BIOCHEMICAL EVALUATION OF RETICULOCYTOSIS IN THE RABBIT

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ABSTRACT

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IN THE RABBIT

by Andrew J. Maturen, Jr.

The reticulocyte count has long been the most practical method of assessing marrow erythropoietic activity. As a laboratory procedure, the reticulocyte count possesses simplicity and rapidity but has several sources of technical and statistical error. The metabolically active reticulocyte possesses certain enzymes and other biochemical substances in greater concentration than the mature erythrocyte, the concentration of these substances decreasing as the erythrocyte ages. The use of determinations of such substances in a mixed erythrocyte population as an alternative to reticulocyte counting is investigated here. Determinations of magnesium, phospholipid, malic and glucose-6-phosphate dehydrogenases, coproporphyrin and ribonucleic acid are performed in parallel with reticulocyte counts on blood from rabbits in which reticulocytosis had been produced by acute blood loss. Reticulocyte counts versus each biochemical determination chosen for investigation are evaluated statistically: coefficients of

correlations are calculated and equations are derived using the method of least squares by which a reticulocyte count can be calculated from a known biochemical value with a predictable standard error. Each biochemical method used is also evaluated as to its use in the clinical laboratory. This study indicates that erythrocyte magnesium, phospholipid and colorimetric malic dehydrogenase determinations could be quite useful as biochemical indices of reticulocytosis.

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By

Andrew Joseph Maturen, Jr.

A THESIS

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For Virginia

who made it worthwhile

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INTRODUCTION

The examination of bone marrow aspirates and the counting of reticulocytes in the peripheral blood have long been the established methods of evaluating the erythropoietic activity of the bone marrow. Bone marrow aspiration is a minor surgical procedure and as such is not as free of risk as is a venipuncture. The reticulocyte count is a technically simple, rapid procedure requiring only a few drops of peripheral blood, but it is beset with a number of possibilities for technical and statistical error.

Various investigators of erythrocyte metabolism have determined that certain biochemical substances involved in the metabolism of the cell are still present in appreciable quantities when maturation has progressed to the reticulocyte stage. Such substances then disappear partially or completely as the cell becomes fully mature. The rate of disappearance roughly parallels the rate of maturation.

This work was designed to determine whether or not chemical measurements might provide simpler and more accurate indices of marrow activity.

The biochemical components of the erythrocyte chosen to be measured here were some of those known or thought to be increased in the immature erythrocyte and decreasing or disappearing with increasing cell age. The biochemical components measured included: ribonucleic acid (RNA) (Burt, Murray and Rossiter, 1951); phospholipid (Weed and Reed, 1966); magnesium (Lohr and Waller, 1961); coproporphyrin (Schwartz et al., 1956); glucose-6-phosphate dehydrogenase (Marks, 1958); and malic dehydrogenase (Lohr and Waller, 1961).

REVIEW OF LITERATURE

The reticulocyte differs biochemically from the mature erythrocyte in a number of ways, most of which are related to the maturation process of the cell.

According to the unitarian theory of hematopoiesis, the erythrocyte is derived from a primitive stem cell in the bone marrow. The stem cell gives rise by differentiation to the proerythroblast, which divides to form two basophilic normoblasts. Successive cell divisions yield polychromatophilic normoblasts and orthochromic normoblasts, after which the maturation to the reticulocyte and erythrocyte involves no further mitotic division (London, 1961). The nucleated precursor of the erythrocyte is apparently capable of most of the metabolic reactions characteristic of cells of other tissues. It synthesizes both deoxyribonucleic and ribonucleic acids (DNA and RNA), lipids, protein, and heme. Carbohydrates are metabolized by the Embden-Meyerhof glycolytic pathway, the hexose monophosphate shunt and the Krebs tricarboxylic acid cycle. As the normoblast matures to form a reticulocyte, the nucleus disappears and the ability to synthesize DNA is lost. The reticulocyte retains the capacity to synthesize hemoglobin

due to the preservation of intact mitochondria and endoplasmic reticulum.

"Reticulum" for which the reticulocyte is named is a threadlike accumulation of granular particles made visible by supravital dyes. Evidence offered by Seno (1962) indicates that this structure is an artifact composed of stain-precipitated mitochondria and endoplasmic reticulum. The reticulocyte loses most of its major biosynthetic pathways as it matures to an adult erythrocyte; the granular endoplasmic reticulum disappears, leaving little or no RNA in the mature cell. Synthesis of heme cannot occur due to loss of mitochondria and products of the Krebs cycle (Kruh and Borsook, 1956), and lipid synthesis is also absent or insignificant (London and Schwarz, 1953).

The bone marrow of man contains a reticulocyte population approximately equal to the population of nucleated erythrocyte precursors (Reiff et al., 1958). Erythrocytes are released into the circulation chiefly as reticulocytes with some mature cells. Reticulocytes in the circulation require about one day to reach maturity (Finch, 1959) and therefore may be regarded as the "one-day-old" cells of the erythrocyte mass. Thus during the steady state the counting of reticulocytes yields a rough estimate of mean age of the total erythrocyte mass.

Reticulocyte counts thus provide a rather sensitive index of erythropoietic activity of the bone marrow (Cline and Berlin, 1963), but these measurements are subject to many technical and statistical errors. The procedure itself involves supravital staining of a sample of peripheral blood with a dye such as methylene blue (Brecher, 1949). A granular or thread-like reticulum appears within the cell as a result of the dye action on the cytoplasmic organelles (Dustin, 1943; Burt et al., 1951; Caspersson, 1955). This substance is said to be RNA on the basis of ultraviolet microscopy, and also by extinction of staining by treatment with ribonuclease (Brachet, 1947).

The percentage of reticulocytes is estimated by counting the proportion of stained cells among a given total number of erythrocytes. Although the procedure is technically simple and requires only a few drops of peripheral blood, it is beset with a number of possibilities for technical error. Some of the factors which may affect a reticulocyte count are: (a) superposition of platelets or dye precipitate over mature erythrocytes, causing them to appear reticulated; (b) presence of Heinz bodies, Howell-Jolly bodies and other cellular inclusions which may be confused with granular reticulum; and (c) crenation of erythrocytes when exposed to the dye solution. Crenation is said by Davidson (1930) to obstruct the passage of dye into the cell, and by others to render the cell more

refractile, the points of refracted light being mistaken for reticulum. Physical and chemical factors such as temperature, pH, and drying, and substances such as glucose and sodium salts, may also affect the amount and appearance of reticulum formed (Wintrobe, 1967).

The statistical error in a reticulocyte count is large, since reticulocyte counts follow the Poisson distribution, in which a relatively small sample is taken from a very large population (Lewis, 1966). The standard deviation of a reticulocyte count is calculated as the square root of n. Thus, 95% of results will be within 2 n. Ordinarily either 500 or 1000 cells are taken as the sample size, but n as used here is the number of reticulocytes seen, not the sample size. Although the standard deviation increases as the number of reticulocytes counted increases, it decreases when expressed as a proportion of the number seen (i.e., the % error decreases). Most reticulocyte counts in normal and pathologic states are in the range of 1 to 20% (i.e., n = 10 to n = 200) and it is in this area that the error is greatest. For example, the standard deviation of a 1% reticulocyte count (i.e., n = 10) is n or 3.16. Ninety-five percent confidence limits are then 63.2%. Thus, a 1% count as reported could actually reflect a true count of 0.4 to 1.6% (Lewis, 1966).

Reticulocytes have been shown to be slightly larger (Heath and Deland, 1930) than mature erythrocytes and of slightly lower specific gravity (Key, 1921). For these reasons, reticulocytes rise to the top of a column of centrifuged erythrocytes and can be separated from the mature cells. Biochemical analyses of such fractions of an erythrocyte column indicate that some substances involved in cell metabolism are present in greater concentration in the reticulocyte than in the mature erythrocyte. Schwartz and his co-workers (1956) have found this to be true of coproporphyrin, and Hallinan and Eden (1962) have done similar work with phospholipids.

The reticulocyte will continue some of its maturation processes when incubated *in vitro* at 37 C. (Rubinstein *et al.*, 1956; Seno *et al.*, 1964). The reticulocytes of a given blood sample may drop as much as 60% in 24 hours at 37 C. Rubinstein (1956) and his co-workers have demonstrated by serial determinations at various incubation times that the activity of certain dehydrogenase enzymes and the concentration of RNA in a sample of reticulocytes fractioned from mature erythrocytes decreases as the reticulocyte count decreases *in vitro*.

