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COMMENTARY

SCOPE AND LIMITATION IN DOPAMINE β-HYDROXYLASE MEASUREMENT

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It is beyond doubt that the important role attributed to dopamine-β-hydroxylase (DBH) in the dynamic events of neurotransmission originates mainly from the three following features: firstly, it forms the last step leading to the synthesis of noradrenaline; secondly, this enzyme is specifically localized within the granules or vesicles containing catecholamines and finally, like the catecholamines, it flows down along the axon of neurons (axonal flow) and may be released from nerve terminals or chromaffin cells by exocytosis.

It often happens that the growing interest in a particular field of experimental research coincides with the introduction of a new and more sensitive method. Dopamine-β-hydroxylase provides a good example. Indeed, since 1971, the number of studies devoted to this enzyme has increased tremendously. Until then the main efforts had been restricted to the adrenal medulla or, occasionally, to certain sympathetic nerves.

The purpose of the present paper is not to provide a survey (for this we refer the reader to excellent reviews [1–3]) on DBH but rather to draw the attention to some critical aspects of the methods used in studying DBH as well as to the interpretation of results, in order to prevent, as far as possible, further inopportune and uncontrolled studies on DBH, particularly in serum under normal or pathological conditions. More specific problems concerning exocytosis and axonal flow have been extensively discussed elsewhere [4, 5] and will not be reconsidered in this paper.

1. REMARKS ON METHODOLOGY

Until 1971, DBH could only be measured in tissues containing high enzyme activities as in adrenal medulla or certain sympathetic nerves. A first group of assays using dopamine or epinine as substrate [6, 7] had to be mainly restricted to DBH measurements from purified enzyme preparations since the high amount of catecholamines present in the crude extracts might mask the appearance of the reaction products. Despite its limitation, this method has revealed the fundamental mechanisms of the enzymatic reaction [1]. Moreover, the great advantage of these studies was the use of natural substrates like dopamine and even epinine, as recently suggested [8].

By using tyramine as substrate, DBH may be measured from crude extracts [9, 10]. However, this assay has sometimes been simplified by using labelled tyramine and omitting the prior isolation of reaction products upon ion exchange chromatography. Although simple and rapid, this method has lost its specificity. Indeed, in addition to labelled *p*-hydroxybenzaldehyde formed by periodate cleavage, the reaction products yielded by monoamine oxidase (MAO) could be extracted together in the same way. Tyramine is known to be a very good substrate for MAO [11], which is always present in amounts higher than DBH in nearly all tissues. Consequently the use of such a method for unpurified enzyme preparation necessitates a complete inhibition of MAO activity. In this regard, tranylcypromine or pargyline is generally used; yet one must make sure that MAO activity is absolutely blocked, as even 2 per cent residual MAO is enough to create the illusion of a DBH activity. This represents a possible source of errors which can be easily overcome by passing the reaction mixture through an ion exchange column as described in the original method [9, 10].

Recently, a more sensitive method has been introduced to measure very low DBH activities [12, 13]. It is based on a coupled enzymatic assay; in the first reaction, tyramine or phenethylamine is converted to its respective β-hydroxylated derivatives which in turn, are *N*-methylated in a second step, by adding to the incubation mixture purified *N*-methyltransferase (PNMT) and radioactive *S*-adenosylmethionine (Fig. 1). By means of this method, it has been possible to measure DBH in tissues like brain, nerves, rat adrenal medulla or serum for which the other methods were inadequate. However, unless the appropriate precautions are taken, this coupled enzymatic assay can give rise to erroneous interpretations of the results. At first sight it seems evident that a procedure requiring two enzymatic reactions is more complicated than that based on a single one.

Firstly, it must be emphasized that phenethylamine can be *N*-methylated by PNMT, especially when the substrate concentration is high (10^{-3} M) as in the case of experimental conditions for the DBH determination (unpublished results). Therefore, as the substrate specificity for PNMT is not as high as firstly described [14],

