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Assay of tyrosine hydroxylase in tissue homogenates: effects of Triton-X, sodium, calcium and cyclic AMP

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The use of the Nagatsu method [1] for the determination of tyrosine hydroxylase (L-tyrosine, tetrahydropteridine: oxygen oxidoreductase [3-hydroxylating] EC 1.14.16.2.; TH) activity has proved unsatisfactory for the assay of TH in tissue homogenates in this laboratory [2, 3]. Although this has been solved in part by the use of acetone dried powders for large samples, there remains a need for a method of assaying TH in whole homogenates and small tissue samples. Previous reports have shown that TH from acetone powders may be activated by Na^+ and K^+ ions [3], while work by other authors [4, 5] had indicated that TH can also be activated by Ca^{2+} ions and cyclic AMP. It has also been shown that these two compounds have only a small effect on TH from acetone powders [6]. The work reported here examines the effect of NaCl, $CaCl_2$, Triton X-100 and cyclic AMP on homogenates of guinea pig caudate nucleus. From the results obtained, a suitable method for the assay of TH in homogenates and small pieces of tissue is described.

Assay procedure. The assay procedure was essentially that reported by Mann [3]. Homogenates of guinea pig caudate nucleus were made in 0.2 M dimethyl glutarate/NaOH buffer, pH 6.0. When necessary, supernatants were prepared by centrifuging homogenates at 84,000 *g* for 2 hr. Samples of homogenate were normally incubated for 30 min at 37.5° in the presence of L (sidechain 2, 3-^{3}H)-tyrosine with a specific activity of approximately 18mCi/mmole. The L-dopa formed was separated from the remaining tyrosine using alumina columns as previously described [3]. The incubation mixture consisted of 40 mM dimethyl glutarate/NaOH buffer (pH 6.0), 0.2 mM $FeSO_4$, 4 mg/ml bovine serum albumin, 2.5 mM dithiothreitol (DTT) and 2500 units/ml catalase. The incubation was started by the addition of enzyme or labelled tyrosine. In the case of additions such as Ca^{2+} and cyclic AMP, these were part of the incubate before the addition of enzyme to start the incubation procedure; there was thus no preincubation procedure. The optimum concentrations of tyrosine and tetrahydrobiopterin (BpH_4) (kindly donated by Dr. K. J. M. Andrews, Roche Products) were 0.3 and 2.5 mM, respectively. The final volume of the mixture was 100 μl.

Preparation of small tissue samples. Small samples of brain, or the posterior lobes of rat pituitary (each weighing approximately 0.5 mg) were homogenized in small glass tubes containing 80 μl incubation mixture without tyrosine and tetrahydrobiopterin; the samples were then incubated following the addition of these two ingredients. Alternatively, acetone powders were prepared in similar tubes by breaking up the tissue in the presence of acetone at −10°. Each sample was washed in its tube with 4 × 0.5 ml acetone and then dried in a desiccator under continuous vacuum for 5 hr at +4°. Incubation mixture was then added to the tube to extract the enzyme and the normal procedure followed. No difference was observed when the tubes were spun to remove the insoluble material. All activities of tyrosine hydroxylase, including those for acetone powders, have been expressed as μmoles L-dopa synthesized.

Activation by Triton X-100 and cations. Samples of homogenate supernatant and whole homogenate were incubated in the presence of 0.5% Triton X-100, 0.5 mM $CaCl_2$ or 400 mM NaCl (+50 mM Na^+ as sodium glutarate) and 0.1 mM cyclic AMP. Combinations of these compounds were also used. A significant increase in enzyme activity was observed when NaCl was incubated with homogenate supernatant (Table 1) but as previously reported [3], no increase was observed with whole homogenate. A significant increase was also observed when the whole homogenate was incubated with Triton X-100; this effect was in turn enhanced by the presence of NaCl which had not activated the whole homogenate in the absence of Triton X-100. It can be seen from the values in Fig. 1 that tyrosine hydroxylase in supernatants may be only 20 per cent of the total. It thus appears that freely accessible TH can be activated by cations and that Triton X-100 and the acetone powder procedure are both good methods of making the enzyme easily accessible to substrates.

