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INHIBITION OF CATECHOLAMINE UPTAKE AND RETENTION IN SYNAPTOSOMAL PREPARATIONS BY TETRAHYDROISOQUINOLINE AND TETRAHYDROPROTOBERBERINE ALKALOIDS*

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Abstract—The effects of racemic mixtures of representative tetrahydroisoquinoline and tetrahydroprotoberberine alkaloids on the mechanisms of catecholamine uptake and retention were studied in synaptosomal preparations from whole rat brain. The synaptosomes were incubated with (^{14}C)-*d,l*-norepinephrine or (^{14}C)-dopamine in the presence of various concentrations of salsolinol (SAL), tetrahydropapaveroline (THP), 2,3,10,11-tetrahydroxyberbine (THB), or 2,3,10,11-tetramethoxyberbine (TMB). Levels of radioactivity in synaptosomes preloaded with labeled norepinephrine were significantly diminished by the addition of 10^{-4} M THP, THB or SAL to approximately 42·7 per cent ($P < 0{\cdot}001$), 85·8 per cent ($P < 0{\cdot}02$) and 85·9 per cent ($P < 0{\cdot}01$), respectively, of control preparations. THP, 10^{-5} M, also significantly decreased synaptosomal retention of the labeled neuroamine. (^{14}C)-dopamine was used in an analysis of alkaloid effects on catecholamine uptake kinetics. K_i values obtained were: $0{\cdot}7 \times 10^{-5}$ M (THP); $3{\cdot}5 \times 10^{-5}$ M (THB); $1{\cdot}25 \times 10^{-4}$ M (SAL); and $2{\cdot}2 \times 10^{-4}$ M (TMB). These results have been interpreted to suggest that the affinity of these amine-derived alkaloids for the catecholaminergic uptake mechanisms, although not marked when compared to that of dopamine or norepinephrine, may be sufficient under conditions of highly localized formation and accumulation to have important physiological sequelae.

There is increasing evidence that, under certain circumstances, endogenous catecholamines of mammalian systems and aldehydic substances can condense to form tetrahydroisoquinoline (THIQ) alkaloids. Since the pioneering work of Falck *et al.* [1], it has been recognized that, in isolated mammalian tissues, *in situ* catecholamines react with formaldehyde vapor to form the THIQ intermediates requisite to their fluorescent visualization. More recently, attention has been directed to the formation *in vivo* of amine–aldehyde condensation products and to the pharmacological implications of this chemical event.

The formation of simple THIQ alkaloids, condensation products of catecholamines and formaldehyde or acetaldehyde, has been demonstrated in rat adrenal glands during methanol intoxication [2, 3], and in acetaldehyde-perfused bovine adrenal glands [4, 5]. Salsolinol (SAL; 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline), the cyclization product of dopamine (DA) and acetaldehyde, has been demonstrated in rat liver and brain homogenates during metabolism of ethanol [6]. Additionally, SAL has been identified [7] in the DA-rich areas of brain after acute ethanol intoxication in pyrogallol-treated rats.

A more complex THIQ alkaloid, tetrahydropapaveroline [THP; 1,2,3,4-tetrahydro-6,7-dihydroxy-1-(3′,4′-dihydroxybenzyl)-isoquinoline; norlaudanosoline], the condensation product of DA and its aldehyde metabolite, 3,4-dihydroxyphenylacetaldehyde, has been detected in preparations of guinea pig liver after incubation with DA [8, 9]. Formation *in vitro* of THP from DA in rat brain and liver homogenates [10] is augmented by the addition of alcohol or acetaldehyde [11]. The small quantity of THP found in the brain of rats treated chronically with 3,4-dihydroxyphenylalanine (dopa) or DA was increased in animals given a combination of dopa and ethanol [12]. Identification of SAL and THP in the urine of Parkinsonian patients being treated with L-dopa provided evidence of formation *in vivo* of THIQ alkaloids in humans [13].

