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COMPARATIVE ASPECTS OF TYROSINE HYDROXYLASE AND TRYPTOPHAN HYDROXYLASE INHIBITION: ARTERENONES AND DIHYDROXYPHENYLACETAMIDE (H 22/54)

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Abstract—A bovine adrenal tyrosine hydroxylase preparation and a mouse mast cell tumor tryptophan hydroxylase have previously been shown to require a reduced pteridine cofactor. Both enzymes are inhibited *in vitro* by analogs of arterenone or β-keto-dihydroxyphenethylamines, and by H 22/54, a dihydroxyphenylacetamide, by an identical mechanism, i.e. competition with the reduced pteridine. Inhibition of tyrosine hydroxylase by H 22/54 was reduced by deleting exogenous Fe^{2+} from the incubate, but the inhibition by various arterenones and by α-methyltyrosine was not significantly altered. Both U-24, 274A, an arterenone analog, at 2000 mg/kg, and H 22/54 at 500 mg/kg reduced mouse brain norepinephrine. However, neither compound decreased mouse brain serotonin (5-HT); administration of either compound in combination with *p*-chlorophenylalanine (PCPA) also failed to diminish 5-HT levels below that produced by PCPA alone. Factors which may affect the specificity of enzyme inhibition *in vivo* are discussed.

A RAT LIVER preparation similar to that of Freedland *et al.*[1] has been employed in many of the previous studies concerned with the inhibition of tryptophan hydroxylase. Tryptophan and phenylalanine hydroxylating activities in these preparations were stimulated by several nicotinamide–adenine nucleotides[2–5] and by a reduced pteridine, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$).[2] Tryptophan hydroxylating activity of a mouse mast cell tumor, initially demonstrated by Levine *et al.*,[6] has an absolute requirement of $DMPH_4$,[7] and, when purified, a substrate specificity for tryptophan.[8] This similarity in cofactor requirements with those in brain tissue[9, 10] suggested that this preparation might be closely related to the tryptophan hydroxylating system in the brain.

The fact that H 22/54, a dihydroxyphenylacetamide, inhibited a beef adrenal tyrosine hydroxylase preparation *in vitro* by competing with $DMPH_4$[11] suggested a similar mechanism for its inhibition of tryptophan hydroxylase. During the course of our study, Levitt *et al.* noted that the inhibition *in vitro* of the adrenal tyrosine hydroxylase by arterenone and adrenalone was also overcome with increased concentrations of $DMPH_4$.[12, 13] These latter compounds were ineffective in producing inhibition of tyrosine hydroxylase *in vivo*. Rate-limiting steps in the synthesis of norepinephrine (NE) and serotonin (5-HT), tyrosine hydroxylase[14] and very likely tryptophan hydroxylase[15] respectively are both dependent upon reduced pteridine cofactors.[7, 9, 16] The possibility of affecting inhibition *in vivo* of one or both of these pathways by competing with the cofactor in these rate-limiting reactions was investigated.

This report compares the inhibition *in vitro* of these two reduced pteridine-requiring enzymes with β-keto-dihydroxyphenethylamines and H 22/54 and examines the effect of these catechols on the synthesis of mouse brain amines *in vivo*.

MATERIALS AND METHODS

Arterenone and the *w*-aminoacetophenone analogs were synthesized by Drs. B. D. Aspergren, R. V. Heinzelman, R. E. Strube, R. B. Moffett, J. B. Wright and M. E. Speeter of the Chemistry Laboratories, The Upjohn Company. U-2556, U-12,005E, and U-24,274A were generously provided by Drs. F. F. Blicke and J. B. Burckhalter, College of Pharmacy, University of Michigan. We wish to thank Dr. H. Corrodi, Hassle Laboratories, and the Merck Institute for Therapeutic Research for making available 3,4-dihydroxyphenylpropylacetamide (H 22/54) and L-α-methyltyrosine (α-MT), respectively. All other compounds were obtained from commercial sources. Tumor fluid was very generously supplied to us by Dr. Walter Lovenberg.

Enzyme isolation.

Tyrosine hydroxylase activity was isolated from a bovine adrenal medullary preparation as described by Udenfriend *et al.*[11, 16] Tryptophan hydroxylase from a mouse mast cell tumor was purified through the ammonium sulfate precipitation step of Lovenberg *et al.*[7] Guinea pig kidney 5-hydroxytryptophan (5-HTP) decarboxylase activity was also purified through the ammonium sulfate precipitation (step 3) reported by Clark *et al.*[17]

Enzyme assay.

