Influence of Mouse Age and Erythrocyte Age on Glutathione Metabolism

By EDATHARA C. ABRAHAM,* JOHN FULLER TAYLOR and CALVIN A. LANG†
*Department of Biochemistry and the Biomedical Aging Research Program, University of Louisville,
Health Sciences Center, Louisville, KY 40232, U.S.A.*

(Received 23 December 1977)

In order to determine whether the biological age of a mouse influences erythrocyte metabolism and erythrocyte aging *in vivo*, blood samples were collected from male C57/BL6J mice of different biological ages ranging from mature (10 months) to 'very old' (37 months). In the very old mouse, compared with the mature mouse, the erythrocyte survival time was decreased, erythrocyte densities were increased, the concentrations of total free thiol and reduced glutathione, and glutathione reductase activity were decreased. Erythrocytes were separated into different density (age) groups by phthalate ester two-phase centrifugation or by albumin density-gradient centrifugation. The density–age relationship of erythrocytes was established by pulse-labelling with ^{59}Fe *in vivo* and by subsequent determinations of specific radioactivity of erythrocyte fractions of different densities prepared during a chase period of 60 days. The age of erythrocytes in mice of all ages was directly related to density. Also, in older erythrocytes compared with younger erythrocytes, decreased concentrations of total free thiol and reduced glutathione, and decreased glutathione reductase activity were observed. These were the lowest in the old erythrocytes of very old mice. These results in aging erythrocytes from aging mice suggest that the glutathione status of the erythrocyte may be an index of aging, not only of the cell but also of the organism.

Aging at the molecular, cellular and organismic levels and the interrelationships among these are extremely complex problems. For a study of the cellular age-dependent changes in metabolism and their relationship to the age of the organism, the erythrocyte has proved to be a very good system in itself and as a model, especially owing to its fairly short life and its characteristic inability to reproduce itself. In the past, most of the investigations of the biochemical modification of erythrocytes during aging *in vivo* have been performed on human cells. However, for such a study the genetics and the aging of the organism have to be taken into account. Therefore we used C57/BL6J mice, a genetically controlled inbred strain of mice of known biological age maintained in a well-controlled environment.

Glutathione contributes largely to the reducing potential of erythrocytes. It is also known that GSH can play an important role in protecting the thiol groups of haemoglobin, structural proteins of the erythrocyte membrane and several glycolytic enzymes [for a review of these see Fornaini (1967)]. Moreover,

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione.

* Present address: Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30901, U.S.A.

† To whom reprint requests should be addressed.

evidence from our laboratory indicates that NADPH content and activity of NADP⁺-linked enzymes decrease with increasing biological age of the mouse and the mosquito (Stephan *et al.*, 1966; Lang & Acree, 1967; Lang & Stephan, 1967).

The present study was undertaken to determine if the age of the organism influences erythrocyte metabolism and erythrocyte aging *in vivo*. In the present paper we report (1) the changes in the erythrocyte lifespan, erythrocyte density distribution, concentrations of total free thiols and GSH, and glutathione reductase activity in unfractionated erythrocytes from mice of various ages; (2) the changes in the concentrations of thiols and GSH and in glutathione reductase activity during aging *in vivo* of erythrocytes in aging mice.

Materials and Methods

Chemicals

NADPH and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from Nutritional Biochemical Division, ICN Pharmaceuticals, Cleveland, OH, U.S.A. GSSG and yeast glutathione reductase [NAD(P)H-oxidized glutathione oxidoreductase, EC 1.6.4.2] were from Calbiochem, La Jolla, CA, U.S.A.; *p*-chloromercuribenzoic acid, GSH and bovine serum albumin (Cohn fraction V) were purchased from

Sigma Chemical Co., St. Louis, MO, U.S.A. Dibutyl phthalate and dimethyl phthalate were from Matheson, Coleman and Bell, Norwood, OH, U.S.A. A sterile solution of [^{59}Fe]ferrous citrate ($25\mu\text{Ci}/\mu\text{g}$ of Fe) was obtained from Abbott Laboratories, Cincinnati, OH, U.S.A. Imferon (iron dextran) was a product of Lakeside Laboratories, Milwaukee, WI, U.S.A.

