BBA 75188

# PHOSPHORUS METABOLISM DURING TRANSPORT OF CALCIUM

E. NEVILLE AND E. S. HOLDSWORTH
*Biochemistry Department, University of Tasmania, Hobart (Tasmania)*
(Received May 24th, 1968)

SUMMARY

1. The role of phospholipid turnover in the stimulation of calcium translocation by vitamin D has been investigated.

2. Pretreatment of rachitic chickens with vitamin D stimulated the incorporation of $^{32}P_i$ from the lumen into phospholipids of the ileum mucosa, provided that the lumen also contained $Ca^{2+}$. When the lumen did not contain added $Ca^{2+}$ pretreatment with vitamin D had no effect, although the rate of incorporation of label was in this case greater than it was when calcium was added. Pretreatment with vitamin D had no effect on incorporation of [$^{14}$C]ethanolamine or [$^{14}$C]serine into phospholipids under the conditions in which it had an effect on $^{32}P$ incorporation.

3. Vitamin D in the presence of calcium stimulated $^{32}P$ incorporation into mucosal acid-soluble phosphate, mucosal ATP, blood and bone, but in the absence of added calcium, it had little or no effect.

4. It is concluded that vitamin D has no effect on phospholipid metabolism, but that in the presence of calcium it increases phosphate translocation and the specific activities of mucosal phosphate, ATP, and of substances derived from them. Omission of calcium appears to increase the rate of utilization of mucosal phosphate.

## INTRODUCTION

HOKIN AND HOKIN[1] found an increase in the rate of incorporation of $^{32}P_i$ into membrane phospholipids after stimulation of certain ion translocations or secretory processes and have suggested that phospholipid is involved in some transport processes. It is generally accepted that vitamin D increases the rate of translocation of calcium by the intestine[2], and various reports have suggested that phospholipid might be involved in this phenomenon. THOMPSON AND DE LUCA[3] found that treatment of vitamin D-deficient rats with vitamin D increased the rate of incorporation of $^{32}P_i$ into phospholipids of kidney slices and suspended cells of intestinal mucosa, but that incorporation by liver slices was not affected. HÜBSCHER[4] found that calcium stimulated the incorporation of serine into phospholipid of rat liver microsomes. HOSOYA, WATANABE AND FUJIMORI[5] reported that treatment of rachitic rats with vitamin D resulted in an increased rate of incorporation of [$^{14}$C]serine *in vitro* into the phospholipid of liver mitochondria and microsomes provided that calcium was added to the incubation medium. They also reported that addition of vitamin D *in vitro* to the mitochondria from vitamin D-deficient rats resulted in an increased incorpo-

ration of [$^{14}$C]serine into phospholipid provided that calcium was also added, and they suggested that vitamin D in the presence of calcium was a cofactor for phospholipid synthesis.

We have used the ileum of chickens to study the fate of $^{32}P_i$ after absorption from the lumen when calcium translocation was low (by using rachitic birds or by omitting calcium from the lumen) or high (after treating with vitamin D).

## METHODS

### *Chickens*

White crossbred (Leghorn × Australorp) cockerels were obtained on the day of hatching, and were reared for 4 weeks in artificial light on a diet deficient only in vitamin D (ref. 6). The legs of the 4-week-old chicks were photographed by X-rays and only those birds with satisfactory signs of vitamin D deficiency were used. To study the effect of vitamin D, chicks were given 200 I.U. of vitamin $D_3$ in peanut oil through a stomach tube, or 400 I.U. in propylene glycol by intramuscular injection, 16 h before the experiment. Control chicks were given the solvent only.

### *Experiments in vivo*

Closed sacs of ileum were prepared under ether anaesthesia as described by COATES AND HOLDSWORTH[7]. The solution which was injected into the lumen was 0.5 ml 0.9 % NaCl containing various additives which are indicated in the tables. The abdomen was sewn up and the bird was allowed to regain consciousness. Blood samples were taken from the wings into heparinized syringes. At the end of the experimental period the bird was anaesthetised, the ileal sac was cut out and the serosa was rinsed with saline. When nucleotides were to be isolated, the rinsed sac *plus* contents was immediately dropped into liquid $N_2$ so that it was frozen within 10 sec of cutting the blood supply. For other experiments, after rinsing the serosa, the contents of the lumen were washed into a receiver with 1 ml 0.9 % NaCl. The ileum was then slit longitudinally and laid, serosa downwards, on a glass plate. The mucosal cells were scraped off with the edge of a microscope slide. Immediately after the death of the animal the left tibia was removed.

