STUDIES ON INHIBITION OF L-DOPA DECARBOXYLASE *IN VITRO* AND *IN VIVO*[1]

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A number of investigators have studied the inhibition of L-3,4-dihydroxyphenylalanine (dopa) decarboxylase *in vitro* and *in vivo* as affected by various physiological and nutritional conditions and by chemical compounds.

In vitro, inorganic anions in general either have no effect, including SCN (69), pyrophosphate (36, 69), fluoride (31, 36), arsenite, iodide, sulfate (36), or else activate, for example, phosphate and arsenate (36), although there is disagreement about the latter (31). Metal chelating agents have no effect (36, 69) but thiol inactivating organic mercurials inhibit (36, 66, 89a) and glutathione prevents or reverses this (36). Mg^{++} and Mn^{++} did not seem to be cofactors (Hartman, 1951, unpublished). Hence, Hartman (36 and unpublished) concluded that the enzyme may be heavy metal independent and contains essential thiol groups (36). There is a report that Zn^{++} is a cofactor (89a).

Reducing compounds such as bisulfite (50, 93), aldehydes, ascorbic acid and glutathione (29, 31) have been reported to inhibit the enzyme although Hartman *et al.* found no inhibition by the latter two agents (36). Thiopropionate (69) had no effect, while methylene blue produced inhibition (31).

Quinone and hydroquinone (50, 93) are stated to inhibit, although there is disagreement about the latter (31), and catechol and pyrogallol either are inert (31) or slightly accelerate (61) decarboxylation.

Folic acid antagonists and analogues in general are fairly potent inhibitors (31, 58, 60, 82) although there are exceptions (82). The mechanism and structural requirements for this are unknown and have not been studied *in vivo*.

It is claimed that substances accelerating tyrosinase oxidation of adrenaline also accelerate dopa decarboxylation *in vitro* (61) but no data were given and the methods are questionable. Substances with increasing affinity for monoamine oxidase also have been claimed to have a corresponding increasing inhibitory effect on dopa decarboxylase (30, 75), but the action of some of them such as the hydroxyphenylalkylamines has been explained by inactivation of the coenzyme of dopa decarboxylase, pyridoxal-5-phosphate, as discussed below.

If precautions, such as working in the cold, are not taken, solvents such as acetone inactivate the enzyme (66). Pyridine is either inert or slightly accelerator (32) and so are octyl alcohol (43) and aniline (30).

Miscellaneous nonspecific inhibitors include Suramin (8, 59), which like qui-

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nones and some benzoic acid derivatives may denature the protein through interaction with amino groups.

Miscellaneous inert substances include azide (8, 69), urea, urethane, acetamide, malonate (31), *o*-toluidine (30), carbon monoxide (8) and 2,4-dinitrophenol (36).

Para-phenylenediamine also is inert according to one group of workers who gave no data (61), although it has been reported to react with dopa *in vitro* and *in vivo* (76, 77). This needs exploration.

Several investigators showed that excess substrate or reaction product, or certain of their analogues, were inhibitory to dopa decarboxylase (8, 30, 52, 75, 83–85, 87, 88, 106), and others (41) showed that excess cofactors inhibited. Schott and Clark (84, 85) showed that this was due to a stoichiometric reaction of such analogues with the cofactor pyridoxal-5-phosphate, to form a tetrahydroisoquinoline by ring closure of the side chain of the analogue with the aldehyde moiety of the coenzyme, inactivating the coenzyme, or the substrate if coenzyme is in excess. The reaction worked best with *m*-hydroxyphenylalkyl primary amines and amino acids. This was confirmed and extended by Sourkes (87, 88) for dopa decarboxylase, Holtz and Westermann (49) for glutamic acid decarboxylase, and Yuwiler *et al.* (106) for 5-hydroxytryptophan (5-HTP) decarboxylase as well as dopa decarboxylase.

Cyanide inhibits dopa decarboxylase (8, 16, 43, 44, 50, 93), possibly by inactivating the cofactor through the formation of a cyanohydrin (17, 80, 81).

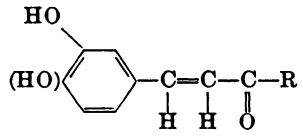
Carbonyl trapping agents such as hydrazides inhibit by inactivation of the coenzyme, for example, by forming inactive hydrazones, a general phenomenon for most decarboxylases and transaminases requiring pyridoxal-5-phosphate. This inhibition occurs with semicarbazide (16, 99), hydrazine (31), hydroxylamine (14, 19, 82), nicotinic and isonicotinic acids and nicotinamide (32), hydrazides including isonicotinic acid hydrazide (Isoniazid) (19, 32, 68) and the monoamine oxidase inhibitors such as N-isopropylisonicotinic acid hydrazide (iproniazid, Marsilid) (35), and the enzymatic synthesis of dopamine is inhibited by 1-phenyl-2-hydrazinopropane-HCl (Lakeside Laboratories "JB-516") (12). It is interesting that it has been observed (32, 68; and *cf.* Holtz, THIS SYMPOSIUM) that isonicotinic acid hydrazide in smaller amounts, by forming a hydrazone, prevents the inactivation of cofactor by substrate as discussed above, yet by slow hydrolytic release of cofactor, permits the reaction to go to completion, although slowly.

Other inhibitory substances which may be classed as carbonyl trapping agents include 2-hydrazino-5-phenylpyridazine (69), 1-hydrazinophthalazine (Apresoline) and 1,4-dihydrazinophthalazine (69, 101) 2,4-dihydrazinoquinazoline, 1-hydrazinoisoquinazoline, 1-hydrazino-4-methylphthalazine, 2-hydrazinoquinoline and aminoguanidine (101). Of interest is the report that the hypoglycemic sulfonylureas such as N-sulfanilyl-N′-n-butylcarbamide (Carbutamide, "BZ-55") not only deplete catecholamines in the adrenal glands of the intact animal as does insulin, but unlike insulin inhibit dopa decarboxylase *in vitro* through some mechanism other than coenzyme inactivation (32, 33).

