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A Simple Procedure for the Separation of Viable Blood Cells, Suitable for Long-Term *In Vitro* Experiments

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There are numerous reports in the literature on the separation from blood of leukocytes (e.g., 1–8) and of platelets (e.g., 9, 10). Existing procedures for the separation of the various cell types from the same sample of blood are rather elaborate and require relatively large amounts of blood (e.g., 11–13). In course of our study on the cellular transport of CNS drugs (e.g., 14–16), we became aware of the need for a reliable and simple procedure for the separation of erythrocytes, leukocytes, and platelets from small samples of blood. The procedure should yield cellular fractions with minimum cross contamination by the other blood cells, and supply cells with unchanging viability over prolonged incubation at 37° in order to allow a variety of *in vitro* experiments to be carried out. Furthermore, the procedure should render possible the separation of all the three major cell types from the same sample of blood.

In accordance with the above described goals, this paper reports a procedure for the separation, from single samples of human and rat blood, of cellular fractions of erythrocytes, leukocytes, and platelets. Special emphasis was placed on ascertaining the viability and the metabolic capacity of the separated blood cells and on the simplicity of the procedure.

MATERIALS

Plasmagel, a modified gelatin fluid containing NaCl and $CaCl_2$ was obtained from HTI Corporation, Buffalo, N. Y. Biochemicals used for the determination of ATP[1] were purchased from Boehringer Mannheim

[1] List of abbreviations: ATP = adenosine-5′-triphosphate; ADP = adenosine-5′-diphosphate; EDTA = ethylenediaminetetraacetic acid; Tris = Tris(hydroxymethyl) aminomethane; $NADP^+$ = nicotinamide adenine dinucleotide phosphate; BSA = bovine serum albumin.

Corporation, New York, N. Y. and Sigma Chemical Company, St. Louis, Mo., respectively. The latter company was also the supplier of ADP and Trypan Blue. All other chemicals used were of reagent grade.

METHODS

Collection of Blood. Human blood was drawn by venipuncture from healthy male and female volunteers who were not taking any medications. Rat blood was obtained from the venae cavae of lightly anesthetized (diethylether) male, Sprague–Dawley rats, weighing 300 g.

Standard Incubation Medium. If not otherwise indicated, the suspending and incubation medium contained the following final millimolar concentrations: Na^+, 155.7; K^+, 49; Mg^{2+}, 1.2; Cl^-, 131.3; SO_4^{2-}, 1.2; HPO_4^{2-}, 13.3; $H_2PO_4^-$, 2.7; and glucose, 10. The ionic balance of this medium showed 163 cationic and anionic charges, respectively. In calculating this figure, the additional amounts of Cl^- and Na^+ ions needed

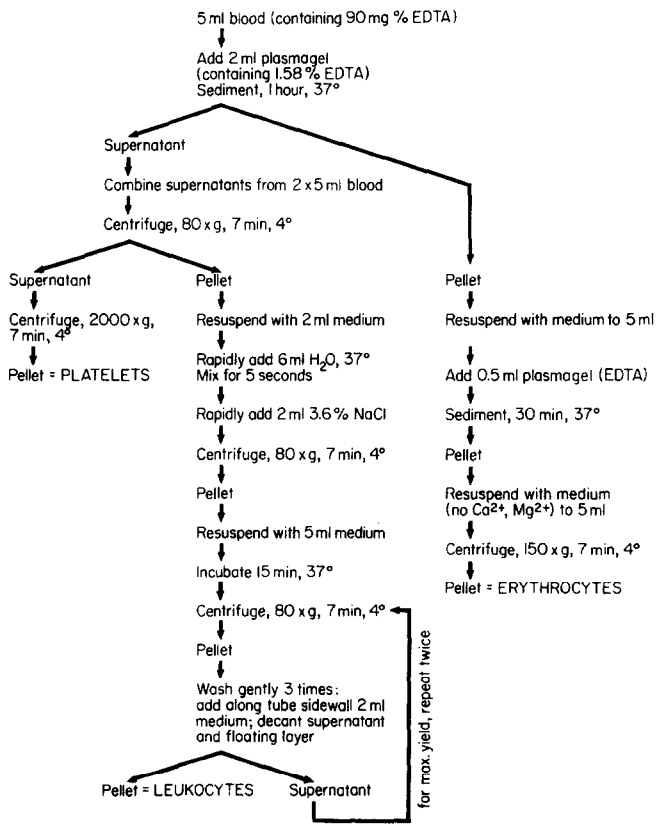

FIG. 1. Scheme for the separation of human blood cells.

