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SOLUBILIZED RECEPTORS FOR [^{3}H]DOPAMINE (D_3 BINDING SITES) FROM CANINE BRAIN

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Abstract—The objective of the present study was to solubilize the D_3 site which binds [^{3}H]dopamine, using the same digitonin method that had been successful in solubilizing the D_2 dopamine receptor. Canine brain striatal membranes were solubilized by a final concentration of 1% digitonin. The specific binding of [^{3}H]dopamine to the soluble D_3 binding sites was measured using Sephadex G-50 gel filtration. The density of D_3 sites was identical in the membrane and soluble preparations (82–90 fmoles/mg protein), although the dissociation constant (K_D value) went from 1.2 nM in the membranes to the value of 3.4 nM in the soluble material. The concentrations of various drugs which inhibited the binding of [^{3}H]dopamine were similar in the two preparations. The agonists [dopamine, apomorphine and (±)-6,7-dihydroxy-2-aminotetralin (ADTN)] all inhibited the binding of [^{3}H]dopamine by 50% at concentrations between 2 and 20 nM in both the intact and soluble preparations. The neuroleptics were all equally weak in inhibiting the binding of [^{3}H]dopamine, with IC_{50} values in the micromolar concentration range, values typical for the D_3 site. Approximately 36% of the D_3 sites were recovered from the original tissue. Since the densities and recoveries of the D_2 and D_3 sites differed upon digitonin solubilization, this provided further indirect evidence that these two sites are distinct and separate entities which might ultimately be separated.

Although there is considerable indirect evidence for the concept of multiple receptors for dopamine [1, 2], it is now desirable to isolate these different receptors to search for direct evidence for their possible separate existence. Toward this objective, therefore, the D_2 receptor for dopamine has been solubilized from canine striatum [3–6] and human putamen [7, 8], in all cases using [^{3}H]spiperone to label the D_2 receptor. Rat and calf striatum [9, 10] are not good sources of soluble dopamine receptors since primarily non-dopaminergic binding sites are solubilized [11].

Four distinct types of dopamine-sensitive sites occur in the brain [12]. The term D_1 conveniently designates dopamine-stimulated adenylate cyclase which is affected by micromolar concentrations (1–10 μM) of both dopamine and neuroleptics [2]. The term D_2 refers to a dopaminergic receptive site (labeled by any ^{3}H-ligand) which is sensitive to micromolar concentrations of dopamine, but nanomolar concentrations (0.1–30 nM) of neuroleptics [12–16]. The term D_3 designates a site sensitive to nanomolar concentrations of dopamine (1–10 nM), but micromolar concentrations of neuroleptics [12–16]. The D_4 site is sensitive to nanomolar concentrations of both dopamine and neuroleptics [12]. Although the majority of dopaminergic behaviours (e.g. emesis, rotation, locomotion) are primarily associated with D_2 sites [12], about 50% of the D_3 sites are located presynaptically and appear to function as dopamine autoreceptors [12].

The physically independent existence of the D_3 site [12–16] is based on many observations. These include the fact that the D_3 site (as labeled by [^{3}H]dopamine, for example) is occupied by nanomolar concentrations of dopamine (1–10 nM), but micromolar concentrations of neuroleptics, thus distinguishing it from the D_2 site (see above). The D_2 and D_3 sites also differ in thermal sensitivity [17], have different densities within the tissue [15], can be differentially alkylated [18], can be differentially centrifuged [19], can be selectively labeled [20, 21], and are differentially altered in brain disease [22–25] or following selective lesions [26, 27]. Two other laboratories have since confirmed the rank order of dopaminergic agonists and antagonists at this D_3-type binding site [27–29].

In order to pursue the goal for direct evidence for the possible separate existence of the D_2 and D_3 sites, therefore, the present study was done to solubilize the D_3 site from the canine striatum. The results indicate that this can be successfully achieved using digitonin, such that the properties of the solubilized D_3 are identical to those for the D_3 site in the native membranes.

