- Bathurst, N. O. (1954a). *J. exp. Bot.* **5**, 253.
 Bathurst, N. O. (1954b). *J. exp. Bot.* **5**, 258.
 Bond, G. (1951). *Ann. Bot., N.S.*, **15**, 447.
 Bond, G. (1958). *Proc. 5th Easter Sch. Agric. Sci. Univ. Nottingham*.
 Elliott, W. H. (1953). *J. biol. Chem.* **201**, 661.
 Hirs, C. W. H., Moore, S. & Stein, W. H. (1952). *J. biol. Chem.* **195**, 669.
 Hunt, G. E. (1951). *Amer. J. Bot.* **38**, 452.
 Leaf, G., Gardner, I. C. & Bond, G. (1958). *J. exp. Bot.* **9**, 320.
 Mardashev, S. R. & Lestrovaya, N. N. (1951). *Dok. Akad. Nauk. S.S.S.R.*, **78**, 547.
 Miettinen, J. K. & Virtanen, A. I. (1952). *Physiol. Plant.* **5**, 540.
 Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
 Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 893.
 Oland, K. & Yemm, E. W. (1956). *Nature, Lond.*, **178**, 219.
 Pucher, G. W., Vickery, H. B. & Leavenworth, C. S. (1935). *Industr. Engng Chem. (Anal.)*, **7**, 152.
 Sen, S. P. & Burma, D. P. (1953). *Bot. Gaz.* **115**, 185.
 Steward, F. C. & Pollard, J. K. (1956). In *Inorganic Nitrogen Metabolism*. Ed. by McElroy, W. D. & Glass, B. Baltimore: *The Johns Hopkins Press*.
 Steward, F. C. & Pollard, J. K. (1957). *Annu. Rev. Pl. Physiol.* **8**, 65.
 Steward, F. C. & Pollard, J. K. (1958). *Nature, Lond.*, **182**, 828.
 Steward, F. C., Thompson, J. F. & Pollard, J. K. (1958). *J. exp. Bot.* **9**, 1.
 Virtanen, A. I. & Miettinen, J. K. (1953). *Biochim. biophys. Acta*, **12**, 181.
 Webster, C. G. & Varner, J. E. (1955). *J. biol. Chem.* **215**, 91.
 Yemm, E. W. & Folkes, B. F. (1958). *Annu. Rev. Pl. Physiol.* **9**, 245.

Oxidative Enzyme Systems in Ossifying Cartilage

BY R. G. WHITEHEAD AND S. M. WEIDMANN

Biological Research Unit, School of Dentistry, University of Leeds

(Received 11 December 1958)

Previous studies into the metabolism of cartilage have been directed mainly towards the anaerobic phase of carbohydrate metabolism and little is known about the presence of aerobic enzyme systems in this tissue. Early work indicated that cartilage was almost anaerobic in nature, and Bywaters (1937) and Lutwak-Mann (1940) showed that in articular cartilage oxygen consumption was virtually absent. The latter author was also unable to demonstrate histochemically the presence of the cytochrome system, a finding which was subsequently confirmed by Follis & Berthrong (1948). Cartilage before ossification shows little vascularization and Cartier (1951a) stated that the resultant low oxygen concentration accounted for the relatively high concentration of lactic acid in cartilage. Dixon & Perkins (1952) demonstrated the presence of the condensing enzyme and aconitase in cartilage, but found that isocitric dehydrogenase activity was very low. This, it was claimed, explained the accumulation of citrate in hard tissues.

Hiatt & Shorr (1953) were, however, able to provide histochemical evidence for the existence of dehydrogenase systems in rachitic rat cartilage by using 2:3:5-triphenyltetrazolium chloride. Follis & Melanotte (1956) confirmed this finding, and in addition showed that the reduction of the dye was accelerated by certain intermediates of the Krebs cycle.

Various attempts to demonstrate manometrically

the presence of dehydrogenase systems have been made and Boyd & Neuman (1954) demonstrated the presence of succinic dehydrogenase in embryonic chicken cartilage. Laskin & Engel (1956) confirmed this finding, but their results showed that glucose, pyruvate, citrate, α -oxoglutarate and fumarate did not affect the respiration of young metaphyseal bone slices. These results would indicate the absence of the Krebs cycle as an entity. The presence of succinic dehydrogenase has also been shown by Castellani & Zambotti (1954), who used a colorimetric procedure.