Tyrosine hydroxylase. A standard incubate of 1 ml total volume contained the following: 200 μmole Na acetate buffer, pH 6·0; 2 mg protein; 0·5 μmole $FeSO_4$; 1·7 μmole $DMPH_4$; 100 μmole 2-mercaptoethanol (2-ME); and 0·1 μmole 3,5-^{3}H-L-tyrosine, 6–10 × 10^5 cpm/μmole (New England Nuclear Corp.). The assay procedure of Nagatsu *et al.*[18] was employed after a 15-min incubation period at 37°.

Tryptophan hydroxylase. The standard incubate contained the following: 100 μmole Tris-HCl, pH 8·0; 0·4-1 mg protein; 0·5 μmole $DMPH_4$; 50 μmole 2-ME; 0·1 mg pargyline; and 0·06 μmole L-tryptophan containing 2–4 × 10^5 cpm DL-tryptophan-2-^{14}C (New England Nuclear Corp.). The total volume was adjusted to 1·0 ml and incubated for 15 min at 37°. The incubate was then boiled, centrifuged, and the supernatant was decanted into a second tube. 5-HTP decarboxylase protein (2 mg), 0·5 μmole pyridoxal phosphate, and 0·5 μmole 5-HTP were then added and the mixture was incubated for 30 min. The assay procedure of Lovenberg *et al.*[9] was then used to determine the amount of radioactivity in 5-HT.

Inhibition studies

The inhibitor was dissolved in aqueous solution containing dilute acid, dilute base, dimethylformamide or ethanol, and added to the incubation. These solvents did not produce inhibition of either enzyme at the volumes or concentrations used. No preincubation period was used. Control assays were run in duplicate with each assay. The standard inhibitor in each tyrosine hydroxylase assay was H 22/54, which provided 65–75 per cent inhibition at 10^{-4} M. U-0603 (Table 1) was employed in each tryptophan hydroxylase assay as a standard inhibitor affording 70–80 per cent inhibition at 5 × 10^{-6} M. Compounds which inhibited the incorporation of label into

5-HT were then assayed in the decarboxylase portion of the assay to determine their inhibition of the 5-HTP decarboxylase. 5-HT formed from 1×10^{-3} M 5-HTP was assayed by the direct acid assay.[19] α-Methyl-DOPA inhibited the decarboxylation of 5-HTP 70-80% at 10^{-4} M.

Studies in vivo

To determine the effect of adrenalone derivatives and H 22/54 on enzyme inhibition *in vivo*, U-24,274A and H 22/54 were dissolved in 0·25% aqueous methylcellulose. α-MT and *p*-chlorophenylalanine (PCPA) were each suspended with the aid of sonication in the same vehicle. All compounds were administered i.p. to CF-1 male mice (18–22 g) as follows: (1) Mice received 500 mg/kg U-24,274A every 2 hr for a total of 2000 mg/kg. α-MT (50 mg/kg) was administered at 6 hr to the U-24,274A-treated mice or to mice similarly injected with diluent. All mice were sacrificed at 8 hr. (2) Mice received 500 mg/kg or 1000 mg/kg H 22/54 in one or two injections of 500 mg/kg 2 hr apart. Mice were sacrificed 2 hr after the last dosing. (3) Mice received 500 mg/kg PCPA at 0 hr and 24 hr later. A dosing procedure for U-24,274A similar to that described in (1) above was started at 16 hr, 40 hr, or 64 hr. All mice were sacrificed at 72 hr. (4) PCPA was similarly administered at 0 hr and 24 hr and H 22/54, 500 mg/kg, was administered at 70 hr.

Mice were sacrificed by decapitation and brains were placed on dry ice. Two brains were pooled, homogenized in 3 vol. of 0·01 N HCl and extracted.[20] Aliquots of the aqueous extract were assayed for NE[21] and 5-HT.[19] Amine content was determined by comparison of the fluorescence of duplicate tissue-containing internal standards. These results were not corrected for recovery in the extraction procedure.

