meters from the unbleached edge to the measuring slit, and $t_{1/2}$ the time to half recovery evaluated from records such as Fig. 2a. Thirty records similar to Fig. 2a give $t_{1/2} = 15.4 \pm 3$ seconds for mud puppy and 4.0 ± 0.5 seconds for frog (*13*). The computed diffusion coefficients are $(4.7 \pm 0.9) \times 10^{-9}$ cm^2/sec for the former and $(5.5 \pm 0.6) \times 10^{-9}$ cm^2/sec for the latter.

Some of our 420-nm measurements in mud puppy suggest that bleached visual pigment molecules may diffuse only half as fast as unbleached molecules. However, we cannot be exact about this because the records must be corrected for a variable rate of loss from the cells of dehydroretinol, which also absorbs at 420 nm, after bleaching.

Finally, results such as those given in Fig. 1b show that mud puppy cone pigment also undergoes rapid lateral diffusion. Since electron micrographs show that cone lamellar membranes (unlike those of rods) are longitudinally continuous through hairpin turns on one edge and through the cell envelope membrane on the other, it was expected that fast longitudinal diffusion would also be found. Surprisingly, no diffusion occurred during 20 minutes. We conclude that motion of cone pigment protein is impeded at the highly curved membrane bends.

Using the Stokes-Einstein relation for diffusing spheres (*14*) together with our value for the diffusion coefficient and an assumed spherical visual pigment radius of 23 Å, we calculate an effective membrane viscosity of about 2 poise, in close agreement with that calculated from rotational relaxation measurements on the same assumptions (*15*). However, we do not conclude from this agreement that visual pigment is a sphere completely immersed in lipid, for the same agreement would occur if it were any partially or completely immersed object of revolution (*16*). The calculated membrane viscosity of 2 poise therefore constitutes a lower limit. Evaluation of the actual viscosity from our kind of experiment awaits more precise information on shape and degree of immersion of visual pigment.

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Stability of Catecholamines Immobilized on Glass Beads

M. S. Yong (*1*) gives the impression that biological activities for catecholamines covalently attached to glass beads (*2, 3*) are due to pharmacological concentrations of catecholamines leaching off the glass beads. The evidence is that [^{14}C]catecholamines are released from glass beads washed only with distilled water. This is not the washing procedure we outlined (*3*) and used (*2, 3*) prior to our biological assay of catecholamines immobilized on glass beads. Instead, the bead-bound catecholamines were extensively washed with several liters of 0.1*N* HCl to remove any noncovalently bound catecholamines. In addition, the beads were washed extensively with 0.8 percent NaCl immediately prior to use, to remove the acid and any free catecholamines that might have accumulated since the acid washing. During this procedure, the beads were washed with at least 12 liters of 0.1*N* HCl, and then with 18 liters of 0.8 percent NaCl or distilled water, prior to biological assay (*4, 5*).

The results of washing 1 g of [^{14}C]epinephrine–glass beads with acid (*4*) illustrate that when the [^{14}C]epinephrine-glass beads are washed slowly with 20 liters of 0.1*N* HCl over a 6-day period, 16 percent of the [^{14}C]epinephrine is removed in the first 10 liters of acid, and less than 1 percent is released in the next 10 liters of acid. The total amount of catecholamine liberated in the first 10 liters of acid would more than account for the free catecholamine that was reported by Yong (*1*).

The release of [^{14}C]epinephrine from [^{14}C]epinephrine–glass beads placed in an oxygenated Krebs solution was studied subsequent to washing in either 0.1*N* HCl or distilled water. Total radioactivity bound to the glass beads was determined by the hydrogen fluoride and dithionite techniques (*4, 5*). The release of [^{14}C]epinephrine from acid-washed glass beads was compared to the release from glass beads washed only with distilled water. Two grams of epinephrine–glass beads had bound a total of 0.10 mc of [^{14}C]epinephrine. One gram of these beads was washed with 5 liters of distilled H_2O, and 1 g with 12 liters of 0.1*N* HCl at a flow rate of 3 liters per day. Each gram was placed in 10 ml of oxygenated Krebs solution at 37°C and 50-μl samples were counted in 10-ml of Aquasol. The [^{14}C]epinephrine–glass beads that were washed only with distilled water released 4 percent of the labeled epinephrine in the first 24 hours, whereas the acid-washed beads released only 0.19 percent of the [^{14}C]epinephrine in the same period. There is a substantial difference in the observed rate of release, dependent upon the washing procedure used, indicating the necessity of acid washing.

