MOLECULAR PHARMACOLOGY, 11, 1-9

The Structure and Quantitation of Catecholamines Covalently Bound to Glass Beads

J. CRAIG VENTER, LYLE J. ARNOLD, JR., AND NATHAN O. KAPLAN

Departments of Medicine and Chemistry, University of California, San Diego, La Jolla, California 92037

(Received July 3, 1974)

SUMMARY

VENTER, J. CRAIG, ARNOLD, LYLE, J. JR. & KAPLAN, NATHAN O. (1975) The structure and quantitation of catecholamines covalently bound to glass beads. *Mol. Pharmacol.*, **11**, 1-9.

Methods are described which enable accurate determination of the amounts of catecholamines covalently bound to glass beads. For isoproterenol and epinephrine these amounts are 1.68 and 1.63 μmoles, respectively, per gram of arylamine glass beads. Distillation experiments indicate that more than 80% of the tritium label released from [^{3}H]catecholamine bound to glass is due to tritium exchange. Catecholamines labeled with tritium at position 7 should not be used to quantitate the extent of release of catecholamines coupled to glass beads, since this proton undergoes slow exchange when the [^{3}H]catecholamine is diazotized to glass beads. Using [^{14}C]catecholamines, the true stability of the glass bead-catecholamine complex was examined. Only 0.008% of the catecholamines are released per hour at 37°, and less than 7×10^{-5}%/hr at 4°. Under the experimental conditions for testing the biological activity of the immobilized catecholamines, less than 8 attomoles of soluble catecholamine are calculated to be released per minute into the bathing medium of the physiological preparations. Methods are also described for synthesis of amino-substituted catecholamines by reduction of the azo linkage of the catecholamine covalently coupled to solid supports. The exact structure and point of catecholamine substitution were determined by proton magnetic resonance; the results are consistent with substitution at position 6 on the catechol ring. Biological data are presented which show the amino-substituted and soluble azo-substituted catecholamines to be active when tested on isolated cat papillary muscles.

INTRODUCTION

Drugs and catecholamines covalently bound to glass beads have been shown to exhibit biological activity in a number of systems, including heart cells in culture, chicken embryo hearts, hearts in open-chested, anesthetized dogs, glial tumor cells, cat papillary muscles (1, 2), and isolated, perfused guinea pig hearts.[1] The immobilized catecholamines have also been used as an affinity column to partially purify the *beta* adrenergic receptor-adenylate cyclase complex from turkey erythrocytes (3).

With these systems it has been established that physical contact between glass bead-bound catecholamines and the effec-

This work was supported by a grant from Hoffmann-La Roche, Inc.

[1] W. F. Riker, personal communication.

tor tissue is absolutely necessary for biological activity and that the immobilized catecholamines exhibit prolonged biological effects as compared to soluble catecholamines. It has also been established that the biological effects of the immobilized catecholamines are immediately and completely reversible by removal of the glass beads from the systems (1, 2).

The observation that cocaine will potentiate the activity of the immobilized catecholamines, including immobilized isoproterenol, indicates a possible postjunctional site of action for cocaine (2) and is an example of the potential use of these immobilized agents for determination of their sites as well as their mechanisms of action. It also emphasizes the importance of determining the exact mode of biological action of immobilized catecholamines.

The coupling procedure we have utilized for attaching catecholamines to glass beads involves their reaction with an aryldiazo compound chemically attached to glass. One would predict that the most likely position for electrophilic substitution of the diazonium salt on the catecholamine would be the aromatic C-5 or C-6 (1); however, until now the point of substitution of the catecholamine has not been precisely determined. The structure and point of substitution are important in order to determine whether the amines are in fact biologically active when such a substitution occurs.

The quantitation of catecholamines bound to glass beads and the rates of their release are presented here, together with data showing their point of attachment and preliminary biological tests of soluble substituted catecholamines.

