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Investigations on Vitamin D Esters Synthesized in Rats

TURNOVER AND SITES OF SYNTHESIS

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1. The tissue contents of vitamin D alcohol and ester were estimated in rats 5, 10, 24, 48 and 72hr. after peroral administration of [1-^{3}H]cholecalciferol. 2. The total vitamin D in liver decreased in an exponential fashion from 19% of the dose at 5hr. to 0·6% at 72hr., but the ester content remained at a relatively constant low value from 5hr., so that by 72hr. it represented 67% of the total vitamin D. Vitamin D ester in kidney increased slowly to 48hr., but by 72hr. it was only 10% of the total vitamin D. 3. The small intestine, unlike liver and kidney, contained a higher content of vitamin D ester 10hr. after administration than at later times, and it is postulated that some vitamin D was esterified during absorption from the alimentary tract. 4. Plasma contained vitamin D ester at all time intervals, and it is suggested that ester found in liver and kidney could have been transported to these sites in the blood. 5. Thoracic-duct lymph was found to transport 43% of a peroral dose of vitamin D in 12hr., of which 1·4% was esterified. The fatty acid components of the lymph vitamin D ester, determined by two-dimensional thin-layer chromatography, were mainly palmitate (31%), stearate (25%), oleate (16%) and linoleate (16%). This pattern was similar to that previously found in liver.

The discovery and identification of vitamin D esters in rats given [1-^{3}H]cholecalciferol by gastric intubation has been reported (Fraser & Kodicek, 1965, 1968). Lund & DeLuca (1966) and Bell (1966) also found these esters after giving labelled vitamin to rats. In most experiments the esterified vitamin in tissues was estimated at one interval of 24hr. after administration. Ester at this time represented 5–11% of the total vitamin in liver and 4·5–20% of that in kidney (Fraser & Kodicek, 1968).

The total vitamin D after distribution to various organs decreases in an apparently exponential fashion over several days, with the most rapid decline taking place in the first 48hr. (Cruickshank, Kodicek & Armitage, 1953, 1954; Murray, Day & Kodicek, 1966). As the mode of formation or removal of esterified vitamin D during this period is unknown, an interpretation of its biological significance cannot be properly undertaken. An experiment was therefore designed to investigate the fate of vitamin D and its ester in tissues at intervals up to 3 days after peroral dosing with vitamin D. From the results it is postulated that some esterification of vitamin D occurred during absorption from the alimentary tract, and the identification of vitamin D ester in thoracic-duct lymph supported this hypothesis. This work has been partly reported in a preliminary communication (Fraser & Kodicek, 1966).

EXPERIMENTAL

Materials. Rachitic vitamin D-deficient hooded rats were raised as described in the preceding paper (Fraser & Kodicek, 1968). [1-^{3}H]Cholecalciferol was prepared from the dinitrobenzoate ester by saponification and was purified by thin-layer chromatography (Wilson, Lawson & Kodicek, 1967). Radioactivity was measured by liquid-scintillation spectrometry with an efficiency for tritium of 20–21%.

Synthesis of vitamin D ester in vivo. Five rachitic rats, 73–84g. in weight, were fitted with tail cups for the collection of faeces and prevention of coprophagy (Barnes, Fiala & Kwong, 1963). After 12hr. they were given 0·5mg. of [1-^{3}H]cholecalciferol (specific radioactivity 6·19mc/m-mole) in 0·1ml. of arachis oil by gastric intubation, and one rat was killed at each time (5, 10, 24, 48 and 72hr. afterwards) by exsanguination from cardiac puncture. Tissues were collected and stored at −15°. The lumen of the small intestine was washed with ice-cold 0·9% NaCl and the intestine was divided into equal proximal and distal halves. Plasma was obtained from the heparin-treated blood before it was frozen.

