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The effect of tropolone on the formation of 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid in the brain of the mouse

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1. The development of a very sensitive and specific fluorimetric assay for 3,4-dihydroxyphenylacetic acid has made it possible to measure how inhibitors of the enzyme catechol-O-methyl transferase affect the relative concentrations of this acid and its O-methylated derivative 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) in the brains of mice treated with L-3,4-dihydroxyphenylalanine or probenecid.

2. It was found that tropolone and tropolone-4-acetamide reduce the concentration of homovanillic acid in the brains of the treated mice to an extent dependent on the dose.

3. The concentration of 3,4-dihydroxyphenylacetic acid in the brain was increased by the administration of tropolone or tropolone-4-acetamide but the dose and response were not simply related to one another.

4. The results suggest that, *in vivo*, the formation of 3,4-dihydroxyphenylacetic acid is not always a simple alternative to the formation of homovanillic acid when the enzyme catechol-O-methyl transferase is inhibited.

The ability of some compounds to inhibit the enzyme catechol-O-methyl transferase (COMT) in the central nervous system *in vivo* has been studied by Carlsson, Lindqvist, Fila-Hromadko & Corrodi (1962), and by Carlsson, Corrodi & Waldeck (1963), who found that such compounds cause a fall in the concentration of 4-hydroxy-3-methoxyphenylethylamine (methoxytyramine) in the brains of mice treated with L-3,4-dihydroxyphenylalanine (L-DOPA) and a monoamine oxidase inhibitor. The activity of the enzyme in the brains of living mice has also been measured by Ross & Haljasmaa (1964a,b) with a radioisotopic method.

The development of a very sensitive specific fluorimetric assay for 3,4-dihydroxyphenylacetic acid (Sharman, Poirier, Murphy & Sourkes, 1967) has enabled us to study the ability of drugs to inhibit catechol-O-methyl transferase in the central nervous system. To do this we have observed the effects of such drugs on the relative concentrations of 3,4-dihydroxyphenylacetic acid and its methylated derivative homovanillic acid, in the brains of mice treated with L-DOPA or probenecid.

Methods

The following chemicals and reagents were used: homovanillic acid (Calbiochem); 3,4-dihydroxyphenylacetic acid (L. Light & Co. Ltd.; recrystallized from benzene); L-3,4-dihydroxyphenylalanine (L-DOPA; Calbiochem); Tris (hydroxymethyl) aminomethane (Tris; L. Light & Co. Ltd.); L-cysteine hydrochloride (Hopkin & Williams Ltd.; recrystallized from ethyl alcohol); Tropolone (2-hydroxy-2:4:6-cycloheptatriene-1-one; Aldrich Chemical Co. Inc.; recrystallized from light petroleum); Tropolone-4-acetamide (A. B. Hässle; recrystallized from dilute acetic acid); 1,2-diaminoethane distilled three times and kept at 4° C; *n*-butyl acetate distilled once and washed once with water. All other reagents and chemicals were of analytical reagent quality except for hydrochloric acid which was of microanalytical reagent quality. Glass distilled water was used throughout.

Female albino mice weighing 15–35 g were used. Drugs were injected intraperitoneally, dissolved in 0·9% (w/v) sodium chloride solution, except for L-DOPA which was dissolved in sterile distilled water.

