The Alleged Effect of Acid Phosphate on the Anion Permeability of Erythrocytes

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Keilin & Mann (1941) described an optical method of studying the penetration of erythrocytes by the bicarbonate ion and were able to demonstrate in an elegant manner the activity of carbonic anhydrase within the cells and the link between this activity and Hamburger's chloride shift. Their method consists in converting the haemoglobin inside the cell to methaemoglobin and then observing the transformation of the methaemoglobin from the acid to the alkaline form by the entering bicarbonate. In their experiments sodium carbonate solution is added to a suspension of methaemoglobin cells in

Keilin & Mann (1941) and Keilin & Hartree (1946) studied the change of acid to alkaline methaemoglobin spectroscopically and found it to be practically complete after 90 sec. They also noticed that cells washed previously in acid phosphate or in phosphate, phthalate or citrate buffers of pH 6 do not change colour in this way. This phenomenon, which is the subject of the present paper, will be referred to as the Keilin & Mann effect. These workers suggested that such washing destroys the permeability of the cells to anions. A second possible explanation of the effect is that the cell has lost its rapidly diffusing chloride ions in exchange for slowly moving phosphate, phthalate or citrate ions and is thus unable to accept incoming bicarbonate.

These two hypotheses have been tested, in so far as they apply to phosphate, by spectroscopic observations, measurement of cell shrinkage and chemical analyses, using conditions in which the cells developed the Keilin & Mann effect gradually. The link between the change of methaemoglobin colour and cell shrinkage was confirmed, and the Keilin & Mann effect studied by photometric measurement of the shrinkage. Cells were treated with phosphate buffer and examined at different times for (a) the Keilin & Mann effect, (b) the phosphate content, and (c) the chloride exuded. A consistent relationship was found between these quantities, while no changes in cell permeability were observed.

METHODS

Solutions. These had the following compositions. Saline, 0.154M-NaCl; NaNO_3 , 0.154M; Na_2CO_3 , 0.2M; phosphate buffer, pH 6, 87.5 ml. 0.2M- NaH_2PO_4 + 12.5 ml. 0.2M- Na_2HPO_4 ; glucose, 0.296M.

Preparation of methaemoglobin cells. Defibrinated horse blood was stored at 5° and used 1–2 days after collection. The separated erythrocytes were washed three times with saline, once with NaNO_3 solution and four times with saline. The volume of washing fluid was always twice the volume of the centrifuged cells. The washed cells were finally mixed with an equal volume of saline to give a concentrated suspension.

Photometric study of cell shrinkage. 1 drop of the concentrated suspension was mixed with saline in the cell of a King colorimeter using full aperture and no filter. The mixture was diluted gradually with saline until the instrument gave a reading of +3 scale divisions. The volume was adjusted to 5 ml. by withdrawing the excess, 5 drops of Na_2CO_3 solution

Fig. 1. The changes that occur when Na_2CO_3 solution is added to a suspension of methaemoglobin cells. The loss of water is accompanied by cell shrinkage.

sodium chloride solution and the following changes occur: (1) external bicarbonate exchanges rapidly for internal chloride, a process completed within 2 sec. (Dirkin & Mook, 1931; Luckner & Lo-Sing, 1938); (2) acid methaemoglobin reacts with bicarbonate forming alkaline methaemoglobin and carbonic acid; (3) carbonic acid is dehydrated, a reaction catalysed by intracellular carbonic anhydrase; (4) carbon dioxide and water leave the cell, and it shrinks (Warburg, 1922; Ponder, 1948). These changes are illustrated in Fig. 1.

were added, a stop-watch started, the suspension mixed well and readings of the photometer deflexion taken at 5 or 10 sec. intervals until they became steady. This occurred after 90–120 sec. The total deflexion of between +10 and +25 scale divisions (depending on the previous treatment of the cells) was reproducible to within ± 1 scale division. The experiment was repeated once or twice and the mean total deflexion noted. The technique is similar to that used by Parpart, Jacobs & Dzienmian (1937) in following chloride-sulphate exchanges of erythrocytes.

The initial adjustment of the concentration of the cell suspension until it gave a reading of +3 in the photometer was adopted as a convenient method of preparing a standard suspension. A subsidiary experiment showed that the further dilution caused by adding 5 drops of saline to 5 ml. of such a suspension brought the photometer reading from +3 to 0. Thus the method also allows for the dilution caused by the addition of 5 drops of Na_2CO_3 solution.

