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TYROSINE HYDROXYLASE IN SNAIL (*HELIX POMATIA*) NERVOUS TISSUE

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Abstract—Snail nervous tissue synthesizes [^{14}C]dopamine and [^{14}C]dihydroxyphenylalanine (DOPA) from [^{14}C]tyrosine. The K_m value for the overall conversion of [^{14}C]dopamine was 6×10^{-4} M. The enzyme converting [^{14}C]tyrosine to [^{14}C]DOPA, tyrosine hydroxylase, has the following characteristics. Approximately 85–90 per cent of the enzyme is soluble, and the enzyme of the nervous tissue, isolated by ammonium sulfate fractionation, had the highest activity in the 25–40 per cent fraction. The enzyme has a pH optimum of 6·5 in Tris–HCl, sodium acetate and potassium phosphate buffers. The enzyme requires a tetrahydropteridine cofactor. K_m values toward various tetrahydropteridines such as 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$), 2-amino-4-hydroxy-6-methyltetrahydropteridine ($6MPH_4$), and 2-amino-4-hydroxy-6-(L-erythro-1,2-dihydroxypropyl)tetrahydropteridine (L-erythro-tetrahydrobiopterin) (BH_4) are 5×10^{-4} M, $3{\cdot}2 \times 10^{-4}$ M and $1{\cdot}1 \times 10^{-4}$ M respectively. The K_m values for tyrosine at 10^{-3} M BH_4 or $6MPH_4$ are $1{\cdot}4 \times 10^{-4}$ M and $4{\cdot}2 \times 10^{-5}$ respectively. The enzyme is markedly stimulated by Fe^{2+} and catalase. The activity is drastically inhibited by dopamine, 6-hydroxydopamine, 5-hydroxytryptophan (5-HTP), noradrenaline and sodium dodecyl sulphate. Analogues of tyrosine also slightly inhibit the activity while Triton X-100, homovanillic acid, dihydroxyphenylacetic acid (DOPAC), reserpine, tyramine, pargyline and sucrose have little effect. The properties of the snail tyrosine hydroxylase are compared with those of the vertebrates.

A great deal of evidence has accumulated during the past few years to suggest that dopamine serves as a neurotransmitter substance in the gastropod (e.g. *Helix pomatia*) nervous system [7, 11, 33]. This is supported by the high concentration of this amine within certain neurons [24, 30], its localisation within synaptic type vesicles [28], the ability of dopamine-containing neurons alone to synthesize the amine from tyrosine [26], and the effects of its iontophoretic application upon the activity of certain neurons [1]. Moreover, data from experiments on the release of dopamine from a single neuron containing the amine [2] and its accumulation by an uptake mechanism into nervous tissue [27, 28] all tend to substantiate the hypothesis that dopamine is a transmitter in gastropod nervous tissue.

The present paper describes results of experiments made to investigate the presence, level and properties of tyrosine hydroxylase (E.C. 1.14.6.2) in the snail brain. This enzyme regulates the formation of dopamine in the vertebrates [15]. The properties of the invertebrate tyrosine hydroxylase are compared with those reported for the mammalian enzyme.

MATERIALS AND METHODS

L-[U-^{14}C]Tyrosine (522 mCi/m-mole) was purchased from Amersham Buchler Company and purified before use by t.l.c. on Silica gel 60 plates (Merck) with the solvent system butanol–pyridine–glacial acetic acid–water (15:2:3:5 v/v). The strip corresponding to tyrosine was eluted with methanol and dried. 2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) and 2-amino-4-hydroxy-6-methyltetrahydropteridine ($6MPH_4$) were obtained from Calbiochem; catalase (3900 units/mg) from Sigma. 2-Amino-4-hydroxy-6-(L-erythro-1,2-dihydroxypropyl)tetrahydropteridine (L-erythrotetrahydrobiopterin) (BH_4) was the generous gift of Dr. K. J. M. Andrews of Roche Products Ltd., Welwyn Garden City, UK.

