MOLECULAR PHARMACOLOGY, 10, 450-456

[^{3}H]-Propranolol Binding Sites in Myocardial Membranes: Nonidentity with *Beta* Adrenergic Receptors

DOROTHY E. VATNER AND ROBERT J. LEFKOWITZ[1]

Cardiology Division, Department of Medicine, and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

(Received October 10, 1973)

SUMMARY

VATNER, DOROTHY E., AND LEFKOWITZ, ROBERT J.: [^{3}H]Propranolol binding sites in myocardial membranes: nonidentity with *beta* adrenergic receptors. *Mol. Pharmacol.* **10,** 450-456 (1974).

[^{3}H]Propranolol binds rapidly and reversibly to sites in membranes prepared from canine ventricular myocardium. Two orders of sites were identified. The higher-order sites have a value (equilibrium constant) of 4.57×10^4 M^{-1} and a binding capacity of 3.2×10^{-8} mole/mg of protein. For the lower-order sites $K = 4.32 \times 10^2$ M^{-1} and binding capacity is 8.4×10^{-7} mole/mg of protein. Nine adrenergic antagonist drugs were tested for their ability to block [^{3}H]propranolol binding and block isoproterenol activation of adenylate cyclase in dog heart membranes. No clear correlation between the two functions was found. *d*- and *l*-Propranolol competed with equal effectiveness for the propranolol binding sites, but *l*-propranolol was 50 times more potent than the *d* isomer in blocking cyclase activation. The beta adrenergic blocking agent dichloroisoproterenol was a very weak inhibitor of [^{3}H]-propranolol binding. Chlorpromazine and haloperidol inhibited both propranolol binding and cyclase activation. It is concluded that the [^{3}H]propranolol binding sites studied here are unrelated to the myocardial *beta* adrenergic receptors and may be involved in mediating the more general membrane or local anesthetic effects of propranolol. *Beta* adrenergic receptor binding sites presumably represent too small a fraction of myocardial membrane sites capable of binding propranolol to be revealed by binding studies of this type.

INTRODUCTION

Catecholamines stimulate the enzyme adenylate cyclase in myocardial membranes via interaction with *beta* adrenergic receptors (1), leading to an increased rate of generation of the "second messenger" cyclic 3′,5′-AMP[2]. Cyclic AMP is said to augment the force of cardiac muscle contraction (3) and may be involved in mediating the inotropic effects of catecholamines.

Beta adrenergic antagonists, such as propranolol, block the inotropic and chronotropic effects of catecholamines on intact hearts (4). Propranolol also blocks catecholamine-induced stimulation of myocardial adenylate cyclase (5). The precise manner in which *beta* adrenergic agonists

This research was supported by National Institutes of Health Grants HL-5196, HL 16037, and SCOR HE-14150 and by a grant-in-aid from the American Heart Association, with funds contributed by the North Carolina Heart Association.

[1] Established Investigator of the American Heart Association.

[2] The abbreviation used is: cAMP, adenosine cyclic 3′,5′-monophosphate.

and antagonists interact with *beta* adrenergic receptors to cause effects on adenylate cyclase activity is unknown.

Recently, work from this and other laboratories has described the characteristics of specific binding sites in membranes from heart (6) and several other tissues (7–10) which bind *beta agonist* catecholamines. These sites, which have been studied with [^{3}H]norepinephrine (1, 6, 10), [^{3}H] epinephrine (7, 8), and [^{3}H]isoproterenol (9), are characterized by a predominant specificity for the catechol portion of the *beta* agonist molecule (1). Stereospecificity of the side chain did not influence binding of the labeled catecholamines. Potent *beta* antagonists, such as propranolol, were also very weak inhibitors of binding at these catechol-specific sites.

