Changes in the alkaline phosphatase (*EC* 3.1.3.1) and inorganic pyrophosphatase (*EC* 3.6.1.1) activities of rat tissues during magnesium deficiency. The importance of controlling feeding pattern

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1. The adoption of a meal-eating pattern of feeding by rats altered the alkaline phosphatase (*EC* 3.1.3.1) activity in serum and liver. It was therefore necessary to regulate the feeding pattern of both magnesium-deficient rats and control animals receiving a Mg-adequate diet in order to study the effect of the deficiency.

2. Mg deficiency decreased the activities of alkaline phosphatase and inorganic pyrophosphatase (*EC* 3.6.1.1) in serum, kidney and tibia, but increased them in spleen.

3. Addition of a standard concentration of exogenous Mg to tissue extracts usually increased the activity of corresponding enzymes from Mg-deficient and control rats by the same proportion, indicating that the main effect of the deficiency was on the amount of enzyme present rather than on the efficiency of its operation.

4. Certain quantitative differences in the response to exogenous Mg and the activity ratio, alkaline phosphatase: inorganic pyrophosphatase were found between tissues from Mg-deficient and control rats. The significance of these are discussed in relation to the association of the two enzymic activities with the same protein molecule, and the possible occurrence of isoenzymes.

The reduced activity of alkaline phosphatase (*EC* 3.1.3.1) found in plasma from magnesium-deficient cattle (Larvor, Girard, Brochart, Parodi & Sevestre, 1964), rats (Heaton, 1965) and mice (Hamuro, 1971) is one of the few disturbances in enzymic activity that has been unequivocally established during Mg deficiency in vivo, although the activating effect of the metal on many enzymes is well recognized in vitro (Mahler, 1961; Wacker, 1969). Addition of Mg to plasma from Mg-deficient rats increases the activity of the enzyme, but does not increase it to the level found in control animals (Heaton, 1965; Pimstone, Eisenberg & Stallone, 1966), which suggests that the reduced activity is due partly to a decreased efficiency of operation by the enzyme in plasma and partly to a reduction in the amount of enzyme.

The present study was undertaken to determine the effect of Mg deficiency on alkaline phosphatase in tissues that are likely to release the enzyme into the blood, and as it was not possible to estimate the amount of enzyme directly, its activity in tissue extracts was measured both with endogenous Mg alone and in the presence of exogenous Mg, to provide uniform conditions for activation. Many mammalian alkaline phosphatases combine orthophosphatase and pyrophosphatase (*EC* 3.6.1.1) activities in the same molecule (Cox, Gilbert & Griffin, 1967; Melani & Farnararo, 1969), and as Mg affects the activity towards both types of substrate in vitro (Eaton & Moss, 1967; Fernley & Walker, 1967), the ability of the tissue extracts to hydrolyse inorganic pyrophosphate was also studied.

Mg-deficient rats develop anorexia during the depletion period and the normal practice in this situation is to pair-feed control rats, on a daily basis, with an amount of food equal to that consumed by the Mg-deficient animals. Although this procedure equalizes the total food intake of the rats, when food restriction becomes severe the controls eat all their food within about 30 min after it is provided and then fast for the remainder of the day, whereas the Mg-deficient rats nibble their food at frequent intervals throughout the 24 h in a normal manner. The pattern of intermittent feeding, known as meal-eating, has been found to influence the activity of several enzymes involved in carbohydrate and lipid metabolism (Fábry, 1967; Leveille, 1966, 1972) and its effect on alkaline phosphatase activity was therefore also determined in this study.

EXPERIMENTAL

Animal management

Thirty-six male Wistar albino rats weighing about 90 g were randomly allocated to three groups which received Mg-deficient (3 mg Mg/kg) (Mg-deficient group) or Mg-adequate (800 mg Mg/kg) (control and meal-fed groups) diets for 16 d. The animals were housed three to a cage and all received an amount of food equal to the mean amount consumed by the Mg-deficient rats, which gave a mean food intake of 9 g/rat per d. Distilled water was provided *ad lib.* The Mg-deficient and control groups received the Mg-deficient and Mg-adequate diets respectively from an automatic feeding apparatus (Loveless, Williams & Heaton, 1972) to ensure that food was available at frequent intervals throughout the 24 h. Meal-fed rats received their daily amount of Mg-adequate diet all in one meal at 10.00 hours and consumed it entirely within about 30 min during the later stages of the experiment. The rats which were fed automatically with Mg-adequate diet therefore served as a control group for both the Mg-deficient and meal-fed animals. The synthetic diets contained (g/kg): casein 200, sucrose 660, arachis oil 80, cod-liver oil 20, salt mixture 40, and purified vitamins, as described previously (Heaton & Anderson, 1965), and they were identical in composition except for the addition of $MgCl_2$ to the Mg-adequate diet.