Since certain substances are more concentrated in the reticulocyte and decrease as the reticulocyte matures, some correlation must exist between concentration of such substances and the reticulocyte count itself at various

levels. On this basis, the following substances were chosen for investigation.

Magnesium. Streef (1939) has reported that human erythrocytes contain 3.4-5.6 mg. of magnesium per 100 ml. of packed cells. There are no known disease states in which the magnesium concentration of erythrocytes is markedly affected, and the effect on erythrocytes of fluctuations in plasma magnesium has not been conclusively determined (Behrendt, 1957). Magnesium is important as a cofactor in many enzymatic reactions, including: (a) the synthesis of delta-amino levulinic acid, the first step in the synthesis of heme (Shemin, 1957); (b) the ribosomal synthesis of the globin portion of hemoglobin (Nathans and Lippman, 1961); (c) the activity of glucose-6-phosphate dehydrogenase; and (d) ATP-coupled phosphorylations, as illustrated by the hexokinase reaction in anaerobic glycolysis. Magnesium ions also function in active transport across the cell membrane (Passow, 1964).

Since magnesium acts mainly as an enzyme activator, a decrease in magnesium concentration may be expected when the enzymatic processes of the cell decrease. Lohr and Waller (1961) have reported that this is indeed the case.

Phospholipid. The endoplasmic reticulum and the mitochondrion are cellular organelles which are preserved through the reticulocyte stage. These possess membranes rich in phospholipid (Hallinan and Eden, 1962). The cell membrane

of the erythrocyte itself is also rich in phospholipid (Weed and Reed, 1966). No de novo synthesis of phospholipid occurs in the mature erythrocyte (Van Deenen and De Gier, 1964). Phospholipid of the organelle membranes is lost as these organelles disappear, and phospholipid of the cell membrane itself diminishes as the cell ages and the membrane becomes more permeable (Weed and Reed, 1966). In vivo aging is, then, associated with loss of total lipid (Westerman et al., 1963). To date, the only clear-cut abnormalities of erythrocyte lipid distribution are the rare syndromes, beta-lipoproteinemia and acanthocytosis (Ways, Reed and Hanahan, 1963). Erythrocyte phospholipid in normal humans, assuming a cross section of cells of all ages represented in the total mass, is estimated at 278 to 318 mg. per 100 ml. packed erythrocytes by Farquhar and Oette (1961) and at 196 mg. per 100 ml. packed erythrocytes by Kirk (1938).

Ribonucleic acid. A number of workers have discussed the nature and function of nucleic acid in reticulocytes (Brachet, 1947; Burt et al., 1951; Claude, 1949; Holloway and Ripley, 1952; Kruh, 1967). The RNA in reticulocytes is associated with retained cytoplasmic structures, especially the ribosomes of the endoplasmic reticulum. Mature erythrocytes contain little if any nucleic acid (Behrendt, 1957). The RNA in normal human erythrocytes has been quantitated on the basis of phosphorus content as 3.5-5.2

mg. per 100 ml. packed cells (Mandel and Metais, 1947). This is attributable to the reticulocytes present.

Coproporphyrin. Coproporphyrin is one of the intermediates in the synthesis of heme (London, 1961). Coproporphyrin is increased in the red cell mass during increased erythropoietic activity, and 90% of the coproporphyrin content of a column of centrifuged, reticulocyte-rich erythrocytes is in the upper reticulocyte fraction (Schwartz et al., 1956). The erythrocyte continues synthesis of heme through the reticulocyte stage (London, 1961).

Malic dehydrogenase. The Krebs tricarboxylic acid cycle, of which the reaction catalyzed by malic dehydrogenase is one step, is linked intimately with the mitochondrion (Bishop, 1964). Others (Ackerman and Bellios, 1955) have noted the presence of mitochondria up to the reticulocyte stage. Rubinstein (1956) and his co-workers have reported, using measurement of optical density change, that the enzymes of the Krebs cycle are all active in the reticulocyte and most of them disappear in the mature erythrocyte.

Glucose-6-phosphate dehydrogenase. This enzyme catalyzes one step of the "pentose phosphate pathway" of carbohydrate metabolism, and is said to be retained in the mature erythrocyte (Rubinstein et al., 1956). The pentose phosphate pathway accounts for only 10% of the metabolism of glucose in mature erythrocytes in vitro (Murphy, 1960). This

pathway, however, along with G6PD in particular, is also thought to be concerned with the production of glutathione and maintenance of the cell membrane (Carson and Frischer, 1966). G6PD then is active in the mature erythrocyte but has also been reported to be increased in activity in reticulocyte-rich blood (Marks, 1958; Marks, 1964).

Many studies have been reported in which erythrocytes, with or without an enhanced reticulocyte population, have been centrifuged and separated into "top" and "bottom" layers designated as "young" and "old" cells, respectively. When differences in enzyme activity or some other cellular biochemical component are found between the two layers, the conclusion is usually reached that enzyme activity or concentration of the substance under study decreases with increasing cell age. Very few such studies cite reticulocyte counts for comparison (Bishop, 1964). The statistical correlation of fluctuations in the reticulocyte count with fluctuation in the levels of substances which can be quantitated chemically should help to provide some insight into the nature of the reticulocyte and at the same time evaluate these measurements as chemical predictions of reticulocyte content.

MATERIALS AND METHODS

Preliminary Procedures

Reticulocytosis was produced in 6 rabbits by withdrawing approximately 25 ml. of blood by cardiac puncture from each rabbit daily for 4 days. From the 5th day until the 17th day, at which time the reticulocyte counts had returned to near-normal levels, only sufficient blood for testing (3 to 5 ml.) was drawn from each rabbit each day. In order to prevent development of an iron deficiency during the anemic and recovery periods, ferrous sulfate was added to the drinking water in a concentration of 0.4 to 0.5 mg. per 100 ml.

Reticulocyte counts and hemoglobin determinations were done on all 6 samples every day. After this each sample was prepared for biochemical testing as follows:

The erythrocytes were washed 3 times and packed in cold 0.85% sodium chloride. The leukocyte layer was removed after the first wash, taking care to remove as few erythrocytes as possible from the reticulocyte-rich layer immediately under the buffy coat. After the final packing of erythrocytes, the last saline wash was removed, and the cell column was mixed well by inversion to break the

age-size gradient produced by centrifugation and to redistribute cells at random.

The hematocrit of each sample of washed, packed cells was recorded and all biochemical determinations were corrected by an appropriate factor. After packing and mixing, the cells were kept at 4 C. in stoppered tubes and analyzed within 12 hours.

One of the 6 biochemical tests selected was performed serially each day on the packed erythrocytes from 1 of the 6 rabbits, blood from the same rabbit being used for the same test each day; i.e., reticulocyte counts and phospholipid determinations were done each day on 1 rabbit, reticulocyte counts and magnesium determinations each day on another, etc.

Only 1 biochemical determination of those chosen was done on the blood of each rabbit each day. Because of the size of the animal, drawing enough blood from each rabbit to do all 6 tests would have represented a large blood loss and introduced extraneous, unrecognized effects into the study.

Reticulocyte counts were done using the method of Brecher (1949), which utilizes new methylene blue N as a supravital dye. The number of reticulocytes among 1000 erythrocytes was enumerated.

Biochemical Tests

Magnesium content was determined using a modification of the method of Spare (1962) for serum magnesium. In this serum method a dilute solution of Titan yellow dye is added to the serum. Upon alkalinization with sodium hydroxide, Titan yellow reacts specifically with magnesium ions to form a dye lake which is stabilized with polyvinyl alcohol. The resulting colored complex is read spectrophotometrically at 540 mμ.

In order to adapt this serum magnesium method to measurements on erythrocytes, the cells were lysed with distilled water, and hemoglobin and other proteins were precipitated with 20% trichloroacetic acid. The dye reaction was then carried out on the protein-free filtrate. Precipitation of proteins is unnecessary in the serum determination, but is necessary here to eliminate the interfering color due to hemoglobin. Spare (1962) has suggested that if a protein precipitant is used, distilled water should be added first to free magnesium bound to protein. In this case, addition of distilled water accomplishes this purpose as well as that of hemolysis, and recovery of magnesium is good.

