Biochemical Pharmacology, Vol. 25, pp. 2493–2497. Pergamon Press, 1976. Printed in Great Britain.

ACTIVATION OF TYROSINE HYDROXYLASE IN RAT STRIATAL SLICES BY K^+-DEPOLARIZATION—EFFECT OF ETHANOL*

GONZALO BUSTOS

Department of Neurobiology, Institute of Biological Sciences, Catholic University, Casilla 114-D, Santiago, Chile

and

ROBERT H. ROTH and VICTOR H. MORGENROTH, III

Department of Pharmacology, Yale University School of Medicine, New Haven, CT. 06510, U.S.A.

(*Received* 5 *November* 1975; *accepted* 21 *April* 1976)

Abstract—Slices from rat corpus striatum were incubated for 10 min at 37° in freshly oxygenated Krebs–Ringer phosphate (KRP) media or KRP-high K^+ (55 mM) media both in the presence and absence of ethanol (0.2 to 0.8%, w/v). Thereafter, the slices were homogenized and tyrosine hydroxylase activity and kinetic parameters were determined in the 105,000 *g* supernatant fraction. The presence of K^+ (55 mM) in the incubation media increased about 3-fold the activity of striatal tyrosine hydroxylase, assayed in the presence of subsaturating concentrations of tyrosine and pterin cofactor, when compared to that found in striatal slices incubated in normal KRP media. Incubation of striatal slices in a KRP-high K^+ media also produced changes in the kinetic properties of tyrosine hydroxylase. The K_m of the enzyme for 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine HCl ($DMPH_4$) was decreased from 0.82 to 0.09 mM and the K_i of the enzyme for dopamine (DA) was increased from 0.13 to 3.52 mM. Ethanol (0.2 to 0.8%, w/v) added directly to the KRP-high K^+ media markedly blocked the K^+-induced activation of tyrosine hydroxylase as well as the kinetic alterations in the enzyme observed after K^+-depolarization of the striatal slices. In contrast, the presence of ethanol did not modify the activity and kinetic characteristics of tyrosine hydroxylase isolated from slices incubated in normal KRP media. The results reported in this work suggest that the increase in DA synthesis observed in striatal slices after K^+-depolarization might be mediated in part via an allosteric activation of tyrosine hydroxylase. This activation appears to be mediated by an increase in the affinity of the enzyme for the pterin cofactor and a decreased affinity for the end-product inhibitor DA. Also, the blocking effect of ethanol upon the kinetic activation of tyrosine hydroxylase after K^+-depolarization seems to offer a likely explanation for the inhibitory effect of ethanol on K^+-induced increase in DA synthesis reported recently by Gysling *et al.* (*Biochem. Pharmac.* **25**, 157 (1976)).

Recent experiments conducted in one of our laboratories have shown that ethanol added *in vitro* specifically blocks the K^+-induced increase in the conversion of labeled tyrosine to dopamine (DA) by striatal slices [1]. These results were difficult to interpret in terms of the commonly accepted mechanisms for regulation of DA synthesis. Until very recently the increase in DA synthesis observed after K^+-depolarization was believed to arise primarily as a result of the removal of end-product inhibition subsequent to the release of a small pool of endogenous or newly formed DA which normally acts to partially inhibit tyrosine hydroxylase [2, 3]. However, ethanol was found to block the K^+-induced increase in the synthesis of DA, while having no effect on K^+-induced release of DA [1].

Roth *et al.* [4–6] have recently shown that an increase in impulse flow in the nigro-neostriatal pathway of the rat causes an increase in DA synthesis, which is mediated in part via an allosteric activation of striatal tyrosine hydroxylase. This activation seems to be mediated by an increase in the affinity of the enzyme for both substrate and pterin cofactor and a decrease in affinity for the end-product inhibitor dopamine. These results raised the possibility that the increase in DA synthesis observed after K^+-depolarization [1, 2] might be, in part, the consequence of kinetic alterations in tyrosine hydroxylase similar to those observed upon electrical stimulation of the nigro-neostriatal pathway [6]. Moreover, ethanol could be blocking the K^+-induced increase in DA synthesis [1] by preventing in some way the kinetic activation of tyrosine hydroxylase observed after K^+-depolarization. The purpose of this paper, therefore, is to study the activity and kinetic characteristics of tyrosine hydroxylase prepared from rat striatal slices incubated in normal and high K^+ media (55 mM) both in the presence and absence of ethanol.

