#30

The Pharmacology of Reserpine in the Horse. I. Effects of Reserpine and Imipramine on Platelet Uptake of Serotonin *In Vitro* and *In Vivo*

C.G. White, M.Sc.
W.E. Woods, M.Sc.
T. Tobin, D.V.M., Ph.D.

From the Kentucky Equine Drug Research and Testing Programs, and the Graduate Program in Toxicology, Department of Veterinary Science, College of Agriculture, University of Kentucky, Lexington, KY 40546.

Publication No. 39 from the Kentucky Equine Drug Research and Testing Programs, Department of Veterinary Science, College of Agriculture, University of Kentucky, Lexington, Ky 40546. Published as Kentucky Agricultural Experiment Station Article No. 78-4-123 with the permission of the Dean and Director, College of Agriculture. The advice and assistance of Drs. Peter Henson, National Jewish Hospital & Research Center, Denver, CO, J.W. Blake and J. Dougherty, University of Kentucky, is gratefully acknowledged.

Supported by grants from the United States Trotting Association and the Kentucky Equine Research Fund.

A rapid, reliable method for measuring uptake of [^{3}H] serotonin (5-HT) in horse platelets was developed. Using this method, [^{3}H] serotonin uptake in horse platelets was found to be highly temperature sensitive, blocked by high concentrations of imipramine and unlabeled serotonin and partially inhibited by high concentrations of reserpine. These results are consistent with platelet uptake of serotonin being due to an active transport mechanism at the plasma membrane which is inhibited by imipramine and a granular binding mechanism which is inhibited by reserpine. A large dose of reserpine administered *in vivo* to a horse produced substantial *in vitro* inhibition of platelet serotonin uptake, suggesting that this inhibition may be a useful biochemical correlate of the pharmacological actions of reserpine in the horse.

Introduction

Reserpine is an alkaloidal drug obtained from a climbing plant indigenous to India and neighboring countries. It was introduced into western medicine in the 1950s for the treatment of psychoses and hypertension. Because of severe side effects associated with its use, it is currently used in human medicine only in patients resistant to other forms of therapy.[13]

Because reserpine is a very potent and effective tranquilizer, small doses are sometimes used to calm hyperexcitable race, trotting and show horses. Until recently, no suitable analytical method was available for the very small ($< 2\ \mu g/kg$) doses of reserpine used in the horse. This led us to examine the pharmacological effects of reserpine on neurohormone metabolism as a possible marker for the presence of reserpine in the equine system and also as a possible biochemical correlate of the pharmacological effects of reserpine.

Reserpine produces its pharmacological effects by blocking the storage of norepinephrine, dopamine and serotonin in storage granules throughout the nervous system.[10] Platelets also possess the same serotonin uptake and storage mechanism as serotonergic neurons,[11] so it appeared to us that measurement of the effects of reserpine[a] on platelet serotonin metabolism might be a useful biochemical indicator of the presence of reserpine[7] in the horse.

This paper characterizes the effects of reserpine on serotonin metabolism in equine platelets *in vitro*. A procedure for measurement of serotonin[b] uptake in horse platelets was devised, and the sensitivity of this uptake to specific inhibitors, reserpine and imimpramine,[4] was determined. Preliminary experiments with reserpine administered *in vivo* to horses suggest that the presence of the drug in equine platelets causes substantial inhibition of the normal rate of uptake of serotonin.

Materials and Methods

Sample Collection and Drug Administration

Mature Thoroughbred mares, weighing between 400 and 550 kg, were used. These animals were housed in individual loose boxes and fed hay, oats and water *ad libitum*. All drug administration and sample taking was done in the animals' loose boxes.

