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THE DECARBOXYLATION OF AMINO ACIDS RELATED TO TYROSINE AND THEIR AWAKENING ACTION IN RESERPINE-TREATED MICE

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Recent work on the formation of catechol amines in mammals has shown that the amino acid L-DOPA (β-3:4-dihydroxy-L-phenylalanine) is the immediate precursor of adrenaline, noradrenaline and dopamine (β-3:4-dihydroxyphenylethylamine). DOPA is decarboxylated by DOPA decarboxylase, an enzyme present in many tissues, including chromaffin and nervous tissue (see Blaschko, 1959).

Although the molecule of DOPA is believed to be devoid of intrinsic pharmacological activity in intact animals, it has *in vivo* some sympathomimetic actions which are believed to be due to the catechol amines produced by the decarboxylation of L-DOPA. Also, in mice and monkeys tranquillized by reserpine, large doses of DOPA have a remarkable awakening effect (Carlsson, Lindqvist & Magnusson, 1957; Everett & Toman, 1959), caused probably by the catechol amines which are formed from it.

Much information is available on the substrate specificity of DOPA decarboxylase. This enzyme acts not only on 3:4-DOPA, presumably its natural substrate, but also on a number of other amino acids, mostly hydroxylated derivatives of phenylalanine. These observations, summarized elsewhere (Blaschko, 1950; Sourkes, 1955), have shown that the 2:5- and the 2:3-isomers of DOPA are readily decarboxylated by preparations of rat and guinea-pig tissues. L-Tyrosine is not decarboxylated, but its *meta*-hydroxy analogue (hereafter called metatyrosine) is a substrate of the mammalian enzyme, and it has recently been shown that in normal mice, rats, rabbits and dogs metatyrosine has excitatory actions, which are also thought to be due to the amine formed by the decarboxylation of the amino acid (Mitoma, Posner, Bogdanski & Udenfriend, 1957).

In the present study we have first tested the substrate specificity of the DOPA decarboxylase in the mouse and then examined a number of amino acids related to 3:4-DOPA for their awakening action in mice tranquillized

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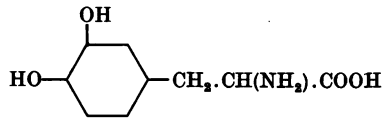
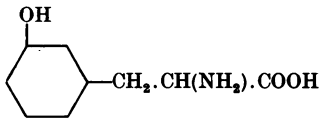
by reserpine, including β-3:4-dihydroxyphenylserine, since this amino acid is slowly decarboxylated by the mammalian enzyme (Blaschko, Burn & Langemann, 1950).

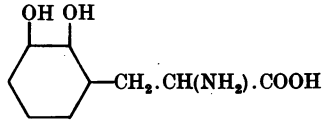
The formulae of the amino acids tested are shown below.



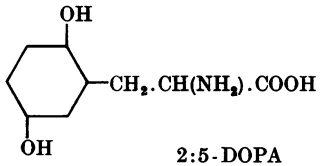
3:4-DOPA



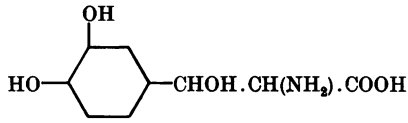
Metatyrosine



2:3-DOPA



2:5-DOPA



3:4-Dihydroxyphenylserine

METHODS

Substances used. The sample of *m*-hydroxy-DL-phenylalanine (metatyrosine) was that prepared by Dr H. R. Ing and used in an earlier study (Blaschko, Holton & Sloane Stanley, 1949); 2:5-dihydroxy-DL-phenylalanine was kindly given to us by Messrs Merck, Sharp and Dohme; 2:3-dihydroxy-DL-phenylalanine was a specimen prepared and previously used by Blaschko & Langemann (1951). The *threo*-3:4-dihydroxyphenyl-DL-serine, from Farbwerke Höchst, was a gift from Professor H. J. Schümann, Frankfurt a.M. The reserpine, given by Dr H. J. Bein, Basel, was dissolved in 20% ascorbic acid, to give a 0·5% stock solution which was further diluted for the injection.

Manometric determination of the DOPA decarboxylase activity. Mouse liver, kidney or brain were ground with sand in a cooled mortar and 0·067 M sodium phosphate buffer of pH 6·5 was added, 1 ml. for each gram of fresh tissue. The suspension was centrifuged at 27,000 *g* for 20 min and the slightly opaque supernatant fluid was used in the experiments.

