Erythrocyte Osmotic Fragility of Rats Receiving the Thermostable Plasma Erythropoietic Factor

By James W. Linman and Martha J. Long

The erythropoiesis stimulatory activity of "anemic" and "polycythemic" plasmas and plasma extracts is now well established. The nature, site of production, and modus operandi in normal and abnormal states relative to erythropoiesis of the substance or substances responsible for this phenomenon have not yet been clearly defined. Current data indicate the coexistence of more than one factor. One is heat stable, ether soluble, and most likely a lipid. The other appears to be relatively thermolabile, insoluble in ether, and probably protein in nature. When administered to the normal rat, the former stimulates cellular division of marrow erythrocytic precursors with a resultant erythrocytosis due to microcytes. There is no associated increase in circulating hemoglobin or red cell mass. The heat labile factor augments hemoglobin synthesis as measured by the incorporation of Fe⁵⁹ in hemoglobin. The observations described in this report are concerned with the response in the normal rat to the heat stable plasma erythropoietic factor.

It has been postulated that the microcytes, produced in the rat by this type of erythropoietic stimulation, have a shortened survival time. This would explain the rapid return of all values to normal following discontinuation of the plasma extract injections. In view of the association of impaired cell viability with decreased resistance to lysis in hypotonic salt solutions, the evaluation of erythrocyte osmotic fragility patterns of rats injected with the plasma erythropoietic factor was undertaken in an effort to obtain experimental support for the above hypothesis.

Materials and Methods

The source of the erythropoietic stimulating factor was plasma from adult rabbits rendered anemic by the daily subcutaneous injection of 1 ml. of a 2.5 per cent solution of phenylhydrazine. After approximately seven such injections, in every instance with hemoglobin levels below 7 Gm. per cent, these animals were exsanguinated via the abdominal aorta. The plasma was obtained by centrifugation and a boiled and perchloric acid precipitated extract was prepared and reconstituted to the original volume of the plasma according to the method we have previously used.

Twelve female Carworth-Wistar rats weighing from 150 to 160 Gm. were divided into two groups of six each. One group received 10 daily subcutaneous injections over a period of two weeks (Saturday and Sundays excepted) of the "anemic" plasma extract. Each injection was equivalent to 2 per cent of their body weight. The other six animals were given Ringer's solution in similar amounts and served as the controls.

Routine hematologic studies were obtained prior to the first injection and weekly thereafter for four weeks. They consisted of hemoglobins determined by the cyanmethemoglobin method.

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technic, microhematocrits, hemacytometer erythrocyte counts, and reticulocytes which were enumerated per 1000 red cells on dried, brilliant cresyl blue coverslip films counterstained with Wright's stain. Cell diameters were measured using Wright's stained coverslip films and a micrometer disc. Price-Jones curves were then constructed and the mean corpuscular diameters calculated.

Erythrocyte osmotic fragility determinations were carried out at the same time intervals as listed above. Two different quantitative methods were performed simultaneously on all animals. One technic was the photocolorimetric quantitative method of Bethell. Twenty cu. mm. of blood were obtained from the tail vein of the rat and pipetted into each of four tubes containing 5 ml. of 0.65, 0.55, and 0.45 per cent sodium chloride solutions and distilled water respectively. The tubes were allowed to stand at room temperature for two hours, centrifuged for a few minutes, and then read in a photoelectric colorimeter. The reading obtained in the tube containing the distilled water was used as the maximal reading indicating complete hemolysis. The per cent hemolysis for each concentration of sodium chloride was then calculated.

The second method consisted of enumerating the number of erythrocytes remaining intact in the varying hypotonic salt solutions. Red cell pipettes were filled as for a standard erythrocyte count using a 1:200 dilution with 0.85, 0.65, 0.55, and 0.45 per cent sodium chloride solutions, respectively, as diluents. The pipettes were allowed to stand at room temperature for two hours, shaken by machine at a uniform rate for three minutes, and the red cells were counted by hemacytometer. It was then possible to calculate the per cent hemolysis based on the actual number of cells lysed. Although a greater number of hypotonic salt solutions would have yielded more detailed fragility curves, the possible import of such data did not appear sufficient to warrant the increased blood loss which might alter the erythropoietic activity.