Chemical Methods

Equine blood was drawn into vacutainer tubes[c] containing Na_2 EDTA, and immediately centrifuged at 250 x g for 15 minutes at 2°C. Aliquots of plasma directly above the red cell layer, platelet-rich plasma (PRP),[7] 1.0 ml per 5.0 ml blood, were removed and incubated without shaking for two minutes at 37°C. All uptake experiments were started by adding 3.6×10^{-8} M [^{3}H] serotonin[d] (1 Ci/ml) to this PRP. The uptake reaction was followed by removing 1.0 ml aliquots of the plasma after appropriate time periods into a filtration manifold[e] (0.45μ filters) followed by washing with 20.0 ml of ice-cold normal saline. The filters were removed from the manifold, air-dried in the dark, and counted in a liquid scintillation cocktail[f] in a counter.[g] Silanized glassware was used throughout this procedure. Platelet counts on the platelet-rich plasma were performed on a photomicroscope[h] using a hemocytometer,[i] and were averaged from four separate observations. Unless otherwise noted, all experimental values are the means ± SEM of determinations on at least four experimental plasma samples. In all experiments the initial rate of [^{3}H] serotonin uptake was estimated by fitting a least squares slope to the first four points of the uptake curve. All data are plotted as the number of μmoles of [^{3}H] serotonin taken up by 10^{11} platelets/minute after the uptake reaction was started.

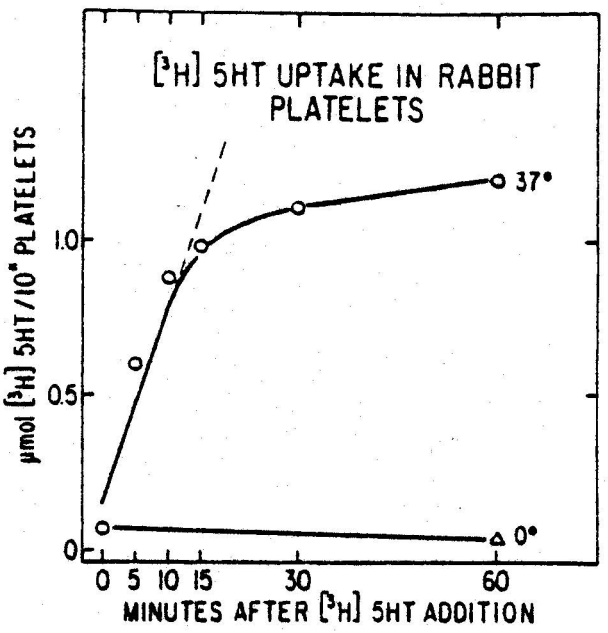

Figure 1. [^{3}H] uptake in rabbit platelets. Freshly drawn rabbit platelets were incubated at 37°C (open circles, O – O) and 0°C (triangle, Δ) with 3.6×10^{-8} M [^{3}H] serotonin and the uptake reaction stopped at the indicated times. All data points are individual determinations on a single plasma sample and are typical of experiments on a number of different rabbit plasma samples. The dashed line represents a least squares fit of the slope of the initial four data points of the uptake curve.

Results

A number of published methods for analyzing [^{3}H] serotonin uptake in platelets[1,5,7] were applied to the measurement of 5-HT uptake in horse platelets with little success. On the advice of Dr. Peter Henson[g] the Millipore filtration method outlined above was developed and found suitable for both rabbit and horse platelets. Figure 1 shows 5-HT uptake measured in rabbit platelets by this method.

Figure 2 shows the initial rates and effect of temperature on uptake of [^{3}H] 5-HT in horse platelets as measured by this method. At 37°C platelets took up [^{3}H] 5-HT from plasma at an initial rate of 1.01 μmol/10^{11} platelets/minute. This rate is about 20 times the initial rate observed in rabbit platelets. If the temperature of the incubation system was reduced, the initial rate of uptake of 5-HT was markedly depressed to 0.22 μmol/10^{11} platelets/minute at 20°C and 0.028 mol/10^{11} platelets/

[a] Serpasil, Sigma Chemical Co., St. Louis, MO.
[b] Sigma Chemical Co., St. Louis, MO.
[c] Becton Dickinson, Rutherford, NJ.
[d] New England Nuclear, Boston, MA.
[e] Millipore Corp., Bedford, MA.
[f] Research Products 3a70 liquid scintillation cocktail, Research Products, Elk Grove Village, IL.
[g] Beckman LS 230 counter, Beckman Instruments, Inc., Irvine, CA.
[h] Zeiss Photomicroscope II, Carl Zeiss, Inc., NY.
[i] American [illegible] Corp., Buffalo, NY.