The manometric procedure was similar to that described earlier (Blaschko, 1942), with 1·6 ml. of fluid in the main compartments of the conical manometer flasks and 0·4 ml. in the side bulbs. Initial substrate concentration after tipping was 2×10^{-3} M with L-amino acids as substrates, and 4×10^{-3} M with DL-amino acids. The temperature was 37·5° C, and the gas phase N_2. In some of the experiments 25 μg of the calcium salt of pyridoxal-5-phosphate was added to each flask in order to compare the rate of the enzymic reaction in the presence and absence of excess of co-decarboxylase. For the calculation of enzymic activity,

the initial linear part of the reaction was used; activities are expressed in terms of q_{CO_2}, i.e. μl. CO_2 formed by 1 mg of fresh tissue in 1 hr. In most experiments, the CO_2 retention was determined at the end of the experiment, but in the experiment with brain extract this was omitted.

Registration and evaluation of motor activity. We are indebted to Messrs D. Groves and O. B. Saxby, of this Department, for the construction of the cage used in the recording of motor activity of mice. The principle of the apparatus is shown in Fig. 1. A polystyrene box, $5 \times 5 \times 2\frac{3}{4}$ in. (12·7 × 12·7 × 7 cm), with a perforated lid, stands on a Perspex platform supported by four brass rods (diameter $\frac{1}{16}$ in. (1·6 mm), length $5\frac{1}{2}$ in. (14 cm)). A central rod is connected by a C-shaped spring wire, to a Rochelle salt crystal, and the output from the crystal is amplified in a two-stage amplifier and fed to a Kelvin–Hughes pen writing on a smoked drum. This arrangement responds to both horizontal and vertical movements of the animal.

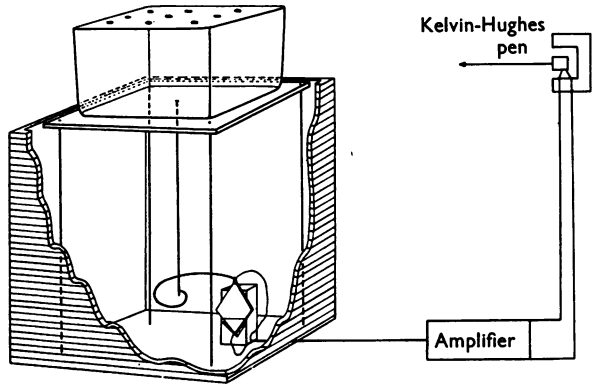


Fig. 1. Apparatus for recording motor activity of a mouse.
For details see text.

For the evaluation of the results the tracing was varnished and a given length of it, for example, one equivalent to 10 min, was selected for measurement of its light transmittance with a selenium photo-electric cell. Results were expressed as percentage of normal activity, that is the mean of several readings taken from the same animal before the application of any drug.

RESULTS

Decarboxylation of phenylalanine derivatives by mouse tissue extracts

The DOPA decarboxylase activity was studied by using L-DOPA as substrate, both with and without added pyridoxal-5-phosphate. The results of these experiments on liver, kidney and brain are shown in Table 1.

It can be seen that the enzymic activity was highest in the liver and much less in the kidney and brain extracts. Using rat tissue extracts without added pyridoxal phosphate, Sloane Stanley (1949) found a mean q_{CO_2} for liver of 0·69 and for kidney of 0·07; in the mouse the corresponding figures are 0·51 for liver and 0·07 for kidney. In the mouse brain without added pyridoxal phosphate the q_{CO_2} was 0·06.

The addition of pyridoxal-5-phosphate (co-decarboxylase) *in vitro* did not significantly alter the rate of decarboxylation of L-DOPA by liver extracts but with kidney extracts the decarboxylation was significantly speeded up in most of the experiments, indicating that in the presence of added pyridoxal-5-phosphate the concentration of free co-decarboxylase was a rate-limiting factor (see Table 2). Using ox brain, Holtz & Westermann (1956) found an increase in the rate of decarboxylation when pyridoxal phosphate was added, but in our experiment with mouse brain there was no increase.

