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Studies on Agranulocytosis. III.
The Reduced Glutathione (GSH) Content of Leukocytes of Normals and Patients Recovered from Agranulocytosis

By ANTHONY V. PISCIOTTA AND MARY DALY

LITTLE is known about the reduced glutathione (GSH) content of leukocytes in leukopenic states. Parker and Kracke[1] found a decrease in GSH of whole blood in rabbits made leukopenic and anemic by benzol. Similar data were reported by Kuzell et al.[2] in a patient with agranulocytosis and anemia which followed treatment with phenylbutazone. Inasmuch as GSH is completely intracellular, it is not certain whether these low values represented development of the anemic state as opposed to a true fall in value for GSH in leukocytes. Further studies[3] have shown that diets low in the sulfhydryl amino acids (cysteine and methionine) frequently result in a drop in the leukocyte count of experimental animals. Furthermore, the anemia and leukopenia which result from vitamin B-12 and folic acid deficiency has been associated with diminished GSH values in erythrocytes.[4]

The unusually high incidence of agranulocytosis induced by chlorpromazine among the mentally ill, is probably a function of the large dosage of drug administered these patients.[5] However, the GSH index of erythrocytes of persons with psychosis was found to be lower than that observed in normals.[6] A similar abnormality in leukocytes from mentally ill patients could possibly correlate with the peculiar sensitivity of this group to agranulocytosis. In view of these observations, it seemed desirable to investigate the problem of susceptibility to agranulocytosis in terms of sulfhydryl metabolism of leukocytes.

Previous studies from this laboratory[7] failed to demonstrate a block in influx of sulfur^{35} labeled L-cystine and L-methionine into leukocytes of persons susceptible to agranulocytosis *in vitro* and *in vivo*, even when challenged with the drug to which they were sensitive. Indeed, there was a marked increase in the uptake of these substances during recovery periods. In this study, we have taken up the possible correlation of sensitivity to agranulocytosis and the glutathione content of leukocytes.

Information relative to content of GSH in leukocytes has been limited because of the lack of a completely satisfactory method to estimate this substance. Bichel[8] used the whole blood to determine GSH by the iodometric method. Contopoulos and Anderson[9] determined GSH on the leukocytes isolated from whole blood in relatively pure suspension and reported GSH in

From the Blood Research Laboratory, Department of Medicine, Marquette University School of Medicine and Milwaukee County Hospital, Milwaukee, Wisconsin.

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terms of wet weight of leukocytes. They noted values above normal in leukemia and in one patient with aplastic anemia, subnormal values were found. Hardin, Valentine, Follette and Lawrence[10] used a modification of the sodium nitroprusside method on leukocytes isolated from whole blood. They corrected for erythrocyte contamination by subtracting the values for GSH in erythrocytes, from the total amount found in the leukocyte erythrocyte suspension. By this technic they reported the GSH content of normal leukocytes to be about 5 mg. per 10^{10} WBC, and described diminished values for GSH in acute leukemia and in chronic lymphocytic leukemia, but no significant difference in content of GSH in normals and leukocytes of chronic granulocytic leukemia.

An improved method for the direct analysis of GSH in leukocytes is desirable. We have used a method employing a purified suspension of leukocytes, free of erythrocyte and platelet contamination. GSH was determined by the highly sensitive Alloxan "305" method[11] on the alkaline digest of the white cells. GSH values for normal leukocytes were compared with those obtained in mentally ill patients and persons recovered from agranulocytosis.

MATERIALS AND METHODS

Blood representing normal individuals was drawn from house officers, medical students, technicians and patients with nonhematologic illnesses. Blood from patients with psychoses was tabulated as a separate category because of the possibility that mental disease might affect cellular content of GSH.[6] The group under observation consisted of nine patients who had had agranulocytosis and now were recovered. Eight of these patients had agranulocytosis induced by chlorpromazine and one was attributed to ristocetin.

