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ANALYSIS OF DENSITY-FRACTIONATED RAT RED BLOOD CELLS OF DIFFERENT AGES BY PARTITIONING IN TWO-POLYMER AQUEOUS PHASE SYSTEMS

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SUMMARY

Rats were injected with radioactive iron and bled at different times thereafter. This gave rise to cell populations in which the bulk of labeled cells corresponded in age to the time elapsed between injection and bleeding. Such cell populations were centrifuged and the 7–10% least dense and the 7–10% most dense cells subjected to counter-current distribution in a charge-sensitive two-polymer aqueous phase system which fractionates cells on the basis of surface properties. While it is known that there is a tendency for cell density to increase with cell age and that rat red cells of different ages have characteristic partititon ratios, we were able, by applying partitioning analysis to density-separated cells, to gather data not obtainable by use of either method alone. These include: (1) the demonstration of two very young cell populations having different surface properties in the most dense fraction; (2) the finding that in the least dense layer labeled mature erythrocytes (at least those labeled 18 days and beyond) and unlabeled red cells have overlapping distribution curves which indicate that the label in the top fraction can represent young or early middle-aged but never old cells; and (3) the presence, as judged by surface properties, of some middle-aged red cells, in addition to old cells, in the most dense cell layer.

INTRODUCTION

To study the senescence of red blood cells many investigators have attempted cell fractionations based on age-associated alterations in physical properties [for a recent review, see ref. 1]. Cohort labeling (*e.g.*, with ^{59}Fe) of the youngest cells in the population and tracing, at different subsequent times, the location of the bulk of labeled cells (which approximate in age the time elapsed between injection and bleeding) in the separatory method indicates whether an age-dependent fractionation has occurred. Thus centrifugation has revealed that at least a general correlation exists

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between increasing cell age and density in some species (*e.g.*, human, rat) while counter-current distribution of rat red cells in charge-sensitive two-polymer aqueous phase systems[2] indicates that the surface charge-associated properties of reticulocytes increase rapidly as these cells become young mature erythrocytes while the latter undergo a systematic diminution in partition ratios during their entire life-span.

As the labeled cells age they predominate, as indicated above, in fractions corresponding to cells of higher density. However, cells (except when very young) never band in a narrow density range but rather tend to spread with cell age[3]. Even in the case of young cells small quantities (order of 0.5%) of the labeled cells can also be found in the 7–10% highest density cell layer. Whether labeled cells in a layer other than the one in which they are concentrated are similar to the bulk of labeled cells or represent a distinct cell subpopulation is subject to conjecture. We therefore studied an additional physical parameter of labeled cells, obtained at different times following isotope injection, in high and low density fractions: their surface properties by counter-current distribution in a charge-sensitive two-polymer aqueous phase system.

EXPERIMENTAL

Animal injection and bleeding

Male Sprague-Dawley rats (Charles River Labs., Wilmington, MA, U.S.A.), weighing 275–400 g, were used. Injection of rats with 15–80 μCi of [^{59}Fe]ferrous citrate (ICN, Irvine, CA, U.S.A.) via the saphenous vein as well as the ^{59}Fe-counting procedures used have previously been detailed[4]. Rats were exsanguinated by heart puncture at different times (16 h to 49 days) after injection. About 4 ml of blood were collected in each of two blue-top (sodium citrate) vacutainer tubes.

