

## Association of Inorganic Pyrophosphatase Activity with Normal Calcification of Rat Costal Cartilage *in vivo*

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1. Dialysed extracts of rat costal cartilage were shown to possess an enzyme that hydrolyses inorganic pyrophosphate. 2. Inorganic pyrophosphatase activity assayed in the presence of 2mM substrate was maximal at pH 6.8. 3.  $Mg^{2+}$  was essential for activity, which was greatest with 10mM or higher concentrations of  $Mg^{2+}$ . 4. Extracts prepared from cartilage taken from suckling rats (<20g.) showed little or no hydrolytic activity, but as rat weight increased inorganic pyrophosphatase activity was detected, increased to a maximum in tissue from animals weighing about 40g., and then rapidly declined. 5. The increase in inorganic pyrophosphatase activity was associated with an increase in the uptake of  $^{45}Ca$  by the cartilage *in vivo*. 6. Accumulation of calcium, inorganic phosphate and magnesium occurred when inorganic pyrophosphatase activity was at its maximum. 7. Alkaline phosphatase activity, measured in the same extracts used to determine pyrophosphatase activity, was highest in the tissues of the animals weighing <20g., and decreased as inorganic pyrophosphatase activity increased to its maximum. 8. There was no direct relationship between alkaline phosphatase activity and the onset of calcification.

Inorganic pyrophosphate, a by-product of many biosynthetic reactions, may be hydrolysed enzymically by pyrophosphatases. Inorganic pyrophosphatases (pyrophosphate phosphohydrolase, EC 3.6.1.1) are widely distributed in mammalian tissues and are usually associated with alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) (Kay, 1928). Recent studies (Cox & Griffin, 1965; Moss, Eaton, Smith & Whitby, 1967; Cox, Gilbert & Griffin, 1967) on purified extracts suggested that the alkaline phosphatase from some mammalian tissues is an inorganic pyrophosphatase. In addition, enzymic hydrolysis of pyrophosphate has been suggested as a prerequisite for the initiation of calcification in bone and other tissues (Fleisch & Neuman, 1961; Fleisch & Bisaz, 1962*a,b*; Fleisch, Russell & Straumann, 1966). Direct evidence for the association of inorganic pyrophosphatase activity with calcification *in vivo* has not previously been reported. The present investigation was designed to study alkaline phosphatase activity and inorganic pyrophosphatase activity before, during and after the onset of normal calcification *in vivo*, in order to assess the relationship between the two enzyme activities and mineralization.

### MATERIALS AND METHODS

*Animals.* Litters of female rats (Charles River Laboratories, North Wilmington, Mass., U.S.A.) were used in all experiments. The animals were weaned when they weighed 45g. and were then given a diet of Purina laboratory chow. The weight ranges of the animals used in various experiments are specified below.

*Preparation of costal cartilage for enzyme studies.* Rats were killed by exsanguination and costal cartilage was freed of muscle. A portion of the cartilage (about 1mm. long) adjacent to the chondrocostal junction was discarded to avoid contamination from the adjoining rib bone. The cartilage was minced into small pieces with scissors and stored in a sealed container at  $-20^{\circ}$  until required for extraction and assay as follows. The minced cartilage was homogenized in ice-cold water (10mg. of tissue/1.0ml. of water) with a pre-cooled mortar and pestle. The homogenate remained at  $4^{\circ}$  overnight, and was then centrifuged at 10000g for 20min. at  $4^{\circ}$ . The supernatant was used for pyrophosphatase and alkaline phosphatase activities in initial studies. Thereafter the supernatant was dialysed against 0.2mM-EDTA, pH 6.5, for 16hr. at  $4^{\circ}$ . The non-diffusible residue was used for enzyme assay.

*Enzyme assays.* Extracts from pooled cartilages from a number of rats (40–50g.) were used to determine inorganic pyrophosphatase activity at various pH values and  $Mg^{2+}$  concentrations. In the initial studies the assay

medium contained 2 mM- $\text{Na}_4\text{P}_2\text{O}_7$ , 2 mM-added  $\text{MgCl}_2$ , and 0.1 ml. of cartilage extract in a total volume of 1.0 ml. The medium used in later studies contained 10 mM- $\text{MgCl}_2$ . The buffers used (50 mM) were: sodium acetate, pH 3.7–5.6, tris-maleate, pH 5.2–8.0, and tris-HCl, pH 7.6–9.0. Incubation was for 30 min. at 37°. The reaction was stopped by the addition of 1.5 ml. of 2.3 M-sodium acetate buffer, pH 4.0, and the liberated inorganic phosphate was determined colorimetrically by the method of Sumner (1944). In each assay a tube was included to which tissue extract was not added until after the addition of 2.3 M-sodium acetate buffer. Negligible non-enzymic hydrolysis of pyrophosphate occurred under these conditions provided that the free inorganic phosphate was determined without delay.

