to hydrolyze. Our attempts to block synthesis of polysaccharide with dinitrophenol were unsuccessful.

Kendall, Friedemann & Ishikawa (1930) have also reported incomplete recovery of carbon from alanine, glucose and pyruvate when resting cells of *Staph. aureus* supplied with these substrates were aerated with CO_2-free air in tall cylinders. They report as both aerobic and anaerobic end products: lactate, volatile acid, CO_2 and small quantities of pyruvate. Test for lactate by the method of Barker & Summerson (1941) revealed less than 0·4 μmol. after oxidation of 50 μmol. of glucose by 10 hr. cells of *Staph. aureus* NRRL-B313.

SUMMARY

1. In microrespiration experiments resting cells of *Staphylococcus aureus* from the logarithmic period of growth (10 hr. cells) oxidized glycerol and glucose almost completely to CO_2 and H_2O. Younger organisms (6 hr. cells) oxidized these substrates to acetate, CO_2 and H_2O.

2. In these products 10% of the glycerol and 15% of the glucose did not appear. Lactate was not formed in appreciable quantity, but synthesis of polysaccharide might account for the missing source of carbon. Encapsulation of young cells favours this explanation, but direct quantitative evidence in its support was not obtained.

3. Study of this strain of *Staph. aureus* at different stages of growth established that only after the organism was well in the logarithmic phase did resting suspensions oxidize acetate. The effect was not one of simple adaptation but was associated with some as yet unknown change in the growth medium.

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Stability of Haemoglobin and of Certain Endoerythrocytic Enzymes *in vitro*

By D. KEILIN and Y. L. WANG, *Molteno Institute, University of Cambridge*

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In the present paper we propose to deal with those aspects of the stability of haemoglobin and of certain enzymes which can be expressed in terms of time during which these proteins may retain their specific biological activities and therefore their structural integrity. The study of this aspect of protein stability, which can be described as the life span of a protein, can hardly be based upon experiments solely designed for this purpose since it must depend to a very great extent upon material collected a considerable time ago and for different purposes.

MATERIAL

The material used in the present investigation was composed of samples of whole blood obtained from different sources:

(A) Samples of citrated horse blood collected by Dr E. G. D. Murray in 1922.

(B) Samples of horse blood received by the late Prof. G. H. F. Nuttall in 1904.

(C) Samples of guinea-pig blood collected by Nuttall in 1902.

(D) Samples of blood of other animals (jackal, ant bear, sea lion and Himalayan bear) received by Nuttall in 1901–3.

These samples of blood were investigated in respect of the properties of (1) haemoglobin, (2) carbonic anhydrase, (3) catalase, (4) glyoxalase and (5) choline esterase. Not every specimen was examined under all these headings, but in every case (except blood samples D) when the properties of haemoglobin or the activities of an enzyme were determined they were invariably compared with those of a corresponding sample of fresh blood.

METHODS

Haemoglobin and its derivatives

The methods applied to this study can be summarized as follows:

The haemin content of the blood was estimated as pyridine haemochromogen using as standard a pyridine haemochromogen prepared from pure haemin crystals. The determinations were carried out by means of (*a*) a microspectroscope and double wedge trough, (*b*) a Hilger-Nutting spectrophotometer, or (*c*) a King photoelectric colorimeter (1946).

For this purpose a stock solution of alkaline haematin containing 1 mg. haemin/ml. was prepared from pure haemin crystals dissolved in 0·1 N-NaOH. Of this stock solution 1 ml. was mixed with 4 ml. pyridine, brought to 25 ml. with distilled water and reduced with a few mg. of $Na_2S_2O_4$ to give a standard solution of pyridine haemochromogen. Samples of horse blood were converted to haemochromogen as follows: 2 ml. of 1 in 100 solution of blood were mixed with 0·4 ml. N-NaOH and 0·8 ml. pyridine, the mixture being diluted to 5 ml. with water and reduced with a few mg. $Na_2S_2O_4$. The pyridine haemochromogen thus obtained was compared with the standard solution by the above methods.

The absorption spectra of different samples of haemoglobin were determined in the usual way with the Hilger-Nutting spectrophotometer, using for this purpose blood diluted 1 in 100. As the haemoglobin in old samples of blood has a tendency to oxidize gradually to methaemoglobin, spectrophotometric studies were also carried out on samples treated with $Na_2S_2O_4$ and CO.

The oxygen capacity of blood samples was determined in Barcroft differential manometers. The right-hand flasks of the manometers receive 1 ml. of blood mixed with 2 ml. of distilled water and, in the case of fresh blood, a few mg. of saponin are added to assure the complete lysis of the red blood corpuscles. A volume of 0·3 ml. of a saturated solution of $K_3Fe(CN)_6$ is delivered from the dangling tube after the equilibration of the temperature and closing of the manometer taps. The left-hand flask in these experiments contains 3·3 ml. water.