Animals

Male C57/BL6J mice of various ages from our aging colony were used. These mice were obtained originally from Jackson Laboratory, Bar Harbor, ME, U.S.A., as retired breeders or as weanlings and were maintained on a Purina Laboratory Chow diet with free access to food and water. Mice used for this study weighed between 28 and 40 g. The median survival time of this mouse population was 30 months. From this information we have classified the 11–19-month-old mice as 'mature', 27–30-month-old mice as 'old' and 33–37-month-old mice as 'very old'.

Determination of erythrocyte lifespan

After dilution of the stock ^{59}Fe with 0.9% NaCl, a tracer dose ($0.02\mu\text{Ci/g}$ body wt.) was injected intraperitoneally into each mouse. Blood samples ($25\mu\text{l}$) were drawn by puncturing the retro-orbital sinus with heparinized capillary tubes under light anaesthesia (chloroform/ether). A few animals in each age group were used to establish the number of days required to reach the maximum erythrocyte radioactivity. From these animals blood samples were taken at 6 h and days 1, 2, 4, 6 and 8 after the injection of the tracer (with all age groups the uptake of ^{59}Fe had reached a maximum on day 4). The remaining animals were bled on day 4 after injection of ^{59}Fe and every 2 or 3 weeks thereafter. Starting on day 4 each mouse was given a weekly intraperitoneal injection of non-radioactive iron dextran (0.14mg/g body wt.). Radioactivity of $25\mu\text{l}$ of blood was measured with a gamma scintillation spectrometer (model 132 from Nuclear-Chicago) with a counting efficiency of 5%. Values for c.p.m./ml of cells were calculated by taking the radioactivity on day 4 as 100%. Examples of erythrocyte survival curves are shown in Fig. 1.

Density distribution of erythrocyte population

This was determined by the phthalate-microcapillary technique of Danon & Marikovsky (1964). Phthalate ester mixtures of relative densities ranging from 1.090 to 1.118 with increments of 0.002 between solutions were used to separate the erythrocytes in microcapillary tubes. The median density (d_{50}) values were calculated from density-distribution curves (examples are shown in Fig. 2).

Two-phase centrifugation with phthalate esters

Erythrocytes were separated into different fractions according to their relative density by using phthalate ester mixtures as separating fluids (Danon & Marikovsky, 1964; Brok *et al.*, 1966). The heparinized blood sample was layered over a column of about 2 ml of phthalate ester mixture (relative density usually 1.108) in a silicone-treated Sorvall centrifuge tube (internal diam. 13 mm $\times$ 100 mm) and centrifuged at 25°C and 20000g for 20 min in a model B20 (IEC) centrifuge. The top fraction was further fractionated by successive centrifugations over phthalate ester mixtures of decreasing densities. The top fraction of the final tube (usually d 1.100) was re-centrifuged over a fluid of 1.062 relative density to remove thrombocytes and leucocytes. Erythrocyte fractions were resuspended in 0.9% NaCl.

Bovine serum albumin density-gradient centrifugation

Solutions of 27 and 37% (w/w) bovine serum albumin (Cohn fraction V) were used to prepare a continuous 9 ml gradient in a cellulose nitrate tube (1.5 cm $\times$ 7.6 cm; Beckman) as described by Bishop & Prentice (1966). Heparinized blood (0.1 ml) was layered on top of the gradient, and the tube was centrifuged at 4°C and 60000g for 60 min in a Beckman LS 35 ultracentrifuge. Fractions (1 ml) were collected from the top of the tube with a syringe. Samples were run in duplicate or triplicate to confirm the reproducibility of the technique.

Relationship between the density and the physiological age of erythrocytes

In mice this relationship was established by labelling of erythrocytes *in vivo* with ^{59}Fe . At various days after ^{59}Fe injection, blood samples were collected by killing the animals, and the erythrocytes were separated into different density fractions by albumin-density-gradient centrifugation or by phthalate ester, two-phase centrifugations, and the radioactivity (c.p.m./mg of haemoglobin) of the erythrocyte fractions and of the total erythrocytes were determined. The ratio of the specific radioactivity of the fractions to that of the total cells (fractionation ratio) was plotted against the density of the fraction (Bishop & Prentice, 1966).