MATERIALS AND METHODS

Preparation and incubation of rat striatal slices. Striatal tissue slices (0.18 mm in thickness) were prepared with a Sorvall tissue chopper, from the striatum of adult, male Sprague–Dawley rats. Tissue slices, weighing about 30–40 mg, were incubated for 10 min

* A preliminary report of this work was presented at the meetings of the Fifth Latinoamerican Congress of Pharmacology, Lima, Peru, October 27–31, 1974.

at 37° in 5.0 ml of pre-warmed Krebs–Ringer phosphate (KRP) pH 7.4, or KRP-high K^+ (55 mM), pH 7.4, saturated with 95% O_2 + 5% CO_2 and in the absence and presence of ethanol. At the end of the incubation period, the slices were transferred to a 5.0-ml lucite chamber with a nylon mesh bottom (pore size = 35 microns) which permitted a complete and rapid (10 sec) separation between slices and medium [7, 8]. The nylon mesh bottom containing the striatal slices was immediately frozen on dry ice and stored at −70° until the time of tyrosine hydroxylase assay. Striata were either assayed singly or pooled for determination of tyrosine hydroxylase kinetics.

Tyrosine hydroxylase assay. At the time of the assay, frozen striatal slices were homogenized in 10 vol. of ice-cold 0.05 M Tris–acetate buffer, pH 6.0, and centrifuged at 105,000 *g* for 90 min at 4°. The supernatant served as the source of soluble tyrosine hydroxylase. Tyrosine hydroxylase activity was assayed according to the method described by Morgenroth *et al.* [9, 10]. In this procedure, the production of ^3H_2O from L-[3,5-^{3}H]tyrosine is used as a measure of the amount of L-3,4-dihydroxyphenylalanine formed. The reaction was carried out in a total volume of 1.0 ml; 0.1 ml of supernatant was added to a reaction mixture containing 200 μmoles of acetate buffer, pH 6.0; 0.1 μmole of 3-hydroxy-4-bromobenzyloxyamine dihydrogen phosphate, an aromatic amino acid decarboxylase inhibitor; 3300 units of catalase; 0.1 ml (2.2 mg protein) of partially purified sheep liver dihydropteridine reductase; 1.0 μmole of NADPH and 0.1 μmole of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine HCl ($DMPH_4$). After a 5-min preincubation, the reaction was started by the addition of 1 μCi L-[3,5-^{3}H]tyrosine (0.1 Ci/m-mole) in a volume of 0.05 ml. After a 45-min incubation period at 37°, the reaction was stopped by the addition of 0.05 ml of glacial acetic acid. Blanks consisted of complete incubation mixtures to which 0.05 ml of glacial acetic acid had been added prior to addition of substrate.

Analysis of the tritiated water formed during the reaction was carried out by ion exchange chromatography through Dowex 50 × 8 (H^+) columns [10]. Protein was determined according to the method of Lowry *et al.* [11] using bovine serum albumin as a standard, and tyrosine hydroxylase activity was expressed as pmoles dopa formed/mg of protein/min. The tyrosine hydroxylase reaction was linear with time for up to 1 hr and with protein concentrations from 50 to 800 μg.

Kinetics were determined on the linear portion of the time course and protein concentration curves. Estimations of K_m for $DMPH_4$ were performed according to the method of Lineweaver and Burk [12] using a saturating concentration of tyrosine (0.1 mM) and six different $DMPH_4$ concentrations. The K_i of dopamine was determined by the method of Dixon [13] at three $DMPH_4$ concentrations. Data were analyzed using a paired *t*-test. All calculations were performed on a Hewlett Packard Programmable Calculator model 9832.