All animals were weighed weekly and the amount of the materials injected adjusted accordingly.

Results

The rats injected with "anemic" plasma extract developed erythrocytosis and reticulocytosis without significant change in their hemoglobin or hematocrit levels (fig. 1). The microcytes were evident in the hemacytometer chamber and on the stained films, and their presence was demonstrated graphically by Price-Jones curves (fig. 2). The mean cell measurements for all animals in each group are listed in table 1. The mean corpuscular hemoglobin concentration remained unchanged while all of the other values decreased in the animals exhibiting the peripheral erythrocytosis.

Erythrocyte osmotic fragility studies with the photoelectric colorimeter (fig. 3) failed to reveal significant variation between the rats injected with the plasma erythropoietic factor and the controls given Ringer's solution. When osmotic fragility was evaluated by direct cell counting, different results were obtained (fig. 4). By this method it was possible to demonstrate a decrease in resistance to hypotonic salt solutions in the rats with the erythrocytosis. At the end of the two-week injection period the average values of these animals in 0.65, 0.55, and 0.45 per cent sodium chloride solutions were 29.1, 40.2, and 74.5 per cent hemolysis respectively with similar determinations of 3.4, 20.8, and 65.3 per cent in the controls. The development of this abnormality in osmotic behavior in each of the hypotonic media used and the return to normal after the injections were stopped are shown in figure 5. The average erythrocyte counts in the different hypotonic diluents throughout the experimental period are tabulated in table 2 with their standard deviation to show the distribution...
Fig. 1.—Erythrocytosis and reticulocytosis without evidence of augmented hemoglobin synthesis in the rats injected with the "anemic" plasma extract. Average determinations of 6 rats in each group.

about the mean and to emphasize the similarity in the individual counts from which the means were derived. The values obtained in 0.85 per cent salt solution were used to calculate the per cent hemolysis occurring in the hypotonic diluents. This was believed desirable in order to obviate any possible change due to more rapid settling of the cells in the counting chamber. These erythrocyte counts, however, did not differ significantly from those diluted with standard red cell diluting fluid.

After the injections were discontinued, the return to normal was prompt and complete in two weeks. All determinations in the control group were constant throughout the period of observation.

The animals in both groups remained healthy and showed no adverse effects from the injections.
The erythropoietic response in this experiment was identical to that which we have uniformly observed in normal rats given the thermostable, ether soluble plasma factor. The finding in such animals of erythrocytosis without significant change in the other erythroid determinations is at variance with
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Fig. 3.—Average erythrocyte osmotic fragility curves determined by a quantitative photocolorimetric method showing no significant variation between the stimulated and control animals.

Fig. 4.—Decreased erythrocytic resistance to hypotonic salt solutions as measured by the direct cell enumeration technic at the end of the injection period in the animals with the erythrocytosis. Two weeks after the injections were stopped this abnormality was no longer demonstrable. Average determinations of 6 rats in each group.

some investigators who have reported associated increases in the hemoglobins and hematocrits. This and other apparent discrepancies in experimental results obtained by various workers studying the humoral control of erythropoiesis are, we believe, due to the existence of two plasma factors with different physical, chemical, and physiologic characteristics.
Table 2.—Average Erythrocyte Counts (Millions per cu. mm.) and the Standard Deviation from the Mean