Accordingly, the studies to be reported here were undertaken in an attempt to determine whether distinct binding sites could be identified using [^{3}H]propranolol, a *beta antagonist drug*. In addition to reporting the characteristics of these [^{3}H]propranolol binding sites in myocardial membranes, we have also compared the ability of various adrenergic antagonists to compete for the [^{3}H]propranolol binding sites and to block catecholamine-stimulated myocardial adenylate cyclase. If the [^{3}H]propranolol binding measured by our procedures did in fact occur to the physiologically significant *beta* adrenergic receptors, a good correlation would be expected between the ability of the adrenergic antagonists to block [^{3}H]propranolol binding and to interfere with activation of adenylate cyclase by catecholamines.

METHODS

Sources of materials. *l*-Propranolol (Ayerst) was tritiated by New England Nuclear Corporation. The tritiated product (specific activity, 5.0 Ci/mmole) was purified by high-voltage electrophoresis on paper in a Gilson Electrophorator, with pyridine-acetate buffer at pH 3.6. Electrophoresis was carried out for 50 min at 3500 V. The sample of [^{3}H]propranolol migrated 19 cm from the origin toward the cathode. Only a negligible amount of background contamination (less than 0.5%) was noted. The electrophoretic profile of the [^{3}H]propranolol corresponded to the mobility of unlabeled propranolol in the same system. [^{3}H]Propranolol prepared in this way has been shown to retain biological activity (11). To evaluate this point further, we tested the ability of [^{3}H]propranolol to block activation of the myocardial adenylate cyclase by isoproterenol (see below). Its activity in this regard was identical with that of unlabeled *l*-propranolol, indicating no decrease in the *beta* adrenergic blocking activity of the compound subsequent to the tritiation. It is not, of course, possible to test directly the biological effectiveness of only those molecules which actually bear the ^{3}H label (and which presumably represent only about 1% of all the propranolol molecules).

Isoproterenol and *dl*-propranolol were obtained from Sigma Chemical Company. *d*-Propranolol was obtained from Ayerst; chlorpromazine, from Smith Kline & French; phentolamine, from Ciba; dichloroisoproterenol, from Lilly; haloperidol, from McNeil, nylidrin, from USV Pharmaceutical Company; sotalol, from Mead-Johnson; and butoxamine, from Burroughs Wellcome. The Millipore filters used were 0.45 μm in pore size and 25 mm in diameter (HAWP 02500). [^{32}P]ATP was purchased from International Chemical and Nuclear Corporation, and [^{3}H]cAMP,[2] from Schwartz-Mann. Other chemicals were of the highest purity obtainable from commercial sources.

Preparation of myocardial membranes. Differential centrifugation of ventricular homogenates in 0.25 M sucrose was performed as previously described (7). The 78,000 $\times$ *g* fraction was routinely used for binding and cyclase studies, since we have found that the specific activity of fluoride- and catecholamine-stimulated cyclase is highest in this fraction. Protein was determined by the method of Lowry *et al.* (12).

Binding assay. [^{3}H]propranolol, 0.1 μM (500,000 cpm/ml), was incubated with 1 ml of myocardial membranes (approximately 60 μg of protein) at 37° for 30–60 min. Binding of [^{3}H]propranolol was quantitated by rapid Millipore filtration of the samples. After the filtration, the Millipore filters

were routinely washed with 10 ml of buffered 0.25 M sucrose, followed by liquid scintillation counting (13). Experiments were done to determine nondisplaceable binding of [^{3}H]propranolol. These were performed by incubating membranes with [^{3}H]propranolol in the presence of a large excess (100 μg/ml) of *dl*-propranolol and determining binding after Millipore filtration. The small number of counts of [^{3}H]-propranolol found on the Millipore filters under such conditions (1–2% of the added tracer) was subtracted from all experimental values, which generally were about 10 times higher. Nonspecific background was also minimized by soaking all Millipore filters in 10 mg/ml of *dl*-propranolol prior to use. This procedure reduced by several fold the amount of [^{3}H]propranolol adsorbed to the Millipore filters. In competition studies, adrenergic blocking drugs were incubated with the membranes for 30 min at 37° prior to the addition of [^{3}H]propranolol. Control membranes were initially incubated without added drugs.