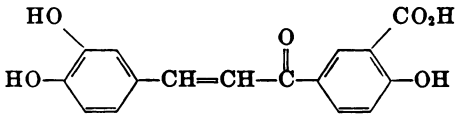
With the aim of preventing the formation of catecholamines in the body, Mar-

tin *et al.* (59) in 1950 made the first systematic study of structural analogues of dopa as possible competitive inhibitors of dopa decarboxylase *in vitro*, and reported good inhibition by several analogues including *p*-hydroxyphenylpyruvic acid. They also found these compounds depressed blood pressure acutely in dogs and postulated a correlation between the depressor effect and the enzyme inhibitory effect *in vitro*. Unfortunately, their data are questionable because of the low yield of CO_2 and the relatively high concentrations of inhibitors used in their manometric experiments, as pointed out by Schales and Schales (82); because of the results we reported with the same compounds (36, 37); and because more recent knowledge makes it unlikely that inhibitors of dopa decarboxylase *in vivo* would have an immediate depressor effect because of such inhibition. Nevertheless their report was provocative and stimulated subsequent work. Gonnard (30) confirmed Martin *et al.*, that phenylalanine and tyrosine were poor inhibitors.

Three years later, after having worked since 1950 on the problem, Hartman *et al.* (37) reported on the activity of many dopa analogues as possible substrates or inhibitors of dopa decarboxylase, and in 1955 published the effects of some 200 compounds on this enzyme system *in vitro* (36). This led to the elucidation of structures which competitively and fairly specifically inhibit dopa decarboxylase, many of which were not described before and were synthesized for these studies. The best inhibitors have the structure,



where R = OH, O-alkyl, or aryl, the activity increasing in that order. 5-(3,4-Dihydroxycinnamoyl)-salicylic acid, which also can be called 3,4,4′-trihydroxy-3′-carboxychalcone,



produces 90% inhibition at a molar concentration of 1×10^{-3} that of the substrate dopa. Martin *et al.* (59) used approximately equimolar concentrations. The inhibition was not due to quinoid structure formation or nonspecific inhibition, since: a) an oxygen-free nitrogen atmosphere was used in addition to cysteine with the substrate in the side arm and glutathione with enzyme in the main compartment of the Warburg vessel prior to equilibration; b) a single *meta*-hydroxyl (or *meta*-mercapto) group alone sufficed at molar concentrations 1×10^{-2} that of the substrate; c) the Lineweaver–Burk relationship held for the inhibitors subjected to such studies, except for *p*-hydroxyphenylpyruvic acid of Martin *et al.*, which was noncompetitive, and 3,5-diiodosalicylic acid, which was pseudo-competitive at concentrations one hundred times that of 5-(3,4-dihydroxycinnamoyl)salicylate; and d) no inhibition occurred with bacterial tyrosine

decarboxylase, histidine decarboxylase and glutamic acid decarboxylase, mammalian melanoma tyrosinase, succinic dehydrogenase or with respiration of surviving rat liver slices, indicating good specificity.

Inhibition of 5-hydroxytryptophan (5-HTP) and *erythro*-DL-3,4-dihydroxyphenylserine (*e*-dops) decarboxylation occurs with the inhibitors at the same concentrations effective on dopa decarboxylase, the former having been observed by Yuwiler *et al.* (106) and the latter by Hartman (unpublished). Yuwiler *et al.* and Westermann *et al.* (105; *cf.* Holtz, THIS SYMPOSIUM) discuss the possibility that 5-HTP and dopa decarboxylase are the same enzyme. C. T. Clark *et al.* (14) maintain otherwise. While Werle *et al.* (100, 102) postulated dopa and dops decarboxylases are 2 different enzymes, Holtz and Westermann disagree (47, 48; *cf.* Holtz, THIS SYMPOSIUM). Resolution of the problem must await further purification of the enzymes.

A few substances were tested by Schott (unpublished) as inhibitors of *o*-tyrosine decarboxylation and were found to behave in the same manner as when dopa was substrate except that the *o*-tyrosine reaction was more sensitive to competitive inhibitors. The enzyme dissociation constant for *o*-tyrosine was about 6 times that for dopa. The order of effectiveness of the competitive inhibitors tested, namely *o*-hydroxycinnamic acid, *m*-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid (caffeic acid) and its quinic acid ester (chlorogenic acid) was the same with both substrates. On the other hand, *p*-hydroxyphenylpyruvic acid, a noncompetitive inhibitor was about equally effective at relatively high concentration with *o*-tyrosine, *m*-tyrosine and dopa as substrates.

Hartman *et al.* showed that the presence of substituted or unsubstituted amino groups in the side chain markedly decreased the activity of the dopa decarboxylase inhibitors, *e.g.*, α-methyldopa. Substituents in the prime ring of the chalcone structures (cinnamoylsalicylates) caused no marked change in activity except those which enhanced water solubility, in which case a favorable effect was seen. That the *meta*-hydroxyl (or mercapto) group was essential was borne out by the inactivity of other *meta*-substituted cinnamic acids such as *m*-methyl, *m*-amino, *m*-halogen and *m*-methoxy derivatives.

In one series of compounds studied, α,β-unsaturation increased activity over the saturated analogues. Thus, although β-(3-hydroxyphenyl)propionic acid was totally inactive, 3-hydroxycinnamic acid inhibited 48% at a molar concentration 0.4 that of substrate. In contrast, β-(3,4-dihydroxyphenyl)propionic acid was as active as 3,4-dihydroxycinnamic acid (caffeic acid).

Sourkes in 1954 (87) reported dopa decarboxylase inhibition by 22 substrate analogues including α-benzamido-3,4-dimethoxycinnamic acid, N-α-diacetyl-3,4-dihydroxyphenylethylamine hydrate, N-benzoyl-3,4-dimethoxyphenylalanine, N-acetyl-3,4-dimethoxyphenylalanine, *d*-catechin and others. In contrast, the experiments of Hartman *et al.* showed that the 3-hydroxyphenyl group had to be free. Sourkes found highest activity with α-methyldopa and α-methyl-3-hydroxyphenylaline. At concentrations less than 10^{-5} M, these 2 compounds stimulated decarboxylation by an unknown mechanism, although Sourkes has discussed various possibilities, as has Umbreit (95). Sourkes incubated the inhibitor with

the enzyme for 15 minutes before adding substrate, whereas Hartman *et al.* added substrate and inhibitor simultaneously to the enzyme. The inhibitions are much greater than those reported previously (36, 37) when Sourkes' technique is employed.

Fellman in 1956 (24) reported that phenylpyruvic, phenyllactic and phenylacetic acids inhibit beef adrenal dopa decarboxylase. We found no activity of phenylacetic or 3-hydroxyphenylacetic acids, although 3,4-dihydroxyphenylacetic acid inhibited 88 % at a concentration equimolar to substrate. Davison and Sandler (20) found that the same compounds Fellman used also inhibited 5-hydroxytryptophan decarboxylase.