Phospholipid. The method used for phospholipid determination was based on that of Youngburg and Youngburg (1930) as modified by Caraway (1966) (See Appendix I for this

method). In this procedure, phospholipids are extracted with an organic solvent. An aliquot is evaporated to dryness and digested with sulfuric acid and hydrogen peroxide to destroy organic matter and convert lipid phosphorus to inorganic phosphorus. Inorganic phosphorus is then determined by the method of Fiske and Subbarow (Annino, 1964). The only modifications of Caraway's method used here were the substitution of 0.2 ml. of packed cells for 0.2 ml of serum and the use of 30% chloroform in methanol as an extracting solution rather than isopropyl alcohol, since certain phospholipids of the erythrocyte, i.e. lecithin and cephalin, are not soluble in alcohol (Behrendt, 1957).

Coproporphyrin. Determinations of erythrocyte coproporphyrin were performed using the method of Schwartz et al. (1956), originally described by Grinstein and Watson (1943). One milliliter of packed cells was used for analysis, and the optical density of the final extract was measured at 400 mμ.

Ribonucleic acid. The procedure used was based on the method used by Kruh (1967) for preparation of total RNA from reticulocytes, and on that of Scott et al. (1956) for microdetermination of RNA in tissues (see Appendix I for details of method). In this method, erythrocytes are hemolyzed in distilled water and proteins and hemoglobin are precipitated with a phenol solution which leaves RNA in the supernatant. The RNA is then precipitated at 0 C. with absolute ethyl

alcohol and the precipitate is washed twice with ethanol and finally with ether to remove all traces of phenol. The precipitate is then dissolved in 1 N sodium hydroxide and the absorption of ultraviolet light was measured at 260 mμ.

The final extract is free from interfering substances such as phenol and hemoglobin; its maximum absorption was at 260 mμ, and its minimum at 230 mμ, as specified for RNA (Davidson, 1960). Recovery of RNA was 82 to 85%. The preparative method from which this analytical method was adapted was scaled down to use 1 ml. of packed erythrocytes.

Dehydrogenases. Glucose-6-phosphate dehydrogenase and malic dehydrogenase activity in erythrocytes was measured by the method of Fennell (1968) (see Appendix I for details of method). The principles of this method are as follows: enzymes present in the erythrocytes act on added substrate at 37 C. with addition of appropriate coenzymes, cofactors and buffers. The reduced coenzyme formed in the enzymatic reaction is then reoxidized with simultaneous reduction of a tetrazolium salt. The purple formazan complex of the reduced tetrazolium is extracted into ethyl acetate and read at 525 mμ. In performing this determination, the author found that hemoglobin present in the incubation mixture was also extracted into ethyl acetate, giving a dark-colored opaque, gelatinous mass of denatured hemoglobin which could not be read spectrophotometrically. Addition of a small

amount of powdered barium carbonate was found to prevent extraction of hemoglobin and allowed selective extraction of the formazan complex.

Standard curves were prepared for each of the biochemical tests, treating standard solutions as blood samples. Where standards were not available, as for dehydrogenases, tetrazolium salts in appropriate concentrations were reduced to formazan complexes with phenazine methosulfate (see Appendix I) and a standard curve was prepared. From each standard curve, a factor relating optical density to concentration was calculated using the method of averages.

Reticulocyte counts and chemical determinations were done on the blood of 6 rabbits for 17 days, the first day on which the rabbits were bled being considered as Day 1. Coefficients of rank correlation between the series of reticulocyte counts and biochemical tests done on each rabbit were calculated and equations relating biochemical concentrations to reticulocyte counts were derived using methods discussed by Lewis (1967). Statistical evaluation of the biochemical methods used may be found in Table 1. Where applicable, recovery of added standard was also evaluated, and results appear in Table 8.

Table 1.--Statistical evaluation of biochemical methods.

Test	1 S.D.	Average	Sample Size
Magnesium	± 0.08 mg./100 ml.	5.04 mg./100 ml.	33
Phospholipid*	± 0.07 mg./100 ml.	417.90 mg./100 ml.	18
Dehydrogenases	± 0.74 µg. formazan/0.1 ml.	55.00 µg. formazan/0.1 ml.	48
Coproporphyrin	± 0.38 µg./100 ml.	12.50 µg./100 ml.	24
RNA	± 1.68 mg./100 ml.	89.10 mg./100 ml.	18

*The portion of the method evaluated was the terminal determination of inorganic phosphorus, since a source of phospholipid standard was not available.

RESULTS

The results of reticulocyte counts and biochemical determinations for the test period of 17 days are recorded in Tables 2 through 7. Rank correlation coefficients (r'), equations of line by the method of least squares ($y = ax \pm b$) and standard error of estimate calculated from the data are shown with the tables.

Graphic representations of reticulocyte counts vs. biochemical determinations appear in Figures 1 through 6.

The tables of results are interpreted as follows: the reticulocyte count (y) may be calculated with the stated standard error (SEE) by applying the biochemical value as determined (x) to the equation given.

Table 2.--Comparison of reticulocyte counts and erythrocyte magnesium determinations.

Day	Reticulocyte % (y)	Magnesium mg./100 ml. (x)
1	1.9	4.9
2	2.8	5.3
3	6.1	6.0
4	8.7	6.5
5	20.1	8.0
6	36.8	10.0
7	29.4	9.2
8	20.5	8.5
9	16.5	7.0
10	13.3	7.0
11	11.9	6.7
12	10.2	6.2
13	8.5	6.5
14	6.8	5.5
15	5.9	5.1
16	4.6	5.0
17	3.2	4.6

$r' = 0.9657$ ($p < 0.01$)

$y = 6.04\,x - 27.62$

SEE $= \pm 2.7\%$

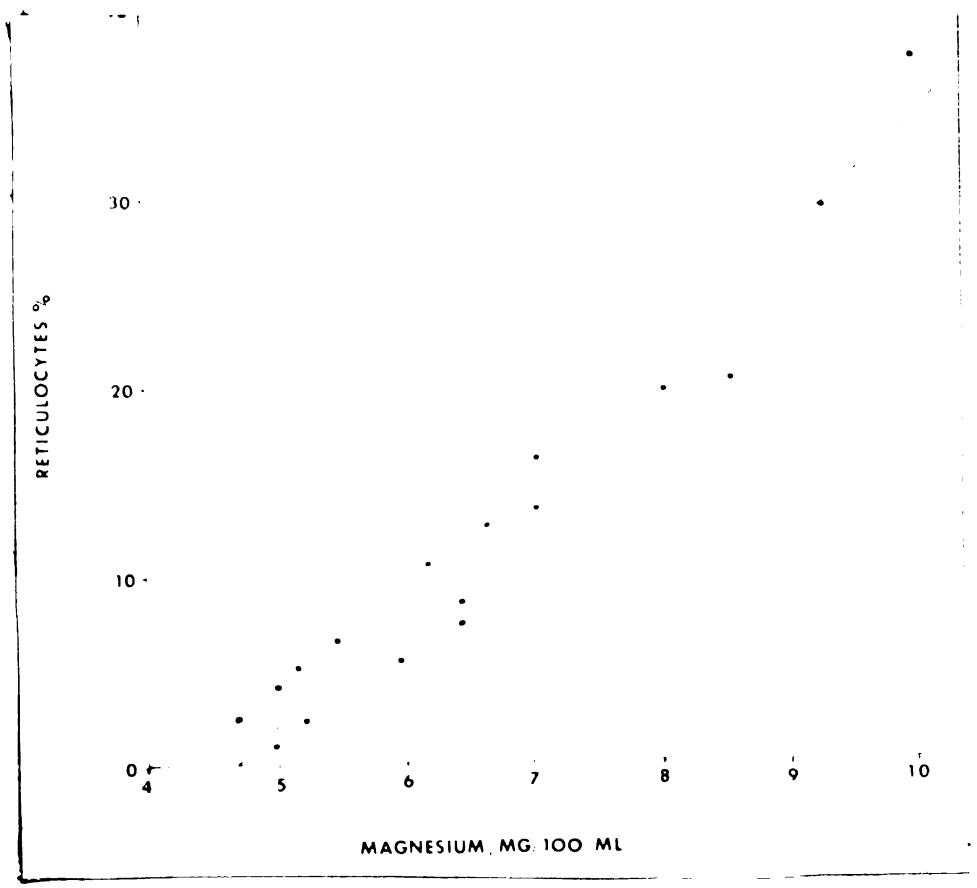

Figure 1.--Graphic representation of correlation between reticulocyte count and erythrocyte magnesium concentration. Equation of line calculated by method of least squares.

