Determination of the Molecular Size of Frog and Turkey Erythrocyte β-Adrenergic Receptors by Radiation Inactivation†

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ABSTRACT: The β_1- and β_2-adrenergic receptors from turkey and frog erythrocyte plasma membranes, respectively, have been solubilized with digitonin and purified by sequential affinity and gel-permeation high-performance liquid chromatography. Examination of these preparations after labeling with $Na^{125}I$/chloramine T or with the photoaffinity reagent ^{125}I-labeled (*p*-azidobenzyl)carazolol, by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, reveals the β-adrenergic receptor to be composed entirely of M_r 58 000 subunits. In contrast, for the β_1-adrenergic receptor of turkey erythrocytes, two distinct receptor peptides of M_r 40 000 and 45 000 were identified. These peptides represent the ligand binding site of these receptors since they interact with adrenergic ligands with the expected β_1 and β_2 specificity. In this study, in order to examine the functional size of both frog and turkey erythrocyte β-adrenergic receptors, particulate membrane and purified preparations were subjected to irradiation. Under these conditions, a molecular weight of 54 000 was obtained for both the membrane-bound and purified β_2-adrenergic receptors of frog erythrocytes. For the β_1 receptor from turkey erythrocytes, molecular weights of 41 000–53 000 were obtained in the membrane. In purified preparations, a slightly higher value of M_r 54 000–55 000 was obtained. These data indicate that the peptides identified as the receptor subunit by purification and photoaffinity labeling contain a single ligand binding site for both the frog erythrocyte β_2-adrenergic receptor and the turkey erythrocyte β_1-adrenergic receptor.

β-Adrenergic receptors which stimulate the enzyme adenylate cyclase are grouped into subtypes termed β_1 and β_2 on the basis of the relative potencies of adrenergic agonists. Epinephrine and norepinephrine are approximately equipotent agonists for β_1 receptors such as those found in mammalian heart and turkey erythrocytes. On the other hand, epinephrine is considerably more potent than norepinephrine for β_2-adrenergic receptors such as those in smooth muscle and frog erythrocytes. Recently, the biochemical characterization of both of these adrenergic receptor subtypes and their mode of coupling to the enzyme adenylate cyclase have become the focus of considerable investigation. Several laboratories have reported purification of the β-adrenergic receptors from model systems such as avian and amphibian erythrocytes and mammalian lung (Shorr et al., 1982a,b; Benovic et al., 1982; Fraser & Venter, 1980; Vauquelin et al., 1977; Homcy et al., 1983). Estimates of receptor molecular size or subunit composition have been based on the visualization of the purified proteins usually after $Na^{125}I$ labeling with chloramine T followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] and radioautography. Additionally, identification of the receptor binding subunit by photoaffinity labeling of purified or particulate receptor preparations with several radioiodinated potent β-adrenergic receptor antagonists has been reported. In frog erythrocytes, a subunit molecular weight of 58 000 has been identified for the β_2-adrenergic receptor whereas for the turkey β_1 receptors two peptides of M_r 40 000 and 45 000 have been identified (Rashidbaigi & Ruoho, 1981; Burgermeister et al., 1982; Lavin et al., 1982). Little data, however, are available on the functional size of β-adrenergic receptor subtypes in the membrane. We have, therefore, applied the technique of radiation inactivation to the determination of the functional size of both frog and turkey erythrocyte β-adrenergic receptor in situ and in purified preparations.

Experimental Procedures

Materials

^{125}I-Labeled cyanopindolol (^{125}I-CYP), ^{125}I-labeled (*p*-azidobenzyl)carazolol (^{125}I-pABC), and carrier-free $Na^{125}I$ were obtained from New England Nuclear, Boston, MA. Alkaline phosphatase (*Escherichia coli*) type III-S was obtained from Sigma. Other reagents and chemicals as well as high-performance liquid chromatography columns were obtained from the same sources as previously described by Shorr et al. (1982a,b).

Methods

Preparation of Purified Membranes. Purified frog erythrocyte membranes were prepared essentially as described by Caron et al. (1979) except that benzamidine (10^{-4} M), bacitracin (100 μg/mL), soybean trypsin inhibitor (10 μg/mL), and phenylmethanesulfonyl fluoride (3×10^{-5} M) were included at each step as protease inhibitors. Once prepared, membranes were stored at −90 °C in the presence of protease inhibitors. Turkey erythrocyte membranes were prepared as described by Stadel et al. (1980) except that protease inhibitors were included as above. Membranes were used immediately or stored at −90 °C in 25 mM $MgCl_2$/75 mM Tris buffer (pH 7.4)/250 mM sucrose/1 mM DTT. Protein concentrations for both membrane preparations were estimated by the method of Lowry et al. (1951).

Preparation and Characterization of Purified β-Adrenergic Receptor. Purified β_2-adrenergic receptor from frog erythrocytes was prepared essentially by the rapid high-yield method

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[1] Abbreviations: ^{125}I-CYP, ^{125}I-labeled cyanopindolol; ^{125}I-pABC, ^{125}I-labeled (*p*-azidobenzyl)carazolol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)-aminomethane; DTT, dithiothreitol; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography.

0006-2960/84/0423-0747$01.50/0

described by Shorr et al. (1982a). Purified β_1-adrenergic receptors were prepared by essentially similar methods as described by Shorr et al. (1982b). Characterization of purified preparations by radioiodination, isoelectric focusing, and SDS–PAGE was also as described previously (Shorr et al., 1982a,b). Reaction of particulate or purified β-adrenergic receptor with the photoaffinity probe ^{125}I-labeled (*p*-azidobenzyl)carazolol was as previously described (Lavin et al., 1982).

