THE UPTAKE OF INJECTED RADIOACTIVE PHOSPHORUS IN THE SKELETON OF THE GROWING WHITE RAT*

III. THE METABOLISM OF SKELETAL ^{32}P IN EXPERIMENTS OF LONGER DURATION, PERFORMED WITH RACHITIC AND CONTROL ANIMALS

by

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It is a long known though not clearly understood fact that ^{45}Ca and ^{32}P, once incorporated in the skeleton, seem to stay there, sometimes for weeks, without returning to the circulation to any great extent[1–6]. At the same time the radioactivity in the plasma falls to a fraction of the value present during the first few hours when the skeleton reached the isotope level which persists for so long a time. To explain this, the concept has been brought forward that the isotope, either by diffusion or recrystallization, is incorporated in the inner regions of the bone mineral crystal and is thus more or less protected against losses arising from an exchange with the inactive ions present in the circulation[2,6].

In the course of experiments on the uptake of intravenously injected ^{32}P, reported earlier[7], we saw during the first 24 hours following injection a substantial uptake of ^{32}P in the femur which we partially ascribed to deposition of active bone material from the bloodstream. Combining these with other data obtained in this laboratory we calculated that over a longer period the increase in ^{32}P content of the skeleton due to skeletal accretion must be quite substantial in the young animal. In the meantime one of us (Cl) had found that on the diets used in our experiments, even during a four week period, the ^{32}P content of the tibia of young albino rats stayed almost at the level it had attained during the first 24 hours following the injection of radiophosphate[8]. This led us to the idea that in these experiments a possible loss of ^{32}P due to exchange with the circulating phosphate had been compensated by a gain caused by skeletal accretion and that the observed steady state was really one of dynamic equilibrium. The same could then be said of most of the experiments mentioned in the literature, as practically all of them were done with young, growing animals.

To test this hypothesis we injected rats subcutaneously with ^{32}P (as Na_2HPO_4) and compared the actual increase in ^{32}P content of the tibia during a 14-day period with the increase that could be expected from skeletal accretion alone. To do this one of the hindlegs was removed by surgery three days after the injection and the tibia

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References p. 53.

obtained. 14 days later the animals were sacrificed and the other tibia was taken out. During this experimental period the specific activity of the plasma inorganic phosphate was followed by taking blood samples from the tail every four or five days. In this way all the data were derived from the same group of animals. The difference in phosphate content between the two tibiae combined with the specific activity (activity/mg P) of the plasma inorganic phosphate made it possible to calculate the increase of the ^{32}P content caused by skeletal accretion during the experimental period. This value was then compared with the difference in ^{32}P content actually found. It was assumed that the phosphate ions incorporated during the formation of the bone crystals either were derived directly from the plasma inorganic phosphate or otherwise were in direct rapid equilibrium with this fraction.

EXPERIMENTAL

Methods

The experiments were performed with young albino rats, weighing about 120 gram. After weaning the rats subsisted on a modified Steenbock-Black diet, containing 1.20% of Ca and 0.30% of P. The control animals received a protecting amount of calciferol orally (6 I.U. per day). Rickets was determined by X-ray once a week. At the beginning of the experiment the phosphorus content of one tibia was around 11 mg in the control group and about half of that in the rachitic group. Each animal received a subcutanous injection of 50 μC of radioactive phosphate which contained a negligible amount of carrier phosphate. Three days later one of the hindlegs was amputated just above the knee-joint. The tibia was taken out and used for analysis. The animals seemed to take this operation quite well. Nothing abnormal was observed and the weight increase was normal during the next fourteen days. After these fourteen days the animals were sacrificed. The second tibia was then obtained and analyzed for inorganic P and ^{32}P, which were also determined in the plasma.

During the 14-day experimental period three 0.4 ml blood samples were obtained from the tail of each animal (Fig. 1). In each group these samples were pooled and centrifuged for plasma which was used for P and ^{32}P determinations. We did not succeed in collecting enough blood during the amputation of the hindleg.

"Inorganic" (acid soluble) P and ^{32}P in plasma and tibia were determined in the usual way, as described earlier[7]. The results of the radio activity measurements are given in $^0/_{00}$ of the total dose or as specific activity (s.a.) in $^0/_{00}$ dose/mg P.

