

THE AGEING OF RED CELLS

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Ever since the demonstration of the uniform life span of the red cell from the linear curve of disappearance of transfused normal cells in healthy recipients, it has been clear that their disappearance from the circulation is a result of an ageing process. Many people have looked for differences in behaviour and constitution which might reflect the heterogenous nature of the cells of a normal blood sample.

Differences in density between reticulocytes and mature red cells have been known for some time, and it has been claimed that by centrifugation young and old cells can be separated (Borun, Figueroa & Perry, 1957). In the present investigation blood samples were centrifuged in 30% bovine albumen, a medium in which it was found that small differences in red cell density were readily demonstrable, and the cells at different depths of the centrifuged column were analysed by a variety of chemical procedures.

METHODS

Twenty millilitres of blood was taken from donors into 10% ethylenediamine tetra-acetic acid (EDTA) and centrifuged at 2000 g for 10 min. The supernatant plasma and white cell layer were removed and the packed cells mixed with an equal volume of 30% bovine albumen in a narrow tube (diam. 1.0 cm.). When fully suspended the mixture was centrifuged at 4000 g for 10 min and then at 10,000 g for 5 min at 4° C. After this the supernatant plasma was completely removed with a fine pipette and appropriate samples of cells withdrawn from the top and bottom of the column of packed cells. These samples were finally recentrifuged under the same conditions and a top fraction from the original top sample, and a bottom fraction from the original bottom sample, used for analyses.

⁵⁹Fe labelling of cells. Healthy donors were given 7.5 μc of ⁵⁹FeCl₃ intravenously and blood taken from them 5 days after injection. At this time 90% of the radio-iron is found in circulating red cells (Joske, McAlister & Prankerd, 1956). The blood was then treated as above. After centrifuging at 10,000 g, 0.5 ml. of packed cells was removed from both top and bottom of the cell column. These were haemolysed in 3.5 ml. of water and their radioactivity assayed in a well-type scintillation counter; at least 1000 counts were recorded on each sample. To correct for any discrepancy between the packing of cells at the top and bottom of the centrifuged column the haemoglobin concentration of the final solution of haemolysed cells was estimated by the cyan-

haematin method of King & Gilchrist (1947). These haemoglobin concentrations were usually in close agreement. When there was a difference in haemoglobin concentration between the cell suspensions it was assumed that this was due to variable cell packing, and the final cell analyses were corrected to comparable cell concentrations.

Osmotic fragility. After centrifuging the cells at 10,000 *g* and removing the supernatant fluid, 0.25 ml. of cells was removed from the top and bottom of the centrifuged column and added to 0.25 ml. of bovine albumen. The suspension was well mixed and then one drop was added to a series of saline solutions ranging from 0.85 to 0.2% NaCl. These were stored at 4° C during the night, and the following day, after they had been gently mixed and then centrifuged, the degree of haemolysis at different saline concentrations was estimated in the supernatant by the cyan-haematin method.

The same procedure for estimating osmotic fragility was also carried out on the cells after they had been removed from the centrifuged column, suspended in their original plasma under sterile conditions and incubated at 37° C for 24 hr.

Phosphate esters. 0.5 ml. of packed cells was removed from the centrifuged column of cells and the phosphate esters extracted from these with 10% trichloroacetic acid and analysed by methods previously described (Prankerd & Altman, 1954). The error of duplicate estimations by these methods is $\pm 5\%$.

Sodium and potassium estimations. 0.25 ml. of cells removed from the centrifuged column was suspended in 10 ml. isotonic saline in graduated receptacles (Harris & Prankerd, 1953) and the cells re-centrifuged at 3000 *g* for 10 min. The supernatant was removed and the receptacle carefully washed with distilled water which was gently drawn over the surface of the centrifuged cells. No haemolysis occurs during this operation (Harris & Prankerd, 1953). After drying the receptacle with absorbent paper the cells were haemolysed in distilled water and their Na and K contents estimated by flame photometry using an 'EEL' flame photometer. The standard error of these estimations on five samples of blood was Na $\pm 0.75\%$, K $\pm 0.61\%$.

Lipid analyses. 1.0 ml. of packed cells was removed from the top and bottom of the centrifuged column and extracted in 25 ml. of ethanol:ether (2:1) at room temperature for 12 hr. The extract was made up to standard volume and filtered and the filtrate analysed for total fat by drying to constant weight, cholesterol by the method of Bloor (1916), phospholipids by the method of Youngberg & Youngberg (1930) and lipid nitrogen by the method of Koch & McMeekin (1924).

Glutathione. This was estimated in 0.5 ml. of packed cells removed from the top and bottom of the centrifuged column. The method used was that of Grunest & Phillips (1951).

RESULTS

Density

To bring out any differences in density between the heterogeneous group of red cells obtained at venepuncture the previously packed cells were resuspended in 30% albumen, the sp.gr. of which closely approximates to that of red cells. This was found to prove more effective than saline in establishing a density gradient from the top to the bottom of a column of packed cells.