The total galvanometer deflexion obtained in such an experiment is dependent on both the shrinkage of the cells and their colour change. The contribution of each phenomenon to the total effect was determined as follows.

Direct counting showed that the standard suspension contained 9.7×10^4 cells/ μl . For a suspension of this concentration in a 1 cm. cell, Drabkin & Singer (1939) have shown that

$$E_T = E_S + E_P,$$

where E_T is the total extinction, E_S is the scattering extinction, and E_P is the extinction of the haemolysed suspension, i.e. that due to the total pigment of the cells when free in solution. Thus, the total change of extinction upon adding Na_2CO_3 is given by the expression

$$\Delta E_T = \Delta E_S + \Delta E_P$$

and the resulting change of colorimeter reading Δx_T is given by the expression

$$\Delta x_T = \Delta x_S + \Delta x_P.$$

Δx_P was measured indirectly by haemolysing a suspension three times as concentrated as a standard suspension and noting the change of colorimeter reading on the addition of Na_2CO_3 to the haemolysate. Allowing for the dilution effect as before, the value of Δx_P for a standard suspension was calculated to be $+2.6 \pm 0.2$ scale divisions. The total deflexion Δx_T of untreated cells was 20–25 scale divisions. About 10% of the total deflexion was thus due to the pigment colour change and about 90% to cell shrinkage.

Analysis of cell suspensions for phosphate. A sample (2 ml.) of the well shaken suspension was haemolysed by adding 5 ml. of water and 0.1 ml. of saponin solution, 2 ml. of 25% (w/v) trichloroacetic acid were added, the volume made up to 10 ml. and the whole stirred and filtered. Between 0.5 and 7 ml. of the clear filtrate were analysed for inorganic phosphate by the method of Lohmann & Jendrasik (1926).

The concentration of cells in the suspension was determined by adding 0.5 ml. to a haematocrit tube and reading the volume of packed cells after centrifuging for 15 min. at 1500 g. The phosphate content was calculated in terms of $\mu\text{moles of P/ml. of packed cells}$.

Subsidiary experiments in which the volume of a sample of cells was determined before and after treatment with phosphate buffer followed by saline washing as described above showed that no net change in volume was produced by this treatment.

Analysis of chloride exuded from the cells. Washed, centrifuged cells were mixed with about seven times their volume of phosphate buffer. At intervals 15 ml. of the well shaken suspension were withdrawn and centrifuged, and two 5 ml. samples of the supernatant analysed by Volhard titration following the procedures of Peters & Van Slyke (1932) for urine.

RESULTS

The shrinkage of cells upon the addition of carbonate

When 5 drops of sodium carbonate solution were added to a dilute suspension of methaemoglobin cells untreated with acid buffers they gave a gradually increasing photometer deflexion reaching a final value of 20–25 scale divisions depending upon the blood sample. The time course of the deflexion, which mainly represents cell shrinkage, followed the curve shown in Fig. 2, curve A. It is practically complete after 90 sec. and quite complete after 120 sec.

Fig. 2. The time curve of cell shrinkage when Na_2CO_3 solution is added to a dilute suspension of methaemoglobin cells in a colorimeter cell. Curve A, cells suspended in saline. Curve B, cells suspended in saline containing 5×10^{-4} M-sulphanilamide.

The shrinkage and colour change are linked with the reaction catalysed by carbonic anhydrase, as illustrated in Fig. 1. This was confirmed by the effect of treating the cells with sulphanilamide, which is a specific inhibitor of this enzyme (Keilin & Mann, 1940), before adding the sodium carbonate. In the presence of 5×10^{-4} M-sulphanilamide the photometer deflexion was greatly slowed (Fig. 2, curve B).

Fig. 3. The effect of pre-treatment with acid phosphate upon the shrinkage of methaemoglobin cells when sodium carbonate is added to a dilute suspension. A concentrated suspension of washed methaemoglobin cells was mixed with an equal volume of phosphate buffer, pH 6. After standing at room temperature (20°) for a measured time the cells were centrifuged, washed twice with saline and resuspended in twice their volume of saline. The shrinkage of these cells on the addition of carbonate was studied as described in the text.

Fig. 4. The quantitative measure of the Keilin & Mann effect.

