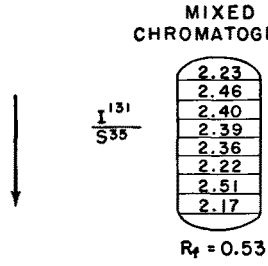
tryptamine was not reported by these authors, but one would expect it to be a precursor of the N-methylated derivatives. Isotopic and chromatographic techniques have måde it possible to demonstrate the presence of 5-hydroxytryptamine to the extent of about 0·1 per cent of the dry weight of *Bufo marinus* venom.

Approximately 2 g of dried venom obtained from 6 toads[1] were ground in a Potter glass homogenizer with a total of 400 ml of 0·02 N HCl in 75 per cent ethanol. The extract was evaporated *in vacuo* at 45° to about 80 ml and poured while still warm into 350 ml of 0·01 N HCl. After standing several hours to permit complete precipitation, the solids were removed by centrifugation and washed with 30–40 ml of 0·01 N HCl. The combined acid solution and washings were extracted twice with equal volumes of n-butanol and the butanol discarded.



Chromatogram of a mixture of S^{35}-labeled pipsyl derivative obtained from *Bufo marinus* venom and the I^{131}-labeled pipsyl derivative of an authentic sample of 5-hydroxytryptamine.

After cooling to 10–15°, the aqueous phase was adjusted with dilute NaOH to pH 10 and extracted three times with equal volumes of n-butanol. To the combined butanol extracts (about 1 l) was added 1 l of heptane. Water from the butanol layer separated out at this step. Three milliliters of 6 N HCl were added and the mixture shaken in a separatory funnel. The acid layer was withdrawn and the organic phase was re-extracted with 60 ml of water. The combined aqueous extracts were evaporated, under nitrogen, to approximately 10 ml, washed with an equal volume of n-butanol, and further evaporated to approximately 1 ml. The entire solution was deposited (as a line) across the tops of 2 sheets of WHATMAN No. 3 filter paper, and one dimensional chromatograms were developed using a mixture of butanol, propionic acid, and water in the proportions 5:2:3. The band of 5-hydroxytryptamine (R_f about 0·25) was located by means of its characteristic pink fluorescence in ultraviolet light. This fluorescent band was cut from each sheet and eluted with 0·1 N HCl. The eluate was adjusted to a pH of 8·95 and a volume of 8 ml and subjected to 24 transfer counter-current distribution[2] between n-butanol and 0·1 *M* borate buffer, pH 8·95. In this system, pure 5-hydroxytryptamine has a partition ratio of about 1. Tubes 8 to 16 which contained the bulk of material possessing spectral characteristics similar to 5-hydroxytryptamine were pooled and concentrated to about 2 ml. A half milliliter portion containing about 100 μg of apparent 5-hydroxytryptamine was treated with S^{35} labeled p-iodophenylsulfonylchloride (pipsyl chloride) to yield the corresponding derivative. An authentic sample of 5-hydroxytryptamine[3] was treated with I^{131} labeled pipsyl chloride forming the corresponding I^{131} labeled derivative. The S^{35} labeled material obtained from the toad venom and the I^{131} labeled derivative of pure 5-hydroxytryptamine exhibited identical R_f values on paper chromatograms and a mixture of the two yielded a single spot[1]. Homogeneity was further established by demonstrating that I^{131}/S^{35} ratios in consecutive transverse segments of the spot on the mixed chromatogram were constant throughout (see Figure)[2].

The finding of 5-hydroxytryptamine in the toad increases the number of animal species in which this material has now been found and further suggests its general physiological importance. Its presence in the toad gland in relatively large amounts should make the toad a useful experimental animal in which to study its biosynthesis. The presence of the methylated derivatives suggests that methylation may be a pathway for metabolism of 5-hydroxytryptamine in other animals. Until now, the methylated derivatives of 5-hydroxytryptamine have been considered peculiar to the toad; they may prove to be of more widespread physiological importance.

S. UDENFRIEND, C. T. CLARK, and E. TITUS

Section on Chemical Pharmacology, National Heart Institute, National Institutes of Health, Public Health Service, Federal Security Agency, Bethesda, Maryland, July 5, 1952.

Zusammenfassung

5-Hydroxytryptamin (Serotonin, Enteramin, Thrombocytin), das in neuerer Zeit aus Blut oder Gewebe verschiedener Tiere isoliert wurde, konnte nun auch unter Verwendung von Isotopentechnik und Papierchromatographie als unmethylierter Vorläufer der schon bekannten Krötenbasen im Giftextrakt von *Bufo marinus* im Ausmasse von 0,1 % (bezogen auf trockenen Giftextrakt) nachgewiesen werden.

