into a four-membered ring ether, as well as by the fact a cyclic benzylidene acetal only formed from ecgoninol and not from *pseudo*-ecgoninol.

The third logical step for establishing the configurations of the ecgonines consisted of a direct proof of the position of the C_2-$COOCH_3$ group in cocain to the ring nitrogen. This was realized by intramolecular cyclization of N-carbamyl-*nor*-cocain into an ureide.

The configuration of cocain as 2β-carbomethoxy-3β-benzoxy-tropane, and that of psicain as 2α-carbomethoxy-3β-benzoxy tropane, are therefore unequivocally proved. For the "third racemate" of ecgonine, the structure of 2α-carboxy-3α-hydroxy-tropane has been predicted, based upon its ready dehydration due to *trans*-elimination. The way to the synthesis of the two hitherto unknown diastereomers of cocain is now disclosed.

In order to establish the configurations of the tropane alkaloids carrying oxygen function(s) at the ethylene bridge (scopolamine, oscine, valeroidine, meteloidine) theoretical deductions, as well as a new method, have been adopted.

(a) The ready conversion of scopolamine into oscine was interpreted in terms of an internal rearward nucleophilic attack of the *anti*-placed C_3-OH group toward the *syn*-placed epoxide ring at $C_{6,7}$. As a control of this deduction, hydrogenolysis of scopolamine into dl-3,6-dihydroxy-tropane has been achieved; the levorotatory form of the latter proved identical with the alkamine from valeroidine.

(b) All the alkaloids mentioned have at $C_{6(7)}$ *syn*-placed oxygen function(s) to the ring nitrogen as indicated by the use of a new method. The tertiary bases were converted by means of ethyl iodoacetate into the quaternary salts of the lactones derived from N-carboxymethyl-oscine, 3,6-dihydroxy-tropane and teloidine, respectively. These intramolecular bridge formations supply unambigous evidence for the neighbourhood of the $C_{6(7)}$ OH groups to the ring N or, more strictly speaking, to the unbound electron pair of this atom.

The selective and "reverse" quaternerization of tropines has been interpreted in terms of the Pitzer effect of the ethylene bridge upon the N-CH_3 group, which favours the position of this group as inclining to the piperidine ring. The quantummechanical concept of the tetrahedral valency orientation of tricovalent nitrogen was taken into consideration.

It is hoped that the investigations reported may help to make possible a more profound analysis of the mode of action of tropane alkaloids of different steric structure on the appropriate bioreceptors. Perhaps the shape of prosthetic groups of some of these receptors may also be visualised on the basis of the knowledge of the morphology of these reactants.

Brèves communications - Kurze Mitteilungen Brevi comunicazioni - Brief Reports

Electron Microscopy of the Bone Ground Substance Using the Pseudo-Replica Technique

The ultrafine morphology of the bone ground substance and its relationship to the inorganic or crystalline fraction, has been so far incompletely investigated by electron microscopy. The problem has already been approached by one of us (ASCENZI[1], ASCENZI and BENEDETTI[2]) observing bone tissue treated in the Waring blendor.

In order to bring a more adequate contribution to this subject, a specific study has been carried out using the pseudo-replica technique (see WYCKOFF[3]) so that a very thin layer of material is directly investigated.

Material and method.–From ox femur diaphysis (fresh or fixed in 10% formol solution), rectangular samples (5 × 1 × 1 cm) were prepared, the maximal dimension of which being orientated parallel to the longitudinal axis of the bone.

The pseudo-replicas were prepared as follows:

(1) One surface of the sample was highly polished.

(2) The surface was etched either with a 1% HNO_3 solution, or with a 0·5% trypsin solution, pH 8·5, at 37°C. The optimal time was 10 s for HNO_3 and 45 min for trypsin.

(3) The surface was washed with distilled water and allowed to dry.

(4) The surface was metal-shadowed by oblique evaporation of chromium.

(5) A Formvar solution (500 mg Formvar in 100 ml dioxan) was dropped on to the surface and the excess liquid was drained off. Then the film was allowed to dry thoroughly.

(6) Some isolated drops of gelatine solution (10 g gelatine in 80 ml water) were placed on the formvar film and allowed to dry. The shrinking of the dried gelatine drops facilitated the stripping off of the pseudo-replica.

(7) The pseudo-replica was stripped off at the level of the gelatine drops using a very fine knife.

(8) The pseudo-replica together with the gelatine drops was then floated (replica side up) on hot distilled water at 60°C. In this way only the gelatine drops were removed.

