Senescent erythrocytes: Isolation of *in vivo* aged cells and their biochemical characteristics

(biotinylation/aging/adenosine 5'-triphosphate/glycolytic enzymes)

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ABSTRACT Rabbit erythrocytes were covalently labeled with biotin and then infused into the donor animal. Up to 60 days after infusion, the biotinylated cells were selectively isolated by their affinity for avidin. These aged erythrocytes, which are within 10 days of death, were analyzed for several biochemical parameters. The 2,3-bisphosphoglycerate and glutathione levels of these cells were constant with age; however, the adenosine 5'-triphosphate concentrations increased approximately 75% as the cells approached the end of their life span. The activities of several glycolytic enzymes did not change with age.

The mechanisms of erythrocyte senescence are poorly understood (1). Clearly, erythrocytes have a well-defined life span; however, the study of the aging processes in these cells has been hindered by the inability to definitively isolate senescent erythrocytes. A myriad of publications have reported age-related differences in various biochemical parameters of erythrocytes (for a review, see ref. 1). However, the majority of these are based on the separation of cells over density gradients with the underlying assumption that the most dense fractions represent the old cells, an assumption that has been questioned (2, 3).

Two methods that are independent of the physical separation techniques outlined above have been reported for the isolation of senescent erythrocytes. Allison and Burn (4) transfused blood group O erythrocytes into normal blood group A individuals. At subsequent time points, the O cells were recovered after agglutination of the host A erythrocytes. Unfortunately, the technique was hindered by technical difficulties. More recently, Ganzoni *et al.* (5) developed a clever procedure for hypertransfusing rats that allows the isolation of a highly enriched fraction of aged erythrocytes. Although this technique has been utilized by Mueller *et al.* (6), it is quite labor intensive and consumes large numbers of experimental animals.

Recently, we described a technique for the isolation of specific subpopulations of rabbit erythrocytes (7). In this procedure, erythrocytes are biotinylated by reaction with *N*-hydroxysuccinimidobiotin (NHS-biotin); these cells have a normal survival upon replacement into the rabbit. The biotin label is stable on the erythrocyte membrane and allows the selective recovery of these cells any time before their *in vivo* death. Recovery is achieved by attachment of the biotinylated cells to avidin on a solid support. In the present report, we utilize this technique to isolate senescent rabbit erythrocytes within 10 days of their normal *in vivo* death and describe several biochemical parameters of these senescent cells.

MATERIALS AND METHODS

NHS-biotin was purchased from Pierce; gelatin was from Difco; polystyrene beads (no. 4023; 200-400 mesh, 12% cross-linked) were from Polyscience (Warrington, PA); dimethylformamide was from Eastman Kodak; potassium [¹⁴C]cyanate and [³²P]phosphate were from Amersham; and avidin was obtained from Calbiochem. All other biochemicals were from Sigma.

Preparation of Young Erythrocyte Cohorts. In some experiments, young erythrocyte populations were used for biotinylating and replacement into donor rabbits. The advantage of using a young cohort of cells is that a larger percentage of the cells will still be circulating 50-60 days after replacement and therefore will allow recovery of greater numbers of senescent cells. Two procedures were used to produce young cell populations—phenylhydrazine treatment and repeated phlebotomy. Phenylhydrazine hydrochloride (25 mg) was subcutaneously injected into a rabbit on days 0, 2, and 3. On day 13, 50 ml of blood was collected in heparin for biotinylating. The reticulocyte count for these preparations averaged more than 50% on day 8. For the repeated phlebotomy procedure, 50-60 ml of blood was drawn from the rabbit on day 0, 2, and 4. The sample for biotinylating was taken on day 14. To protect against iron deficiency during repeated phlebotomy, each rabbit received 50 mg of iron-dextran intramuscularly on days -7, -5, -3, 0, 2, and 4.

Preparation and Infusion of Biotinylated Erythrocytes. Rabbit erythrocytes were washed, labeled with [¹⁴C]cyanate, treated with NHS-biotin, and infused into the donor rabbit exactly as described (7). The average level of biotinylating was approximately 25,000 biotin molecules per erythrocyte.

