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METABOLIC ASPECTS OF THE UPTAKE OF OXYGEN BY HAEMOGLOBIN IN ERYTHROCYTES

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SUMMARY

Spectrophotometric measurements have been made of how the uptake of O_2 and CO by haemoglobin in intact human erythrocytes varies with time. A decay, from a fast initial rate, is observed with O_2 which varies with temperature, storage, and the presence of O_2. No decay is observed with CO unless O_2 is also present. The fast rate with O_2 can be inhibited by lowering the pH, but cyanide, azide, fluoride, iodoacetate and methylene blue have no significant effect. After decay, the rate can be temporarily increased by the addition of adenosine. It is suggested that the membrane permeability is altered by aerobic metabolism.

INTRODUCTION

The rate at which O_2 is taken up by reduced haemoglobin in erythrocytes has been interpreted by ROUGHTON[1,2] in terms of diffusion through the cell membrane, followed by diffusion through and reaction with the internal haemoglobin. The chemical reaction of O_2 with haemoglobin has been extensively investigated by ROUGHTON and co-workers, a review of which has been given by GIBSON[3]. Measurements have also been made by LONGMUIR AND ROUGHTON[4], later confirmed by KLUG, KREUZER AND ROUGHTON[5], of the diffusion coefficient of gases through haemoglobin. Using these two parameters it is possible to calculate from experimental observations on intact cells a factor, λ, directly related to the membrane permeability and thickness[2]. The first measurements on the O_2 uptake by erythrocytes were made by HARTRIDGE AND ROUGHTON[6] and suggested that the membrane had a significant resistance. Later an interesting exception was found by LEGGE AND ROUGHTON[7] and analysed by NICOLSON AND ROUGHTON[8], of a ram, in which the membrane permeability was effectively infinite. Though this result might be in error due to non-linearity of the split-beam detecting system, as discussed by SIRS AND ROUGHTON[9], further work by SIRS[10,11] suggested that the membrane permeability to gases did vary with the time elapsed from collection and the cell metabolism. The present results represent an investigation into the nature of this variation and the metabolic pathway involved.

METHODS

The rate of O_2 uptake by the haemoglobin in human erythrocytes has been measured by the constant- and stopped-flow methods of SIRS AND ROUGHTON[9]. In principle, a small sample of whole blood is drawn by venipuncture, and then diluted with an isotonic solution. The O_2 present is quickly removed by repeated evacuation and flushing with N_2. The reduced cell suspension is then rapidly mixed, by the method of HARTRIDGE AND ROUGHTON[12] with a similar isotonic solution containing a known O_2 concentration. With the constant-flow system, the mixture is driven, by gas pressure, at a steady velocity through a uniform-bore glass tube. The percentage saturation of the internal cell haemoglobin with O_2 is observed spectrophotometrically at 4 or 5 points along the tube, corresponding to different elapsed times since mixing. From these measurements a curve relating the percentage saturation with time can be drawn and the initial rate of uptake calculated. Though this system is in some respects easier to calibrate and can be used with elapsed times of the order 0.5 msec, it has the disadvantage that large quantities of reagents are required. For this reason, it has been used in the following experiments mainly as reference method, and the majority of the results were obtained with the much more economical stopped-flow system. This involves stopping the flow suddenly, and observing the rapid uptake at a fixed distance, approx. 1 cm from the mixing point, with a spectrophotometric detector, an oscilloscope and a camera. In detail the techniques are exactly the same as described by SIRS AND ROUGHTON, except that with the constant-flow system, measurements were first made with the oxygenated suspension flowing to the mixing chamber via inlet A and the reduced cells by inlet B; and then with the connections reversed, so that the reduced cells entered A and the oxygenated by B. By comparing the mixture deflections a check could be maintained to ensure that no blockage had occurred in the mixing jets, and that any non-linearity was of spectrophotometric origin.