[1] A. ASCENZI, Rendic. Ist. super. San. *12*, 893 (1949).

[2] A. ASCENZI and E. L. BENEDETTI, Arch. Sci. biol. *38*, 234 (1954).

[3] R. W. G. WYCKOFF, *Electron Microscopy, Technique and applications* (Interscience Publ., New York, 1949).

(9) Finally the pseudo-replica, floating face up, was mounted on the supporting disc of the electron microscope.

For examination of unetched bone surface, the second and third steps were omitted.

We obtained somewhat sharper structural details using KOSSA's method of staining after step 3, the bone surface being etched with HNO_3 solution.

The pseudo-replicas were examined under the electron microscope of the "Istituto Superiore di Sanità" in Rome.

Results. (A) In pseudo-replicas of unetched bone, the calcified ground substance has a poorly granulated structure (see also ROUILLER, HUBER, and RUTISHAUSER[1]). No crystalline features can be observed (see also RUTISHAUSER and MAJNO[2]).

(B) In pseudo-replicas obtained from bone previously etched with HNO_3 (Fig. 1), the ground substance is made up of small globules or particles apparently spherical in shape. The particles are frequently arranged either end to end, building up single fibres, or side to side, forming a two or three-dimensional network. It is possible to demonstrate a relationship between the globules of the ground substance and the collagen fibres. In Figure 2

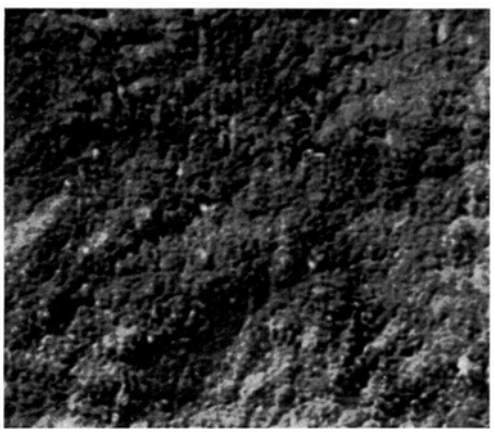

Fig. 1.–Magnification, 36,000 ×.

(left and downwards) the globules appear aligned in agreement with the collagen period. The diameter of single globules ranges from 200 to 250 Å.

The above-mentioned features of the ground substance can also be observed in preparations of decalcified bone treated in the Waring blendor (ASCENZI and BENEDETTI[3]).

(C) Pseudo-replicas prepared from bone previously digested by trypsin show a structure consisting of a very fine framework (Fig. 3*A*). The meshes are irregular in shape and delimit spaces from 200 to 250 Å in diameter. The thickness of the single meshes is about 150 Å. This particular structure can be regarded as that of the inorganic bone fraction obtained after removal of the ground substance by trypsin digestion. No evident crystalline features could be detected anywhere.

The pseudo-replica pattern is very similar to the structure of the trypsin-digested bone, directly observed under the electron microsope. In fact, Figure 3*B* shows a thin fragment of digested bone which was accidentally removed from the sample in stripping off the replica; its structure has the appearance of a very fine framework delimiting round spaces. The latter can be regarded as the spaces previously occupied by the spherical granules of the ground substance.

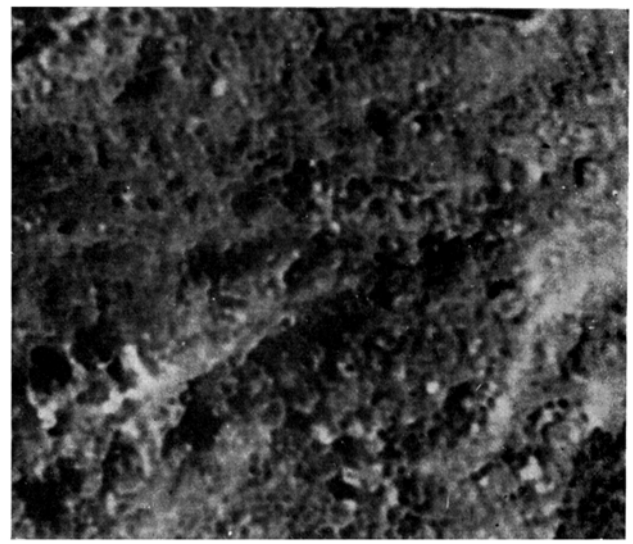

Fig. 2.–Magnification, 42,000 ×. The preferential oblique orientation (from above to bottom and from left to right) of the organic ground substance appears to correspond to the collagen period.