Recent work (Whitehead & Weidmann, 1959) on the effect of parathyroid extract on metabolism of adenosine triphosphate indicated the possible importance of oxidative phosphorylation in cartilage, but owing to the uncertainty in the literature as to the presence of an oxidative metabolism it was difficult to ascertain the significance of this suggestion. To clarify this situation, in the following work oxidative systems in ossifying cartilage were studied. The distribution of dehydrogenase and cytochrome oxidase systems was examined histochemically, and the nature of the various enzymes involved deduced from manometric studies. Oxidative phosphorylation in cartilage was also investigated by examining the effect *in vivo* of the injection of 2:4-dinitrophenol on the incorporation of ^{32}P into adenosine triphosphate.

METHODS

The material used in the present work was taken from the limb cartilages of young kittens aged 2-3 weeks.

Distribution of dehydrogenase activity. Slices about 0.2 mm. thick were cut, free-hand, longitudinally from the cartilage at the distal end of the femur and then placed in small flasks containing 10 ml. of Ringer phosphate (Umbreit, Burris & Stauffer, 1957) and 0.5% 2:3:5-triphenyl-tetrazolium chloride (Hiatt & Shorr, 1953). The vessels were equilibrated for 5 min. with either oxygen or nitrogen, and then incubated at 37° with occasional shaking for about 1 hr. The slices were examined immediately for red formazan deposition in the cells and then photographed. The colour fades quickly on exposure to light.

Distribution of cytochrome oxidase. The method used was the Moog (1943) modification of the Nadi reaction. Hand-cut slices similar to those described above were placed in the Nadi reagent, which was prepared by combining, immediately before use, equal parts of 0.01M-*p*-amino-*NN*-dimethylaniline and 0.01M- α -naphthol, each in 1% NaOH and 0.066M-phosphate buffer (NaH_2PO_4 adjusted to pH 5.8 with NaOH). Control slices were first washed in aq. 0.09% NaCl containing 5 mM-sodium azide, then placed in a modified Nadi reagent also containing 5 mM-sodium azide. The low pH used was to facilitate the entry of azide into the cells. Moog (1943) showed that the uninhibited results were identical with those obtained at pH 7.2. The slices were incubated at 37° for about 30 min., then examined for indophenol-blue deposition in the cells and photographed.

Manometric studies. Longitudinal slices again about 0.2 mm. thick were cut from the limb cartilages and about 150 mg. (wet wt.) was transferred to a manometer flask, containing 2.8 ml. of Ringer phosphate (pH 7.4), and KOH in the centre well. Before equilibration of the flasks at 37° the system was flushed with oxygen. The various substrates used—citrate, α -oxoglutarate, succinate, malate, pyruvate (10^{-3} M) and glucose (5 mM)—were contained in 0.5 ml. of Ringer phosphate (pH 7.4) and added from the side arm after equilibration. The inhibitors, sodium arsenite (5 mM) and sodium malonate (10^{-3} M), were added in 0.1 ml. of Ringer phosphate directly to the main compartment of the flask. Readings were taken every 10 min. and the calculations for oxygen consumption based on the first hour of observation. On completion of the manometric measurements the weight of the cartilage sample was measured after drying to constant weight at 105°. The average amount was 20-30 mg.

Dinitrophenol injection. Litter-mate pairs of kittens were selected and the experimental kittens injected intraperitoneally with 20 mg. of 2:4-dinitrophenol in aq. 0.09% NaCl and the control animals with aq. 0.09% NaCl. Both groups were then returned to their mothers. Certain of the pairs were killed 24 hr. later; the remainder were given two subsequent daily injections of 2:4-dinitrophenol and killed 24 hr. after the final treatment. Two hours before death each animal was injected with 330 μC of carrier-free $\text{H}_3^{32}\text{PO}_4$ /kg. body wt. Blood samples were taken under anaesthetic immediately before death, and the serum was analysed for inorganic phosphate and ^{32}P content as previously described (Rogers, Weidmann & Jones, 1953). The limb cartilages were analysed for adenosine triphosphate and orthophosphate, as recently reported (White-

head & Weidmann, 1957). The units of specific activity are expressed as the ratio:

$$\frac{(\text{Counts/min./mg. of P}) \times 10^9}{\text{Administered counts/min./kg. body wt.}}$$