In addition to these reports of the formation *in vitro* and *in vivo* of both simple and benzyl-THIQs, it has now been demonstrated for the first time that THP is utilized by mammalian systems in the formation of more complex alkaloids [14]. Rats injected with THP are able to insert a 1-carbon unit into this benzyl-THIQ molecule, thus forming the tetracyclic structure of the tetrahydroprotoberberine (THPB) class of alkaloids (Fig. 1). The major metabolite excreted in rat urine after THP administration was identified as 2,3,10,11-tetrahydroxyberbine (THB). There were smaller amounts of 2,3,9,10-tetrahydroxyberbine as well as two *O*-methylated THPB alkaloids. Moreover, THPB alkaloids were identified in the urine of two Parkinsonian patients receiving 4–5 g L-dopa daily.

Certain members of the THPB and THIQ classes of alkaloids are pharmacologically active compounds. Studies of a homologous series of alkaloids related to SAL demonstrated both vasopressor and depressor properties, various smooth muscle effects, and central nervous system depression or stimulation [15–21].

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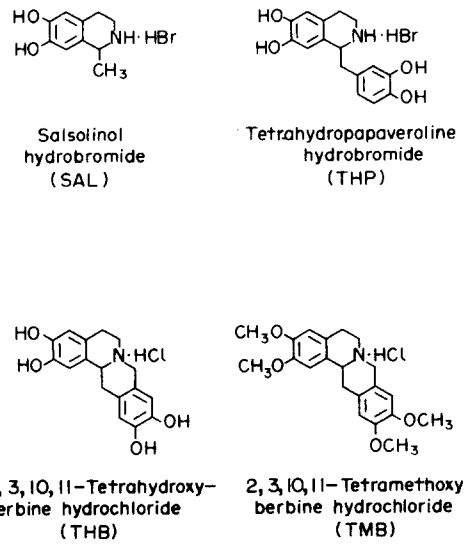

Fig. 1. Structures of four tetrahydroisoquinoline and tetrahydroprotoberberine alkaloids used in the study.

The affinity of two THIQs, viz. SAL and 6,7-dihydroxy-THIQ, for catecholaminergic mechanisms is evident in their accumulation in the amine-binding granules of acetaldehyde-perfused adrenal glands [22], in synaptosomal preparations of rat brain [23], and in the nerve terminals of adrenergically innervated tissues [24–27].

Since the pharmacological activity of the benzyl-THIQ, THP, was first described by Laidlaw [28] in 1910, a number of studies [29–35] attest its β-sympathomimetic effects. Its pharmacological profile is characterized by lipolysis, respiratory stimulation and a hypotensive effect that occurs concomitantly with increased myocardial activity and is presumed due to peripheral vasodilation [30, 34]. The β-sympathomimetic effects can be antagonized by drugs that block β-adrenergic receptors [29, 32, 33] but not by prior reserpinization of experimental animals [31].

Sedative effects have been described in pharmacologic studies of 2,3,10,11-tetramethoxyberbine (TMB; xylopinine) [36–39] and its isomer 2,3,9,10-tetramethoxyberbine (tetrahydropalmatine) which produces additional analgesic and tranquilizing effects [40, 41]. Tranquilizing properties have also been attributed to several other substituted THPBs [42–45].

In the present study, representative compounds of the THIQ and THPB alkaloids (Fig. 1) were examined for their effects on the catecholaminergic mechanisms of uptake, storage and release in the central nervous system. Synaptosomal preparations (sheared-off nerve endings) of rat brain were used, their suitability for studies *in vitro* having been demonstrated in numerous reports (see reviews, Refs. 46 and 47).