Ligand Binding Assays. Soluble receptor assays using ^{125}I-labeled (+)- and (−)-cyanopindolol were performed as described in Shorr et al. (1982b). The separation of bound from free ligand was accomplished by chromatography on Sephadex G-50. Particulate assays were carried out by the method of Caron et al. (1976). For most of the experiments, saturation curves were carried out in duplicate at each irradiation point, and the data were analyzed by computer-based methods (De Lean et al., 1982). Alkaline phosphatase (*E. coli*) used as a standard for irradiation was assayed (25 °C) according to Garen & Levinthal (1960).

Preparation of Receptor Samples for Irradiation. Aliquots (500 μL) of purified frog or turkey erythrocyte membranes (~2–3 mg/mL) to which was added 400–500 units of alkaline phosphatase (*E. coli*) were placed into 2-mL glass vials and frozen in liquid nitrogen, and the tubes were sealed under flame. Purified receptor was aliquoted similarly except that bovine serum albumin was added to 3 mg/mL in addition to alkaline phosphatase (400–500 units/vial). For both purified soluble receptor preparations and membrane preparations, the controls consisted of frozen and sealed vials which did not undergo irradiation.

Irradiation of Particulate and Purified β-Adrenergic Receptor Preparations. Samples were exposed to a beam of 13 MeV electrons produced by a linear accelerator (Armed Forces Radiobiology Research Institute, Bethesda, MD). Dose rates, measured by thermoluminescent dosimeters, ionization chambers, and/or radiochromic dye films, were 15 or 30 Mrad/h, depending on which pulse rate the machine was set. Samples were maintained by a stream of cold nitrogen gas at −135 °C, monitored continuously with a platinum resistance probe or thermistor during exposure.

Analysis of Radiation Inactivation Data. The simplest approach to protein size determination in membranes is the method of target size analysis (Kempner & Schlegel, 1979) based on random probable hits and destruction of a given protein. Briefly, the rationale of inactivation is that the deposition of ionization energy within a protein molecule results in the loss of biological activity; since the ionization occurs randomly in the mass of the protein, the larger the protein the greater the chance of a hit and inactivation. Surviving activity can then be assayed and examined by using the equation

$$A = A_0 e^{-\mu D}$$

where A is the measured activity, A_0 is the activity prior to irradiation, D is the radiation dose (in rads), and μ is a factor proportional to the mass of the particular functional unit. For an activity due to a single size component, a plot of ln (A/A_0) vs. D (the inactivation curve) results in a straight line with a slope = μ. For irradiations at −135 °C, the target size can be calculated (Nielsen et al., 1981) from the relation

$$M_r = (17.9 \times 10^{11})\mu$$

In receptor studies where reversible ligand binding assays are used, it is necessary additionally to establish that irradiation truly results in the loss of active units and not a change in the dissociation constant. For this reason, at each radiation dose, samples were analyzed in duplicate by performing saturation binding isotherms.

Table I: Dissociation Constants of ^{125}I-Cyanopindolol in Frog and Turkey Erythrocyte β-Adrenergic Receptor Preparations as a Function of Radiation Doses[a]

dose (Mrad)	frog erythrocytes		turkey erythrocytes	
	membrane K_D (pM)	purified K_D (pM)	membrane K_D (pM)	purified K_D (pM)
0	27	26	52	58
12	38	15	43	74
24	23	50	35	44
36	34	50	52	38
48	33	62	50	48
60	35	27	41	63
72	62		40	40
84	41		34	55
96			30	31

[a] Saturation curves using the β-adrenergic reversible antagonist ^{125}I-CYP were constructed (see Figures 1 and 3), and data were analyzed to determine the apparent K_D for both particulate and purified preparations of frog (β_2) and turkey (β_1) erythrocytes. Values shown were determined from a single experiment performed in duplicate but are representative of three such experiments in the case of frog erythrocytes and two experiments for turkey erythrocytes.

Results

Irradiation of Frog Erythrocyte Membranes. In order to estimate the size of the β-adrenergic receptor in membranes from frog erythrocytes, membrane preparations were subjected to increasing doses of radiation ranging from 12 to 96 Mrad. When saturation binding curves were generated at each radiation dose by using the β-adrenergic antagonist ^{125}I-CYP, levels of receptor activity were found to decrease with increasing amounts of radiation (Figure 1A, top panel). As shown, little change occurred in nonspecific ^{125}I-CYP binding defined in the presence of 10^{-5} M alprenolol (shaded region in the lower portion of the panels in Figure 1A). Computer analysis (De Lean et al., 1982) of the data and calculation of dissociation constants (K_d) revealed no significant change in K_d with increasing radiation dose (Table I). These data then suggest that the loss of binding activity is due to receptor inactivation rather than to changes in ligand affinities. When the estimated total number of receptors (B_{max}) for each saturation curve was plotted as described under Experimental Procedures, a straight inactivation line was obtained (Figure 1B). Calculation of the receptor size (as described under Experimental Procedures) resulted in an estimate of the molecular weight of 54 000. In order to compare the functional size of the membrane-bound receptor with that in purified preparations, purified receptor preparations of frog erythrocyte obtained after affinity chromatography and sequential high-pressure liquid chromatography steps as described by Shorr et al. (1982a) were subjected to irradiation. The saturation binding isotherms of ^{125}I-CYP at each radiation dose are shown in the bottom panel of Figure 1A. As shown in Figure 1B, identical slopes and molecular weight estimates were obtained for both the particulate and the purified β-adrenergic receptor preparations.

