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FLICKER PHENOMENON IN HUMAN ERYTHROCYTES

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In 1890, Browicz described vibratory movements in human erythrocytes, and these observations were confirmed by Cabot in 1901. Fifty years later, Pulvertaft (1949) rediscovered the phenomenon using the phase-contrast microscope, and drew attention to the earlier work of Browicz and Cabot. In view of the demonstration of active cation transport in human erythrocytes by Harris (1941) and by Maizels (1948, 1949), it was thought of interest to see if red-cell flicker were affected by the same agencies as active transport.

METHOD

This is substantially that of Pulvertaft (1949), with minor modifications. To avoid rouleaux formation in cells to be examined all dilutions are made with serum or defibrinated plasma of the appropriate group. The following are used: (*a*) 40% suspension of red cells in serum or defibrinated plasma; (*b*) a mixture of serum or plasma and of reagent, the proportions of each being adjusted to give the required concentration of reagent. 1 ml. cell suspension and 9 ml. of the diluted reagent are mixed, incubated and submitted to the following investigations: (i) a drop of the suspension is examined with the phase-contrast microscope and the percentage of flickering cells counted; (ii) 9 ml. are centrifuged, the cells lysed in 4 vol. of water and the pH of the haemolysate measured (Maizels, 1949); (iii) 0·2 ml. suspension is gently centrifuged and the cells re-incubated with 4 ml. normal serum: the object of this last procedure is to show how far cells deprived of their flicker by incubation with noxious agents may recover when the agent is removed and the cells re-incubated in normal serum.

RESULTS

Experiments were carried out at 37° C. and, broadly speaking, two types of cell change were seen: the first occurred on the addition of water to give a hypotonic system and also on the addition of acids; the second type occurred on the addition of alkali, certain metabolic poisons and also with haemolytic agents.

Normally, the red cell is biconcave with a pale centre and it is at the junction of the outer dark and pale central area that flicker is best seen as a very rapid shadowy flame-like movement, comparable in character to the shimmering of heat-haze on a sunny road. In such normal preparations a few erythrocytes are

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likely to show early crenation, the cells being polygonal instead of round, but with flicker fully active.

The first type of cell abnormality, induced by hypotonicity or by acidity, passes through the following stages: the shading becomes diffuse instead of peripheral and the flicker, feeble or absent; when counting the flickering cells, these varieties present difficulties. Next, the evenly shaded cell changes to a cell showing a small bright round refractile area, which may be centric or excentric and, when marginal, is seen to correspond to the reflexion of light from the bottom of a small invagination of the cell surface. But sometimes, the cupping is so marked that the erythrocyte resembles a punctured rubber ball that has been squeezed. It is probable that most of the so-called balloon cells are really cupped. These cells ultimately become ghosts, with or without an intermediate spherocytic stage.

The second type of cell change occurs in the presence of metabolic poisons. The first stage is an increase in the number of polygonal cells over that seen in unpoisoned samples; these change to coarse crenated cells, each cell having a few large dark spikes. The bases of the spikes are shaded and the intervening areas by contrast appear refractile. These cells show small general rocking movements without displacement of the cell as a whole and since they are not due to currents in the intercellular solution, they are probably Brownian in nature and due to the impact of water molecules on the large spikes: owing to this and to the alternation of light and dark areas, it is not always possible to be sure that flicker is absent from all these cells, though absence is probably the rule. The coarse crenated cell turns into the spherical finely crenated cell which shows neither flicker nor rocking movements and this in turn changes to the spherocyte, a small spherical cell diffusely shaded except for a small light rim round the periphery. The spherocyte haemolyses and becomes a ghost.

Both the above types of change (shown semi-diagrammatically in Fig. 1) if they have not lasted too long, may be reversed, in part at least, by returning the cells to normal plasma or serum. It must be added that crenated cells and spherocytes have been fully discussed by Ponder (1948), who also gives a full bibliography.

Quantitative assessment of cell changes with inhibitory agents is not easy, because these changes are not all clear-cut, while fatigue and autosuggestion intrude and make it desirable for records to be kept by two observers. For these reasons, erythrocytes have been classified into active cells and inactive cells, the latter showing only very slight shimmering or none at all.

All experiments, except those on the effects of reaction on flicker, were conducted at about pH 7.

Time and flicker. If blood is incubated at 37° C. in the presence of glucose, flicker is fully active for 2 or 3 days, but, after this, cells become progressively inactive, crenated and finally spherical.

