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Electron microscopic x-ray microanalysis of normal and leukemic human lymphocytes

(lymphocytic leukemia/erythrocyte analysis/nuclear elements/intracellular zinc)

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ABSTRACT **A comparative study of the elemental content of normal and leukemic cells was undertaken on a few subjects, using electron microscopic x-ray microanalysis. Phosphorus, sulfur, chlorine, calcium, copper, and zinc were detected in intracellular loci. The concentration of some of the above elements appeared to be disease related. In leukemic lymphocytes, the nuclear zinc was significantly lower than that recorded in normal lymphocytes, while the phosphorus was only moderately decreased. This suggests a faulty zinc uptake or binding in leukemic cells. The possible consequences of intracellular zinc deficiency are discussed.**

In previous studies (1–4) electron microscopic x-ray microanalysis was employed to explore the role of calcium and zinc in the pathogensis of disease. This technique is particularly useful in studying nonhomogenous tissues where very small amounts of elements can be measured and localized within the cell with ultrastructural accuracy under direct visual monitoring. In preliminary experiments to determine the optimal method for preparing specimens for x-ray analysis (4), it was found that glutaraldehyde-fixed tissues, when embedded directly in resin, retained many endogenous elements in concentrations which were detectable in ultrathin sections. Localized elemental changes related either to altered experimental conditions or to pathological states were also found to be measurable with the above technique.

The present study was undertaken to examine the possibility of employing electron microscopic x-ray microanalysis of blood leukocytes in the diagnosis and research of leukemias and related blood disorders. Comparative electron probe analyses of normal and leukemic lymphocytes were attempted on a few subjects and the preliminary results are reported here.

MATERIALS AND METHODS

The blood cells were obtained from two healthy adult males, two patients with chronic lymphocytic leukemia, one patient with monoblastic leukemia, and a single patient with polycythemia vera. In the cases of leukemia and polycythemia vera, 8 ml of venus blood whose coagulation was prevented with heparin was centrifuged at 200 × *g* for 8 min and the buffy coat was collected. In the two controls, lymphocytes were concentrated from the peripheral blood using the Ficoll–Hypaque gradient centrifugation technique (5). The cell pellets were resuspended in 0.1 M cacodylate buffer at room temperature and centrifuged again at 200 × *g* for 5 min. The supernatant was discarded and 2.5% (wt/vol) glutaraldehyde in 0.1 M cacodylate was gently layered onto the cell pellet. After 1 hr, the pellet was cut into small pieces, resuspended in the fixative for another half hour, and increasing concentrations of glutaraldehyde were added. Five minutes were allowed in 5, 10, and 25% solutions followed by two changes of 10 min each in 50% glutaraldehyde. Pure Epon 812 was then introduced and, after 1 hr, replaced by the full resin mixture. Capsules were prepared on the next day and were kept in an oven at 60° for 2–3 days.

Sections, 120 nm thick (gold), were placed on formvar- and carbon-coated nickel grids. These were examined unstained with an electron microscopic x-ray analyzer, the EMMA 4 (AEI, England), at an accelerating voltage of 40 kV. The current on the specimens was kept at 200 nA; the probe diameter was about 250 nm, and the counting time was 50 sec.

Most of the examinations were done with a Kevex Si/Li energy dispersive spectrometer; in addition to the elemental spectrum from each spot, the characteristic peak minus background ($p - b$) x-ray counts were noted for phosporous (2.01 keV), sulfur (2.31 keV), calcium (3.69 keV), and zinc (8.63 keV).

The continuum or "white" radiation was also recorded in an energy band devoid of characteristic peaks and this served as a relative measure of local specimen mass (W) [an adjustment was made for the supporting film (Wb)]. The final result was expressed as the relative mass fraction R which was calculated from the formula $(p - b)/(W - Wb)$. In some cases, the crystal spectrometer (LiF as the crystal) was employed for "in depth" zinc estimations (4, 6).

RESULTS

In the simplified method of specimen preparation, the section thickness (120 nm) and lack of stain result in images somewhat lacking in contrast and intracellular resolution (Figs. 1 and 2). Nevertheless, the different cells and some of their organelles were easily recognized, and focusing the electron beam onto areas of interest for analysis posed no problem. Fig. 1A–C shows elemental spectra from normal cells after 50 sec of analysis. The variations in emissions from different spots are easily seen. When the probe is on the embedding medium close to a cell (Fig. 1C), only peaks of the supporting material and instrumentation are seen. With the probe on the cell cytoplasm (Fig. 1B), small peaks of phosphorus, sulfur, chlorine, calcium, and zinc appear. No attempt was made in this preliminary study to localize the beam onto specific intracytoplasmic organelles, although this is technically possible.