In previous experiments it has been shown that a plot of enzyme concentration against activity was not linear when tissue homogenates were used. In the present work,

Table 1. Activation of tyrosine hydroxylase in caudate nucleus homogenates*

	Supernatant		Homogenate	
	μmoles/g/hr	%	μmoles/g/hr	%
Control	0.111	100	0.495	100
NaCl (400 mM)	0.411	370‡	0.582	117
Triton X-100 (0.5%)	0.120	108	0.793	160†
$CaCl_2$ (0.5 mM)	0.122	110	0.506	102
cAMP (0.1 mM)	0.109	98	0.519	105
NaCl + Triton X	0.411	370§	2.056	415§
$CaCl_2$ + Triton X	0.094	85	0.828	161

* Tissues were incubated in subsaturating concentrations of tyrosine (66 μM) and tetrahydrobiopterin (317 μM).
† $P > 0.02$.
‡ $P > 0.01$.
§ $P > 0.001$.

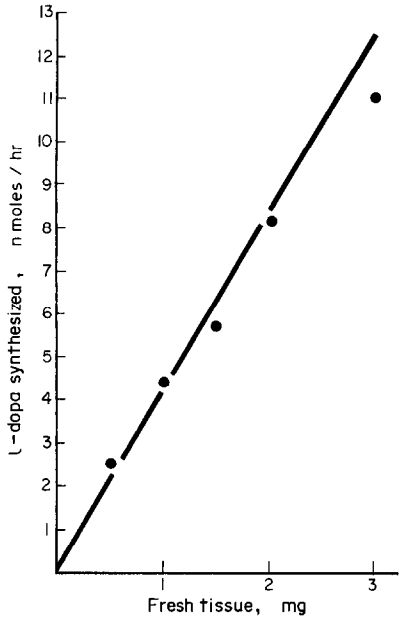

Fig. 1. Synthesis of L-dopa by a homogenate of guinea pig caudate nucleus in the presence of Triton X-100 and 125 mM NaCl.

as in work previously reported [3], the production of L-dopa was linear with time over a period of 1 hr. Figure 1 shows a plot obtained with increasing amounts of guinea pig caudate nucleus homogenate in the presence of 100 mM NaCl and Triton X-100. This plot is clearly linear up to 2 mg of caudate nucleas tissue and is therefore satisfactory for the assay of TH within this tissue range. The value obtained for TH in the caudate nucleus under optimal conditions was 4.5 μmoles/g/hr compared with the previous figure of 2.5μmoles/g/hr [3]. Experiments in which Triton X-100 was used in the absence of NaCl or in which the NaCl was replaced by $CaCl_2$ also gave rise to linear plots. The addition of calcium chloride produced maximal activation at 25 mM; above this concentration $CaCl_2$ was inhibitory.

Effect of calcium and cyclic AMP on kinetic constants. The effects of $CaCl_2$ and cyclic AMP were examined both in homogenates and homogenates activated with Triton X-100. There was a tendency for the Michaelis constants to be lower for BpH_4 in the activated homogenates (Table 2), but this was not true of the K_m' for tyrosine which had a value of 27 μM. There was also a tendency for the K_m' for BpH_4 to be lowered by Ca^{2+} and cyclic AMP in the activated homogenates, while no effect on the K_m' was observed with NaCl. This effect, however, is very much smaller than that observed by other workers [5]. The values given in Table 2 were obtained in a subsaturating concentration of tyrosine, while the concentration of oxygen was that which was available from the air at atmospheric pressure which gives a concentration of 200–355 mM [6]. The values for V_{max}' are therefore not the true maximal velocities (V_{max}), while the values of K_m' are not the values which would be obtained at infinite concentrations of second and third substrates (i.e. K_m); the values obtained here are sometimes referred to as (K_s).

