ELECTROCHEMICAL BEHAVIOR OF BLOOD. I.
A VOLTAMMETRIC STUDY OF METABOLIZING ERYTHROCYTES

M. J. ALLEN*

Shell Research Ltd,
Borden Microbiological Laboratory,
Laboratory of Biophysics,
Sittingbourne, Kent, England

Received May 5th, 1970

The results of a voltammetric study performed on washed suspension of intact human erythrocytes (RBC) in a pH 7·4 isotonic phosphate buffer suggested that at a dropping mercury electrode there was an interaction between the electrode and the sulfhydryl groups associated with the RBC membrane. This assumption was based on the findings that this wave was not observed when using a rotating platinum or glassy carbon electrode, or with the DME after exposure of the RBC to a sulfhydryl blocking agent. Incubation of the RBC suspension (37°C — 18 h) in the presence of glucose gave an I_d greater than that observed prior to incubation ($+\Delta I_d$). In the absence of this substrate a $-\Delta I_d$ was obtained. This observation was applied to studies on the change in metabolic viability with the storage age of packed RBC under normal preservation conditions. The results suggested a rapid decrease in metabolic viability of the RBC during the first 8—10 days after collection from the donor followed by a considerably slower decrease.

Earlier reports demonstrated that electrochemical data could be obtained which reflected the metabolic behavior or bacterial systems under various conditions[1-3]. Therefore the objective in these studies was to extend the knowledge gained from these systems to intact erythrocytes (RBC) and establish electrochemical parameters which could be related to their metabolic viability. Having accomplished this, these parameters were then utilized as an indication of the metabolic storage-life of human RBC under normal preservation conditions, *i.e.* in ACD (acid citrate dextrose) solution A, U.S.P., at 4—5°C. Normally the storage-life of RBC in the ACD solution is 21 days. This figure is based primarily on studies of the post-transfusion survival of ^{51}Cr labeled RBC[4].

* Former address: Department of Physiology & Biophysics, Georgetown University Medical School, Washington, D. C., USA.

EXPERIMENTAL

The packed RBC used in these studies were prepared from whole blood obtained from "normal" adults which had been collected into vacuum units containing the ACD solution. These cells were stored at 4–5°C and used as needed.

The washed RBC were prepared for electrochemical studies by first diluting 25 ml of the packed RBC with 25 ml pH 7·4 isotonic Na_2HPO_4–KH_2PO_4 buffer and then shaking gently to distribute the cells uniformly. The diluted sample was centrifuged for 10 min at 3000 *g* (4°C) and the supernatent liquid siphoned from the packed RBC. The washing and centrifugation process was repeated an additional 4 times and finally the packed RBC diluted with phosphate buffer (40–50 ml) so as to yield a hematocrit of 40 ± 1%.

All studies were performed on deaerated aliquots from a mixture consisting of 5 ml of the washed RBC diluted with 45 ml phosphate buffer or 0·0445M glucose in the phosphate buffer. The concentration–I_d relationships were determined on appropriate dilutions of RBC in the glucose–buffer medium.

A Radiometer PO 4 Polariter in conjunction with a drop life timer and an E 65 mercury electrode assembly (DME) was used in this study as well as a rotating platinum (1000 RPM) and glassy carbon electrode (Chemtrix, Inc.). A scan rate of 0·2 V/min was used to obtain the recordings. With the DME at a reservoir height of 50·0 cm, the drop life time was adjusted to give $t = 1{\cdot}0$ s ($m = 1{\cdot}629$ mg s^{-1}) with 50% blanking. All voltammograms were recorded at 22–23°C.

RESULTS AND DISCUSSION

Deaerated aliquots obtained from a number of buffer diluted samples of freshly washed packed RBC gave a wave (DME), $E_{1/2} = -0{\cdot}220 \pm 0{\cdot}01$ V *vs* S.C.E., $n \approx 1$ and $I_d = 0{\cdot}700-0{\cdot}900$ μA. This I_d value was found to vary from donor to donor and with the storage age of the RBC. The supernatant liquid obtained by centrifugation of the buffered RBC suspensions demonstrated no polarographic wave. This strongly suggests that the substituent group being detected is associated with the erythrocyte membrane.

The fact that this wave was not observed when using a rotating platinum or glassy carbon electrode suggested the following reaction:

$$RSH + Hg \rightarrow RSHg + H^+ + e\,.$$

Suspension of the RBC in a pH 7·4 phosphate buffered 10^{-3}M solution of N-ethyl maleimide, a sulfhydryl blocking agent[5], for 3 h at 24°C followed by washing with the buffer yielded no decernable wave. Under similar conditions in the absence of the blocking agent, the usual polarographic wave was obtained. This finding supported the suggested interaction of the mercury with sulfhydryl groups.