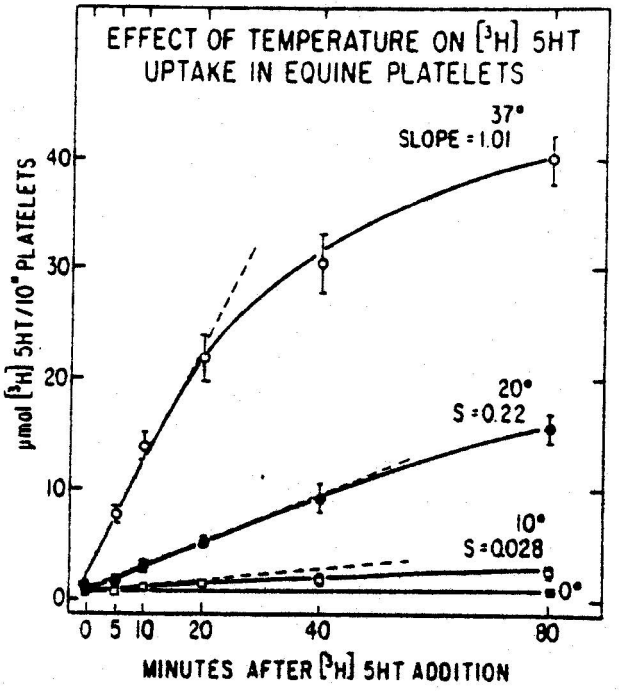

Figure 2. Effects of temperature on [³H] 5-HT uptake in equine platelets. Freshly drawn horse platelets were incubated with [³H] 5-HT as outlined in "Materials and Methods," and the uptake reaction stopped at the indicated time periods. The open circles (○ – ○) show [³H] 5-HT uptake at 37°C; the solid circles (● – ●) at 20°C; the open squares (□ – □) at 10°C, and the solid squares (■ – ■) at 0°C. All data points are the means ± SEM of six separate experiments, and the dashed lines represent the initial uptake rates at each temperature.

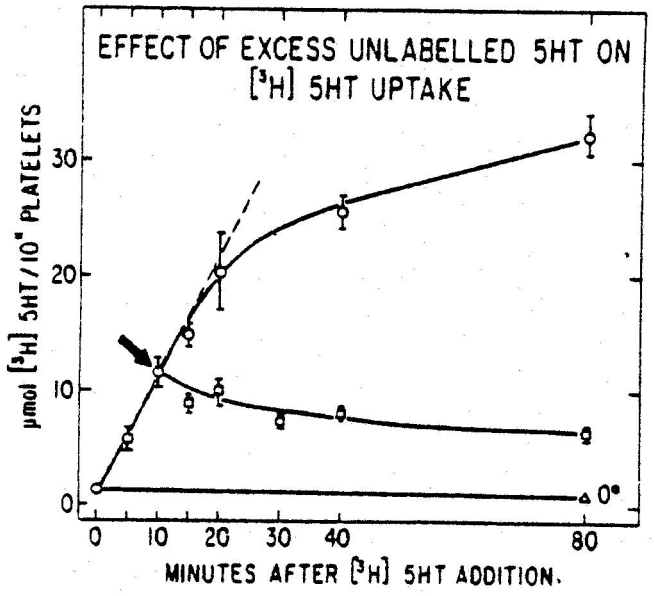

Figure 3. Effect of excess unlabeled 5-HT on [³H] 5-HT uptake. The open circles (○ – ○) show the rate of uptake of [³H] 5-HT in horse platelets *in vitro* at 37°C. The open squares (□ – □) show uptake of [³H] 5-HT after addition of excess (1×10^{-5} M) unlabeled 5-HT at 10 minutes. All points are the means ± of five separate experimental determinations.