The oxygen dissociation curves of haemoglobin were determined spectroscopically by a method previously described (Keilin & Wang, 1946). It consists in matching the absorption spectra of a solution of haemoglobin equilibrated at different pressures of O_2 in special microtonometer tubes with the absorption spectra of optical mixtures of oxyhaemoglobin and reduced haemoglobin kept separately in the two compartments of a double wedge trough. For this purpose, 1 ml. of blood is mixed with 3 ml. water and when lysis is complete (in the case of fresh blood) 1 ml. 0·25 M-phosphate buffer of pH 7·3 is added to the mixture. The stromatin is centrifuged off to give a clear solution in which the haemoglobin concentration is one fifth of that of the original blood. The spectroscopic measurements were carried out by means of a microspectroscope outfit and a double wedge trough placed close to the side aperture of the spectroscope on a brass platform attached to the microscope. The microtonometers used in these experiments were of the bulb-capillary type devised by Fox (1945) for the study of the oxygen affinities of invertebrate haemoglobins. The diameters of the capillary portions of these tonometers were carefully determined and one drop of blood was introduced into each bulb. Gas mixtures of the desired O_2 pressure were prepared by diluting air with oxygen-free nitrogen in a graduated mercury gasometer. The gas mixtures were passed through a series of bubble counters containing blood of the same concentration and treated with $K_3Fe(CN)_6$ in order to remove the O_2 bound to haemoglobin. About 400 ml. gas was then passed during 10 min. through the tonometer, which was occasionally rotated. Five tonometers filled with different gas mixtures were rotated for 15 min. in a water-bath at 21°. The blood was then drawn into the capillary and matched spectroscopically against the optical mixture of oxyhaemoglobin and deoxygenated haemoglobin in the double wedge trough (see Keilin & Wang, 1946).

Enzymes

The estimations of the activities of carbonic anhydrase, catalase, glyoxalase and choline esterase in fresh and old samples of blood were carried out manometrically. The brief descriptions of the methods used which follow are based upon the study of fresh samples of blood. The slight modifications of these methods introduced for certain samples of blood will be given later on.

Carbonic anhydrase. The activity of carbonic anhydrase was determined with the aid of the boat apparatus as described by Meldrum & Roughton (1933). One side of the glass boat receives 2 ml. of phosphate solution prepared by mixing equal volumes of 0·2 M-Na_2HPO_4 and 0·2 M-KH_2PO_4 while in the other side are placed 2 ml. of 0·2 M-$NaHCO_3$ in 0·02 M-NaOH. In the non-catalyzed reaction, which is used as a blank, the phosphate side of the boat receives 0·2 ml. water; in the catalyzed reaction it receives 0·2 ml. of blood diluted 1 : 200. The experiments were carried out at 0° with the boats shaken longitudinally 300 times/min.; the readings being taken every 5 sec. for about 1 min.

Catalase. The activity of catalase was determined both with differential manometers at room temperature and with the boat method at 0°. In the first case the right-hand flasks of the manometers received 0·3 ml. of 0·5% (v/v) blood, 2·7 ml. 0·1 M-phosphate buffer pH 6·5, while 0·3 ml. of 1 vol. H_2O_2 was put in a dangling tube. The left-hand flasks received 3·3 ml. water. The dangling tube was dislodged after equilibration of the temperature and the readings were taken every min. for 5 min., the manometers being shaken rapidly (about 200 strokes/min.).

In experiments with the boat method one side of the boat received 1·8 ml. water and 0·2 ml. of 1% (v/v) blood whereas the other side received 1 ml. of 2 vol. H_2O_2 mixed with 1 ml. 0·25 M-phosphate buffer pH 6·8. The activities were calculated from the evolution of O_2 during the first 3 min. of the reaction, the readings being taken every 10 sec. during the first minute and every 30 sec. during the last 2 min.

Glyoxalase. The activity of this enzyme was estimated in differential manometers by the rate of evolution of CO_2 from a bicarbonate buffer by lactic acid formed from methyl glyoxal in the presence of glutathione. In these experiments, carried out at room temperature, the right-hand flask of the manometer receives 0·2 ml. blood, 0·5 ml. of 0·18 M-$NaHCO_3$, 2·3 ml. water containing a few mg. glutathione, and 0·2 ml. of 5% (v/v) methyl glyoxal was put in a dangling tube. The left-hand flask receives the same

reagents, except the blood, which is replaced by 0·2 ml. water. The manometers are filled with the gas mixture composed of 95% N_2 and 5% CO_2. After the temperature equilibration the taps of the manometers are closed, the dangling tubes are dislodged and the readings are taken during 40 min.

Choline esterase. The activity of this enzyme was estimated by the liberation of CO_2 from bicarbonate by the acetic acid formed during the catalytic hydrolysis of acetylcholine. In this case the method adopted was similar to that used for the estimation of glyoxalase activity. The right-hand flask of the manometer receives 0·2 ml. blood, 0·5 ml. of 0·18 M-$NaHCO_3$, 2·3 ml. water and the dangling tube contains 0·3 ml. of 0·087 M-acetylcholine. The manometers are filled with N_2 containing 5% CO_2.

RESULTS

Horse blood A preserved 24 years

These blood samples were in several sealed ampoules of 2·5 ml. capacity, each containing about 2 ml. citrated horse blood collected aseptically in October 1922 by Dr E. G. D. Murray* then working in the Field Laboratories near Cambridge. The blood was specially collected for one of us (D.K.) in connexion with certain physiological experiments on the larvae of *Gastrophilus* parasitic in horses.

The ampoules were kept in cotton wool and enclosed in tin boxes deposited in a cold room at +1° in the Low Temperature Research Station, Cambridge. At the end of the year only one box containing eight ampoules remained from previous experiments. In June 1923 the box was transferred to the Molteno Institute where it remained exposed to the usual variations of room temperature. This box, although occasionally moved, was seldom opened so that the ampoules were well protected from light.