MATERIALS AND METHODS

Synaptosomal preparations of rat brain. Male Sprague–Dawley rats (TEX/SDD: 230–300 g) were decapitated; the brains were quickly removed, rinsed with cold isotonic saline and then kept chilled during the procedures preceding incubation. The choroid plexus and the larger blood vessels were removed before the brain tissue was weighed and homogenized in 9 volumes of ice-cold 0·32 M sucrose. A glass homogenizer (Kontes 88600) having a clearance of 0·25 mm between the mortar and the Teflon pestle was used to prepare the homogenate. The homogenate was centrifuged at 1000 *g* for 10 min at 4°; the residue was discarded and aliquots of the supernatant (crude synaptosomal preparation) were used in the various experiments.

Incubations were carried out in 25-ml Erlenmeyer flasks containing; 7·5 ml Krebs bicarbonate buffer (NaCl, 118·5 mM; KCl, 4·75 mM; $CaCl_2$, 1·30 mM; KH_2PO_4, 1·20 mM; $MgSO_4$, 1·20 mM; $NaHCO_3$, 26·5 mM), pH 7·2, modified by the addition of pargyline hydrochloride (0·125 mM), glucose (11 mM), disodium ethylenediaminetetraacetate (0·134 mM), and ascorbic acid (1·14 mM) [48]. Alkaloid solution, or water, the alkaloid vehicle, was added in 0·5-ml volumes.

Solutions of radiolabeled dopamine (dopamine-2-^{14}C hydrochloride, 55 mCi/m-mole, Amersham/Searle Corp.) or norepinephrine (NE; *d,l*-norepinephrine-carbinol-^{14}C bitartrate, 54 mCi/m-mole, Amersham/Searle Corp.), prepared in the modified buffer, were added to the flasks in 1-ml volumes at the beginning of incubation at 37° in a metabolic shaker. After 5 min, a 1-ml aliquot of the crude synaptosomal preparation was added to each flask and incubations were continued. The total volume of the incubation mixture was 10 ml. Aliquots of the preparation used to determine synaptosomal pellet weights were included in the incubation step of each experiment. The mean value and standard error was 20·6 $\pm$ 2·3 mg (N = 29).

At the end of the incubation period, the flasks were removed and quickly chilled in an ice bath. The contents were transferred to chilled tubes and centrifuged at 27,000 *g* for 30 min at 4°. The supernatant from each tube was then discarded.

The precipitated particulate (synaptosomal pellet) was rinsed twice with 4 ml chilled 0·9% saline and quickly frozen (dry ice/isopropanol bath) to facilitate transfer to the scintillation vial. A solubilizing agent, 0·5 ml NCS (Amersham/Searle Corp.), was added to the synaptosomal pellets. The vials were warmed gently and the brain tissue was completely solubilized before 15 ml toluene phosphor was added. The scintillation fluid consisted of a 2:1 mixture of toluene and Triton X-100 containing 4 g of 2,5-diphenyloxazole and 0·2 g of 1,4,-bis-2-(5-phenyloxazolyl) benzene/liter. Prior to counting, the samples were kept for 48 hr in the dark at 5° to avoid counting errors resulting from chemiluminescence. Radioactivity in the preparations was then measured in a Packard Tri-Carb liquid scintillation spectrometer, model 3390.

Compounds used. Salsolinol HBr was purchased from Aldrich Chemical Co. and tetrahydropapaveroline [1,2,3,4-tetrahydro-6,7-dihydroxy-1-(3′,4′-dihydroxybenzyl)-isoquinoline] HBr from Burroughs-Wellcome & Co. The protoberberine compounds used in the study, 2,3,10,11-tetrahydroxy- and 2,3,10,11-tetramethoxyberbine, were prepared in our laboratory by Dr. Kenneth McMurtrey.

Statistical procedures. The techniques described by Goldstein [49] were used in the statistical analyses of the data.