RESULTS

Distribution of dehydrogenase and cytochrome oxidase activity. In the histochemical studies, the reduction of 2:3:5-triphenyltetrazolium chloride produced deposition of red formazan only in those slices incubated in 100% nitrogen; those in oxygen remained colourless, thus indicating that the ultimate hydrogen acceptor is normally molecular oxygen. The formazan deposition, which indicates dehydrogenase activity (Mattson, Jenson & Dutcher 1947), occurred mainly in the hypertrophic zones of cartilage. In young kittens, aged about 7 days, the only definite red coloration was in the hypertrophic cells adjacent to the metaphysis; in older kittens when ossification centres have developed in the middle of the epiphyseal cartilage the hypertrophic cells in these areas also showed formazan deposition. In the remainder of the cartilage little or no red formazan was visible (Plate 3a).

The distribution of cytochrome oxidase was very similar to that of the dehydrogenases. Formation of granules of indophenol blue resulting from the oxidative coupling of α -naphthol and *p*-amino-*NN*-dimethylaniline, which according to Keilin (1933) indicates cytochrome oxidase activity, was apparent only in the hypertrophying cells (Plate 3b). In other areas no blue deposition could be seen. To confirm that the formation of indophenol blue was due to cytochrome oxidase activity, the effect of sodium azide was tested; this was observed to retard the reaction. From these two histochemical tests it would appear that the hypertrophic cells of cartilage are able to support an oxidative metabolism.

Manometric studies. To obtain more definite information about the oxidative systems in cartilage, the respiration of cartilage slices was investigated. Oxygen consumption, although low compared with that of liver slices from the same animals, was much greater than that reported by earlier workers. Using articular cartilage, Bywaters (1937) found Q_{O_2} values of 0.005 $\mu\text{l.}$ of oxygen consumption/mg. dry wt./hr.; in the present work the corresponding values were about 100 times as much, i.e. between 0.5 and 0.9 for 2-3-week-old kittens. Boyd & Neuman (1954) found similar high values in embryonic chicken cartilage. Sodium cyanide (mM) almost completely inhibited the oxygen consumption of the cartilage slices.

To obtain information on the nature of the dehydrogenase systems and the possible operation of the Krebs cycle in cartilage, the stimulatory effect on respiration of various components of the cycle

(a) Band of hypertrophic cartilage cells, adjacent to the metaphysis, showing intense red-formazan deposition. This is indicative of dehydrogenase activity. (b) Hypertrophic cartilage cells showing indophenol-blue deposition within the cytoplasm. This is indicative of cytochrome oxidase activity.

R. G. WHITEHEAD AND S. M. WEIDMANN—OXIDATIVE ENZYME SYSTEMS IN OSSIFYING CARTILAGE

(Facing p. 668)

Table 1. *Effect of various substrates of the Krebs cycle on the respiration of cartilage slices*

All calculations for consumption are based on the first hour of observation.

Animal (no.)	Substrate	Endogenous respiration (μl. of O ₂ /mg. dry wt./hr.)	Respiration with substrate (μl. of O ₂ /mg. dry wt./hr.)
1	10 ⁻² M-Citrate	0.17	0.64
2		0.19	0.69
3		0.50	0.90
4		0.37	0.92
5		0.62	1.60
6	10 ⁻² M-α-Oxoglutarate	0.62	2.30
7		0.55	1.72
8		0.74	1.50
9	10 ⁻² M-Succinate	0.86	1.51
10		0.71	1.50
11		0.60	1.36
12		0.50	1.10
13	10 ⁻² M-Malate	0.50	0.90
14		0.60	1.71
15		0.60	1.40
16		0.56	2.06
17	10 ⁻² M-Pyruvate	0.70	2.20
18		0.90	1.77
19		0.53	1.21
20		0.55	1.41
21	5 mM-Glucose	0.88	0.87
22		0.52	0.40
23		0.66	0.70
24		0.77	0.96

Fig. 2. Effect of arsenite on the respiration of α-oxoglutarate by slices of epiphyseal cartilage taken from a 10-day-old kitten. Δ, Respiration in the presence of 10⁻²M-α-oxoglutarate; ○, respiration in the presence of 10⁻²M-α-oxoglutarate and 5 mM-arsenite; ●, endogenous respiration.

