The estimation of whole-body zinc and Zn turnover in rheumatoid and osteoarthritis using ^{65}Zn tracer

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1. A method of estimating whole-body zinc, and Zn balances using a two-compartment model in combination with whole-body counting of ^{65}Zn, is described. The method is applied to patients with rheumatoid and osteoarthritis.

2. The results suggested that there was not a wide variation in whole-body Zn and Zn turnover in individuals with these two diseases and no clearcut difference between patients with one or the other.

The observation that the concentration of zinc in plasma tends to be low in patients with rheumatoid arthritis (Kennedy, Fell, Rooney, Stevens, Dick & Buchanan, 1975) is of considerable interest in view of possible therapeutic implications. Its significance, however, is doubtful because plasma Zn may or may not be low when the Zn balances are negative (Spencer, Rosoff, Lewin & Samachson, 1966) and that it may indeed increase despite considerable urinary and faecal losses (Vallee, 1964) calls into question conclusions on Zn metabolism deduced entirely from plasma Zn measurements. Simkin (1976) has reported significant clinical improvement in rheumatoid patients treated for 6 months with oral zinc sulphate, but also concluded that there was no apparent correlation between the extent of clinical improvement and the consequent increase in serum Zn concentration. A more direct measurement of whole-body Zn status is, therefore, called for, in the anticipation that this will correlate better with disease states and response to Zn therapy, and we have investigated the use of the ^{65}Zn radio-isotope with 245 d half-life.

A considerable amount of information on ^{65}Zn metabolism, after oral or intravenous administration, has been amassed by measurements on blood, urine and faeces (Vallee & Gibson, 1948; Graig & Siegel, 1960), by external organ counting and postmortem tissue analysis (Ross, Ebaugh & Talbot, 1958; Spencer, Rosoff, Feldstein, Cohn & Gusmano, 1965; Spencer *et al.* 1966) and by whole-body counting (Richmond, Furchner, Trafton & Langham, 1962; Spencer *et al.* 1965; Hussain & Bessent, 1974; Lombeck, Schnippering, Ritzl, Freinemdegen & Bremner, 1975; Henkin & Aamodt, 1975). A simple extension of some of these techniques, using the two-compartment model, has been applied in the present study to provide an estimate of whole-body Zn stores in arthritic patients.

MATERIALS AND METHODS

Nineteen female patients undergoing treatment at the Outpatient Department of the Centre for Rheumatic Diseases, Glasgow, were included in this study. Of these, twelve suffered from 'definite' or 'classical' rheumatoid arthritis (Ropes, Bennet, Cobb, Jacox & Jessar, 1959), six were receiving corticosteroid therapy, and six were receiving only non-steroidal anti-inflammatory drugs. Five suffered from osteoarthritis and were not receiving specific

treatment. The last two patients could not on final diagnosis be assigned to any of the arthritic groups.

All patients were over 40 years of age and the nature of the study was explained in detail to each patient, with particular reference to the use of the radio-isotope, and her consent was obtained.

Permission to carry out the study was granted by the Ethical Committee of Glasgow Royal Infirmary and approval of the Isotope Advisory Panel of the Medical Research Council was obtained for the administration of the ^{65}Zn radio-isotope.

The age, height (H) and weight (W) of each patient were noted and total body water (TBW) and lean body mass (LBM) were calculated from the W and H of each subject as follows:

$$\text{TBW (l)} = 0{\cdot}1838 \times W\,(\text{kg}) + 0{\cdot}3466 \times H\,(\text{cm}) - 35{\cdot}2701 \quad \text{(Hume \& Weyers, 1971);}$$

$$\text{LBM (kg)} = \text{TBW} \times (100/73) \quad \text{(Pace \& Rathbun, 1945).}$$

Each patient was given a 5 μCi oral dose of carrier-free (0·05 μg Zn) $^{65}ZnCl_2$ in 0·1 M-hydrochloric acid and after 15 mins whole-body radioactivity was measured with a shadow-shield whole-body monitor (James Girdler & Co., Acton, London, and Nuclear Enterprises Ltd, Edinburgh). After 1 week, whole-body radioactivity was measured again. Thereafter at intervals of 1–2 months patients were recalled for further whole-body activity measurements over a total period of up to 7 months. In addition, for twelve patients, two further measurements were made at approximately 20 months after the dose of $^{65}ZnCl_2$. The values for whole-body fractional retention from 7 d onwards were fitted to a double exponential function of the form:

$$W(t) = W_1 \exp(-m_1 t) + W_2 \exp(m_2 t),$$

where W_1, W_2, m_1 and m_2 are constants and t is time (d). In each instance a least squares iterative procedure was used to fit the actual exponential function to the values.