A small quantity, usually 6.5 ml, of human blood was collected from the author by venipuncture and then diluted with 500 ml of Ringer–Locke solution (9 g NaCl, 0.42 g KCl, 0.24 g $CaCl_2$ and 0.2 g $NaHCO_3$ per l). In some experiments the Ringer–Locke was replaced by Gev and Gev solution (5.61 g NaCl, 0.38 g KCl, 2.27 g $NaHCO_3$, 0.15 g $CaCl_2$, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.14 g $Na_2HPO_4 \cdot 2H_2O$, 0.027 g KH_2PO_4, 0.074 g $MgSO_4 \cdot 7H_2O$ and 10.0 g glucose per l, equilibrated to pH 7.4 with 4 cm Hg partial pressure CO_2). The cell concentration is not critical, the initial rate being independent of its value[9]. In the majority of experiments, heparin was used in the collecting syringe to prevent clotting; a few samples were defibrinated with glass beads and in one case no agent was used. The rates obtained did not vary with the method or lack of defibrination. The diluted cell suspension was then reduced as quickly as possible by the process of repeated evacuation and flushing with N_2. This usually involved a loss of time (up to 30 min), between the collection of the blood sample and the first measurement. When a shorter time-loss was required, the 6.5 ml of venous blood was injected through rubber pressure tubing direct into Ringer–Locke previously reduced and equilibrated with N_2. In this case only 5 min elapsed between collection and measurement though a small quantity of O_2 (less than 10 mm partial pressure) remained. Approx. 30 ml of the reduced suspension being stirred magnetically was drawn under N_2 pressure from the 500-ml reservoir to flush and fill

the stopped-flow syringe. 8–10 O_2-uptake curves were then recorded by mixing with a similar oxygenated solution within the next 2 min. After a given interval (10–30 min), a further sample was drawn from the main reduced suspension and the process repeated. This was continued for several hours until the rate reached its lowest value. A similar series of control experiments, by mixing CO with the reduced cells, was also done, in parallel with the main experiment.

Temperatures varied between 0° and 40° using a thermostatic control previously described[9]. The temperature of the reacting mixture in the observation tube was checked to $\pm 0.2°$ by a thermocouple inserted just below the observation point. The pH was measured with a standard glass electrode and pH meter. Small samples were taken directly from the reduced cell suspension before an experiment and from the outlet after an experiment to be centrifuged and checked to ensure the absence of haemolysis. Microscopic examination was also made to ensure no significant crenation had occurred.

RESULTS

The objective of the experiments was to observe the effect of different factors on the decay from a fast rate of O_2 uptake to its slower, passive, form. A comparison of these uptakes, corresponding to the extreme fast and slow rates, obtained with the constant-flow apparatus, under similar conditions, is shown in Fig. 1. The stopped-flow system, over the slow and intermediate phases, gave similar values for k_e' (ref. 9), within the experimental error of $\pm 5\%$. The factor k_e' is, as defined by FORSTER *et al.*[13], the initial rate of O_2 uptake, relative to the total amount of O_2 that combines with the haemoglobin, divided by the concentration of the O_2 surrounding the cells immediately after mixture. A similar factor k_c' is used for the case of CO. Throughout this paper both k_e' and k_c' are given in units of $mM^{-1} \cdot sec^{-1}$ and have the dimensions of a bimolecular velocity constant. At the higher rates, however, the agreement is not so good. The reason for this is that within the time elapsed from mixing to observation with the stopped-flow system, corresponding to approx. 1 cm distance, the reaction has already proceeded some 10 %. Extrapolation to the initial mixture point, using the elapsed time, is too inaccurate. The alternative process of using a calibration solution, corresponding to the initial unreacted mixture, has the disadvantage that it must be done after the curves have been obtained, not simultaneously as with the constant-flow technique. This again leads to small differences which, due to the steepness of the initial slope, makes for greater inaccuracies. The constant-flow system is not particularly suitable, however, for the repetitive type of experiment necessary to follow the decay and requires a longer preparation time. The higher values of k_e' given later and obtained with the stopped-flow system must therefore be regarded as relative rather than absolute values, and indicative that the faster type of uptake illustrated in Fig. 1 pertains.

Another difficulty with these observations is that the faster rates are not always obtained, even within 5 min of collection. The reason for this is not known. The curves in Fig. 1 for example were obtained on different samples, after a 2-week interval, from the same donor, all other factors being the same. The slower curve occurred after some difficulty in drawing the blood sample, and in consequence much higher apprehension on the part of the donor. The influence of anxiety on blood coagulability has been reported by OGSTON *et al.*[14], but it is difficult due to its subjective nature to

say it was a decisive factor in this case. The results reported here thus represent the fraction of measurements on cell suspensions in which the faster rates were obtained initially.