[1] We wish to thank Dr. C. BERNARD LEWIS of the Institute of Jamaica for supplying the specimens of *Bufo marinus*.

[2] L. C. CRAIG, J. Biol. Chem. *155*, 519 (1944).

[3] 5-Hydroxytryptamine, as the creatinine sulfate complex, was generously supplied by the Abbott Laboratories, North Chicago, Illinois, U.S.A.

[1] The radioactive tryptamine derivatives were located on the chromatograms by radioautography.

[2] The radioactivity of the samples, which were essentially weightless, was measured with a 1·8 mg/cm² mica window G. M. counter. The amounts of I^{131} and S^{35} in each sample were determined by measurement of radioactivity with and without an aluminum absorber as described by A. S. KESTON, S. UDENFRIEND and M. LEVY, J. Amer. Chem. Soc. *72*, 748 (1950) and S. UDENFRIEND, J. Biol. Chem. *187*, 65 (1950).

Further Experiments on the Fixation *in vitro* of Radiocalcium to Sections of Bone

Historadiography of ground sections of total bone shows that minerals are unevenly distributed in the structures of second order of bone compacta and spongiosa, viz. the Ca content is lower in bone tissue of recent formation[1]. No changes of the degree of the X-ray absorption in recently laid down and old structures occur when organic components of bone are removed by microincineration (at 700°C for 3 h)[2] or by treatment with glycol/K hydroxide (GABRIEL's method).

The investigation has been further extended and the new data can be summarized as follows:

(1) Ground sections of total bone (40 to 50 μ in thickness) decalcified from 18 to 24 h in highly diluted

[1] A. ENGSTRÖM and R. AMPRINO, Exper. *6*, 276 (1950); R. AMPRINO and A. ENGSTRÖM, Acta Anat. *15*, 1 (1952).

[2] R. AMPRINO, Z. Zellforsch. *37*, 144 (1952).

nitric acid (from 1:20,000 to 1:30,000, pH from 3·8 to 4·5) lose from $^1/_2$ to $^2/_3$ of their mineral content. This partial decalcification does not substantially modify the differences in the relative degree of calcification of the various structures (quantitative micro-photographic-photometric study of the X-ray absorption).

(2) Autographs were made from ground sections of fresh, fixed in ethanol or macerated bone which were kept in slightly acid solutions of Ca^{45} chloride (0·1 g $Ca^0/_{00}$, pH 6·6 circa, Sp. activ. 1400 counts per minute/ml). The radioactivity after the treatment is much higher in recently formed and less calcified structures (e.g. recently laid down HAVERSIAN systems, inner and outer circumferential layers) than in relatively more calcified older structures[1]. This differential uptake of radiocalcium is already apparent after treatment of the section with radiocalcium for a few minutes (from 10′ to 15′). When the bone sections are kept in the radioactive solution for several hours (Fig. 1) or days (up to 18 days), the amount of labelled Ca fixed to the bone ground substance increases progressively according to a rate which is high at first and decreases with time. The *relative* distribution of radioactivity in the various structures does not show any detectable shifts after prolonged treatment with radiocalcium (cf. Fig. 1 *C* and 3 *A*).

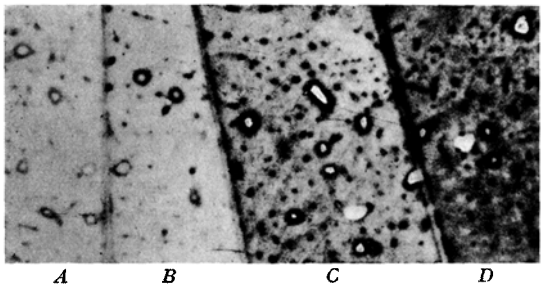


Fig. 1.–Calf, metatars. Successive sectors of the same ground section of total bone treated with Ca^{45} for 20′, 70′, 280′, 420′. Autographs 12 ×.

The photometric analysis of the degree of calcification of newly formed HAVERSIAN systems (relatively to the calcification of the old primary bone) made on microradiographs of the same ground section before and after a long treatment with Ca^{45} (18 days) reveals no increment of minerals in the secondary bone[2]. The failure of historadiography to detect the increment of Ca in less calcified HAVERSIAN systems may depend on the low sensitivity of the method.

(3) Autographs show that a different uptake of Ca^{45} also occurs in recently formed and in old structures when ground sections of fresh bone undergo one of the following procedures before treatment with radiocalcium:

(*a*) boiling in neutral distilled water for 10′ to 20′;

(*b*) digestion from 1 to 3 h in a buffered 0·5% solution of hyaluronidase (pH 6·4) at 38°C;

(*c*) treatment with a buffered 1% solution of phosphowolframic acid (pH 6);

(*d*) partial decalcification in extremely diluted nitric acid to remove circa 30% of the mineral content.