Murphy centrifugation procedure

A somewhat modified Murphy centrifugation procedure[5] for the fractionation of red blood cells of different ages was used. The Sorvall centrifuge (RC-5) rotor (SS-34) was incubated overnight at 30°C and then spun for 1 h with the centrifuge set to the same temperature. The blood, collected as described above, was first centrifuged (using a 15 ml disposable plastic centrifuge tube) on a table model at 1200 *g* for 30 min in the cold room. The plasma was removed and saved. The buffy coat was aspirated and the plasma added back to the cells. The latter were resuspended and centrifuged once again in a similar manner. The plasma was aspirated and the cells were well mixed. A 0.2-ml volume of cells was taken as the "unfractionated" sample. The remaining red cells were transferred into round-bottom polycarbonate tube (77 × 10.9 mm) (Damon/IEC Division, Needham Heights, MA, U.S.A.) and centrifuged in the Sorvall unit at 26 900 *g* for 60 min at 30°C. The small amount of remaining plasma was aspirated and the top 7–10% of the cells was removed with a 1-ml plastic pipette and represents the "top" fraction. Cells were then slowly aspirated. Prior to aspirating the last 0.5 ml of cells to be discarded, the sides of the tube were rinsed down with isotonic aqueous salt solution (saline) using a Pasteur pipette. The wash solution was aspirated as was the last 0.5 ml of cells to be discarded. With a 1-ml plastic pipette the 7–10% of cells on the bottom were collected and represent the "bottom" fraction. The cells constituting the top, bottom and unfractionated samples were washed three times with saline and finally suspended in 10 ml saline. Aliquots of

these cell suspensions were counted electronically while others were used in counter-current distribution experiments and/or to determine the specific activity ratios of cells in top/cells in unfractionated (or top/bottom) fractions (see below).

Electronic cell counting

Aliquots of washed cells, suspended in saline were electronically counted using a Celloscope (Particle Data, Chicago, IL, U.S.A.) operating on the Coulter principle and fitted with a 76-μm orifice tube.

Determination of specific radioactivity of Murphy-method fractionated cells

Aliquots of top, unfractionated and bottom cell suspensions, each containing $2 \cdot 10^8$ cells, from a Murphy experiment, were centrifuged, the supernatant solution discarded and the cells lysed in 10 ml of a 20 mos*M* sodium phosphate buffer, pH 7.2. After high-speed centrifugation (to remove the stroma) the absorbance of the supernatant solution was read at 540 nm on a Gilford spectrophotometer. ^{59}Fe radioactivity was determined by counting a known aliquot (2.5–3 ml) on a Beckman scintillation well-counter set for ^{59}Fe.

Preparation of phase system

A phase system, selected and prepared as previously described, was used[2]. It contained 5% (w/w) dextran T500, lot No. LL 01035 (Pharmacia LKB, Piscataway, NJ, U.S.A.), 5% (w/w) poly(ethylene glycol) 8000 (PEG, "Carbowax 8000", Union Carbide, Long Beach, CA, U.S.A.), 210 (or 220) mos*M* sodium phosphate buffer, pH 7.4, 60 (or 46) mos*M* sodium chloride and 5% (w/w) heat-inactivated fetal bovine serum (FBS). Such a phase system has an electrostatic potential difference between the phases (top phase positive) and is charge-sensitive (see refs. 2 and 6 for detailed discussion).

The phase system was permitted to equilibrate in a separatory funnel at 4–5°C. Top and bottom phases were then separated.

Counter-current distribution of red blood cells

Our counter-current distribution apparatus (Workshop, Chemical Center, University of Lund, Sweden) consists of two circular Plexiglas plates, one stator and one rotor, having 120 cavities with a bottom phase capacity of 0.7 ml[7]. Counter-current distribution of cells was carried out as previously described[4]. All cavities received 0.5 ml of bottom phase. Aliquots of "unfractionated" cells and "top" and "bottom" cells (from a Murphy centrifugation, see above) corresponding to $2 \cdot 10^9$ cells were centrifuged and the supernatant solutions discarded. The cells were resuspended, using a Pasteur pipette, in 2.3 ml of top phase ("load mix"). Cavities 0–2 received 0.7 ml of one load mix, cavities 40–42 received 0.7 ml of the second load mix and cavities 80–82 the third load mix. All other cavities received 0.7 ml of top phase. Counter-current distribution was then carried out at 4–5°C using a 6-min settling and a 27.5-s shaking time. Thirty or 39 transfers (see Figures) were completed.