<table>
<thead>
<tr>
<th>Diluent Groups</th>
<th>Baseline Mean</th>
<th>SD</th>
<th>1 Week Mean</th>
<th>SD</th>
<th>2 Weeks Mean</th>
<th>SD</th>
<th>3 Weeks Mean</th>
<th>SD</th>
<th>4 Weeks Mean</th>
<th>SD</th>
<th>0.85% NaCl Mean</th>
<th>SD</th>
<th>0.65% NaCl Mean</th>
<th>SD</th>
<th>0.55% NaCl Mean</th>
<th>SD</th>
<th>0.45% NaCl Mean</th>
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<tbody>
<tr>
<td>Plasma Erythropoietic Factor</td>
<td>8.08</td>
<td>0.46</td>
<td>9.23</td>
<td>0.73</td>
<td>10.06</td>
<td>0.22</td>
<td>8.81</td>
<td>0.38</td>
<td>8.13</td>
<td>0.15</td>
<td>7.94</td>
<td>0.47</td>
<td>9.11</td>
<td>0.54</td>
<td>9.96</td>
<td>0.33</td>
<td>8.77</td>
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<td>Ringer's Solution</td>
<td>7.93</td>
<td>0.44</td>
<td>8.04</td>
<td>0.56</td>
<td>7.82</td>
<td>0.41</td>
<td>7.73</td>
<td>0.37</td>
<td>7.82</td>
<td>0.16</td>
<td>7.77</td>
<td>0.48</td>
<td>8.14</td>
<td>0.46</td>
<td>7.85</td>
<td>0.37</td>
<td>7.78</td>
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<td>Plasma Erythropoietic Factor</td>
<td>7.52</td>
<td>0.42</td>
<td>6.90</td>
<td>0.60</td>
<td>7.03</td>
<td>0.59</td>
<td>8.13</td>
<td>0.53</td>
<td>7.59</td>
<td>0.58</td>
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<td>7.88</td>
<td>0.50</td>
<td>7.58</td>
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<td>0.47</td>
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<td>5.94</td>
<td>0.53</td>
<td>6.81</td>
<td>0.74</td>
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<tr>
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<td>0.86</td>
<td>6.20</td>
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<td>6.21</td>
<td>0.34</td>
<td>6.57</td>
<td>0.16</td>
<td>2.52</td>
<td>1.15</td>
<td>2.62</td>
<td>0.82</td>
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<td>0.59</td>
<td>3.04</td>
<td>0.78</td>
</tr>
<tr>
<td>Ringer's Solution</td>
<td>2.77</td>
<td>0.74</td>
<td>2.20</td>
<td>0.45</td>
<td>2.71</td>
<td>0.63</td>
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<td>2.20</td>
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<td>2.71</td>
<td>0.63</td>
<td>2.78</td>
<td>0.57</td>
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</table>

* Injections discontinued.

As previously discussed, the most apparent explanation for the rapid return of the red cell and reticulocyte counts and Price-Jones curves to normal following cessation of the injections is that the microcytes responsible for the erythrocytosis possess markedly impaired viability. The abnormal behavior of these cells in hypotonic salt solutions lends considerable support to this theory. The more common technics used to determine erythrocyte osmotic fragility are based on the assumption that there is a direct relationship in a given quantity of blood between the number of cells hemolysed and the color imparted to the supernatant whether noted visually or read in a photoelectric colorimeter. Since the erythrocytosis in the present study was not accompanied by augmented hemoglobin synthesis, this assumption is not valid under the conditions of this experiment and a considerably greater number of small cells containing less hemoglobin could be lysed without significantly altering the reading in the photoelectric colorimeter. We have failed on repeated occasions in the past to demonstrate significant variations in osmotic fragility between the control and
Fig. 5.—The development of the abnormal erythrocyte osmotic behavior and the return to normal as measured by the direct cell counting method in the animals injected with the erythropoietic factor for each of the hypotonic diluents used. All values in the control group remained constant throughout the period of observation.

experimental animals by this technic, and these observations were confirmed by the present study (fig. 3).

The determination of erythrocyte osmotic fragility by direct cell counting was described by Simmel in 1923. This method has received only limited use chiefly because of its time consuming aspects, the possibility of laboratory error in the erythrocyte counts, and the fact that it offers no definite advantage in the clinical evaluation of hemolytic states. As an investigative tool, however, it is ideally suited to an experimental situation such as the one just described.

