recoveries of added counts (as [^{14}C]glycine) from tissue and protein solutions. [^{14}C]Glycine dissolved in these solutions was counted at the same efficiency as in water solution.

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The Zinc Content of Erythrocytes and Leucocytes of Blood from Normal and Leukaemic Subjects

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The biological importance of zinc is now well established, and the significance of zinc in the plasma and cellular components of blood has been studied by many investigators. Human leucocytes contain as much as 3% of the whole-blood zinc (Vallee & Gibson, 1948), and the individual leucocyte is relatively rich in this metal and probably contains more zinc than does any other cell in the body (Vikbladh, 1951). For example, normal human leucocytes contain about twenty-five times as much zinc per cell as do erythrocytes (Vallee & Gibson, 1948; Wolff, 1956). A protein which contains 0·3% of zinc, and which accounts for about 80% of the zinc of the leucocytes, has been extracted from normal human leucocytes (Hoch & Vallee, 1952; Vallee, Hoch & Hughes, 1954). Although it is suggested that this zinc-protein is probably an enzyme, the nature of its enzymic activity is unknown.

In investigations on the transport of zinc in the blood and other aspects of zinc 'metabolism', we have become interested in the zinc content of

human leucocytes and the variation in the amount of zinc present in these cells in various diseases, particularly the leukaemias. Vallee and his colleagues (Gibson, Vallee, Fluharty & Nelson, 1950) studied five cases of myeloid and three cases of lymphatic leukaemia, and found that the zinc content of the leukaemic leucocytes (expressed as μg. of zinc/10^6 cells) was without exception abnormally low, ranging between 10 and 40% of the normal values. There was a tendency for the zinc content of the leukaemic leucocytes to rise to normal limits under successful therapy, and the authors concluded that 'one characteristic of the blood dyscrasias known as leukaemias is an inability of the leucocytes to attain a normal zinc content'.

Although the zinc content of leucocytes is relatively high compared with that of erythrocytes, the determination of the amount of zinc in separated leucocytes presents many problems. The leucocytes must first be separated completely, or almost completely, from plasma, erythrocytes and platelets, and the procedure should be sufficiently mild to avoid the risk of removing zinc from the zinc-containing proteins and possible other complexes in the leucocytes. Separation of the 'buffy layer' from centrifuged blood by customary methods usually yields a suspension containing many erythrocytes, and this contamination renders difficult an accurate measurement of the leucocytic zinc, for erythrocytes contain appreciable amounts of zinc in carbonic anhydrase. Various flotation methods have been advocated, but we did not find them satisfactory for the preparation of leucocyte suspensions largely free from erythrocytes and containing most of the leucocytes originally present in the blood sample. However, as advocated by Wildy & Ridley (1958), the addition of dextran to the blood to increase the erythrocyte sedimentation rate was found to be satisfactory. The use of fibrinogen to facilitate separation of the erythrocytes, according to the method described by Buckley, Powell & Gibson (1950), also led to good separation of leucocytes and erythrocytes, but when it was used for leukaemic bloods there was often excessive clumping of leucocytes; thus it was difficult to measure representative samples for assay for zinc and nitrogen, and for cell counts. The fibrinogen method of separation was used for a few of the normal bloods examined in our earliest studies recorded here, but all the leukaemic and most of the normal bloods were dealt with by the dextran method.

Another problem was the selection of methods of assay for zinc and nitrogen which would give reliable duplicate results with the small amounts of washed leucocytes which could be separated from about 10 ml. of human blood, i.e. a total amount of a suspension of 10^7–10^8 leucocytes containing 100–1000μg. of nitrogen and 0·2–0·6μg. of zinc. The leucocyte suspension from 10 ml. of human blood had thus to serve for the provision of samples for two zinc determinations, two total nitrogen determinations and a measurement of the erythrocyte and leucocytes contents, and it was obvious that special micromethods were required. For the zinc determinations we developed a method of neutron-activation analysis (Banks, Tupper, White & Wormall, 1959) which proved completely satisfactory for the present studies, and we have also found it possible, using the colorimetric phenol–hypochlorite method of Russell (1944), to determine the nitrogen content of these suspensions. For comparisons of the zinc content of leukaemic and normal human leucocytes, we wished to record the zinc contents per leucocyte and also per unit amount of total nitrogen. These zinc contents might preferably have been measured with reference to the nucleic acid content, but with the small amounts of material available this was not possible.

This paper records the results of these determinations of the zinc content of the whole blood, erythrocytes and leucocytes of 29 normal and 27 leukaemic individuals, and a few observations about the stability of the zinc in human leucocytes. A preliminary account of some of these investigations has been given (Dennes, Tupper & Wormall, 1960).

MATERIALS

'Zinc-free' (or zinc-depleted) materials and apparatus. It is essential when estimating small amounts of Zn that one should pay special attention to the possibility of contamination from glassware, laboratory dust and the chemicals used. These precautions are particularly important during the collection of the blood samples and their treatment before the irradiation of the fractions or whole-blood samples in the atomic pile, but the subsequent part of the Zn determinations does not require these rigid precautions; in fact, it is advisable that a large amount of carrier Zn should be added to the samples after they have been irradiated to facilitate complete separation of all the radioactive Zn from them. All glassware, polythene bottles, pill packs and envelopes used in the pre-irradiation part of the procedure were soaked in 2N-HCl for at least 24 hr. and then thoroughly rinsed with Zn-free water. The blood samples were drawn from an arm vein into an all-glass or a nylon syringe, and each sample was immediately transferred to a polythene bottle containing heparin (0·4 ml. of a 2·5% solution in 0·9% NaCl soln. for 20 ml. of blood).