MATERIALS AND METHODS

Preparation of glass-bound catecholamines. Glass beads (GZO-3940) were obtained from Corning Glass Works, and the alkyl- and arylamine derivatives were synthesized by the method of Venter and Dixon (4). *l*-Epinephrine and *dl*-isoproterenol (Sigma) were covalently coupled to arylamine glass by the method of Venter *et al.* (2, 4). *dl*-[7-^{3}H]Isoproterenol (New England Nuclear Corporation) had a specific activity of 2.11 Ci/mmole. One gram of activated arylamine glass was added to a mixture of 2.0 ml of *dl*-[^{3}H]isoproterenol (1 mCi/ml) and 5 mg of unlabeled *dl*-isoproterenol in 3 ml of 0.1 M phosphate buffer, pH 7.5; the reaction was allowed to proceed overnight at 0° in the dark. The supernatant extract was then decanted, and the glass beads were packed into a small column and washed continuously at 23° with 12 liters of 0.1 N HCl, followed by 18 liters of distilled water at a rate of 3 liters/day.

The [^{14}C]epinephrine–glass bead complex was prepared by allowing 0.35 mg of *dl*-[7-^{14}C]epinephrine bitartrate (0.1 mCi) in 2.0 ml of 0.1 M phosphate buffer, pH 7.5, to react with 1.0 g of activated arylamine glass overnight in the dark at 0°. For biological testing, various dilutions were made with unlabeled epinephrine and similarly coupled. The ^{14}C-labeled epinephrine-beads were then washed as described above.

Fluorescamine was a gift of Hoffmann-La Roche, Inc.

Determination of radioactivity on glass beads. The total radioactive catecholamine bound to glass beads was determined by two methods. (a) Dithionite (50 mg) was added to 10 mg of [^{3}H]- or [^{14}C]catecholamine–glass beads (dry weight) in 10 ml of distilled water for 5 min; 50-μl aliquots of the supernatant solution were then removed and counted in 10 ml of Aquasol (New England Nuclear Corporation) in a liquid scintillation counter. (b) Either [^{3}H]- or [^{14}C]catecholamine–glass beads (10 mg, dry weight) were placed in a plastic scintillation vial, and 0.1 ml of 40% hydrogen fluoride was added. When the glass beads had totally dissolved, 9.9 ml of distilled water were added; 50-μl aliquots were removed and counted as above.

The amount of [^{3}H]- or [^{14}C]catecholamine released into solution from the glass bead complexes was determined by placing 1 g of each complex in 10 ml of distilled H_2O and incubating it at 4°, 23°, or 37° as

indicated below. Fifty-microliter samples of the supernatant solution were removed at the appropriate times, care being taken to ensure that no glass particles were present, and counted as indicated above.

Preparation of amino-substituted catecholamines for proton magnetic resonance studies. Amino-substituted catecholamines were obtained for proton magnetic resonance studies by reductively cleaving the catecholamines from 5.0 g of glass-bound catecholamines with 10 ml of 50 mM sodium dithionite in D_2O. The catecholamine eluate was adjusted to pH 2.0 with concentrated DCl (MSD Isotope Products, 99% deuterium), 10 mg of sodium metabisulfite were added, and the sample was lyophilized. The resulting dry solid was extracted with 3 ml of methanol, and the extract was evaporated, dissolved in 99.8% D_2O, lyophilized, and redissolved in a 1:1 solution of Wilmad 100% D_2O-99.5% [*U*-^{2}H]methanol. In addition, 5 μl of concentrated DCl were added to maintain the isoproterenol derivative in the acid form. Samples of *dl*-isoproterenol were prepared in a similar manner, but without the addition of DCl, since they were already in the acid form. An internal standard of tetradeuterotrimethylsilyl sodium proprionate was employed, and the catecholamine compounds were used at a concentration of 0.1 M. Sample volumes were 0.25 ml, and Wilmad Teflon plugs were used to prevent eddying. The PMR spectra were obtained with a field-sweep Varian HR 220 spectrometer, and the precise chemical shifts of the aromatic resonance were determined by generating modulation side bands from TSP[2] with a Wavetek 131A oscillator. In order to improve resolution, the spectra were obtained at 40°.

RESULTS

Yields of radioactive catecholamines. The acid wash profile of 1 g of [^{14}C]-epinephrine–glass bead complex is shown in Fig. 1. The [^{14}C]epinephrine–glass beads were packed into a small column and washed continuously for 6 days with 0.1 N HCl, and the cumulative percentage of ^{14}C per liter of 0.1 N HCl was determined. As shown in this figure, 16% of the total radioactivity on the glass was eluted in the first 10 liters of acid wash. Later a sustained, much slower rate of release was observed, with less than 1% eluted in an additional 10 liters of acid wash.