None of these workers made a Lineweaver–Burk analysis for competitive inhibition, or studied enzyme specificity.

The extension of such investigations to the intact animal has been limited because of the difficulties encountered. In discussing the literature on the subject, the reviewer will include the results of some work which has been performed in the past eight years which is unpublished except in abstract form (15, 71–73) and which of necessity must be condensed from several papers in preparation.

Some of this work has been based on blood pressure assays, so it is necessary to discuss briefly this aspect of the investigations.

Earlier investigators reported no pressor effects of dopa administered to normal animals (3–7, 25, 34, 65) although Holtz *et al.* (40, 41; *cf.* Holtz, THIS SYMPOSIUM), Page and Reed (67) and the reviewer and colleagues (15, 71–73) demonstrated the typical long-lasting pressor effect of dopa administered intravenously to several species of animals, with the exception of guinea pigs and rabbits, which show a depressor response to dopa and dopamine (41), the mechanism of which remains to be elucidated (24a, 49a).

When 1 to 5 or more mg/kg L-dopa is administered intravenously to pithed cats and rats, or to dogs given hexamethonium, a slow-rising pressor response occurs, which is enhanced by cocaine and which lasts for several minutes, associated with tachycardia. It is unnecessary to inject the dopa into the ischemic kidney and subsequently release the formed dopamine from the clamped kidney as described by Bing and Zucker (4–7) and Beyer (3).

We found that the pressor response was not affected by differences in intravascular routes of administration in pithed cats, whether intraarterial or intravenous. Removing the kidneys from the circulation usually, but not always, enhanced and prolonged the response somewhat, in confirmation of Page and Reed (67). Clamping the hepatic portal vein decreased the response moderately. Partial hepatectomy in rats one day previously, however, did not decrease the response, but even prolonged it. Removal of the entire gastrointestinal tract in cats usually decreased the response, although not always, and restoration to normal was obtained in a few instances by intravenous pyridoxal hydrochloride, although this observation needs confirmation. Complete evisceration usually but not always decreased the response to dopa, but not to pressor amines. In these experiments, it was important to maintain the temperature of the animal, to replace lost blood and to observe all possible precautions to maintain the ani-

mal in good condition, as emphasized by others who have studied the effects of pressor amines on partially or completely eviscerated animals (53, 70, 98).

Clark *et al.* reported in 1953 (15; final ms. in preparation) that the thyroid hormone affected dopa decarboxylation *in vivo*. Samiy (78)[2] found that prolonged administration of L-thyroxine to rats resulted in a marked and fairly sustained pressor response to intravenous dopa, which seemed to be greater in males. This seemed to be due largely if not altogether to enhanced receptor sensitivity to the formed dopamine, as might be expected from the extensive literature on enhanced sensitivity to sympathomimetic drugs in hyperthyroidism. At first, Samiy thought decarboxylation to dopamine by the intact animal was enhanced, as judged by chemical and bioassays of the urine, but this remains to be established unequivocally. Analyses of all the visceral organs by manometric and microbiological assays (*Clostridium* tyrosine decarboxylase manometrically; *Lactobacillus* delbrückii growth; direct dopa decarboxylase manometrically) showed that hyperthyroidism had no measurable effect on the apoenzyme or coenzyme content of any organs except liver and kidney. Hyperthyroidism decreased dopa decarboxylation in liver and increased it in kidney, the former apparently due to an effect on the apoenzyme rather than the coenzyme.

Since normal liver and kidney tissues contain 4 to 5 μg of vitamin B_6 per g wet tissue, an experiment using liver and kidney tissue as a source for B_6 for bacterial tyrosine decarboxylase was performed to show the relative amounts of B_6 in normal and hyperthyroid tissues. The results showed the hyperthyroid tissues contained as much or more B_6 than normal tissues, thus providing additional evidence that the increases and decreases in dopa decarboxylase shown previously were due to changes in apoenzyme rather than changes in the amount of cofactor present (Hartman, Schott and Masuoka, unpublished).

Thyroxamine and triiodothyronamine also enhanced the pressor responses to dopa, but without the long lag period required for the thyroxine effect (Pogrund, unpublished). Thibault (90–92) has shown a similar lack of lag period in the effect of these decarboxylated thyroid hormones on adrenaline responses.

Holtz *et al.* (45, 104) showed that in hyperthyroid guinea pigs and rats, liver dopa decarboxylase decreased, as it also did in starvation, and that this was reversed in the latter case but not the former, by pyridoxal-5-phosphate, added to the liver *in vitro*. They too concluded the thyroid effect was on the liver apoenzyme. Spinks and Burn (89) found hyperthyroidism in rabbits decreased liver monoamine oxidase and hypothyroidism increased it, and would explain enhanced sensitivity to pressor amines in hyperthyroidism by this decrease. Holtz *et al.* (45, 104) took issue with this since they found hyperthyroidism increased liver monoamine oxidase in rats and guinea pigs.

Goldstein *et al.* (27) found that hyperthyroidism increased the urinary excretion of radioactive adrenaline and noradrenaline formed from labeled dopamine and decreased what apparently were its O-methylated metabolites.

Polonovsky *et al.* (74) found that guinea pig kidney dopa decarboxylase decreased in the winter or when the animals were subjected to low environmental

[2] Submitted as a dissertation from this laboratory.

temperature. Holtz *et al.* (45, 104) observed a decreased liver dopa decarboxylase in starved rats, apparently due to coenzyme, since addition of pyridoxal-5-phosphate *in vitro* normalized the reaction. Weil-Malherbe (97) reported a reversible decrease in dopamine excretion in a pheochromocytoma patient on a low protein intake which could have been due to a decreased dopa decarboxylation, although a decreased catabolism also could be the explanation.

Hawkins and Walker (39) found a decreased liver dopa decarboxylase in colchicine-treated rats, which was not affected by addition of pyridoxal-5-phosphate *in vitro*. Anderson *et al.* (1) reported decreased pressor amines in the adrenals of X-irradiated rats, and a decreased liver dopa decarboxylase, which was partially prevented by dietary vitamin B_6.

Blaschko *et al.* (9–11) and West (103) found that vitamin B_6 deficiency decreased rat liver dopa and cysteic acid decarboxylases and the biogenesis of adrenal catecholamines. Similar observations have been made for other decarboxylases requiring pyridoxal-5-phosphate as coenzyme, *e.g.*, glutamic acid and 5-hydroxytryptophan decarboxylases.