When hypotonic salt solutions were used as diluents for erythrocyte counts, it was possible to demonstrate definite abnormalities in the red cell osmotic behavior of the rats injected with the heat stable plasma erythropoietic factor (fig. 4) as compared not only to the control group but to their own baseline and four-week values. This finding, without significant change in osmotic fragility by the photocolorimetric technic, would indicate that only the microcytes possessed the increased susceptibility to lysis in hypotonic sodium chloride solutions. This interpretation is supported by comparing the counts of the experimental and control animals in the different hypotonic solutions (table 2). The erythrocyte counts of the control animals throughout the experimental period in all of the diluents were quite constant. The counts in 0.65 per cent salt solution, 7 and 14 days after the injections were started, were slightly lower in the stimulated animals than in the controls, although the values of the former group in 0.85 per cent salt solution and routine red cell diluting fluid were considerably higher. This finding would be anticipated if normal erythropoiesis was largely replaced by the formation of microcytes with decreased osmotic resistance secondary to accelerated erythroblastic division.
occaisioned by the administration of the plasma factor. Such erythropoietic stimulation would result in a greater number of cells containing essentially the same amount of hemoglobin as the smaller number of normal cells that would have been formed from the erythrocytic precursors involved had their rate of cellular division not been altered. This would preclude increased diversion of the multipotential marrow reticulum cells into red cell production. If this were not true, an increase in circulating red cell mass and hemoglobin would of necessity accompany the erythropoietic stimulation evoked by the thermostable factor and augmentation in hemoglobin synthesis would be evident. The slightly lower counts in the experimental group with 0.65 per cent sodium chloride solution as the diluent could then be explained on the basis of the expected removal of a certain number of cells with a normal survival time during the injection period. It cannot be assumed, however, that normal erythropoiesis was completely replaced by the formation of microcytes with decreased osmotic resistance. Based on a normal life span of 58 days,11 approximately 25 per cent of the cells present prior to the administration of the factor would have been removed by the end of the 14-day injection period. If these cells had all been replaced by microcytes with abnormal osmotic behavior, the latter would have contained 25 per cent of the circulating hemoglobin. Fragility abnormalities should then have been readily apparent with the photocoolorimetric technic. In addition, at the end of the injection period the erythrocyte counts in 0.65 per cent salt solution would have been expected to be considerably lower in the stimulated animals than in the controls, and a greater number of small cells should have been demonstrable. The possibility that all of the microcytes did not display decreased osmotic resistance must be considered. The Price-Jones curves in the stimulated animals (fig. 2) afford evidence against this. At the end of the injection period this curve showed two definite peaks. One represented the microcytes and the other the cells of normal size. The microcytes comprised 30.6 per cent of the mean erythrocyte count or 3,080,000 per cu. mm. If all of these cells were lysed in 0.65 per cent salt solution, the expected per cent hemolysis and erythrocytes per cu. mm. with this hypotonic diluent would be 30.6 and 6,980,000 respectively. The actual values (fig. 4, table 2) were 29.1 per cent and 7,030,000 per cu. mm.

Therefore, in the experimental situation just described, all of the marrow erythrocytic precursors would not appear to have been involved in the accelerated cellular division. The microcytes probably contributed only a small amount to the total circulating hemoglobin. The dosage and number of injections are important factors in this regard. The erythrocytosis increases progressively during the administration of boiled plasma extracts, and past observations have indicated that the extent of the erythropoietic response is directly related to the amount of material given.12 Small doses continued for a long period of time are capable of evoking essentially the same degree of erythropoietic stimulation as larger daily amounts given over a shorter period.

There remains the possibility that the erythropoietic factor may, in some way, alter the resistance of the circulating red cells to hypotonic salt solution or cause them to become spherocytic. Such an effect would be expected to involve cells regardless of size, thereby producing abnormalities in osmotic frag-
ility detectable by both methods employed. The lack of parallelism between the increase in red count and the other erythroid determinations excludes the possibility that the microcytes were otherwise normal cells that had assumed a spheroidal shape. Although the diameter of such erythrocytes would be decreased, the hemoglobin content would remain constant.

The sources of error inherent in the technic of red cell counting are important in the interpretation of experimental data based on this procedure. Although the possibility and frequency of such laboratory error is well established, erythrocyte counts performed by a single individual with strict observance of all details of the technic can be reproduced with a high degree of accuracy. In addition, the magnitude of the changes (table 2), similar findings in each of the rats in the experimental group, and the constancy of both the individual and average counts in the control group throughout the period of observation would appear to contribute to the significance of these data. Two other factors must be considered in the direct cell enumeration method for determining osmotic fragility. The first is the possibility that lysis in hypotonic salt solution may not be an all or none phenomenon, and the second is the presence of erythrocyte fragments or ghosts. There exists evidence that partial lysis does not occur. If, however, one or both of these factors had been operating in the present study, they would have diminished rather than accentuated the observed changes.

