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THE BINDING OF CATECHOLAMINES TO HUMAN SERUM PROTEINS

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Abstract—The binding of catecholamines at physiological concentrations to human serum and human serum proteins has been studied by equilibrium dialysis and gel filtration chromatography. Serum in equilibrium with a free catecholamine concentration of 100 pg/ml, binds 51 pg (−)-noradrenaline/ml and 28 pg (±)-adrenaline/ml. The binding is rapid and is only partially reversed by prolonged dialysis. Fractionation of the serum proteins by gel filtration chromatography revealed that 63 per cent of the bound (−)-noradrenaline is associated with albumin, 13 per cent with lipoprotein, principally very low density lipoprotein, 7 per cent with IgM and 5 per cent with α_2-macroglobulin. A further 12 per cent is associated with unidentified proteins. The major difference in the binding of (±)-adrenaline is its reduced affinity for IgM and albumin. The constants for the binding of catecholamines to purified IgM, very low density lipoprotein and albumin have been measured. Serum contains a non-dialysable inhibitor of the binding of catecholamines to albumin. The possible physiological consequences of binding are discussed.

Catecholamines have been reported to exist in a partially bound form in human plasma [1, 2] and in serum [3]. Some workers have reported that catecholamines bind with serum albumin in man [3, 4], in ox [5, 6] and in rabbit [7], whilst other workers have failed to find any such interaction either in man [8] or in dog [9]. There have also been reports that catecholamines bind to α-globulin in rabbit [7] and to β-globulin in rabbit [7] and man [8].

The present investigation was designed to study the interaction between catecholamines at physiological concentrations and human serum and serum proteins, by equilibrium dialysis and gel filtration chromatography.

MATERIALS AND METHODS

The binding of catecholamines by serum was studied by dialysing 1-ml aliquots of fresh serum obtained early in the morning from non-fasted human volunteers, in sacs of Visking dialysis tubing, inflated diameter 6·4 mm (Scientific Instrument Centre) against 500 volumes of NaCl 150 mM, phosphate buffer 2 mM, pH 7·4, containing either [7-^{3}H](−)-noradrenaline, methylene-[^{14}C](+)-noradrenaline or [7-^{3}H](±)-adrenaline, 100 ng/l (Radiochemical Centre, Amersham), for 18 hr at 4° with continuous stirring. The child sera used were those remaining after the routine biochemical analysis of samples obtained from children not receiving drugs, on their admission to hospital. Preliminary experiments revealed that additional ions as normally found in serum were not necessary for binding. Phosphate was found to provide adequate buffering without affecting binding. Ascorbic acid 10 mg/l, which had no effect upon binding, was added to retard the autooxidation of catecholamines. Preliminary experiments also revealed that 18 hr was adequate for complete equilibration. Aliquots of serum and of a similarly treated saline blank were taken for liquid scintillation counting. Binding was measured as the increased concentration of catecholamine in the serum compared to the concentration in the saline blank. A correction was made for dilution of the serum by measuring the protein concentration before and after dialysis, by the method of Lowry *et al.* [10]. In studies to investigate the characteristics of the binding of catecholamines to purified plasma proteins, protein concentrations of 3 mg/ml and catecholamine concentrations between 10^{-9} and 5×10^{-3} M were employed.

The binding of catecholamines to individual serum proteins was studied by applying 5 ml serum to a 3·8 × 90-cm column of Sephadex G-200 (Pharmacia Fine Chemicals) previously equilibrated with NaCl 150 mM, phosphate buffer 2 mM, pH 7·4, containing [7-^{3}H](−)-noradrenaline or [7-^{3}H](±)-adrenaline 100 ng/l. The proteins were eluted with the same buffer at a flow rate of 0·5 ml/min and at 4°, collecting 5-ml fractions of the eluate. Aliquots of the eluate were taken for liquid scintillation counting and protein determination as described previously. Bound catecholamine was apparent as peaks of increased radioactivity over the background activity. Pooled human serum (Biocult Laboratories) stored at −20°, was used in these and in later studies. This was necessary because of the large quantities of serum required for the preparation of the individual serum proteins. There was however no difference in the binding of catecholamines to stored or fresh serum as determined by dialysis or by gel filtration chromatography.

