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BIOCHEMICAL CHARACTERIZATION OF DENSITY-SEPARATED HUMAN ERYTHROCYTES

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SUMMARY

A simple, reproducible method for the separation of human erythrocytes, described recently (Murphy, J R (1973) J Lab Clin Med 82, 334–341) has been utilized for the purpose of obtaining a wide range of biochemical data on these cells Using phthalate ester density centrifugation of the fractions obtained by Murphy's method, we established that the cells were separated exclusively on the basis of their densities Data on a wide range of biochemical and hematological parameters, when compared with previously reported density separation procedures showed that this simple technique can be used to fractionate the cells according to their densities (age) in their own plasma

Cells of increasing density consistently and reproducibly exhibited an increase in hemoglobin concentration, a moderate elevation in Na^+ and a decrease in the following K^+, acetylcholinesterase, sialic acid, membrane protein, 2,3-diphosphoglycerate, ATP, cholesterol, phospholipid, mean corpuscular volume and critical hemolytic volume, However, no change in mean corpuscular hemoglobin was evident. The observed differences were not artifacts of the centrifugation process This was determined in recentrifuged top fractions from which new top and bottom cells were obtained The latter cells resembled the top fraction from which they were obtained, rather than the original bottom fraction

Whereas the parameters mentioned above exhibited consistency and reproducibility, such was not the case with the ATPase values Depending on the cell density group examined and/or buffer as well as other conditions, significant variability in the activity levels of the ouabain sensitive, as well as the Ca^{2+}-stimulated ATPase, was observed Use of these enzyme activities as indicators of cell age must be viewed with caution

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INTRODUCTION

The importance of the mammalian erythrocyte in the study of (plasma) membrane structure has been well documented [1, 2] Yet concern has been expressed that the sample of erythrocytes normally collected for membrane preparation may not present a completely valid picture because of the considerable span in the age of these cells [2] Consequently it was of import to investigate routes to the collection (or separation) of cells of a more precisely defined age group for detailed biochemical and physiological studies

Many methods for the separation of erythrocytes according to age-dependent density changes have been devised [3, 8] Separation by centrifugation alone [3, 4] has not provided the resolution achieved by the use of artificial gradients such as bovine serum albumin [5–7], but use of these media introduces questions concerning the biochemical data derived from the separated cells Very precise separation can be achieved by the use of phthalate esters [8], but this method again provides only two fractions per centrifugation, and presents the difficulties inherent to any foreign medium All of the techniques involving artificial media involve rather long preparative procedures, and many are limited in the volumes which can be handled Reasonably good separations were achieved by O'Connell et al [9] by means of centrifugation of erythrocytes in plasma combined with a precisely designed and controlled apparatus for removing the fractions

Recently, however, Murphy [10] demonstrated that successful separation of erythrocytes could be achieved by the simple method of centrifugation in plasma at 30 °C (to decrease blood viscosity) in an angle rotor (to facilitate circulation of the tube contents) at 39 000 $\times g$ The attractiveness of the method led us to examine in detail whether or not separation of erythrocytes was achieved on the basis of their densities and to subject these separated cells to biochemical and hematological evaluations The results of these studies are reported in detail and show that this method of fractionation is capable of separating human erythrocytes in their own plasma according to their density (and presumably age)

METHODS

Blood was obtained from healthy adults utilizing either heparin or citrate/phosphate/dextrose as an anticoagulant The method for separating the cells was essentially the same as that described by Murphy [10] The blood was centrifuged at 3500 rev /min for 20 min at 4 °C in a Sorvall Model RC2B with an SS-34 rotor The plasma was removed, the buffy coat was aspirated with as few of the upper red cells as possible, and the cells were resuspended in the plasma This was followed by a second centrifugation and buffy coat removal The cells were then placed at high hematocrit (90 %) in polypropylene tubes, which measured either 13 × 98 mm, or 38 mm × 103 mm (internal diameter) Separation was accomplished by centrifuging the tubes for 1 h at 39 000 $\times g$ at 30 °C in the SS-34 rotor The remaining plasma was removed and discarded, and the desired fractions were removed carefully from the top of the tube with a 1 ml disposable syringe as described by Murphy [10] Usually the fractions were taken as the top 10 %, bottom 10 % and the remainder divided into four equal fractions These cells were resuspended in the original plasma

prior to any further study Control samples were handled in a manner identical to that used for the separated fractions, expect that another aliquot of the total blood sample was centrifuged for 1 h under identical conditions, resuspended in plasma and considered to be a control On occasion, uncentrifuged blood was used as an additional control with no evident major differences