To minimize non specific agglutination of leukocytes and platelets, all blood was drawn with siliconized syringes and arquad treated needles and transferred into each of two siliconized test tubes 23 × 1.4 cm. Each tube contained 1.0 ml. of 1 per cent sodium sequestrene in 0.85 per cent saline, to which was added 10 ml. of whole blood. One tube of each contained the drug (chlorpromazine or ristocetin) which had previously induced agranulocytosis in the person whose blood was tested. The drug was dissolved in 1.0 ml. of 0.85 per cent saline in a concentration of 0.2 μm of the drug for each 1 ml. of blood. The other tubes contained 1.0 ml. of 0.85 per cent saline, as control. Blood from hematologically normal individuals were tested simultaneously utilizing the same test drug solutions.

After 30 minutes of incubation at 37 C., the leukocytes were recovered by sedimentation of the erythrocytes with 1.25 ml. of 3.1 per cent bovine fibrinogen in 0.85 per cent saline. To eliminate contaminating erythrocytes by hemolysis, the leukocytes were washed at least once in phosphoric acid-sodium acetate buffer, pH 7.45,[12*] and twice with a 0.85 per cent saline. If contaminating erythrocytes persisted as judged by hemoglobin coloration of the supernate, or by red cell contamination of the leukocyte button, another washing with hypotonic buffer was employed. In most cases, one such washing was enough. Leukocytes obtained by this technic remained viable as judged by motility, redox activity and phagocytic activity. The washed leukocytes were resuspended in 0.5 ml. of 0.85 per cent saline and counted in duplicate using Toisson's solution as diluent. A measured quantity of well suspended leukocyte suspension was transferred to a plastic centrifuge tube and sufficient distilled water was added to make a total volume of 3.6 ml. The leukocytes were digested with 0.2 ml. of 0.5 M NaOH and the mixture gently shaken until clear.

*Phosphate-acetate buffer: sodium acetate, 2 gm., 85 per cent phosphoric acid, 1.42 ml. adjust pH to 7.45 with 5 per cent NaOH. Dilute with distilled water to 1000 ml.

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Table 1

Reagents	Milliliters (mb) of solution added A	A-1	B	B-1
Blood filtrate or standard (in 1% metaphosphoric acid)	0.0	0.0	1.0	1.0
1% Metaphosphoric acid	1.0	1.0	0.0	0.0
Distilled water	1.0	1.2	1.0	1.2
0.1 M Alloxan	0.2	0.0	0.2	0.0
0.5 M Phos. buffer, pH 7.5	0.2	0.2	0.2	0.2
Equivalent (0.5 M.) NaOH	0.2	0.2	0.2	0.2
Standing time (min.)	10	10	10	10
Equivalent (0.5 M.) NaOH	0.4	0.4	0.4	0.4
Total volume (ml.)	3.0	3.0	3.0	3.0

A is the value for the absorption of the nonspecific decomposition products of Alloxan and is subtracted from all test readings.
A-1 is a reagent to set absorbance values at zero in determination of value for A.
B is the total value for "305" product, including nonspecific decomposition products of Alloxan in the sample, determined under A.
B-1 is the nonspecific reagent blank to set the spectrophotometer at zero for the determination of B.
B-A = amount of GSH in the sample.

Digestion with alkali was found to be more efficient than mechanical homogenization, to disrupt leukocytes in order to liberate their contents and consistently led to increased GSH values. The leukocyte suspension cleared immediately after addition of NaOH. The NaOH was neutralized by the addition of 1.2 ml. of 5 per cent metaphosphoric acid, which also precipitated the protein. After shaking, the mixture was centrifuged in a refrigerated centrifuge at 32,700 x G. for 10 minutes. The protein-free supernatant represents a 1 to 5 dilution.

