hexoses in the carbohydrate-rich fraction is emphasized.

5. The possibility is considered that the treatment of our seromucoid with sodium sulphate at 37° also results in the detachment of a carbohydrate-rich fraction.

6. The bearing of these preliminary findings upon

current views concerning the nature of the serum glycoproteins is briefly discussed.

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The Reduction of Methaemoglobin in Red Blood Cells and Studies on the Cause of Idiopathic Methaemoglobinaemia

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It is well established that methaemoglobin produced in the blood of normal animals by the injection of drugs disappears quite rapidly on withdrawal of the drug (Dennig, 1900; Stadie, 1921), and that its disappearance is due to reactions taking place in the red cell itself (Cox & Wendel, 1942). The last named authors showed that reduction of methaemoglobin taking place *in vivo* continued at the same speed in the shed blood, and did not depend on reducing substances in the plasma. Drug methaemoglobinaemias in man appear to be closely similar to those in animals, the foreign pigment usually disappearing within 24 hr. after the last dose of the drug.

In idiopathic methaemoglobinaemia, a rare disease which may be familial, it has been found that there is little or no tendency for the methaemoglobin to disappear from the shed blood (Lian, Frumusan & Sassier, 1939; Barcroft, Gibson, Harrison & McMurray, 1945). This observation has recently been confirmed by Sievers & Ryon (1946) who showed that the stability of the methaemoglobin probably does not depend on an abnormality of the plasma, since plasma from their patient did not

affect the rate of reduction of methaemoglobin in normal human erythrocytes, nor did normal plasma alter the behaviour of methaemoglobin in erythrocytes from the case.

The work described here has been carried out on blood from six persons belonging to two families in which idiopathic methaemoglobinaemia occurred. It has been possible to obtain evidence of a specific deficiency in the enzyme system of erythrocytes from the cases, and also to draw conclusions concerning the reactions involved in the reduction of methaemoglobin in the blood of normal persons. Apart from their application to the study of idiopathic methaemoglobinaemia, the results are of interest since they throw light on the mechanism by which the haemoglobin of normal persons is maintained in a functional state. The mechanism of regeneration of haemoglobin from methaemoglobin is of importance as it has been suggested that methaemoglobin is constantly being formed in the blood (Peters & Van Slyke, 1931), and Paul & Kemp (1944) have shown that methaemoglobin is present in small amounts in normal human blood.

METHODS

Materials. Hexosediphosphate was prepared by the method of Morgan & Robison (1930). For use, the neutral Ba salt was rubbed up in a small glass mortar with the theoretical amount of $N-H_2SO_4$, and neutralized with NaOH after removal of $BaSO_4$. Hexosemonophosphate (Robison ester) was isolated from a yeast fermentation mixture as described by DuBois & Potter (1943). Final purification was effected through the Pb salt as described by Robison (1922). As the salt is soluble, Ba was removed at pH 8 as the phosphate. The acid Ba salt of D-3-phosphoglyceric acid was prepared from hexosediphosphate as described by Neuberg & Vercellone (1935). The purity of the specimen of the crystalline salt was checked by the polarimetric method of Meyerhof & Schulz (1938). Na pyruvate was prepared from the freshly distilled acid by the method of Peters (1938) and recrystallized twice from aqueous acetone. Diphosphopyridine nucleotide (DPN) was prepared from brewer's yeast by the method of Green & Brosteaux (1936). Later the isolation of larger quantities from baker's yeast was undertaken, using the simplified method of Green & Williamson (1940). The DPN content of the preparations was determined manometrically by measuring the CO_2 evolution on reduction with hydrosulphite in bicarbonate solution (Warburg, Christian & Griese, 1935). Coenzyme factor was prepared from ox heart in the form of the Straub flavoprotein (Straub, 1939). Purification was carried to the stage of elution from alumina C_{γ} .

Analytical methods. Venous blood samples of 30–50 ml. were taken from the subjects 2 hr. after breakfast and defibrinated by stirring with glass wool. Before use, the cells were washed three times with 5 vol. 0.9% (w/v) NaCl containing 0.01M-phosphate buffer pH 7.4, (phosphate saline). It is important that the cells be washed with saline shortly after the blood is drawn, otherwise rapid reduction of methaemoglobin (MHb) may be observed in the controls without substrate.

In the experiments with normal blood and in some of the experiments with blood from the cases of idiopathic methaemoglobinemia, it was necessary to form MHb in the cells. This was effected by treating the red cells with 3 vol. 0.075% (w/v) amyl nitrite in phosphate saline. The mixture was allowed to stand for 10–15 min. at room temperature, and the cells freed from excess nitrite by repeated washing with phosphate saline. This treatment converts about half of the haemoglobin to MHb. It was noted that in the absence of phosphate the formation of MHb by amyl nitrite takes place much less rapidly. The total pigment in the cell suspensions was usually about 14 g./100 ml. All results, however, have been corrected to refer to a suspension containing 15.6 g./100 ml. to facilitate comparison of the figures in the tables.