Dissection and extraction of tissues

The mice were stunned and then decapitated. The brain was rapidly removed and placed on a glass plate in an ice bath. In the experiments in which DOPA was injected, the brain stem was divided at the posterior border of the hypothalamus and all of the tissue rostral to the section, but excluding the olfactory lobes, was extracted. The tissue from one mouse was sufficient for the analysis of both dihydroxyphenylacetic acid and homovanillic acid. In the investigations on normal mice, the striatal region was dissected out (Sharman, 1966) and the tissue from three mice combined for the estimation of both the acids. The tissue samples were homogenized in 2 ml. of ice cold 0·1 N hydrochloric acid in a cooled, all glass homogenizer. The homogenate was transferred to a polypropylene or cellulose nitrate centrifuge tube (7 ml. capacity) and the homogenizer washed twice with 1 ml. of ice cold distilled water, the washings being combined and mixed with the homogenate. The centrifuge tube was then dipped into liquid nitrogen so that the homogenate was frozen. The frozen homogenate was placed in a refrigerator at 4° C until all the tissue samples to be extracted had been homogenized. The homogenates were then allowed to thaw at room temperature and concentrated perchloric acid (0·12 ml.; specific gravity, 1·72) was added to and mixed with each sample. Solid potassium chloride was then added in an amount just in excess of that required to saturate the homogenate, which was then thoroughly mixed. This procedure removes the perchlorate ion as insoluble potassium perchlorate while leaving the solution acid. The procedure is endothermic and this helps to keep the homogenate cold, also ensuring a good precipitation of the potassium perchlorate. The homogenate was then centrifuged at 0° C for 5 min with a radial acceleration at the tip of the tube of 147,000 m sec^{-2} (15,000 *g*). The clear supernatant was transfered to a glass stoppered test tube (15 ml. capacity) and a few crystals of potassium chloride were added to ensure that the solution was saturated. *n*-Butyl acetate (10 ml.) was added and the tube shaken by hand for 5 min. The tube was centrifuged at room temperature for 1 min to separate the two layers and two portions (4·5 ml. each) of the *n*-butyl acetate extract were taken. One portion was added to 2 ml. of Tris solution (6 g/l. in distilled water) contained in a glass stoppered test tube (10 or 15 ml. capacity). The other was added to a 2·2 ml. portion of a mixture of 35 ml. water,

1 ml. 2 N hydrochloric acid and 1·5 ml. of 1,2-diaminoethane, also in a glass stoppered test tube. The tubes were cooled in ice, shaken for 3 min and then centrifuged for 1 min. The butyl acetate layers were discarded. Homovanillic acid was estimated in the Tris extract and dihydroxyphenylacetic acid in the 1,2-diaminoethane extract.

Estimation of homovanillic acid

Homovanillic acid was estimated fluorimetrically essentially as described by Andén, Roos & Werdinius (1963). Three portions (0·6 ml. each) of the Tris extract were taken. To one of them was added a known amount of homovanillic acid (usually 0·1 μg) and to another 0·2 ml. of a freshly prepared, solution of cysteine (1 mg/ml.). The latter tube served as a blank and the former enabled the development of the fluorescence to be checked and gave a standard for the calculation of the amount of homovanillic acid present. Potassium ferricyanide (1 ml. of 20 mg/l. in 5 N ammonium hydroxide) was then added to each tube and mixed. After exactly 4 min, 0·2 ml. of the cysteine solution was added to the two tubes which contained no cysteine and the contents of all tubes were mixed thoroughly. The fluorescence of the solutions was then measured in an Aminco-Bowman spectrophotofluorometer. The activating light wavelength was 315 mμ and the fluorescence light wavelength was 430 mμ. An interference filter with maximum transmission at 426·2 mμ and a band width of 9·9 mμ and 44% transmission (Grubb Parsons type G.P.) was placed in the fluorescent light path. Slits 5 mm wide were placed, immediately adjacent to the cuvette, in both the activating and fluorescent light paths. This arrangement yielded the smallest blank readings in comparison with the fluorescence developed from homovanillic acid.

Estimation of 3,4-dihydroxyphenylacetic acid

Two samples (1·0 ml. each) of the 1,2-diaminoethane extract were taken. To one of them was added a known amount of authentic 3,4-dihydroxyphenylacetic acid (usually 0·1 μg in a volume of 0·01 ml.). The solutions were then heated in a water bath at 60° C for 20 min in the dark. At the end of this time the tubes were cooled in an ice bath and hydrochloric acid (0·3 ml. of a 1:1 v/v dilution of concentrated hydrochloric acid (36% w/v) in distilled water) was added and mixed. The tubes were left in the ice bath for 10 min and neutralized by the addition of 1,2-diaminoethane (0·3 ml. of a 1:9 v/v dilution in distilled water). The fluorescence of the solution was immediately measured in an Aminco-Bowman spectrophotofluorometer. The activating light wavelength was 385 mμ and the fluorescence light wavelength was 450 mμ (uncorrected for any errors in the instrument). A Corning 3389 glass filter was placed in the fluorescence light path.

