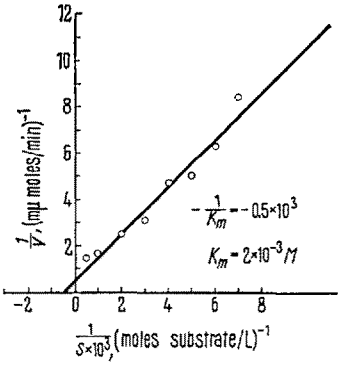
Enzymatic 5-Hydroxylation of 3-Methoxytyramine

Hydroxylation of phenylalanine to tyrosine[1], and subsequently to DOPA[2], by oxygenating enzyme systems present in many sources of mammalian tissue, have been demonstrated to be the key initial steps for the biosynthesis of catecholamines[3]. The existance of these enzymes and the necessary cofactors have been demonstrated by extensive in vitro and in vivo studies[4]. The only report of in vivo hydroxylation of a catecholamine to a trihydric phenolic amine was the demonstration by SENOH et al.[5] that 2,4,5-trihydroxy-β-phenethylamine was excreted as a minor labeled urinary metabolite when dopamine-8-C^{14} was administered to rats. LEETE[6] showed that tyrosine-8-C^{14} is converted to mescalin-8-C^{14} in the tissues of the cactus *Anhalonium lewinii*. This is direct evidence for the biological transformation of a monohydric phenol to a completely *O*-methylated trihydric phenolic amine by a plant organism. However, further hydroxylation of catecholamines or other dihydric phenols to 3,4,5-trihydric phenols has not previously been reported to occur in mammals.

We have found that hydroxylation of 3-methoxytyramine (4-hydroxy-3-methoxy-β-phenethylamine) to 3-methoxy-4,5-dihydroxy-β-phenethylamine does occur when 3-methoxytyramine is offered as a substrate to the hydroxylating enzyme systems in rat or rabbit liver homogenates. There is no appreciable difference in the rate of hydroxylation between the liver homogenates of these species. 3-Methoxytyramine has been shown by KUEHL et al.[7] to be a normal metabolite of dopamine, which is converted to 3-methoxytyramine by purified rat liver catechol-*O*-methyltransferase. 3-Methoxytyramine was chosen as the substrate for our initial studies because it could be readily labeled in the 5-position with tritium. Using this tritium-labeled amine as a substrate, hydroxylation was found to occur in the 5-position by measurement of the amount of tritiated water formed using the technique described by POMERANTZ[8].

The 3-methoxytyramine-5-H^3 was obtained by catalytic tritium exchange with 5-bromo-3-methoxy-4-hydroxy-β-phenethylamine[9], which was obtained by reduction of 5-bromo-4-hydroxy-3-methoxy-β-nitrostyrene, m.p. 184–185 °C, with lithium aluminum hydride. Bromination of vanillin to 5-bromovanillin followed by a methylamine-catalyzed condensation with nitromethane afforded the intermediate nitrostyrene. The 3-methoxytyramine-5-H^3, which was stored in 0.01 N HCl solution, was radiochromatographically homogeneous and showed the same Rf value on a silica gel TLC chromatogram as an authentic sample of 3-methoxytyramine using *n*-butanol-acetic acid-water (4:1:5) as the developing solvent.

Homogenates prepared from the livers of Sprague-Dawley rats (250–300 g) in potassium phosphate buffer (0.1 M; pH 7.4; 2 ml/g of tissue) were used in this study; identical results were obtained when homogenates were prepared in an identical manner from albino rabbit livers. In typical experiments, mixtures containing homogenate, buffer, and 2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridine as a cofactor were incubated in air at 37 °C with 3-methoxytyramine-5-H^3 (tracer level) as a substrate. Since the radiolabeled portion of the substrate undergoes a slow tritium exchange in aqueous medium, it was necessary to measure the quantity of tritiated water present in identical control mixtures which had been inactivated by means of 1 M metaphosphoric acid prior to incubation; this reagent was also used to inactivate samples after appropriate incubation times. Both samples and controls were first centrifuged (2100 g) and the supernatant passed through a column of 1:1 Celite-Norit A (Pfanstiehl Laboratories) to remove organic matter[8]. The effluent solution of tritiated water and inorganic ions was diluted to a standard volume (10 ml) with water, and a 0.5 ml aliquot was counted in BRAY's solution[10] to determine the tritium content, which was proportional to the extent



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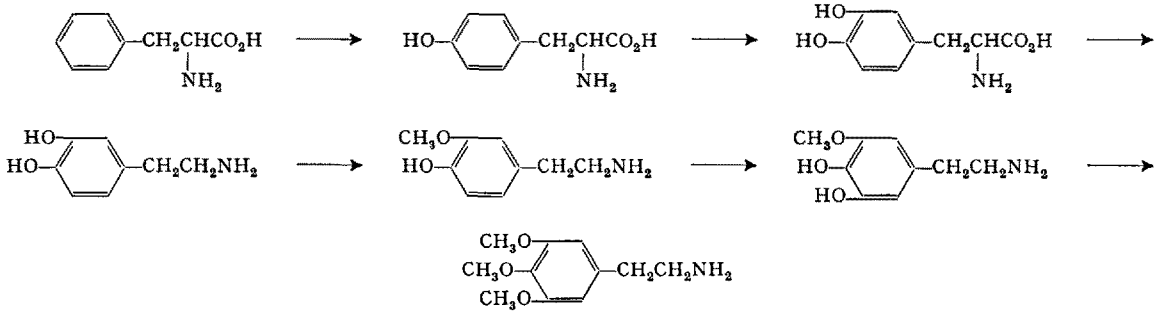

of 5-hydroxylation occurring in the substrate[11]. In order to minimize the quantity of tritiated water present initially in the samples and controls, the total volume of the labeled substrate solution to be used in a particular run was evaporated to dryness in a stream of nitrogen gas immediately before use and reconstituted to the initial volume with deionized water.

