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An Avidin-Biotinyl-Propranolol Complex for β-Adrenergic Receptor Characterization

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The synthesis of biotinyl-hexaglycyl-NEDA (abbreviation:BGN), a biotinyl derivative of propranolol, is described. This bifunctional molecule binds with high affinity to the biotin-binding protein, avidin. The duck erythrocyte was used as a model β-receptor system. Formation of an avidin-BGN-β-receptor complex was demonstrated in intact erythrocytes, in erythrocyte ghosts, and in the digitonin-solubilized β-receptor. The avidin-BGN complex will be used for localization and purification of the β-receptor.

Key words: avidin-biotin complex, β-adrenergic antagonist, β-adrenergic receptor, adenylate cyclase, duck erythrocytes, biotinyl derivatives of hormones

The distribution of hormone receptors on cell surfaces may provide important information on the mechanism by which hormones function. To this end, biotinyl derivatives of hormones and other ligands are potentially useful tools in receptor research. Avidin, a protein isolated from egg white, binds with very high affinity to biotin and biotin derivatives [1]. Avidin can be labeled with fluorescein [2] and ferritin [3] for use in receptor localization. In addition, avidin-Sepharose columns may be prepared [4] for affinity chromatography of receptors bound to biotinyl ligands. While this work was under way, the syntheses of biotinyl derivatives of insulin were reported [5, 6]. This paper describes the synthesis and characterization of a biotinyl derivative of the β-adrenergic antagonist propranolol. This compound will be utilized for the localization and purification of the β-receptor.

MATERIALS AND METHODS

Materials

Avidin, (+)-biotin, L-isoproterenol-D-bitartrate, 4-hydroxyazobenzene-2′-carboxylic acid, N-hydroxysuccinimide, and hexaglycine were obtained from the Sigma Chemical Co. 1,1′-Carbonyldiimidazole was obtained from Aldrich Chemical Co. [^{14}C]-Biotin and

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[α-^{32}P]-ATP (adenosine triphosphate) were from Amersham/Searle Corp. [^{3}H]-Dihydroalprenolol, cAMP radioimmunoassay kits and Aquasol were purchased from New England Nuclear. Duck blood was collected at the C and D Duck Co, Franksville, Wisconsin.

Synthesis of Biotinyl-hexaglycine

Biotinyl-N-hydroxysuccinimide was prepared according to the method of Jasiewicz, Schoenberg, and Mueller [7]. Biotinyl-N-hydroxy-succinimide (0.624 g, 1.8 mmole) in 6 ml dimethylformamide (distilled from barium oxide) was added to a solution of hexaglycine (0.6 g, 1.7 mmole) and Na_2CO_3 (0.352 g, 3.3 mmole) in 20 ml distilled H_2O at 40°C. The mixture was stirred for 14 h at 25°C. The product appeared as a white precipitate, which was removed by centrifugation at 1000g, then washed several times with dilute HCl (pH 2). The supernatant was acidified with HCl and the resulting precipitate was washed as described above. This was repeated until no further precipitate appeared on acidification. The product was dried over P_2O_5 in vacuo to an off-white powder (0.415 g, 50%, mp: 215–225 with decomposition). Following hydrolysis for 20 h at 100°C in 6 N HCl, the glycine content was determined in a Beckman amino acid analyzer to be 6.02 ± 0.89 moles glycine per mole product (586 g/mole). Avidin titration indicated (see below) the presence of 0.94 mole of biotin per mole biotinyl-hexaglycine (BG).