Preparation of haemolysate

The erythrocytes were washed three times with 0.9% NaCl solution. The washed cells were haemolysed by addition of 1 vol. of water and 0.5 vol. of toluene, followed by shaking for 8 min and centrifugation at 20000g for 30 min. The clear haemolysate was siphoned off, and the insoluble debris and toluene layers were discarded. The haemolysates were used immediately or stored at 4°C and used within 24 h.

Determination of GSH

The method of Beutler *et al.* (1963), as modified by Prins & Loos (1969), was adapted for samples of 25–100 µl of blood. After 1 min for complete colour development the A_{412} was measured in semi-micro-cuvettes in a Beckman DU spectrophotometer. A standard curve was prepared by substituting 0.02 mM-, 0.05 mM- and 0.10 mM-GSH for the extract and a reagent blank with water.

Determination of total free thiols

Free thiols were determined by spectrophotometric titration with *p*-chloromercuribenzoic acid by the procedure of Boyer (1954). When an undialysed erythrocyte haemolysate was used for these determinations, the total thiols included those from haemoglobin (2.0 mol of thiol/mol of haemoglobin) and GSH.

Glutathione reductase assay

The method of Long & Carson (1961) was used, based on the rate of NADPH oxidation determined spectrophotometrically at 340 nm. The reaction cuvette contained 0.5 ml of 0.4 M-Tris/HCl, pH 7.6, 0.2 M-EDTA, 0.2 ml of 0.033 M-GSSG and 0.1 ml of haemolysate of known haemoglobin concentration to which 0.1 ml of 1.8 mM-NADPH was added to start the reaction. The change in A_{340} was measured at 25°C against a reagent blank for 5 min in a Cary 14 recording spectrophotometer. The rate of oxidation of NADPH under the same conditions in the absence of haemolysate was also determined and subtracted from the reaction rate with the haemolysate. Control experiments were performed by using yeast glutathione reductase. The glutathione reductase activity was expressed as units/mol of haemoglobin (1 unit = 1 µmol of NADPH oxidized/min at 25°C).

Other methods

Haematological data from several mice were obtained by standard laboratory procedures (Wintrobe, 1967). Haemoglobin concentrations of

blood, erythrocyte suspensions or haemolysates were determined by converting haemoglobin into cyan-methaemoglobin by treatment with Drabkin's reagent (Meites & Faulkner, 1962). Student's *t* test was used to evaluate the significance of the difference between means.

Results

A summary of haematological data is given in Table 1. The mouse erythrocytes are considerably microcytic and hypochromic compared with human erythrocytes (mean corpuscular volume = 45 versus 87 fl in human); moreover, the mouse erythrocytes are dense (mean corpuscular haemoglobin concentration > 40%). The 35–37-month, very old mice were anaemic with a packed cell volume of 0.35 (v/v) and a haemoglobin concentration of 13.6 g/100 ml as compared with the corresponding values of 0.42 and 20.8 in 8–11-month-old mice.

The erythrocyte survival curves for mice of various ages are shown in Fig. 1. The 50% survival times (see

Fig. 1. Erythrocyte survival curves for mice of various ages. Percentage radioactivity was calculated by taking the maximum c.p.m./ml of cells as 100%. The peak radioactivities for 11–19-, 30- and 36-month-old mice were $39\,359 \pm 4632$, $50\,544 \pm 4632$ and $41\,965 \pm 4397$ c.p.m./ml of cells respectively. ●, 11–19 months; □, 30 months; ○, 36 months. The arrow indicates the beginning of the chase period on day 4.

Table 1. Haematological findings in mice of different ages
Values are means of the results of three mice in each group. Methods used are described in the text.