Adenylate cyclase activity was assayed by a modification (6) of the method of Krishna *et al.* (14), which follows the conversion of [α-^{32}P]ATP to [^{32}P]cAMP in the presence of an ATP-regenerating system. Substrate ATP concentration was 1.5 mM. Under these conditions mean stimulation by 10 μM isoproterenol was 70%, and by 100 μM isoproterenol, 100%, above basal levels. Incubations were routinely performed for 10 min at 37°. The sensitivity of the myocardial adenylate cyclase to the *beta* agonist isoproterenol is demonatrated in Fig. 1. Adrenergic antagonists were tested for their ability to block the stimulation of the myocardial cyclase by isoproterenol (10 μM). In reporting the results of these studies, 100% of "maximum response" refers to the amount of cAMP generated above basal levels in the presence of 10 μM isoproterenol; 0% therefore represents complete blockade of the isoproterenol effect, indicating basal cyclase activity.

RESULTS

[^{3}H]Propranolol bound rapidly and reversibly to sites in the myocardial membranes (Fig. 2). Addition of excess unlabled propranolol to incubations after equilibrium had been achieved was followed by rapid dissociation of all the bound [^{3}H]propranolol, which was complete within 1 min. Scatchard plots (15) of the binding data were constructed by computer analysis (16) (Fig. 3). The data could not be fitted by a model based on a single, homogeneous class of sites (16). Two orders of sites gave a good fit, with the higher-order site having $K_1 = 4.57 \times 10^4$ M^{-1} and the lower-order site having $K_2 = 4.32 \times 10^2$ M^{-1}. Binding capacities of the two orders of sites correspond to 3.2×10^{-8} mole/mg of protein for the high-order sites and 8.4×10^{-7} mole/mg of protein for the low-order binding sites. Adequacy of fit for models with higher numbers of sites was not evaluated.

In order to determine the structural requisites for binding of drugs to the [^{3}H]pro-

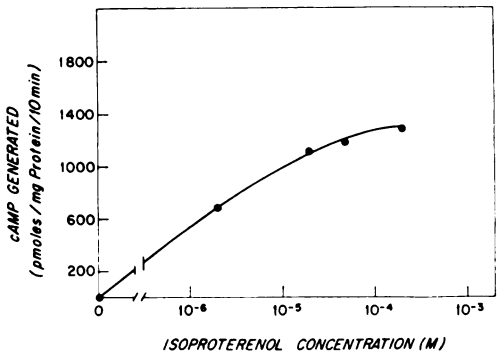

FIG. 1. *Stimulation of myocardial adenylate cyclase by isoproterenol*

Assays were performed according to Krishna *et al.* (14). Incubation mixtures contained the following in a total volume of 50 μl: ATP, 1.5 mM; Tris-HCl buffer, 30 mM (pH 7.5); $MgCl_2$, 5 mM; cAMP, 0.1 mM; phosphopyruvate kinase, 40 μg/ml; myokinase, 20 μg/ml; and isoproterenol at the indicated concentrations. Incubations were conducted for 10 min at 37°. Recovery of [^{32}P]-cAMP during chromatography on Dowex 50W-X2 (14) was monitored with [^{3}H]cAMP added to each assay tube. Appropriate corrections for this recovery were made in each experimental value. cAMP generated refers to the increment above basal levels due to isoproterenol. Each point is the mean of triplicates. In this and the experiments depicted in Fig. 5, basal activity ranged between 1100 and 1200 pmoles of cAMP per milligram of protein in 10 min.

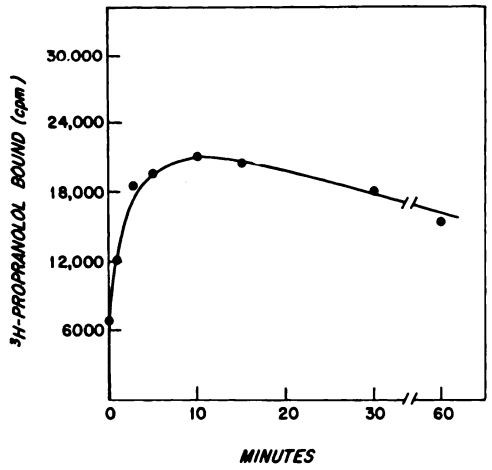

FIG. 2. *Time course of binding of [³H]propranolol to cardiac membranes*

Bound [³H]propranolol was isolated by Millipore filtration at various intervals. Each point is the mean of duplicates. The earliest point tested was 30 sec. [³H]Propranolol was present at 0.1 μM.

