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Vitamin D and the Incorporation of [1-^{14}C]Acetate into the Organic Acids of Bone

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A relationship between vitamin D and citrate metabolism in bone was first observed by Dickens (1941), who found that the citrate content of bones from vitamin D-deficient animals was about 50% that of normal. This relationship was subsequently confirmed by Nicolaysen & Nordbø (1943), Waasjö & Eeg-Larson (1951*a*, *b*), Steenbock & Bellin (1953), and Carlsson & Hollunger (1954). In other work it has been shown that dietary vitamin D increases the citrate content of many tissues (Steenbock & Bellin, 1953; Freeman & Chang, 1950) as well as inducing a citraturia (Bellin & Steenbock, 1952; Harrison & Harrison, 1951). However, a detailed understanding of the mechanism of the accumulation of citrate after the administration of vitamin D is still lacking.

In this Laboratory it was demonstrated that the addition of vitamin D to the diet of rats lowered the oxidation rates of citrate and isocitrate but not that of other tricarboxylic acid-cycle intermediates by kidney homogenates and mitochondria (DeLuca, Gran & Steenbock, 1957*a*; DeLuca, Gran, Steenbock & Reiser, 1957*b*). This was also attainable by the addition of vitamin D to systems *in vitro* (DeLuca & Steenbock, 1957). Electron microscopy of isolated rat-kidney mitochondria revealed that dietary vitamin D had a marked protective effect on the structural integrity of the organelles (DeLuca, Reiser, Steenbock & Kaesberg, 1960). It was then thought that the diminished oxidation of citrate observed previously could be explained in terms of a physical inhibition of citrate penetration into the mitochondria.

On the other hand, Patwardhan and co-workers (Ramalingaswami, Sriramachari, Dikshit, Tulpule & Patwardhan, 1954; Dikshit, Joshi & Patwardhan, 1956; Joshi, Dikshit & Patwardhan, 1957) and later Meyer, Bolen & Antin (1959) have suggested that vitamin D increases the synthesis of citrate from acetate in bone. From continued investigations, the former group has concluded that the administration of vitamin D induces an increase in the citrogenase activity of cartilage (Joshi *et al.* 1957), which would then account for the increased synthesis of citrate.

In an attempt to resolve the divergent views on the accumulation of citrate after the administration of vitamin D, the effect of vitamin D on the metabolism of [1-^{14}C]acetate to other tricarboxylic acid-cycle intermediates and to carbon dioxide has been studied in bone slices. The results reported in the present paper demonstrate that vitamin D does not increase citrate synthesis from acetate, but rather decreases the conversion of citrate into the subsequent intermediates of the tricarboxylic acid cycle.

EXPERIMENTAL

Animals and diets. Young male rats of the Holtzman strain weighing 60–70 g. were used in all experiments. They were housed in hanging wire cages and given food and water *ad libitum*. Two semi-synthetic diets were given: diet 11, which induced approximately normal skeletal development; diet 23, which was strongly rachitogenic. The basal diet consisted of: cottonseed oil (Wesson), 10%; roughage (Celluflour), 3·0%; calcium-free and phosphate-free salts, 2·0%; all vitamins except vitamin D; glucose monohydrate, 66·9%; choline hydrochloride, 0·1%; protein, 18%. In diet 11 the protein source was 'vitamin-free' casein (General Biochemicals Inc.) supplemented with 0·2% of L-cystine (at the expense of glucose), and in diet 23 it was steamed egg-white. The desired calcium and phosphate concentrations were obtained by the addition of $CaCO_3$ or an equimolar mixture of K_2HPO_4 and KH_2PO_4 at the expense of glucose. Diet 11 contained 0·47% of calcium and 0·3% of phosphate, and diet 23 contained 0·47% of calcium and 0·016% of phosphate.

Administration of vitamin D. All rats on diet 11 received a supplement of 75 i.u. of vitamin D every 3 days administered orally as a solution of crystalline calciferol in 0·1 ml. of cottonseed oil (Wesson). After the rats had been given diet 11 for 25 days, and had gained 90–100 g., they were killed by decapitation. The rats given diet 23 received no vitamin D until the fifteenth day. During this period they gained 20–30 g. and became severely rachitic, as revealed by a visual examination of sectioned tibia. On the fifteenth day these animals were divided into two equal groups. One group continued to receive no vitamin D for the duration of the experiment. Each rat in the other group received 25 i.u. on the fifteenth day, 2000 i.u. in the morning and again in the afternoon of the sixteenth day, and 1000 i.u. in the morning and again in the afternoon of the seventeenth day. Thus the total amount of vitamin D_2 administered was 6025 i.u. per rat. The doses of vitamin D_2 were always dissolved in 0·1 ml. of cottonseed oil. The rats from both groups were killed by decapitation on the morning of the eighteenth day.