RESULTS

Table 1 summarizes the comparative inhibition of tyrosine hydroxylase and tryptophan hydroxylase with the *w*-aminoacetophenones and H 22/54 (D). The phenolic derivatives of the acetophenones or arterenones (A), were inhibitory in both assays, but the methoxy (B), and the unsubstituted analogs (C) (U-14,177E) were without or possessed very little inhibitory activity in either assay. The relative inhibitory activity of the two catechol-containing groups varied greatly in the two assays. H 22/54 was the most potent inhibitor of the tyrosine hydroxylase, but it was a weaker antagonist of the hydroxylation of tryptophan than were the arterenones (A).

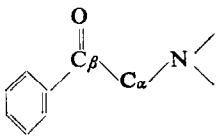
The mechanism of inhibition of both enzymes by the arterenones and H 22/54 was determined. Figs. 1 and 2 show the reversal of tyrosine hydroxylase and tryptophan hydroxylase inhibition by U-12,696A with increased concentrations of $DMPH_4$. Udenfriend *et al.* have previously reported a similar mechanism of inhibition of tyrosine hydroxylase by H 22/54.[11] Both catechol groups represented by H 22/54 and U-12,696A respectively, inhibit the hydroxylases by the same mechanism, i.e. successfully competing with the reduced pteridine.

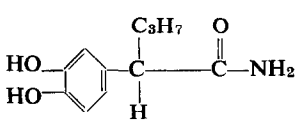
Although exogenous Fe^{2+} was added only to the tyrosine hydroxylase incubate, a role for this cation in both hydroxylating mechanisms has been inferred.[9, 16] A possible mechanism of inhibition of the tyrosine hydroxylation via chelation of the Fe^{2+} by the catechol-containing inhibitors was next investigated. Results in Table 2 indicate that omission of Fe^{2+} from the incubate, while markedly decreasing the efficiency of the hydroxylation reaction, does not appreciably affect the extent of

inhibition by the arterenones or α-MT. However, inhibition by H 22/54 was markedly decreased in the absence of exogenous Fe^{2+}.

U-12,696A was without effect upon mouse brain amines after 72 hr on a chronic mouse feeding procedure previously described[22] in which approximately 500 mg/kg/day was ingested. At the very high cumulative i.p. dose of 2000 mg/kg, U-24,274A decreased mouse brain NE 23 per cent and further reduced NE levels after α-MT by the

TABLE 1. INHIBITION OF TYROSINE AND TRYPTOPHAN HYDROXYLASE BY *w*-AMINOACETOPHENONES INCLUDING NORADRENALONE DERIVATIVES—EFFECT OF SUBSTITUTION AND COMPARISON WITH H 22/54



	U-No.	*Para*	*Meta* 3 5	*Ortho* 2 6	α	N	Tyrosine hydroxylase inhibitor concentration (M 10^{-4}	Tryptophan hydroxylase inhibitor concentration (M 5×10^{-5}	5×10^{-6}
							% Inhibition		
(A)	0603	OH	OH,H	H,H	H,H	H,H	49		72
	2134	OH	OH,H	H,H	H,H	H,CH_3	55		65
	2556	OH	OH,H	H,H	H,H	—$(CH_2)_6$—	46		74
	2956	OH	OH,H	H,H	H,H	—$(CH_2)_4$—	46		82
	12,005E*	OH	OH,H	H,H	H,H		61		60
	12,696A†	OH	OH,H	H,CH_3	H,H	H,CH_3	60	83	50
	12,844A†	OH	OH,H	H,H	H,H	H, —$CH(CH_3)_2$	38		65
	24,274A†	OH	OH,H	H,H	H,H	CH_3,CH_3	40		75
(B)	12,995A†	OCH_3	OCH_3,H	H,H	H,H	H,CH_3	8	2	
	15,802E*	OCH_3	OCH_3,H	H,H	CH_3,H	H,CH_3	8	9	
	16,332	OCH_3	OCH_3,H	OCH_3,H	H,H	H,CH_3	13	2	
	24,038	OCH_3	OCH_3, OCH_3,	H,H	H,H	CH_3, —$CH_2C_6H_5$	11	5	
(C)	14,177E†	H	H,H_3	H,H	H,H	H,H	5	15	
(D)	H22/54						78	74	23

* Inhibitors assayed as the HCl monohydrate salt. Conditions of incubation and assay are described in Materials and Methods.

† Inhibitors assayed as the HCl salt.

same extent (Table 3). H 22/54 at both 500 mg/kg and 1000 mg/kg decreased brain NE concentrations to 69 and 47 per cent of control levels, respectively. Although dopamine values are not reported here, since the determination of this amine was not carried out in all experiments, dopamine levels were also reduced after U-24,274A and H 22/54. Mouse brain 5-HT levels were not affected by either compound under these conditions.