Avidin-Coated Polystyrene Beads. The avidin-coated polystyrene beads for the recovery of biotinylated erythrocytes were prepared by a modification of the previously published procedure (7). Polystyrene beads (500 mg) were coated with biotinylated-gelatin and avidin as described (7) except that 500 µg of avidin was used instead of 750 µg. The beads were then treated briefly with succinic anhydride, which was found to lower the nonspecific erythrocyte binding. The 500 mg of avidin/biotinylated-gelatin-coated polystyrene beads in approximately 4 ml of phosphate-buffered saline (PBS) (0.15 M NaCl/10 mM sodium phosphate, pH 7.4) was incubated with 0.18 mg of succinic anhydride dissolved in dioxane (25 mg/ml). The mixture was rotated at 4°C for 10 min, after which the beads were washed four times with PBS.

Recovery of Biotinylated Cells. At various times after infusion of the biotinylated erythrocytes into the donor

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Abbreviation: NHS-biotin, *N*-hydroxysuccinimidobiotin.

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animal, blood samples were drawn; erythrocytes were freed of leukocytes (8) and then washed three times with PBS. One milliliter of packed erythrocytes was added to 0.5 g of succinylated-avidin/biotinylated-gelatin-coated polystyrene beads in a 50-ml beaker; the total volume was approximately 2 ml and contained 10 mM glucose. The slurry was mixed gently on a rocking platform at room temperature for 60 min. The avidin-coated beads with bound erythrocytes were then washed four times with PBS by gentle centrifugation at $30 \times g$ for 5 sec. To remove the attached erythrocytes from the avidin-coated beads, 2 mg of collagenase (900 units) in 10 ml of PBS was mixed with the beads and incubated at 37°C for 15 min. The mixture was briefly centrifuged at $30 \times g$ for 5 sec, and the supernatant containing the released erythrocytes was collected. This supernatant was centrifuged twice at $30 \times g$ for 5 sec to remove contaminating beads. The released, biotinylated erythrocytes were then washed three times with PBS. [^{14}C]Cyanate/hemoglobin ratios were determined as detailed (7). Control erythrocytes for each experiment were unfractionated cells from the same rabbit.

Erythrocyte Intermediates. Biotinylated erythrocytes, recovered as detailed above, were lysed in water. The absorbance of an aliquot of this hemolysate was immediately measured at 410 nm for the estimation of hemoglobin. One milliliter of the hemolysate was then mixed with 0.5 ml of ice-cold 20% perchloric acid. After mixing and a brief centrifugation, 1 ml of the supernatant solution was neutralized with 3 M K_2CO_3 as described (9). ATP concentrations were determined fluorometrically by the methods of Williamson and Corkey (10) and Beutler (9). ATP concentrations were calculated from the fluorescence reading before and after the addition of hexokinase; ATP standards were used to calibrate the assay in each experiment. The concentration of 2,3-bisphosphoglycerate was also determined on this perchloric acid extract using the catalytic cycling method of Beutler (9). ATP levels in perchloric acid extracts were verified by HPLC analysis by the method of McKeage and Brown (11).

The total glutathione concentration of the hemolysate was measured spectrophotometrically by the cycling method of Tietze (12). The NADPH solution for this assay was freshly prepared, and the concentration was measured spectrophotometrically at 340 nm. Oxidized glutathione (glutathione disulfide; GSSG), which was used as a standard, was quantitated by the standard glutathione (GSH) reductase assay (9). For the actual glutathione cycling assay, 100 μl of 1.2 mM 5,5'-dithiobis(2-nitrobenzoic acid), 20 μl of glutathione

reductase at 10 units/ml, and the unknown sample or various amounts of glutathione disulfide standard were mixed in a total volume of 0.9 ml. After incubation at 37°C for 10 min, 100 μl of 2 mM NADPH was added, and the reaction was monitored at 412 nm for 10 min.

For the ATP and glutathione determinations, seven rabbits were monitored. Four had been pretreated by repeated phlebotomy, and three were pretreated with phenylhydrazine. The 2,3-bisphosphoglycerate levels were determined over time in three rabbits that were treated with phenylhydrazine.

Erythrocyte Enzyme Levels. Hexokinase, glucose phosphate isomerase, phosphofructokinase, aldolase, phosphoglycerate kinase, pyruvate kinase, lactic dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase were measured by the method of Beutler (9).

[^{32}P]Phosphate Incubations. Biotinylated erythrocytes were recovered 51 days after reinfusion. Approximately 15 μl of packed erythrocytes were washed two times in 0.15 M NaCl/10 mM Hepes, pH 7.4, and left in 2 ml of the same buffer. Glucose (5 mM) was added along with 15 μCi of carrier-free [^{32}P]phosphate. The mixture was incubated at 37°C for 15 min and then stopped with 2 ml of 4% perchloric acid; at this point, 50 μl of control erythrocytes was added to act as carrier during the centrifugation of the protein precipitate and subsequent chromatography step. Precipitated protein was spun down, and the perchloric acid supernatant was neutralized as detailed above. The extract was chromatographed as detailed (13).