Effect of temperature. The variation of the rate of decay of k_e' with time has been observed at different temperatures and is shown in Fig. 2. These measurements are in agreement with the qualitative statements of SIRS[11], that at higher temperatures the decay is more rapid and at temperatures below approx. 14° no fast rates have been observed. This explains to some degree the difficulty in obtaining reproducible fast rates after each collection. If the blood sample is left for long at body temperature, the rate of decay is so rapid that no effect will be observed. Another aspect of these observations is that the maximum rate of uptake does not appear to vary over the temperature range 20–37°. The variation of pH between 7.4 and 8.0, under these conditions, also does not have any significant effect. A change of O_2 concentration from 0.138 mM to 0.296 mM, after mixture, only increased the value of the fast k_e' by 7%. Although this is not regarded as significant a similar comparison, as the cell uptake decayed, suggested that at the higher O_2 concentration the faster rate was maintained for a longer period.

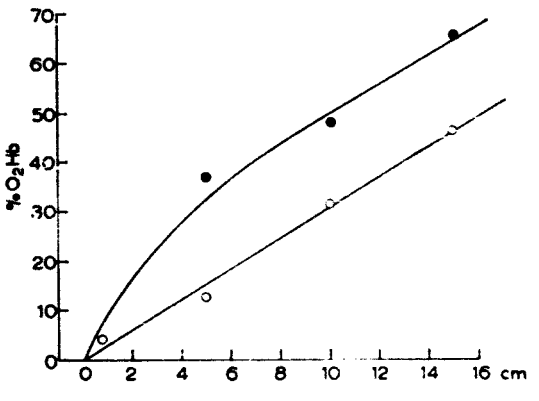

Fig. 1. A comparison, using the constant-flow system, of the fast and slow rates of O_2 uptake by erythrocytes diluted 1:200 in Ringer–Locke (pH 8.0). ●, O_2 concn. 0.21 mM (mixed); temperature 20.9°; flow rate 2.29 m/sec. ○, O_2 concn. 0.23 mM (mixed); temperature 21.5°; flow rate 2.55 m/sec.

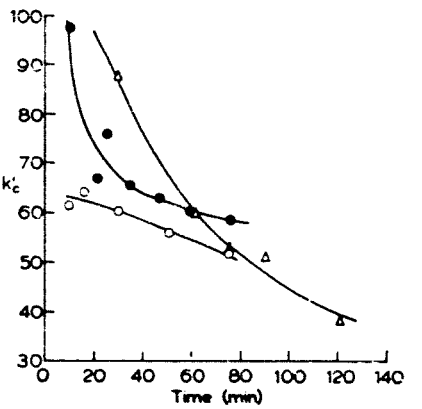

Fig. 2. The effect of temperature on the decay rate, 1/80 cells in Ringer–Locke using the stopped-flow apparatus. Blood sample collected at zero time. ●, O_2 concn. 0.137 mM; temperature 35.9°. △, O_2 concn. 0.143 mM temperature 23°. ○, O_2 concn. 0.197 mM; temperature 18.4°.

The influence of storage. The rapid rate at which the uptake decayed at 37° suggested that some advantage could be gained by cooling the cells relatively quickly. Cells which had been cooled and stored overnight in a refrigerator at 4°, were found to exhibit an induction period, of the order of 1 h, during which high values of k_e' were maintained, before decaying to a lower value. A typical series of results of this type is shown in Fig. 3. A search of the literature revealed that a similar phenomenon had been observed by DAVSON AND DANIELLI[12] when studying sodium transport. Use has been made of this observation to obtain and maintain cells with a high rate of O_2 uptake. Immediately upon collection the cells are injected into ice-cold Ringer–Locke or Gey and Gey solution. They are warmed to approx. 20° just prior to the

experiment. In this way the high rates have been maintained long enough to examine the effect of the introduction of various agents as given below. This method again does not ensure that a high rate will always be obtained.

Comparison of O_2 and CO uptake. That the decay is specifically associated with O_2 can be seen from Fig. 4. The full-line curves represent simultaneous measurements of the rate of O_2 and CO uptake, by the haemoglobin in the intact cells, in Ringer–Locke at room temperature and pH 8.0. While the O_2 values, k_c', fall steadily, the CO rate, l_c', remains constant. This implies that the change observed with O_2 is not due simply to physical changes in the cell structure. In contrast when the cells are placed in Gev and Gev solution only partially reduced and equilibrated with 2 cm partial pressure O_2, both the O_2 and CO uptakes are found to vary with time. Another aspect of this decay is that the CO rate falls in advance of the O_2 change. If these curves are compared with the rate of decay in Ringer–Locke given in Fig. 2, the O_2 decay rate is much slower than would be expected for a temperature of 33°. Some advantage can thus be obtained by suspending the cells in a suitable medium, but even when suspended in their own plasma it was found[11] that the cells normally reach a passive level within 4 h.

