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Binding of amines to purified bovine adrenal medullary storage vesicle membranes*

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THE MEMBRANES of adrenal medullary storage vesicles play an active role in the maintenance of catecholamine stores. The transport of amines into the vesicles is stimulated by ATP and magnesium and inhibited by reserpine or *N*-ethylmaleimide;[1,2] the latter also reduces the ATPase activity of the membranes as well as the incorporation of ^{32}P from labeled ATP into membrane components.[3,4] Recent studies have suggested that "uptake" and "storage" of amines in the vesicles are two separate and distinct processes: Taugner[5,6] has shown that, when suspended in isotonic medium, isolated vesicle membranes can re-form into "empty" vesicles which still accumulate amines by an ATP and magnesium-stimulated mechanism, but which do not store amines stably. Similarly, amine uptake without stable storage can occur in newly formed storage vesicles *in vivo*.[7,8] Although serotonin is stored less stably than catecholamines in isolated vesicles, it is taken up to a greater extent, which suggests that serotonin has a greater affinity for "uptake" but a lower affinity for "storage".[9] Metaraminol, on the other hand, is both taken up and stored to a lesser extent than are catecholamines.[7,9,10] Studies of the uptake of small molecules by other systems[11] indicate that a membrane-bound macromolecular carrier is involved in the transport of the small molecule across the membrane. The studies reported here show that epinephrine and other amines which are taken up by isolated storage vesicles of the adrenal medulla are bound to the vesicle membranes and suggest that this binding is to a carrier involved in the uptake process.

Bovine adrenal glands were obtained from a local slaughterhouse and the cortices were stripped from the medullae. The latter were homogenized in 10 vol. of isotonic sucrose and purified storage vesicles were obtained by a combination of differential and discontinuous sucrose density gradient centrifugation as described by Smith and Winkler.[12] The vesicles were lysed by resuspension in a small volume of distilled water, dialysed overnight against 3 l water, and sedimented by centrifugation at 130,000 *g* for 30 min. The pellet was washed and resedimented three times, resuspended in a small volume of water, and layered over 1 M sucrose. After 1 hr at 100,000 *g*, the purified membranes were collected from the interface of the water and sucrose layers and washed once with distilled water. The final suspension contained 2 mg protein[13] per ml.

Incubations used in the binding studies each contained 0·1 ml of the membrane preparation and 0·9 ml of 10 mM Tris buffer (pH 7), with bivalent cations, adenine nucleotides, radioactive amines (1–5 μCi epinephrine-7-^{14}C, metaraminol-7-^{3}H or serotonin-2-^{14}C) and drugs in varying concentrations. Blanks were treated identically but contained an additional 0·1 ml Tris instead of the membrane preparation. Incubations were for 15 min at 30° unless otherwise indicated. The membranes were sedimented at 26,000 *g* for 10 min and the supernatant was saved for the determination of the specific activity of the labeling medium. The pellet was washed and resedimented twice, and then dissolved

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in 0·5 of 1 M hydroxide of Hyamine in methanol by heating to 60° for 1 hr. The solution was acidified by adding 0·2 ml of 3 N HCl and then counted by liquid scintillation spectrometry. Binding was calculated from the following equation:

amount bound = dis/min in pellet/specific activity of labeling medium.

Additional washes of the membrane pellet prior to dissolution in hydroxide of Hyamine resulted in no further decreases in radioactivity, indicating that the radioactivity in the pellet after two washes reflected binding to the membranes.

TABLE 1. EQUILIBRATION OF PURIFIED ADRENAL VESICLE MEMBRANES WITH (0·01 mM) EPINEPHRINE IN THE PRESENCE AND ABSENCE OF ATP AND MAGNESIUM*

	Epinephrine bound (p moles)	
Time (min)	with ATP + Mg^{2+}	Without ATP + Mg^{2+}
0	28	48
5	39	52
15	46	73
30	45	67
45	44	72
60	47	76

* Each sample contained 0·2 mg protein. Samples were incubated at 30° for the time indicated. Data are from individual determinations.