Subsequent to centrifugation as described above, five equal fractions from top to bottom were resuspended in plasma to a hematocrit of 20–30 % and each one was further fractionated on 12 mixtures of dibutyl phthalate and dimethyl phthalate with densities ranging from 1 0946 to 1 1128 [11] After centrifugation in micro-hematocrit capillary tubes at room temperature in an Adams Autocrit centrifuge for 15 min, the percentage of top cells in each phthalate mixture was plotted against its density, and the density at which 50 % of total cells were distributed below and above the phthalate mixture was considered as an average density of that fraction

Hemoglobin was determined by the cyanmethemoglobin method described by Kachmar [12] Cell counts were performed on a Coulter Model ZBI (Coulter Electronics, Inc , Hialeah, Fla) Hematocrits were measured in tubes spun for 5 min in an Adams Micro-Hematocrit centrifuge (Clay Adams, Inc , New York) Cell Na^+, K and Mg^{2+} were assayed by atomic absorption spectroscopy on a Beckman Model 403 unit after washing 0 2 ml of cells twice with 5 ml of 0 172 M Tris buffer, pH 7 6, 0 15 M NaCl, or 0 12 M $MgCl_2$ Cell membranes were prepared according to the method of Dodge et al [13], except that a Tris HCl buffer, pH 7 6, was used Sialic acid determinations on these membranes were accomplished by the method described by Warren [14], after acid hydrolysis 0 05 M H_2SO_4, 80 °C, 1 h) Membrane protein was measured by the method of Lowry et al [15] Cell lipids were isolated as described by Hanahan and Ekholm [16] and cholesterol was assayed by the technique of Rudel and Morris [17] King's microphosphorus assay [18] was used for determination of phospholipid phosphorus

ATP and 2,3-diphosphoglycerate were measured on samples prepared as follows (Kim, H D , personal communication) 1 ml of cell suspension (hematocrit 15–50 %) was mixed with 4 ml of 0 56 M $HClO_4$ After centrifugation, 4 ml of the supernatant were titrated to neutral pH with 5 62 M K_2CO_3 This mixture was centrifuged again and the supernatant used immediately, if necessary, it was frozen and stored at −20 °C ATP was assayed by measuring $NADP^+$ reduction in the hexokinase reaction as reported by Williamson and Corkey [19] and 2,3-diphosphoglycerate by the method outlined by Keitt [20] On occasion, the procedure described by Rose and Liebowitz [21] for 2,3-diphosphoglycerate was employed with comparable success Depending on the sample size, either fluorometry or spectrophotometry was used for assay purposes.

Total and ouabain-sensitive ATPase was determined essentially by the technique of Brewer et al [22] In the determination of Ca^{2+}-stimulated ATPase activity, washed cells were suspended to 15 % hematocrit, freeze-thawed three successive times in a solid CO_2-acetone bath Immediately thereafter 0 2 ml of the cell hemolysate was transferred to a tube containing 1 5 ml of Ca^{2+}-ATPase assay mixture A or B in an ice bath At this point the concentration of the reagents in the A mixture was as follows histidine/imidazole (1 1, molar ratio), pH 7 6, 88 mM, NaCl, 90 mM, KCl, 36 mM, EDTA, 0 55 mM, $CaCl_2$ 0 55 mM, $MgCl_2$, 2 0 mM, ATP, 2 1 mM Assay mixture B was the same, except that Tris HCl, pH 7 6, 88 mM, was substi-

tuted for the histidine/imidazole buffer Then, as the sample was added, each tube was placed at 44 °C for 2 h The reaction was stopped by the addition of 1 5 ml cold 10 % trichloroacetic acid The P_i release was measured and activity expressed on a g hemoglobin basis

Acetylcholinesterase was assayed by the dithiobisnitrobenzoate method as outlined by Chow and Islam [23], except that the enzyme unit value is 10^3 higher than that of Chow and Islam [23]

