The Uptake and Metabolism of [^{32}P]Pyrophosphate by Mouse Calvaria *in vitro*

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In order to study the uptake and metabolism of PP_i by bone, $^{32}\text{PP}_i$ was added to the medium surrounding explanted mouse calvaria maintained in organ culture. Most of the PP_i was hydrolysed during incubation, but there was a measurable entry of intact PP_i into bone. When $^{32}\text{P}_i$ was added to the medium, synthesis of PP_i and organic phosphates from P_i was observed in bone. There was no detectable passage of PP_i from bone into the medium. These results are discussed in terms of two models of pyrophosphate hydrolysis and exchange. Some quantitative estimates about the fate of PP_i in bone were made.

Bone contains inorganic pyrophosphate (PP_i) (Perkins & Walker, 1958; Cartier, 1959) and it has been proposed that variations in the PP_i content of bone brought about by hydrolysis by pyrophosphatases may influence the rates of mineral deposition and mineral removal in bone (Fleisch & Russell, 1970).

This hypothesis is based on the observation that PP_i inhibits crystal formation and dissolution *in vitro* (Fleisch & Neuman, 1961; Fleisch *et al.*, 1966a,b) and ectopic calcification in animals (Schibler & Fleisch, 1966; Schibler *et al.*, 1968; Gabbiani *et al.*, 1966). In addition, plasma PP_i is increased in hypophosphatasia and osteomalacia, two disorders characterized by a defect in mineralization (Russell *et al.*, 1971).

However, PP_i has no effect on bone mineralization when injected into rats (Irving *et al.*, 1966), or on bone resorption in tissue culture or in intact rats (Russell *et al.*, 1970). In contrast, the structurally closely related but more stable diphosphonates inhibit all these processes (Russell *et al.*, 1970; Schenk *et al.*, 1973). The lack of effect of PP_i may be due to a rapid hydrolysis of PP_i or a failure of PP_i to reach its potential sites of action. Another possibility is that the PP_i in bone is synthesized locally and is not in free exchange with the PP_i in extracellular fluid. In this case the endogenous PP_i in bone could still be important in regulating crystal growth and dissolution, even though exogenous PP_i failed to have any effect on these processes.

In an attempt to determine why PP_i fails to inhibit the resorption of living bone, we have studied the fate of PP_i added to the culture medium surrounding explanted mouse calvaria. We have measured the hydrolysis of $^{32}\text{PP}_i$, the distribution of non-hydrolysed $^{32}\text{PP}_i$ between medium and bone and the movement of PP_i between medium and calvaria.

Materials and Methods

Sources of materials

The reagents for the organ culture medium were obtained from the same sources as described previously (Russell *et al.*, 1970). All chemicals were obtained from the Merck Chemical Co., Darmstadt, Germany, and were of highest available purity.

Radiochemicals. [^{32}P]Pyrophosphate was obtained as the tetrasodium salt (greater than 99% chemical and radiochemical purity) from New England Nuclear, Dreieichenhain, Germany, with specific radioactivity, 2000–5000 mCi/mmol. It was stored frozen in 1% Na_2CO_3 and the purity checked by the 2-methylpropan-1-ol-extraction procedure described below.

[^{32}P]Orthophosphate was obtained as H_3PO_4 , carrier-free, from Institut für Reaktorforschung, Würenlingen, Switzerland.

Tissue-culture technique

The technique was based on that of Reynolds & Dingle (1970). Frontal and parietal bones were dissected out under sterile conditions from the calvaria of 4-day-old mice of the NMRI strain, taking care to keep the periosteum intact. For each experiment the calvaria were taken from littermates. The calvaria were divided into right and left halves and separate pools of right and left halves made.

In each experiment, six half calvaria (three from each pool) were placed on metal grids so that they lay on the surface of 5.5 ml of medium in a glass Petri dish. The medium was a synthetic one but with 5% (v/v) heat-inactivated rabbit serum added, after the original formulation of Biggers *et al.* (1961), modified by Reynolds (1966, 1972). The dishes were

incubated in McIntosh jars at 37°C, gassed with CO₂ + air (5:95).