Temperature and flicker. Effects here have not been fully controlled because of the difficulty of maintaining anything like a constant temperature on the microscope stage, but it is clear that when blood is examined immediately after cold-storage many of the cells are crenated and inactive, and that these become bi-concave and active after a few minutes at 37° C.

Tonicity and flicker. If distilled water is added to blood and the sample examined after 5 min., the cells are seen to have become plumper but activity is not decreased. When, however, the volume of cell suspension and water added are nearly equal, flicker begins to fail and 'diffusely shaded' and 'balloon' cells become numerous. When the cell suspension is diluted with an equal quantity of water less than 30% of the cells flicker, most of the cells are bal-

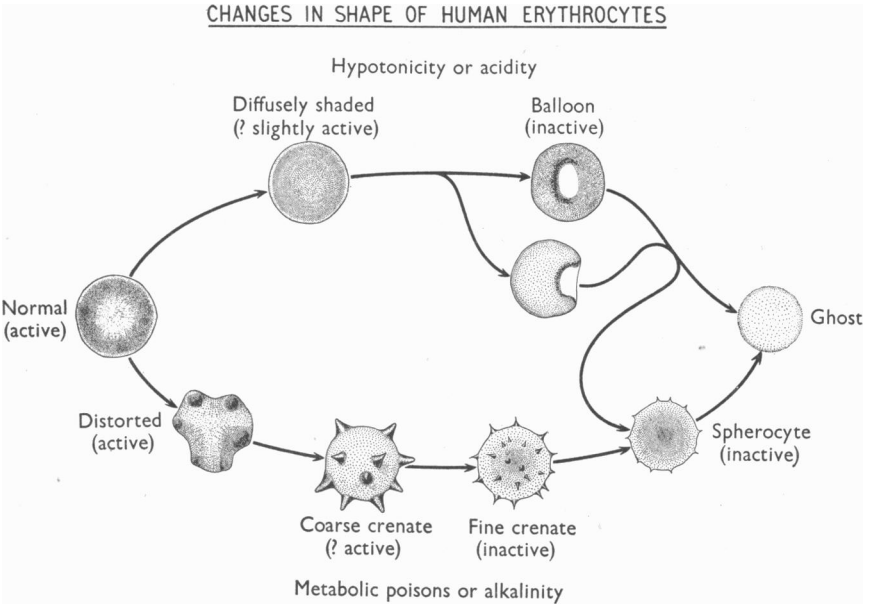

Fig. 1.

looned, while a few spherocytes are seen and a fair number of ghosts. With further dilution flicker disappears and lysis becomes marked.

pH *and flicker* (Table 1). Decinormal hydrochloric acid or sodium hydroxide was mixed with cell suspension, and the samples examined after 5 min. and again at 4½ hr. The shorter time gives more correctly the pH range of activity because it tends to avoid permanent cell damage. With short exposure, the number of flickering cells was much reduced above pH 9·6. Between pH 9 and 5·5, flicker appeared to be active throughout, and though there may well have been loss of power at the extremities of this range, this was not perceptible. Between pH 5·5 and 5, an undoubted general decrease in the activity of flicker could be seen, with the appearance of diffusely shaded and ballooned cells:

below pH 5, flicker is no longer seen. With regard to the effects of restoration to normal plasma: of cells completely inactivated at pH 10·2, 10% recovered while of cells largely inactivated at pH 9·6 nearly all recovered in normal plasma as did cells inactivated at pH 5·2; cells inactivated at pH 5 showed poor recovery.

TABLE 1. Effects of pH on red-cell flicker

Exp.	Period of exposure (min.)	Cells pH	Lysis	Cells active (%)	Cells restored to normal plasma for 1½ hr. (% active)
1	5	10·2	+	Very few	10
	5	9·6	±	15	95
	5	9·3	0	50	—
	5	9·0	0	90	—
	5	6·0	0	100	—
	5	5·5	0	95	—
	5	5·2	0	80 (weak)	100 (normal)
	5	5·0	±	0	10
2	270	9·1	±	10	55
	270	8·9	0	43	75
	270	7·2	0	85	—
	270	6·0	0	77	—
	270	5·8	0	47	67
	270	5·5	0	10	52
	270	5·2	±	0	47

(± = slight.)

As might be expected, the pH range of flicker was narrower when the period of exposure was extended to 4½ hr.—9·2–5·5, but even here considerable recovery of inhibited cells followed the transference to normal plasma.