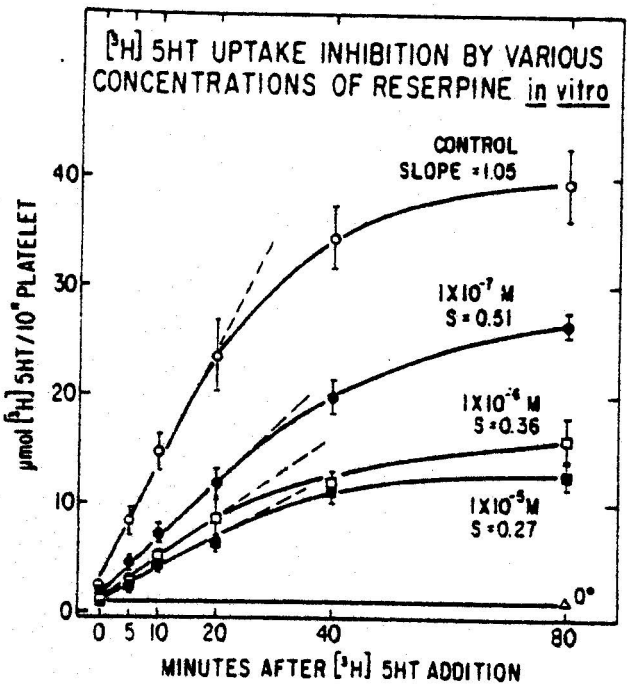

Figure 4. [³H] 5-HT uptake inhibition by various concentrations of reserpine *in vitro*. The open circles (○ – ○), show the uptake of [³H] 5-HT by freshly drawn horse platelets at 37°C. The solid circles (● – ●), open squares (□ – □) and solid squares (■ – ■) show inhibition of [³H] 5-HT uptake by 1×10^{-7}M, 1×10^{-6}M, and 1×10^{-5}M reserpine, respectively. Reserpine was added 10 minutes before the 5-HT. In each case all points are the means ± SEM of six experimental determinations.

minute at 10°C. In all experiments, essentially no uptake was observed at 0°C, and binding of [³H] 5-HT under these conditions was taken as background binding.

Figure 3 shows that the addition of excess unlabeled 5-HT to the incubation system resulted in an abrupt halt in the uptake of [³H] 5-HT and a subsequent slow decline in the level of bound [³H] 5-HT. The relatively slow decline in bound [³H] 5-HT after the addition of excess unlabeled 5-HT suggests that the binding is relatively stable and that minimal changes in the level of bound [³H] 5-HT occur during the filtration process.

Figure 4 shows the effects of reserpine addition on this uptake of [³H] 5-HT by horse platelets. The indicated concentrations of reserpine were added to the incubation system 10 minutes prior to the addition of [³H] 5-HT. Reserpine at 1×10^{-7}M produced about 50% inhibition of the initial rate of uptake of 5-HT, while the remaining uptake was relatively resistant to inhibition by increasing concentrations of reserpine.

Imipramine[1] has been shown to inhibit the transport of 5-HT across the platelet plasma membrane, and Figure 5 shows the effects of increasing concentrations of imipramine on the initial rate of uptake of [³H] 5-HT. While I

[1] Tofranil NCI, Ciba-Geigy Corporation, Summit, NJ.