Synthesis of Biotinyl-hexaglycyl-NEDA

1-(1-Napthoxy)-3-N-(ethylamino)propan-2-ol (abbreviation: NEDA) was made in our laboratories; the synthesis will be detailed elsewhere. To a suspension of biotinyl-hexaglycine (0.16 g, 0.054 mmole) in 150 ml dimethylsulfoxide (dried over molecular sieves) at 80°C was added 1,1′-carbonyldiimidazole (0.044 g, 0.054 mmole). The mixture was allowed to cool to 25°C and was stirred for 3 h. To this mixture was then added NEDA-HCl (0.086 g, 0.27 mmole) and triethylamine (0.08 ml, 0.57 mmole) in 4 ml dry dimethylsulfoxide. This mixture was stirred at 25°C for 40 h. The solvent was removed by vacuum distillation (5 mm Hg). An oily brown residue remained which was washed, using centrifugation at 1,000g, several times each with ethanol, 1% Na_2CO_3, and distilled H_2O. The product was dried in vacuo, and an off-white powder remained (0.122 g, 37%, mp: 227–235 with decomposition). Glycine content was 5.4 ± 0.07 moles per mole product (829 g/mole). Avidin titration (see below) indicated the presence of 0.85 mole of biotin per mole of product.

The product, biotinyl-hexaglycyl-NEDA (abbreviation: BGN) (Fig 1) was soluble only in dimethylsulfoxide (DMSO), and all subsequent experiments incorporated the appropriate DMSO controls.

Avidin Titrations

The biotin-binding sites of avidin were titrated according to the method of Green [8]. An aliquot of avidin in 50 mM Tris-HCl buffer, pH 7.4, containing 1.2×10^{-8} mole biotin-binding sites as determined from the OD_{282} (ϵ = 25,000), was placed in a spectrophotometer at 25°C. The OD_{500} was measured as 1 μl aliquots of 10 mM hydroxyazobenzene-2′-carboxylic acid (abbreviation: HABA) were added. After 10 μl of the dye had been added, 1-μl aliquots of 2 mM biotin or 1 mM BGN were added. Saturation of the binding sites was indicated when there was no further change in the OD_{500}.

Unless otherwise noted, in all subsequent experiments the avidin-BGN complex (A-BGN) was prepared as a stoichiometric complex with 4 moles of BGN per 1.1 mole of avidin tetramer (ie, with a 10% molar excess of biotin-binding sites over BGN). The

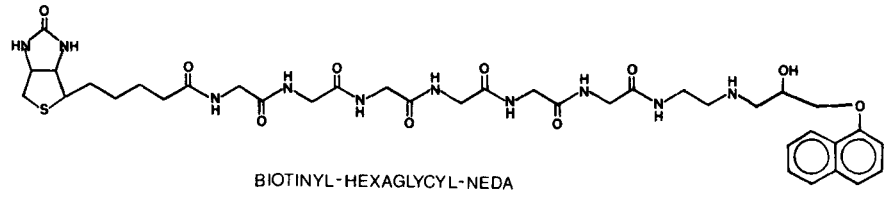

Fig 1. Structure of biotinyl-hexaglycyl-NEDA (abbreviation: BGN).

avidin concentration is expressed in terms of the number of biotin-binding sites present (four per tetramer).

Avidin-BGN Dissociation Assay

A solution of 10 mM biotin containing 50 μCi [^{14}C]-biotin per milliliter was prepared in dilute NaOH (pH 10). To 500 μl of 0.1 mM avidin in 50 mM Tris-HCl, pH 7.4, was added 50 μl of a 1 mM solution of BGN. This mixture was incubated for 10 min at 25°C, and then 50 μl of the [^{14}C]-biotin solution was added. Incubation was continued at 4°C, 25°C, or 37°C. Aliquots of 100 μl were removed from the mixture after varying incubation times, and bound and free [^{14}C]-biotin were separated on a Sephadex G-50 column. Maximal [^{14}C]-biotin binding was determined in the absence of BGN. In subsequent experiments, the A-BGN complex was freshly prepared prior to addition to incubation mixtures.

Adenylate Cyclase Assay — Intact Erythrocytes

Heparinized whole duck blood was centrifuged at 4°C at 1,000 g and the serum and buffy coat were removed. The packed red cells were washed twice at 4°C with phosphate-buffered saline (PBS), pH 7.4. The packed cells were then diluted fivefold in cold PBS to a concentration of 1.2×10^9 cells per milliliter. The assay incubation mixture contained 250 μl of the cell suspension, 250 μl of 4 mM theophylline in PBS, and varying concentrations of isoproterenol in the presence and absence of BGN or A-BGN. This mixture was incubated for 10 min at 37°C. The incubation was terminated by the addition of 4.5 ml of cold 2 mM theophylline followed by heating to 100°C. The tubes were centrifuged for 30 min at 1,000 g, and 100 μl of the supernatant was removed and assayed for cAMP content by radioimmunoassay [9].