Analysis of cells after counter-current distribution

After counter-current distribution the cells in each cavity were collected, by use of a fraction collector, directly into plastic centrifuge tubes. Saline (0.7 ml) was added

to each tube thereby reducing the polymer concentrations and giving rise to a single homogeneous suspending medium. Cells in each three adjacent tubes were pooled. They were centrifuged at 1200 *g* for 10 min, the supernatant solution was discarded, and the cells were lysed by addition of 3 ml of 20 mos*M* sodium phosphate buffer, pH 7.2. The tubes were then centrifuged at high speed to remove the stroma and the hemoglobin absorbance and ^{59}Fe radioactivity was determined as described above.

Presentation of data

The fractionation of rat labeled red blood cells by the Murphy centrifugation procedure is given as the ratio of specific activity (cpm/hemoglobin absorbance) of cells in the top layer to cells in the original, unfractionated cells, T/U[8]. For longer times after injection the ratio of specific activity of cells in the bottom layer to cells in the original, unfractionated cells, B/U, is also presented.

Counter-current distribution curves of total cells are shown in terms of hemoglobin absorbance (at 540 nm). Distributions of labeled (^{59}Fe) cell populations are in counts/min (cpm). A relative specific activity is also presented through the distribution curves and reflects, most sensitively, the extent of displacement and, hence, of difference between the labeled and unlabeled red cell populations. It is defined as:

$$\frac{\text{cpm/unit hemoglobin absorbance in a given cavity}}{\text{cpm/unit hemoglobin absorbance in the original cell population prior to counter-current distribution}}$$

The data depicted in the graphs are representative of at least three separate experiments with individual rats.

RESULTS AND DISCUSSION

Cell age-related fractionation of rat red blood cells by centrifugation

Rats were injected with [^{59}Fe]ferrous citrate and bled at different times (from 16 h to 49 days) thereafter. Erythrocyte populations were thus obtained in which the predominantly labeled cells corresponded in age to the time elapsed between injection and bleeding (*i.e.*, reticulocytes, mature young to old erythrocytes)[1].

Such cell populations were subjected to a Murphy-method centrifugation. The specific radioactivity (in cpm/hemoglobin absorbance) of the original, unfractionated cells as well as of cells constituting the top 7–10% of the centrifuged cell column was determined as a ratio of specific activities of top cells/unfractionated cells (T/U). As previously reported[8], this ratio was approximately constant at 3–4 (range 3.1–3.9) during the first 7 days of the experiment suggesting that reticulocytes (constituting the primary labeled cell population for at least 24 h after isotope injection) and mature young erythrocytes (at the later bleeding times) are both enriched in the top 7–10% layer of centrifuged red cells[a]. Cells in the bottom fraction contained about 0.5% of the labeled young cells.

[a] It should be noted that if one were to express data as specific activity ratios of 7–10% of cells in top/7–10% of cells in bottom at *short* times after isotope injection they would be of the order of 50–250. This variability in the ratio is caused by the low specific activities associated with the bottom fraction at such short times after injection and has prompted us to present specific activity ratios of cells in top/cells in unfractionated cells at these earlier times.

The T/U ratio diminished with time after isotope injection being about 2.2 at 13–14 days, 1.6 at 18–21 days, 0.6 at 28–33 days and 0.4 at 40–49 days[8]; while the analogous B/U ratio increased, from about 0.2 at 18–21 days to 0.8 at 35–36 days and 1.6 at 40–49 days.

Surface properties of rat labeled red blood cells, at different times after ^{59}Fe injection, in top and bottom fractions obtained by Murphy-method centrifugation: analysis by counter-current distribution

Murphy-method fractionated cells as described above were subjected to counter-current distributions. Results of a typical series of such experiments (with cells obtained at different times after injection of rats with isotope) are depicted in Figs. 1–5. The upper panel of each figure presents the counter-current distribution pattern of unfractionated cells, U, the middle panel the counter-current distribution of cells from the top layer, T, of a Murphy-method centrifugation and the lower panel the counter-current distribution of cells from the bottom layer, B, of the centrifugal column.