Methods

Disappearance of ³²PP_i from the medium. Experiments (nos. 1–3, Table 1) were done with three different amounts of PP_i added to 5.5 ml of medium, namely 8, 24 and 89 nmol. The amount of ³²P added as ³²PP_i varied between 3.7 and 19 μCi in 5.5 ml of medium. In the 46 h after the addition of PP_i, 10–12 samples of medium (50 μl) were taken at time-intervals, after gentle mixing of the medium with a magnetic stirrer. Each 50 μl sample of medium was deproteinized at 0°C with 0.90 ml of 10% (w/v) trichloroacetic acid, after addition of 50 μl of non-radioactive 0.03 M-orthophosphate solution. After centrifugation the total radioactivity of the supernatant was measured in 10 ml of 0.5 M-HCl by using Čerenkov radiation in a Packard 3375 scintillation counter. Radioactivity due to ³²P_i was separated from radioactivity due to ³²PP_i and other ³²P-labelled organic compounds by extracting orthophosphate as a phosphomolybdic acid complex into 2-methylpropan-1-ol (Hall, 1963; Bisaz *et al.*, 1968). The 2-methylpropan-1-ol layers (0.5 ml) were counted by Čerenkov radiation after addition to 10 ml of 2-methylpropan-1-ol. Appropriate corrections by using internal standards were made for the different counting efficiencies in HCl and 2-methylpropan-1-ol.

Disappearance of ³²P_i from the medium. Experiments (nos. 4–6, Table 2) with 10 μCi of carrier-free ³²P_i added to the medium were essentially similar to those with ³²PP_i, and the radioactivities of 50 μl samples of medium taken at various intervals were counted as above.

Distribution of ³²P-labelled compounds between calvaria and medium after incubation. After 46 h incubation, i.e. after the last sample was taken, the six half calvaria were rinsed in a cold Tyrode solution and extracted three times in a Potter homogenizer in 1.5 ml of 10% (w/v) trichloroacetic acid, centrifuging between extractions. The medium was deproteinized with a final concentration of 10% (w/v) trichloroacetic acid. These and subsequent steps were all done at 4°C. In both trichloroacetic acid extracts (medium and calvaria) the orthophosphate content was determined by a modification (Bisaz *et al.*, 1968) of the technique of Chen *et al.* (1956). The radioactivity due to ³²P_i, ³²PP_i and organic ³²P was measured by the following techniques:

(i) Total ³²P was measured in 50 μl of the extracts by Čerenkov radiation.

(ii) ³²P_i was extracted into 2-methylpropan-1-ol as a phosphomolybdic acid complex as described above and counted for radioactivity. The ³²P remaining in the water layer represented ³²PP_i plus a fraction of non-extractable ³²P that was assumed to be organic and denoted organic ³²P.

(iii) ³²PP_i was separated by specific precipitation as its Mn²⁺ salt, by a modification of the technique of Heinonen (1970) described below. A known amount (4 μmol) of PP_i was added to 3.5 ml of trichloroacetic acid extract, and the pH adjusted to 4.0 with NaOH, after addition of 2 ml of 1 M-sodium acetate buffer. To each volume of this solution was added 2 vol. of Heinonen's (1970) reagent (20 ml of propan-2-ol; 10 ml of 1 M-sodium acetate buffer at pH 4.0; 2 g of MnCl₂·4H₂O; 10 ml of water). After 30 min the mixture was centrifuged and the supernatant discarded. As the proportion of PP_i to P_i was very low, to obtain a pure precipitate of manganous [³²P]pyrophosphate free of ³²P_i it was necessary to repeat this procedure three to four times, adding 6.5 μmol of unlabelled orthophosphate each time before addition of Heinonen's reagent. To check the removal of ³²P_i during the successive precipitations, the radioactivity of each supernatant was checked until it remained constant at a very low amount. The last precipitate was dissolved in HCl and the radioactivity, which represents the ³²PP_i, measured by using Čerenkov radiation. To verify that only ³²PP_i was present, an extraction with 2-methylpropan-1-ol was made to show that less than 1% of the radioactivity had moved into the 2-methylpropan-1-ol phase. On the same solution, the total PP_i content was measured colorimetrically (Bisaz *et al.*, 1968) to determine the recovery through the various stages of the technique. Between 90 and 97% of the added PP_i (4 μmol) was recovered.

(iv) The organic ³²P was calculated from the difference between the ³²P in the aqueous layer from (ii) above and the ³²PP_i determined in (iii).