Glycerol lysis time was measured by the technique of Gottfried and Robertson [24] The behavior of cells on incubation in isotonic Tris HCl buffer, pH 7 6, was examined by the procedure described by Luthra et al [25]

RESULTS

A General

At least 24 different separation experiments have been performed in 16 mm × 98 mm tubes (10 ml of cells per tube), and a minimum of 2 experiments in 38 mm × 103 mm tubes (35 ml of cells per tube) In all of these procedures a similar curve was obtained for the distribution of hemoglobin/ml packed cells among the different fractions Results of four typical experiments with 16 mm × 98 mm tubes are shown

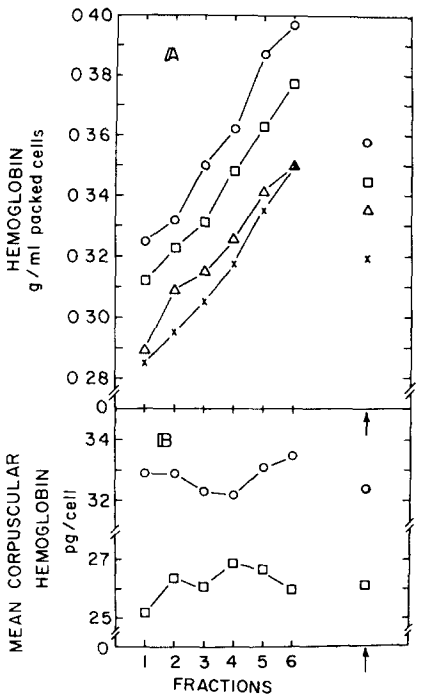

Fig 1 (A) Hemoglobin concentration in separated erythrocyte fractions The results shown are from four different experiments (○, △, ◇, □) using 13 98 mm separation tubes (B) Mean corpuscular hemoglobin of separated cells The results shown are from two typical experiments The arrow (↑) refers to values obtained on unseparated cells, whereas data collected on the separated cell samples are identified by the following numbers 1, top 10 %, 2–5, succeeding 20 % layers, 6, bottom 10 %

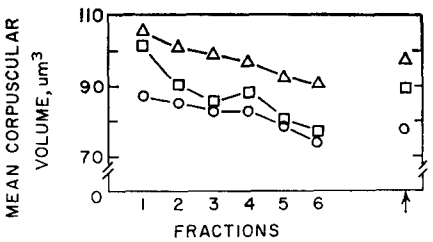

Fig 2 Mean corpuscular volume of separated cells The results shown are from three different experiments using 13 × 98 mm separation tubes Identification of samples is same as in Fig 1

in Fig 1 In these studies the ratio of bottom top values ranged from 1 17 to 1 23 with a mean of 1 21 In the case of the larger tubes (35 ml) the ratios from 2 experiments were 1 15 and 1 19 Better results were obtained with heparinized samples than with blood anticoagulated with citrate/phosphate/dextrose because the buffy coat was more easily removed in heparinized blood In citrate/phosphate/dextrose blood more of the uppermost red cells were sacrificed in the preliminary centrifugations in an effort to remove the leukocytes

Although hemoglobin/ml packed cell increased with increasing cell density (see Section C below), calculation of the mean corpuscular hemoglobin and mean corpuscular volume indicated that mean corpuscular hemoglobin was similar in all fractions while mean corpuscular volume decreased with increasing cell density (Figs 1 and 2, respectively) For this reason, calculations for all subsequent data were based on hemoglobin rather than on volume of packed cells

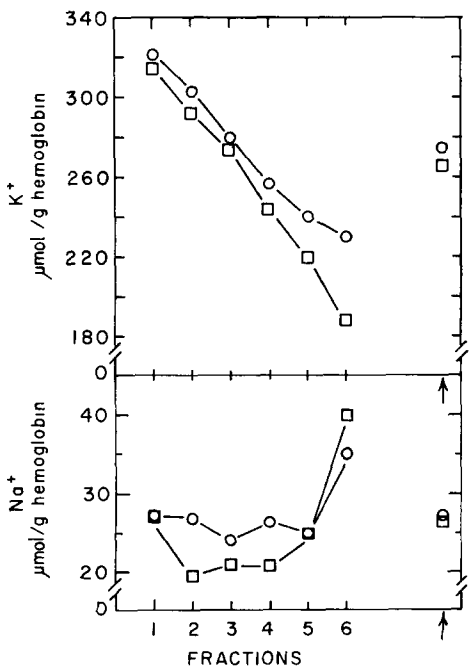

Fig 3 The K^+ and Na^+ content of separated cells The results shown here are from two different experiments See Fig 1 for identification of fractions