A modification of the method of Kind & King (1954) was used to determine the alkaline phosphatase activity of extracts. The assay medium contained 0.05 M-sodium carbonate-sodium hydrogen carbonate buffer, pH 10.0, 5 mM-disodium phenyl phosphate as substrate, and 10 mM- $\text{MgCl}_2$ . Phenol liberated during the incubation of 0.1 ml. of tissue extract in a total volume of 2.15 ml. for 30 min. at 37° was determined colorimetrically.

**Determination of calcium, magnesium and phosphate.** A sample of minced cartilage was dried and weighed, extracted overnight in 5% (w/v) trichloroacetic acid containing 10% (w/v) acetic acid (5 mg. dry wt. of cartilage/ml. of acid), and then shaken in a Vortex mixer for 3 min. Less than 1% of the total calcium and magnesium and less than 0.5% of the inorganic phosphate remained in the washed sediment after the acid extraction. Calcium and magnesium were determined by atomic-absorption spectrophotometry by methods based on those of Willis (1960, 1961). Inorganic phosphate was measured on a sample of the acid extract by the method of Sumner (1944).

**Measurement of the uptake of  $^{45}\text{Ca}$  by cartilage.** An injection (5  $\mu\text{C}$ /100 g. body weight) of  $^{45}\text{Ca}$  ( $^{45}\text{CaCl}_2$ , specific radioactivity 17.4 mc/g. of calcium) was made subcutaneously into rats 4 hr. before they were killed. Costal cartilage was dried, weighed and extracted with trichloroacetic acid-acetic acid mixture as described above for the determination of calcium. A sample (0.5 ml.) of the acid extract was used for the counting of  $^{45}\text{Ca}$  by liquid scintillation (Alcock, 1960) in the solution described by Herbert (1960). An internal standard was added to correct for quenching. Counting efficiency in the samples was 65%.

## RESULTS

**Optimum pH for pyrophosphatase activity.** The highest rate of hydrolysis of inorganic pyrophosphate by the supernatant and also by the dialysed supernatant was observed at pH 6.8. No other peak of activity occurred in the range pH 3.7–9.0. The activity at pH 3.7 was zero and that at pH 9.0 was 17% of the activity at pH 6.8. Subsequent determinations of pyrophosphatase activity were carried out at pH 6.8.

**Effects of divalent cations on inorganic pyrophosphatase activity.** No activity was detected in dialysed extracts in the absence of added  $\text{Mg}^{2+}$ . The dialysis residue contained <0.07 mM-mag-

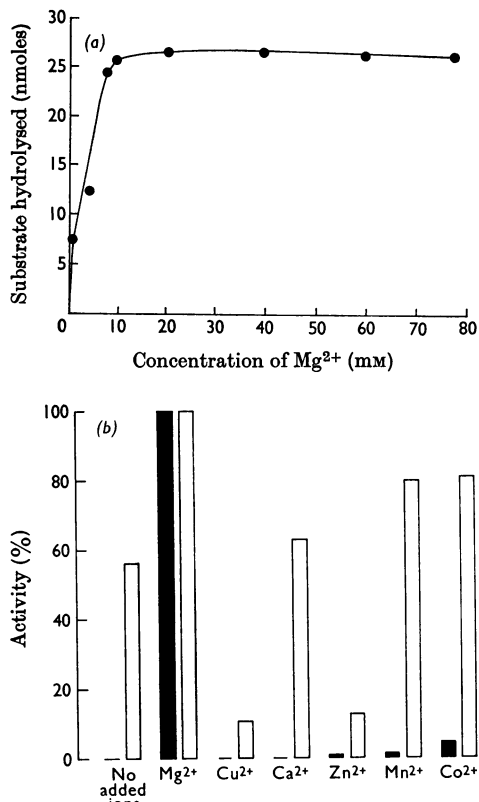


Fig. 1. (a) Effect of  $\text{Mg}^{2+}$  concentration on inorganic pyrophosphatase activity at pH 6.8 of dialysed rat costal cartilage extracts. (b) Effects of divalent metal ions at 1.0 mM on inorganic pyrophosphatase activity at pH 6.8 (■) and alkaline phosphatase activity at pH 10.0 (□) of dialysed rat costal cartilage extract. The activity with each ion is expressed as a percentage of that found in the presence of 1.0 mM- $\text{Mg}^{2+}$ . For experimental details, see the text.