P_i and PP_i content of calvaria. The left halves of six calvaria were frozen at -20°C and the right halves were incubated for 46 h in 5.5 ml of medium. In a series of such experiments 8, 24 and 89 nmol of PP_i were added to the medium. After incubation, the six half calvaria were rinsed with a cold Tyrode solution. The incubated and non-incubated calvaria were extracted in trichloroacetic acid as described above and a tracer amount of ³²PP_i was added, so that the PP_i content could be determined by isotope dilution (Russell *et al.*, 1971; Bisaz *et al.*, 1968). The trichloroacetic acid extract was made alkaline until a precipitate of calcium phosphate formed. After being centrifuged the precipitate was dissolved in 0.5 M-HCl and precipitated again, to increase its purity. To remove Ca²⁺, the dissolved precipitate was poured on to a small column in a Pasteur pipette containing 500 mg of a cation-exchange resin (Dowex 50 W, X4, 200–400 mesh, Na⁺ form). The separation of ortho- and pyro-phosphate was achieved by using an anion-exchange resin as described by Bisaz *et al.* (1968) with slight modifications [volume of Dowex 1 (X10), 5 ml; approx. length of column 11 cm; elution of P_i with 50 ml of

HCl (0.045M); elution of PP_i with HCl (0.25M) in 1ml fractions]. P content and radioactivity of the fractions were measured, and use was made of the principle of isotope dilution to calculate the concentration of PP_i in the calvaria. The P_i content was determined on the trichloroacetic acid extract (Bisaz *et al.*, 1968).

Changes in the specific radioactivity of PP_i in the medium after incubation. To see whether unlabelled PP_i left the calvaria during incubation, ³²PP_i was added to the medium and a decrease of the specific radioactivity during incubation looked for. ³²PP_i was added to a 40ml stock of medium to produce ³²P concentrations in the range 0.015–0.18 μCi/5.5ml. A portion (5.5ml) of this medium was placed in four different Petri dishes, in each of which six half calvaria were incubated. In a series of experiments the concentration of PP_i in the medium varied between 13 and 89nmol in 5.5ml of medium. The rest of the medium was frozen before PP_i analysis. To avoid excessively low values of PP_i owing to disappearance and hydrolysis, the time of incubation was decreased from 46h to 12–16h, except in the experiment with the highest PP_i concentration. The specific radioactivity of ³²PP_i in the incubated and non-incubated samples of medium was measured after purification through steps involving ultrafiltration, precipitation and finally column chromatography on an anionic-exchange resin (Russell *et al.*, 1971). It is known that in the separation of PP_i from P_i in a ratio of 1:500 to 1:1000 on a 10ml Dowex 1 (X10) column, minute

amounts of P_i are eluted in the PP_i fractions. This produces an error in the calculated specific radioactivity of the fractions. The error is larger the smaller the amount of PP_i eluted. It is assumed that this P_i represents incomplete separation of P_i from PP_i on the columns. To measure this error, some column separations with 10ml of exchange resin were done with 16 μmol of P_i mixed with increasing amounts of ³²PP_i, and the specific radioactivity of the fractions compared with the theoretical radioactivity of PP_i. The technique of separation was slightly changed in the last experiment (where 89nmol of PP_i was added to 5.5ml of medium); the amount of Dowex used for separation was decreased to 5ml. Under these conditions, the correction for contamination of PP_i by trace amounts of P_i is not necessary, because the specific radioactivity of PP_i in the fractions eluted is the same as the specific radioactivity of the PP_i solution added to the medium.

Results

Fate of ³²PP_i added to the medium

Table 1 shows the amount and distribution at the end of the incubation period in the bone and in the medium of ³²PP_i, ³²P_i and organic ³²P after addition of ³²PP_i to the medium. The total amount of radioactivity taken up by the calvaria expressed as % of counts added depended mainly on the mineral content (measured as P_i) of the calvaria and not on the concentration of PP_i in the medium. For this reason

Table 1. *Distribution between calvaria and medium of ³²P-labelled compounds 46h after addition of ³²PP_i to the medium*
Half-life and rate constants of ³²PP_i disappearance from the medium are given.