Recovery experiments

The recovery of authentic homovanillic acid and 3,4-dihydroxyphenylacetic acid was tested by adding amounts ranging from 0·1 μg to 2 μg to tissue homogenates and extraction and estimation as described above. For homovanillic acid the mean recovery was 70% ±2·3% (S.E.M.), in eighteen tests, and for 3,4-dihydroxyphenylacetic acid the mean recovery was 65% ±1·6% (S.E.M.), in seventeen tests.

Results

Notes on the fluorimetric assay of 3,4-dihydroxyphenylacetic acid

The present method for the estimation of this acid is an improvement on previous methods in two respects. First, the fluorescence intensity of the derivative is higher and second, the reaction is more specific for 3,4-dihydroxyphenylacetic acid.

The changes in the fluorescence characteristics are illustrated in Fig. 1. The yellow fluorescence (maximum wavelength 540 mμ) which develops when 3,4-dihydroxyphenylacetic acid is condensed with 1,2-diaminoethane (Valk & Price, 1956) disappears on acidification and is replaced by a green fluorescence of lower intensity at a shorter wavelength (490 mμ when activated at 395 mμ). As the solution is neutralized with ethylene diamine, this latter fluorescence at first increases in intensity, and then a second, more intense, blue fluorescence suddenly appears (maximum at 450 mμ ; wavelength of maximum activation, 385 mμ). As more of the base is added, the fluorescence at 490 mμ gradually decreases and eventually disappears, but the intensity of the fluorescence at 450 mμ is only slightly increased. The conditions selected were those which, in model experiments, were found to give reproduceable results.

The fluorescence at 450 mμ appears to be specific for 3,4-dihydroxyphenylacetic acid and some closely related compounds which have an acid function. For example, if the reaction is carried out on 3,4-dihydroxyphenylalanine (DOPA) or α-methyl-3,4-dihydroxyphenylalanine (α-methyl-DOPA) very little fluorescence is developed.

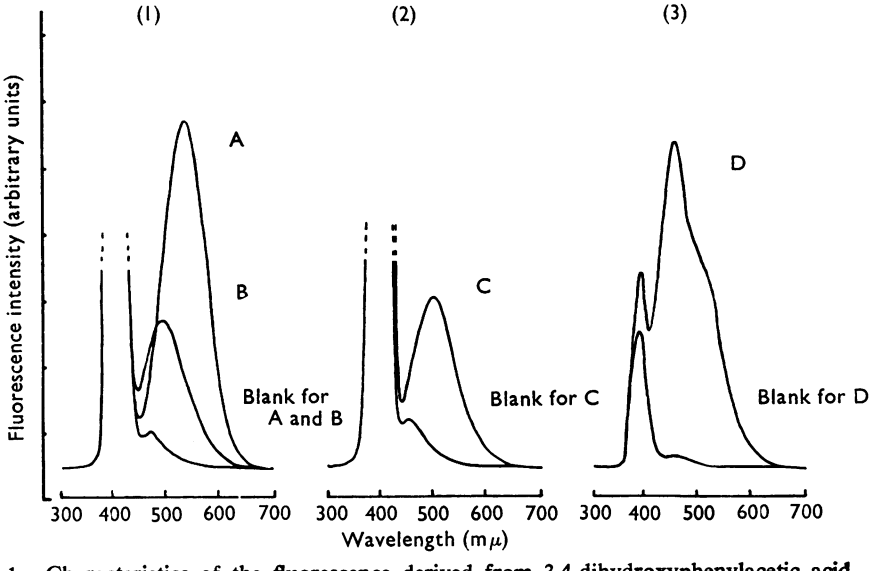

FIG. 1. Characteristics of the fluorescence derived from 3,4-dihydroxyphenylacetic acid. The reaction was carried out as described in the text using 0·2 μg of the acid. Where necessary the volume of the sample was adjusted to 1·6 ml. with water for each scan. (1) The characteristics of the fluorescence derived from 3,4-dihydroxyphenylacetic acid when activated at 405 mμ. (A) After condensation with 1,2-diaminoethane ; (B) After the addition of hydrochloric acid. (2) Activation wavelength 385 mμ. (C) As 1 (B). (3) Activation wavelength 385 mμ. (D) After the addition of 1,2-diaminoethane solution. The scans in (3) were recorded at one tenth of the amplification used for 1 and 2.