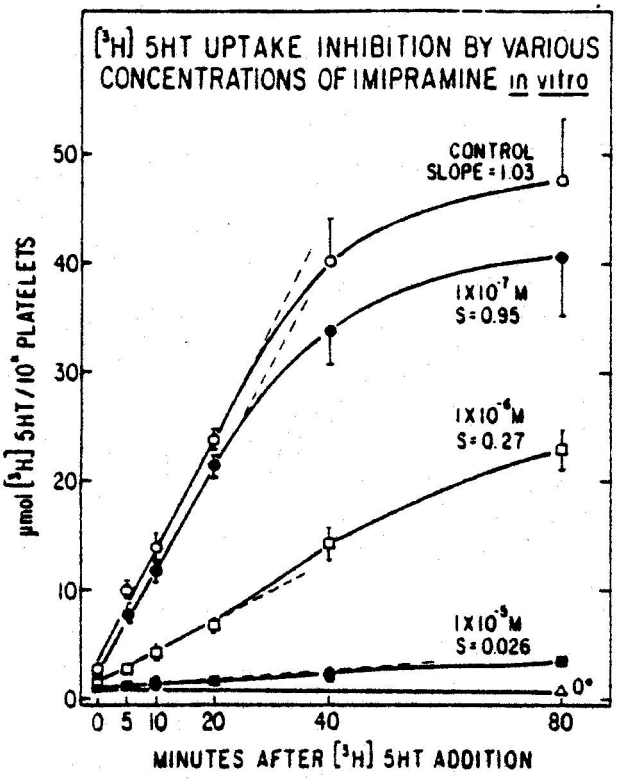

Figure 5. [³H] 5-HT uptake inhibition by various concentrations of imipramine *in vitro*. The open circles (○ – ○) show the uptake of [³H] 5-HT by freshly drawn horse platelets at 37°C. The solid circles (● – ●), the open squares (□ – □), and solid squares (■ – ■) show inhibition of 5-HT uptake by 1×10^{-7}M, 1×10^{-6}M, and 1×10^{-5}M imipramine, respectively. Imipramine was added 10 minutes before the [³H] 5-HT. In each case, all points are the means ± SEM of four separate experimental determinations.

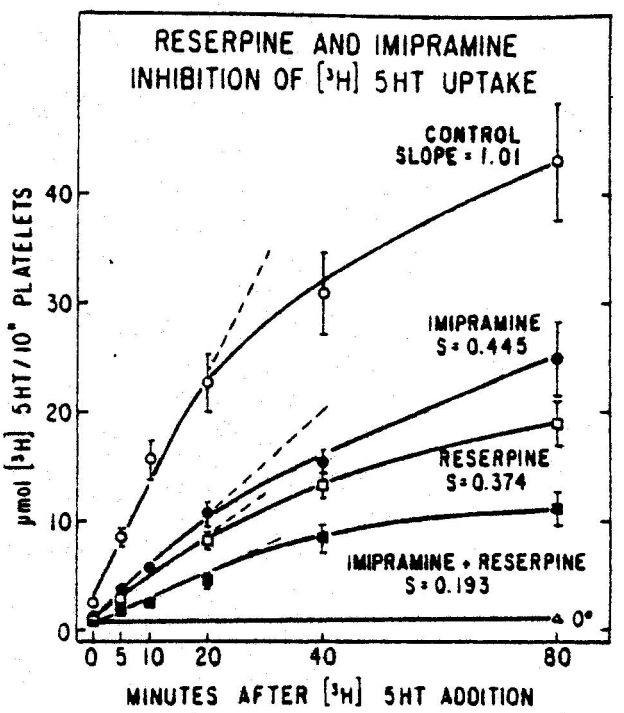

Figure 6. Reserpine and imipramine inhibition of [³H] 5-HT uptake. The open circles (○ – ○) show the uptake of [³H] 5-HT by freshly drawn horse platelets at 37°C. The solid squares (■ – ■) show inhibition of [³H] 5-HT uptake by a combination of 1×10^{-7}M reserpine and 5×10^{-7}M imipramine. Reserpine and imipramine were added 10 minutes before the [³H] 5-HT. In each case all points are the means ± SEM of four separate experimental determinations.

$\times 10^{-7}$M imipramine had relatively little effect on the initial rate of uptake of [³H] 5-HT, higher concentrations (1×10^{-5}M) produced essentially complete inhibition of [³H] 5-HT uptake by platelets at 37°C.