Test Procedure

GSH was determined by measuring the intensity of absorption at 305 millimicrons of a compound formed by the interaction of GSH and an excess of alloxan according to the technic described by Patterson and Lazerow.[11]

The reagents listed in table 1 were added in the order indicated. The phosphate buffer and equivalent (0.5 M.) sodium hydroxide were dispensed from micro burettes at intervals of 20 seconds. At the end of 10 minutes, 0.4 ml. of 0.5 M. NaOH was added to each tube at 20 second intervals in order to stop the reaction and to stabilize the "305" reaction product. The mixtures were then read in the Beckman DU spectrophotometer at 305 mμ.

A standard curve was prepared which contained 5, 10, 15, 20 and 25 micrograms of GSH per ml.* A duplicate curve was prepared simultaneously in which 0.2 ml. of 0.5 M. sodium hydroxide was added to standard solutions of GSH and then neutralized with equivalent amounts of metaphosphoric acid. The points on the curve derived from the standard GSH pretreated with NaOH were found to conform exactly to the values determined at each point of the untreated standard solution of GSH. NaOH was added to plasma, serum and fibrinogen in the same proportions as above. When the proteins were precipitated with metaphosphoric acid, no alloxan 305 reaction product was found in the protein-free filtrate. These observations demonstrated that treatment with NaOH had no effect on the determination of GSH by the alloxan 305 mehod and that it did not split off sulfhydryl groups from soluble proteins.

*Standard reduced glutathione was obtained from Schwarz Biochemicals, Inc., Mt. Vernon, N. Y. Chromatographic analysis and thiol titration with N-ethyl maleimide disclosed that it was more than 98 per cent pure. Electrolytic reduction was not carried out.

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Results

The results of all studies are illustrated in figure 1.

Normal subjects: Observations were made on 25 apparently normal individuals. Values for GSH were calculated per 10^{10} leukocytes. A range of 3.0 to 6.4 mg. GSH was found per 10^{10} WBC. The mean value for GSH was 5.2 mg. ± a standard deviation of 0.9 mg. per 10^{10} WBC. The adjacent bar graph depicts the leukocyte GSH content following incubation with chlorpromazine. For the sake of simplicity, paired determinations were not connected by a line. There was no effect of chlorpromazine on increase or decrease in GSH values. After incubation with this drug, mean GSH content of leukocytes remained 5.2 ± standard deviation of 1.0 mg./10^{10} WBC.

Mentally ill subjects: Leukocyte GSH content was determined in 17 persons with mental disease. Values following incubation with saline and with chlorpromazine were identical; 5.3 mg. ± standard deviation of 1.4 mg. per 10^{10} WBC. Thus no trends which differed from those observed in normals could be demonstrated.