B Electrolytes

Cellular K^+ measurements consistently showed that the content of this cation varied inversely with cell density but that the Na^+ content of cells in the different fractions was rather uniform except for the bottom fraction (Fig 3) Cells in this fraction were high in Na^+ In another series of subjects (male or female) the same general pattern held, i e a significant change in the level of monovalent cations in different density cells For example, in an unseparated sample, the cell K^+ was 282 mol/g hemoglobin, whereas in the separated cells the values were distributed as follows top 10 %, 310, middle 80 %, 276, bottom 10 %, 252

C Density measurement of various fractions

In a typical experiment, five equal fractions were recovered from top to bottom of the tube after centrifugation by the Murphy technique [10] Each fraction was further separated on various phthalate mixtures Results presented in Figs 4A and 5 show that the average density of top cells was lower than bottom cells and the

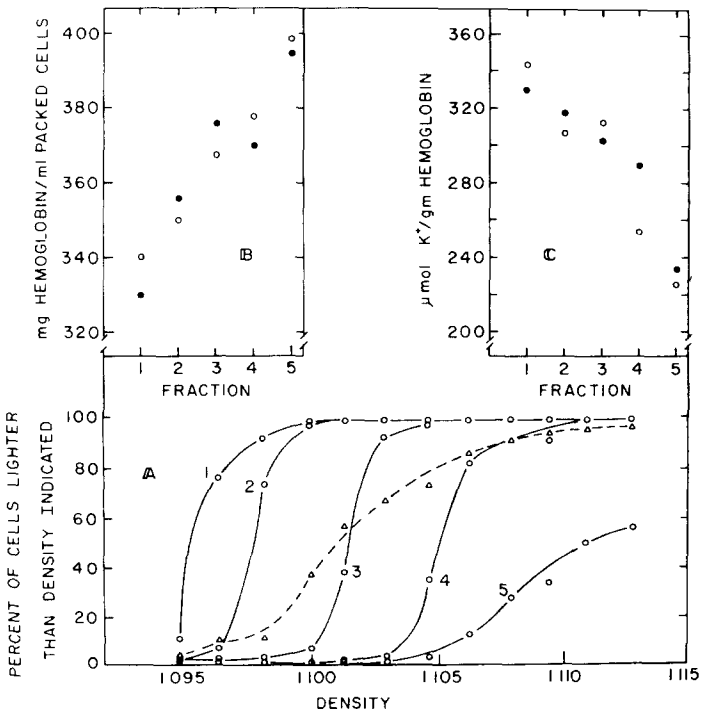

Fig 4 Density profile of separated human erythrocytes The cells were separated by centrifugation as described in Methods Five equal fractions (1–5) from top to bottom were collected and resuspended in plasma An aliquot from each fraction and total unseparated cells (△– △) was further layered on various mixtures of dibutyl phthalate and dimethyl phthalate and centrifuged (see Methods) Percentage of cells on top of the phthalate layer was plotted against its indicated density (A) The other aliquot of each fraction was collected under mixture of phthalate at density of 1 0946 The tube was broken and cells were hemolyzed with water for K^+ (B) and hemoglobin (C) measurement The levels of K^+ and hemoglobin were compared both before (○) and after (●) phthalate centrifugation