Time, temperature and concentration dependence. The binding of epinephrine to purified vesicle membranes reached equilibrium within 15 min of incubation at 30° (Table 1). Approximately 70 pmoles were bound per sample, corresponding to 350 pmoles/mg of protein at 0·01 mM epinephrine. The substantial degree of binding at zero time (sample kept on ice) suggested that most of the binding was independent of both time and temperature; subsequent studies (Table 2(b)) confirmed that binding at 0° was two-thirds of that at 30°. The concentration dependence of the binding is represented in the form of a Scatchard plot[14] covering the concentration range from 1 μM to 10 mM epinephrine (Fig. 1). Two sites participate in the binding of epinephrine: one with a high affinity and low capacity for epinephrine, and the other with a low affinity and high capacity. From the slope and intercept of the high concentration data, the low affinity binding site has $K_{diss} = 6{\cdot}1$ mM, with a binding capacity of 78 nmoles/mg of protein. The parameters of the high affinity site are obtained by replotting the low concentration data (Fig. 2). For this site, $K_{diss} = 0{\cdot}121$ mM, with a capacity of 4·4 nmoles/mg; for comparison, human serum albumin can bind only about 3 pmoles catecholamines/mg.[15] By utilizing 0·01 mM epinephrine in the binding studies, the system is far below saturation of the high affinity site and the results reflect binding to that site only.

Effects of adenine nucleotides and bivalent cations. In the presence of ATP and magnesium, equilibrium was reached within 15 min of incubation at 30°, but at all times the binding was less than with epinephrine alone (Table 1). The decreased binding could be produced by the addition solely of ATP (Table 2(c)). Similar experiments were performed utilizing ATP and 2 mM EDTA (not shown) to rule out any interaction with endogenous magnesium; the results were identical to those obtained with ATP alone. Magnesium by itself had no effect on the binding (Table 2(d)), and the effect of ATP and magnesium was identical to the effect of ATP alone (Table 2(e)). The ATP effect was not temperature dependent (Table 2(f)), nor did increasing the concentration of ATP produce a further decrement in binding (Table 2(g)). Calcium did not alter the binding of epinephrine (Table 2(h)) and did not alter the effect of ATP (Table 2(i)). ADP was less effective than ATP in reducing the binding of epinephrine (Table 2 (j)) and AMP was totally ineffective (Table 2(k)).

The addition of ATP and magnesium reduced the binding of epinephrine at all concentrations of epinephrine (Fig. 1). The parameters describing the binding of epinephrine to the low affinity site

TABLE 2. BINDING OF AMINES TO PURIFIED ADRENAL VESICLE MEMBRANES*

Amine (0·01 mM)		Nucleotide	Bivalent cation	Drug (0·01 mM)	Temperature (°C)	Amount bound† (pmoles)	No. of determinations	Significance (*t*-test)
Epinephrine	a				30	73 ± 5	9	
	b				0	49 ± 1	3	P < 0·001 vs a
	c	0·5 mM ATP			30	42 ± 4	5	P < 0·001 vs a
	d		0·5 mM Mg^{2+}		30	73 ± 8	4	not significant (P > 0·05) vs a
	e	0·5 mM ATP	0·5 mM Mg^{2+}		30	48 ± 4	5	P < 0·005 vs a
	f	0·5 mM ATP	0·5 mM Mg^{2+}		0	31 ± 2	3	P < 0·001 vs b
	g	5 mM ATP	5 mM Mg^{2+}		30	46 ± 4	2	P < 0·005 vs a, not significant vs e
	h		0·5 mM Ca^{2+}		30	73 ± 6	2	not significant vs a
	i	0·5 mM ATP	0·5 mM Ca^{2+}		30	47 ± 2	2	P < 0·001 vs a
	j	0·5 mM ADP			30	58 ± 3	2	P < 0·05 vs a, P < 0·05 vs c
	k	0·5 mM AMP			30	72 ± 4	2	not significant vs a
	l			Reserpine	30	63 ± 9	4	not significant vs a
	m		0·5 mM Mg^{2+}	Reserpine	30	43 ± 5	3	P < 0·05 vs a
	n	0·5 mM ATP		Reserpine	30	45 ± 5	3	P < 0·05 vs a, not significant vs c
	o	0·5 mM ATP	0·5 mM Mg^{2+}	Reserpine	30	21 ± 2	4	P < 0·001 vs e
	p	0·5 mM ADP	0·5 mM Mg^{2+}	Reserpine	30	36 ± 4	3	P < 0·05 vs j
	q			*N*-ethylmaleimide	30	61 ± 8	2	not significant vs a
	r	0·5 mM ATP	0·5 mM Mg^{2+}	*N*-ethylmaleimide	30	14 ± 1	2	P < 0·001 vs e
Metaraminol	s				30	48 ± 2	2	P < 0·001 vs a
	t	0·5 mM ATP	0·5 mM Mg^{2+}		30	15 ± 3	2	P < 0·02 vs s, P < 0·002 vs e
	u	0·5 mM ATP	0·5 mM Mg^{2+}	Reserpine	30	18 ± 2	2	not significant vs t
Serotonin	v	0·5 mM ATP	0·5 mM Mg^{2+}		30	64 ± 2	2	P < 0·02 vs e

* Each sample contained 0·2 mg protein. Incubations lasted 15 min at the temperature indicated, except in samples containing *N*-ethylmaleimide, where membranes were preincubated with the drug for 10 min at 30° followed by 15 min of incubation with the other additions.