Expt.	PP _i added (nmol/ 5.5 ml of medium)	Six half calvaria					Medium (4.9 ml)				Half- life (h)	Rate constant (h ⁻¹)
		P _i (μmol)	Total counts as % of counts added†	Percentage of total counts in calvaria as			Total counts as % of counts added†	Percentage of total counts in medium as				
				³² PP _i	³² P _i	Organic ³² P		³² PP _i	³² P _i	Organic ³² P		
1	8	16.2	24.7	30.8	48.2	21.0	59.4	50.0	44.6	5.4	31	0.022
	8	15.8	21.9	32.5	47.5	20.0	57.0	51.5	41.6	6.9	32	0.022
	24	15.5	23.4	32.8	44.0	23.2	56.2	54.3	37.2	8.5	30.5	0.023
	89*	15.2	19.3	33.2	43.5	23.3	64.2	60.3	28.5	11.2	47	0.015
2	24	23.9	40.2	27.8	61.8	10.4	42.2	12.2	87.0	0.8	11	0.063
	89	23.1	39.2	32.9	61.5	5.6	45.0	10.8	88.5	0.7	11	0.063
3	8	17.7	37.8	50.5	42.3	7.2	52.7	36.4	59.9	3.7	15	0.046
	8*	17.4	35.9	43.0	50.6	6.4	51.2	22.8	74.6	2.6	10.5	0.066
	24	19.2	35.2	49.7	42.0	8.3	51.8	41.2	54.3	4.5	17	0.041
	89	19.2	33.5	47.2	46.5	6.3	55.7	40.9	54.9	4.2	16.5	0.042

* These two assays show the shortest and longest half-lives for the disappearance of ³²PP_i and were used for the calculation in the models.

† The recovery of counts in medium plus calvaria does not add up to 100% because by the end of the incubation a total of 0.6ml of medium had been removed for counting. Therefore only 4.9ml of the initial 5.5ml of medium remained. Since the correction for the samples taken is not straightforward no attempt has been made to correct for it.

the individual values are shown rather than the means. There was a large disappearance of $^{32}\text{PP}_i$ from the medium, part of which appeared in the calvaria. The greatest part of the radioactivity in the system was as $^{32}\text{P}_i$. This indicates that extensive hydrolysis occurs. A considerable fraction of the ^{32}P from the $^{32}\text{PP}_i$ added was ultimately found in the calvaria as organic ^{32}P -labelled compounds, and smaller amounts were present in the medium.

The hydrolysis of $^{32}\text{PP}_i$ depends on the presence of the calvaria, since if medium was incubated without calvaria much less hydrolysis occurred. Thus 26.8, 17.1 and 12.7% of added $^{32}\text{PP}_i$ was recovered as $^{32}\text{P}_i$ after incubation for 46 h in medium to which no calvaria had been added and in which the PP_i added (per 5.5 ml) was 8, 24 and 89 nmol. $^{32}\text{PP}_i$ added to the medium without calvaria disappeared by hydrolysis according to a single exponential function. The rate constant of disappearance varied between 0.003 h^{-1} (for the highest concentration of PP_i , 89 nmol in 5.5 ml of medium) and 0.007 h^{-1} (for the lowest concentration of PP_i , 8 nmol in 5.5 ml of medium). The effect of calvaria on speeding up hydrolysis was not due to release of hydrolytic enzymes into the medium since the rate of hydrolysis in medium preincubated for 5 h with calvaria was the same as in fresh medium.

Fig. 1 shows on a semi-log scale the disappearance from the medium of $^{32}\text{PP}_i$ added as a function of incubation time in three assays of Expt. 1 (Table 1). The concentration of radioactivity denoted ' $^{32}\text{PP}_i$ ' is that remaining after 2-methylpropan-1-ol extraction of the $^{32}\text{P}_i$, and includes organic ^{32}P -labelled compounds as well as $^{32}\text{PP}_i$ (see Table 1). In all cases, the disappearance could be described by a single exponential function.

Table 1 also shows that the half-lives of the $^{32}\text{PP}_i$ varied between 11 and 47 h and the rate constants of disappearance varied between 0.015 and 0.066 h^{-1} .

These variations are partly dependent on the mineral content of the calvaria, so that the larger the calvaria the greater was the disappearance. The rate constants due to the calvaria alone could be obtained by subtracting from these values the rate constants of hydrolysis by the medium incubated without calvaria, which varied between 0.003 and 0.007 h^{-1} as stated above.

Synthesis of $^{32}\text{PP}_i$ from $^{32}\text{P}_i$

To find out whether the $^{32}\text{PP}_i$ found in the calvaria originates from direct entry of the $^{32}\text{PP}_i$ present in the medium, or from synthesis via the $^{32}\text{P}_i$ produced in the medium by hydrolysis, we studied the fate of $^{32}\text{P}_i$ added to the medium.

Table 2 shows the amount and distribution at the end of the incubation period of $^{32}\text{P}_i$, $^{32}\text{PP}_i$ and organic ^{32}P in the bone and in the medium after addition of $^{32}\text{P}_i$ to the medium. About one-third of the radioactivity was found in the calvaria, mainly

Fig. 1. Disappearance of $^{32}\text{PP}_i$ added to the medium as a function of incubation time (Expt. 1)

Initial amount of PP_i in 5.5 ml of medium: ●, 8 nmol; ■, 24 nmol; ○, 89 nmol.

