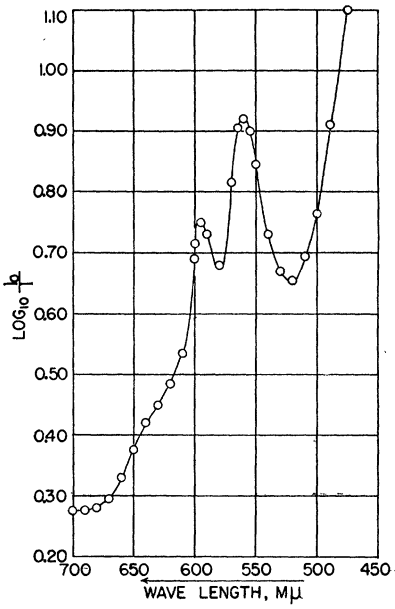

FIG. 2. Absorption spectrum of lyophilized catalase, Kat. f. = 14,000 (same sample as used for △, Fig. 1), after reduction with $Na_2S_2O_4$: 26.4 mg in 4 ml of M/10 phosphate buffer, pH 8.0.

had not been lyophilized, whereas M/10 phosphate buffer of pH 8.0 without NaCl was used as solvent for the lyophilized material. NaCl was omitted where it was not needed to dissolve the material, because it inevitably caused the gradual formation of a precipitate in the solution. Moreover, the curves were not adjusted so as to coincide at any particular point, as they were in the earlier work.

Fig. 2 shows the absorption spectrum of the lyophilized catalase sample which had been reduced with $Na_2S_2O_4$. In this case, also, the solvent was M/10 phosphate buffer of pH 8.0, without NaCl. A two-banded spectrum is obtained which is characteristic of ferroheme compounds. It seems certain that the catalase has really been reduced, since treatment of lyophilized catalase with sodium boron hydride, $NaBH_4$, gradually causes the production of a spectrum with bands at the same location as those observed with hydrosulfite reduction. Moreover, if carbon monoxide is passed into a solution of lyophilized catalase which has been reduced with hydrosulfite, the bands are shifted from 595 mμ and 560 mμ to approximately 576 mμ and 544 mμ.

It can be seen from Fig. 2 that the amount of residual unaltered catalase left over after reduction with hydrosulfite must be very low, as judged by the smallness of the bulge in the curve at about 640 mμ. In spite of this, the Kat. f. of the lyophilized material was approximately 14,000 as compared with a Kat. f. of about 23,000 before lyophilization. Thus a major portion at least of the lyophilized catalase must be active, and no more than 5%, or at most 10% of the lyophilized material can be in the form of undenatured catalase. The only way to escape the conclusion that the lyophilized, reducible catalase is active would be to assume a great enhancement in activity of the small portion of catalase that may have escaped change during lyophilization. Even if the Kat. f. of this material were doubled, the activity of the lyophilized sample could not be explained in this manner. We feel quite safe, therefore, in reiterating our original conclusion (*1*) that lyophilized beef liver catalase is changed in some way so as to render it reducible. As stated previously (*1*), completely heat-denatured catalase behaves in quite a different manner.

The most likely explanation of the behavior of lyophilized catalase reported here and previously (*1*) is that a mild sort of denaturation has loosened the attachment of the iron atom to the protein part of the molecule so that the iron becomes reducible but still remains active catalytically. A detailed investigation of this phenomenon would be worth while to someone working on the mechanism of catalase action or on the phenomenon of protein denaturation. For the benefit of any such investigator, it should be noted that the flask in which the enzyme is being lyophilized must be kept in the air during evaporation of the water and not in a cooling bath. If a cooling bath of ice and water is used, the enzyme can be lyophilized without any apparent change in its behavior.

The Kat. f. values for our recrystallized catalase samples are somewhat lower than usual but this fact does not alter the arguments presented.

References

1. DOUNCE, A. L. and HOWLAND, J. W. *Science*, 1943, **97**, 21.
2. KEILIN, D. and HARTREE, E. F. *Biochem. J.*, 1945, **39**, 148.
3. SUMNER, J. B. *Adv. Enzymol.*, 1941, **1**, 163.
4. VON EULER, H. and JOSEPHSON, K. *Liebig's Ann. der Chem.*, 1927, **452**, 158.