RESULTS

Fig. 1a shows the general experimental scheme for the biotinylation, infusion, and subsequent recovery of aged rabbit erythrocytes (7), while Fig. 1b schematically diagrams the beads used to recover the biotinylated erythrocytes. The polystyrene beads were coated with biotinylated-gelatin and succinylated-avidin; the gelatin anchor is cleavable by collagenase, allowing the eventual recovery of intact erythrocytes from the beads (7).

The selectivity of the recovery procedure is shown in Table 1. At the time of biotinylation, the erythrocytes were also labeled with [^{14}C]cyanate, which has been shown to be a stable tag for erythrocytes (14). Before infusion of the biotinylated, ^{14}C -labeled rabbit erythrocytes, the ratio of [^{14}C]cyanate to hemoglobin was determined. While the total

FIG. 1. (A) Overall experimental scheme for biotinylation and recovery of rabbit erythrocytes as described in the text. (B) Avidin-coated beads used to bind biotinylated erythrocytes. The bead-gelatin linkage is cleavable with collagenase.

Table 1. Biotinylated erythrocytes recovered after *in vivo* circulation

Rabbit	$[^{14}\text{C}]$ Cyanate after infusion, cpm per mg of Hb (% of control)			
	Day 0	Day 50	Day 56	Day 60
1	128.7 (100%)	ND	92.3 (71.7%)	94.6 (73.5%)
2*	269.8 (100%)	253.9 (94.1%)	ND	225.3 (83.5%)
3*	304.2 (100%)	285.4 (93.8%)	ND	292.0 (96.0%)

Data are expressed as cpm of $[^{14}\text{C}]$ cyanate per mg of hemoglobin; the percentages within the parentheses are the percentage of the day 0 sample. The data shown for day 0 were obtained on samples before replacement of erythrocytes in donor animals.

*The erythrocytes for biotinylation in these cases were obtained after phenylhydrazine-induced anemia and, therefore, initially represented a young population of erythrocytes. ND, not determined.

level of circulating ^{14}C will decrease as cells are sequestered by the animal, this ^{14}C /hemoglobin ratio should remain constant for the surviving biotinylated cells during the entire experiment. These data are shown in Table 1 and indicate that the recovered, biotinylated cells up to 60 days after infusion have a nearly constant amount of $[^{14}\text{C}]$ cyanate per gram of hemoglobin. In addition, these data show that the nonspecific adsorption of nonbiotinylated erythrocytes to the avidin recovery beads is minimal because any nonspecific adsorption would dramatically alter the ^{14}C /hemoglobin ratio.

Biotinylated erythrocytes were recovered and monitored for the levels of several erythrocyte intermediates and enzyme activities over the latter life span of these cells. The concentrations of ATP, glutathione, and 2,3-bisphosphoglycerate in control and aged cells were measured between 32 and 60 days after infusion of biotinylated cells into a rabbit. The glutathione and 2,3-bisphosphoglycerate levels did not change over the life span of the erythrocytes (Fig. 2); however, the ATP concentration gradually increased from day 42 through day 60. At day 60 the ATP level for the recovered cells was 8.34 ± 0.54 $\mu\text{mol/g}$ of hemoglobin (mean $\pm$ 1 SEM; $n = 7$), whereas the control level was 4.74 ± 0.13 $\mu\text{mol/g}$ of hemoglobin. This difference was significant at the $P < 0.001$ level.

The ATP concentrations in Fig. 2 were determined enzymatically with yeast hexokinase; since hexokinase can react with other nucleoside triphosphates (15), it was necessary to verify the identity of the ATP. Recovered erythrocytes, at day 51, were briefly incubated with $[^{32}\text{P}]$ phosphate, followed by perchloric acid precipitation and Dowex-1 chromatography by standard techniques (13). The chromatographic profiles shown in Fig. 3 demonstrate that the senescent cells do not contain any abnormal triphosphate peaks, supporting the observation that the increased ATP concentration measured enzymatically is in fact adenosine 5'-triphosphate. An unidentified peak, labeled "X," occurs in these chromatograms. Control experiments documented that this peak is a variable contaminant of the commercial $[^{32}\text{P}]$ phosphate and is not a metabolic product. In addition, the ATP levels in several of the perchloric acid extracts were also quantitated by high-pressure liquid chromatography (11). These separate ATP determinations agreed with the enzymatic analyses (data not shown).

