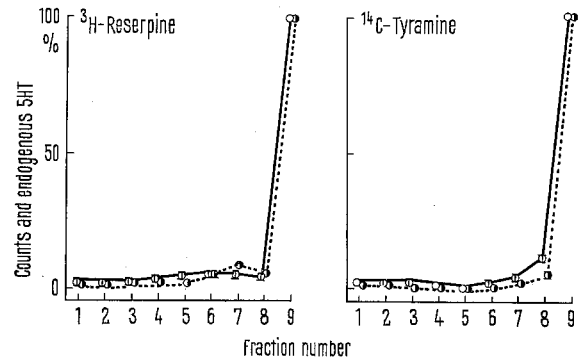
Different Localization of Reserpine and Tyramine within the 5-Hydroxytryptamine Organelles of Blood Platelets

Reserpine and tyramine liberate endogenous aromatic monoamines, e.g. norepinephrine, from sympathetic nerve endings and 5-hydroxytryptamine from blood platelets[1,2]. Both drugs are thought to affect the intracellular storage of the amines; their mode of action, however, seems to be different. In isolated blood platelets, for instance, reserpine acts in concentrations as low as 10^{-6} *M* and less, whereas tyramine is equieffective in at least 1000 times higher concentrations. Thereby, one molecule of reserpine taken up by the intracellular storage organelles[3] replaces several thousand molecules of liberated 5-hydroxytryptamine, but 1 molecule tyramine only a few molecules of 5-hydroxytryptamine[4].

In this paper, the subcellular localization of reserpine and tyramine in blood plateiets has been compared.

Experimental. Blood platelets of rabbits have been isolated as previously described[5]. In the in vivo experiments, the animals received 1 mg/kg ^{3}H-reserpine (methyl-*o*[3,4,5-trimethoxybenzoyl-2,6-$^3H_2$]reserpate)[6] (spec. act. 1.85 mC/mg) i.p. or 400 µg/kg tyramine-α-^{14}C (HCl) (spec. act. 68 µC/mg) i.v. 16 and 1 h resp. before exsanguination. In the in vitro experiments, isolated platelets of untreated animals were incubated at 37 °C for 1 h in plasma supplemented with 2 µg/cm^3 ^{3}H-reserpine (spec. act. 1.03 mC/mg) or 350 µg/cm^3 ^{14}C-tyramine (HCl) (spec. act. 0.86 µC/mg). The isolated platelets were then homogenized and subjected to density gradient centrifugation as described earlier[7], whereby the 5-hydroxytryptamine organelles sedimentated in virtually pure form in the bottom layer (fraction 9 of the Figure). The isolated organelles (0.5–2 µl) were taken up in 0.5 ml distilled water, subjected to freezing and thawing and thereafter to ultracentrifugation. By this procedure, the content of the organelles could be separated from their membranes, since virtually all the 5-hydroxytryptamine and adenosine-triphosphate (97–99%) were found in the supernatant. In the membrane sediment as well as in the supernatant, the total radioactivity was determined with a liquid scintillation counter. Furthermore, estimations of the total radioactivity, the endogenous 5-hydroxytryptamine[8], and in some instances the protein and adenosine-triphosphate content[9,10] were carried out in the isolated intact organelles as well as in various other fractions of the density gradient. Thin layer chromatography of the organelles (dissolved in about 0.1 ml H_2O) from the reserpine experiments (silicagel F 254, Merck; chloroform:acetone:diethylamine 50:40:10) and paper chromatography from the tyramine experiments (Whatman no. 1; isopropanol:H_2O:NH_3, 80:18:2) showed that the radioactivity consisted in principle of unchanged ^{3}H-reserpine and ^{14}C-tyramine respectively.

Results and discussion. The subcellular distribution studies demonstrate that within the platelets ^{14}C-tyramine like ^{3}H-reserpine[11] is mainly concentrated in the 5-hydroxytryptamine organelles (Figure). This strongly supports the hypothesis that at least in the platelets both substances act at the level of the intracellular storage organelles. Their distribution within the organelles differs, however, markedly. Thus, the in vitro as well as the in vivo experiments show that the bulk of ^{14}C-tyramine is localized in the supernatant (Table). Similar results are obtained if the platelets have been incubated with 10 µg/ml instead of 350 µg/ml ^{14}C-tyramine (HCl). In contrast, almost all the ^{3}H-reserpine remains in the membranes of the organelles (Table) from which it cannot easily be removed. Thus, after 3 washings (each with 0.5 ml H_2O) only about $^1/_3$ of the radioactivity has disappeared from the membrane fraction. If, however, isolated membranes of organelles of untreated platelets are incubated for 1 h at 37 °C with small amounts of



Subcellular distribution of ^{3}H-reserpine and ^{14}C-tyramine in blood platelets of rabbits in comparison with the endogenous 5-hydroxytryptamine. ^{14}C-tyramine and ^{3}H-reserpine were injected 1 and 16 h resp. before exsanguination. The endogenous 5-hydroxytryptamine as well as the radioactivity were calculated in µg/µg proteins and are indicated in percent of the respective values found in the organelles (= 100%). Fraction 9 represents the pure 5-hydroxytryptamine organelles. Each point of each curve represents an average with S.E. of 2–4 experimental values. —, radioactive substances; ..., endogenous 5-hydroxytryptamine.