If these two amino-acids are first acetylated to give the O,O,N-triacetyl derivatives, converting the amino group to a neutral amide group, then a blue fluorescence develops. 3,4-Dihydroxymandelic acid yields only a very small amount of fluorescence so that this acid will not interfere with the assay of 3,4-dihydroxyphenylacetic acid.

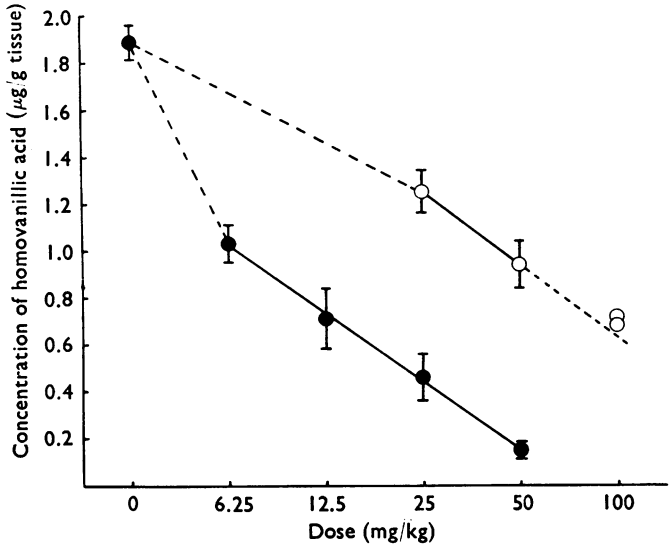

FIG. 2. Effect of tropolone and tropolone-4-acetamide on the concentration (±S.E.M.) of homovanillic acid on the brains of mice treated with L-DOPA. ●——●, Mice treated with L-DOPA (100 mg/kg) and tropolone for 1 hr; ○——○, mice treated with L-DOPA (100 mg/kg) and tropolone-4-acetamide for 1 hr.

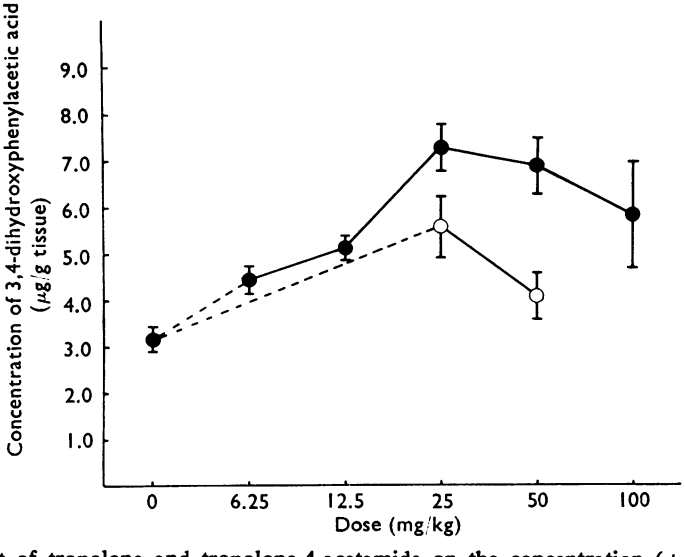

FIG. 3. Effect of tropolone and tropolone-4-acetamide on the concentration (±S.E.M.) of 3,4-dihydroxyphenylacetic acid in the brains of mice treated with L-DOPA. ●——●, Mice treated with L-DOPA (100 mg/kg) and tropolone for 1 hr. ○——○, mice treated with L-DOPA (100 mg/kg) and tropolone-4-acetamide for 1 hr.

The method, in common with many other fluorimetric methods, suffers from the difficulty of determining accurately the blank fluorescence. The fluorescence derived from extracts of cerebral cortex of rabbits and mice was slightly higher than that derived from the reagents alone. This increased fluorescence was found to be equivalent to 3,4-dihydroxyphenylacetic acid in a concentration of 0·03 μg/g tissue when approximately 0·5 g of brain tissue was extracted. In the present experiments the reagent blank has been used in the calculation of the concentration of 3,4-dihydroxyphenylacetic acid in the tissues from experiments in which L-DOPA was administered, because the error introduced by doing this is negligible. In the estimation of the endogenous 3,4-dihydroxyphenylacetic acid, however, the values might be over-estimated by about 10% if the reagent blank is used. It has not been proved that the fluorescence derived from the cortex is due to the presence of 3,4-dihydroxyphenylacetic acid, so a correction for the fluorescence from the tissue has been applied to these estimates.

