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,

they imply that the burden of proof should be placed on the assumption that such insect preparations lack a JH generating capacity, whereas we believe that the burden of proof should be placed on the demonstration that these preparations can generate a physiologically significant JH level in response to JH mimics.

The evidence presented by Slade and Wilkinson (*1*) is not adequate to judge the validity of the synergist hypothesis, as it is based only on effects obtained in tissue homogenates of the moth *Prodenia eridania*, without any evidence as to whether such effects are relevant to the activity of these compounds in vivo. The concentrations (~ 30 parts per million) of JH mimics in Slade and Wilkinson's incubation medium are much higher than the 10 parts per billion for ZR-515 or 1 ppb for ZR-512 that are required for systemic activity in a JH bioassay in the moth *Manduca sexta*, involving penultimate larvae with neck ligatures (*5*), which suggests that the effects demonstrated in vitro may be unrelated to the basic mode of action of these compounds. Even smaller doses of these compounds are required for localized juvenilization around the site of application in the *Manduca* assay. Recently, it has been found that hemolymph from *Manduca* larvae of the stage used in this assay lacks JH esterase activity, in contrast to the hemolymph from last instar larvae (*6*). Can the JH esterase activity of the tissues, in particular that of the epidermal cells during this stage, account for the supposed synergistic action of ZR-515 and ZR-512? Additional evidence for inhibition in vitro of epoxide hydrase, important in JH inactivation (*7*), by JH mimics and nonmimetic compounds has appeared (*8*). Some of the information in that study suggests anomalies in the synergist hypothesis. For instance, several potent inhibitors of epoxide hydrase were said to synergize exogenous JH in whole insects, yet had no JH activity when applied alone. Also, triphenyl phosphate was a strong competitive inhibitor of epoxide hydrase in vitro and is also a well-known esterase inhibitor in vivo (*9*), suggesting it might be expected to help stabilize natural JH's by inhibiting JH esterases as well. Despite its proven ability to penetrate insect tissues and an appreciable in vivo stability, this compound had no JH activity on the two test species.

Slade and Wilkinson suggest that an important reason for favoring the synergist hypothesis is the lack of structural resemblance of many JH mimics to the natural JH's. However, the steric requirements for intrinsic JH activity have not been established. If we assume that only an approximation of the steric properties of natural JH's is one prerequisite for intrinsic activity, all of the compounds they studied except propyl-2-propynylphenyl phosphonate (which had little activity in their assay) and probably piperonyl butoxide (which is a relatively weak JH mimic on most insects tested) (*3*, *10*) might by this criterion alone be predicted to have significant intrinsic activity. Construction of space-filling structural models of these compounds reveals their steric resemblances (*11*).

In conclusion, while we recognize the possibility that some JH mimics may exert at least part of their action through synergism of endogenous hormone, the evidence presented by Slade and Wilkinson is not adequate to support the statement, "Our data confirms the view . . . that the activity of some JHA [juvenile hormone analog] is synergistic rather than intrinsically hormonal." Perhaps experiments in vivo with low morphogenetically effective doses of JH mimics would reveal their effect on JH metabolism and the concentration of JH in the tissues. Doses in excess of the minimum required for morphogenetic activity should be avoided, since esterase and epoxidase inhibition may be dependent on the JH mimic concentration. If a given JH mimic can be shown to induce physiologically significant JH levels in allatectomized insects lacking a measurable (by current assays) JH level, then the credibility of the synergist hypothesis will be vastly increased—although the possibility would not be excluded that these compounds also have physiologically significant intrinsic activity as well. In view of the close physical and steric resemblances with natural JH's exhibited by most of the demonstrated potent JH mimics, we suggest that it is not unreasonable to retain the hypothesis that most of these compounds have intrinsic activity which fully or partly is responsible for their activity in vivo, until the time that a reliable biochemical means of determining intrinsic activity becomes available or incontrovertible evidence for an alternative hypothesis is accumulated. The recent demonstration of a tissue receptor protein with a high affinity for a JH analog (*12*) suggests that a specific assay for intrinsic activity may be attainable, provided that a functional relation between binding affinity and specific biological or biochemical effects of JH can be established.

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