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TYROSINE HYDROXYLASE:—EXAMINATION OF CONDITIONS INFLUENCING ACTIVITY IN PHEOCHROMOCYTOMA, ADRENAL MEDULLA AND STRIATUM*

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Abstract—Soluble tyrosine hydroxylase from human pheochromocytoma, bovine adrenal medulla and rat striatum was studied with regard to factors influencing enzyme activity. The pH optimum was different for tyrosine hydroxylase from the three tissues examined: pheochromocytoma, pH 7.8; adrenal medulla, pH 6.8; and striatum, pH 6.0. In preparations of tyrosine hydroxylase from each tissue, the presence of Mg^{2+}, ATP and cyclic AMP resulted in a significant activation of the enzyme. On the other hand, addition of catecholamines to the reaction mixture produced a marked inhibition which became more pronounced with increasing pH. In all three tissues, the pH for maximum per cent stimulation by Mg^{2+}, ATP and cyclic AMP was different from the pH optimum for tyrosine hydroxylase. In addition, the magnitude of this stimulation, as well as the basal activity, was dependent on the buffer present in the reaction mixture.

Since tyrosine hydroxylase is generally believed to be the rate-limiting enzyme in catecholamine biosynthesis [1], it has been the focus of considerable attention in recent years. The activity of tyrosine hydroxylase in brain slices [2] and synaptosomes [2–4] is increased after exposure to dibutyryl cyclic AMP, and the activity of soluble preparations of tyrosine hydroxylase is increased in the presence of Mg^{2+}, ATP and cyclic AMP [5–7]. It has been suggested that this activation of tyrosine hydroxylase may be mediated by a cyclic AMP-dependent protein kinase [2, 5, 8, 9]. In addition, calcium has also been implicated in the regulation of this enzyme [10–14]. The assay of tyrosine hydroxylase is generally carried out in a reaction mixture containing the enzyme, pterin cofactor, an enzymatic regenerating system (or mercaptoethanol), radiolabeled tyrosine and buffer. The buffer generally used has been acetate (Tris or Na) or phosphate. Acetate has the disadvantage of having little buffering capacity at pH 6–7, the pH range in which the assay is generally carried out. Phosphate buffer, on the other hand, does have sufficient buffering capacity in the pH range of 6–7, but it has been suggested that it inhibits cyclic AMP-dependent protein kinase [15]. Furthermore, due to the relative insolubility of calcium phosphate, this buffer may be a poor choice for studying the regulatory effects of calcium on tyrosine hydroxylase and may explain some of the discrepancies which exist in the literature.

In order to establish suitable assay conditions for studying the regulation of soluble tyrosine hydroxylase, we have examined this enzyme from bovine adrenal medulla, rat striatum and human pheochromocytoma, a tumor characterized by a high rate of catecholamine formation and secretion [16].

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MATERIALS AND METHODS

Preparation of soluble tyrosine hydroxylase. Human pheochromocytoma tumors obtained from two patients (white females, ages 30 and 51) were immediately placed on ice, dissected free from the adrenal gland and external capsule, minced, and frozen at −80° until used. Frozen bovine adrenal glands were obtained from Pel-Freeze, and immediately before homogenization they were thawed and the adrenal medullas removed for use. Frozen rat striata (obtained from male Sprague–Dawley rats), pheochromocytoma and medulla were each weighed and homogenized in 5 vol. of 0.13 M potassium phosphate buffer, pH 7.4. Striata were homogenized in a glass homogenizer fitted with a Teflon pestle, whereas pheochromocytoma and medulla were homogenized with an ultraturrax. The homogenates were centrifuged at 100,000 *g* for 1 hr and the supernatant fluid was dialyzed at 4° against 300–400 vol. of 0.154 M KCl. The dialysate was changed three times and the total time of dialysis was 24 hr. Aliquots of each dialyzed enzyme were then stored at −80° until the time of assay. The enzyme preparations could be stored at −80° for at least 3 months without appreciable loss of activity.