Weissbach *et al.* (99) reported that semicarbazide inhibits dopa decarboxylase in mice, apparently by inactivating the coenzyme. Similar observations have been made for glutamic acid decarboxylase with carbonyl trapping agents.

Clark *et al.* announced in 1953 (15; final ms. in preparation) that vitamin B_6 affects dopa decarboxylase *in vivo*. Vitamin B_6 deficiency induced in rats by a pyridoxine-deficient diet with and without administration of deoxypyridoxine depressed the pressor response to intravenous dopa, which was unaffected by acute intravenous administration of pyridoxine, pyridoxal or pyridoxal-5-phosphate. Manometric assays of all organs showed no changes except in liver and kidney, both of which showed decreased dopa decarboxylation. This was restored to normal *in vitro* by the addition of coenzyme to the kidney extracts but not liver, indicating a decreased apoenzyme in the latter organ.

We also found (73; and in preparation) that the pressor effect of 3-hydroxy- and 3,4-dihydroxyphenylpyruvic acid (see later discussion) is decreased by vitamin B_6 deficiency, as might be expected since both the transamination and decarboxylation steps are B_6-dependent.

An incidental observation may be of interest here, in connection with the inactivation of dopa decarboxylase coenzyme by substrates and their analogues discussed above. Noradrenaline and dopamine readily form tetrahydroisoquinolines with pyridoxal-5-phosphate, and these have no effect on the cardiovascular system (85). It was found that the reaction occurred on incubation in blood as well as in aqueous media, but *in vivo*, the pressor responses to 10–20 μg *l*-noradrenaline were little affected by previous or simultaneous intravenous administration of 5,000 to 10,000 times more pyridoxal-5-phosphate (Ca or Na salts) or pyridoxal-HCl in pithed cats, and the LD50 of *l*-noradrenaline in mice was not affected by Na pyridoxal-5-phosphate or pyridoxal-HCl given immediately previously intravenously or intraperitoneally in doses up to 100 mg/kg. Presumably noradrenaline and pyridoxal-5-phosphate do not react to form the pharmacologically inert tetrahydroisoquinoline *in vivo*, at least fast enough to prevent the

pressor and toxic effects of noradrenaline. To establish that the reaction does not occur at all would necessitate identification of the reaction product in the urine.

Without benefit of statistical analysis, it has been our impression in working with over a thousand animals over the past years, that male cats and rats have a greater pressor responsiveness to dopa than females, and this may vary seasonally, in agreement with Polonovski *et al.* (74). In a preliminary attempt to study this further (Pogrund, unpublished), it was found that prolonged administration of testosterone did not increase the responsiveness of male or female rats, although the latter did appear more sensitive when previously ovariectomized. Sloane-Stanley (86) observed greater cysteic acid decarboxylase activity in male animals.

Adrenalectomized rats maintained in good health on salt, showed little or no pressor response to dopa, although they responded normally to adrenaline, noradrenaline and dopamine. Maintenance therapy with hydrocortisone, but not an acute injection, appeared to restore the response to normal (Pogrund and Clark, unpublished). Page and Reed (67) found that adrenalectomy abolishes the pressor response of rats to dopa, which was restored to normal after 2 weeks' treatment with desoxycorticosterone. Studies are in progress to elucidate the mechanisms involved.

The dopa analogues which were found to inhibit dopa decarboxylase *in vitro*, also inhibited it *in vivo* (15, 71–73). Page and Reed (67) believed the pressor effect of dopa might not be due to decarboxylation alone because catechol and acetaldehyde also produced pressor responses. The changes in blood pressure and heart rate responses probably are, however, due to the dopamine formed [(−)-noradrenaline in the case of *threo*-dops and (+)-noradrenaline in the case of *erythro*-dops (38)] and to a decreased formation of dopamine in the presence of inhibitors, and not to dopa itself, since:

(a) Adrenergic blocking drugs such as the benzodioxanes, *e.g.*, piperoxan (Benodaine) prevent the dopa response;

(b) Responses to dopamine, adrenaline and noradrenaline are not influenced by the inhibitors, whereas the pressor and cardioacceleratory responses to dopa, *t*-dops and *m*-tyrosine are abolished, as exemplified by Fig. 1 which shows the effect on dopa but not dopamine;

(c) Cocaine plus iproniazid (Marsilid) enhances the nictitating membrane and pressor response to dopa; and ganglionic blocking drugs such as hexamethonium (Bistrium) enhance the pressor responses just as they do the dopamine response;[3]

(d) The dopamine formed from the dopa is excreted in the urine, and has been isolated, purified, and identified by paper chromatography, color reactions, bioassays, and melting point of the crystalline product in confirmation and extension of the results of Holtz *et al.* (43) who isolated the benzoyl derivative, and this excretion is decreased by the inhibitors, as illustrated in Table 1;

[3] Page and Reed (67) found no effect of piperoxan or cocaine on the pressor response of rats to dopa, whereas Holtz *et al.* (43) found that cocaine enhances dopamine responses and ergotamine blocks them.

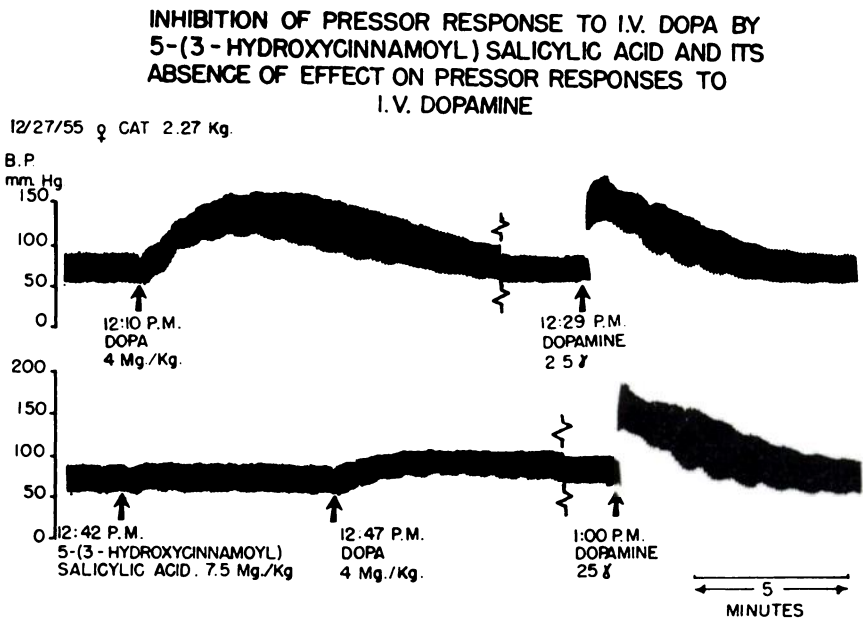


FIG. 1. Inhibition of pressor response to i.v. dopa by 5-(3-hydroxycinnamoyl)salicylic acid and its absence of effect on pressor responses to i.v. dopamine.