RESULTS

Effects of alkaloids added to synaptosomal preparations preloaded with (^{14}C)-d,l-norepinephrine. Figure 2 illustrates the synaptosomal accumulation of (^{14}C)-*d,l*-NE from the incubation mixture (10^{-8} M) as a function of time. It also demonstrates the effects of alkaloids added to synaptosomes preloaded with radioactive neuroamine. Labeled NE continued to accumulate in the synaptosomal pellets for 30 min, radioactivity remaining at approximately the same level for at least 10 min longer.

At a final concentration of 10^{-4} M, every alkaloid except TMB decreased pellet radioactivity to levels lower than control levels were at the time of alkaloid addition—i.e. at the 10-min point on the curve. At the 10^{-4} M concentration, THP, THB and SAL decreased pellet content of labeled NE to approximately 42·7 per cent ($P < 0.001$), 85·8 per cent ($P < 0.02$) and 85·9 per cent ($P < 0.01$) of controls respectively. At 10^{-5} M concentration of alkaloids, only THP-treated preparations were significantly different from controls ($P < 0.001$). The marked effect of THP (10^{-4} M) on pellet radioactivity was also significantly greater than that of SAL ($P < 0.001$) or the equimolar concentration of THB ($P < 0.001$).

Addition of an equal vol. of water, the alkaloid vehicle, did not significantly alter the radioactivity level in synaptosomes preloaded with labeled NE.

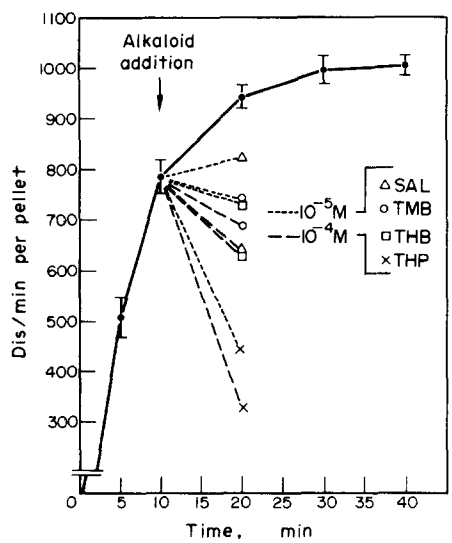

Fig. 2. Accumulation of (^{14}C)-*d,l*-norepinephrine in rat brain synaptosomal preparations as a function of time and effect of alkaloid addition on existing levels of radioactive amine. Unbroken line represents radioactive NE accumulation by synaptosomal pellet during incubation with 10^{-8} M amine. After 10 min of incubation, alkaloids were added to other samples to obtain a final concentration in incubation mixture of 10^{-5} or 10^{-4} M. Each point on the graph represents a minimum of three separate experiments run in duplicate. Standard errors ranging from 20 to 72 dis./min/pellet of mean values of alkaloid-treated preparations were omitted for sake of clarity. The two-tail *t*-test was used to compare radioactivity in synaptosomal pellets after 10-min of exposure to the alkaloid with radioactivity present in controls at time of alkaloid addition, i.e. the 10-min point on the time curve. Statistically significant differences obtained for 10^{-4} M alkaloid concentrations are: THP ($P < 0.001$); SAL ($P < 0.01$); and THB ($P < 0.02$); for 10^{-5} M THP ($P < 0.001$).

Kinetics of (^{14}C)-dopamine uptake by synaptosomal preparations. Several concentrations of labeled DA, ranging from 10^{-8} M to 10^{-7} M, were incubated for 5 min at 37° with aliquots of the synaptosomal preparations. The data from these experiments were converted to double-reciprocal values for the Lineweaver–Burk [50] plot (Fig. 3). The K_m obtained graphically was 0.96×10^{-7} M, and the V_{max} was 4·9 nmoles/g of pellet/5 min.