MATERIALS AND METHODS

The methods were essentially those of Madras *et al.* [6]. Individual striata (canine brain) were homogenized (10% w/v) in 0.25 M sucrose and centrifuged at 1,100 *g* for 10 min to remove nuclear (P_1) material. After washing the pellet once, supernatants fractions were pooled and centrifuged at 105,000 *g* for 60 min. This combined mitochondrial and microsomal membrane fraction was resuspended in 100 vol. (of the original wet weight) of TEAN buffer

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[50 mM Tris–HCl, 5 mM disodium EDTA, 0.01% (w/v) ascorbic acid, 10 μM nialamide, pH 7.4 at 4°] and centrifuged at 24,000 *g* for 25 min. The resulting pellet was washed and pelleted a further three times in 100 vol. of TEAN buffer. This procedure was used to remove endogenous substances which might interfere with or inhibit the binding of [^{3}H]dopamine. Final resuspension was in 25–30 vol. of buffer for the membrane preparation and 10 vol. for the soluble material.

The soluble material was obtained by incubating 2 vol. of the final membrane preparation with 1 vol. of 3% digitonin (in TEAN buffer containing 0.01% sodium azide; 1% digitonin final concentration) for 30 min at 0–4°. The non-solubilized material was removed by centrifugation at 105,000 *g* for 60 min. Electron microscopy (negative staining with phosphotungstic acid) confirmed the absence of any particulate matter or lamellated profiles in the supernatant fraction of this soluble fraction.

Binding of [^{3}H]*dopamine to the* D_3 *sites of the intact membranes.* The specific binding of [^{3}H]dopamine to D_3 sites in the intact (i.e. not solubilized) membranes was measured as described elsewhere [13, 14] with minor modifications. The incubation mixture (final vol. of 0.6 ml) consisted of 0.2 ml buffer, 0.2 ml [^{3}H]dopamine (0.8 nM final concentration; sp. act. 43.0 Ci/mmole; Amersham), and 0.2 ml of membrane material (containing 0.14–0.3 mg protein, as determined by a modified Lowry method [30, 31]). The purity of the [^{3}H]dopamine was periodically checked by thin-layer chromatography (solvent system: butanol–acetic acid–water, 4:1:5; $R_f = 0.49 \pm 0.04$) since appreciable breakdown of the ^{3}H-ligand can occur at this specific activity. The drug solutions were prepared in TEAN buffer. The ^{3}H-ligand was used at a purity exceeding 90%. The final mixture was incubated for 3 hr at 0.5° followed by rapid filtration (under vacuum) of a 0.5 ml aliquot through a glass fiber filter (Whatman GF/B). The filter was washed with 10 ml of ice-cold buffer. Independent experiments in this laboratory (unpublished) confirmed that the bound ^{3}H-ligand was over 98% [^{3}H]dopamine. Radioactivity was counted by liquid scintillation spectrometry (efficiency of 38–47%) after overnight equilibration in 9 ml Aquasol (New England Nuclear Corp., Boston, MA). Specific binding of [^{3}H]dopamine was defined as that inhibited by the presence of 1 μM dopamine or 1 μM apomorphine. At a final concentration of 0.8 nM [^{3}H]dopamine, the total number of cpm (counts per min) bound (after subtracting the