TABLE 1. DOPA decarboxylase activity of mouse tissue extracts. Substrate: 2×10^{-3} M L-DOPA (no pyridoxal phosphate added). Enzymic activity expressed as q_{CO_2} (μl. CO_2 formed by 1 g fresh tissue in 1 hr).

Tissue	No. of expts.	Mean value of q_{CO_2}
Liver	9	$0{\cdot}51 \pm 0{\cdot}16$
Kidney	7	$0{\cdot}07 \pm 0{\cdot}015$
Brain	3	$0{\cdot}06 \pm 0{\cdot}029$

TABLE 2. Effect of adding pyridoxal-5-phosphate on enzymic decarboxylation of L-DOPA in mouse tissue extracts

Tissue	Pyridoxal-5-phosphate omitted q_{CO_2}	Pyridoxal-5-phosphate added q_{CO_2}
Liver	0·52	0·54
	0·52	0·56
Kidney	0	0·13
	0·11	0·20
	0·12	0·13
	0·04	0·09
	0·03	0·04
Brain	0·09	0·03

No CO_2 formation occurred when L-DOPA was added to extracts of mouse spleen or heart (ventricles).

Substrate specificity of mouse liver decarboxylase. Since the liver was the most active source of enzyme in the mouse, we have used extracts of this tissue to study the decarboxylation of the other amino acids which was compared with that of L-DOPA.

Metatyrosine was readily decarboxylated by the mouse-liver enzyme, with a mean rate of 0·95, only slightly less than that of L-DOPA. The other two isomeric forms of dihydroxyphenylalanine were also readily decarboxylated, 2:3-DOPA at 0·87 times, and 2:5-DOPA at 0·91 times the rate of L-DOPA. These results show that the mouse-liver enzyme resembles that in rat liver and guinea-pig kidney (Blaschko *et al.* 1949; Blaschko & Langemann, 1951).

It may be mentioned here that earlier observations have not revealed any difference in the rates of decarboxylation of L-DOPA and DL-DOPA; it therefore seems likely that the rates of CO_2 formation from the racemic amino acids used in the present study represent the true rates of decarboxylation of the L-isomers.

Awakening effects of phenylalanine derivatives in mice

Normal animals

The intraperitoneal injection of 500 or 1000 mg/kg of L-DOPA in normal mice led to an increased activity of the animals for 1–4 hr, proportional to the dose administered. With 500 mg/kg the animals showed an increase in their normal motor activity and in their respiratory rate. With 1000 mg/kg of L-DOPA, over-activity was much more evident. About 30 min after the injection there was a great change in the behaviour of the animals: they jumped; they fought with each other; when touched, they attacked. Two of the animals went into convulsions. This over-activity lasted for about 4 hr. All the animals survived.

The effect of the amine oxidase inhibitor, iproniazid, was also studied by Carlsson *et al.* (1957). In agreement with their findings, an enhancement of the action of L-DOPA was observed after an injection of iproniazid. In one experiment, ten animals each received 500 mg/kg of L-DOPA alone. In another group of ten this was preceded by an injection of 100 mg/kg of iproniazid intraperitoneally. Five of these animals received the iproniazid 1 hr, the other five 2 hr, before the injection of L-DOPA. In all the iproniazid-treated animals the increase of motor activity was more pronounced and after about 2 hr the mice appeared to be exhausted. Of the ten mice treated with iproniazid, three died in the following 12 hr, whereas all the animals treated with L-DOPA alone survived.

Injections of D-DOPA (500 mg/kg) did not cause any increase in motor activity; if anything, the activity of the treated animals was less than that of the control animals.

Neither 2:3-DOPA nor 2:5-DOPA had an effect on motor activity in a dose of 500 mg/kg, but with 1000 mg/kg of 2:3-DOPA motor activity was diminished.

Metatyrosine (500–1000 mg/kg) caused an increase in motor activity of the mice similar to that caused by L-DOPA, although of somewhat less intensity. Like the response to L-DOPA, it started after about 30 min and lasted for 2–4 hr, as described by Mitoma *et al.* (1957).

Animals treated with reserpine

The effect of L-DOPA. The plan of the experiments was similar to that of Carlsson *et al.* (1957). To determine the appropriate dose of reserpine preliminary experiments were carried out with 10, 20, 30 and 40 mg/kg,

respectively, of reserpine, administered intraperitoneally. After 20 mg/kg of reserpine the mice were still spontaneously active, whereas after 40 mg/kg they had the appearance of heavily anaesthetized animals. A dose of 30 mg/kg was therefore chosen for the subsequent experiments and L-DOPA was given 16–18 hr after the reserpine; when iproniazid was also administered this was done 2 hr before the amino acid.