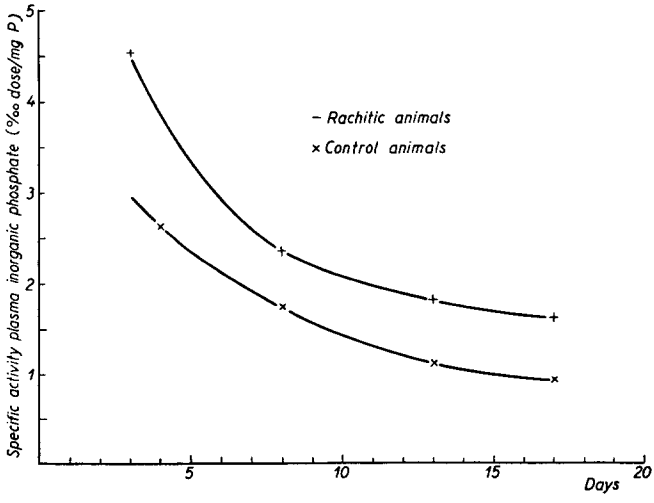

Fig. 1. Specific activity of the plasma inorganic phosphate during the experimental period (3rd until 17th day after the injection of ^{32}P).

References p. 53.

RESULTS

The results of the plasma determinations were calculated as specific activities and are given in Fig. 1. Here the usual decline is seen during the experimental period. The values in the rachitic group were always higher than the corresponding values in the control group.

TABLE I

^{31}P AND ^{32}P CONTENT OF THE TIBIA OF RACHITIC AND CONTROL ANIMALS AT THE BEGINNING AND AT THE END OF THE EXPERIMENTAL PERIOD (VALUES ± STANDARD ERROR)

Days after injection of ^{32}P		*rachitic (8)*	*control (6)*
	^{32}P content in $^0/_{00}$ dose		
3		7.4 ± 0.5	15.6 ± 1.2
17		9.3 ± 0.5	17.3 ± 0.4
	difference	1.9 ± 0.7	1.7 ± 1.3
	Inorg. phosphate in mg		
3		5.5 ± 0.2	11.2 ± 0.8
17		7.0 ± 0.4	16.8 ± 0.8
	difference	1.5 ± 0.4[5]	5.6 ± 1.1
	increase/day	0.107 ± 0.032	0.400 ± 0.078
	Specific activity $^0/_{00}$ dose/mg P		
17	tibia	1.39 ± 0.03[5]	1.04 ± 0.04
17	plasma	1.63 ± 0.12	0.95 ± 0.04

In Table I the results of the P and ^{32}P determinations in the tibia are put together. For comparison the specific activity of the plasma at the end of the experimental period is given. The blood samples collected at that time were analyzed separately and average values and standard errors of the specific activity of the inorganic phosphate fraction calculated, which give an idea of the accuracy of the values obtained in the pooled samples.

TABLE II

^{32}P BALANCE OF TIBIA (VALUES ± STANDARD ERROR)

	Rachitic	*Control*
	$^0/_{00}$ of injected dose	
1. Uptake of ^{32}P at a hypothetical rate of calcification of 1 mg P/day, calculated from graph 1	32.9	22.8
2. Calculated uptake of ^{32}P at rate of calcification actually found (Table I)	3.5 ± 1.3*	9.1 ± 2.3*
3. Actual uptake of ^{32}P	1.9 ± 0.7	1.7 ± 1.3
Difference 2 and 3 = loss by exchange	1.6 ± 1.5	7.4 ± 2.7

* In this calculation the standard error of the values 32.9 and 22.8 (see under 1) was taken at 6%, this being an average of the standard errors of the specific activities of the plasma 17 days after injection. Contrary to the other blood samples these last samples had not been pooled (see text).

References p. 53.

Table II gives a calculation of the increase in ^{32}P content which could be expected from skeletal accretion alone. These values were calculated by evaluating the area under the curves in Fig. 1. Here it was assumed that the skeletal accretion proceeds linearly with time during the experimental period.

DISCUSSION

The data in Table I show that though a substantial accretion of phosphate has occurred in the tibiae of the control animals during the experimental period and a small but definite gain is seen even in the rachitic animals, the ^{32}P content has hardly increased in both cases (there are no reasons to expect a maximum in the ^{32}P content of the tibiae to occur during the experimental period[6,9]). Calculations given in Table II prove that this small increase in ^{32}P content is lower than could be expected from the net amount of phosphate deposited. Especially in the case of the control animals the uptake as calculated from the graph is $7.4^0/_{00}$ dose higher than the value actually found. This means that the tibiae in the latter group, while being constantly on a level of about $16^0/_{00}$ dose (Table I), have lost *ca.* $7.4^0/_{00}$ or nearly 50% of this value during the experimental period. For the rachitic animals this value is much lower.