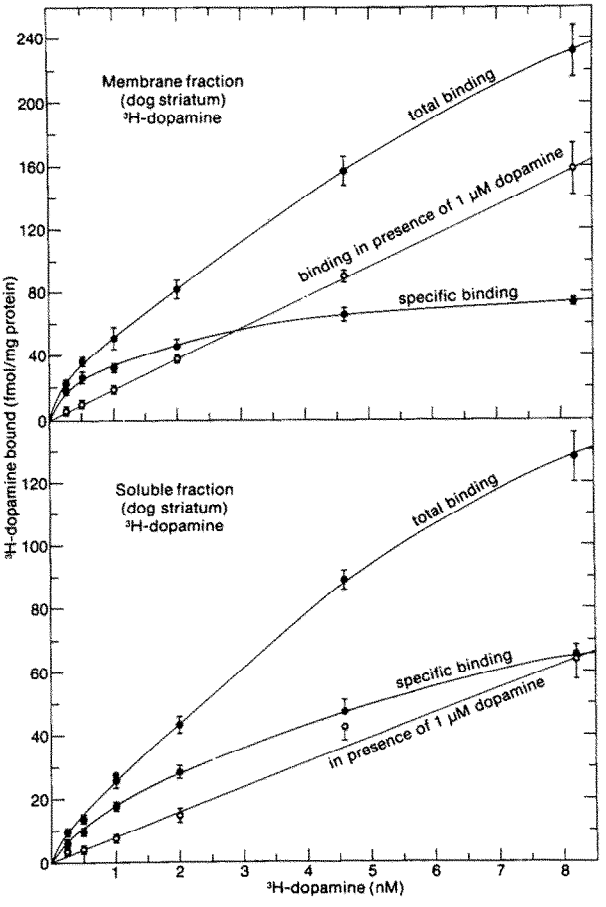

Fig. 1. Saturation of [^{3}H]dopamine binding to membrane and soluble preparations of dog striata. A range of 0.25 to 8 nM [^{3}H]dopamine was used for both the membrane and the soluble material. Specific binding of [^{3}H]dopamine was defined as that inhibited by 1 μM dopamine.

amount bound to the filter) was 500 cpm, of which 65% was specific. The amount that was bound non-specifically to the filter was approximately 150 cpm.

Binding of [^{3}H]*dopamine to the soluble fraction.* The specific binding of [^{3}H]dopamine to the solubilized material was carried out in almost exactly the same way as for the intact membranes (above), except that the period of incubation was about 16 hr (overnight) at 0–4°. The bound and free fractions of [^{3}H]dopamine were separated using Sephadex G-50 (fine) gel filtration. Aliquots (0.3 ml) of the incubation mixture were layered onto columns (0.5 × 12 cm) pre-equilibrated with buffer at 4°. Flowing at 0.3 to 0.4 ml/min, 1 min fractions were collected and monitored for ^{3}H as above. Bound [^{3}H]dopamine eluted in the void volume (third to fifth fractions) whilst unbound [^{3}H]dopamine eluted in subsequent fractions. Specific binding was defined as that amount of bound [^{3}H]dopamine which was inhibited by the presence of 1 μM dopamine or 1 μM apomorphine. At a final concentration of 0.8 nM [^{3}H]dopamine, the amount of specific binding in the soluble material was consistently 180 cpm, which represented 65% of the total binding in the soluble fraction. Previous work in this laboratory (unpublished) had established that prolonged incubation at 0° (overnight) resulted in a loss of [^{3}H]dopamine, but the bound ^{3}H-ligand was still over 98% [^{3}H]dopamine. Background was 35 cpm. Radioactivity was counted immediately after addition of 9 ml Aquasol.

Drug sources were as follows: digitonin, Fisher, Pittsburgh, PA, U.S.A.; [^{3}H]dopamine, Amersham Corp.; (+)-butaclamol, Ayerst Research Laboratories, Montreal, Canada; and haloperidol, Janssen Pharmaceutica, Beerse, Belgium. All other reagents were obtained from commercial sources.

RESULTS

Figures 1 and 2 compare the saturation curves and corresponding Scatchard analyses obtained for [^{3}H]dopamine binding to membrane and soluble preparations. For both preparations, 1 μM dopamine or apomorphine was used to define the baseline for calculation of specific binding. The affinity of [^{3}H]dopamine decreased approximately 3-fold after solubilization (K_D increased from 1.2 to 3.4 nM), while the density of the binding sites remained similar to that for the membrane preparation (Table 1). The receptor and protein recoveries are indicated in Table 1.

The pharmocological characteristics of the D_3 sites were retained after solubilization. Figure 3 and Table 2 show the inhibition data and the corresponding IC_{50} values (using 0.8 nM [^{3}H]dopamine) for dopamine agonists, neuroleptics and other aromatic amine neurotransmitters. Dopamine, (±)-6,7-dihydroxy-2-aminotetralin (ADTN), and apomorphine all inhibited the ^{3}H-ligand with very high potency (nanomolar) in both the membrane and soluble preparations. Noradrenaline was approximately 6-fold less potent than dopamine while very high concentrations of 5-hydroxytryptamine were required to inhibit [^{3}H]dopamine binding. The neuroleptics were also very weak competitors for the binding and appeared to interact with only about one-half of the specifically labeled sites. The correlation between the membrane and soluble IC_{50} (or K_i) values was excellent ($r^2 = 0.98$, slope = 0.98). The characteristics of the membrane-bound D_3 sites were very close to those previously described in the calf, rat and human striatum [13, 14]. The IC_{50} values were also confirmed using a higher concentration of [^{3}H]dopamine (4 nM) as seen in Table 3, thus confirming the homogeneity of the binding site population.