### *Experiments in vitro with everted sacs*

The ileum was rinsed with warm saline and removed from the bird under ether anaesthesia. Everted sacs were prepared at room temperature and incubated at 37° in Krebs–Henseleit bicarbonate Ringer with added glucose as described by SALLIS AND HOLDSWORTH[6]. Isotopically labelled compounds were added to the mucosal fluid as indicated in the tables. The gas phase was either $O_2$–$CO_2$ (95:5, v/v) or $N_2$–$CO_2$ (95:5, v/v). At the end of incubation, the mucosa was rinsed with 0.9 % NaCl. The serosal fluid was washed into a receiver with 1 ml 0.9 % saline. The mucosal cells were scraped off as described for experiments *in vivo*.

### *Experiments in vitro with suspended mucosal cells*

The ileum was rinsed with warm saline and removed from the bird under ether anaesthesia. Mucosal cells were scraped off as described for experiments *in vivo*, and were resuspended by means of a syringe and 18-gauge needle in Krebs–Henseleit

bicarbonate Ringer containing 200 mg glucose per 100 ml, gassed with $O_2$–$CO_2$ (95:5, v/v). They were washed and resuspended twice in this medium, and were incubated in the same medium with the additions which are listed in Table VI. At the end of incubation, 1 ml of the suspension was added to 6 ml chloroform–methanol (1/2, v/v) and lipid was extracted as described for mucosal cells. Another 1-ml aliquot was centrifuged, the sedimented cells were extracted with 0.5 M $HClO_4$ and the acid-insoluble material was washed with 0.1 M $HClO_4$. Aliquots of the combined extract *plus* wash were used for the estimation of $^{32}P$, $^{3}H$, and $P_i$. $P_i$ was estimated by a modification of the method of FISKE AND SUBBAROW[8].

*Treatment of tissue samples*

*Whole blood and plasma.* Whole blood and plasma were mixed with 4 vol. of 0.5 M $HClO_4$. Radioactivity in the supernatant was counted.

*Bones.* Tibiae were rubbed clean with paper tissues, extracted with ethanol and ether, then dried to constant weight at 110°. They were ashed at 800° and the percentage ash was calculated. This served as a check that the birds were rachitic, the values ranging from 26–31 % for rachitic birds (38–42 % for normal birds). The ash was powdered, mixed, and 25 mg were dissolved in 1 ml 0.1 M $HClO_4$. A portion of this solution was counted.

*Mucosal cells.* Mucosal cells which had been scraped from the ileum of one bird, after incubation *in vivo* or as an everted sac, were suspended in 0.8 ml water, and 6 ml chloroform–methanol (1:2, v/v) were added. The mixture was shaken frequently for 60 min, then 2 ml chloroform were added. The upper organic phase was removed, the aqueous suspension was washed with 2 ml chloroform and this wash was added to the organic phase.

The combined chloroform extract was washed with 1 ml of 0.1 M $KH_2PO_4$ when $^{32}P_i$ had been given, or with 1 ml 0.1 M ethanolamine–acetic acid at pH 4.5 or with 1 ml 0.1 M serine when $^{14}C$-labelled substrates had been used. The lipid in this washed chloroform extract was fractionated as described below. The defatted aqueous suspension was made 0.5 M with respect to $HClO_4$ and centrifuged. Aliquots of the supernatant were counted, calcium was estimated by atomic absorption spectrometry, and $P_i$ was estimated by a modification of the method of FISKE AND SUBBAROW[8].

*Serosal fluid and mucosal fluid.* Serosal and mucosal fluid from experiments with everted sacs, were made 0.5 M with respect to $HClO_4$. Aliquots of the supernatant were counted, and calcium and $P_i$ were estimated.

*Fractionation of lipid*

The chloroform extracts of the mucosal cells from four birds were pooled and taken to dryness at 40° in a rotary evaporator which had been flushed with $N_2$. The residue was dissolved in light petroleum (b.p. 60–80°) and fractionated on silicic acid (Bio-Sil A, 200–325 mesh, which had been washed with methanol and ether and dried *in vacuo*) by the method of LIS, TINOCO AND OKEY[9]. Fraction IV contained all the $^{32}P$ and was called crude phospholipid.

In most experiments the crude phospholipid was fractionated on silicic acid with a chloroform–methanol gradient by a slight modification of the method described by THOMPSON AND DE LUCA[3]. We were not able to separate phosphatidyl ethanolamine and phosphatidyl serine by this method. Each fraction was counted and

phosphate was determined after wet-ashing with $HClO_4$ by the method of WEIL-MALHERBE AND GREEN[10]. Nitrogen/phosphorus ratios were determined on the peaks, nitrogen being estimated by a modification of the method described by BOISSONAS AND HASELBACH[11].

