Summary

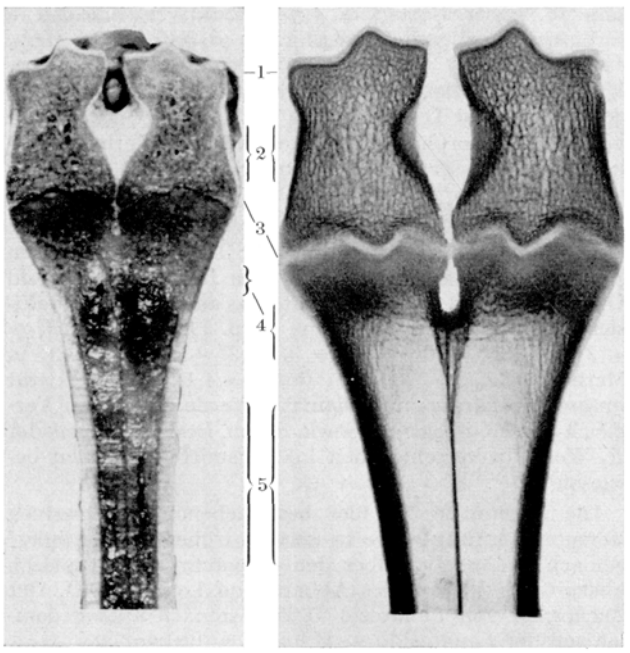
The selective enzymatic degradation of lanatoside-D yields acetyl-diginatin-α, the mild alkaline hydrolysis desacetyl-lanatoside-D. The properties of these glycosides are described; both can be transformed by further degradation into diginatin.

Pyrophosphatases of Bone

Scarce and discordant information is found in the literature as to the presence of pyrophosphatases in ossifying cartilage and bone, although, according to some authors[1], inorganic pyrophosphate seems to play a role in bone formation.

In the present work, we have thought it of interest to study the distribution and properties of pyrophosphatases in the various parts of bone tissue.

Experimental and Results. The anterior metatarsum of a less than 30 days old lamb was excised immediately after the killing of the animal, cooled at – 10°C and freed from the soft parts and periosteum. From this sample, a section of which is shown in Figure 1, the following histologically uniform fractions were prepared: (1) Articular cartilage, (2) Spongiosa of epiphysis, (3) Epiphyseal cartilage plate, (4) Metaphyseal spongiosa, corresponding to newly ossified cartilage, and different from the spongiosa of epiphysis, (5) Compact bone, (6) Bone marrow of diaphysis, (9) Periosteum. Fractions 2 and 4 were centrifuged so as to extrude the marrow contained between the trabeculae, which was separately examined as fractions, (7) Marrow from No. 2, and (8) Marrow from No. 4.



A *B*

Fig. 1.—Photograph (*A*) and microradiograph (*B*) of a section of lamb's anterior metatarsum.

1 = Articular cartilage, 2 = Spongiosa of epiphysis, 3 = Epiphyseal cartilage plate, 4 = Spongiosa of metaphysis, 5 = Compact bone.

1 g of each fraction was added with 5 ml of water and, when necessary, with traces of quartz sand, and homogenized in a mortar. The final volume of the homogenate was adjusted to 24 ml. 0·4 ml of the homogenate were incubated with 0·3 ml 0·15 *M* $Na_4P_2O_7$, 0·1 ml 0·005 *M* $MgCl_2$, 0·5 ml 0·0356 *M* diethylbarbiturate buffer of the desired pH. The final volume was brought to 1·5 ml with H_2O. The pH was checked before and after incubation. Controls were made without addition of the enzyme and of the substrate.

The samples were incubated for 8 min at 38°C with continuous stirring, and the reaction was stopped by heating for 3 min at 100°C or by addition of 1 ml 15% trichloroacetic acid (TCA). After centrifuging, inorganic orthophosphate was determined on an aliquot of the supernatant by the method of FISKE and SUBBAROW[2].

The activity has been assayed over all the pH range from 3·5 to 10·3. Results are given in Figure 2 and 3. No activity was found in the marrow of epiphyseal spongiosa and in periosteum.