Solutions and chemicals. The Krebs–Ringer phosphate (KRP) used had the following composition: NaCl, 128 mM; KCl, 4.8 mM; $CaCl_2$, 0.75 mM; $MgSO_4$, 1.20 mM; glucose, 16 mM; Na_2HPO_3, 16 mM at pH 7.4; sodium ascorbate, 20 mg/l. Krebs–Ringer phosphate-high K^+ (KRP-high K^+) was made by replacing proportions of NaCl with equimolar amounts of KCl.

Dihydropteridine reductase was purified from sheep liver through the first ammonium sulfate fraction according to the method of Kaufman [14]; an excess of the reductase was used in the assay.

Catalase, NADPH and ultrapure Tris were purchased from Schwarz-Mann. 2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine HCl was obtained from CalBiochem. 3-Hydroxy-4-bromobenzyloxyamine dihydrogen phosphate was a gift from Lederle Laboratories. L-[3,5-^{3}H]tyrosine (30 Ci/m-mole) was purchased from New England Nuclear Corp. and purified by passage over a Dowex 50 column and taken to dryness just before use.

RESULTS

Incubation of striatal slices in high potassium (55 mM) Krebs–Ringer phosphate for 10 min resulted in a marked increase in the activity of tyrosine hydroxylase found in the high speed supernatant of homogenates prepared from the striatal slices. An increase in activity of about 300 per cent was observed when compared to the activity of tyrosine hydroxylase found in striatal slices incubated in normal KRP media (Table 1). Ethanol (0.8%, w/v) added directly to the KRP-high K^+ media completely blocked the activation of the tyrosine hydroxylase produced by incubating the striatal slices in this potassium enriched Krebs solution. In contrast, the presence of ethanol (0.8%, w/v) was not able to alter the tyrosine hydroxylase activity found in striatal slices incubated in normal KRP media (Table 1).

In order to analyze further the mechanism involved in the increase in tyrosine hydroxylase activity which results from depolarizing the slices prior to enzyme isolation as well as the inhibitory effects produced by ethanol on this increase in enzymatic activity, the kinetic properties of tyrosine hydroxylase were determined after each treatment. Incubation of the striatal slices for 10 min in a KRP-high K^+ media prior to the isolation of tyrosine hydroxylase resulted in a dramatic change in the kinetic properties of the enzyme. The K_m of the enzyme for $DMPH_4$ was decreased 9-fold, from 0.82 to 0.09 mM, with no significant change in the V_{max} (Fig. 1, Table 2). On the other hand, the K_i of the enzyme for DA was increased about 27-fold, from 0.13 to 3.52 mM (Fig. 2, Table 2). The presence of ethanol (0.8%, w/v) in the KRP-high K^+ media was able to block the kinetic alterations in tyrosine hydroxylase obtained upon K^+-depolarization of the striatal slices. In fact, the K_m and K_i values of the enzyme obtained under these experimental conditions were similar to those obtained after incubating the striatal slices in a normal KRP media with or without ethanol (Table 2). Ethanol (0.8%, w/v) added directly to normal KRP media produced no effect on the kinetic characteristics of tyrosine hydroxylase isolated from slices incubated in this media (Table 2).

As shown in Table 3, ethanol exerts its inhibitory effect on K^+-induced activation of tyrosine hydroxylase enzyme even at concentrations at low as 0.2%

Table 1. Effect of ethanol and potassium depolarization of striatal slices on tyrosine hydroxylase activity

Treatment*	Tyrosine hydroxylase activity† (pmoles dopa/mg protein/min)
KRP	27.2 ± 2.6
KRP + ethanol (0.8%, w/v)	24.5 ± 2.6
KRP-high K^+ (55 mM)	87.3 ± 0.3‡
KRP-high K^+ (55 mM) + ethanol (0.8%, w/v)	28.0 ± 0.3§

* Striatal slices were prepared by means of a Sorvall tissue chopper and incubated for 10 min at 37° in the various media. Thereafter, the slices were frozen on dry ice and subsequently homogenized and assayed for tyrosine hydroxylase.

† Tyrosine hydroxylase activity was determined in the 105,000 *g* supernatant fraction. Results are expressed as the mean ± S. E. M. of three different experiments (assayed in triplicate). Assays were conducted in the presence of 10 μM tyrosine and 0.1 mM $DMPH_4$.