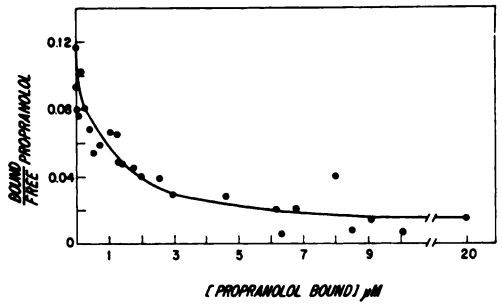

FIG. 3. *Scatchard plot of binding of [³H]propranolol to cardiac membranes (15)*

Increasing amounts of unlabeled *l* propranolol from 0.1 μM to 10 mM were added. Incubations were conducted at 37° for 30 min. Values are the means of triplicates.

pranolol binding sites, nine adrenergic antagonist drugs were tested for their ability to inhibit [³H]propranolol binding. The most effective inhibitors were *l*-propranolol, *d*-propranolol, chlorpromazine, and haloperidol. The inhibition curves obtained with these drugs are depicted in Fig. 4A. The other drugs tested were significantly less effective in blocking [³H]propranolol binding (Fig. 4B and C).

Each of the adrenergic blocking agents was next tested for its ability to inhibit isopro-

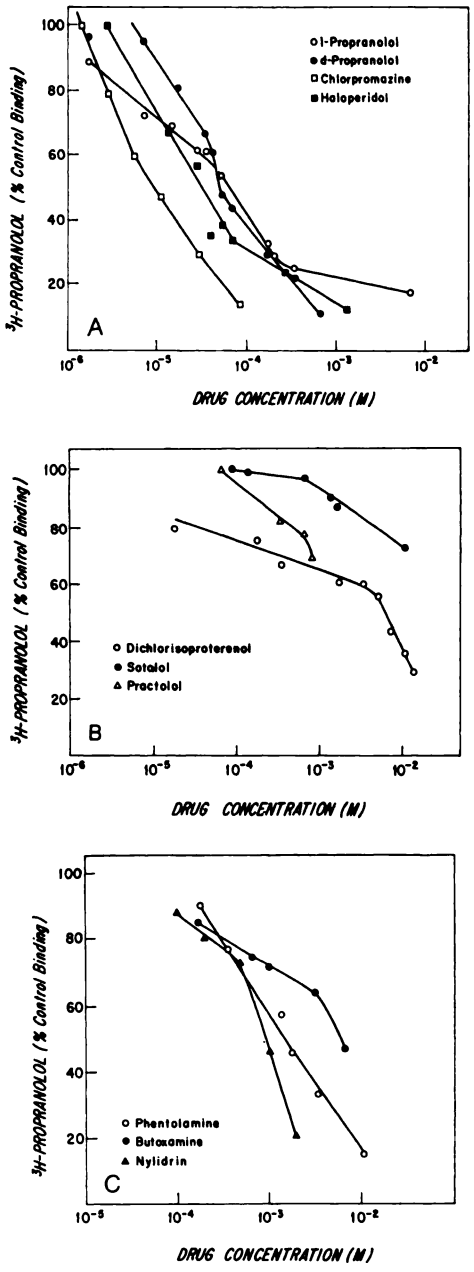

FIG. 4. *Inhibition of binding of [³H]l-propranolol to cardiac membranes by adrenergic antagonists*

A. *l*-Propranolol, *d*-propranolol, chlorpromazine, and haloperidol. B. *dl*-Dichloroisoproterenol, *dl*-sotalol, and *dl*-practolol. C. Nylidrin, *dl*-butoxamine, and phentolamine. Incubation conditions are described under METHODS. Each point is the mean of triplicates. Control binding refers to the amount of [³H]*l*-propranolol bound in the absence of added drugs.

terenol-stimulated myocardial adenylate cyclase. These data are presented in Fig. 5A–C.