TABLE 1

Comparison of dopamine content in extracts of urine from dopa-treated cat with and without inhibitor

Urine Extract	Bioassay (μg dopamine)	Chemical (μg dopamine)
+ Inhibitor	450	480
− Inhibitor	2280	2352

Bioassay in pithed rat vs. dopamine. Chemical determination by modification from Euler and Hamberg (23).

(e) Guinea pigs given an intravenous dose of 25 to 50 mg/kg[4] of 5-(3-hydroxycinnamoyl)salicylate show decreased dopa decarboxylation in the subsequently removed, saline-perfused blood-free liver and kidney homogenates;

(f) Compounds structurally related to dopa but not having the necessary configuration for acting either as substrates or inhibitors of dopa decarboxylase *in vitro*, are not active either as pressor substances themselves, in the concentrations tested, or as inhibitors. The analogues we studied included 3,4-dihydroxyphenylglycine, 3,4-dihydroxyphenylacetic acid, D-dopa, catechol, pyrogallol and chloroacetocatechol;[5]

(g) Neither the compounds listed above nor dopa nor dops affects the isolated rabbit ileum or the isolated perfused frog heart.

[4] The LD50 intraperitoneally in mice is 380 mg/kg.

[5] Disparate reports have been made on the pressor effects of catechol, pyrogallol and chloroacetylcatechol, although the majority of workers observed slight sympathomimetic effects of large doses (2, 18, 26, 51, 54–57, 64, 67).

Page and Reed (67) found a similar lack of effect of dopa on perfused rat hind limbs. These results may be explained by a lack of sympathomimetic activity in the concentrations tested, and by a failure of decarboxylation of dopa and dops in the isolated preparations used.

In examining various compounds discussed in (f) and (g) above, it was found that 3,4-dihydroxyphenylpyruvic and 3-hydroxyphenylpyruvic acids were pressor when given alone. This was unexpected since they are not dopa decarboxylase substrates, and are in fact slightly inhibitory *in vitro*. An extensive study was made of this phenomenon *in vivo* by Pogrund *et al.* (73; and in preparation), and it was demonstrated that both 3-hydroxyphenylpyruvic and 3,4-dihydroxyphenylpyruvic acids are transaminated to their corresponding amino acids, *m*-tyrosine and dopa, which subsequently are decarboxylated to *m*-tyramine and dopamine. These compounds were isolated and identified. Dopa decarboxylase inhibitors block the pressor responses presumably by interference at the decarboxylation step.

Thus a new possible metabolic pathway for the biosynthesis of noradrenaline and adrenaline may include phenylpyruvic acid and its ring-hydroxylated derivatives. These observations may also be of interest in connection with phenylpyruvica oligophrenia.

In extending our studies of dopa (and dops) decarboxylase inhibitors *in vitro* to the intact animal, a satisfactory assay procedure was developed, based on the pressor responses to dopa. In general, it was found that those compounds active *in vitro* also were active *in vivo* in all species studied, including rats, cats, dogs, guinea pigs and rabbits, although rats and particularly cats were used in the majority of experiments. Exceptions were 3-hydroxy-4-methoxycinnamic (isoferulic) acid and 3-hydroxyhydrocinnamic acid which inhibited *in vivo* but little or not at all *in vitro*. The effects of these compounds might be explained by demethylation and dehydrogenation, respectively to active inhibitors *in vivo*.

Table 2 lists the compounds tested, along with the results previously obtained *in vitro* (36, 37) for comparison.

Scrutiny of Table 2 and Table 4 (see below) shows that it was not possible to relate quantitatively the comparative activity *in vivo* to that *in vitro*. Table 3 shows a few of the structural relationships of active and inactive compounds *in vivo*.

Considerable data were obtained on 50% inhibition values *in vivo*. These were obtained graphically by starting at low doses of inhibitor and gradually increasing the doses to maximum inhibition somewhere near 100%. Results of averages from several such experiments showed that 6.6 mg/kg of 5-(3-hydroxycinnamoyl)salicylate and 50 mg/kg of caffeic acid caused 50% inhibition. The activity ratio on a mg/kg basis thus was 7.6.

Because of the observation that responses can vary considerably from animal to animal, the method described above was considered to be less reliable than one involving the use of animals in which a standard reference inhibitor, 5-(3-hydroxycinnamoyl)salicylic acid as the Na salt, as used for comparison with the inhibitor to be tested, in the *same* animal, and plotting at least 2 or 3 different