AnalaR chemicals were used wherever possible, and many of these were freed from traces of Zn as described below. All the reagents were made up with Zn-free water. Measurements, by the neutron-activation method of analysis, showed that the heparin used contained only a small amount of Zn (8·5 μg./100 mg. of heparin, i.e. 0·04 μg. of Zn in the heparin added to each ml. of blood); the fibrinogen (7 μg. of Zn/g. of powder) and the dextran con-

tained small amounts only and did not contribute significantly to the Zn content of the blood–fibrinogen or blood–dextran mixtures. Thus, there could be no possibility that the use of either fibrinogen or dextran to facilitate separation of erythrocytes and leucocytesled to any significant addition of Zn to the cells during the process of separation. Further, preliminary tests with normal human blood showed that the separated leucocytes obtained by the fibrinogen method had precisely the same Zn content as those obtained by the dextran method. For this reason, the results we obtained in the early determinations with six samples of blood treated with the fibrinogen are included in Table 2, although the dextran method was used for all subsequent normal samples and all the leukaemic samples.

Zinc- and ammonia-free water. This was prepared by passing distilled water through a column of mixed ion-exchange resins (Elgastat water purifier; Elga Products Ltd.). This water was used for the preparation of all the solutions.

Zinc-free 0·9% sodium chloride solution. A 0·9% (w/v) NaCl solution was prepared with AnalaR NaCl; Zn was removed by shaking the solution with portions of a solution of dithizone in CCl_4 (100 mg./l.) until the dithizone remained green.

Preparation of zinc–glycine solutions containing ^{65}Zn. Glycine (5 mg.) was added to a solution of $ZnCl_2$ containing ^{65}Zn (0·05 ml. of a solution obtained from The Radiochemical Centre, Amersham, Bucks). Water (0·5 ml.) was added and the solution adjusted to pH 7·4 by the addition of 0·1N-NaOH (0·1 ml.). More water was then added to give a final volume of 1·5 ml., and the solution was made iso-osmotic with blood cells by the addition of 12·9 mg. of NaCl. This solution contained 300 μg. of Zn with a total activity of 72 μc of ^{65}Zn.

Veronal buffer, pH 7·4. A 0·05M-veronal buffer, pH 7·4, prepared as described by Gomori (1955), was made iso-osmotic with erythrocytes and leucocytes by adding 0·75 g. of NaCl/100 ml.

Veronal buffer, pH 7·4, containing ethylenediaminetetraacetic acid or o-phenanthroline. Ethylenediaminetetraacetic acid (EDTA) or *o*-phenanthroline was added to the veronal buffer described above to give a final concentration of 0·001M.

METHODS

Zinc determinations. The Zn in the leucocytes, erythrocytes and whole blood was determined by the neutron-activation method of Banks *et al.* (1959). For the calculation of the Zn content of the leucocyte samples, allowance was made for the small amount of Zn present in the erythrocytes contained in these samples; this allowance was reasonably small, representing 5–10% of the total Zn in the suspensions of leukaemic leucocytes, and only slightly more for the normal leucocytes, where the ratios of leucocytes to erythrocytes in the leucocyte preparations were lower. By this method it is possible to measure accurately the Zn content of samples of about 0·2 ml. of whole blood or of suspensions of 10^7–10^8 leucocytes (containing 0·2–0·6 μg. of Zn and 100–1000 μg. of N). In all cases, duplicate determinations gave good agreement and the values for Zn and N recorded here for any blood or cellular fraction are the mean values for duplicates.

Nitrogen determinations. Leucocyte and erythrocyte samples were digested, respectively, with 0·2 and 0·5 ml. of H_2SO_4–SeO_2 mixture [prepared as described by King (1951)]. Digestion was carried out in Pyrex centrifuge tubes placed in holes in an electrically-heated copper block. The N was determined by the Russell (1944) colorimetric phenol–hypochlorite method after diffusion of the NH_3 in Conway units. The erythrocyte digests were diluted to 100 ml. with NH_3-free water, and 1 ml. of the diluted digest was placed in the outer well of the Conway unit. Usually the whole of the leucocyte digest was transferred to the Conway unit, but if the digest was expected to contain more than 40 μg. of N it was suitably diluted, and 1 ml. of the diluted digest containing 5–20 μg. of N was placed in the unit. 0·01N-HCl (1 ml.) was placed in the centre well of the Conway unit and KOH solution (50%, w/v, 1 ml.) was added to the contents of the outer well. Diffusion was allowed to take place overnight. The contents of the centre well were then transferred to test tubes with three washings of NH_3-free water (0·5, 0·25 and 0·25 ml.); the N was then determined by the Russell (1944) method, with a Unicam SP. 600 spectrophotometer.

Separation of leucocytes. Dextraven (a solution of 6% of dextran in 0·9% NaCl; Benger Laboratories) (3·3 ml.) was added to the heparinized blood (10 ml.) in a 6 in. × $\frac{5}{8}$ in. test tube, the contents of the tubes were well mixed and kept at room temperature until sedimentation of the erythrocytes was complete (usually 30 min. for normal blood; 15–20 min. for leukaemic blood).

The supernatant leucocyte suspension was pipetted off, transferred to a centrifuge tube and spun at 50 *g* for 3 min. The supernatant solution was removed, and the pellet of leucocytes was suspended in 0·9% NaCl soln. (2 ml.) and centrifuged. This washing of leucocytes was repeated once and the cells were then resuspended in 0·9% NaCl soln. The volume of 0·9% NaCl soln. used in the preparation of the final suspension was 0·6–2·0 ml., depending upon the number of leucocytes present. In general the suspension obtained from a normal subject contained 25 000–50 000 leucocytes/mm.^{3} and from a leukaemic patient 60 000–900 000/mm.^{3} The ratio of leucocytes to erythrocytes in the leucocyte suspension from a normal subject was usually about 1:4. With the suspensions from leukaemic bloods, ratios of about 1:1 were most commonly obtained, and on some occasions the ratio was as good as 16:1.