The top panels of Figs. 1–5 show the previously reported charge-associated surface alterations that accompany rat red blood cell maturation and aging in the peripheral blood[2]. At 16 h after isotope injection (Fig. 1, U) it is the reticulocytes that are labeled. They have a lower partition ratio (presumably lower surface charge-associated properties) than the bulk of the peripheral red cells as evidenced by the position of the distribution of labeled cells (open circles) to the left of the distribution of unlabeled cells (solid circles). The extent of displacement is clearly indicated by the relative specific activity curve (solid triangles).

The partition ratio of reticulocytes increases rapidly[9] during maturation to the youngest erythrocytes (42 h, Fig. 2, U) and, subsequently, slowly diminishes (Figs. 3–5, U) over the entire 55-days life-span of the mature erythrocyte. The oldest erythrocytes in the peripheral blood have a partition ratio that is close to that of the reticulocytes (compare Figs. 1, U, and 5, U, and see ref. 2).

As is evident from all five figures, the least dense cells (T) have a higher partition ratio (*i.e.*, are to the right), most dense cells, B, a lower partition ratio and the original, unfractionated cells, U, an intermediate partition ratio[10–12]. This result (indicated, for easier viewing, by the arrows in Fig. 1) is in line with the fact (see above) that young mature erythrocytes have a high, old mature erythrocytes a low partition ratio[a].

To study the surface properties of labeled red blood cells associated, at different times during their life-span, with cell populations of low and high mean densities we compared the relative partition ratios (*i.e.*, positions in the graphs) of labeled cells from the top and bottom of a cell column after a Murphy-method centrifugation and of unfractionated samples.

In Fig. 1, the reticulocytes associated with centrifuged cells from the top, T, have the same partition ratio as reticulocytes in the unfractionated cell population, U, implying that their charge-associated surface properties, as reflected by partitioning, are the same. The labeled cells in the centrifugate from the bottom, B (representing the small percentage of total labeled cells as indicated by the relative cpm scales on the

[a] The youngest reticulocytes, which have a low partition ratio, constitute a very small percentage of the total peripheral red cell population. They thus do not affect the higher partition ratio of cells of lower density (primarily mature younger erythrocytes) obtained on Murphy centrifugation.