A small sample of blood was taken from the tail on the 15th day for assay of leucocyte alkaline phosphatase activity, and all animals were given a final meal containing half the daily ration of food, then fasted for 18 h and killed by exsanguination from the heart under diethyl ether anaesthesia. The serum was separated, and the liver, kidneys, spleen, small intestine and both tibiae were removed as soon as possible, and stored separately at $-35°$ until required for analysis. The lumen of the small intestine was flushed with distilled water to remove any contents.

Extraction of enzyme from tissues

The complete small intestine, spleen, both kidneys and approximately 0·8 g tissue from the left lateral lobe of the liver were weighed and homogenized separately with 5 ml distilled water and 3 ml butan-1-ol at 0° for 15 min (Morton, 1953), using a top-drive homogenizer (MSE Scientific Instruments, Crawley, Sussex). After standing at 37° for 15 min the homogenate was centrifuged and the lower aqueous layer was

separated and retained for enzyme assay. Enzyme was extracted from bone by allowing the intact left tibia from each rat to autolyse in 10 ml distilled water saturated with chloroform for 3 d at room temperature (Conyers, Birkett, Neale, Posen & Brundenell-Woods, 1967) and the clear extract obtained on centrifugation was used for assay.

Analytical methods

Alkaline phosphatase activity was measured at pH 10·0 in appropriately diluted serum and tissue extracts by a modification of the phenyl phosphate hydrolysis method described by Wootton (1964). Each sample was assayed after addition to a Mg-free medium, and in a similar medium containing 20 mg exogenous Mg/l to provide uniform conditions for activation of the enzyme. The amount of endogenous Mg from the tissue extracts was negligible at the dilutions used. The inorganic pyrophosphatase activity was assayed, with and without added Mg in the same way, by hydrolysis of sodium pyrophosphate at pH 8·5 (Moss, Eaton, Smith & Whitby, 1967), the orthophosphate liberated being determined by the method of Panusz, Graczyk, Wilmanska & Skarzynski (1970). One unit of enzyme activity is defined as the amount which hydrolysed 1 μmol substrate/min and it is expressed on a per ml serum, per g liver, kidney or spleen, per mg small intestine nitrogen or per g dry tibia basis. Leucocyte alkaline phosphatase activity was estimated histochemically by a modification (Dacie & Lewis, 1970) of the Kaplow (1955) method. Depth of staining was scored visually on a three-point scale (0–2) and thirty leucocytes were counted from each rat to obtain an average value for the animal. Slides from Mg-deficient and control rats were examined in a random sequence.

N was determined in homogenates of small intestine by the micro-Kjeldahl method and the right tibia from each rat was dried to constant weight at 105°. Mg was estimated by atomic absorption flame photometry in serum deproteinized with trichloroacetic acid solution (100 g/l), and in diets that had been dry ashed at 500° and dissolved in 2 M-hydrochloric acid. Unknown and standard solutions contained 0·1 M-HCl to prevent interference by other constituents of the sample.

The statistical significance of differences was assessed by Student's *t* test.

RESULTS

Influence of meal-feeding

The gain in body-weight was similar in the meal-eating rats and control (continuously-fed) animals, and averaged 37 g/rat during the experimental period. Meal-eating animals had significantly lower levels of alkaline phosphatase activity in the serum and higher levels in the liver than the controls. This difference was statistically significant for both tissues when the activities were measured in the presence of added Mg (Table 1), but only for liver in the absence of added Mg (Table 2). The addition of exogenous Mg produced a smaller increase in activity with alkaline phosphatase from the small intestine and inorganic pyrophosphatase from the tibia of meal-fed than control rats (Table 3), but it had a greater effect with inorganic pyrophosphatase from the liver of the former group of animals. The biological significance of these differences is not clear.