Methods of Preparing Bone and Tooth Samples for Viewing in the Electron Microscope[1]

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One of the main characteristics of bone is its resistance to bending, torsion, compression, and tensile stresses. This resistance is offered by a combination of

[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

[2] Grateful acknowledgment is made for technical assistance to Mrs. Bessie L. Jacobson, research associate, The University of Rochester Atomic Energy Project, in making the electron micrographs; and to Miss B. Kindig, of the Research Laboratories of the Eastman Kodak Company, for the ultrasonic dispersion of the various preparations.

mineral and organic substances. The inorganic particles have not been seen hitherto.

Since the advent of the electron microscope the range of possible visualization has increased to the 20-to-100-A range, in which the individual inorganic units of bone should be seen. Henschen (*5*, *6*) stated that bone in the mineral phase exists as crystals 2×10^{-6} to 2×10^{-8} cm long. Cagliotti (*4*) suggested 10^{-6} cm as the crystal length. (Bale, Hodge, and Warren [*1*] postulated that the crystal size in dentine is 2.4×10^{-6} cm and in enamel 2.7×10^{-5} cm. Moeller and Troemel [*7*] gave 10^{-4} cm as the size of enamel particles and 10^{-5} to 10^{-6} cm as dentine particle size.)

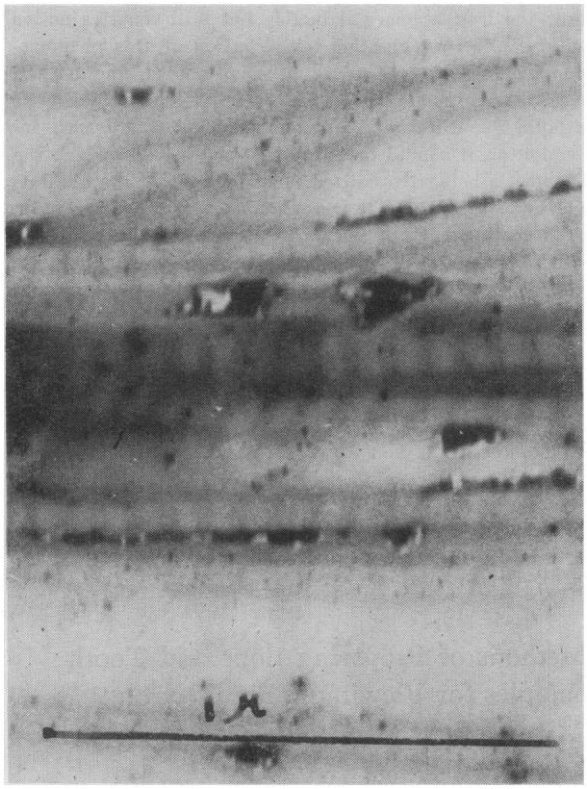

FIG. 1. Micrograph of fresh tendon, agitated in chemically pure water in Waring Blendor for 10 min and subjected to ultrasonics at 400 kc for 10 min. Magnification × 62,500.

During the past seven months satisfactory methods of preparing bone samples for the electron microscope were developed by the authors. Three methods were found most useful:

(1) Fresh cortical bone is cleaned and then scraped with a sharp knife. The scrapings are placed in a Waring Blendor in 100 ml of chemically pure distilled water (in ampules). This sample is blended for 10–15 min and then poured into a meticulously cleaned test tube. The fluid appears gray and cloudy, owing to a fine dispersion of the bone scrapings. A drop of this fluid is placed on an electron microscope specimen screen which was previously coated evenly with silicon (*3*). Similar treatment of tendon shows that it leaves collagen fibers intact and that striations at 640 A, as described by Schmitt (*8*), are clearly seen. Therefore, this treatment should not destroy the collagen in bone (Fig. 1).