Both sets of data (inhibition of [^{3}H]propranolol binding and inhibition of isoproterenol-activated cyclase) are tabulated in Table 1. Little correlation between binding and cyclase inhibition was found. The potent *beta* agonist isoproterenol did not displace [^{3}H]propranolol from the sites at concentrations less than 1 mM. In separate experiments it was determined that only dichloroisoproterenol at the concentrations tested affected basal cyclase (slight depression). All other drugs were without effect.

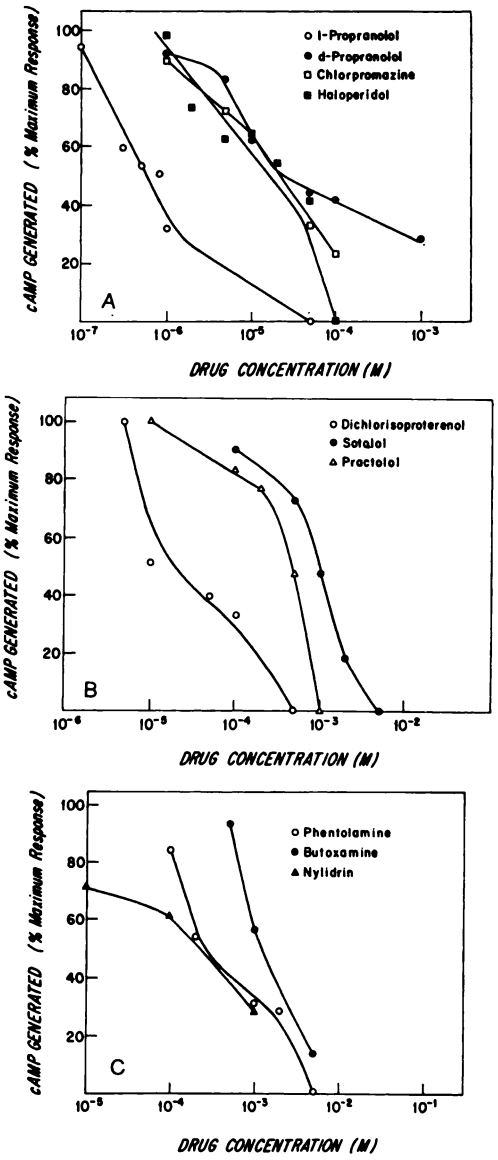

FIG. 5. *Inhibition of isoproterenol-stimulated adenylate cyclase by adrenergic antagonists in canine ventricular membranes*

A. *l*-Propranolol, *d*-propranolol, chlorpromazine, and haloperidol. B. *dl*-Dichloroisoproterenol, *dl*-sotalol, and *dl*-practolol. C. Nylidrin, *dl*-butoxamine, and phentolamine. Assays were performed as described under METHODS. Each value is the mean of three separate experiments.

TABLE 1

Inhibition of [^{3}H]propranolol binding and isoproterenol-activated adenylate cyclase in myocardial membranes by adrenergic antagonists

Drug	Half-maximal inhibition	
	[^{3}H]Propranolol binding	Isoproterenol-stimulated cyclase
l-Propranolol	60 μM	600 nM
d-Propranolol	50 μM	30 μM
Chlorpromazine	9 μM	20 μM
Haloperidol	30 μM	20 μM
dl-Dichloroisoproterenol	6 mM	10 μM
dl-Sotalol	10 mM	1 mM
dl-Practalol	2 mM	500 μM
dl-Nylidrin	900 μM	200 μM
dl-Butoxamine	6 mM	1 mM
Phentolamine	20 mM	300 μM

DISCUSSION

A number of attempts have been made to specifically label *alpha* adrenergic receptors with radioactive irreversible *alpha* blocking agents. These attempts have generally been unsuccessful, in that the labeled sites had characteristics other than those to be expected of the receptors (17–19).