Tyrosine hydroxylase assay. Enzyme activity was assayed according to the method of Shiman *et al.* [17], with minor modifications. The reaction was carried out in a total volume of 0.3 ml; 0.125 ml of striatal enzyme or 0.01 ml of adrenal medulla or pheochromocytoma enzyme was added to a reaction mixture containing 15 μmoles morpholinopropane sulfonic acid (MOPS) buffer (unless otherwise noted), 3700 units catalase, 0.02 ml (ca. 0.45 mg protein) of sheep liver dihydropteridine reductase purified through the first ammonium sulfate fraction [18], 0.15 μmole NADPH and 0.075 μmole DL-6-methyl 5,6,7,8-tetrahydropterine HCl (6-MPH_4). After a 5-min preincubation at 37°, the reaction was initiated by the addition of 3 μCi of L-[3,5-

^{3}H] tyrosine (1 Ci/m-mole) previously purified according to the method of Coyle [19] and evaporated to dryness just prior to use. After a 15-min incubation at 37°, the reaction was terminated by the addition of 0.2 ml of 10% trichloroacetic acid (w/v). Blank values were determined by running the assay in the absence of enzyme. Analysis of tritiated water formed during the reaction was carried out by ion exchange chromatography using Dowex 50 × 8 (H^+) columns. Tyrosine hydroxylase activity is expressed as pmoles of DOPA formed/min/mg of protein. The results obtained by this method were identical to the results we obtained by direct measurement of DOPA [20] by alumina column chromatography. When the reaction mixture contained $MgCl_2$, ATP and cyclic AMP, the amounts added were 3 μmoles, 0.3 μmole and 3 nmoles respectively. In all cases, ATP was neutralized with KOH prior to adding it to the incubation reaction. In the studies where buffer and pH were varied, the various buffers were carefully adjusted to the indicated pH at 37° on a pH meter. Subsequently, the pH of the complete reaction mixture at 37° was checked with "narrow range" pH paper and found to remain unaltered.

Protein determination. Protein was determined by the method of Lowry *et al.* [21] using bovine serum albumin as a standard.

MOPS, NADPH, ATP and cAMP were obtained from Sigma Chemical Co., St. Louis, MO. Catalase was from Boehringer-Mannheim, Indianapolis, IN, 6-MPH_4 was from Calbiochem, La Jolla, CA, and L-[3,5 ^{3}H] tyrosine (1 Ci/m-mole) was purchased from Amersham, Arlington Heights, IL.

RESULTS

Striatal tyrosine hydroxylase was assayed alone, in the presence of Mg^{2+}, ATP and cyclic AMP or in the presence of 10 μM dopamine, at various pH values. Morpholinoethanesulfonic acid (MES) buffer (50 mM) was employed in the pH range 5.6 to 6.8, and MOPS buffer (50 mM) was used in the pH range 6.4 to 8.4. Enzyme activity was the same whether assayed in MES buffer or in MOPS buffer. For the striatal enzyme, the

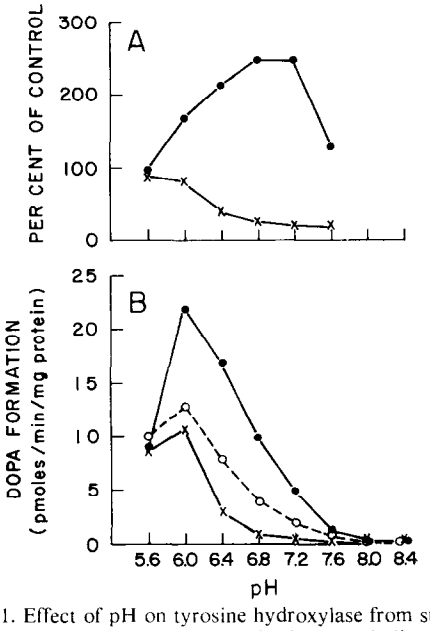

Fig. 1. Effect of pH on tyrosine hydroxylase from striatum. Tyrosine hydroxylase was assayed using morpholinosulfonic acid buffer (50 mM) at the pH values indicated: control (O), 10 μM dopamine (×), and Mg^{2+}, 10 mM; ATP, 1 mM; and cyclic AMP, 10 μM (●). The data are representative of three separate experiments assayed in triplicate.