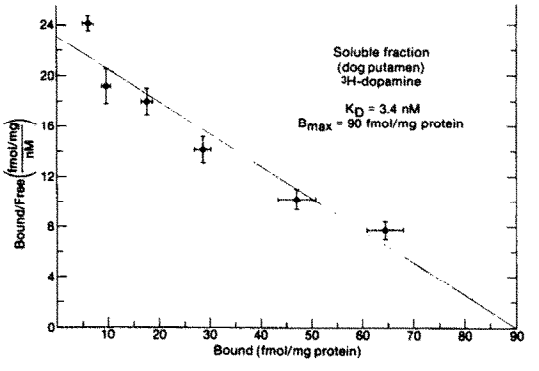

Fig. 2. Scatchard analyses of the specific binding of [^{3}H]dopamine to the soluble preparation of dog striata. A range of 0.25 to 8 nM [^{3}H]dopamine was used with 1 μM dopamine to define specific binding.

Table 1. Recovery of [^{3}H]dopamine binding sites and protein after digitonin solubilization

	Protein (mg/ml)	Volume (ml)	Total Protein (mg)	Receptor density (B_{max}) (fmol/mg protein)	Kd (nM)	Total Receptors (fmol)
Membrane	4.0	1	4	82	1.2	328
Soluble	1.0	1.5*	1.5	79	3.4	118
Recovery			37%	96%		36%

Binding assays for both the membrane and soluble fractions were described under Materials and Methods. Values are representative of six experiments.

* Membrane preparation (1 ml) was solubilized by the addition of 0.5 ml digitonin.

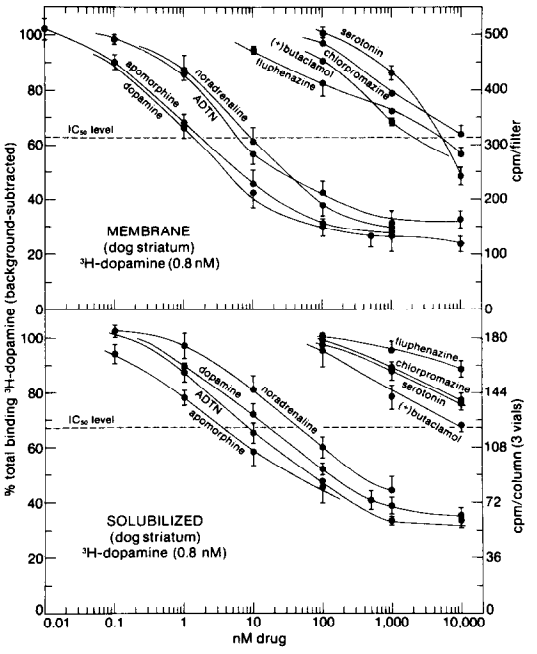

Fig. 3. Inhibition of [³H]dopamine binding (0.8 nM) to membrane and soluble preparations of dog striatum by dopamine agonists and neuroleptics. Specific binding was defined by 1 μM dopamine or apomorphine, and represented 65–75% of the total binding in both fractions. Points are mean ± S.E.M. of at least three experiments, each performed in triplicate. ADTN is (±)-6,7-dihydroxy-2-aminotetralin.

DISCUSSION

[³H]Dopamine binding has now been examined in four species: calf [15], rat [12], human [13] and dog [32, 33]. In all these studies the D_3 site consistently had a high affinity for dopamine agonists and a very low affinity for neuroleptics (100–2000 nM). The present results for [³H]dopamine binding to the solubilized D_3 site in the canine striatum qualitatively agree with those reported by Sano *et al.* [32] and Nishikori *et al.* [33], although these workers used the term D-2 (instead of D_3) for the high-affinity site for [³H]dopamine. However, the original definition of D-2 (see Kebabian and Calne [2]) defined dopaminergic sites which were not linked to adenylate cyclase, a definition which causes difficulties when one considers the fact that the binding of [³H]sulpiride (which does not inhibit dopamine-sensitive adenylate cyclase) to dopamine receptors is markedly affected by nucleotides [34]. Thus, the concentrations (or IC_{50} values) of dopamine and neuroleptics effective on the present D_3 site correspond very well to those found by Sano and Nishikori and co-workers [32, 33] on their D-2 site (our D_3 site). As outlined elsewhere in detail [12], it is most clear and simple to use the concentrations (IC_{50} values) to define the binding sites rather than the affinity constants (or K_i values), since often the ³H-ligand binds to muliple sites and it is not correct to use the K_D value for the ³H-ligand on the assumption that it is binding to a single set of sites.