Table 1. *Alkaline phosphatase* (EC 3.1.3.1) *and inorganic pyrophosphatase* (EC 3.6.1.1) *activities in tissues from magnesium-deficient, control and meal-fed rats, in the presence of exogenous Mg* (20 *mg/l*)†

(Mean values with their standard errors for twelve determinations for serum, and for six determinations for other tissues)

	Alkaline phosphatase activity						Inorganic pyrophosphatase activity					
Group‡ ...	Meal fed		Control		Mg-deficient		Meal-fed		Control		Mg-deficient	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Tissue												
Serum (U§/ml)	0·251	0·014*	0·300	0·017	0·173	0·012***	0·129	0·013	0·145	0·011	0·099	0·008**
Liver (U§/g)	0·606	0·040**	0·390	0·024	0·442	0·042	0·090	0·013	0·087	0·007	0·064	0·009
Kidney (U§/g)	20·4	1·2	22·4	2·0	16·6	1·3*	0·515	0·035	0·773	0·196	0·487	0·108
Spleen (U§/g)	1·29	0·11	1·54	0·13	3·75	0·64**	0·026	0·013	0·046	0·012	0·145	0·031**
Small intestine (U§/mg nitrogen)	1·55	0·45	1·28	0·26	0·99	0·12	0·531	0·193	0·301	0·067	0·272	0·085
Tibia (U§/g dry wt)	83·5	4·5	73·1	4·3	48·8	4·2**	4·61	0·22	5·34	0·50	2·43	0·42**

Value significantly different from that for control group: $*P<0·05$, $**P<0·01$, $***P<0·001$.

† For details of assay procedures, see p. 489.

‡ Meal-fed and control groups received the Mg-adequate diet, and Mg-deficient group received the Mg-deficient diet; for details of diets and feeding regimens, see p. 488.

§ One unit of activity is defined as the amount of enzyme which hydrolysed 1 μmol substrate/min.

Table 2. *Alkaline phosphatase* (EC 3.1.3.1) *and inorganic pyrophosphatase* (EC 3.6.1.1) *activities in tissues from magnesium-deficient, control and meal-fed rats in the absence of exogenous Mg*†

(Mean values with their standard errors for twelve determinations for serum and leucocytes, and for six determinations for other tissues)

	Alkaline phosphatase activity						Inorganic pyrophosphatase activity					
Group‡ ...	Meal-fed		Control		Mg-deficient		Meal-fed		Control		Mg-deficient	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Tissue												
Serum (U§ml)	0·242	0·015	0·283	0·015	0·161	0·011**	0·115	0·011	0·116	0·009	0·075	0·005**
Liver (U§/g)	0·562	0·058*	0·403	0·038	0·422	0·032	0·054	0·016	0·077	0·010	0·064	0·016
Kidney (U§/g)	15·5	0·9	18·5	1·5	14·6	1·5	0·666	0·122	0·854	0·077	0·589	0·115
Spleen (U§/g)	1·00	0·09	1·18	0·09	2·99	0·59*	0·090	0·035	0·054	0·016	0·140	0·019**
Small intestine (U§/mg nitrogen)	1·17	0·33	0·924	0·236	0·738	0·138	0·466	0·160	0·462	0·248	0·200	0·062
Tibia (U§/g dry wt)	59·9	3·8	52·7	2·5	31·5	2·9**	5·07	0·22	5·03	0·37	2·65	0·36**
Leucocytes: depth of staining (arbitrary units‖)		—	0·843	0·069	1·180	0·085**		—		—		—

Value significantly different from that for control group: $*P < 0.05$, $**P < 0.01$.

† For details of assay procedure, see p. 489.

‡ Meal-fed and control groups received the Mg-adequate diet, and Mg-deficient group received the Mg-deficient diet; for details of diets and feeding regimens, see p. 488.

§ One unit of activity is defined as the amount of enzyme which hydrolysed 1 μmol substrate/min.

‖ For details, see p. 489.