Preparation of Duck Erythrocyte Ghosts

All steps were performed at 4°C. Heparinized whole duck blood was diluted in an equal volume of washing buffer (146 mM NaCl, 10 mM Tris-HCl, 20 mM glucose, pH 7.4). The cells were centrifuged at 500 g and the supernatant and buffy coat were removed. The packed cells were washed twice more in washing buffer, and then lysed with stirring in lysis buffer (10 mM Tris-HCl, 4 mM $MgCl_2$, pH 7.4). The resulting lysate was centrifuged at 20,000 g and the supernatant was removed. The lysed cells were stored at −80°C. The protein contents of the whole ghost preparation and of the ghosts without hemoglobin were determined by the Lowry method [10]. Ghosts without hemoglobin were prepared by washing the thawed ghosts with cold lysis buffer (three times) until the pellet was white.

Adenylate Cyclase Assay — Erythrocyte Ghosts

The assay was performed by a modification of the method of Salomon, Londos, and Rodbell [11]. The incubation mixture contained 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 70 U/ml creatine phosphokinase, 20 mM creatine phosphate, 0.1 mM cAMP, 3 mM [α-^{32}P]-ATP (5-10 cpm/pmole), 0.1 mM dithioerythritol, and varying concentrations of isoproterenol in the presence and absence of BGN, A-BGN, or propranolol. The reaction was initiated by the addition of 50 μl of the ghost preparation. The reaction was terminated, after incubation for 10 min at 37°C, by the addition of 900 μl of "stopping reagent" containing 5 mM ATP, 0.18 mM cAMP, and 19,500 dpm/ml [^{3}H]-cAMP in 10 mM Tris-HCl, pH 7.5. This was followed by heating to 100°C (3 min). The tubes were centrifuged for 45 min at 1000 g. Cyclic [^{32}P]-AMP was purified from 800 μl of the supernatant by ion-exchange chromatography on Dowex AG50 WX4 followed by chromatography over aluminum oxide. The amount of [^{32}P]-cAMP and the recovery of [^{3}H]-cAMP were determined in a Packard Dual Channel liquid scintillation counter under dual isotope counting using 12 ml of Aquasol in each counting vial.

Solubilization of the β-Adrenergic Receptor

The beta-receptor was solubilized from duck erythrocyte ghosts by a modification of the procedure of Caron and Lefkowitz [12]. Duck erythrocyte ghosts were washed twice with lysis buffer, then frozen in a dry ice/acetone bath. After thawing, the ghosts were washed once with 1 mM phosphate buffer, pH 7.4, and then resuspended in the same buffer. Digitonin was added to give a final concentration of 1% (w/v) and the mixture was incubated for 5 min. The mixture was diluted with an equal volume of buffer and incubated at room temperature for 1 h, then cooled to 4°C and centrifuged for 1 h at 100,000 g. The supernatant was concentrated in an ultrafiltration apparatus at 4°C and stored at −80°C. Approximately 18 ml of concentrated extract were obtained from 35 ml of packed ghosts.

Solubilized Receptor Binding Assays

A 10-μl aliquot of the concentrated digitonin extract in 100 μl of 50 mM Tris-HCl (pH 7.4) was incubated at 4°C with varying concentrations of BGN (10 min), A-BGN (20 min), BG (10 min), avidin (20 min), or propranolol (10 min). [^{3}H]-Dihydroalprenolol (abbreviation: [^{3}H]-DHAP) was then added to give a final concentration of 5×10^{-8}M, and the mixture was incubated at 4°C for 10 min. Bound and free [^{3}H]-DHAP were separated on a Sephadex G-50 column [12]. "Specific" binding refers to [^{3}H]-DHAP binding protectable by 10^{-5}M propranolol, and was routinely found to be 90–95% of the total binding.