Distribution of ^{3}H-reserpine and ^{14}C-tyramine in isolated 5-hydroxytryptamine organelles of rabbit platelets

Drugs		Membrane	Supernatant
in vitro	Reserpine	76.0 ± 4.8	24.0 ± 4.8
	Tyramine	3.0 ± 0.8	97.0 ± 0.8
in vivo	Reserpine	91.5 ± 1.1	8.5 ± 1.1
	Tyramine	1.8 ± 0.2	98.2 ± 0.2

In the in vitro experiments, isolated platelets were incubated with these drugs for 1 h in plasma at 37 °C. In the in vivo experiment, ^{14}C-tyramine and ^{3}H-reserpine were injected 1 and 16 h resp. before exsanguination. The figures represent averages with S.E. of 3–4 experiments and are indicated in percentage of the radioactivity contained in the whole organelles.

[1] P. A. Shore, Pharmac. Rev. *14*, 531 (1962).
[2] A. Carlsson, in *Handbuch der experimentellen Pharmakologie* (Springer Verlag, Berlin, Heidelberg 1966), vol. 19, p. 529.
[3] J. P. Tranzer, M. Da Prada and A. Pletscher, Nature *212*, 1574 (1966).
[4] M. Da Prada and A. Pletscher, in preparation.
[5] G. Bartholini, A. Pletscher and K. F. Gey, Experientia *17*, 541 (1961).
[6] Synthetized by Drs. J. Würsch and R. Barner, Department of Physics, F. Hoffmann-La Roche & Co. Ltd., Basle (Switzerland).
[7] M. Da Prada and A. Pletscher, Br. J. Pharmac. *34*, 591 (1968).
[8] D. F. Bogdanski, A. Pletscher, B. B. Brodie and S. Udenfriend, J. Pharmac. exp. Ther. *117*, 82 (1956).
[9] O. H. Lowry, N. J. Rosenbrough, A. L. Farr and S. Randall, J. biol. Chem. *193*, 265 (1951).
[10] H. Holmsen, I. Holmsen and A. Bernhardsen, Analyt. Biochem. *17*, 456 (1966).
[11] M. Da Prada and A. Pletscher, Europ. J. Pharmac., in press (1969).

^{3}H-reserpine (corresponding to those taken up by the membranes of intact organelles), only about 30% of the drug go to the membrane, whereas 70% remain in the supernatant. The different distribution of ^{3}H-reserpine and ^{14}C-tyramine within the 5-hydroxytryptamine organelles of platelets subjected to the drugs in vitro and in vivo might therefore indicate that reserpine acts mainly at the level of the membrane and tyramine preferentially in the interior of the 5-hydroxytryptamine organelles.

According to recent findings[12], 5-hydroxytryptamine and adenosine-triphosphate of the organelles of platelets form mixed micelles of high molecular weight. Tyramine might replace the 5-hydroxytryptamine of these micelles. Reserpine, in contrast, possibly acts by altering the membrane of the organelles. The nature of this postulated change as well as its possible consequences with regard to the stability of the 5-hydroxytryptamine-adenosine-triphosphate micelles remains to be investigated. Up to now, no evidence exists that the storage of 5-hydroxytryptamine is a process depending on an active (i.e. energy-dependent) transport through the membrane of the organelles which would be impaired by reserpine[13,14].

In conclusion, the experiments with subcellular fractionation of blood platelets show that reserpine probably acts at the level of the membrane, whereas tyramine seems to have its site of action in the interior of the 5-hydroxytryptamine organelles.

Zusammenfassung. Die subzelluläre Lokalisation von Reserpin und Tyramin in Blutplättchen von Kaninchen erfolgt in vitro und in vivo grösstenteils in den 5-Hydroxytryptamin (5HT)-Organellen. Dabei reichert sich Reserpin vorwiegend in der Membran, Tyramin hauptsächlich im Inneren der Organellen an. Es wird vermutet, dass Reserpin in bezug auf 5HT-Freisetzung an der Membran und Tyramin im Inneren der 5HT-Organellen angreift.