During the 3 d preceding each attendance for whole-body measurements, patients collected a total of approximately 2 l urine whenever it was convenient for them to do so. ^{65}Zn activity in the urine was measured with a 125 × 75 mm sodium iodide scintillation detector, with correction for volume dependence of the counting efficiency, after which the urine was assayed for stable Zn.

Approximately 3 months after the start of the study blood samples were obtained from a non-occluded vein from each patient and used for estimation of plasma Zn levels. Plasma and urinary Zn were estimated by atomic absorption spectrophotometry (Peaston, 1973), great care being taken to avoid contamination of specimens by the use of appropriate syringes, needles and specimen tubes.

Significant differences between groups were sought using the Wilcoxon rank-sum test (Bradley, 1968) which does not rely on assumptions about the distributions of the populations being tested.

Principles of estimation of whole-body zinc content and its daily excretion

The absorbed fraction of a ^{65}Zn oral dose enters the blood pool, whence it is redistributed throughout the body's Zn stores by exchange with stable Zn. Unabsorbed ^{65}Zn is lost in the faeces in the first week.

Since the whole-body activity *v.* time curve thereafter is a good fit to a double exponential

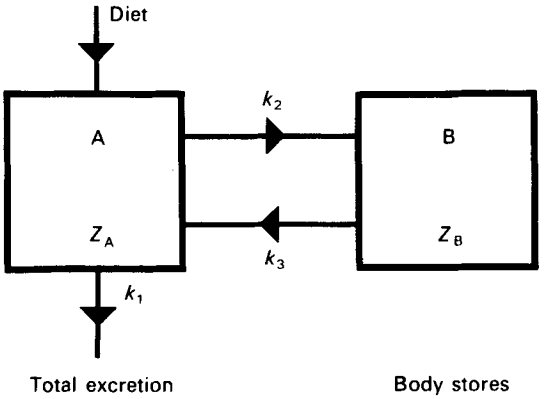

Fig. 1. The two-compartment model for total whole-body zinc where A and B are the two pools and Z_A and Z_B are the stable Zn contents of the two pools respectively, k_1, k_2, k_3 are constants.

curve a two-compartment model is required to fit the data and the simple system of Fig. 1 is used. A rapidly exchanging entry pool, A, which includes the plasma, exchanges more slowly with a second pool B under control of the rate constants k_2 and k_3. Total excretion of Zn, in both urine and faeces, is assumed to come from pool A and the urinary specific activity is assumed equal to that in pool A. The dietary input to pool A is only that proportion of dietary Zn which is actually absorbed into plasma. Unabsorbed Zn is excluded from this model. However, the unabsorbed Zn makes it impossible to use faeces to measure the specific activity in pool A, even though there is much higher excretion by this route, since the unabsorbed Zn will lower the specific activity of faecal Zn by up to 70 % of the value in pool A.

From the four parameters, W_1, W_2, m_1 and m_2 of the whole-body activity curve, the parameters k_1, k_2 and k_3 together with the functions describing the activity in pools A and B may be derived. From these, values for stable Zn content of pool A: stable Zn content of pool B may be derived and, from the urinary specific activity at any time, the stable Zn content of pool A and hence of the total whole-body may be calculated. The model also permits calculation of total endogenous daily loss of Zn. The details of the derivations are outlined in the appendix.

RESULTS

The rheumatoid patients not receiving corticosteroid therapy had had their disease a little longer than the other rheumatoid group, but not as long as the osteoarthritic group. In age distribution the three groups were comparable. The calculation of LBM from H and W for each patient revealed no significant difference in the mean value for each group.

The plasma levels and values for whole-body stable Zn and ^{65}Zn retention for the three arthritic groups of patients are given in Table 1, together with separate results for the two other patients.

It can be seen that the highest mean value for whole-body Zn in total or per unit LBM was found for the osteoarthritic group although the highest individual value was for the patient with metatarsalgia. This group had a significantly higher value ($P < 0{\cdot}05$) for total whole-body Zn than the rheumatoid group not receiving steroids. The level of significance increased to $P < 0{\cdot}02$ for whole-body Zn expressed per unit LBM between these two groups and the coefficient of variation for the osteoarthritic group was halved. No other intercomparisons of whole-body Zn values showed significant differences and there were no significant differences between groups for any of the other parameters given in Table 1.