Haemoglobin in blood A

The haemoglobin of these samples was examined for the first time by one of us (D.K.) in November 1930, i.e. when the samples were about 8 years old. It was then noted that the blood was laked and of a purple colour showing the characteristic absorption band of deoxygenated haemoglobin when examined spectroscopically through the narrow portion of the ampoule. On opening an ampoule and transferring its contents to a test tube the haemoglobin was rapidly oxygenated to oxyhaemoglobin showing a normal colour and absorption spectrum. The oxygen capacity of this blood sample measured in the usual way was found to be approximately the same as an average capacity of three samples of freshly collected horse blood.

The blood in these ampoules was probably lysed and deoxygenated within the first few weeks of storage. The order of events as indicated by freshly stored samples is a gradual lysis beginning about 2 weeks after sealing the ampoule, accompanied by the deoxygenation of oxyhaemoglobin and its oxidation to methaemoglobin which is ultimately reduced back to haemoglobin. The oxygen is slowly used in oxidation processes within the cellular elements of the blood. The autoxidation of haemoglobin to methaemoglobin during the gradual disappearance of O_2 confirms some of the previous observations by Neill & Hastings (1925) and Brooks (1935) who found that the rate of oxidation of haemoglobin by molecular oxygen is greater at a small partial pressure of oxygen.

The ampoules were again examined between August 1945 and November 1946, i.e. after being stored for about 24 years. Before opening them it was ascertained that haemoglobin was in the deoxygenated state showing the characteristic colour and absorption spectrum. On opening an ampoule and transferring its contents to an open vessel, the haemoglobin became fully oxygenated without formation of methaemoglobin. The oxyhaemoglobin thus formed has the normal colour and absorption spectrum. It can easily be deoxygenated by diluting it with water and boiling it at room temperature in a Thunberg vacuum tube or by treating it with $Na_2S_2O_4$. In both cases it forms a typical haemoglobin showing one wide and somewhat diffuse band at about 550–560 mμ. The fact that there is no marked reinforcement at 557 mμ. denotes the absence of a haemochromogen which would appear, especially in presence of $Na_2S_2O_4$, had the haemoglobin undergone a partial denaturation. One may naturally ask whether the denatured fraction of haemoglobin, if formed, has undergone a further degradation beyond the stage at which it may still give a haemochromogen. However, this was ruled out by the fact that the total iron of these samples estimated with the $\alpha\alpha'$-dipyridyl method as previously described (Keilin & Hartree, 1945) was found to be within 3% of the amount calculated from the haemin content of the blood.

Before we enter into a discussion of the haemin content and the oxygen capacity of blood A as compared with those of fresh samples of horse blood it is important to mention that in both cases blood was drawn from the jugular veins of horses kept at the Field Laboratory and bled at more or less regular intervals. It is therefore not surprising that the fresh blood samples show a certain variation in their haemoglobin content. The haemin content and the oxygen capacity of blood A and of five different samples of fresh horse blood were determined as described above (p. 492). The results of these determinations, which are summarized in Table 1, clearly show that there is little difference in this respect between the 24-year-old blood A and the freshly

* Now Professor of Bacteriology, McGill University, Montreal.

collected material. Moreover, the O_2 binding capacity of blood/mg. of haemin which is 32·0 μl. for the old blood agrees well with the average of the values found for fresh blood (32·2) and is only about 6 % below the theoretical value of 34·2 μl.

Table 1. *O_2 capacity and haemin content of a 24-year-old sample of blood A and of fresh samples of horse blood*

(Blood samples 1, 2, 4, citrated; 3, 5, 6, defibrinated)

Samples of blood	O_2 capacity (μl. O_2/ml. blood)	Total haemin of blood (mg./ml.)	μl. O_2/mg. haemin
1. Old blood A	200	6·25	32·0
2. Fresh blood	190	6·40	29·7
3. Fresh blood	231	6·80	34·0
4. Fresh blood	202	6·50	31·5
5. Fresh blood	170	5·30	32·1
6. Fresh blood	176	5·09	34·7

HbO_2 were found to be approximately the same (Table 2).

Table 2. *Heights of the α- and β-bands of oxyhaemoglobin in blood A and in two samples of fresh blood*

(Values expressed as the molecular extinction coefficient $\epsilon_{mol.} = 1/cl \log_{10} I_0/I$, where c = molar conc. of blood haemin, l = optical depth and I_0 and I are the intensities of incident and transmitted light.)

Samples of blood	$\epsilon_{mol.} \times 10^{-4}$ α—578 mμ.	β—542 mμ.
1. Old blood A	1·34	1·30
2. Fresh blood	1·38	1·35
3. Fresh blood	1·39	1·36

The oxygen dissociation curves of oxyhaemoglobin in the old sample A and in a fresh blood sample diluted five times were determined spectroscopically at 21° (cf. p. 492). These curves, represented in Fig. 1, show only small differences, the curve of the old sample being somewhat less S-shaped. The O_2 pressure (P_{50}), corresponding to 50 % HbO_2 at 21° is 5·1 mm. Hg for fresh and 4·8 mm. for