Groups of at least five mice were used for each dose level; the animals in each group were kept together in one cage. In addition, one animal was usually placed in the cage shown in Fig. 1, for records of its movements. The normal motor activity was recorded for at least 1 hr, just before administration of the reserpine. On the following day, the same animal was again placed in the cage, and records were taken both before and after the amino acid was administered intraperitoneally.

The awakening effect of L-DOPA observed in these experiments fully confirms the findings of Carlsson *et al.* (1957) and of Everett & Toman (1959). Before the amino acid was administered the mice all presented the appearance of being asleep, motionless and with eyelids closed. After the administration of L-DOPA the eyelids opened and within 20–30 min the animals resumed their normal motor activity. Then followed a period of over-excitement, during which the animals were more active than normal, untreated mice. After 3–5 hr the increased activity gradually disappeared and the animals returned to the motionless and sleeplike condition of the reserpinized control animals.

Of twenty-five animals treated with reserpine and subsequently with 500 mg/kg L-DOPA, one died. When iproniazid was also administered, four out of sixteen mice died. This increase in toxicity was probably related to the increased over-activity in the iproniazid-treated animals (see Figs. 2 and 3).

Amino acids related to L-*DOPA.* D-DOPA had no awakening action comparable to that of L-DOPA in reserpine-treated animals. However, 24 hr after 500 mg/kg of D-DOPA, seven out of eleven mice moved spontaneously and reacted briskly when touched, whereas in the control group treated with reserpine alone such a response was present in only two animals.

The two isomers of dihydroxyphenylalanine, 2:3-DOPA and 2:5-DOPA (1000 mg/kg), did not have any awakening action in the reserpinized animals even after iprioniazid.

When metatyrosine (500 mg/kg) was administered to the reserpine-treated animals, an awakening effect was seen similar to that of L-DOPA. A typical tracing is shown in Fig. 4, and the evaluation of another tracing from a similar experiment in Fig. 5. In the experiment of Fig. 5 almost 1 hr elapsed before spontaneous movements became evident. Under the influence of metatyrosine the animals resumed their normal activity, but

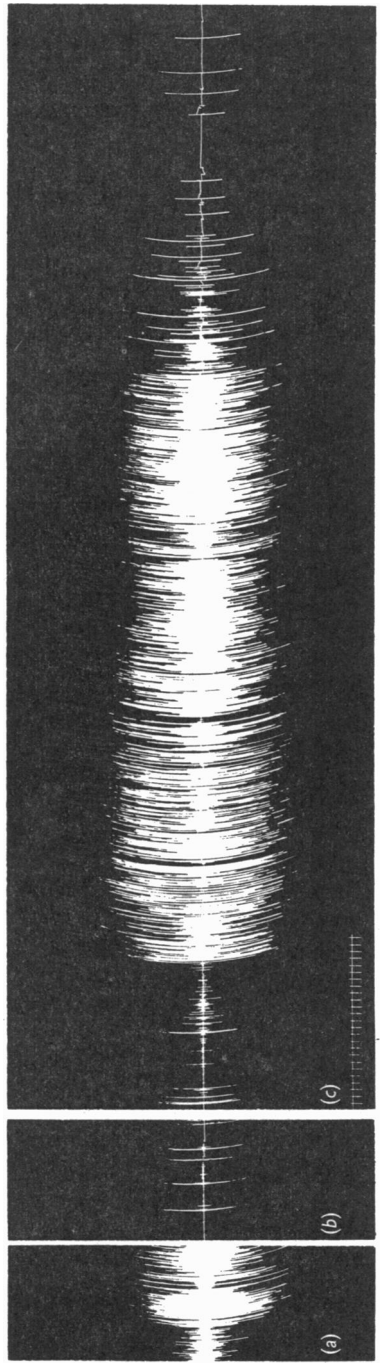


Fig. 2. Tracing taken with the apparatus of Fig. 1; effect of L-DOPA on the motor activity of a reserpine-treated mouse: (*a*) before reserpine; (*b*) 16 hr after 30 mg/kg of reserpine I.P. and 2 hr after 100 mg/kg of iproniazid I.P.; followed immediately by (*c*) after 500 mg/kg of L-DOPA I.P. Timemarker: 1 min.

this period was sometimes followed by one of over-activity. The effect of pre-treatment with iproniazid was less pronounced than in the experiment with iproniazid and L-DOPA, where the characteristic signs of over-activity were always stronger after pre-treatment with iproniazid. However, when a larger amount of metatyrosine was given (1000 mg/kg), the enhancement of the effect of the amino acid by iproniazid was more evident.