TABLE 2

Inhibition of dopa decarboxylase

Compound	In vitro		In vivo
	R.M.	% Inhib.	
A. Active Compounds			
5-(3,4-Dihydroxycinnamoyl)salicylic acid	0.001	85	C-120-(97 → 81)-110
5-(3,4-Diacetoxycinnamoyl)acetylsalicylic acid	0.001	62	C-50-100-30 (P. G.)
5-(3-Hydroxycinnamoyl)salicylic acid	0.01	84	C-35-100-30
5-(3-Acetoxycinnamoyl)acetylsalicylic acid	0.01	50	C-100-(92 → 64)-180
3-Mercaptocinnamic acid	0.05	78	R-20-42-30
5-(3-Hydroxy-4-nitrocinnamoyl)salicylic acid	0.1	87	C-60-84-30
5-(3-Hydroxyhydrocinnamoyl)salicylic acid	—	—	C-6-50-30
5-(3-Hydroxyhydrocinnaomyl)salicylic acid	—	—	C-25-100-60
3,4-Dihydroxycinnamic. acid (caffeic acid)	0.22	74	C-50-100-30
Chlorogenic acid (Quinic acid ester of caffeic acid)	0.1	70	C-50-50-30
3,4-Dihydroxyhydrocinnamic acid	0.22	63	R-50-70-30
3,4-Diacetoxycinnamic acid	0.2	31	C-100-52-30
Dimethylaminoethyl 3,4-diacetoxycinnamate	0.2	55	C-50-32-30
3,4-Dihydroxyphenylacetic acid	0.1	70	C-50-61-
3-Hydroxy-4-methoxycinnamic (isoferulic) acid	1.0	19	C-50-80-30
3-Hydroxycinnamic acid	0.4	48	R-50-56-30
3-Hydroxycinnamic acid	—	—	C-35-61-30
3-Hydroxyhydrocinnamic acid	1.0	0	C-40-(65 → 47)-60
α-Ethyl-3-hydroxycinnamic acid	0.2	27	C-50-21-30
α-Methyl-3-hydroxycinnamic acid	0.2	31	C-100-52-30
3-Hydroxyphenoxyacetic acid	0.1	30	C-50-70-
3-Hydroxy-ω-nitrostyrene	1.0	73	C-50-(100 → 61)-180 (alk.)
3-Hydroxyphenylacetic acid	1.0	11	R-90-11-
3,5-Dibromosalicylic acid	0.1	33	C-20-38-30
3,5-Dibromo-2-hydroxycinnamic acid	0.2	65	C-50-23-30
d-Catechin (3,5,7,3′,4′-pentahydroxyflavan)	0.1	50	C-5-50-
d-Epicatechin (isomer of above)	0.1	50	C-5-55-48 (alk.)
Gossypin (3,5,7,8,3′,4′-hexahydroxyflavane-8-glucoside)	0.1	70	C-21-50- (alk.)
B. Inactive Compounds			
2′,3,4-Trihydroxychalcone	0.1	90	C-35-0-(alk.)
5-(2-Hydroxy-3,5-dibromocinnamoyl)salicylic acid	0.1	65	C-40-0-
α-Phenyl-3,5-diodo-2-hydroxy-hydrocinnamic acid	0.2	100	C-100-0-(P. G.)
Cynarine (1,4-dicaffeic acid ester of quinic acid)	0.1	45	R-60-0-
3′-Amino-3-hydroxychalcone	0.1	30	C-50-0-(P. G.)
3-Hydroxybenzoic acid	1.5	0	Syn.
4-Hydroxycinnamic acid	0.25	7	Syn.
Cinnamic acid	1.0	0	R-100-0-
Acetylsalicylic acid	—	—	R-100-0-
4-Hydroxyphenylpyruvic acid	1.0	60	C-100- Syn.
Quercetin-6′-sulfonic acid (3,3′,4′,5,7-pentahydroxyflavone-6-sulfonic acid)	0.05	60	C-10-43-Syn.
3-Methoxy-4-hydroxycinnamic (ferulic) acid	1.0	5	C-50-100-Syn. (P. G.)

TABLE 2—*Continued*

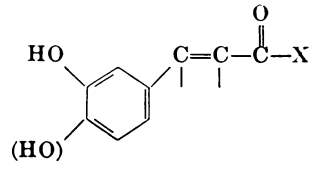
Compound	In vitro		In vivo
	R.M.	% Inhib.	
B. Inactive Compounds—*Continued*			
Protocatechuic acid	1.0	0	C-100-25-
4-Methoxycinnamic acid	—	—	C-50-100-Syn.
2,4-Dihydroxycinnamic acid	1.0	9	C-60-25-Syn.
Phenylbutyric acid	1.0	10	C-100-100-Syn.
Phenylacetic acid	1.0	0	C-50-0-
2,4-Dihydroxyhydrocinnamic acid	1.0	75	C-100-0-
3-Hydroxychalcone	0.01	24	C-50-0-(alk.)
3-Hydroxychalcone	0.001	8	—
3,3′-Dithiocinnamic acid	0.1	51	R-37-0-
Hydrocinnamic acid	1.0	8	R-100-0-
4-Hydroxyhydrocinnamic acid	1.0	9	R-100-0-
4-Hydroxyphenyllactic acid	1.0	24	R-100-0-
3-Hydroxyphenylacetic acid	1.0	11	R-90-11-
Phenyllactic acid	1.0	0	R-100-0-
α-Aminophenylacetic acid	1.0	0	R-100-0-(alk.)
o-Coumaric acid	1.0	52	R-35-18-(P. G.)
(+)-3-Hydroxyphenylhydracrylic acid	—	—	R-50-93-Syn.
(−)-3-Hydroxyphenylhydracrylic acid	—	—	R-50-12-Syn.
β-N-(3-Hydroxy-6-pyridone)-α-aminopropionic acid (Leucanol)	1.0	0–18	R-50-10-Syn. (alk.)
β-(3-Hydroxyphenyl)-β-alanine	4.0	0	R-50-0-
β-(2-Thienyl)acrylic acid	1.0	0	C-50-0-
3-Nitro-L-tyrosine	1.0	0	R-25-0-(alk.)
2,3,3′,4,4′,5,7-Heptahydroxyflavan glucoside	—	—	R-20-18-(P. G.)
4-Hydroxyisophthalic acid	—	—	C-50-0-
Histidine	1.0	0	C-50-24-Syn.
Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside) piperazine	—	—	C-100-0-
Lemon peel extract flavonoids	—	—	C-100-0-
"Hesperidine methyl chalcone"	—	—	C-100-0-
3,4-Dihydroxyphenylglycine	1.0	6	C-100- Syn.
Hydroferulic acid	—	—	C-50- Syn.
Epimerized *d*-catechin (80% *d*-catechin + 20% *d*-epicatechin)	—	—	C-10-0-(P. G.)
3-Fluorotyrosine	1.0	0	C-50-23-(alk.)
3-Aminotyrosine-HCl	1.0	0	C-50-9-Syn.
4-Chlorocinnamic acid	1.0	8	C-50-32-Syn.
β-(2-Furyl) serine	1.0	6	C-50-11-Syn.
erythro-3-Nitrophenylserine	1.0	0	C-50-0-(acid)
3,4-Dimethoxyphenylacetic acid	1.0	18	C-50-0-
3,4-Methylenedihydroxymandelic acid	1.0	0	C-50-15-Syn.

KEY: *In vitro* data from Hartman *et al.* (36); *cf.* for conditions. R. M. = mols of inhibitor relative to 1.0 mol of dopa. Code used in expressing results *in vitro*, *e.g.*, C-35-100-30; C = cat, (R = rat), 35 = mg/kg, free acid given as Na salt i.v. (P. G.: 25–100% propylene glycol, alk. = alkalinized), 100 = 100% inhibition of pressor response to L-dopa test dose (1–10 mg/kg, usually 5), 30 = 30 min duration of inhibition, *i.e.*, response to test dopa is normal again in 30 min (100 → 61) means 100% inhibition right after test dose of dopa, declining to 61% at the time indicated. Syn. = % synergized.