Table 1. *Plasma zinc and whole-body Zn in groups of female patients undergoing treatment for rheumatoid and osteoarthritis after oral administration of* ^{65}Zn

(Mean values and standard deviations)

Group*	No. of patients	Plasma Zn (μmol/l) Mean	SD	Whole-body retention Mean	SD	Calculated whole-body Zn (g) Mean	SD	Lean body mass† (mg Zn/kg) Mean	SD
Rheumatoid arthritis:	6	11·5	2·7	0·67	0·12	1·2	0·4	31	9
On steroids									
Not on steroids	6	10·7	2·0	0·62	0·19	1·0	0·3	27	6
Osteoarthritis	5	11·3	2·0	0·66	0·07	1·5	0·2	35	2
Arteritis	1	10·6		0·41		0·8		19	
Metatarsalgia	1	13·8		0·66		2·7		59	

* For details see p. 115.

† TBW × (100/73), where TBW is total body water (l) (TBW = 0·1838 × weight (kg) + 0·3466 × height (cm) − 35·2701 (Hume & Weyers, 1971).

Table 2 gives the mean values of the parameters k_1, k_2 and k_3 derived on the basis of the two-compartment model and also the calculated daily loss of endogenous Zn. The Wilcoxon rank-sum test revealed no significant differences between any pairs of groups for any parameter.

DISCUSSION

Results

Measurements of whole-body Zn are scarce and have been obtained previously by chemical analysis of cadavers (Widdowson, McCance & Spray, 1951). Values obtained by these authors ranged from 1·4 to 2·3 g and are slightly higher than the values obtained in the present study but comparison of the values suggests that the two-compartment model is an acceptable method for the estimation of whole-body Zn.

The calculated values of whole-body Zn of patients in the rheumatoid arthritis group on steroids were not significantly different from those of patients in the group not on steroids nor from the osteoarthritic group. There was, however, a difference ($P < 0·05$) between the osteoarthritic patients and the non-steroid-treated group of rheumatoid arthritic patients. The latter group of patients had lower levels of whole-body Zn than the osteoarthritic group, although an influence of the non-steroidal anti-inflammatory drugs cannot be discounted. There were no significant differences in plasma Zn concentrations between the groups although previous studies (Kennedy, Fell *et al.* 1975) have shown that rheumatoid arthritic patients treated with corticosteroid therapy have lower levels of plasma Zn than untreated patients, in contrast to our whole-body Zn results. There was no correlation ($r < 0·1$) between plasma Zn and calculated whole-body Zn as total content or per unit LBM. Thus it appears that plasma Zn concentration is not a good index of whole-body Zn. A similar independence occurs with potassium (Flear, Cooke & Quinton, 1957). The combination of a low serum level of an element together with normal or increased body stores occurs in rheumatoid arthritic patients with low serum iron or even hypochromic anaemia while there are still high stores of Fe in the form of ferritin in the liver, spleen and especially synovium; (Freireich, Ross, Bayles, Emerson & Finch, 1957; Bennett, Holt & Lewis, 1974). Similarly, low plasma copper together with increased deposition of Cu at various sites, in addition to other anomalies in Cu metabolism, is observed in Wilson's disease (Walshe, 1956, 1963, 1967, 1970).

Table 2. *Daily zinc loss and values for Zn balance based on two-compartment model* in groups of female patients undergoing treatment for rheumatoid and osteoarthritis after oral administration of ^{65}Zn*

(Mean values and standard deviations)

Group†	No. of patients	Calculated daily Zn loss (mg)		k_1*(/d)		k_2*(/d)		k_3*(/d)		pool A* / pool B*		Whole-body specific activity / Urinary specific activity	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Rheumatoid arthritis: On steroids	6	2·8	0·6	0·0082	0·0037	0·014	0·007	0·0063	0·0022	2·6	1·8	1·3	0·1
Not on steroids	6	2·5	0·6	0·012	0·006	0·024	0·013	0·0063	0·0022	3·7	1·3	1·3	0·1
Osteoarthritis	5	3·4	1·0	0·0083	0·0031	0·017	0·011	0·0060	0·0008	2·9	1·6	1·3	0·1
Metatarsalgia	1	4·2		0·0079		0·015		0·0037		4·1		1·4	
Arteritis	1	2·1		0·0063		0·0077		0·0062		1·2		1·3	

* See Fig. 1 and Appendix.
† For details, see p. 115.