Purified or partially purified serum macroglobulins were applied to a 3 × 40-cm column of Sepharose 6B (Pharmacia Fine Chemicals) previously equilibrated with buffer containing [7-^{3}H](−)-noradrenaline 100 ng/l, and eluted with the same buffer at a flow rate of 0·5 ml/min and at 4°. Binding was determined as previously described.

Lipoproteins were prepared by flotation on KBr solutions of different density as described by Radding and Steinberg [11], using a Beckman Model L-2 ultracentrifuge. The fractions prepared were chylomicrons, very low density lipoprotein (VLDL), density < 1·006 g/ml, low density lipoprotein (LDL), density 1·006–1·063 g/ml, and high density lipoprotein (HDL),

density 1·063–1·21 g/ml. Protein fractions were concentrated by dialysis against the high molecular weight synthetic polymer Ficoll (Parmacia) at 4°. Individual serum proteins were identified by immunoelectrophoresis on 1% agarose by the micro method of Sargent [12]. Goat antisera to whole human serum and human albumin, IgG, IgM, IgA, α_2-macroglobulin, haptoglobulin, ceruloplasmin, low density lipoprotein and high density lipoprotein were obtained from Miles Laboratories. Prior to immunoelectrophoresis column eluates were concentrated to give a volume similar to that of the serum from which they were derived. Immunoglobulins were assayed quantitatively by radial immunodiffusion using a commercially available test combination (ICL Scientific). Total lipid was measured by the method of Zöllner and Kirsch [13]. Purified very low density lipoprotein and IgM were prepared by a combination of preparative flotation and gel filtration chromatography on Sephadex G-200 and Sepharose 6B. Crystalline human albumin was obtained from Miles Laboratories and fatty acids removed as described by Russell and Doty [2].

RESULTS

Binding to whole serum. Sera obtained from adult males, in equilibrium with a free catecholamine concentration of 100 pg/ml bound 50·8 ± 2·7 pg (−)-noradrenaline/ml (n = 12), 9·6 ± 1·9 pg (+)-noradrenaline/ml (n = 6) and 27·6 ± 1·2 pg (±)-adrenaline/ml (n = 12). Sera obtained from adult females bound significantly less (−)-noradrenaline 42·6 ± 1·8 pg/ml (n = 12, P < 0·02) and (±)-adrenaline 23·2 ± 1·4 pg/ml (n = 12, P < 0·05). The binding of (−)-noradrenaline to sera from children prior to puberty was not significantly different to the adult values, male 47·5 ± 1·8 (n = 11, P > 0·20) and female 41·2 ± 1·8 (n = 10, P > 0·20), although the difference between the sexes was still apparent (P < 0·05).

Analysis of the radioactivity in the serum after dialysis by two dimensional thin layer chromatography [14], failed to reveal any autooxidation or metabolic degradation of the catecholamines. The binding of catecholamines to serum was particularly strong. Dialysis of adult male sera previously equilibrated with catecholamine against three changes of buffer for 18 hr each only reduced the binding of (−)-noradrenaline by 13·5 ± 2·0 per cent (n = 4, P > 0·10) and (±)-adrenaline by 30·0 ± 3·3 per cent (n = 4, P < 0·01). It was not possible to measure the rate at which binding occurred as equilibration across the dialysis sac took several hours. However, preliminary studies with a centrifugal ultra-filter (Gelman Hawksley) revealed that equilibrium was attained within at least 10 min of mixing, the shortest time interval studied.

Binding to serum proteins. The binding of catecholamines to serum proteins fractionated by gel filtration chromatography on Sephadex G-200 is shown in Fig. 1. A total of 242 pg (−)-noradrenaline was bound by 5 ml serum; 25 per cent was associated with the first protein peak eluted, containing lipoproteins (VLDL and LDL) [15], IgM and α_2-macroglobulin [16, 17, 18], 12 per cent with the middle protein peaks and 63 per cent with the final protein peak, which contains mostly albumin and a little transferrin and α-globulin [16]. A total of 151 pg (±)-adrenaline was bound and the main difference in binding lay with the final protein peak where only half as much (±)-adrenaline as (−)-noradrenaline was bound.

