

## ZINC IN HUMAN BLOOD CELLS: NORMAL VALUES AND ABNORMALITIES ASSOCIATED WITH LIVER DISEASE \*

By ROBERT E. FREDRICKS, KOUICHI R. TANAKA AND WILLIAM N. VALENTINE,  
WITH THE TECHNICAL ASSISTANCE OF MARYLU MATTSON

(From the Department of Medicine, University of California Medical Center at Los Angeles,  
and the Wadsworth Hospital, Veterans Administration Center, Los Angeles, Calif.)

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Studies of the biochemistry of zinc have produced results which are widely divergent because of difficulties in methodology. However, techniques have evolved so that general agreement now exists that dithizone methods give reliable and reproducible measurements of zinc in erythrocytes and sera, and that acid extraction of the zinc from these biological materials produces more consistent and accurate results than wet-ashing or dry-ashing methods of preparing specimens for dithizone determination of zinc (1-4). Although normal values for erythrocyte and serum zinc have been established, the zinc content of leukocytes from normal persons using the acid extraction-dithizone method has not been evaluated in the past.

Patients with hepatic cirrhosis have been found to have low serum zinc levels (5, 6), zincuria (7), and decreased concentrations of zinc in liver tissue (6, 7). Vallee, Wacker, Bartholomay and Hoch (7) have postulated that these disturbances of zinc metabolism may be due to inhibition and perhaps to degradation of the zinc metalloenzymes, alcohol and glutamic dehydrogenases, because of excessive ethanol ingestion. However, there has been no direct evidence of altered intracellular zinc levels in patients with liver disease.

The purposes of this study were to extend the application of the acid extraction-dithizone method for the determination of zinc to human leukocytes, to re-examine critically the levels of zinc in blood cells of normal persons, and to investigate the zinc content of the erythrocytes and leukocytes of patients with cirrhosis.

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### MATERIALS AND METHODS

All reagents were prepared, glassware cleaned, and blood collected with the special precautions required for trace metal analysis (4). Venous blood (about 30 to 40 ml) was drawn from the subjects in the morning, without regard to fasting or nonfasting state. A small aliquot of blood was taken for routine hematological determinations and the remainder was mixed with about an equal volume of polyvinylpyrrolidone-heparin sedimenting solution. After about 45 minutes the leukocyte-rich supernatant was pipetted off. This leukocyte suspension and a suspension of erythrocytes were washed three times with normal saline, and then diluted with saline so that the erythrocyte suspension contained about 500,000 red blood cells per mm<sup>3</sup> and the leukocyte-rich suspension had approximately 40,000 to 50,000 white blood cells per mm<sup>3</sup>. An aliquot of each suspension was then removed for quadruplicate counting of erythrocytes and leukocytes in each suspension with a Coulter counter. Random checks of leukocyte counts were also made in hemocytometers.

Berfenstam's method for the acid extraction of zinc from blood was modified slightly and used routinely (2). For some experiments used to evaluate methods, wet-ashing for zinc extraction, as described by Wolff (3) and by Sandell (8), were also used. The determinations of zinc were made according to the dithizone method of Malmstrom (4), modified by using a 1.00 mg per cent dithizone-carbon tetrachloride solution and reading in a Beckman DU spectrophotometer at 520 m $\mu$ . Duplicate zinc-free water blanks and duplicate zinc standards (2 or 3  $\mu$ g) were processed with each set of samples.

In this dithizone method the zinc standard curves obey Beer-Lambert's law to a concentration of 3.5  $\mu$ g of zinc per sample. It would be possible, therefore, to read the zinc content of a sample directly from a standard curve. However, because of slight variations in standard curves from day to day, probably due to differences in room temperature and slight variations in the dithizone solution concentrations, the zinc content of each sample was determined by using a calibration constant determined for each set. Duplicate determinations were made on each sample in most cases. The values of all duplicate determinations were averaged, and a single result, cor-

TABLE I

*Serial determinations of zinc in 3 ml aliquots of the same erythrocyte-rich suspension*

Date	μg Zn/3 ml sample
9/10/59	1.41
9/17/59	1.48
9/21/59	1.43
9/22/59	1.45
9/24/59	1.51
9/30/59	1.45
10/5/59	1.50
10/28/59	1.41
10/29/59	1.53

rected for erythrocyte or leukocyte contamination, is shown for each sample tested.