In initial experiments, snail circumoesophageal ganglia were dissected free of most connective tissue and incubated for various lengths of time at 25° in snail saline [18] containing [^{14}C]tyrosine (20 μCi/ml), pyridoxal-5-phosphate (15 μg/ml), pargyline (150 μg/ml) and $DMPH_4$ (15 μg/ml). The ganglia were then freeze-dried and subsequently homogenized in 0·01 N HCl–acetone (1:2 v/v), centrifuged, and the supernatant, together with carrier amounts of dopamine, dihydroxyphenylalanine (DOPA), and tyrosine applied to 20 × 20 cm Silica gel 60 plates. After chromatography with the solvent system butanol–pyridine–glacial acetic acid–water (15:2:3:5 v/v), the plates were sprayed first with ninhydrin and then with potassium ferricyanide in 20% ammonium hydroxide solution in order to localize the various substances. Autoradiograms were also prepared. The areas corresponding to tyrosine, DOPA and dopamine were eluted with methanol, dried, and the activity measured in a Packard Tricarb 3380 Liquid Scintillation Counter.

In other experiments, circumoesophageal ganglia were dissected into ice-cold snail saline, and homogenized in 0·25 M sucrose. Soluble tyrosine hydroxylase was partially purified according to Nagatsu *et al.* [21]. The 25–40% ammonium sulfate fraction was

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used as the enzyme source for the kinetic studies. Alternatively, selected ganglia were homogenized in 0·32 M sucrose and centrifuged at 800 *g* for 5 min at 4° to remove connective tissue material and cell debris, the supernatant then being centrifuged at 17000 *g* for 30 min to give a crude mitochondrial pellet fraction and a soluble cytoplasmic fraction.

Tyrosine hydroxylase activity was analysed by a modification of the method of Nagatsu *et al.* (1965). A total reaction volume of 30 μl contained: 3·3 μmole of potassium phosphate buffer pH 6·5; 30 nmole $6MPH_4$; 90 nmole $FeSO_4$; 1·5 nmole tyrosine containing $1{\cdot}7 \times 10^5$ dpm; 50 μmole mercaptoethanol; 10 μl enzyme preparation. The mixture was incubated at 30° for 30 min and then stopped by the addition of 0·5 ml 0·4 N perchloric acid containing 2 μg DOPA. After the addition of 3 ml of a solution containing 1% EDTA, 0·05 M KPO_4 buffer pH 9·0 (citric acid, KH_2PO_4, H_3BO_3 and diethylbarbituric acid), and 0·067 M NaOH, the solution, now at pH 9·0, was applied to a column of 200 mg acid-washed alumina. The column was washed twice with 30 ml deionized water under a slight vacuum. The labelled DOPA was eluted with 1·5 ml 0·5 N acetic acid. 10 ml Bray's scintillator [3] was added and the activity was measured in a Packard Tricarb Liquid Scintillation Counter with external standardization. Typical counting efficiency was 78 per cent. DOPA recovery was checked by alumina absorption and elution of a known amount of L-[1-^{14}C]DOPA and ranged from 65–72 per cent. 70 per cent was used as a standard recovery in all calculations. Protein was analysed according to Lowry *et al.* [16].

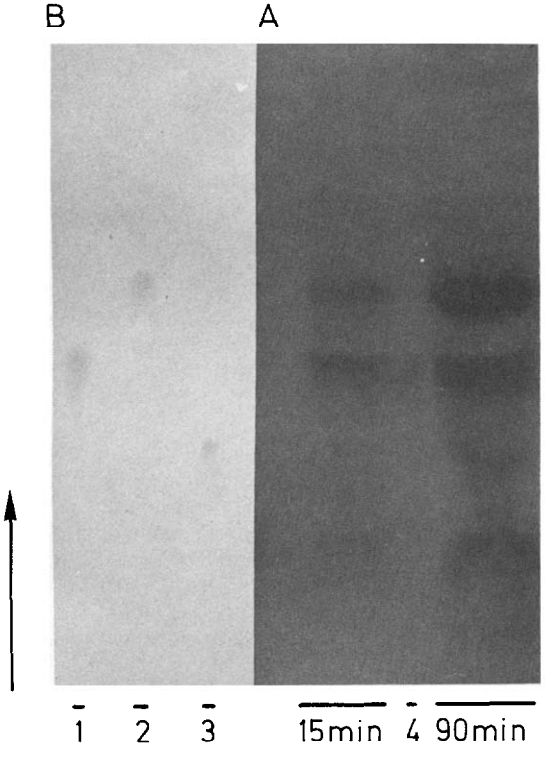

Fig. 1. An autoradiogram (A) and a stained thin layer chromatogram (B) to show the separation and the incorporation of radioactivity from [^{14}C]tyrosine into [^{14}C]DOPA and [^{14}C]dopamine. The conditions of the experiments are described in Materials and Methods. The autoradiogram indicates the content of radioactive products present in snail nervous tissue after incubation for 15 and 90 min. in [^{14}C]tyrosine. 1 = tyrosine, 2 = dopamine, 3 = DOPA, 4 = [^{14}C]tyrosine. The direction of chromatography is indicated by the arrow.