Table 3.--Comparison of reticulocyte counts and erythrocyte phospholipid determinations.

Day	Reticulocyte % (y)	Phospholipid mg./100 ml. (x)
1	0.9	222.0
2	2.3	238.6
3	4.8	260.0
4	6.2	285.0
5	20.6	387.2
6	27.9	415.0
7	42.0	466.0
8	32.8	380.0
9	21.7	378.1
10	15.6	340.0
11	11.8	320.2
12	10.2	307.8
13	7.8	278.7
14	7.4	319.8
15	4.2	270.0
16	4.0	247.5
17	3.1	237.8

$r' = 0.9706$ ($p < 0.01$)

$y = 0.163\,x - 38.01$

SEE $= \pm 3.5\%$

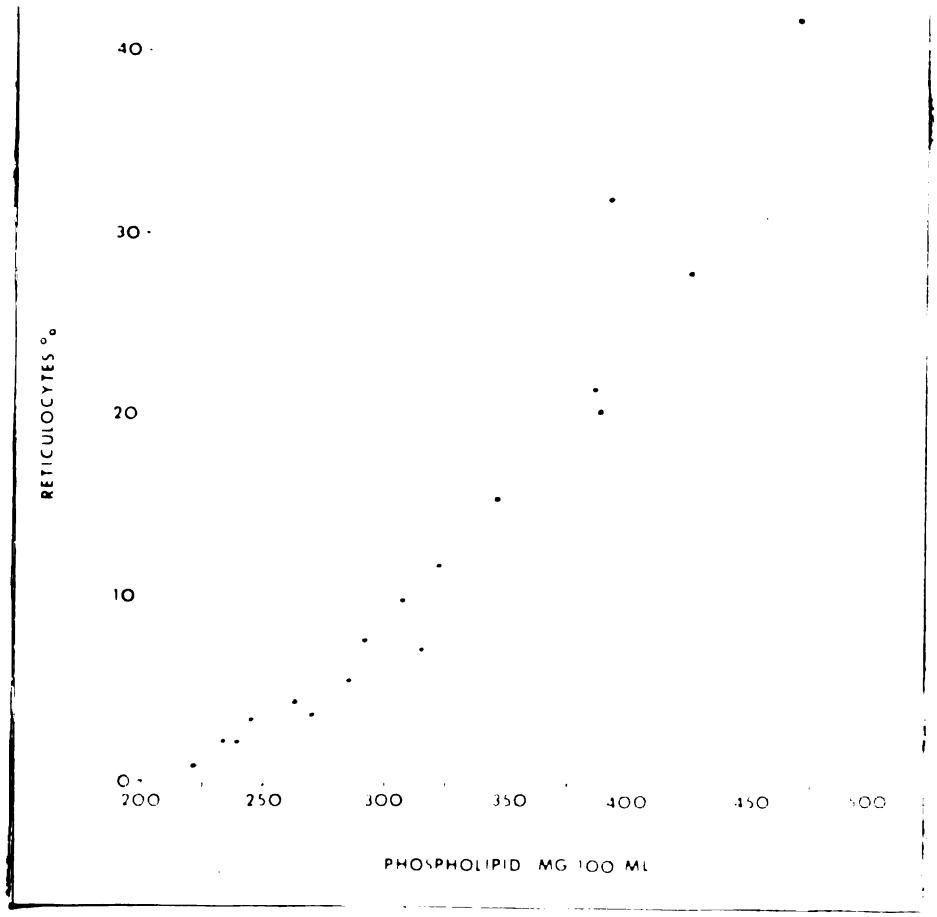

Figure 2.--Graphic representation of correlation between reticulocyte count and erythrocyte phospholipid concentration. Equation of line calculated by method of least squares.

Table 4.--Comparison of reticulocyte counts and erythrocyte malic dehydrogenase determinations.

Day	Reticulocyte % (y)	Malic Dehydrogenase µg. formazan/ 0.1 ml. (x)
1	1.6	38.7
2	3.8	76.0
3	6.4	52.0
4	9.2	52.0
5	14.1	120.0
6	28.8	120.0
7	36.7	230.0
8	30.2	162.8
9	28.4	92.2
10	20.3	67.7
11	19.1	89.7
12	16.2	78.6
13	16.2	83.7
14	11.8	57.6
15	7.8	63.2
16	6.0	39.8
17	5.8	27.3

$r' = 0.8487$ ($p < 0.01$)

$y = 0.189\ x - 0.718$

SEE = ±5.57%

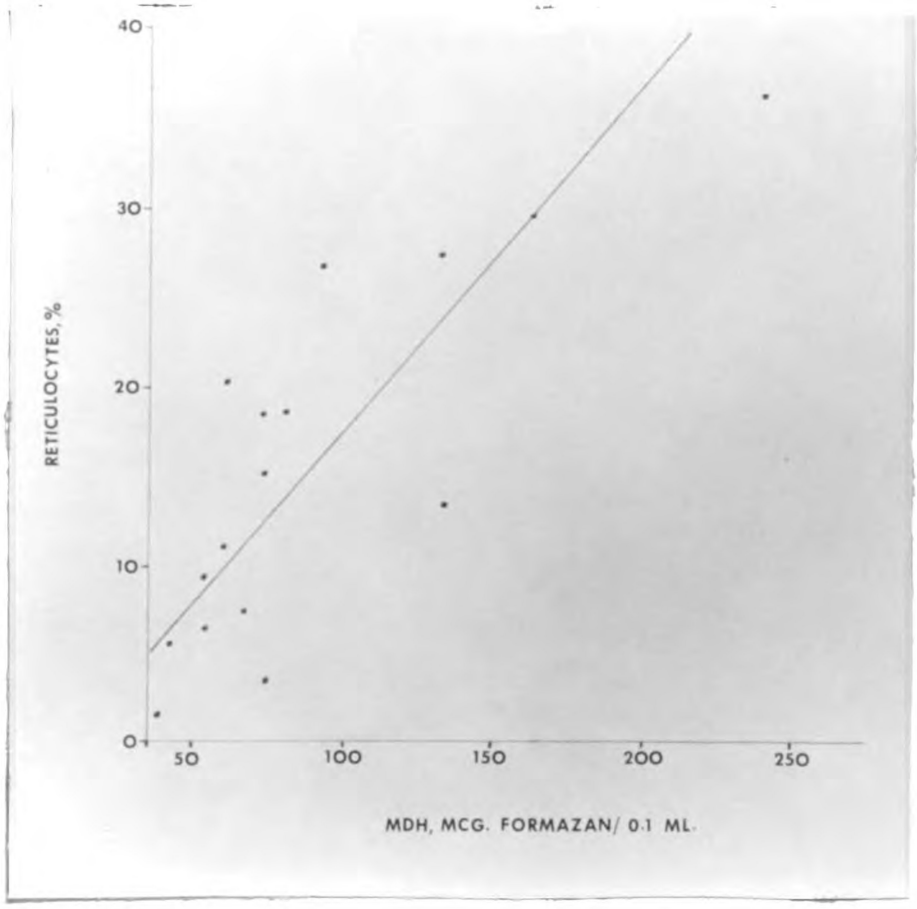

Figure 3.--Graphic representation of correlation between reticulocyte count and erythrocyte malic dehydrogenase activity. Equation of line calculated by method of least squares.

Table 5.--Comparison of reticulocyte counts and erythrocyte glucose-6-phosphate dehydrogenase determinations.