[1] R. AMPRINO, Exper. *8*, 20 (1952).

[2] The degree of calcification of newly laid down HAVERSIAN systems is from 20 to 25% lower than that of old bone tissue. The progressive increment of calcification of recently deposited ground substance takes place at a very slow rate *in vivo*. R. AMPRINO and A. ENGSTRÖM, Acta Anat. *15*, 1 (1952).] The full calcification is attained in a period of at least a few months. [R. AMPRINO, Z. Zellforsch. *37*, 144 (1952).]

The total amount of Ca^{45} absorbed by partially decalcified sections is somehow greater than the amount of Ca^{45} absorbed by non-decalcified control sections.

Fully decalcified bone sections do not fix radiocalcium at all.

(4) Removal of the organic components brings forth remarkable changes of the calcium-binding capacity of bone ground substance; however, the results vary if different methods are used to remove organic matter.

The total amount of Ca^{45} absorbed by the whole bone section is greatly reduced after microincineration[1]; moreover, the differences of radioactivity in the various structures—as apparent in autographs of sections which were not ashed—become very small. In some instances no differences at all are detectable. The differences in the radiocalcium uptake fail almost entirely to show up in autographs from sections incinerated at 500°C for one hour and are not appreciable after incineration at 700°C for 3 h.

The uptake of radiocalcium is almost uniform in the various structures of bone also after treatment of the sections with boiling glycol/K hydroxide (GABRIEL); however, the total amount of Ca^{45} absorbed by the whole section increases considerably after treatment with this method (Fig. 2 *B*). In sections treated according to GABRIEL's procedure, the diffusion of radiocalcium seems to become an important controlling factor of the amount of Ca^{45} absorbed by ground substance; in fact, the Ca^{45} uptake is greater on the fringe of the sections and at the margins of the vascular channels than at some distance: a regular dropping of radioactivity from a maximum to a minimum is apparent in the regions mentioned (Fig. 2 *B*).

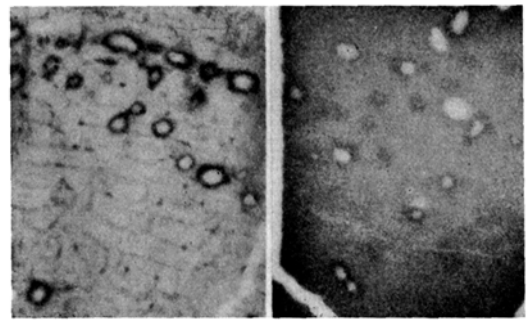


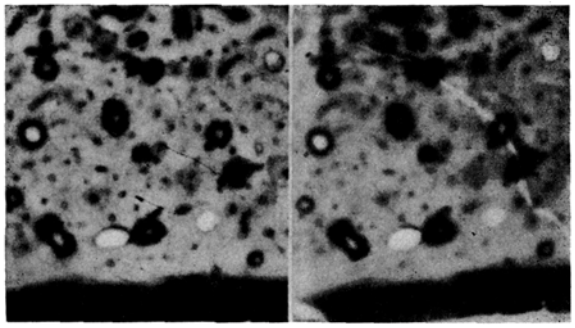
Fig. 2.–Calf, metatars. *A*, section of total bone treated with Ca^{45} then ashed (GABRIEL); *B*, control section ashed (GABRIEL) then treated with Ca^{45}. Autographs 15 ×.

The radioactivity of sections of total bone ashed by incineration *and* treatment with GABRIEL's method (or reciprocally, treatment with GABRIEL's method *and* incineration), thoroughly washed and treated with radiocalcium, is only slightly higher than the activity of sections of the same thickness which did not undergo ashing.

(5) The radiocalcium absorbed in different amounts in recently formed and old structures of bone sections fails to be removed by ashing (incineration or treatment with GABRIEL's method) and prolonged washing in distilled water (48 h; Fig. 3 *B*). The *distribution* of radioactivity does not undergo any changes when ground

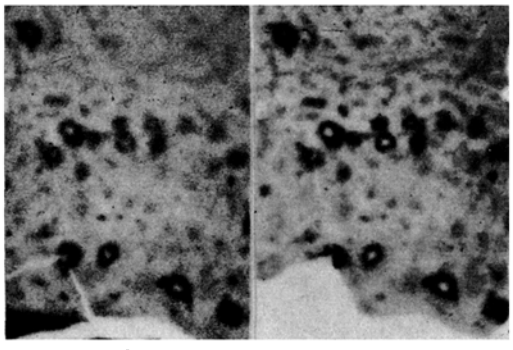
[1] R. AMPRINO, Z. Zellforsch. *37*, 240 (1952); cf. also W. F. NEUMAN, M. W. NEUMAN, E. R. MAIN, and B. J. MULRYAN, J. biol. Chem. *179*, 335 (1949); W. P. NORRIS and W. KISIELESKI, Cold Spring Harbour Simp. Quant. Biol. *13*, 164 (1948).

sections of total bone treated with radiocalcium are partially decalcified (or incinerated and decalcified) to remove from 1/10 to 1/8 circa of their mineral content. The total radioactivity of the section is thus greatly reduced, but in a rather uniform degree in the various structures (Fig. 4.)