for pH adjustments have been considered. The medium was prepared as follows: 0.77 M NaCl, 200 ml; 0.77 M KCl, 8 ml; 0.77 M $MgSO_4$, 2 ml; 0.5 M Na_2HPO_4 buffer, adjusted to pH 7.4 with HCl, 40 ml; H_2O, 1000 ml. Glucose, in amounts to give 10 mM final concentration, was added in solid form to aliquots of the above medium. The pH of the medium after preparation was approximately 7.3. It was adjusted to pH 7.4 with NaOH.

Separation of Cellular Fractions. Details of the experimental procedures are outlined in Figs. 1 and 2. Throughout the separation, polyethylene tubes were used. After the addition of Plasmagel, the content of the tubes was mixed by inversion.

Yield and Characterization of Cells. The yield of cells was determined both by manual and electronic cell counting. Manual counts and quantitative examination of blood smears were carried out using an AO Spenser Bright Line hemacytometer. The blood smears were prepared

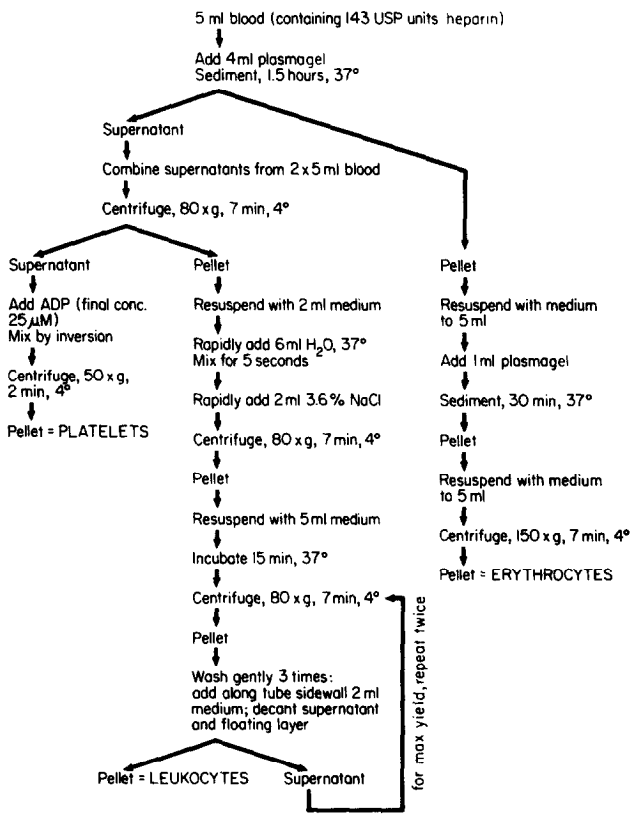

FIG. 2. Scheme for the separation of rat blood cells.

using Wright's stain (17). Phase contrast microscopic examination of the cellular populations was accomplished with an AO series 10, Phase–Star microscope. Electronic counting was carried out in a Cytograph, model No. 6301 (Bio/Physics Systems, Inc., Mahopac, N. Y.). Using the latter instrument, the cellular fractions were characterized by their size distribution and the results presented as corresponding histograms (Fig. 3). In addition, using the cytograph, differential counts were obtained of viable and injured cells. For that purpose, cell counts were obtained after an incubation of the cells with 0.05% Trypan Blue for 20 minutes at 37° (18, 19). The number of stained cells was expressed as percent of the total cell count, obtained without prior exposure to the dye.

Protein Determination. The method of Lowry *et al.* (20) was used.

Determination of Cellular Sodium and Potassium Concentrations. Aliquots of the cellular suspensions, representing 1–3 × 10^7 cells were