In vitro experiments on decalcified bone have shown that such bone has an affinity for Zn two to three times greater than for calcium (Samachson, Dennis, Fowler & Schmitz, 1967). The generalized decalcification of bone present in rheumatoid arthritis (Kennedy, Smith, Anton & Buchanan, 1975) may therefore result in increased uptake of Zn by bone. In addition, the Zn content of synovial fluid is markedly increased in rheumatoid arthritis patients compared with normal controls (Bonebrake, McCall, Hunder & Polley, 1972). Both these factors may tend to produce an over-all increase in whole-body Zn in patients with rheumatoid arthritis.

The values of retention of the oral dose of ^{65}Zn obtained in the present study have a wide range of values from 0·28 to 0·89 similar to those of previous measurements (Spencer *et al.* 1966). Our measurements give an average retention of 0·64. Dietary protein and phytate content may be a cause of the wide variation of Zn retention observed in such studies, but pathological malabsorption or the effect of drugs must also be considered.

The final ^{65}Zn whole-body half-life of the nineteen patients averaged 412 d, in agreement with previous studies. The early, more rapid phase for these patients had an average half-life of 26 d and accounted for about one-quarter of the retained ^{65}Zn activity.

Most of the Zn lost by the body is excreted via the faeces. Endogenous faecal Zn excretion is of the order of ten times urinary zinc excretion (Spencer *et al.* 1965). Faecal Zn has been found to be in the range 5–10 mg/24 h (Vallee, 1959). For a patient whose Zn metabolism is in balance and whose absorption of dietary Zn is 0·50, endogenous faecal Zn will be typically 2·5–5 mg/24 h. The values of endogenous Zn loss obtained (by calculation) in the present study are within this range, or slightly below, with a mean value of 2·9 mg/d, and because of the small number of patients in each group no significant difference is discernible between groups.

Method

The two-compartment model is well known but has not previously been applied to Zn metabolism. The use of the model enables whole-body Zn estimates to be made from urine specific activity measurements made before equilibrium throughout the body is established. Indeed Table 2 shows that even for very long periods uniform specific activity does not exist throughout the body and an estimate based on this assumption and using measurements of urine specific activity and whole-body activity would overestimate whole-body Zn by 15–40 % with a mean excess of 30 %.

For each patient four or five estimates of whole-body Zn were obtained from urine specific activities up to 200 d. The mean coefficient of variation was 25 for the estimates of an individual patient's whole-body Zn. While this indicates only moderate precision, the results obtained after different periods of study did not in general show a systematic drift, indicating a failure in the model. Rather the different values for each patient were randomly scattered as a result of the low ^{65}Zn activity in the urine samples and the errors in estimating the stable Zn content.

It may be objected that a two-compartment model is unduly simple for Zn metabolism and that uniform mixing within the pools, especially pool B which includes bone, is unlikely. However, the quantity of information on each patient will not support a more complex model and the validity of the model used is enhanced by the order of values obtained which were in the range found by other workers.

The values of the k parameters found are small as a direct consequence of the high values for time constants of the whole-body activity *v.* time curve. This results in a poor coupling between the two pools and shows why a normal plasma Zn, especially after a dietary intake of Zn, could give a misleading impression of the Zn status in pool B. The values for the pool size ratio show a surprisingly large range, 1·0–4·8 with a mean of 2·9, and indicated that only approximately 25 % of the total whole-body Zn was in rapid exchange with the plasma.

In order to define the whole-body activity *v.* time curve adequately, measurements need to be made over at least 200 d. This is probably too long a period to make the method practicable for routine clinical estimation of whole-body Zn stores but the technique should be of considerable value in the research situation. Much effort has been expended in attempting to correlate plasma Zn levels with disease states and the general conclusion is that there is an efficient homoeostatic mechanism which maintains a more or less normal plasma Zn level in many situations where low body Zn stores are suspected.