When the probe is moved on to a heterochromatic part of the nucleus of normal lymphocytes (Fig. 1A), the phosphorus peak is very high and the calcium and zinc peaks become more prominent. Chlorine and potassium peaks were often high, but too variable for even semiquantitative conclusions. Small iron and copper peaks were also recorded occasionally. The nucleolus of normal cells had especially high concentrations of elements, with calcium and zinc more abundant here than in any other location. The euchromatic portions of the nucleus in normal cells appeared to have much less calcium, but only a little less phosphorus and zinc, than other nuclear locations.

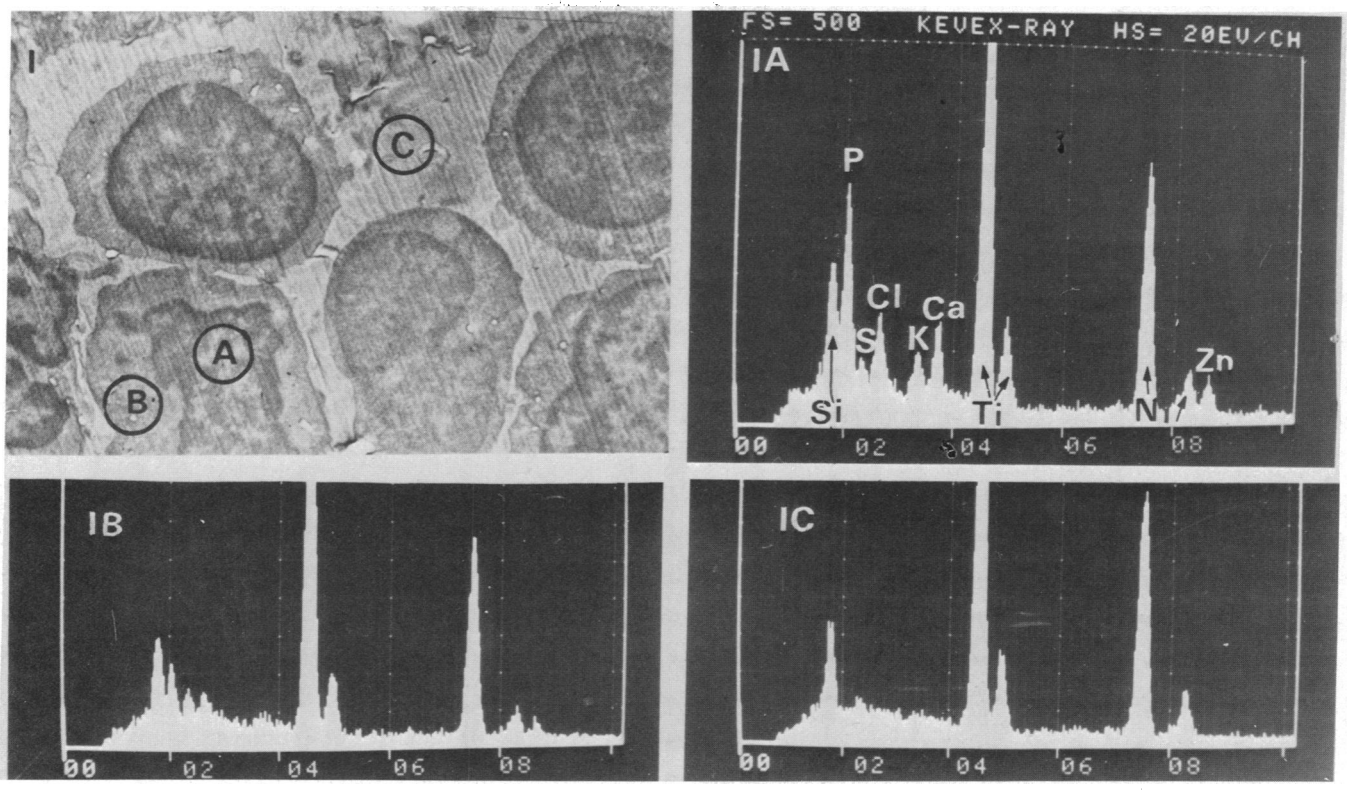

FIG. 1. Micrograph of mononuclear blood cells from normal control case. The material was fixed in glutaraldehyde and embedded directly in Epon. Sections are unstained and 120 nm thick. The circles indicate the analyzed spots whose elemental spectra are shown in (A–C). (A) Probe on nucleus of a lymphocyte (A in Fig. 1). The phosphorus (P) and calcium (Ca) peaks are high; sulfur (S), chlorine (Cl), potassium (K), and zinc (Zn) peaks are well seen. The silicon (Si), titanium (Ti), and nickel (Ni) peaks are instrumental (compare with panel C). The abscissa is marked in keV. (B) Probe on cytoplasm of lymphocyte (B in the micrograph). The peaks of phosphorus, sulfur, chlorine, calcium, and zinc are still present but smaller. (C) Probe on Epon between cells (C in the micrograph). The high peaks are of silicon from the instrument, titanium from the grid holder, and nickel from the grid.

Table 1 shows averages of relative mass fractions of different elements in the nuclei and cytoplasm of several normal and leukemic lymphocytes. It is evident that, in normal lymphocytes obtained from different individuals, except for calcium, there is fair agreement in the different elemental concentrations. No significant differences in elemental concentrations relating to cell size were detectable in normal or leukemic cells.

Erythrocytes showed great variability of elemental content, but, as can be seen from Figs. 2B, 3, and 4, interesting spectra with a number of good peaks were obtained from these cells.

In one case of terminal chronic lymphocytic leukemia where an overall decrease in zinc content occurred, a high copper peak appeared at 8 keV in both erythrocytes and lymphocytes (Figs. 2, 2A, and 2B).