The effect of acid phosphate buffer on cell shrinkage and on the colour change of methaemoglobin

Methaemoglobin cells treated with phosphate buffer showed a reduced photometer deflexion on the addition of sodium carbonate, depending upon the period of treatment with phosphate (Fig. 3). The velocity of the shrinkage was unaffected, but its extent was reduced by such treatment. These cells also exhibited only a partial change in absorption spectrum when studied in the microspectroscope of Keilin & Mann (1941). When the total deflexion resulting from sodium carbonate addition had been reduced to about 10 scale divisions by sufficiently long treatment with phosphate no change in absorption spectrum was observed on adding carbonate. The reduction in photometer deflexion caused by treatment with phosphate buffer was taken as a quantitative measure of the Keilin & Mann effect (see Fig. 4). Prolonged treatment with phosphate failed to reduce the deflexion below 8 scale divisions.

The relation between the Keilin & Mann effect and the phosphate-chloride exchange

The phosphate content of cells treated for various periods with phosphate buffer is plotted in Fig. 5 against the Keilin & Mann effect shown by the same cells. In Fig. 6 the chloride content of the supernatant phosphate buffer obtained and analysed as described is plotted against the Keilin & Mann effect exhibited by cells separated from these supernatants. The Keilin & Mann effect is seen to rise steadily with the phosphate absorbed and also with the chloride exuded by the cells.

Reversibility of phosphate penetration

Keilin & Hartree (1946) found that the effect of acid phosphate treatment in preventing the entry of bicarbonate into the cells was irreversible. By following the phosphate content of cells for several hours, it has now been possible to show that this apparent irreversibility is due to the very slow rate at which phosphate leaves the cell. Methaemoglobin cells suspended in saline were found to be too fragile for such protracted experiments and normal erythrocytes were therefore studied.

The rate of penetration of phosphate into normal erythrocytes suspended in phosphate buffer pH 6 was first studied by direct chemical analysis. The results, given in Fig. 7, curve A, show that the phosphate content rises logarithmically with time, attaining equilibrium in 4–5 hr. The rate of loss of phosphate from erythrocytes that had been washed three times in phosphate and then suspended in saline pH 7.3 was also studied by direct analysis. Fig. 7, curve B, shows that the phosphate content falls logarithmically with time, but that the rate of exit is about one-tenth that of entry.

DISCUSSION

The unchanged permeability of cells washed with acid phosphate

The rate of cell shrinkage and colour change on the addition of carbonate depends on the overall rate of the process shown in Fig. 1. Any reduction of the

action is also dependent upon the penetration of the cell by bicarbonate.

Keilin & Hartree (1946) found that citrate and phthalate buffers of pH 6 produced the same effect as acid phosphate. It remains to be shown whether these buffers act by the same mechanism as phosphate or whether some other mechanism is involved.

Fig. 5. The relation between the phosphate content of cells and their Keilin & Mann effect. The cells were washed free of intercellular phosphate before analysis.

rate of transfer of bicarbonate or chloride ions into and out of the cell will appear only if the rate of this ionic exchange becomes the limiting factor of the whole process. The results illustrated in Fig. 3 indicate that washing with acid phosphate buffer has no such effect. Thus it may be concluded that the permeability of the cells to these anions is not destroyed and is probably unaffected by such washing.

The phosphate-chloride exchange

The results given in Figs. 5 and 6 show that an exchange of external phosphate for internal chloride occurs when cells are treated with acid phosphate buffer. As Booth (1938) has pointed out, if rapidly diffusing chloride ions inside the cell are replaced by slowly diffusing phosphate ions, the amount of bicarbonate ion which can then enter the cell rapidly is limited by the extent of the replacement. This has now been confirmed by the direct relation shown to exist between the extent of the phosphate-chloride exchange and the reduction in the cell shrinkage, which is itself linked with the entry of bicarbonate (Fig. 1).

It is clear that this replacement is responsible for the action of acid phosphate in decreasing and finally abolishing the acid methaemoglobin $\rightarrow$ alkaline methaemoglobin reaction since this re-

Fig. 6. The relation between the chloride content of the suspending fluid and the Keilin & Mann effect of the cells. Cells were transferred from saline to phosphate buffer. The rise in the chloride content of the phosphate buffer indicates a progressive loss of chloride from the cells. The cells were not washed free of intercellular chloride before suspension in phosphate. The intercept on the ordinate indicates the original intercellular chloride.

Fig. 7. Curve A, the time course of phosphate entry into washed horse erythrocytes suspended in phosphate buffer, pH 6.3, temp. 20°. Curve B, the time course of phosphate exit from phosphate-treated horse erythrocytes suspended in saline, pH 7.3, temp. 20°.

Mechanisms of cell shrinkage caused by carbonate

The shrinkage of saline-washed methaemoglobin cells upon the addition of carbonate to a saline suspension seems to be comprised of two parts.