Thoracic-duct cannulation. One male rat, raised on the rachitogenic diet of Steenbock & Black (1925) but with a vitamin D supplement, was surgically prepared with a thoracic-duct cannula by the method of Bollman, Cain &

Grindlay (1948). The animal was placed in a restraining cage (Bollman, 1948) and allowed access to food, and was supplied with drinking water containing 0·9% NaCl and 3% glucose. The weight of the rat at the beginning of the operation was 122g. and 95ml. of lymph was collected during the first 24hr. This interval allowed recovery from surgery and ensured that any tendency for clots to form in the cannula had ceased. For administration of vitamin D the rat was briefly anaesthetized with diethyl ether and a dose of 0·47mg. of [1-^{3}H]cholecalciferol (specific radioactivity 5·35mc/m-mole) in 0·15ml. of arachis oil was given by gastric intubation. Lymph was collected in fractions at 0·5hr. intervals for the first 3hr., at hourly intervals from 3 to 12hr. and at 12hr. intervals after that. The animal was killed 48hr. after the administration of vitamin D, and its liver and small intestine were removed and stored at −15°.

Extraction of tissues and estimation of vitamin D ester. Diethyl ether extracts of Na_2SO_4-dried powders of liver, kidney and small intestine were prepared as described in the preceding paper (Fraser & Kodicek, 1968). Samples containing approximately 2000 counts/min. of radioactivity were applied to silica gel G thin layers with standard vitamin D and vitamin D stearate as side-markers. The chromatograms were developed with $CHCl_3$ to a height of 16cm., 32 bands of 0·5cm. from the origin to the solvent front were scraped directly into counting vials and shaken vigorously with scintillator solution, and the radioactivity was estimated. From the radioactivity distribution on the chromatograms and from the total estimated radioactivity, the amounts of vitamin D and vitamin D ester were calculated.

All the individual lymph fractions were shaken with 25vol. of acetone–ethanol (1:1, v/v) for 60min. and then filtered. This solvent mixture was successfully used in the extraction of vitamin D from lymph by Schachter, Finkelstein & Kowarski (1964). Total radioactivity in the extracts was measured and the presence of ester was sought by the one-dimensional thin-layer-chromatography method described above. The lymph extracts were also chromatographed on floridin earth to isolate vitamin D esters, and these were identified by two-dimensional thin-layer chromatography (Fraser & Kodicek, 1968).

Total radioactivity/ml. of plasma was estimated by spotting samples on Millipore filter disks as suggested by Nirenberg & Leder (1964), drying in a stream of air and counting directly in 5ml. of scintillator solution. The presence of ester was determined on acetone–ethanol (1:1, v/v) extracts, as for lymph, by the one-dimensional thin-layer chromatography method described above.

RESULTS

Tissue distribution studies. A graphical representation of changes with time in tissue free and esterified vitamin D after peroral administration of [1-^{3}H]cholecalciferol is shown in Fig. 1.

In liver, free and esterified vitamin D were found at all time intervals (Fig. 1*a*). As in previous experiments (Cruickshank *et al.* 1953, 1954; Murray *et al.* 1966), vitamin D absorbed from the alimentary tract and deposited in liver rapidly decreased as absorption ceased. By 5hr. the maximum content had occurred, when 19% of the dose was present; this fell away to 0·6% of the dose at 72hr. Vitamin D ester, in contrast, changed only slightly after 5hr. In consequence the amount of ester remaining by 72hr. represented 67% of the total vitamin D, although at 5hr. it had been a relatively minor proportion (Table 1).

Unlike liver, kidney accumulated vitamin D slowly and the maximum content of 1·1% of the dose was not reached until 48hr. after administration (Fig. 1*b*), in agreement with results of Kodicek (1956). This pattern also applied to the ester, which increased both absolutely and relatively up to 48hr.; however, the relative increase in ester was not as great as that in liver. The fall in vitamin D from 48hr. to 72hr. might not be significant, as these values were obtained from only one animal.