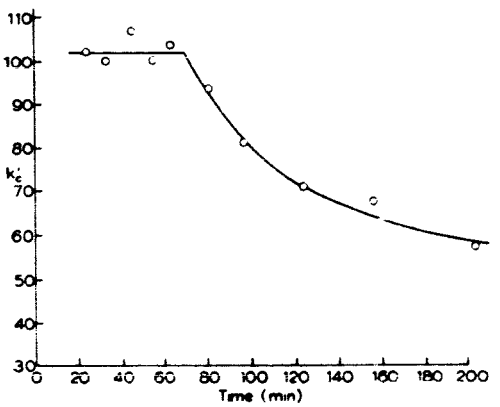

Fig. 3. A decay curve obtained on cells which had been stored for 20 h at 4°. O_2 concn. 0.15 mM in mixed solution at 23.3°.

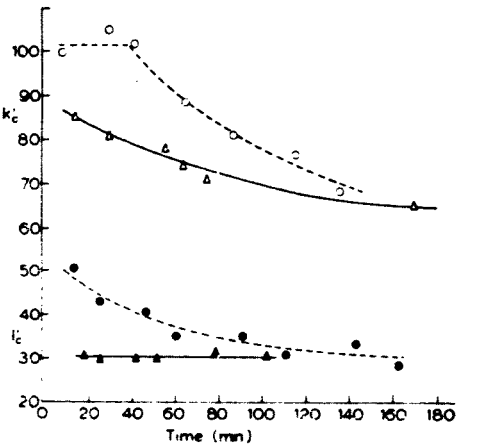

Fig. 4. A simultaneous comparison of the variation of O_2 and CO uptake with time. Cells in Ringer–Locke (pH 8.0) at 21.5°. △, O_2 concn. 0.23 mM. ▲, CO concn. 0.15 mM. Cells in Gev and Gev solution (pH 7.4) and all solutions equilibrated with 2 cm partial pressure O_2 at 33.5°. ○, O_2 concn. 0.10 mM. ●, CO concn. 0.19 mM.

Stimulation. The results obtained so far are suggestive of a metabolic function as the source of the decay. If this was the case it should be possible to stimulate the uptake by addition of suitable substrates. A curve obtained by adding first adenosine and then 4.5 ml Ringer–Locke equilibrated with O_2 is shown in Fig. 5. These solutions were injected into the reduced cell suspension through a piece of rubber pressure tubing supplying the N_2 pressure. The stimulating effect of a low O_2 concentration had been observed in previous experiments and care was taken to ensure that it was excluded when introducing the adenosine sample.

Inhibition. A variety of inhibitors have been similarly injected, after removing any O_2, via a syringe into the reduced suspension reservoir to examine their effect on the decay rate. Sodium azide (concn. 5 mM in reduced suspension reservoir), urethane (10 mM), and methylene blue had no immediate effect on the decay curve. The addition of sodium cyanide (10^{-4} M) does not inhibit the process and there is some evidence that it helps to maintain the higher rate of uptake. With the addition of ether a striking increase was observed, which appeared to be associated with a structural alteration of the cell. Immediate inhibition was observed upon injecting iodoacetic acid (2 mM) into the reduced suspension, as is illustrated in Fig. 6. However, the solution was not neutralized in this experiment and further examination disclosed that a similar effect could be obtained by addition of 1 ml of 0.5 M HCl to the cell suspension, changing the pH from 8.0 to 6.3. With neutralized iodoacetate no change was observed. The action of NaF (1–20 mg/l) is erratic. In one series of experiments[11] definite inhibition was observed but this has not been confirmed with the human cells used on this occasion. It is possible that anaerobic glycolysis was involved in the previous experiments.