RESULTS

Formation and Stability of the Avidin-BGN Complex

The titration of avidin with biotin and BGN is shown in Figure 2. BGN formed a stoichiometric complex with avidin. When the biotin-binding sites of avidin had been saturated with BGN, further addition of biotin had no effect, indicating that all of the binding sites accessible to biotin were capable of binding BGN. This stoichiometric titration of avidin further establishes the chemical structure of BGN (ie, the concentration of BGN could be calculated from the known concentration of avidin binding sites).

In order to be useful, the A-BGN complex must be stable during experimental manipulations. Accordingly, experiments were performed to assess the stability of the

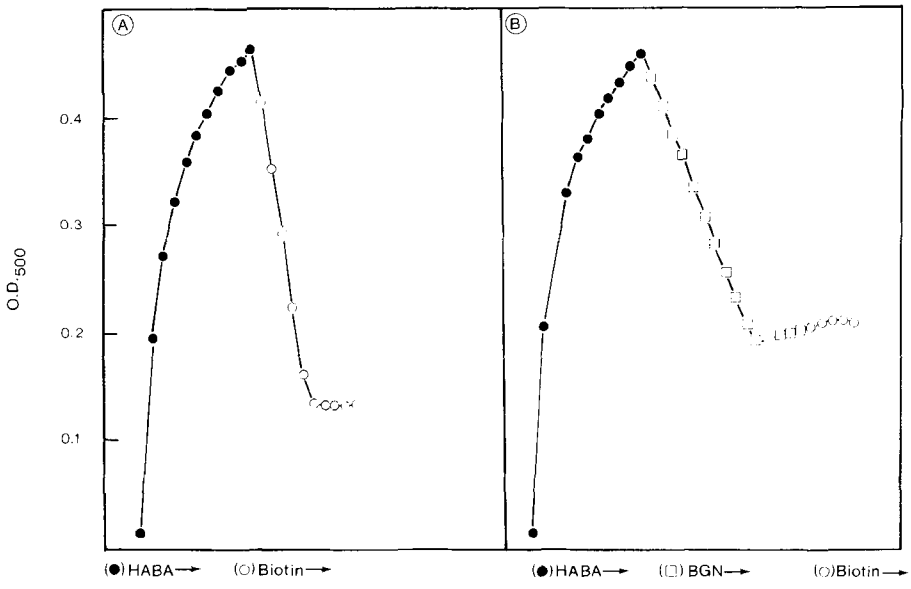

Fig 2. Titration of avidin with biotin and BGN. In each experiment, the OD_{500} was measured as 1-μl aliquots of 10 mM HABA were added to an aliquot of avidin containing 1.3×10^{-8} moles biotin-binding sites (as determined from the OD_{282}). In experiment A, titration was continued with 1-μl aliquots of 2 mM biotin. In experiment B, 1-μl aliquots of 1 mM BGN were added. Saturation was observed after addition of 1.2×10^{-8} moles biotin or 1.1×10^{-8} moles BGN. Addition of biotin after saturation with BGN produced no further decrease in the OD_{500}.

A-BGN complex at various temperatures. The results are shown in Figure 3. At 4°C, no measurable dissociation of BGN from avidin was observed during a 2-h incubation. At 25°C, the half-life of the A-BGN complex was 15.5 h. At 37°C, the half-life was 2 h. Less than 10% of the BGN dissociated in 10 min at 37°C. No measurable dissociation of biotin from avidin occurred during a 3-h incubation at 37°C (data not shown). In the experiment performed at 4°C, the incubation mixture contained digitonin-extracted β-receptor. The inclusion of erythrocyte ghosts in the 37°C incubation mixture did not increase the dissociation rate (data not shown).

