Reports

Stability of Catecholamines and Propranolol Covalently Bound to Sepharose and Glass Beads

Abstract. *Catecholamines and propranolol were chemically bound to Sepharose and glass beads and washed extensively. The complexes were unstable and bound ligands were gradually released into the supernatants.*

Enzymes or small molecules apparently can be covalently bound to insoluble materials with partial retention of activity (*1*). The immobilized ligands have been used as specific adsorbents for biochemical separation by affinity chromatography. In our study norepinephrine and propranolol were covalently attached to Sepharose and the stability of the complexes were examined. While this work was in progress, several papers appeared in which norepinephrine was immobilized to succinyldiaminodipropylsuccinyldiaminodipropyl (SDSD) Sepharose (*2*) and to arylamine glass beads (*3, 4*). Consequently, we included complexes of these types with epinephrine and norepinephrine for comparison.

Succinylated and *p*-aminobenzoylated aminoethyl Sepharose were prepared by the method of Cuatrecasas and Anfinsen (*5*). [^{14}C]Norepinephrine succinylaminoethyl Sepharose was prepared by reacting succinylaminoethyl (SAE) Sepharose (1.0 g) with [^{14}C]norepinephrine (0.076 m*M*; 65 μc/mmole) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (0.23 m*M*) in distilled water at *p*H 4.8 to 5 for 20 hours at room temperature. [^{14}C]Propranolol and [^{14}C]norepinephrine *p*-aminobenzamidoethyl (PABE) Sepharose were prepared as follows. The PABE Sepharose (0.2 g) was suspended in 0.5*N* HCl and diazotized at 0°C with 0.1*M* sodium nitrite. The diazonium intermediate of PABE Sepharose was reacted with either [^{14}C]propranolol (0.019 m*M*; 49 μc/mmole) or with [^{14}C]norepinephrine (0.076 m*M*; 65 μc/mmole) in 0.2*M* sodium borate at 4°C for 16 hours. SDSD Sepharose was prepared and reacted with [^{14}C]norepinephrine (*2*). Arylamine glass beads were prepared from silica glass beads containing reactive primary amine groups (GAO-3940; Corning) (*6*). The arylamine glass (0.139 g) was diazotized and then coupled to [^{14}C]norepinephrine (0.017 m*M*; 299 μc/mmole), [^{14}C]epinephrine (0.017 m*M*; 295 μc/mmole), or [^{14}C]propranolol (0.019 m*M*; 49 μc/mmole) (*3*).

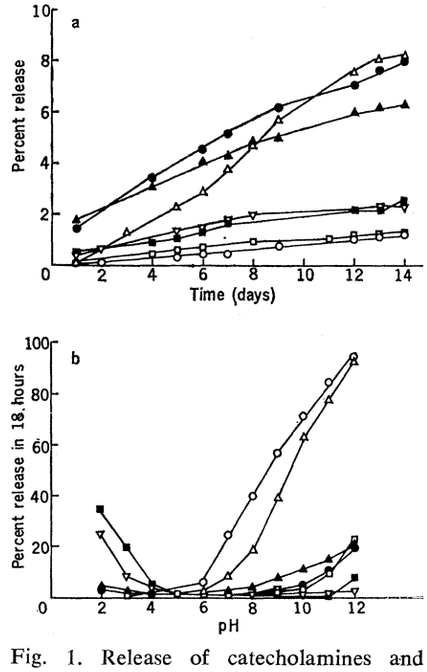

Fig. 1. Release of catecholamines and propranolol covalently bound to Sepharose and glass beads (a) in distilled water and (b) at different *p*H's. The complexes were stored in distilled water at 4°C or incubated with citrate buffer (*p*H 2 to 4), phosphate buffer (*p*H 5 to 8), and glycine-NaOH buffer (*p*H 9 to 12) at 4°C for 18 hours. ○, Norepinephrine SAE Sepharose; △, norepinephrine SDSD Sepharose; □, norepinephrine PABE Sepharose; ▽, propranolol PABE Sepharose; ●, norepinephrine bound to glass; ▲, epinephrine bound to glass; ■, propranolol bound to glass.