Day	Reticulocyte % (y)	G6PD µg. Formazan/ 0.1 ml. (x)
1	0.4	38.5
2	1.3	41.5
3	1.7	30.0
4	2.9	41.5
5	6.8	67.5
6	7.3	41.0
7	13.5	43.0
8	16.2	62.6
9	13.7	50.6
10	10.8	47.2
11	8.5	86.5
12	4.2	43.0
13	4.0	59.7
14	3.8	44.6
15	3.6	23.7
16	2.7	77.2
17	1.9	46.5

$r' = 0.4860$ $(p < 0.05)$

$y = .0245\ x + 7.30$

SEE = ±7.98%

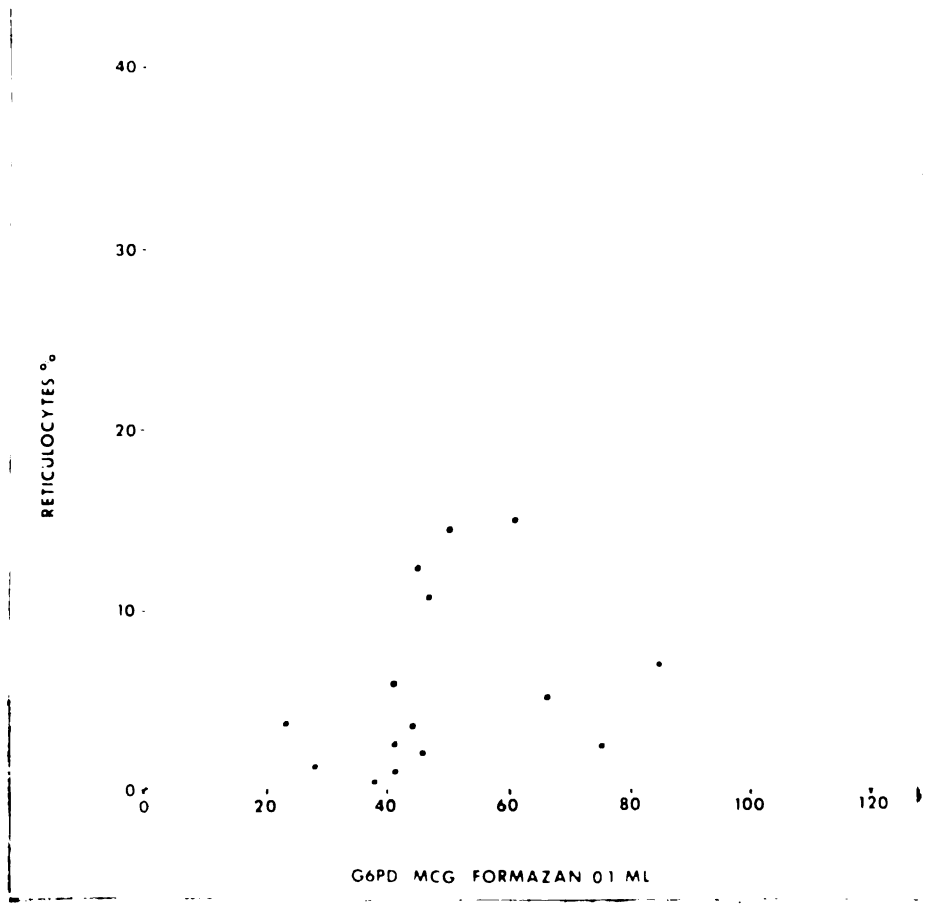

Figure 4.--Graphic representation of correlation between reticulocyte count and erythrocyte glucose-6-phosphate dehydrogenase activity. Equation of line calculated by method of least squares.

Table 6.--Comparison of reticulocyte counts and erythrocyte coproporphyrin determinations.

Day	Reticulocyte % (y)	Coproporphyrin µg. % (x)
1	1.2	12.0
2	3.4	3.7
3	6.0	17.6
4	6.7	43.0
5	17.0	19.3
6	24.6	45.0
7	31.4	32.0
8	23.8	21.8
9	22.0	30.0
10	19.6	4.6
11	14.4	29.6
12	9.5	25.0
13	8.2	52.5
14	7.6	7.2
15	7.5	37.0
16	5.9	31.5
17	4.1	5.2

$r' = 0.3579$ ($p > 0.05$)

(correlation not significant at 5% level)

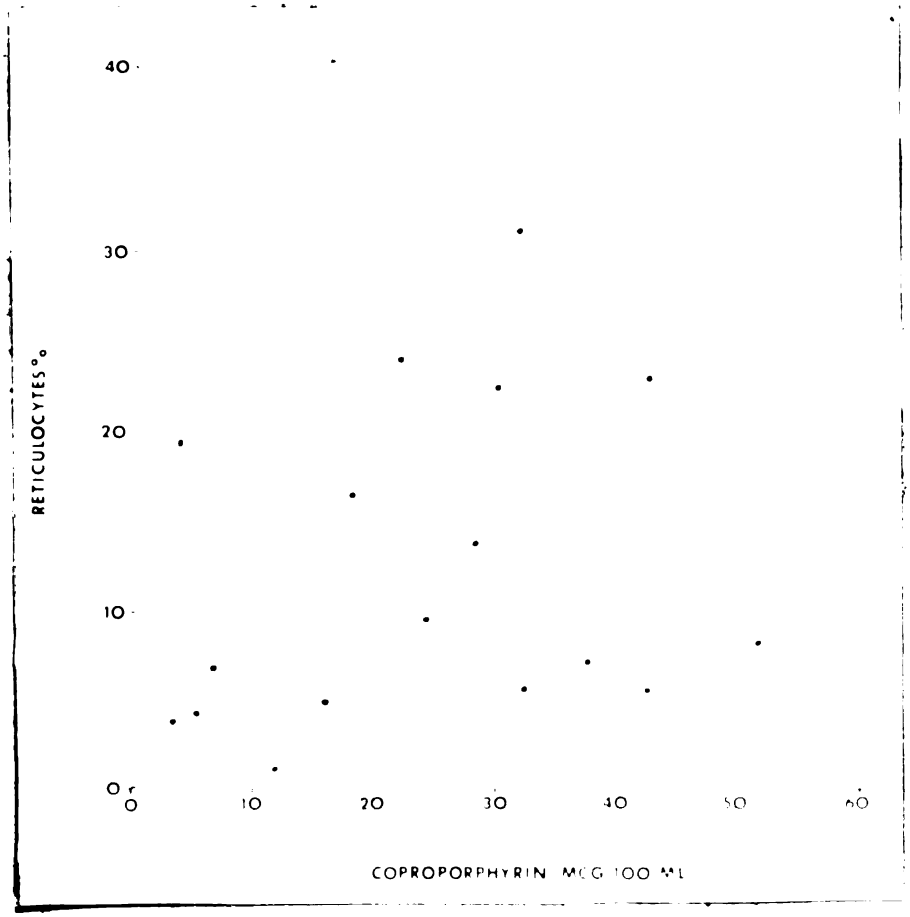

Figure 5.--Graphic representation of correlation between reticulocyte count and erythrocyte coproporphyrin concentration. Correlation not significant.

Table 7.--Comparison of reticulocyte counts and erythrocyte ribonucleic acid determinations.

Day	Reticulocyte % (y)	RNA, mg./100 ml.
1	1.0	5.2
2	1.8	5.6
3	2.6	19.8
4	4.9	6.7
5	7.8	7.9
6	14.9	23.4
7	26.7	67.6
8	19.6	47.5
9	17.0	6.7
10	17.2	11.1
11	16.0	56.2
12	14.7	21.9
13	12.0	7.9
14	7.9	26.7
15	6.4	33.8
16	5.7	19.8
17	3.0	7.6

$r' = -.6200$ ($p < 0.01$)