As a control for this estimate, a protein of known molecular size, alkaline phosphatase (*E. coli*), was included along with receptor preparations (Experimental Procedures), subjected to the irradiation procedures, and assayed. This enzyme has been purified to homogeneity (Stadtman, 1961) and shown to be a dimer of M_r 84 000 composed of two M_r 43 000 subunits (Lazdunshi & Lazdunshi, 1969). As shown in the inset to Figure 1B, a semilogarithmic plot of A/A_0 vs. radiation dose

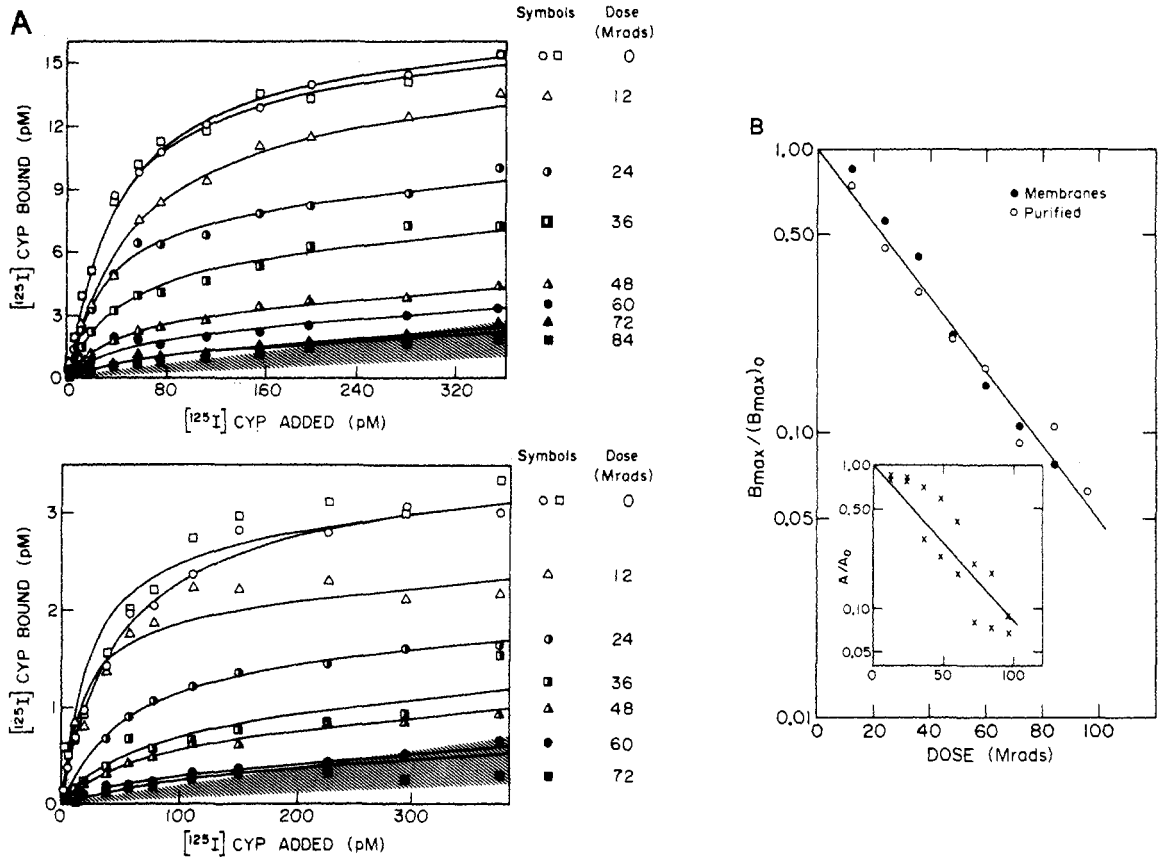

FIGURE 1: Radiation inactivation of particulate and purified frog erythrocyte β-adrenergic receptor. (A) (Top panel) Aliquots (0.5 mL) of frog erythrocyte membranes containing exogenous alkaline phosphatase and adrenergic receptors (15 pM) were irradiated (−135 °C) with increasing doses of radiation from 0 to 96 Mrad. Samples were then thawed and aliquots assayed for receptor binding by using the ligand ^{125}I-labeled cyanopindolol. Bound ligand was separated from free by using GF/C filters under reduced pressure as described under Methods. Bound radioactivity was measured with a Packard Auto 800 γ counter at 78% efficiency. Saturation binding isotherms of ^{125}I-CYP were constructed at each radiation point. Data were analyzed by computer-based methods as described by De Lean et al. (1982). (Bottom panel) Data similar to those shown in the top panel but obtained by using receptor (3 pM) solubilized from the membrane with digitonin and purified to apparent homogeneity by sequential affinity and high-performance liquid chromatography as described previously (Shorr et al., 1982b). Samples contained 3–4 mg/mL BSA to minimize receptor losses on the glass vial surface during irradiation. Saturation curves for the binding of ^{125}I-labeled cyanopindolol were generated as described above except that bound ligand was separated from free by gel filtration on Sephadex G-50. Binding data were analyzed by computer-based methods. In both the top and bottom panels, the range of nonspecific binding determined for each concentration in the presence of 10 μM (±)-alprenolol is shown as the shaded portion at the bottom of each figure. In (B), radiation inactivation data are plotted as $B_{max}/(B_{max})_0$ on a logarithmic scale vs. radiation dose for both membrane-bound (●) and purified (○) receptors. B_{max} represents the maximum binding calculated from the data in (A) at a given radiation dose whereas $(B_{max})_0$ is the maximum specific binding in the control sample. As shown, a single straight line can accomodate the data for both receptor preparations. Determination of molecular weight as described under Methods yields 54 000 in each case. Inset: Radiation inactivation data expressed as log percent control vs. radiation dose for the enzyme alkaline phosphatase, included in both purified and membrane-bound receptor preparations, as a standard. Determination of functional size yields M_r 49 000. The published molecular weight for this enzyme monomer is 43 000 (Stadtman, 1961).