The serum proteins from the first peak were prepared in a separate experiment using buffer containing no catecholamine, and the lipoproteins separated from the other macroglobulins, by preparative flotation. The binding of (−)-noradrenaline to the different protein fractions thus obtained is shown in Figs. 2 and 3. The lipoproteins prepared from 10 ml serum bound 330 pg (−)-noradrenaline, of which 75 per cent was bound to VLDL (Fig. 2). The remaining macroglobulins bound 280 pg (−)-noradrenaline of which 57 per cent was associated with the first protein peak eluted (Fig. 3). This peak contained no detectable lipid and immunoelectrophoretic analysis revealed a single component reacting with antiserum to human IgM. Quantitative analysis by radial immunodiffusion showed that the major constituent of this peak was IgM with 6% IgG, probably present as polymerized IgG.

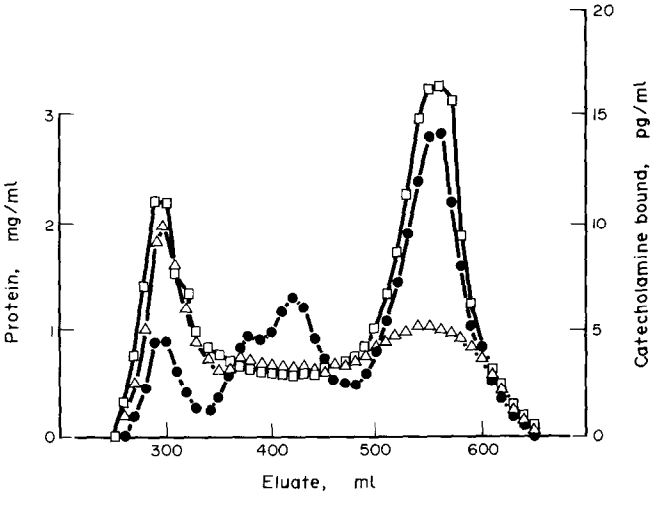

Fig. 1. Binding of (−)-noradrenaline (□) and (±)-adrenaline (△) to human serum proteins (●). Pooled human serum 5 ml, was applied to a 3·8 × 90-cm column of Sephadex G-200 equilibrated with buffer containing catecholamine 100 pg/ml and eluted with the same buffer at a flow rate of 0·5 ml/min and at 4°.

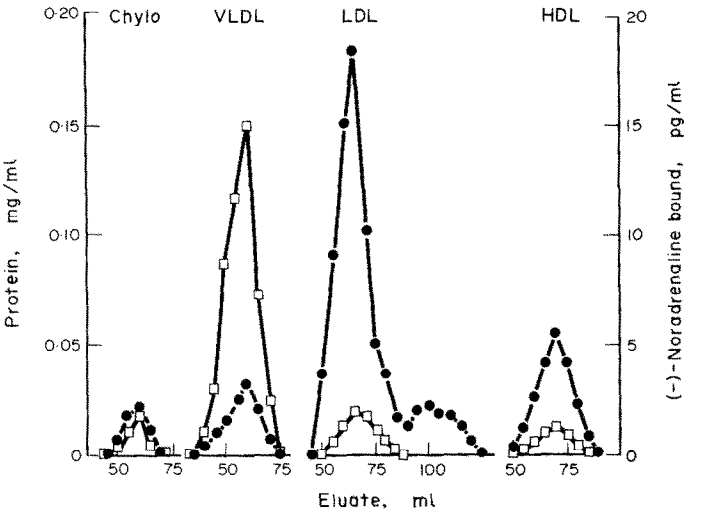

Fig. 2. Binding of (−)-noradrenaline (□) to high molecular weight lipoproteins (●). Chylo = chylomicrons, VLDL = very low density lipoprotein, LDL = low density lipoprotein and HDL = high density lipoprotein, prepared from 10 ml pooled human serum. Lipoprotein fractions were applied individually to a 3 × 40-cm column of Sepharose 6B equilibrated with buffer containing (−)-noradrenaline 100 pg/ml and eluted with the same buffer at a flow rate of 0·5 ml/min, and at 4°.

Immunoglobulin-G has a low molecular weight but is particularly liable to form high molecular weight polymers under experimental conditions [19]. Further fractionation failed to remove the IgG contaminant and in later studies it was ignored. Immunoglobulin-G itself did not bind (−)-noradrenaline and it was considered unlikely that the high molecular weight form would account for much binding.