M. DA PRADA and A. PLETSCHER

Forschungsabteilung der F. Hoffmann-La Roche and Co. AG, CH-4002 Basel (Switzerland), 22 May 1969.

[12] K. H. BERNEIS, M. DA PRADA and A. PLETSCHER, Science, submitted for publication.
[13] A. PLETSCHER, M. DA PRADA and J. P. TRANZER, in *Progress in Brain Research*, in press (1969).
[14] M. DA PRADA and A. PLETSCHER, Life Sci. *8*, 65 (1969).

Calcification in Implants of Tendon

In vitro studies[1-5], conducted over a decade ago, demonstrate that calf tail tendon and reconstituted collagen fibers could nucleate and crystallize mineral, chemically and radiographically similar to bone salt, from metastable solutions of calcium and phosphate ions. When nuclei are present, secondary nucleation and crystal growth follows, even at calcium and phosphate ion concentrations nearly as low as in normal blood serum[6,7]. The theory is that the formation of crystals of hydroxyapatite occurs by heterogeneous nucleation[1-9], but the question whether a collagen-calcium, or a collagen-phosphate, or a collagen-calcium-phosphate complex, or collagen itself is the nucleation site cannot be answered by experiments with solutions containing both calcium and phosphate ions in systems free of inhibitors in vitro.

Some of the objections to in vitro methods can be overcome by experiments on calcification of implants of normal tendon in rats with normal body fluid organic constituents and ion concentrations of calcium and phosphate of only 1 mmole/l. Implants of KCl-extracted tendon and various forms of reconstituted tendon collagen do not calcify with any degree of consistency, or to any appreciable extent, under these conditions in vivo. Implants of tendon calcify consistently when implanted in the anterior eye chamber of the rat after they are exposed to solutions containing 25 mmoles Ca^{++}/l, but not after exposure to phosphate ion concentrations even as high as 500 mmoles/l[10-12]. These observations on calcification in implants of calcium-treated tendon in vivo are corroborated by WADKINS[13] in a recent report of experiments on calcification in vitro.

To quantitate the process of calcification, experiments were performed to determine the relative amount of mineral per total mass of tendon deposited in uncalcified, partially calcified, and completely calcified implants after periods as long as 35 days. In addition, experiments were also performed to determine the effect of nuclei of apatite produced in vitro upon the quantity of mineral deposited in vivo in tendon over a similarly long period of time.

Samples of Achilles tendon of young New Zealand rabbits were cut into segments, about 1 cm in length, and equilibrated at 2 °C: (a) in 1.5, 5.0, 10.0 and 15.0 mmolar solutions of calcium for 10 h, or (b) in nucleating and precipitating mixtures of calcium and phosphate ions, pH 7.4 and ionic strength 0.16, for 10 days. Nucleation and precipitation were assessed by methods presented in previous publications[2,4]. Experimental and control (0.9% NaCl-treated) preparations of tendon were aseptically implanted into pouches in the anterior abdominal wall muscles of allogeneic rabbits as previously described[14]. Implants were recovered at 5–7 weeks after

[1] B. STRATES and W. F. NEUMAN, Fedn Proc. *1195*, 365 (1956).
[2] B. S. STRATES, W. F. NEUMAN and G. J. LEVINSKAS, J. phys. Chem. *61*, 279 (1957).
[3] M. J. GLIMCHER, A. J. HODGE and F. O. SCHMITT, Proc. natn. Acad. Sci. U.S.A. *43*, 860 (1957).
[4] B. STRATES and W. F. NEUMAN, Proc. Soc. exp. Biol. Med. *97*, 688 (1958).
[5] M. J. GLIMCHER, Rev. mod. Phys. *31*, 359 (1959).
[6] C. C. SOLOMONS and W. F. NEUMAN, J. biol. Chem. *235*, 2502 (1960).
[7] H. FLEISCH and W. F. NEUMAN, Am. J. Physiol. *200*, 1296 (1961).
[8] B. N. BACHRA and A. E. SOBEL, Arch. Biochem. *85*, 9 (1959).
[9] B. N. BACHRA, A. E. SOBEL and J. W. STANFORD, Arch. Biochem. *84*, 79 (1959).
[10] M. R. URIST and J. M. ADAMS JR., Arch. Path. *81*, 325 (1966).
[11] M. R. URIST and J. M. ADAMS JR., Ann. Surg. *166*, 1 (1967).
[12] M. R. URIST and J. L. ABERNETHY, Clin. Orthop. rel. Res. *51*, 255 (1967).
[13] L. L. WADKINS, Calc. Tiss. Res. *2*, 214 (1968).
[14] M. R. URIST, T. A. DOWELL, P. H. HAY and B. S. STRATES, Clin. Orthop. rel. Res. *59*, 59 (1968).