The reduction of MHb in the red cells was followed by measuring the uptake of CO consequent on the reduction of MHb to haemoglobin. Samples of 2 ml. of the red cell suspension were measured into the main chamber of Barcroft-Dixon flasks of about 40 ml. capacity. 0.2 ml. 2N-KOH and a roll of filter paper were placed in the inset and substrate added, after equilibration in the water bath, from Keilin tubes. The flasks were filled with 20% (v/v) CO in N_2 by repeated evacuation, the CO being prepared from formic and sulphuric acids. The results in all cases were corrected for the condensation of CO with the KOH in the

insets to form formate (Warburg & Christian, 1931). The temperature in all experiments was 37°.

Haemolysates were prepared by washing red cells with 0.9% (w/v) NaCl, and cooling the concentrated corpuscular cream to -2°. Two vol. of ice-cold water were then added, and the mixture allowed to stand for 10 min. A volume of 0.5M-phosphate buffer pH 7.4 equal to that of the blood corpuscles was added and the cloudy liquid centrifuged at high speed for 15 min. The clear supernatant fluid was sucked off and used in the manometers. Pyruvic acid was determined on trichloroacetic acid filtrates of blood by the method of Long (1942). Phosphoglyceric acid was determined by the method of Rapoport (1936). When required, MHb was determined by the method of Evelyn & Malloy (1938), and total pigments by the method of Clegg & King (1942), standardized according to Gibson & Harrison (1945). Where necessary, the quantities of reagents taken for these colorimetric estimations were slightly altered to suit the Hilger Spekker absorptiometer.

RESULTS

Glucose and lactate as substrates in the reduction of methaemoglobin in erythrocytes

The reduction of methaemoglobin (MHb) produced by the action of nitrite on red cells from normal persons is slow when they are suspended in saline. The reduction is greatly accelerated by the addition of glucose (Table 1, cols. A, B). This confirms previous observations of Warburg, Kubowitz & Christian (1930), using rabbit erythrocytes. The effect of lactate is closely similar (Table 1, cols. A, C). Glucose and lactate together do not produce additive effects, which suggests that some stage in the overall reaction is common to both substrates. In the case of lactate it is found that the net molecular ratio *pyruvate formed/MHb reduced* is approximately 0.5 (Table 1, cols. G–E/C–A), in agreement with the equation

With glucose as substrate, the position is less clear, since there are several possible pathways for the reaction. The main problem is whether glucose acts before splitting, during glycolysis, into 3-carbon compounds, or after the splitting has taken place. In the first case the reactions would be of the type described by Dickens (1938), involving phosphorylation followed by step-wise oxidation of the molecule through phosphogluconic acid to phosphoketohexonate and decarboxylation to phosphopentose (Fig. 8, steps 1 and 2). The enzymes catalyzing these reactions have been shown to occur in horse blood and require coenzyme II. The other possibility is that reduction of MHb takes place after splitting of the glucose molecule to two triosephosphate molecules. The reactions possible would then be

Table 1. *The reduction of methaemoglobin in nitrite-treated erythrocytes from normal persons and from cases of idiopathic methaemoglobinaemia*

(Each manometer contained 2.0 ml. cell suspension, total pigment 15.6 g./100 ml. Cells suspended in 0.9% (w/v) NaCl made 0.01M with phosphate buffer pH 7.4. Substrates added from Keilin tubes at the end of the equilibration period. Reaction period 4 hr. at 37°, gas phase 20% (v/v) CO in N₂. KOH papers in insets. Final concentrations, glucose 0.01M, lactate 0.02M.)

	MHb reduced (μmol.)			Pyruvate (μmol.)			Ratio Net pyruvate Net MHb		
	Cells A	Cells + glucose B	Cells + lactate C	Cells initial D	Cells final E	Cells + glucose F	Cells + lactate G	Cells + glucose H	Cells + lactate I
Normal bloods									
1	1.0	3.9	3.9	0.5	3.6	2.4	5.1	0.49	0.52
2	0.9	3.0	3.5	0.3	2.1	1.8	3.3	0.50	0.46
3	0.8	3.6	4.1	0.3	3.5	2.0	5.5	0.47	0.49
4	1.0	3.2	—	0.4	2.9	2.0	—	0.50	—
5	1.2	2.7	3.0	0.5	2.4	1.8	3.4	0.48	0.55
6	2.0	6.0	6.6	0.5	4.4	3.8	6.8	0.53	0.52
Cases									
1	0.7	1.6	0.8	0.2	3.3	1.0	3.5	—	—
2	0.5	0.8	0.7	1.0	4.0	0.9	4.0	—	—
3	0.7	0.8	0.7	0.4	2.8	0.4	2.9	—	—
4	0.4	0.6	0.7	0.2	3.2	0.5	3.4	—	—
5	1.0	1.7	1.7	—	—	—	—	—	—
6	1.2	1.8	1.7	—	—	—	—	—	—

or glycolysis to lactate may be completed and reaction (1) follow. These reactions require coenzyme I. It is unfortunately impossible to test these reactions directly by trying out the intermediates postulated since the envelope of the erythrocyte is impermeable to the phosphorylated compounds, and the reduction of MHb effectively ceases on haemolysis.