Because imipramine and reserpine act at different points on platelet uptake of serotonin,[8] we hoped that simultaneous use of both inhibitors might result in a potentiation of inhibition of 5-HT uptake by reserpine. Figure 6 shows that 5×10^{-7}M imipramine and 1×10^{-7}M reserpine each produced about 60% inhibition of [³H] 5-HT uptake. However, addition of reserpine plus imipramine produced only about a further 50% inhibition of [³H] 5-HT uptake, showing that the effect of drugs was simply additive.

Figure 7 shows the effects of administration of 100 mg reserpine intravenously to a Thoroughbred mare on the initial rate of 5-HT uptake by her platelets. Prior to administration of reserpine, the initial rate of uptake of 5-HT by platelets was 1.5 µmol/10^{11} platelets/minute, while three hours postreserpine the uptake had been reduced by more than 66%.

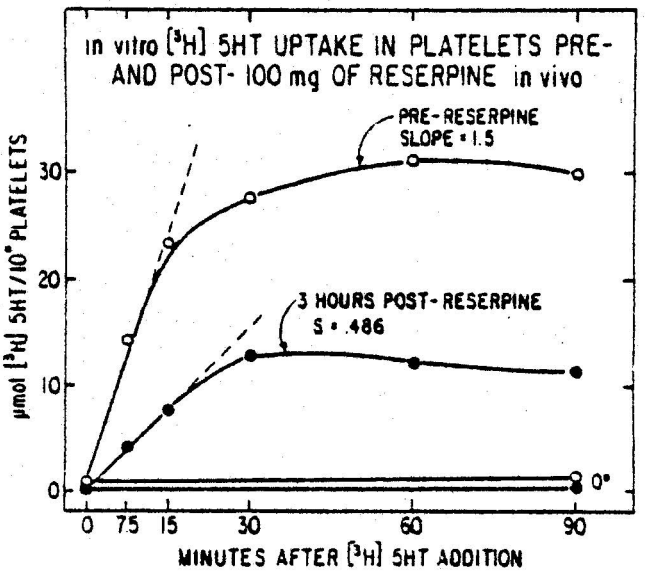

Figure 7. *In vitro* [³H] uptake in platelets pre- and post-100 mg reserpine *in vivo*. The open circles (○ – ○) show uptake of [³H] 5-HT by control platelets at 37°C. The solid circles (● – ●) show uptake of [³H] 5-HT in a plasma sample taken three hours after this horse was treated with 100 mg reserpine by rapid intravenous injection. The initial rate of uptake of the control (slope = 1.5) was reduced more than 60% in the postreserpine sample (slope = 0.486).

Discussion

The methods and results reported here present a rapid and reliable method for measuring serotonin uptake in platelets. With this method, platelet-rich plasma can be prepared and the initial rate of uptake over a 20-minute period determined within one hour of drawing the blood sample. No repeated centrifugation or resuspension of the platelets as used in other methods was required.[1,2] Since we observed that equine platelets lose motility and begin to lose the ability to take up [^{3}H] serotonin within 30 minutes of incubation at 37°C, the advantages of this rapid method are substantial. A further advantage is that the background levels of uptake observed were very low in contrast to the substantial backgrounds observed when we employed adaptations of methods used by other workers.[7,9]

Using this method, the 5-HT uptake by rabbit (Figure 1) and horse platelets was readily demonstrated. In rabbit platelets the initial rate of [^{3}H] 5-HT uptake using this method was found to be about one-half the rate reported by other workers.[5] In equine platelets a 20-fold higher initial rate of 1.0 μmol/10^{11} platelets/minute was observed, and this initial rate was consistent throughout the whole series of experiments. The experiment shows that though horses have fewer platelets than rabbits, the rate of uptake of serotonin by these platelets is extremely high.

The uptake of serotonin by horse platelets has a number of characteristics of an active transport system (Figure 8). It is highly sensitive to temperature (Figure 2) and was almost completely eliminated at 0°C. This strict temperature dependence is characteristic of active transport systems, while uptake systems dependent on diffusion are not so substantially inhibited by cooling to 0°C.