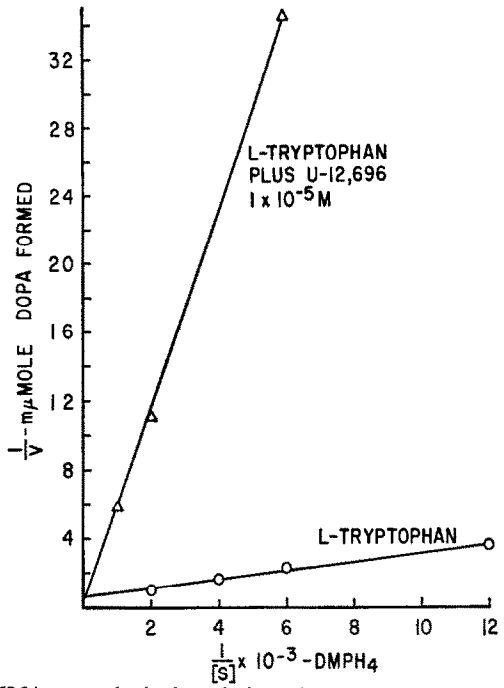


FIG. 1. Effect of U-12,696A upon the hydroxylation of tyrosine. $DMPH_4$ concentrations were varied from $1{\cdot}7 \times 10^{-3}$ M to 5×10^{-5} M. Both tyrosine and inhibitor concentrations were kept at 1×10^{-4}M.

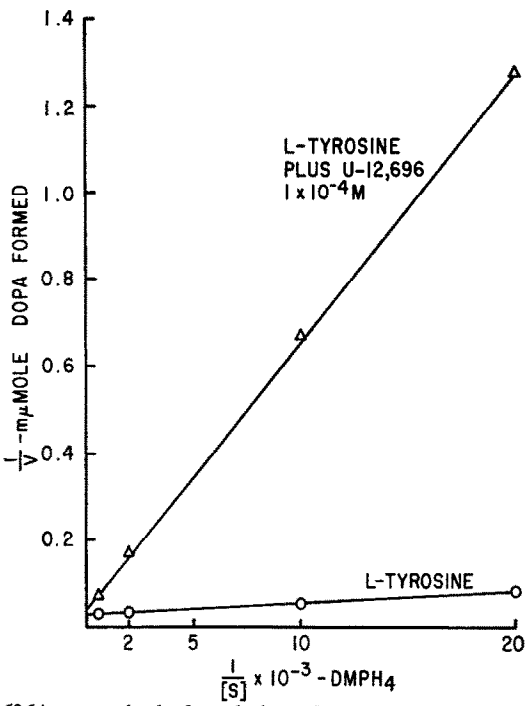


FIG. 2. Effect of U-12,696A upon the hydroxylation of tryptophan. $DMPH_4$ concentrations in the hydroxylase incubate varied from 5×10^{-4}M to $8{\cdot}3 \times 10^{-5}$M. Tryptophan concentration was kept at 6×10^{-5}M.

PCPA inhibits the hydroxylation of tryptophan.[23] As a result of its administration brain levels of 5-HT and its metabolites are reduced, while brain catecholamines are not affected. This inhibitor is less effective in mice than in other species, but repetitive dosing will produce substantial decreases in mouse brain 5-HT.[23] Not wishing to deplete mouse brain 5-HT as extensively as Koe and Weissman did with 300 mg/kg/day

TABLE 2. EFFECT OF Fe^{2+} ON CATECHOL INHIBITION OF TYROSINE HYDROXYLASE*

	Fe^{2+}			
	0		0·5 μmole	
	(μmole)	(% inhibition)	(μmole)	(% inhibition)
Control	8·3		23·1	
H 22/54	5·3	36	8·3	64
U-0603	6·1	26	17·1	26
U-2134	5·9	30	16·3	29
U-2556	6·1	26	16·6	28
U-12,696A	5·7	31	13·5	41
U-12,005E	4·8	42	10·0	56
U-24,274A	4·7	43	15·9	31
α-Methyltyrosine	3·3	60	8·2	65

* Data were from two separate experiments described under assay conditions of Materials and Methods, except that ferrous sulfate was omitted from one series. All inhibitor concentrations were 1×10^{-4} M.