Some indirect evidence is however available that reactions (1) and (2) are principally concerned.

(a) In glycolysis the oxidation of triosephosphate is coupled with the reduction of pyruvate so that the latter part of the scheme may be written

Owing to the coupling of triosephosphate and pyruvate in the oxido-reduction, there can be no increase in the sum of the phosphoglycerate and pyruvate unless either triosephosphate or lactate is oxidized by some reaction outside the scheme. If the reduction of MHb takes place at the expense of either triosephosphate or lactate, then an increase in phosphoglycerate or pyruvate will occur. The figures in Table 1, col. H, i.e. cols. (F-D)/B, show that an amount of pyruvate equivalent to the amount of MHb reduced is formed in normal erythrocytes during the reduction of MHb by glucose. Net values, however, cannot be obtained by comparison with controls without substrate, because red cells

contain a large amount of phosphoglycerate (Jost, 1927), which breaks down in the absence of glucose to give pyruvate (Table 1, cols. D, E). The cells from the cases of methaemoglobinaemia may be used as controls, since in them little reduction of MHb is taking place, and no accumulation of pyruvate occurs in the presence of glucose, while in the absence of glucose, pyruvate is formed in an amount similar to that found in normal cells (Table 1, cols. D, F, and D, E).

(b) In the presence of fluoride, the breakdown of phosphoglycerate is inhibited (Lohmann & Meyerhof, 1934), so that reduction of MHb by means of reaction (2) would be accompanied by the formation of an equivalent amount of phosphoglycerate. It has been found that reduction of MHb in the presence of glucose is not inhibited by 0.01M-NaF, and that in its presence phosphoglycerate accumulates to a significant extent only when MHb is being reduced (Table 2). In the estimation of phosphoglycerate by the method of Rapoport (1936) it was found that with blood there was a tendency to give a greyish brown colour as compared with the blue of the standard, probably due to charring of organic material during heating with H₂SO₄ (Meyerhof & Schulz, 1938). For this reason, and also because of the large number of subtractions needed to obtain net values for comparison with the amounts of MHb reduced, close stoichiometric equivalence was not obtained, but the figures show that both MHb and glucose are required for substantial formation of phosphoglycerate.

Table 2. *The formation of phosphoglycerate in normal erythrocytes during the reduction of methaemoglobin in the presence of 0.01M-NaF*

(Conditions as in Table 1, except that each manometer contained, in addition, 0.01M-NaF. Incubation for 3 hr., except for the manometer containing methylene blue, which was incubated for 1 hr. only.)

	Phosphoglycerate (μ mol.)		MHb reduced (μ mol.)
	Washed cells	Nitrite-treated cells	
Initial	2.2	2.2	—
Final	2.8	3.2	2.5
Final + glucose	3.4	5.9	4.7
Final + glucose + 5×10^{-6} M-methylene blue	—	6.1	12.0

(c) Monoiodoacetate is known to inhibit the triose-phosphoric enzyme. If MHb reduction depends on the oxidation of triosephosphate, it would be expected that iodoacetate would have an inhibitory effect, and, as shown in Table 3, it has been found

Table 3. *The effect of iodoacetate on the reduction of methaemoglobin in normal erythrocytes in the presence of glucose and lactate*

(Conditions as for Table 1.)

	MHb reduced (μ mol.)			
	Gross		Net	
	Control	Iodoacetate 0.002M	Control	Iodoacetate 0.002M
Cells	0.8	0.8	—	—
Cells + glucose	3.5	1.0	2.7	0.2
Cells + lactate	4.0	4.0	3.2	3.2

that small concentrations of iodoacetate produce a 90% inhibition of MHb reduction. This effect is not due to a non-specific inhibitory action of iodoacetate, since reduction of MHb by lactate is unaffected.

Reduction of methaemoglobin by glucose and lactate in cells from cases of idiopathic methaemoglobinemia

It has been found (Table 1, cols. A, B, C; Fig. 1) that there is relatively little tendency for MHb to disappear from the red cells of cases of idiopathic methaemoglobinemia either in the presence or absence of glucose or lactate, and that there is correspondingly little formation of pyruvate during the experimental period (Table 1, cols. D, F and E, G). As the work on normal cells which has just been described had suggested that MHb reduction under these conditions depends chiefly on the triose-phosphoric and lactic enzymes, the cells from the

Fig. 1. Reduction of MHb by erythrocytes from a normal person and from a case of idiopathic methaemoglobinemia. The ordinate gives the amount of MHb reduced, expressed in μ l. CO absorbed by 2.0 ml. of a cell suspension containing 15.6 g./100 ml. total pigments, incubated at 37°. The clear symbols refer to the normal, the shaded symbols to the case. The conditions are the same as in Table 1.