More convincing evidence that the uptake system is a carrier-mediated active transport is presented in Figure 3. In this experiment an excess of unlabeled 5-HT was added to the system 10 minutes after the uptake was started. If the uptake was dependent on simple diffusion through the plasma membrane, the entry of radiolabeled 5-HT into the platelets would not be affected by the presence of the unlabeled 5-HT. If the uptake was carrier-mediated, however, both the labeled and unlabeled 5-HT would compete for the limited number of carrier sites. Because of the great excess of unlabeled 5-HT, the uptake of [^{3}H] 5-HT essentially ceased, and the bulk of the uptake then presumably became unlabeled drug as seen in Figure 3.

Figure 3 also shows that once transported into the platelet, [^{3}H] 5-HT was not readily lost. This was an important point to establish for the time course experiments,

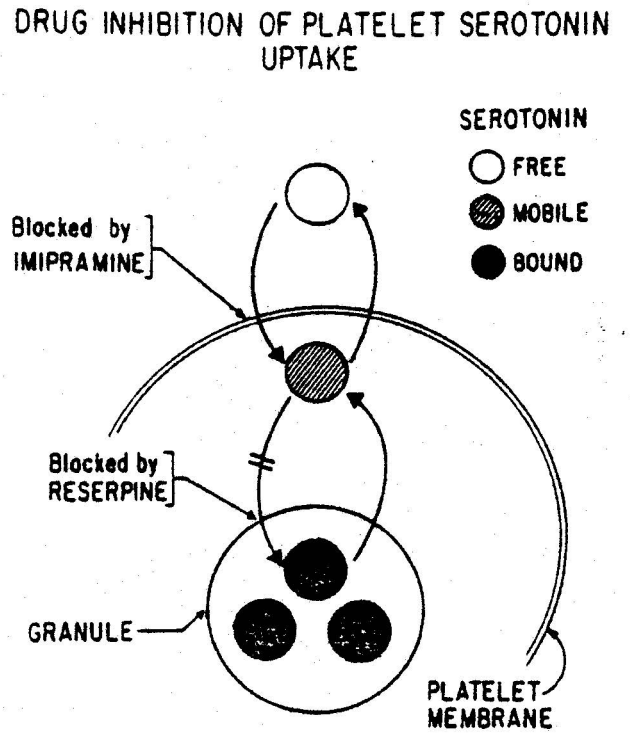

Figure 8. Sites of inhibition of platelet serotonin uptake by imipramine and reserpine.

since it shows that small variations in the washing step in the filtration procedure did not introduce variability into the experimental results.

Studying the uptake of [^{3}H] 5-HT by human platelets, Stahl and Meltzer[12] presented evidence that [^{3}H] serotonin uptake proceeds by three interdependent pathways. Transport through the plasma membrane proceeds via a carrier-mediated active transport system (Figure 8) and also by simple passive diffusion. At low concentrations of 5-HT the carrier-mediated process is dominant, so at low 5-HT concentrations, one is essentially studying the active transport system. This hypothesis is in good agreement with the observations of Figure 5, where the uptake of 5-HT was 95% inhibited by imipramine, suggesting a minimal involvement of the passive diffusion pathway at the 5-HT concentrations used in these experiments.

Most experimental work on the mechanism of reserpine suggests that its primary action on serotonin binding and storage is at the granular binding sites, and that its effect is to block a binding reaction (Figure 8).[8] Thus, Stahl and Meltzer[11] have suggested that the best way to isolate and measure the effect of reserpine on uptake is to greatly increase the concentration of [^{3}H] 5-HT in the medium and increase the passive diffusion pathway to the point that it overcomes ("swamps out") the active transport pathway. Under these conditions, the initial rate of

uptake should be primarily dependent on the reserpine-inhibitable binding mechanism and minimally influenced by the active transport system.[11]

This theoretical approach presents a problem in that the initial rate of uptake of [^{3}H] 5-HT at very high 5-HT concentrations would be too fast to follow using the methods available to us. We therefore elected to take the approach of inhibiting the active transport step with imipramine[4] in the hope that that this inhibition would increase the sensitivity of the uptake system to reserpine. Unfortunately, as shown in Figure 6, this procedure did not appear to increase the sensitivity of the system to reserpine, as reserpine produced about the same fractional inhibition of uptake in the presence and absence of imipramine.