Metabolic poisons and flicker. Fluoride: Pulvertaft (1949) added 0·1 ml. M-sodium fluoride to 0·9 ml. blood and observed inhibition of flicker after 5 min. The addition of molar fluoride solution must result in hypertonicity of Pulvertaft's blood systems and in the present experiments (Table 2) with isotonic systems, even high concentrations of fluoride failed to inhibit flicker after only 5 min. incubation, though after 4 hr. inhibition was marked with all concentrations down to 5 mM., initial crenation being followed by spherocytosis. Even when inhibition was marked, however, washing and replacement in fluoride-free plasma led to considerable recovery of flicker, more especially in cells exposed to weaker concentrations of inhibitor: recovery after strong fluoride was much less, indicating permanent cell damage. On the other hand, addition of pyruvate to fluoride systems did not prevent loss of flicker (Table 3).

With mono-iodoacetate (Table 4) cell flicker was unaffected by high concentrations applied for 5 min., but definite inhibition was seen at 1 hr. With rather weaker concentrations (15 – 10 mM.) inhibition was slight at 1 hr., but marked at 3 hr. and even quite low concentrations down to 0·15 mM. inhibit slightly at 3½ hr., while after 8 hr. incubation some inhibition was perceptible when iodoacetate was only 0·02 mM. Absence of flicker was always associated with marked crenation and sphering.

Carbon monoxide did not inhibit flicker after 4 hr. exposure—an observation

which agrees with Pulvertaft's (1949) earlier findings. Cyanide and azide acting for 5 hr. at 10 mM. or less also failed to inhibit: high concentrations, on the other hand (over 30 mM.), caused considerable depression with crenation and sphering.

TABLE 2. Effects of fluoride on cell flicker

NaF (mM.)	Period of exposure (min.)	Cells active (%)	Cells restored to normal plasma for 2 hr. (% active)
80	5	66	—
80	60	28	—
80	240	8	16
80	330	3	8
40	5	58	—
40	60	37	—
40	240	10	33
40	330	4	7
20	5	61	—
20	60	49	—
20	240	9	33
20	330	7	16
10	5	70	—
10	60	61	—
10	240	9	67
10	330	8	35
5	5	95	—
5	60	72	—
5	240	37	76
5	330	16	47
0	5	95	—
0	330	90	—

TABLE 3. Effects of fluoride and pyruvate on cell flicker

NaF (mM.)	Na pyruvate (mM.)	Period of exposure (min.)	Cells active (%)
40	0	120	25
40	13	120	33
40	0	240	4
40	13	240	3
20	0	120	60
20	13	120	53
20	0	240	10
20	13	240	13
10	0	120	80
10	13	120	72
10	0	240	40
10	13	240	36
5	0	120	87
5	13	120	80
5	0	240	85
5	13	240	84
0	0	240	98

Mepacrine had little effect with levels at 0·4 mM. or less, but more than 1 mM. gave clear inhibition with diffusely shaded and ballooned forms, crenated cells and spherocytes.

TABLE 4. Effects of mono-iodoacetate on cell flicker

Exp.	Mono-iodoacetate (mM.)	Period of exposure (min.)	Cells active (%)	Cells restored to normal plasma for 2 hr. (% active)
1	36	5	66	—
	36	60	24	—
	36	180	4	23
	36	270	1	—
	24	5	63	—
	24	60	20	—
	24	180	23	33
	24	270	11	—
	15	5	76	—
	15	60	67	—
	15	180	25	37
	15	270	14	—
	11	5	84	—
	11	60	78	—
	11	180	40	55
	11	270	25	—
	0	270	96	—
2	40	210	18	—
	40	480	0	—
	20	210	29	—
	20	480	3	—
	10	210	35	—
	10	480	8	—
	2·5	210	47	—
	2·5	480	14	—
	0·6	210	47	—
	0·6	480	9	—
	0·15	210	52	—
	0·15	480	21	—
	0·04	210	77	—
	0·04	480	21	—
	0·02	210	71	—
	0·02	480	35	—
	0·01	210	83	—
	0	210	80	—
	0·01	480	45	—
	0	480	50	—

TABLE 5. Effects of cyanide, azide and mepacrine on cell flicker

Reagent	mM.	Percentage cells active after 60 min.	180 min.	300 min.
Cyanide	61	46	39	19
	38	50	38	28
	13	76	69	65
	6·5	84	80	81
	0	87	—	80
Azide	68	66	50	20
	27	85	53	35
	13	82	67	68
	6·5	86	68	70
	0	85	80	78
Mepacrine	2	30	—	30
	1·2	45	—	38
	0·4	78	—	76
	0	75	—	72