reveals a single straight line for the inactivation of alkaline phosphatase activity irradiated in the presence of either particulate or purified receptor. Determination of enzyme size as described above indicates a molecular weight of 49 000, in close agreement with the known subunit size.

Purified receptor from frog erythrocytes was prepared from a batch of membranes similar to those which were irradiated, and the purified receptor was labeled with Na^{125}I/chloramine T and subjected to SDS–PAGE and autoradiography. The autoradiogram shown in Figure 2A revealed a single band centered at M_r 58 000. Shown in Figure 2B is the autoradiogram of the labeling pattern of a similar frog erythrocyte membrane preparation with the photoactive antagonist ^{125}I-labeled (*p*-azidobenzyl)carazolol (left lane) in the presence of alprenolol (10 μM) (middle lane) or isoproteronol (10 μM) (right lane). The photoaffinity labeling of frog erythrocyte membranes resulted in visualization of only a single specifically labeled broad band centered at M_r 58 000, suggesting that the iodinated protein seen in purified preparations contains the active center for binding adrenergic ligands. Identical results were obtained for photoaffinity labeling of either particulate (membrane) or purified preparations. Thus, the molecular size estimates (M_r 58 000) obtained by SDS–PAGE of purified or photoaffinity-labeled receptor and target irradiation (M_r 54 000) are in good agreement.

Irradiation of Turkey Erythrocyte β-Adrenergic Receptor. As in the case of the frog erythrocyte β_2-adrenergic receptor, irradiation of turkey erythrocyte membrane preparations and construction of ^{125}I-CYP saturation curves revealed a radiation-dependent loss of binding activity (Figure 3A, top panel). Again as for the frog erythrocyte, little change occurred in nonspecific ^{125}I-CYP binding as shown by the shaded portion at the bottom of the panels in Figure 3A. Quantitative analysis of the specific binding activity showed essentially no change in the K_d of ^{125}I-CYP for the receptor (Table I). The irradiation curve plotted as described under Experimental Procedures revealed a single-component inactivation line for receptor activity but with a slope different than that obtained for the frog erythrocyte receptor. Calculation of receptor size for the turkey erythrocyte membrane system resulted in an estimate of M_r 48 000 (molecular weight range of 41 000–53 000, $n = 6$). A slightly higher estimate of receptor size (M_r

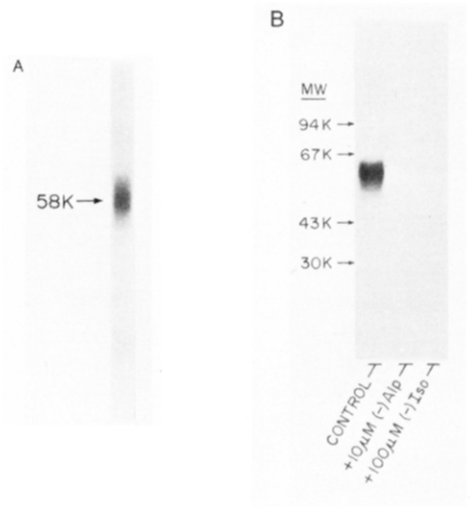

FIGURE 2: SDS–PAGE of purified iodinated and photoaffinity-labeled frog erythrocyte β-adrenergic receptor. (A) Frog erythrocyte β-adrenergic receptor prepared by affinity and high-performance liquid chromatography was iodinated with $Na^{125}I$/chloramine T (Shorr et al., 1982a) and subjected to a final HPLC pass to remove labeled detergent. Those ^{125}I-labeled fractions corresponding to receptor activity were then pooled, and an aliquot was subjected to SDS–PAGE and autoradiography. Exposure was for 3 days (–90 °C), and Kodak XAR-5 films were developed manually according to Kodak Instructions. (B) Frog erythrocyte membranes (25–30 pM receptor concentration) were incubated with ^{125}I-pABC (25–30 pM) for 90 min at 25 °C in the absence (control) and presence of 10^{-5} M (–)-alprenolol or 10^{-4} M (–)-isoproterenol. At the end of the incubation, the samples were washed 3 times with 0.5% BSA in incubation buffer (25 mM Tris-HCl/2 mM $MgCl_2$) and once with incubation buffer alone. Photolysis was carried out for 90 s after which the samples were solubilized in 50 mM Tris-HCl, pH 6.8, 10% SDS, 5% mercaptoethanol, and 10% glycerol. Electrophoresis was performed on an 8% polyacrylamide slab gel. The gel was then dried and exposed to Kodak XAR-5 film for 3 days with intensifying screens. Standards shown as arrows to the left of the figure (molecular weights in parentheses) were phosphorylase *b* (94 000), BSA (67 000), ovalbumin (43 000), and carbonic anhydrase (30 000) and were iodinated with $Na^{125}I$/chloramine T as described for the receptor by Shorr et al. (1982a,b).