nesium and <0.015 mM-calcium. Activity increased rapidly with increasing  $\text{Mg}^{2+}$  concentration from 0 to 10 mM- $\text{Mg}^{2+}$ , with no significant change from 10 mM- to 80 mM- $\text{Mg}^{2+}$  (Fig. 1a). The assay medium was therefore supplemented with 10 mM- $\text{Mg}^{2+}$  for all assays carried out on dialysed extracts. The effect of replacement of  $\text{Mg}^{2+}$  by other divalent cations is shown in Fig. 1(b).  $\text{Cu}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$  ions (0–1.0 mM) failed to activate inorganic pyrophosphatase; slight activity occurred with  $\text{Co}^{2+}$  ion (0.2–1.0 mM).

**Alkaline phosphatase activity.** Alkaline phosphatase reached maximum activity when the concentration of  $\text{Mg}^{2+}$  was 1 mM and remained constant between 1 mM- and 10 mM- $\text{Mg}^{2+}$ . In the absence of added  $\text{Mg}^{2+}$  the extract showed considerable activity. The other ions tested had various effects

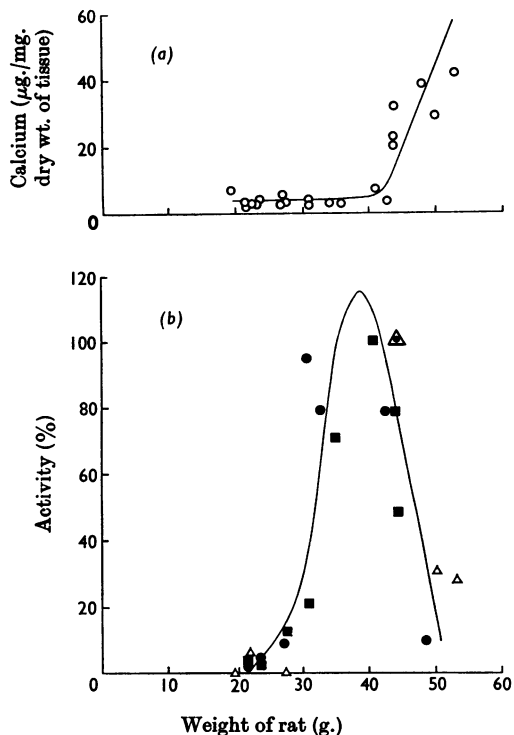


Fig. 2. Changes in rat costal cartilage calcium concentration and inorganic pyrophosphatase activity with increasing rat weight. (a) Relation between calcium concentration in dry cartilage and rat weight. Calcium was determined in samples of the same cartilage used for enzyme assay. (b) Activity of inorganic pyrophosphatase in dialysed extracts of rat costal cartilage with increasing rat weight.  $\Delta$ , Expt. 1;  $\bullet$ , Expt. 2;  $\blacksquare$ , Expt. 3. In each experiment the results are expressed as a percentage of the activity of the extract prepared from the rats weighing 41–44g. For experimental details, see the text.

on alkaline phosphatase activity;  $Zn^{2+}$  and  $Cu^{2+}$  ions actually inhibited activity in the absence of added  $Mg^{2+}$  (Fig. 1b). Of the ions tested  $Mg^{2+}$  was the most effective as an activator, but  $Mn^{2+}$  and  $Co^{2+}$  ions were 80% as effective as  $Mg^{2+}$  at the same concentration.

*Inorganic pyrophosphatase activity, rat weight and calcification of cartilage.* Inorganic pyrophosphatase activity was hardly detectable in dialysed extracts of costal cartilage from very young rats (< 20g.), rose rapidly to a maximum in extracts from rats weighing 35–40g., and then declined to a very low value in extracts from rats weighing 50g. (Fig. 2b). The concentration of calcium in samples of individual cartilages taken from the same animals was constant in animals weighing < 40g. When the rat weight was about 40g., which was the weight at

