Studies on Inhibition of Tyrosinase. (21088)

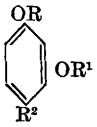
FREDERICK C. KULL, MADELON R. GRIMM, AND RUDOLF L. MAYER.
From the Microbiological Research Laboratories, CIBA Pharmaceutical Products, Inc., Summit, N. J.

A number of substances including thioureas, cyanides, sodium azide, and hydroxyquinolines are known to poison phenoloxidase. Of these compounds, phenylthiourea(1,2) is probably the most potent inhibitor and, according to Chodat(1), prevents the *in vitro* activity of potato phenoloxidase at a concentration of 1×10^{-3}. Using a particularly sensitive method, Mayer, Grimm and Kull(3) were able to detect antiphenoloxidase activity of phenylthiourea at a concentration as low as 1×10^{-10} using tyrosine, β-(3,4-dihydroxyphenyl)-L-alanine (DOPA), epinephrine or norepinephrine as substrates. The antiphenoloxidase activity of thioureas and certain other substances is attributed to their ability to form complexes with copper, the essential metal component of the enzyme. The observation that these compounds exhibit similar inhibitory activity against other metal-containing enzyme systems supports this thesis. A second group of phenoloxidase poisons has been claimed to act by a different mechanism of enzyme inactivation, namely, competitive inhibition. To this group belong p-nitrophenol(4), 4-nitrocatechol(5), several 4-acyl tyrosines(6) and m-hydroxybenzoic acid(7). Except for the convincing kinetic demonstration of the competitive action of m-hydroxybenzoic acid presented by Warner(7), the mode of action of the other compounds within this group has not been supported by experimentation but inferred from the structural similarity between substrate and inhibitor. A large number of related aromatic compounds were studied which might on the basis of structural similarity, compete with the substrate for the enzyme.

Method. The filter paper method for the measurement of phenoloxidase activity described by Mayer, Grimm and Kull(3) was used. In this procedure the compounds to be tested for enzyme inhibition are dissolved in an appropriate solvent such as water, alcohol or chloroform, with the addition of hydrochloric acid or sodium hydroxide to dissolve bases or acids as required. Serial dilutions of these solutions are deposited in 0.05 ml portions upon large filter paper sheets. After drying, a phenoloxidase-substrate mixture is sprayed upon the paper in a stream of nitrogen. The enzyme-substrate mixture contains potato phenoloxidase mixed with equal parts of saturated solutions of L-tyrosine, 0.2% DOPA, epinephrine or norepinephrine in Sorensen buffer pH 7.0. The wet sheet is immediately suspended in a humid atmosphere and observed for color changes. Melanin formation rapidly proceeds through the various color changes, starting with the bright red hallachrome and terminating with a gray-black color when tyrosine or DOPA are used or red-brown in the case of epinephrine or norepinephrine. Interference with the oxidation is quantitatively indicated by round white spots. Since the extent of visible inhibition at the hallachrome stage is often considerably greater than the final degree observed when melanin formation has been completed, two endpoints may be established: that of a maximal but transient or temporary inhibition; and that of the definitive inhibition. Unlike the inhibitors, substances representing potential enzyme substrates produce spots darker in color than the developed melanin background. *Substances investigated.* Approximately 400 compounds including simple and substituted phenols, diphenols, aminophenols, amines and diamines were investigated. It is not possible to list all the substances tested and only those yielding the most interesting results are recorded in Table I.

Results. As seen in Table I, resorcinol at a concentration of 5×10^{-4} inhibits melanin formation from tyrosine or DOPA. This is in agreement with Baur's(8) findings. The monobenzoate and monostearate derivatives were 20 times more active than free resorcinol. Neutral resorcinol derivatives were inactive, whereas the diacetate had the same activity as resorcinol. The highest degree of

TABLE I. Inhibition of Potato Phenoloxidase (Tyrosinase) by Various Resorcinol Derivatives.