Table 4. *The disappearance of pyruvate from normal erythrocytes and from erythrocytes from cases of idiopathic methaemoglobinemia*

(Values given are μ mol. pyruvate present/vessel. Each contained 2.0 ml. cell suspension in 0.9% (w/v) NaCl containing 0.01M-phosphate buffer, pH 7.4. Total pigment 15.6 g./100 ml., 0.1 ml. 0.5M-NaF, 0.2 ml. 0.029M-Na pyruvate, and 0.2 ml. 0.1M-glucose, or 0.2 ml. 0.9% (w/v) NaCl. Incubation at 37° in air for 1 hr.)

	Pyruvate (μ mol.)		
	Initial	Final	Final + glucose
Normals			
1	6.8	6.4	2.4
2	6.8	6.3	2.0
3	6.8	6.4	3.0
4	6.8	6.5	2.0
5	6.8	5.8	3.0
Cases			
1	6.8	6.3	2.0
2	6.8	6.4	2.8
4	6.8	6.2	2.6

cases were investigated for a possible deficiency of these enzymes. To test this point, the disappearance of pyruvate in the presence of glucose and NaF was measured in normal and pathological cells. This reaction (Dische, 1937) involves both the triosephosphoric and lactic enzymes and diphosphopyridine nucleotide (DPN). The results given in Table 4 show that there is no significant difference, in this respect, between the normal and pathological cells. The cells from the cases are therefore not deficient in these enzymes and DPN (Fig. 8 A, 1-4).

The effect of methylene blue on the reduction of methaemoglobin by glucose and lactate

The finding of active triosephosphoric and lactic enzymes in the cells from cases of idiopathic methaemoglobinaemia, suggested that the failure of these cells to reduce MHb might be due to a deficiency of some oxidation-reduction carrier intervening between the pyridine nucleotides and MHb. In view of the effect of methylene blue in accelerating the reduction of MHb *in vivo* (Cox & Wendel, 1942), its action *in vitro* was examined. It was found, using normal cells (Table 5), that small amounts of the

Table 5. Reduction of methaemoglobin in the presence of 5×10^{-5} M-methylene blue by cells from cases of idiopathic methaemoglobinaemia and by cells from normal subjects

(The figures show the amount of MHb reduced/hour. In comparing with Table 1, it should be noted that the figures there are for a period of 4 hr. The conditions are as for Table 1, except that each manometer contained 5×10^{-5} M-methylene blue in addition.)

Normals	MHb reduced (μ mol./hr.)		
	Cells	Cells + 0.01 M-glucose	Cells + 0.02 M-lactate
1	0.8	8.2	3.0
2	0.9	13.0	—
3	1.1	16.2	3.9
4	1.3	—	4.5
5	1.8	9.6	6.1
Cases			
1	0.7	12.7	1.0
2	0.4	11.8	0.6
3	0.3	10.5	0.5
4	0.4	12.2	0.8
5	0.8	15.7	1.0
6	0.5	19.2	0.8

dye accelerated the reduction of MHb by glucose more than ten times. The rate of reduction by lactate was approximately doubled. Dewan & Green (1938) have shown that the reduction of methylene blue by reduced DPN is catalyzed by 'coenzyme factor', while Corran, Green & Straub (1939) have shown that 'coenzyme factor' is probably identical with the flavoprotein isolated by

Straub (1939) from heart muscle. In the light of these findings it is probable that methylene blue acts as a carrier between this or a similar flavoprotein and MHb.

The results with the pathological cells are in striking contrast to the results with normal cells (Table 5). In the abnormal cells, the rate of reduction in the presence of methylene blue and glucose is accelerated in a manner similar to that found in normal cells. With lactate, however (Table 5; Fig. 2), the rate of MHb reduction is scarcely changed on the addition of methylene blue. This finding is notably different from that in normal blood, where the rate of reduction with lactate is substantially accelerated.

Fig. 2. MHb reduction by erythrocytes from normal persons and from cases of idiopathic methaemoglobinaemia in the presence of lactate and methylene blue. Each manometer contained 2.0 ml. nitrite-treated cell suspension (total pigment 15.6 g./100 ml.), 0.02 M-Na lactate and 5×10^{-5} M-methylene blue. The results are corrected for reduction of MHb taking place in controls without substrate. Temp. 37°, gas phase 20% (v/v) CO in N₂, reaction period 1 hr.

The pathway of the reaction in the presence of glucose and methylene blue is uncertain. The following evidence is available.

(a) The reaction does not appear to proceed via the triosephosphoric or lactic enzymes, since the amount of pyruvate accumulating is far below that

which would be expected if lactate were the ultimate reductant of MHb (Table 6), while the amount of phosphoglycerate accumulating in the presence of fluoride is similarly too small to account for the methaemoglobin reduced (Table 2).