(2) Bone is cleaned and autoclaved at 27-lb pressure (269.8° F) for 2 hr (*4*). The bone is then washed in distilled water and placed in the Waring Blendor in 100 ml chemically pure distilled water for 10–15 min. The

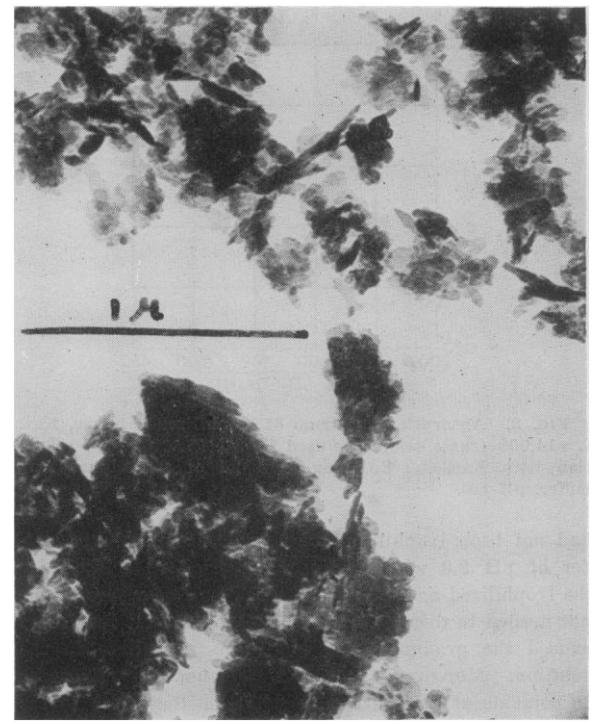

FIG. 2. Micrograph of cortical bone, autoclaved 2 hr at 27 lb (270° F), agitated in Waring Blendor for 15 min. Magnification × 34,500.

organic matter having been largely removed, the collagen fibers are not seen in these samples. The fluid appears cloudy gray and a drop of it is placed on a specimen screen, previously silicon-coated. The micrographs obtained are uniformly clean and, when properly focused, the crystal units of the bone are plainly seen, most of the crystals remaining in clumps of characteristic appearance (Fig. 2).

(3) Samples obtained by the two preceding methods are subjected to ultrasonics, approximately 400 kc, for 5, 10, and 15 min (*2*). In our experience, collagen so resonated for more than 10 min appears to disintegrate. Therefore, when attempting to observe collagen fibers in bone, the sample is resonated for shorter periods but when inorganic crystals are to be observed, longer periods (15 min) have been used.

The best preparation for viewing the inorganic portion of bone is obtained by autoclaving clean bone for 2 hr, blending for 20 min, and finally resonating for 15 min. By these procedures a fine, clean dispersion of the crystalline units of inorganic bone is obtained for observation (Fig. 3). Cancellous bone so treated produced

fair preparations but it was more difficult to find clean fields, probably due to the larger amount of organic material present. Deciduous teeth were crushed, autoclaved, blended, and resonated. Glycol-ashed bone was blended and subjected to ultrasonics. Adequate micrographs were obtained in both instances. Bone thus prepared shows the picture of normal bone in the x-ray diffraction pattern.

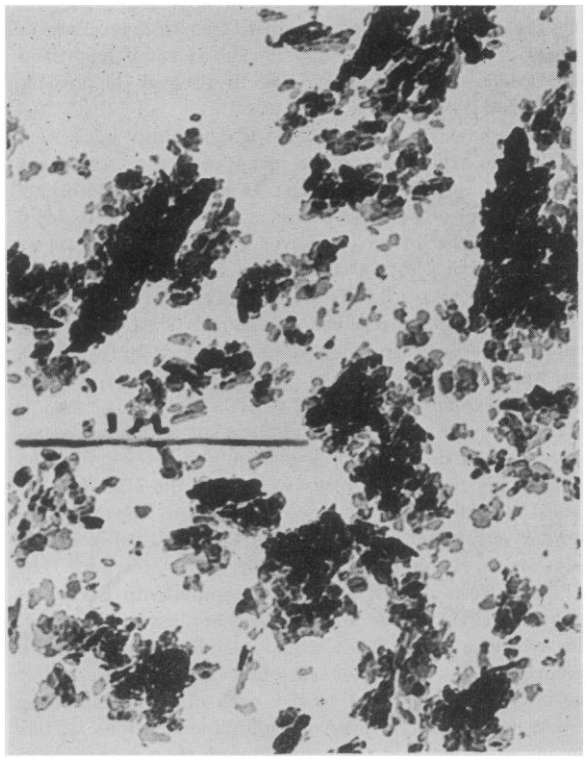

FIG. 3. Micrograph of cortical bone, autoclaved 2 hr at 27 lb (270° F), agitated in Waring Blendor for 15 min, and subjected to ultrasonics at 400 kc for 15 min. Magnification × 34,500.