The figures represent the averages of several animals, usually 3 but sometimes exceeding 10, and several tests are made in each animal.

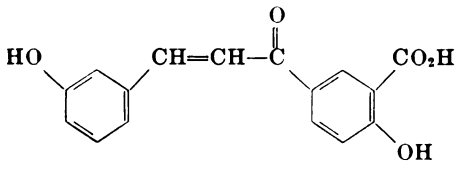
TABLE 3

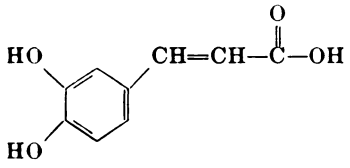
Structural requirements for dopa decarboxylase inhibitors in vivo

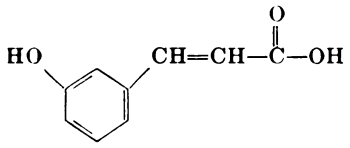


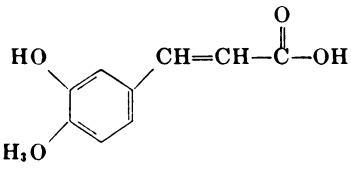
where X = OH
O—Alkyl
Alkyl
Aryl

Active

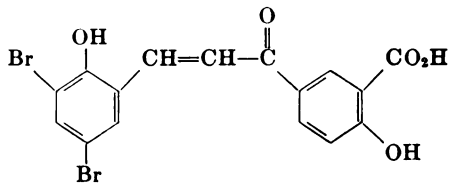


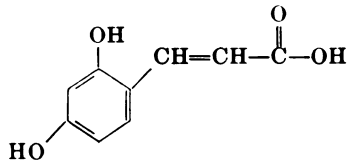


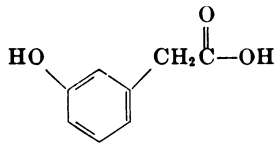


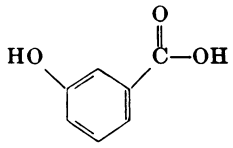


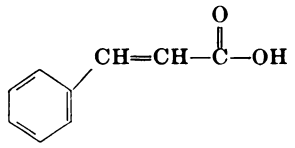
Inactive

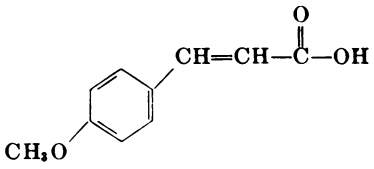












doses for each against % inhibition, drawing a line intersecting the 50% inhibition values of both, and thus obtaining the activity ratio in a way which would compensate for animal variations. Such experiments were laborious and yielded data for only one inhibitor per animal. Since an average of at least 3 or 4 cats

TABLE 4

Relative activity for 50% inhibition

Compound	In vitro	In vivo	
	Molar basis	Molar basis	Weight basis
(1) 5-(3-Hydroxycinnamoyl)salicyclic acid	1.0	1.0	1.0
(2) 5-(3,4-Dihydroxycinnamoyl)salicylic acid	2.60	0.41	0.42
(3) Chlorogenic acid	0.35	0.17	0.21
(4) Caffeic acid	0.048	0.20	0.13
(5) 3-Hydroxycinnamic acid	0.0076	0.48	0.28
(6) *d*-Catechin	0.026	1.18	1.20
(7) Gossypin	0.052	0.21	0.35
(8) 3-Hydroxyphenylpropionic acid	<0.001	0.35	0.20

5-(3-Hydroxycinnamoyl)salicylic acid is taken as the standard unit.

was used for each inhibitor and all preparations were not satisfactory, such work was time-consuming and limited the numbers of compounds which could be tested. The results from animal to animal were surprisingly uniform, however, and the average results are shown in Table 4, along with those obtained *in vitro*.

From Tables 2 and 4 it is apparent that quantitatively the more active compounds *in vitro* are not necessarily so *in vivo*. Thus for example, 5-(3-hydroxycinnamoyl)salicylate was the most active compound *in vivo* whereas its catechol analogue, 5-(3,4-dihydroxycinnamoyl)salicylate was most active *in vitro*.

In the experiments *in vitro*, it was shown that the inhibition was competitive by the Lineweaver–Burk graphic method. It was noticed in some experiments *in vivo* that, shortly after giving inhibitor, large doses of dopa seemed capable of displacing inhibitor, so that the duration of given smaller doses of dopa decreased, suggesting competitive inhibition *in vivo*. Although the results are difficult of interpretation and may not be biochemically meaningful, experiments then were designed to see if "competitive inhibition" could be graphically depicted by the Lineweaver–Burk analysis used *in vitro*. This was done by measuring responses at small increasing doses of dopa and then repeating these measurements in the presence of a given dose of the inhibitor 5-(3-hydroxycinnamoyl)salicylate. Plotting the reciprocals of response units against reciprocals of dopa doses in the absence and presence of the inhibitor yielded converging straight lines, as in a Lineweaver–Burk analysis. Nickerson, elsewhere in THIS SYMPOSIUM, discusses similar analyses for other drug actions *in vivo*.

It is seen from Table 2 that in most cases, the dopa decarboxylase inhibitors were rather rapidly detoxified, since the responses usually were normal again within 30 to 40 minutes after intravenous administration. Attempts to prolong this effect included repeated injections of smaller doses, constant intravenous infusion, intravenous administration of inhibitors "complexed" with dextran and polyvinylpyrrolidone, intramuscular injection in oil, and intraduodenal administration of large doses. It was observed that 20 mg 5-(3-hydroxycinnamoyl)-salicylate/kg, i.v., in addition to 100 mg/kg multiple intramuscular injections in

oil prolonged the effect for more than 4 hours in cats, and so did 100 mg/kg intraduodenally, but not orally. This latter effect may be due to precipitation in the stomach of the more insoluble free acid. The acetylated compound also gave inhibition lasting more than 3 to 5 hours when given intravenously or intraduodenally at 100 mg/kg in cats. Moreover, 3-hydroxy-ω-nitrostyrene at 50 mg/kg intravenously in cats gave inhibition for more than 3 hours. Since the latter compound is toxic and relatively insoluble and the former requires 100 mg/kg for the prolonged effect, it is indicated that different structures are required which will inhibit at lower concentrations and resist detoxication or elimination. The chalcone mentioned above, the most active inhibitor found to date *in vivo*, will inhibit 50% at 5 to 15 mg/kg. The acute LD50 intravenously in mice is 400 mg/kg; hence the "therapeutic index" is very favorable if repeated doses over a long time are not required, which, however, is not the case.