Table 6. *The relation between methaemoglobin reduced and pyruvate formed in the presence of glucose and methylene blue*

(Conditions as in Table 1, except that each manometer contained 5×10^{-5} M-methylene blue. The figures for MHb are gross quantities reduced, the figures for expected pyruvate are calculated on the assumption that 1 mol. pyruvate corresponds to 2 mol. MHb. Incubation 4 hr.)

Normals	MHb reduced (μ mol.)	Pyruvate formed (A) (μ mol.)	Pyruvate expected (B) (μ mol.)	100 A/B
1	9.4	1.09	4.7	23
2	7.5	0.80	3.8	21
3	10.6	1.28	5.3	24
4	6.4	1.05	3.2	32
5	12.6	1.46	5.3	23
Cases				
1	7.6	0.60	3.8	16
2	17.0	1.72	8.5	20
3	4.8	0.44	2.4	18
4	5.5	0.87	2.7	32

(b) Phosphorylation of glucose probably precedes the reduction of MHb, since arsenate, which interferes with phosphate transfer in the oxidation of triosephosphate (Needham & Pillai, 1937), produces a marked inhibition of MHb reduction. Oxalate, which is believed to inhibit the transfer of phosphate from phosphopyruvate (Lohmann & Meyerhof, 1934), produces less inhibition than arsenate, but the effect of the two together is more than additive. An experiment of this type is summarized in Fig. 3.

(c) When minimal quantities of glucose are present, the amount of MHb reduced/mol. glucose added exceeds the theoretical maximum possible, if oxidation of glucose were taking place after splitting of the sugar molecule to two 3-carbon compounds. If triosephosphate or lactate were the substrates concerned, the maximum possible ratio would be 4 mol. MHb/mol. glucose. In the presence of fluoride or oxalate, it has been found that this ratio may be substantially exceeded. An experiment of this kind is shown in Fig. 4. It should be noted that ratios of this kind represent minimum values, since any loss of glucose by side reactions such as glycolysis would tend to depress them.

The marked difference between normal and pathological bloods in the presence of glucose only, and the similarity of their behaviour in the presence of glucose and methylene blue, suggests that the addition of methylene blue virtually opens up a new

Fig. 3. The effect of arsenate and oxalate on the reduction of MHb in nitrite-treated erythrocytes in the presence of glucose and methylene blue. Each manometer contained 2.0 ml. cell suspension (total pigments 15.6 g./100 ml.), 0.01 M-glucose and 5×10^{-5} M-methylene blue. The glucose was added after equilibration at 37° from Keilin tubes. Gas phase 20% (v/v) CO in N_2 .

Fig. 4. The figure shows the amount of MHb reduced/mol. glucose added, after various periods of time. Each manometer contained 2.0 ml. nitrite-treated cell suspension in phosphate saline, 5×10^{-5} M-methylene blue, and 1 μ mol. glucose which was added from a Keilin tube. The results are corrected for the reduction of MHb which took place in controls without added glucose. The theoretical maximum amount of MHb which could be reduced after splitting to two three-carbon compounds is indicated by the dotted line.

reaction pathway, rather than that it acts by accelerating a reaction which is already proceeding. These results may be accounted for by supposing that in the presence of methylene blue, stepwise oxidation of sugar occurs according to the scheme of Dickens (1938), who has shown that the necessary enzymes are present in the blood of the horse as well as in yeast (Fig. 8, col. B). In this step-wise breakdown of carbohydrate it would be possible for at least 8 mol. MHb to be reduced/mol. glucose used, and phosphorylation would precede the utilization of the sugar. Further, the enzymes in Dickens's (1938) scheme require triphosphopyridine nucleotide, as opposed to the enzymes taking part in the reduction of MHb in normal cells in the absence of methylene blue, which require DPN.

Coenzyme factor activity in normal red cells and in cells from cases of idiopathic methaemoglobinaemia

The finding that the cells from the cases of methaemoglobinaemia were unable to reduce MHb appreciably faster in the presence of lactate + methylene blue than in the presence of methylene blue alone (Table 5, Fig. 2) together with evidence that there are adequate amounts of the triosephosphoric and lactic enzymes in these cells (Table 4) suggested that the cells might be deficient in coenzyme factor activity. Measurements were therefore made of the rate of oxidation of triosephosphate in haemolysates prepared from normal blood and from the blood of the cases. It was found that under the conditions used (0.1M-phosphate buffer, pH 7.4, 7×10^{-3} M-Na hexosediphosphate, 5×10^{-4} M-DPN, 2×10^{-4} M-methylene blue), the limiting factor governing the rate of oxidation of triosephosphate was the coenzyme factor when this was added in the form of a solution containing the Straub flavoprotein (Straub, 1939).

The results of assays of 'coenzyme factor' activity in twelve consecutive normal bloods and in the blood of the cases are given in Fig. 5, where the blackened portions correspond to the oxygen uptakes by the system specified. The total heights of the columns (shaded + unshaded) represent the uptakes in one-third of the time by the system + Straub flavoprotein where this uptake was determined.