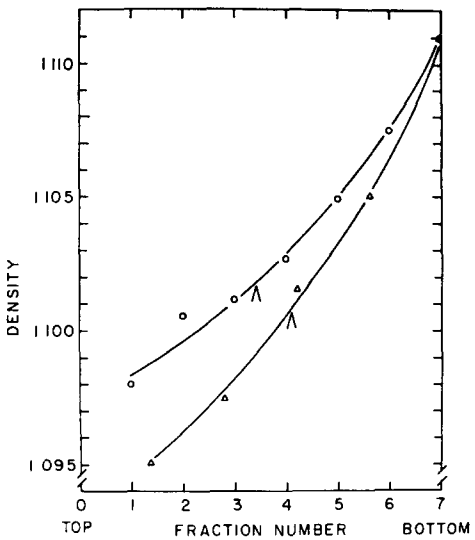

Fig 5 Relationship of density of cells to various fractions Average densities (see text) of various fractions of cells from top to bottom were taken from Fig 4 and plotted against the number of the fraction Arrow (∧) indicates the average density of unseparated cells

density of the cells increased almost linearly when plotted against length of the tube (Fig 5) The linear relation between density and length of tube was consistent in each individual but the slope was found to vary in different individuals as depicted in Fig 5 It is noted that phthalate itself did not change the density profile since the hemoglobin/ml packed cells (Fig 4B) and μmol K^+/g hemoglobin (Fig 4C) showed the same relation before and after phthalate centrifugation

D Enzyme activities

1 *Total ATPase* A reasonably consistent pattern of decreasing levels of total ATPase activity from the top (low-density) to bottom (high-density) cells was obser-

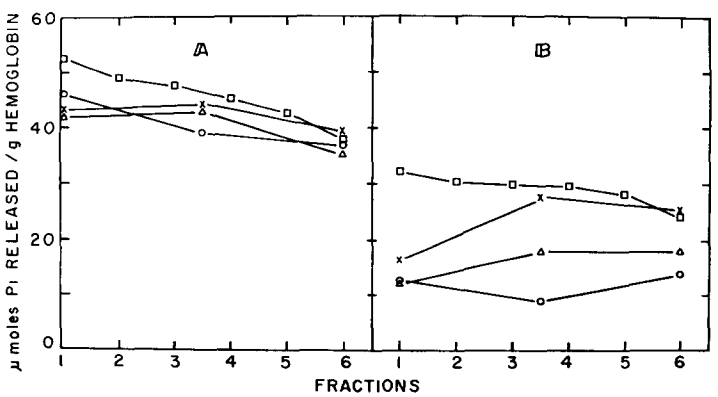

Fig 6 $(Na^+ + K^+ + Mg^{2+})$-ATPase values of separated cells A, total, B, ouabain-sensitive Identification of samples is the same as in Fig 1

TABLE I

Ca^{2+}-STIMULATED ATPase LEVELS OF SEPARATED ERYTHROCYTES WASHED AND ASSAYED IN A VARIETY OF MEDIA

In each experiment, 0 5 ml aliquots of a 50 % plasma cell suspension of unseparated or control cells (C), as well as the separated cells (T, top 10 %, M, middle 80 %, B, bottom 10 %), were washed twice with 5 ml aliquots of the indicated media The cells were frozen and thawed and assayed for ATPase activity in either histidine/imidazole or Tris buffer system Other details are provided in the text

Washing media	ATPase activity (μmol Pi released/g hemoglobin per 2 h at 44 C)							
	Tris buffer				Histidine/imidazole buffer			
	T	M	B	C	T	M	B	C
0 155 M NaCl	61	139	134	137	56	155	157	152
0 172 M Tris HCl, pH 7 6	56	138	124	127	54	160	154	147
0 31 M histidine, pH 7 6	85	144	130	132	112	180	162	164
0 31 M sucrose	114	152	98	113	128	174	116	125

ved in all samples separated by this technique (Fig 6A) However, these observations must be tempered by the fact that on a comparative basis considerable variability was noted in the activity values of the top cells

2 *Ouabain-sensitive ATPase* This ATPase activity, which is considered representative of the Na^+/K^+-stimulated component in the human erythrocyte, showed widely varying levels without a particularly distinctive pattern (Figs 6B)

3 *Ca^{2+}-stimulated ATPase* Cells (separated as well as unseparated) were washed twice with an isomolar solution of NaCl (unbuffered), Tris HCl, pH 7 6, histidine, pH 7 6, and sucrose and assayed either in the histidine/imidazole buffer (see Methods) or in a similar mixture with 0 172 M Tris HCl, pH 7 6, in place of