Preparation of tissue samples. Immediately after death, the rear legs were cut from the carcass and packed in ice. The adhering tissue was quickly cleaned from the proximal half of the tibiae, and each bone was then split lengthwise. The half bones from the rats given diet 23 were divided into four sections, i.e. the epiphysis, cartilage, spongiosa and trabecular bone (bone shaft). The half bones from the animals given diet 11 were divided only into epiphysis, spongiosa and trabecular bone, since the very small amount of uncalcified cartilage produced by this diet could not be conveniently and quantitatively removed. The tissue fragments were washed in ice-cold Krebs bicarbonate solution to remove the marrow. The epiphysis, spongiosa and cartilage were sliced into thin sections with a Stadie–Riggs microtome. All other bone samples were cut repeatedly into very small segments with a pair of surgical scissors. Samples (200–400 mg.) from the same experimental category were pooled, blotted, weighed on a torsion balance and transferred to a 25 ml. Erlenmeyer flask containing the incubation medium.

Incubation medium. The incubation medium consisted of 3·0 ml. of Krebs–Ringer bicarbonate buffer (Umbreit, Burris & Stauffer, 1957), 6·0 μc of [1-^{14}C]acetate equivalent to 100000 counts/min. (specific activity 2·56 mc/m-mole) and 30 μmoles of oxaloacetate. Cartilage incubation mixtures always had 30 μmoles of malate in place of the oxaloacetate. With cartilage as a tissue source it was found in preliminary experiments that, when malate was used as a primer substrate, there was about five times as much radioactivity incorporated into the various organic acids as when oxaloacetate was used. This enhancement of incorporation was not observed when epiphysis, spongiosa or bone shaft was incubated with malate.

Incubations. Before the introduction of the sample, the flasks containing incubation medium were gassed for 10 min. with O_2+CO_2 (95:5) and equilibrated at 37° for 15 min. The tissue samples were incubated at 37° in a Dubnoff shaker for 30 min. The tissue samples and media were frozen in an acetone–solid CO_2 bath. They were stored at −14° until used for analysis. As the tissue samples were desired for chromatographic analysis, they were removed from the freezer and allowed to thaw in the presence of 3 ml. of N-H_2SO_4. After being thawed they were ground in a mortar with a pestle to a liquid paste which was then extracted three times with 5 ml. of N-H_2SO_4. The residue was centrifuged off each time and the combined extracts were used for chromatographic analysis.

Separation of organic acids from bone salts. To prevent the excess of bone salts present in the H_2SO_4 extract from overloading the ion-exchange resin subsequently used to resolve the organic acids, a procedure was developed to separate the organic acids from the inorganic salts by partition chromatography. The combined extracts of one tissue sample were mixed in a beaker with Celite 545 (Johns–Manville Co., New York, U.S.A.) in the proportion of 1 ml. of extract to 2 g. of dry Celite. The Celite was then packed into a column (35 cm. × 1·8 cm.). Technical-grade diethyl ether, pre-equilibrated with N-H_2SO_4, was found most suitable as an eluent for the organic acids since large volumes are easily evaporated. The ether (1·5 l.) was allowed to flow by gravity through the column, and was collected in a beaker containing 300–400 ml. of redistilled water maintained at 35–36° to evaporate the ether. Preliminary experiments demonstrated that this volume of ether quantitatively removed all the expected organic acids applied to columns of this size, e.g. glutamic acid, lactic acid, succinic acid, malic acid, citric acid, fumaric acid and α-oxoglutaric acid.

Preparation of Dowex 1 resin. The desalted samples of organic acids were evaporated to 5 ml., neutralized to pH 8·0 and applied to a column (1·0 cm. × 13·5 cm.) of Dowex 1 (X8; 200–400 mesh). The resin had previously been converted into the formate form by exhaustive washing with 7 N-formic acid until a negative chloride test was obtained, and then washing with water to give an eluent with pH 5·0. The resin was always discarded after a single use.