There would, therefore, on the basis of the experimental observations described, appear to be a double red cell population in the normal rat given the heat stable, ether soluble plasma erythropoietic factor. These cells are microcytes with decreased resistance to lysis in hypotonic salt solutions and normal erythrocytes.

The assumption that such in vitro osmotic behavior indicates an in vivo decrease in viability of these cells appears to be justified. Although not analogous to the mechanisms of intravascular hemolysis or to the demonstration of decreased cell survival by radioisotopic methods in the living organism, the correlation between increased osmotic fragility and these abnormalities is well accepted in experimental and clinical medicine. Decreased cell survival can exist without demonstrable osmotic fragility changes but the reverse probably does not occur in the intact experimental animal or man.

The relationship between the basic configuration of an erythrocyte and its transformation from a biconcave disc to a sphere, with an increase in volume but not surface area, prior to lysis in hypotonic media has been extensively studied. A number of variables are involved in the osmotic behavior of red cells and fragility is not determined by shape alone. Intracellular and extracellular osmotic pressures are important, and altered erythrocyte osmotic fragility can be produced by a variety of physical, chemical, and immunologic agents capable of injuring the inelastic red cell membrane.

The abnormality responsible for the observations reported above is not yet apparent. Although the microcytes appeared spheroidal on dried, stained films, the measurements failed to reveal a decrease in length: thickness ratio. It should be emphasized, however, that the slight changes listed in table 2 are mean, and in many instances derived, measurements of a distinctly bimodal
population. They are significant only in showing the effect of the small cells on the average cell size and do not reflect the true size or shape of the microcytes. There exist several possible explanations for the behavior of these cells in hypotonic media, such as their altered size or shape, injury or change in surface membrane, intracellular metabolic abnormalities, a possible combination of the above, or some more obscure factor. It cannot be assumed to be a specific effect of the thermostable plasma erythropoietic factor. Current data indicate that this agent exerts important regulatory control over erythropoiesis in normal and certain anemic states where neither microcytes nor abnormal osmotic behavior are demonstrable. In these situations the combined physiologic activities of both stimulatory factors result in the production of normal cells. Therefore, the decrease in osmotic resistance observed in the experiment herein described would certainly appear to be a manifestation of accelerated erythroblastic cellular division in the absence of comparable augmentation in the relatively thermolabile factor which stimulates hemoglobin synthesis.

The relationship between erythrocyte age and osmotic resistance has been the subject of considerable investigation. The majority of these experiments have dealt with the behavior of reticulocytes in hypotonic salt solutions and the results have been conflicting. The osmotic resistance of newly formed cells has been reported to be significantly less with technics employing radioactive iron. More recent experiments with glycine- C as a label of newly formed rabbit erythrocytes and measurement of dehydrogenase activity have shown that young cells are more resistant to lysis in hypotonic media than are older cells. Alterations in osmotic resistance of young cells would not seem to alter the significance of the findings just described. Even if there existed conclusive evidence that reticulocytes and other young erythrocytes are less resistant to osmotic stress, the number of cells of the stimulated rats that were lysed in 0.65 per cent salt solution was three times greater than the number of reticulocytes present.

The rapid return to normal erythrocytic equilibrium after the administration of the plasma erythropoietic factor was stopped would certainly appear to be due to the removal from the peripheral blood of the short-lived microcytes. One week after the injections were stopped, the average erythrocyte count had decreased considerably (fig. 1) and at two weeks closely paralleled the baseline and control values. In view of the recent evidence described above, indicating increased osmotic resistance of young erythrocytes, the failure to demonstrate abnormal fragility one week after the injections were discontinued (fig. 5, table 2) may be a reflection of increased resistance to osmotic stress of the newly formed normal cells, thereby obscuring the abnormal behavior in hypotonic media of the relatively small number of microcytes remaining.