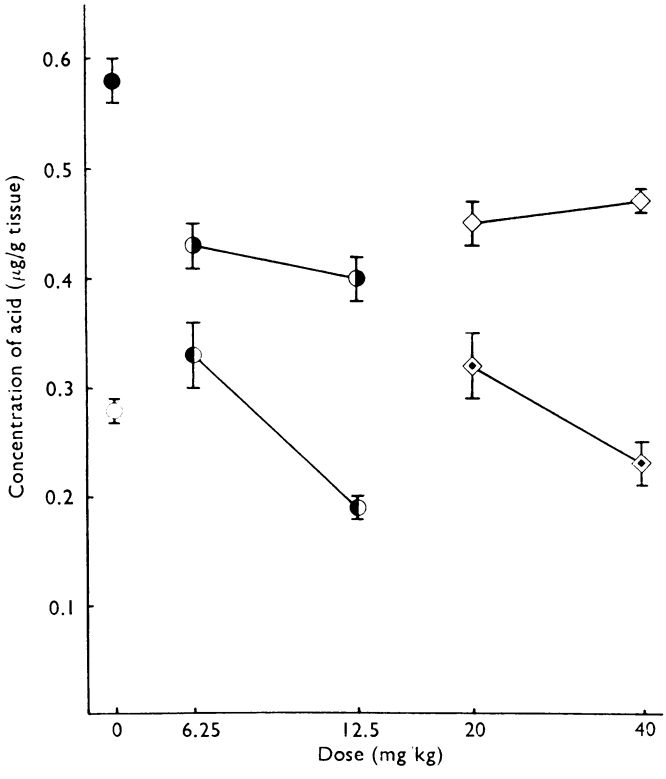

FIG. 4. Effect of tropolone and tropolone-4-acetamide on the concentrations (±S.E.M.) of homovanillic acid and 3,4-dihydroxyphenylacetic acid in the striata of mice treated with probenecid. All mice received probenecid (200 mg/kg intraperitoneally) and were killed after 1·5 hr. ●, Control homovanillic acid; ◐——◐, homovanillic acid after tropolone; ◈——◈, homovanillic acid after tropolone-4-acetamide; ○, control 3,4-dihydroxyphenylacetic acid; ◑——◑, 3,4-dihydroxyphenylacetic acid after tropolone; ◇——◇, 3,4-dihydroxyphenylacetic acid after tropolone-4-acetamide. The two regression lines for the fall in the concentration of homovanillic acid with the two drugs do not deviate significantly from parallel.

Effect of tropolone and tropolone-4-acetamide on the concentration of homovanillic acid and 3,4-dihydroxyphenylacetic acid in the mouse brain

After treatment with L-DOPA

Two groups of mice were injected with L-DOPA (100 mg/kg intraperitoneally), followed immediately by an intraperitoneal injection of tropolone or tropolone-4-acetamide in the test group. One hour later the mice were killed, the brains removed and analysed for their content of homovanillic acid and 3,4-dihydroxyphenylacetic acid. The effect of different doses of the two tropolone compounds on the concentration of homovanillic acid is shown in Fig. 2 and the effect on the concentration of 3,4-dihydroxyphenylacetic acid is shown in Fig. 3.

Figure 2 shows that both drugs reduce the concentration of homovanillic acid, 1 hr after the administration of L-DOPA, and that the reduction bears a linear relation to the logarithm of the dose of the drug used. Figure 3 shows that the concentration of 3,4-dihydroxyphenylacetic acid at first increases with increasing doses of tropolone, but that with the higher doses of both tropolone and tropolone-4-acetamide the concentration of the acid tends to fall. A comparison of the abilities of tropolone and tropolone-4-acetamide to reduce the concentration of homovanillic acid shows that, on a weight basis, tropolone is 6·5 times as active as tropolone-4-acetamide. No homovanillic acid could be detected when tropolone was injected in a dose of 100 mg/kg or more.

In normal mice treated with probenecid

Normal mice were injected with tropolone or tropolone-4-acetamide intraperitoneally. Immediately afterwards, they were given probenecid (200 mg/kg intraperitoneally) to prevent the egress of acid metabolites from the brain (Neff, Tozer & Brodie, 1964 ; Sharman, 1966).