Chromatography of organic acids. Initially the ion-exchange chromatographic procedures of Busch, Hurlbert & Potter (1952) and Palmer (1955), with a hyperbolic gradient of formic acid, were utilized, but they were found to give unsatisfactory resolution of all the acids found in our samples. As a consequence a linear-gradient procedure was devised according to the procedures developed by Bock & Ling (1954). Two narrow cylinders (3·5 cm. × 50 cm.), one containing initially 300 ml. of 6·15 N-formic acid and the other containing initially 300 ml. of deionized water, were interconnected by a siphon system. As liquid was withdrawn from the water chamber, 6·15 N-formic acid flowed in, giving a linearly increasing gradient of formic acid. Vertical mixing in the water chamber to ensure a smooth reproducible gradient was provided by an air bubbler controlled by a Nullmatic pressure regulator. The liquid eluent was withdrawn from the water chamber and pumped by a Minipump (Milton Roy Co., Philadelphia, Pa., U.S.A.) through the ion-exchange columns at a rate of 1·0 ml./min. Three hundred 2·0 ml. fractions were collected on stainless-steel planchets. After the solvent had evaporated, the radioactivity was measured at infinite thinness in a Nuclear–Chicago Corp. model D–47 gas-flow counter. The reproducibility of this counting procedure was ±5%. The standard error for the slowest counting sample was 10%. The overall recovery of radioactive citrate from initial extraction through the counting procedure was 96–102%.

The linear-gradient procedure was standardized by chromatographing groups of organic acids known to occur in bone and by measuring their recovery by titration with 0·01 N-NaOH to the phenol red end point according to the procedure of Palmer (1955). Essentially 100% recovery was obtained with glutamic acid, lactic acid, succinic acid, malic acid, citric acid, fumaric acid and α-oxoglutaric acid.

Identification of organic acids. The radioactive organic acids were identified by use of the following three methods: (*a*) Their positions in the ion-exchange chromatogram were compared with the positions of standard acids. (*b*) Radioactive samples from a particular peak were recovered from the planchets and rechromatographed on the ion-exchange resin with a known acid thought to correspond to the radioactive acid. Each fraction from the fraction collector was divided to permit counting of the radioactivity and titration of the added acid. (*c*) The recovered radioactivity was also chromatographed with standards in a one-dimensional ascending paper-chromatography system. Two solvent systems were used: ether–88% formic acid–water (70:1:9, by vol.) (Denison & Phares, 1952) and methylCellosolve–aq. NH_3 (sp.gr. 0·88)–water (16:1:3, by vol.). The acids were detected by a spray consisting of 1 ml. of aniline and 1 g. of glucose dissolved in 60 ml. of butan-1-ol,

20 ml. of ethanol and 20 ml. of water. The radioactivity was measured by cutting the chromatograms into segments and counting in a gas-flow counter. When the three techniques agreed, an acid was considered identified.

Collection of $^{14}CO_2$. Essentially the procedure of Snyder & Godfrey (1961) and Eisenberg (1959) was used to obtain quantitative information on the production of $^{14}CO_2$ from [1-^{14}C]acetate by cartilage and spongiosa.

After the tissues had been sliced and weighed as described above, they were introduced into a single-arm Warburg flask containing the same media as described above for the production of radioactive organic acids, except that the specific activity of the [1-^{14}C]acetate was 1·3 mc/m-mole. The total amount of [1-^{14}C]acetate used was maintained at 6·0 μc. The slices were incubated for 30 min. in a Dubnoff shaker, and after the incubation the $^{14}CO_2$ produced was released by acidification and collected in a solution of hyamine placed in a removable vial supported by the centre well of the Warburg flask. The hyamine was then added to a solution containing diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene and counted directly in a Packard liquid-scintillation counter. Counting rates of triplicate samples were within 2% of each other. The standard error for the lowest counting rate was 5%.

RESULTS AND DISCUSSION

Chromatographic separation of radioactive acids. Fig. 1 depicts the chromatogram profile of the ^{14}C-labelled organic acids formed from [1-^{14}C]acetate by epiphysis from rats given the normal diet no. 11. This chromatogram is representative both with regard to the separation of the various organic acids attained and to the type and number of radioactive acids found. The combined Celite and Dowex 1 method has proved more convenient and more reproducible than, and has more capacity than, the silicic acid-column method of Lees & Kuyper (1957). The linear gradient of formic acid on Dowex 1 has also been used to separate the organic acids produced by incubation of slices from other tissues with [1-^{14}C]acetate.

