STABILITY OF ISOPROTERENOL BOUND TO CYANOGEN BROMIDE-ACTIVATED AGAROSE.

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SUMMARY. The chemical stability and release of isoproterenol bound by diazotation to an insoluble agarose matrix has been investigated. It is demonstrated, by a double labeling procedure ([^{3}H]isoproterenol and [^{14}C]-spacer arm), that the bound ligand is readily released in a soluble form. This occurs primarily through a chemical hydrolysis of the arm-linked ligand from the cyanogen bromide-activated agarose, and can be observed under extensive washing procedures as well as under more "physiological" incubation conditions. The instability of the agarose-arm linkage should lead to a critical analysis of physiological effects obtained with agarose bound hormones *in vitro*.

Peptides and small molecules coupled directly, or through a single spacer arm, to a solid matrix such as porous glass or agarose beads, are reportedly released from this matrix during storage or incubation under physiological conditions (1-7). Such a phenomenon has shed a new and critical light upon several technical approaches (8-10) whereby immobilized hormones were used to stimulate various cellular or subcellular systems *in vitro*. This release has been explained either by the simple desorption of free ligand (4), or by the cleavage of the azo-bond used for coupling the ligand to the spacer arm (11). Cleavage of linkage between spacer arm and matrix (glass beads or agarose) was also suggested (6, 11). Experiments by Tesser *et al* (6) strongly supported this hypothesis, suggesting a "solvolyse" of this link at pH above 5. Finally, tritium exchange from the labeled ligand with the incubation medium was recently reported for [^{3}H]catecholamines coupled to glass beads (12). Using differently labeled arm and isoproterenol, we report here that arm and hormone are released together, which suggests that the release from the agarose matrix, in our conditions, is mostly due to the instability of the agarose-arm linkage.

EXPERIMENTAL PROCEDURE. *MATERIALS*. Sepharose 4B was obtained from Pharmacia. *dl*-isoproterenol was from Aldrich. Dimethylformamide and cyanogen bromide were from Merck. *p*-Nitrobenzylazide was from Eastman. All other reagents and solvents were of the purest grade available. [7-^{3}H] *dl*-isoproterenol (2.11 Ci/mmole) was obtained from New England Nuclear Co. and [U-^{14}C]-ethylenediamine, HCl (7.95 Ci/mmole), from the Radiochemical Centre (Amersham, England).

METHODS. Preparation of isoproterenol-agarose conjugates. Agarose was activated by the cyanogen bromide method. One gram (wet weight) of washed Sepharose 4B was suspended in 10 ml water. 250 mg cyanogen bromide, dissolved in a minimal amount of dimethylformamide, were added to the gently stirred slurry at 5-10° and adjusted around pH 11 by addition of 1 N NaOH. After 20 min, the gel was rapidly washed on a glass funnel with 25 ml of cold, 0.5 M carbonate buffer pH 10, and added to 50 mg of ethylenediamine dissolved in 5 ml of the same buffer. This suspension was gently stirred for 24 h at 4°. The diazotized *p*-aminobenzamidoethyl-agarose was then prepared according to Cuatrecasas (13). Isoproterenol-agarose conjugates were prepared by the addition of 18.1 mg of *dl*-iosproterenol dissolved in 20 ml of 0.1 M acetate buffer, pH 4, to the previously obtained diazotized *p*-aminobenzamidoethyl-agarose. This solution was stirred in the dark at 4° for 2-3 days and washed with 50 ml distilled water. In order to block unreacted diazonium groups (14), the gel was suspended in 10 ml of 0.1 M phosphate buffer (pH 8) containing 75 mg glycine, stirred in the dark at 4° for 24 h and then washed with 100 ml distilled water. Labeled gels were obtained by adding [^{3}H] -isoproterenol and [^{14}C] -ethylenediamine to the cold compounds at the corresponding steps of the gel preparation. Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer. The release of the bound ligand was followed under various washing and incubation conditions as depicted in the legends to the figures. After washing, gelswere stored at 4° in the dark, in 100 mM EDTA, pH 4.8, containing 0.5 % *n*-butanol (v/v).

RESULTS. Data from the literature report the coupling of catecholamines, such as epinephrine and isoproterenol, to diazonium agarose and diazonium glass beads at pH 7.5 (2,9,15). However, under these conditions, the coupling is accompanied by several, unexpected side-reactions leading to the formation of pyrocatechol azoderivative as well as other by-products, whereas the highest yield of isoproterenol azoderivative is observed at pH 4 (data to be published). Thus in order to obtain a maximal amount of

the expected conjugate (9), the coupling step was routinely performed at pH 4.