Alkaloid effects on (^{14}C)-dopamine uptake. Alkaloid inhibition of labeled DA uptake by rat brain synaptosomal preparations was assessed by the method of Dixon [51]. Synaptosomes were incubated for 5 min with either 10^{-8} M or 10^{-7} M labeled DA and various concentrations of each alkaloid. For each concentration of the labeled compound, a regression line was calculated from the reciprocals of the DA content of synaptosomes after incubation with the various alkaloid concentrations. The intersection of the regression lines from the two sets of data gives the K_i value for the alkaloid inhibitor directly. In competitive inhibition, the lines intersect at a distance equal to V_{max} above the abscissa. Thus, SAL, THB and TMB inhibit DA uptake competitively, their respective K_i values being 1.25×10^{-4} M, 3.5×10^{-5} M and 2.2×10^{-4} M.

THP interferes with DA uptake in a noncompetitive fashion (Fig. 4); the K_i value is 0.7×10^{-5} M. Reversibility of this inhibitory effect is indicated by the results of an experiment modeled after the method of Ackermann and Potter [52] for distinguishing between reversible and irreversible enzyme inhibitors. Enzyme activity plotted against enzyme concentration produces a straight line through the origin of the graph. A reversible inhibitor produces a straight line, which also passes through the origin but with a lesser slope. In contrast, an irreversible inhibitor produces a line across the abscissa to the right of the origin, which has the same slope as the control data line.

In the present study, the synaptosomal preparation was diluted to obtain 25, 50 and 75% concentrations of the 1000 *g* supernatant. Pellet content of radioactive DA was linear with synaptosomal concentration. A plot of the data (Fig. 5) produced a line passing through the origin of the graph. Similarly, uptake of

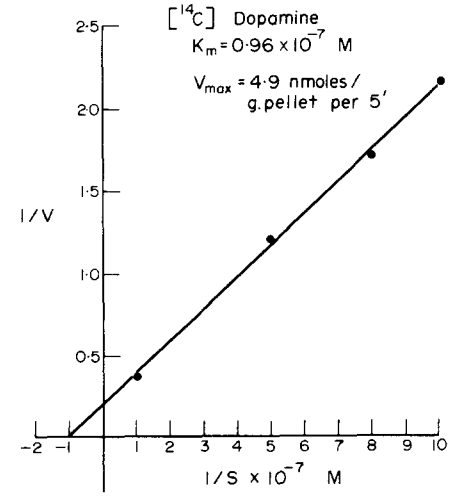

Fig. 3. Analysis of kinetics of (^{14}C)-dopamine uptake by rat brain synaptosomes.