‡ $P < 0.001$ when compared to respective normal KRP control.

§ $P < 0.001$ when compared to KRP-high K^+ (55 mM) without ethanol.

(w/v). Ethanol, at this concentration, produced a 36.7 per cent inhibition of the K^+-induced increase of tyrosine hydroxylase activity. Higher concentrations of ethanol (0.4 and 0.8%, w/v) produced an almost complete block of the K^+-induced effect (Table 3).

DISCUSSION

Potassium depolarization of dopaminergic nerve terminals results in an acceleration of dopamine synthesis due to an increase in the activity of the rate-limiting enzyme tyrosine hydroxylase [2]. This increase in tyrosine hydroxylase activity was previously thought to arise as a result of the removal of end-product inhibition subsequent to the release of a small pool of endogenous or newly formed dopamine which continually depresses tyrosine hydroxylase activity [2, 3]. However, this hypothesis is not consistent with recent results obtained from studies in rat striatal slices in which ethanol was able to block K^+-evoked synthesis of DA while having no effect on K^+-evoked release of DA [1]. The experiments reported in this paper suggest that the increase in DA synthesis observed in striatal slices after K^+-depolarization might be mediated in part via a kinetic activation of striatal tyrosine hydroxylase. Thus, an increased enzyme activity was found when tyrosine hydroxylase prepared from K^+-depolarized slices was assayed *in vitro* in the presence of subsaturating concentrations of tyrosine and pterin cofactor (Table 1).

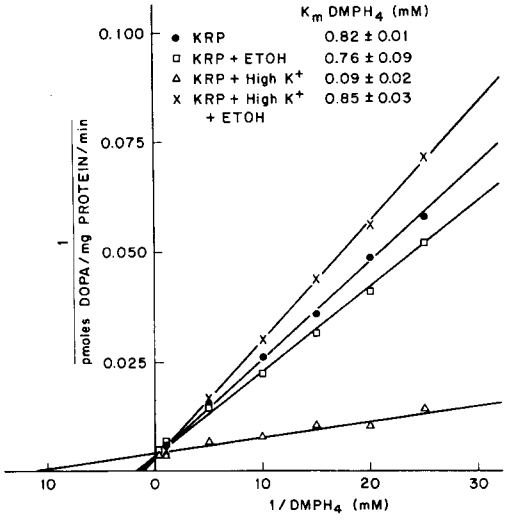

Fig. 1. Effect of potassium depolarization on the K_m of striatal tyrosine hydroxylase for $DMPH_4$. The K_m for $DMPH_4$ was determined by the method of Lineweaver–Burk [12] at a tyrosine concentration of 10^{-4} M and six $DMPH_4$ concentrations ranging from 10^{-3} to 5×10^{-5} M. Each value is the mean of the intercepts generated from three separate lines. Striatal slices were incubated for 10 min at 37° in normal KRP or KRP-high K^+ (55 mM) both in the presence and absence of ethanol (0.8%). The tyrosine hydroxylase kinetics were measured in the 105,000 *g* supernatant obtained from the slices.