Formation of an Avidin-BGN-β-Receptor Complex

In intact duck erythrocytes, BGN and the A-BGN complex inhibited the isoproterenol activation of the adenylate cyclase (Fig 4). The apparent dissociation constants were estimated from the shifts in the isoproterenol dose-response curve [13] to be 1.0×10^{-6}M for BGN, and 2.1×10^{-6}M for A-BGN. Avidin alone (1.5×10^{-5}M) had no effect on the isoproterenol response (data not shown).

In duck erythrocyte ghosts (Fig 5), the apparent dissociation constants were 0.5×10^{-6}M for BGN, 2.5×10^{-6} for A-BGN, and 0.0033×10^{-6}M for propranolol. Neither Avidin alone (10^{-4}M) nor biotinyl hexaglycine (10^{-5}M) inhibited the isoproterenol response (data not shown).

In the digitonin-solubilized β-receptor, both BGN and A-BGN inhibited the binding of [^{3}H]-dihydroalprenolol to the receptor (Fig 6). The apparent dissociation constants were estimated from the concentration of ligand resulting in 50% inhibition of [^{3}H]-

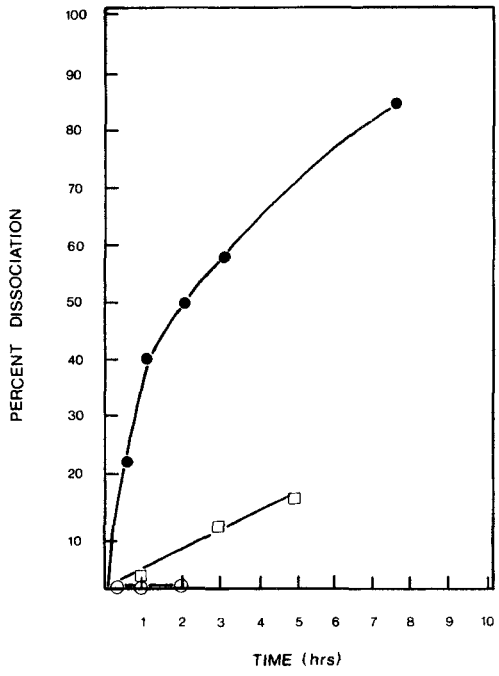

Fig 3. Stability of the A-BGN complex. A stoichiometric A-BGN complex was incubated at various temperatures in the presence of a 10-fold molar excess of [^{14}C]-biotin. At various times after the addition of [^{14}C]-biotin, aliquots of the incubation mixture were removed and the bound and free [^{14}C]-biotin were separated on a Sephadex G-50 column. BGN dissociation was calculated from the percentage of total [^{14}C]-biotin bound to avidin. •) 37°C; □) 25°C; ○) 4°C.

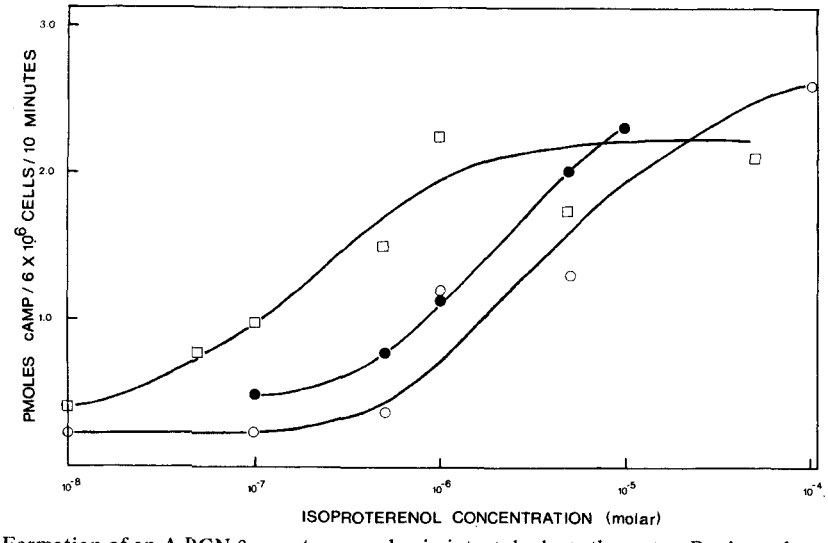

Fig 4. Formation of an A-BGN-β-receptor complex in intact duck erythrocytes. Duck erythrocytes were incubated in the presence of varying concentrations of isoproterenol in the presence and absence of BGN and A-BGN. Adenylate cyclase activity was measured by radioimmunoassay for cAMP. □) Control; ○) 10^{-5}M BGN; •) 10^{-5}M A-BGN. Each point is the mean of two duplicates.