TABLE 6. Effects on red cell flicker of incubation for 18 hr. with various sugars

Sugar added	Cells active (%)
None	0·5
Arabinose (0·2%)	1·0
Fructose (0·2%)	60
Galactose (0·2%)	1·5
Glucose (0·2%)	75
Mannose (0·2%)	79
Lactose (0·2%)	2·5
Maltose (0·2%)	9
Sucrose (0·2%)	2

TABLE 7. Comparison of the effects of various agents on red-cell flicker and on active transport

Agent	Concentration	Period of exposure (hr.)	Flicker index	Active transport index
Tonicity	Total base 188 m.equiv./l.	18	1·00	1·06
	164 ,,	18	1·00	1·0
	136 ,,	18	1·00	0·84
	124 ,,	18	0·90	0·53
pH 5·5	—	6	0·40	0·12
6	—	6	0·69	0·35
6·5	—	6	1·00	0·59
7	—	6	1·00	1·0
7·5	—	6	1·00	0·92
8	—	6	0·94	0·55
8·5	—	6	0·82	0·28
9	—	6	0·60	0·08
9·5	—	6	0·08	0
Fluoride	10 mM./l.	18	0·01	−0·26
	4 ,,	18	0·21	+0·05
	2 ,,	18	0·60	0·39
	1 ,,	18	0·79	0·52
Iodoacetate	0·16 mM./l.	18	0·05	0
	0·08 ,,	18	0·35	0·25
	0·04 ,,	18	0·70	0·68
	0·02 ,,	18	0·98	0·94
Cyanide	11 mM./l.	18	0·85	0·77
	5·5 ,,	18	1·00	0·95
Azide	12 mM./l.	18	0·74	0·66
	6 ,,	18	0·91	0·80
	3 ,,	18	0·95	0·85
Mepacrine	0·5 mM./l.	18	0·61	0·45
	0·25 ,,	18	0·95	0·84
Fructose	500 mg. %	18	0·92	0·93
	167 ,,	18	0·65	0·59
	85 ,,	18	0·14	0·09
Glucose	500 ,,	18	0·90	0·92
	167 ,,	18	0·99	0·95
	85 ,,	18	1·00	1·00
Mannose	500 mg. %	18	0·93	0·86
	167 ,,	18	0·86	0·82
	85 ,,	18	0·83	0·80

Sugars and flicker. Dialysis of blood against saline removes most of the natural glucose after several days, but the cells were seen to be spherocytic and showed little recovery on being incubated with added glucose. The following method gave satisfactory results: 5 ml. samples of blood were cold-stored at 6° C. for 9 days with a variety of sugars. At the end of this time, much of the natural glucose originally present has disappeared. The several samples were then poured into a solution consisting of 3·5 ml. potassium chloride (0·15 N) and 10 ml. sodium chloride (0·15 N), together with enough alkali to prevent the pH of the cells falling below 7 during incubation: the cell suspensions were rocked for 18 hr. at 37° C. (by which time none of the original natural glucose remained), and examined with a phase-contrast microscope. Table 6 shows that in the presence of moderate amounts of glucose, mannose and fructose flicker is well maintained with mannose as a rule slightly less efficient than glucose (this is not seen in Table 6) and fructose definitely less effective. The lower the sugar contents, the greater is the disparity in effectiveness of the various sugars; these findings are illustrated in Table 7 and the matter is discussed more fully later. With arabinose, galactose, lactose, maltose and sucrose, flicker at the end of 18 hr. incubation was slight, and indeed most of the cells had then become spherical (maltose was the least ineffectual of these five sugars; possibly because it contained a trace of glucose as shown by fermentation tests).

Correlation between cell flicker and active cation transport

The present paper shows that flicker is affected by certain agents which are known to affect active cation transport (Maizels, 1951), and an attempt has been made to compare these effects on the respective cell activities. In Table 7, flicker has been expressed as an index related to the degree of flicker seen in a control sample, the latter consisting of a suspension of cells in serum, containing enough glucose to ensure activity throughout the period of observation and free from the addition of any toxic substance. Thus, if the control gives 80% of flickering cells and the inhibitor reduces this to 40% the flicker index is 0·5. Reduction of active transport to an index is less easy, because allowance must be made for the action of passive diffusion in opposing transport, the former amounting roughly to about 8% of the external sodium concentration. This amount of diffusion has therefore been assumed to occur in calculating the active transport index. Thus, if the cold-storage level of sodium is 45 m.equiv./l. and if, after incubation in solutions containing 125 m.equiv. sodium, without and with inhibitors, the cell sodium contents are respectively 20 and 30 m.equiv., the active transport index is $(45+10-30)/(45+10-20)$ or 0·71. The two indices are obtained by very different techniques, but there is a marked qualitative resemblance and a definite quantitative association between the flicker and active transport indices—an association which is manifest even in such details as the effects of reducing the sugar content in incubated systems:

with fructose the reduction causes a decrease both in flicker and transport, while with glucose a similar reduction is without effect (Table 7). On the whole, reduction in the transport index induced by an inhibitor exceeds the corresponding reduction in the flicker index.