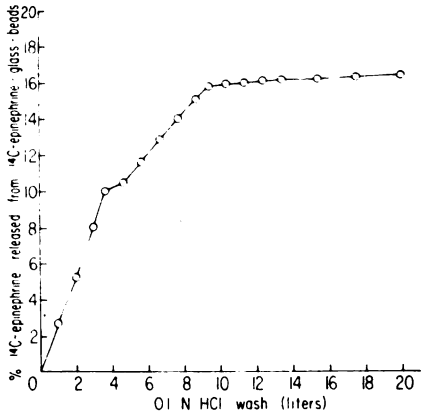

FIG. 1. *HCl wash profile of* [^{14}C]*epinephrine-glass beads*

One gram of [^{14}C]epinephrine-glass beads was packed into a small column and washed continuously for 6 days with 20 liters of 0.1 N HCl. Aliquots of the wash were counted in Aquasol, and the cumulative percentage release was calculated.

The total amounts of catecholamine coupled to the glass beads were determined subsequent to the acid washing procedure. The hydrogen fluoride technique yielded on the average 13% more total radioactivity than the dithionite reduction procedure. As the latter procedure did not give 100% recovery of labeled amine from the porous glass, the HF method appears to be the preferred technique and can be assumed to represent 100% of the radioactivity. Utilizing the HF method, the amounts of isoproterenol and epinephrine bound to glass beads are 419 μg (1.68 μmoles) and 304 μg (1.63 μmoles), respectively, per gram of arylamine glass. Since 1 g of arylamine glass contains approximately 2.5×10^5 glass beads 100–300 μm in diameter, there are approximately 6.7 pmoles of iso-

[2] The abbreviation used is: TSP, tetradeuterotrimethylsilyl sodium proprionate.

proterenol and 6.5 pmoles of epinephrine per glass bead.[3]

Nature of radioactivity released from [^{3}H]isoproterenol-glass bead complex. The total amount of [^{3}H]isoproterenol bound to 1 g of arylamine glass beads subsequent to the acid and water washing procedures was 3.56×10^8 dpm as determined by the HF method. When 1 g of glass was placed in 10 ml of distilled water at 23° and 50-μl aliquots were removed and counted, 1.74×10^5 dpm were released per hour, representing 0.048% of the radioactivity bound to the glass beads.

It was important to determine the precise nature of the radioactivity leached from the labeled catecholamine-glass bead complex in order to determine whether the released radioactivity represented exchanged tritium or catecholamine. One gram of [^{3}H]isoproterenol-glass beads was placed in 10 ml of distilled water for 12 hr at room temperature, and the resulting radioactive supernatant fraction was distilled. This procedure revealed that only 19% (4.0×10^5 dpm) of the total released radioactivity (2.1×10^6 dpm) remained undistilled, whereas 81% (1.62×10^6 dpm) was distilled. As a control, [^{3}H]isoproterenol (5.8×10^6 dpm) was treated under the same conditions, and only 3.8% (2.2×10^5 dpm) was distilled. This experiment was repeated with [^{3}H]-isoproterenol reduced from the glass with dithionite; of this, less than 3% was distilled. These results indicate that approximately 80% of the radioactivity leached from the glass represents exchanged tritium and not released catecholamine. On the basis of this information, we calculate the actual amount of catecholamine released from the glass to be 0.0091%/hr.

In order to test the tritium exchange hypothesis, *dl*-[7-^{14}C]epinephrine was coupled to arylamine glass. After acid washing the total ^{14}C radioactivity on 1 g of glass was 1.91×10^8 dpm. However, only 0.0082% (1.57×10^4 dpm) of this radioactivity was released per hour into 10 ml of distilled water at 23°. This value is slightly smaller than that calculated from the adjusted ^{3}H exchange data, and supports the hypothesis that, while bound to glass, tritium in position 7 of the catecholamine exchanges slowly with its environment.

