RESOLUTION OF TWO POPULATIONS OF RETICULOCYTES BY COUNTER-CURRENT DISTRIBUTION

Harry Walter* and Per-Ake Albertsson

Department of Biochemistry, University of Umeå, Sweden.

Received November 15, 1966

The great current interest in reticulocytes of different ages (Borsook et al., 1962), their ribosomal and polyribosomal content (Glowacki and Millette, 1965; Rowley, 1965) and its relation to their activity in protein biosynthesis (Glowacki and Millette, 1965; Rowley, 1965; Miller and Maunsbach, 1966) prompted us to test for possible heterogeneities of reticulocytes by counter-current distribution. The development by Albertsson (1960) of two-phase systems consisting of a mixture of two aqueous solutions of different polymers for the separation of particles and cells and the successful separation by Walter et al. (1964; 1965; 1966) of red blood cells of different ages, using some of these phase systems, lent promise to such an approach. We wish to report preliminary results on the resolution of at least two populations of reticulocytes (obtained from phenylhydrazine-treated rats) by counter-current distribution.

Experimental Methods

Wistar rats weighing about 200 gms were injected daily for 6 or 7 days (sub-cutaneously) with 0.3 ml of a solution containing 4 mg phenylhydrazine. On the eighth day the animals were exsanguinated and their blood collected in an acid-citrate-dextrose (ACD) solution. The reticulocytes represented from 47 to 98% of the red cells so obtained and only those cell populations con-

*Work performed while HW was on leave from the Laboratory of Chemical Biology, Veterans Administration Hospital, Long Beach, and the Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, California. Requests for reprints should be addressed to HW at the VA Hospital.

taining more than 90% reticulocytes were examined by counter-current distribution in these experiments. The red cells were washed 5 times with an aqueous isotonic salt solution and were then subjected to counter-current distribution as described below.

Buffered dextran-polyethylene glycol, aqueous two-phase systems previously described by Albertsson (1960) were used in this work. Dextran # 500 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and polyethylene glycol was obtained as "Carbowax 6000" from Union Carbide, New York. In the present study stock solutions of appropriate concentrations were prepared (Albertsson and Baird, 1962) and a mixture with the following Polymer and salt composition was then used: 5% (w/w) dextran, 4% (w/w) polyethylene glycol, and equimolar amounts of NaH_2PO_4 and Na_2HPO_4 (total conc. 0.09 M phosphate) and 0.03 M NaCl. The phases were allowed to equilibrate and were then separated.

An automatic thin-layer counter-current distribution apparatus (with 120 cavities) was used in this work (Albertsson, 1965). The use of a standard Craig apparatus is not suitable for the separation to be described because the greater height of the phase system increases the settling time and permits no more than about 50 transfers to be made without undue cell damage. The bottom phase capacity of the apparatus used was 0.7 ml. 0.6 ml of bottom phase (dextran-rich solution) was pipetted into cavities 10 to 119. Cavities 0 to 9 received 0.5 ml of bottom phase and 0.1 ml of packed red blood cells. All cavities were loaded with 0.8 ml of top phase (polyethylene glycol-rich solution) giving rise to 0.1 ml stationary top phase during the separation. The apparatus was set for a cycle consisting of 20 sec shaking followed by a 5 min settling time and transfer. A total of 120 transfers was completed. The procedure was carried out at 22 - 24°.

The red blood cells from each cavity were collected directly into plastic centrifuge tubes and left in the cold-room overnight. They were centrifuged (13,000 x g for 10 min) and the supernatant solution discarded. The cells

were lysed in a small volume of water and the concentration of hemoglobin in each lysate was determined by measuring the absorbance of a suitable aliquot at 540 mμ on a Zeiss spectrophotometer.

In a control experiment performed to determine whether the maturation of reticulocytes to erythrocytes during the course of the counter-current distribution could account for our results (see below) it was found that the storage of reticulocytes in a phase system for 18 hours at room temperature did not alter the reticulocyte count. The cells were resuspended in their original plasma prior to staining.

Results

The results of an experiment with a red cell population containing 97% reticulocytes are presented in Fig. 1. The distribution obtained is superimposed on one of a normal (untreated) rat red blood cell population for comparison.

It is apparent from Fig. 1 that the counter-current distribution of a rat reticulocyte population gives rise to two major peaks and a minor one. The

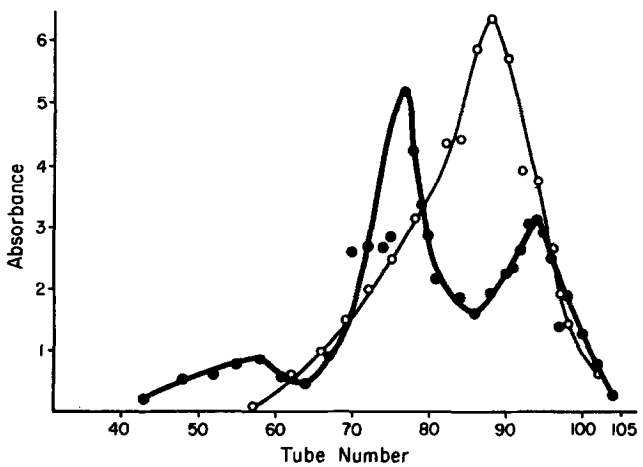

Fig. 1. Counter-current distribution of red blood cells from a rat repeatedly injected with phenylhydrazine and having 97% reticulocytes (● - ●). Other curve (O - O) shows distribution pattern of a normal rat red blood cell population. Red blood cell concentration is given in terms of hemoglobin absorbance.

general distribution patterns observed from experiment to experiment are quite similar, but not identical. Thus the relative magnitude of the two major peaks varies and the left-hand minor peak is sometimes as indicated in Fig. 1 but at others somewhat broader and shifted a little further to the left. We believe that such differences can be expected in such an obviously heterogeneous cell population.