The release of free and bound hormones was studied under various conditions. To elucidate the "true" mechanism of release, we used a double labeling procedure to prepare the gels (Table I): [^{14}C] ethylenediamine for the spacer arm and [^{3}H] isoproterenol for the hormone. Thus gel I contained both [^{14}C] labeled arm and tritiated hormone bound to the agarose. Under this condition, it was possible that part of the radioactivity was not bound, but only adsorded on the gel. Gel II and gel III

	Structure of the agarose conjugate	Labeled products adsorbed	Content in labeled products (μmoles/g gel) before washing	
			[^{14}C]	[^{3}H]
Gel I	≋-*A-B-N_2-*IPR	*A + *IPR	1.85	1.5
Gel II	≋ (agarose alone)	*A	0.03	
Gel III	≋-A-B-NH_2	*IPR		0.35

Symbols used:

*A: [^{14}C]-ethylenediamine; *IPR: [^{3}H]-isoproterenol.

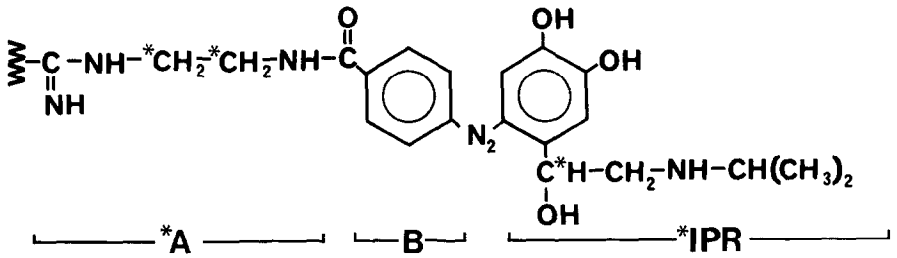

Table I: Structure and labeling of the gels.

Gel I was prepared as described under "Methods". Gel II was prepared by the treatment of inert agarose (not activated by cyanogen bromide) with [^{14}C]-ethylenediamine, followed by the other steps described under "Methods". Gel III was prepared by treatment of *p*-aminobenzamidoethyl agarose (not diazotized) with [^{3}H]-IPR and by the subsequent steps described under "Methods". The specific activity of [^{3}H] isoproterenol was 2.7 Ci/mole for gel I and III and that of [^{14}C] ethylenediamine was 240 and 40 mCi/mole for gel I and II respectively.

were prepared to correct for this possibility: they contained respectively native [^{14}C] ethylenediamine and [^{3}H] isoproterenol which were considered to be only adsorded. This procedure allowed us to measure the "desorption" of unbound material during the various washing and incubation steps and to subtract it from the total release observed from gel I. Thus we could calculate the actual release of bound material.

As shown in Fig. 1, appreciable amounts of isoproterenol were released from gel I and III, during washing, first with 80 percent methanol and then with 10 mM HCl. Almost no release of ethylenediamine was observed from gel II. When the release of chemically bound isoproterenol and spacer

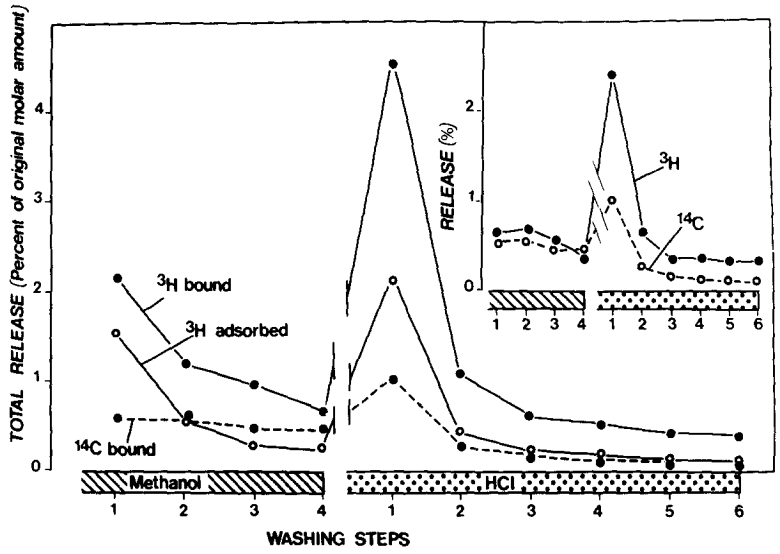

Fig. 1: Ligand release during washing. The washing steps were performed as follows: one g of agarose conjugate (prepared as depicted in Table I) was suspended in 40 ml methanol-water (4:1, v/v), and shaken for 48 h at 4° in the dark. The gel was filtered on a Millipore filter disc (0.45 μ pore size). The filtrate was collected and counted. The washing procedure was repeated in similar conditions for several times with either 40 ml methanol-water (4:1, v/v) or 10 mM HCl. Total amount of [^{14}C] arm (●– – –● for gel I) and [^{3}H] isoproterenol (●——● for gel I, and o– – –o for gel III) present in each washing solution is given as percent of their molar concentration in the original gel before washing. These concentrations were calculated from the specific activity data given in the legend to Table I. No release of [^{14}C] arm adsorded to gel II was observed under this washing procedure.