Chemical compound 

R	R^1	R^2	Name	Lowest conc. inhibiting enzyme
			(A) Unsubstituted resorcinols:	
H	H	H	—	5×10^{-4}
H	C_6H_5CO	H	-monobenzoate	1×10^{-6}
H	$C_{17}H_{35}CO$	H	-monostearate	1×10^{-6}
H	CH_3CO	H	-monoacetate	5×10^{-4}
H	C_2H_5	H	-monoethyl ether	—
H	CH_3	H	-monomethyl ether	—
CH_3CO-	CH_3CO-	H	-diacetate	5×10^{-4}
CH_3-	CH_3-	H	-dimethyl ether	—
C_2H_5-	C_2H_5-	H	-diethyl ether	—
C_6H_5CO	C_6H_5CO	H	-dibenzoate	—
			(B) Substituted resorcinols:	
H	H	Cl	4-Chlororesorcinol	1×10^{-8}
H	H	-CHO	2,4-Dihydroxy-benzaldehyde	5×10^{-4}
H	H	C_6H_{13}-	4-n-Hexylresorcinol	5×10^{-4}
H	H	3,5-di(2',4-dihydroxyphenyl-triazinyl)	2,4,6-Tri-(2',4-dihydroxy-phenyltriazine)	1×10^{-4}
H	H	CH_3CO-	Resacetphenone	5×10^{-4}
H	H	p-Nitrophenylazo-	p-Nitrophenylazoresorcinol	5×10^{-4}
H	H	CH_2CHNH_2COOH	2,4-Dihydroxyphenylalanine	1×10^{-4}
H	H	COOH	2,4-Dihydroxy-benzoic acid	1×10^{-3}
H	H	m-nitrophenylazo	4-(3'-Nitrophenylazo)-resorcinol	—
H	CH_3	CH_2CHNH_2COOH	2-Methoxy-4-hydroxyphenyl alanine	—
CH_3	H	COOH	2-Hydroxy-4-methoxybenzoic acid	1×10^{-3}
			(C) Resorcinol-like compounds:	
			Orcinol	5×10^{-4}
			Phloroglucinol	1×10^{-3}
			Naphthoresorcinol	5×10^{-4}
			2-Nitroresorcinol	1×10^{-2}
			2-Aminoresorcinol	—
			3,5-Dihydroxyphenylalanine	—

inhibition was obtained with 4-chlororesorcinol. An activity in concentrations as low as 1×10^{-8} to 1×10^{-9} was recorded; thus indicating an activity of 2000 to 20,000 times greater than resorcinol. Other substitutions in the 4-position resulted in considerably lower activities or even complete abolition of enzyme blocking activity.

These results obtained with various simple 4-substituted resorcinols prompted the examination of isomers of the physiological substrate DOPA (a 4-substituted catechol), including d,L - 2,4 - dihydroxyphenylalanine.* During the preparation of the latter compound Lambooy(9) described the syntheses of various isomers of DOPA as well as the inhibition of tyrosinase by the 2-4-isomer. As seen from our table we obtained results similar to those of Lambooy. However, the 2,4 DOPA had only 1/10,000 of the activity of 4-chlororesorcinol.

Other simple derivatives of mono- or diphenols which displayed activities identical with that of resorcinol (5×10^{-4}) were orcinol, naphthoresorcinol and hydroquinone monobenzylether (a known melanin inhibitor).

* These substances were prepared by Dr. R. Mizzoni, Chemical Research Laboratories, CIBA, Summit, N. J.

Phloroglucinol, 2-nitro- or 2-amino resorcinol and d,L-3,5-dihydroxyphenylalanine were inactive. Hydroquinone, m- and p-hydroxybenzoic acids yielded slight inhibition, while phenol, pyrogallol and m-methyl anisole had no activity. Catechol, a known substrate for the enzyme, produced dark gray spots of a melanin-like substance in concentrations as low as 1×10^{-5}.

The strongest inhibitor in a series of aromatic amines was m-aminophenol with an activity of 1×10^{-6}. p-Aminophenol was 20 times less active, whereas o-aminophenol was inactive. Of the 3 phenylenediamines only p-phenylenediamine proved to be a powerful enzyme inhibitor. Aniline, and several of its chlorinated derivatives, as well as toluidines showed little if any activity.

In order to compare quantitatively the activities of these new phenoloxidase poisons, a number of compounds previously known to be strong phenoloxidase inhibitors were examined. Mono substituted thioureas, such as the phenyl-, p-phenetyl-, and p-butoxy-phenyl-thioureas, showed activity ranges between 1×10^{-6} to 1×10^{-9}. Their efficacy was thus comparable to that of 4-chlororesorcinol. Definitely less potent was the most active representative of a group of 8-hydroxyquinolines. These compounds were effective in a dilution of 1×10^{-5}.

Discussion. Resorcinol derivatives probably substitute for the substrate or replace it to block the normal reaction. Support for this hypothesis is afforded 1) by the observation that the activity of 4-chlororesorcinol appears to be strongly dependent on the substrate concentration and 2) by the fact that 1,3-dihydroxybenzene derivatives are not likely to inactivate the enzyme by chelation with copper. Experiments are now in progress to analyze more specifically the possible competitive nature of the inhibitory mechanism of these compounds.

Summary. Using a previously described spot-spray technic, more than 400 simple, aromatic compounds were investigated as inhibitors of tyrosinase. 4-Chlororesorcinol inhibited melanin formation from tyrosine, DOPA or epinephrine at a concentration of 10^{-9}; it was thus 20,000 times more potent than resorcinol.

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Age and Susceptibility to Neurotropic Influenza Virus.* (21089)

SHEELAGH O'CONNOR AND ROBERT R. WAGNER.
From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.

Numerous reports have appeared in the literature on the relation between the age of a given host and its susceptibility to various infectious agents. Mice, guinea pigs, and rabbits have been among the experimental animals most commonly used in these investigations. In addition, chick embryos in various stages of development, newly hatched chicks and adult chickens have frequently been employed. Many viral agents have been studied in this connection, among them the viruses of rabies and pseudorabies, eastern

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