~55 000) was obtained on irradiation of purified β_1-adrenergic receptor prepared as described under Methods. In the experiments shown in Figure 3B, however, both sets of data (membrane-bound receptor and purified receptor preparations) could be adequately described by the same line. An estimate of 53 000 for the molecular weight of both receptor preparations was obtained, suggesting that these various estimates are not different. Calculation of enzyme size from the alkaline phosphatase data again indicated a size of M_r 49 000, in agreement with the known structure reported for this enzyme (Figure 3B, inset).

As shown in Figure 4A, purification of the β_1-adrenergic receptor from turkey erythrocytes and labeling with $Na^{125}I$/chloramine T resulted in the visualization of two bands of M_r ~40 000 and M_r 45 000–50 000, respectively, on SDS–PAGE and autoradiography (Shorr et al., 1982b). Photoaffinity labeling using ^{125}I-labeled (*p*-azidobenzyl)carazolol of similar preparations or particulate receptor also resulted in the visualization of these two bands (Figure 4B), which were specifically labeled with a classic β_1-adrenergic specificity [see Figure 4B and Shorr et al. (1982b)]. As described elsewhere (Shorr et al., 1982b), each of these proteins has recently been purified to homogeneity and shown to possess binding properties characteristic of β_1-adrenergic receptors. The presence of these two populations of binding sites in the membranes, however, did not result in a biphasic irradiation curve (Figure 3B). The difference between the apparent size of these two populations of sites is too small to be resolved by the radiation inactivation method.

Thus, the data described above strongly support the identification of a M_r 58 000 polypeptide in β receptor preparations of frog erythrocytes and M_r 40 000–45 000 polypeptides in β receptor preparations of turkey erythrocytes as containing the receptor ligand binding sites *in the membrane* and with a single site per subunit. This observation was further tested for both receptors by examination of receptor specific activities in purified preparations. Thus, if the calculated ligand site sizes from radiation inactivation data are correct, then theoretical specific binding activities for antagonist ligands should approach 17 000 pmol/mg of protein for the frog erythrocyte β receptor and 20 000 pmol/mg of protein for the turkey erythrocyte β-adrenergic receptor preparations. As determined by protein assay (Dolly & Barnard, 1977) and ^{125}I-CYP binding, the specific activity of purified receptor from frog erythrocyte preparations was found to be 11 800 pmol/mg of protein (Shorr et al., 1982a), and in turkey erythrocyte preparations, ~18 700 pmol/mg of protein (Shorr et al., 1982b), approaching theoretical calculations.

Discussion

The β-adrenergic receptor from a number of tissues and cell types has been well characterized pharmacologically and has been classified into two major subtypes each shown to be coupled to the enzyme adenylate cyclase (Minneman et al., 1979). In fact, this pharmacological distinction is based upon the characterization of receptors by utilizing the differential potencies of known agonists in receptor binding studies as well as receptor-mediated stimulation of the adenylate cyclase enzyme. Little is known, however, of the structural basis of these pharmacological differences between receptor subtypes.

Recently in our laboratory, advances in the purification of both the β_2-adrenergic receptor from the frog erythrocyte membrane and the β_1 receptor from turkey erythrocyte have resulted in the identification, respectively, of a M_r 58 000 polypeptide and two polypeptides of M_r 40 000 and 45 000–50 000 as containing the binding subunit of these two receptors for adrenergic ligands (Shorr et al., 1981, 1982b). As mentioned above, the fact that the specific activities of purified receptor preparations approach theoretical values and that the same peptides which can be purified appear to be covalently labeled by photoaffinity ligands suggests that each peptide contains a single ligand binding site. Since size determination of proteins by radiation inactivation gives an estimate of the functional size of the protein assayed, it was of interest to compare this method with the results obtained by purification and photoaffinity techniques by irradiating membrane-bound as well as purified receptor preparations. Utilizing this approach, we have confirmed that receptor molecular weight estimates based on SDS–PAGE of ^{125}I-labeled purified β_1 and β_2 receptor preparations do approximate the functional size of the receptor protein in the intact membrane as assayed by antagonist ligand binding (Table II). These data are supported in addition by studies using photoaffinity probes. In the frog erythrocyte systems using the photoaffinity probes [^3H](*p*-azidobenzyl)carazolol, ^{125}I-labeled (*p*-azidobenzyl)-carazolol (Lavin et al., 1981, 1982), and ^{125}I-labeled (*p*-azidobenzyl)pindolol (Rashidbaigi & Ruoho, 1982), a single peptide of M_r 58 000–60 000 has been identified. These same probes along with ^{125}I-labeled cyanopindolol azide (Burgermeister et al., 1982) have also been used in the turkey erythrocyte to identify two peptides of M_r 40 000 and 45 000–50 000. Thus, by several criteria, the ligand binding