Relatively few studies of binding *in vitro* of labeled *beta* adrenergic blocking agents, such as propranolol, have been reported. Potter (11) studied binding of [^{3}H]propranolol to subcellular fragments derived from guinea pig atria and concluded that the binding observed was "nonspecific" and nonsaturable. He did not, however, use drug concentrations higher than 0.1 μM. As noted here, the kinetics of [^{3}H]propranolol binding is such that saturation would not have been

observed until somewhat higher concentrations than those Potter used were reached. DeRobertis and Fiszer de Plazas (20) observed binding of [^{14}C]propranolol to cat brain synaptic membranes. The binding was inhibited by norepinephrine, but only at very high concentrations (1 mM).

Clearly the [^{3}H]propranolol binding sites which we have studied are not the *beta* adrenergic receptors which mediate stimulation of the myocardial adenylate cyclase or of effects on myocadial contractility. This is demonstrated by several features. (a) The ability of adrenergic blockers to inhibit isoproterenol-stimulated adenylate cyclase was not correlated with ability to inhibit [^{3}H]-propranolol binding. (b) Binding was not stereospecific; thus *d*- and *l*-propranolol were equally effective as inhibitors of [^{3}H]propranolol binding, but *l*-propranolol was considerably more potent for cyclase inhibition. (c) A number of pharmacologically effective *beta* adrenergic blocking agents (e.g., dichloroisoproterenol were poor inhibitors of [^{3}H]propranolol binding.

The physiological significance of these [^{3}H]propranolol binding sites cannot be unequivocally determined from the present experiments. The equivalent affinities of *d*- and *l*-propranolol as well as the effectiveness of chlorpromazine, however, suggest that these sites may be involved in some more general membrane effects of these compounds, such as membrane stabilization or local anesthetic effects. Additional support for such an interpretation is provided by a recent study (21) which showed that chlorpromazine and propranolol competed for the same set of binding sites in dog liver mitochondrial membranes (which would not be expected to contain the *beta* receptors).

The most plausible explanation why the myocardial *beta* adrenergic receptors were not identified in this study is that the number of physiologically significant receptors represents a very small fraction of the total number of membrane sites capable of binding the labeled antagonist drug. Another possible, though less attractive, explanation is that the affinity of receptor sites is very high and the specific activity of the available tracer is not high enough to permit delineation of the sites. With regard to this point, we attempted to perform binding experiments with very low concentrations of [^{3}H]propranolol (1000 cpm = 1 nM). Under these conditions, however, reproducible specific binding could not be demonstrated.

Our findings with regard to blockade of isoproterenol-stimulated adenylate cyclase by the various adrenergic antagonists agree resonably well with results previously reported for more intact cardiac preparations, as well as for cyclase activation in subcellular particles. Koch-Weser (22) found *l*-propranolol > dichloroisoproterenol > sotalol in cat papillary muscles, which agrees with the potency series found here.

In general, somewhat higher concentrations of agonists and antagonists are necessary for effects *in vitro* on adenylate cyclase in broken cell preparations than for comparable effects on more intact preparations (23). We found half-maximal effects of *l*-propranolol on isoproterenol stimulation of cyclase at approximately 600 nM, which is virtually identical with the results obtained by Rosen *et al.* (24) in erythrocyte membranes and Burges and Blackburn (25) in rat heart membranes, though somewhat higher than that reported by Mayer (5) in dog heart membranes. Our data for half-maximal effects of dichloroisoproterenol (24) and practolol (25) also agree with results of other systems *in vitro*. Sotalol was weaker than anticipated, 1 mM being required for half-maximal effects. Sotalol, however, has been found to be only 0.5% as potent as *dl*-propranolol in blocking the effects of isoproterenol on cat papillary muscles (26).

d-Propranolol was only 2% as potent as *l*-propranolol on adenylate cyclase, which is still perhaps more active than might have been anticipated. The reason for this is not apparent, although some racemization to the *l* form might have occurred.