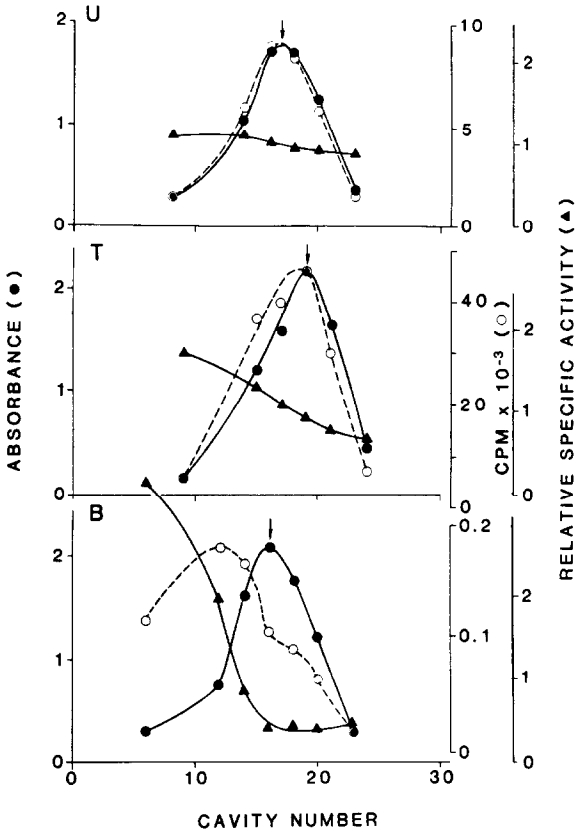

Fig. 1. Rats were injected with [^{59}Fe]ferrous citrate and bled at different times thereafter. This gave rise to rat cell populations in which the bulk of labeled cells corresponds in age to the time elapsed between injection and bleeding. Such cell populations were fractionated, in succession, on the basis of density and surface properties. Cells were centrifuged and the top layer, T, containing predominantly young cells, the bottom layer, B, containing older cells, and an aliquot of unfractionated cells, U, were subjected to counter-current distribution in a charge-sensitive two-polymer aqueous phase system (see text for composition). Thirty transfers were carried out at 4–5°C using 6-min settling and 27.5-s shaking times. The run was analyzed for total cell distribution (●, in terms of hemoglobin absorbance at 540 nm) and labeled cell distribution (○, in terms of cpm). A relative specific activity is also presented (▲) with 1.0 being the specific activity of each sample prior to counter-current distribution. Note that the unlabeled bulk of cells in T have a higher partition ratio (*i.e.*, are to the right) than those in B, while U cells have an intermediate ratio (see arrows above the distribution curves of total cells). In this figure results are depicted with blood collected 16 h after injection when it is the reticulocytes that are labeled and most of the label is in T and least in B (note cpm scales). See text for discussion.

three panels) are composed of two subpopulations. Those with the higher partition ratio consist of cells similar in partitioning behavior to reticulocytes found in the top fraction[a]. An analogous experiment done with blood obtained at 42 h after ^{59}Fe injection (Fig. 2, B) shows that the two cell subpopulations remain in evidence with the left peak diminishing in relative size. This process continues for about 3–4 (some-

[a] For easier viewing this is indicated in Fig. 2, depicting an analogous experiment with red cells collected 42 h after injection, by the broken-line arrows.

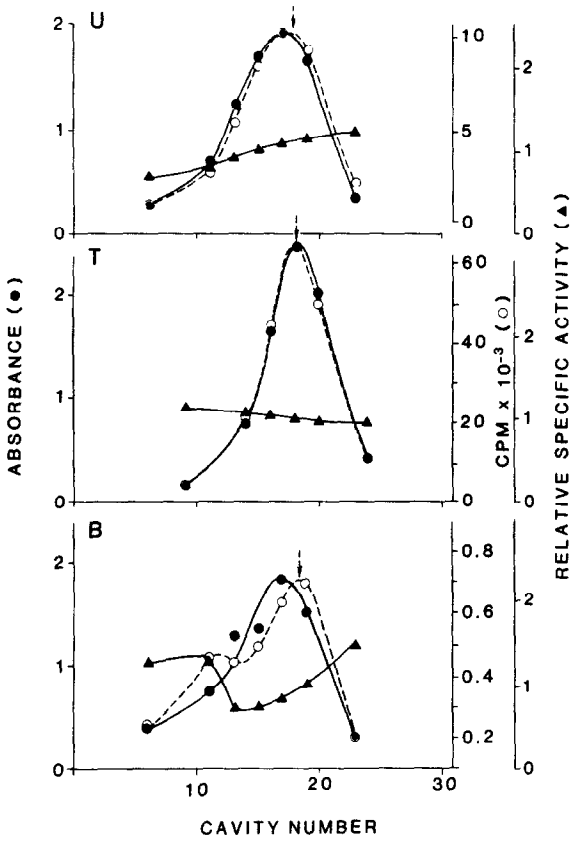

Fig. 2. Experiment as in Fig. 1 except that blood was collected 42 h after injection when the label is predominantly in mature young erythrocytes. For easier visualization of the similar positions of labeled cells in T and U and those having the higher partition ratio in B a broken arrow has been placed over each of the isotope peaks. See text for details.

times even 6) days following isotope injection after which the left peak disappears.

We have previously traced, by partitioning, the surface alterations that accompany erythroid cell differentiation in the rat bone marrow[13] and reticulocyte maturation in the peripheral blood (see ref. 9, Figs. 1 and 2 and discussion above) and have found that these processes are accompanied by increases in the cells' partition ratios. It is therefore tempting to speculate that the labeled red cells with the lower partition ratio associated with the centrifugal bottom cell fraction are representative of the youngest cells in the peripheral blood.