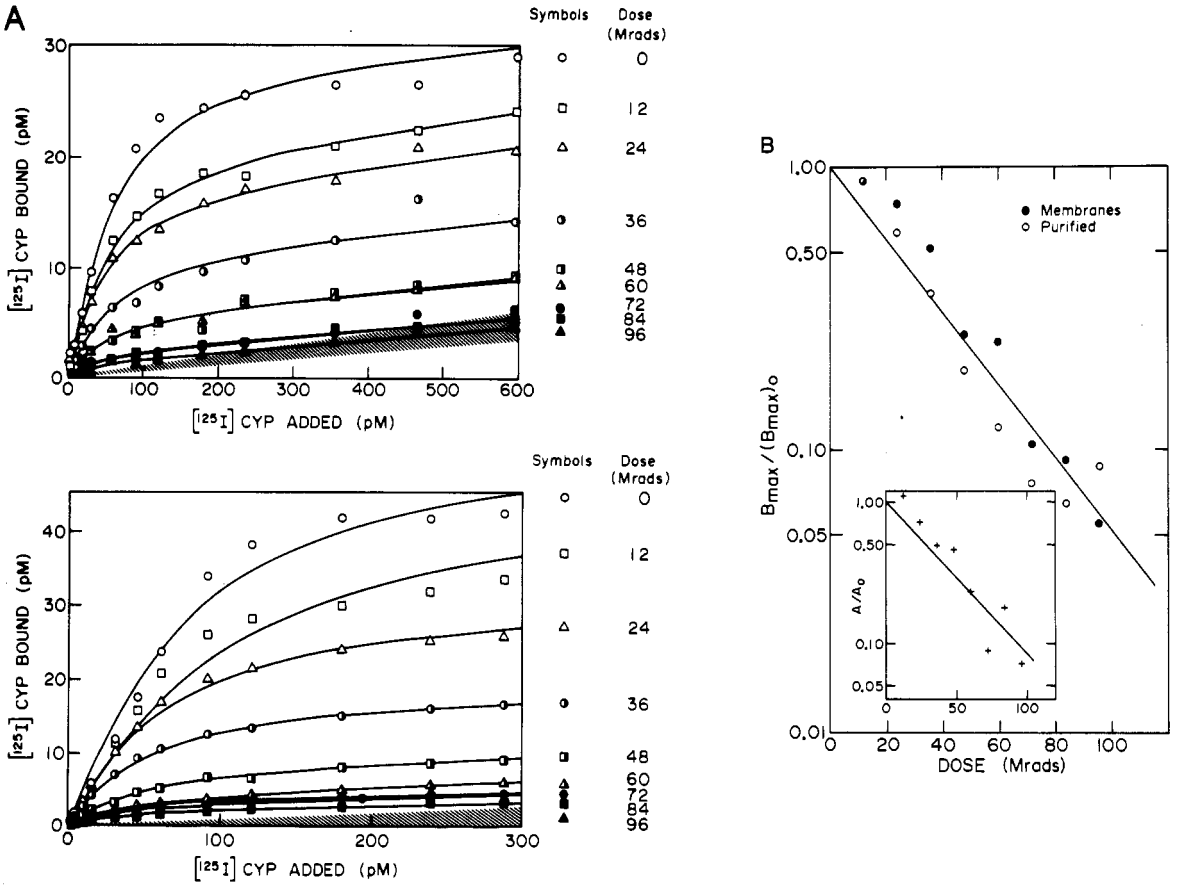

FIGURE 3: (A) (Top panel) Turkey erythrocyte membranes (0.5 mL) containing exogenous alkaline phosphatase were irradiated (−135 °C) with increasing doses of radiation (0–96 Mrad), and receptor activity was assayed (25 °C) by constructing saturation curves using the radioligand ^{125}I-labeled cyanopinolol. As described under Methods, bound ligand was separated from free by filtration using GF/C glass fiber filters. The range of nonspecific binding determined for each concentration of radioligand in the presence of 10^{-5} M alprenolol is shown by the shaded portion at the bottom of the figure. In the bottom panel is shown a similar analysis of purified turkey erythrocyte β-adrenergic receptor obtained by digitonin solubilization and affinity and high-performance liquid chromatography. After radiation, receptor samples with 3–4 mg of BSA/mL were also assayed by constructing saturation curves with the radioligand ^{125}I-CYP except that separation of bound from free ligand was accomplished by filtration using Sephadex G-50. The range of nonspecific binding determined for each concentration of radioligand as in the top panel is shown at the bottom of the figure. In (B), radiation inactivation data of both particulate (●) and purified (○) preparations are presented as $B_{max}/(B_{max})_0$ on a logarithmic scale vs. radiation dose. Calculation of functional size from the common straight line results in an estimated M_r of 53 000. Inset: Radiation inactivation data for alkaline phosphatase are expressed as A/A_0 vs. each radiation dose. Determination of functional size for this enzyme yields a value of M_r 49 000.

Table II: Molecular Weight Estimates of β-Adrenergic Receptors by Radiation, Purification, and Photoaffinity Labeling

method	mol wt	
	frog erythrocyte	turkey erythrocyte
purification-iodination[a]	58 000	40 000, 45 000–50 000
photoaffinity labeling[b]	58 000–62 000	40 000, 45 000–50 000
radiation[c]	54 000 (n = 4)	41 000–55 000 (n = 8)

[a] Taken from Shorr et al. (1982a,b). [b] Taken from Lavin et al. (1982), Rashidbaigi & Ruoho (1982), and Burgermeister et al. (1982). [c] Data obtained in this study; n represents the number of experiments, and the values shown are the range of values obtained.

site of the β_1- and β_2-adrenergic receptors in these two tissues appear to reside on the same peptides which have been isolated.