Effects of temperature on catecholamine release rates. The effect of temperature on the release rate of [^{14}C]epinephrine from its glass bead complex is shown in Fig. 2. One gram of [^{14}C]epinephrine-glass was incubated in 10 ml of distilled water at 4°, 23°, or 37° as indicated in the figure, and the cumulative percentage release was determined at the times shown. A release rate of 0.008% hr was maintained at 37° and 23° but dropped to 6.9×10^{-5}%/hr at 4°; after 120 days the total radioactivity released at 4° was less than 0.2%. A previous study showed that the release rate determined in an oxygenated Krebs solution is not different from that obtained in distilled H_2O (5).

Proton magnetic resonance and chromatography of isoproterenol derivative after removal from glass. The structure of the isoproterenol derivative obtained by dithionite reduction of the isoproterenol-glass complex as described under MATERIALS AND METHODS was determined by proton magnetic resonance and paper chromatography.

The aromatic regions of the PMR spectra of isoproterenol and the isoproterenol derivative are shown in Fig. 3. The parent catecholamine (Fig. 3b) gives the expected *ABX* spectrum. In this case the C_5 and C_6 protons show a typical *A-B* quartet pattern, with an *ortho* coupling constant of 8.3 Hz. The C_6 proton is further coupled to be downfield C_2 proton by a 2.0-Hz *meta* coupling, splitting the upfield portion of the *A/B* quartet. There is no observable coupling between the *para* C_2 and C_5 protons. In contrast, the aromatic region of the isoproterenol derivative in Fig. 3 shows two sharp resonances[4] of equal intensity. This indicates that a substitution has

[3] The previous reports (1, 2) of the amounts of catecholamine per glass bead were based upon an underestimation of glass beads per gram of glass and on unsatisfactory methods of quantitating the total amounts covalently coupled.

[4] The aromatic region of the isoproterenol derivative shows other minor resonances, which appear to account for less than 10% of the aromatic protons.

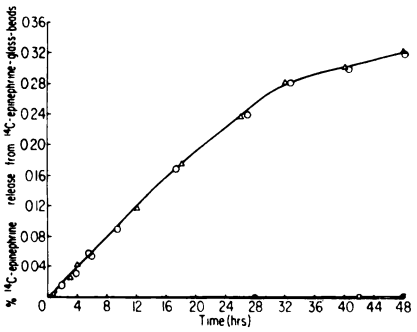

FIG. 2. *Effect of temperature on release rate of [¹⁴C]epinephrine from [¹⁴C]epinephrine-glass beads*

One gram of [¹⁴C]epinephrine-glass beads was incubated in 10 ml of distilled water at 4° (□), 23° (○), or 37° (△), and the cumulative percentage release was determined at the indicated times.

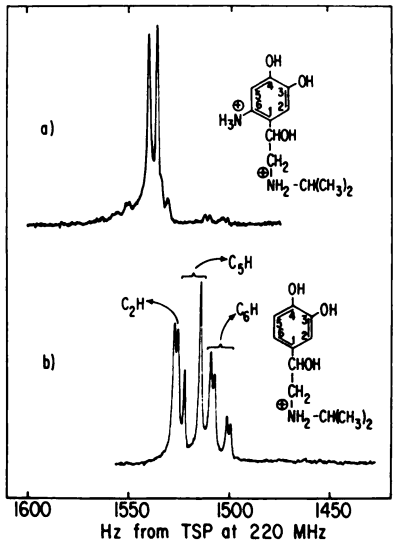

FIG. 3. *Proton magnetic resonance spectrum of aromatic region of isoproterenol derivative (a) and isoproterenol (b) at 220 MHz*

The concentrations were 0.1 M in D_2O-[*U*-²H]-methanol (1:1), the chemical shifts are from TSP and the probe temperature was 40°. The isoproterenol spectrum (b) shows the expected *ABX* spectrum with both *ortho* and *meta* coupling constants. The isoproterenol derivative (a), however, shows no *ortho* or *meta* coupling, which indicates that the two residual aromatic protons are *para* to each other.

taken place on the aromatic ring. The isoproterenol derivative shows no coupling between the two remaining aromatic protons, indicating that they are neither *meta* (approximately 8 Hz coupling) nor *ortho* (approximately 2 Hz coupling) with respect to one another. The two remaining aromatic protons must occupy *para* positions with respect to each other. The substitution on the isoproterenol derivative must therefore be at the C_6 position. The chemical shifts and coupling constants of the isoproterenol and the 6-substituted isoproterenol aromatic protons are shown in Table 1. The PMR spectrum (not shown) demonstrates that the ethanolamine side chain remains intact in this derivative, indicating that only the aromatic region is involved. Furthermore, the isoproterenol derivative, but not isoproterenol, reacts in solution with fluorescamine, indicating the presence of a new primary alkyl- or arylamine group.