Table 2. Distribution between calvaria and medium of ^{32}P -labelled compounds up to 46 h after addition of a tracer amount of $^{32}\text{P}_i$ to the medium

Expt.	Incubation time (h)	P _i (μmol)	Six half calvaria				Medium (4.9ml)			
			Total counts as % of counts added†	Percentage of total counts in calvaria as			Total counts as % of counts added†	Percentage of total counts in medium as		
				³² P _i	³² PP _i	Organic ³² P		³² P _i	³² PP _i	Organic ³² P
4	32	—	33.0	98.7	0.57	0.73	61.7	—	—	—
5	28	—	39.0	—	0.19	—	52.9	—	—	—
6A*	46	19.8	33.7	96.4	0.35	3.25	57.0	99.7	0.035	0.31
6B	46	19.7	35.5	96.5	0.38	3.10	54.4	99.7	0.036	0.29

* The results of this assay were used for the calculation in Appendix 1.

† The recovery of ^{32}P at the end of the experiment in medium plus calvaria does not add up to 100% for the reasons stated in Table 1.

as ³²P_i. The amount of ³²PP_i synthesized was very small and accounted for about 0.1–0.2% of ³²P_i added, and therefore could not contribute significant radioactivity to the amount of ³²PP_i found in the calvaria after addition of ³²PP_i to the medium. (The precision of the methods used is such that these values of ³²PP_i synthesis should be correct within ±10%.) However, the specific radioactivity of ³²P_i in the medium is very low, so that the synthesis of PP_i from stable P_i (the concentration of which was about 1.5 mM) as measured in these experiments works out to be surprisingly large in relation to the total PP_i content of the calvaria.

A minimum estimate of the amount of PP_i synthesized from P_i in 46 h is 13–30 nmol of PP_i per six half calvaria (see Appendix 1). The amount of organic ³²P-labelled compounds derived from ³²P_i was about 10 times greater.

Fig. 2 shows on a semi-log scale the disappearance from the medium of ³²P_i added as a function of incubation time in both assays of Expt. 6 (Table 2).

Fig. 2. Disappearance of ³²P_i added to the medium as a function of incubation time (Expt. 6) in two experiments, A and B

Values of ³²P_i obtained experimentally in Expt. A (■) and Expt. B (□) and values obtained by subtracting values due to the second exponential term from the experimental values in Expt. A (●) and Expt. B (○) are shown.

As the points were not on a straight line on semi-log paper, conventional methods of curve peeling (Riggs, 1971) were used to determine whether the curve could be described by a multi-exponential function and its individual terms calculated. It appeared that, in all experiments, the points were adequately described by the sum of two exponential functions. This contrasts with the disappearance of ³²PP_i, which seems to be mono-exponential. This means, as was expected, that there is a bidirectional flux of P_i between bone and medium and that in the time-course of the experiment only one compartment in bone is demonstrable.

P_i and PP_i content of calvaria before and after incubation

To evaluate the net gains and losses of PP_i and P_i by the calvaria during incubation, the amounts of P_i and PP_i were measured before and after incubation. Table 3 shows that there was a small gain in PP_i during incubation, which seems most marked for the highest concentration of PP_i in the medium. Although the trends were nearly all in the same direction, the precise extent of this increase in PP_i content is hard to determine, because it is in the same range as the precision of the analysis, i.e. ±5%. The P_i content of the calvaria decreased slightly during incubation, presumably because some bone resorption takes place in these bones under these conditions.

Changes in the specific radioactivity of PP_i in the medium after incubation

A significant decrease in the specific radioactivity of ³²PP_i in the medium during incubation would mean that stable PP_i had left the calvaria and passed into the medium. This possibility was tested by incubating calvaria with ³²PP_i and determining the specific radioactivity in the medium before and after incubation. Table 4 shows these results and also the separations of synthetic mixtures of P_i and PP_i