Table 3. *Values for the ratio, activity in the presence of exogenous magnesium* (20 *mg/l*): *activity in absence of exogenous Mg*† *for alkaline phosphatase* (EC 3.1.3.1) *and inorganic pyrophosphatase* (EC 3.6.1.1) *in tissues from Mg deficient, control and meal-fed rats*

(Mean values with their standard errors for twelve determinations for serum, and for six determinations for other tissues)

	Alkaline phosphatase activity						Inorganic pyrophosphatase activity					
Group‡ ...	Meal-fed		Control		Mg-deficient		Meal-fed		Control		Mg-deficient	
Tissue	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Serum	1·04	0·01	1·05	0·01	1·08	0·01	1·15	0·05	1·24	0·03	1·35	0·04*
Liver	1·12	0·02	1·15	0·02	1·21	0·02	2·02	0·34*	1·21	0·17	1·37	0·34
Kidney	1·32	0·03	1·38	0·02	1·32	0·03	0·67	0·04	0·85	0·15	0·61	0·06
Spleen	1·30	0·02	1·30	0·02	1·27	0·04	1·03	0·61	0·73	0·10	1·03	0·11
Small intestine	1·31	0·04*	1·45	0·05	1·41	0·14	1·14	0·07	1·36	0·16	1·34	0·08
Tibia	1·40	0·04	1·39	0·03	1·55	0·03**	0·91	0·03*	1·06	0·05	0·89	0·07

Value significantly different from that for control group: * $P < 0.05$, ** $P < 0.01$.

† For details of assay procedures, see p. 489.

‡ Meal-fed and control groups received the Mg-adequate diet, and Mg-deficient group received the Mg-deficient diet; for details of diets and feeding regimens, see p. 488.

Effect of Mg deficiency

The mean body-weight of the Mg-deficient rats increased by 28 g and they developed severe deficiency as indicated by the serum Mg concentrations of (mean and SE) 0.35 ± 0.01 and 1.05 ± 0.02 mmol/l in the Mg-deficient and control groups respectively. In the absence of added Mg the alkaline phosphatase and inorganic pyrophosphatase activities were lower in serum and extracts of tibia from Mg-deficient rats than in control animals, but the activities of both enzymes were higher in spleen, and the histochemical assay indicated that alkaline phosphatase activity was also increased in leucocytes during Mg deficiency (Table 2). In the presence of added Mg similar effects of deficiency were found in serum, tibia and spleen, and in addition a significant decrease in alkaline phosphatase activity was found in the kidney of Mg-deficient rats (Table 1).

Addition of Mg to the assay medium significantly increased the alkaline phosphatase activity with all tissues and it usually had a similar effect with inorganic pyrophosphatase activity except in kidney (Table 3). The increase in alkaline phosphatase activity varied between tissues from 6% with serum to about 40% with small intestine and tibia, and variations between tissues in the effect on pyrophosphatase activity were also found. The magnitude of the stimulation produced by exogenous Mg was usually similar with extracts from both Mg-deficient and control rats, but there was a significantly greater action with alkaline phosphatase from the tibia and inorganic pyrophosphatase in the serum of Mg-deficient rats than with the corresponding enzymes from control animals (Table 3).

Quantitative differences in the activity ratio, inorganic pyrophosphatase:alkaline phosphatase were also found in the serum and tibia of Mg-deficient and control rats.

Table 4. *Values for activity ratio, inorganic pyrophosphatase* (EC 3.6.1.1):*alkaline phosphatase* (EC 3.1.3.1) *in tissues from magnesium-deficient, control and meal-fed rats in the presence of exogenous Mg* (20 *mg/l*)†

(Mean values with their standard errors for twelve determinations for serum, and for six determinations for other tissues)

Group‡ ...	Meal-fed		Control		Mg-deficient	
	Mean	SE	Mean	SE	Mean	SE
Serum	0·486	0·028	0·479	0·025	0·565	0·027*
Liver	0·150	0·028	0·201	0·032	0·130	0·024
Kidney	0·025	0·001	0·028	0·005	0·025	0·006
Spleen	0·017	0·008	0·033	0·010	0·038	0·005
Small intestine	0·355	0·050	0·328	0·068	0·250	0·046
Tibia	0·056	0·004*	0·074	0·006	0·049	0·007*

Value significantly different from that for control group: $*P < 0·05$.

† For details of assay procedures, see p. 489.

‡ Meal-fed and control groups received the Mg-adequate diet, and Mg-deficient group received the Mg-deficient diet; for details of diets and feeding regimens, see p. 488.