When leukemic and normal lymphocytes are compared (Table 1), the most prominent finding is a much lower zinc concentration in the nuclei and cytoplasm of leukemic cells. The nuclear phosphorus and calcium also show a decrease, while the sulfur appears to be more abundant in both the cytoplasm and nuclei of leukemic cells.

The crystal diffractive analyses of intracellular zinc in leukocytes from three different cases are compared and shown in Table 2. The very low zinc content of leukemic as compared with normal lymphocytes is again obvious, while the difference is less striking in cells from a case of monoblastic leukemia (Fig. 5). Cells from a patient with polycythemia vera were used as controls in this instance, since it is known that they have zinc concentrations comparable to those of normal cells (7).

Perhaps a more reliable way of expressing chemical differences is to compare various elemental emission ratios. Thus, the phosphorus-to-zinc ratio in nuclei of normal lymphocytes is in the vicinity of 8, while in leukemic lymphocytes it is 21. This is due to the much larger decrease in nuclear zinc (60%) than in phosphorus (16%). The nuclear:cytoplasmic ratio for phosphorus is 2.7 in normal lymphocytes and 3.5 in leukemic lymphocytes. The nuclear:cytoplasmic ratio for calcium, although variable, is always much higher in normal lymphocytes (3.3) than in leukemic cells (less than 1).

DISCUSSION

The present study shows that x-ray microanalysis can be successfully applied as an ultrastructural tool in the study of malignant disorders such as the leukemias. Theoretically, this sensitive method is almost unique in its ability to detect elemental quantities as small as 10^{-17} g in localized intracellular areas of less than 200 nm diameter.

In practice, there are two major problems in microanalysis of ultrathin tissue sections. The first relates to the very small concentrations of many intrinsic intracellular elements, while the second concerns the unknown elemental extractions and shifts caused by the required processing of tissue prior to analysis. Work continues on specimen preparation, with particular emphasis on the use of frozen, hydrated samples (8, 9), which may prove to be essential for the successful preservation of tissue chemical composition.

Absolute quantitation depends on comparing emissions obtained from the specimen with those recorded from a standard