RESULTS

Synthesis of dopamine from tyrosine. Figure 1 is a photograph of an autoradiogram of a chromatogram demonstrating the capacity of snail nervous tissue to form [^{14}C]DOPA and [^{14}C]dopamine from [^{14}C]tyrosine. The time course for this metabolism is shown in Fig. 2. From Fig. 2 it is clear that the amount of dopamine formed is linear for the first 90 min and then gradually stabilizes, while the amount of DOPA formed remains more-or-less constant after the first 45 min. By using different concentrations of tyrosine and constructing a Lineweaver–Burk plot it was found that the apparent K_m for the overall conversion of tyrosine to dopamine was approximately 6×10^{-4} M.

Intracellular distribution and partial purification of snail tyrosine hydroxylase. From Table 1 it can be seen that tyrosine hydroxylase is associated mainly with the soluble fraction and occurs with approximately the same activity in both the sub- and supra-oesophageal ganglia masses. Only about 11 per cent of the total enzyme activity remains with the connective tissue and cell debris after centrifugation. Of the remaining activity, only 3–6 per cent remained in the mitochondrial pellet (see Table 1).

When the soluble enzyme was fractionated with ammonium sulfate, the highest activity was observed in the 25–40% fraction.

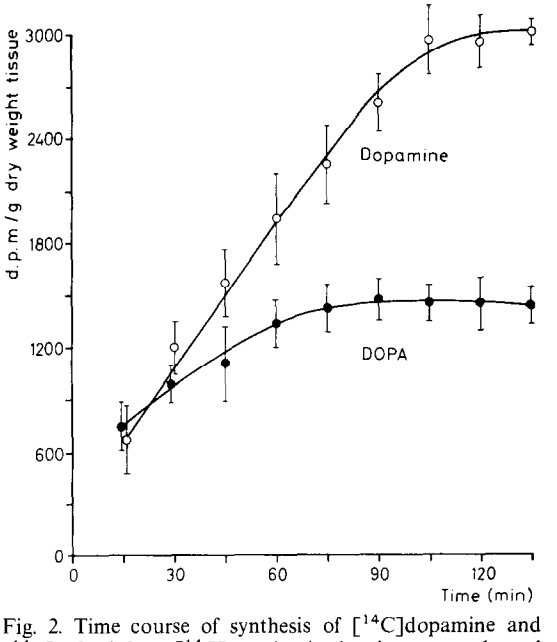

Fig. 2. Time course of synthesis of [^{14}C]dopamine and [^{14}C]DOPA from [^{14}C]tyrosine in the circumoesophageal ganglia of *Helix pomatia*. Each point is the mean ± S.E.M. of four separate determinations, each of which was carried out on a single circumoesophageal ganglion and expressed as dis/min/dry wt tissue. The conditions of the experiments were carried out as described in Materials and Methods.

Table 1. Intracellular distribution of tyrosine hydroxylase activity in various fractions of the snail CNS. Activity was assayed as described in Materials and Methods. Activity is expressed as either nmole/g protein/min or nmole/g tissue/min. The activity of the fractionated enzyme is expressed as nmole/g protein/min and total nmole/min, with the percent of 100,000 *g* supernatant activity in parenthesis. Each value represents the mean of four different experiments