The effects of chlorpromazine and haloperidol on catecholamine-stimulated cyclase are also in agreement with findings by others (27, 28). These effects presumably are not due to competition for occupancy of hormone receptors, but rather to distortion of the normal receptor-cyclase interaction by binding of the drugs to the membranes (27).

ACKNOWLEDGMENT

The authors are indebted to Dr. Edgar Haber, Massachusetts General Hospital, in whose laboratory part of these studies were performed.

REFERENCES

1. Murad, F., Chi, Y., Rall, T. W. & Sutherland, E. W. (1962) *J. Biol. Chem.*, **237**, 1233–1238.
2. Sutherland, E. W. & Robison, G. A. (1966) *Pharmacol. Rev.*, **18**, 145–161.
3. Epstein, S. E., Levey, G. S., & Skelton, C. L. (1971) *Circulation*, **43**, 437–450.
4. Black, J. W., Duncan, W. A. M. & Shanks, R. G. (1965) *Br. J. Pharmacol.*, **25**, 577–591.
5. Mayer, S. E. (1972) *J. Pharmacol. Exp. Ther.*, **181**, 116–125.
6. Lefkowitz, R. J., Sharp, G. W. G. & Haber, E. (1973) *J. Biol. Chem.*, **248**, 342–349.
7. Schramm, M., Feinstein, H., Naim, E., Lang, M. & Lasser, M. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 523–527.
8. Tomasi, V., Koretz, S., Ray, T. K., Dunnick, J., & Marinetti, G. V. (1970) *Biochim. Biophys. Acta*, **211**, 31–42.
9. Bilzekian, J., & Aurbach, G. (1973) *J. Biol. Chem.*, **248**, 5577–5583.
10. Lefkowitz, R. J., O'Hara, D. & Warshaw, J. (1973) *Nat. New Biol.*, **244**, 79–81.
11. Potter, L. T. (1967). *J. Pharmacol. Exp. Ther.*, **155**, 91–100.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
13. Bray, G. A. (1960) *Anal. Biochem.*, **1**, 279–285.
14. Krishna, G., Weiss, B., & Brodie, B. B. (1968) *J. Pharmacol. Exp. Ther.*, **163**, 379–388.
15. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.*, **51**, 660–672.
16. Feldman, H. A. (1972) *Anal. Biochem.*, **48**, 317–338.
17. Moran, J. F., May, M., Kimelberg, H. & Triggle, D. J. (1967) *Mol. Pharmacol.*, **3**, 15–27.
18. Yong, M. S. & Marks, G. S. (1969) *Biochem. Pharmacol.*, **18**, 1619–1626.
19. Yong, M. S. & Nickerson, M. (1973) *J. Pharmacol. Exp. Ther.*, **186**, 100–108.
20. DeRobertis, E. & Fiszer de Plazas, S. (1969) *Life Sci.*, **8**, 1247–1262.
21. Huunan-Seppala, A. (1972) *Acta Chem. Scand.*, **26**, 2713–2733.
22. Koch-Weser, J. (1971) in *Cardiovascular Beta Adrenergic Responses, Effects of Adrenergic Stimulation and Blockade on Myocardial Mechanics* (Kattus, A. A., Gordon, R. & Hall, V. E. eds.), p. 62, University of California Press, Berkeley.
23. Robison, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP*, pp. 37–38, Academic Press, New York.
24. Rosen, O. M., Erlichman, J. & Rosen, S. M. (1970) *Mol. Pharmacol.*, **6**, 524–531.
25. Burges, R. A. & K. J. Blackburn, K. J. (1972) *Nat. New Biol.*, **235**, 249–250.
26. Parmley, W. W., Rabinowitz, B., Chuck, L., Bonorris, G. & Katz, J. (1972) *J. Clin. Pharmacol.*, **12**, 127–135.
27. Wolff, J. & Jones, A. B. (1970) *Proc. Natl. Acad. Sci. U. S. A.* **65**, 454–459.
28. Sheppard, H. & Burghardt, C. R. (1971) *Mol. Pharmacol.*, **7**, 1–7.