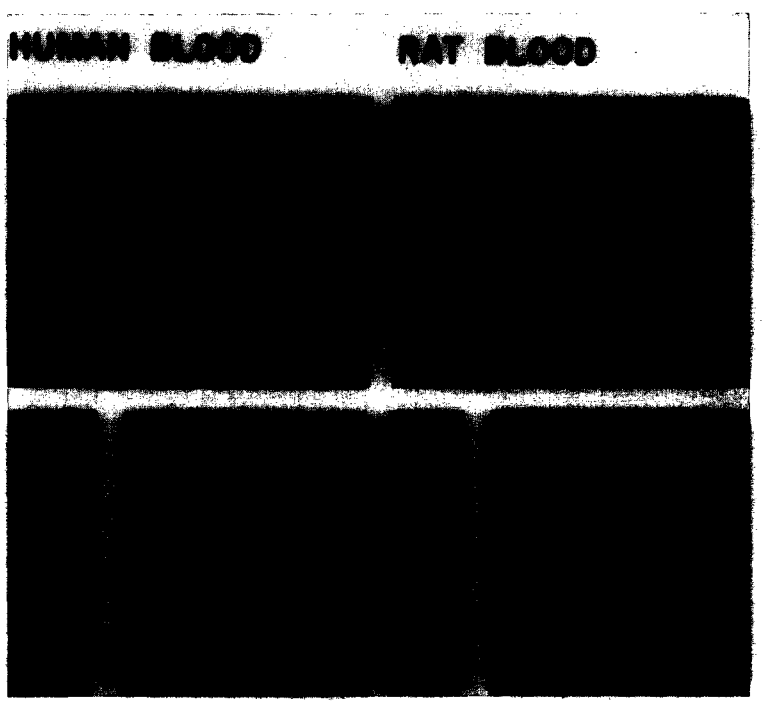

FIG. 3. Histograms of size distribution in various fractions of blood cells. Upper figures: erythrocytes; lower left figures: platelets; lower right figures: leukocytes. The abscissa and ordinate correspond to the cellular diameter and the number of cells of identical size, respectively. The calibration of these coordinates in case of human and of rat blood are not identical. The histograms were generated using a Cytograph, model No. 6301, as described in METHODS.

centrifuged at 12,000g for 3 minutes at 4°. The cells were thoroughly washed with cold 0.25 M sucrose by resuspension and gentle mixing on a Vortex mixer. After renewed centrifugation, the pellets were digested in 50 μl of 70% HNO_3 for 2 hours at 60° (alternatively, 1 hour at 80°). After the addition of 5 ml LiCl reference solution (15 mM), the measurements were carried out in an IL flame photometer.

Determination of Cellular ATP. All the following steps were carried out at 2–4°. Aliquots of the cellular suspension, representing approximately 5×10^7 cells, were centrifuged at 12,000g for 3 minutes. The pellets were treated in the plastic centrifuge tubes with 400 μl of 0.6 M $HClO_4$, thoroughly mixed with a glass rod and the suspensions transferred to 1-ml all-glass Potter–Elvehjem homogenizers. After homogenization, the suspensions were centrifuged in 4-ml tubes at 2,000g for 15 minutes and 250 μl of the supernatants were transferred into fresh 4-ml tubes. Following neutralization to pH 7.0 with 2 M K_2CO_3, the suspensions were kept in ice for 10 minutes and then centrifuged at 2000g for 5 minutes. The clear supernatants were decanted and stored at −70° until analysis.

ATP was determined by measuring the fluorescence of NADPH in a coupled enzymatic reaction utilizing hexokinase and glucose-6-phosphate dehydrogenase. The final millimolar concentrations during the assay were: Tris–HCl (pH 7.5), 100; $MgCl_2$, 5; glucose, 1; $NADP^+$, 0.05; as well as BSA, 0.01%; glucose-6-phosphate dehydrogenase, 2 μg/ml; and hexokinase, 4 μg/ml. The measurements were carried out in a Farrand filter fluorometer at excitation and emission wavelengths of 360 and 470 nm, respectively. To isolate these wavelengths, Corning glass color filters No. 7-37 (glass No. 5860) and, starting from the photocell side, 3-72 (glass No. 3387), 4-70 (glass No. 4303), 5-61 (glass No. 5562), were used.

Measurement of Oxygen Uptake. The rate of respiration of the cellular suspensions was determined in air at 37° using an YSI oxygen electrode connected to a recorder. The volume of the measuring chamber was 3 ml and the concentration of cells was $1\text{-}5 \times 10^7$ cells/ml standard incubation medium. The oxygen capacity of 3-ml incubation medium at 37° was 14.6 μl.