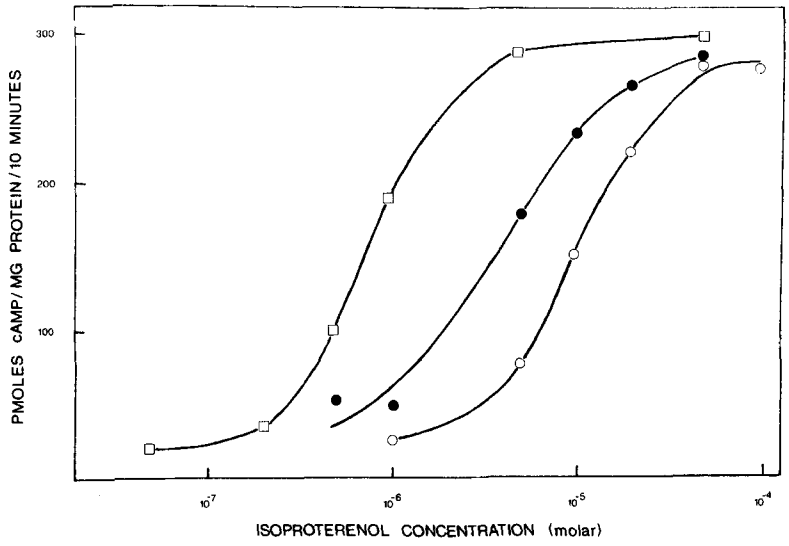

Fig 5. Formation of an A-BGN-β-receptor complex in duct erythrocyte ghosts. Duck erythrocyte ghosts were incubated with [α-^{32}P]-ATP and varying concentrations of isoproterenol in the presence and absence of BGN and A-BGN. The adenylate cyclase activity was determined by measurement of the [^{32}P]-cAMP produced. □) Control; ○) 10^{-5} M BGN; ●) 10^{-5} M A-BGN. Each point is the mean of two duplicates.

dihydroalprenolol binding [14], and were 0.047×10^{-6}M for BGN, 0.32×10^{-6}M for A-BGN, and 0.011×10^{-6}M for propranolol. Similar results were obtained whether the A-BGN complex was prepared as a stoichiometric complex or with a fivefold molar excess of biotin-binding sites over BGN. Neither biotinyl hexaglycine nor avidin alone had any effect on [^{3}H]-dihydroalprenolol binding at cocentrations below 2.5×10^{-5}M. The time course of A-BGN binding to the solubilized receptor is shown in Figure 7. Equilibrium binding was observed within approximately 20 min at 4°C.

These receptor-binding data are summarized in Table I. The affinity of BGN was found to be less than that of the parent compound, propranolol. This may be due to the addition of one or more amide groups to the amino portion of the molecule.

DISCUSSION

In this paper we have described the synthesis and characterization of BGN, a biotinyl derivative of propranolol. This molecule contains a "spacer" sequence of six glycine residues, separating the two ends of the bifunctional reagent so that avidin and the β-receptor may bind simultaneously. This ligand maintains high affinity for the biotin-binding sites as indicated by the ability to titrate the avidin with BGN and by the formation of a stoichiometric complex. Dissociation of the avidin-BGN complex is, however, dependent on the temperature (Fig 3). The reason for this behavior has not been elucidated. The stability characteristics of this complex may be due to the NEDA portion of the molecule solely since biotinyl-NEDA (ie, no hexaglycyl spacer) (data not shown) and BGN exhibit the same dissociation characteristics. The stoichiometric complex of biotin and avidin is stable at all temperatures between 4°C and 37°C.