The major protein constituent of the second peak was found by immunoelectrophoresis to be α_2-macroglobulin. Preliminary purification of the α_2-macroglobulin by ammonium sulphate fractionation [20] showed that this was the major protein responsible for binding in the second peak.

Fractionation of the proteins obtained from the last peak after Sephadex G-200 chromatography of whole serum by further chromatography on Sephadex G-100 revealed that binding occurred exclusively to albumin. The albumin prepared from 10 ml serum bound 302 pg (−)-noradrenaline and 132 pg (±)-adrenaline. Danon and Sapira [3] have suggested that catecholamines might not be bound to albumin itself but to a minor contaminant of the albumin fraction. Attempts to separate the (−)-noradrenaline binding capacity from the albumin by preparative electrophoresis on Sephadex G-200 [21] and by ion exchange chromatography of DEAE-Sephadex, were unsuccessful.

The binding of (−)-noradrenaline and (±)-adrenaline to the three major proteins in serum responsible for the binding of catecholamines, namely IgM, VLDL and albumin, is shown in Fig. 4 plotted according to the method of Scatchard [22], where r = number of moles of catecholamine bound per mole of protein, and D_f = free concentration of catecholamine. Curvature of the plot indicates more than one set of binding sites and obeys the general equation [23]

$$r = \sum_{i=1}^{i} \frac{n_i K_i D_f}{1 + K_i D_f}$$

where n_i is the total number of sites of type i with an intrinsic association constant of K_i. The equation was fitted to a rational function and fitted to the data in Fig. 4 using a rational approximation to a discrete function in the least squares sense on an IBM 370/155 computer. Assuming two mutually independent sites this approach yields the constants shown in Table 1. The molecular weight of IgM was taken as 900,000 [24] and albumin as 69,000 [25]. VLDL is a heterogeneous group of proteins with a molecular weight

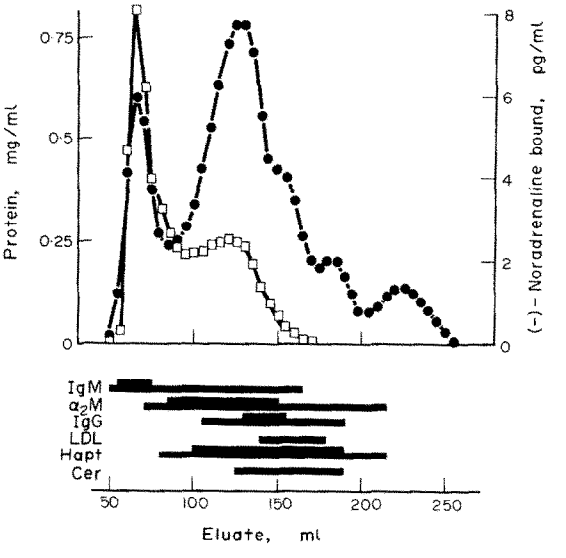

Fig. 3. Binding of (−)-noradrenaline (□) to macroglobulins, other than lipoproteins, prepared from 10 ml pooled human serum (●) applied to a 3 × 40-cm column of Sepharose 6B equilibrated with buffer containing (−)-noradrenaline 100 pg/ml and eluted with the same buffer at a flow rate of 0·5 ml/min, and at 4°. Proteins identified by immunoelectrophoresis were IgM, α_2-macroglobulin (α_2M), IgG, low density lipoprotein (LDL), haptoglobin (Hapt) and ceruloplasmin (Cer). The thickness of the line gives an indication of the amount of each individual protein in the different fractions of eluate, although it gives no indication of the relative amounts of different proteins present.