Fig. 1. Effect of arsenite on the oxidation of citrate by slices of epiphyseal cartilage taken from 9-day-old kittens. Δ, Respiration in the presence of 10⁻²M-citrate; ○, respiration in the presence of 10⁻²M-citrate and 5 mM-arsenite; ●, endogenous respiration.

Fig. 3. Effect of malonate on the respiration of succinate by slices of epiphyseal cartilage taken from 9-day-old kittens. Δ, Respiration in the presence of 10⁻²M-succinate; ○, respiration in the presence of 10⁻²M-succinate and 10⁻²M-malonate; ●, endogenous respiration.

was studied. Citrate, α -oxoglutarate, succinate and malate (all 10^{-2} M) significantly increased the oxygen consumption of the cells two- to three-fold over the first hour of measurement (Table 1). This indicated that isocitric dehydrogenase, α -oxoglutarate oxidative decarboxylase, succinic dehydrogenase and malic dehydrogenase were all present in cartilage. The stimulation of respiration was most prominent over the first $\frac{1}{2}$ hr. After this time the stimulatory effect became less pronounced and ceased after about $1\frac{1}{2}$ hr. (Figs. 1-3). Pyruvate (10^{-2} M) produced a similar stimulation of respiration, but 5 mM-glucose had no significant effect on the oxygen consumption of cartilage.

The actions of the two enzymic inhibitors, sodium malonate (10^{-2} M) and sodium arsenite (5 mM), on the stimulated respiration produced by the various substrates were tested to verify the presence of succinic dehydrogenase and α -oxoglutarate oxidative decarboxylase. Sodium arsenite caused a very marked inhibition of oxygen consumption both with citrate as substrate (Fig. 1) and with α -oxoglutarate (Fig. 2). Sodium malonate produced a partial inhibition with succinate (Fig. 3).

Effect of 2:4-dinitrophenol on the incorporation of 32 P into cartilage orthophosphate and adenosine triphosphate. The injection of 2:4-dinitrophenol did not appreciably alter the growth rate of the animals and they continued to feed quite normally from the mother. Neither single nor multiple injection of 2:4-dinitrophenol had any significant effect on the specific activity of 32 P contained in the serum of experimental animals ($201\,000 \pm 9\,000$) compared with the controls ($201\,000 \pm 9\,000$). The results are expressed as mean values $\pm$ s.e.m. and are calculated for the 9 pairs of animals. The corresponding values for serum inorganic phosphate were similarly unaffected (8.6 ± 0.4 ; 8.8 ± 0.5). Consequently the amount of isotope available at the tissue sites was unchanged by the treatment.

Injection of 2:4-dinitrophenol resulted in a very significant reduction in the incorporation of 32 P into cartilage adenosine triphosphate measured after 24 hr. (Table 2), and this was maintained by multiple injection. On the other hand, the cartilage orthophosphate showed no significant difference in the specific activity of incorporated 32 P between the experimental and control animals after the single injection (Table 3). After repeated injection of the dinitrophenol, however, a significant diminution of isotope incorporation was observed also in the orthophosphate. These results are very similar to those obtained with parathyroid extract (Whitehead & Weidmann, 1959), where again only multiple injections of the hormone affected the orthophosphate specific activity; the adenosine triphosphate showed a significantly reduced turnover after the single injection.