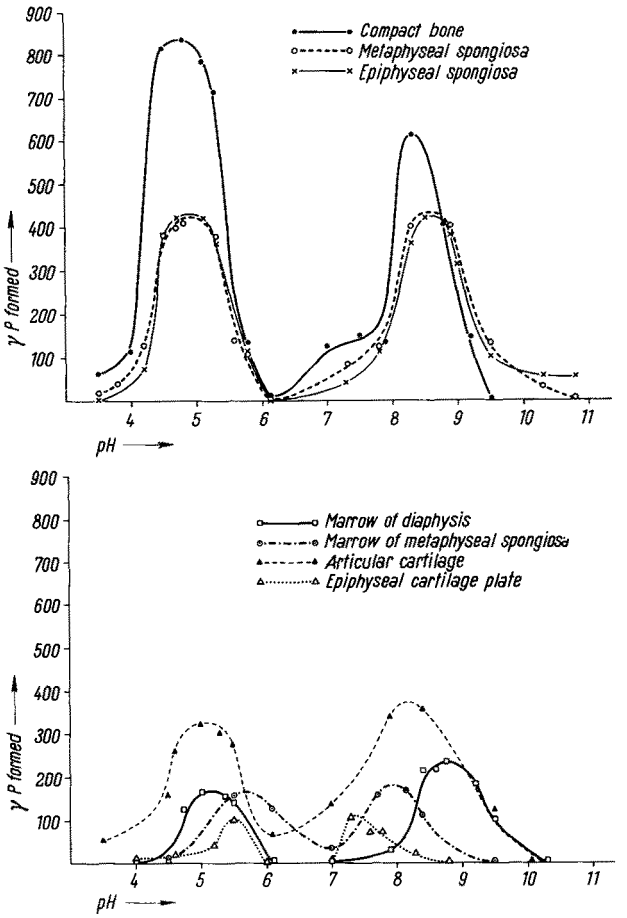


Fig. 2 and 3.—Pyrophosphatase activity at different pH of the various components of bone.

Ordinates give μg orthophosphate (calculated as P) formed in 8 min at 38°C from 2·8 mg P added as $Na_2P_4O_7$. The incubation medium contained also 16·6 mg tissue (wet weight), 0·1 ml 0·005 *M* $MgCl_2$, 0·5 ml 0·0356 *M* diethylbarbiturate buffer of the desired pH. Final volume 1·5 ml with H_2O.

Discussion. Periosteum and the marrow of epiphyseal spongiosa do not show pyrophosphatase activity over all the pH range tested. All the other fractions show two

[1] P. CARTIER and J. PICARD, Bull. Soc. Chim. biol. *37*, 1169 (1955).

[2] C. N. FISKE and Y. SUBBAROW, J. biol. Chem. *66*, 375 (1925).

optima, one at acid and one at alkaline pH. This corresponds to what was found for liver pyrophosphatases by Bo NORBERG[3]. Both optima of articular cartilage and epiphyseal cartilage plate are slightly nearer to neutrality than those of bone and marrows. The absolute amounts of orthophosphate produced by the various bone constituents are quite different. The lowest activity is shown by the epiphyseal cartilage plate, although the presence of a pyrophosphatase is clear. This is in agreement with PERKIN's[4] observation that in rabbits the pyrophosphatase activity in the epiphyseal plate is lower than in the metaphysis. We therefore cannot confirm the data of CARTIER and PICARD[1] who found no pyrophosphatase in the epiphyseal cartilage plate. Moreover, since a considerable activity in the newly formed bone of metaphysis has also been found by these authors, their negative finding in cartilage, unless due to the different age of the animals used (sheep embrios in their experiments), is probably to be ascribed to a lower sensitivity of the methods they employed. A moderate increase of activity in compact bone and the two spongiosas was observed when $MgCl_2$ was omitted from the incubation medium. This result agrees with LIEBKNECHT's[5] data on bone phosphatase.

P. CERLETTI, P. L. IPATA, and G. TANCREDI

National Research Council Unit for Enzyme Studies, Rome and Institute for Clinical Orthopaedics, University of Perugia (Italy), August 4, 1958.

Riassunto

Si è studiata l'attività pirofosfatasica, de terminandone il pH ottimale, in 9 frazioni scheletriche istologicamente uniformi: cartilagine articolare e cartilagine di coniugazione, osso spongioso epi e metafisario, osso compatto, midollo diafisario, midollo dell'osso spongioso epifisario e dell'osso spongioso metafisario, periostio.

[3] Bo NORBERG, Acta chem. scand. *5*, 325 (1951).
[4] H. R. PERKINS, Biochem. J. *57*, XV P (1954).
[5] W. L. LIEBKNECHT, Biochem. Z. *303*, 96 (1939).