In the case of mice the youngest red cells are more dense than older cells during the first day or so after release from the bone marrow and then (possibly after restructuring in the spleen[14]) become less dense[15]. Whether a small percentage of rat reticulocytes behaves in a similar manner is not known. Alternatively, it is possible that the labeled rat cell populations in the bottom are qualitatively representative of those in the top fraction but that, due to differences in physical properties (*e.g.*, a differential tendency to adhere to other cells), cells with lower partition ratios appear to a rela-

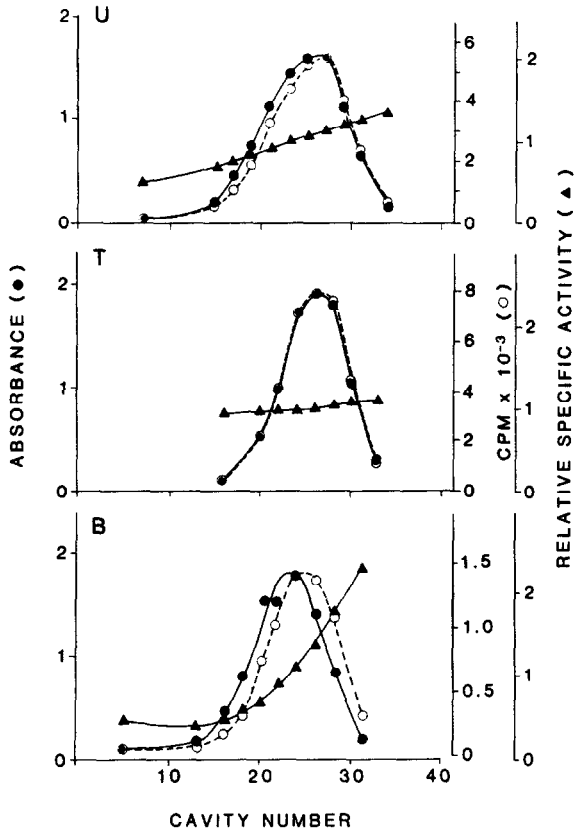

Fig. 3. Experiment as in Fig. 1 except that the blood was collected 18 days after isotope injection when the label is predominantly in early middle-aged cells. Thirty-nine transfers were carried out. See text for discussion.

tively greater extent in the bottom fraction than do those with the higher partition ratios.

At 6–7 days after isotope injection (data not shown) labeled cells in unfractionated, top and bottom fractions have the same partition ratio which is slightly greater (*i.e.*, is displaced to the right) than that of unlabeled cells in the least dense (T) fraction. Hence the labeled cells at this time are younger than the mean age of the 7–10% least dense cells.

Fig. 3 depicts results with rat erythrocytes containing labeled cells 18 days after isotope injection. These have mean surface properties similar to those of the young, lower-density cells in the top fraction in a Murphy-method centrifugation, as indicated by the virtual overlap of labeled and total cell distributions (Fig. 3, T). The labeled cells also have the same partition ratios (*i.e.*, positions) in the bottom fraction and in the unfractionated cell sample (Fig. 3, B and U). Thus, 18-days labeled cells in low- and high-density fractions have the same mean surface properties, those associated with early middle-aged rat erythrocytes[2,16].

At 35 days after isotope injection (Fig. 4) labeled cells overlap not only with the