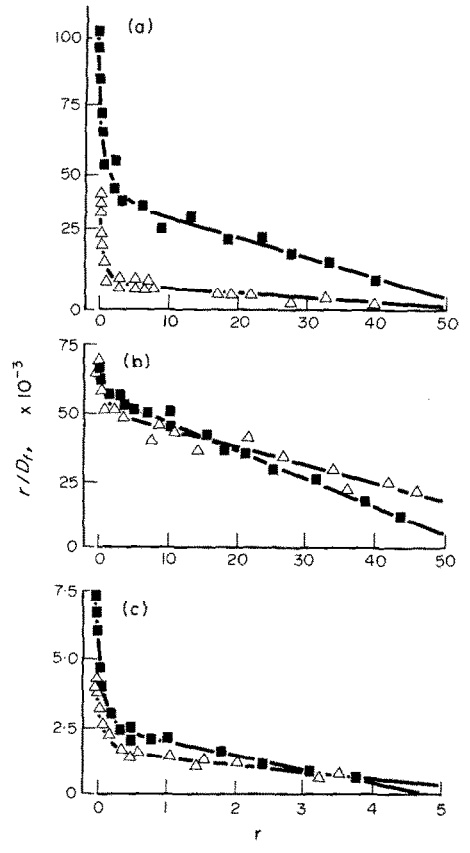

Fig. 4. Binding of (−)-noradrenaline (■) and (±)-adrenaline (△) to purified IgM (a), very low density lipoprotein (b) and albumin (c), plotted according to the method of Scatchard [22]. The continuous line represents a computer plot of the data as described in the text. D_f = free concentration of catecholamine, M; r = number of moles of catecholamine bound per mole of IgM and albumin, or per 10^6 g very low density lipoprotein.

ranging from 3×10^6 to 128×10^6 [26], and in this case r represents moles of catecholamine bound per 1,000,000 g lipoprotein. The lipid content of the VLDL varied between 85 and 91 per cent depending upon the preparation.

Table 1. Constants for the binding of catecholamines to human IgM, very low density lipoprotein (VLDL) and albumin

	Site 1		Site 2	
	K_1	n_1	K_2	n_2
		(−)-noradrenaline		
IgM	99,961	0·730	534	57·6
VLDL	92,073	0·161	949	54·1
Albumin	104,582	0·048	483	4·5
		(±)-adrenaline		
IgM	60,627	0·571	149	56·1
VLDL	91,254	0·210	567	82·5
Albumin	62,240	0·045	275	5·5

The constants are derived from the data shown in Fig. 4 as described in the text. K = intrinsic association constant, l/mole for IgM and albumin, $l/10^6$ g lipoprotein for VLDL; n = number of moles of catecholamine bound per mole of IgM or albumin, or per 10^6 g VLDL.

It can be calculated from the constants shown in Table 1 that at the protein concentrations found in adult male serum [27] and with a free catecholamine concentration of 100 pg/ml, IgM will bind 10 pg (−)-noradrenaline/ml, largely at the high affinity, low capacity site; VLDL will bind 10 pg (−)-noradrenaline/ml, largely at the low affinity, high capacity site; and albumin will bind 416 pg (−)-noradrenaline/ml largely at the high affinity, low capacity site. The binding of (±)-adrenaline calculated in the same manner is IgM 4 pg/ml, VLDL 10 pg/ml and albumin 250 pg/ml. It thus appears that serum contains an inhibitor of binding, which affects primarily the binding of catecholamines to albumin. This inhibitor is not removed by dialysis.

DISCUSSION

There exist conflicting reports in the literature as to the extent of the binding of catecholamines to human plasma [1–3] and the nature of the proteins with which the catecholamines interact [3, 4, 8]. The differences may arise because of the study of binding under non-equilibrium conditions [4, 8] and the use of catecholamine concentrations in excess of those found under physiological conditions [1, 3].

Physiological concentrations of catecholamines in the plasma in man are around 200 pg noradrenaline/ml and 50 pg adrenaline/ml [28, 29]. Equilibrium dialysis at a free catecholamine concentration of 100 pg/ml revealed that sera from adult males bound 51 pg (−)-noradrenaline/ml and 28 pg (±)-adrenaline/ml. The binding studies were carried out at 4° in order to limit the autooxidation of catecholamines which occurs at pH 7·4, even in the presence of ascorbate. It has recently been shown that the binding of adrenaline to human plasma is independent of temperature, between 3° and 37° [2].

Of the noradrenaline bound 63 per cent was held in association with albumin, 13 per cent with lipoprotein, principally VLDL, 7 per cent with IgM and 5 per cent with α_2-macroglobulin. A further 12 per cent was associated with unidentified proteins, which were possibly lipoproteins. It is possible to produce antibodies to catecholamines acting as haptens under experimental conditions [30] and it is conceivable that catecholamines, or their derivatives, might act as haptens under physiological conditions and be bound at the antigen combining sites on IgM which they raise. Immunoglobulin-M has ten potential antigen combining sites [31], although there appears to be less than one high affinity catecholamine binding site per mole of IgM. This may reflect heterogeneity amongst the IgM molecules or heterogeneity amongst the antigen combining sites. The similarity of the binding to IgM with the binding to other serum proteins, with both high and low affinity binding sites, might however suggest that catecholamines bind at sites distinct from the antigen combining sites.