DISCUSSION

According to Pulvertaft (1949) both nucleated and non-nucleated erythrocytes show flicker, but the former alone show a second independent and continuous movement of the surface. Hence, he concludes that in nucleated cells and presumably also in non-nucleated cells flicker is independent of the external face of the cell. In support of this, it may be shown that minute particles in Brownian movement show no special disturbance in the vicinity of erythrocytes, and indeed it is difficult to conceive of a purely surface movement producing the radial flicker of erythrocytes without being transmitted to the cell interior. The movement observed might be due to the Brownian movement of minute particles constrained by structural orientation within the cell, but it is difficult to believe that such physical movement could be inhibited by iodoacetate (in minimal amounts) or by fluoride, and that it would depend for its maintenance on certain monosaccharides and not on others; indeed, the resemblance between flicker and active transport is so close as to lead to the conclusion that the former, like the latter, is due to metabolic activity. These resemblances may now be considered more closely. Both activities persist in the presence of carbon monoxide and with cyanide and azide (up to 0·01 M), and are therefore independent of the normal mechanisms of cell respiration; indeed flicker and transport are both evident even when, because of combination with carbon monoxide, cyanide or azide, oxygen carriage by haemoglobin is no longer possible. On the other hand, fluoride and mono-iodoacetate which inhibit glycolysis both inhibit transport and flicker, nor is this removed by the simultaneous presence of pyruvate. It is true that inhibition by fluoride and iodacetate is not immediate and may take more than an hour to appear, but the initial activity may depend on the presence of existing high-energy phosphorus compounds, ceasing, in the absence of further synthesis, only when these compounds are used up.

The pH range of the two activities are also qualitatively similar, extending from about pH 5 to 9·5, and though depression by pH and other agents is apparently exerted more powerfully on transport than on flicker, the disparity is not great (Table 7), and it may well be that the maintenance of flicker requires less energy than the accomplishment of active cation transport.

A number of metabolic poisons exist besides those already discussed, but of these mepacrine alone was investigated because it appeared to damage the outer surface of erythrocytes (Maizels, 1951), and there was a possibility that this might affect flicker, but not transport. It is known that mepacrine

increases the permeability of red cells (Maizels, 1951), and cells so treated even in isotonic solution show diffusely shadowed forms, ballooning and cupping. Less altered cells, however, showed flicker and, as usual, the general reduction of flicker over that seen in controls was exceeded by the reduction of transport—an apparent excess due in part at least to using the standard figure for sodium diffusion, instead of allowing for the increased cell permeability induced by mepacrine. Mepacrine in quite small amounts inhibits cytochrome reductase (Haas, 1944) and it is probable that effects observed with the highest concentrations used, and absent with weaker concentrations, were due to non-specific damage and not to specific action on cytochrome reductase.

The effects of the sugars are of special interest. Meyerhof & Geliazkowa (1947) showed that with brain homogenates glucose and mannose are actively glycolysed, galactose is inactive, while fructose glycolyses more slowly than glucose when both are present in solution at 0·2%, but both the sugars glycolyse at the same rate when concentrations are 2%. The writers attribute these findings to different affinities of the sugars for hexokinase, differences which are more apparent at low concentrations. Meyerhof & Geliazkowa's findings are paralleled exactly with active transport (Maizels, 1951) and also with red cell flicker (Table 7), for here we see that glucose and mannose in large or small amounts are associated with activity, galactose is always inactive, while cells incubated with solutions rich in fructose are active and in fructose-poor systems they are inactive, although the incubated cells of such systems contain more sugar than do the cells of the corresponding glucose-poor systems, showing that the findings with fructose are not due to premature exhaustion of substrate but to a failure in utilization. The parallelism is so exact as to force the conclusion that active transport and flicker are parallel or successive events both dependent not on physical causes, but on metabolic activities in which glycolysis, but not respiration, is the chief factor. It is tempting to assume that flicker is itself an expression of the activity of 'carrier' molecules engaged in transporting sodium from within the erythrocyte to the exterior and this may well be the case; but the matter is not yet proven and, as already remarked, flicker and cation transport may merely be parallel activities.