Although the action of metatyrosine on the reserpine-treated mice was similar to that of L-DOPA, the onset of the awakening action was somewhat slower and the effects were less pronounced, particularly in the absence of iproniazid. The action of metatyrosine began to decline after 2–3 hr until the animals no longer differed from the reserpine-treated controls.

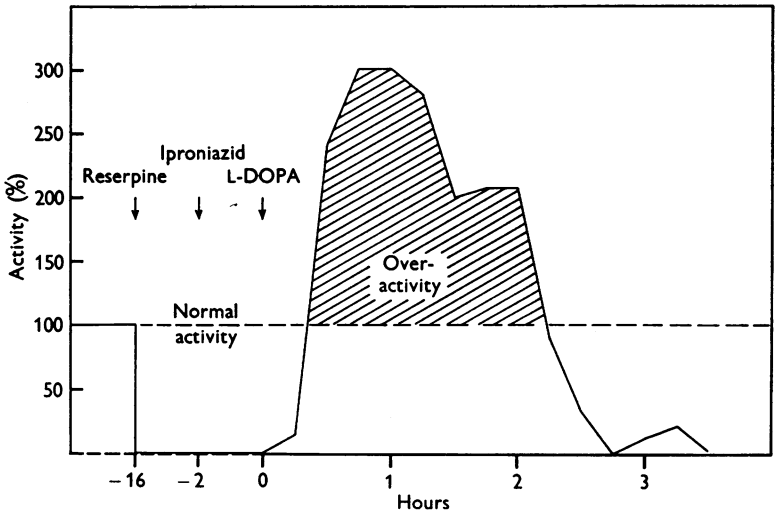


Fig. 3. Evaluation of motor activity of a reserpine-treated mouse after administration of iproniazid (100 mg/kg) and L-DOPA (500 mg/kg), as in the experiment shown in Fig. 2, the level of activity before reserpine (30 mg/kg) being taken as 100%. This animal showed no changes in spontaneous activity in the period of observation before reserpine administration.

Enough *threo*-3:4-dihydroxyphenylserine was available for an experiment in which 500 mg/kg of the amino acid was administered to three reserpine-treated mice. Almost immediately the ptosis of the eyelids disappeared, the respiration rate increased, and in two of the three animals tremors were seen that persisted for 2 to 3 hr.

The rapid onset of these symptoms makes it necessary to consider the possibility that the effects seen were due, at least in part, to a small contamination of the sample with noradrenaline; it is known that samples of adrenaline carboxylic acid, the corresponding *N*-methyl amino acid, are contaminated with adrenaline (see Schmiterlöw, 1951).

No awakening effect of dihydroxyphenylserine was seen.

Dopamine and noradrenaline. Dopamine, the product of the decarboxylation of DOPA, has been tested in mice treated with reserpine and iproniazid, in doses of 500 mg/kg of the hydrochloride. About 3 min after injections of the amine all the mice opened their eyes; the respiration rate was raised for 2–3 hr, and there was a flow of tears, but apart from occasional tremors there was no return of normal motor activity.

Reserpine-treated mice, when injected with 5 mg/kg of noradrenaline bitartrate intraperitoneally, opened their eyes and righted themselves