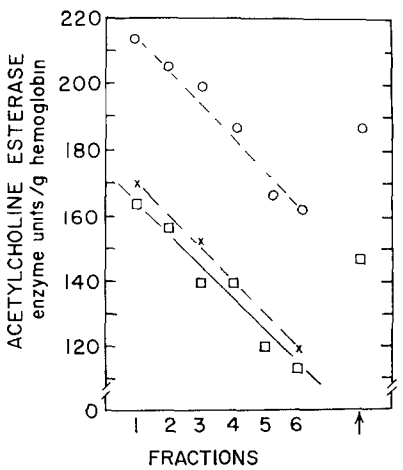

Fig 7 Acetylcholinesterase activities of separated cells The results shown are from three different experiments See Fig 1 for identification of fractions

the histidine/imidazole mixture As shown in Table I, the Ca^{2+}-ATPase showed in histidine/imidazole/buffer a variable pattern, in which the activity level in the top cells was significantly lower in all instances than that of the middle cells The middle cells exhibited comparable or higher levels of those obtained for the bottom cells. A similar trend was noted for the Ca^{2+}-ATPase level in the Tris assay buffer, but with a consistently lower value for all cells in this medium (Table I)

4 *Acetylcholinesterase* As shown in Fig 7, the level of this enzymatic activity bore a consistent and reproducible inverse relationship to the cell density

E Other components

The sialic acid content showed a small but reproducible decrease from top to bottom cells (Fig 8) In a comparable fashion, unseparated cells, which yielded 19 5 mg membrane protein/g hemoglobin, could be fractionated into top cells containing 20 9 mg membrane protein/g hemoglobin and bottom cells with values close to 17 8 membrane protein/g hemoglobin. Cholesterol and phospholipid phosphorus both decreased similarly with increasing cell density with the result that the

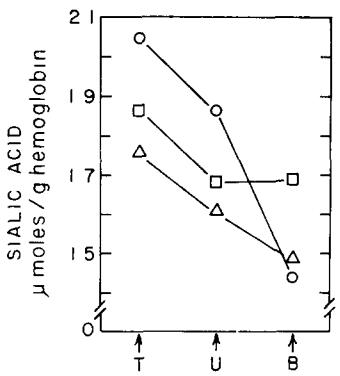

Fig 8 Sialic acid content of top 10 % (T), bottom 10 % (B) and unseparated (U) cells The results shown are from three different experiments

TABLE II

CHOLESTEROL AND PHOSPHOLIPID PHOSPHORUS LEVELS IN SEPARATED ERYTHROCYTES

Results expressed as μmol/g hemoglobin

Fraction	Cholesterol (A)	Phospholipid phosphorus (B)	Molar ratio A/B
1 (top)	10 3	14 9	0 70
2	9 2	13 6	0 67
3	9 4	12 2	0 78
4	8 8	12 8	0 69
5	8 8	12 2	0 72
6 (bottom)	8 0	11 8	0 69
Unseparated cells	9 0	12 2	0 74

TABLE III

ATP AND 2,3-DIPHOSPHOGLYCERATE LEVELS IN SEPARATED HUMAN ERYTHROCYTES

Fractions	Hemoglobin (g/ml packed cells)	ATP		2,3-Diphosphoglycerate	
		(μmol/ packed cells)	(μmol/g hemoglobin)	(μmol/ml packed cells)	(μmol/g hemoglobin)
Subject R M					
Top 10 %	0 295	1 54	5 22	5 62	19 1
Middle 80 %	0 339	1 44	4 25	5 19	15 3
Bottom 10 %	0 365	1 29	3 53	4 10	11 2
Subject P S					
Top 10 %	0 324	1 21	3 74	5 86	18 1
Middle 80 %	0 341	1 10	3 20	4 30	12 6
Bottom 10 %	0 400	1 01	2 52	4 65	11 6

cholesterol phospholipid phosphorus ratios of the different fractions were virtually all the same (Table II) A detailed examination of the distribution of individual phospholipids in each of the fractions in Table II by Dr Masami Gamo (personal communication) revealed no significant differences The pattern of phospholipids present was the same as reported previously [26]

The ATP and 2 3-diphosphoglycerate content of cells from the top fraction were higher than those of cells from the bottom fraction (Table III)

F Lysis behavior

The time required for the lysis of 50 % of a sample of erythrocytes in a standard glycerol solution has been shown to vary from one individual to another