Peaks from these columns, and in some cases the total phospholipid, were fractionated by thin-layer chromatography on Kieselgel G using the solvents: chloroform–methanol–25% ammonia–0.8% NaCl (65:35:1:8, by vol.) and chloroform–methanol–water (65:25:4, by vol.). The chromatograms were stained by the methods used by SKIDMORE AND ENTENMAN[12], and autoradiograms were made. Peaks from columns, and chloroform extracts of zones from thin-layer chromatograms, were dried under $N_2$, and hydrolysed in a sealed tube with 1.3 M HCl in dry methanol for 5 h at 120°. The methanol-soluble hydrolysis products were chromatographed on Kieselgel G plates in methanol–water–25% ammonia (60:35:5, by vol.).

*Isolation and fractionation of nucleotides*

The frozen ileum (see experiments *in vivo*) was wrapped in polythene sheet and powdered by hammering while still frozen. The frozen powder was ground in an agate mortar at 0° with 15 ml ice-cold 0.5 M $HClO_4$. The suspension was allowed to stand in ice until it had thawed, and was centrifuged while still cold. The cold supernatant was neutralised to bromthymol blue with ice-cold KOH. The neutralised material was kept at 5° for 1 h, and centrifuged while it was still cold. The supernatant was stirred for 30 min at room temperature with two successive 0.5-g portions of charcoal which had been washed with acid, pyridine, and ethanolic ammonia. The two portions of charcoal were combined and washed twice with 5 ml 0.1 M $KH_2PO_4$ and twice with 5 ml water and the washes were discarded. The washed charcoal was extracted with three 5-ml portions of ethanol–25% ammonia–water (50:4:46, by vol.) and the combined extracts were centrifuged at 12000 × *g* for 20 min. The extracts were made to 15 ml, and aliquots were counted, and the absorption spectrum was read at pH 1.

The extracts from four birds were pooled, concentrated under reduced pressure and fractionated on a column (1 cm × 20 cm) of Whatman ET 41 ECTEOLA-cellulose by a slight modification of the method of NILSSON AND SJUNNESSON[13]. The buffer was adjusted to pH 6.0 at 1.0 M (the pH drops on dilution), and the gradient was modified to get better separation in the region of ADP. The adenine nucleotides were identified by determining $\lambda_{max.}$ at pH 1 and by chromatography on paper in the solvents: *n*-propanol–25% ammonia–water–0.1 M $Na_2EDTA$ (60:30:9:1, by vol.) and *n*-butanol–glacial acetic acid–water (50:25:25, by vol.). ATP was further identified and the purity determined by means of ATP: D-hexose 6-phosphotransferase (EC 2.7.1.1) and D-glucose 6-phosphate: NADP oxidoreductase (EC 1.1.49)[14].

*Counting of radioactive samples*

The Beckman liquid scintillation counter was used with the scintillator fluid of BRAY[15]. A correction for quenching was applied. When the sample contained two different isotopes, separate channels were used. The overlap of radioactivity was calculated and the correction applied. The radioactivity of a sample was initially expressed as counts/min, and in the case of $^{32}P$, the figures were corrected for decay from day 1 of the experiment. In the experiments with $^{14}C$-labelled substrates, it was found by chromatography that the $^{14}C$ in the serosal fluid and lipid was essentially

all in the form in which it had been given; therefore, counts/min have been converted into $\mu$mole [$^{14}$C]substrate incorporated. Figures for $^{32}$P have been left as counts/min since the $^{32}$P incorporated would have been considerably diluted with $^{31}$P.

## RESULTS

### *Factors affecting the labelling of phospholipid*

When solutions containing $^{32}P_i$ were placed in the lumen of the ileum *in situ*, the phospholipids of the ileal mucosa became labelled. Table I shows that when the solution in the lumen also contained $Ca^{2+}$, the specific activity of the phospholipids at 60 min was greater in vitamin D-treated birds than in rachitic birds. However, when the solution which was placed in the lumen did not contain calcium, pretreatment with vitamin D did not affect the specific activity of the phospholipids at 60 min, although the specific activities were greater under these conditions than they were when calcium was added. Figures for the incorporation of $^{45}$Ca into bone are included to show that pretreatment with vitamin D had stimulated calcium translocation.

TABLE I

INCUBATION OF ILEAL LOOPS *in vivo* WITH $^{32}P_i$

The preparation of the loops is described in METHODS. 0.5 ml of 0.9% NaCl containing 0.1 mg $P_i$-phosphorus (12 200 000 counts/min $^{32}$P) with or without 2.0 mg $Ca^{2+}$ (6 930 000 counts/min $^{45}$Ca) was placed in the lumen, and the loop was incubated *in situ* for 60 min.

| $Ca^{2+}$ | *Pre-treatment with vitamin D* | $^{45}$*Ca in left tibia** (*counts/min* $\times 10^{-3}$) | $^{32}$*P in left tibia** (*counts/min* $\times 10^{-3}$) | *Lipids of ileal mucosa* | | |
|---|---|---|---|---|---|---|
| | | | | *Crude** (*counts/min* $\times 10^{-3}$/*ileum*) | *Pooled phospholipid from four birds* | |
| | | | | | *$\mu$g phosphorus/ileum* | *counts/min per $\mu$g phosphorus* |
| + | − | 13.9 ± 3.2 | 4.0 ± 2.2 | 11.8 ± 9.7 | 322 | 33 |
| + | + | 85.7 ± 43.1 | 40.5 ± 20.0 | 59.0 ± 3.1 | 365 | 147 |
| − | − | — | 72.3 ± 35.5 | 160.0 ± 81.0 | 252 | 562 |
| − | + | — | 77.2 ± 9.3 | 169.0 ± 35.0 | 327 | 426 |

* Each figure is the mean of the values for four birds.