The major point from the studies reported here is that radiation inactivation of membranes and purified receptor preparations from the two systems of the frog and turkey erythrocytes gives the same molecular size estimate for the ligand binding site as that obtained by purification and photoaffinity labeling procedures. Whereas it is likely that the differences in the molecular sizes of the frog erythrocyte β_2 and turkey β_1 receptors may represent differences in the overall primary structures of the proteins themselves, it should be noted that not all β_1- and β_2-adrenergic receptors appear to have important differences in overall molecular weights. Stiles et al. (1983) have shown recently that the ligand binding sites of β_1- and β_2-adrenergic receptors from several mammalian and nonmammalian tissues appear to reside on similar M_r 62 000–65 000 polypeptides. Moreover, the heterogeneity of labeling of the receptor subunit by photoaffinity techniques previously reported in several mammalian tissues (Lavin et al., 1982) appears to be due, at least in certain tissues, to limited proteolytic degradation of the larger molecular weight peptide (M_r 62 000–65 000) (Benovic et al., 1983). In avian erythrocytes, whether the two receptor peptides identified represent separate forms of a common receptor peptide has not been systematically examined. However, the fact that the predominance of one or the other peptide can be changed by varying the membrane preparation suggests that these two forms are closely related (unpublished experiments).

Previously, Nielsen et al. (1981) have determined a radiation target size of M_r 90 000 for binding of another antagonist, ^{125}I-labeled (hydroxybenzyl)pindolol, to the β_1-adrenergic receptor of the turkey erythrocyte. The reason for the discrepancy with the present results is not immediately apparent. Differences in the preparation of the membranes or differences in assay conditions might have contributed to the different estimates. However, it should be noted that the adenylate cyclase of the turkey erythrocyte membranes which have been

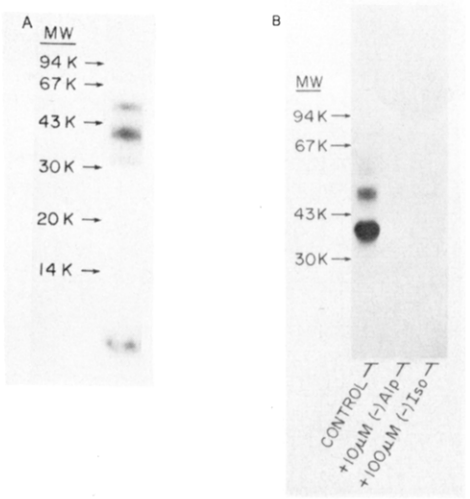

FIGURE 4: SDS–PAGE of purified iodinated and photoaffinity-labeled turkey erythrocyte β-adrenergic receptor. (A) Purified receptor obtained from turkey erythrocytes by digitonin solubilization and affinity/high-performance liquid chromatography was labeled with $Na^{125}I$/chloramine T and subjected to an additional HPLC pass to remove labeled detergent. Those fractions corresponding to receptor activity were then pooled, and an aliquot was subjected to SDS–PAGE and autoradiography. Exposure was for 3 days. Standards shown as arrows to the left of the figure were as in Figure 2. In (B), turkey erythrocyte membranes (25–30 pM receptor concentration) were incubated with ^{125}I-pABC (25–30 pM) for 90 min at 25 °C in the absence (control) and presence of 10^{-5} M (−)-alprenolol or 10^{-4} M (−)-isoproterenol. At the end of the incubation, the samples were washed 3 times with 0.5% BSA in 75 mM Tris-HCl/25 mM $MgCl_2$, pH 7.4, and once with the same Tris buffer alone. Photolysis was carried out for 90 s after which the samples were solubilized in SDS sample buffer (legend to Figure 2). Electrophoresis was done on an 8% polyacrylamide slab gel. The gel was then dried and exposed to Kodak XAR-5 film for 2–3 days with intensifying screens. Molecular weight standards are described in the legend to Figure 2.

used in our experiments retains its full responsiveness to β-adrenergic stimulation. Moreover, the receptor labeled in these membranes by photoaffinity labeling yields two peptides of M_r 40 000 and 45 000–50 000 which are the same as the two peptides purified from this tissue.

Moreover, Fraser & Venter (1982) have reported recently a target size of M_r 109 000 for the binding of ^{125}I-labeled (hydroxybenzyl)pindolol to the β_2-adrenergic receptor of canine lung membranes by radiation inactivation. These data appear to be at variance with the results of labeling of the receptor binding subunit in these membranes which we have done by ^{125}I-labeled (*p*-azidobenzyl)carazolol photoaffinity labeling which reveals a molecular weight of 55 000–58 000 (J. L. Benovic, M. G. Caron, and R. J. Lefkowitz, unpublished experiments). It also differs from results of purification of the β_2 receptor peptide from the same tissue by Homcy et al. (1983); an estimate of M_r 52 000–53 000 was obtained by these authors for the size of the purified peptide which could be covalently labeled with the photoaffinity probe [^3H]acebutolol azide. It is of interest to note that both Fraser & Venter (1982) and Nielsen et al. (1981) have obtained an apparent dimer size for the binding of ^{125}I-labeled (hydroxybenzyl)-pindolol, a ligand which is known in some systems to have partial agonist properties.

It should be stressed that since the assays of receptor binding in the radiation inactivation experiments reported here were performed with an antagonist radioligand, it might have been expected that the estimate of this functional size would correspond to the smallest subunit of the receptor demonstrating the ability to bind specifically adrenergic ligands. The question of whether the β-adrenergic receptor exists in a multisubunit configuration when occupied with agonist and coupled to adenylate cyclase remains unanswered. It has been established that the β-adrenergic receptor does couple to the guanine nucleotide regulatory protein upon interaction with an agonist (Limbird et al., 1980). Nielsen et al. (1981) have also examined the functional size of the adenylate cyclase activated under various conditions. Adenylate cyclase activity stimulated by guanine nucleotide and isoproterenol was estimated to have an overall molecular weight of 250 000–300 000, suggesting interaction of the various components of the system.