The animals were killed 1·5 hr later and the striata dissected out and analysed for homovanillic acid and 3,4-dihydroxyphenylacetic acid. The striata from mice treated only with probenecid (200 mg/kg intraperitoneally ; 1·5 hr previously) were examined at the same time. The experiment was designed as a four-point assay to compare the effects of the two tropolone compounds on the concentrations of the two acid metabolites. The result is illustrated by Fig. 4 which shows that both tropolone and tropolone-4-acetamide reduce the concentration of homovanillic acid in mice treated with probenecid and that tropolone is 3·5 times as active as tropolone-4-acetamide in this test.

Discussion

The enzyme, catechol-O-methyl transferase (COMT), is of importance in the metabolism of dopamine in the central nervous system because the major metabolic product of this amine in the brain appears to be homovanillic acid. The inhibition of this enzyme by tropolone derivatives was postulated and confirmed by Belleau & Burba (1961, 1963), who also showed that these substances could block sympathetic β-receptors, though this observation was not confirmed by Ross & Haljasmaa (1964b).

Tropolone and related compounds have been used in studying the effect of inhibition of COMT on the metabolism of radioactive catecholamines in the brain (Goldstein, 1964), but there have been few comparisons of the ability of different

substances to inhibit the enzyme in the central nervous system of living animals. Carlsson, Corrodi & Waldeck (1963) examined a series of compounds, including tropolone-4-acetamide, by determining the dose of each substance which would reduce the cerebral concentration of 4-hydroxy-3-methoxyphenylethylamine (methoxytyramine), without reducing the cerebral concentration of dopamine, in mice treated with L-DOPA and the monoamine oxidase inhibitor, nialamide. Ross & Haljasmaa (1964b) have measured the COMT activity in supernatants obtained after centrifuging homogenates of brains obtained from mice treated with tropolone derivatives and other compounds. A dose of tropolone-4-acetamide 500 mg/kg was required for these effects. This dose was found by Carlsson, Corrodi & Waldeck (1963) to reduce the concentration of methoxytyramine to below 12% of its normal value and by Ross & Haljasmaa (1964b) to reduce the COMT activity in brain extracts by 50%. The present experiments show that tropolone-4-acetamide prevents the accumulation of homovanillic acid after L-DOPA, presumably as a result of the inhibition of COMT, at dose levels much lower than 500 mg/kg, and that tropolone itself is more active than tropolone-4-acetamide.

In mice treated with L-DOPA, the administration of small doses of tropolone causes a reduction in the concentration of homovanillic acid with a concomitant increase in the concentration of 3,4-dihydroxyphenylacetic acid. The increase in the latter is larger than the decrease in the former and suggests that the rate constant for the removal of 3,4-dihydroxyphenylacetic acid from mouse brain is smaller than that for the removal of homovanillic acid; in other words, it is more difficult for 3,4-dihydroxyphenylacetic acid to get out of the mouse brain than it is for homovanillic acid. After higher doses of tropolone derivatives there is apparently some inhibition of the accumulation of 3,4-dihydroxyphenylacetic acid. The tropolone compounds will inhibit the hydroxylating enzymes involved in the biosynthesis of catecholamines, tropolone-4-acetamide being less active than α-propyldihydroxyphenylacetamide (Carlsson *et. al.*, 1963), but there is no evidence to show that they will inhibit DOPA-decarboxylase, the enzyme involved in the formation of dopamine from L-DOPA. It may be that the larger doses of tropolone interfere with the transport of L-DOPA or its metabolites in the brain. The tropolones can also chelate divalent metal ions (Bryant, Fernelius & Douglas, 1953) and thus the higher concentrations may interfere with many metabolic reactions and processes.

It can be concluded that observations on cerebral metabolism of catecholamines made *in vivo* with doses of tropolone compounds in excess of 100 mg/kg should not, in the mouse, be referred simply to the inhibition of COMT.