The ratio was higher in the serum but lower in the tibia of Mg-deficient rats than control animals (Table 4).

No significant differences in the weights of individual organs were found between Mg-deficient and control rats, except for the spleen, which was appreciably heavier in Mg-deficient rats (4·46 ± 0·15 g/kg body-weight) than in control animals (3·25 ± 0·12 g/kg body-weight) ($P < 0·01$).

DISCUSSION

The results of this study indicate that the development of a meal-eating pattern of feeding alters the alkaline phosphatase activity in serum and liver. Administration of food to fasting rats has been reported to increase the activity of the enzyme in serum and small intestine during the postabsorptive stage (Heaton & Loveless, 1973), but as the meal-fed and control rats in the present study were fasted for 18 h before death and the changes in enzyme activity were of opposite direction in serum and liver, the effect of meal-eating must be distinct from the short-term response to the ingestion of food. When studying the effect of dietary composition on the enzyme it is therefore important to control the feeding pattern as well as the total amount of food consumed, and reliable conclusions about the direct effects of Mg deficiency can only be obtained by comparing the groups of Mg-deficient and control rats which had access to their respective diets with the same normal frequency throughout the day.

Studies with purified enzymes from human liver and small intestine (Eaton & Moss, 1967), calf intestine (Fernley & Walker, 1967), rat kidney (Melani & Farnararo, 1969) and chick cartilage (Majeska & Wuthier, 1975) have indicated that alkaline phosphatase and inorganic pyrophosphatase activities are often properties of the same protein molecule and the findings of the present study are generally consistent with this. Thus Mg deficiency reduced the activities of both enzymes in serum and tibia and increased them in spleen (Tables 1 and 2), and the effect of exogenous Mg was

qualitatively similar with extracts from all organs except the kidney, where stimulated alkaline phosphatase activity but inhibited inorganic pyrophosphatase activity (Table 3). However, the value for the activity ratio, inorganic pyrophosphatase: alkaline phosphatase varied considerably in different organs from the same animal (Table 4) and if both activities are properties of the same protein molecule, this can only be explained by the existence of different molecular species of the protein. The value for the activity ratio in small intestine was closest to that found in serum, which is consistent with the view that much of the serum alkaline phosphatase is of intestinal origin in the rat (Saini & Posen, 1969), but the fact that the value for this ratio in serum was higher than that in any organ studied suggests that serum contained a component from a tissue not included in the present study, or that selective release of enzymes occurs from tissues of origin into the blood. Intestinal alkaline phosphatase appears to be heterogeneous in both the rat and mouse (Saini & Done, 1972; Nayudu & Hercus, 1974) and the latter suggestion is therefore possible.

The reduced alkaline phosphatase and inorganic pyrophosphatase activities found in serum and tibia during Mg deficiency may have been due either to a reduced efficiency of operation by the enzyme or to a reduction in the amount of enzyme present. As it was not possible to determine the amount of enzyme-protein directly, this problem was studied by estimating the activities with endogenous Mg alone and in the presence of a physiological level of exogenous ionic Mg (Heaton, 1973) to provide uniform conditions for activation of the enzyme. The similarity of the values for the ratio, enzyme activity in serum from Mg-deficient: activity in serum from control rats in the presence and in the absence of exogenous Mg, respectively 0·58 and 0·57 for alkaline phosphatase, 0·68 and 0·65 for inorganic pyrophosphatase (from Tables 1 and 2), implies that the reduced activity in Mg deficiency was due predominantly to a decrease in the amount of enzyme present. Similar conclusions apply to the tibia, where the corresponding values were 0·67 and 0·60 for alkaline phosphatase, and 0·46 and 0·53 for inorganic pyrophosphatase in the presence and in the absence of exogenous Mg respectively.

The differences in the magnitude of the stimulation produced by exogenous Mg with the inorganic pyrophosphatase activity in serum and the alkaline phosphatase activity from the tibia of Mg-deficient and control rats (Table 3), together with the differences in the values for the activity ratio found in the same tissues (Table 4), indicate that the effects of Mg deficiency cannot be explained by a reduction in the amount of a single enzyme. If isoenzymes occur they could, however, be explained by Mg deficiency producing different effects on individual molecular species. The alkaline phosphatase activity in both human and rat serum can be separated into three or four bands by electrophoresis (Newton, 1967; Bussel, Vogel & Levy, 1974) and there is evidence, mentioned above, that intestinal alkaline phosphatase is heterogeneous.