Serum Cholesterol Levels in Germ-Free Chickens

The major products of cholesterol catabolism are the bile acids[1]. The role of intestinal bacteria in the turnover of bile acids[2] plus the findings that, under certain conditions, sulfa drugs[3] or antibiotics[4] may alter serum cholesterol levels in cholesterol-fed animals has led us to investigate the serum cholesterol levels of germ-free chickens as a base line for subsequent work.

White Leghorn Chickens were used. They were reared by our own modification[6] of the method of REYNIERS *et al.*[6]. 18-day old embryonated eggs held in a nylon net were immersed for 2 min in a 0·15% (W/V) detergent solution[7] and then for 12 min in a 2% mercuric chloride solution at 37°C. From this bath the eggs were transferred through a tube without exposure to nonsterile air into a previously sterilized germ-free unit. Control eggs from the same hatch were treated in a similar fashion before being transferred to an incubator. Both groups of chicks, germ-free and conventional, were fed an autoclaved semisynthetic diet[8]. The germ-free chickens were given canned sterilized water[9,5] while the conventionally reared birds were given tap water.

The sterility of the chickens in the germ-free units was determined by taking frequent fecal samples and inoculating them into various bacteriological media according to a standard procedure which has been described previously[5].

At the termination of the experiment, the chickens were killed by exsanguination under ether anesthesia and blood samples taken by heart puncture. Sera were analyzed for cholesterol by the method of Trinder[10].

Two groups of birds aged 6 and 8 weeks respectively were studied and the average results (with standard deviations) are tabulated in the Table.

It is evident that while the germ-free chickens showed a better growth rate than the controls confirming earlier findings[5], there were no significant differences in the serum cholesterol levels of the two groups. These data

[1] M. D. SIPERSTEIN and A. W. MURRAY, J. clin. Invest. *34*, 1449 (1955).
[2] B. E. GUSTAFSSON, S. BERGSTROM, S. LINDSTEDT, and A. NORMAN, Proc. Soc. exp. Biol. Med. *94*, 467 (1957).
[3] O. W. PORTMAN, E. Y. LAWRY, and D. BRUNO, Proc. Soc. exp. Biol. Med. *91*, 321 (1956).
[4] D. KRITCHEVSKY, W. C. GRANT, M. J. FAHRENBACH, B. A. RICCARDI, and R. F. J. MCCANDLESS, Arch. Biochem. Biophys. *75*, 142 (1958).
[5] M. FORBES and J. T. PARK, J. Nutrition, in press.
[6] J. A. REYNIERS, P. C. TREXLER, R. F. ERVIN, M. WAGNER, T. D. LUCKEY, and H. A. GORDON, Lobund Reports *2*, 70 (1949).
[7] PN-700 Conditioner, Service Industries, Phila., Pa.
[8] Diet (per 100 g): Cornstarch, 57·0 g; Casein (purified), 25·0 g; Corn oil, 5·0 g; Alphacel, 3·0 g; Glycine, 1·5 g; L-Arginine-HCl, 1·0 g; DL-Methionine, 0·5 g; Choline-HCl, 0·27 g; Thiamine-HCl, 0·1 g; Ca Pantothenate, 10·0 mg; Nicotinic acid, 10·0 mg; Riboflavin, 4·0 mg; Pyridoxine HCl, 2·0 mg; Folic acid, 1·0 mg; Vitamin B_{12}, 0·005 mg; Biotin, 0·1 mg; Menadione, 0·8 mg; Vitamin A, 2,600 IU; Vitamin D_3, 100 ICU; a-Tocopherol, 5 mg; $CaCO_3$, 2·5 g; K_2HPO_4, 1·72 g; Na_2HPO_4 1·40 g; NaCl, 0·5 g; $MgSO_4 \cdot 7\ H_2O$, 0·45 g; $MnSO_4 \cdot H_2O$, 30 mg; $FeSO_4$, 12 mg; $CuSO_4$, 1 mg; $CoCO_3$, 1 mg; $ZnSO_4 \cdot 7\ H_2O$, 1 mg; KI, 0·3 mg.
[9] MacDonald-Bernier Co., Boston, Mass.
[10] P. TRINDER, Analyst *77*, 321 (1952).

Table

Group	No.	Sex M	Sex F	Weight (g)	Serum Cholesterol Mg %	(Range)
Six-Week Experiment						
Germ-free	10	2	8	540 ± 70	107 ± 25	(63–149)
Conventional	11	4	6	487 ± 33	120 ± 21	(86–168)
Eight-Week Experiment						
Germ-free	5	4	1	807 ± 48	144 ± 20	(108–170)
Conventional	5	3	2	579 ± 57	149 ± 17	(135–179)