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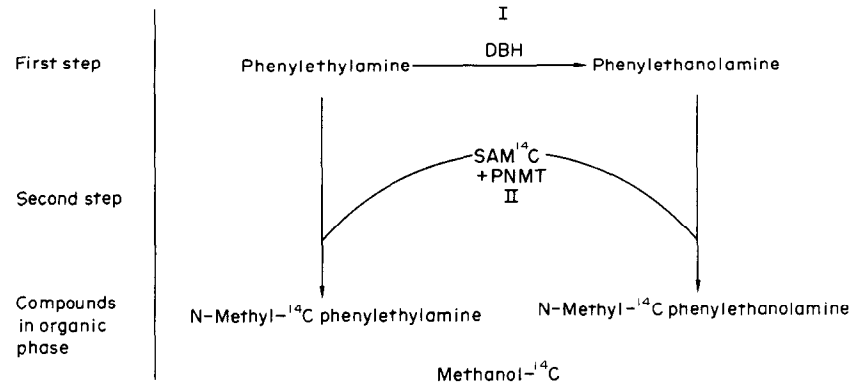

Fig. 1. Schema illustrating the DBH coupled-reaction assay and showing the different reaction products formed and extracted.

it seems to me that the name of this enzyme, phenylethanolamine *N*-methyl transferase, is not quite appropriate and can lead to certain confusion. This *N*-methylation of phenethylamine was also clearly observed in the original paper of Molinoff *et al.* [12], but in this particular case an identical peak of *N*-methylphenethylamine was also detected chromatographically in the blank.

Another difficulty encountered in measuring DBH by the coupled reaction consists of the choice of the blank. Boiled enzyme is commonly used as blank. Therefore, in this case, only the β-hydroxylation is prevented while the second reaction yielding *N*-methylated products may normally occur, since PNMT is added to the incubation mixture of the assay as well as of the blank. However, in these conditions, certain precautions must be taken if the methanol forming enzyme is present together with DBH. Indeed this enzyme may increase the radioactivity extracted in the assay but not in the blank where it has been heat-inactivated. Secondly, if PNMT is present together with DBH in the sample—for instance in adrenal medulla—a higher amount of radioactive products could be extracted in the assay than in the blank because of the higher content of PNMT in the former. These two examples can give rise to overestimated DBH values. In contrast, underestimated DBH values may be obtained if the sample being analyzed contains a PNMT inhibitor which can be denatured by heat in the blank. It is to note that labelled methanol can be also produced from enzyme preparations containing catechol-*O*-methyltransferase, histamine-*N*-methyltransferase or PNMT, even when these enzymes are purified. Therefore in certain conditions, this methanol formation could give rise to a higher amount of extracted radioactivity in the assay than in the blank.

More important is the major drawback that the endogenous inhibitors of DBH represent for the assay. All crude extracts contain one or several endogenous inhibitors. To overcome this difficulty, different copper concentrations or *N*-ethylmaleimide are used. However, as reported by Molinoff *et al.* [12], various enzyme concentrations from rat tissues did not require the same Cu^{2+} concentration to reach an optimal activity. So 1:50, 1:100 and 1:200 dilutions of enzyme gave maximal DBH activity in the presence of 80, 50 and 20 μM copper sulphate, respectively. Moreover, even in these optimal conditions, the 1:200 dilution exhibited a much higher DBH activity than the 1:50 dilution which normally contains four times more enzyme. In this particular case, the reaction was found to follow a linear course with enzyme concentrations up to the 1:400 dilution. Consequently, the range of linearity was extremely narrow, and the assay conditions were so critical that it was necessary to determine the appropriate dilution for each sample belonging to the linear part of the curve. Although certain endogenous inhibitors are believed to be sulfydryl compounds, it seems likely that others remain unknown.

In order to rule out possible sources of error related to the methodology of DBH assay, I suggest that all the papers bearing on DBH activities measured by the coupled reactions mention detailed results on: (1) identification of the reaction products, especially when the experimental conditions are modified by pharmacological drugs or stress reactions. It is somewhat surprising that in one out of two papers describing the coupled reaction [13], nothing about the identification of reaction products was reported; (2) linearity of the reaction by increasing the enzyme concentration; (3) different kinds of blank must be checked; boiled enzyme, ascorbate and fumarate omitted during the first step, enzyme only added after the first step; (4) inclusion of internal standard of phenylethanolamine or octopamine in the assay as recommended by Molinoff *et al.* [12], but also in the blank; (5) influence of several $CuSO_4$ concentrations must be tested for each sample, especially when DBH activities are compared in two different experimental groups, indeed, it seems likely that various factors like drugs, food and emotional and

physiological stress can modify the endogenous inhibitor concentration and therefore artifically change the DBH activity.