Yong's method (*1*) for determining biological activity of the water-washed epinephrine–glass beads consisted of placing 25 mg of the glass [by our estimate, about 6500 glass beads of the size used in (*2, 3*)] into 2 ml of a Krebs solution for 5 minutes, in which time, he reports, approximately 0.5 per-

cent of the catecholamine was released from the beads; when this supernatant was added to a 15-ml muscle bath, the catecholamine concentration was sufficient to cause contractions of the aortic strips. As a result of the washing procedure, sufficient amounts of noncovalently bound catecholamines may have been present in the porous glass. The glass, as well as an incomplete conversion of the alkylamine glass to the arylamine glass, can result in a cation exchange system. Such a system could bind catecholamines, whereas acid washing would remove such binding. We have reported (*2, 3*) numerous control experiments establishing that contact between the glass beads and the hearts, papillary muscles, or heart cells was absolutely necessary to elicit a biological response. These include the experiments in which large amounts of catecholamine glass (125 mg or more) were added to small volumes (2 ml or less) of medium surrounding the biological preparations. The beads had no effect on the tissue when present in the bath but not in contact with the tissue.

Our data on catecholamines released after washing give a calculated value of only 0.008 percent released per hour at 37°C and only 6.0×10^{-5} percent release per hour at 4°C (*4*). Moreover the biological assays we reported (*3*) were carried out with only one to ten beads which, we estimate, weigh 4 to 40 μg. On the basis of our most recent methods for the determination of the total amount of amine bound, we calculate that 6×10^{-12} mole is bound to one glass bead with a 300-μm diameter (*4*). One isoproterenol glass bead gave the same response on a cat papillary muscle as did a 0.1 μM solution of isoproterenol (*3*). If upon addition of the beads to the biological preparation the total amount normally released in 1 hour is 0.008 percent, this would yield only 4.8×10^{-16} mole of catecholamines in a 40-ml bath bubbled continuously with a mixture of 95 percent O_2 and 5 percent CO_2. The half-life of free catecholamines in such a solution is less than 5 minutes (*6*). Even from Yong's value of about 2 percent release per hour, the amount of free catecholamines would be only 1.2×10^{-13} mole/hour from one glass bead, an insufficient amount of free catecholamine to elicit a response in any preparation. Furthermore, we reported (*3*) that there was no detectable difference in the amount of catecholamine bound to ten glass beads before or after incubation with a cat papillary muscle in an oxygenated Krebs solution at 37°C. In addition, we reported (*3*) that the catecholamines bound to glass beads are biologically active more than ten times as long as the soluble catecholamines.

We will report (*7*) a 10,000-fold increase in the amount of free propranolol necessary to inhibit immobilized isoproterenol as compared to free isoproterenol, suggesting that small amounts of catecholamines leaching off the glass into solution cannot be causing the biological effects. We have left open (*2, 3*) the possibility that the action of the immobilized catecholamines is that of an artificial prejunctional site with the volume between the bead and the muscle so small that less than 10^{-16} mole becomes an important concentration, even though this is inconsistent with many of our findings (*2–5, 7*). Excluding this possibility, we feel that the evidence we have obtained is strongly in favor of the catecholamines acting while covalently coupled to the glass beads.

The potential of immobilized hormones for affinity chromatography is not altered by small percentages of hormone leakage; at most, it would only slightly reduce the efficiency of such an affinity column.

It should be clear that we are not dealing with the gross mechanism of action directly implied by Yong's data for the catecholamines bound to glass beads (*1*), and that the exact mechanism of action for any of the immobilized hormones will only be elucidated when more carefully controlled experiments are undertaken.

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Certain criteria must be met before the acceptance of the proposition that drugs or hormones bound to a solid support exert a biological action. The most important criterion is experimental evidence that demonstrates unequivocally that the biological action is not due to drug or hormone liberated from the support.

In the first report by Venter *et al.* (*1*) the epinephrine–glass beads were washed with only 1 liter of distilled water, then washed again just before use; neither the type nor the quantity of solution was specified. In the second report Venter *et al.* (*2*) washed the beads with several liters of 0.1*N* HCl in addition to the 1-liter water wash and then again washed the beads extensively with 0.8 percent NaCl just before use. The quantities used for "several" or for "extensive" are not given except in the above comment where they are 12 and 18 liters, respectively. The wash with the acid was not done, to the best of our knowledge, in the work described in the first report which included biological experiments (*1*).

In the experiments covered in both reports Venter *et al.* (*1, 2*) used tritium labeled catecholamines to demonstrate the stability of attachment to the beads. In the first report (*1*) they placed beads with a total radioactivity of 111,973 count/min in 10 ml of water and at various times withdrew 20-μl samples for isotope determination. They stated that after 76.8 hours only 167 count/min were present in the solution (*1*). However, it is unclear what this figure means. If it represents the counts in the last 20-μl sample, we calculate the release to be 83,500 count/min in 10 ml and the rate of release of 0.975 percent per hour; if it is the total calculated for 10 ml, the counts in any sample would be undetected (*3*).