The presence of reduced sulfhydryl groups in RBC is essential for the maintenance of the integrity of the RBC membrane and the numerous enzyme systems associated with the cell, the integrity of the hemoglobin molecule, and the maintenance of methemoglobin at normal levels[6–8].

As a result of the metabolism of glucose *via* the glycolytic and pentose phosphate pathways, the reduction of nicotinamide-adenine dinucleotide (NAD) to NADH and NAD-phosphate (NADP) to NADPH occurs. It is the availability of NADH and NADPH which is responsible for maintaining the sulfhydryl groups in their reduced form[9]. On this basis then the effect of the metabolism of glucose by the RBC under sterile aerobic conditions for 18 h at 37°C was investigated. A comparison of the polarographic waves obtained before and after incubation in this medium with those obtained in the absence of glucose are shown in Fig. 1.

From a comparison of the two sets of curves it will be noted that the control shows a small $-\Delta I_d$ after incubation, whereas the RBC in the presence of glucose demonstrates a significant $+\Delta I_d$.

In order to demonstrate the effect of RBC concentration on I_d values, samples with increasing hematocrit values were incubated in the buffered glucose medium. The results obtained are plotted in Fig. 2.

The RBC performs its many functions optimally at pH 7·4. Therefore it was not unexpected to find that when washed RBC were incubated in a pH 7·0 phosphate buffered glucose medium, that a $-\Delta I_d$ was obtained. Similar behavior was noted with a hemolyzed sample of RBC in a pH 7·4 buffered glucose solution. Its non-hemolyzed counterpart gave the usual $+\Delta I_d$. Although there is generally no significant deterioration of individual enzymes as a result of hemolysis, there is a destruction of the 'enzymatic transmission network'. As a result, the normal sequence of metabolic events cannot occur and a $-\Delta I_d$ might be anticipated.

The association of the $+\Delta I_d$ with active metabolism was further confirmed by experiments in which the glucose substrate was replaced by fructose, which as fructose-6-phosphate and gluconolactone, as 6-phosphogluconolactone are intermediates in the glycolytic and pentose phosphate pathways respectively. Pyruvate was also incorporated into this series to determine what effect this intermediate, which pre-

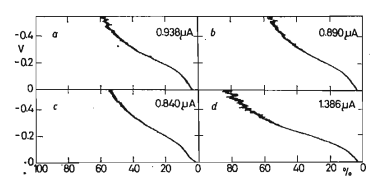

FIG. 1

Comparison between a Washed RBC Sample (Donor 10/17/69 - Washed 10/20) in pH 7·4 Buffer *a* Before incubation, *c* 18h 37°C, and in pH 7·4 buffer containing 0·04M glucose, *b* before incubation, *d* 18 h 37°C. 2 μA sensitivity on recorder.

cedes the formation of the terminal product of metabolism, namely lactate, would have on the ΔI_d value. It was anticipated that pyruvate because of its position in the metabolic scheme would either demonstrate no effect or manifest an adverse effect on the metabolic behaviour of the RBC. The adverse effect might occur due to the absence of sufficient NADH to accomplish the reduction of pyruvate to lactate. Normally the production of NADH accompanies the oxidation of glyceraldehyde-3-phosphate to 1,3-phosphoglyceric acid obtained as a result of the metabolism of a substrate such as glucose either by the glycolytic or pentose phosphate pathways. As a consequence of this NADH deficiency, the sulfhydryl groups could conceivably serve as electron donors to accomplish the requisite reduction. The percentage change in ΔI_d of RBC after incubation with glucose, fructose, gluconolactone and pyruvate was found to be 188, 158, 123 and 79% respectively of a substrateless control. The $E_{1/2}$ values obtained from these polarographic waves were within the range mentioned earlier. This suggests that regardless of the substrate used, a particular sulfhydryl species is apparently being quantitated in all instances. The low value obtained with pyruvate indicates that this substrate did have an adverse effect on the RBC.

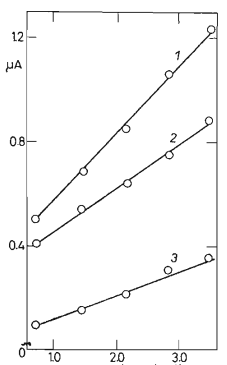

FIG. 2

RBC Concentration Dependence of I_d (in μA) before and after Incubation in pH 7·4 Buffer – 0.04M Glucose Mixture
1 I_d, 18 h, 37°C, *2* I_d, 0 h, *3* ΔI_d.

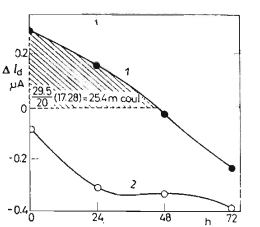

FIG. 3

ΔI_d Obtained Intervals from RBC Aliquots Subjected to Moderate Stress after Initial Washing (Donor 8/12/69 — washed 8/25/69)
1 RBC in pH 7·4 buffer, *2* RBC in pH 7·4 buffered glucose.