That part which is studied in this paper is associated with the bicarbonate-chloride exchange, is abolished by previous treatment with acid phosphate, is slowed by sulphanilamide, and is accompanied by a change of internal pH. It is satisfactorily explained by the processes illustrated in Fig. 1. The other part of the shrinkage is unaffected by sulphanilamide, since in Fig. 2 the initial slopes of curves *A* and *B* are identical, is not abolished by prolonged treatment with acid phosphate, and does not change the colour of intracellular methaemoglobin. It is probably caused by the change in external pH, which according to the theoretical predictions of Jacobs & Stewart (1947) itself produces an osmotic loss of water and cell shrinkage.

The rates of phosphate entry and exit

There have been several demonstrations that phosphate enters the erythrocytes of different species (see Davson & Danielli, 1943, for review). Many of these have been only semi-quantitative and have used indirect methods of following the phosphate transfer.

Quantitative observations were made by Maizels (1933, 1934) who studied the entry of phosphate into human red blood cells and showed that it was most rapid from acid buffers. He also noted that in blood stored for 11 days at 2° phosphate left the cells more slowly than it entered them (Maizels, 1944). Anderson (1942) had also noted this phenomenon in rabbit erythrocytes suspended in plasma at 37°. He followed the phosphate transfer using ³²P and found that entry was three to five times as fast as exit under these conditions. On the other hand, the observations of Booth (1938) suggested a more rapid loss of phosphate from erythrocytes than is given by Anderson (1942) or by the results reported here. Booth (1938) showed that pig cells in which the chloride content had been decreased by shaking with phosphate buffer regained the chloride lost by shaking with saline for the same period. He put this down to entry and exit of phosphate but made no phosphate determinations. If the changes in chloride analyses he observed were in fact due to phosphate-chloride exchanges, the rate of phosphate exit indicated is much higher than is reported here for horse erythrocytes. However, his results may be partially accounted for by bicarbonate-chloride

exchanges since his solutions probably contained sufficient dissolved carbon dioxide to account for at least half the observed changes (Kolthoff, 1926; Booth, private communication). Such an interpretation does not affect the conclusions of Booth (1938) on the importance of intracellular chloride in studying carbonic anhydrase activity in red corpuscles.

Phosphate transfer and cell metabolism

There is no evidence in this work that the mechanism of phosphate transfer necessarily involves an enzyme system coupled with the cell metabolism (cf. Halpern, 1936; Eisenman, Ott, Smith & Winkles, 1940). While the well washed cells are still capable of active metabolism, they are isolated from sources of energy (e.g. glucose) and yet show no deterioration in their phosphate-transferring capacity during the protracted experiments described.

SUMMARY

1. A method is described for studying the water shift accompanying the bicarbonate-chloride exchange in horse erythrocytes.
2. Methaemoglobin cells treated with phosphate buffer, pH 6, exhibit normal permeability to bicarbonate and chloride ions as far as can be observed by this method.
3. The apparent destruction of the anion permeability of methaemoglobin cells by acid phosphate buffer (Keilin & Mann, 1941; Keilin & Hartree, 1946) is shown to be due instead to a phosphate-chloride exchange across the cell boundary.
4. The apparent irreversibility of the effect of acid phosphate buffer on methaemoglobin cells (Keilin & Hartree, 1946) is probably due to the slowness with which phosphate leaves the cells compared to the rate at which it enters them.

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REFERENCES

- Anderson, R. S. (1942). *Amer. J. Physiol.* **137**, 539.
 Booth, V. H. (1938). *J. Physiol.* **93**, 117.
 Davson, H. & Danielli, J. F. (1943). *The Permeability of Natural Membranes*. Cambridge University Press.
 Dirkin, M. N. J. & Mook, H. W. (1931). *J. Physiol.* **73**, 349.
 Drabkin, D. L. & Singer, R. B. (1939). *J. biol. Chem.* **129**, 739.
 Eisenman, A. J., Ott, L., Smith, P. K. & Winkles, A. W. (1940). *J. biol. Chem.* **135**, 165.
 Halpern, L. (1936). *J. biol. Chem.* **114**, 747.
 Jacobs, M. H. & Stewart, D. R. (1947). *J. cell. comp. Physiol.* **30**, 79.
 Keilin, D. & Hartree, E. F. (1946). *Nature, Lond.*, **157**, 210.
 Keilin, D. & Mann, T. (1940). *Nature, Lond.*, **146**, 164.
 Keilin, D. & Mann, T. (1941). *Nature, Lond.*, **148**, 493.