Persons recovered from agranulocytosis: The patients in our group who

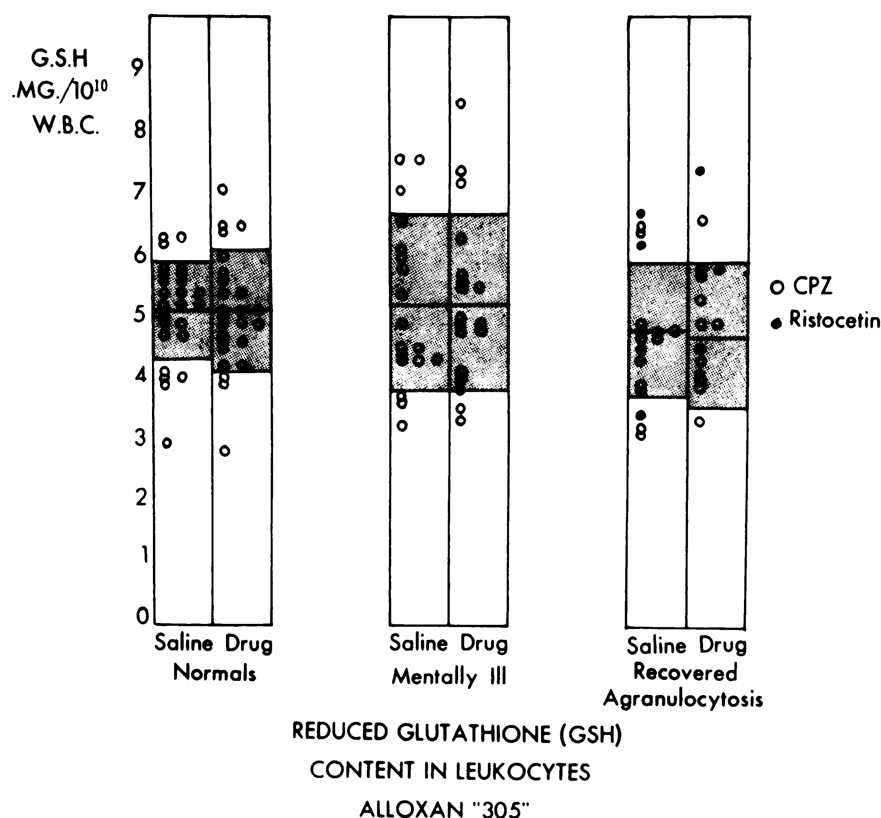

Fig. 1.—Reduced glutathione (GSH) content of leukocytes after incubation with saline (control) or drug (○ chlorpromazine, ● ristocetin). Shaded area equals ± 1 standard divation.

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had agranulocytosis were studied on at least two different occasions. Seventeen observations were made on the leukocytes of nine patients. In these, a range of values of GSH was observed between 3.2 and 6.8 mg. per cent. The mean GSH value was 4.9 ±, a standard deviation of 1.1 mg./10^{10} WBC. Incubation with chlorpromazine (white circles) or with ristocetin (black circles) had no significant effect on these values. In 14 observations, the observed range extended between 3.4 and 7.4 mg. GSH/10^{10} WBC. The mean value was 4.8 mg. with a standard deviation of 1.2 mg. per 10^{10} WBC. It may therefore be stated that the leukocytes of patients known to have had agranulocytosis show no abnormality in GSH content during the time of normal hematologic values. For mechanical reasons, sufficient leukocytes could not be obtained during the time of leukopenia to warrant valid determinations.

Discussion

Several inadequacies which limit the accuracy of determination of glutathione in leukocytes have been eliminated by the method described herein. The use of hypotonic buffer to hemolyze erythrocytes and remove their contents eliminates the necessity of having to correct for GSH contamination by red cells. Comparison of this method of leukocyte recovery, with one in which an isotonic saline wash is used throughout, showed neither a gain nor a loss in GSH content when hypotonic buffer was used in the manner prescribed. With saline, of course, correction had to be made for the GSH content of contaminating red cells. Also, by meticulous centrifugation, one may obtain a leukocyte suspension free of platelet contamination. Second, we have adopted as an arbitrary reference point, 10^{10} leukocytes([10]) in order to avoid variation in number of leukocytes per unit volume of suspension as well as the inaccuracies of wet weight as a point of reference. Third, the question of rupture of leukocytes in order to ensure liberation of GSH for assay is an important determinant for accuracy. We have found mechanical homogenization, alternate freezing and thawing and grinding of a frozen suspension of leukocytes inadequate for the complete liberation of GSH. On the other hand, the addition of 0.5 M. NaOH results in a rapid liquefaction of leukocytes with liberation of contents. NaOH has no effect upon the determination of a standard solution of GSH by the alloxan 305 method nor does it result in the liberation of minus SH groups from soluble proteins. With respect to determination of GSH itself, it has been found that the intensity of color developed by sodium nitroprusside is too faint to allow for a reasonably accurate estimate of the small quantity contained in leukocytes. On the other hand, the alloxan 305 method is sufficiently sensitive to detect GSH in the concentration of 1.0 microgram per 1 ml. of reaction mixture. According to Patterson and Lazerow,[13] glutamylcysteine will absorb 16 per cent while cysteine and cysteinylglycine will absorb only 5 and 4 per cent of the GSH values respectively. Ergothionine and ascorbic acid will remove a negligible amount of alloxan from the reaction mixture.