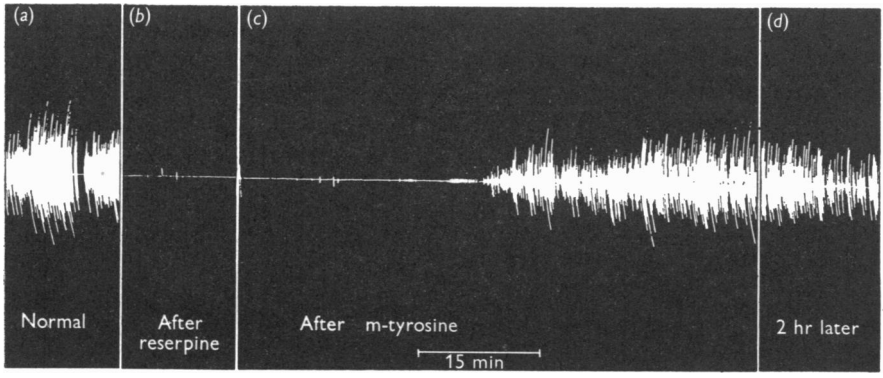


Fig. 4. Effect of metatyrosine on a reserpine-treated mouse. Motor activity (*a*) before reserpine; (*b*) 16 hr after reserpine (30 mg/kg); (*c*) immediately following, after metatyrosine (500 mg/kg); (*d*) 2 hr later.

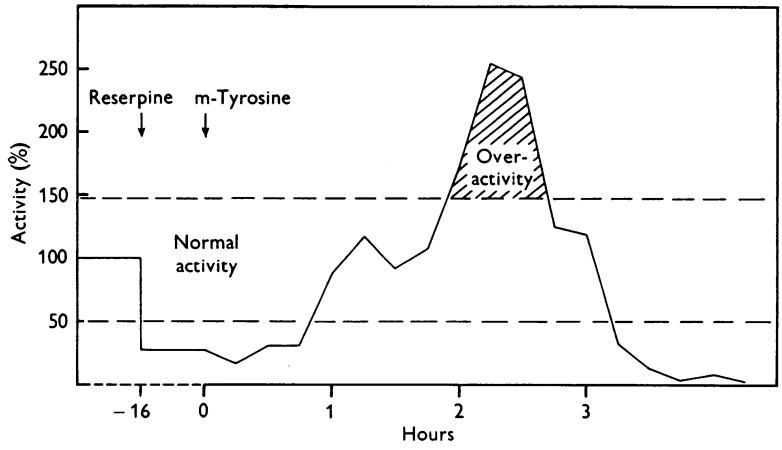


Fig. 5. Effect of metatyrosine on the motor activity of a reserpine-treated mouse. Doses as in the experiment of Fig. 4. This animal showed some changes in spontaneous activity before reserpine.

almost immediately, but did not return to their normal motor activity. With 50 mg/kg there were also occasional tremors, but no return of normal motor activity.

DISCUSSION

The results suggest that the amino acids which were able to awaken the mice treated with reserpine acted after being converted to the corresponding amines. This is supported by the following observations. There was always a time lag between administration of the amino acids and onset of action. Also, D-DOPA, which is not a substrate of the decarboxylase, was inactive as an awakening agent, except for an occasional feeble response 24 hr after its administration; this is of interest, since Holtz & Credner (1944) have found that in the rat D-DOPA can be converted to L-DOPA, probably through a transamination reaction involving 3:4-dihydroxyphenylpyruvic acid.

The different responses of the animals to an injection of the amines and that of the amino acids have been interpreted as due to an inability of the amines to penetrate what is called the blood–brain barrier. It is believed that the amino acids are able to pass this barrier and are then converted to the pharmacologically active amines in the brain, in close vicinity to their sites of action. This view is supported by findings that the administration of DOPA leads to an accumulation of catechol amines in the brain (Raab & Gigee, 1951) and that of metatyrosine to the appearance of metatyramine (Mitoma *et al.* 1957).

Where is this barrier situated? It is impossible to give a precise answer to this question, but it is probably not an anatomically defined barrier that limits the concentration of amine after systemic application. Central actions of adrenaline and other sympathomimetic amines, e.g. upon the electric activity of the brain, are well known. Also, there have been reports that tritium-labelled adrenaline can be recovered from the hypothalamus (Weil-Malherbe, Axelrod & Tomchick, 1959) which must therefore be accessible to adrenaline.

The amino acids differ from the corresponding amines in that the latter only are substrates of amine oxidase. Amine oxidase, together possibly with O-methyl transferase and other enzymes active in amine metabolism, would prevent the amines but not the amino acids from reaching the excitable structures. The acids are decarboxylated near their sites of action, and in this way an effective concentration of amine is built up.