TABLE 3. MOUSE BRAIN NE AFTER U-24,274A, U-24,274A WITH α-MT AND H 22/54

		NE	
	No.*	(μg ± S.D.)	(% of control)
Control	6	0·47 ± 0·02	100
U-24,274A, 2000 mg/kg	6	0·36 ± 0·04	77
α-Methyltyrosine, 50 mg/kg	6	0·34 ± 0·04	72
α-Methyltyrosine + U-24,274A (2+3)	6	0·25 ± 0·06	53
Control	3	0·51 ± 0·04	100
H 22/54, 500 mg/kg	3	0·35 ± 0·05	69
H 22/54, 1000 mg/kg	3	0·24 ± 0·06	47

* Number of determinations.

for 3 days,[23] we chose two daily doses of 500 mg/kg, which decreased 5-HT by approximately 50 per cent (Table 4). Additional treatment with U-24,274A (200 mg/kg) at the times indicated produced no further depletion of 5-HT. In a separate experiment, U-24,274A similarly administered from hour 64 to hour 70, after PCPA at 0 and 24 hr, had no effect on the reduction of 5-HT produced by PCPA. H 22/54, 500 mg/kg, at hour 70, after similar prior treatment with PCPA, also failed to produce any further decreases in mouse brain 5-HT.

DISCUSSION

Inhibition of tyrosine hydroxylase by catechol derivatives has been reported.[11–13] Levitt *et al.* previously demonstrated that in one of these catechol groups, the artere-

nones, phenolic groups in the 3- and 4-position were necessary for tyrosine hydroxylase inhibition.[12] Inhibition of the reduced pteridine-dependent tryptophan hydroxylation by arterenones has now been demonstrated (Table 1). Methylation of both ring hydroxyl groups in the arterenone series reduced almost totally the observed inhibition of both hydroxylases (Table 1B). Substitution in the *o*-position (compare

TABLE 4. EFFECT OF U-24,274A ON MOUSE BRAIN SEROTONIN LEVELS AFTER PRETREATMENT WITH *p*-CHLOROPHENYLALANINE (PCPA)*

Drug Treatment	5HT (μg/g)	Dose schedule PCPA (hr)	Dose schedule U-24,274A (hr)
Control	0·56		
PCPA, 1000 mg/kg	0·27	0,24	
U-24,274A, 2000 mg/kg	0·56		40–46
U-24,274A, 2000 mg/kg	0·52		16–22
PCPA, 1000 mg/kg + U-24,274A, 2000 mg/kg	0·32	0,24	40–46
PCPA, 1000 mg/kg + U-24,274A, 2000 mg/kg	0·25	0,24	16–22

* All animals were sacrificed at 72 hr. Each compound was administered at 500 mg/kg, i.p., at each injection. U-24,274A was administered each 2 hr for 6 hr.

U-12,696A vs. U-2134) or increasing the mass of the substituent upon the nitrogen (compare U-2556 vs. U-0603) did not significantly affect enzyme inhibition. In a comparison of inhibitory activity, U-12,005E and U-12,696A, which were most inhibitory in the hydroxylation of tyrosine, were relatively less active in the tryptophan hydroxylase assay. We can only speculate on the effects of substitution on the methylene carbon of the arterenone series upon hydroxylase inhibition. α-Alkyl substitution in the dopacetamides greatly influenced inhibition *in vitro* of tyrosine hydroxylase;[7] H 22/54, the α-propyl derivative, was the most potent inhibitor in this series.

A role for Fe^{2+} in these hydroxylation reactions has been noted. Data in Table 2 illustrate the stimulation of tyrosine hydroxylation by Fe^{2+}. Although catechols chelate divalent metals,[24] we have not assessed the role of chelation in the inhibition of these hydroxylases, and whether the variable effect upon tyrosine hydroxylase *in vitro* of the two structurally similar catechols may be related to their chelating capability. The competitive inhibition of tyrosine hydroxylase by α-MT was not affected by the deletion of exogenous Fe^{2+}.

The ineffectiveness of the arterenones in reducing brain levels of catecholamines and 5-HT was well illustrated in this experiment. Only after very high doses were NE levels affected by U-24,274A. This relative inability of U-24,274 A to inhibit catecholamine synthesis *in vivo* in comparison to the effects of H 22/54 may be related to their respective resistance to catabolism. H 22/54 has been reported to reduce mouse brain 5-HT and NE after single i.p. doses equal to or slightly less than those used in this study.[2, 25] We have no explanation for our failure to detect a reduction of brain 5-HT with this compound. However, chronic administration of H 22/54 to guinea pigs also failed to alter brain 5-HT or NE concentrations.[26]

Adrenalone will release ^{3}H-NE from mouse heart.[27] However, the concurrent decrease of brain dopamine with NE in U-24,274A-treated mice indicated that this drug was blocking synthesis of the catecholamines and not merely releasing NE from the brain.