Insert: Release of bound [^{3}H] isoproterenol (●——●) and bound [^{14}C] arm (o– – –o) was calculated from data in Fig. 1 by subtracting the amount due to desorption (gel III) from the total amount of release (gel I).

arm was corrected for the desorption and expressed as percent of the original molar amount (Fig. 1, insert), similar proportions of [^{3}H] isoproterenol and [^{14}C] -arm were released in the methanol washing solutions: 0.6 to 0.7 percent release for each washing step. Upon washing with 10 mM HCl, the level of isoproterenol released was about 3 times higher than that of ethylenediamine which decreased from 1 percent for the first washing step to 0.1 percent after five steps. It seems therefore that the cleavage occurred essentially between arm and matrix when 80 % methanol was used for washing the immobilized catecholamine-agarose complex, whereas an important additional part of the hormone release was due to the cleavage of the azo-linkage between arm and bound isoproterenol when 10 mM HCl was used for washing.

Furthermore, this double labeling method indicated that appreciable release of labeled materials also occurred during the short time incubations (10 min at 37°) used for the adenylate cyclase assay (16, 17). As shown in Table II, similar amounts of [^{14}C] arm and [^{3}H] isoproterenol were released (0.17 and 0.18 percent of the initial molar amount). This suggests that, during incubation under "physiological"conditions, as

Radioactivity released	Percent of the original molar amount
[^{3}H] from gel I (total release)	0.23
[^{3}H] from gel III (desorption)	0.06
[^{3}H] from gel I minus [^{3}H] from gel III	0.17
[^{14}C] from gel I	0.18

Table II: Ligand release during incubation.

After the washing steps described in Fig. 1, 60 mg aliquots of gel I and III (prepared as described in the legend to Table I) were suspended in 1 ml of incubation medium containing 50 mM Tris-HCl buffer pH 7.4, 3 mM $MgCl_2$ and 0.5 mM ATP. The suspensions were shaken for 10 min at 37° and the supernatants were removed after centrifugation and counted. The total amount of labeled material present in gel I before incubation was 1.4 µmole/g gel for the [^{14}C] arm, 0.9 µmole/g gel for [^{3}H] isoproterenol, and was referred to as 100 percent. Values depicted are means of four experiments.

well as during washing with 80 percent methanol, a cleavage occurred between arm and matrix. From the data in Table II, it was calculated that the total amount of arm-bound ligand released during the 10 min incubation was sufficient to give a final concentration of 0.1 µM isoproterenol. This represents a contamination susceptible to stimulate the adenylate cyclase system (16, 17).

Gel I and III were also submitted (data not shown) to the extensive washing procedure described by Venter and Kaplan (4). For this purpose, one gram of gel, settled in a column, was washed with a continuous flow of 12 l of 0.1 M HCl and 18 l of 0.8 percent NaCl. During the subsequent incubation under the conditions described in the caption to Table II, desorption of unbound isoproterenol from gel II was minimal (0.02 percent) whereas release of bound material remained comparable to that presently observed. Thus, extensive column washing procedure diminished the level of adsorded material without affecting the release of the bound material.

DISCUSSION. Several authors have reported a release of ligand from insoluble carriers, during incubations especially designed to assess the biological activity of the gels. It was suggested that this release was due to the cleavage of isourea and iminocarbonate linkages between spacer arm and agarose (6,11). In this study, by the use of differently labeled arm and hormone,we provide now direct evidence that the major part of ligand is released together with the spacer arm itself. Thus, whereas desorption of free ligand could be eliminated by extensive washing, release of arm-bound ligand can still occur, due to the instability of the arm-agarose bond, by a "solvolytic" process, already alluded to by Tesser *et al* (6) . An exchange of tritium from the tritiated hormone has been reported by Venter *et al* (12). However, under the conditions described by these authors, it would represent at most 5 percent of the total release of the radioactivity that we observed. This phenomenon could lead to a slight overestimation of the hormone release but it cannot obviously explain the contamination of the supernatant from agarose-bound hormones by free, and physiologically active, material. In fact, since this "chemical" release does occur under physiological incubation conditions(Table II), great caution should be exercized when interpreting experiments where the effect of "bound" ligand is studied in biological systems such as the activation of membrane adenylate cyclase.Release of arm-bound ligand might also be detrimental to affinity chromatography, especially when very small amounts of ligand-binding receptor proteins are to be selectively purified. Alternative methods for binding ligands to insoluble matrix, *e.g.* the use

of macromolecular arms or epoxy-activated Sepharose, are conceivably more suitable, and are now under investigation.

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