Preliminary experiments had shown that the translocation of $^{45}$Ca was linear over a period of 60 min. The figures for $^{32}$P in bone show that the rate of utilization of $^{32}$P by the bird was greatest when calcium was not added to the lumen, and that vitamin D had a stimulating effect only when calcium was added. Thus, the presence or absence of calcium or vitamin D similarly affect the amount of $^{32}$P in mucosal phospholipid at 60 min and the rate of utilization of $^{32}$P by the bird.

In other experiments (Table II) the amounts and proportions of $P_i$ and calcium in the lumen and the time of incubation were varied. In all cases, the concentration of calcium and $P_i$ was such that the solubility product of calcium phosphate would not be exceeded in the lumen. In all experiments, the specific activities of the phospholipid at the end of incubation were in the following descending order: (1) no calcium

added to the lumen, (2) pretreatment with vitamin D *plus* calcium in the lumen, and (3) rachitic *plus* calcium in the lumen. Vitamin D had no effect when calcium was omitted from the lumen.

Table III shows that pretreatment with vitamin D increased the incorporation of $^{32}P_i$ into mucosal phospholipids and the translocation of $P_i$ into serosal fluid when

TABLE II

INCUBATION OF ILEAL LOOPS *in vivo* WITH $^{32}P_i$

Conditions were the same as for Table I except for additions to the lumen and the time of incubation.

| | *Expt. No.* | | | | | |
|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *2* | *3* |
| Additions to lumen | | | | | | |
| $P_i$ (mg P) | Carrier-free | 0.1 | 0.1 | Carrier-free | 0.1 | 0.1 |
| calcium (mg) | 0.2 | 2.0 | 2.0 | 2.0 | 0 | 0 |
| Time of incubation (min) | 20 | 60 | 60 | 60 | 60 | 60 |
| $^{45}Ca$ in left tibia* | 3.6 | 8.0 | 6.2 | 4.3 | — | — |
| Mucosal phospholipid* | 4.1 | 14.0 | 4.5 | 4.0 | 1.5 | 0.8 |
| $^{32}P$ in left tibia* | — | 8.1 | 7.3 | 4.0 | 1.7 | 1.1 |
| $^{32}P$ in plasma* | 2.6 | 2.3 | 5.0 | 3.4 | 2.0 | 1.3 |
| Mucosal acid-soluble phosphate* | — | 1.8 | 1.1 | 2.0 | 1.4 | 1.2 |
| Mucosal acid-soluble calcium ($\mu$atom) | | | | | | |
| rachitic | — | 46.9 | 35.0 | 37.5 | 1.1 | 0.9 |
| vitamin D-treated | — | 11.1 | 23.1 | 18.8 | 0.9 | 1.1 |

* Each figure is the ratio of the mean value of the specific activity of the material from four vitamin D-treated birds to the mean value of the specific activity of the material from four rachitic birds (all samples taken at the end of the incubation period).

TABLE III

INCUBATION OF ILEAL LOOPS *in vitro* WITH $^{32}P_i$

The preparation of the loops is described in METHODS. Initially the mucosal fluid (5 ml) contained 6.15 $\mu$moles $P_i$ (17250000 counts/min $^{32}P$) and 10 $\mu$moles calcium (5550000 counts/min $^{45}Ca$). Serosal fluid (1 ml) contained 1.23 $\mu$moles $P_i$ and 2.0 $\mu$moles calcium initially. The preparations were incubated at 37° for 30 min.

| *Gas* | *Pre-treatment with vitamin D* | *Mucosal cells* | | | *Serosal fluid* | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Phospho-lipid** (*counts/min/$\mu$atom P*) | *Acid-soluble phosphate*** | | *Calcium* | | *Acid-soluble phosphate* | |
| | | | *$\mu$mole* | *$^{32}P$ counts/min $\times 10^{-6}$* | *$\mu$atom/ileum* | *$^{45}Ca$ counts/min $\times 10^{-3}$* | *$\mu$mole* | *$^{32}P$ counts/min $\times 10^{-3}$* |
| $O_2$–$CO_2$ | – | 3400 | 6.2 ± 0.6 | 1.43 ± 0.50 | 1.82 ± 0.28 | 34 ± 16 | 2.62 ± 0.64 | 185 ± 158 |
| $O_2$–$CO_2$ | + | 5300 | 6.4 ± 0.2 | 1.99 ± 0.31 | 1.98 ± 0.12 | 189 ± 36 | 3.60 ± 1.16 | 614 ± 368 |
| $N_2$–$CO_2$ | – | 0 | 7.0 ± 0.6 | 0.87 ± 0.33 | 1.72 ± 0.18 | 31 ± 7 | 2.20 ± 0.24 | 109 ± 48 |
| $N_2$–$CO_2$ | + | 0 | 7.0 ± 0.2 | 0.79 ± 0.16 | 1.74 ± 0.09 | 100 ± 64 | 2.14 ± 0.26 | 143 ± 76 |