In spite of these precautions, the problems arising from the presence of endogenous inhibitors have not been solved yet. It is often believed that the addition of purified DBH to a given sample allows one to estimate the amount of endogenous inhibitors. This is not true as the amount of DBH is critical too. In other words, for the same amount of endogenous inhibitors, a lower DBH activity (i.e. in serum) will be much more affected than a higher one when purified DBH is added. Therefore, it is extremely difficult to ascertain that two samples have an identical amount of endogenous inhibitors, or that this amount was not altered by pharmacological agents or stress reactions.

Another point giving rise to difficulties concerns the K_m of DBH. It is illusive to believe that, by using the coupled reaction assay, it is possible to determine the K_m of DBH. In fact this system involves two reactions with four different substrates, one for the first step (i.e. tyramine) and three for the second (tyramine, octopamine and SAM) (cf. Fig. 1). As the experimental conditions are never optimal for the second step, it is impossible to attach any value to a K_m obtained in this way. The K_m is of quite relative interest, even for a two-substrate enzyme. Therefore, the comparison of two K_m's of DBH is not relevant and cannot be considered as an index of identity for the enzyme provided by two different samples.

More recently, a new method for DBH has been introduced [15, 16]. It is a radio-immunoassay using antibodies against DBH produced in rabbits. At first sight this procedure presents numerous advantages for measuring the total enzyme protein rather than the highly variable residual activity of the enzyme [15]. A real advantage of this method, contrary to the preceding one, is that the endogenous inhibitors do not seem to counteract the enzyme measurement. Further investigation will show whether or not this latter method is preferable to the coupled reaction assay.

2. INTERPRETATION OF DBH RESULTS

In recent years, DBH has been measured in various tissues and in many human sera, therefore it seems useful to recall some of the most important principles to be taken into consideration for a valid interpretation of results bearing on this enzyme.

Which are the factors capable of modifying DBH levels? There are numerous factors of physiological or pharmacological origin which may theoretically affect different steps: protein synthesis, axonal flow, exocytosis and degradation. Change in DBH activity may be induced by change in any one process. Furthermore, as all the DBH measurements are performed by an *in vitro* determination, i.e. the coupled-reaction procedure, it is necessary to control in a critical manner the possible artifacts resulting from the determination itself. Which are the main factors one must keep in mind for the interpretation of DBH results?

(1) The physical condition of animals and patients is most important. Unfortunately it has often been neglected. Since the DBH level presumably depends on the general activity of the adrenergic system, it is conceivable that numerous environmental factors (stress, psycho-social influence, conditions of blood sampling, etc.) can modify the DBH levels, especially in serum samples [17]. A very good example has been suggested by Goodwin [18] in the course of homovanillic acid (HVA) determination in cerebrospinal fluids. HVA was found to be lower in depressed patients than in a control group. In order to evaluate the possible contribution of activity differences to these data, a group of depressed patients was asked to simulate the hyperactivity of mania for four hours preceding a lumbar puncture. In these conditions Goodwin found that the HVA levels of depressed patients were significantly increased as compared with control values. Therefore it was clearly demonstrated that a modification in the psycho-physical condition of such patient just before the puncture might modify the HVA levels of the cerebrospinal fluid. The question remains whether the extremely high variability of the DBH activity in human serum is not due to the difference of the physical or psychological condition of patients. Indeed, it is not exceptional to find a DBH activity ranging from 100 to 2000 units in the same group of patients [19]. Because of its extreme variability, and for many other reasons, the DBH determination in plasma cannot be considered a routine analysis.

Up to now only a few cases, like phaeochromocytoma [20] or neuroblastoma [21], have revealed a significant elevation of their plasma DBH levels. Although some elevated or reduced plasma DBH activities were reported in autosomal dominant torsion dystonia [19] and in familial dysautonomia [22], it is still too early to attribute this change to a genetic defect. Many other factors, direct or indirect, cannot be excluded. Indeed, it does not seem appropriate to assess the nature of a genetic defect merely by measuring the enzyme activity in serum, especially as the release of DBH is itself modulated by various factors as the cholinergic system in the preganglionic fibers and in neurons of the central nervous system, possibly the prostaglandin synthesis [23] and other biochemical events involved in the exocytosis but until now, not yet well defined. Therefore, many factors in addition to the adrenergic system may cause an increase or decrease in plasma DBH levels.