The complexes were placed in Büchner funnels (medium disk) at room temperature and washed with 30 ml of distilled water at 15- to 20-minute intervals. At the end of each washing the complexes were incubated with 1.0 ml of distilled water for 5 minutes and filtered; the filtrates were then counted in Aquasol (10 ml) in a liquid scintillation counter. The counting efficiency, determined by the channels ratio method, was 55 to 60 percent. The washing was repeated until no detectable radioactivity appeared in the filtrate; the amount of washing water required was about 5 to 6 liters. For determination of the amount of coupled ligand, Sepharose complexes were dissolved in 6*N* HCl at 50°C, and the substituted glass beads were reduced with 0.1*M* sodium dithionite at *p*H 9.3. The resulting solutions were counted in Aquasol. The amount of [^{14}C]norepinephrine bound to SAE, PABE, SDSD Sepharose, and glass were 9.5 μmole/g, 12.1 μmole/g, 7.8 μmole/g, and 2.8 μmole/g, respectively. Incorporation of [^{14}C]propranolol to PABE Sepharose and glass was 28.3 μmole/g and 17.7 μmole/g, whereas incorporation of [^{14}C]epinephrine to glass was 3.7 μmole/g.

All complexes were stored in distilled water at 4°C. Butanol (0.5 percent) was added to the Sepharose complexes as an antimicrobial agent. Supernatant was taken from each complex at various intervals and the radioactivity was determined. Radioactivity appeared in the supernatant of all complexes after storage for 1 day and the amount of radioactivity increased with the duration of storage. At the end of 2 weeks, approximately 8 percent of the covalently bound norepinephrine in SDSD Sepharose and glass, and 6.4 percent of epinephrine on glass was released into supernatant as compared to 1.2 percent release of norepinephrine from SAE or PABE Sepharose; about 2.2 percent of the propranolol that was bound to PABE Sepharose and glass was released into the supernatant (Fig 1a). This release of radioactivity could not be attributed to the breakdown of the Sepharose matrix, since SAE Sepharose containing either aminoethyl or succinyl groups that were labeled with ^{14}C did not release radioactivity after storage of up to 1 month.

The complexes (15 mg) were placed in 2.0 ml of one of three buffers: citrate (0.1*M*)-HCl (0.1*N*) buffer (*p*H 2 to 4), phosphate (0.066*M*) buffer (*p*H 5 to 8), or glycine (0.1*M*)-NaOH (0.1*N*) buffer

(*p*H 9 to 12). They were stored at 4°C for 18 hours and samples were taken for radioactivity determinations. Catecholamine complexes were relatively stable from *p*H 3 to *p*H 6; at higher *p*H they were very unstable (Fig. 1b). It appeared that at higher *p*H the norepinephrine and Sepharose complex formed by an amide linkage was less stable than that formed by a diazo linkage. Thus, at *p*H 8, 40 percent of the norepinephrine bound to SAE Sepharose and 20 percent bound to SDSD Sepharose were released, whereas only 2 to 5 percent of norepinephrine or epinephrine bound to PABE Sepharose or glass were released. Propranolol complexes were more stable at high than at lower *p*H's.

The biological activity of norepinephrine SAE Sepharose was determined by its ability to cause the contraction of vascular smooth muscle. Isolated rabbit aortic strips were prepared and bathed in 15 ml of Krebs-Henseleit solution to which 45 m*M* dextrose and 0.26 m*M* disodium ethylenediaminetetraacetate were added. The medium was maintained at 37°C aerated with 95 percent O_2 and 5 percent CO_2 to give a *p*H of 7.4 (*7*). Contractions were recorded on a kymograph. The norepinephrine SAE Sepharose complex caused the strips to contract, but most of the response could be attributed to free norepinephrine released by hydrolysis. In four experiments 36.43 ± 1.11 percent of the bound radioactivity was released into the medium within 30 minutes to give a free norepinephrine concentration of 78.25 ± 2.38 ng per milligram of SAE Sepharose in a milliliter of bath fluid.