Radioactivity labelled (−)-adrenaline was not available at the time this work was carried out. However, the binding of the non-physiological isomer (+)-noradrenaline to serum was much less than that of (−)-noradrenaline. A similar stereoselectivity has been found in the binding of noradrenaline to elastin [32]. A racemic mixture of (−)-noradrenaline and (+)-noradrenaline would have a binding approaching that of

(±)-adrenaline. It is therefore possible that (−)-adrenaline binds to a greater extent than (+)-adrenaline and to a similar extent as (−)-noradrenaline. There have been reports that the binding of (±)-noradrenaline to human plasma [2] and human serum albumin [3] is similar to that of (±)-adrenaline.

Danon and Sapira [3] have suggested that serum might contain an inhibitor of catecholamine binding. In the present study it was found that the binding of catecholamines to crystalline serum albumin was ten times greater than the binding to an equivalent amount of albumin in serum. The binding to purified IgM and VLDL did not appear to be appreciably greater than in serum, although these proteins were not as highly purified as the albumin. Endogenous catecholamines are unlikely to produce such a large inhibition. Fatty acids which bind avidly to serum albumin [33] are known to inhibit the binding of tryptophan [34] and treating plasma with activated charcoal, which will remove fatty acids, has been reported to increase the binding of (±)-adrenaline almost 50 times [2], although in this particular study the binding of catecholamines to untreated plasma was found to be very low.

The physiological implications of the binding of catecholamines to serum proteins remain obscure. Binding to serum proteins may however account for the unusual stability of catecholamines in blood. Catecholamines administered intravenously in relatively small doses to mice, exhibit a biphasic removal [35, 36]. In the first 5 min after administration there is a rapid removal of approximately 70 per cent of the dose, whilst the remaining 30 per cent is more slowly removed over several hours. Relatively more adrenaline than noradrenaline disappears during the first rapid phase of removal [36]. These findings may possibly be explained by the rapid and avid binding of catecholamines by serum proteins. The greater binding of noradrenaline may also explain why the relative levels of noradrenaline in serum are higher than adrenaline, rather than as a result of a greater release of noradrenaline into the blood stream [36]. Besides protecting catecholamines from metabolic degradation, binding may also limit the rate of autooxidation which is quite rapid in protein free solutions at physiological pH and temperature. Catecholamines stored in plasma at 37° are known to be stable for several hours [37].

The binding to serum albumin has been reported to reduce the biological potency of catecholamines [38] and the binding to other serum proteins may be expected to have similar effects. The fact that binding occurs to the high molecular weight proteins IgM and VLDL may suggest a transport function for these proteins. Bound catecholamines will largely be confined to the vascular compartment. Drugs or other conditions which displace catecholamines from their binding sites have been found to increase the biological potency of catecholamines *in vitro* [38], and the same may apply *in vivo*.

Changes in the levels of the various serum proteins might be expected to affect the extent of the binding of catecholamines. The lower serum levels of VLDL in females [26] may account for the lower catecholamine binding capacity. In the present study one child with Crohns disease was found to have an almost 20-fold increased catecholamine binding capacity. The levels of serum albumin 13·3 g/l were low, whilst the levels of α_2-globulin, 10·1 g/l, were elevated compared to normal values. The levels of the other serum proteins were relatively unaltered: α_1-globulins 3·3 g/l, β-globulins 7·7 g/l and γ-globulins 18·5 g/l. In a detailed study of patients with Crohns disease Weeke and Jarnum [39] found decreased levels of serum albumin and α_2-macroglobulin whilst lipoproteins and immunoglobulins were unaltered. It is therefore difficult to account for the increased binding capacity in terms of the proteins known to be responsible for the binding of catecholamines in normal serum. It is possible however that the serum might contain other constituents capable of binding catecholamines or possess less of the inhibitor of binding. The consequences of the changes in catecholamine binding capacity, if any, are however unknown.

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