The metabolic viability of stored packed RBC prepared from whole blood collected in the ACD solution was evaluated on the basis of an electrical capacity factor (coulombs). In accomplishing this measurement the assumption was made that the initial viability of the RBC will be related to its ability to withstand moderate stress. Experiments were performed on freshly washed packed RBC which had been diluted to the required hematocrit value and then stored at 4–5°C until all studies on this sample had been completed. A zero hour ΔI_d was obtained from the I_d of aliquots of the RBC-buffered glucose mixture before and after 18 h incubation. RBC-buffered glucose mixtures prepared from the same sample of washed RBC after 24, 48 and 72 h of refrigeration were also evaluated in this same manner. A representative ΔI_d/time curve is shown in Fig. 3 as well as, for purposes of comparison, a control ΔI_d/time curve. As a $+\Delta I_d$ was considered a manifestation of metabolic activity, only the area under this segment of the curve was integrated to obtain the coulomb value associated with active metabolism.

Eight units of packed RBC stored in the ACD solution at 4–5°C were evaluated by the above method at various time intervals in their storage-life. A plot of millicoulombs *vs* storage days for one of the units of packed RBC studied is shown in Fig. 4.

Although there was a degree of variation from donor to donor in the magnitude of the coulomb values for similar periods of evaluation, they all demonstrated a rapid coulomb decrease within essentially the same period of time. These results therefore suggest that the metabolic viability of the RBC decreases rapidly during the first 8–10 days after withdrawal from the donor under these particular storage conditions and then more slowly thereafter. A comparatively low level of viability could still be detected after 45 days of storage.

As a final note it should be mentioned that although the evidence presented strongly suggests that it is a sulfhydryl group which is being detected by the DME, the identity

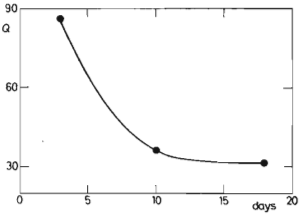

FIG. 4

Typical Plot of Millicoulombs *vs* Days for Packed RBC Collected from Donor II (9/26/69) and Evaluated at Various Time Intervals within its 21 Day Storage-Life Period in the ACD Solution Maintained at 4–5°C

of the chemical compound or compounds containing this substituent group is still to be resolved. It is known that glutathione (GSH) is the major sulfhydryl bearing compound in the RBC [10]. Experimental findings demonstrated a considerable divergence in $E_{1/2}$ values between the RBC suspension (−0·220 V) and a solution of GSH ($1 . 10^{-4}$M) in the pH 7·4 buffer (−0·535 V). This finding might negate the possibility of the sulfhydryl containing compound being GSH were it not for the probability that this substance is bound to the lipoprotein of the cell membrane. As such it would represent an electrochemically reactive species, possibly of different behavior of free GSH with a corresponding $E_{1/2}$ value of its own. Thus with the present information GSH is still only suspect. Further studies will no doubt shed more light on the subject.

Of interest too, are preliminary findings which show that a significant difference exists between the $+\Delta I_d$ values obtained with washed packed RBC from freshly drawn "normal" donors blood, and those $+\Delta I_d$ values obtained from patients with various pathological conditions either directly or indirectly related to metabolic disturbances. The results of this study by the author will appear in a forthcoming publication.

REFERENCES

1. Allen M. J.: Bacterial Rev. *30*, 80 (1966).
2. Allen M. J.: Current Mod. Biol. *1*, 116, 178 (1967); *2*, 329 (1968).
3. Allen M. J.: Electrochim. Acta *15*, 1565 (1970).
4. Ebaugh F. G. jr, Emerson C. P., Ross J. F.: J. Clin. Invest. *32*, 1260 (1953).
5. Sandberg H. E., Bryant R. G., Piette L. H.: Arch. Biochem. Biophys. *133*, 144 (1969).
6. Benesch R. E., Benesch R.: Arch. Biochem. Biophys. *48*, 38 (1954).
7. Jandl J. H., Allen D. W.: J. Clin. Invest. *39*, 1000 (1960).
8. Scheuch D., Kahrig C., Ockel E., Wagenknecht C., Rapopot S. M.: Nature *190*, 631 (1961).
9. Huennekens F. M., Caffrey R. W., Gabrio B. W.: Ann. N. Y. Acad. Sci. *75*, 167 (1958).
10. Prins H. K., Loos J. A.: *Biochemical Methods in Red Cell Genetics* (J. J. Yunis, Ed.), p. 116. Academic Press, London 1969.