A variety of erythrocyte enzymes associated with the glycolytic and hexosemonophosphate pathways were measured in this study. The data presented in Table 2 show that none of these activities changed significantly over the 60-day life span of the rabbit erythrocyte.

FIG. 2. Metabolites from senescent and control erythrocytes. At the days specified after reinfusion, biotinylated senescent erythrocytes (○) were isolated and analyzed for three intermediates, glutathione (Top), 2,3-bisphosphoglycerate (Middle), and ATP (Bottom). Control erythrocytes (●) were assayed concurrently. Each data point represents the mean $\pm$ 1 SEM. For glutathione and ATP, $n = 7$; for 2,3-bisphosphoglycerate, $n = 3$. All intermediates are expressed as $\mu\text{mol/g}$ of hemoglobin.

DISCUSSION

This report details several biochemical properties of senescent erythrocytes isolated by a new technique. The isolation method involves the biotinylation of rabbit erythrocytes with NHS-biotin, the infusion of these derivatized cells into donor animals, and their eventual recovery after *in vivo* aging (7) (Fig. 1). The advantage of this technique is that it allows the definitive isolation of aged erythrocytes. As detailed in the introduction, the study of erythrocyte senescence has been seriously hampered by the lack of procedures for isolating aged cells; as a result, the field has often been confused by contradictory and questionable data. Therefore, the present technique offers an opportunity to study the properties of *in vivo* aged erythrocytes free of many of the past criticisms related to the isolation procedure. One point concerning terminology needs to be clarified. In this report, senescent, biotinylated erythrocytes are often referred to as senescent erythrocytes with the assumption that biotinylation has not adversely affected these cells. At this time there is no indication to the contrary; in fact, the normal survival of the biotinylated erythrocytes (7) strongly argues in favor of their normal function.

FIG. 3. ^{32}P -labeled metabolites from senescent and control erythrocytes. Cells were incubated with [^{32}P]phosphate, and the ^{32}P -labeled metabolites were separated on Dowex-1 as described. (A) Senescent erythrocytes (51 days after reinfusion). (B) Control erythrocytes. The unidentified peak X is a contaminant of the [^{32}P]phosphate and is not a metabolic product.

Vacha (16) reported that the life span of erythrocytes in rabbits is approximately 60 days; however, this limit is often not reached by a given cell because of the significant random destruction of erythrocytes (0.5% per day) that occurs in these animals. Our own data with [^{14}C]cyanate-labeled erythrocytes would suggest that the erythrocyte life span may be a little longer at 60–70 days (data not shown). The data presented in Table 1 confirm the utility of this biotinyl-

ated erythrocyte technique. This table shows the isolation of ^{14}C -labeled, biotinylated erythrocytes 50–60 days after infusion—i.e., within 0 to 10 (or perhaps 0 to 20) days of cell death. The data are presented as the ratio of [^{14}C]cyanate to hemoglobin in the recovered cells; this ratio should remain constant during the life span of the erythrocytes. Additionally, any contamination of the isolated cells with nonbiotinylated and non- ^{14}C -labeled cells will dramatically alter the ^{14}C /hemoglobin ratio. Therefore, these data not only demonstrate the accuracy of the recovery technique but also substantiate our earlier report that the biotin label on the erythrocyte is stable *in vivo* (7).

The current experiments use a procedure for the recovery of biotinylated cells that is slightly modified from our earlier report (7). Succinylation of the avidin on the solid support was found to dramatically reduce the level of nonspecific adherence of control erythrocytes to the support. Earlier reports had shown that succinylated-avidin decreased the nonspecific background in assays using avidin-enzyme conjugates without significantly affecting the avidin's affinity for biotin (17). In contrast, we found that the succinylation protocols used previously resulted in an avidin that worked very poorly in this recovery system. However, if the succinylation was performed at neutral pH and at low temperature, the derivatized avidin was still as effective as the native material for binding biotinylated erythrocytes, and the nonspecific binding was nearly eliminated.