(D) Pseudo-replicas obtained from bone digested by trypsin and stained with KOSSA's method, demonstrate a very fine framework, similar in every respect to that of pseudo-replicas from unstained bone but somewhat sharper.

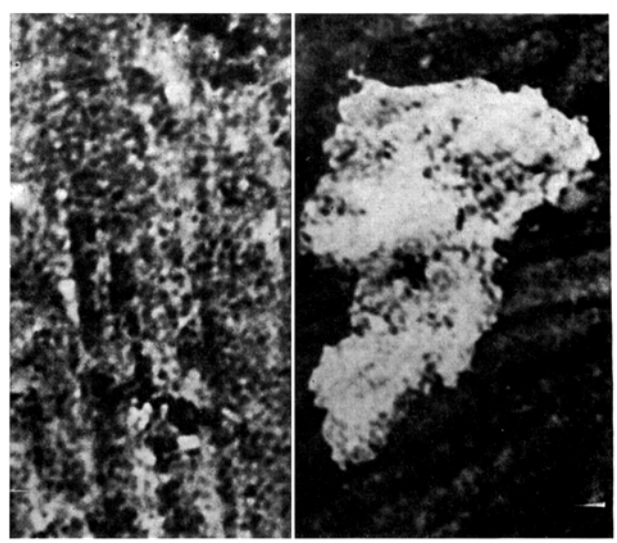

Fig. 3.–Magnification, 40,000 ×.

Conclusion.–With the pseudo-replica method, we have shown that the calcified ground substance of bone has a granular structure due to the organic ground substance enclosed in the meshes of a very fine framework deriving from the inorganic fraction. The granules of organic

[1] CH. ROUILLER, L. HUBER and E. RUTISHAUSER, Acta anat. *16*, 16 (1952).

[2] E. RUTISHAUSER and G. MAJNO, Bull. Hosp. Joint. Dis. (H. L. JAFFE, anniversary volume) *12*, 468 (1951).

[3] A. ASCENZI and E. L. BENEDETTI, Arch. Sci. biol. *38*, 234 (1954).

ground substance show an orientation which corresponds to the period of collagen fibres.

A. ASCENZI and A. CHIOZZOTTO

Institute of Anatomia Patologica, University of Rome, and Istituto Superiore di Sanità in Rome, August 26, 1954.

Résumé

Les auteurs ont étudié au microscope électronique, au moyen de la technique de la pseudo-empreinte, la structure de la substance fondamentale calcifiée d'os à structure lamellaire (fémur de bovidé). Ils ont pu ainsi démontrer que cette substance est formée de globules de nature organique, enclavés dans un fin réseau inorganique. Le diamètre des globules, ainsi que celui des mailles du réseau inorganique, est de l'ordre de 200–250 Å. Les globules organiques sont alignées parallèlement aux fibrilles collagènes.

The Exchange of Bone Calcium with Ca^{45} [1]

Introductory Background. (1) It is well established that KOH-glycol ashed bone will exchange calcium ions when suspended in a calcium solution tagged with Ca^{45} [2]. When equilibrium is obtained 14–15% of the bone calcium is found to enter into the exchange reaction[3].

(2) Calcium phosphates with Ca/P weight-ratios ranging from 1·72 to 2·26 have been described as a series of defect pseudo-apatites with more or less calcium occupying lattice positions[4]. It is possible to fill these missing positions by suspending the material in lime solution[5]. These pseudo-apatites differ chemically and physically from true hydroxyapatite despite the fact that their X-ray diffraction patterns are similar. The compound with the greatest Ca/P (2·26) is similar to the principal inorganic component of bone.

If we prepare this calcium phosphate (Ca/P = 2·26) by suspending a defect pseudo-apatite in lime solution, the various types of calcium ions in the resulting solid are:

(a) the original 9 Ca ions stoichiometrically bound to the phosphates groups,

(b) the additional 1½ Ca ions included in some manner in the pseudo-apatite of Ca/P = 2·26,

(c) moreover, especially in the case of bone salts, some physically adsorbed Ca ions.

(3) Hydrochloric acid can preferentially dissolve the Ca ions bound to the carbonate in bone[1]. In the first stages of the acid action, the Ca/P ratio of the liquid phase is very high, decreasing slowly as the fundamental phosphate (Ca/P = 1·94) goes into solution. The action of HCl on bone is just the reverse of adding Ca from lime solution to a defect calcium phosphate as above. Unfortunately, this acid action is not quite selective and it is not possible to dissolve the calcium ions which fill the defects without destroying the fundamental tricalcium phosphate to some extent.