A *B*

Fig. 3.–Calf, metatars. *A*, section of total bone treated with Ca^{45} for 18 days; *B*, the same after ashing (GABRIEL). Autographs 16 ×.



A *B*

Fig. 4.–Calf, metatars. *A*, section of total bone treated with Ca^{45} then ashed; *B*, the same after partial decalcification. Exposure times of autographs 1:3. 13 ×.

(6) Autographs made from strips of periosteum peeled off from bone compacta (fixed in ethanol) and treated with radiocalcium show radioactivity only in limited areas which correspond to small spicules of bone adherent to the fibrous tissue. R. AMPRINO

Institute of Anatomy, University of Turin, July 15, 1952.

Résumé

L'auteur s'est proposé de préciser les conditions qui règlent les différences quantitatives de fixation du radiocalcium dans la structure des os à différents stades de formation. De minces lamelles d'os usées et polies ont été soumises *in vitro* à des traitements divers (micro-incinération à 500° ou à 700°C, gabriélisation, décalcification partielle, traitement par la hyaluronidase, par l'acide phosphowolframique, etc.) et traitées en suite avec une faible solution de chlorure de Ca^{45}. L'étude des autoradiographies démontre que seule la destruction totale des composants organiques de la matrice osseuse entraîne des modifications appréciables de la distribution du radiocalcium.

Researches on the Chemical Composition of the Erythrocyte Membrane

At present the chemical composition of the erythrocyte membrane is still little known. Our researches are intended to contribute further data and clarity on the protein composition of the normal erythrocyte membrane, and at a future stage to see if any deviation from the normal exists in anemiae due to haemolysis and erythrocyte fragmentation.

From erythrocyte shadows, precipitated from 50 to 100 cc of blood with the CO_2 method, stromatin (a fibrous protein) was extracted with EDSALL-WEBER's liquid[1]. This extracted substance was precipitated several times to remove all the possible Hb present. All the aforesaid operations took place at low temperature. The precipitate was again dissolved in EDSALL-WEBER's liquid and chromatographed on WHATMAN's No. 1 filter paper using pyridine (80) + water (20) as solvent: ascensional method, time employed 12–18 h.

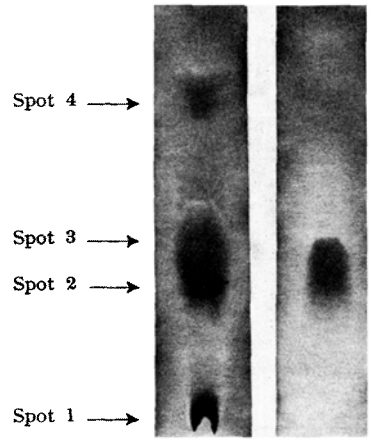


Fig. 1.

The Iodine evidence showed up 4 different spots (Fig. 1*A*) besides the frontal one which was partly due to the impurities of solvents, paper and partly due to traces of Hb.

Figure 1*B* represents the chromatogram of the top liquid after the first precipitation operation. It shows a spot corresponding to a substance which was not submitted to further researches.

In order to obtain and study a certain quantity of substances of which the 4 spots are made up, we chromatographed the substance by placing it on the paper along a horizontal line at a distance of about 3 cm from the edge immersed in the solvent. After drying the paper 3 strips were cut parallel to the course followed by the liquid (2 at the lateral edges and 1 central); the test was made with Iodine (Fig. 2).

Using these strips as guides we cut the strips corresponding to the 4 spots. The substances were eluated with EDSALL-WEBER's liquid according to CONDSEN, GORDAN, MARTIN's method[2]. After dialysis through cellophan, the 4 substances were hydrolized for 8–10 h. with 20% HCl and after removing the HCl, the hydrolizate was

[1] M. CIGADA, P. CITTERIO, A. ORLANDI, S. RANZI, and L. TOSI, Rend. Istit. Lombar. Sci. Lett. (Cl. Scienze) *82*, 351 (1949).

[2] R. CONSDEN, A. H. GORDON, and A. J. P. MARTIN, Biochem. J. *41*, 590 (1947).