In most of the studies so far, DBH activity was measured in serum and compared to control values. It would be much more appropriate to perform "longitudinal" studies in which DBH is repeatedly determined in the serum of the same patients for a long period. This kind of study has been carried out on hypertensive patients [24] or during a treatment with fusaric acid [25]. The conclusion was that serum DBH is not a satisfactory index of the sympathetic function and,

moreover, activity levels do not correlate with prevailing blood pressure [24]. Here again the plasma DBH activities were reported to vary from 3 to 581 units/ml in a group of 168 untreated adults [24]. However, these values tended to remain in a narrow range for an individual subject during a period of several months.

From the previous data it seems unlikely that the DBH determination in the serum of patients will become useful to validate a clinical diagnosis.

(2) Age. A striking feature of DBH is that, during the first years of life, its serum levels are very low and even reach the lower sensivity limits of the assay [26]. Afterwards, a continuing rise with age was observed to attain a maximum value throughout late adulthood [26]. Since adrenalectomy does not lower the serum DBH levels in rats, one may assume that most of the circulating DBH originates from extramedullary sympathetic tissues. Similarly, DBH activity was found to increase gradually in the rat brain during gestation, but also from birth to adulthood [27].

Nevertheless, although one of the characteristics of DBH activity is an increase during maturation, which can be considered as an index for the synapse formation, it is not known whether, during older age, DBH levels in brain and in blood remain unaltered. It is, therefore, advisable to compare DBH values in patients belonging to identical age groups. In a recent report dealing with DBH deficits in the brain of schizophrenic patients [28], a too high variation of age made the comparison of DBH values unconvincing.

(3) Influence of drugs. The direct or indirect influence of drugs on the DBH activity in sera or tissues may occur in four different ways. Firstly, the drug may interfere directly through the enzyme assay, not only during the β-hydroxylation itself, but also in the course of the *N*-methylation, if the coupled reaction method is used. Secondly the drug may induce a change in endogenous inhibitor concentration in such a way that the DBH values can be indirectly affected by it. Thirdly a drug may interact with the normal pathway of DBH at the stage of biosynthesis, axonal flow or release. Finally, a drug may be responsible for an increased degradation of DBH, especially after a long-term administration. DBH values in schizophrenic patients receiving phenothiazine for 10 or 20 years cannot be compared with those of untreated patients as reported by Wise and Stein [28]. The fact that DBH in rat brain remained unchanged after treatment with chlorpromazine for 5 or 12 weeks does not prove that DBH from patients treated during a period of several years should be not affected. Even if both DBH's (rat and human) were identical, a fact which remains to be proved, the relatively short duration of treatment in rats is not comparable with the long-time treatment (often more than 10 or 20 years) of schizophrenic patients.

(4) Enzyme kinetics. It is useless to compare two enzyme activities if the preliminary kinetic experiments necessary to reach optimal conditions have not been performed. In papers dealing with DBH levels, this point of view has been often neglected. As the linearity range is very narrow and depends on the Cu^{2+} concentration [12], with increasing enzyme concentrations it is necessary to mention the limits of this linearity in any paper concerning this enzyme. Therefore, more so than for any other enzyme, the elementary kinetic experiments as function of time and enzyme and substrate concentration are of prime importance for DBH.

In a recent paper [28], already quoted, a very surprising picture was presented, showing DBH activities as a function of the substrate concentration. The results were plotted r vs s and a nearly straight line was obtained for the control brains, as opposed to an upward concave curve for the brains of the schizophrenic group. Surprisingly, an identical K_m value was found for both groups. It is quite impossible for the equation of Michaelis and Menten to be graphically expressed by such an upward concave curve, which is the exact reverse of a saturation curve. Therefore, the calculation of a K_m value which, in any case, is already worthless for the DBH measured by the coupled reaction, was unfeasible in this particular case where even the elementary conditions of saturation were not acquired.

(5) Endogenous inhibitors. As already stressed previously, the endogenous inhibitors of DBH represent not only one of the major limiting factors but also a great drawback for the DBH assay when measured biochemically. The radioimmuno-assay is not hampered by this inconvenience. The presence of endogenous inhibitors was first shown in the adrenal medulla where they are believed to be sulphydryl compounds because the inhibition can be overcome by addition of *N*-ethyl-maleimide of Cu^{2+} to the incubation mixture [29, 30]. In contrast, such a reversal of the inhibition by *N*-ethyl-maleimide was not observed in bovine heart [31]. Recent experiments to isolate these inhibitors led to the conclusion that their sulphydryl nature was due to the presence of cysteine [32]. Since this amino-acid is in various proteins, it is easy to imagine that the endogenous inhibitors of DBH are widely distributed through the cell.