	Sp. act. (nmole/g protein/min)	Sp. act. (nmole/g tissue/min)	Total act. (nmole/min)
Sub-oesophageal ganglia			
Homogenate	10·06	0·438	—
17,000 *g* mitochondrial pellet	2·21	0·013	—
17,000 *g* supernatant	19·08	0·411	—
Supra-oesophageal ganglia			
Homogenate	14·32	0·665	—
17,000 *g* mitochondrial pellet	4·62	0·038	—
17,000 *g* supernatant	20·89	0·622	—
Ammonium-sulfate fractionation			
100,000 *g* supernatant	15·30	—	2842
0–25% $(NH_4)_2SO_4$	7·84	—	328 (11·5%)
25–40% $(NH_4)_2SO_4$	32·56	—	1600 (56·2%)
40–66% $(NH_4)_2SO_4$	5·09	—	264 (9·3%)

Properties of snail tyrosine hydroxylase. The ammonium sulfate fractionated enzyme (25–40% fraction) had an absolute requirement for a reduced pteridine cofactor. $DMPH_4$, a synthetic cofactor used by most earlier researchers, gave only 33% activity when compared with $6MPH_4.BH_4$, the probable natural cofactor for mammals [10, 32], gave 118% activity when used instead of $6MPH_4$.

Analysis of Lineweaver–Burk plots gives K_m values for the pteridine cofactors at 10^{-4} M tyrosine as $1{\cdot}1 \times 10^{-4}$ M, $3{\cdot}2 \times 10^{-4}$ M and 5×10^{-4} M for BH_4, $6MPH_4$, and $DMPH_4$, respectively (see Figure 3). For tyrosine, the K_m values were $1{\cdot}4 \times 10^{-4}$ M and $4{\cdot}2 \times 10^{-5}$ M at 10^{-3} M $6MPH_4$ and 10^{-3} M BH_4 respectively (see Figure 4).

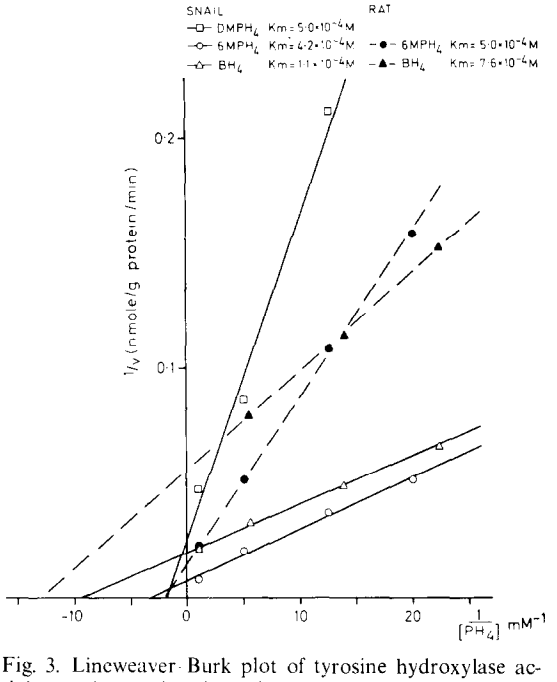

Fig. 3. Lineweaver–Burk plot of tyrosine hydroxylase activity against reduced pteridine concentration with partially purified enzyme preparations from both rat (cerebrum and brainstem) and snail nervous tissue using various reduced pteridines as cofactor. The assay was carried out as described in Materials and Methods. Tyrosine hydroxylase activity is expressed in nmole/g protein/min. PH_4 indicates reduced pteridine. Each value represents the mean of two determinations from two separate preparations.

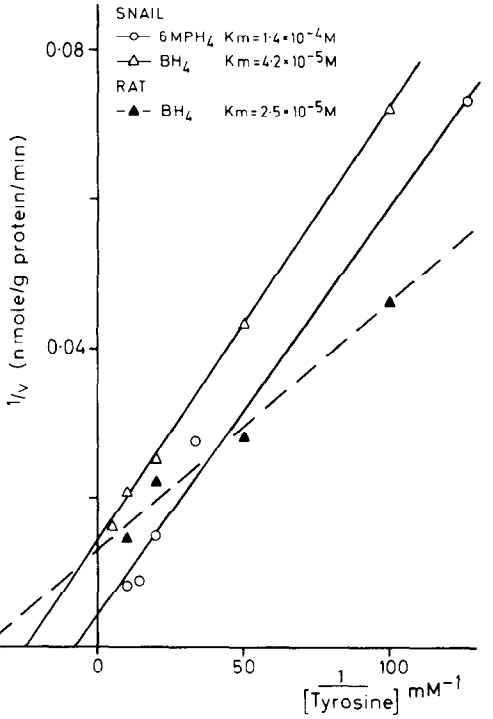

Fig. 4. Lineweaver–Burk plot of tyrosine hydroxylase activity against tyrosine concentration with partially purified enzyme preparations from both rat (cerebrum and brainstem) and snail nervous tissue using various reduced pteridines as cofactors. The assay was carried out as described in Materials and Methods. Tyrosine hydroxylase is expressed in nmole/g protein/min. Each value represents the mean of two determinations from two separate preparations.