Table 3. P_i and PP_i content of calvaria before and after incubation

PP _i added (nmol)	Incubation time (h)	P _i in six half calvaria (μmol)			PP _i in six half calvaria (nmol)		
		Before incubation	After incubation	ΔP _i (%)	Before incubation	After incubation	ΔPP _i (%)
0	39	—	—	—	72.7	75.0	+ 3.2
8	46	18.1	18.1	0	52.3	58.1	+11.1
		16.3	15.8	− 3.0	41.9	43.5	+ 3.8
		19.1	17.1	−10.5	60.0	51.6	−14.0
		19.5	19.0	− 2.6	56.6	58.9	+ 4.1
24	46	20.4	19.4	− 4.9	—	63.1	—
		18.3	17.1	− 6.6	53.1	57.4	+ 8.1
		16.5	15.3	− 7.3	43.5	46.0	+ 5.7
		18.8	17.3	− 8.0	56.9	63.5	+11.6
89	46	17.1	16.9	− 1.2	44.8	47.7	+ 6.5

Table 4. *Specific radioactivity of PP_i in medium before and after incubation*

PP _i added to 5.5ml of medium (nmol)	Incubation time (h)	Volume of Dowex 1 used for separation (ml)	Without incubation		With incubation		Sp. radio- activity of incubated samples as % of non- incubated samples
			Sp. radio- activity (c.p.m./nmol)	PP _i eluted (nmol)	Sp. radio- activity (c.p.m./nmol)	PP _i eluted (nmol)	
12.7	12	10	1850	—	1700	8.4	92
26.8	16	10	580	43.5	500	8.2	86
29.4	11	10	950	41.9	820	16.1	86
89	46	5	2180	43.5	2120	4.0	97

Separation on a column (10ml, Dowex 1) of 16 μ mol of P_i with increasing amounts of ³²PP_i

PP _i eluted (nmol)	Percentage of theoretical sp. radioactivity
10.5	85
13.1	86
31.3	99.5
37.1	100

Fig. 3. *Models representing two extreme possibilities of entry of PP_i into bone and hydrolysis of PP_i*

The square represents the total bone, the line represents the surface. See the text for details. Abbreviations: Hydr., hydrolysis; Exch., exchange.

with 10ml of Dowex 1 (X10) as explained in the Materials and Methods section. There was no significant decrease in specific radioactivity during incubation in the experiment with 5ml of Dowex.

The decrease in specific radioactivity in the experiments with 10ml of Dowex is small and can be explained by the contamination of PP_i with trace amounts of P_i (see Table 4). This suggests that there is no detectable movement of stable PP_i from the calvaria to the medium.

Calculation of the fluxes of PP_i between medium and bone

The rate of entry of PP_i into bone and the rate of hydrolysis of PP_i were calculated by using two different models (Fig. 3). In model I, which gives the maximum possible fluxes of PP_i into bone, it is assumed that all the PP_i that disappears from the medium enters the interior of the bone where some of it is hydrolysed. The P_i formed by hydrolysis then mixes with the exchangeable P_i of bone and thence is free to exchange with the P_i in the medium. In model II, which gives the minimum possible flux of PP_i from medium to bone, it is assumed that all hydrolysis of PP_i occurs in the medium, at the surface of the bone. The non-hydrolysed PP_i, which is assumed to enter the bone, is then the difference between the total disappearance of PP_i from the medium and the hydrolysis taking place at the surface of the bone. The ³²P_i formed by hydrolysis in the medium mixes with the P_i of the medium and exchanges with some P_i of the bone.

The principles of calculation for the two models are developed in Appendices 2 and 3. The calculation is based on the use of convolution integrals (Riggs, 1971) by introducing into the appropriate equations values of ³²P in the medium (or ³²P in the medium plus bone) observed in the experiments. This gives rate constants of hydrolysis of PP_i at the surface (model II) or in the interior of the bone (model I),

Table 5. Rate constants (h^{-1}) of disappearance of PP_i from the medium (k_T), of hydrolysis of PP_i (k_h) and of entry of PP_i into bone (k_e) during incubation

These values were calculated on the basis of models I and II for two extreme disappearance curves of $^{32}\text{PP}_i$ ($k_T = 0.012$ and 0.059). The % of initial dose of $^{32}\text{PP}_i$ found in the bone after incubation is given and compared with the theoretical amount calculated according to the two models.

k_T	Observed % of initial $^{32}\text{PP}_i$	Model I		Calculated % of initial $^{32}\text{PP}_i$	Model II		Calculated % of initial $^{32}\text{PP}_i$
		k_h	k_e		k_h	k_e	
0.012 (min.)	7.4	0.011	0.012	34	0.006	0.006	21
0.059 (max.)	15.4	0.010	0.059	67	0.050	0.009	14

and rate constants of entry from medium to bone. This allows calculation, according to the appropriate models, of the theoretical amount of PP_i that should be present in the calvaria at the end of the experiments.