### RESULTS

*Evaluation of the method.* As a check on the day-to-day reproducibility of results, many 3 ml aliquots of erythrocyte-rich suspensions were prepared at one time and stored. A single sample of such a control lot was randomly introduced and the zinc content determined with a batch of other cell samples from time to time. The results of such a control study are shown in Table I and serve to demonstrate the reliability of the method. In this set of control samples, as in other sets, some of the erythrocyte-rich suspensions were stored in zinc-free water added after the final saline wash, and some in hydrochloric acid-trichloroacetic acid mixture. No significant difference in results was introduced by varying the storage state.

Because of the possibility that the acid extraction method of preparing leukocytes for the dithizone determination might not result in complete extraction of the cellular zinc, several experiments were performed using concurrent wet-ashing and

TABLE II

*Comparison of zinc levels in leukocyte-rich suspensions as determined using wet-ashing and acid extraction methods*

Subj.	Cells	Wet-ashing	Acid extraction
		μg Zn/3 ml WBC-rich suspension	
R. D.	1,111	2.02	1.94
A. B.	1,117	1.28	1.11
R. S.	1,124	1.06	1.10

acid extraction methods to prepare aliquots of the same leukocyte-rich cell suspensions. As revealed in Table II, there were no significant differences between the leukocyte zinc values determined by these two methods.

The calculation of leukocyte zinc would be simplified if the leukocyte-rich sample were completely free of contaminating erythrocytes. Wolff lysed the erythrocytes with 2.5 per cent acetic acid (3) and thus obtained a pure leukocyte preparation. Using the method, we found that most or all of the leukocyte zinc was leached from the cells (Table III). In a single experiment the acidity of the saline wash solution was reduced to pH 3.7 with zinc-free hydrochloric acid, and a single washing with this solution resulted in about 50 per cent loss of leukocyte zinc. When either a 2.5 per cent acetic acid or a 1 per cent saponin solution was used to lyse the erythrocytes, the remaining leukocytes tended to be clumped rather markedly, making accurate enumeration of the cells impossible.

*Values in normal persons.* In 38 simultaneous determinations of erythrocyte and leukocyte zinc concentrations of 32 normal persons (Table IV), using the acid extraction and dithizone determina-

TABLE III

*Data showing effect on zinc of washing leukocyte-rich suspension once with 2.5 per cent acetic acid \**

Subj.	Cells	Washed thrice with normal saline	Washed once with 2.5% acetic acid and twice with normal saline		Zinc in supernatant acetic acid wash sol.
			μg Zn/10 <sup>10</sup> WBC	μg Zn/3 ml WBC-rich susp.	
W. T.	930	61.5	1.78	0.0	0.00
W. B.	105	57.6	0.83	0.0	0.05
R. D.	11,111	64.0	1.94	13.3	0.20
A. B.	1,116			0.0	0.00
A. B.	1,117	33.1	1.11	0.0	0.00
R. S.	1,124	72.2	1.10	6.6	0.08

\* Cells used in these experiments were not from normal persons.

TABLE IV  
*Normal values for zinc in leukocytes and erythrocytes*

Subjects		No.	No. of determinations	Range	Mean	SD	Range	Mean	SD
Sex	Age range								
Males	22-41 yrs	24	29	9.5-14.4	12.0	1.25	56.8-168	102.0	26.5
Females	25-50	8	9	9.3-15.5	12.2	2.14	57.7-122	105.0	21.5
Both	22-50	32	38	9.3-15.5	12.1	1.51	56.8-168	103.0	25.5

TABLE V  
*Comparison with normal subjects of the range and mean of values for leukocyte and erythrocyte zinc in 15 cirrhotics, and for serum zinc in 9 cirrhotics*

	Normals			Cirrhotics		
	Range	Mean	SD	Range	Mean	SD
μg Zn/10 <sup>10</sup> leukocytes	56.8-168.0	103.0	25.5	39.9-106	72.9	19.9
μg Zn/10 <sup>10</sup> erythrocytes	9.3-15.5	12.1	1.51	7.9-18.0	12.5	3.16
μg Zn/100 ml serum	84.0-163.0	124.0	15.9 (11)	36.0-80.0	64.2	13.1

tion method, we found a mean of 12.1  $\mu\text{g}$  of zinc per  $10^{10}$  erythrocytes ( $SD \pm 1.51$ ) and 103  $\mu\text{g}$  of zinc per  $10^{10}$  leukocytes ( $SD \pm 25.5$ ).