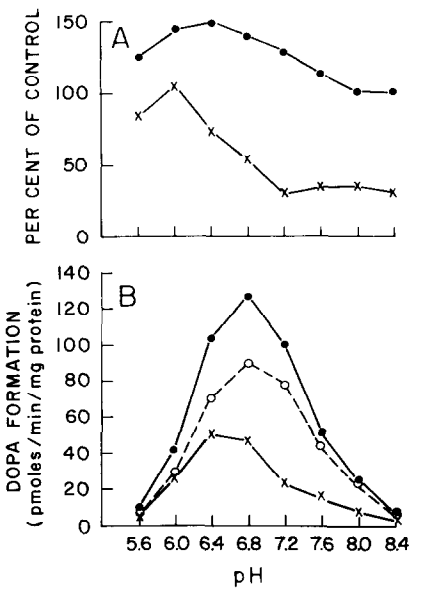

Fig. 2. Effect of pH on tyrosine hydroxylase from adrenal medulla. Tyrosine hydroxylase was assayed using morpholinosulfonic acid buffer (50 mM) at the pH values indicated: control (O), 60 μM norepinephrine (×), and Mg^{2+} 10 mM; ATP, 1 mM; and cyclic AMP, 10 μM (●). The data are representative of three separate experiments assayed in triplicate.

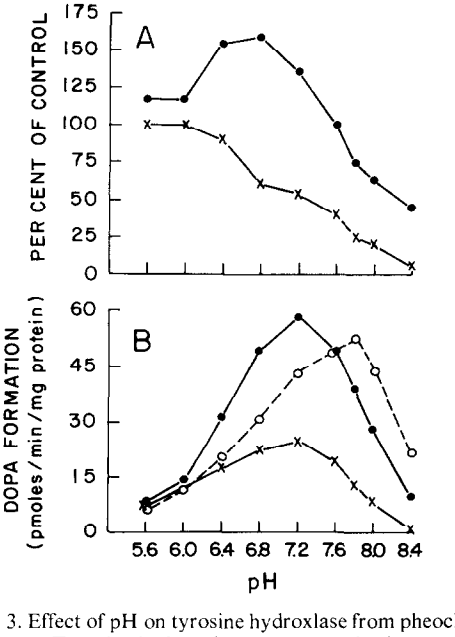

Fig. 3. Effect of pH on tyrosine hydroxlase from pheochromocytoma. Tyrosine hydroxylase was assayed using morpholinosulfonic acid buffer (50 mM) at the pH values indicated: control (O), 60 μM norepinephrine (×), and Mg^{2+}, 10 mM; ATP, 1 mM; and cyclic AMP, 10 μM (●). The data are representative of three separate experiments assayed in triplicate.

Table 1. pH Optima for tyrosine hydroxylase from pheochromocytoma and striatum in the presence or absence of Mg^{2+}, ATP and cAMP in different buffers*

Buffer	Additions	Pheochromocytoma pH optimum	Pheochromocytoma Tyrosine hydroxylase activity*†	Striatum pH optimum	Striatum Tyrosine hydroxylase activity*†
Morpholinosulphonic acid	Control	7.8	52	6.0	13
Morpholinosulphonic acid	Mg^{2+}, ATP, and cAMP	7.2	59	6.0	24
Tris–acetate	Control	7.8	50	5.8	9
Tris–acetate	Mg^{2+}, ATP, and cAMP	7.2	57	5.8	16
Potassium phosphate	Control	6.6	58	5.6	22
Potassium phosphate	Mg^{2+}, ATP, and cAMP	6.6	67	5.6	29

* Tyrosine hydroxylase was assayed as described in Materials and Methods in the absence and presence of Mg^{2+}, 10 mM; ATP, 1 mM; and cAMP, 10 μM.

† Values are expressed as pmoles DOPA/min/mg of protein and are the means of three experiments which differed by less than 10 per cent.

maximum per cent activation by Mg^{2+}, ATP and cyclic AMP, as well as the maximum per cent inhibition by 10 μM dopamine, occurred at pH 6.8 to 7.2 (Fig. 1A). The pH optimum for striatal tyrosine hydroxylase under the three conditions studied was 6.0, and marked decreases in enzyme activity were observed at both higher and lower pH values (Fig 1B).