The first three cases of methaemoglobinaemia examined gave results which are markedly lower than those obtained with normal blood. In the last two cases, however, the difference is much less striking. The assays of the first three cases were interpolated among assays of normal bloods, while the last two were carried out some time afterwards. The assays of the pathological bloods were in all cases repeated upon two separate blood samples taken at different times, with results which did not differ by more than 10%. After the assays of the last two cases had given higher results than for the

other abnormal bloods, one of the earlier patients was recalled and the assay repeated. The result of this experiment confirmed the original low figure for this patient, so there does not appear to, be any

Fig. 5. The solid rectangles represent the amount of O_2 absorbed by 2.0 ml. of a haemolysate containing 5.6 g./100 ml. total pigment, in the presence of 0.1M-phosphate buffer pH 7.4, 0.007M-Na hexosediphosphate, 5×10^{-4} M-diphosphopyridine nucleotide, and 2×10^{-4} M-methylene blue. The heights of the whole columns (shaded + unshaded) represent the amount of O_2 absorbed by the same system in the presence of 0.4 ml. of a preparation of the Straub flavoprotein. The methylene blue was added from Keilin tubes. The figures for the shaded portions give the uptakes from 15 min. after the addition of the dye until 60 min. afterwards. The whole columns represent the uptakes in the first 15 min. after the addition of methylene blue. Incubation at 37° in air. In the last two cases examined the preparation of Straub flavoprotein had become relatively inactive. It produced a large increase in both cases, but the final figures were not considered reliable and so have been omitted.

doubt either of the low figures for the first three cases examined, or of the higher figures for the last two. It should be noted that the last two cases were much less severely affected than the others, having initial blood MHb levels of less than 2 g./100 ml. while the first three had initial MHb levels ranging from 5.1 to 7.8 g./100 ml. The last two cases, nos. 5 and 6 in Table 1, also showed a higher enzyme activity in the

intact cells. Taking the results as a whole, it would seem justifiable to conclude that a definite deficiency in the coenzyme factor activity of the blood of persons suffering from idiopathic methaemoglobinemia has been demonstrated. The coenzyme factor which was tested in these experiments is coenzyme factor I reacting with DPN. The deficiency of this factor in the cells from the cases provides a satisfactory explanation of the failure of methylene blue to accelerate lactate oxidation in the intact pathological cells.

Correction required in coenzyme factor assays. In carrying out these experiments it was assumed that the rate of reaction between methylene blue and reduced DPN is negligible in the absence of flavoprotein, but later work showed that this is by no means true, and that the figures obtained required correction for the oxygen transferred by direct reaction between reduced DPN and methylene blue. As the size of this correction had an important bearing on the comparisons made between normal and abnormal bloods it was determined with some care. Reduced DPN was prepared by the method of Ohlmeyer (1938) and allowed to react with methylene blue in phosphate buffer in air at 37°. The reaction was followed manometrically. The results, plotting the logarithm of reduced DPN concentration against time for two different methylene blue concentrations, show that the reaction is apparently bimolecular (Fig. 6). This allows of the calculation of the correction for any required amounts of DPN and methylene blue.

(a) In the lactic enzyme system prepared from acetone powder of heart muscle extract, varying amounts of DPN were added in the presence of 0.065M-HCN, 0.08M-phosphate buffer pH 7.4, and 0.016M-Na lactate. The results of several experiments gave a mean value for the correction of 28 $\mu\text{l./hr.}$

(b) Experiments in which varying amounts of DPN were added to blood haemolysates showed that the addition of the amount required to give a final concentration of $6 \times 10^{-5}\text{M}$ approximately doubled the oxygen uptake, while further additions up to a concentration of 10^{-3}M gave uptakes which increased slowly and gradually beyond this point. These results suggested that the enzyme system was almost effectively saturated at the lower concentration and that the slow increase on further addition of DPN was due to the direct reaction between reduced DPN and methylene blue. The correction derived from these experiments was 26 $\mu\text{l./hr.}$ These independent methods of determining the correction lead to practically the same result, and the average value has been used in correcting the coenzyme factor assays.

It will be noted that in Fig. 5 the oxygen uptakes have been recorded over a period of 45 min. which

began 15 min. after the addition of methylene blue from a Keilin tube. The readings in the initial period were disregarded. This was done for two reasons, namely that in the controls without substrate, a small amount of MHb is formed by oxidation by the

Fig. 6. Oxidation of reduced diphosphopyridine nucleotide by molecular O_2 in the presence of methylene blue. Each manometer contained 5.9 mg. reduced diphosphopyridine nucleotide (CoH_2), in 0.1M-phosphate buffer, pH 7.4. The quantity of methylene blue shown on the graph was added from a Keilin tube after temperature equilibration. Temp. 37°, gas phase air. The ordinate shows the amount of reduced diphosphopyridine nucleotide present at any time expressed as the log. of the equivalent amount of O_2 . As H_2O_2 is the end product under the conditions used, the figures are twice those which would be observed in the presence of catalase.

methylene blue, and that when the blood from the cases was used, which already contained a large amount of MHb, rapid reduction occurred as soon as the methylene blue was added and continued until an equilibrium amount of MHb was present. As the transfer of 1 electron will lead to the uptake of 1 mol. O_2 when applied to the reduction of MHb, whereas 4 electrons must be transferred when H_2O is the end product, it is desirable to postpone the initial reading until reasonable time has been allowed for equilibrium with regard to MHb, and the reaction has settled down to a steady rate.