The amount of reduced glutathione in normals per 10^{10} leukocytes, by

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this technic, is found to compare favorably with the data expressed by Hardin, et al.[10] With the methods outlined above, we have confirmed that the average GSH content of normal leukocytes is 5.2 ± 1.0 mg. per 10^{10} WBC. In our hands, comparison of our technic with that of Hardin shows recovery of about 20 per cent greater value for GSH by our method. The same values were found in the blood cells of patients with mental disease and in the leukocytes of persons who had recovered from agranulocytosis. It was not possible to estimate the quantity of GSH in leukocytes during leukopenic periods, nor do we have any information regarding the GSH content of leukocyte precursors in the bone marrow. Inasmuch as agranulocytosis induced by chlorpromazine result from temporary aplasia of granulocyte precursors, these studies do not rule out a possible metabolic defect of leukocyte precursors in the bone marrow.

Summary

1. A method for the direct determination of GSH in leukocytes is described. Treatment with alkali (0.5 M. NaOH) effects complete solution of the white cells and after deproteinization (5 per cent HPO_3), the GSH is determined by the sensitive "alloxan 305" procedure. Control studies showed that under the conditions of the test, GSH is not affected by the alkali and no splitting of soluble minus SH from protein occurs.

2. Using this method, the amount of reduced glutathione content of normal leukocytes was found to be 5.2 ± 1 mg. per 10^{10} WBC.

3. No differences from normal levels were detected for the leukocytic GSH of patients with mental disease and those susceptible to agranulocytosis. Incubation of whole blood with the drug which caused agranulocytosis had no effect upon the GSH content of leukocytes.

Summario in Interlingua

1. Es describite un methodo pro le determination directe de reducite glutathiona in leucocytos. Tractamento con alcali (0,5 M de NaOH) effectua un complete solution del leucocytos. Post disproteinisation (5 pro cento de HPO_3), le reducite glutathiona es determinate per medio del sensibile procedimento a "alloxano 305." Studios de control monstrava que sub le conditiones del test, reducite glutathiona non es afficite per le alcali e que il occurre nulle fission del solubile SH negative ab le proteina.

2. Le contento de reducite glutathiona in leucocytos normal, determinate per medio de iste methodo, esseva 5,2 ± 1 mg per 10^{10} leucocytos.

3. Nulle deviation ab le nivellos normal del reducite glutathiona leucocytic esseva detegite in patientes con morbos mental o in patientes susceptibile a agranulocytosis. Le incubation de sanguine total con le droga que causava agranulocytosis habeva nulle effecto super le contento de reducite glutathiona in le leucocytos.

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EFFECT OF ASCORBIC ACID ON NITRITE-INDUCED METHEMOGLOBINEMIA. *L. Magos, S. Mario and M. Szirtes.* Országos Munkaegészségügyi Intézet, Budapest. Kisérl.Orvostud. *11*:63–66, 1959.

Ascorbic acid, then sodium nitrite were added to erythrocyte suspensions. It was found that at lower ascorbic acid concentrations methemoglobin formation was slowed down, whereas at higher concentrations this inhibitory action was reduced. In rats pretreated with ascorbic acid, sodium nitrite caused a less marked methemoglobinemia than in the rats treated with sodium nitrite alone.—*S. R. H.*

PHOSPHO-HEXOKINASE ACTIVITY IN STORED BLOOD. *J. Hořejší, L. Mirčevová and J. Vosyková.* From the Institute of Hematology and Blood Transfusion, Prague. Vnit.lék. *4*:1080–1082, 1958.

The ability of erythrocytes from ACD stored blood to convert glucose into trioses was investigated. The glycolytic activity of erythrocytes was also studied. It was found that erythrocytes even after 35 days storage have sufficient endogenous ATP. Though the amount of trioses produced remains practically unchanged during storage, total glycolytic activity declines.—*L. D.*

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