The results reported here document that for the two systems of the frog and turkey erythrocytes, the size of the β-adrenergic receptor estimated by radiation inactivation of antagonist binding is the same as that obtained by purification and photoaffinity labeling.

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Effect of Ethanol, Phenol, Formamide, Dimethyl Sulfoxide, Paromomycin, and Deuterium Oxide on the Fidelity of Translation in a Brain Cell-Free System†

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ABSTRACT: The effects of six different agents (ethanol, phenol, formamide, dimethyl sulfoxide, heavy water, and a misreading-inducing antibiotic, paromomycin) on the activity and the accuracy of poly(U) translation have been compared under a range (2.5–12 mM) of Mg^{2+} concentrations in a rat brain cell-free system. The effect of most of these agents was remarkably sensitive to the Mg^{2+} concentration under which the assay was made. Ethanol decreased the fidelity of translation, and the efficiency of ethanol was increased 3–10-fold by higher Mg^{2+} concentrations. The effect of paromomycin was identical with that of ethanol, despite its very different structure. Formamide, a "RNA denaturant", increased the accuracy of translation under all Mg^{2+} concentrations tested. Dimethyl sulfoxide, another type of RNA denaturant, decreased the accuracy of translation under all Mg^{2+} concentrations tested. Phenol increased the accuracy of translation at high Mg^{2+} concentrations but decreased it at low Mg^{2+} concentrations. D_2O did not change to any appreciable extent the accuracy of translation, at all the Mg^{2+} concentrations used. There exists a cooperativity between the effects of Mg^{2+} and ethanol, Mg^{2+} and paromomycin, and Mg^{2+} and dimethyl sulfoxide on the fidelity of translation; no such cooperativity was detected between Mg^{2+} and formamide and between Mg^{2+} and D_2O. The differential effects of dimethyl sulfoxide and formamide are interpreted in terms of their different dielectric constants. The dielectric constant of dimethyl sulfoxide is higher than that of water, while that of formamide is lower.

The phenomenon of misreading mRNA has not been extensively studied in mammalian cell-free systems (Laughrea, 1981a). Nevertheless, it is widely believed that the accuracy of translation in mammalian systems is not markedly sensitive to environmental changes, in contrast to the high sensitivity of bacterial systems (Schlanger & Friedman, 1973; Burrans & Kurtz, 1977; Kurtz, 1979). This belief is implicitly based on the assumption that the effect of an environmental change (e.g., temperature, pH, organic solvents, etc.) is independent of the ionic conditions under which it is studied. In only two instances were both prokaryotic and mammalian systems compared under identical ionic conditions; the data were made largely inconclusive by the presence of a high level of endogenous RNA directed incorporation in the poly(U)-directed cell extracts (Laughrea, 1981b).

In this paper, we investigated some parameters of the translation fidelity in a rat brain cell-free extract directed by poly(U). The effects of six different agents (ethanol, phenol, formamide, dimethyl sulfoxide, deuterium oxide, and a misreading-inducing antibiotic, paromomycin) on the activity and the accuracy of translation were compared under a range of Mg^{2+} concentrations. The experimental results indicate that the effect of most of these agents is remarkably sensitive to the Mg^{2+} concentration under which the assay is made, that ethanol and dimethyl sulfoxide decrease the accuracy of translation, that formamide and phenol can increase the accuracy of translation, and that the presence of deuterium oxide does not affect the fidelity of translation.

Materials and Methods

Animals. Male rats, either Sprague-Dawley or Fisher 344, were used. No significant difference was seen between extracts from either breed.

Chemicals. Poly(U), ATP (disodium salt), GTP (disodium salt), creatine phosphate (disodium salt), calf liver tRNA, tRNA from brewers' yeast specific for phenylalanine ($tRNA^{Phe}$), all enzymes, and antibiotics were purchased from Boehringer Mannheim. Paromomycin sulfate was a gift from Dr. J. J. Brossard, Parke-Davis, Canada. Spermine tetrahydrochloride and spermidine trihydrochloride were from Sigma. Deuterium oxide (D_2O) was purchased from Merck Sharp & Dohme, Canada. Radioactive [^{3}H]leucine (40–60 Ci/mmol) and [^{14}C]phenylalanine (>450 mCi/mmol) were purchased from both Amersham and New England Nuclear. Some of the [^{3}H]leucine batches contained, on arrival, higher than specified levels of impurities, resulting in a doubling or even a tripling of the background incorporation. These batches were discarded or, later in the study, purified by paper chromatography using 25:4:10 1-butanol:acetic acid:water or 7:7:6 pyridine:isoamyl alcohol:water as the solvent system. The discarded impurities were more toluene soluble than leucine itself. Most of them moved faster than leucine, but both faster moving and slower moving impurities contributed to the background incorporation. All acceptable [^{3}H]leucine batches

† From the Lady Davis Institute for Medical Research of the Sir Mortimer B. Davis—Jewish General Hospital, Montreal, Quebec, Canada H3T 1E2. *Received June 3, 1983.* This work was supported by grants from the Medical Research Council of Canada and the Conseil de la Recherche en Santé du Québec. M.L. is a recipient of a research career development award from the Conseil de la Recherche en Santé du Québec.

0006-2960/84/0423-0753$01.50/0