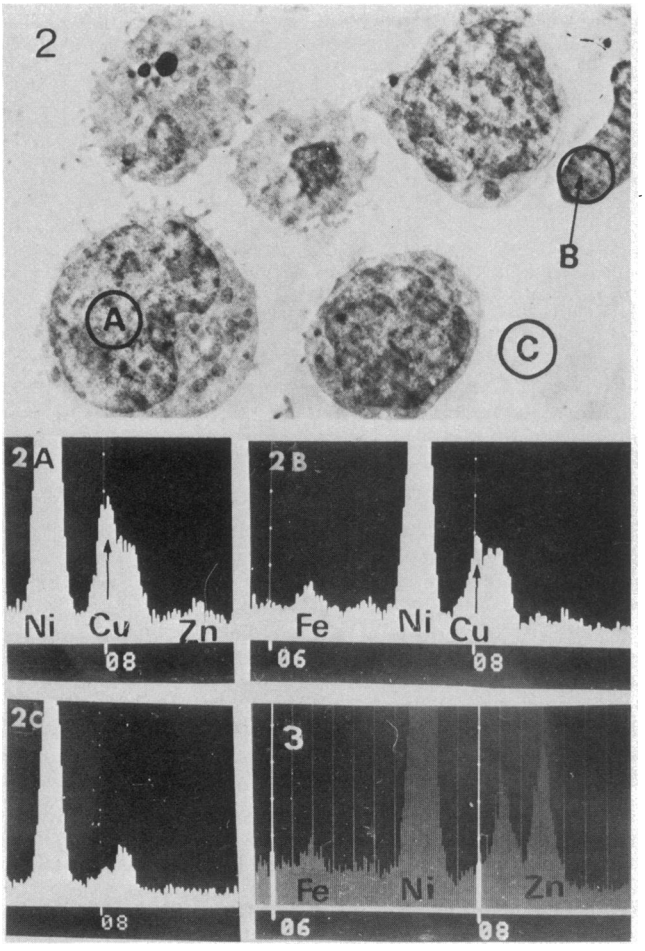

FIG. 2. Cells from case of chronic lymphocytic leukemia prepared as described in Fig. 1. Sections are unstained and 120 nm thick. (A) Analysis of nucleus showing very little zinc, but a high copper (Cu) peak (A in the micrograph). (B) Analysis of an erythrocyte from the same case which also shows a high copper (Cu) and low zinc (Zn) emission. Note small iron (Fe) peak (B in the micrograph). (C) Analysis of Epon nearby shows no prominent copper or zinc peaks (C in the micrograph).

FIG. 3. Analysis of an erythrocyte from a case of polycythemia vera showing a high zinc peak and no copper.

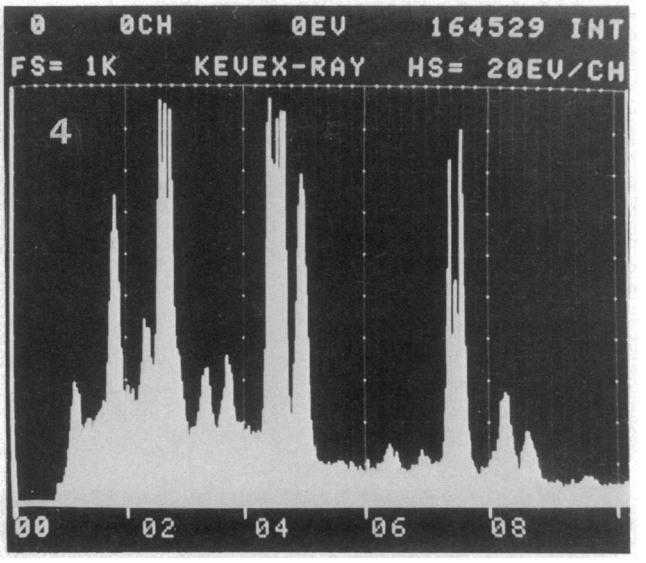

FIG. 4. Probe on an erythrocyte from a case of monoblastic leukemia showing good emissions from various elements, including zinc.

of known composition examined under the same instrumental conditions (6). To eliminate the need for checking many standards and controls, only comparisons between various specimens were sought in this preliminary work. Thus, relative mass fractions and a few ratios were sufficient, while some technical artifacts became less important.

The EMMA-4 can detect elemental concentrations as low as 10^{-4} (or 100 ppm), which may at first appear to be inadequate for biological studies because many trace metals occur in much smaller amounts within cells. Nevertheless, as in previous studies (1–4), we succeeded in detecting zinc, calcium, and other elements. Reproducible differences relating to intracellular loci and pathological states were also recorded. The discrete elemental concentrations within cells were much higher than anticipated and it is possible that fixation and, perhaps, the drying and incinerating effects of the instrumentation enhance the readings. Whatever the reasons for the good results, this study shows that blood cells can be studied to advantage by x-ray microanalysis.

It appears that zinc will provide a fruitful field for microprobe work in pathologic states, as the changes reported here are impressive, reproducible, and in agreement with results obtained by other methods of investigation (7, 10, 11). Zinc is a firm component of several metalloproteins essential for biological processes and its dietary deficiency leads to definite clinical syndromes (12–17). From reported studies concerning zinc levels in the blood and urine, it seems that disease-related patterns do exist (10, 18–21), but, in most cases, no satisfactory explanation of the underlying mechanisms has yet been found.