A decline of vitamin D alcohol and ester occurred with time in both halves of the small intestine (Fig. 1*c*). The distal half contained more vitamin D than did the proximal between 5 and 24hr. After this the values were almost equal, although there was always a little more in the distal half. This is in disagreement with Kodicek & Ashby (1960) who found more vitamin D in the upper half of the small intestine after 24hr., although at 5 and 10hr. the reverse applied. Cruickshank & Kodicek (1958) showed in histograms that with non-radioactive ergocalciferol there was slightly more vitamin D in the lower than in the upper small intestine at 24hr. The differences at this time were not great in any experiment and it is unlikely that any significance can be attributed to them.

From the much larger amounts of vitamin D present between 5 and 24hr. in the tissue of the distal compared with the proximal small intestine it might appear that the distal absorbs more than the proximal half (Kodicek, 1958). However, the distal part of the small intestine has had less time than the proximal half to transfer the absorbed vitamin from the mucosa, and this process may also be slower in the distal region, thus producing there a relative accumulation of vitamin D. Evidence from intestinal loop experiments (Schachter *et al.* 1964) points to the proximal small intestine as the site of maximal vitamin D absorption.

Vitamin D ester content was at its highest value at 5hr. in the proximal small intestine and fell away to a low value by 72hr. (Fig. 1*d*). In the distal region the ester remained at its highest amount between 5 and 10hr. and then dropped, to decline as that in the proximal part. The intestine was the only tissue found to contain a higher content of ester shortly after administration of vitamin D than at later times. The inflexions in these curves may not be significant, as the amount of radioactivity is very small. The general implication is that most vitamin D ester is present in the period when vitamin D is being absorbed and that the ester then decreases