In a subsequent report Venter *et al.* (*2*) presented evidence for the stability of [^{3}H]isoproterenol glass beads. In this report they introduced the acid wash for the beads and tested their biological activity on horizontally mounted cat papillary muscles. Ten [^{3}H]isoproterenol glass beads were placed on the papillary muscle in an organ bath of unspecified volume. After 30 minutes of contact with the tissue the radioactivity of the beads was not significantly changed and no label was found in a 100-μl sample of bath fluid. These data were taken as the strongest evidence in favor of the

hypothesis that catecholamines produce their actions while covalently bound to the glass beads (*2*). If we assume that the bath used had a volume of 10 ml it can be calculated that a 1 to 2 percent [^{3}H]isoproterenol release from the beads in 30 minutes would give 0.9 to 1.8 count/min in 100 μl (1/100 of the total volume), a level of radioactivity too low to be detected with the random background variation of 43 to 46 count/min reported (*2*). In view of these inadequate and insensitive techniques for tritium determination reported by Venter *et al.* (*1*, *2*) we feel that the validity of their conclusion in regard to the site of action and the mechanism of action of immobilized catecholamines must be questioned.

Venter and Kaplan now present evidence from studies with immobilized [^{14}C]epinephrine of a continuous release of label from beads previously washed with 12 liters of 0.1*N* HCl. These findings are in contradiction to those given in their previous two reports where biological activity was demonstrated (*1*, *2*). They argue that the calculated release of 4.8×10^{-16} mole per bead per hour is too small to cause a discernible effect if the tissue is suspended in a 40-ml organ bath. Their argument is, however, untenable since the glass beads are placed in contact with the tissue and the released amine is distributed into a small volume, not the entire 40 ml. It is the concentration of drug which is important for receptor activation, not the amount. It has been estimated that the quantity of norepinephrine released per stimulus from one varicosity of an adrenergic nerve is small, 400 to 9000 molecules (*4*). A quantity of 400 to 4000 molecules of norepinephrine at a close contact varicosity with an area of 2 μm^2 and a depth of 0.02 μm can give a maximal concentration in the range of 5.9×10^{-6} to $5.9 \times 10^{-5}M$ (*5*) and is capable of triggering a biological response. In fact, the volume of distribution is probably greater than the minimum calculated. If we assume that 50 percent of the surface area of one catecholamine bead (the surface area of a bead of diameter 300 μm is 282,744 μm^2) is in close contact with the muscle and that the distance between the bead and the muscle is 0.1 μm, then the volume of this space is 1.41×10^{-8} ml. A release of as little as 2.4×10^{-16} mole of amine from one bead would give a concentration of $1.7 \times 10^{-5}M$, or 1/60 of this in a minute, which is enough to cause a response, in a coupled system like the heart. It is therefore not surprising to note that contact between the glass beads and the tissue is absolutely necessary to elicit a biological response and that a very high concentration of propranolol is needed to block the effect of isoproterenol beads in contact with the myocardium.

One of us has reported on the unstable nature of catecholamine glass beads and presented evidence to show that the biological activity of these beads could best be explained on the basis of amine release (*6*). To further investigate this problem we have coupled [^{14}C]epinephrine to arylamine glass beads prepared from different types and batches of silica glass manufactured by Corning Glass. In addition, [^{14}C]-epinephrine and 5-[^{14}C]hydroxytryptamine were coupled to CPG-arylamine (*7*). After the reaction the beads were washed with more than 18 liters of either distilled water or 0.1*N* HCl for 1 to 2 weeks. After the final wash radioactivity appeared in the storage solution and the amount increased with time. No definite advantage was found for acid over water wash. The biological activity of these complexes on isolated tissues was found to closely parallel release of amine from the beads (*7*).

We agree with Venter and Kaplan that there is a potential use for immobilized hormones in affinity chromatography or immunoassay where release from the matrix would not interfere too much with the technique. However, in determining the site or mechanism of action of a drug or hormone, the evidence for stability is critical. We do not feel that the evidence presented by Venter *et al.* is adequate to show that the immobilized catecholamines act while covalently bound to glass beads. The biological activity they described can be explained by the release of amine from the beads.

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Juvenile Hormone Synergists: A Possible Case of Hasty Conclusion?

Slade and Wilkinson (*1*) have suggested that many juvenile hormone (JH) analogs probably act by synergizing subthreshold levels of endogenous JH, rather than by possessing intrinsic activity. We believe that this conclusion is based on several questionable assumptions which are not supported by the evidence presented.

As Slade and Wilkinson pointed out, the evidence in support of the intrinsic activity hypothesis is admittedly circumstantial and rests mainly on two observations:

1) Most of the more active JH mimics discovered in extensive structure-activity screenings in many laboratories bear a strong resemblance to the natural JH's in their size, shape, stereoisomerism, and polarity distribution (*2*). However, this observation suffers several limitations, including (i) a probable bias toward most intensive screening of compounds with physical and chemical properties resembling those of natural JH's, (ii) the difficulty of rationalizing the activity of some compounds such as propyl-2-propynylphenyl phosphonate (*3*) on this basis, and (iii) the lack of reliable biochemical means of distinguishing intrinsic JH activity from other possible modes of action.

2) Also, some of these compounds are active on insect preparations which lack corpora allata and therefore presumably lack a significant JH level or generating capacity (*4*). As Slade and Wilkinson point out, these assumptions lack rigorous verification. However,