RESULTS

Separation of Blood Cells. The principal step in the separation of leukocytes from erythrocytes was a sedimentation in the presence of Plasmagel as described earlier (14). Special attention was required to avoid contamination of the other cellular fractions by platelets. With rat blood, heparin was used as anticoagulant and the platelets were ag-

gregated with ADP prior to their pelleting by centrifugation (Fig. 2). Human platelets, however, showed different characteristics: it was necessary to replace heparin with EDTA and, in addition, to include quite high concentrations of the latter compound during sedimentation in order to chelate the Ca^{2+} present in Plasmagel (Fig. 1). The concentration of unbound EDTA during sedimentation was approximately 2 mM. The yield of cells as well as the ratios protein/cell count are summarized in Table 1.

Characterization of the Cellular Fractions. As the size distribution in Fig. 3 shows, there was no evidence for cross contamination in the individual cellular fractions. The two populations visible in the leukocyte fractions correspond to the heterogeneity of these blood cells and to the reversed ratio of granulocytes and lymphocytes in human and rat blood, respectively. The virtual absence of cross contamination shown by cytographic analysis, was confirmed by microscopic examination under phase contrast and by differential manual counts of cells treated with Wright's stain. In all of the latter experiments, the contamination of a particular fraction by any other cell type was less than 5%. The presence of minute amounts of irregularly shaped small fragments was noticeable in the erythrocyte and leukocyte fractions from both human and rat blood. This contamination which, based on protein, in all cases was less than 1%, probably represents protein fragments resulting from some cellular damage which occurred in course of the separation procedure. Strong evidence against a cellular nature of these particles was obtained by the complete staining of the fragments after incubation with Trypan Blue.

For methodological reasons, the uptake of Trypan Blue by erythrocytes was not determined. In all other cellular preparations less than 3% of the total cells were stained at any time during the 3-hour incubation at 37°, as determined by both microscopic and electronic differential cell counts.

TABLE 1
YIELD OF BLOOD CELLS AND PROTEIN EQUIVALENT OF CELL COUNTS

	Yield of cells per milliliter blood (Cell number and percent)				mg protein/10^9 cells	
Blood cells	Human		Rat		Human	Rat
Erythrocytes	3.4×10^9	67 ± 7%	6.0×10^9	85 ± 12%	33 ± 7	19 ± 5
Leukocytes	2.1×10^6	42 ± 11%	8.5×10^6	59 ± 7%	101 ± 5	67 ± 11
Platelets	1.1×10^8	54 ± 8%	3.2×10^8	45 ± 11%	7 ± 2	8 ± 1

Sodium and Potassium Content. Erythrocytes and platelets from both human and rat blood showed no change in their content of sodium and potassium during a 3-hour incubation period at 37° (Figs. 4 and 5). Whereas the concentration of these monovalent cations did not change significantly in rat leukocytes, a decrease of potassium and a corresponding increase of sodium was noticed after the second hour of incubation in the human white blood cells (Fig. 4). These changes amounted to approximately 10% of the corresponding initial values.

Cellular ATP Concentration. The mean concentration of ATP in all three cellular fractions from both human (Fig. 6) and rat (Fig. 7) blood did not change during the 3-hour incubation period.

Uptake of Oxygen. For obvious reasons, erythrocytes were excluded from these experiments. Although the oxygen consumption of leukocytes, particularly those from the rat, showed somewhat greater variability when compared to platelets (Figs. 8 and 9), no significant change of the mean respiration rate of both of these cell types occurred during 4 hours of incubation at 37°.

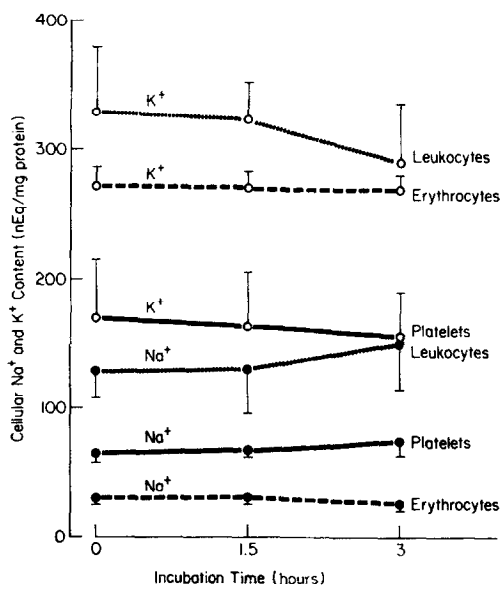

FIG. 4. Sodium and potassium content in human blood cells. Immediately after the isolation, aliquots of the cellular suspensions corresponding to approximately 3×10^7 cells/ml of standard incubation medium were incubated at 37° for the times indicated. The further treatment of the cells as well as the measurement of the monovalent cations was as described in METHODS. Presented are means ± SD of six determinations using different preparations of the particular cells.