Erythrocytes were isolated 30–60 days after infusion and analyzed for several key intermediates and enzymes. It should be mentioned that the population of cells isolated 30 days after biotinylation and infusion represents a wide range of ages—i.e., 30–60 days old. However, the use of young cohorts of erythrocytes does skew this distribution towards the younger age limit. Only at the end of the experiment do the isolated cells represent a relatively narrow range of ages. However, even the 55-day sample will contain cells that are from 55 to 60 days old, and this fairly narrow range of ages may represent a time period of dramatic changes in the aging erythrocyte. Fig. 2 shows the data for three key intermediates in the senescent erythrocytes. As mentioned above, the total glutathione and 2,3-bisphosphoglycerate levels do not change during the aging of these cells. These results are contradictory to earlier reports of declines in both of these metabolites in "aged" human erythrocytes isolated by density gradient centrifugation (18–22). These discrepancies may be related to species differences or to the inadequacies of the density gradient separation methods. The current data would suggest that the antioxidant defenses of the senescent erythrocyte are not impaired by a lack of glutathione, nor are the cell's oxygen dissociation characteristics affected by a diminution of 2,3-bisphosphoglycerate levels.

Table 2. Enzyme activities of senescent erythrocytes

Enzyme	Enzyme activity, $\mu\text{mol}/\text{min}$ per g of hemoglobin	
	Control	Aged
Hexokinase	0.97 ± 0.19	1.10 ± 0.40 ($n = 5$)
Glucose phosphate isomerase	162.8 ± 5.8	137.6 ± 27.8 ($n = 4$)
Phosphofructokinase	8.27 ± 2.58	6.45 ± 3.30 ($n = 4$)
Aldolase	4.63 ± 2.13	3.75 ± 1.02 ($n = 3$)
Phosphoglycerate kinase	135.6 ± 25.5	162.3 ± 25.0 ($n = 5$)
Pyruvate kinase	6.64 ± 0.78	5.84 ± 2.11 ($n = 4$)
Lactate dehydrogenase	217.7 ± 44.3	230.0 ± 51.7 ($n = 3$)
Glucose-6-phosphate dehydrogenase	11.27 ± 1.99	10.25 ± 0.70 ($n = 3$)
6-Phosphogluconate dehydrogenase	4.23 ± 0.37	4.14 ± 0.17 ($n = 4$)

Enzyme values are the mean and one SD. Aged cells were isolated between 50 and 60 days after biotinylation and infusion. Two of the rabbits analyzed were initially phenylhydrazine-induced; the remainder of the rabbits were untreated before biotinylation. There was no difference in the enzyme values for the aged erythrocytes between the phenylhydrazine and untreated rabbits.

Also shown in Fig. 2 are the ATP levels for the senescent erythrocytes. Surprisingly, these levels increase rather dramatically during the latter stages of the cell's life. Again, this is contradictory to the results published previously with density-fractionated human erythrocytes, which showed both lowered (20, 21) and normal values (23, 24) for ATP in aged cells. Of particular concern in the determination of ATP levels is the possible impact of the isolation procedure. However, the erythrocytes were isolated in the presence of glucose to allow them to maintain ATP. In addition, the control levels of ATP shown in Fig. 2 agree with earlier reports (24, 25).

The increased levels of ATP shown in Fig. 2 are too large to result simply from the conversion of existing ADP or AMP to ATP (25) but rather must represent the net synthesis of adenine nucleotides as the cell ages. The rabbit erythrocyte is similar to the human cell in that it cannot synthesize adenine nucleotides *de novo* but does have effective salvage pathways that will allow it to use available adenine or adenosine to synthesize these nucleotides (26). Actually, the rabbit erythrocyte is more flexible than the human cell in that it can also use inosine for the synthesis of adenine nucleotides (27). It is interesting that a group of disorders exists in humans that is characterized by high ATP levels in erythrocytes. Some of these are caused by high pyruvate kinase levels (28), while most are of unknown etiology (29, 30). However, no clear connection has ever been established between the elevated ATP and any disease state.

Table 2 presents the activities from control and senescent erythrocytes of nine enzymes related to energy metabolism. No changes are seen in these enzymes with age. As before, these data are in contrast to previous reports of enzyme levels changing with erythrocyte age for density-fractionated cells (21, 31, 32) but do agree with a report by Ganzoni *et al.* (33), where very modest decreases in pyruvate kinase and glucose-6-phosphate dehydrogenase were found in aging rat erythrocytes. Our results are in agreement with the proposal of Beutler (3) that, after reticulocyte maturation, the decline in erythrocyte enzyme levels with age is very gradual—in this case, not detectable.

One goal of this project has been to find an enzyme level that is a true indicator of erythrocyte age. So far this aim has not been achieved, although the increased level of adenine nucleotides suggests a key area for investigation. These data are, however, a step towards a definitive assessment of the various metabolic capabilities of senescent erythrocytes from rabbits and may help to define the factors involved with the death of these cells.