The stimulation of alkaline phosphatase activity in extracts of spleen by exogenous Mg appears paradoxical in view of the increase in both alkaline phosphatase and inorganic pyrophosphatase activity produced by Mg deficiency in the organ. This effect of the deficiency is distinct from that found in any other organ, and when considered

with the accompanying increase in weight of the spleen and the histochemical evidence for an increase in leucocyte alkaline phosphatase activity, it suggests that Mg deficiency has an action on the reticulo-endothelial system that is quite different from its effect on the remainder of the body.

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REFERENCES

Bussell, N. E., Vogel, J. J. & Levy, B. M. (1974). *Proc. Soc. exp. Biol. Med.* **145**, 574.
Conyers, R. A. J., Birkett, D. J., Neale, F. C., Posen, S. & Brundenell-Woods, J. (1967). *Biochim. biophys. Acta* **139**, 363.
Cox, R. P., Gilbert, P. & Griffin, M. J. (1967). *Biochem. J.* **105**, 155.
Dacie, J. V. & Lewis, S. M. (1970). *Practical Haematology*, 4th ed., p. 91. London: J. & A. Churchill.
Eaton, R. H. & Moss, D. W. (1967). *Biochem. J.* **105**, 1307.
Fábry, P. (1967). In *Handbook of Physiology*. Section 6: *Alimentary Canal*, vol. 1, p. 31 [C. F. Code, editor]. Washington, DC: American Physiological Society.
Fernley, H. N. & Walker, P. G. (1967). *Biochem. J.* **104**, 1011.
Hamuro, Y. (1971). *J. Nutr.* **101**, 635.
Heaton, F. W. (1965). *Nature, Lond.* **207**, 1292.
Heaton, F. W. (1973). *Biochem. Soc. Trans.* **1**, 67.
Heaton, F. W. & Anderson, C. K. (1965). *Clin. Sci.* **28**, 99.
Heaton, F. W. & Loveless, B. W. (1973). *Proc. Nutr. Soc.* **32**, 18A.
Kaplow, L. S. (1955). *Blood* **14**, 975.
Larvor, P., Girard, A., Brochart, M., Parodi, A. & Sevestre, J. (1964). *Annls Biol. anim. Biochim. Biophys.* **4**, 345.
Leveille, G. A. (1966). *J. Nutr.* **90**, 449.
Leveille, G. A. (1972). *J. Nutr.* **102**, 549.
Loveless, B. W., Williams, P. & Heaton, F. W. (1972). *Br. J. Nutr.* **28**, 261.
Mahler, H. R. (1961). In *Mineral Metabolism*, vol. 1, part B, p. 743 [C. L. Comar & F. Bronner, editors]. New York and London: Academic Press.
Majeska, R. & Wuthier, R. E. (1975). *Biochim. biophys. Acta* **392**, 51.
Melani, F. & Farnararo, M. (1969). *Biochim. biophys. Acta* **178**, 93.
Morton, R. K. (1953). *Biochem. J.* **55**, 786.
Moss, D. W., Eaton, R. H., Smith, J. K. & Whitby, L. G. (1967). *Biochem. J.* **102**, 53.
Nayudu, P. R. V. & Hercus, F. B. (1974). *Biochem. J.* **141**, 93.
Newton, M. A. (1967). *Q. Jl Med.* **36**, 17.
Panusz, H. T., Graczyk, G., Wilmanska, D. & Skarzynski, J. (1970). *Analyt. Biochem.* **35**, 494.
Pimstone, B., Eisenberg, E. & Stallone, W. (1966). *Proc. Soc. exp. Biol. Med.* **123**, 201.
Saini, P. K. & Done, J. (1972). *Biochim. biophys. Acta* **258**, 147.
Saini, P. K. & Posen, S. (1969). *Biochim. biophys. Acta* **177**, 42.
Wacker, W. E. C. (1969). *Ann. N.Y. Acad. Sci.* **167**, 717.
Wootton, I. D. P. (1964). *Microanalysis in Medical Biochemistry*, 4th ed., p. 101. London: J. & A. Churchill.

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