In the few experiments when the fibrinogen method was used, the heparizined blood (10 ml.) was mixed with 0·9% NaCl soln. (3·4 ml.) and 4% fibrinogen (1·8 ml.), and the mixture was kept until erythrocyte sedimentation was apparently complete. The subsequent treatment of the leucocyte suspension was as described for the dextran method. The sedimented erythrocytes were washed with 0·9% NaCl soln. (2 × 5 ml.) and centrifuged at 800 *g* for 3 min. after each washing. Duplicate samples (0·25 ml.) of the leucocyte suspensions and the packed erythrocytes (0·1 ml.) were introduced into polythene pill packs for total Zn determinations. Samples of the leucocyte suspensions (0·05 ml.) and the packed erythrocytes (0·1 ml.) were also taken for total-N determinations. The samples for Zn determinations were dried in a desiccator over conc. H_2SO_4.

Cell counts. Leucocyte and erythrocyte counts were made on the whole blood, the final leucocyte suspension and the packed erythrocytes. A smear of the blood was stained with Leishmann's stain and a differential count was made on the leucocytes.

Treatment of erythrocytes and leucocytes in vitro *with ethylenediaminetetra-acetic acid or o-phenanthroline*

Treatment of leucocytes. A leucocyte suspension, prepared as described above, was made up to a total volume of 3 ml. with 0·9% NaCl soln. Portions (1 ml.) of this suspension were pipetted into each of three centrifuge tubes. Saline (0·9%, w/v, NaCl, 2 ml.), veronal buffer, pH 7·4, (2 ml.) and veronal buffer, pH 7·4, plus EDTA (2 ml.) were added to tubes 1, 2 and 3 respectively. The contents of each tube were well mixed and left at room temperature for 1 hr. with occasional shaking. The tubes were then centrifuged at about 70 *g* for 3 min. and the supernatant solution was pipetted off and discarded. The cells in each tube were washed with 0·9% NaCl soln. (1 ml.), the tubes were then centrifuged, and the supernatant solutions were discarded. The cells were resuspended in 0·9% NaCl soln. (0·9 ml.). Samples were removed from each tube for total Zn and N determinations and for cell counts. A similar series of experiments was made substituting *o*-phenanthroline for EDTA in the buffer.

Treatment of erythrocytes. The erythrocytes were treated in the same way as has been described for the leucocytes, packed washed erythrocytes (1 ml.) being used instead of the leucocyte suspension.

Labelling of erythrocytes and leucocytes in vitro *with* ^{65}Zn

A solution (0·3 ml.) of ^{65}Zn-containing Zn–glycine complex at pH 7·4 was added to freshly drawn and heparinized blood (10 ml.) in a test tube. The contents of the tube were well mixed and kept for 2 hr. at room temperature with occasional remixing as the cells settled out. At the end of this period, samples (0·1 ml.) of the labelled blood were withdrawn for ^{65}Zn determinations. Dextraven (3·3 ml.) was added to the remaining blood in the tube and the contents were mixed thoroughly by repeated inversion of the tube; the mixtures were then kept until the erythrocytes had sedimented and the erythrocytes and leucocytes were separated as described above. The cells were washed three or four times with 0·9% NaCl soln. instead of twice as in the normal separation procedure. Samples of the final leucocyte and erythrocyte suspensions were taken for ^{65}Zn and total-N determinations and for cell counts.

RESULTS

Most of the normal blood samples were obtained from blood-transfusion donors. Through the kindness of Dr H. F. Brewer, an extra 12 ml. of blood was obtained for our use from each donor. All the patients with leukaemia, with one exception, had been receiving various forms of treatment, and blood samples were taken from them at the regular out-patient clinic. The diagnosis of the type of leukaemia was made in the Hospital and the necessary information supplied to us.

Zinc determinations were made on the whole blood samples, and zinc and nitrogen determinations on the separated erythrocytes and leucocytes from 29 normal subjects and 27 leukaemic subjects. The subjects with leukaemia were divided into two groups, those with chronic lymphatic leukaemia (17) and those with chronic myeloid leukaemia (10). Blood samples were obtained from some of the subjects on more than one occasion, and the total number of samples analysed was 30 from the normal and 43 from the leukaemic subjects. The nitrogen content of erythrocytes and leucocytes from normal and leukaemic subjects is given in Table 1. Blood was taken on two separate occasions from eight of the leukaemic subjects, and on four and six separate occasions at intervals over a period of several months from two subjects (subjects nos. 13 and 14, Tables 3 and 4).

In Tables 1–4 the mean values given are means of the results from every blood sample taken. These particular values have not been used in the calculation of the significance of differences, as it was considered that the inclusion of more than one value for subjects from whom several blood samples were taken would unduly weight the statistical analysis. Therefore, where more than one result was available for any subject, the results have been averaged to give one value for that subject for use in the statistical analysis. Similarly, the mean values and standard errors quoted in this section have been calculated by using only one value for each subject. The mean values obtained in this way were slightly higher than those given in the Tables.

Zinc content of leucocytes from normal and leukaemic subjects. The zinc content of the leucocytes has been related to cell number and to cell nitrogen. The values expressed as μg. of zinc/10^9 cells have been given to enable an easy comparison to be made between our results and those of other workers who have invariably reported their results in this form (Vallee & Gibson, 1948; Wolff, 1956).