In patients with leukemias, especially chronic lymphocytic leukemia, the zinc in serum and in leukocytes is much lower than in normal subjects (8, 10, 22). It is unclear, however, whether the low serum zinc values lead to the low intracellular concentrations or vice versa.

Table 1. Energy-dispersive x-ray microanalysis of elements in normal and leukemic lymphocytes

	Normal control 1		Normal control 2		Lymphocytic leukemia	
Element	Nucleus (8)	Cytoplasm (3)	Nucleus (15)	Cytoplasm (3)	Nucleus (18)	Cytoplasm (6)
Phosphorus	100 ± 7	32 ± 5	103 ± 9	21 ± 2	84 ± 6	23 ± 2
Sulfur	11 ± 1	12 ± 1	8 ± 2	10 ± 1	14 ± 2	18 ± 2
Calcium	25 ± 4	12 ± 6	13 ± 2	2 ± 2	4 ± 1	5 ± 1
Zinc	13 ± 1	9 ± 1	12 ± 1	15 ± 1	4 ± 1	3 ± 1

The figures are averages of relative mass fractions R (see *text*) $\times 10^3 \pm$ SEM. Figures in parentheses represent the number of cells examined.

Table 2. Relative concentrations of intracellular zinc in lymphocytes and monocytes measured with the crystal diffractive x-ray detector

Polycythemia (control)		Monoblastic leukemia		Lymphatic leukemia	
Nucleus (10)	Cytoplasm (4)	Nucleus (10)	Cytoplasm (4)	Nucleus (20)	Cytoplasm (5)
16 ± 3	13 ± 2	10 ± 1	0	6 ± 2	0

The figures are relative mass fractions $R \times 10^3 \pm$ SEM. Figures in parentheses represent the number of cells examined.

Red and white blood cells readily take up radioactive zinc against steep chemical gradients, both *in vivo* and *in vitro* (8, 11, 12, 23); leukemic cells have lower zinc uptake and concentrations, but the difference is not related to the degree of cellular differentiation, cell mass, leukocyte alkaline phosphatase, or other enzymes so far examined (8, 17).

If the causes of low intracellular zinc in leukemia are obscure, the consequences of such a state are easier to speculate upon. With zinc insufficiency, a number of different biological processes can be affected, the most important of which, in relation to lymphocytes, are a decrease in thymidine kinase activity (21), retardation of nucleic acid synthesis and cell division (24, 25), and inhibition of mitogen-induced lymphocyte transformation (26–28).

The relation of zinc to the other elements analyzed here is also of interest. It is known that the zinc present in nucleic acid often binds to phosphate groups of the DNA molecule (24). However, the phosphorus content in the cell nuclei does not decrease as much as the zinc, suggesting insufficient zinc availability or a faulty binding mechanism in the nucleus.

Zinc and sulfur also have a great affinity for each other (12, 13, 17), and the high sulfur and low zinc levels in leukemic cells may also be suggestive of faulty entry or binding of zinc in these cases. It has been noted previously that there is generally an inverse relation between the serum levels of copper and zinc. A high serum copper is a poor prognostic sign in cases of leukemia and lymphoma, and when patients are in remission, the serum copper levels drop while the zinc increases (10). In our case of end-stage leukemia, the copper-zinc inversion within red and white cells was easily recorded.

Many more cases of leukemia will have to be studied in order to establish whether the alterations in intracellular elemental concentrations reported here occur consistently. In the future we envisage more extensive studies of blood disorders utilizing x-ray microanalysis with scanning as well as transmission electron microscopy, striving towards a better structure-function correlation. This would undoubtedly contribute towards further understanding of the dynamic metabolism of the leukemic cell.

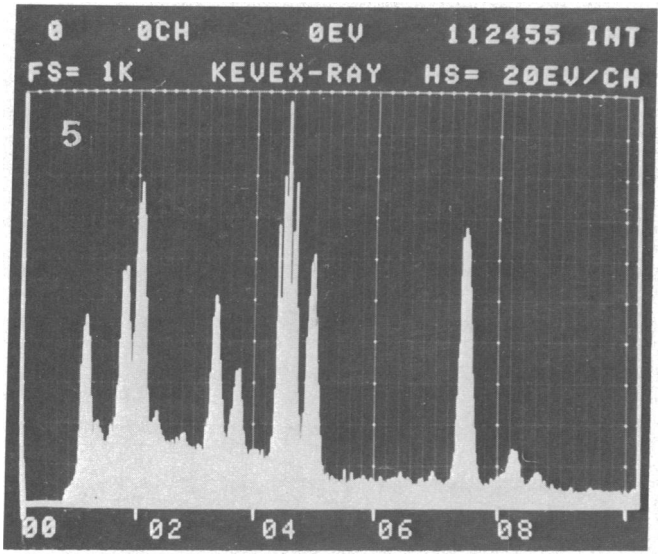

FIG. 5. Probe on the nucleus of a leukemic monoblast showing the presence of various elements and only a small zinc peak.