When similar experiments were carried out using tyrosine hydroxylase from adrenal medulla, the maximum per cent stimulation by Mg^{2+}, ATP and cyclic AMP occurred over the pH range, 6.0 to 6.8, whereas the maximum per cent inhibition by 60 μM norepinephrine occurred at pH 7.2 and higher (Fig. 2A). The pH optimum for the adrenal medulla enzyme occurred at pH 6.8 under all conditions studied (Fig. 2B).

Pheochromocytoma tyrosine hydroxylase showed a maximum per cent stimulation by Mg^{2+}, ATP and cyclic AMP at pH 6.4 to 6.8, and a maximum per cent inhibition by 60 μM norepinephrine was observed at pH 8.4. In Fig. 3A it can be seen that the per cent inhibition by norepinephrine in the pheochromocytoma continues to increase markedly as the pH is raised above 6.4. The pH optimum for the pheochromocytoma enzyme under control conditions was 7.8, whereas the pH optimum in the presence of Mg^{2+}, ATP and cyclic AMP or in the presence of 60 μM norepinephrine was 7.2 (Fig. 3B).

The pH optima for tyrosine hydroxylase from striatum and pheochromocytoma were determined in the presence and absence of Mg^{2+}, ATP and cyclic AMP using different buffers (Table 1). Tyrosine hydroxylase from pheochromocytoma exhibited similar activity at a given pH in either morpholinosulfonic acid buffer or Tris–acetate buffer. However, in both buffers the pH optimum was lower in the presence of Mg^{2+}, ATP and cyclic AMP than under control conditions. When the enzyme was assayed in potassium phosphate buffer, the pH optimum was lower than that observed in the other two buffers, but no shift in pH optimum occurred in the presence of Mg^{2+}, ATP and cyclic AMP. Under control conditions, striatal tyrosine hydroxylase exhibited small differences in pH optima in the different buffers employed. However, there were no shifts in pH optima in the presence of Mg^{2+}, ATP and cyclic AMP in any of the buffers examined.

Since maximum stimulation did not occur at the pH optimum for the enzyme, it was of interest to determine the pH at which maximum activation by Mg^{2+}, ATP and cyclic AMP took place. The pH for maximum stimulation by Mg^{2+}, ATP and cyclic AMP was different for the two enzymes, and appeared to be dependent on the buffer used in the assay (Table 2). The pH for maximum stimulation of tyrosine hydroxylase from pheochromocytoma was higher in potassium phosphate buffer than in either morpholinosulfonic acid buffer or Tris–acetate buffer. Moreover, the per cent increase was considerably less when potassium phosphate buffer was used. On the other hand, the pH for

Table 2. Optimal pH for maximal stimulation of tyrosine hydroxylase from pheochromocytoma and striatum by Mg^{2+}, ATP and cAMP*

Buffer	Pheochromocytoma pH for maximal stimulation	Pheochromocytoma Maximal stimulation (% increase)	Striatum pH for maximal stimulation	Striatum Maximal stimulation (% increase)
Morpholinosulfonic acid	6.4–6.8	60	6.8–7.2	150
Tris–acetate	6.6–6.8	55	7.0–7.2	90
Potassium phosphate	7.0–7.4	22	6.0–6.2	120

* Tyrosine hydroxylase was assayed as described in Materials and Methods in the absence and presence of Mg^{2+}, 10 mM; ATP, 1 mM; and cAMP, 10μ M. The data are representative of three separate experiments which varied by less than 8 per cent.

maximum stimulation of tyrosine hydroxylase from striatum was lower in potassium phosphate buffer than in either of the other two buffers studied. The per cent increase caused by Mg^{2+}, ATP and cyclic AMP was greater in the striatal enzyme than in the pheochromocytoma enzyme, regardless of the buffer utilized.

DISCUSSION

Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis [1], has been studied extensively by a number of laboratories. While the basic assay system is similar in most of these studies, some differences do exist, not only in the pH of the assay mixture, but also in the actual buffer employed. For example, potassium phosphate, sodium acetate, Tris–hydrochloride, and Tris–acetate have been used, and pH values for the assay have ranged from 5.5 to 7.1 [2, 6, 8, 19, 22, 23.]. Unfortunately, some of the buffers employed do not have significant buffering capacity at the pH chosen for the assay. Consequently, when the addition of a test substance results in an increase or decrease in enzyme activity, it may, in fact, be due in part to a change in the pH. In the course of our investigations on tyrosine hydroxylase, this type of artifact was indeed noted when small amounts of unneutralized ATP were added to reaction mixtures using Tris–acetate in the pH range of 6.0 to 7.0. Therefore, we undertook a systematic study of buffer and pH conditions which would be appropriate for use in the assay of tyrosine hydroxylase.