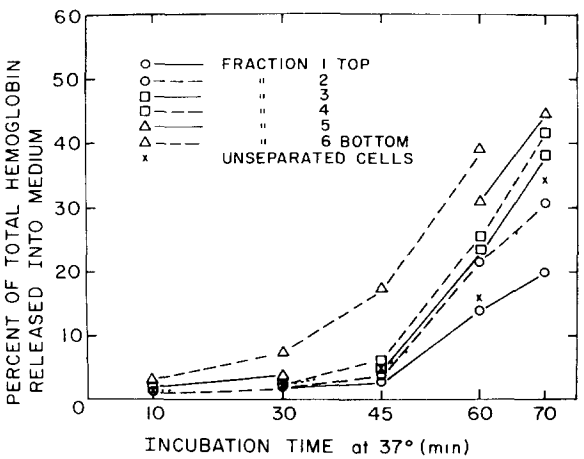

Fig 9 Hemolysis of separated cells in 0 172 M Tris HCl, pH 7 6, at 37 C Initial hematocrits ranged from 12 9 to 15 1 % At the times indicated, aliquots were removed and centrifuged The supernatant was removed and assayed for hemoglobin The results shown are from a typical experiment

and in certain hematological disorders [24] The factors determining glycerol lysis time are not fully understood at present, but it was conceivable that some differences might be found in cells of differing densities The glycerol lysis of top, bottom, and control cells from a given sample of blood, however, was always identical as measured by Gottfried and Robertson technique [24]

Luthra et al [25] have described the swelling and subsequent hemolysis of normal erthrocytes incubated at 37 °C for 1 h in isotonic (0 172 M) Tris buffer, pH 7 6 Similar incubation of separated erythrocytes revealed that the cells in the different fractions swelled at approximately the same rate, but that hemolysis occurred first in the high density or bottom cells and last in the low density or top cells (Fig 9), suggesting that the denser cells had a lower hemolytic volume under these conditions

G Characteristics of separated cells on recentrifugation

In order to determine if the cells in the bottom fraction of a separation tube were intrinsically different from cells in the top fraction or if they had been altered as a result of being at the bottom of the tube, recentrifugation experiments were performed Cells were separated into the top and bottom 10 % and the top and bottom 20 %, and aliquots of these fractions were assayed for hematocrit, mean corpuscular hemoglobin, mean corpuscular volume, K^+ and acetylcholinesterase The top 20 % fraction was recentrifuged for another hour at 39 000 $\times g$, 30 °C, in the SS-34 rotor, and the top and bottom 10 % fractions were again taken, i e the "top of the top" and the "bottom of the top" These were used for the same assays In general, the top of the top and the bottom of the top cells displayed values similar to the original top 10 % and top 20 % cells (comparable to Figs 1, 2, 3 and 7) Thus, recentrifugation of the top fraction did not result in typical top and bottom cells, the differences between the original top and bottom cells were not artifacts of the centrifugation process

DISCUSSION

The results described here confirm Murphy's [10] observations that his method consistently provided reliable cell separations, as judged by increased hemoglobin concentration per unit volume of the bottom fraction compared to the top fraction Murphy found that the ratio of hemoglobin concentration in the bottom 5 % to that in the top 5 % was about 1 24 In the present study the use of 10 % fractions provided a mean ratio of 1 21 and it was also established that separation was purely dependent on cell density, and was not an artifact of the centrifugation procedure The separation method is relatively rapid and simple, and involves minimal handling of the blood and no exposure of the cells to foreign media Moreover, it is possible to obtain large quantities of each fraction, thus providing sufficient material for a variety of biochemical and physiological studies These advantages make possible the accumulation of data on a wide range of biochemical parameters in a single separation experiment In most studies of erythrocyte separations, sample sizes have been restricted by the physical limitations of the separation method

Several reports have demonstrated the relationship of increasing cell density to increasing cell age [3, 4, 6, 7] The rise in hemoglobin concentration with increasing cell density, and the concomitant decrease in mean corpuscular volume de-

scribed here are similar to those found by Murphy [10] A linear decrease in mean corpuscular volume was also reported by Rahman et al [7] utilizing Ficoll gradients, and by Piomelli et al [6], on bovine serum albumin gradients