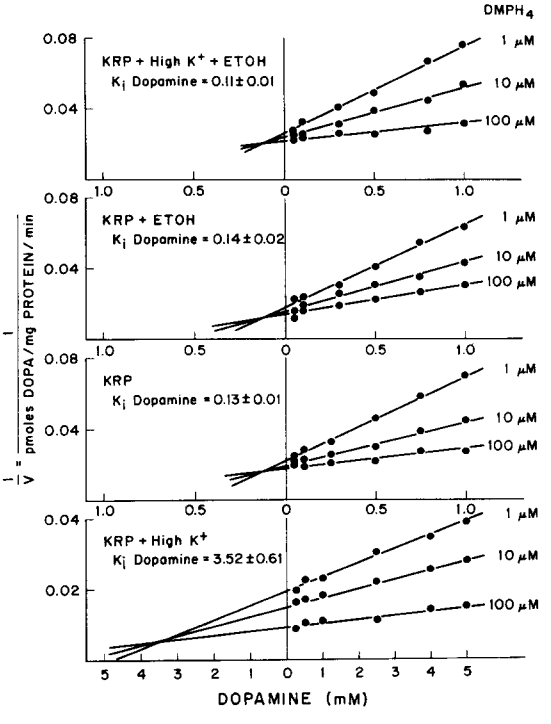

Fig. 2. Effect of potassium depolarization of the K_i of striatal tyrosine hydroxylase for dopamine. The K_i for dopamine was determined by the method of Dixon [13] at six dopamine concentrations (5×10^{-3} to 10^{-5} M) and three $DMPH_4$ concentrations (10^{-4} to 10^{-6} M). Each value is the mean of the intercepts generated from three separate lines. Incubation conditions of the striatal slices and tyrosine hydroxylase assay were as described under Fig. 1.

Table 2. Effect of ethanol and potassium depolarization on the kinetics of striatal tyrosine hydroxylase

Treatment*	K_m tyrosine (μM)	K_m $DMPH_4$† (mM)	K_i DA† (mM)
KRP	41.9 ± 3.7	0.82 ± 0.01	0.13 ± 0.01
KRP + ethanol (0.8%, w/v)	46.3 ± 3.4	0.76 ± 0.09	0.14 ± 0.02
KRP-high K^+ (55 mM)	19.4 ± 1.6	0.09 ± 0.02	3.52 ± 0.61
KRP-high K^+ (55 mM) + ethanol (0.8%, w/v)	39.9 ± 2.8	0.85 ± 0.03	0.11 ± 0.01

* Striatum was dissected out and slices were prepared by means of a Sorvall tissue chopper. Incubation conditions in the various media were as described under Table 1.

† Striatal slices were homogenized and tyrosine hydroxylase was assayed in the 105,000 g supernatant fraction. The K_i for DA was determined by the method of Dixon [13] at six dopamine concentrations (5×10^{-3} to 10^{-5} M) and three $DMPH_4$ concentrations (10^{-4} to 10^{-6} M). Each value is the mean ± S. E. M. of the intercepts generated from three separate lines. The K_m for tyrosine and $DMPH_4$ were determined according to the method of Lineweaver and Burk [12] in the presence of saturating concentrations of $DMPH_4$ and tyrosine respectively.

This activation appears to be mediated by an increase in affinity of the enzyme for substrate and pterin cofactor and a decreased affinity of the enzyme for the end-product inhibitor dopamine (Table 2).

Recent experiments in one of our laboratories [4–6] have demonstrated that an increase in impulse flow in the nigro-neostriatal pathway of the rat causes kinetic alterations in striatal tyrosine hydroxylase similar to those reported in this work. Thus, short-term regulation of striatal tyrosine hydroxylase during augmented impulse flow in dopaminergic neurons seems to occur in part through changes in the affinity of the enzyme for both substrate and pterin cofactor and for the end-product inhibitor dopamine. We now show that it is possible to reproduce partially these phenomena *in vitro* employing brain slices.

It is possible that the activating effect on tyrosine hydroxylase reported above is not the consequence of changes associated with K^+-depolarization of the slices but is the result of a direct activating effect of K^+ ions upon the enzyme. We feel, however, that this is not the case, since it is possible to demonstrate the K^+-induced activation of tyrosine hydroxylase after manipulations which involve freezing, thawing and homogenization of the slices. By the time the assay of tyrosine hydroxylase is performed the enzyme has been diluted a 100-fold. Therefore, even if we assume that slices incubated in potassium enriched medium equilibrate rapidly with K^+ ions, the final concentration of this ion in the enzyme assay will not be more than 0.55 mM. It is unlikely that this very low K^+ concentration will induce by itself the activation of tyrosine hydroxylase reported in this work. Furthermore, it has been previously shown [15] that soluble striatal tyrosine hydroxylase activity is not modified by the addition of K^+ ions (5 and 10 mM) to the enzyme assay. Recent experimental evidence is consistent with the possibility that alterations in endogenous levels of cAMP which occur during depolarization [16] might in part be responsible for the increase in DA synthesis [1, 2] and for the kinetic activation of tyrosine hydroxylase produced after incubating striatal slices in a K^+-enriched medium (Tables 1 and 2). Thus, DA synthesis in striatal slices is increased by the addition of dibutyryl cAMP to the incubation media [17]. Moreover, tyrosine hydroxylase in high speed supernatant prepared from rat striatum is activated by cAMP [18, 19]. This acti-