* Each figure is the value for the pooled phospholipid from four birds.
** Each figure is the mean value for four birds.

the ileum was aerobically incubated *in vitro*. Although $^{45}Ca$ appeared in the serosal fluid, there was a net loss of calcium from the serosal fluid. This loss was greatest in tissues incubated anaerobically and least in tissues from vitamin-D-treated birds which were incubated aerobically. Thus, for these preparations incubated *in vitro* in the presence of calcium, the conditions which diminished calcium and phosphate translocation also diminished the labelling of phospholipid, just as occurred *in vivo* in the presence of calcium.

The major components of the mucosal phospholipid obtained from ileum incubated *in vivo* or *in vitro* were phosphatidyl ethanolamine and phosphatidyl choline. The amounts of the various components were identical in tissue from rachitic and vitamin D-treated birds. Phosphatidyl ethanolamine *plus* phosphatidyl serine together always contained $38 \pm 3\%$ of the lipid phosphorus and $69 \pm 3\%$ of the lipid-$^{32}P$ (mean figures for 16 rachitic and 16 vitamin D-treated birds incubated with and without calcium) although the specific activity varied by more than four-fold in these experiments. All other phospholipid fractions had lower specific activity. Phosphatidic acid and phosphoinositides were not identified, but both in column and thin-layer chromatography, the material with the $R_F$ expected for these compounds had a low specific activity.

TABLE IV

INCUBATION OF ILEAL LOOPS *in vivo* WITH [2-$^{14}C$]ETHANOLAMINE

Conditions were the same as for Table I except that the solution placed in the lumen contained 2.0 mg $Ca^{2+}$ (16969596 counts/min $^{45}Ca$) and 1 $\mu$mole [2-$^{14}C$]ethanolamine (5 $\mu$C).

| *Pre-treatment with vitamin D* | *$^{45}Ca$ in left tibia** (*counts/min* $\times 10^{-3}$/*tibia*) | *Mucosa* | | |
|---|---|---|---|---|
| | | *Crude lipid** (*m$\mu$mole [$^{14}C$]-ethanolamine*) | *Phospholipid*** (*m$\mu$mole [$^{14}C$]-ethanolamine/$\mu$g P*) | *Acid-soluble $^{14}C$** (*m$\mu$mole [$^{14}C$]-ethanolamine*) |
| − | 31 ± 8 | 221 ± 29 | 813 | 380 ± 50 |
| + | 200 ± 10 | 197 ± 33 | 763 | 410 ± 40 |

* Each figure is the mean of the values from four birds.
** Each figure is the value for the pooled phospholipid from four birds.

Although pretreatment with vitamin D resulted in a greater incorporation of $^{32}P_i$ into phospholipid when the lumen contained calcium, it had no effect on the incorporation of [$^{14}C$]serine or [$^{14}C$]ethanolamine under the same conditions *in vivo* (Table IV) and *in vitro* (Table V). The appearance of $^{14}C$ in the serosal fluid *in vitro* was not affected by pretreatment with vitamin D. Thus, although vitamin D in the presence of added calcium stimulated both the incorporation of $^{32}P_i$ into mucosal phospholipid and the translocation of $^{32}P$ to the serosa both *in vivo* and *in vitro*, vitamin D under the same conditions had no effect on either the incorporation of $^{14}C$ from ethanolamine or serine into phospholipid or the translocation of $^{14}C$ to the serosa. 90% (*in vivo*), 100% (*in vitro*) of the lipid-$^{14}C$ given as ethanolamine was recovered as the ethanolamine residue of phosphatidyl ethanolamine in both rachitic and vitamin D-treated birds. 80% of the lipid-$^{14}C$ given as serine was recovered as the serine residue of phosphatidyl serine, most of the remainder being in phosphatidyl ethanolamine.