Because we were unable to increase the sensitivity of the [^{3}H] 5-HT uptake system to reserpine, we elected to do our initial determination of the efficacy of this method *in vivo* with a very large (100 mg) dose of reserpine. As shown in Figure 7, a very substantial (approximately 66%) inhibition of the initial rate of uptake of [^{3}H] serotonin was observed. Though this particular dose of reserpine was very large, there are good theoretical reasons for believing that platelet uptake of serotonin may be a very sensitive indication of reserpinization. Principal among them is the fact that reserpine must displace a relatively large fraction of norepinephrine from the granule before pharmacological effects are seen.[3] Therefore, any dose of reserpine which effects behavior in the horse has already produced a very large effect on the granular binding of norepinephrine and serotonin. In keeping with these predictions, experiments currently in progress in our laboratory show very substantial inhibition of the initial rate of [^{3}H] 5-HT in horse platelets after doses of reserpine as low as 1.0 mg (2.0 μg/kg) and less.[13]

References

1. Bartholini, G., Pletscher, A., and Gey, K.F.: Dimunition of 5-Hydroxytryptamine in Thrombocytes *in vitro* by Chlorpromazine and Related Compounds. *Brevi Comunicazioni*, 15,(1961): 541-542.
2. Boullin, D.J. and O'Brien, R.A.: Abnormalities of 5-Hydroxytryptamine Uptake and Binding by Blood Platelets from Children with Down's Syndrome. *J Physiol*, 212,(1971): 287-297.
3. Cooper, J.R., Bloom, F.E., and Roth, R.H.: *The Biochemical Basis of Neuropharmacology*. 3rd ed., Oxford University Press, New York (1978).
4. Costa, J., Silber, S., and Murphy, D.: Effects of Reserpine and Imipramine on Vesicular Serotonin Uptake and Storage in Intact Human Platelets. *Life Sci*, 21, (1977): 181-188.
5. DaPrada, M. and Pletscher, A.: Isolated 5-Hydroxytryptamine Organelles or Rabbit Blood Platelets: Physiological Properties and Drug-Induced Changes. *Brit J Pharmacol*, 34, (1968): 591-597.
6. Henson, P.M.: *Personal communication*. Nat'l Jewish Hosp & Res Ctr, Denver, CO, (1977).
7. Hilton, B.: Effects of Reserpine and Chlorpromazine on 5-HT Uptake of Platelets from Migrainous and Control Subjects. *J Neurol, Neurosurg, Psychiat*, 37, (1974): 711-714.
8. Markwardt, F.: Influence of Drugs and Enzymes on Platelet 5-Hydroxytryptamine. *Ann Med Exp Fenn*, 46, (1968): 407-415.
9. Rudnick, G.: Active Transport of 5-Hydroxytryptamine by Plasma Membrane Vesicles Isolated from Human Blood Platelets. *J Biol Chem*, 252, (1977): 2170-2174.
10. Slotkin, T.A.: Neuropoisons: Their Pathophysiological Actions. Vol 2. L.L. Simpson and D.R. Curtis, Eds. Plenum Press, New York (1974).
11. Stahl, S.: The Human Platelet. *Arch Gen Psychiat*, 34, (1977): 509-516.
12. Stahl, S.M. and Meltzer, H.Y.: A Kinetic and Pharmacologic Analysis of 5-Hydroxytryptamine Transport by Human Platelets and Platelet Storage Granules: Comparison with Central Serotonergic Neurons. *J Pharm Exp Therap*, 205, (1978): 116-132.
13. Tobin, T.: A Review of the Pharmacology of Reserpine in the Horse. *J Eq Med Surg*, 10, (1978): 433-438.
14. White, C.G., Woods, W.E., and Tobin, T.: The Pharmacology of Reserpine in the Horse. II. Biochemical and Behavioral Effects of Reserpine. (1979): In preparation.