DISCUSSION

Cartilage is poorly vascularized, resulting in a low concentration of oxygen in the cells. It possesses a high rate of anaerobic glycolysis (e.g. Roche & Deltour, 1943; Gutman & Yü, 1949). These facts, together with the importance attached to alkaline phosphatase and the hydrolysis of glycolytic phosphate esters in calcification (Robison, 1923), have resulted in attention being centred almost completely on anaerobic metabolism. Most of the work on aerobic metabolism was done with non-calcifying articular cartilage, and the conclusions reached from this tissue indicated that the aerobic metabolism of cartilage was almost negligible.

The results in the present paper provide definite evidence for the existence of an oxidative metabolism in actively ossifying cartilage. The histochemical distribution of dehydrogenase and cytochrome oxidase activity indicates that this is

Table 2. *Effect of 2:4-dinitrophenol on the specific activity of adenosine triphosphate in cartilage measured 2 hr. after the injection of 32 P*

Litter no.	Specific activity		Effect (%)
	Experimental	Control	
	Multiple injections		
1	18 500	32 600	-43
2	18 500	32 600	-43
3	13 500	24 800	-46
4	16 000	29 000	-45
Average			-44*
	Single injections		
5	10 700	22 600	-53
6	16 700	25 000	-34
7	13 500	28 000	-52
8	19 000	33 000	-42
9	15 000	30 000	-48
Average			-44†

* Significant at $P < 0.01$.
† Significant at $P < 0.001$.

* Significant at $P < 0.01$.

† Significant at $P < 0.001$.

Table 3. *Effect of 2:4-dinitrophenol on the specific activity of orthophosphate in the cartilage epiphyses of kittens measured 2 hr. after the injection of 32 P*

Litter no.	Specific activity		Effect (%)
	Experimental	Control	
	Multiple injections		
1	51 400	73 500	- 31
2	42 000	73 500	- 43
3	27 400	44 400	- 38
4	40 000	65 000	- 40
Average			- 38*
	Single injections		
5	62 200	60 400	+ 3
6	44 000	44 800	- 2
7	68 000	67 500	+ 1
8	72 000	74 000	- 3
9	79 100	78 800	0

* Significant at $P < 0.01$.

particularly true in the hypertrophic cartilage cells. These areas have a rich blood supply and, presumably, the oxygen concentration is relatively high in the cells. That cells away from this area show no dehydrogenase or cytochrome oxidase activity indicates a low concentration of oxygen, which would be expected from the relatively avascular nature of the tissue. It is the hypertrophic region which subsequently undergoes calcification, and these ossifying regions are equipped with an active oxidative-enzyme system. This finding of a preferential distribution of dehydrogenases in the calcifying region differs from the histochemical results of Hiatt & Shorr (1953) and Follis & Melanotte (1956), who found the activity distributed evenly throughout rachitic-rat cartilage. According to the latter authors, as a consequence of their even distribution the oxidative enzymes would appear to have no bearing on the calcification mechanism. This statement requires re-examination in the light of the present results. Castellani & Zambotti (1954) found the concentration of succinic dehydrogenase highest in the layer of hypertrophic cells, and subsequently Castellani & Bianco (1956) showed that its activity was much higher in calcifying metaphyseal cartilage than in costal cartilage. Aerobic metabolism is more favourable than anaerobic metabolism for the synthesis of adenosine triphosphate, and Cartier (1951*b*) has demonstrated the probable importance of this compound in endochondral calcification. Cartier & Picard (1955) have shown, in addition, that the concentration of this compound is particularly high in the mineralizing zone.

The preferential distribution of oxidative enzymes in the narrow hypertrophic region may possibly explain the relatively low respiration of cartilage slices. It is probable that the majority of the respiration measured results from this area and the true oxygen consumption values should be attributed only to the hypertrophic cells. If this were the case then the corrected values would be substantially higher than those reported and possibly comparable with those of the liver.