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1. Yarom, R., Peters, P. D., Hall, T. A., Kedem, J. & Rogel, S. (1974) *Micron* **5**, 11–20.
2. Yarom, R., Peters, D. P. & Hall, T. A. (1975) *Experientia* **31**, 154–157.
3. Yarom, R. (1975) *Proc. Joint Meeting of Microbeam Analysis Society and Electron Microscopy Society of America*, Las Vegas, pp. 46A–46C.
4. Yarom, R., Maunder, C. A., Hall, T. A. & Dubowitz, V. (1975) *Histochemistry* **45**, 44–50.
5. Bøyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **21** (Suppl.), 97, 31–50.
6. Hall, T. A. (1971) in *Physical Techniques in Biological Research*, ed. Oster, G. (Academic Press, New York), Vol. 1, part A.
7. Fredricks, R. E., Tanaka, K. R. & Valentine, W. N. (1964) *J. Clin. Invest.* **43**, 304–315.
8. Moreton, R. B., Echlin, P., Gupta, B. L., Hall, T. A. & Weis-Fogh, T. (1974) *Nature* **247**, 113–115.
9. Hall, T. A. (1972) in *X-ray Microscopy in Clinical and Experimental Medicine*, eds. Hall, T. A., Röckert, H. O. E. & Saunders, R. L. de C. H. (Charles C Thomas, Springfield, Ill.), pp. 164–165.
10. Delves, H. T., Alexander, F. W. & Lay, H. (1973) *Br. J. Haematol.* **24**, 525–531.
11. Dennes, E., Tupper, R. & Wormall, A. (1961) *Biochem. J.* **78**, 578–587.
12. Chvapil, M., Elias, S. L., Ryan, J. N. & Zukoski, C. F. (1972) "Neurobiology of the trace metals zinc and copper," ed. Pfeiffer, C. C. in *International Review of Neurobiology Supplement 1* (Academic Press, New York).
13. McBean, L. D., James, T., Dov, J. T., Halsted, J. A. & Smith, J. C. (1972) *Am. J. Clin. Nutr.* **25**, 672–676.
14. Pekarek, R. S., Kluje, R. M., Du Pont, H. L., Wannemacher, R. W., Hornick, R. B., Bostian, K. A. & Beisel, W. R. (1975) *Clin. Chem.* **21**, 528–532.
15. Parisi, N. & Valle, B. L. (1969) *Am. J. Nutr.* **22**, 1222–1239.
16. Prasad, A. S. (1969) *Am. J. Clin. Nutr.* **22**, 1215–1221.
17. Vallee, B. L. (1959) *Physiol. Rev.* **39**, 443–444.
18. Auerbach, S. (1965) *J. Lab. Clin. Med.* **65**, 628–637.
19. Dennes, E., Tupper, R. & Wormall, A. (1962) *Biochem. J.* **82**, 466–476.
20. Fell, G. S., Flack, A. & Cuthbertson, D. P. (1973) *Lancet* **i**,

280–282.
21. Prasad, A. S., Oberleas, D. & Miller, E. R. (1971) *J. Lab. Clin. Med.* **77**, 144–152.
22. Rosner, F. & Gorfien, P. C. (1968) *J. Lab. Clin. Med.* **72**, 213–219.
23. Cox, R. P. & Ruckerstein, A. (1971) *J. Cell. Physiol.* **77**, 71–81.
24. Falchuk, F. H., Fawcett, D. W. & Vallee, B. L. (1975) *J. Cell Sci.* **17**, 57–78.
25. Rubin, H. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 712–716.
26. Berger, N. A. & Skinner, A. M. (1974) *J. Cell Biol.* **61**, 45–55.
27. Chester, J. K. (1972) *Biochem. J.* **130**, 133–139.
28. Williams, R. O. & Loeb, L. A. (1973) *J. Cell Biol.* **58**, 594–601.