What is the physiological role of these endogenous inhibitors? Since the conversion of dopamine to noradrenaline by DBH is exclusively confined to the granules or vesicles, an important regulatory function could be attributed to these inhibitors if their presence was detected within the organelles containing catecholamines. In early studies [29, 30] such an identity of localization for DBH and endogenous inhibitors was apparently demonstrated. However, subsequent analysis of subcellular fractions obtained from bovine adrenal medulla ruled out the possibility that these endogenous DBH inhibitors are contained in the catecholamine granules [33]. As they were found in the supernatant fraction, a possible role in the regulation of catecholamine biosynthesis could be excluded.

More recently, our results [33] were interpreted by Kuzuya and Nagatsu [34] as being due to an inhibitory effect of Triton X-100 on DBH. However, in their experiments, the use of an Ultraturrax for tissue homo-

genization gave rise to an artificial redistribution of these inhibitors because the great shearing forces of this homogenizer disrupted not only the chromaffin cells and partially the subcellular structures, but even the framework or connective tissue which normally sedimented in the nuclear fraction instead of contaminating all the other fractions. Therefore, it may be concluded that these endogenous inhibitors of DBH are certainly not specific for the chromaffin granules but are provided by structures, proteins or small molecules containing sulphydryl groups.

The interpretation of two different DBH values implies a possible difference in the endogenous inhibitor content. For this reason, it is essential to determine the DBH activity in the presence of different concentrations of copper or *N*-ethyl-maleimide [12]. In spite of these recommendations, one can read this sentence in a recently written paper: "It is also unlikely that the deficit of DBH in the schizophrenic group is due to an excess of endogenous inhibitors. These would have been inactivated in the assay and dialysis of homogenate did not affect the activity of the enzyme" [28]. Such a statement is quite inadequate. Only a part of the natural inhibitors of bovine adrenal medulla were found to be dialyzable but very unstable [29]. The non-dialyzable parts are most probably proteins. Moreover, these inhibitors are stable to boiling for 10 min [30] and those isolated from bovine heart were reported to be heat stable [31]. Therefore, Wise and Stein [28] have underestimated the importance of endogenous inhibitors when interpreting their results.

(6) Inhibition of PNMT. Another source of error in the DBH results lies in a possible inhibition of PNMT, the second step in the coupled reaction method. Here again, inhibition could be caused by a drug or even by an endogenous compound. As noted by Molinoff *et al.* [12], at least a part of this inhibition by endogenous substances should be due to noradrenaline, or even adrenaline, both which are known to be potent inhibitors of PNMT. The inclusion of internal standards of phenylethanolamine or octopamine in the incubation mixture allows one to overcome this difficulty for assaying DBH.

(7) *N*-Methylation of tyramine and phenethylamine. Both amines may be converted to their *N*-methylated derivatives which can pass into the organic phase (Fig. 1). The use of an appropriate blank may eliminate this source of error. However, as previously noted, it is highly recommended to identify the reaction products formed in the course of the DBH assay, especially when experimental conditions are not comparable.

(8) Methanol-forming enzyme. In the PNMT assay, the blank, performed without substrate, may gradually increase as a function of time. It has been shown that the radioactive product extracted in these conditions is due to the formation of methanol [35]. Therefore, one has to choose an appropriate blank. Boiled PNMT is not to be used because a difference between the assay and the blank could only be due to a methanol formation.

To summarize briefly, measuring DBH is not easy and, for this reason, must be done and interpreted by specialized teams. The recent introduction of more sensitive methods for measuring DBH activity has allowed its detection, in tissues or liquids where it had not been possible before. In this regard, the field of the adrenergic neurotransmission has largely taken advantage of this contribution. Nevertheless, until now DBH levels cannot be considered as a biological test for some adrenergic defects or psychiatric diseases. Therefore, it is not useful to advertise the systematic use of the DBH assay in hospitals or psychiatric institutions as it is sometimes done. Many problems remain to be solved, such as the extreme variability of DBH activities in human serum.