Complexes of norepinephrine and epinephrine on glass (25 mg) were incubated in Krebs-Henseleit buffer (2 ml) aerated with 95 percent O_2 and 5 percent CO_2 at 32°C, and the released radioactivity was determined at intervals. Approximately 0.5 percent of the bound radioactivity was released after only 5 minutes of incubation (Fig. 2). This radioactivity represents 72 ng of amines. Moreover, the medium induced rabbit aortic strips to contract, indicating that the soluble radioactivity included considerable amounts of intact norepinephrine or epinephrine.

These data do not support the proposition that catecholamines chemically bound to glass exert their pharmacological effects on isolated tissue as covalently bound complexes (*3*, *4*). Our results indicate that, although catecholamines and propranolol can readily be bound to either Sepharose or glass beads, a significant portion of the bound ligands can be gradually released into the supernatants. Furthermore, the rate of release depends on the *p*H and conditions used in biological experiments. It is not clear how a ligand complexed through a diazo linkage to a solid support is released, but partial hydrolysis can contribute to the release of a ligand complexed through an amide linkage. Recent studies have demonstrated that the apparent biological activity of bovine growth hormone and Sepharose complex (*8*) and of insulin and Sepharose complex (*9*) could largely be attributed to the release of free hormones from the Sepharose matrix. These limitations must be considered when "immobilized" hormones and drugs are tested for biological activity or used as specific adsorbents in affinity chromatographic separation.

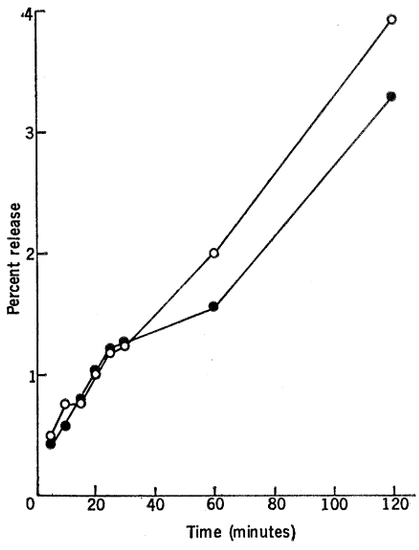

Fig. 2. Release of catecholamines covalently bound to glass beads in Krebs-Henseleit solution. The complexes were incubated in Krebs-Henseleit solution aerated with 95 percent O_2 and 5 percent CO_2 at 32°C. ○, Norepinephrine bound to glass; ●, epinephrine bound to glass.

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The 25-km Discontinuity: Implications for Lunar History

Abstract. *The lunar velocity profile and laboratory data on terrestrial and lunar rocks are constraints on models of lunar history. They show that shock-induced microcracks are absent from the rocks present in the moon today at depths of 25 to 60 kilometers. All possible causes of this observation are examined, and the most likely explanations are that either the rocks at depths of 25 to 60 kilometers formed after the major impacts ceased or the microcracks have annealed at temperatures of about 600°C over geologically long times.*

Startling implications for lunar science are hidden in the combined analysis of the lunar seismic velocity profiles and the laboratory data on elastic properties of shocked rocks. Comparison of the two sets of data shows that the rocks in situ at depths greater than 25 km in mare regions do not now contain microcracks. If both sets of data are essentially correct, then only two explanations of this remarkable fact appear to be possible: (i) the rocks below 25 km have never been shock metamorphosed, or (ii) the rocks below 25 km have been shock metamorphosed but the shock effects have been removed by some process. Before discussing these two possibilities, we describe the data briefly and argue for their validity.

The velocity profile of Toksoz *et al.* (*1*, *2*), shown in Fig. 1, does not differ in essential features from the earlier profile of Toksoz *et al.* (*3*), but includes additional data. The velocity profile is based mainly on the travel times of pulses from artificial impacts but is also consistent with the data