Table 2. Inhibition of [³H]dopamine (0.8 nM) binding to membrane and soluble preparation of dog striata*

	IC_{50} (nM)
Dopamine	7 ± 2
Apomorphine	6 ± 4
ADTN	15 ± 3
(-)Norepinephrine	25 ± 8
5-Hydroxytryptamine	5,000 ± 800
(+)Butaclamol	>1,000
Fluphenazine	>1,000
Chlorpromazine	>1,000

* The IC_{50} values were determined from 3–5 curves obtained from at least three individual brain preparations. Each consisted of at least four points, each performed in triplicate. Apomorphine (1 μM) was used to define specific binding.

Table 3. Displacement of [³H]dopamine (4.4 nM) binding to membrane preparations of dog striata*

	IC_{50} (nM)	
	MEMBRANE	SOLUBLE
Dopamine	1.5 ± 0.2	5.0 ± 2.0
Apomorphine	1.8 ± 0.5	1.3 ± 0.7
ADTN	5.4 ± 0.2	3.0 ± 1.1
(-)Norepinephrine	10 ± 2.3	15 ± 4.0
5-Hydroxytryptamine	5,500 ± 1,000	8,000 ± 1,000
(+)Butaclamol	2,000 ± 300	1,700 ± 300
Fluphenazine	5,500 ± 800	>10,000
Chlorpromazine	>10,000	10,000 ± 2,000

* Membrane preparations were assayed as described in Materials and Methods. Specific binding was 56 fmoles/mg protein, which represented 37% of the total. Total binding in the assay was 760 cpm after subtraction of the filter background (210 cpm). Antagonists displace a maximum of 20% of the total binding with very shallow slopes.

The density of the [³H]dopamine binding sites was consistent with the other species: rat 81 fmoles/mg; calf, 184 fmoles/mg; human 60 fmoles/mg; and dog, 82 fmoles/mg protein. The affinity constant (K_D) of the canine D_3 receptor for [³H]dopamine was also on the same order of magnitude as the other species.

Comparison of the characteristics of [³H]dopamine binding to membrane and soluble fractions of dog striata indicated that the digitonin maintained the integrity of the D_3 binding site. The solubilized D_3 site had the same rank order of drug action with little change in affinity or specificity following disruption of the membrane. The high affinity for dopamine agonists and low affinity for neuroleptics was retained. The receptor affinity for dopamine decreased by a factor of 3 in the soluble form and the IC_{50} values for agonists differed by no greater than 1.5-fold.

The recovery of the D_3 receptor after solubilization can be considered in terms of density and total number of binding sites. The ratio of receptor protein to the rest of the membrane proteins remained constant after release into solution. The recovery of the

protein was 37%, a value which may be determined by the extent of washing of the membrane preparation as well as the starting concentration of the protein. The recovery of soluble protein from unwashed membrane was higher [5, 6], perhaps because hydrophilic proteins may be released into the wash buffer during the washing procedure.

About twice as many D_3 sites were released into solution as the D_2 sites prepared under the same conditions [5, 6]. The density of the D_3 site remained the same whereas the D_2 site fell from 400 fmoles/mg protein to 194 fmoles/mg. Therefore, the ratio of D_3 to D_2 sites has increased in the soluble preparation. Either the D_2 site is more labile in digitonin or more hydrophobic and thus less soluble than the D_3 site.

Thus, both the D_2 receptors [5, 6] and the D_3 sites have retained their properties found in the native membranes. Preliminary observations in our laboratory suggest that the two sites can be physically separated (see also Titeler *et al.* [35]).