Assay of small samples of tissue. Tissue samples from Wistar rats were assayed as described in the methods. Values of 0.29 μmoles/g/hr were obtained for the posterior lobe of the pituitary using Triton X-100 NaCl incubation mixture. Using the acetone powder technique, values of 0.11 μmoles/g/hr were obtained. Small samples of basal hypothalamus (1.0–3.0 mg) were also assayed and values of 0.11 μmoles/g/hr and 0.04 μmoles/g/hr were obtained for fresh tissue and acetone powders, respectively. The incubation medium that gave good results for homogenates is, therefore, also successful with small pieces of tissue.

There have been a number of reports that tyrosine hydroxylase is activated by either cyclic AMP, Ca^{2+} ions or both. Roth *et al.* [5] indicatated that this activation, which mimicked nerve stimulation, resulted in the lowering of the Michaelis constants for both tyrosine and the pterin cofactor with a consequential increase in the inhibitory constant for catechols. The work on TH previously reported from this laboratory [6] has indicated that no significant effects of these compounds could be found when TH from acetone powders was used. The results reported here show that Ca^{2+}, like Na^+, activates TH in that it can increase the observed velocity but does not substantially affect the kinetic constants. Nevertheless, the tendency for the K_m of BpH_4 to decrease in the presence of Ca^{2+} and cyclic AMP, together with the decrease in K_m' in the presence of Triton X-100 (from 803 to 361 μM) indicates that the soluble enzyme may be different from the bound enzyme. However, 25 mM Ca^{2+} increased the observed velocity both in the whole homogenate and that treated with Triton X-100. Weiner *et al.* [7] have suggested an indirect action of Ca^{2+} on TH *in vivo,* but clearly the results in Tables 1 and 2 show that it is possible for a direct action to occur *in vitro.* It is difficult, therefore, to explain the lack of effect of Ca^{2+} and cyclic AMP on the kinetics of TH which has been reported here compared with the substantial effects which have been reported by others [4, 5]. The enzyme activity of the activated homogenates is high and it is not unreasonable to expect that little improvement would occur in such samples. There was no effect with cyclic AMP in the unactivated homogenates; although there was some increase in velocity with Ca^{2+}, there was no effect on the K_m'. It may be that there is a distinct physical difference between the TH from peripheral sources where noradrenaline is synthesized and central sources where dopamine is synthesized, but this would be unusual. It is probable that Ca^{2+} and cyclic AMP may well have different quali-

Table 2. The effect of Ca^{2+} and cyclic AMP on tyrosine hydroxylase in homogenates of caudate nucleus*

	Homogenate		Homogenate + Triton X-100	
	K_m' BpH_4	V_{max}'	K_m' BpH_4	V_{max}'
Control	629 ± 96	1.165 ± 0.14	505 ± 75	2.152 ± 0.19
$CaCl_2$ (25 mM)	803 ± 99	1.778 ± 0.15	361 ± 106	3.3 ± 0.56
cAMP (0.1 mM)	648 ± 166	1.251 ± 0.20	437 ± 125	2.17 ± 0.35

* Samples were incubated in the absence of added NaCl with a tyrosine concentration of 66 μM and an oxygen concentration of between 200 and 355 mM.

tative and quantitative effects *in vivo* from those observed *in vitro* and that the conditions used here are not suitable for these substances to act as they would in the intact nerve ending.

The values obtained using Triton X-100 and activating ions on whole homogenates have again shown that the amounts of TH in the caudate nucleus are greater than earlier observations (4.5 compared with 2.5 μmoles/g/hr [3], both of which were greater than values reported by other authors [8, 9], indicating that the caudate nucleus has a considerable capability for the synthesis of L-dopa under optimal conditions. It is, possible however, that the enzyme *in vivo* may not be present in its most active form.