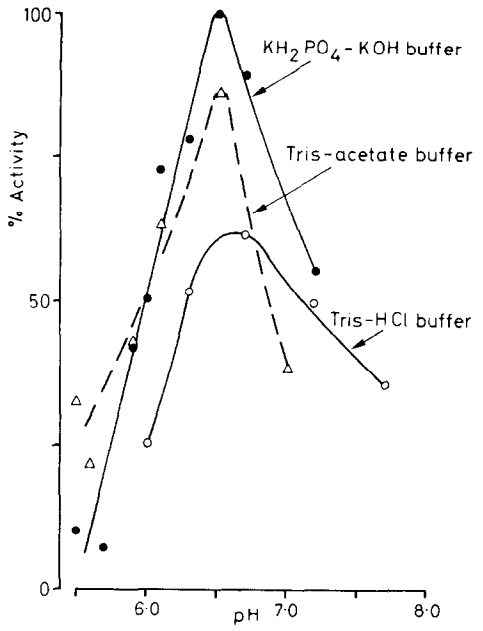

Fig. 5. Effect of pH and buffer on tyrosine hydroxylase activity in the snail. The assay was carried out as described in Materials and Methods, with phosphate buffer pH 6·5 being replaced by the appropriate buffer and pH. Activity is expressed as per cent of phosphate buffer, pH 6·5. Each value represents the mean of two determinations from two separate experiments.

The enzyme showed maximum activity at pH 6·5 for the three buffer systems tried (see Figure 5) with potassium phosphate giving the highest activity. Furthermore, the enzyme had an optimum temperature of 30°, at which temperature the reaction was linear for 30 min, as shown in Figure 6. The Q_{10} (20–30°) was 3·5. Above 30° the enzyme was rapidly inactivated. No measurable activity was found at 0° even after 60 min incubation time.

The effects of various substances on snail tyrosine hydroxylase activity are listed in Table 2. Fe^{2+} and catalase increased the activity in an additive fashion. There was a small but significant activity (34%) without Fe^{2+}. Diethyldithiocarbamate, a chelating agent, reduced the activity to the level found when Fe^{2+} was not included. Cu^{2+} inhibited the enzyme, having the same effect as the chelater. Two tyrosine derivatives (α-methyl-*p*-tyrosine methylester and α-methyl-*m*-tyrosine) inhibited the enzyme, but to a somewhat minor degree in view of the concentrations used. Of the catechol compounds tested, 6-hydroxydopamine and dopamine were the most effective inhibitors (50% inhibition at 0·4 mM). 5-Hydroxytryptophan (5-HTP) was a strong inhibitor (60% inhibition at 0·4 mM), while 5-hydroxytryptamine had a little effect. The main metabolites of dopamine in gastropods, homovanillic acid and dihydroxyphenylacetic acid (DOPAC) [25, 26], also had little influence as had tyramine, reserpine and pargyline. Of the two detergents tested, sodium dodecyl sulphate (SDS), even in