The oxidation of hexosemonophosphate in haemolysates

In view of the suggestion that the reduction of MHb in the presence of glucose + methylene blue may proceed by means of coenzyme II dehydrogenases, it was of interest to examine the oxidation of hexosemonophosphate in the blood of normal persons and in the blood of the cases. Owing to the difficulty of preparing coenzyme II, fully detailed

studies were not carried out, but it was found, as would be expected if this suggestion is correct, that the rate of oxidation of hexosemonophosphate was substantially similar in both normal and abnormal bloods (Fig. 7).

Fig. 7. Oxidation of hexosemonophosphate in haemolysates of normal blood and of blood from cases of idiopathic methaemoglobinaemia. Each manometer contained 2.0 ml. haemolysate (total pigment 5.6 g./100 ml.), 2×10^{-4} M-methylene blue, 5×10^{-4} M-diphosphopyridine nucleotide and 0.07 M-Na hexosemonophosphate. The methylene blue was added from Keilin tubes after temperature equilibration. The height of the columns represents the amount of O₂ absorbed in 1 hr. at 37° after the addition of the methylene blue. Gas phase air.

DISCUSSION

On the basis of the work which has been described, the following tentative explanation is offered for the occurrence of idiopathic methaemoglobinaemia, and of the normal mechanism for the reduction of MHb in the blood.

(a) MHb reduction requires coenzyme I dehydrogenases, particularly the lactic and triosephosphoric dehydrogenases. Associated with these is coenzyme factor I. It is assumed that coenzyme II dehydrogenases do not play any very significant part in the reduction of MHb under normal circumstances, probably because coenzyme factor II does not react with MHb.

(b) On the addition of methylene blue, which is capable of reacting rapidly with both coenzyme factors I and II and MHb, the reduction of MHb is greatly speeded up both by an acceleration of the existing pathway via coenzyme I dehydrogenases and the opening up of new pathways via coenzyme II dehydrogenases in Dickens's scheme. These reactions are presented schematically in Fig. 8.

Fig. 8. Diagrammatic scheme of the reactions taking place in the reduction of MHb in erythrocytes. Column A reactions occurring in the absence of methylene blue; B in the presence of methylene blue. In cases of idiopathic methaemoglobinaemia where there is a deficiency of coenzyme factor I, the reactions in column A do not occur, and MHb reduction is very slow.

(c) MHb reduction fails to occur in the cases of idiopathic methaemoglobinaemia because of the deficiency of coenzyme factor I. The addition of methylene blue speeds up MHb reduction as with normal blood because of the presence of coenzyme II dehydrogenases and coenzyme factor II, together with glucose. In the presence of lactate, there is no marked effect on adding methylene blue because the lactic enzyme requires coenzyme I and coenzyme factor I which is present only in small amounts.

(d) MHb does not continue to increase in amount in the blood of persons affected with idiopathic methaemoglobinaemia to an unlimited extent, because, as the concentration of MHb rises, its rate of reaction with non-specific reducing agents in the blood will also rise and an equilibrium will be established. In this respect it is probable that ascorbic acid and glutathione are the most important

reducing agents in the blood. The results of Gibson (1943) suggest that ascorbic acid reacts directly with MHB and the dehydroascorbic acid formed is again reduced by glutathione. The action of large amounts of ascorbic acid in bringing about a new equilibrium with much smaller amounts of MHB in the blood of these cases (Lian *et al.* 1939; Deeny, Murdock & Rogan, 1943; Barcroft *et al.* 1945; Sievers & Ryon, 1946) is probably due to a mass action effect of this kind.

It should be pointed out that direct evidence is not available for all the assumptions and suggestions made in this scheme. It has, however, the merit of accounting for all the reactions observed in the abnormal cells by means of a single deficiency, which has been demonstrated with reasonable assurance, namely the deficiency of coenzyme factor I. The difficulties met with in the work have been of two kinds: first, it has not been possible to obtain blood samples from the patients as often and at the times which would have been desirable, because none of the patients lived in Belfast, but had to travel for considerable distances to the laboratory; and secondly, it has been impossible to put the theories about the intermediates involved in the various reactions to a direct experimental test, since the red cells are impermeable to most of the substrates involved, and laking the cells stops the reduction of MHB.