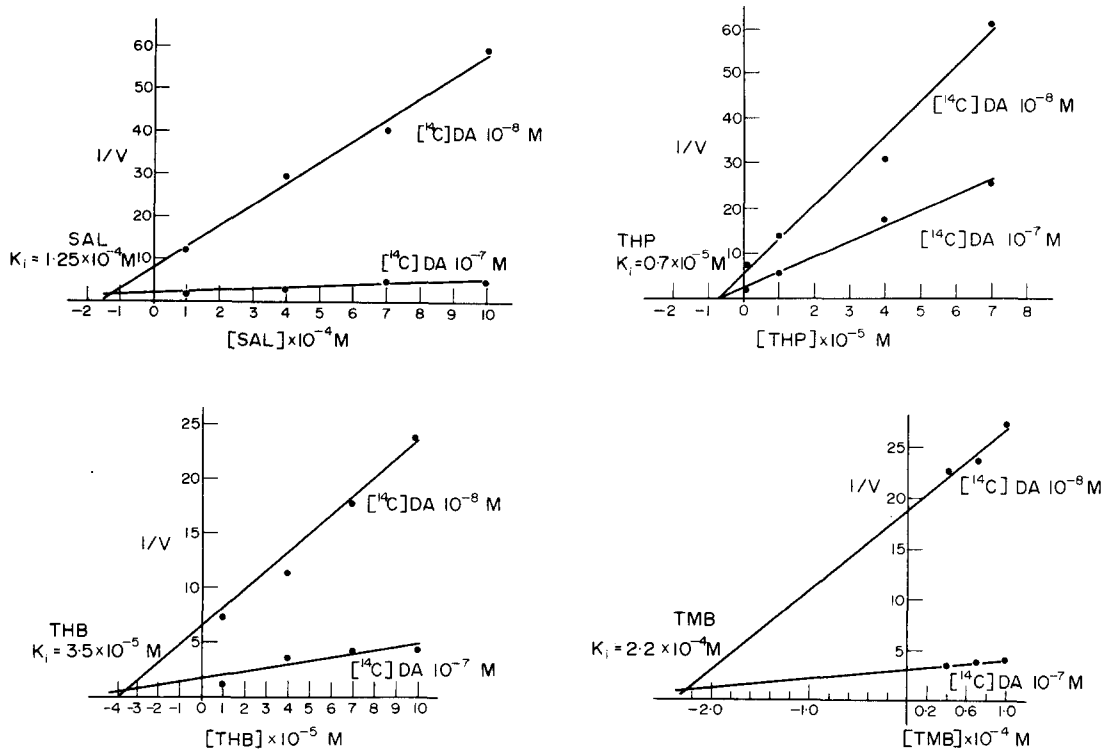

Fig. 4. Analysis by the method of Dixon [51] of (^{14}C)-dopamine uptake inhibition by salsolinol (SAL), tetrahydropapaveroline (THP), 2,3,10,11-tetrahydroxyberbine (THB) and 2,3,10,11-tetramethoxyberbine (TMB). Alkaloid concentration is shown on the abscissa of each graph. Reciprocals of V, which represents amine uptake expressed as dis./min ($\times 10^{-4}$)/synaptosomal pellet, are shown on the ordinate. Each point is the mean of data from a minimum of three experiments run in duplicate.

(^{14}C)-DA was linear in the presence of THP, but the line passing through the origin was of a lesser slope, suggesting that noncompetitive inhibition by THP is reversible.

DISCUSSION

According to current concepts, two processes—a transport mechanism located at the neuronal membrane, often called the amine pump, and a vesicular storage mechanism within the nerve terminals—contribute to the accumulation of catecholamines by adrenergic nerve endings (see review, Ref. [53]). Thus any compound which alters uptake in preparations *in vitro* may do so by an effect on the initial membrane transport, on the intracellular binding, on the efflux of amine from the synaptosomes, or by a combination of these effects. Conversely, drugs which alter retention of amine may do so by an effect on the intracellular storage or outward mobility of the amine, or by inhibiting the re-uptake of amine released into the incubation medium.

The inhibitory effect of the THIQ alkaloid, SAL, on norepinephrine accumulation in rat brain synaptosomal preparations (sheared-off nerve endings) has been demonstrated in other reports. Experimental procedures and preparations have differed according to the laboratories involved, precluding exact comparison. In preparations incubated with 10^{-8} M labeled norepinephrine for 5, 10 or 15 min, 10^{-4} M SAL produced, respectively, 50 [54], 33 [55] or 42 per cent [22] inhibition of amine accumulation.

To obtain information about possible alkaloid effects on retention of labeled NE by synaptosomes, experiments were carried out with preparations preloaded with this catecholamine. In control samples, radioactivity in the synaptosomal pellet increased progressively with time and reached a plateau after a 30-min incubation at 37° (Fig. 2). After a 10-min incubation of synaptosomes with the labeled amine, alkaloids were added and pellet radioactivity was determined 10 min later. Comparison of pellet amine content in the alkaloid-treated preparations with levels of radioactivity present in the untreated controls at the time of alkaloid addition (10-min point on the time curve) demonstrated significant decreases for 10^{-4} M SAL ($P < 0{\cdot}01$), 10^{-4} M THB ($P < 0{\cdot}02$) and 10^{-4} or 10^{-5} M THP ($P < 0{\cdot}001$). On the basis of this statistical comparison, 10^{-4} M TMB had no significant effect on levels of radioactivity in the pellet. However, by comparing the TMB-treated samples with controls which were also incubated an additional 10 min (20-min point on the time curve) and thus allowing the increment of increase in these latter preparations in the statistical analysis, a significant decrease ($P < 0{\cdot}001$) in pellet radioactivity was demonstrated for this alkaloid as well as for SAL, THB and THP at the same 10^{-4} M concentration. However, the extent to which these amine losses can be attributed to an alkaloid action on retention instead of an effect on re-entry into the synaptosome depends, of course, on the magnitude of spontaneous amine efflux in untreated preparations, since such