The most simple manner which offers itself is to ascribe these losses to exchange reactions between the tibia and the plasma phosphate. Some loss might also occur due to the dissolution of some recently deposited bone material still containing a high amount of ^{32}P, but in all probability this effect is comparatively small.

As we stated in an earlier paper[7] a rapid uptake by exchange of the injected ^{32}P in the skeleton occurs during the first 5 to 15 minutes. There we also indicated that uptake or loss by exchange may be seen as governed by the equation

$$\mathrm{d}\,(^{32}P) = k \cdot C_{pl} \cdot S\,(sa_{pl} - sa_{bs})\,\mathrm{d}t$$

where C_{pl} means the concentration of the inorganic phosphate in the plasma, S the surface available for exchange and sa_{pl} and sa_{bs} stand for the specific activities of the inorganic phosphate of the plasma and on the bone crystal surface respectively. During the first minutes after the administration of the radiophosphate sa_{pl}, governed mainly by turnover reactions outside the skeleton, decreases rapidly. Meanwhile sa_{bs} increases steadily. After some time the value of sa_{pl} crosses the average value of sa_{bs} and as sa_{pl} gets smaller than sa_{bs} loss of ^{32}P from the tibia sets in. By that time the decrease of sa_{pl} is much slower and sa_{bs} may be expected to follow sa_{pl} closely. The rate at which the tibia will lose ^{32}P by exchange will depend to a considerable extent on the phosphate content of the plasma (C_{pl}) and its rate of renewel by exogenous phosphate which influences sa_{pl}.

NEUMAN AND MULRYAN[6] have shown that the availability for exchange (*in vitro*) of ^{32}P taken up in *vivo* by the skeleton decreases as more days elapse between the administration of ^{32}P and the sacrifice of the animal. This may be explained by assuming that ^{32}P taken up at the surface of the bone crystals, gradually migrates into the interior until all ^{32}P would be spread out evenly. As a matter of fact this state is never reached, for in the meantime the plasma, with its specific activity steadily decreasing, starts removing ^{32}P from the surface of the crystals. At the beginning of the experiment the ^{32}P is situated on the crystal surface, after some weeks we may expect to find the isotope concentrated more in the centre.

References p. 53.

In the young animal this effect may be increased by the fact that in the meantime material of a much lower specific activity is deposited, thus isolating to a certain extent the ^{32}P already incorporated. For these reasons we may expect the loss by exchange to get less and less in the growing animal. In our experiments (as in many other) this decrease is counterbalanced, however, by a steady decrease of the specific activity of the plasma phosphate which enters the femur by active calcification.

The data in Table II show that, though in both rachitic and control animals only a slight increase in ^{32}P content during the experimental period was found, the difference from the values calculated from the graph (loss by exchange) is much smaller for the rachitic group than it is for the control group (*ca.* 20% and *ca.* 50% of the average level). This might be ascribed to the lower inorganic phosphate level (C_{pl}) of the rachitic plasma (around 30 microgram/ml against 60 microgram/ml in the control group) which will give rise to a lower exchange rate between inactive inorganic plasma phosphate and the radioactive phosphate situated at the surface of the bone crystals.

Comparing our data with the work of NEUMAN AND MALRYAN[6] we see that, instead of the small increase in ^{32}P content seen in our experiments, a loss of about 25% of the incorporated tracer occurs during a comparable experimental period. The diets used by us, however, are essentially rachitogenic with an abnormal Ca/P ratio (4:1) and result in an inadequate supply of phosphorus for the rachitic animals, modified to a certain extend for the controls. But in both cases the phosphorus supply is so low that urinary excretion is negligible. The diet used by NEUMAN AND MULRYAN has an adequate supply of phosphate. Thus the sa_{pl} will decrease faster due to a greater dilution with exogenous phosphate. As a result the uptake of ^{32}P by skeletal accretion will be lower and the loss by exchange higher.

A similar effect is seen in the work of COPP *et al.*[5] with ^{45}Ca. On the diets used in these experiments, no skeletal growth was possible during the experimental period. There were even signs of decalcification. This resulted in a very rapid loss of ^{45}Ca initially taken up by exchange. In their controls, fed on a diet with normal calcium and phosphorus content, the ^{45}Ca content of the skeleton reached during the initial exchange period persisted in the course of the following 16 days.