The leucocytes from leukaemic subjects contained approximately half the amount of zinc found in normal leucocytes (Tables 2–4). The mean leucocyte zinc content was $8·9 \pm 1·4$ and $14·2 \pm 2·0$ μg. of zinc/10^9 cells for leukaemic and normal leucocytes respectively. The corresponding values related to cell nitrogen were $0·93 \pm 0·08$ and $1·97 \pm 0·31$ μg. of zinc/mg. of nitrogen. Statistical analysis of these results showed that the zinc content of the leucocytes from the leukaemic subjects was significantly lower than that of leucocytes from normal subjects.

Table 1. *Nitrogen content of leucocytes and erythrocytes of normal and leukaemic subjects*

Type of blood	Nitrogen content (μg./10^6 cells) Erythrocytes	Leucocytes
Normal	$4·61 \pm 0·08$ (28)	$10·0 \pm 1·3$ (28)
Chronic myeloid leukaemia	$4·42 \pm 0·12$ (20)	$11·7 \pm 1·3$ (20)
Chronic lymphatic leukaemia	$4·45 \pm 0·13$ (23)	$8·0 \pm 1·1$ (23)

For the values measured on a cellular basis, P was 0·02 and for the values calculated as μg. of zinc/mg. of nitrogen, P was between 0·01 and 0·02.

The normal leucocyte contains, per cell, about fifteen times as much zinc as does the normal erythrocyte and, on a nitrogen basis, about ten times as much zinc. These ratios are considerably lower for leukaemic individuals. In the leukaemic subjects studied, the average leucocyte contained about eight times as much zinc as did the average erythrocyte, and three to four times as much per mg. of nitrogen. Although the mean values for the zinc content of lymphatic leukaemic leucocytes differed slightly from those for the leucocytes of the subjects with myeloid leukaemia, the differences were not statistically significant.

The number of leucocytes in the blood from leukaemic patients varied over a wide range (3×10^3–8×10^5/mm.^{3}) and it seemed possible that the zinc content of the leucocytes might be related in some way to the number of leucocytes. Such a relationship was found in the group of subjects with lymphatic leukaemia. The leucocyte zinc content decreased as the number of leucocytes increased, and calculation of the coefficient of correlation showed that this was a significant relationship ($0·05 > P > 0·02$). A similar relationship was not found in normal subjects or in the smaller group with myeloid leukaemia. It may be significant that a large number of the blood samples from the patients with lymphatic leukaemia contained more than 90% of lymphocytes in the leucocyte fraction; the suspensions prepared from these blood samples represented an almost pure population of one cell type.

Zinc content of erythrocytes from normal and leukaemic subjects. The erythrocytes from the leukaemic subjects had a higher zinc content than had those from the normal subjects. The mean values for the erythrocytes from normal subjects were 0·97 ± 0·03μg. of zinc/10^9 cells and 0·21 ± 0·01μg. of zinc/mg. of nitrogen, compared with corresponding values 1·12 ± 0·05μg. and 0·25 ± 0·01μg. for the erythrocytes from leukaemic subjects. The difference in zinc content between normal erythrocytes and those from leukaemic blood was statistically significant. If the results were expressed on a

Table 2. *Zinc content of erythrocytes and leucocytes of blood of normal individuals*

RBC, Erythrocytes; WBC, leucocytes; A, ratio on a cellular basis; B, ratio on a nitrogen basis.

Subject no.	Zinc in whole blood (μg./g.)	Zn content of cells (μg. of Zn/10^9 cells) RBC	WBC	(μg. of Zn/mg. of N) RBC	WBC	Ratio (Zn in WBC/Zn in RBC) A	B
1	6·0	0·92	6·0	0·19	5·35	6·5	28·2
2	5·1	1·43	16·8	0·32	7·80	11·8	24·2
2	7·1	0·78	3·7	0·17	0·49	4·8	2·9
3	5·1	0·84	20·1	0·22	2·71	24·5	12·3
4	6·7	0·77	8·4	0·16	1·14	10·9	7·1
5	5·4	1·02	30·0	0·22	1·78	29·4	8·3
6	8·0	0·96	17·5	0·21	0·63	18·2	3·0
7	5·1	1·26	11·4	0·28	1·54	9·1	5·5
8	4·8	0·95	10·4	0·19	1·33	10·9	7·0
9	4·6	0·77	8·6	0·18	1·15	11·2	6·4
10	6·9	1·17	9·4	0·27	1·54	8·0	5·7
11	6·1	1·06	10·3	0·20	1·39	9·7	7·0
12	5·1	0·89	4·8	0·18	0·51	5·4	2·8
13	5·7	1·03	5·8	0·19	4·30	5·6	22·6
14	6·2	0·90	6·6	0·19	0·87	7·3	4·6
15	5·5	0·85	14·5	0·20	1·80	17·0	9·0
16	5·8	1·42	32·0	0·28	4·00	22·6	14·3
17	4·5	0·81	7·3	0·17	6·25	9·0	37·0
18	4·7	0·78	3·9	0·19	0·29	5·0	1·5
19	5·0	1·13	5·8	0·22	0·72	5·1	3·3
20	5·3	1·06	4·6	0·28	0·33	4·3	1·2
21	3·9	0·77	6·8	0·21	0·53	8·8	2·5
22	5·2	0·92	14·9	0·20	2·06	16·2	10·3
23	5·9	1·01	43·5	0·21	2·79	43·5	13·4
24	5·6	0·97	20·0	0·21	1·44	20·6	6·9
25	5·8	0·86	14·2	0·20	1·09	16·5	5·5
26	5·0	0·75	41·6	0·18	2·12	55·5	11·8
27	5·4	1·01	13·8	0·20	1·50	13·7	7·6
28	—	0·80	13·7	—	—	17·1	—
29	—	1·24	14·6	—	—	11·8	—
Mean ± S.E.	5·6±0·2 (28)	0·97±0·03 (30)	14·0±1·9 (30)	0·21±0·01 (28)	2·05±0·35 (28)	14·6±2·1 (30)	9·7±1·6 (28)

Table 3. *Zinc content of erythrocytes and leucocytes of blood of leukaemic subjects (chronic lymphatic leukaemia)*

For abbreviations see Table 2.