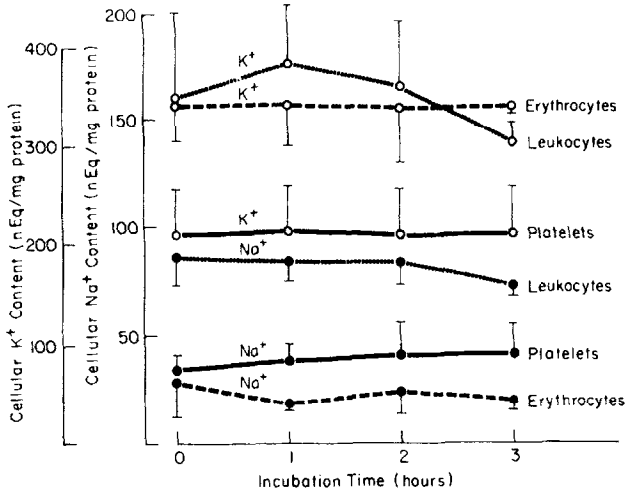

FIG. 5. Sodium and potassium content in rat blood cells. Immediately after the isolation, aliquots of the cellular suspensions corresponding to approximately 3×10^5 cells/ml of standard incubation medium were incubated at 37° for the times indicated. The further treatment of the cells as well as the measurement of the monovalent cations was as described in METHODS. Presented are means ± SD of six determinations using different preparations of the particular cells.

DISCUSSION

Blood cells represent valuable tissue compartments which can be used to study a wide range of biochemical interactions. In view of the lack of other easily accessible cellular preparations from humans, the convenient availability of blood cells offers the opportunity to use these cells as models to gain information on biochemical processes localized in tissues, which are not readily accessible in man. Most of the available methods for the separation of blood cells are focusing on a particular cell type. Furthermore, because of the low peripheral leukocyte count, the existing procedures frequently require considerable amounts of blood. The method described in this study allows the isolation of the three major cell types from a single, small sample of both human and rat blood. The separation is completed in 1.5 to 2 hours and does not require sophisticated instrumentation: a water bath and a table-top centrifuge capable of delivering 2000*g*, placed in a cold room (4°), is sufficient. The individual cellular fractions showed a cross contamination of less than 3% and the obtained yields for leukocytes and platelets of both human and rat blood ranged from 42 to 59%. The purity of the fractions and the cell counts were established both visually and electronically. In view of difficult preferences in expressing biochemical findings, a cor-

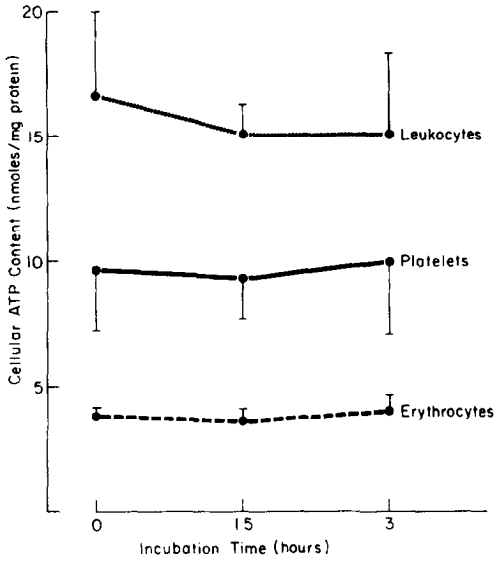

FIG. 6. ATP concentration in human blood cells. Aliquots of the cellular suspensions corresponding to approximately 5×10^7 cells/ml of standard incubation medium were incubated at 37°. At the times indicated the tubes were placed in ice. The further treatment of the cells as well as the determination of ATP was as described in METHODS. Presented are the means ± SD of six determinations using separate preparations of the particular cells.

relation of cell counts with the protein content was provided. An attempt to correlate various reported biochemical parameters of blood cells creates considerable difficulties. In expressing their results individual authors vary, in using as a reference, between wet and dry weight, volume of freshly packed cells, volume of intracellular water, as well as cell count. On the other hand, cellular protein content can be estimated by precise chemical determination and, if coupled with cell count, provides a convenient and markedly reproducible reference for expressing data of biochemical investigations in blood cells.