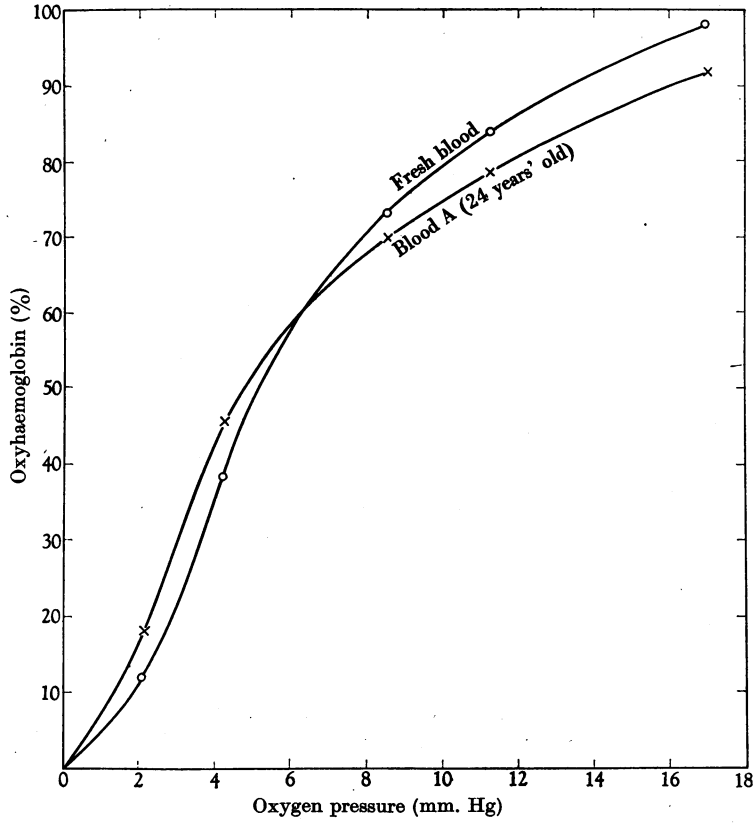

Fig. 1. O_2 dissociation curves of horse haemoglobin in a 24-year-old sample of blood A and in a fresh sample of blood at 21°.

The spectrophotometric curves of HbO_2 and HbCO in blood A and in fresh horse blood were almost indistinguishable and absolute values of the absorption in the region of the α- and β-bands of

the old blood A. Since an O_2 dissociation curve was obtained with only one sample of fresh blood, slight differences in the shape of curves shown in Fig. 1 may be partly due to individual variations.

Enzymes in blood A

Carbonic anhydrase. This enzyme, which catalyzes the reversible reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$, is found in blood only within the red blood corpuscles. Its activity was determined by the boat method at 0° and expressed in mm. of manometric fluid corrected by the readings obtained in determinations of blanks or non-catalyzed reactions. Two experiments carried out with two different samples of blood A and with fresh blood gave the following results: In the first experiment the reaction catalyzed by fresh blood and by blood A gave readings of 205 and 165 mm. respectively, which shows that blood A retained 80 % of the normal activity. In the second experiment the corresponding values were 126 and 104 mm., indicating the retention of about 82 % of the normal activity.

Catalase. This enzyme, which catalyzes the decomposition of H_2O_2 to molecular O_2 and water ($2H_2O_2 = 2H_2O + O_2$), is also localized within the erythrocytes. Several samples of blood A were tested for the activity of this enzyme and compared with the catalase activity of fresh defibrinated, citrated or oxalated horse blood. The activities of one defibrinated sample of fresh horse blood and of one of the samples of blood A determined by the boat method at 0° and expressed in mm. of manometric fluid were 78 and 36 mm. respectively, hence the catalase activity in this sample of A was about 46 % that of the fresh blood. It is important to note that this ampoule was only two-thirds filled with blood. The catalase activity of a second sample of blood A, which almost completely filled the ampoule, as well as that of a fresh sample of oxalated blood were compared in differential manometers at 20°. In this case the evolution of O_2 in fresh blood and in the sample A during the first 2 min. of reaction were 83 and 72 μl. respectively, which shows that blood A has retained about 87 % of its catalase activity.

Glyoxalase, which is also an endoerythrocytic enzyme, catalyzes the conversion of methyl glyoxal into lactic acid ($CH_3COCHO + H_2O = CH_3CHOHCOOH$). The activities of this enzyme in fresh oxalated blood and in a sample of blood A were estimated manometrically in terms of CO_2 liberated from bicarbonate buffer in 10, 20 and 30 min. gave the following values: (*a*) for fresh blood 118, 257 and 372 μl. and (*b*) for samples A 58, 125 and 182 μl., which indicates that blood sample A retained about 50 % of its glyoxalase activity.

Choline esterase. In experiments on the estimation of choline esterase no distinction was made between the specific and non-specific esterases of corpuscles and plasma respectively. The activity of choline esterase was determined only by hydrolysis of acetylcholine and was expressed in μl. of CO_2 liberated from bicarbonate buffer. The volumes of gas evolved in 5, 10, 20 and 30 min. were as follows: (*a*) fresh oxalated blood: 36, 72, 120 and 170 μl. and (*b*) blood A: 15, 29, 57 and 75 μl. Blood A contains therefore about 42 % of its original choline esterase activity.

Blood samples in Nuttall's collection

This material was found among a large number of specimens of blood, sera and anti-sera collected and preserved by the late Prof. G. H. F. Nuttall between 1901 and 1904 and kept in boxes at room temperature. They were left over from his extensive serological investigations, the results of which were incorporated in the classical monograph on 'Blood immunity and blood relationships' published in 1904. In addition to blood specimens B and C, description of which will follow, Nuttall's collection contains a few other specimens of whole blood. Unfortunately some samples of whole blood which he received were not collected under aseptic conditions. They were sealed by him in a number of glass tubes with or without previous treatment with chloroform. We shall examine them separately as samples D. The anti-sera, obtained mainly from the blood of immunized rabbits and occasionally of guinea-pigs, as well as the sera of different animals were collected by him aseptically and preserved in sterile sealed tubes without addition of antiseptics. Many specimens were heated by him to 55°. Other samples of sera not aseptically collected had been passed through Chamberland filters before being sealed. Detailed descriptions of his methods for the collection and preservation of these interesting materials are given in his monographic study.