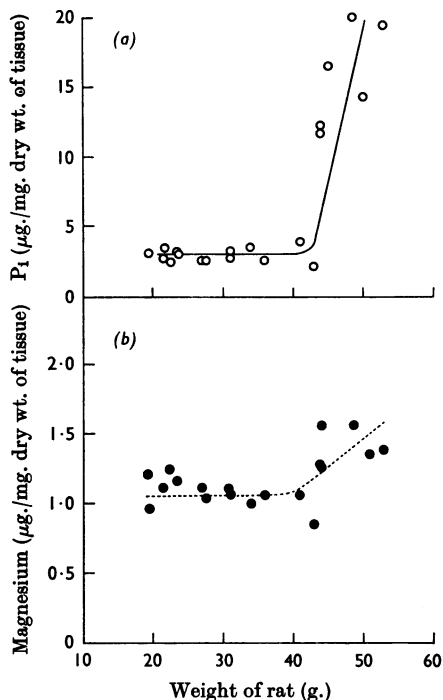


Fig. 3. Changes with increasing rat weight, in (a) inorganic phosphate concentration and (b) magnesium concentration in rat costal cartilage. For experimental details, see the text.

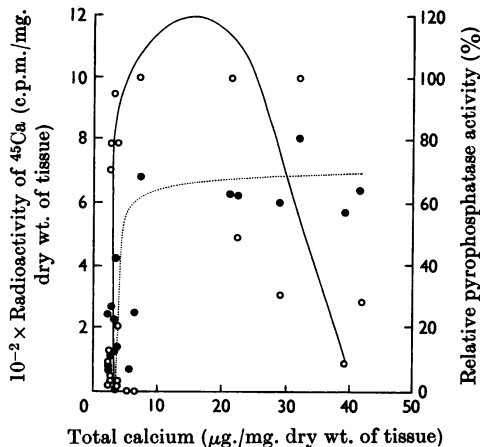


Fig. 4. Relation between inorganic pyrophosphatase activity of dialysed extracts of rat costal cartilage ( $\circ$ — $\circ$ ) and uptake of  $^{45}Ca$  *in vivo* ( $\bullet$ ···· $\bullet$ ), and total calcium concentration of the same tissues. For experimental details, see the text.

which inorganic pyrophosphatase activity was at its maximum, the calcium concentration in the cartilage suddenly increased (Fig. 2a) and continued

to do so as inorganic pyrophosphatase activity rapidly declined. The increase in inorganic phosphate and magnesium concentrations that occurred simultaneously with the increase in calcium concentration showed that mineralization of the tissue was occurring (Fig. 3).

There was a close correlation between the pyrophosphatase activity of the cartilage extracts and the uptake of  $^{45}\text{Ca}$  by costal cartilage *in vivo* during the period when pyrophosphatase activity was increasing. The rapid rise in these two parameters preceded the accumulation of calcium in the cartilage (Fig. 4).

*Relation between inorganic pyrophosphatase and alkaline phosphatase activities.* Alkaline phosphatase activity was highest in dialysed cartilage extracts of rats weighing <20g.; inorganic pyrophosphatase activity of the same extracts was negligible. As inorganic pyrophosphatase activity increased with rat weight, alkaline phosphatase activity showed a definite decrease, but the inverse relationship between the two activities measured in all extracts from rats weighing 19–40g. was not statistically significant. Alkaline phosphatase activity of extracts of cartilage from animals weighing >40g. in weight was negligible.

#### DISCUSSION

The possible involvement of a phosphatase in the mechanism of calcification has been considered since the work of Robison (1923) and Robison & Rosenheim (1934) in which alkaline phosphatase was associated with mineralization. However, no convincing explanation of a direct role of this enzyme has been provided. Cartier & Picard (1955*a,b,c,d*) concluded that a 'non-extractable' enzyme that required magnesium for activation

was present in sheep embryonic cartilage slices, and that inactivation of the enzyme inhibited mineralization. These workers found that ATP was the most efficient phosphate donor of a number tested. They postulated that calcium pyrophosphate was formed and that its hydrolysis by inorganic pyrophosphatase permitted mineralization. They were able to detect pyrophosphate in mineral deposits. Perkins & Walker (1958) demonstrated the presence of small amounts of pyrophosphate (1% of the total phosphate) in normal rat and rabbit bone salt. Mineral deposited *in vitro* in rachitic cartilage had a similar pyrophosphate content to the normal salt only if ATP was the source of phosphate. In addition, Perkins & Walker (1958) found twice as much inorganic pyrophosphatase activity in hypertrophic rachitic cartilage as alkaline phosphatase activity, a finding consistent with the activities in the extracts of pre-calcified costal cartilage in the present work.