Particular importance in this study was given to investigate the viability and metabolic state of the cells immediately after the isolation and during a prolonged incubation at 37°. The selected 3-hour incubation period should allow a variety of *in vitro* experiments to be carried out. It should be emphasized that, from the beginning of isolation to the end of incubation, the cells were actually 4.5 to 5 hours under *in vitro* conditions. As an indicator of the integrity of the plasma membrane, the cellular contents of sodium and potassium were estimated. In addition, the exclusion of Trypan Blue was used to ascertain cellular integrity. The

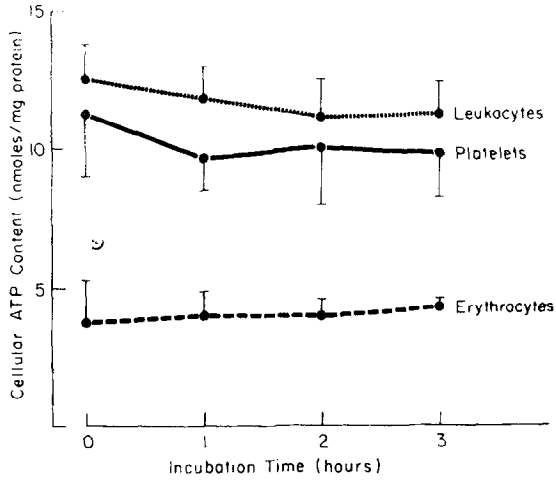

FIG. 7. ATP concentration in rat blood cells. Aliquots of the cellular suspensions corresponding to approximately 5×10^7 cells/ml of standard incubation medium were incubated at 37°. At the times indicated the tubes were placed in ice. The further treatment of the cells as well as the determination of ATP was as described in METHODS. Presented are the means ± SD of six determinations using separate preparations of the particular cells.

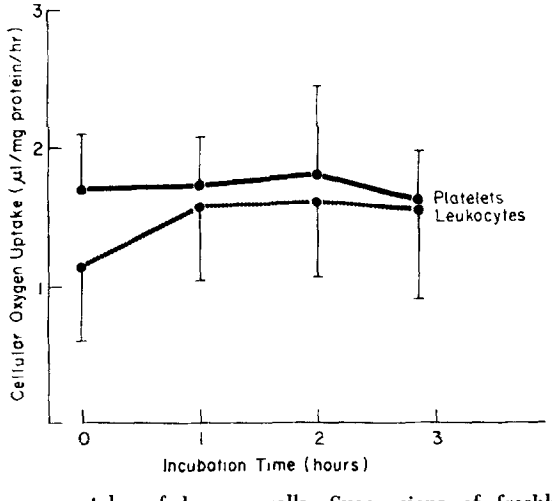

FIG. 8. Oxygen uptake of human cells. Suspensions of freshly isolated cells ($1–5 \times 10^7$/ml) were incubated at 37° in the standard incubation medium. At the indicated times the rate of oxygen uptake of these cellular suspensions was determined as described in METHODS. The results represent means ± SD of six separate measurements using different preparations of the particular cells.

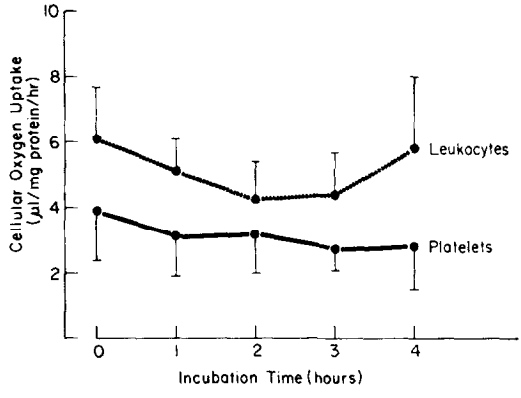

FIG. 9. Oxygen uptake of rat blood cells. Suspensions of freshly isolated cells ($1–5 \times 10^7$/ml) were incubated at 37° in the standard incubation medium. At the indicated times the rate of oxygen uptake of these cellular suspensions was determined as described in METHODS. The results represent means ± SD of six separate measurements using different preparations of the particular cells.

energetic state of the cells was studied by determining the concentrations of ATP. Furthermore, the metabolic capacity was investigated by measuring the oxygen consumption of the cellular suspensions. The latter parameter was found to be a sensitive indicator of cellular viability, whereas vital staining of leukocytes occurred at a later stage of membrane damage (21).