The method we have described provides a potentially accurate estimate of whole-body Zn stores and it should be valuable in investigating the relationship between Zn status and many pathologies in which it is thought to be relevant. It could also demonstrate the effect of deliberate attempts to increase body Zn stores, as by zinc sulphate supplement, or inadvertent depletion caused by treatment for other conditions, for example, penicillamine therapy or long-term parenteral nutrition.

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APPENDIX

The two-compartment model

Our model represented in Fig. 1 is the open, two-compartment, mamillary system amongst those treated by Matthews (1971) who states the solutions of the differential equations and indicates that the rate constants may be derived from measurements on pool A. The solutions also assume that the whole of the administered activity is in pool A at the start of the study. Since, in the present paper, we are concerned with measurements of the whole-body activity (i.e. pool A plus pool B) and we assume that only the absorbed fraction of the ^{65}Zn is in pool A at time zero, we indicate below the details of the solution which relates the constants of the model to the whole-body measurements.

The ^{65}Zn activity in pools A and B varies with time and, as a fraction of the administered dose, is represented as A(t) and B(t). The stable Zn contents of the two pools are assumed constant and represented by Z_A and Z_B grams. The input dietary flow of stable Zn is assumed constant and specific activity is assumed uniform in each pool at all times.

The system is described by the simultaneous differential equations

$$dA/dt = -(k_1+k_2)A+k_3B, \tag{1}$$

$$dB/dt = k_2A-k_3B, \tag{2}$$

where k_1, k_2 and k_3 are the rate constants. The solution for A as a function of time is the double exponential:

$$A(t) = A_1e^{-m_1t}+A_2e^{-m_2t}, \tag{3}$$

where the exponents m_1 and m_2 are such that:

$$m_1m_2 = k_1k_3, \tag{4}$$

$$m_1+m_2 = K, \tag{5}$$

where

$$K = k_1+k_2+k_3. \tag{6}$$

The activity in pool B is of similar form to equation (3), with coefficients B_1 and B_2. By substituting for A and B in equation (2) we obtain the relations:

$$B_1 = A_1(k_1+k_2-m_1)/k_3, \tag{7}$$

$$B_2 = A_2(k_1+k_2-m_2)/k_3. \tag{8}$$

The whole-body activity is of the form of equation (3) with coefficients W_1 and W_2 and is equal to the sum of activities in pools A and B. Thus:

$$W_1 = A_1+B_1 \tag{9}$$

$$W_2 = A_2+B_2 \tag{10}$$

From equations (7) and (9) and equations (8) and (10), using equations (5) and (6) and rearranging we obtain:

$$A_1 = k_3 W_1/m_2, \tag{11}$$

$$A_2 = k_3 W_2/m_1. \tag{12}$$

At time zero all the activity is in pool A, thus:

$$W_1+W_2 = A_1+A_2 \tag{13}$$

and, using equations (11) and (12) we obtain:

$$k_3 = (W_1+W_2)/(W_1/m_2+W_2/m_1). \tag{14}$$

The constants W_1, W_2, m_1 and m_2 are all derived from the least squares fit to the whole-body results, measured as fractions of the administered activity, and W_1+W_2 is taken to equal the fractional absorption of ^{65}Zn.

k_3 is calculated from equation (14), k_1 from equation (4) and k_2 from equations (5) and (6). A_1 and A_2 are given by equations (11) and (12) and B_1 and B_2 by equations (7) and (8), enabling the activities in the two pools, $A(t)$ and $B(t)$, to be calculated at any time.

If the specific activity (S) of urine, and hence of pool A, at time t, $S(t)$, is expressed as the fraction of administered ^{65}Zn activity/g stable Zn then:

$$Z_A = A(t)/S(t). \tag{15}$$

Since the stable zinc in pools A and B is in equilibrium:

$$k_2 Z_A = k_3 Z_B. \tag{16}$$

Thus whole-body Zn is given by

$$Z_A+Z_B = (1+k_2/k_3)\, A(t)/S(t) \tag{17}$$

$S(t)$ is measured for each urine sample and $A(t)$ is calculated for the appropriate value of t. The rate of loss by excretion of endogenous stable Zn is given simply by $k_1 Z_A$.

Knowing Z_A and Z_B, the ratio, specific activity in A (urinary S): mean specific activity of the whole-body (mean total S) may be calculated for any time t:

$$\frac{\text{mean total } S}{\text{urinary } S} = \frac{W(t)}{A(t)} \times \frac{k_3}{k_2+k_3}. \tag{18}$$

This function increases rapidly for small values of t reaching an asymptotic value at high values of t.