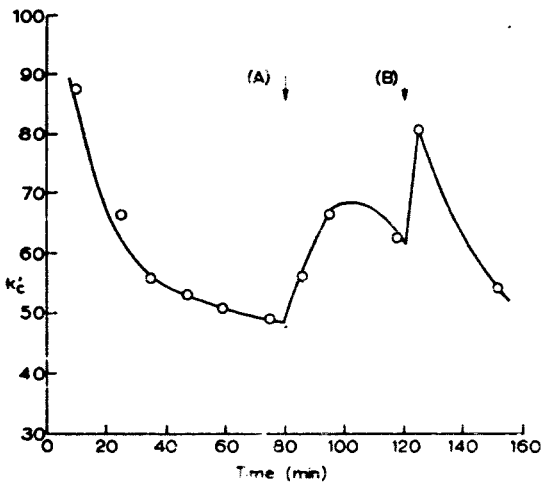

Fig. 5. The uptake of O_2 (0.14 mM) by human red cells diluted 1:100 in Ringer–Locke (pH 8.0) at 35.9°, after the addition to the reduced suspension of (A) adenosine (2 mM in reduced suspension reservoir) and (B) 4.5 ml Ringer–Locke, equilibrated with 25 cm partial pressure O_2.

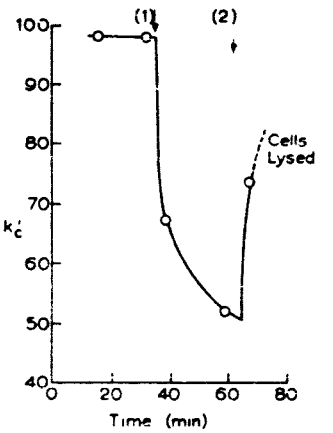

Fig. 6. Effect on the uptake of O_2 (0.19 mM) by human cells diluted 1:100 in Ringer–Locke (pH 8.0) at 21°, of iodoacetic acid. (1) concn. 2 mM in reduced suspension; (2) 4 mM

DISCUSSION

The variation of the rate of O_2 uptake, by the haemoglobin of the erythrocyte with time, temperature, presence of glucose and O_2, storage, its activation by adenosine and inhibition by pH change suggest the process is metabolically controlled. The relative constancy of the CO uptake, while the O_2 rate varies, indicates that no direct physical change has occurred in the cell structure and the metabolic pathway must be aerobic. The invariance of the maximum rate of O_2 uptake, k_c', with temperature (20–37°) and pH also support this view. Reviews of the metabolic pathways of

the erythrocyte have been given by LEMBERG AND LEGGE[16], PRANKERD[17] and LONDON[18]. Two main aerobic pathways may be considered, via glutathione or methaemoglobin. Direct oxidation of glutathione has been demonstrated by MELDRUM AND DIXON[19] but there is no evidence of its occurring in the intact cell[20]. Inhibition of this process also occurs with HCN (ref. 19) but no effect of cyanide was observed. On the other hand the inhibition of catalase by fluoride, azide and cyanide would favour the formation, via H_2O_2, of oxidized glutathione[21,22]. These agents would also combine with methaemoglobin and inhibit its reduction to haemoglobin[23,24]. The formation of methaemoglobin would therefore also tend to increase. No significant difference was found after the addition of fluoride, azide and cyanide, nor, as might be expected, does the CO-uptake rate alter. The formation of H_2O_2 and methaemoglobin is probably limited by the anaerobic conditions under which the cells are stored. One tenth or less of the normal metabolism occurs via the pentose shunt[25]. Methylene blue considerably increases the activity of this system[26-29] with a corresponding oxidation of both glutathione and haemoglobin. Addition of methylene blue to the cell suspension had no effect on the rate of O_2 uptake. It is possible that methylene blue by-passes or does not directly involve the source of energy utilized in these observations. Iodoacetic acid, however, is known to directly inhibit glyceraldehyde-3-phosphate dehydrogenase[24,30] and so prevent the metabolic sequence via DPN to haemoglobin–methaemoglobin and O_2. Though no direct action of iodoacetate was observed, none would be expected if inhibition had already occurred at the haemoglobin–methaemoglobin link. If the process is associated with present known metabolic pathways, the evidence would suggest the autoxidation of haemoglobin to methaemoglobin as the most likely mechanism.

Physically it has been shown by SIRS[11] that the alteration in the rate of O_2 uptake must be associated with a change in the membrane permeability. If the process involves the utilization of energy, as the biochemical mechanism indicates, the enhanced rate after storage at 4° would suggest the accumulation of an energy reserve. That the cell metabolism does continue, though at a slower rate, has been shown for example by BICK[31] and PRANKERD[32]. It is possible to assume that the leakage of Na^+ and K^+ at low temperatures is due to the low production of ATP being unable to meet the energy requirements necessary to pump the Na^+ out. This would imply that the energy produced is still being used. An alternative explanation, more consistent with the above observations and those of DAVSON[15], would be that the low temperature prevents the utilization of this energy, and for a time any ATP or other energy source formed is accumulated.

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