Table 3. Effect of different doses of ethanol on K^+-depolarization-induced activation of striatal tyrosine hydroxylase

Treatment*	Tyrosine hydroxylase activity† (pmoles dopa/mg protein/min)	Inhibition of high K^+ effect on tyrosine hydroxylase activity‡ (%)
Normal KRP	27.2 ± 2.6	
KRP-high K^+ (55 mM)	87.2 ± 0.3§	0
KRP-high K^+ (55 mM) + ethanol (0.2%, w/v)	65.2 ± 3.2‖	36.7
KRP-high K^+ (55 mM) + ethanol (0.4%, w/v)	28.7 ± 3.5‖	97.5
KRP-high K^+ (55 mM) + ethanol (0.8%, w/v)	27.8 ± 0.4‖	99

* Striatum was dissected out and slices were prepared by means of a Sorvall tissue chopper. Incubation conditions in the various media were as described under Table 1.

† Striatal slices were homogenized and tyrosine hydroxylase was assayed in the 105,000 g supernatant fraction. Results are expressed as the mean ± S. E. M. of three different experiments (assayed in triplicate). Assays were conducted in the presence of 10 μM tyrosine and 0.1 mM $DMPH_4$.

‡ High K^+ effect on tyrosine hydroxylase activity is equal to tyrosine hydroxylase activity obtained from slices incubated in KRP-high K^+ media minus the activity obtained from slices incubated in normal KRP.

§ $P < 0.001$ when compared to respective normal KRP control.

‖ $P < 0.001$ when compared to KRP-high K^+ (55 mM) without ethanol.

vation is associated with a decrease in the K_m for tyrosine and for the pterin cofactor, while no change in the V_{max} for either the substrate or cofactor is observed when the assay is conducted in a system identical to that employed in this paper. The activation of tyrosine hydroxylase produced by cAMP is similar to that reported in this paper after K^+-depolarization of the striatal slices. Thus, it seems possible that cAMP accumulation during K^+-depolarization might be responsible for the activation of tyrosine hydroxylase observed after exposure of the slices to high K^+. Of course, the assumption inherent in this hypothesis is that endogenous cAMP is synthesized within the dopaminergic nerve terminal, since cAMP formed postsynaptically is unlikely to diffuse across the synaptic cleft and penetrate into the presynaptic neurons. However, at present no direct evidence is available to prove that cAMP is formed in the dopaminergic nerve terminals.

Previous results have shown that ethanol (0.2 to 0.8%, w/v) specifically blocks the activating effect K^+-depolarization has on DA synthesis while having no effect on DA synthesis measured in non-depolarized striatal slices [1]. We show now that ethanol (0.2 to 0.8%, w/v) blocks the kinetic activation of tyrosine hydroxylase produced by K^+-depolarization while having no effect on tyrosine hydroxylase prepared from non-depolarized slices (Tables 1 and 2). The results described in this paper offer then a very likely explanation for the inhibitory effect of ethanol on K^+-induced DA synthesis reported previously.