Mouse age (months)	Packed cell volume (v/v)	Haemoglobin (g/100 ml)	Mean corpuscular volume (fl/cell)	Mean corpuscular haemoglobin (pg/cell)	Mean corpuscular haemoglobin concentration (g/100 ml)	$10^{-6} \times$ No. of erythrocytes (per µl)	$10^{-3} \times$ No. of leucocytes (per µl)
8–11	0.42	20.8	47.5	21.8	45	7.7	10.0
16–19	0.40	18.6	47.7	22.0	45	9.4	7.8
27–30	0.38	16.3	43.1	20.2	47	8.0	8.5
35–37	0.35*	13.6*	41.5	20.6	50	6.9	13.4

* $P < 0.05$ compared with 8–11-month-old group.

Table 2. *Changes in characteristics of whole erythrocyte populations from mice of different ages*
The numbers in parentheses denote the number of mice used. Methods used are described in the text. Results are expressed as mean values $\pm$ standard error of the mean.

Mouse age (months)	(Survival time) ₅₀ (days)	d_{50}	Total free thiols (mol/mol of haemoglobin)	GSH (mmol/litre of erythrocytes)	$10^{-5} \times$ Glutathione reductase activity (units/mol of haemoglobin)
10	52 $\pm$ 2 (6)	1.1078 $\pm$ 0.0007 (7)	2.77 $\pm$ 0.06 (14)	2.34 $\pm$ 0.11 (12)	2.06 $\pm$ 0.05 (15)
19	52 $\pm$ 2 (3)	1.1065 $\pm$ 0.0006 (4)	2.77 $\pm$ 0.08 (5)	2.71 $\pm$ 0.15 (7)	2.26 $\pm$ 0.10 (6)
30	38* $\pm$ 1 (4)	1.1089 $\pm$ 0.0011 (4)	2.65 $\pm$ 0.07 (9)	2.33 $\pm$ 0.04 (6)	2.68 $\pm$ 0.18 (6)
33		1.1103 $\pm$ 0.0008*(3)	2.34 $\pm$ 0.04†(5)	1.80 $\pm$ 0.07*(5)	1.57 $\pm$ 0.07†(3)
37	20† $\pm$ 1 (4)	1.1117 $\pm$ 0.0010†(4)	2.21 $\pm$ 0.01†(3)	1.38 $\pm$ 0.04†(6)	1.27† (2)

* $P < 0.01$ compared with 10-month-old group.

† $P < 0.001$ compared with 10-month-old group.

Fig. 2. Typical density distribution curves for erythrocytes of mice of different ages

(a) ●, 19-, △, 30- and ○, 37-month-old mice. From the height of the erythrocyte column under and above the separating fluid in the microcapillary tube the percentage of cells (cumulative percentage of cells) under the separating fluid was calculated and plotted against the density of the separating fluid. (b) From the density distribution curves the density corresponding to 50 cumulative percentage (median density or d_{50}) is plotted against mouse age. Each point is the mean of the results from three to six mice.

values in Table 2) determined from the curves are 52, 38 and 20 days respectively for 11–19-month-old (mature), 30-month-old (old) and 36–37-month-old (very old) mice. There is a decrease in these erythrocyte-survival times of about 25% in old mice and 60% in very old mice. Contrary to what is generally observed in the human, the erythrocyte survival curves in the mouse show an early exponential disappearance of radioactivity. This might mean that a significant number of erythrocytes in mice is randomly destroyed.

Erythrocyte density distribution curves were

Fig. 3. Changes in total free thiols (○), GSH (●), glutathione reductase activity (△), and erythrocyte lifespan (□) with increasing mouse age

The percentage change was calculated, based on the results for 10-month-old mice taken as 100%. For absolute values refer to Table 2. Each point is the mean $\pm$ S.E.M. of the results from three to fourteen mice.

obtained for mice of ages 10–37 months. Fig. 2(a) shows three such curves, obtained for a 19-, a 30- and a 37-month-old mouse. The curves for the two last are shifted to the right, to a higher density. The median density (d_{50}) values were obtained from such curves for each mouse, and the data indicate (Fig. 2b) that there is a significant increase in d_{50} from 30 months. Compared with 10-month-old mice, at 30, 33 and 37 months old, there is an increase in d_{50} of 0.0011, 0.0025 ($P < 0.01$) and 0.0039 ($P < 0.001$) respectively (Table 2).