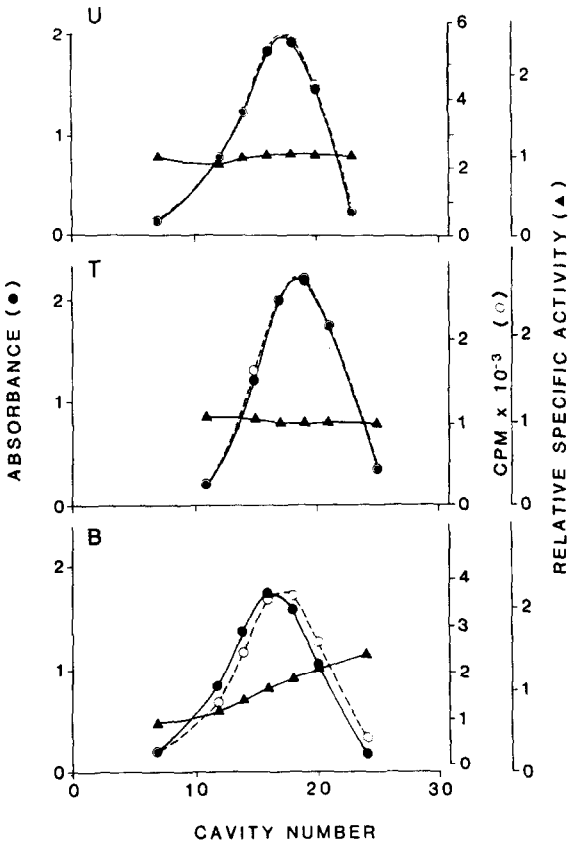

Fig. 4. Experiment as in Fig. 1 except that the blood was collected 35 days after isotope injection when the label is predominantly in middle-aged cells. See text for discussion.

distribution of unfractionated cells (Fig. 4, U) but also with that of low-density (younger) cells (Fig. 4, T) as indicated by specific activity ratios of 1.0 through the distribution curves in both cases. Since red blood cells in unfractionated and low-density fractions have different partition ratios (see discussion above and arrows in Fig. 1), the labeled cells in the two parts of the figure must represent different cell subpopulations. The labeled cells in the low-density fraction are younger than the mean age of those in the unfractionated cells. Reutilization of ^{59}Fe (refs. 1 and 16) in the formation of young cells, as older cells break down and release their ^{59}Fe, is the most likely basis for this result. The labeled unfractionated cells (Fig. 4, U) have the same partition ratio (associated with middle-aged erythrocytes[2,16]) as the labeled cells in the high-density fraction (Fig. 4, B).

Fig. 5 shows results obtained with erythrocytes 48 days after isotope injection. At this time labeled and unlabeled cells are found to overlap in both the least dense (Fig. 5, T) and most dense (Fig. 5, B) fractions. Since the partition ratios of these cells differ (again see discussion above and arrows in Fig. 1) it is apparent that the labeled cells in these two fractions also differ. They reflect subpopulations of different cell age:

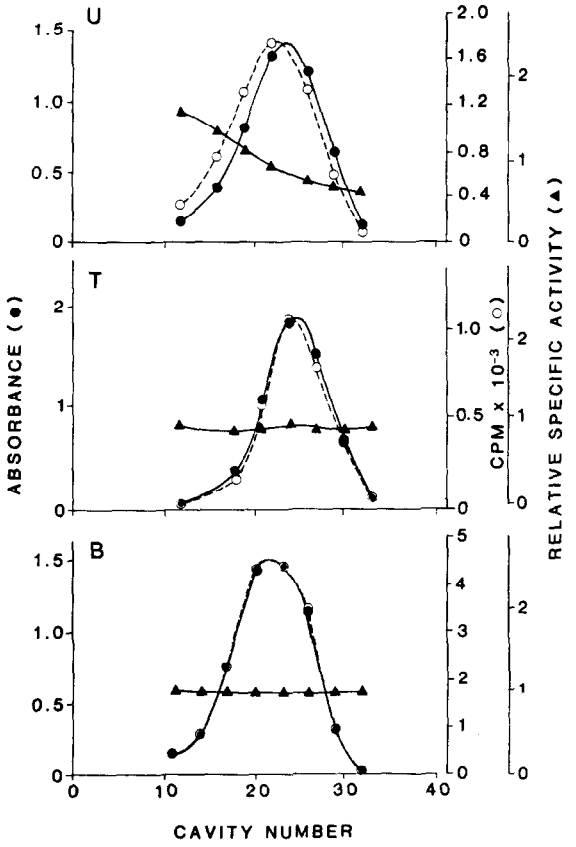

Fig. 5. Experiment as in Fig. 3 except that the blood was collected 48 days after isotope injection when the label is predominantly in old cells. See text for discussion.

younger cells with higher partition ratio (Fig. 5, T) and older cells with lower partition ratio (Fig. 5, B). Labeled cells in the unfractionated sample (Fig. 5, U) have the same partition ratio as those in the most dense fraction as might be expected since more of the label is now in the older cells and the unfractionated sample represents a mean.

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