Some compounds that inhibit both tyrosine hydroxylase and tryptophan hydroxylase *in vitro* by competing with the cofactor shared by these two enzymes lower levels of NE but not of 5-HT in the brain. This difference in their effects *in vivo* cannot be explained with the aid of the data now at hand.

REFERENCES

1. R. A. FREEDLAND, I. M. WADZINSKI and H. A. WAISMAN, *Biochem. biophys. Res. Commun.* **5**, 94 (1961).
2. S. B. ROSS and O. HALJASMAA, *Acta Pharmac. tox.* **24**, 55 (1966).
3. R. W. FULLER, *Life Sci.* **4**, 1 (1965).
4. W. P. BURKHARD, K. F. GEY and A. PLETSCHER, *Med. pharmac. Exp.* **13**, 388 (1965).
5. S. B. ROSS and O. HALJASMAA, *Life Sci.* **3**, 579 (1964).
6. R. J. LEVINE, W. LOVENBERG and A. SJOERDSMA, *Biochem. Pharmac.* **13**, 1283 (1964).
7. W. LOVENBERG, R. J. LEVINE and A. SJOERDSMA, *Biochem. Pharmac.* **14**, 887 (1965).
8. S. HOSODA and D. GLICK, *J. biol. Chem.* **241**, 192 (1966).
9. W. LOVENBERG, E. JEQUIER and A. SJOERDSMA, *Science* **155**, 217 (1967).
10. D. G. GRAHAME-SMITH and L. MOLONEY, *Biochem. J.* **96**, 66P (1965).
11. S. UDENFRIEND, P. ZALTZMAN-NIRENBERG and T. NAGATSU, *Biochem. Pharmac.* **14**, 837 (1965).
12. M. LEVITT, J. W. GIBB, J. W. DALY and M. A. LIPTON, *Fedn Proc.* **25**, 259 (1966).
13. M. LEVITT, J. W. GIBB, J. W. DALY, M. LIPTON and S. UDENFRIEND, *Biochem. Pharmac.* **16**, 1313 (1967).
14. M. LEVITT, S. SPECTOR, A. SJOERDSMA and S. UDENFRIEND, *J. Pharmac. exp. Ther.* **148**, 1 (1965).
15. M. DAY and J. P. GREEN, *J. Physiol., Lond.* **164**, 210 (1962).
16. T. NAGATSU, M. LEVITT and S. UDENFRIEND, *J. biol. Chem.* **239**, 2910 (1964).
17. C. T. CLARK, H. WEISSBACH and S. UDENFRIEND, *J. biol. Chem.* **210**, 139 (1954).
18. T. NAGATSU, M. LEVITT and S. UDENFRIEND, *Analyt. Biochem.* **9**, 122 (1964).
19. F. BOGDANSKI, A. PLETSCHER, B. B. BRODIE and S. UDENFRIEND, *J. Pharmac. exp. Ther.* **117**, 82 (1956).
20. P. A. SHORE and J. S. OLIN, *J. Pharmac. exp. Ther.* **122**, 295 (1958).
21. U. S. VON EULER and I. FLODING, *Acta. physiol. scand.* **33**, *suppl.* **118**, 45 (1955).
22. G. A. JOHNSON, E. G. KIM, W. VELDKAMP and R. R. RUSSELL, *Biochem. Pharmac.* **16**, 401 (1967).
23. B. K. KOE and A. WEISSMAN, *J. Pharmac. exp. Ther.* **154**, 499 (1966).
24. R. R. GRINSTEAD, *Biochemistry, N.Y.* **3**, 1308 (1964).
25. A. CARLSSON, H. CORRODI and B. WALDECK, *Helv. chim. acta* **46**, 2271 (1963).
26. S. SPECTOR, A. SJOERDSMA and S. UDENFRIEND, *J. Pharmac. exp. Ther.* **147**, 86 (1965).
27. J. W. DALY, C. R. CREVELING and B. WITKOP, *J. med. Chem.* **9**, 273 (1966).