The density differences between cells containing ^{59}Fe at varying stages after the injection of the isotope are shown in Table 1. No isotopic iron exchanges with the haemoglobin of mature circulating cells (Hahn, Bale, Ross, Hettig & Whipple, 1940), and after injection it starts to appear in newly formed mature cells after 2-3 days (Joske *et al.* 1956). Labelled cells appear to be lighter than unlabelled cells and the radioactivity is concentrated in the upper

part of the centrifuged column. As 95% of the injected radioactivity is in newly formed red cells at the end of 5 days, any change in density of these cells as they age will be apparent from the radioactivity descending the column of packed cells. This was found to occur.

TABLE 1. Distribution of radioactivity in top and bottom layers of column of centrifuged red cells

Sample	5 days after injection of ^{59}Fe		90-105 days after injection of ^{59}Fe	
	Counts/min/ml. cells	% total counts in column of cells	Counts/min/ml. cells (corrected for decay)	% total counts in column of cells
1 Top	914	56	104	6
1 Bottom	179	11	663	41
2 Top	745	57	251	19
2 Bottom	102	8	725	55
3 Top	1150	64	345	19
3 Bottom	96	6	986	55
4 Top	954	50	188	10
4 Bottom	121	7	541	28
5 Top	641	47	91	7
5 Bottom	182	3	499	36

Osmotic fragilities

Only slight differences in osmotic fragilities were found on testing cell suspensions from the top and bottom of the centrifuged cell column (Table 2a), but differences could be brought out by incubating the cells resuspended in their own plasma under sterile conditions. During this procedure cells gain total base, mainly sodium, with some loss of potassium, and as a result undergo a degree of swelling and therefore become haemolysed more readily in higher concentrations of saline (Selwyn & Dacie, 1954). It appears that the swelling that occurs is different in degree between old and young cells under these conditions (Table 2b), the younger cells swelling less and showing less increase in osmotic fragility. Thus they would appear to be more resistant to any electrolyte changes brought about by incubation.

Phosphate esters

It was expected that the resistance to the cation changes found during incubation might be reflected in the cells by differing metabolic activities which might be detected by analyses of phosphate esters within the different layers of cells. However, Table 3 shows that no such difference could be found.

Sodium and potassium content

In looking for a cause of the density differences between young and old cells it was decided to estimate their cation content. Differences in this respect of different layers of centrifuged cells were found. Thus there was a greater

content of Na in the lower and presumably older cells (Table 4), whilst in younger cells the K content was greater. These changes are small, however, and not likely to contribute much towards the density differences.

Lipid analyses

A more likely cause for the differences in densities was detected in the lipid contents of cells at the top and bottom of the centrifuged column. Cells at the

TABLE 2a. Osmotic fragility of top and bottom layers of unincubated blood

Saline concentration (%) ...	0.6	0.55	0.5	0.45	0.4	0.35	0.3
Sample							
1 Top	—	—	—	35	85	88	100
Bottom	—	—	10	59	85	93	100
2 Top	—	—	—	32	82	90	99
Bottom	—	—	—	55	85	88	100
3 Top	—	—	—	30	80	95	100
Bottom	—	—	5	61	90	100	100
4 Top	—	—	—	44	90	95	100
Bottom	—	—	12	48	90	92	100
5 Top	—	—	—	38	78	86	100
Bottom	—	—	8	55	86	94	100

Figures represent percentage of cells haemolysed.

TABLE 2b. Osmotic fragility of top and bottom layers of blood after 24 hr incubation

Saline concentration (%) ...	0.7	0.65	0.6	0.55	0.5	0.45	0.4	0.35	0.3
Sample									
1 Top	—	—	12	30	54	93	96	96	100
Bottom	—	12	35	45	52	91	97	100	100
2 Top	—	5	12	45	62	90	90	100	100
Bottom	—	20	40	54	68	100	100	100	100
3 Top	—	—	7	34	56	98	96	98	100
Bottom	—	28	28	48	66	90	96	100	100
4 Top	—	—	20	45	60	88	100	100	100
Bottom	—	10	45	53	65	90	96	100	100
5 Top	—	1	8	24	50	88	95	100	100
Bottom	—	15	35	40	62	96	95	100	100

Figures represent percentage of cells haemolysed.

TABLE 3. Phosphate ester contents of red cells from top and bottom layers of centrifuged column of red cells

Sample	DPG	ATP	ADP	IP
1 Top	155	47	17	31
Bottom	130	45	12	27
2 Top	170	39	10	26
Bottom	161	43	15	24
3 Top	120	58	20	25
Bottom	119	54	18	17
4 Top	142	45	17	27
Bottom	132	51	13	33
5 Top	150	50	12	28
Bottom	166	46	17	29

Values given as $\mu\text{g P/ml}$. packed cells.

bottom of the column consistently had rather less lipid per unit packed cell than those at the top and the deficit appeared largely to be in the phospholipid fraction (Table 5).