Paper chromatography in butanol–acetic acid–water (25:4:10) shows the catecholamine derivative reduced from glass to have an R_F value of approximately 0.14 based on its blue fluorescence, its reaction with both ninhydrin and fluorescamine, and its blue color upon oxidation. In contrast, the parent compound has an R_F value of 0.44 as determined by its ninhydrin reaction and its slight yellow fluorescence. Isoproterenol gives no reaction with fluorescamine and does not undergo a marked color change upon oxidation. These results (summarized in Table 2) indicate that isoproterenol and its 6-amino derivative have strikingly different chemical characteristics.

Biological activity of 6-substituted catecholamines. 6-Aminoisoproterenol, 6-aminoepinephrine, and 6-[*p*-nitrophenylazo]epinephrine, synthesized and purified as described in MATERIALS AND METHODS and Fig. 4, exert positive inotropic effects in a dose-related manner when added to the baths of isometrically contracting cat papillary muscles. The dose-response relationships for the 6-aminocatecholamines, 6-[*p*-nitrophenylazo]epinephrine, and the parent catecholamines are shown in Fig. 4. 6-Aminoisoproterenol appears to be less active than isoproterenol and slightly less active than *l*-epinephrine. 6-Aminoepinephrine appears less potent than either epinephrine or norepinephrine.

TABLE 1

Proton magnetic resonance measurements

The chemical shifts are from TSP, and the coupling constants of isoproterenol and 6-aminoisoproterenol are in Hertz at 220 MHz. The chemical shifts are accurate to ±0.5 Hz, and the coupling constants are accurate to ±0.2 Hz. See Fig. 3 for other details.

Compound	C_2H	C_5H	C_6H	J_{2-5}	J_{2-6}	J_{5-6}
Isoproterenol	1527.3	1517.5	1505.9	<0.5	2.0	8.3
6-Aminoisoproterenol	1537.2[a]	1541.2[a]		<0.5		

[a] Assignments are arbitrary.

TABLE 2

Chromatographic characteristics

Ascending paper chromatography of isoproterenol and the isoproterenol derivative was carried out in butanol-acetic acid-H_2O (25:4:10). The fluorescence was determined immediately after chromatography with a standard ultraviolet light. The ninhydrin test was performed by a standard dip procedure (6), and the Fluorescamine test was done by spraying the chromatogram with an aerosol of 10 mg of fluorescamine per 100 ml of acetone (7). Oxidation of the chromatographic material was carried out by placing the chromatographic strips in the atmosphere for several hours. The reactive groups of these compounds were determined by simultaneously chromatographing several "spots" of each compound on a single chromatogram; individual spots were isolated by cutting the chromatogram into strips and were assayed by the various methods.

Compound	R_F	Fluorescence	Ninhydrin	Fluorescamine	Oxidation
Isoproterenol	0.44	Light yellow	Pink-brown	No reaction	Colorless
Isoproterenol derivative	0.14	Light blue	Blue	Yellow	Blue

In contrast, 6-[*p*-nitrophenylazo]epinephrine appears to be slightly more active than *l*-epinephrine in developing positive inotropic responses in the isolated cat cardiac muscle.

The 6-aminocatecholamines appear to have lower efficacies than their respective parent catecholamines, although they exhibit a greatly increased duration of biological action. *l*-Epinephrine (1 μM), although attaining a higher peak isometric force on a molar basis, displayed a shorter duration of action with diminution of the response to control levels within 30 min (Fig. 5). In contrast, 6-aminoepinephrine (2 μM), tested under identical conditions, produced positive inotropic responses that were sustained for 8 hr ± 30 min (n = 10), demonstrating a consistent prolongation of the biological action. 6-Aminoisoproterenol and 6-[*p*-nitrophenylazo]epinephrine gave a positive inotropic time course (not shown) similar to that for 6-aminoepinephrine. The positive inotropic effects in response to the 6-aminocatecholamines and 6-[*p*-nitrophenylazo]epinephrine could be completely blocked by the *beta* adrenergic antagonist *dl*-propranolol (10 μM).