When the pH optima for tyrosine hydroxylase from pheochromocytoma, adrenal medulla and striatum were determined in morpholinosulfonic acid buffer, each showed a different pH optimum. The maximum enzyme activity occurred at pH 7.8 for the pheochromocytoma, at pH 6.8 for the adrenal medulla, and at pH 6.0 for the striatum. Previous studies have reported pH optima for tyrosine hydroxylase from human pheochromocytoma of 6.3 in acetate buffer [24], and from bovine adrenal medulla of 6.2 in dimethylglutarate or phosphate buffer [17]. For rat striatal tyrosine hydroxylase, a pH optimum of 5.8 has been reported, using Tris–acetate buffer [25]. The differences in pH optima obtained in the present study from those previously reported may be due, perhaps, to the preparation of the enzyme or the method of assay. In addition, the present data indicate that different pH optima are obtained when different buffers are utilized in the assay (Table 1). This has also been observed by Numata and Nagatsu [23] using bovine mandibular nerve preparation.

A number of recent reports have described the activation of tyrosine hydroxylase by the addition of Mg^{2+}, ATP and cyclic AMP to soluble preparations of the enzyme [2, 5, 6, 8]. When Mg^{2+}, ATP and cyclic AMP were added to soluble preparations of striatal tyrosine hydroxylase, the pH optimum was the same as that obtained under control assay conditions. A similar situation was also observed with soluble tyrosine hydroxylase from adrenal medulla. However, the enzyme from pheochromocytoma exhibited different pH optima under control and activating conditions. In the three enzymes studied, the pH for the maximum per cent stimulation by Mg^{2+}, ATP and cyclic AMP differs from the pH at which one observes maximum enzyme activity. Similar observations have been reported previously on the soluble enzyme from rat striatum [2]. It is interesting to note that above pH 7.6 the addition of Mg^{2+}, ATP and cyclic AMP to pheochromocytoma tyrosine hydroxylase results in an inhibition, rather than an activation of enzyme activity (Fig. 3, panels A and B). This was also observed when the reaction was carried out in Tris–acetate buffer (data not shown). A similar inhibitory effect was also observed below pH 5.6 when Mg^{2+}, ATP and cyclic AMP were added to striatal tyrosine hydroxylase (data not shown). This inhibitory effect of Mg^{2+}, ATP and cyclic AMP was independent of the buffer used, but was dependent on the pH of the reaction mixtures. When we investigated the effect of Mg^{2+}, ATP and cyclic AMP individually or in combination, the presence of Mg^{2+} seemed to be responsible for the observed inhibition. A complete study examining the effects of Mg^{2+}, ATP, cAMP and protein kinase alone and in combination in these three tissues has been published elsewhere [26].

While the studies above demonstrate clear differences in the behaviour of tyrosine hydroxylase activity in crude supernatant preparations of the three tissues examined, these studies do not answer the question of whether the enzyme itself is different or whether other factors present in the crude supernatant fluid influence enzyme activity.

A number of years ago it was demonstrated that catecholamines exert feedback inhibition on the rate-limiting enzyme, tyrosine hydroxylase [27]. Generally, the inhibitory effects have been studied at the pH which was optimum for the enzyme (usually pH 6.0). However, for the three enzymes examined in the present study, the per cent inhibition increased as the pH increased (Figs. 1–3). Thus, the potency of catecholamines as inhibitors of tyrosine hydroxylase may, in fact, be much greater at physiologic pH than at the pH most commonly used for the assay, i.e. pH 6.0 [3, 5, 10, 14, 22].