By comparing these values with the actual amounts found (as is shown in Table 5 for two extreme values of PP_i disappearance) it appears that the results favour model II. More of the PP_i is hydrolysed at the surface and some of it is actually entering the bone, where it remains unchanged. However, some experiments show that there is less PP_i actually found after incubation than expected by the model II (Table 5); this means that there is probably some hydrolysis within the bone, in addition to the hydrolysis occurring at the surface.

Discussion

These experiments are a first attempt to define the dynamic relationships between PP_i in bone and its bathing fluids. The results show that PP_i passes from medium into bone but probably not in the reverse direction. Further, synthesis and destruction of PP_i seem to occur within bone itself or on its surface.

The concentration of PP_i in the plasma varies between 1.2 and 5.6 nmol/ml (Russell *et al.*, 1970), and in these assays the concentration of PP_i in the medium was between 1.5 and 16 nmol/ml. In this organ-culture system, however, there is a continual disappearance of PP_i from the medium, in contrast with the steady-state concentrations of PP_i in living organisms (Jung *et al.*, 1970).

When $^{32}\text{PP}_i$ was added to the medium, some entered the bone, but the greater part of it was hydrolysed. The incubation of bone in the medium was not associated with a change of the specific radioactivity of $^{32}\text{PP}_i$ in the medium, which suggests that there was no detectable flow of PP_i from the bone to the medium.

The statements so far about the behaviour of PP_i in bone do not depend on any complex mathematical interpretations. However, estimates of the rates of entry of PP_i into bone and the rate of hydrolysis of PP_i can be made more quantitative if the results are calculated in terms of two different, but simple, models. In model I the hydrolysis of PP_i is assumed to take place within the calvaria, whereas in model II hydrolysis is assumed to take place in the medium at the surface of the bone. Model II offers a somewhat better fit to the data, which indicates that most of the hydrolysis occurs at the surface of the bone. However, there is probably also some hydrolysis of PP_i in the bone itself.

The experiments with [^{32}P]orthophosphate show that the disappearance curve consists of the sum of two exponential functions; this indicates that P_i turnover is best represented by a two-compartment model (one compartment being the medium, the other one the exchangeable P_i of bone), with a bidirectional flux of P_i between bone interior and medium. Further, there is a synthesis of PP_i as well as of organic phosphates from P_i . It is quite possible that the synthesis of PP_i from P_i represents movement of ^{32}P through nucleotide triphosphates followed by liberation of PP_i during biosynthetic pyrophosphorylatic reactions. However, the fact that the specific radioactivity of $^{32}\text{PP}_i$ in bone is much smaller than the specific radioactivity of $^{32}\text{P}_i$ in the medium suggests that only a part of the PP_i pool is metabolically active (Fleisch & Russell, 1970).

On the basis of calculations from the two models, the following semi-quantitative estimates of the fate of PP_i can be made. (1) Of the PP_i present initially in the medium, 7.5–21 % is found in the bone after 46 h. This amounts to 1.6–16 nmol for six half calvaria. More PP_i than this probably enters the bone but is hydrolysed in the interior. (2) During the 46 h incubation, at least 13 nmol of PP_i per six half calvaria is synthesized by the bone from P_i initially present in the medium. (3) Most of the PP_i added is

hydrolysed during incubation, the greater part at the surface of the bone.

The experiments show that bone pyrophosphate is subject to turnover and that its amount could therefore be altered to regulate the speed of bone mineralization and destruction. Such a regulation could be obtained by way of synthesis, hydrolysis or entry into the bone. However, PP_i entering into the bone seems not to reach the regions of bone resorption, since in tissue culture PP_i is unable to inhibit bone resorption (Russell *et al.*, 1970). On the other hand, it seems to reach the sites of mineralization, since PP_i has been shown to inhibit mineralization in femurs of chick embryo grown *in vitro* (Fleisch *et al.*, 1966c). Interestingly, the diphosphonates EHDP (ethane-1-hydroxy-1,1-diphosphonate) and Cl_2MDP (dichloromethylenediphosphonate) compounds closely related to PP_i in structure, but resistant to enzyme destruction, are able to inhibit the resorption processes (Russell *et al.*, 1970; Reynolds, 1972; Schenk *et al.*, 1973). From preliminary experiments, EHDP and Cl_2MDP , studied in our system, were shown to enter the calvaria at a rate of the same order of magnitude as the rate of entry of PP_i . It is possible that their effect on resorption is due to their resistance to enzymic hydrolysis.