Incorporation of ^{14}C *into organic acids of tissue from normal and rachitic rats.* Table 1 shows the results for the incorporation of ^{14}C from [1-^{14}C]-acetate into the organic acids of tissues from rats given a normal purified diet. All the intermediate

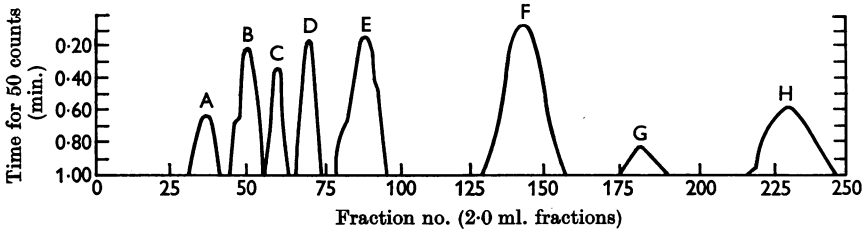

Fig. 1. Chromatogram profile of radioactive organic acids resulting from the incubation of [1-^{14}C]acetate with approx. 200 mg. of epiphysis from rats given a normal diet. The incubation medium and the chromatographic procedure are described in the text. A, Glutamic acid; B, lactic acid; C, unidentified; D, succinic acid; E, malic acid; F, citric acid; G, fumaric acid; H, α-oxoglutaric acid.

Table 1. *Incorporation of* ^{14}C *from* [1-^{14}C]*acetate into the organic acids of bones from normal rats*

The tissues slices were obtained from the tibiae of rats given a normal diet. Each sample represents tissue from three to four rats. The incubation medium is described in the text. Slices (200–400 mg.) were added to separate flasks and the contents shaken under $O_2 + CO_2$ (95 : 5) at 37° for 30 min. The results for citric acid are expressed as means ± S.D. —, Not detected.

	^{14}C incorporated (counts/min./g. wet wt. of tissue)		
Acid	Epiphysis	Spongiosa	Trabecular bone
No. of samples ...	5	5	5
Fore run	—	650	—
Glutamic acid	2550	900	130
Lactic acid	2320	1950	1280
Unidentified	2500	2170	530
Succinic acid	7070	5400	—
Malic acid	4860	2780	1610
Unidentified	600	—	500
Citric acid	15000 ± 1910	15600 ± 3100	2820 ± 140
Fumaric acid	2690	3500	—
α-Oxoglutaric acid	5660	8850	700
Total acids	43200 ± 3900	41800 ± 3810	7570 ± 360

acids of the tricarboxylic acid cycle to be expected are found in radioactive form. Oxaloacetic acid and oxalosuccinic acid are not stable to the isolation procedures described above and are therefore not detected. The equilibrium concentrations of aconitic acid and isocitric acid are likely to be lower than the sensitivity of the analytical method. Further, isocitric acid is not separated from citric acid by this chromatographic procedure. Glutamic acid is probably formed via the reductive amination of α-oxoglutaric acid or by transamination. The reductive amination of oxaloacetic acid to yield aspartic acid is not observed, perhaps because of dilution by the large amount of unlabelled oxaloacetate used as a C_4 primer.

There is obviously considerable tricarboxylic acid-cycle activity present in epiphysis, spongiosa and trabecular bone, since the observed labelling occurred in only 30 min. Although Lees & Kuyper (1957) also observed radioactivity incorporated into organic acids from [1-^{14}C]acetate by cancellous bone, their incubations were for 5 hr. and resulted in a total incorporation of about one-quarter of that observed in the present experiments. Approx. 30 % of the total radioactivity found in the organic acids is associated with citric acid. The other 70 % of the radioactivity exists in acids that are produced metabolically from citric acid. Our results indicate that, on a wet-weight basis, spongiosa and epiphysis are 5–6 times more active metabolically than trabecular bone; but this difference is probably a reflexion of the number of cells per unit weight in the respective tissues rather than a metabolic difference.