TABLE V

INCUBATION OF ILEAL LOOPS *in vitro* WITH [2-$^{14}$C]ETHANOLAMINE OR [3-$^{14}$C]SERINE

Conditions were the same as for Table III except that the mucosal fluid initially contained in addition either 1 $\mu$mole (5 $\mu$C) [2-$^{14}$C]ethanolamine or 2 $\mu$moles (10 $\mu$C) DL-[3-$^{14}$C]serine. The preparations were gassed with $O_2$–$CO_2$.

| [$^{14}$C]*Substrate* | *Pre-treatment with vitamin D* | *Mucosa* | | | *Serosal fluid** (*m$\mu$mole* [$^{14}$C]*substrate*) |
|---|---|---|---|---|---|
| | | *Crude lipid** (*m$\mu$mole* [$^{14}$C]*substrate*) | *Phospholipid*** (*m$\mu$mole* [$^{14}$C]*substrate*/*$\mu$g P*) | *Acid-soluble** (*m$\mu$mole* [$^{14}$C]*substrate*) | |
| Ethanolamine | − | 19.0 ± 5.1 | 0.047 | 68.7 ± 6.1 | 32 ± 8.4 |
| Ethanolamine | + | 22.8 ± 5.2 | 0.050 | 81.2 ± 12.6 | 30 ± 7.8 |
| Serine | − | 5.77 ± 0.61 | 0.0112 | 165.0 ± 5 | 208 ± 36 |
| Serine | + | 5.21 ± 0.80 | 0.0114 | 159 ± 32 | 226 ± 43 |

* Each figure is the mean of the values obtained from four birds.
** Each figure is the value for the pooled phospholipid from four birds.

*Factors affecting the labelling of cell phosphate*

By incubating a suspension of ileal mucosa cells in the presence of [$^3$H]inulin and $^{32}P_i$, it was possible to calculate the inulin space and hence the specific activity of the cellular acid-soluble phosphate when $^{32}$P was being incorporated into phospholipid. Table VI shows that the rate of incorporation of $^{32}$P into both cell acid-soluble phosphate and phospholipid was greater when the birds had received vitamin D than it was in tissue from rachitic birds (the incubation medium contained calcium). Also, the specific activity of the acid-soluble phosphate was greater than that of phospholipid during the experimental period, and reached its maximum value earlier than that of phospholipid.

Attempts to measure the specific activity of the mucosal cellular acid-soluble phosphate *in vivo* (by merely rinsing the mucosa with saline at the end of incubation, scraping off the mucosa and extracting it with acid) showed no consistent difference between rachitic and vitamin D-treated tissue. The specific activity of the extracted acid-soluble phosphate from vitamin D-treated tissue was always greater than or

TABLE VI

INCUBATION OF SUSPENDED MUCOSAL CELLS *in vitro* WITH $^{32}P_i$

A suspension of cells of the mucosa of the ileum was prepared as described in METHODS. 1.0 ml incubation mixture contained 12.5 mg protein, 5 $\mu$C [$^3$H]inulin, 0.25 $\mu$mole (1.5 $\mu$C) $P_i$.

| *Pretreatment with vitamin D* | *Incubation* | | *Phospholipid* (*m$\mu$C*/*$\mu$atom P*) | *Cell (non-inulin space) acid-soluble phosphate* (*$\mu$C*/*$\mu$mole*) |
|---|---|---|---|---|
| | *Temperature* | *Time (min)* | | |
| − | 0° | 30 | — | 0.15 |
| − | 37° | 10 | 0.25 | 0.67 |
| − | 37° | 30 | 0.98 | 0.42 |
| + | 0° | 30 | — | 0.22 |
| + | 37° | 10 | 0.71 | 1.7 |
| + | 37° | 30 | 5.8 | 1.2 |

equal to that from rachitic tissue, but the ratio was variable (Table II). Presumably there was variable contamination of the mucosal cells with phosphate of high specific activity from the lumen.

*Factors affecting the labelling of nucleotides*

ATP and ADP were the major components of the nucleotide fraction in the mucosa of the ileum. The amounts and proportions of the various nucleotides were unaltered by the treatments applied. All other components had lower specific activity than ATP. Table VII shows that the specific activity of ATP was always greater when the bird had been treated with vitamin D, but the ratio of the specific activity of the material from vitamin D-treated birds to the specific activity of the material from rachitic birds was greater when calcium was included in the lumen. This is a similar pattern to that found for incorporation of $^{32}P$ into phospholipid, blood and bones. It differs only in that for ATP, the ratio of values for vitamin D-treated to rachitic is slightly greater than 1 in the absence of added calcium; whereas for phospholipid, blood and bone, it was close to 1 in the absence of added calcium.