$y = 0.22x + 5.78$

SEE $= \pm 5.63\%$

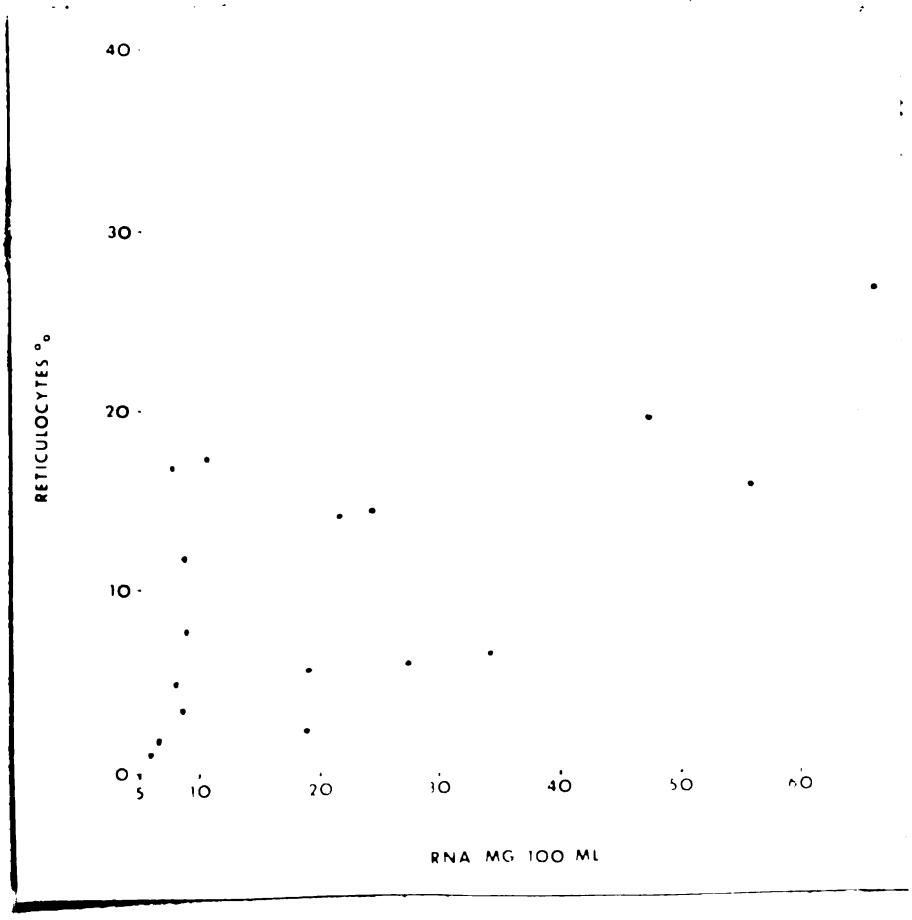

Figure 6.--Graphic representation of correlation between reticulocyte count and erythrocyte ribonucleic acid concentration. Equation of line calculated by method of least squares.

DISCUSSION

A test to substitute for the reticulocyte count as an index of marrow activity must: (a) utilize a small sample of peripheral blood; (b) equal or surpass the technical simplicity of the reticulocyte count with less inherent statistical error; (c) be acceptable as to sensitivity and specificity; and (d) correlate exclusively with reticulocyte counts at various levels. Biochemical tests used are summarized with respect to these criteria in Table 8.

With the exception of erythrocyte coproporphyrin, all of the other biochemical components tested here correlated with the reticulocyte count at a 5% level of significance or better. The biochemical test which correlated best was phospholipid, followed by magnesium, malic dehydrogenase, G6PD and RNA, in that order.

Erythrocyte phospholipid determinations correlated with reticulocyte counts slightly better than did erythrocyte magnesium. However, the magnesium procedure is more desirable from the standpoint of performance in the clinical laboratory. It requires about 15 minutes to complete after washing and packing of cells and does not involve the use of caustic reagents and an open flame, while the phospholipid

Table 8.--Evaluation of biochemical tests investigated as indices of reticulocytosis.

Test	Vol. RBC, ml.	Test Time*	1 S. D. (See Table I)	Recovery %	Specificity	Correlation with Ratio (r') (See Tables 2-7)
Magnesium	1.0	15 min.	±0.08 mg%	98-99	Good	0.9657
Phospholipid	0.2	30 min.	±0.07 mg%	96-98**	Good	0.9706
MDH	0.1	45 min.	±0.74 g formazan/ 0.1 ml.	-----	Questionable	0.8487
G6PD	0.1	45 min.	±0.74 g formazan/ 0.1 ml.	----	Questionable	0.4860
Coproporphyrin	1.0	1-1/2 hr.	±0.38 g%	80-82	Poor	0.3579
RNA	1.0	3 hr.	±1.68 mg%	82-85	Good	0.6200

*Following washing and packing of erythrocytes

**For inorganic phosphorus

determination requires about 30 minutes and is most safely performed in a chemical hood. Both determinations are subject to error from glassware contamination due to hardness of water; this may account for some values which are higher than expected (such as magnesium, Day 17, and phospholipid, Day 14).

In the phospholipid determination, complete conversion of lipid phosphorus to inorganic phosphorus may not have been achieved in all cases. This step is rather subjective and may account for some values being lower than expected.

In both of these procedures uniform cleaning of glassware with a final acid wash, together with careful attention to the lipid oxidation step in the phospholipid determination could probably lower the standard error of estimating a reticulocyte count. Both of these procedures closely approximate the reticulocyte count as to performance time. Determination of magnesium by atomic absorption spectrophotometry (Hansen and Freier, 1967) is said to be less subject to interference than the Titan yellow dye-lake method. This technique might prove to be more accurate and faster, but atomic absorption spectrophotometers are not universally available.

There may be several reasons for the erratic data yielded by the dehydrogenase determinations, especially G6PD. Wilkinson (1962) reported that the activity of

erythrocyte dehydrogenases is markedly decreased even by short periods of exposure to room temperature. Although blood samples for this study were kept at 4 C. as consistently as possible, there were inevitable, variable periods during washing and packing when the cells came to room temperature. Also, the phenomenon referred to as "nothing dehydrogenase" by Zimmerman and Pearse (1959) may be involved here; this refers to nonspecific reduction of tetrazolium salts to formazan complexes without the action of enzyme on substrate. Effects of this phenomenon may be somewhat overcome by incorporation of hemolysate blanks without added substrate in the procedure. These were not included consistently, but when used very little nonspecific formazan formation was noted.

The addition of barium carbonate in the ethyl acetate extraction step of this procedure is an improvement which makes it possible to adapt the original tissue extract method for use with hemolysates. This development may be of some importance in quantitative assay of G6PD, since this enzyme is of considerable pathological significance (Gross, 1963; Tizianello et al., 1963).

It may be possible to facilitate performance of this dehydrogenase procedure by advance preparation of dry vials of mixtures of the reagents involved, to which substrate and hemolysate could be added at the time of the test.

Determinations of malic dehydrogenase (Table 4) generally correlate well with the reticulocyte count, being significant at the 1% level. Perfection of this dehydrogenase method (which at present can be performed in about 45 minutes), together with use of a refrigerated centrifuge and consistent running of hemolysate blanks, could render assay of malic dehydrogenase a valuable alternative to reticulocyte counting. This is particularly true since this enzyme is virtually absent from mature erythrocytes.

Determinations of G6PD are less desirable for this purpose since this enzyme is present in the mature cell and its level is affected in various pathological states. As is evident here, G6PD correlates rather poorly with the reticulocyte count and its levels are not appreciably affected by changes in mean cell age.

Coproporphyrin determination did not correlate well with simultaneous reticulocyte counts, as can be seen in Table 6. The large amount of variation in this series of determinations is probably due to the small amount of blood used. Adaptability of the coproporphyrin method of Schwartz et al. (1956) to clinical diagnosis depends on successfully scaling it down to use with a small amount of blood. Schwartz and his co-workers used 50 ml. of blood for these determinations, and a great deal of sensitivity was lost in adapting the method to use with small amounts of blood.

Also, the specificity of the method is in question since absorbance of hemoglobin traces at 400 mμ cannot be ruled out. The minimum time required for the series of extractions involved in this procedure is 1-1/2 hours, which precludes its efficient use as an alternative to the reticulocyte count.

The correlation of erythrocyte RNA and reticulocyte counts as reported in Table 7 is significant at the 1% level, but inspection of the data in Table 7 shows that an individual determination may not reflect the reticulocyte count with much sensitivity. This is probably partly due to the small volume of blood used. The method used here for RNA determination was adapted from a method for purification of RNA from large volumes of reticulocyte-rich erythrocytes (Kruh, 1967) and apparently lost considerable sensitivity in scaling down. Recovery of RNA in this method was only 82 to 85% and occasional technical error may have considerably reduced even this percentage at certain times. The specificity of the method is not in question, since hemoglobin, other proteins and the phenol extractant do not interfere in the final reading of the extract at 260 mμ. Generally, incomplete recovery and poor precision, implying poor sensitivity, are common in any microdetermination of a substance present in small amounts when several extraction steps are involved. With the present method satisfactory results require the use

of large amounts of blood. Rubinstein (1956) and his co-workers have stated that the level of erythrocyte RNA may not correlate significantly with the reticulocyte count because cells classed as reticulocytes in a count contain varying amounts of RNA-rich reticulum; that is, two identical reticulocyte counts may represent different levels of RNA since some cells counted contained more reticulum than others. It is impossible to compensate for this error, since compensation would involve subjective classification of cells on the basis of amount of reticulum and lead to even more error.