Specific radioactivities of the organic acids are not reported since large amounts of organic acids are present in the bone crystal (Lees & Kuyper, 1957). If the large pool of organic acids were an integral component of the bone crystal or were either adsorbed or exchanged on the crystal surface, then the acids would probably not be in rapid equilibrium with metabolic events occurring inside the bone cell. Thus a quotation of the specific radioactivity of an organic acid would be meaningless for the metabolic state of the bone cells even if an accurate chemical measurement of acids were possible.

Effect of the administration of vitamin D on the incorporation of ^{14}C *into organic acids.* Table 2 shows the results for the incorporation of ^{14}C from [1-^{14}C]acetate into the organic acids of bone from rats given a rachitogenic diet. The differences in total incorporation between the tissues from animals given the normal diet (Table 1) and the comparable tissues from vitamin D-treated animals given the rachitogenic diet are most evident in the spongiosa. The bone from rats given the rachitogenic diet and vitamin D (Table 2) is in a different state from that from animals given the normal diet 11 (Table 1). The gross details of the difference are described by Steenbock & Herting (1955). Thus the differences between Table 1 and Table 2 are not unexpected, and are revealing with regard to the dangers encountered in such a study.

The administration of vitamin D to the rachitic rats only 2 days before they were killed was considered to be the most favourable condition for study of the alleged effect of vitamin D on citrate synthesis. This technique would allow sufficient time for the metabolic effects of vitamin D to appear and still minimize secondary changes such as those indicated in the preceding paragraph. It appears likely that the changes reported by Meyer *et al.* (1959) to be due to vitamin D might be at least in part explicable in terms of secondary morphological and cytological differences between the tissues from the rachitic rats and those from animals given a continual supply of vitamin D. For example, the uncalcified area in the ends of long bones from rachitic rats is made up of resting, proliferating and hypertrophic cartilage cells as well as osteoid tissue, whereas the same area in a rat receiving a continual supply of vitamin D contains predominately resting and proliferating cartilage. It would not be surprising if these cell types differed in their metabolism of citrate and other intermediates.

As our results demonstrate, the epiphysis, spongiosa, cartilage and trabecular bone from vitamin D-deficient animals incorporated, within the limits of experimental error, as much radioactivity from [1-^{14}C]acetate into the organic acids as do the corresponding tissues from animals that had received vitamin D on the fifteenth day. Thus it appears that the administration of vitamin D only shortly before the rats were killed has not greatly changed the distribution of cell types and therefore the overall metabolic activity of the tissue.

The tissues from the rachitic rats that had been given vitamin D have 40–50 % (bone and cartilage) to 100 % (epiphysis and spongiosa) more radioactivity in citric acid. On the other hand, the tissues from rachitic rats not given vitamin D have more ^{14}C in α-oxoglutaric acid and in most cases more in glutamic acid (except in cartilage). In general the administration of vitamin D has little or no effect on the appearance of ^{14}C in the other organic acids, i.e. succinic acid, malic acid, 'unidentified' and lactic acid (except in trabecular bone). Since both types of tissues incorporate the same total amount of radioactivity, the administration of vitamin D causes a significant increase in the amount of radioactive citric acid, while causing an approximately equal decrease in the sum of the radioactivity in all the remaining acids.

Effect of vitamin D on the conversion of [1-^{14}C]*acetate into* $^{14}CO_2$. To study further the alleged vitamin D-stimulated synthesis of citrate, an investigation was made of the production of $^{14}CO_2$ from [1-^{14}C]acetate. On the basis of an increased synthesis, the administration of vitamin D should cause an increase in the production of carbon dioxide from acetate. However, the decreased-destruction theory would predict a diminution of the production of $^{14}CO_2$ from [1-^{14}C]acetate after the administration of vitamin D, because radioactive citrate would not be converted so rapidly into the other acids of the tricarboxylic acid cycle. The results (Table 3) indicate that the $^{14}CO_2$ produced by spongiosa from vitamin D-deficient rats is 20% greater than that produced by the spongiosa from rats given vitamin D. Similar results were also obtained with cartilage tissue.