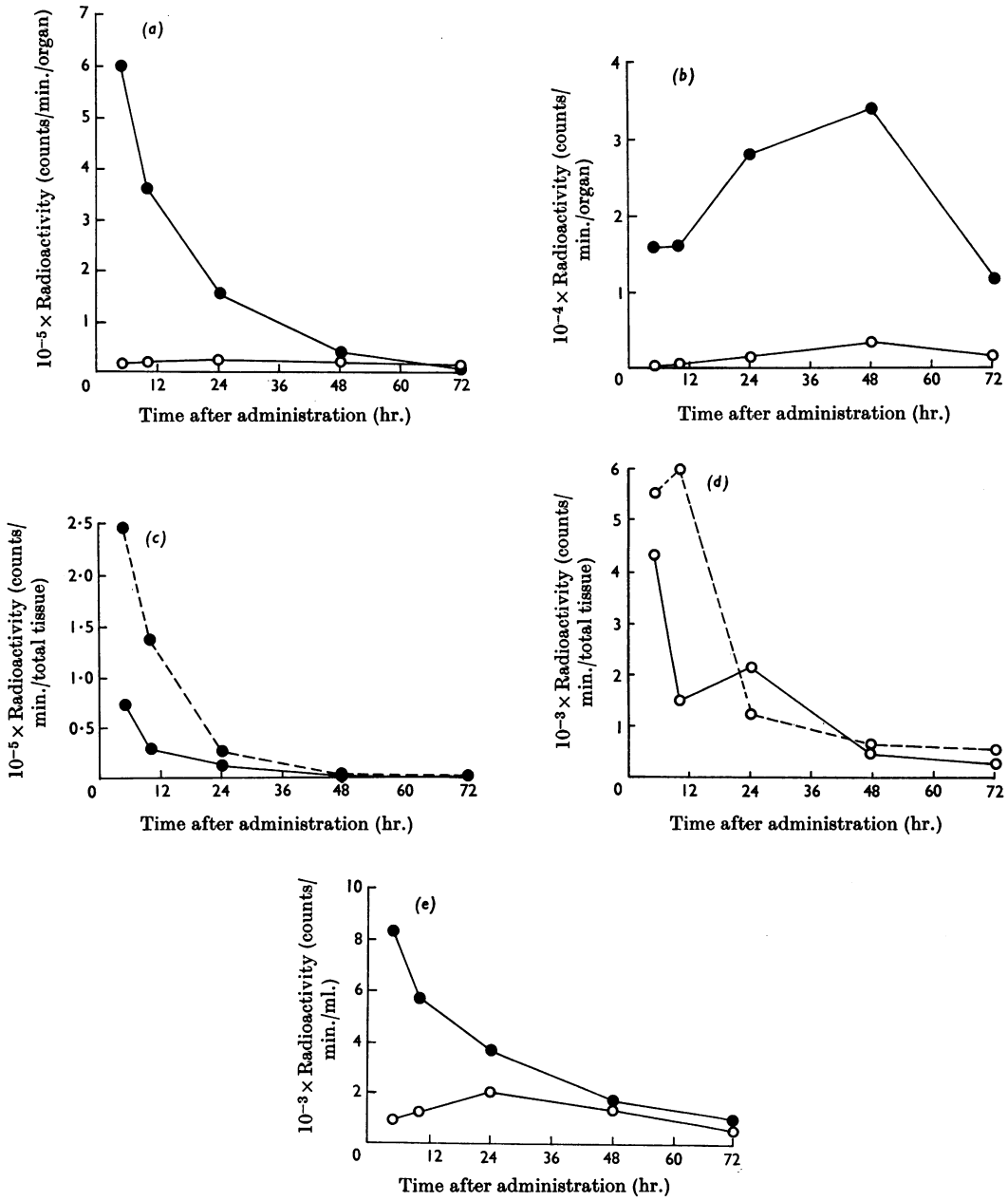

Fig. 1. Changes in the radioactivity of tissue vitamin D alcohol (●) and ester (○) after a peroral dose of 0·5mg. of [1-^{3}H]cholecalciferol (6·19mc/m-mole). (*a*) Liver vitamin D; (*b*) kidney vitamin D; (*c*) small intestine vitamin D alcohol: ●——●, proximal half; ●----●, distal half; (*d*) small intestine vitamin D ester: ○——○, proximal half; ○----○, distal half; (*e*) plasma vitamin D.

with time, although not as rapidly as free vitamin D (see Table 1).

Plasma contained vitamin D ester at all intervals, but its proportion of the total circulating vitamin rose from 11% at 5hr. to 43% at 48hr. Vitamin D alcohol itself declined continuously from 5hr. and the increasing concentration of ester was partly related to the loss of free vitamin. However, the

Table 1. *Tissue vitamin D ester after a peroral dose of 0·5 mg. of [1-^{3}H]cholecalciferol/rat*

One rat was used for each time interval. The percentage of esterified vitamin D was calculated from thin-layer chromatograms of diethyl ether extracts of tissues.

Time after administration (hr.)	Vitamin D ester (% of vitamin D recovered in the tissue)				
	Liver	Kidney	Proximal half of small intestine	Distal half of small intestine	Plasma
5	3	1	6	2	11
10	5	3	5	4	18
24	14	4	14	4	36
48	34	9	17	15	43
72	67	10	23	20	36

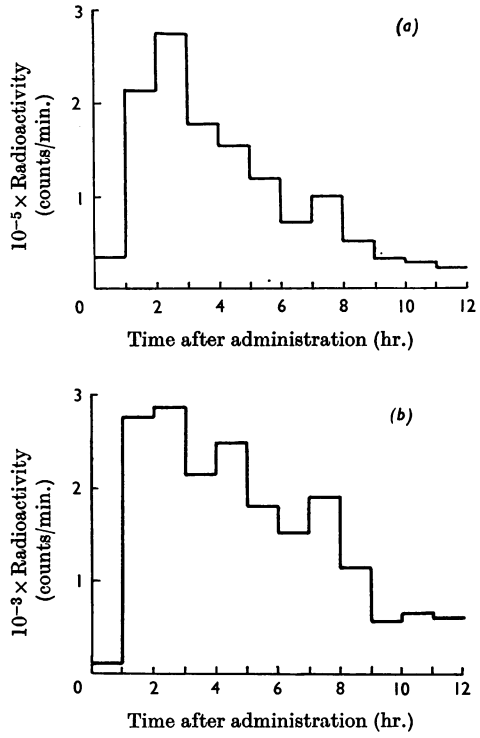

Fig. 2. Absorption of vitamin D into thoracic-duct lymph after administration of 0·47 mg. of [1-^{3}H]cholecalciferol (sp. radioactivity 5·35 mc/m-mole) by gastric intubation. Radioactivity is shown in total hourly fractions. (*a*), Total vitamin D; (*b*), esterified vitamin D.

absolute concentration of ester rose linearly to 24 hr. and declined thereafter at a rate similar to that of non-esterified vitamin D.