Experimental procedure. Combining the techniques of radioactive exchange and HCl attack, we were able to obtain information regarding the nature of exchangeable calcium in bone mineral. The starting material for our experiment was KOH-glycol ashed bone which was exposed for a month to $Ca^{45}Cl_2$ solution and then filtered and dried to constant weight at 105°C. The specific activity of this stock bone ash was $9{\cdot}48 \times 10^3$. Fractions of this material (500 mg) were suspended for 10 min in 25 ml of varying HCl solutions. The amount of HCl ranged from 0·2 mE to 6·25 mE. The amount of HCl sufficient fully to dissolve 500 mg of bone ash was 6·85 mE. After filtration and drying at 105°C, each sample was weighed; the calcium, phosphorus and specific activity were determined for both the liquid and residual solid phase. See Table for a tabulation of the results.

Results and discussion. The weight loss of the samples in HCl solution increased with the increase of HCl employed. As the HCl concentration increased, the specific activity of the liquid, very high for the small HCl quantities, decreased. However, the specific activity was always higher in the liquid than in the corresponding residual solid phase. For each experiment, the liquid phase always contained more calcium than required for 9 Ca per 6 P (Ca/P = 1·94). This excess represents the additionnal Ca ion content of bone structure. On the other hand, the Ca/P weight-ratio in the solid phase decreased slowly until it reached a value of 1·98.

Instead of regarding the ratio (counts/m)/total Ca, which decreases in both phases, we can calculate the ratio: (counts/m)/excess Ca. This turns out to be a constant value (61·9 ± 5·2) independent of the extent of HCl attack on the solid.

We feel that the Ca^{45} exchanged by bone mineral is exclusively the excess 1½ moles of calcium of the saturated pseudo-apatite. These calcium ions are not only on the surface, but are contained in the structure of the solid, for they are still present after severe HCl attack. This is supported by a recent experiment in this laboratory which studied the uptake of Ca^{45} by a defect pseudo-apatite of Ca/P = 1·94. The material was dried at 105°C and exposed to a solution containing *low* calcium concentrations. Only pseudo-apatites with a Ca/P greater than 1·94, such as bone, can exchange in the presence of small Ca concentrations.

The exchange reaction of the Ca in excess of 9 moles per unit cell in bone mineral is rapid, with ¾ of the equilibrium value being attained in 10 min and full equilibrium being attained in a few hours. Thus these excess Ca positions do not behave chemically in the same way as the original 9 Ca ions of the fundamental neutral phosphate.

[1] This work was sponsored by the *Air Research and Development Command*, United States Air Force, through its European Office: contract n° AF 61(514)-647 C, and the Centre Interuniversitaire des Sciences Nucléaires of Belgium.

[2] H. C. HODGE, Proc. Metabolic Interrelations Conference (J. Macy Jr. Fd., New York, 1949, p. 49. – W. F. NEUMAN, Proc. Metabolic Interrelations Conference (J. Macy Jr. Fd., New York, 1950), p. 32. – M. FALKENHEIM, E. E. UNDERWOOD, and H. C. HODGE, J. Biol. Chem. *188*, 805 (1951). – E. E. UNDERWOOD and H. C. HODGE, J. Dent. Res. *31*, *64* (1952). – W. MINDER and T. GORDONOFF, Schweiz. Med. Wschr. *63*, 825 (1953). – W. F. NEUMAN, Chem. Rev. *53*, *1* (1953).

[3] H. C. HODGE, Proc. Metabolic Interrelations Conference (J. Macy Jr. Fd., New York, 1949), p. 49. – M. FALKENHEIM, E. E. UNDERWOOD, and H. C. HODGE, J. Biol. Chem. *188*, 805 (1951).

[4] A. S. POSNER, C. FABRY, and M. J. DALLEMAGNE, Biochim. Biophys. Acta *15*, 304 (1954). – M. J. DALLEMAGNE, C. FABRY, and A. S. POSNER, J. Physiol. *126*, 18 (1954).

[5] C. FABRY, Biochim. Biophys. Acta *14*, 401 (1954); ibidem 1954 (in print).

[1] M. A. LOGAN and H. L. TAYLOR, J. Biol. Chem. *125*, 391 (1938). R. KLEMENT, Z. physiol. Chem. *184*, 132 (1929). – R. KLEMENT and G. TRÖMEL, Klin. Wschr. *12*, 292 (1933). – M. J. DALLEMAGNE and J. MELON, Arch. internat. Physiol. *58*, 188 (1950). – P. CARTIER, Bull. Soc. Chim. Biol. *30*, 65 (1948).