Horse blood B preserved 42 years

The blood B, although found in the above serological collection, was labelled in Nuttall's handwriting 'Horse sickness, received 13. vi. 1904, Gray'. It is possible that this sample was sent to him from South Africa in connexion with his work on blood parasites, especially piroplasmosis, in which he became interested soon after the completion of his serological study. This specimen of blood was found in two corked tubes each containing about 17 ml. leaving only 1–1·5 ml. air space. The corks were carefully sealed in with a thick layer of red sealing wax.

Haemoglobin in blood B

Examination of a thin film of blood near the air space showed that it was completely laked, giving a clear purple solution characteristic of a deoxygenated haemoglobin. On opening one of these tubes

and transferring about 2 ml. of the contents to another vessel the solution gradually turned red and showed distinct bands of oxyhaemoglobin, which on standing gradually oxidized to methaemoglobin, recognized by the appearance of an absorption band at 630 mμ.

A preliminary spectrophotometric study of this sample of blood revealed a marked deficiency in the haemoglobin content. Since this blood was obtained from a case of 'horse sickness' which, at that time, may have been confused with horse piroplasmosis or some other diseases, the low haemoglobin content of this blood sample could be ascribed to anaemia. However, the viscosity of this sample and the fact that it failed to dry when spread upon a slide suggested that the blood was diluted, probably with glycerol. To decide between these two possibilities 3 ml. of this blood were dialyzed against distilled water. Already after a few hours the water of dialysis contained much glycerol while the blood within the dialyzing sac rapidly increased in volume. After completion of dialysis the dry weight of the blood, corrected for the dilution, was found to be 75 mg./ml., whereas the value for a fresh sample of blood treated in a similar way was 230 mg./ml. This clearly indicates that the main reason for the low haemoglobin content of blood B was its dilution with glycerol which was added as a preservative.

The blood was then examined for the haemin content, oxygen capacity and absorption spectrum. Since it had a tendency to oxidize to methaemoglobin the spectrophotometric study of the sample was carried out after reduction with $Na_2S_2O_4$ and treatment with CO, followed by filtration through a dry kieselguhr bed. The results of these estimations (Table 3), confirm the previous conclusion, namely that the blood was diluted almost threefold.

Enzymes in blood B

Since the blood B was found to be diluted about three times, the quantities of this blood used for estimations of enzyme activities were proportionately increased to contain the same final concentrations of haemin as were present in the control experiments with fresh blood.

Carbonic anhydrase tested by the boat method and very rapid shaking at 0° gave the following results expressed in μl. CO_2 evolved between the 10th and the 40th seconds of the reaction: blank, 150 μl.; blood B, 605 μl.; and fresh blood, 690 μl. The relative catalytic activities of blood sample B and of fresh blood are therefore (605–150)/150 = 3·0 and (690–150)/150 = 3·6 respectively. The old blood thus retained about 83 % of its original carbonic anhydrase activity.

Catalase activity was determined both by the boat method at 0° and in differential manometers at 20°. In both series of experiments the rate of the reaction catalyzed by blood B was found to be from 75 to 92 % of the values obtained with different samples of fresh blood.

Glyoxalase activity was determined at 20° and expressed in terms of μl. CO_2 liberated from bicarbonate buffer by the lactic acid formed. In 10, 20 and 30 min., the CO_2 production was as follows: (*a*) fresh citrated blood: 174, 363 and 495 μl. and (*b*) an equivalent amount of blood B: 125, 246 and 340 μl. The glyoxalase activity of blood B is therefore about 70 % of that found in fresh blood.

Choline esterase activity was estimated at 20° in 10, 25 and 40 min. and gave the following values: (*a*) fresh oxalated blood: 60, 138 and 192 μl., and (*b*) an equivalent amount of blood B: 51, 120 and 180 μl. Blood B retained therefore about 85 % of the normal choline esterase activity.

Table 3. *Properties of haemoglobin in blood B (42-year-old sample) and fresh blood*

(Dry weight determined after dialysis; O_2 capacity determined manometrically; haemin as pyridine haemochromogen; $\epsilon_{mol.} = 1/cl \log_{10} I_0/I$.)

Blood	Dry weight (mg./ml.)	O_2 capacity (μl. O_2/ml.)	Total haemin (mg./ml.)	O_2 capacity (μl. O_2/mg. haemin)	HbCO $\epsilon_{mol.} \times 10^{-4}$ α – 570 mμ.	HbCO $\epsilon_{mol.} \times 10^{-4}$ β – 539 mμ.
Blood B 42 years old	75	70	2·30	30·3	1·34	1·35
Fresh blood	228	218	6·50	33·5	1·32	1·34

The fact that the value for the O_2 capacity of blood B determined manometrically is about 10 % below the value obtained by calculation from the haemin content shows that a small fraction of pigment was present in the form of methaemoglobin. This is more clearly shown by the normal values of the molecular extinction coefficient ($\epsilon_{mol.}$) of the HbCO, especially in the presence of $Na_2S_2O_4$.