Thin-layer chromatography in butanol-acetic acid-water 66:17:17 showed that the 6-[*p*-nitrophenylazo]epinephrine molecule remains intact throughout the biological tests. 6-Aminoepinephrine was not produced, further indicating that the azo linkage remained intact in this molecule.

A more detailed study of the biological and chemical activity of these new compounds is under way.[5]

DISCUSSION

Structural determination of the catecholamines covalently bound to glass beads is simplified by the fact that dithionite readily cleaves azo linkages to amino groups (Fig. 6). Determination of the point of substitution of the new amino group on the catecholamine gives the site of the diazo substitution. This procedure also

[5] B. R. Venter, J. C. Venter, and N. O. Kaplan, manuscript in preparation.

produces a substituted catecholamine analogue which has been found to be biologically active.

The properties of the isoproterenol derivative are consistent with an amino substitution on the aromatic ring. The ability of this derivative to react with fluorescamine indicates that it possesses a primary alkyl- or arylamine group or groups (8). The altered reaction of the isoproterenol derivative with ninhydrin also indicates the incorporation of a "new" amino group, and the reduction in the R_F value of this compound with respect to isoproterenol furnishes evidence for an additional positive charge (7). From this information we may reasonably conclude that the C-6 substitution revealed by PMR studies is a protonated amine.

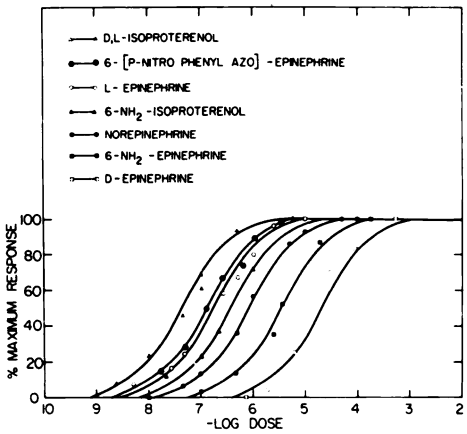

FIG. 4. *Effects of various concentrations of catecholamines, their 6-amino derivatives, and 6-[p-nitrophenylazo]epinephrine on force of contraction of isometrically contracting cat papillary muscles*

Right ventricular cat papillary muscles were mounted horizontally in a Lucite muscle bath and arranged to contract isometrically. One end of the muscle was held by a Lucite clip attached to a force transducer, and the tendinous end was tied by 4-0 silk thread to a micrometer. The muscle bath contained Krebs solution, pH 7.4, at 30°, bubbled continuously with 95% O_2–5% CO_2. 6-Aminocatecholamines were synthesized by vacuuming the appropriate catecholamine glass to near dryness in a sintered glass funnel. Then 2 ml of distilled water were added to make a slurry, to which 1–5 mg of sodium dithionite were added, and the slurry was mixed until the color disappearance was homogeneous (indicating azo bond reduction). The eluent was collected, and the glass was washed twice with 10–50 ml of ice-cold 0.01 N HCl. The eluent was immediately lyophilized, then extracted four times with 1–3 ml of methanol. Concentrations were determined with [^{14}C]catecholamines. 6-[*p*-Nitrophenylazo]epinephrine was synthesized by reacting *p*-nitroaniline (221 mg) in 4 ml of 1 N HCl at −5° with 110 mg of $NaNO_2$ for 2 min. Exactly 0.8 ml of 1 N NaOH was added to reach pH 7.0. This solution was immediately added to 146 mg of *l*-epinephrine in 20 ml of phosphate buffer, 0.1 M, pH 7.4, containing *dl*-[^{14}C]epinephrine. The reaction was continued for 10 min, and the entire mixture was then lyophilized. The 6-[*p*-nitrophenylazo]epinephrine was purified over a neutral alumina column, eluting with butanol-acetic acid-H_2O 66:17:17. Purity was determined utilizing paper and thin-layer chromatography.

The acid wash profile shown in Fig. 1 clearly demonstrates the necessity of acid-washing the glass bead-immobilized catecholamines prior to their quantitation or biological assay. It has been suggested (5) that there is an initial ionic interaction between the catecholamines and the glass beads which is reversed by the acid wash. If such an ionic interaction does exist, it does not appear to contribute to the biological activity of the glass bead-immobilized catecholamines, as soaking arylamine glass beads in catecholamine solutions does not result in biologically active glass beads (1). It is also of interest that the catecholamine-glass complex has the same apparent biological activity both before and after acid washing.