We determined the concentrations of GSH, total free thiols (GSH + haemoglobin-SH) and glutathione reductase activity in the erythrocytes from the mice of different ages. The data are presented in Fig. 3 and Table 2. After 30 months, there are significant

decreases in total free thiol and GSH contents and glutathione reductase activity, which correlate well with the increase in erythrocyte density and the decrease in erythrocyte lifespan. It is noteworthy that GSH increases between 10 and 20 months and then gradually decreases whereas glutathione reductase activity increases until 30 months and rapidly decreases thereafter. The magnitude in the percentage change in total free thiols is smaller than that of GSH because the total free thiol values of an undialysed haemolysate include the reactive thiol groups of haemoglobin (2.0 mol/mol of haemoglobin) and GSH. However, the total free thiol values of dialysed haemoglobin solution did not show any significant change with age of the mouse (results not shown).

Erythrocyte density is often considered to be directly related to erythrocyte age in man. We have confirmed this relationship in mice by labelling *in vivo* with ^{59}Fe and by both albumin density-gradient centrifugation and phthalate ester two-phase centrifugation for fractionation of erythrocytes. The data presented in Fig. 4 shows that as the cells age from 4 to 65 days, most of the radioactivity moves from a lower density to a higher density, thus establishing a direct density-age relationship of erythrocytes in mice.

Fig. 4. Density-age relationship of erythrocytes in the mouse

(a) At various time intervals ranging from 4 to 65 days after pulse-labelling with ^{59}Fe , erythrocytes from a 10-month-old mouse were separated into different density classes by the phthalate ester method. The ratio of the specific radioactivity of a fraction to that of the total erythrocytes (specific-radioactivity ratio) is plotted against the density of the fraction. ●, 4 days; □, 30 days; ○, 65 days. (b) A portion of the same blood sample used in the phthalate ester method was subjected to albumin density-gradient centrifugation. Specific-radioactivity ratio is plotted against the density of the fraction determined by pycnometry. ●, 4 days; □, 26 days; △, 43 days; ○, 65 days.

Fig. 5. Changes in the concentrations of total free thiol and GSH and in glutathione reductase activity with increasing erythrocyte age *in vivo*

Erythrocytes from 10- and 37-month-old mice were separated into different age groups by the phthalate ester method. Total free thiol (a), GSH (b) and glutathione reductase (c) were determined for each fraction. ●, Results from a 10-month-old mouse; ○, results from a 37-month-old mouse.

Various cell fractions separated according to their density (cellular age) by the phthalate ester procedure were analysed for total free thiol, GSH and glutathione reductase activity. The data obtained for a 10-month-old mouse and a 37-month-old mouse are given in Fig. 5. The old cell population (highest density) of the 37-month-old mouse have an average density higher than the old cells of the 10-month-old mouse. Similarly, the young cells of the former have a higher density than those of the latter. This phenomenon has caused the unique frame shift in density patterns of the cell fractions. This supports the erythrocyte density distribution data presented in Fig. 2. Moreover, in both the mature and the very old mice, with increasing erythrocyte age there is a decrease in the concentrations of free thiols and GSH and of glutathione reductase activity; the decrease was nearly 50% in the old erythrocyte populations. Interestingly, the lowest values for thiols, GSH and glutathione reductase were found in the old cells of very old mice, and the highest values in the young cells of mature mice. By these criteria, the young cells of very old mice are similar to the old cells of mature mice.