To support the evidence for the presence of vitamin D ester, the 5 hr. plasma extract was refluxed for 30 min. with 5 ml. of 2 N-potassium hydroxide in methanol. The unsaponifiable material,

Table 2. *Thoracic-duct-lymph vitamin D ester after administration of 0·47 mg. of [1-^{3}H]cholecalciferol by gastric intubation*

Time after administration (hr.)	Vitamin D ester (% of recovered vitamin D)
0 –0·5	0·3
0·5–1	0·7
1 –1·5	0·7
1·5–2	1·5
2 –2·5	0·9
2·5–3	1·1
3 –4	1·2
4 –5	1·6
5 –6	1·5
6 –7	2·1
7 –8	1·9
8 –9	2·2
9 –10	1·7
10 –11	2·2
11 –12	2·6

Tissue content		
Tissue		
Liver	48	<0·1*
Small intestine	48	<0·1†

* Total vitamin D = 0·2% of dose.
† Total vitamin D = 0·5% of dose.

Table 3. *Percentage composition of thoracic-duct-lymph vitamin D ester*

Time after administration (hr.) ... ... Vitamin D ester	Percentage composition			
	1·5–2	2·5–3	5–8	Mean
Acetate	1·7	3·7	3·6	3·0
Stearate	19·2	27·4	27·2	24·6
Palmitate	43·4	32·5	17·0	31·0
Myristate	5·1	5·3	3·0	4·5
Laurate	2·2	2·5	1·7	2·1
Oleate	14·2	8·7	24·0	15·6
Palmitoleate	1·1	2·8	3·8	2·6
Linoleate	12·6	16·2	19·6	16·1
Linolenate	0·5	0·7	0·1	0·4

Table 4. *Thoracic-duct lymph*

A comparison of percentage composition of fatty acids esterified with vitamin D, cholesterol and retinol is shown.

Acyl group	Vitamin D ester (present results)	Cholesteryl ester (Swell *et al.* 1960)	Retinyl ester (Huang & Goodman, 1965)
$C_{14:0}$	5	0·5	—
$C_{16:0}$	31	34	45–48
$C_{16:1}$	3	2·7	—
$C_{18:0}$	25	9	22–25
$C_{18:1}$	16	16	13–25
$C_{18:2}$	16	27	8–17
Others	4	10·8	—

Table 5. *Comparison of percentage composition of vitamin D esters in different sites in the rat*

Acyl group	Liver	Thoracic-duct lymph	Kidney
$C_{14:0}$	5	5	9
$C_{16:0}$	39	31	49
$C_{18:0}$	22	25	31
$C_{18:1}$	13	16	3
$C_{18:2}$	16	16	4
Others	7	7	4

on thin-layer chromatography, produced only one radioactive component in the region of the standard vitamin D marker, indicating the hydrolysis of ester to vitamin D alcohol.

Thoracic-duct lymph. At 40min. after vitamin D had been given to the rat with a thoracic-duct cannula, the appearance of the lymph changed from an opalescent to a milky fluid, indicating absorption of the arachis oil. This was reflected in the absorption of radioactivity, since very little appeared until the 0·5–1hr. fraction (Fig. 2). A maximum rate of transfer into lymph was reached between 2 and 3hr. from administration, and this declined gradually, so that by 12hr. 43·4% of the dose had been recovered. In the period 12–24hr. only 1·9% of the dose was found. The 24hr. total absorption of 45% via intestinal lymph was in good agreement with the values found by Schachter *et al.* (1964).