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REFERENCES

1. M. Titeler, P. Weinreich, D. Sinclair and P. Seeman, *Proc. natn. Acad. Sci. U.S.A.* **75**, 1153 (1978).
2. J. W. Kebabian and D. B. Calne, *Nature, Lond.* **277**, 93 (1979).
3. H. Gorissen, G. Aerts and P. M. Laduron, *Fedn Eur. Biochem. Soc. Lett.* **100**, 281 (1979).
4. H. Gorissen and P. M. Laduron, *Nature, Lond.* **279**, 72 (1979).
5. B. K. Madras, A. Davis and P. Seeman, *Soc. Neurosci. Abstr.* **6**, 240 (1980).
6. B. K. Madras, A. Davis, P. Kunashko and P. Seeman, in *Psychopharmacology and Biochemistry of Neurotransmitter Receptors* (Eds. H. Yamamura, R. W. Olsen and E. Usdin), pp. 411–19. Elsevier North Holland, New York (1980).
7. A. Davis, B. K. Madras and P. Seeman, *Soc. Neurosci. Abstr.* **6**, 500 (1980).
8. A. Davis, B. K. Madras and P. Seeman, *Eur. J. Pharmac.* **70**, 321 (1981).
9. H. Gorissen and P. M. Laduron, *Life Sci.* **23**, 575 (1978).
10. S. Tam and P. Seeman, *Eur. J. Pharmac.* **52**, 151 (1978).
11. H. Gorissen, B. Ilein, G. Aerts and P. M. Laduron, *Fedn Eur. Biochem. Soc.* **121**, 133 (1980).
12. P. Seeman, *Pharmac. Rev.* **32**, 299 (1980).
13. S. List, M. Titeler and P. Seeman, *Biochem. Pharmac.* **29**, 1621 (1980).
14. M. Titeler, S. List and P. Seeman, *Commun. Psychopharmac.* **3**, 411 (1979).
15. S. List and P. Seeman, in *Transmitter Biochemistry of Human Brain Tissue* (Eds E. Usdin and P. Riederer), pp. 195–202. Macmillan, London (1980).
16. S. List and P. Seeman, *Soc. Neurosci. Abstr.* **6**, 240 (1980).
17. J. Y. Lew and M. Goldstein, *Eur. J. Pharmac.* **55**, 429 (1979).
18. M. Titeler, *Soc. Neurosci. Abstr.* **6**, 240 (1980).
19. M. Titeler, P. Seeman and F. Henn, *Eur. J. Pharmac.* **51**, 459 (1978).
20. P. M. Whitaker and P. Seeman, *Eur. J. Pharmac.* **56**, 269 (1979).
21. M. Titeler and P. Seeman, *Eur. J. Pharmac.* **56**, 291 (1979).
22. T. Lee, P. Seeman, A. Rajput, I. J. Farley and O. Hornykiewicz, *Nature Lond.* **273**, 59 (1978).
23. T. Lee, P. Seeman, W. W. Tourtellotte, I. J. Farley and O. Hornykiewicz, *Nature Lond.* **274**, 879 (1978).
24. T. Lee and P. Seeman, *Am. J. Psychiat.* **137**, 191 (1980).
25. T. Lee, P. Seeman, O. Hornykiewicz, J. Bilbao, J. Deck and W. W. Tourtellotte, *Brain Res.* **212**, 494 (1981).
26. J. I. Nagy, T. Lee, P. Seeman and H. C. Fibiger, *Nature Lond.* **274**, 278 (1978).
27. P. Sokoloff, M-P. Martres and J-C. Schwartz, *Nature Lond.* **288**, 283 (1980).
28. G. Arana and R. J. Baldessarini, *Soc. Neurosci. Abstr.* **6**, 239 (1980).
29. P. Sokoloff, M-P. Martres and J-C. Schwartz, *Naunyn-Schmiedeberg's Archs Pharmac.* **315**, 89 (1980).
30. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
31. M. A. K. Markwell, S. M. Hass, L. L. Bieber and N. E. Tolbert, *Analyt. Biochem.* **87**, 206 (1978).
32. K. Sano, O. Noshiro, K. Katsuda, K. Nishikori and H. Maeno, *Biochem. Pharmac.* **28**, 3617 (1979).
33. K. Nishikori, O. Noshiro, K. Sano and H. Maeno, *J. biol. Chem.* **255**, 10909 (1980).
34. S. B. Freedman and G. N. Woodruff, *Brit. J. Pharmac.* (Proceedings June 30), 129P (1980).
35. M. Titeler, G. L. Van Loon, P. Seeman and G. M. Brown, *Eur. J. Pharmac.* **71**, 143 (1981).