Subject no.	Zinc in whole blood (μg./g.)	Zn content of cells (μg. of Zn/10^9 cells) RBC	Zn content of cells (μg. of Zn/10^9 cells) WBC	Zn content of cells (μg. of Zn/mg. of N) RBC	Zn content of cells (μg. of Zn/mg. of N) WBC	Ratio (Zn in WBC/Zn in RBC) A	Ratio (Zn in WBC/Zn in RBC) B
1	3·9	0·84	6·0	0·23	1·05	7·1	4·6
2	4·3	1·45	2·8	0·29	0·58	1·9	2·0
3	5·1	1·22	8·1	0·28	1·03	6·6	3·7
4	4·3	1·08	4·3	0·27	0·51	4·0	1·9
5	5·9	1·30	3·3	0·29	0·52	2·5	1·8
5	4·5	1·43	2·7	0·30	0·70	1·9	2·2
6	4·0	1·06	3·2	0·29	0·59	3·0	2·0
6	—	1·37	4·8	0·34	0·91	3·5	2·7
7	6·5	1·46	5·0	0·31	1·17	3·4	3·8
7	6·1	1·45	1·5	0·27	0·36	1·0	1·3
8	5·0	0·93	3·3	0·22	1·90	3·5	8·6
9	4·3	1·13	24·0	0·24	0·93	21·2	3·9
9	—	1·01	22·4	0·25	1·30	22·2	5·2
10	5·5	1·00	6·7	0·32	0·67	6·7	2·1
11	6·4	1·47	3·9	0·30	0·69	2·7	2·3
11	—	1·63	3·6	0·29	0·68	2·2	2·3
12	2·6	0·52	3·0	0·14	0·51	5·8	3·6
12	3·2	0·54	3·9	0·13	0·49	7·2	3·8
19	4·6	0·90	3·0	0·18	0·95	3·3	5·3
20	5·3	0·98	14·5	0·22	1·25	14·8	5·7
21	4·2	0·82	17·0	0·20	1·50	20·8	7·5
25	4·1	0·76	3·4	0·16	0·38	4·5	2·4
26	5·1	0·90	21·2	0·17	1·74	23·6	10·2
Mean ± S.E.	4·8±0·2 (20)	1·10±0·06 (23)	7·5±1·5 (23)	0·25±0·01 (23)	0·89±0·09 (23)	7·5±1·5 (23)	3·9±0·5 (23)

Table 4. *Zinc content of erythrocytes and leucocytes of blood of leukaemic subjects (chronic myeloid leukaemia)*

For abbreviations see Table 2.

Subject no.	Zinc in whole blood (μg./g.)	Zn content of cells (μg. of Zn/10^9 cells) RBC	Zn content of cells (μg. of Zn/10^9 cells) WBC	Zn content of cells (μg. of Zn/mg. of N) RBC	Zn content of cells (μg. of Zn/mg. of N) WBC	Ratio (Zn in WBC/Zn in RBC) A	Ratio (Zn in WBC/Zn in RBC) B
13	5·1	1·06	3·4	0·21	0·54	3·2	2·6
13	5·0	0·89	11·2	0·22	0·96	12·6	4·4
13	4·4	0·92	8·0	0·21	0·44	8·7	2·1
13	3·8	0·94	11·9	0·20	0·44	12·7	2·2
14	6·9	1·16	6·1	0·33	0·54	5·3	1·6
14	7·2	1·42	6·8	0·40	0·81	4·8	2·0
14	6·6	1·80	5·1	0·36	0·80	2·8	2·2
14	6·6	1·47	4·7	0·38	0·64	3·2	1·7
14	7·0	1·28	6·2	0·30	0·58	4·8	1·9
14	6·1	1·34	5·9	0·29	0·63	4·4	2·2
15	5·3	1·14	9·4	0·28	1·22	8·3	4·4
16	5·5	1·48	6·9	0·29	0·88	4·7	3·0
17	4·7	1·44	4·8	0·29	1·33	3·3	4·6
17	4·1	1·32	5·8	0·28	0·42	4·4	1·5
18	7·2	1·28	5·3	0·25	0·45	4·1	1·8
22	7·1	0·86	22·0	0·24	1·82	25·6	7·6
23	7·0	1·19	6·0	0·27	0·57	5·0	2·1
23	—	1·65	10·6	0·39	0·83	6·4	2·1
24	4·5	0·84	8·2	0·17	0·63	9·8	3·7
27	6·9	1·27	30·0	0·29	1·20	23·6	4·1
Mean ± S.E.	5·8±0·3 (19)	1·24±0·06 (20)	8·9±1·4 (20)	0·28±0·01 (20)	0·79±0·08 (20)	7·9±0·8 (20)	2·9±0·3 (20)

cellular basis, $0{\cdot}01 > P > 0{\cdot}001$, and on a nitrogen basis, $0{\cdot}01 > P > 0{\cdot}001$. One subject with lymphatic leukaemia (no. 12, Table 3) had a very low erythrocyte zinc content, about half that of normal erythrocytes. This low value was found on two different occasions.