The re-establishment of normal erythropoietic activity was not accompanied by significant change in the hemoglobin or hematocrit levels. With cessation of the increased rate of erythroblastic cellular division and rapid destruction of the circulating microcytes, the failure to observe a decrease in these erythroid determinations deserves comment. The peripheral blood was not examined for seven days after the injections were discontinued in order to keep the blood
loss uniform throughout the entire period of observation. Possible changes in the immediate postinjection period cannot, therefore, be excluded. As previously discussed, however, the microcytes under the conditions of this experiment most likely contributed a relatively small amount to the total circulating hemoglobin and red cell mass. In addition, the available evidence indicates that although these cells possess impaired viability, their life span is probably several days. Replacement with normal cells could then prevent any discernible drop in hemoglobin. In view of the shortened life span of the microcytes, it would seem necessary to postulate the development of some augmentation in hemoglobin synthesis during the period of erythropoietic readjustment. Marrow examinations of stimulated animals show a three to fourfold increase in erythrocytic elements with a roughly proportional increase in all recognizable precursors. It would appear reasonable to assume that following the removal of the stimulus to accelerated cellular division, significant numbers of these precursors would mature in an essentially normal manner. This could result in a sufficient increase in hemoglobin production to keep up with the natural destruction of normal cells and replace that lost by removal of the microcytes.

The hazards associated with attempts to explain, on the basis of experimental observations in the normal animal, the role played by the plasma erythropoietic factors in the maintenance of normal erythroid values and in disorders of altered erythropoiesis should be re-emphasized. Polycythemia vera and secondary polycythemia are the hematologic disorders which, with the exception of the duration of the erythropoietic stimulation, most closely simulate the experimentally induced accelerated erythropoiesis in the normal rat given the plasma factor or factors. Stimulatory activity of the types attributable to both the heat stable and the relatively thermolabile erythropoietic factors has been demonstrated in the plasma of patients with both primary and secondary polycythemia. Studies now in progress indicate that erythropoietic activity similar to that seen in the normal rat injected with the heat stable plasma factor is present in patients with primary and secondary polycythemia. Microcytes are prominent and readily demonstrable by Price-Jones curves, and osmotic fragility determinations by the direct enumeration technic are abnormal.

The renewed interest by many investigators in the humoral control of erythropoiesis has, in recent years, greatly advanced our knowledge of red cell production. Continued study should make possible the practical application of some of these basic principles toward furthering our understanding of certain hematologic disorders in man.

**Summary**

1. The thermostable erythropoietic factor in “anemic” rabbit plasma stimulates cellular division of marrow erythrocytic precursors in the normal rat producing erythrocytosis due to microcytes without evidence of augmented hemoglobin synthesis.

2. Study of erythrocyte osmotic behavior in these animals with a quantitative photocolorimetric method failed to demonstrate significant variation from the controls.

3. Osmotic fragility determinations by a direct cell enumeration technic re-
ERYTHROCYTE OSMOTIC FRAGILITY OF RATS

It was observed that the microcytes in the normal rat injected with the heat stable plasma erythropoietic factor comprised two distinct cell types. They are microcytes with shortened survival times and normal erythrocytes.

The decreased viability of these microcytes is the apparent explanation for the rapid re-establishment of normal erythrocytic equilibrium after the "anemic" plasma extract injections are stopped.

SUMMARIO IN INTERLINGUA

1. Le thermostabile factor erythropoietic que se trova in le plasma de conilios "anemic" stimula le division cellular de precursores erythrocytic in le medulla de rattos normal. Illo produce erythrocytose per microcytos, sin evidentia de un augmentate synthese de hemoglobin.

2. Le studio del comportamento osmotic del erythrocytos in tal animales per medio de un quantitative methodo photocolorimetric demonstrava nulle significative variation in comparation con le animales de controlo.

3. Determinaciones del fragilitate osmotic per medio de un technica de numeracion cellular directe revelava un reducite resistencia del microcytos contra le lyse in solutiones hypotonic de chioruro de natrium.

4. Super le base de iste datos, le conclusion es formulate que le erythrocytos in le ratto normal in que le thermostabile factor erythropoietic de plasma esseva injicite representa duo distincte typos de cellula. Isto es microcytos con reducite tempore de superviventia e erythrocytos normal.

5. Le reducite viabilitate de iste microcytos es apparentemente le explication pro le rapide restablimento de un normal equilibrio erythrocytic post que le injectiones de "anemic" extractos de plasma es discontinuate.

REFERENCES


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