mixtures of CO-haemoglobin and oxyhaemoglobin are open to the criticism levelled above against his method of determining the ratio of the two compounds. It is doubtful, whether the slight increase of the ESI percentage in haemoglobin after partial adsorption on alumina, observed by Barkan [1936], is actually due to a preferential adsorption of haemoglobin on alumina; if one remembers the ease with which small amounts of choleglobin are obtained from haemoglobin, it is quite likely that a slight oxidation of haemoglobin to choleglobin occurs during the adsorption experiments; Barkan has omitted to show that the haemoglobin recovered from the alumina contained less ESI.

The majority of Barkan's and our observations can be explained satisfactorily on the assumption that ESI is derived from haemoglobin by oxidation during the acidification. Inert gases or CO would prevent this reaction by transforming oxyhaemoglobin into reduced haemoglobin or CO-haemoglobin, while ascorbic acid would prevent the oxidative removal of iron from the haemoglobin molecule by being oxidized instead, in the same way as it protects added bile pigment. Once the acid denaturation has taken place under exclusion of O_2, aeration would no longer cause oxidative removal of iron, in agreement with the experiment of Barkan & Berger mentioned above. The transformation of oxyhaemoglobin into methaemoglobin does not decrease the yield of ESI [Barkan & Berger, 1928], since the oxidative reaction setting free the iron would occur during the methaemoglobin formation.

One observation in our experiments would still remain unexplained, namely the presence of the usual percentage of ESI in solutions of synthetic methaemoglobin, but this observation too speaks against the theory of Barkan and favours the assumption that some ESI is derived from haemoglobin itself, since any bile pigment-iron compounds have been excluded by the method of preparation.

While the greater part of ESI, about two-thirds, would thus have to be considered as an artefact, there would still remain the one-third (ESI_{CO}) which can be split off even in inert gases. Here our experiments support Barkan's view of the bile pigment-haemoglobin nature of at least a part of this fraction. In Part 5 we shall describe the isolation of small amounts of biliverdin and biliviolin from red cells. We have not yet been able to decide whether or not the detachment of iron during the acid denaturation of haemoglobin, leads to formation of bile pigment. If the porphyrin part of the haemoglobin molecule is oxidized to substances other than bile pigment, the yield of bile pigment indicates that almost the total ESI_{CO} fraction is bile pigment-haemoglobin. Otherwise a part of the bile pigment found would also be an artefact, certainly, however, not all, since in the presence of ascorbic acid biliverdin was obtained from red cells.

A certain part of E′ must also be the blood catalase. We have recently proved [Lemberg, Norrie & Legge, 1939] that a bile pigment-haematin group occurs in catalase. A simple calculation shows, however, that the blood catalase constitutes only a small fraction of the total E′. The monomolecular reaction constant k of rabbit's blood catalase multiplied by the dilution is 1500 (own unpublished experiments). If the catalase factor of pure catalase ($=k$/g. catalase in 50 ml.) is 35,000, this corresponds to a catalase concentration of 86 mg. catalase in 100 ml. of blood. Catalase contains one equivalent of bile pigment-haematin iron in a molecule of mol. wt. 248,000. The ESI derived from blood catalase is, therefore, about 20 μg. per 100 ml. of blood, while the total ESI_{CO} (E′-iron) is about $\frac{15{,}000{,}000 \times 55{\cdot}8 \times 1{\cdot}5}{16{,}700 \times 100} = 750$ μg. per 100 ml. Catalase forms,

therefore, less than 3% of the E. Barkan & Schales [1936] have, indeed, separated catalase from E′ by adsorption on alumina, and with the slight qualification that their separation means separation of catalase from the main bulk of E′, their experiments are in agreement with the above calculation.

Summary

The findings of Barkan & Schales are confirmed that blood and haemoglobin solutions contain 5–6% of the total iron in the form of 'labile iron' (ESI_{O_2}) and that about two-thirds of this ($ESI_{O_2}-ESI_{CO}$) are split off only in the presence of O_2 and in the absence of reducing substances while the remaining third is split off also in inert gases and CO (ESI_{CO}).

In contradistinction to Barkan & Schales we explain the fraction $ESI_{O_2}-ESI_{CO}$ as an artefact, arising by oxidation of the prosthetic group of the haemoglobin molecule by the O_2 evolved from oxyhaemoglobin by acids.

The fraction ESI_{CO} is at least partially attributable to a bile pigment-haemoglobin which gives rise to bile pigments when treated with acids. Only a small part of ESI_{CO} is due to the blood catalase.

The increase of labile iron occurring during the coupled oxidation of haemoglobin and ascorbic acid is proportional to the concentration of choleglobin formed, the latter being estimated spectrophotometrically. About 66% of the choleglobin iron is split off by 16 hr. incubation with $N/10$ hydrochloric acid, while the remainder is found as unsplit cholehaematin in the precipitate of denatured protein. Neither CO nor reducing substances appear to inhibit the acid splitting of choleglobin.

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