† Values expressed as mean ± S. E.

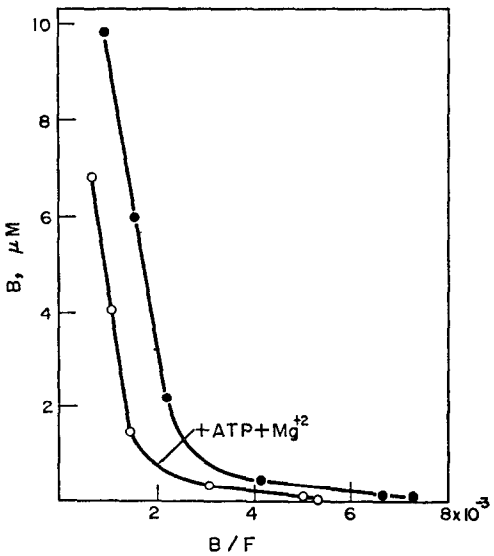

FIG. 1. Scatchard plot showing binding at 30° of epinephrine to purified bovine adrenal medullary vesicle membranes in the presence and absence of 0·5 mM ATP and magnesium. Protein concentration was 0·2 mg/ml. B = concentration of amine bound; F = concentration of free amine. Concentrations of binding sites are obtained from B-intercepts; K_{dlss} are obtained from slopes.

were altered in the presence of ATP and magnesium: K_{dlss} = 7·1 mM, capacity = 59 nmoles/mg of protein. For the high affinity site (Fig. 2), only the capacity was altered: K_{dlss} = 0·127 mM, capacity = 3·5 nmoles/mg of protein.

Effects of drugs. Reserpine alone produced little change in the binding of epinephrine (Table 2(l)), but the addition of reserpine and magnesium reduced the binding (Table 2(m)). Reserpine alone did not alter the effect of ATP in reducing binding (Table 2(n)), but when magnesium was added the

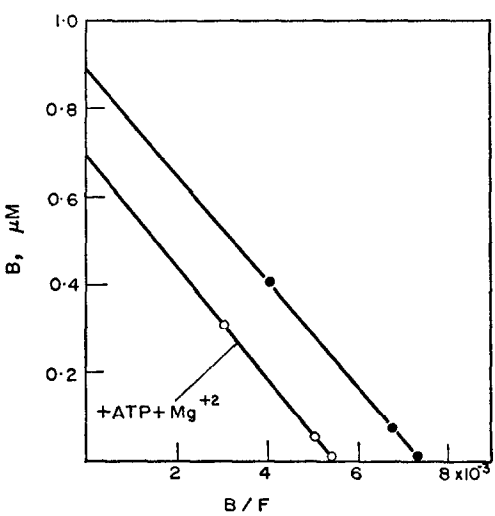

FIG. 2. Low concentration data of Scatchard plot from Fig .1.

binding of epinephrine was additively decreased (Table 2(o)). Reserpine and magnesium similarly had an additive effect with ADP to decrease the binding of epinephrine to the membranes (Table 2(p)). The effects of *N*-ethylmaleimide were similar to those of reserpine: *N*-ethylmaleimide alone had no effect (Table 2(g)) but in combination with ATP and magnesium produced a large decrement in binding (Table 2(r)).

Other amines. Metaraminol was bound to a substantially lower degree than was epinephrine (Table 2(s)), and the effect of ATP and magnesium was even more pronounced with metaraminol (reduction in binding = 2/3; Table 2(t)) than with epinephrine (reduction = 1/3). Reserpine had no additional effect on the reduction of metaraminol binding in the presence of ATP and magnesium (Table 2(u)). Serotonin was bound to a greater extent in the presence of ATP and magnesium than was epinephrine (Table 2(v)).

Discussion. The effects of adenine nucleotides on binding of amines to purified vesicle membranes did not correlate with the effects on uptake and storage in intact vesicle preparations[1,2] or in re-formed "empty" vesicles.[5,6] ATP, which in either of the latter two preparations stimulates uptake in combination with magnesium, decreased the binding of epinephrine to purified membranes. In intact vesicles, the ATP-dependent uptake is associated with an ATPase and with phosphorylation of membrane components; it is magnesium- and temperature-dependent and is blocked by *N*-ethylmaleimide.[3,4] With the present membrane preparation, the effect of ATP was magnesium- and temperature-independent and was not blocked by *N*-ethylmaleimide. These studies indicate that the binding reported here is not due to uptake by re-formed vesicles and indicates that the ATP–magnesium stimulated uptake of amines by storage vesicles is not due to an ATP–magnesium-mediated increase in the affinity for a transport molecule.