The enhancement by iproniazid of the awakening action of L-DOPA, as well as the higher mortality, is of interest in view of recent work on the inactivation of catechol amines. It seems doubtful whether amine oxidase determines the time course of action of noradrenaline or adrenaline, but there can be little doubt that it is an important factor in the inactivation

of dopamine (Blaschko, 1956). An increased accumulation of dopamine might either have intrinsic awakening action or speed up the formation of noradrenaline and adrenaline.

That L-DOPA is the normal precursor of the catechol amines in the body is now generally accepted (see Blaschko, 1959). Its effects upon the reserpinized animal are so profound that it is tempting to assume that the catechol amines are in some way essential for the maintenance of wakefulness in the normal animal. Other observations support this view, e.g. the awakening actions of amphetamine and of other sympathomimetic compounds. It is of interest that these effects are most pronounced in amines immune from attack by amine oxidase. Further, there are well-known 'arousal' reactions caused by adrenaline and related compounds, and the changes in the electroencephalogram resemble those seen in wakefulness (see Rothballer, 1959).

L-DOPA is a substance normally occurring in mammals, but other substances may also have awakening actions in reserpine-treated animals. Gaddum & Vogt (1956) have found that in cats morphine administered intraventricularly counteracts the effects of reserpine given intraperitoneally. However, in mice, morphine did not produce effects comparable to those produced by L-DOPA (Chruściel, unpublished observation).

The decarboxylation of 3:4-dihydroxyphenylserine by the mouse-liver enzyme was not tested, but it is known that the acid is slowly decarboxylated by the mammalian decarboxylase (Blaschko *et al.* 1950; Holtz, 1959). On decarboxylation the acid would give rise to noradrenaline, bypassing the dopamine stage. If the serine derivative had produced awakening effects comparable to those of L-DOPA, this might have been valuable as an indication that noradrenaline rather than dopamine was responsible for the effects observed after giving L-DOPA. The relatively low efficacy of the serine derivative need not necessarily be due to the omission of the dopamine stage; it might well be due to the slow rate of decarboxylation of this amino acid.

Although metatyrosine is not normally found in the organism, it is able to awaken reserpinized mice. Mitoma *et al.* (1957) have already noted excitatory actions of this compound in normal mice. The amino acid is readily decarboxylated by the mammalian enzyme *in vitro* and presumably *in vivo*.

How do the excitatory actions of metatyrosine arise? It seems likely that the first step is again the formation of the corresponding amine. It is conceivable that metatyramine is converted to dopamine by the introduction of a second phenolic hydroxyl group, but there is nothing to suggest that a reaction of this kind can occur in the mammalian body. Other alternatives are either an intrinsic pharmacological action of metatyramine

exerted on the structures normally excited by the catechol amines, or an action mediated through an increase of the catechol amines normally formed in the body, either by their release or by an interference with their inactivation. In the reserpinized animal, where the stores of catechol amines are depleted, a release seems unlikely but can at present not be definitely excluded. The possibility that metatyramine itself has intrinsic pharmacological activity is worth considering, since it is known that phenylethylamine derivatives hydroxylated in the *meta* position are usually more potent sympathomimetic agents than the *para*-hydroxy compounds (see Bovet & Bovet-Nitti, 1948). An intrinsic awakening action of metatyramine would be of interest since it is a compound not normally occurring in the body.

That rapid decarboxylation alone is not a sufficient requisite for an awakening action is shown by the absence of any awakening effect with 2:3-DOPA or 2:5-DOPA which are both rapidly decarboxylated. This suggests that an amino acid must satisfy two specificity requirements for the awakening effect, first, that of the amine-forming enzyme, and secondly, that of the excitable structures upon which the amines act.

SUMMARY

1. The activity of the DOPA decarboxylase of the mouse on a number of amino acids related to tyrosine has been measured.

2. Extracts of liver, kidney and brain decarboxylated not only L-DOPA, but also metatyrosine, 2:3-DOPA and 2:5-DOPA.

3. The effect of the intraperitoneal administration of a number of amino acids on the motor activity of normal and reserpine-treated mice was studied.

4. A method for the evaluation of the motor activity is described.

5. L-DOPA and metatyrosine had strong awakening effects upon mice treated with reserpine.

6. D-DOPA, 2:3-DOPA, 2:5-DOPA and *threo*-3:4-dihydroxyphenylserine had no awakening actions in reserpine-treated mice.

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