Discussion

We have observed that in mice with increasing erythrocyte age the GSH and total free thiol concentrations decrease considerably. In addition, we found that the overall reducing potential of the erythrocyte decreases with increasing biological age of the mouse. It is unknown whether this is due to a decreased synthesis of GSH *de novo* or to an increased oxidation of GSH (maybe as a result of decreased glutathione reductase and increased peroxidase activity). It is noteworthy that erythrocytes of sheep with glutathione deficiency have a concomitant decrease in γ -glutamylcysteine synthetase activity (Smith *et al.*, 1973). It has been reported that in the newborn human the concentration of GSH is greater than in the maternal blood (Minnich *et al.*, 1974); however, no relationship has been found between GSH concentration and glutathione synthetase activity. This means GSH oxidation may be the reason for the decreased concentrations of GSH observed with increasing erythrocyte age and mouse age. In addition, our observation that there is a concomitant decrease in glutathione reductase activity with erythrocyte aging *in vivo* and with biological aging of the mouse, shows that enhanced oxidation of GSH to the oxidized form (GSSG) may be ultimately responsible for the decreased concentrations of GSH. Determination of the concentrations of GSSG might prove this point; however, GSSG is known to combine with cellular proteins by disulphide-interchange reactions (Fornaini, 1967; Huisman & Dozy, 1962; Scheuch *et al.*, 1961; Srivastava & Beutler, 1970) or GSSG may be transported across the erythrocyte membrane (Srivastava & Beutler, 1969) and thus a large proportion may be lost from the erythrocyte in spite of its enhanced formation.

GSH plays a central role in erythrocyte metabolism. It is known to stabilize a chain of thiol-enzymes in the metabolic regulation of erythrocytes (Scheuch *et al.*, 1961). For instance, hexokinase, glutathione reductase and glucose 6-phosphate dehydrogenase are inactivated under conditions of oxidation of GSH (Beutler *et al.*, 1957). In this context it is noteworthy that glutathione reductase, which itself is a thiol-containing enzyme, can be inactivated by decreasing quantities of GSH; the inactivation of this enzyme leads to further oxidation of GSH, thus forming a vicious cycle. Beutler (1969) has shown that oxidation of glutathione or decreased glutathione concentrations usually is accompanied by a shorter erythrocyte lifespan. Thus it is not surprising that we observed a significant decrease in the erythrocyte lifespan in very old mice, whose erythrocyte GSH concentrations have decreased to almost 40% of those in the mature mice. Even though it is generally considered that GSH might play a significant role in

maintaining the structural integrity of the erythrocyte membrane, it is difficult to determine whether a decreased GSH concentration is primarily responsible for the decreased erythrocyte survival. Glutathione metabolism is interdependent on several other intracellular metabolic processes. For instance, NADPH concentrations and activities of NADP⁺-linked enzymes should influence the concentration of GSH. In this respect, our observation (Stephan *et al.*, 1966) that NADPH and NADP⁺-linked enzymes decrease with increasing mouse age is significant, especially because NADPH through the glutathione reductase systems controls the concentration of GSH.

Aging of erythrocytes *in vivo* has been previously studied to a limited extent in several animal models such as the rat (Tochner *et al.*, 1975), ox (Smith *et al.*, 1972), dog (Johnson & Schwartz, 1971), rabbit (O'Connell *et al.*, 1965) and rather extensively in human (Bonsignore *et al.*, 1964; Fornaini, 1967; Oski, 1970; Ganzoni *et al.*, 1971; Abraham *et al.*, 1975). Erythrocyte aging in general seems to be associated with a decline in activity of enzymes, such as glucose 6-phosphate dehydrogenase, hexokinase, pyruvate kinase, 6-phosphogluconate dehydrogenase, NADP⁺-dependent isocitrate dehydrogenase and glutathione reductase. In addition, cellular constituents such as ATP, 2,3-bisphosphoglycerate, NADPH and GSH also decrease. Modifications of mechanisms involved in maintenance of the energy potential and the oxidoreduction equilibrium might lead to haemolysis. Moreover, the cell volume decreases and the mean corpuscular haemoglobin concentration increases with aging *in vivo*. It is obvious that the functional units of erythrocytes, the membrane, haemoglobin, the intracellular enzymes, cofactors and substrates act in an interrelated fashion.

Erythrocyte metabolism is influenced by both the environment of the organism and its own plasma milieu. This milieu shows different metabolic states at different stages in the life of the organism, and GSH in aging mice is a typical example of this. The decreases in GSH concentration and glutathione reductase activity with aging of the erythrocyte and of the mouse may be fundamental metabolic alterations that affect a variety of related biochemical systems.