REFERENCES

1. S. Kaufman and S. Friedman, *Pharmac. Rev.* **17**, 71 (1965).
2. P. Molinoff and J. Axelrod, *A. Rev. Biochem.* **40**, 465 (1971).
3. L. B. Geffen and B. G. Livett, *Physiol. Rev.* **51**, 98 (1971).
4. A. D. Smith and H. Winkler, in *Catecholamines* (Eds. H. Blaschko and E. Muscholl), p. 538. Springer, Berlin (1972).
5. P. Laduron, in *Dynamics of Degeneration and Growth in Neurons* (Ed. Fuxe, Olson and Zotterman), p. 245. Pergamon Press, Oxford (1974).
6. E. Y. Levin, B. Levenberg and S. Kaufman, *J. biol. Chem.* **235**, 2080 (1960).
7. W. F. Bridgers and S. Kaufman, *J. biol. Chem.* **237**, 526 (1962).
8. P. Laduron, *Archs intern. Pharmacodyn. Thér.* **195**, 197 (1972).
9. J. J. Pisano, C. R. Creveling and S. Udenfriend, *Biochim. biophys. Acta* **43**, 566 (1960).
10. C. R. Creveling, J. W. Daly, B. Witkop and S. Udenfriend, *Biochim. biophys. Acta* **64**, 125 (1962).
11. Z. Bacq, *Pharmac. Rev.* **1**, 1 (1949).
12. P. B. Molinoff, R. Weinshilboum and J. Axelrod, *J. Pharmac. exp. Ther.* **178**, 425 (1971).
13. M. Goldstein, L. S. Freeman and M. Bonnay, *Experientia*, **27**, 632 (1971).
14. J. Axelrod, *Pharmac. Rev.* **18**, 95 (1966).
15. R. R. Rush and L. B. Geffen, *Circulation Res.* **31**, 444 (1972).
16. L. B. Geffen, R. A. Rush, W. J. Louis and A. E. Doyle, *Clin. Sci.* **44**, 617 (1973).
17. Y. Hashimoto, Y. Kurobe and K. Hirota, *Biochem. Pharmac.* **23**, 2185 (1974).
18. F. K. Goodwin and R. L. Sack, in *Frontiers in Catecholamine Research* (Eds. E. Usdin and S. Snyder), p. 1157. Pergamon Press, Oxford (1973).
19. G. F. Wooten, E. Roswell, J. Axelrod and R. S. Stern, *New Engl. J. Med.* **288**, 284 (1973).
20. L. B. Geffen and R. A. Rush, in *Frontiers in Catecholamine Research* (Eds. E. Usdin and S. Snyder), p. 483. Pergamon Press, Oxford (1973).
21. M. Goldstein, L. S. Freedman, C. Bohuon and F. Guerinot, *New Engl. J. Med.* **286**, 1123 (1972).
22. R. Weinshilboum and A. Axelrod, *New Engl. J. Med.* **285**, 938 (1971).

23. L. Stjärne, in *Frontiers in Catecholamine Research* (Eds. E. Usdin and S. Snyder), p. 491. Pergamon Press, Oxford (1973).
24. D. Horwitz, R. W. Alexander, W. Lovenberg and H. R. Keiser, *Circulation Res.* **32,** 594 (1973).
25. T. Nagatsu, T. Kato, H. Kuruya, T. Okada, H. Umezawa and T. Takeuchi, *Experientia* **28,** 779 (1972).
26. L. S. Freedman, T. Ohuchi, M. Goldstein, F. Axelrod, I. Fish and J. Dancis, *Nature, Lond.* **236,** 310 (1972).
27. J. T. Coyle and J. Axelrod, *J. Neurochem.* **19,** 449 (1972).
28. C. D. Wise and L. Stein, *Science, N.Y.* **181,** 344 (1973).
29. T. Nagatsu, H. Kuzuya and H. Hidaka, *Biochim. biophys. Acta* **139,** 319 (1967).
30. D. S. Duch, O. H. Viveros and N. Kirshner, *Biochem. Pharmac.* **17,** 255 (1968).
31. I. W. Chubb, B. N. Preston and L. Austin, *Biochem. J.* **111,** 243 (1969).
32. D. S. Duch and N. Kirshner, *Biochim. biophys. Acta* **236,** 628 (1971).
33. F. Belpaire and P. Laduron, *Biochem. Pharmac.* **19,** 1323 (1970).
34. H. Kuzuya and T. Nagatsu, *Biochem. Pharmac.* **21,** 740 (1972).
35. R. D. Ciaranello, H. J. Danders and J. D. Barchas, *Mol. Pharmac.* **8,** 311 (1972).