A simple computer program was written to calculate the k values from the whole-body activity curve constants and to compute the resulting activity v. time curves for pools A and B together with the ratio, mean whole-body specific activity: pool A specific activity, as

a function of time. Whole-body Zn estimates were obtained from equation (17) using the several 'spot' values of urinary specific activity and the mean value was taken for each patient.

REFERENCES

Bennett, R. M., Holt, P. J. & Lewis, S. M. (1974). *Ann. rheum. Dis.* **33**, 147.
Bonebrake, R. A., McCall, J. T., Hunder, G. G. & Polley, H. F. (1972). *Proc. Staff Meet, Mayo Clin.* **47**, 746.
Bradley, J. V. (1968). In *Distribution-Free Statistical Tests*, p. 105. New Jersey: Prentice-Hall.
Flear, C. T., Cooke, W. T. & Quinton, A. (1957). *Lancet* i, 458.
Freireich, E. J., Ross, J. F., Bayles, T. B., Emerson, C. P. & Finch, S. C. (1957). *J. clin. Invest.* **36**, 1043.
Graig, F. A. & Siegel, E. (1960). *Proc. Soc. exp. Biol. Med.* **104**, 391.
Henkin, R. I. & Aamodt, R. L. (1975). *Lancet* i, 1379.
Hume, R. & Weyers, E. (1971). *J. clin. Path.* **24**, 234.
Hussain, S. L. & Bessent, R. G. (1974). In *Clinical Applications of Zinc Metabolism*, p. 168 [W. J. Pories, W. H. Strain, J. M. Hsu and R. L. Woosley, editors]. Springfield, Illinois: Thomas.
Kennedy, A. C., Fell, G. S., Rooney, P. J., Stevens, W. H., Dick, W. C. & Buchanan, W. W. (1975). *Scand. J. Rheumatol.* **4**, 243.
Kennedy, A. C., Smith, D. A., Anton, H. C. & Buchanan, W. W. (1975). *Scand. J. Rheumatol.* **4**, 209.
Lombeck, I., Schnippering, H. G., Ritzl, F., Feinemdegen, L. E. & Bremner, H. J. (1975). *Lancet* i, 855.
Matthews, C. M. E. (1971). In *Radioisotopes in Medical Diagnosis*. [E. H. Belcher and H. Vetter, editors]. London: Butterworths.
Pace, N. & Rathbun, E. N. (1945). *J. biol. Chem.* **158**, 685.
Peaston, R. T. (1973). *Med. Lab. Technol.* **30**, 249.
Richmond, C. R., Furchner, J. E., Trafton, G. A. & Langham, W. H. (1962). *Hlth Phys.* **8**, 481.
Ropes, M. W., Bennet, G. A., Cobb, S., Jacox, R. & Jessar, A. R. (1959). *Ann. rheum. Dis.* **18**, 49.
Ross, J. F., Ebaugh, F. G. & Talbot, T. R. (1958). *Trans. Ass. Am. Physns* **71**, 322.
Samachson, J., Dennis, J., Fowler, R. & Schmitz, A. (1967). *Biochim. biophys. Acta* **148**, 767.
Simkin, P. A. (1976). *Lancet* ii, 539.
Spencer, H., Rosoff, B., Feldstein, A., Cohn, S. H. & Gusmano, E. (1965). *Radiat. Res.* **24**, 432.
Spencer, H. V., Rosoff, B., Lewin, I. & Samachson, J. (1966). In *Zinc Metabolism*, p. 339 [A. S. Prasad, editor]. Springfield, Ill.: C. C. Thomas.
Vallee, B. L. (1959). *Physiol. Res.* **39**, 443.
Vallee, B. L. (1964). In *Mineral Metabolism*, p. 443 [C. L. Comar and F. Bronner, editors]. London: Academic Press.
Vallee, B. L. & Gibson, J. G. (1948). *J. biol. Chem.* **176**, 445.
Walshe, J. M. (1956). *Lancet*, i, 25.
Walshe, J. M. (1963). *Clin. Sci.* **25**, 405.
Walshe, J. M. (1967). *Brain* **90**, 149.
Walshe, J. M. (1970). *Br. J. Hosp. Med.* **4**, 91.
Widdowson, E. M., McCance, R. A. & Spray, C. M. (1951). *Clin. Sci.* **10**, 113.

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