It must be remembered in using any of these methods that aging of the cells *in vitro* at or above room temperature (Seno, *et al.*, 1964) and failure to mix specimens thoroughly after centrifugation may yield distorted results.

SUMMARY AND CONCLUSIONS

In an attempt to circumvent the technical and statistical error involved in use of the reticulocyte count as an index of erythropoiesis, a reliable biochemical method of simplicity comparable to that of the reticulocyte count was sought. Six biochemical methods were chosen for evaluation in this respect: (1) magnesium, an enzyme cofactor whose concentration is increased in the metabolically active reticulocyte; (2) phospholipid, present in the membrane of various erythrocytic organelles and decreasing in concentration as the cell ages; (3) malic dehydrogenase, an enzyme of the Krebs cycle which is active in the reticulocyte and disappears as the cell matures; (4) glucose-6-phosphate dehydrogenase, an enzyme of the pentose phosphate pathway present in all erythrocytes but thought to be increased in the reticulocyte; (5) coproporphyrin, an intermediate in the synthesis of heme not present in the fully hemoglobinized adult cell; and (6) ribonucleic acid, itself the characteristic basophilic substance of the erythrocyte. Each method was evaluated with respect to sensitivity and specificity, ease and rapidity of performance and correlation with the reticulocyte count at various levels.

Generally, the methods which are most specific and performed most easily are those which correlate best with the reticulocyte count at various levels, and from which the corresponding reticulocyte count may be estimated with the smallest standard error. This is because simpler methods, involving fewer steps and omitting complicated series of extractions may be performed with more precision and accuracy. Of the biochemical methods evaluated as alternatives to the reticulocyte count, the determination of magnesium, followed by phospholipid and malic dehydrogenase, respectively, are most desirable. Glucose-6-phosphate dehydrogenase is not desirable because it does not decrease appreciably as the cell ages and is also affected by other disease states. Coproporphyrin and RNA determinations as performed here by a series of extractions are not acceptable as test procedures because a large amount of sensitivity is lost when an erythrocyte sample of the small size necessary for clinical use is subjected to analysis.

With further investigation and application of some of the additional precautions discussed here, the procedures for magnesium, phospholipid, and malic dehydrogenase might be adaptable to future use in the clinical laboratory. It might then be possible to accurately estimate a reticulocyte count by a single determination of one of these substances.

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APPENDIX I

Biochemical Methods Used in This Study

Magnesium

Reagents:

1. Stock magnesium standard. Dissolve 8.458 gm. $MgCl_2 \cdot 6H_2O$ in distilled water and make up to 1 liter.

2. Working magnesium standard. Dilute 1.0 ml. of stock to 100 ml. with distilled water (1 mg./100 ml.).

3. Polyvinyl alcohol, 0.1% w/v. Dissolve 1.0 gm. PVA (with heat) in distilled water. Store in polyethylene bottle at 4C.

4. Stock Titan (Clayton) yellow, 0.5%. Dissolve 0.5 gm. Titan yellow in distilled water and make up to 100 ml. Store in brown bottle at 4C.

5. Working Titan yellow, 0.01%. Prepare fresh each day by dilution of 2ml. stock to 100 ml. with distilled water.

6. NaOH, 7.5% w/v. Dissolve 15.0 gm. NaOH pellets in distilled water and make up to 200 ml.

Calibration:

Make the following dilutions of the working standard in distilled water:

Equivalent mg./100 ml. Magnesium	ml. working std.	ml. distilled water
0 (blank)	0.0	10.0
0.5	0.5	9.5
1.0	1.0	9.0
2.0	2.0	8.0
3.0	3.0	7.0
4.0	4.0	6.0
5.0	5.0	5.0
6.0	6.0	4.0
7.0	7.0	3.0
8.0	8.0	2.0
9.0	9.0	1.0
10.0	10.0	0.0

Then treat 1.0 ml. of each dilution as 1.0 ml. of packed cells in the procedure below. Plot % T vs. concentration.

Test:

1. Do not draw blood in EDTA anticoagulant.

2. Lyse 1 ml. packed erythrocytes in 4 ml. distilled water.

3. Precipitate proteins with 5 ml. 20% trichloroacetic acid. Centrifuge.

4. Place in 19 mm. cuvets in order:

	Blank	Test
Distilled water, ml.	3.0	1.0
Protein-free supernatant, ml.	---	2.0
0.1% polyvinyl alcohol, ml.	0.5	0.5
0.01% Titan yellow, ml.	0.5	0.5
7.5% NaOH, ml.	1.0	1.0

5. Mix after each addition, let stand 5 minutes and read test against blank at 540 mμ. Obtain values from calibration curve.

Phospholipid

Reagents:

1. Hydrogen peroxide, 30%
2. 30% chloroform in absolute methanol
3. Sulfuric acid, concentrated
4. 1 N sodium hydroxide
5. Ammonium molybdate reagent. 10% ammonium molybdate in 10 N sulfuric acid
6. Sulfonic acid reagent (Harleco)
7. Stock standard, 10 mg./100 ml. P. Transfer 0.4394 gm. pure dry KH_2PO_4 to a 1-liter flask and dissolve in distilled water.
8. Working standard, 0.5 mg./100 ml. Dilute stock standard 1:20 with 10% trichloroacetic acid.

Calibration: (for determination of inorganic phosphorus)

Make the following dilutions of the working standard in distilled water:

Equivalent mg./100 ml. inorganic phosphorus	ml. working std.	ml. distilled water
0.0 (blank)	0.0	5.0
1.0	0.5	4.5

Equivalent mg./100 ml. inorganic phosphorus	ml. working std.	ml. distilled water
2.0	1.0	4.0
3.0	1.5	3.5
4.0	2.0	3.0
6.0	3.0	2.0
8.0	4.0	1.0
10.0	5.0	0.0

To each add 0.5 ml. ammonium molybdate reagent, 0:2 ml. sulfonic acid reagent, let stand 10 minutes and read each against blank at 660 mμ. Plot % T vs. concentration.

Test:

1. Pipet exactly 0.20 ml. of packed cells into a 15 x 125 mm. test tube.

2. Blow in 5.0 ml. of 30% chloroform in methanol rapidly from a volumetric pipet. Toward the end, allow the pipet to drain to obtain accurate measurement. Stopper, shake vigorously, and centrifuge.

3. Pipet 2 ml. of supernatant to a 19 x 150 mm. PYREX or KIMAX test tube. Pipet 2 ml. of isopropyl alcohol to a second tube for a blank. Evaporate to dryness in a boiling water bath.

4. To the dry residue add 0.5 ml. of water and 0.1 ml. of concentrated sulfuric acid.

5. Heat carefully, with shaking, over a small flame until the solution chars, the volume is reduced to about 0.1 ml., and dense white fumes of SO_3 appear. Do not overheat. Remove from flame, wait about 20 seconds, then add 1 drop of 30% hydrogen peroxide directly into the solution. Heat the tube again until dense white fumes appear. If the digest is still brown, add another drop of H_2O_2 and reheat until fumes appear and solution is clear and colorless. Let cool for about one minute.

6. Add 3.0 ml. of water and 2.0 ml. of 1 N NaOH, mix, and place in a boiling water bath for 3 minutes. Cool in a cold water bath to room temperature.

7. For the Fiske-Subbarow method add:

0.5 ml. ammonium molybdate reagent. Mix.

0.2 ml. sulfonic acid reagent. Mix.

Let stand 10 minutes.

Set to 100% T with the blank at 660 mμ and read the test

8. Obtain value in mEq/L from the calibration curve or chart as prepared from inorganic phosphorus calibration.

9. Chart value in mg./100 ml x 81 = mg. phospholipid in 100 ml. packed erythrocytes.

Dehydrogenases

Calibration

Reagents:

1. MTT tetrazolium, 10 mg./100 ml. in distilled water.
2. DPNH, 15 mg. in 6 ml. distilled water.
3. Phenazine methosulfate (PMS), 10 mg. in 4 ml. distilled water (protect from light).
4. $CoCl_2$, 0.1 M.