Guinea-pig blood C preserved 44 years

Blood C comprised three 1·5 ml. ampoules each containing about 1 ml. of guinea-pig blood collected and preserved aseptically by Nuttall. They were placed by him, together with similar ampoules of sera containing different amounts of haemoglobin, into a box labelled 'Anti-reindeer. Guinea-pig, bled

17. iii. 1902.' Each of the three ampoules contained laked blood which was fluid, clear and of the purple colour characteristic of deoxygenated haemoglobin. This was confirmed by spectroscopic examination of the blood within the narrow parts of the ampoule.

It is well known that guinea-pig haemoglobin is one of the most easily crystallizable proteins. The ease of crystallization as well as the solubility depends, however, upon the state of the haemoglobin (Granick, 1942). Thus, oxyhaemoglobin of guinea-pig is very insoluble and easily crystallizes, forming characteristic tetrahedral crystals. Methaemoglobin is slightly more soluble and forms similar crystals, whereas haemoglobin is very soluble and does not crystallize under similar conditions. On deoxygenation crystals of HbO_2 rapidly dissolve and reform when the solution is reoxygenated. Crystallization of a protein is one of the properties which depend upon the integrity of its molecular structure. This property, however, could not be easily investigated in the old samples of horse blood A and B in which the haemoglobin was intimately mixed with the serum proteins. The old sample of guinea-pig blood, although laked and available only in very small quantities, offered an exceptional opportunity for the study of this property of haemoglobin in solutions preserved for more than 44 years.

On opening one of the ampoules of old guinea-pig blood (C) and transferring its contents to another vessel, the haemoglobin, thus exposed to air, slowly underwent oxygenation as shown by the change in its colour and its absorption spectrum. At the same time the solution became increasingly opalescent and viscous until it had practically solidified. Microscopic examination revealed a thick suspension of characteristic tetrahedral crystals varying greatly in size, the largest being almost visible to the naked eye. On reduction with $Na_2S_2O_4$ the crystals of HbO_2 were gradually deoxygenated and dissolved. The solution did not show any signs of denaturation.

Other specimens (D) of whole blood in Nuttall's collection

In addition to blood specimens B and C described above, Nuttall's serological collection contains a few other specimens of whole blood labelled as follows:

1. 'Common jackal (*Canis aureus*), received 27. xi. 1901, India.'
2. 'Ant bear, received 5. iii. 1903, removed to R.T. and sealed 9. iii. 1903.'
3. 'Sea lion (*Otaria californica*) received 24. vii. 1902, removed to R.T. and sealed 12. xi. 1902.'
4. 'Himalayan bear (*Ursus tibetanus* F. Cuv.) E. Asia, 27. xii. 1901 + chloroform, removed to R.T. and sealed 10. iii. 1903.'

These specimens of blood were collected apparently without aseptic precautions and after being kept in the cold they were transferred to room temperature (R.T.) and sealed in tubes. The blood of the Himalayan bear was the only specimen which was protected from putrefaction by treatment with chloroform.

(1) The 45-year-old samples of jackal blood were found to be in a fluid state and, before the tubes were opened, showed the colour and absorption spectrum of deoxygenated haemoglobin. Since there was no reinforcement in the region of the haemochromogen bands, the haemoglobin appeared to be solely in the native state.

On opening these tubes the blood was found to be saturated with gases and had a pungent odour of putrefaction. Exposed to air, the haemoglobin underwent oxygenation which was accompanied by slow oxidation to methaemoglobin. However, even after standing for several hours, the amount of methaemoglobin formed did not represent more than 10% of the total haemoglobin.

The oxygen capacity of this blood was estimated manometrically by the ferricyanide method as already described. As the blood was saturated with CO_2 it was found necessary to use rolls of filter paper impregnated with 10% KOH in the central tubes of the manometer flasks. Estimated in this way 1 ml. of blood was found to evolve 184 μl. O_2 which, corrected for about 10% methaemoglobin formed before the estimation, would give an oxygen capacity of 202 μl./ml. If we assume that the normal oxygen capacity of fresh jackal blood does not differ markedly from that of other mammals, the haemoglobin in the 45-year-old sample had not undergone any appreciable deterioration. This was corroborated by spectrophotometric determinations of the CO-haemoglobin in these samples.

(2) The 43-year-old blood of the 'ant bear' or 'aard-vark' (*Orycteropus capensis*) contained much solid material consisting of denatured proteins arising probably from a heavy bacterial infection present. The fluid fraction of the blood showed the colour and absorption spectrum of deoxygenated haemoglobin. The blood was saturated with gases and smelt very strongly of putrefaction. After filtration and boiling *in vacuo* in a Thunberg tube the clear solution of this blood was reoxygenated. During these manipulations about 10–12% of haemoglobin was oxidized to methaemoglobin. The oxygen capacity of this blood, estimated in the usual way and corrected for the methaemoglobin present, was 64 μl. O_2/ml. This blood sample therefore retained about 30% of its original oxygen capacity.

(3) The 44-year-old blood of the sea lion (*Otaria californica*), which also contained much solid material consisting of denatured proteins due to putrefaction, retained not more than 18% of its original oxygen capacity.

(4) The 45-year-old samples of bear's blood treated with chloroform were of a distinctly brown colour showing a diffuse spectrum of parahaematin which on reduction gave haemochromogen. The concentration of the latter was, however, too low to account for the complete disappearance of haemoglobin and for the loss of the oxygen binding property of the blood. This shows that the degradation of haemoglobin in this sample had proceeded beyond the stage of haemochromogen or even of the free haem.