It is possible that ethanol is producing its inhibitory effect on K^+-induced activation of tyrosine hydroxylase by exerting a direct effect upon the enzyme. However, this is not supported by the experimental evidence available: (1) the activity and kinetic characteristics of tyrosine hydroxylase isolated from striatal slices incubated in normal KRP were not modified by the addition of ethanol (0.8%, w/v) to normal KRP (Tables 1 and 2), and (2) slices incubated in the presence or absence of ethanol were submitted to homogenization and dilution procedures before the tyrosine hydroxylase present in the slices was assayed. Therefore, the final amount of ethanol present in the tyrosine hydroxylase assay never was higher than 0.008% (w/v). It is unlikely that this low ethanol concentration can produce any direct inhibitory effect upon the enzyme, since ethanol (0.8%, w/v) was found to be without effect upon tyrosine hydroxylase activity determined in homogenates from rat corpus striatum [14]. Thus, it seems most likely that ethanol is inhibiting the K^+-induced activation of tyrosine hydroxylase by acting upon some stage located between K^+-depolarization of the slices and activation of the enzyme. Nerve membrane changes and ion fluxes associated with K^+-depolarization seem not to be affected by concentrations of ethanol used in this work. Only very high concentrations of ethanol (2–4%, w/v) produce any significant effect upon nerve membranes that lead to an increased passive permeability for Na^+ and K^+ ions during the action potential [20–22]. Ethanol (0.4 to 0.8%, w/v) was found not to alter the K^+-induced release of DA from striatal slices [1], a process which is dependent on the presence of Ca^{2+} in the medium and blocked by Ca^{2+} removal [8]. As discussed above, cAMP which accumulates after K^+-depolarization might be responsible for the kinetic activation of tyrosine hydroxylase which occurs during depolarization of dopaminergic terminals. It is possible then that ethanol is inhibiting K^+-induced activation of tyrosine hydroxylase by altering cAMP production and/or its action. We are currently investigating this possibility.

Acknowledgement—This work was supported in part by grant 18/73 from the Catholic University Research Fund to G.B. and by N.I.H. grants MH-14092 and NS-10174 to R.H.R.

REFERENCES

1. K. Gysling, G. Bustos, I. Concha and G. Martinez, *Biochem. Pharmac.* **25,** 157 (1976).
2. J. E. Harris and R. H. Roth, *Molec. Pharmac.* **7,** 593 (1971).
3. F. Javoy, Y. Agid, D. Bouvet and J. Glowinski, *J. Pharmac. exp. Ther.* **182** 454 (1972).
4. R. H. Roth, J. R. Walters, L. C. Murrin and V. H. Morgenroth, III, in *Pre- and Postsynaptic Receptors* (Eds. E. Usdin and W. E. Bunney, Jr.), pp. 5–48. Marcel Dekker (1975).
5. L. C. Murrin, V. H. Morgenroth, III and R. H. Roth, *Pharmacologist* **16,** 128 (1974).
6. L. C. Murrin and R. H. Roth, *Molec. Pharmac.* **12,** 463 (1976).
7. G. Bustos and R. H. Roth, *Br. J. Pharmac.* **44,** 817 (1972).
8. G. Bustos and R. H. Roth, *Br. J. Pharmac.* **46,** 101 (1972).
9. V. H. Morgenroth, III, M. C. Boadle-Biber and R. H. Roth, *Proc. natn. Acad. Sci. U.S.A.* **71,** 4283 (1974).
10. V. H. Morgenroth, III, M. C. Boadle-Biber and R. H. Roth, *Molec. Pharmac.* **11,** 427 (1975).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
12. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56,** 658 (1934).
13. M. Dixon, *Biochem. J.* **55,** 170 (1953).
14. S. Kaufman, *Meth. Enzym.* **5,** 812 (1962).
15. G. Bustos, P. Baeza, O. H. Viveros and I. Concha, *Proc. Chilean biol. Soc.* **16,** R11 (1974).
16. S. Kakiuchi, T. Rall and H. McIlwain, *J. Neurochem.* **16,** 485 (1969).
17. M. Goldstein, B. Agnoste and C. Shirron, *J. Pharm. Pharmac.* **25,** 351 (1973).
18. J. Harris, V. Morgenroth, III, R. H. Roth and R. Baldessarini, *Nature, Lond.* **252,** 156 (1974).
19. J. E. Harris, R. J. Baldessarini, V. H. Morgenroth, III and R. H. Roth, *Proc. natn. Acad. Sci. U.S.A.* **72,** 789 (1975).
20. C. M. Armstrong and L. B. Binstock, *J. gen. Physiol.* **48,** 256 (1964).
21. J. W. Moore, W. Ulbricht and M. Takata, *J. gen. Physiol.* **48,** 279 (1964).
22. P. Rosenberg and T. R. Podleski, *Biochim. biophys. Acta* **75,** 104 (1963).