Thin-layer chromatography revealed low concentrations of esterified vitamin D, which was positively identified by pooling several lymph extracts and isolating the ester by floridin-earth chromatography. Saponification of the ester fraction gave radioactivity that co-chromatographed with vitamin D alcohol. The highest concentration of ester was in the 11–12hr. fraction, namely 2·6% of the lymph vitamin D, with a mean value over 12hr. of 1·4% (Table 2).

The ester composition was determined by two-dimensional thin-layer chromatography; three chromatograms were prepared from the ester derived from several lymph samples (Table 3). As observed in liver and kidney, the main esters were palmitate, stearate, linoleate and oleate, but there was a wide variation, particularly in the proportion of palmitate and oleate. Because of this variation there was no great difference between the mean values for vitamin D esters and those quoted for rat thoracic-duct-lymph cholesteryl esters (Swell, Law, Field & Treadwell, 1960) and retinyl esters (Huang & Goodman, 1965) (Table 4). However, when the vitamin D-ester pattern of the thoracic-duct lymph is compared with those for vitamin D in liver and kidney, there is a closer similarity between lymph and liver than between lymph and kidney distribution (Table 5).

DISCUSSION

Observations on the tissue content of free and esterified vitamin D after peroral administration of the vitamin were made on only one animal at each time interval. The experiment was so designed because the cost and scarcity of the radioactive vitamin imposed restrictions on the scale of the experiment, and the laborious and time-consuming nature of the analyses would have made any elaboration of the design difficult to carry out. The results appear to be valid, for not only do the values fit smooth curves in time, they are also in line with previously published data (Cruickshank *et al.* 1953, 1954; Murray *et al.* 1966).

In all tissues examined, the relative amount of vitamin D ester compared to vitamin D alcohol is much smaller early after administration than at later periods, when it becomes a more significant proportion of total vitamin D. This change is due mainly to a faster decrease in the content of vitamin D alcohol. Up to 24hr., although the ester has reached by this time its highest amounts in most tissues, it is relatively insignificant. However, by 72hr. esterified vitamin D is a greater proportion of the total, and the ester in liver is the largest pool of vitamin D in any of the tissues examined. Because the bulk of a single oral dose of vitamin D is metabolized and excreted, the residual ester may be a storage form of the vitamin and protected from catabolism.

It is concluded that the small intestine is a site of vitamin D esterification, and it is suggested that this takes place during the process of absorption. Thus it was observed that the highest content of ester in the intestinal wall was during the 10hr. of maximum absorption, and that in other tissues vitamin D ester did not alter in such a way as to be indicative of synthesis there. For instance, liver and kidney accumulated little ester after 5hr. and therefore no

evidence was obtained that they are able to esterify vitamin D. This agrees with negative results obtained in attempts to synthesize vitamin D esters with these tissues *in vitro* (D. R. Fraser & E. Kodicek, unpublished work). Also the fact that in the liver of the rat with a cannulated thoracic duct there was less than 0·1% of ester after 48hr. (Table 2), whereas in an intact rat at this time after administration, 34% of vitamin D was esterified, is again evidence that this organ does not synthesize the ester. Since the proportion of ester to free vitamin D in liver is the same 24hr. after 500μg. or 10μg. peroral doses of vitamin D (Fraser & Kodicek, 1968), the low amount of vitamin D in the liver of the cannulated rat should still be partly esterified if the liver possessed esterifying properties.

The presence of vitamin D ester in plasma indicates a means of transport to the other organs where ester is found, and it appears to accumulate in plasma at a rate faster than its removal up to 24hr. It is also possible that vitamin D ester is synthesized in, as well as being transported by, plasma, as is the case with cholesterol ester (Swell & Treadwell, 1950). Certainly in the period of highest vitamin D concentration the ester is rising while the free vitamin falls. Subsequent experiments (D. R. Fraser & E. Kodicek, unpublished work) have shown that some esterification of vitamin D takes place in plasma.