In order to understand something about the duration of inhibition, some preliminary studies were made of the renal excretion, blood levels, sites of detoxication and possible metabolites.

First of all, the slope of the recovery curves from inhibition has not been determined, since this requires applying statistics to large numbers of animals. This is due to the fact that the dopa pressor effects last up to 10–12 minutes before returning to normal, so that only 2 or at most 3 points can be obtained before recovery. In addition each dose of dopa given presumably may competitively displace some inhibitor. Hence, one dose of inhibitor would have to be given to large numbers of animals, repeatedly testing with a constant dose of dopa at different times. In view of the time and expense involved, it was not considered sufficiently important at this time to determine the slope of the "detoxication curves" from pressor response data.

However, some information was obtained on blood level[6] curves which suggests the same thing, and which more or less confirms the pressor inhibition results—that is, the blood levels were quite high at first and were negligibly low in 30 minutes in the case of caffeic acid, a typical inhibitor. No other inhibitors have been studied in this way as yet. Renal ligation increased the duration of inhibition and the blood levels about 2-fold, but no more, indicating that detoxication occurs rapidly even when renal elimination cannot occur.

Paper chromatography of the urine concentrates, of the caffeic treated cats revealed 2 phenolic substances, one of which, on elution, gave a positive naphthoresorcinol test for glucuronic acid, and the other more prominent spot had the same R_f and color tests as authentic caffeic acid. A sample of 5-(3-hydroxycinnamoyl)salicylic acid was sent to Dr. Floyd De Eds, U. S. Dept. of Agriculture, Albany, Calif., who administered it to rabbits and examined the urine for metab-

[6] The method is based on the development of red color in the diluted plasma or urine, by ferric ion in strong acid solution, which is bleached quantitatively by hydroxylamine in acid. The excess ferric ion is removed by the hydroxylamine and the blanks fade much more than the test samples. The sensitivity, *e.g.*, for caffeic acid, is ca. 10 μg, and the recoveries are good from urine and serum but not plasma or protein free filtrates of tissues. The specificity was not studied.

olites by paper chromatography. He found only one substance, which had the same R_f and color reactions as an authentic sample of the saturated analogue, 5-(3-hydroxyhydrocinnamoyl)salicylic acid. *In vivo* the latter compound has only one-half the activity of the unsaturated analogue, and the same duration of action. Hence, at least one route of detoxication involves saturation of the side chain.

No effect on the duration of inhibition was found with *p*-benzoic acid, *p*-aminosalicylic acid, *p*-(dipropylsulfamyl)benzoic acid (Probenecid), β-diethylaminoethyldiphenylpropyl acetic acid (SKF-525A), 3-hydroxycinchoninic acid or cysteine, in amounts up to near toxic levels administered intravenously in one or repeated doses.

It is well established (13, 79, 94, 96) that the resynthesis of adrenal medullary noradrenaline and adrenaline after depletion by, for example, insulin hypoglycemia, is a slow process, requiring several days. Early in our work, and before others had published on this phenomenon, we applied the method to see if repeated injections of a dopa decarboxylase inhibitor to depleted rats would prevent such resynthesis. About 800 rats were used in the total study. A study was made of the effect of varying repeated doses of caffeic acid as the sodium salt on the resynthesis of adrenaline in rats after insulin depletion. A definite inhibitory effect was seen of doses from 5 to 50 mg/kg given intraperitoneally every 6 hours for 48 hours, and this was proportional to the dose. The inhibition by 50 mg/kg doses was 4-fold. The experiment was not repeated with more potent inhibitors, since it was found subsequent to these experiments, that detoxication occurred in less than an hour with the usual effective doses of inhibitors (see Table 2).

No effect of the inhibitors has been seen on the fatigability of the nictitating membrane response to constant electrical stimulation of the postganglionic superior cervical sympathetic nerve in the cat, which one might expect if the stores of noradrenaline at the nerve endings were released and prevented from replenishment by synthesis from precursors. Presumably it is difficult if not impossible to exhaust the pool even in the presence of the inhibitor. Similar studies are planned on rabbits whose stores of nerve noradrenaline have been depleted with reserpine and related releasers of central and peripheral nerve catecholamines.

Our results on dopa decarboxylation *in vivo* have been confirmed by Westermann *et al.* (105), by Dengler and Reichel (21, 21a; *cf.* Holtz, THIS SYMPOSIUM), using α-methyldopa, which Sourkes (87) and Hartman *et al.* (37) had found to inhibit *in vitro*, although poorly (37).[7] The results also have been confirmed by Yuwiler *et al.* (106), using some of the inhibitors studied by Hartman *et al.*, as well as some cinnamoyl analogues of 5HTP.

Summary

Studies relating to the inhibition *in vitro* are reviewed, with some emphasis on inactivation of coenzyme by the substrate and its analogues, and on carbonyl

[7] Preliminary comparative bioassays, illustrated in Table 4, indicate that α-methyldopa is of the same order of activity as 5-(3-hydroxycinnamoyl)salicylic acid.

trapping agents, but more specifically on analogues of certain hydroxycinnamic acids which inhibit dopa-, dops- and 5-HTP-decarboxylases. The structural features of such inhibitors are analyzed, as well as their specificity and competitive nature.

The nature and characteristics of dopa decarboxylation *in vivo* are discussed, particularly as affected by the absence of visceral organs, and by the thyroid hormone, adrenal cortex, sex and vitamin B_6.

A review and analysis is made of the work pointing to changes in apoenzyme and coenzyme caused by hyperthyroidism and vitamin B_6 deficiency.

Chemical and pharmacological evidence is presented which shows that the substances which inhibit dopa decarboxylase *in vitro* also are generally effective *in vivo*. Twenty-five *in vitro* inhibitors and 50 structurally related noninhibitors have been assessed in several hundred animals. This has led to an understanding of the structural requirements for effective inhibition *in vivo*, which are essentially the same as for inhibition *in vitro*.

A possible alternate biosynthetic route to noradrenaline and adrenaline may be by transamination of hydroxyphenylpyruvic acids and subsequent decarboxylation of the resulting amino acids, which is blocked by the inhibitors at the decarboxylation step.

The duration of inhibition and the detoxication of the inhibitors have been studied, and various structural modifications and pharmacological procedures have been investigated for prolonging the duration of the inhibition.

One inhibitor was shown to inhibit the biosynthesis of adrenal medullary adrenaline in insulin-treated rats.

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