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- Clark, M. R. & Shohet, S. B. (1985) *Clin. Haematol.* **14**, 223–257.
- Morrison, M., Jacuson, C. W., Mueller, T. J., Huang, T., Docktor, M. E., Walker, W. S., Singer, J. A. & Edwards, H. H. (1983) *Biomed. Biochim. Acta* **42**, 107–111.
- Beutler, E. (1985) *Br. J. Haematol.* **61**, 377–384.
- Allison, A. C. & Burn, G. P. (1955) *Br. J. Haematol.* **1**, 291–303.
- Ganzoni, A. M., Oakes, R. & Hillman, R. S. (1971) *J. Clin. Invest.* **50**, 1373–1378.
- Mueller, T. J., Jackson, C. W., Docktor, M. E. & Morrison, M. (1987) *J. Clin. Invest.* **79**, 492–499.
- Suzuki, T. & Dale, G. L. (1987) *Blood* **70**, 791–795.
- Beutler, E., West, C. & Blume, K. G. (1976) *J. Lab. Clin. Med.* **88**, 328–333.
- Beutler, E. (1983) *Red Cell Metabolism: A Manual of Biochemical Methods* (Grune & Stratton, Orlando, FL), 3rd Ed.
- Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol.* **13**, 434–513.
- McKeag, M. & Brown, P. R. (1978) *J. Chromatogr.* **152**, 253–254.
- Tietze, F. (1969) *Anal. Biochem.* **27**, 502–522.
- Bartlett, G. R. (1968) *Biochim. Biophys. Acta* **156**, 221–230.
- Eschbach, J. W., Korn, D. & Finch, C. A. (1977) *J. Lab. Clin. Med.* **89**, 823–828.
- Purich, D. L., Fromm, H. J. & Rudolph, F. B. (1973) *Adv. Enzymol.* **39**, 249–326.
- Vacha, J. (1983) in *Red Blood Cells of Domestic Mammals*, eds. Agar, N. S. & Board, P. G. (Elsevier, New York), pp. 67–132.
- Finn, F. M., Titus, G., Montibeller, J. A. & Hofmann, K. (1980) *J. Biol. Chem.* **255**, 5742–5746.
- Abraham, E. C., Taylor, J. F. & Lang, C. A. (1978) *Biochem. J.* **174**, 819–825.
- Imanishi, H., Nakai, T., Abe, T. & Takino, T. (1985) *Mech. Ageing Dev.* **32**, 57–62.
- Cohen, N. S., Ekholm, J. E., Luthra, M. G. & Hanahan, D. J. (1976) *Biochim. Biophys. Acta* **419**, 229–242.
- Fornaini, G., Magnani, M., Fazi, A., Accorsi, A., Stocchi, V. & Dacha, M. (1985) *Arch. Biochem. Biophys.* **239**, 352–358.
- Sass, M. D., Caruso, C. J. & O'Connell, D. J. (1965) *Clin. Chim. Acta* **11**, 334–340.
- Kirkpatrick, F. H., Muhs, A. G., Kostuk, R. K. & Gabel, C. W. (1979) *Blood* **54**, 946–950.
- Shojania, A. M., Israels, L. G. & Zipursky, A. (1968) *J. Lab. Clin. Med.* **71**, 41–47.
- Ohyama, H. & Minakami, S. (1967) *J. Biochem.* **61**, 103–107.
- Lowy, B. A., Williams, M. K. & London, I. M. (1961) *J. Biol. Chem.* **236**, 1439–1441.
- Mager, J., Hershko, A., Zeitlin-Beck, R., Shoshani, T. & Razin, A. (1967) *Biochim. Biophys. Acta* **149**, 50–58.
- Staal, G. E. J., Jansen, G. & Roos, D. (1984) *J. Clin. Invest.* **74**, 231–235.
- Brewer, G. J. (1965) *Biochem. Biophys. Res. Commun.* **18**, 430–434.
- Beutler, E. (1978) in *Hemolytic Anemia in Disorders of Red Cell Metabolism*, ed. Wintrobe, M. M. (Plenum, New York), pp. 199–248.
- Seaman, C., Wyss, S. & Piomelli, S. (1980) *Am. J. Hematol.* **8**, 31–42.
- Jansen, G., Hepkema, B. G., van der Vegt, S. G. L. & Staal, G. E. J. (1986) *Scand. J. Haematol.* **37**, 189–195.
- Ganzoni, A. M., Barras, J. P. & Marti, H. R. (1976) *Vox Sang.* **30**, 161–174.