A point we wish to emphasize is that the two major reticulocyte peaks are associated with the opposite ends of the normal distribution curve of red blood cells obtained from an untreated rat.

Discussion

Red blood cells of different ages from a number of different species can be separated by counter-current distribution (Walter et al., 1964; 1965; 1966). The basis for this separation seems to be, at least in part, the continuous alteration of red blood cell surface charge during its life-span (Walter et al., 1966). The combination of counter-current distribution of normal red cell populations with radioactive isotope labeling techniques has led to the conclusion that reticulocytes are associated with the left portion of the distribution, mature young erythrocytes with the right portion and erythrocytes of increasing age are in sequential order from the right to the left of the distribution (Walter et al., 1966).

From the results depicted in Fig. 1 it now appears that cells giving the appearance of reticulocytes under the microscope fall into at least two groups: one associated with the left portion of the distribution of a normal red cell population (reported previously to contain reticulocytes) and one with the right portion (previously thought to contain only mature young erythrocytes). It follows that the surface properties (probably surface charge) of reticulocytes become altered before the reticulum is lost. Because of the previously reported correlation between the partition of red blood cells and their electrophoretic mobilities (Walter et al., 1965; Walter et al., in press) we would predict that a similar resolution of reticulocytes

could be effected by electrophoresis. Further, because of the correlation between the separation of red blood cells of different ages by counter-current distribution and their lysates by a serial osmotic hemolysis procedure (Simon and Topper, 1957; Walter et al., 1964) it seems not unlikely that the two major reticulocyte populations described here could also be partially resolved by differential hemolysis.

Just as erythrocytes can be separated according to their age using density gradient centrifugation (Leif and Vinograd, 1964), reticulocytes can be similarly fractionated (Borsook et al., 1962). Thus Glowacki and Millette (1965) and Rowley (1965) reported, on the basis of such a fractionation, that young reticulocytes (obtained from phenylhydrazine-treated rabbits) are richer in ribosomes and polyribosomes than the older reticulocytes and that there is an increasing fraction of inactive polyribosomes with progressive cell maturation. Electron microscopic autoradiography of rabbit reticulocytes revealed some to be active and others inactive in protein synthesis. A direct correlation between loss of protein-synthesizing activity with loss of ribosomes could not be demonstrated (Miller and Manusbach, 1966). Inactive reticulocytes were shown to contain ribosomes and even polyribosomes indicating that these were not rate-limiting in protein biosynthesis. Of great interest to us was the description of active cells showing deep invaginations and the reference to the work of Bessis and Bricka (1952) who described young reticulocytes as motile cells with amoeboid-type movements. Such differences in surface characteristics may well be responsible for the resolution of reticulocytes by counter-current distribution reported here.

These results, together with those reported earlier (Walter et al., 1966), lead us to think that the "active, young" reticulocytes are those associated with the left end of the normal red cell distribution curve while the "inactive, old" reticulocytes are those found on the right. The dramatic change(s) that must occur to cause these cells to, essentially, jump from one side of the normal distribution curve to the other is still open to specula-

tion. Perhaps the interaction of active cells with transferrin which subsequently leaves the cells (Morgan, 1964) contributes to this change in surface charge and morphology.

In conclusion: we have found that reticulocytes from phenylhydrazine-treated rats are highly heterogeneous and can be resolved by counter-current distribution into at least two cell populations, one of which has the surface properties of mature young erythrocytes. We believe, although this is subject to future investigation, that we have separated reticulocytes by counter-current distribution into populations representing different degrees of maturation, a subject which has found much discussion in the recent literature (Borsook et al., 1962; Glowacki and Millette, 1965; Rowley, 1965; Miller and Maunsbach, 1966).

Acknowlegments

We thank Miss Agneta Persson for expert technical assistance during the course of this work. This investigation was supported by the Veterans Administration, by a research grant (No. HE 08304) from the National Heart Institute, United States Public Health Service and from Malmfonden-Swedish Foundation for Scientific and Industrial Development.

References

Albertsson, P. Å., Partition of cell particles and macromolecules, John Wiley and Sons, New York (1960).
Albertsson, P. Å. and Baird, G. D., Exptl. Cell Research 28, 296 (1962).
Albertsson, P. Å., Analyt. Biochem. 11, 121 (1965).
Bessis, M. and Bricka, M., Rev. Hematol. 7, 407 (1952).
Borsook, H., Lingrel, J. B., Scaro, J. L. and Millette, R. L., Nature 196, 347 (1962).
Glowacki, E. R. and Millette, R. L., J. Mol. Biol. 11, 116 (1965).
Leif, R. C. and Vinograd, J., Proc. Nat. Acad. Sci. U.S. 51, 520 (1964).
Miller, A. and Maunsbach, A. B., Science 151, 1000 (1966).
Morgan, E. H., Brit. J. Haematol. 10, 442 (1964).
Rowley, P. T., Nature 208, 244 (1965).
Simon, E. R. and Topper, Y. J., Nature 180, 1211 (1957).
Walter, H., Selby, F. W. and Brake, J. M., Biochem. Biophys. Res. Commun. 15, 497 (1964).
Walter, H., Winge, R. and Selby, F. W., Biochim. Biophys. Acta 109, 293 (1965).
Walter, H. and Selby, F. W., Biochim. Biophys. Acta 112, 146 (1966).
Walter, H., Selby, F. W. and Garza, R., Biochim. Biophys. Acta (in press).