The present results show that quite small doses of tropolone and tropolone-4-acetamide will reduce the concentration of homovanillic acid in the striatum of normal mice, not treated with L-DOPA, but given probenecid to prevent the active transport of acid metabolites out of the brain. The reduction in the concentration of homovanillic acid again, is directly proportional to the logarithm of the dose of the COMT-inhibitor. In this test, tropolone-4-acetamide was more active relative to tropolone than in the first test, and it can be inferred that the test using probenecid-treated mice gives a better assessment of the inhibition of COMT at the sites where it is normally acting. In this experiment the increase in 3,4-dihydroxyphenylacetic acid was smaller than the fall in homovanillic acid and was similar for all the doses of the tropolones. This is unlikely to be a result of an accelerated removal of 3,4-dihydroxyphenylacetic acid because of the probenecid treatment, which was intended to

block the active transport of acid metabolites from the brain. The finding suggests that in the normal mouse the formation of 3,4-dihydroxyphenylacetic acid is not a simple alternative to the formation of homovanillic acid in the metabolism of dopamine in the striatum, but if the test is to be used as a method for examining the ability of compounds to inhibit catechol-O-methyl transferase, there should be no decrease below the normal concentration of 3,4-dihydroxyphenylacetic acid, because this would indicate an interference in the metabolism of dopamine elsewhere in the metabolic pathway.

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REFERENCES

ANDÉN, N.-E., ROOS, B.-E. & WERDINIUS, B. (1963). On the occurrence of homovanillic acid in brain and cerebrospinal fluid and its determination by a fluorometric method. *Life Sci., Oxford*, **2**, 448–458.

BELLEAU, B. & BURBA, J. (1961). Tropolones: a unique class of potent non-competitive inhibitors of S-adenosylmethionine-catechol methyl transferase. *Biochim. biophys. Acta*, **54**, 195–196.

BELLEAU, B. & BURBA, J. (1963). Occupancy of adrenergic receptors and inhibition of catechol-O-methyl transferase by tropolones. *J. Med. Chem.*, **6**, 755–759.

BRYANT, B. E., FERNELIUS, W. C. & DOUGLAS, B. E. (1953). Formation constants of metal complexes of tropolone and its derivatives. I. Tropolone. *J. Am. chem. Soc.*, **75**, 3784–3786.

CARLSSON, A., CORRODI, H. & WALDECK, B. (1963). α-Substituierte Dopacetamide als Hemmer der Catechol-O-methyl-transferase und der enzymatischen Hydroxylierung aromatischer Aminosäuren. In den Catecholamin-Metabolismus eingreifende Substanzen. 2. Mitteilung. *Helv. chim. Acta*, **46**, 2271–2285.

CARLSSON, A., LINDQVIST, M., FILA-HROMADKO, S. & CORRODI, H. (1962). Synthese von Catechol-O-methyl-transferase-hemmenden Verbindungen. In den Catecholamin-Metabolismus eingreifende Substanzen. 1. Mitteilung. *Helv. chim. Acta*, **45**, 270–276.

GOLDSTEIN, M. (1964). Cerebral metabolism of DOPA-C^{14} in rats. *Int. J. Neuropharmac.*, **3**, 37–43.

NEFF, N. H., TOZER, T. N. & BRODIE, B. B. (1964). Indole metabolism Part II. A specialised transport system to transfer 5-HIAA directly from brain to blood. *Pharmacologist*, **6**, 194.

ROSS, S. B. & HALJASMAA, Ö. (1964a). Catechol-O-methyl transferase inhibitors. *In vitro* inhibition of the enzyme in mouse brain extract. *Acta pharmac. tox.*, **21**, 205–214.

ROSS, S. B. & HALJASMAA, Ö. (1964b). Catechol-O-methyl transferase inhibitors. *In vivo* inhibition in mice. *Acta pharmac. tox.*, **21**, 215–225.

SHARMAN, D. F. (1966). Changes in the metabolism of 3,4-dihydroxyphenylethylamine (dopamine) in the striatum of the mouse induced by drugs. *Br. J. Pharmac. Chemother.* **28**, 153–163.

SHARMAN, D. F., POIRIER, L. J., MURPHY, G. F. & SOURKES, T. L. (1967). Homovanillic acid and dihydroxyphenylacetic acid in the striatum of monkeys with brain lesions. *Can. J. Physiol. Pharmac.*, **45**, 57–62.

VALK, A. de T. JR. & PRICE, H. L. (1956). The chemical estimation of epinephrine and norepinephrine in human and canine plasma. 1. A critique of the ethylene diamine condensation method. *J. clin. Invest.*, **35**, 837–841.

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