The presence of pyrophosphate in bone and in mineral deposits *in vitro*, coupled with the observation that pyrophosphates inhibited nucleation of crystal formation with various collagen preparations (Fleisch & Neuman, 1961) provided the basis for the theory that pyrophosphate may inhibit calcification *in vivo*. Further evidence that pyrophosphate inhibits biological calcification stems from the presence in urine (Thomas & Howard, 1959) of an inhibitor to calcification of rachitic cartilage *in vitro*. This inhibitor was later identified by Fleisch & Bisaz (1962*a,b*) as pyrophosphate. This substance and other polyphosphates when administered to rats were shown to be associated with a decrease in induced calcification (Fleisch, Schibler, Maerki & Frossard, 1965) and also to inhibit mineralization of bone in tissue culture (Fleisch *et al.* 1964). Further, the precipitation of calcium phosphate in

Table 1. *Optimum pH values reported for inorganic pyrophosphatases from various sources*

The presence or absence of alkaline phosphatase activity is also shown.

Source of inorganic pyrophosphatase activity	Reported pH optimum	Alkaline phosphatase activity	Reference
Yeast	6.9–7.0	Absent	Bailey & Webb (1944)
Human erythrocytes	7.1	Absent	Bloch-Frankenthal (1954)
Human erythrocytes	7.7	Absent	Pynes & Younathan (1967)
Sheep embryonic cartilage	8.0	Absent	Cartier & Picard (1955 <i>a,b,c,d</i> )
Mammalian bone, kidney, lung and duodenal mucosa	7.2–7.8	Present	Kay (1928)
HeLa cell	8.5	Present	Cox <i>et al.</i> (1967)
Rat bone	8.5	Present	Cox & Griffin (1965)
Calf scapula cartilage	4.4; 9.0	Present	Jibril (1967)
Human liver and intestine	8.0	Present	Moss <i>et al.</i> (1967)
Rat costal cartilage	6.8	Variably present, depending on age of rat	Present work

synthetic systems was shown to be inhibited by the presence of pyrophosphate (Fleisch *et al.* 1966).

The present investigation provides evidence that increased inorganic pyrophosphatase activity is associated in time with an increase in the uptake of  $^{45}\text{Ca}$  by costal cartilage. However, it is possible that the two phenomena are independent of each other. Since  $^{45}\text{Ca}$  uptake increased before the accumulation of calcium in the tissue (Fig. 4) a change in calcium turnover is evident. This finding is consistent with observations by Urist & Adams (1967), who noted that an increase in  $^{45}\text{Ca}$  uptake by transplants of aorta occurred in human beings before calcification. If under these conditions calcium is bound to pyrophosphate the induction of pyrophosphatase activity could lead to hydrolysis of the pyrophosphate. The enzyme associated with calcification in the work described here is easily extracted; complete extraction from minced tissue has been shown to occur in water at  $4^\circ$  in 1 hr. (N. W. Alcock, unpublished work).

The discrepancies in pH optima reported for inorganic pyrophosphatases are summarized in Table 1 and may be due to variations in the assay conditions used by the various authors, or to the fact that entirely different enzymes were studied. It had been suggested by many (Kay, 1928; Cox & Griffin, 1965; Moss *et al.* 1967; Cox *et al.* 1967) that a single enzyme is responsible for both alkaline phosphatase and inorganic pyrophosphatase activities. The variable presence or absence of alkaline phosphatase activity in association with inorganic pyrophosphatase activity in the cartilage extracts in the present work is striking. Extracts from very young rats showed relatively high alkaline phosphatase activity when inorganic pyrophosphatase activity was absent, and inorganic pyrophosphatase activity increased as alkaline phosphatase activity decreased with rat growth, suggesting that either major changes occurred with age at the active sites of a single protein, or alternatively that two entirely distinct enzymes exist.

The apparent specific requirement of inorganic pyrophosphatase for magnesium demonstrated in the present work has been reported by a number of workers (Bailey & Webb, 1944; Bloch-Frankenthal, 1954; Jenner & Kay, 1931; Pynes & Younathan, 1967). The failure to demonstrate inhibition of enzyme activity in the presence of high magnesium concentration suggests that this is a different enzyme from that associated with mammalian alkaline phosphatases described by Eaton & Moss (1967).