Zinc content of the whole blood from normal and leukaemic subjects. The mean values for the zinc content of whole blood from normal and leukaemic subjects were respectively $5{\cdot}5 \pm 0{\cdot}02\mu$g. and $5{\cdot}2 \pm 0{\cdot}02\mu$g. of zinc/g. of whole blood. No statistically significant difference was found between these two values. As might be expected, the zinc content of the whole blood is closely connected with the erythrocyte count. Since the erythrocytes account for about 75% of the total zinc in the blood, a low erythrocyte count will usually result in a low zinc value for whole blood.

Nitrogen content of erythrocytes and leucocytes from normal and leukaemic subjects. The mean value for the nitrogen content of leucocytes from normal blood was $10{\cdot}0 \pm 1{\cdot}3\mu$g. of nitrogen/$10^6$ cells (Table 1), compared with $9{\cdot}7 \pm 0{\cdot}9\mu$g. of nitrogen for the average values for the leucocytes from all the leukaemic subjects. No significant difference was found between the nitrogen content of the normal leucocytes and the leukaemic leucocytes.

If the nitrogen content of leucocytes from subjects with myeloid leukaemia ($11{\cdot}7 \pm 1{\cdot}3\mu$g. of nitrogen/$10^6$ cells) is compared with that of leucocytes from patients with lymphatic leukaemia ($8{\cdot}0 \pm 1{\cdot}1\mu$g. of nitrogen/$10^6$ cells), the leucocytes from myeloid subjects have a significantly greater nitrogen content ($0{\cdot}05 > P > 0{\cdot}02$). The mean value for the erythrocyte nitrogen content of normal subjects was $4{\cdot}61 \pm 0{\cdot}08\mu$g. of nitrogen/$10^6$ cells and that of the leukaemic subjects $4{\cdot}43 \pm 0{\cdot}09\mu$g. of nitrogen/$10^6$ cells. The difference between the two values is not significant.

Effect of ethylenediaminetetra-acetate or o-phenanthroline on the zinc content of erythrocytes and leucocytes in vitro. Erythrocytes or leucocytes from normal or leukaemic subjects lost negligible amounts of zinc when treated for 1 hr. *in vitro* with veronal buffer, pH 7·4, containing either EDTA or *o*-phenanthroline in 0·001M concentration. The results obtained on leucocytes from six leukaemic subjects are given in Table 5. To study whether the buffer alone had any effect on the zinc content of the leucocytes or erythrocytes, the cells were treated *in vitro* for 1 hr. with the buffer. No change in the zinc content of the cells was observed.

Labelling in vitro *with* ^{65}Zn *of erythrocytes and leucocytes from normal and leukaemic subjects.* More ^{65}Zn was found in the leucocytes than in the erythrocytes of whole blood labelled *in vitro* for 2 hr. with a zinc–glycine solution, pH 7·4, containing the isotope. The ratio of ^{65}Zn/10^6 leucocytes to ^{65}Zn/10^6 erythrocytes was 57 ± 26 (7) and 30 ± 13 (6) respectively for normal and leukaemic subjects. In agreement with the finding that leukaemic leucocytes contain very much less zinc than do normal leucocytes, it can be seen that under the conditions of these experiments leukaemic leucocytes take up about half as much ^{65}Zn as do the normal leucocytes. The results show a wide range and this is probably due largely to differences in viability of the leucocytes, with a consequent effect on the uptake of ^{65}Zn.

Table 5. *Effect of treatment* in vitro *with ethylenediaminetetra-acetic acid or o-phenanthroline on the zinc content of leucocytes from leukaemic subjects*

Expts. nos. 1–4 were with EDTA and nos. 5 and 6 with *o*-phenanthroline. *A*, Untreated cells; *B*, cells treated with chelating agent.

	Zinc content of leucocytes			
	(μg./10^9 cells)		(μg./mg. of N)	
Expt. no.	*A*	*B*	*A*	*B*
1	4·8	7·7	0·91	0·88
2	10·6	6·8	0·83	0·57
3	8·5	8·3	0·71	0·68
4	6·8	7·4	0·23	0·21
5	23·2	27·2	2·20	2·57
6	11·6	11·9	1·86	1·73

DISCUSSION

One of the chief difficulties associated with the study of tumour metabolism lies in the heterogeneous nature of the tumour tissues, which makes it almost impossible to obtain a sample of tumour cells for analysis uncontaminated with non-neoplastic tissues. This difficulty does not arise in the leukaemias and collection of the leukaemic blood cells is comparatively easy. The leukaemias are therefore particularly suitable for the investigation of trace element metabolism in neoplasia.

In studies of this type, it is useful to have a suitable reference standard, and, if sufficient material had been available, we should have measured the deoxyribonucleic acid-phosphorus content of our cell samples, for this value can serve as a useful standard of reference for revealing changing metabolic patterns of cell activity in normal growth and differentiation (Davidson & Leslie, 1950; see also Davidson, 1960). However, as it was not possible for us to measure the amount of deoxyribonucleic acid in our leucocyte samples, we used, as reference, the amount of nitrogen in the leucocytes and erythrocytes, and recorded the results in amounts of zinc per cell and per mg. of nitrogen. Since, however, the amount of deoxyribonucleic acid in leucocytes is fairly high in relation to that of other constituents, we shall try to use this reference standard in further studies on differences between leukaemic and normal leucocytes.