The ability of A-BGN to inhibit the β-adrenergic receptor cannot be accounted for by the dissociation of BGN from the avidin. This is especially the case in the digitonin-sol-

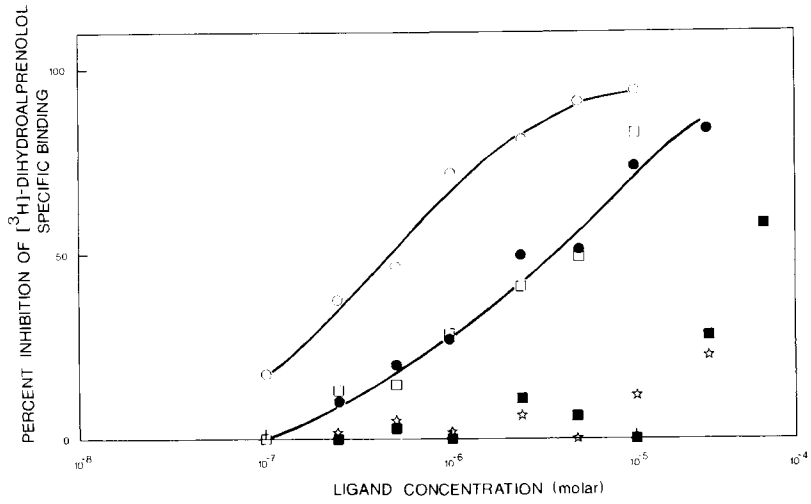

Fig 6. Formation of an A-BGN-β-receptor complex in the digitonin-solubilized duck β-receptor. After preincubation of receptor with avidin, A-BGN, BGN, or BG, [^{3}H]-dihydroalprenolol was added and the incubation was continued for 10 min. Bound and free [^{3}H]-DHAP were then seperated on a Sephadex G-50 column. ○) BGN; ●) A-BGN (1 mole biotin-binding sites per mole BGN); □) A-BGN (5 moles biotin-binding sites per mole BGN); ■) avidin; ☆) BG. Each point is the mean of two duplicates.

ubilized β-receptor preparation, where no measurable dissociation occurred under the assay conditions at 4°C. In the most unfavorable case, a 10 min adenylate cyclase assay at 37°C, it can be calculated that less than 10% of the avidin BGN complex has dissociated. With this amount of free BGN (10^{-6}M) no detectable shift in the isoproterenol activation curve would have been detected. This means that the shift in the isoproterenol activation curve was due to inhibition by the intact avidin-BCN complex. The dissociation rate of the avidin-BGN complex was not accelerated by the presence of soluble receptor or ghosts. These data support the conclusion that the avidin-BGN complex interacts directly with the β-receptor.

The data presented in Figures 4 and 5 demonstrate parallel shifts in the isoproterenol dose-response curves. This indicates that both BGN and the avidin-BGN complex are competitive inhibitors of the β-adrenergic receptor in duck erythrocytes. Both BGN and the avidin-BGN complex can also competitively displace [^{3}H]-dihydroalprenolol from the digitonin-solubilized β-receptor. In all these systems, the apparent affinity of BGN for the receptor was reduced by binding this ligand to avidin. The reasons for this apparent decrease in affinity have not been determined. One possibility, especially in the case of the intact erythrocyte and erythrocyte ghost, is that carbohydrate moieties on the cell surface and the carbohydrate on avidin prevent, for steric reasons, close apposition of the avidin molecule with the receptor molecule embedded in the membrane bilayer.