Dialysis was considered necessary in the present investigation to attain a constant concentration of magnesium and also to remove calcium and inorganic phosphate, which were present in the tissue in variable concentrations owing to different degrees

of calcification. Dialysis against EDTA has been reported to inhibit alkaline phosphatase activity of some preparations (Conyers, Birkett, Neale & Posen, 1966). Although the alkaline phosphatase activity of the individual tissue extracts was not determined before dialysis in the present work due to lack of sufficient material, no activity could be demonstrated in either dialysed or undialysed extracts obtained from a number of rats weighing 40–50 g. used in other experiments.

A rise in inorganic pyrophosphatase activity was closely associated with an increase *in vivo* in the uptake of  $^{45}\text{Ca}$  by rat costal cartilage. When pyrophosphatase activity reached a maximum, mineralization of the cartilage commenced. The findings are consistent with the theory that hydrolysis of pyrophosphate may be a necessity for the initiation of calcification. There is no evidence that pyrophosphatase from rat costal cartilage is also an alkaline phosphatase.

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## REFERENCES

- Alcock, N. W. (1960). Ph.D. Thesis: University of London.  
 Bailey, K. & Webb, E. C. (1944). *Biochem. J.* **38**, 394.  
 Bloch-Frankenthal, L. (1954). *Biochem. J.* **57**, 87.  
 Cartier, P. & Picard, J. (1955a). *Bull. Soc. Chim. biol., Paris*, **37**, 661.  
 Cartier, P. & Picard, J. (1955b). *Bull. Soc. Chim. biol., Paris*, **37**, 485.  
 Cartier, P. & Picard, J. (1955c). *Bull. Soc. Chim. biol., Paris*, **37**, 1159.  
 Cartier, P. & Picard, J. (1955d). *Bull. Soc. Chim. biol., Paris*, **37**, 1169.  
 Conyers, R. A. J., Birkett, D. J., Neale, F. C. & Posen, S. (1966). *Aust. J. Sci.* **29**, 83.  
 Cox, R. P., Gilbert, P., jun. & Griffin, M. J. (1967). *Biochem. J.* **105**, 155.  
 Cox, R. P. & Griffin, M. J. (1965). *Lancet*, ii, 1018.  
 Eaton, R. H. & Moss, D. W. (1967). *Biochem. J.* **102**, 917.  
 Fleisch, H. & Bisaz, S. (1962a). *Nature, Lond.*, **195**, 911.  
 Fleisch, H. & Bisaz, S. (1962b). *Amer. J. Physiol.* **203**, 671.  
 Fleisch, H. & Neuman, W. F. (1961). *Amer. J. Physiol.* **200**, 1296.  
 Fleisch, H., Russell, R. G. G. & Straumann, F. (1966). *Nature, Lond.*, **212**, 901.  
 Fleisch, H., Schibler, D., Maerki, J. & Frossard, I. (1965). *Nature, Lond.*, **207**, 1300.  
 Fleisch, H., Straumann, F., Schenk, R., Bisaz, S., Frei, E., Maerki, J. & Allgower, M. (1964). *Helv. physiol. Acta*, **22**, c71.  
 Herberg, R. J. (1960). *Analyt. Chem.* **32**, 42.  
 Jenner, H. D. & Kay, H. D. (1931). *J. biol. Chem.* **93**, 733.  
 Jibril, A. O. (1967). *Biochim. biophys. Acta*, **141**, 605.  
 Kay, H. D. (1928). *Biochem. J.* **22**, 1446.

- Kind, P. R. N. & King, E. J. (1954). *J. clin. Path.* **7**, 322.
- Moss, D. W., Eaton, R. H., Smith, J. K. & Whitby, L. G. (1967). *Biochem. J.* **102**, 53.
- Perkins, H. R. & Walker, P. G. (1958). *J. Bone Jt Surg.* **40B**, 333.
- Pynes, G. D. & Younathan, E. S. (1967). *J. biol. Chem.* **242**, 2119.
- Robison, R. (1923). *Biochem. J.* **17**, 286.
- Robison, R. & Rosenheim, A. H. (1934). *Biochem. J.* **28**, 684.
- Sumner, J. B. (1944). *Science*, **100**, 413.
- Thomas, W. C. & Howard, J. E. (1959). *Trans. Ass. Amer. Physons*, **72**, 181.
- Urist, M. R. & Adams, J. M. (1967). *Ann. Surg.* **166**, 1.
- Willis, J. B. (1960). *Spectrochim. Acta*, **16**, 257.
- Willis, J. B. (1961). *Analyt. Chem.* **33**, 556.