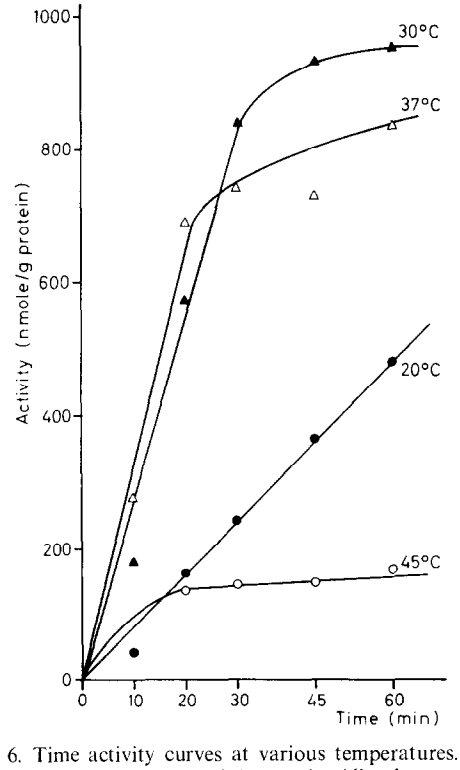

Fig. 6. Time activity curves at various temperatures. The activity is expressed as nmole/g protein. All volumes given in Materials and Methods were increased and aliquots were taken after the specified time and then handled as usual. Each point represents the mean of duplicate determinations.

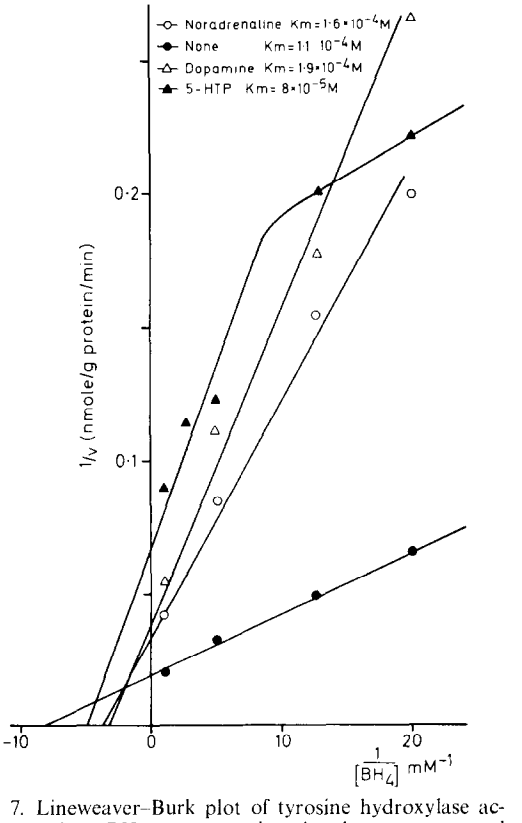

Fig. 7. Lineweaver–Burk plot of tyrosine hydroxylase activity against BH_4 concentration in the presence and absence of various inhibitors. The inhibitors were at a concentration of 0·2 mM. The assay was carried out as described in Materials and Methods.

Table 2. Effect of various drugs and inhibitors on tyrosine hydroxylase activity. The complete system consisted of 3·3 μmole potassium phosphate pH 6·5, 30 nmole $6MPH_4$, 90 nmole $FeSO_4$, 1·5 nmole tyrosine containing $1·7 \times 10^5$ dpm, 5 nmole mercaptoethanol, 10 μl enzyme preparation. The activity was assayed as described in Materials and Methods. Each point represents the mean of duplicate determinations

Compound	Concentration	Activity %
Complete System		100
Minus $6MPH_4$		0
Minus $6MPH_4$ plus $DMPH_4$	1 mM	33
Minus $6MPH_4$ plus BH_4	1 mM	118
Plus catalase	400 units	151
Minus Fe^{2+}		34
Minus Fe^{2+} plus catalase	400 units	132
Minus Fe^{2+} plus Cu^{2+}	3 mM	4
Plus Cu^{2+}	3 mM	30
Plus diethyldithiocarbamate	3 mM	31
Plus α-methyl-*p*-tyrosine methylester	0·4 mM	78
Plus α-methyl-*m*-tyrosine	0·4 mM	92
Plus 6-hydroxydopamine	0·4 mM	49
Plus dopamine	0·4 mM	50
Plus L-DOPA	0·4 mM	75
Plus noradrenaline	0·4 mM	63
Plus 5-HTP	0·4 mM	39
Plus 5-Hydroxytryptamine	0·4 mM	93
Plus homovanillic acid	0·4 mM	100
Plus DOPAC	0·4 mM	95
Plus tyramine	0·4 mM	100
Plus reserpine	0·4 mM	100
Plus pargyline	0·4 mM	100
Plus SDS	0·50%	0
Plus SDS	0·10%	1
Plus SDS	0·05%	9
Plus triton X-100	0·50%	87
Plus triton X-100	0·10%	94
Plus triton X-100	0·05%	93
Plus sucrose	50 mM	85
Plus sucrose	100 mM	88
Plus sucrose	150 mM	94

low concentrations (0·05%), resulted in almost complete inhibition of activity while Triton X-100 had little effect upon the enzyme. Sucrose had the interesting effect of inhibiting the enzyme slightly at low concentrations, but had less influence at higher concentrations.