Haemoglobin in laked blood is therefore less stable in presence of an organic solvent such as chloroform than in presence of micro-organisms producing putrefaction. The fact that the oxygen capacity of jackal blood was almost undiminished whereas those of the ant bear and of the sea lion were reduced to 30 and 18 % respectively may be ascribed to differences in the bacterial flora responsible for the putrefaction. Attempts to study enzymes in these samples were abandoned since it appeared that they harbour viable micro-organisms.

Previous records of protein stability

The proteins which can be considered under this heading fall into three categories: (*a*) haemoglobin, (*b*) enzymes and (*c*) plant virus.

(*a*) *Haemoglobin.* Donaldson, Harding & Wright (1943), while discussing the permanence of the colour in standard tubes, write as follows: 'A tube which is the property of Dr Price-Jones and with which he carried out work on haematological standards (Price-Jones, Dill & Wright, 1931) was compared with the present standard. This tube was originally made by Dr Haldane more than thirty years ago, and had since been carefully preserved in the dark when not in use....' 'The agreement with the present standard is very close indeed. Although the exact colour of the tube when it was made is unknown, it is unlikely that it can have changed much when it still remains so close to other similarly prepared tubes.' However, since Haldane's standard tube contains CO-haemoglobin an appreciable denaturation of the pigment accompanied by the formation of CO-haemochromogen could hardly cause any marked change in the colour. Yet the results of our observations related above support the view that the haemoglobin in Haldane's standard tube remained unchanged from the time of its preparation.

(*b*) *Enzymes.* It was found by White (1909) that 'diastatic, fibrin-digesting and ereptic ferments' in seeds of wheat, maize, barley, oats and rye 'retain their activity without appreciable change in stored dry seeds for 20 or more years, that is long after the power of germination has been lost.'

More recently Steppuhn & Utkina-Ljubowzowa (1930) claimed to have demonstrated the presence of proteolytic enzymes in tissues of Egyptian mummies about 3000 years old and in those of a mammoth 30,000–100,000 years old. Unfortunately they give no quantitative data in support of these very interesting claims. Nor do they record any examination of the tissues for micro-organisms which might have invaded the tissues at various times and might have been responsible for the proteolytic activity.

(*c*) *Plant virus.* Thornberry, Valleau & Johnson (1938) have shown that 'Tobacco mosaic virus in cured tobacco that had been kept 52 years in a box, in a dry barn, was found to have retained some virulence.' Later Johnson & Valleau (1940) found that tobacco-mosaic-affected green leaves ground to a pulp and mixed with water retained about one-third of their original activity after 14 years, especially in presence of benzene or xylene.

DISCUSSION

The results of observations recorded in this paper show that the haemoglobin in old samples of blood (A and B) collected aseptically and kept for 24, 42 and 44 years retained its original properties such as oxygen binding capacity, the shape of the dissociation curve, spectroscopic properties and even the property of crystallization (blood C). Small quantitative differences between the old and fresh samples were well within the individual variations of fresh blood. There is therefore no indication that haemoglobin in the whole blood preserved for more than 44 years has undergone any appreciable change. In the same samples of old blood (A and B), enzymes such as carbonic anhydrase, catalase, glyoxalase and choline esterase retained up to 85 % of their original catalytic activities.

All this shows that native proteins like haemoglobin and certain enzymes may retain their specific biological properties and therefore the integrity of their structure for more than 44 years in blood at room temperature.

The stability of haemoglobin and of endoerythrocytic enzymes *in vitro* contrasts markedly with their instability *in vivo*. In fact, since the life span of red blood corpuscles determined by different methods is approximately 120 days, our organism loses daily about 25 ml. of erythrocytes, which corresponds to about 8 g. of haemoglobin or 305 mg. of haemin. Yet neither free haemoglobin or its derivatives, nor any one of the endoerythrocytic enzymes such as carbonic anhydrase, catalase, glyoxalase or choline esterase are found free in the body. However, this rapid elimination of all components of the destroyed corpuscles is not due to the instability of these components but to active and well regulated processes of their degradation involving a series of catalyzed reactions localized in certain cells of the organism.

The analysis of conditions under which the above old samples (A, B and C) of blood were kept enables us to determine the factors which were either favourable or unfavourable for their preservation.

The four main factors which contributed to the stabilization of these blood samples were: (1) freedom from bacterial infection, (2) protection from light, (3) protection from oxygen and (4) high protein content.

(1) Freedom from bacterial infection of blood samples A and C was assured by their aseptic collection and preservation. In the case of the blood sample B it was partly due to dilution with glycerin.

(2) All samples were protected from light by being kept in closed tin boxes. On rare occasions and only for short periods were they exposed to light.

(3) The oxygen present in preserved samples of blood is derived partly from the small amount of air sealed within the ampoule or tube and partly from oxygen fixed by haemoglobin or dissolved in the fluid. Thus a 2·5 ml. ampoule containing 2·2 ml. blood A would contain 60 μl. O_2 in the air space, about 400 μl. O_2 combined with haemoglobin and 10–12 μl. dissolved in the fluid. The total amount of O_2 in different ampoules at the time of sealing probably varied between 450 and 500 μl. This O_2 was probably used up within a couple of weeks in normal respiratory processes of cellular constituents of the blood and very little of it remained for oxidative deterioration of native proteins or of their prosthetic groups. The same applies to blood specimens B and C, but not to blood samples D.

The factors unfavourable to preservation of these blood samples were: (1) bacterial infection in D, (2) the fluid state of the medium with its high water content which would facilitate any of the denaturation reactions likely to take place, (3) presence of hydrolytic enzymes liberated from lysed cellular elements, (4) a comparatively high temperature of storage and variations of this temperature, (5) presence of a small amount of O_2, at least during the initial period of storage, and (6) contact with glass.