Enzymatic activity has been used as an indicator of cell age, with lowered levels commonly associated with older cells Sass et al [27] assayed different levels of a column of centrifuged human erythrocytes for various enzyme activities and concluded that glutamic-oxaloacetic transaminase provided the most sensitive reflection of age, with significantly decreased activities in the denser (older) cells A decrease in erythrocyte acetylcholinesterase activity with cell age has been reported by several investigators (reviewed by Herz and Kaplan [28]), and this was confirmed by the results described here It is not evident from the data available whether this loss represents an inactivation of the enzyme with age or a loss of membrane sites In the current study, a loss of total membrane protein was also noted Rahman et al [7] have reported a slight loss of protein with increasing age of erythrocytes These workers were measuring whole cell protein however, rather than membrane protein

Increasing cell density correlated well with the observed decrease in cell K^+ content from top to bottom fractions as well as the increased Na^+ content of the cells in the bottom fraction Similar results were obtained by M Luthra (unpublished observations) on cells separated on a bovine serum albumin density gradient Recently Astrup [29] separated human erythrocytes into the different density fractions by centrifugation, presumably at 4 °C, and found the K^+ level to be higher in top cells and lower in bottom cells together with a definitive change in the cell water content On the other hand, Astrup concluded that the Na^+ content was not influenced by cell density but as a consequence of water loss, Na^+ concentration increased Of the cations examined here, Mg^{2+} values were lower in bottom cells as compared to top cells, and this was in agreement with LaCelle et al [30]

The ATPase activities of the separated cells displayed a complex pattern No consistent sequential change in levels emerged, as might have been expected from the other parameters under examination Not only was there variability, as expected from individual to individual, as to levels of activity, but the trends in the total, ouabain-sensitive and Ca^{2+}-stimulated ATPase values varied inexplicably as to density of the cells as well as to the type of buffer employed in the wash solution and in the assay media

Low ATP values have been reported in old cells by LaCelle et al [30] and by Brok et al [31] Differences in ATP content of young and old cells found by the latter workers were much greater than those observed in the present study since they reported values in young cells to be three times those found in old cells The levels of 2,3-diphosphoglycerate decrease with increasing cell density The differences found between high-density (bottom) cells and unseparated cells are similar to those reported by LaCelle et al [30]

A loss of phospholipid with cell age has been described by Westerman et al [32] and these workers found no change in phospholipid content per unit surface area Similarly, the present data indicate a decrease in both cholesterol and phospholipid, as well as membrane protein, and no alteration in the cholesterol phospholipid phosphorus ratio A small decrease in total lipid content of erythrocytes with age has been described by Winterbourn and Batt [33], but these authors found no age-

related alterations in the relative amounts of the major membrane lipids Another loss with cell age found in the present study concerned sialic acid residues which displayed a consistent decrease with increasing cell density

The present results strongly suggest that, during the aging (as indicated by increased cell density) of the human erythrocyte, portions of the membrane components are lost Whether these losses are uniform or whether a discrete portion of the membrane is removed is not clear Inasmuch as there was no evident change in distribution of phospholipids in the young as compared to the old cells it seems most likely that the loss was a general phenomenon (probably removal as a lipoprotein) The implication from these observations, together with the fact that there is a decrease in many other parameters of the membrane, is that with the reduced surface area in the older cells there could be a reordering or restructuring of membrane components within the cell Obviously this is an important area for further investigation

The observations presented here provide a wide range of data on several erythrocyte parameters The method utilized provides a simple, reproducible way to obtain sufficient quantities of the separated cells to perform a wide variety of assays on the same sample The data obtained after reseparation of the top fraction indicate that the differences observed between top and bottom cells are inherent to the cells and are not the result of an artifact of the separation method Certainly the important features of the Murphy technique [10] center on the temperature of centrifugation, i e 30 °C and the configuration of the centrifuge tubes As noted in our results, one can achieve a linear density separation by this procedure In a similar sense, the data collected by O'Brien et al [34] on the influence of the angle of the centrifuge tube or vessel, but not the cross sectional area, on the erythrocyte sedimentation rate illustrates further the importance of physical configuration on behavior of these cells Though a temperature variation of 1–2 °C did not have apparent influence on the sedimentation rates, it is interesting to note that O'Brien et al [34] conducted all their experiments at 22 °C

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