Studies by Schachter *et al.* (1964) on the absorption of radioactive vitamin D from the intestine into lymph showed 13–28% of the lymph radioactivity appearing on thin-layer chromatograms in a region where vitamin D ester would run. Vitamin D esters were identified by Bell (1966) in thoracic-duct lymph of rats given [4-^{14}C]cholecalciferol orally, and their composition was partly determined by fractionation according to the degree of unsaturation of the esterified fatty acid. A wide variation in composition was reported between rats and it is difficult to compare the mean values with those reported in the present paper (Table 6). In both instances, however, most of the esterified vitamin D contained either saturated or mono-unsaturated fatty acids. The similarity of the lymph and liver vitamin D esters supports the hypothesis that the liver vitamin D ester is synthesized in the intestinal mucosa and is taken up rapidly from chylomicrons derived from intestinal lymph. On the other hand, the kidney ester, which appears more slowly, could have an altered composition as a result of either differential absorption from the circulation or of change in the composition of the circulating ester.

From the comparable patterns with other lymph esters (see Table 4) it seems reasonable that either the cholesterol or the retinol esterifying systems in mucosa could be responsible for the small amount of vitamin D ester. Enzymic studies (D. R. Fraser & E. Kodicek, unpublished work) have demonstrated that vitamin D can be esterified by a cholesterol-esterifying enzyme.

Table 6. *Thoracic-duct lymph vitamin D ester*

A comparison with values in the literature is shown.

Fatty acid	% composition: Present results	Bell (1966)
Saturated	61 } 80	48·5 } 79
Mono-unsaturated	19 }	30·5 }
Di-unsaturated	16	4·8
Tri-unsaturated	0·4	5·6
Tetra-unsaturated	?	10·6
Other	4·6	—

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REFERENCES

Barnes, R. H., Fiala, G. & Kwong, E. (1963). *Fed. Proc.* **22**, 125.
Bell, N. H. (1966). *Proc. Soc. exp. Biol., N.Y.*, **123**, 529.
Bollman, J. L. (1948). *J. Lab. clin. Med.* **33**, 1348.
Bollman, J. L., Cain, J. C. & Grindlay, J. H. (1948). *J. Lab. clin. Med.* **33**, 1349.
Cruickshank, E. M. & Kodicek, E. (1958). *Abstr. 4th int. Congr. Biochem., Vienna*, p. 90.
Cruickshank, E. M., Kodicek, E. & Armitage, P. (1953). *Biochem. J.* **54**, 337.
Cruickshank, E. M., Kodicek, E. & Armitage, P. (1954). *Biochem. J.* **58**, 172.
Fraser, D. R. & Kodicek, E. (1965). *Biochem. J.* **96**, 59P.
Fraser, D. R. & Kodicek, E. (1966). *Biochem. J.* **100**, 67P.
Fraser, D. R. & Kodicek, E. (1968). *Biochem. J.* **106**, 485.
Huang, H. S. & Goodman, DeW. S. (1965). *J. biol. Chem.* **240**, 2839.
Kodicek, E. (1956). *Ciba Found. Symp.: Bone Structure and Metabolism*, p. 161. Ed. by Wolstenholme, G. E. W. London: J. and A. Churchill Ltd.
Kodicek, E. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, p. 198. London: Pergamon Press Ltd.
Kodicek, E. & Ashby, D. R. (1960). *Biochem. J.* **75**, 17P.
Lund, J. & DeLuca, H. F. (1966). *J. Lipid Res.* **7**, 739.
Murray, T. K., Day, K. C. & Kodicek, E. (1966). *Biochem. J.* **98**, 293.
Nirenberg, M. & Leder, P. (1964). *Science*, **145**, 1399.
Schachter, D., Finkelstein, J. D. & Kowarski, S. (1964). *J. clin. Invest.* **43**, 787.
Steenbock, H. & Black, A. (1925). *J. biol. Chem.* **64**, 263.
Swell, L., Law, M. D., Field, H. & Treadwell, C. R. (1960). *Proc. Soc. exp. Biol., N.Y.*, **104**, 7.
Swell, L. & Treadwell, C. R. (1950). *J. biol. Chem.* **185**, 349.
Wilson, P. W., Lawson, D. E. M. & Kodicek, E. (1967). *Biochem. J.* **103**, 165.