It is important to note that although the 45-year-old samples of jackal blood showed marked signs of putrefaction the oxygen capacity was not far from that of fresh mammalian blood. On the other hand, in blood samples (Himalayan bear) protected from putrefaction by treatment with chloroform, haemoglobin was completely destroyed.

It is surprising that, although the conditions for the preservation of these blood samples were not ideal, the haemoglobin and the endoerythrocytic enzymes retained the major part of their specific biological activities and therefore the integrity of their complex molecular structure even after 44 years' storage. However, even under these comparatively unfavourable conditions we are still far from the upper limit of the life span of these proteins.

SUMMARY

1. Properties of haemoglobin and of certain endoerythrocytic enzymes were examined in 24- and 42-year-old samples of horse blood (A and B respectively) collected aseptically and preserved in the dark at room temperature.

2. Blood in these samples was laked and haemoglobin completely deoxygenated. The fact that no bands of haemochromogen could be detected even on addition of $Na_2S_2O_4$ indicates that no partial denaturation of haemoglobin occurred.

3. The O_2 capacity of these samples/mg. dry weight or/mg. haemin was approximately the same as that of fresh horse blood.

4. The O_2 dissociation curve of haemoglobin in sample A was found to be S-shaped and very little different from that of fresh blood, the P_{50} for blood A and for fresh horse blood being 5·1 and 4·8 mm. Hg respectively.

5. Absolute absorption spectra of oxyhaemoglobin in blood A and of carboxyhaemoglobin in blood B are indistinguishable from those of fresh horse blood.

6. From a 44-year-old sample of guinea-pig blood (C) collected and preserved aseptically, crystals of oxyhaemoglobin were obtained of the characteristic tetrahedral type similar in every respect to those prepared from fresh guinea-pig oxyhaemoglobin.

7. The endoerythrocytic enzymes such as carbonic anhydrase, catalase, glyoxalase and choline esterase in different samples of blood specimens A and B retained up to 85% of their activities when comparison was made with fresh horse blood.

8. The factors which favoured the preservation of haemoglobin and of the enzymes in these blood samples were mainly protection from bacterial infection, absence of oxygen, protection from light and a high concentration of proteins.

9. Since the conditions of preservation of these blood samples were not perfect, 44 years is probably very far below the possible life span of these proteins *in vitro*.

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Activity of the Cytochrome System in Heart Muscle Preparations

By D. KEILIN and E. F. HARTREE, *Molteno Institute, University of Cambridge*

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The best material for the study of the cytochrome system *in vitro* is the heart muscle preparation which we have previously described (Keilin & Hartree, 1938, 1940). The preparation consists of a uniform colloidal suspension of highly active material completely devoid of haemoglobin and suitable both for manometric and spectroscopic studies. In addition to the complete cytochrome system this preparation contains a powerful succinic dehydrogenase, α-glycerophosphoric dehydrogenase, fumarase and variable amounts of diaphorase, catalase and certain other enzymes, all bound to a protein the nature of which is not yet fully determined. This preparation is often referred to as cytochrome oxidase or as succinic dehydrogenase according to the nature of the substrate under investigation. The cytochrome oxidase activity of this preparation is determined by the rate of the catalyzed O_2 uptake in presence of an excess of cytochrome *c* and of a substance such as *p*-phenylenediamine, hydroquinone, adrenaline or ascorbic acid which, by reducing cytochrome *c*, ensure a constant supply of substrate for the oxidase. The activity of our preparation estimated in this way and expressed as Q_{O_2}, i.e. μl. O_2/mg. dry weight/hr. at 39° is about 1400 (Keilin & Hartree, 1940). Although this activity is well below that of several completely purified oxidizing enzymes all attempts to raise it have so far failed. More recently, by introducing a new method of preparation, Haas (1943) claimed to have extracted material containing 15 times more enzyme and being six times more active than the preparation obtained by our method. However, this claim is not supported by his own observations since the Q_{O_2} of his enzyme preparation was only 203 at 17° and was calculated by him to be 812 at 39° assuming that the activity of the preparation increases fourfold with this rise in temperature. The calculated activity of Haas's material is therefore not six times higher but 40 % lower than that of our preparation.

Since heart muscle preparations are often used for the study of cytochrome in relation to other intracellular catalytic systems we propose to compare the main properties of our preparation with that of Haas. We shall also examine the claims put forward by Haas (1943, 1944) (*a*) that the use of ultrasonic vibrations has led to the production of a soluble cytochrome oxidase and (*b*) that the oxidase may be separated into two protein components.

EXPERIMENTAL

Methods

O_2 uptakes. These were measured in Barcroft differential manometers at 39°. The oxidase sample was diluted to 3 ml. with 0·3 ml. cytochrome *c* (Keilin & Hartree, 1945) and phosphate buffer (0·1 M, pH 7·3). The substrate (*p*-phenylenediamine or hydroquinone) was added as 0·3 ml. aqueous solution from dangling tubes.

Enzyme units. In order to work out a balance sheet of the enzyme content at different stages of our preparation, samples of successive fractions were withdrawn, ground with sand and diluted with phosphate buffer (0·1 M, pH 7·3) to a known volume. The enzyme content was expressed in arbitrary units corresponding to the quantity of enzyme which catalyzes an O_2 uptake of 100 ml./hr. under the above standard conditions.