TABLE 4. Sodium and potassium content of red cells from top and bottom layers of centrifuged column

Sample	Na	K
1 Top	15.3	122.4
Bottom	20.8	107.8
2 Top	19.6	112.1
Bottom	23.0	96.4
3 Top	16.4	115.7
Bottom	19.5	108.3
4 Top	12.0	112.8
Bottom	17.8	100.2
5 Top	12.6	119.7
Bottom	18.4	109.0

Values given as m-equiv cation/l. cells.

TABLE 5. Lipid content of red cells from top and bottom layers of centrifuged column

Sample	Total lipid	Cholesterol	Lipid P	Lipid N
1 Top	11.5	1.39	0.110	0.088
Bottom	9.0	1.34	0.95	0.083
2 Top	11.0	1.39	0.132	0.134
Bottom	10.5	1.22	0.96	0.087
3 Top	12.5	1.33	0.110	0.150
Bottom	10.0	1.24	0.87	0.105
4 Top	11.5	1.30	0.117	0.096
Bottom	10.5	1.31	0.89	0.112
5 Top	12.5	1.37	0.121	0.091
Bottom	12.0	1.26	0.104	0.103
Mean normal values \pm s.e.	12.0 \pm 0.5	1.28 \pm 0.06	0.105 \pm 0.003	0.12 \pm 0.02

Values given as mg/ml. cells.

TABLE 6. Glutathione concentration of top and bottom layers of red cells from centrifuged column

Sample ...	(1)	(2)	(3)
Top	73	Top 55	Top 64
Bottom	86	Bottom 57	Bottom 60

Values as mg/100 ml. red cells.

Glutathione

Glutathione was estimated in the different layers of centrifuged cells because there is evidence that it is an important factor in certain haemolytic anaemias (Beutler, Dern, Flanagan & Alving, 1955) and Fegler has claimed that it is an important factor in red cell stability. The results, showing no difference between top and bottom layers of cells, are given in Table 6.

DISCUSSION

The distribution of ^{59}Fe in the column of centrifuged red cells clearly shows that the method is a satisfactory means of separating old from young cells, and that the top 0.5 ml. of cells consists mainly of cells not more than 5 days old. As these cells age they become more dense and ultimately sink to the bottom of the centrifuged column. The cause of the differences in density of the two groups of cells would appear to lie in their different lipid content.

Younger cells have appreciably more lipid, which makes them lighter than older cells which have lost lipid. The lipid lost appears to be largely phospholipid. Chromatography of the phospholipids of old and young cells does not, however, show the loss of any one specific phospholipid, but a general decrease in all fractions (Prankerd, unpublished).

In considering how these changes are brought about, a factor common to both could be a decrease in energy potential within the cell. It is well known that the uptake of potassium and extrusion of sodium are metabolically controlled processes and if metabolism is stopped the concentration of red cell cations alters in a manner similar to, but to a greater extent than, those observed here. Furthermore, there is evidence from experiments with labelled acetate that lipid synthesis can occur in the mature red cell and that the acetate is largely incorporated in the phospholipid fraction (Altman, 1953). Thus both the observed changes could be a result of falling energy potential within the cell. However, when one looks for evidence of this by examining intracellular phosphate esters there does not appear to be any observable change in nucleotide phosphate concentration, or such changes as a fall in 2:3-diphosphoglycerate and increase in organic phosphate which are commonly found in metabolically ailing cells (Prankerd & Altman, 1954). This may appear surprising, as Gabrio & Finch (1954) have reported differences in hydrolysable phosphate partition in cell populations with a young age distribution, produced by bleeding.

Finally, some further indirect light is cast on the changes in young cells by the results of the osmotic fragility tests. Immediately after separation the osmotic fragility patterns of the two groups of cells are very similar, but if the cells are incubated in their original plasma for 24 hr changes occur which increase to a greater extent the osmotic fragility of the older cells (Selwyn & Dacie, 1954); if it is accepted that the osmotic fragility test measures the diameter:thickness ratio of the cell, then clearly there has been no swelling as the cells aged *in vivo*. The changes occurring in red cells, on incubation *in vitro* for 24 hr, are partly known. Once all available glucose has been used the cell utilizes its own energy stores (Prankerd & Altman, 1954); as these fall the ability of the cell to maintain its normal cation concentration gradients fails and it swells. At the end of 24 hr there has been about 30% swelling (Selwyn

& Dacie, 1954). The osmotic fragility test after incubation may therefore be regarded as one of metabolic endurance. It would appear that the aged cell has less endurance than the younger in this respect.

The principal cause of the removal of aged red cells from the circulation remains a mystery, but it is possible that the loss of lipid associated with age is an important factor, as cells artificially denuded of lipid survive but a short time after transfusion (Harris, McAlister & Pranker, 1957).

SUMMARY

1. Red cells of differing ages have been separated by centrifuging in 30% albumen solution. The separation has been confirmed by labelling with ^{59}Fe .
2. Young cells are less dense than old cells, owing to an increase in lipid content, and are found mainly at the top of a centrifuged column of cells.
3. Young cells contain more potassium and less sodium than old cells, but there is no difference in phosphate ester or glutathione content.
4. Osmotic fragility of young and old cells is similar, but after incubation old cells are more fragile.

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