TABLE VII

INCORPORATION OF $^{32}P_i$ INTO NUCLEOTIDES BY ILEAL LOOPS *in vivo*

Conditions of incubation were the same as for Table I except for variation of dose and incubation time. At the end of incubation the gut was frozen and nucleotides extracted as described in METHODS. Results are expressed as counts/min per $\mu$mole ($D_3$-treated)/counts/min per $\mu$mole (rachitic).

| *Additions to lumen* | | *Incubation time (min)* | *ATP* | *ADP* | *AMP* |
|---|---|---|---|---|---|
| *Calcium (mg)* | *$P_i$ (mg P)* | | | | |
| 2.0 | 0.10 | 20 | 3.9 | 2.4 | 0.1 |
| 0.2 | 0.10 | 30 | 3.7 | — | — |
| 0 | 0.10 | 20 | 1.3 | 1.1 | 1.3 |
| 0 | 0.10 | 20 | 2.0 | 1.5 | — |

The specific activity of the ATP was 1–3.8 % of the specific activity of the $^{32}P_i$ which was placed in the lumen. In the experiments reported in Table II, the specific activities of acid-soluble phosphate and phospholipid were respectively 9.9–38.8 % and 0.031–0.46 % of that of the $^{32}P_i$ in the lumen. These results are consistent with a transfer of phosphate from the lumen to phospholipid *via* cell acid-soluble phosphate and ATP in that order.

## DISCUSSION

When chick ileum was incubated *in vivo* or *in vitro* with $^{32}P_i$ *plus* $Ca^{2+}$ in the lumen, pretreatment of the bird with vitamin D resulted in an increased $^{32}P$ incorporation into the mucosal phospholipid. Similarly, THOMPSON AND DE LUCA[3] found that pretreatment of vitamin D-deficient rats with vitamin D resulted in increased incorporation of $^{32}P_i$ into the phospholipids of kidney slices and suspended intestinal mucosa cells. As vitamin D is believed to affect primarily the translocation of calcium

by the small intestine, and as HOKIN AND HOKIN[1] have found that stimulation of certain secretory or translocation processes results in an increased incorporation of $^{32}P_i$ into phospholipid, these effects of vitamin D might be related to calcium translocation.

However, we find that the increase in the specific activity of the phospholipids is a general effect on all phospholipids. THOMPSON AND DE LUCA[3] previously showed that the specific activities of all the phospholipids of suspended cells of intestinal mucosa were increased equally by pretreatment of vitamin D-deficient rats with vitamin D. In contrast to this, the increase in specific activity of phospholipid which was found by HOKIN AND HOKIN[1] when they selectively stimulated ion translocation or certain secretory processes, was not a general effect on all phospholipids but was confined to phosphatidic acid and phosphoinositide. Thus, the effect of vitamin D on the labelling of phospholipid must be a different kind of phenomenon from the effects found by HOKIN AND HOKIN[1].

THOMPSON AND DE LUCA[3], using suspended cells of intestinal mucosa, were not in a position to measure calcium translocation concurrent with the labelling of phospholipid. In our preparations of ileal loops, the effect of vitamin D on $^{32}P$ incorporation into phospholipid when calcium was added to the lumen was accompanied by increased calcium translocation. Vitamin D had no effect on the labelling of phospholipid when calcium translocation was low due to omission of calcium from the lumen. This would suggest that the effect of vitamin D on phospholipid labelling is mediated through its effect on calcium translocation, but it is clear that there is no obligatory coupling of calcium translocation and phospholipid labelling since the incorporation of $^{32}P$ into phospholipid was greatest when calcium translocation was least due to lack of calcium in the lumen (Table I). Furthermore, vitamin D stimulated $^{32}P$ incorporation into phospholipid by ileal loops *in vitro* when there was no net translocation of calcium from mucosa to serosa, although $^{45}Ca$ was being transported to the serosa (Table III).

The effect of vitamin D in the presence of calcium on the incorporation of $^{32}P$ into phospholipid might be an effect of vitamin D, either directly or indirectly *via* the effect on calcium translocation, (1) on the rate of synthesis of all phospholipid molecules, or (2) on the turnover of the phosphoryl part of all the phospholipids, or (3) on the specific acitivity of the precursors of the phospholipid-phosphoryl groups. In case (1) one would expect that vitamin D *plus* calcium would have a similar effect on the incorporation of other phospholipid precursors, but [$^{14}C$]serine and [$^{14}C$]-ethanolamine, which are precursors of the phospholipids which had the highest specific activity when labelled with $^{32}P$, were incorporated to the same extent in rachitic and vitamin D-treated chicks, both *in vivo* and *in vitro* (Tables IV and V). Similarly, THOMPSON AND DE LUCA[3] found that vitamin D did not affect the incorporation of [$^{14}C$]glycerol or [$^{14}C$]serine by preparations in which $^{32}P$ incorporation was stimulated by vitamin D. Furthermore, both Mechanisms (1) and (2) might be expected to result in a change in the distribution of $^{32}P$ between the phospholipids since they are synthesised by different routes, but both we and THOMPSON AND DE LUCA[3] found no change in the $^{32}P$ distribution. Mechanism (3) therefore seems the most likely. Furthermore, HARRISON AND HARRISON[16] have shown that vitamin D stimulates the uptake of $P_i$ by isolated loops of rat small intestine, and that calcium is necessary for this $P_i$ transport. Therefore, the effect of vitamin D in the presence of

calcium on $^{32}P$ incorporation into mucosal phospholipid might be mediated by way of the effect of vitamin D *plus* calcium on $P_i$ transport.