The manometric investigations indicated the presence in cartilage of isocitric dehydrogenase, α -oxoglutarate oxidative decarboxylase, succinic dehydrogenase and malic dehydrogenase. Previously only succinic dehydrogenase has been demonstrated with certainty, and the results of Laskin & Engel (1956) indicated that it was the only dehydrogenase present. Since citrate was used as the substrate to show isocitric dehydrogenase activity this provides indirect evidence for the presence of aconitase. Pyruvate also stimulated respiration and this demonstrates that the condensing enzyme is possibly present in cartilage. Sodium arsenite inhibited oxygen consumption

both with citrate and α -oxoglutarate as substrate, and since arsenite inhibits the oxidative decarboxylation of α -oxoglutarate to succinate it can be said with certainty that the portion of the Krebs cycle between citrate and succinate is present in entirety in cartilage cells. The effect of malonate also confirms the existence of succinic dehydrogenase, and since malate and pyruvate are oxidized by cartilage the most likely inference is that the whole of the Krebs cycle is operative in hypertrophic cartilage cells.

To couple the Krebs cycle with oxidative phosphorylation involves the cytochromes and cytochrome oxidase, but their presence in cartilage cells has previously been denied (Lutwak-Mann, 1940; Follis & Berthrong, 1948). The present results, however, revise the position. The existence of cytochrome oxidase has been demonstrated by a positive Nadi reaction in the hypertrophic cells. The results with 2:3:5-triphenyltetrazolium chloride are also indicative of the presence of cytochromes since the reduction potential of the dye is in the same range as the cytochromes with which it interacts in the reduction to red formazan. The cyanide sensitivity of the respiration provides confirmatory evidence for the presence of the cytochrome system in cartilage.

It is generally assumed that 2:4-dinitrophenol uncouples oxidative phosphorylation. If this is true, the present studies *in vivo* with dinitrophenol on the uptake of ^{32}P into adenosine triphosphate indicate the possible existence of oxidative phosphorylation in cartilage cells.

Stoner, Threlfall & Green (1952) and Stoner & Threlfall (1958), who studied the effect of inhibitors of oxidative phosphorylation in brain and skeletal muscle metabolism *in vivo*, were unable to show a decreased incorporation of ^{32}P into adenosine triphosphate, even when the dosage was so great that the animal was at the brink of death. These results were explained by suggesting that isotopic incorporation is normally so rapid that a partial inhibition of the process would not be apparent. This is apparently not true of cartilage.

As was observed with a preparation of parathyroid hormone (Whitehead & Weidmann, 1959), dinitrophenol affects orthophosphate specific activity only after a period of two or three daily injections, although the adenosine triphosphate turnover is significantly reduced after only one injection. It has been suggested that adenosine triphosphate is responsible for the initiation of calcification by the formation of a primary seed, which then stimulates the deposition of bone salt independently of cellular metabolism (Cartier & Picard, 1955); the relationship found between the ^{32}P incorporated into cartilage bone salt and adenosine triphosphate is in agreement with this

hypothesis. To explain the present results, it is suggested that there is normally an excess of seed present; consequently at short time-intervals after the injection of 2:4-dinitrophenol no significant reduction in bone-salt deposition is apparent. The reduced seed formation caused by the diminished synthesis of adenosine triphosphate does become manifest eventually and a reduced bone-salt deposition results. This is accompanied by a decrease in the specific activity of the orthophosphate owing to the presence of less of the newly formed bone salt available for ionic exchange.

Although previous studies of the calcification of cartilage were concerned almost exclusively with glycolysis, certain results were difficult to explain on the grounds of complete anaerobiosis. Gutman & Yü (1949) showed that 2:4-dinitrophenol (10^{-4} M) and gramicidin markedly inhibited calcification *in vitro*. Although it was stated that these inhibitors were known to dissociate inorganic phosphate uptake from hydrogen transfer in oxidative phosphorylation, it was difficult to see the importance of this relationship in the calcification of cartilage owing to its anaerobic nature. Several workers have shown that the calcification of cartilage slices *in vitro* is cyanide-sensitive; although this would indicate the importance of the cytochrome system in the calcification of cartilage, Goldenberg & Sobel (1951) state that the absence of this system in cartilage rules out the role of cyanide as an inhibitor of oxidative systems. No alternative suggestion could be made. Although adenosine triphosphate has been shown to be of great importance in calcification *in vitro*, Picard & Cartier (1956) have said that the high glycolytic activity compared with the low respiratory activity means the former system must provide the necessary adenosine triphosphate. The present results have shown that, whereas in the non-calcifying areas of cartilage the metabolism appears to be anaerobic, the region of cartilage which is undergoing rapid calcification is equipped with all the systems required for an active aerobic metabolism. On these grounds the previous studies *in vitro* require reinterpretation.