The effect of three of the strongest inhibiting substances, 5-HTP, dopamine and noradrenaline, on snail tyrosine hydroxylase was further examined kinetically (see Figures 7,8). Dopamine and noradrenaline were competitive with respect to BH_4 and essentially uncompetitive with respect to tyrosine. 5-HTP exhibited rather unusual characteristics, being partially competitive with BH_4 at high concentrations but uncompetitive at lower concentrations.

DISCUSSION

The present data show clearly that *Helix pomatia* nervous tissue has the enzymatic capacity for making dopamine from tyrosine and supports previous findings on a related gastropod [26]. Since gastropod nervous tissues are known to convert DOPA to dopamine [4], these data provide additional proof that DOPA is an intermediary product in the synthesis of dopamine from tyrosine. The radioactivity associated with DOPA remains more or less constant, whereas that associated with dopamine increases with the length of incubation, suggesting that the hydroxylation of tyrosine by tyrosine hydroxylase is the rate-limiting step in the formation of dopamine [15].

In general, the present results indicate that the characteristics of snail tyrosine hydroxylase are similar to those of the vertebrate enzyme [5, 6, 12, 13, 17, 21, 23]. Though some distinctive features have been found for snail tyrosine hydroxylase, the differences are no greater than those differences described between the various vertebrate tyrosine hydroxylase.

Like the vertebrate enzyme (for a review see Musacchio and Craviso 1973), snail tyrosine hydroxylase is a soluble enzyme. Moreover, BH_4, the probable natural cofactor in the vertebrates [10, 32], gives the highest activity in the snail. The interesting observation that the V_{max} value for tyrosine hydroxylase with BH_4 is lower than that with $6MPH_4$ is probably not of physiological importance since several authors [8, 19] have suggested that the reduced pteridine cofactor limits the reaction velocity in the *in vivo* system. At lower pteridine concentrations, BH_4 gives a considerably higher activity than does $6MPH_4$ or $DMPH_4$. A comparison of the K_m values of tyrosine hydroxylase for varying concentrations of $6MPH_4$ and BH_4 for the rat brain and snail nervous tissue,

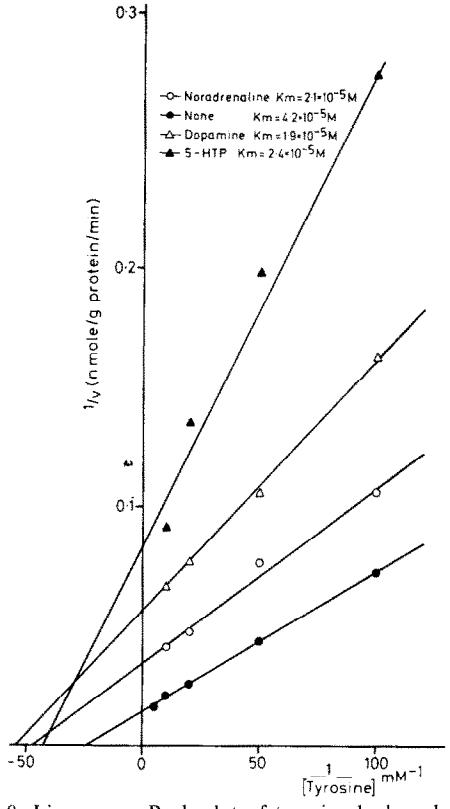

Fig. 8. Lineweaver–Burk plot of tyrosine hydroxylase activity against tyrosine concentration in the presence and absence of various inhibitors. The inhibitors were at a concentration of 0·2 mM. The assay was carried out as described in Materials and Methods. Each point represents the mean of duplicate determinations.

as determined in this study, shows some variation (see Figure 3) i.e. 5×10^{-4} M and $3{\cdot}2 \times 10^{-4}$ M for $6MPH_4$, and $7{\cdot}6 \times 10^{-5}$ M and $1{\cdot}1 \times 10^{-4}$ M for BH_4 for the rat and the snail respectively. In both instances the ratio of $V_{max}6MPH_4/V_{max}BH_4$ is similar. The K_m values determined for the different pteridine cofactors are difficult to compare with those published by other authors for various reasons, though the values reported by Shiman *et al.* [31] for bovine adrenal tyrosine hydroxylase (1×10^{-4} M for BH_4 and $3{\cdot}3 \times 10^{-4}$ M for $6MPH_4$) are very similar.