Discussion of the mechanism of the accumulation of citrate after the administration of vitamin D. The present results do not exclude the possibility that initially there is a larger unlabelled intracellular citrate pool in the bone cells from rats given vitamin D than in those from vitamin D-deficient rats. Then the larger pool would dilute the radioactive citrate, resulting in the 'trapping' of more radioactivity in citrate but less radioactivity in other acids and carbon dioxide, which follow citrate in the metabolic sequence. However, it is even more difficult to measure the size of various 'compartments' within the cells of bone and the apparent citrate concentration in those 'compartments' than it is to measure specific radioactivities of the organic acids in bone cells. Thus the present investigation agrees with but does not prove the hypothesis that there is decreased destruction of citrate

Table 2. *Incorporation of* ^{14}C *from* [1-^{14}C]*acetate into the organic acids of bones from rachitic rats*

The tissue slices were obtained from the tibiae of rats given a rachitogenic diet. Each sample represents tissue from three to four rats. The administration of vitamin D to the rats and the incubation medium are described in the text. Slices (200–400 mg.) were added to each flask and the contents shaken under $O_2 + CO_2$ (95:5) at 37° for 30 min. Results are given for rachitic rats ('−vitamin D') and for rachitic rats that had been given vitamin D ('+vitamin D'). The results for glutamic acid, citric acid and α-oxoglutaric acid are given as means ± S.D. —, Not detected.

	^{14}C incorporated (counts/min./g. wet wt. of tissue)			
	Cartilage		Epiphysis	
	−Vitamin D	+Vitamin D	−Vitamin D	+Vitamin D
	No. of samples			
Acid	8	8	8	7
Fore run	1180	220	—	—
Glutamic acid	700±220	950±220	3020±260*	1600±170*
Lactic acid	5040	4350	6020	6640
Unidentified	—	430	3800	4580
Succinic acid	2160	2450	4770	4820
Malic acid	650	640	910	790
Citric acid	1790±230*	2340±180*	1460±205*	3910±130*
α-Oxoglutaric acid	1470±310*	680±110*	1490±110*	890±90*
Total acids	11990±1320	12060±1600	21460±1520	23110±1515
Total acids minus citric acid	10200±1150	9720±1580	20000±1450	19210±1290

	^{14}C incorporated (counts/min./g. wet wt. of tissue)			
	Spongiosa		Trabecular bone	
	−Vitamin D	+Vitamin D	−Vitamin D	+Vitamin D
	No. of samples			
Acid	11	6	4	4
Fore run	—	—	—	—
Glutamic acid	460±100*	300±100*	800±110*	260±75*
Lactic acid	1860	1920	2430	1570
Unidentified	—	—	—	—
Succinic acid	2830	2570	2860	2280
Malic acid	580	740	—	420
Citric acid	2110±780*	5210±600*	1380±110*	2020±110*
α-Oxoglutaric acid	2830±92*	1580±97*	—	—
Total acids	10670±850	12300±970	7470±700	6500±1140
Total acids minus citric acid	8560±580	7100±490	6090±850	4520±1090

* Significant difference ($P < 0.01$) between '−vitamin D' and '+vitamin D' samples.

Table 3. *Incorporation of* ^{14}C *from* [1-^{14}C]*acetate into carbon dioxide by spongiosa and cartilage from rachitic rats*

The tissue slices were obtained from the tibiae of rats given a rachitogenic diet. The administration of vitamin D to rats and the incubation medium are described in the text. Slices (80–100 mg.) were added to each flask and the contents shaken under O_2+CO_2 (95:5) at 37° for 30 min. Results are given for rachitic rats ('−vitamin D') and for rachitic rats that had been given vitamin D ('+vitamin D'). The results are given as means ± S.D. for six samples.

	^{14}C incorporated (counts/min./mg. wet wt. of tissue)	
	−Vitamin D	+Vitamin D
Spongiosa	285±38*	238±32*
Cartilage	136±33†	108±25†

* $P < 0.02$ for the difference between '−vitamin D' and '+vitamin D' samples.
† $P < 0.10$ for the difference between '−vitamin D' and '+vitamin D' samples.

after the administration of vitamin D. It also provides strong evidence that vitamin D does not increase the synthesis of citrate from acetate, as had been suggested previously (Dikshit *et al.* 1956; Joshi *et al.* 1957; Meyer *et al.* 1959).