SUMMARY

1. Calcifying cartilage possesses both dehydrogenase and cytochrome oxidase systems, present predominantly in the hypertrophic cells.

2. Ossifying cartilage shows a much greater respiratory activity than has been reported for non-ossifying articular cartilage. The respiration is cyanide-sensitive.

3. The respiration of cartilage was stimulated by citrate, α -oxoglutarate, succinate, malate and pyruvate. It was inhibited by arsenite in the

presence of citrate or α -oxoglutarate and by malonate in the presence of succinate.

4. The synthesis of adenosine triphosphate *in vivo*, measured by incorporation of ^{32}P , is inhibited by 2:4-dinitrophenol; uptake of isotope into the bone salt was inhibited only after longer treatment with dinitrophenol.

5. The possible significance of oxidative phosphorylation in calcification has been discussed.

Thanks are due to Mr L. Jepson for the photography. This work has been supported by a grant from the Medical Research Council.

REFERENCES

- Boyd, E. S. & Neuman, W. F. (1954). *Arch. Biochem. Biophys.* **51**, 475.
 Bywaters, E. G. L. (1937). *J. Path. Bact.* **44**, 247.
 Cartier, P. (1951a). *C.R. Soc. Biol., Paris*, **145**, 274.
 Cartier, P. (1951b). *Bull. Soc. Chim. biol., Paris*, **33**, 161.
 Cartier, P. & Picard, J. (1955). *Bull. Soc. Chim. biol., Paris*, **37**, 1169.
 Castellani, A. A. & Bianco, L. (1956). *R.C. Ist. lombardo*, **90**, 302.
 Castellani, A. A. & Zambotti, V. (1954). *Boll. Soc. ital. Biol. spar.* **30**, 453.
 Dixon, T. F. & Perkins, H. R. (1952). *Biochem. J.* **52**, 260.
 Follis, R. H. & Berthrong, M. (1948). *Amer. J. Path.* **24**, 685.
 Follis, R. H. & Melanotte, P. H. (1956). *Proc. Soc. exp. Biol., N.Y.*, **93**, 382.
 Goldenberg, H. & Sobel, A. E. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 719.
 Gutman, A. B. & Yü, T. F. (1949). *Trans. 1st Macy Conf. Metabolic Interrelations*, **1**, 11.
 Hiatt, H. H. & Shorr, E. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 543.
 Keilin, D. (1933). *Ergebn. Enzymforsch.* **2**, 239.
 Laskin, D. M. & Engel, M. B. (1956). *Arch. Path.* **62**, 296.
 Lutwak-Mann, C. (1940). *Biochem. J.* **34**, 517.
 Mattson, A. M., Jenson, C. D. & Dutcher, R. A. (1947). *Science*, **106**, 294.
 Moog, F. (1943). *J. cell. comp. Physiol.* **22**, 223.
 Picard, J. & Cartier, P. (1956). *Bull. Soc. Chim. biol., Paris*, **38**, 697.
 Robison, R. (1923). *Biochem. J.* **17**, 286.
 Roche, J. & Deltour, G. H. (1943). *Bull. Soc. Chim. biol., Paris*, **25**, 1261.
 Rogers, H. J., Weidmann, S. M. & Jones, H. G. (1953). *Biochem. J.* **54**, 37.
 Stoner, H. B. & Threlfall, C. J. (1958). *Biochem. J.* **69**, 376.
 Stoner, H. B., Threlfall, C. J. & Green, H. N. (1952). *Brit. J. exp. Path.* **33**, 398.
 Umbreit, W. W., Burris, R. H. & Stauffer, J. H. (1957). *Manometric Techniques*, p. 149. Burgess Publishing Co. Minneapolis.
 Whitehead, R. G. & Weidmann, S. M. (1957). *Nature, Lond.*, **180**, 1196.
 Whitehead, R. G. & Weidmann, S. M. (1959). *Biochem. J.* **71**, 312.