The K_m value of snail tyrosine hydroxylase for tyrosine differed from that of the rat as determined by this study (Figure 4). With BH_4 as cofactor, the K_m value for the rat was $2{\cdot}5 \times 10^{-5}$ M and for the snail $4{\cdot}2 \times 10^{-5}$ M, almost a 2-fold difference. The significance of this observation is not clear, though it is of interest to note that the K_m value for bovine adrenal tyrosine hydroxylase quoted by Shiman *et al.* [31], ($2{\cdot}1 \times 10^{-5}$ M) is similar to that found for the rat in this study and the value reported by Nagatsu *et al.* [22], also for bovine adrenal enzyme (4×10^{-5} M), is similar to that found for the snail enzyme. Also of interest is that substrate inhibition by tyrosine using BH_4 as cofactor was not observed, even at 10-fold K_m levels. Shiman *et al.* [32] found substrate inhibition with the bovine adrenal enzyme at 2-fold K_m levels. It is thus very difficult to compare critically the kinetic data of the snail tyrosine hydroxylase with the vertebrate tyrosine hydroxylase, though they appear to be very similar.

The pH optimum of 6·5 for the snail tyrosine hydroxylase activity in phosphate, acetate and Tris–HCl buffers is also in general agreement with what a number of authors have found for the vertebrate enzyme, though there are minor differences. For example, Numata and Nagatsu [23] found maximum activity in bovine peripheral nerve extracts at pH 6·5 when using Tris–HCl or phosphate buffer, but a pH of 5·9 was required for maximum activity when using acetate buffer.

As in the vertebrate, snail tyrosine hydroxylase is drastically inhibited by dopamine and noradrenaline, suggesting a possible feed-back regulation of catecholamine biosynthesis in the snail. 5-HTP also had a potent inhibitory effect upon the snail enzyme, which is of special interest since it may indicate a possible mutual regulation between catecholamine and indolamine biosynthesis. Evidence for such an interregulation between various transmitter pathways can be found in the inhibition of tyrosine hydroxylase by 5-HTP [23] and the inhibition of tryptophan hydroxylase by various catechols [9]. The functional significance of this mutual inhibition suffers from the proposal that the catechol inhibition of tryptophan hydroxylase is due to chelation of Fe^{2+}; this area should be investigated more closely, however. In this context is of interest to note that other metabolites of catecholamines viz. DOPA, homovanillic acid, DOPAC and 5-hydroxytryptamine, had little effect upon the snail enzyme. This has also been reported for vertebrates [23]. As in vertebrate preparations, in the snail, catecholamines are competitive with respect to the reduced pteridine cofactor and not with substrate tyrosine [23]. The mechanism of the effect of 5-HTP for the snail enzyme seems to be similar to the bovine peripheral enzyme [23].

Of a number of other substances tested, 6-hydroxydopamine and sucrose had interesting effects and should be further analysed, while reserpine, pargyline and tyramine had no effect. Triton X-100, used to liberate tyrosine hydroxylase from tissue [5], had a minor inhibitory influence while SDS, even at concentrations of 0·05%, almost completely inhibited the enzyme. Of interest is to note that a recent report has shown that very low concentrations of SDS (0·006%) can activate rat striatal tyrosine hydroxylase [14]. Catalase had the effect of stimulating the enzyme to greater degree than did Fe^{2+}. Contradictory data on the relative effects of catalase and Fe^{2+} have been reported. In general, however, authors who have used phosphate buffers [5, 32] have, as here, found a greater stimulatory effect with catalase than with Fe^{2+}; those who used other buffer systems have found the reverse [5, 23]. These contradictory reports may result from the formation of the insoluble $FeSO_4$ salt. In any event, a higher purification of snail enzyme is necessary before a definite conclusion can be reached.

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