It must be presumed that diffuse agitation at the inner face of the red-cell membrane is propagated centrally, causing disturbances of refraction, and it is possible that the inertia of the cell contents suffices to make these disturbances visible with the phase-contrast microscope, or it may be that visibility depends on a constraint imposed by molecular orientation within the cells. Alternatively, flicker may be due to variations in refraction produced by the liberation of metabolic products in incubated cells. Pulvertaft has attempted, without success, to count flicker by a stroboscopic method. According to this worker, too, flicker is not observed in cells other than erythrocytes and this must be ascribed to a less fluid type of contents or to 'damping' of the flicker

by intracellular phase boundaries. It is, however, possible that motility in certain cells may have something in common with red-cell flicker, and certainly spermatozoa seem to depend on fructolysis for activity: whether motility in bacteria is similarly based on glycolysis, perhaps in association with active transport, is more speculative.

Cell shape and flicker. With a little experience it is possible to guess from the shape of a cell alone whether it will or will not show flicker, and closer inspection will almost always confirm the surmise. It is probable that erythrocytes which have lost their biconcavity and are even slightly biconvex still flicker, but gross departure from the normal shape by crenation, ballooning or cupping, is incompatible with flicker or active transport, while on the other hand, normal looking cells without flicker have not been met. It therefore follows that retention by the red cell of its normal biconcavity may, like flicker and active transport, depend on metabolism and presumably on glycolysis. Alternatively, normal cell metabolism may itself depend on the persistence of physical forces able to maintain cell biconcavity; in this connexion it will be recalled that haemolysis results in inhibition of glycolysis and phosphorylation and in acceleration of phosphorolysis, and it may well be that mere disturbance of shape has similar effects.

With regard to the first possibility: the dependence of cell shape on metabolism might seem to be excluded by the observations that cell shape in sickle cell anaemia seems to depend on oxygen tension, while flicker and active transport are independent of respiration. But in fact sickling *in vitro* is dispersed by the admission of either oxygen or carbon-monoxide (Ponder, 1948), and it seems that sickling depends not on the absence of respiration but on the presence of uncombined haemoglobin. In this connexion it is interesting to note that in a case of sickle cell anaemia recently examined, flicker persisted in moderately deformed cells but was not seen in cells whose outlines were grossly spiked or serrated.

According to Ponder (1948) the biconcave shape of the human erythrocyte is the resultant of some of several possible physical forces: surface tension at the external cell face, surface tension at the inner cell face, inter-molecular forces in the surface 'ultra-structure', expansive force of contained non-penetrating substances and orientation of haemoglobin molecules within the cell: to these must be added possible action depending on cell metabolism. The last two factors might seem to be excluded by the observation that biconcavity may persist in ghosts (Ponder, 1948). But this is not necessarily so, for in the case of haemoglobin at least the protein exerts a negative effect: normal haemoglobin molecules are randomly disposed within the erythrocyte and do not oppose its biconcavity; the abnormal haemoglobin molecules of sickled cells, on the other hand, are thought to be partially alined 'resulting in the erythrocyte's becoming birefringent, and the cell membrane's being distorted

to accommodate the now relatively rigid structures within its confines' (Pauling, Itano, Singer & Wells, 1949). Moreover, though mere physical forces might suffice to keep a ghost biconcave, in the case of the unhaemolysed cell with its osmotically active contents, such physical agencies might not suffice unless supported by metabolic forces. The possibilities therefore remain that the biconcave shape of the normal human erythrocyte depends on physical forces or on metabolic activity or on both.

SUMMARY

1. Normal human erythrocytes examined with the phase-contrast microscope show flickering movements in the cell interior. This flicker closely correlates with active cation transport, both depending on the retention of normal cell shape and being limited by a pH range between 5 and 9·5. Both activities are constrained by agencies inhibiting glycolysis but are practically unaffected by substances which inhibit respiration.

2. Glucose and mannose energize red-cell flicker. Fructose is equally effective in high concentration, but much less effective at low concentration. Galactose, arabinose and the disaccharides do not energize flicker. These sugars bear an identical relation to active cation transport.

3. There is an association between flicker and normal cell shape, but while the former is clearly related to cell metabolism, the latter may depend on metabolic activity or on simple physical agencies or on both.

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