Only limited data are available concerning the here investigated parameters in blood cells of the rat, e.g., the oxygen uptake of rat lymphocytes was reported to be 5 $\mu l/10^7$ cells/hour (22). The corresponding value for rat leukocytes in our study was 3.6 $\mu l/10^7$ cells/hour (Fig. 9). Pertinent information for human blood cells is available in the literature, although there is, to our knowledge, no study in which all the here-presented parameters were investigated in one and/or more types of blood cells. Furthermore, even in studies directed specifically towards isolation and characterization of a particular blood cell, e.g., leukocyte (1), the changes of cellular characteristics during a subsequent incubation period were not investigated.

Using sedimentation techniques to isolate human leukocytes, average yields of 24–45% were reported (1, 5, 6). In these studies, experiments on Trypan Blue exclusion resulted in 5% stained cells.

Cellular sodium and potassium contents of human blood cells were reported as (in nEq/mg protein, calculated from the originally presented expressions): erythrocytes, 35 and 250 (23–25); leukocytes, 90 and 300 (26); platelets, 83 and 230 (27). It should be noted that due

to the necessary use of various conversion factors to allow a comparison of the reported data, the accuracy of the values calculated above inevitably suffered. Changes in leukocyte sodium and potassium during incubation at 37° were measured by Rosenberg and Downing (5). During 45-minutes incubation at 37° the ratio of cellular sodium/potassium gradually decreased, indicating a recovery of the initially impaired transmembrane distribution of these ions. The increase, in our study, of sodium and loss of cellular potassium in human leukocytes after 2 hours of incubation (Fig. 4) seems to be the result of the rather high concentrations of EDTA during the separation procedure. On the other hand, lowering the concentration of this compound resulted in impaired separation of the cellular fractions. Owing to the limited extent of this change in cellular ion content and because of the fact that it only occurred after the second hour of incubation, no correcting attempts were made. In rat leukocytes, separated in the absence of EDTA, the ratio cellular sodium/cellular potassium remained constant over the entire incubation period. Pertoft *et al.* (12) reported, that in order to preserve cell functions of blood cells separated by density gradient centrifugation, the presence of sulfhydryl compounds was essential.

Our results of ATP determinations in human blood cells are within the concentration ranges of this nucleotide reported in the literature (in nmoles/mg protein, calculated from the originally presented expressions): in erythrocytes, 3.5–4, (28–31) and in platelets, 7–8.5, (32, 33). In human leukocytes, the measured ATP content was lower than the 51 nmoles/10^7 cells reported by Frei (30). Other corresponding data, summarized by Seitz (34), are not unequivocal due to the diverse expression of results. In earlier studies, data for ATP levels in biological material were often too high due to a lack in specificity of the analytical procedure.

Finally, the rate of oxygen uptake of human leukocytes and platelets correlates favorably with the following reported values for the respiration of these cells (in μl O_2/mg protein, calculated from the originally presented expressions): 1.7 (1) and 1.5 (27), respectively.

In addition to the favorable correlation of the determined parameters with corresponding reported data, it is of particular significance to emphasize the constancy of these parameters in our cellular preparations during a prolonged incubation at 37°. These findings suggest, that during a 3-hour incubation period following the isolation, the integrity of the plasma membrane, the energetic state, and metabolic capacity of these cells remained unchanged. This manyfold proven viability, together with the simplicity of the isolation of the highly pure fractions of various cells from a single small sample of blood, should contribute to the labo-

ratory investigation of these valuable tissue compartments. Additional versatility is provided by presenting data for both human and rat blood cells.

SUMMARY

A simple procedure is presented for the isolation of erythrocytes, leukocytes and platelets from single small samples of both human and rat blood. The separation of cells is based on sedimentation and low-speed centrifugation. The obtained yields for the various cells ranged from 42–85%. The cross contamination of the individual cellular fractions was less than 5%. The purity of the cellular fraction was ascertained by microscopic examination and electronic size distribution. Cellular viability, energetic state, and metabolic function was investigated by Trypan Blue exclusion, by determination of cellular contents of sodium, potassium, and ATP, as well as by measurements of oxygen consumption. The obtained data compared favorably with those reported previously and the investigated parameters remained essentially constant over a 3-hour incubation period at 37°.

ACKNOWLEDGMENT

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