The exact stoichiometry of the receptor-avidin-BGN interaction has not been determined. However, in the "solubilized" system (Fig 6) increasing the avidin/BGN ratio (and thereby reducing the number of BGN molecules bound to each tetramer) did not increase the affinity of the BGN-avidin complex. This suggests that each BGN molecule in the tetrameric complex with an avidin molecule is capable of interacting with the dihydroalprenolol binding site of the β-receptor. If only one of the four BGN molecules in any tetramer was capable of interacting with the receptor, one would then expect a

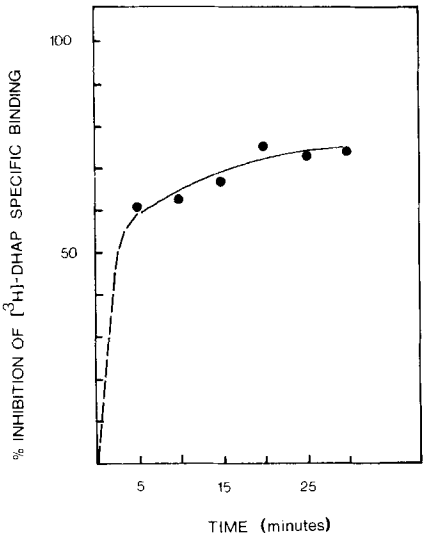

Fig 7. Time course of A-BGN binding to the digitonin-solubilized duck β-receptor. A-BGN (10^{-5}M) was added to solubilized receptor and incubated for various time periods prior to addition of [^{3}H]-DHAP. After 10 min further incubation, bound and free [^{3}H]-DHAP were separated on a Sephadex G-50 column. Each point is the mean of two duplicates.

TABLE I. Summary of Kinetic Constants for β-Receptor Ligands

Receptor system	$K_{D_{app}}$(× 10^{-6}M)		
	BGN	A-BGN	Propranolol
Intact duck erythrocytes	1.0	2.1	. . .
Duck erythrocyte ghosts	0.50	2.5	0.0033
Digitonin-extract of duck erythrocyte ghosts	0.047	0.32	0.011

The apparent dissociation constants for BGN, A-BGN, and propranolol were determined, as described in the text, for each of the β-receptor assay systems.

more potent inhibitor (expressed as BGN equivalents) when only one of four of the avidin binding sites was occupied with BGN. The avidin-BGN complex as described in this paper may, therefore, be a multifunctional ligand for the β-receptor. Formation of a monofunctional avidin complex should be possible by appropriate manipulation of the avidin/BGN ratio [6].

These studies indicate that it is possible to form an avidin-ligand-β-receptor complex in situ. This should allow the use of this complex as a β-receptor probe utilizing fluorescein-avidin [2], fluorescein- or ferritin-labeled anti-avidin antibody, avidin-coupled microspheres [15], avidin-coupled virus [16], or avidin-coupled peroxidase [17] to visualize the distribution of β-receptor on the surface of responsive cells. Recent evidence [18], based on the concept of receptor mobility on cell surfaces [19–21], has suggested that some hormone receptors, notably the glucagon receptor, occur as large complexes on the sur-

face of liver cells. Upon activation by glucagon and guanyl nucleotides, these complexes appear to diminish in size. One might predict that the changes in physical state of the glucagon receptor could be monitored by visualizing the surface distribution of this receptor. Changes in the apparent physical size of the β-receptor in frog erythrocytes have also been demonstrated upon addition of agonist [22]. One cannot differentiate, at this time, between cis (ie, interactions in the plane of the bilayer) and trans (ie, interaction across the bilayer) in these agonist-induced changes in the receptor size. The use of the avidin-BGN complex should make it possible to detect surface rearrangements of the β-receptor under various agonist and antagonist states [23] of the receptor. By analogy, biotinyl derivatives of glucagon or other hormones (eg, insulin [6]) will similarly be of use in this regard.

Especially important, based on the evidence shown in Figure 6 for the interaction with the digitonin-solubilized receptor, is the potential application of the avidin-BGN complex for β-receptor purification using affinity chromatography. The bifunctional ligand, BGN, may, under appropriate conditons, allow an efficient elution of β-receptor from avidin-agarose affinity columns because both ends of the molecule (ie, the NEDA portion and the biotinyl portion) are potentially dissociable.

Experiments designed to investigate the surface distribution of the β-receptor and the use of the avidin-BGN complex in receptor purification are underway.

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