The exact role of citrate in the action of vitamin D on mineral metabolism is not known. Carlsson & Hollunger (1954) postulated that local concentrations of citrate induced by vitamin D may be responsible for the stimulation by vitamin D of the mobilization of bone minerals. Such a mechanism has also been suggested by Neuman & Neuman (1958) for parathyroid hormone. However, work by Guroff, DeLuca & Steenbock (1963) and by Harrison, Harrison & Park (1958) has shown that the elevation of extracellular citrate concentrations by vitamin D can be prevented by vitamin deficiency or by cortisol without interfering with the action of vitamin D on calcium and phosphate metabolism. It therefore appears that the action of vitamin D on citrate is not the primary action, but may be a consequence of its action on subcellular membrane function. Such an explanation is suggested by recent work on the binding and release of calcium by subcellular fractions from normal and vitamin D-deficient rats (DeLuca & Engstrom, 1961; Engstrom & DeLuca, 1962).

SUMMARY

1. A method for the extraction and chromatographic separation of organic acids of bone has been developed.

2. Slices of epiphysis, cartilage, spongiosa and bone shaft incubated with [1-^{14}C]acetate produce significant amounts of radioactive citrate, succinate, malate, α-oxoglutarate, lactate, glutamate and an unidentified acid.

3. The administration of vitamin D to rachitic rats before they were killed resulted in an increase in the amount of radioactive citrate, a decrease in the amount of radioactivity in the other organic acids and a decrease in the amount of $^{14}CO_2$ from the [1-^{14}C]acetate. These results demonstrate that vitamin D does not increase the synthesis of citrate from acetate in bone. Instead, they support the alternative hypothesis that the vitamin decreases the rate of conversion of citrate into subsequent intermediates in the tricarboxylic acid cycle.

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Studies on Human Casein Preparations from Single Milk Samples

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The reported values for the sialic acid content of human casein vary widely. Malpress (1961, 1962) has given a maximum value of 2·1 % for samples prepared from milk collected from the fourth to the eighth day after parturition, and a range of 0·30–0·38 % for samples from mature milk. Other values given for mature milk are 0·76 % (Johansson & Svennerholm, 1956), 0·8 and 0·37 % (Alais & Jollès, 1962*a*, *b*).

Discrepancies are also present in reports on the extent of rennin action on casein samples: Malpress (1962) found the release in combined form of 30 % of the total sialic acid by this enzyme; Alais & Jollès (1962*b*) obtained a glycopeptide, containing sialic acid, from some but not all of their casein preparations as the result of rennin action.

These variations could be the result of the different procedures used for preparing casein. Alternatively they might indicate true individual variations in caseins from different mothers, or from the same mother at different stages of lactation; the complexity of human casein shown by its heterogeneity on electrophoresis in the presence of urea (Malpress, 1962; Alais & Jollès, 1962*a*) suggests possibilities of variation in support of this alternative view.

The problem has now been further studied by an investigation of caseins, prepared by a standard procedure, from 80 single milk samples taken from 47 mothers and covering all stages of lactation.

MATERIALS AND METHODS

Preparation of casein. Milk samples (3–100 ml.) were stored at 2° and used for the preparation of casein within 1 week of collection. (In a few exceptional cases the samples were kept frozen for up to 1 month before use.) The milks were centrifuged at 10 000*g* at 5° for 15 min. to remove fat. The skimmed milks (pH 6·5–7·5) were filtered through cotton wool, adjusted to pH 4·7 with HCl and kept at 2° overnight; they were then warmed to 25° for 30 min. (cf. Maeno & Kiyosawa, 1962) and centrifuged at 35 000*g* at 10° for 30 min. The precipitated caseins were dispersed and recentrifuged twice in 0·01 M-acetate adjusted to pH 4·7 with N-NaOH. The volume of the skim-milk supernatants was measured; they were then filtered through cotton wool and retained for the estimation of 'non-casein sialic acid'.

The washed casein precipitates were dissolved in water with minimum amounts of 0·1 N-NaOH, care being taken to keep the pH less than 10, and the solutions (50–100 ml.) were filtered through a cotton-wool plug and adjusted to pH 4·7 with HCl. After storage overnight at 2° the caseins were collected by centrifuging at 15 000*g* at 10° for 15 min. They were washed twice with ethanol–ether (1:1, v/v) and twice with ether, and dried in air.

In the present paper 'casein' is defined as the protein prepared from human milk samples by the foregoing method, and 'mature casein' refers to the preparations derived from milk taken on or after the tenth day of lactation.

Starch-gel electrophoresis. This was carried out at pH 8·6 by using the discontinuous tris–citrate–borate buffer system of Poulik (1957) in the presence of 7 M-urea as described by Wake & Baldwin (1961). All analyses were made at 2° in a