*Values in patients with cirrhosis.* The results, which are summarized in Table V, show that the

leukocyte zinc values in 15 cirrhotic patients range from 39.9 to 106  $\mu\text{g}$  per  $10^{10}$  white cells with a mean of 72.9  $\mu\text{g}$  per  $10^{10}$  white cells. These values are distinctly lower than normal, and the differences can not be explained on the basis of

TABLE VI  
*Summary of zinc determinations and of clinical and laboratory data at the time of study for each of 15 patients with hepatic cirrhosis*

Patient	Age, race, sex	Bilirubin	BSP*	RBC	WBC	Serum	PCV†	MCV‡	Leukocyte counts§	
									μg Zn/10 <sup>10</sup>	μg Zn/100 ml
W. B.	39 WM	13.0		15.1	66.6		42	121	10,850	Normal
W. C.	40 NM	0.62	14	11.4	97.0	63	51	89	11,000	83.5% Neutrophils
P. F.	65 WM	0.50		12.1	48.1	36	33	82	6,030	Normal
I. G.	65 WM		28	8.0	95.2	72	41	102	9,370	Normal
R. H.	38 WM	1.66	32	12.7	59.4	68	32	84	7,970	Normal
A. J.	50 WM	14.1		15.2	60.3		35	121	35,000	92.5% Neutrophils
I. M.	68 WM	15.6	49	15.1	39.9		29	106	15,300	92.5% Neutrophils
A.R.	40 WM	25.0		14.9	45.5		29	115	23,000	89.0% Neutrophils
E.R.	49 WM	2.28	45	16.4	76.2	80	41	104	9,290	Normal
R.S.	37 WM	3.25		11.9	87.8		47	94	7,980	Normal
O.S.	65 WM	2.72	67	8.1	60.5	48	48	103	8,230	Normal
C.T.	43 WM	0.76	24	7.9	80.5	66	27	78	5,880	Normal
O.W.	48 WM	1.98		11.5	106.0		34	89	5,940	Normal
W.W.	57 WM	3.90		18.0	94.2	75	33	112	6,470	Normal
W.J., 1	39 WM	0.65	23	8.6	81.6	60	48	89	4,870	Normal
W.J., 2	39 WM	0.65	29	8.8	70.8	80	41	87	4,530	Normal

\* Per cent retention of sulfobromophthalein 45 minutes after injection.

† Packed cell volume or hematocrit.

‡ Mean corpuscular volume of erythrocytes.

§ Total counts of leukocytes in 1 cu mm of peripheral blood, and differential counts of 200 leukocytes in smear of peripheral blood.

|| Alcoholic cirrhosis.

¶ Post-hepatitic cirrhosis.

the total or differential leukocyte counts or on any morphological feature of the leukocytes.

The range of erythrocyte zinc in these patients is broader than normal, 7.9 to 18.0  $\mu\text{g}$  per  $10^{10}$  red cells, although the mean value, 12.5  $\mu\text{g}$  per  $10^{10}$  red cells, is not significantly different from the normal mean (Table V). As seen in Table VI, the content of zinc in erythrocytes tends to vary directly with the mean corpuscular volume of erythrocytes.

Ten determinations of serum zinc were made on samples from 9 of the patients with cirrhosis. The values range from 36 to 80  $\mu\text{g}$  with a mean of 64.2  $\mu\text{g}$  of zinc per 100 ml serum for the 9 patients.

#### DISCUSSION

The erythrocyte zinc levels reported here agree closely with those reported by other investigators (1-3, 9). However, the zinc levels of normal leukocytes which are presented here vary greatly from those previously reported, being only about 30 to 50 per cent of the values given by Vallee and Gibson (9), who used a dry-ashing process, and by Wolff (10), who used a wet-ashing method (Table VII).

Vallee and Gibson's zinc values for normal leukocytes were calculated on the basis of the assumption that 100 per cent recovery of uncontaminated leukocytes from the peripheral blood was obtained, i.e., the actual number of leukocytes in each sample was not determined directly, and there is a possibility that recoveries were incomplete. It should also be mentioned that the values for serum zinc reported by Vallee and Gibson (9) and by Hoch and Vallee (1) have been "corrected" downward about 50 per cent in subsequent work by Vikbladh (11), Berfenstam (2), Wolff (10), and Vallee and co-workers (5). It is possible that the same

unknown technical difficulty that resulted in the erroneously high serum zinc values also contributed to the high values for zinc in normal leukocytes reported by Vallee and Gibson.