In the Figure there is shown the mean rate of hydroxylation as a function of substrate concentration (10^{-2} to $10^{-4}M$); these data exhibit typical Lineweaver-Burke behaviour and the Michaelis-Menton constant is $2 \times 10^{-3}M$ with respect to the substrate. Mean velocity constants at 37 °C were 0.31 mμmole/min (pH 6.0), 0.40 mμmole/min (pH 7.4) and 0.45 mμmole/min (pH 8.0) at a substrate concentration of $5 \times 10^{-4}M$, indicating only a moderate change in the rate with pH. Consequently, all subsequent runs were made at a pH of 7.4. The hydroxylating enzyme appeared to be saturated with respect to the substrate at a concentration of 5×10^{-3} to $10^{-2}M$.

Four substances which are known to exert an inhibitory action on certain oxidative enzymes were examined in our system: (1) aminopterin, which markedly inhibits phenylalanine hydroxylase at a concentration of $5 \times 10^{-4}M$[12] failed to inhibit our enzyme to any appreciable extent, (2) 2-phenylisopropylhydrazine at $10^{-3}M$ brought about a 63% inhibition, (3) sodium ethylenediaminetetraacetate caused 63% inhibition at $10^{-3}M$, and (4) equimolar concentrations of ATP and Mg^{++} together failed to change the reaction velocity.

The ability of enzyme systems present in liver homogenates to hydroxylate catecholamines to 3,4,5-trihydric phenolic amines lends added significance to the psychotomimetic activity of the hallucinogen mescalin. If a catecholamine, such as dopamine or its metabolite 3-methoxytyramine, is hydroxylated in the 5-position through an alternate metabolic pathway instead of being detoxified by the usual mechanisms, a potential psychotogenic substance could be formed. DALY et al.[13] have shown previously that 5-methoxy-3,4-dihydroxy-β-phenethylamine can be converted to 5-hydroxy-3,4-dimethoxy-β-phenethylamine by the action of *O*-methyltransferase; the remaining methylation step to mescalin has not been demonstrated in mammalian tissue. Our enzymatic conversion of 3-methoxytyramine to 3-methoxy-5-hydroxytyramine brings closer the possibility of a biochemical link between phenylalanine and mescalin by the following route:

It is, of course realized that neither 3-methoxy-4,5-dihydroxy-β-phenethylamine nor its *O*-methyl derivatives nor the corresponding oxidative deamination products have been reported as normal or abnormal urinary or other metabolites from mammals. Since the hydroxylation of our substrate occurs at a very low specific rate, it is anticipated that new procedures will be required which will permit the isolation of sufficient 3-methoxy-4,5-dihydroxy-β-phenethylamine for chemical identification[14].

Résumé. Les homogénats de foie de rat et de foie de lapin contiennent un systéme enzymatique capable d'hydroxyler en position 5 la 4-hydroxy-3-méthoxy-phénéthylamine. Ce type d'oxydation pourrait représenter une voie biologique pour la transformation de catécholamines en substances analogues à la mescaline.

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Affinity of Various Cations for *Staphylococcus aureus* Cell-Wall

It has been found that *Staphylococcus aureus* cell-wall can bind metal ions[1]. Its binding capacity is greater for divalent ions (Ca^{++} and Mg^{++}) than for monovalent ones (K^+ and Na^+), it depends on the pH of medium, and it is not affected by the anion to which the cation is bound[1]. Potentiometric titration curves for the cell-wall in the presence of cations show a definite pH shift, greater for Ca^{++} and Mg^{++} than for Na^+ and K^+[1]. It has been postulated that there is a relationship between the binding capacity and the cell-wall poly-ionic structure: specifically it seems that teichoic acid is able to confer this feature to the cell-wall. This paper deals with the association constants of various cations for *S. aureus* isolated cell-wall.

The apparent constants of association enable us to measure the relative affinity of the ions for the cell-wall binding sites and therefore give us information on the cell-wall ion exchange capacity.

Materials and methods. S. aureus cell-wall (strain 22 Istituto Sieroterapico Italiano) was prepared by SALTON's scheme[2] and further purified with $0.1M$ ethylenediamine tetra-acetic acid (pH 7.5)[1].

The hydrogen ion binding capacity of *S. aureus* cell-wall was studied by suspending 200 mg of lyophilized cell-wall in 50 ml solution of HCl at 0.01–0.000001 N or NaOH at 0.001–0.000001 N. The binding of metal ions has been studied by a titration method similar to tha reported by GILBERT and MYERS[3].

The potentiometric titration curves of lyophilized cell-wall were carried out using a HCl solution in the presence of metal ions at suitable molar concentrations, so that the medium ionic strength remained at 0.1. After equilibration for 5 h at 25 °C with shaking in a nitrogen atmo-

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