These methods are being used to investigate the submicroscopic structure of bone. A paper is being prepared which will detail the findings, interpretations, and conclusions obtained from electron micrographs of material prepared by these methods.

References

1. BALE, W. F., HODGE, H. C., and WARREN, S. L. *Amer. J. Roentg. rad. Therap.*, 1934, **32**, 369.
2. BERGMAN, LUDWIG. *Ultrasonics and their scientific and technical application.* Translated by Hatfield, H. S. New York: John Wiley, 1938. Pp. 202–26.
3. BISHOP, F. W. *Rev. sci. Instr.*, 1949, **20**, 529.
4. CAGLIOTTI, V. *Atti Congr. Nazionale Chim. Pura Appl.*, 1935, 325.
5. HENSCHEN, H. "The Crystalline Structure of Bone." Lectures, University of Manchester, England, 1936.
6. ———. *Schweiz. med. Wschr.*, 1937, **67**, 153.
7. MOELLER, V. H. and TROEMEL, G. *Naturwiss.*, 1933, **21**, 346; quoted in REYNOLDS, L. *et al. Amer. J. Roentg.*, 1938, **39**, 103.
8. SCHMITT, F. O., HALL, C. E., and JAKUS, M. A. *J. cell. comp. Physiol.*, 1942, **20**, 11.

Arrest of Development of *Plasmodium gallinaceum* in Mosquitoes (*Aedes aegypti*) by Radiation Effect of P^{32} [1,2]

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During the course of experiments on the radioactive tagging of sporozoites of the avian malaria parasite *Plasmodium gallinaceum*, it was observed incidentally that ingestion of P^{32} by host mosquitoes during the period of extrinsic incubation caused development of the parasite to be arrested. The present report deals with this observation.

One hundred fifty *Aedes aegypti* were allowed to engorge on chicks that were infected with *P. gallinaceum* and that showed many gametocytes in their peripheral blood. Thereafter these mosquitoes were provided daily with 5% glucose solution containing radioactive sodium acid phosphate, the total radioactivity approximating 30 mc. This material was soaked in a cotton pledget which was moistened occasionally with water. One hundred other mosquitoes, exposed to infection at the same time and in the same way, were subsequently provided with glucose solution only. Radioactivity measurements were made on a counting-rate meter (*1*) employing a thin mica window, bell-type Geiger-Müller tube.

Nine days following the blood feeding, ten mosquitoes from each group were dissected for determination of the presence of oöcysts in their stomachs; all ten of those receiving glucose and P^{32} contained oöcysts, as did eight of those receiving glucose alone (see Table 1). No difference in average numbers or stage of development of oöcysts was noted in the two groups of mosquitoes. Five days later, mosquitoes from each group were dissected to determine the presence of sporozoites in the salivary glands. No sporozoites were found in 93 mosquitoes given P^{32}, whereas 11 out of 15 given glucose alone showed these forms (see Table 1).

Measurements of radioactivity were made on the salivary glands of 87 of the mosquitoes given P^{32}. These measurements were corrected for decay to the time of first feeding of the P^{32} glucose solution. The lowest activity for a salivary gland was 334 cpm, the highest was 14,000 cpm, and the average of the 87 was 2,400 cpm. In the absence of a reliable figure for the average amount of tissue involved in the salivary glands, it is difficult to

[1] This study was carried out at the suggestion of Dr. Robert Briggs Watson, then of the Tennessee Valley Authority, who arranged for the partial financial support from that source. Miss Lois Seamans, of the Tennessee Valley Authority, performed many of the final salivary gland dissections.

[2] The radioactive phosphorus (P^{32}) used in this study was prepared in the cyclotron of the Massachusetts Institute of Technology.