Wolff's zinc values for normal leukocytes were determined from leukocyte-rich samples which had been purified by lysing the erythrocytes with 2.5 per cent acetic acid wash solution. As mentioned earlier in this paper, this process apparently leaches the zinc from leukocytes and also makes sampling and counting of the cells very difficult.

Our acid extraction method was compared with the wet-ashing method of Wolff, and the values for zinc in leukocytes by the two methods were in good agreement. The methods used in this study were not evaluated in reference to the dry-ashing method of liberating zinc from biological materials, since dry-ashing has been shown by skilled investigators to be more subject to errors due to contamination and losses (2, 4, 10).

The previously reported evidence for disturbed zinc metabolism in patients with liver disease is corroborated by the finding of low serum zinc levels in our group of cirrhotics. Our mean of 64.2 ( $SD \pm 13.1$ )  $\mu\text{g}$  of zinc per 100 ml serum is similar to the 66 ( $SD \pm 19$ )  $\mu\text{g}$  of zinc per 100 ml serum in 28 patients with advanced cirrhosis described by Vallee and colleagues (5), and the 64.4 ( $SD \pm 19.0$ )  $\mu\text{g}$  zinc per 100 ml serum in 12 cirrhotics reported by Rechenberger (6).

The definitely decreased levels of zinc in the leukocytes of our patients with cirrhosis afford the first direct evidence that the disturbance of zinc metabolism in cirrhosis is reflected by changes of intracellular zinc. Knowledge concerning the zinc in leukocytes is limited. A zinc metalloprotein, estimated to account for 80 per cent of their zinc, has been described, but no biochemical function has been ascribed to it (12). The activity in leukocytes of carboxypeptidase and certain dehydrogenases, which are known to be zinc metalloenzymes in other animal and plant tissues, does not correlate with the zinc content of human white cells (12). Leukocyte alkaline phosphatase is also thought to be a zinc metalloenzyme (13), but studies in this laboratory have not shown any relationship between leukocyte alkaline phosphatase activity and leukocyte zinc values. Indeed, no correlation of zinc levels in leukocytes with any enzy-

TABLE VII  
Previously reported values for zinc in leukocytes of normal persons

Author	Subjects	Range	Mean
$\mu\text{g Zn}/10^{10} \text{ WBC}$			
Vallee and Gibson (9)	17 males	120-650	290
Vallee and Gibson (9)	18 females	170-620	350
Wolff (10)	24 males	152-348	225
Wolff (10)	20 females	160-332	226

matic or other biochemical aberrations has yet been established. However, it seems most reasonable to suspect that the significance of abnormalities in zinc levels is dependent upon a zinc metalloenzyme. Correlative trace metal analysis and enzymatic studies of the easily biopsied leukocytes offer a likely route to the understanding of the biochemical and physiological roles of zinc in the human organism.

Our most advanced knowledge concerning zinc in humans relates to erythrocytes. There is excellent evidence that the level of zinc is directly related to the activity and the content of the zinc metalloenzyme, carbonic anhydrase, in the red cells (10, 14, 15). Therefore, the erythrocyte zinc values for the cirrhotic patients might be assumed to correlate with their carbonic anhydrase activity, but they can not be correlated with the degree of anemia, with the severity of the illness as judged by clinical or biochemical findings, or with the serum or leukocyte zinc values. These sub-to supra-normal levels of red cell zinc tend to correlate with the mean corpuscular volumes of the erythrocyte samples. A marked elevation of zinc per erythrocyte in pernicious anemia has been demonstrated by several investigators (10, 14-17), but the elevation in this disease exceeds the level that might be anticipated on the basis of macrocytosis alone. It has been stated that the unit red cell zinc is normal in other anemias, with the possible exception of sickle cell anemia (16). Since this study has not shown a conclusive correlation of unit erythrocyte zinc to cell size, further study is necessary to clarify this relationship.

#### SUMMARY

1. An acid extraction method of liberating zinc for determination by a dithizone method has been applied to the study of leukocytes, and evidence for its reliability has been offered.
2. Normal values for zinc in human leukocytes and erythrocytes, using the method, are reported.
3. A decrease of serum zinc concentration in cirrhosis is confirmed, and the evidence for disturbed zinc metabolism in these cases is extended by the finding of decreased intracellular zinc in the leukocytes of these patients.
4. Erythrocyte zinc ranges from below to above

normal in the group of cirrhotics, apparently varying directly with mean corpuscular volume.

5. The biochemical and physiological significance of zinc in leukocytes and of the disturbed zinc metabolism in patients with cirrhosis remains to be defined.

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