The Scatchard plot indicated that the effect of ATP on the binding of epinephrine was of the non-competitive type for the high affinity binding site (change in the number of binding sites without alterations in K_{diss}); this was confirmed by the observation that increasing the ATP concentration did not produce further decreases in binding. There were ATP-induced shifts both in K_{diss} and in the number of binding sites for the low affinity site, indicating a partially competitive inhibition of binding; the competitive component may reflect the formation *in vitro* of epinephrine–ATP complexes at high epinephrine concentrations.[16] Despite the fact that ATP decreased the binding of amines in absolute terms, it increased the specificity of the binding as determined by the ratio of metaraminol to epinephrine bound to the membranes. The ratio was about 2:3 in samples with only the amines present, but when ATP and magnesium were added, the ratio dropped to 1:3.

The studies reported here demonstrate a high affinity binding site in the membranes whose amine specificity and sensitivity to reserpine and *N*-ethylmaleimide behave in the manner predicted for an amine carrier responsible for uptake into the vesicles.[9] In the presence of ATP and magnesium, the rank order of binding of amines to the membranes was the same as that for the uptake of amines by intact vesicles,[9] viz. serotonin > epinephrine > metaraminol. The reserpine sensitivity of the binding of epinephrine and metaraminol correlated with the differential effect of reserpine on the uptake of the two amines: reserpine blocks epinephrine uptake into intact vesicles[1] and, in the presence of magnesium, blocks the binding to vesicle membranes. Metaraminol uptake into intact vesicles is markedly reserpine-insensitive[9,10,17,18] and reserpine had no effect on the binding of metaraminol in the presence of ATP and magnesium. *N*-ethylmaleimide, which also blocks stimulated uptake into intact vesicles,[1] behaved in a manner similar to reserpine in blocking the binding of epinephrine.

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Binding of phenothiazines to proteins—Measurement of binding based on the inhibition of the hemolytic activity of phenothiazines on sheep red blood cells

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THERE HAVE been several reports that phenothiazine tranquilizers are bound to blood proteins. Salzman and Brodie[1] found that chlorpromazine in dogs was highly bound to certain organs, such as brain, spleen and kidney, whereas a relatively low concentration of this drug was found in plasma, where most of the chlorpromazine was bound to plasma proteins. Mellinger *et al.*[2] reported that in serum of patients receiving oral forms of thioridazine, an accumulation of the drug occurred within 3–4 days, followed by a well maintained blood level. During our studies *in vitro* on protection of blood cells from lysis by hemolytic phenothiazines,[3] it was found that the extent of hemolysis of blood cells was remarkably reduced in the presence of serum. From these observations, it appeared that there was an affinity for binding between phenothiazines and serum proteins, which occurred both in a highly concentrated system, such as blood plasma, and in a less concentrated system, such as dilute aqueous solutions of serum and serum fractions.

An indication of the importance of the interaction of phenothiazines with blood proteins has been obtained by measuring the activity of normally occurring proteins in the blood after the administration of these drugs. It has been found, for example, that some of the phenothiazine derivatives inhibit the cholinesterase activities of normal human serum[4] and the activities of blood phosphatase and cholinesterase[5] in studies *in vivo*, and also inhibit the hemolytic activity of serum complement in studies *in vitro*.[6]

This report presents a simple and rapid method for testing the patterns of binding of phenothiazines by serum and serum fractions. The method is useful in determining which plasma proteins are involved in the interaction and possibly to relate these results to previous reports about the inactivation of enzymes[4,5] and complement[6] by structurally related phenothiazines.

Materials. Sera were prepared by processing blood freshly drawn from healthy human subjects and animals, and were stored at −55° until use. Serum proteins and fractions were obtained from Mann Research Laboratories. The solutions of serum fraction used were made by weight per cent in physiological saline unless otherwise mentioned. Only freshly prepared solutions were used for each experiment. Chlorpromazine and trifluoperazine were supplied by Smith, Kline & French Laboratories; promazine was supplied by Wyeth Laboratories. The chemicals were dissolved in physiological saline and adjusted to pH 7·3 to 7·4 with 1 M Na_2HPO_4. Since phenothiazines are not very soluble in saline and form colloid-like substances upon standing, 5 mM was the highest concentration used, and the solutions were introduced into the test system as quickly as possible. Sheep blood was collected in modified Alsever's solution and processed as described by Plescia *et al.*[7] A suspension of washed cells in physiological saline was standardized spectrophotometrically, to contain 2×10^9 cells/ml as a stock suspension. The concentration of washed sheep cells used in the experiments was usually 5×10^8 cells/ml, and it was used within 48 hr of storage at 4°.