This work was supported in part by research grants from the National Institutes of Health (AM02831 to J. F. T., 5P01 HDO 5701 to C. A. L.) and from the National Science Foundation (GB 25034X to J. F. T.).

References

- Abraham, E. C., Walker, D., Gravelly, M. & Huisman, T. H. J. (1975) *Biochem. Med.* **13**, 56-77
- Beutler, E. (1969) *Pharmacol. Rev.* **21**, 73-103
- Beutler, E., Robson, M. & Bittenwieser, E. (1957) *J. Clin. Invest.* **36**, 617-628

- Beutler, E., Duron, O. & Kelley, B. M. (1963) *J. Lab. Clin. Med.* **61**, 882-888
- Bishop, C. & Prentice, T. C. (1966) *J. Cell. Physiol.* **67**, 197-208
- Bonsignore, A., Fornaini, G., Fantoni, A., Leoncini, G. & Segni, P. (1964) *J. Clin. Invest.* **43**, 834-842
- Boyer, P. D. (1954) *J. Am. Chem. Soc.* **76**, 4331-4337
- Brok, F., Ramot, B., Zwang, E. & Danon, D. (1966) *Isr. J. Med. Sci.* **2**, 291-296
- Danon, D. & Marikovsky, Y. (1964) *J. Lab. Clin. Med.* **64**, 668-674
- Fornaini, G. (1967) *Ital. J. Biochem.* **16**, 254-330
- Ganzoni, A. M., Oakes, R. & Hillman, R. S. (1971) *J. Clin. Invest.* **43**, 834-842
- Huisman, T. H. J. & Dozy, A. M. (1962) *J. Lab. Clin. Med.* **60**, 302-319
- Johnson, L. W. & Schwartz, S. (1971) *Proc. Soc. Exp. Biol. Med.* **138**, 871-875
- Lang, C. A. & Acree, D. W. (1967) *Gerontologist* **7**, 13 (abstr.)
- Lang, C. A. & Stephan, J. K. (1967) *Biochem. J.* **102**, 331-338
- Long, W. K. & Carson, P. E. (1961) *Biochem. Biophys. Res. Commun.* **5**, 394-399
- Meites, S. & Faulkner, W. R. (1962) *Manual of Practical Micro and General Procedures in Clinical Chemistry*, pp. 82-85, Charles C. Thomas, Springfield
- Minnich, V., Smith, M. E., Rajanasathit, C. & Majerus, P. W. (1974) *Biol. Neonate* **24**, 128-133
- O'Connell, D. J., Caruso, C. J. & Sass, M. D. (1965) *Clin. Chem.* **11**, 771-781
- Oski, F. A. (1970) in *Blood Cells as a Tissue* (Holmes, W. L., ed.), pp. 99-129, Plenum Press, New York
- Prins, H. K. & Loos, J. A. (1969) in *Biochemical Methods in Red Cell Genetics* (Yunis, J. J., ed.), pp. 115-137, Academic Press, New York
- Scheuch, D., Kahrig, C., Ockel, E., Wagenknecht, C. & Rapoport, S. M. (1961) *Nature (London)* **190**, 631-632
- Smith, J. E., McCants, M., Parks, P. & Jones, E. W. (1972) *Comp. Biochem. Physiol. B* **41**, 551-558
- Smith, J. E., Lee, M. S. & Mia, A. S. (1973) *J. Lab. Clin. Med.* **82**, 713-718
- Srivastava, S. K. & Beutler, E. (1969) *Biochem. J.* **114**, 833-837
- Srivastava, S. K. & Beutler, E. (1970) *Biochem. J.* **119**, 353-357
- Stephan, J. K., Acree, D. W. & Lang, C. A. (1966) *Gerontologist* **6**, 14 (abstr.)
- Tochner, Z., Benbassat, J. & Hershko, C. (1975) *Scand. J. Hematol.* **14**, 377-384
- Wintrobe, M. M. (1967) *Clinical Hematology*, 6th edn., pp. 410-440, Lea and Febiger, Philadelphia