These data indicate that the catecholamine–glass bead complexes are extremely stable under the conditions described. It seems unlikely that the 0.008% release rate is a result of the reduction of the azo bond, since this is a highly stable linkage. More likely it is the result of removal of the entire side chain from the glass because of actual dissolution of the glass, as has been shown for glass bead-immobilized enzymes at neutral and basic pH (9).

The stability of the glass bead-immobilized catecholamines give additional evidence that the catecholamine–glass bead complexes do not exert their biological actions by release of the amines into solution in sufficient concentrations to account for the pharmacological responses seen. The amount of catecholamine released per

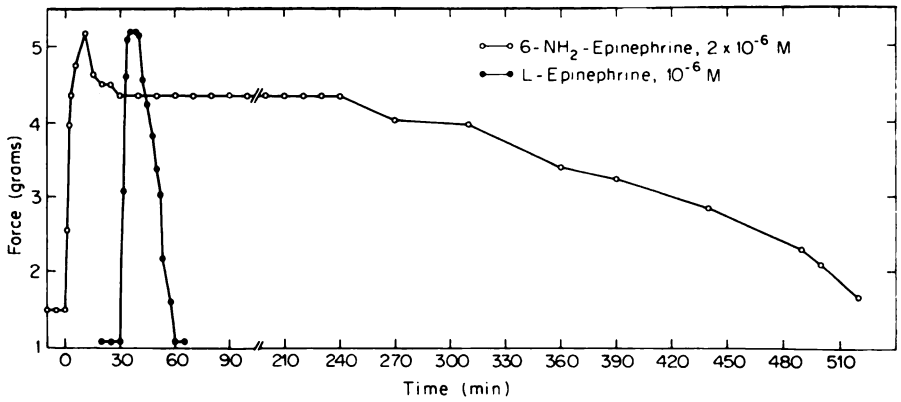

FIG. 5. *Effect of l-epinephrine and 6-aminoepinephrine on force of contraction of isometrically contracting cat papillary muscles at various times following exposure to drug*

The experimental procedure was the same as outlined in Fig. 4. Both drugs were tested under identical conditions.

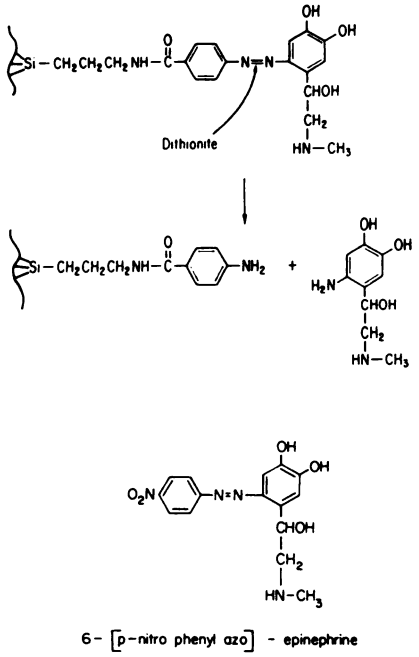

FIG. 6. *Azo bond reduction of glass bead immobilized catecholamines and the structures of 6-aminoepinephrine and 6-[p-nitrophenylazo]epinephrine.*

Dithionite readily cleaves azo linkages to amino groups. This procedure gives an amino-substituted catecholamine. Determination of the point of substitution of the new amino group gives the site of the diazo substitution. Below: the structure of 6-[*p*-nitrophenylazo]epinephrine. This new derivative, which is biologically active, may serve as a useful model compound for further study of immobilized catecholamines.