The recorded data show that in both groups a considerable loss of ^{32}P, ascribable to exchange between inactive inorganic plasma phosphate and the radioactive phosphate of the bone, was apparently more than compensated by the incorporation of ^{32}P caused by accretion of bone salt. Here, as in the experiments recorded elsewhere[7], we do not see much difference between the behaviour of the normal and rachitic skeleton as such. An explanation of the available data can be given based on the difference in plasma inorganic phosphate between the rachitic and the control group. This difference then accounts for both the difference in skeletal accretion and the difference in the rate of loss of ^{32}P from the skeleton by exchange with the plasma phosphate. In both groups these two effects seem to compensate each other quite well, so that only a slight change in net ^{32}P content of the tibia is seen during the experimental time.

SUMMARY

Isotopic phosphate was injected subcutaneously into rachitic and control rats. After three days one of the hindlegs of each rat was amputated, the tibia taken out and its P and ^{32}P content determined. During the following 14-day experimental period the specific activity of the plasma

References p. 53.

inorganic phosphate in each group was followed by drawing blood samples from the tail. At the end of this period the rats were sacrificed and the second tibia was obtained.

Comparison of the P and ^{32}P content of the tibiae at the beginning and at the end of the experimental period showed an increase in P content due to skeletal accretion of 5.6 mg in the control and of 1.5 mg in the rachitic group. The increase in ^{32}P content, however, was only small and much less than could be expected on the base of skeletal accretion alone. This led us to the conclusion that in both groups considerable loss of ^{32}P from the skeleton occurred during the experimental period, caused by the exchange between inactive plasma inorganic phosphate and the radiophosphate of the bone. This loss counterbalanced the effect of the skeletal accretion and gave rise to the apparently constant level of ^{32}P in the skeleton during the experimental period.

RÉSUMÉ

Un phosphate marqué est injecté par voie sous-cutanée à des rats rachitiques et à des rats témoins. Après trois jours, on ampute une des pattes postérieures de chaque rat et l'on détermine la teneur en P et en ^{32}P du tibia prélevé. Pendant les 14 jours suivants l'activité spécifique des phosphates minéraux du plasma de chaque groupe est suivie sur du sang prélevé sur la queue. A la fin de cette période, on sacrifie les rats et l'on prélève le second tibia.

La comparaison de la teneur en P et en ^{32}P des tibias au début et à la fin de l'expérience montre une augmentation de la teneur en P due à la croissance du squelette. Cette augmentation est de 5.6 mg pour les témoins et de 1.5 mg pour les rats rachitiques. Mais la teneur en ^{32}P n'augmente que faiblement et beaucoup moins que ne permet de le prévoir la seule croissance du squelette. Les auteurs en concluent que dans les deux groupes les échanges entre les phosphates minéraux inactifs du plasma et les phosphates radioactifs des os provoquent une perte considérable en ^{32}P dans le squelette pendant la durée de l'expérience. La perte équivaut au gain entraîné par la croissance squelettique et la teneur en ^{32}P du squelette reste donc apparemment constante au cours de l'expérience.

ZUSAMMENFASSUNG

Rachitische Ratten und Kontrollratten erhielten subkutane Phosphat-isotopinjektionen. Nach 3 Tagen wurde eines der Hinterbeine jeder Ratte amputiert, das Schienbein herausgenommen und sein P- und ^{32}P-Gehalt bestimmt. Während der folgenden 14 Tage der Versuchsperiode wurde die spezifische Aktivität des anorganischen Plasmaphosphats in jeder Tiergruppe durch den Entzug von Blutproben aus dem Schwanz verfolgt. Am Ende dieser Periode wurden die Ratten getötet und das zweite Schienbein untersucht.

Ein Vergleich des P- und ^{32}P-Gehalts des Schienbeins am Anfang und am Ende der Versuchsperiode zeigte einen Anstieg des P-Gehaltes, der einem Skelettzuwachs von 5.6 mg in den Kontrolltieren und von 1.5 mg in den rachitischen Tieren zu zuschreiben war. Das Ansteigen des ^{32}P-Gehaltes war jedoch nur gering und viel geringer als allein auf Grund des Skelettzuwachses erwartet werden konnte. Das führte uns zu dem Schluss, dass bei beiden Tiergruppen ein beträchtlicher Verlust des Skelettes an ^{32}P während der Versuchsperiode auftrat, der durch den Austausch von inaktivem anorganischen Plasmaphosphat und dem radioaktiven Phosphat der Knochen verursacht wird. Dieser Verlust glich den Skelettzuwachseffekt aus und verursachte den scheinbar konstanten ^{32}P-Stand im Skelett während der Versuchsperiode.

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