There seems to be no doubt, however, that idiopathic methaemoglobinaemia belongs to the group of diseases due to an inborn error of metabolism and should be classified along with alcaptonuria and cystinuria rather than under enterogenous cyanosis. Of the alternative explanations possible for the accumulation of MHB in the blood of these cases, impermeability of the erythrocytes to glucose may be dismissed, since glucose is rapidly oxidized by the cells in the presence of methylene blue, while a rapid rate of formation of MHB also seems to be improbable, otherwise reducing systems would lead to the disappearance of the pigment from the shed blood as occurs in drug methaemoglobinaemias where the cells are normal. In this connexion it should be noted that Sievers & Ryon (1946) examined the blood of a case with great care for MHB-forming agents without finding any evidence of their presence.

The results bearing on the mechanism of MHB reduction in normal red cells are of considerable interest, since it is probable that MHB is constantly being formed in the blood of normal persons (Peters & Van Slyke, 1931). The low level which is present in normal blood, about 1% of total pigment (Gibson & Harrison, 1945), would presumably represent the amount required to give equilibrium between the rates of formation and removal of the pigment. If there is such an equilibrium between formation and removal, the enzymatic mechanism described would account for the removal of MHB and the maintenance of the haemoglobin in a functional state in normal persons. Failure of any part of this enzyme system to function would lead to methaemoglobinaemia of the type exemplified by the patients described in this paper.

SUMMARY

1. Methaemoglobin reduction in normal erythrocytes takes place through the oxidation of triose-phosphate and lactate.

2. The system requires coenzyme factor I in addition to the enzymes and coenzyme I.

3. Coenzyme II dehydrogenases do not play a part in the normal mechanism of methaemoglobin reduction, but on the addition of methylene blue, they take the principal part in bringing about the great acceleration of methaemoglobin reduction which is observed.

4. Methaemoglobin present in the blood of persons suffering from idiopathic methaemoglobinaemia is not reduced with appreciable speed in the presence of glucose or lactate.

5. Blood from cases of idiopathic methaemoglobinaemia contains less coenzyme factor I activity than does normal blood.

6. It is suggested that, owing to the deficiency of coenzyme factor I in the blood of patients suffering from idiopathic methaemoglobinaemia, the enzymatic reducing activity is lower than in normal cells, leading to a displacement of the equilibrium $\text{Hb} \rightleftharpoons \text{MHB}$ to the right and the consequent accumulation of abnormal amounts of MHB in the blood.

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Studies on Glyoxalase

1. A NEW FACTOR

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The experiments to be described took origin from a personal observation by one of us (E.J.M.). He found that the enzyme glyoxalase was well adsorbed on calcium phosphate gel at pH 5-6, the filtrate showing no activity at all in the presence of methylglyoxal and glutathione. When, however, a proportionate amount of the filtrate was added to the eluate from the calcium phosphate the activity of this was largely increased.

EXPERIMENTAL

General methods

The method was that used in our previous paper (Hopkins & Morgan, 1945), namely, the determination of the rate of CO₂ evolution from NaHCO₃ solution, due to lactic acid production from methylglyoxal. We employed Barcroft differential manometers provided with standardized flasks. Each flask contained a measured quantity of the enzyme solution or other preparations, 0.6 ml. of 0.2M-NaHCO₃, with 2 mg. of glutathione (GSH) in 0.3 ml. water in a Keilin cup. To one flask 2.1 mg. of methylglyoxal were added, and the total contents of each flask made up to 3 ml. with distilled water (any variation of these quantities is given in the text). The apparatus was filled with a gas mixture of 95 vol. N₂ + 5 vol. CO₂, the GSH tipped in and the manometer shaken in a bath at 25°. The stopcocks were left open for 4 min. and then closed.

The majority of our experiments were carried out with ox heart, as this was the most convenient tissue to obtain fresh in sufficient quantities. Acetone preparations of the hearts were prepared. In making these, owing to the scarcity of acetone, smaller quantities were used than usual. The hearts freed from fat were minced, and extracted at 0° with three times their weight of acetone for 30 min., filtered, and extracted a second time for 15 min., using half the previous volume of acetone. The extracted heart was filtered off and dried in large vacuum desiccators. It was then ground to a fine powder in a mill. This acetone heart powder if kept dry keeps its activity for several months.

Separation of the new factor from the enzyme

The following separation was repeated several times; the results are shown in Table 1.

1st stage. 80 g. of acetone heart powder were extracted by stirring with 400 ml. of distilled water for 1 hr. and then centrifuged. The supernatant, which was highly pigmented, measured 234 ml. Of this, 0.3 ml. (dry wt. 9.90 mg.) gave 303.2 μ l. CO₂ in 20 min.

2nd stage. Fractional precipitation with ammonium sulphate. To 220 ml. of the supernatant, solid ammonium sulphate (68.64 g.) was slowly added to give 50% saturation. After standing 2 hr. the precipitate was centrifuged off and discarded.