Most investigators have employed an acetate buffer in the assay of tyrosine hydroxylase at a pH at which acetate has little buffering capacity. While one observes similar activity of tyrosine hydroxylase in an acetate system as in a morpholinosulfonic acid buffer system, the buffering capacity of the latter occurs over the pH range of 5.6 to 7.8. Since tyrosine hydroxylase has a relatively sharp pH optimum, it becomes especially important to maintain the pH of the reaction upon the addition of test substances if reliable results are to be obtained. Moreover, the observations that pH and buffer influence tyrosine hydroxylase activity both quantitatively and qualitatively may, in part, account for some of the differences in results on tyrosine hydroxylase obtained in various laboratories.

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REFERENCES

1. M. Levitt, S. Spector, A. Sjoerdsma and S. Udenfriend, *J. Pharmac. exp. Ther.* **148**, 1 (1965).
2. M. Goldstein, R. L. Bronauch, B. Ebstein and C. Roberge, *Brain Res.* 109, 573 (1976).

3. J. E. Harris, R. J. Baldessarini, V. H. Morgenroth, III and R. H. Roth, *Nature, Lond.* **252**, 156 (1974).
4. J. E. Harris, R. J. Baldessarini, V. H. Morgenroth, III and R. H. Roth, *Proc. natn. Acad. Sci. U.S.A.* **72**, 789 (1975).
5. V. H. Morgenroth, III, L. R. Hegstrand, R. H. Roth and P. Greengard, *J. biol. Chem.* **250**, 1946 (1975).
6. W. Lovenberg, E. A. Bruckwick and I. Hanbauer, *Proc. natn. Acad. Sci. U.S.A.* **72**, 2955 (1975).
7. N. Weiner, E. Barnes and F. L. Lee, in *Neuroregulators and Psychiatric Disorders* (Eds E. Usdin, D. A. Hamburg and J. D. Barchas), p. 75. Oxford University Press, New York (1977).
8. T. Lloyd and S. Kaufman, *Biochem. biophys. Res. Commun.* **66**, 907 (1975).
9 J. D. Raese, A. M. Edelman and J. D. Barchas, *Trans. Am. Soc. Neurochem.* **8**, 147 (1977).
10. V. H. Morgenroth, III, M. C. Boadle-Biber and R. H. Roth, *Proc. natn. Acad. Sci. U.S.A.* **71**, 4283 (1974).
11. V. H. Morgenroth, III, M. C. Boadle-Biber and R. H. Roth, *Molec. Pharmac.* **11**, 427 (1975).
12. R. H. Roth, V. H. Morgenroth, III and P. M. Salzman, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **289**, 327 (1975).
13. N.N. Osborne and V. Neuhoff, *Hoppe-Seyler's Z. physiol. Chem.* **357**, 1271 (1976).
14. R. H. Roth and P. M. Salzman, in *Structure and Function of Monoamine Enzymes* (Eds E. Usdin, N. Weiner and M. B. H. Youdim), p. 149. Marcel Dekker, New York (1977).
15. Y. Takai, K., Nishiyama, H. Yamamura and Y. Nishizuka, *J. biol. Chem* **250**, 4690 (1975).
16. H. Blaschko, *Clin. Sci.* **34**, 453 (1968).
17. R. Shiman, M. Akino and S. Kaufman, *J. biol. Chem.* **246**, 1330 (1971).
18. S. Kaufman, *Meth. Enzym.* **5**, 812 (1962).
19. J. T. Coyle, *Biochem. Pharmac.* **21**, 1935 (1972).
20. M. Boadle-Biber and R. H. Roth, *Br. J. Pharmac.* **46**, 696 (1972).
21. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
22. B. Zivkovic, A. Guidotti and E. Costa, *Molec. Pharmac.* **10**, 727 (1974).
23. Y. Numata and T. Nagatsu, *J. Neurochem.* **24**, 317 (1975).
24. T. Nagatsu, K. Mizutani, Y. Sudo and I. Nagatsu, *Clinica. Chim. Acta* **39**, 417 (1972).
25. R. Kuczenski, *Life Sci.* **13**, 247 (1973).
26. J. R. Simon, L. R. Hegstrand and R. H. Roth, *Life Sci.* **22**, 421 (1978).
27. S. Udenfriend, P. Zaltzman-Nirenberg and T. Nagatsu, *Biochem. Pharmac.* **14**, 837 (1965).