- Kolthoff, I. M. (1926). *Biochem. Z.* **176**, 101.
 Lohmann, K. & Jendrassik, L. (1926). *Biochem. Z.* **178**, 419.
 Luckner, H. & Lo-Sing (1938). *Pflüg. Arch. ges. Physiol.* **239**, 278.
 Maizels, M. (1933). *J. Physiol.* **77**, 22 P.
 Maizels, M. (1934). *Biochem. J.* **28**, 337.
 Maizels, M. (1944). *Quart. J. exp. Physiol.* **32**, 143.
 Parpart, A. K., Jacobs, M. E. & Dzienmian, A. J. (1937). *Biol. Bull. Woods Hole*, **73**, 381.
 Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, Vol. 2. London: Baillière, Tindall and Cox.
 Ponder, E. (1948). *Haemolysis and Related Phenomena*. London: Churchill.
 Warburg, E. J. (1922). *Biochem. J.* **16**, 153.

The Reaction between Metmyoglobin and Hydrogen Peroxide

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Transient complexes with well defined spectroscopic characteristics play an important role in the reaction of the haemoprotein enzymes, catalase and peroxidase, with hydrogen peroxide. Two and sometimes three different complexes can be observed depending on the experimental conditions. For example, Keilin & Mann (1937) obtained two red complexes with peroxidase and hydrogen peroxide, and later Theorell (1941) showed that a green complex preceded the formation of the first red complex.

This system has been studied intensively by Chance (1949*a, b*) who extended the observation to systems of catalase and hydrogen peroxide or alkyl hydroperoxides (1949*c*). There is, however, very little information from which structural differences accounting for the various complexes can be inferred. The transient nature of the complexes leads to experimental difficulties.

These difficulties were absent in the case of the complex formed with metmyoglobin (MetMb) and hydrogen peroxide. The existence of a well defined red complex in this system and with haemoglobin too is well established. Keilin & Hartree (1935) found that the formation requires 1 molecule of hydrogen peroxide per haematin iron atom. With reducing agents the complex is reduced back to metmyoglobin or methaemoglobin. As far as is known, only this single complex is formed and because it shares certain properties with the secondary complexes of peroxidase and catalase, a detailed knowledge of its behaviour is valuable for the further elucidation of enzyme reaction mechanisms.

In this paper experiments are described on the formation of the metmyoglobin-hydrogen peroxide complex over a wide pH range which reveal that only between pH 8 and 9 is the formation unattended by oxidative side reactions affecting the porphyrin ring. Reduction of the complex with

ferrocyanide shows it to have 1 oxidizing equivalent, and magnetic measurements suggest that it may be a compound containing quadrivalent iron. A preliminary account of these experiments has been published (George & Irvine, 1951).

MATERIALS AND METHODS

Metmyoglobin. The metmyoglobin (MetMb) used in these experiments was obtained from horses' hearts, the method of preparation following that of Theorell (1932). Briefly, the minced hearts are extracted with water at 0° and muscle globulins removed by addition of basic lead acetate to the extract, the pH of which has been previously adjusted to 7.0 with 1*N*-NaOH. The excess Pb is removed as phosphate with Na₃PO₄, and haemoglobin precipitated by adding (NH₄)₂SO₄ to 75% saturation. MetMb is precipitated when the solution is saturated to 100% with (NH₄)₂SO₄ and is taken up in the minimum quantity of 3*M*-phosphate buffer, pH 6.8. Phosphate is removed by dialysis against distilled water and MetMb again salted out. It is then taken up in 0.6*M*-phosphate buffer, pH 5.7, passed through a Seitz filter and stored at 0°.

Quantities of this stock solution were thoroughly dialysed against distilled water before use and the concentration determined following the method of de Duve (1948).

Hydrogen peroxide. Pure 97% (w/w) H₂O₂, free from inhibitors, was used. This was kindly supplied by Laporte Chemicals Ltd. Before each experiment a stock solution (approx. 0.1*N*) was standardized against KMnO₄ and then diluted to the desired concentration.

Buffer solutions. In the pH range 5.8–7.0 phosphate buffers (Green, 1933) were used. For the pH range 7.8–10.0 borate buffers were used. Experiments at pH 11.1 were carried out in a glycine buffer, while at higher pH values dilute alkali was used. The pH values of all buffer solutions were measured on a Cambridge pH meter.

Methods

Colorimetric or spectrophotometric determinations of optical density were made in the majority of the experiments. Because of its easier and more rapid application, the