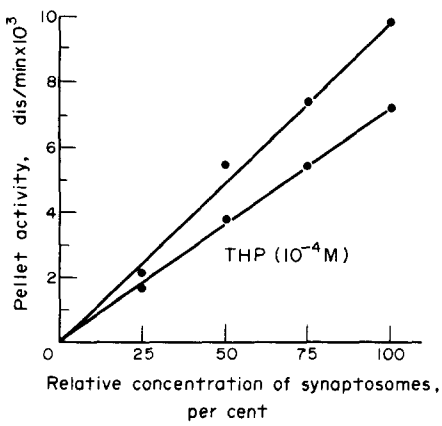

Fig. 5. Effect of synaptosomal concentration on inhibition of (^{14}C)-dopamine uptake by tetrahydropapaveroline (THP). Upper line represents uptake of (^{14}C)-DA, 10^{-7} M, in the absence of alkaloid inhibitor.

amines would then be vulnerable to the inhibition of their synaptosomal re-uptake by the added alkaloid. But at least 88 per cent of amine content of the synaptosomes must be lost from the 10-min controls and then regained during the next 10 min in order for the action of THP, for example, to be directed solely to an effect on amine transport. Although no precise information is available on the intrasynaptosomal–extrasynaptosomal exchange in such preparations, this value seems extremely high. It is more probable that the alkaloids do modify retention, in an unknown manner, as well as inhibit transport of catecholamines. Evidence that 6,7-dihydroxy-tetrahydroisoquinoline can be taken up, stored, and then released from peripheral adrenergic nerve terminals by nerve stimulation [27] suggests that the closely related alkaloid, SAL, may also diminish NE stores in synaptosomal preparations through the mechanism of displacement.

Although the brain preparations for these procedures contained a mixed population of synaptosomes, those from both dopaminergic and noradrenergic, as well as from other neuronal tracts, the study of catecholamine uptake kinetics in several brain regions by Snyder and Coyle [48] strongly suggests that both DA and NE use the same transport mechanism of either catecholaminergic neural tract. In the brain regions studied, including the striatum, NE and DA competitively inhibited the other's uptake with K_i values similar to their respective K_m values. However, the affinity of DA for the uptake mechanisms exceeded that of NE, being 5-fold greater in all brain areas except the striatum, where its affinity was 2-fold greater. In the present study, utilizing synaptosomes from whole rat brain, the K_m of DA, determined graphically according to the method of Lineweaver and Burk [50] was $0{\cdot}96 \times 10^{-7}$ M, a value quite similar to one of the two Michaelis constants reported above for DA uptake in synaptosomes from rat brain cortex [48]. This investigation demonstrated a K_m of $0{\cdot}8 \times 10^{-7}$ M for low DA concentrations in the range used in experiments reported herein, and a K_m of $1{\cdot}4 \times 10^{-6}$ M at higher DA concentrations ranging upward to $1{\cdot}2 \times 10^{-6}$ M.