Although it is now 12 years since Vallee & Gibson (1948) showed that the human leucocyte is relatively rich in zinc, we still have no definite information concerning the function of the metal in this cell. It has been observed that eosinophils contain large amounts of zinc in the granules (Mager, McNary & Lionetti, 1953; Wolff, 1956). Cram & Rossiter (1948) found that the concentration of alkaline phosphatase in granulocytes was 1000 times that in serum. Alkaline phosphatase of swine kidney has been shown to be a zinc-metalloenzyme (Mathies, 1958) and it is possible that some of the zinc in leucocytes is associated with alkaline phosphatase. In this connexion, it may perhaps be significant that in chronic and acute leukaemias the alkaline phosphatase activity of the white cells is decreased (Beck & Valentine, 1951; Valentine & Beck, 1951; Valentine & Lawrence, 1956).

A peptidase which is found in leucocytes is known to require zinc as a cofactor in the hydrolysis of glycyl-L-leucine (Fleisher, 1956). It is not known whether the activity of this enzyme is below normal in leukaemia. According to Vallee (1959) there is no correlation between the zinc content of human leucocytes and alcohol dehydrogenase, carboxypeptidase, lactic dehydrogenase, malic dehydrogenase, glutamic dehydrogenase and rhodanese activity. Hoch & Vallee (1952) (cf. Vallee *et al.* 1954) were able to isolate a protein from human leucocytes containing 80% of the leucocyte zinc, but they were unable to demonstrate any associated enzyme activity.

This lack of knowledge concerning the function of zinc in the normal leucocyte hampers any interpretation of results obtained on the leukaemic leucocyte. Our observations that the leukaemic leucocyte contains about half as much zinc as does the normal leucocyte confirms the earlier observations of Gibson *et al.* (1950) and Wolff (1956). The results given here show that this lower zinc content is relative not only to the number of leucocytes but also to the cellular nitrogen. In the two types of leukaemia studied, namely chronic myeloid and chronic lymphatic, there was no significant difference between the nitrogen content of normal and leukaemic leucocytes. It would appear that no significant change takes place in the nitrogen content of the leucocytes when they become leukaemic. The decrease in zinc content of the leukaemic cells is therefore not paralleled by a fall in the nitrogen content of the cells. The significantly lower nitrogen content of leucocytes from subjects with chronic lymphatic leukaemia compared with that of leucocytes from subjects with chronic myeloid leukaemia may be attributed to the smaller size of the lymphocytes compared with the myeloid cells.

The low zinc content of leukaemic leucocytes may not be a characteristic of the leukaemic state but may be related to the immaturity of the cells. On the other hand, although in chronic myeloid leukaemia large numbers of immature cells are found in the blood, in chronic lymphatic leukaemia the majority of cells are mature lymphocytes which show the same low average zinc content as do the immature myeloid cells. The incorporation of zinc into the primitive developing leucocytes in the leukaemic bone marrow may be faulty, but whether this is a cause or a consequence of the neoplastic state cannot be decided from the existing evidence. A low tumour zinc content compared with that of the normal tissue of origin is not an invariable finding, several workers having reported high tumour zinc contents (Cristol, 1923; Labbé & Nepveux, 1927; Sugai, 1939, 1940; Addink, 1950).

Attempts to treat leukaemia by administration of zinc gluconate have been unsuccessful (Gibson *et al.* 1950). The low zinc content of the leukaemic leucocytes may be due to lack of a factor which is responsible for the incorporation of zinc into the cell, in which case administration of zinc would not necessarily lead to an increase in leucocyte zinc.

The higher zinc content of the leukaemic erythrocytes is of interest. Anaemia of various degrees of severity was present in nearly all the leukaemic subjects from whom we obtained blood. Vallee (1959) states that in anaemias other than pernicious anaemia, and leukaemia, the erythrocyte zinc value varies directly with the haematocrit level and haemoglobin concentration. However, in our studies we have observed no similar correlation. The possibility of association of zinc with haemoglobin in the erythrocyte must be considered, for there is evidence that, in the equine erythrocyte labelled *in vitro* with ^{65}Zn, the zinc is bound to haemoglobin (Sivarama Sastry, Viswanathan, Ramaiah & Sarma, 1960). It is therefore possible that erythrocyte zinc not present in the carbonic anhydrase may be partly associated with haemoglobin, but from our results it would appear that in leukaemic subjects the zinc content of the erythrocytes is not directly proportional to the haemoglobin concentration. The range of the erythrocyte zinc concentration is much greater in leukaemic subjects than in normal subjects. It appears that in leukaemia there is an imbalance of zinc metabolism of the haemopoietic system which is reflected in the raised erythrocyte zinc and lowered leucocyte zinc.

The concentration of zinc in the whole blood of the leukaemic subjects was not significantly lower than that of normal subjects. In leukaemia and other neoplastic conditions the serum zinc level is below normal (Vikbladh, 1951), but, as the amount of zinc in the serum is small compared with that in the cells, this does not have much effect on the whole-blood zinc level.

Both EDTA and 1:10-phenanthroline are powerful chelating agents for zinc, but *in vitro* they will not remove zinc from erythrocytes or leucocytes. The cell wall of the erythrocyte is impermeable to EDTA (Foreman & Trujillo, 1954) and inability to penetrate the cell walls may explain the inability of this compound to affect the zinc content of normal and leukaemic erythrocytes and leucocytes. Sivarama Sastry *et al.* (1960) showed that ^{65}Zn bound *in vitro* to equine erythrocytes was only removed very slowly by EDTA, and that the compound completely inhibited uptake of ^{65}Zn by the erythrocytes. Thus, the removal of zinc from combination with proteins and other constituents of leucocytes can probably be effected only by drastic treatment involving the disruption of the cell before treatment with a chelating agent.