If Mechanism (3) were operating, then vitamin D *plus* calcium should cause an increase in the specific activity of cell $P_i$ and nucleoside triphosphate or of nucleoside triphosphate alone. Although we have not obtained reliable figures for cell acid-soluble phosphate *in vivo* (Table II), the specific acitivities of cell acid-soluble phosphate *in vitro* (Table VI) and of adenine nucleotides *in vivo* (Table VII) were raised by vitamin D in the presence of calcium. Furthermore, the relative specific activities of cell acid-soluble phosphate, ATP and phospholipid were such that cell $P_i$ could be the precursor of ATP which could be a precursor of phospholipid. Therefore it seems likely that the effect of vitamin D in the presence of calcium on $^{32}P$ incorporation into phospholipid is not an effect on phospholipid metabolism, but is an indirect result of an effect of vitamin D on $P_i$ translocation and hence on the specific activities of the precursors of the phosphoryl groups of phospholipids. Recently[17] it has been suggested that the changes in incorporation of $^{32}P_i$ into mitochondrial phospholipid which are brought about by various agents such as parathyroid hormone might be due to changes in the turnover rate of ATP.

However, these findings do not explain the greater incorporation of $^{32}P$ into phospholipid, ATP, blood and bone in the absence of added calcium. HELBOCK, FORTE AND SALTMAN[18] reported that omission of calcium decreased phosphate flux in the short-circuited rat duodenum in the absence of magnesium without altering the flux ratio. Therefore, one might expect that the specific activities of cellular $P_i$ and of substances derived from it would be lower in the absence of calcium; we, however, find them to be higher in the absence of calcium. Presumably the fate of $P_i$ is determined by other factors besides the rate of $P_i$ uptake from the lumen. The concentration of calcium in the lumen may be one such factor, since *in vivo* it had a large effect on the concentration of calcium in the cell (Table II). Thus the specific activities of mucosal phospholipid, ATP, and blood and bone would be the result of the balance between two variables: (1) the specific activity of cell phosphate which is increased by vitamin D *plus* calcium, and (2) the rate of mobilisation of cell phosphate which is apparently greater when the concentration of calcium in the lumen is low.

ACKNOWLEDGEMENTS

We gratefully acknowledge a grant from the National Health and Medical Research Council which enabled us to purchase equipment for measuring radioactivity.

REFERENCES

1 L. E. HOKIN AND M. R. HOKIN, *Ann. Rev. Biochem.*, 32 (1963) 553.
2 H. RASMUSSEN AND H. F. DE LUCA, *Ergeb. Physiol. Biol. Chem. Exptl. Pharmakol.*, 53 (1963) 109.
3 V. W. THOMPSON AND H. F. DE LUCA, *J. Biol. Chem.*, 239 (1964) 984.
4 G. HÜBSCHER, *Biochim. Biophys. Acta*, 57 (1962) 555.
5 N. HOSOYA, T. WATANABE AND A. FUJIMORI, *Biochim. Biophys. Acta*, 84 (1964) 770.
6 J. D. SALLIS AND E. S. HOLDSWORTH, *Am. J. Physiol.*, 203 (1962) 497.
7 M. E. COATES AND E. S. HOLDSWORTH, *Brit. J. Nutrition*, 15 (1961) 131.
8 H. H. TAUSSKY AND E. SHORR, *J. Biol. Chem.*, 202 (1953) 675.
9 E. W. LIS, J. TINOCO AND R. OKEY, *Anal. Biochem.*, 2 (1961) 100.
10 H. WEIL-MALHERBE AND R. H. GREEN, *Biochem. J.*, 49 (1951) 286.

11 R. A. BOISSONAS AND C. H. HASELBACH, *Helv. Chim. Acta*, 36 (1953) 576.
12 W. D. SKIDMORE AND C. ENTENMAN, *J. Lipid Res.*, 3 (1962) 471.
13 R. NILSSON AND M. SJUNNESSON, *Acta Chem. Scand.*, 15 (1961) 1017.
14 M. D. JOSHI AND V. JAGANNATHAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 9, Academic Press, New York, 1966, p. 371.
15 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
16 H. E. HARRISON AND H. C. HARRISON, *Am. J. Physiol.*, 201 (1961) 1007.
17 M. FANG AND H. RASMUSSEN, *Biochim. Biophys. Acta*, 153 (1968) 88.
18 H. J. HELBOCK, J. G. FORTE AND P. S. SALTMAN, *Biochim. Biophys. Acta*, 126 (1966) 81.