Procedure:

1. Make dilutions of MTT standard as follows:

Equivalent µg. formazan	ml. 10 mg./ 100 ml. MTT	ml. distilled water
0 (blank)	0.00	5.00
10	0.10	4.90
16	0.16	4.84
22	0.22	4.78
28	0.28	4.72
34	0.34	4.66
40	0.40	4.60
46	0.46	4.54

Equivalent µg. formazan	ml. 10 mg./ 100 ml. MTT	ml. distilled water
52	0.52	4.48
58	0.58	4.42
64	0.64	4.36
70	0.70	4.30
76	0.76	4.24
82	0.82	4.18
88	0.88	4.12
94	0.94	4.06
100	1.00	4.00

2. Then add to each MTT dilution:

0.1 ml. $CoCl_2$, 0.1 M (above)

0.3 ml. DPNH solution (above)

0.2 ml. PMS solution (above)

3. Shake and add 5 ml. ethyleacetate. Stopper and shake vigorously.

4. Settle out at 4C., 10 minutes

5. Transfer ethyl acetate layer to 19 mm. cuvets and read against blank at 525 mµ, Coleman Jr.

6. Plot % T vs. µg formazan formed.

Test

Reagents:

1. 1.0 M substrate; malate, glucose-6-phosphate, etc. (malate substrate must be buffered to pH 7.0).

2. TPN (for C6PD) or DPN (for MDH), 2.5 mg./ml.

3. MTT tetrazolium, 1 mg./ml.

4. 0.05 M $MgCl_2$.

5. 0.1 M $CoCl_2$.

6. 0.2 M Tris, pH 7.2.

7. 0.2 M Tris, pH 10.1.

8. 0.1 M NaN_3.

Procedure:

1. Keep packed cells at 4C. until hemolysate is prepared.

2. Lyse 0.1 ml. packed cells in 4.9 ml. cold distilled water. Return hemolysate to refrigerator while substrate is prepared.

3. To prepare substrate mixture, add in order (prepare one tube for each hemolysate and one extra for blank):

1.0 M. substrate, 0.3 ml.

0.2 M Tris, pH 7.2, 1.0 ml.

0.1 M $CoCl_2$, 0.1 ml.

0.1 M sodium azide, 0.5 ml.

0.05 M $MgCl_2$, 0.5 ml.

DPN or TPN, 0.5 ml.

MTT tetrazolium, 0.5 ml.

0.2 M Tris, pH 10.1, 0.05 ml.

4. Add entire quantity of hemolysate to substrate mixture. To substrate mixture in one tube, add 5 ml. distilled water as a blank.

5. Add a small amount of barium carbonate powder to each tube.

6. Incubate 30 minutes at 37C.

7. Add 5 ml. ethyl acetate, shake vigorously and let settle out at 4C. for 10 minutes.

8. Read ethyl acetate layer of test against that of blank at 525 mμ.

9. Obtain value in μgm. formazan formed from calibration curve and report as μgm. formazan formed/0.1 ml. packed erythrocytes.

Coproporphyrin

Reagents:

1. 3:1 ethyl acetate:glacial acetic acid.
2. 10% NaOH
3. Concentrated HCl
4. Glacial acetic acid
5. Ethyl ether
6. 0.5% HCl

Calibration:

Reconstitute 1 5.0 µgm. vial of Sigma coproporphyrin with 10 ml. 0.5% HCl (equivalent to 50 µgm./100 ml. coproporphyrin). Dilute as follows:

Equivalent µgm./100 ml. coproporphyrin	ml. standard	ml. 0.5% HCl
0.0 (blank)	0.0	5.0
1.0	0.1	4.9
2.0	0.2	4.8
3.0	0.3	4.7
4.0	0.4	4.6
5.0	0.5	4.5
10.0	1.0	4.0

Equivalent µgm./100 ml. coproporphyrin	ml. standard	ml. 0.5% HCl
25.0	2.5	2.5
50.0	5.0	0.0

Read each at 400 mµ. Beckman DU spectrophotometer, against the blank and plot concentration vs. % T.

Test:

1. To 1 ml. packed cells, in a glass-stoppered tube, add 10 ml. 3:1 ethyl acetate:glacial acetic acid.
2. Shake 10 minutes on Kahn shaking machine.
3. Centrifuge 5 minutes at 1000 x g.
4. Remove supernatant.
5. Repeat steps 2 and 3; pool supernatants.
6. Extract supernatant with two 5 ml. aliquots of 10% NaOH. Centrifuge each time and discard organic layer. Pool NaOH layers.
7. Add concentrated HCl drop by drop to NaOH layer to pH 4.0 (about 25 drops).
8. Add 2 ml. glacial acetic acid.
9. Extract with 10 ml. ethyl ether.
10. Extract ether layer with 0.5% HCl.
11. Read 0.5% HCl extract against a 0.5% HCl blank in quartz covets at 400 mµ. Beckman DU spectrophotometer.
12. Obtain value in µg./100 ml. from a calibration curve or chart.

Ribonucleic Acid

Reagents:

1. 82% phenol in 0.01% K_2EDTA
2. 20% $KC_2H_3O_2$, pH 5.0.
3. Absolute ethanol
4. Absolute ethyl ether
5. 3:1 absolute ethanol:distilled water
6. 1 N NaOH
7. 6 N HCl
8. 0.85% NaCl.

Calibration:

Dissolve 200 mg. Sigma Grade XI (highest purity) Ribonucleic acid in 0.85% NaCl at 56 C. Make dilutions in 0.85% NaCl to represent 100, 75, 50, 25 and 10 mg./100 ml. RNA. Treat 1 ml. aliquots of these dilute standards as 1 ml. of packed erythrocytes. Plot % T vs. concentration.

Test:

1. Lyse 1 ml. packed erythrocytes in 5 ml. cold distilled water in glass-stoppered tube.
2. Add 5 ml. 82% phenol reagent.

3. Shake 30 minutes on Kahn shaking machine.

4. Cool in at 0C. 5 minutes.

5. Centrifuge 15 minutes at 1000 x g.

6. Decant aqueous upper layer. Discard precipitate.

7. Recentrifuge aqueous upper layer 5 minutes at 1000 x g.

8. Remove 2 ml. from top of supernatant, place in a conical centrifuge tube and add 0.2 ml. 20% $KC_2H_3O_2$, pH 5.0.

9. Add 10 ml. cold absolute ethanol.

10. Cool 15 minutes at 0C.

11. Centrifuge 10 minutes at 1000 x g.

12. Discard supernatant.

13. Wash precipitate by suspension in and centrifugation from (in order): 3 ml. cold 3:1 ethanol:distilled water; 3 ml. cold absolute ethanol; 3 ml. cold absolute ethyl ether.

14. Remove ether wash and allow precipitate to dry at room temperature.

15. Dissolve precipitate in 5 ml. 1 N NaOH by standing 1 hour at room temperature.

16. Add 1 ml. 6 N HCl, centrifuge briefly at 1000 x g; decant supernatant.

17. Read supernatant in silica cuvets at 260 mμ (Beckman DU spectrophotometer) against a blank prepared from 5 ml. 1 N NaOH and 1 ml. 6 N HCl.

18. Obtain value in mg./100 ml. from calibration curve.

VITA

The author was born in Flint, Michigan, on September 30, 1944. He graduated from St. Matthew High School, Flint, in June, 1962, and enrolled at Michigan State University in September, 1962. He received his B.S. degree in Medical Technology from Michigan State University in June, 1966, and completed professional training in Medical Technology at St. Joseph Hospital, Flint, in June, 1967. In August, 1967, he was registered as a Medical Technologist with the American Society of Clinical Pathologists.

After working one summer in the pathology laboratory of the Michigan State University Veterinary Clinic, he enrolled in a program of graduate study in Clinical Laboratory Science in the Department of Pathology, Michigan State University, in September, 1967.

The author was married to Virginia Brice of Alma, Michigan, on July 1, 1967. He is a member of the Detroit, the Michigan, and the American Societies of Medical Technologists.

In September, 1968, he accepted a position as Instructor of Medical Technology in the School of Medicine, Wayne State University, Detroit, Michigan.