The effect of chelating agents might not directly lead to the removal of zinc bound to proteins, for it has been shown that the treatment of the zinc-metalloenzyme alcohol dehydrogenase with *o*-phenanthroline causes inactivation of the enzyme with the formation of enzymically inactive zinc–*o*-phenanthroline–enzyme complexes; 1 mole of *o*-phenanthroline reacts with the zinc of the enzyme, the chelating agent competing with the cofactor (reduced or oxidized diphosphopyridine nucleotide) for the active centre of the enzyme (Vallee & Coombs, 1959).

The cellular components of rabbit blood show a similar difference in their capacity to take up added zinc. In some of our earlier experiments, the addition of ^{65}Zn to rabbit blood was followed by fairly rapid uptake of this isotope by leucocytes and erythrocytes, with the average leucocyte taking up about eight times as much ^{65}Zn as did the average erythrocyte (Tupper, Watts & Wormall, 1951). This ratio was much lower than that recorded in this paper for human blood labelled *in vitro* with ^{65}Zn, but the difference in the ratios may be due largely to differences in the experimental conditions. In the earlier studies, the separation of leucocytes and erythrocytes was effected by the flotation method of Vallee, Hughes & Gibson (1947), and this separation was not as satisfactory as was that effected by the method used in the present paper, and, further, radioactive zinc of higher specific activity has been used for the experiments on human blood described here. It is possible that the capacity of leucocytes of rabbit blood, relative to that of the erythrocytes, is as high as that for human blood, and this view is confirmed by some labelling studies *in vivo* that we are now making. For example, the intravenous injection of ^{65}Zn into a rabbit resulted in the labelling of the blood cells with this isotope, and in samples of blood collected 24 hr. after the injection, the leucocytes contained, per cell, about sixty times as much ^{65}Zn as did the average erythrocyte (E. Dennes, R. Tupper & A. Wormall, unpublished work); this ratio is the same as that found in the *in vitro* labelling experiments with normal human blood described in this paper. A similar high ratio (of 30) was found for the ratio leucocyte ^{65}Zn/erythrocyte ^{65}Zn in the cells obtained from the bone marrow of these rabbits killed 24 hr. after the injection of ^{65}Zn.

Much more work remains to be done on the metabolism of zinc in leucocytes and erythrocytes of normal and leukaemic subjects, and particularly on the abnormalities in zinc metabolism which occur in the leukaemias. There is evidence that the serum activity of the zinc-metalloenzyme lactic dehydrogenase is elevated during acute and chronic human leukaemia (Friend & Wróblewski, 1956), and there are other indications of disturbances of zinc metabolism in this disease. In general, however, there is a lack of precise information about the zinc content of bone marrow under normal conditions and about its variation in disease. A study of the zinc in leucocytes from subjects with leucocytosis not caused by neoplastic conditions would be of great value in establishing whether the low zinc concentration in leukaemic leucocytes is indeed connected with the neoplastic state of the cells.

SUMMARY

1. Neutron-activation analysis has been used to measure the amount of zinc in whole blood, erythrocytes and leucocytes from 29 normal subjects and from 27 subjects suffering from chronic leukaemia (17 suffering from lymphatic and 10 from myeloid leukaemia).

2. The zinc content of the leucocytes from the leukaemic subjects, expressed either as μg. of zinc/10^9 cells or /mg. of nitrogen, was significantly lower than that of leucocytes from normal subjects, the mean values being $8{\cdot}9 \pm 1{\cdot}4\mu$g. of Zn/$10^9$ cells and $0{\cdot}93 \pm 0{\cdot}08\mu$g. of zinc/mg. of nitrogen for the leukaemic leucocytes, and $14{\cdot}2 \pm 2{\cdot}0\mu$g. of zinc/$10^9$ cells and $1{\cdot}97 \pm 0{\cdot}31\mu$g. of zinc/mg. of nitrogen for the normal leucocytes. Statistical analysis of the results showed that these differences were significant with $0{\cdot}02 > P > 0{\cdot}01$ for the values measured per mg. of nitrogen.

3. The zinc content of the leucocytes from the subjects with lymphatic leukaemia was approximately inversely proportional to the number of leucocytes in the blood.

4. The erythrocytes showed a slight but statistically significant difference in the opposite direction, the leukaemic erythrocytes containing more zinc than did normal human erythrocytes (average values of $0{\cdot}25 \pm 0{\cdot}01$ and $0{\cdot}21 \pm 0{\cdot}01\mu$g. of zinc/mg. of nitrogen, respectively).

5. There was no significant difference between the whole blood zinc of normal and leukaemic subjects.

6. There was no significant difference between the average nitrogen contents of leucocytes from normal and leukaemic subjects, but the leucocytes from the subjects with myeloid leukaemia contained significantly more nitrogen than did the leucocytes from the subjects with lymphatic leukaemia ($0{\cdot}05 > P > 0{\cdot}02$).

7. No removal of zinc occurred when normal or leukaemic leucocytes and erythrocytes were exposed *in vitro* to buffer solutions containing a zinc-chelating agent (ethylenediaminetetra-acetic acid or *o*-phenanthroline).

8. The cells of normal and leukaemic bloods have been labelled *in vitro* with ^{65}Zn. With the normal bloods the average leucocyte took up about 60 times as much zinc as did the average erythrocyte. With the leukaemic bloods the ratio of leucocyte ^{65}Zn/erythrocyte ^{65}Zn was also high, but average ratios were only about half those for the normal bloods.

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