minute from one 300-μm glass bead is calculated to be 8 attomoles. One isoproterenol-glass bead with only picomole amounts of isoproterenol is sufficient to elicit the same positive inotropic response on a cat papillary muscle as that produced by a 0.1 μM solution of isoproterenol on the same muscle (2). In a 40-ml muscle bath, 8 attomoles would yield only a 0.2 fM solution, which is less by a factor of 10^8 than the minimal concentration necessary to elicit a biological response. We have also calculated that, of the 8 attomoles released per minute, less than 0.08 attomole would be available for direct absorption into the papillary muscle tissue because of the small surface area of contact between the glass bead and the muscle. If such minute concentrations can be proven responsible for the activities seen, important information will be gained concerning the site and mechanism of action of catecholamines on cardiac muscle. On the other hand, equally important information would be provided by proof that the biological action of the catecholamines is due only to the covalently coupled species.[6] However, if the 0.08 attomole is responsible for the biological activity, it would be difficult to explain some of the reported differences in activities (1, 2) and

[6] J. C. Venter and G. Weiland, manuscript in preparation.

responses to cocaine (2) that the immobilized catecholamines display with respect to catecholamines in solution. Furthermore, diffusion and inactivation would help to limit the possible contribution of a soluble form of catecholamines. Unless we are dealing with heretofore unrecognized mechanisms of action for catecholamines on cardiac tissue, it is extremely difficult to explain the biological activity of the glass bead-immobilized catecholamines in terms of release from glass beads.

The chemical structures of the catecholamines bound to glass via the azo linkage are of theoretical as well as practical importance, as is the demonstration of biological activity of the soluble, 6-substituted catecholamines (Figs. 4 and 5). Synthesis of 6-aminocatecholamines by the method presented here indicates that the parent catecholamines were originally covalently coupled via an azo linkage to position 6 on the catechol ring. Had the point of substitution been to a hydroxyl group at position 3 or 4, it would have been difficult to explain how the immobilized catecholamines could exhibit biological activity, since the hydroxyl groups are essential for biological activity (10). The ethanolamine side chain is also thought to be essential for biological activity, and we have shown that norepinephrine is in fact biologically inactive when coupled to glass beads through the primary amine of the side chain (2). The observation that the 6-[*p*-nitrophenylazo]epinephrine derivative is biologically active (Fig. 4) further indicates that the epinephrine molecule exists on the glass side arm in a potentially active conformation. The 6-[*p*-nitrophenylazo]epinephrine derivative may be a useful model compound for further study of the chemistry and pharmacology of immobilized catecholamines (Fig. 6, lower section).

We feel that the data we have accumulated (1–5) are all highly consistent with the catecholamines being biologically active while covalently coupled via an azo linkage to glass beads. The *p*-nitrophenyl derivatives continue to be used for large-scale synthesis of 6-aminocatecholamines, amino-substituted 5-hydroxytryptamine, and 2- and 4-aminohistamine. The methods utilized to synthesize the 6-aminocatecholamines might well be applied to the synthesis of amino derivatives of other labile aryl pharmacological agents.

REFERENCES

1. Venter, J. C., Dixon, J. E., Maroko, P. R. & Kaplan, N. O. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 1141–1145.
2. Venter, J. C., Ross, J., Jr., Dixon, J. E., Mayer, S. E. & Kaplan, N. O. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, **70**, 1214–1217.
3. Venter, J. C. & Kaplan, N. O. (1974) in *Colowick's and Kaplan's Methods in Enzymology Volume 38* (O'Malley, B. W. & Hardman, J. G., eds.), Academic Press, New York, in press.
4. Venter, J. C. & Dixon, J. E. (1974) in *Colowick's and Kaplan's Methods in Enzymology Volume 38* (O'Malley, B. W. & Hardman, J. G., eds.), Academid Press, New York, in press.
5. Venter, J. C. & Kaplan, N. O. (1974) *Science*, **185**, 459–460.
6. Dregor, W. J. & Byrom, E. (1967) in *Colowick's and Kaplan's Methods in Enzymology* (Hirs, C.H.W., ed.), Vol. 11, p. 32, Academic Press, New York.
7. Baily, J. L. (1971) *Techniques in Protein Chemistry*, American Elsevier, New York.
8. Udenfriend, S., Stein, S., Böhlen, P., Dairmen, W., Leimgruber, W. & Weigele, M. (1972) *Science* **178**, 871–872.
9. Weetall, H. H. & Havewala, N. B. (1972) *Enzyme Engineering*, pp. 241–266, Wiley, New York.
10. Innes, I. & Nickerson, M. (1970) in *The Pharmacological Basis of Therapeutics* (Goodman, L. S. & Gilman, A., eds.), Ed. 4, pp. 478–570, New York.