To analyze the inhibitory effects of the representative alkaloids on amine uptake by the method of Dixon [51], two concentrations of radioactive DA were incubated with the synaptosomes and alkaloids in varying concentration. The reciprocals of pellet radioactivity were plotted against alkaloid concentration. In the graphs shown in Fig. 4, intersection of the regression lines, obtained from the two sets of data for each alkaloid, gives their respective K_i values directly. These dissociation constants of the enzyme–inhibitor complex (or the reciprocals of alkaloid binding affinity for the transport mechanism) are as follows: THP, $0{\cdot}7 \times 10^{-5}$ M; THB, $3{\cdot}5 \times 10^{-5}$ M; SAL, $1{\cdot}25 \times 10^{-4}$ M; and TMB, $2{\cdot}2 \times 10^{-4}$ M. Also, the Dixon plots indicate that SAL, THB and TMB inhibit DA uptake in a competitive fashion. The regression lines in the graphs intersect above the abscissa, as is characteristic of competitive enzyme inhibitors. On the other hand, the regression lines for the THP data intersect at the abscissa, suggesting that THP interferes with DA uptake in a noncompetitive fashion.

These effects of selected DA-derived alkaloids, although not marked in degree, are sufficient to suggest that their localized formation *in vivo* in certain brain areas might have important consequences. Two aspects of this study suggest that the inhibitory effects of any endogenously formed alkaloid on catecholaminergic mechanisms may be underestimated in the present report. The inhibition of uptake in experiments with the neuroamine possessing the greater affinity for the transport mechanism, that is, dopamine instead of norepinephrine, presumably understates alkaloid action against the latter amine which is considered the catecholamine neurotransmitter in most brain areas. In addition, only racemic mixtures of the representative alkaloids were available for use in these experiments. The pharmacological activity of an optical pair frequently differs qualitatively and quantitatively from that of the individual members. Activity may reside largely in one compound, or one antipode may actually oppose the actions of the other. Since formation *in vivo* of catecholamine-derived alkaloids may favor the production of one enantiomer over the other, the true affinities of endogenously formed alkaloids for the catecholamine transport mechanism may be greater than these estimated herein. Resolution of the isomers of selected alkaloids is now underway in our laboratory.

The derivation of THP from DA, and the characteristics of DA metablism, suggest that it may be important to study the enantiomers of this alkaloid in synaptosomes from DA-rich areas in which the catecholamine uptake mechanism is kinetically [48] and pharmacologically distinct [56]. Horn *et al.* [56] have demonstrated differential effects of several drugs on the two catecholamine populations of nerve terminals in synaptosomes from regional preparations of rat brain. A 1000-fold difference between the marked potency of desipramine in inhibiting NE uptake by hypothalamic synaptosomes and the relative lack of effect in inhibiting DA uptake by striatal synaptosomes was demonstrated. In addition, the inhibition by benztropine, trihexyphenidyl, and imipramine in the cerebral cortex and hypothalamus was competitive, whereas in the striatum noncompetitive kinetics were observed.

Moreover, the metabolism of DA differs somewhat from that of norepinephrine. In contrast to the alternative pathways available to the aldehyde metabolite of NE, that is, oxidation to an acid or reduction to a glycol, only the former route appears available to the aldehyde derived centrally from DA [57, 58]. This lack of a second route for the metabolism of the side chain of 3,4-dihydroxyphenylacetaldehyde suggests that inhibition of the enzyme normally catalyzing this reaction might result in high local accumulation of the DA-derived aldehyde and theoretically enhance the possibility for the formation *in vivo* of THP in brain areas where DA is concentrated. Thus, the limited capacity of the alkaloids, as represented by their racemic mixtures, in inhibiting the catecholamine uptake mechanism *in vitro* does not negate the possibility that their formation *in vivo* may have important physiological sequelae. In addition to the possibility that alkaloid formation in the brain may be quite localized and yield concentrations of alkaloids sufficient to modify amine transport or storage, certain of these compounds are known to alter other aspects of catecholaminergic neurophysiology. For example, THP and SAL moderately inhibit the oxidative deamination of the catecholamines and serve as excellent substrates for the enzyme catalyzing the *O*-methylation of these neuroamines [59].

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