The uptake of PP_i by bone is also interesting in relation to the current use of ^{99m}Tc -Sn-pyrophosphate complexes as efficient bone-scanning agents in clinical studies (Bok *et al.*, 1973; Hosain *et al.*, 1973). The finding of an entry of PP_i would explain why ^{99m}Tc -Sn-pyrophosphate is taken up. Whether the binding of Sn and Tc increases the rate of entry is under investigation.

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APPENDIX 1

Estimation of the synthesis of PP_i in the calvaria from the P_i in the medium

The synthesis of PP_i from P_i is probably the end of a chain of reactions (Fleisch & Russell, 1970). For this reason the constant k_s in the following

equation represents only a minimal estimate of the rate constant of synthesis:

$$^{32}PP_i = k_s \int_0^t ^{32}P_i dt$$

where $^{32}PP_i$ is the amount of $^{32}PP_i$ synthesized in

bone at time t and $^{32}\text{P}_i$ is the function of disappearance of P_i from the medium. For the calculation of k_s for Expt. 6, the disappearance curve was determined by peeling (Fig. 2, main paper) (Riggs, 1963):

$$0.0035 \times 0.337 = k_s \int_0^{46} (0.19e^{-0.12t} + 0.81e^{-0.046t}) dt$$

$$k_s = 3.4 \times 10^{-5} \text{ h}^{-1}$$

The calculated k_s for the four experiments 4–6

varied between 3.4×10^{-5} and $7.8 \times 10^{-5} \text{ h}^{-1}$, which represents a minimum synthesis of 0.28 nmol/h.

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APPENDIX 2

Calculation of the rate of hydrolysis of total PP_i in model I

By using model I, the flux $pp(t)$ of $^{32}\text{PP}_i$ from medium to bone is:

$$pp(t) = k_e {}^{32}\text{PP}_i(t) = k_T {}^{32}\text{PP}_i(t)$$

where $^{32}\text{PP}_i$ is the amount of $^{32}\text{PP}_i$ in the medium, k_e is the rate constant of entry and k_T is the rate constant of disappearance. The total amount of $^{32}\text{P}_i$ formed in bone and medium by hydrolysis would be:

$$^{32}\text{P}_i(t) = \int_0^t pp(t-\tau) h(\tau) d\tau$$

where $h(\tau)$ is the equation of disappearance of a pulse (delta function) of $^{32}\text{PP}_i$ added in the bone at time 0. $h(t) = e^{-k_h t}$, because PP_i in the calvaria disappears in this model by hydrolysis only, with a rate constant k_h . We can then write:

$$\frac{{}^{32}\text{P}_i(t)}{({}^{32}\text{PP}_i)_{t=0}} = k_e \int_0^t e^{-k_e(t-\tau)} e^{-k_h \tau} d\tau = k_e \left(\frac{e^{-k_e t} - e^{-k_h t}}{k_h - k_e} \right)$$

For Expt. 1, where $k_e = 0.012$ and the total $^{32}\text{P}_i$ is 28% of initial radioactivity, $k_h = 0.011 \text{ h}^{-1}$.

APPENDIX 3

Calculation of the rate of hydrolysis in the medium of PP_i in model II

The appearance of P_i in the medium would be:

$$^{32}\text{P}_i(t) = k_h \int_0^t {}^{32}\text{PP}_i(t-\tau) g(\tau) d\tau \quad (1)$$

where k_h is the rate constant of hydrolysis, $^{32}\text{PP}_i$ is the function of disappearance of $^{32}\text{PP}_i$ in the medium and $g(t)$ is the equation of disappearance of $^{32}\text{P}_i$ when it is added to the medium. $g(t)$ was calculated from the means of the rate constants derived from the individual equations of the four experiments 4–6 by the method of Skinner *et al.* (1959):

$$g(t) = 0.14e^{-0.256t} + 0.86e^{-0.008t}$$

For Expt. 3, where $k_T = 0.059$:

$$^{32}\text{PP}_i(t) = ({}^{32}\text{PP}_i)_{t=0} e^{-0.059t}$$

These values were put into eqn. (1) and integration gave the following expression:

$$\frac{{}^{32}\text{P}_i(t)}{({}^{32}\text{PP}_i)_{t=0}} = k_h (16.1e^{-0.059t} - 0.71e^{-0.256t} + 16.8e^{-0.008t})$$

This equation was solved for k_h at the 12 different experimental values of t . The mean $\pm$ S.D. for k_h is 0.050 ± 0.003 .

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