From the results obtained with homogenates and small tissue samples it is clear that the Triton X-100/NaCl incubation mixture is suitable for the assay of TH in all cases where the measurement of maximal activity is required.

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Maturation of sympathetic neurotransmission in the rat heart—III. Developmental changes in reserpine inhibition of norepinephrine uptake into isolated synaptic vesicles*

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Reserpine inhibits the uptake of catecholamines into storage vesicles by competitive, irreversible blockade of the ATP-Mg^{2+}-dependent transport mechanism [1–5], resulting in profound depletion of neurotransmitters from the vesicles and interference with sympathetic neurotransmission. After administration of reserpine to the rat, the drug disappears biphasically: most of the drug is eliminated very rapidly, but a small amount disappears extremely slowly [6, 7] due to irreversible binding of reserpine to sites on the vesicles [7, reviews 8, 9]. Disappearance of the bound reserpine, along with the appearance of new, unoccupied reserpine binding sites, has been used to estimate the rate of synthesis and axonal transport of newly formed vesicles; complete recovery of binding sites in the adult rat heart requires 7–8 weeks [10]. This closely approximates the time required for full recovery of endogenous heart norepinephrine content after reserpine treatment [11].

Another index of new vesicle arrival in the terminal after reserpine treatment [5] is the recovery of vesicular uptake capabilities. Recently, a technique has been developed to measure uptake of norepinephrine into synaptic vesicles isolated from small amounts of rat heart [12]. Using this method, rapid increases in rat heart vesicular uptake sites have been demonstrated during the first few days of postnatal life [13], possibly corresponding to increased rates of vesicle synthesis and/or down-transport in the neonates compared to adults. In support of this explanation of the postnatal increases, studies in rat brain have found that a single injection of reserpine into neonatal rats results in inhibition of norepinephrine uptake lasting only a few days, compared to more than 2 weeks in adults, despite the fact that neonates display a greater initial sensitivity to the drug [5]. In the present study, this approach has been extended to the developing peripheral sympathetic nervous system by measuring the time required for recovery of cardiac vesicular norepinephrine uptake after giving reserpine to rats of different ages. The results confirm that the ontogenetic increases in transmitter vesicular uptake sites are associated with accelerated arrival of new vesicles.

Timed pregnant Sprague–Dawley rats (Zivic-Miller, Allison Park, PA) were housed individually in breeding cages and allowed food and water *ad lib*. Pups from all litters were randomized at birth and redistributed to the nursing mothers, with litter sizes kept at 8–11 pups to maintain a standard nutritive status. Additionally, for each experiment, pups of both sexes were selected from several different cages. Neonates were given 250 μg/kg reserpine subcutaneously on days 1, 9, 17 and/or 30 and were killed at intervals from 3 hr to several days after each reserpine injection. Adult male 250 g rats were given a single subcutaneous injection of reserpine (either 250 or 50 μg/kg) and decapitated 3 hr, 24 hr, 4, 7, 14, 21 or 28 days after the injection. All control animals received equivalent volumes of vehicle (1 ml/kg).

For studies of uptake of norepinephrine into cardiac sympathetic vesicle, hearts were weighed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) immediately in 4 vol. of ice-cold 300 mM sucrose buffered with 25 mM Tris (pH 7.4) containing 10 μM iproniazid. During the first 2 weeks of postnatal development, hearts from several animals were pooled to obtain at least 200 mg of tissue for analysis. A crude fraction containing synaptic vesicles was prepared from the heart homogenate by the method adapted from Seidler *et al.* [14] by Bareis and Slotkin [12]. The homogenate was centrifuged at 1000 *g* for 15 min and the pellet discarded. The supernatant fraction was recentrifuged at 20,000 *g* for 30 min and this supernatant fraction was sedimented at 100,000 *g* for 30 min in a Beckman Type 40 fixed angle rotor. The crude vesicle pellet (from preparation to preparation consistently con-

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