weight significantly lower than that produced by both the thiouracil control ($P > 0.99$) and by 3.0 mcg. of L-thyroxine ($P > 0.95$), and not significantly different from the maximal reversal produced by 4.5 mcg. of L-thyroxine. This 4′-amino analog of 3,5,3′-triiodo-L-thyronine is, therefore, at least 1.5% as active as L-thyroxine. Additional graded dose assays will be required to fix the exact level of activity. The thyromimetic activity shown by two 4′-amino analogs of thyroxine indicates that the amino group is capable of functioning in place of the phenolic hydroxyl group in reversing thiouracil-induced goiter in the rat. This would support a mechanism of action related to electron transport, and present evidence against a mechanism involving simple interaction between phenoxide ion and biological receptor. The additional requirement for appropriate substitution *ortho* to the amino group (iodine or dimethyl) for minimal activity, when supplemented by additional examples, may help to clarify the role of the "prime ring" and its substituents in the thyroxine-like response.

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Studies on Monoamine Oxidase Inhibitors. I. The Autoxidation of β-Phenylisopropylhydrazine as a Model Reaction for Irreversible Monoamine Oxidase Inhibition

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The cupric ion catalyzed autoxidation of β-phenylisopropylhydrazine has been studied by preparative and kinetic methods under conditions resembling those existing in biological systems. The data obtained point to a radical mechanism for the autoxidation, initiated by transfer of one electron from the hydrazine group to a cupric ion. A mechanism for irreversible monoamine oxidase inhibition is proposed, involving transfer of one electron from the hydrazine to the enzyme and subsequent oxidation of the hydrazine radical by molecular oxygen.

The intermediate radicals react with the enzyme reactive center producing irreversible changes in its structure.

The mechanism of the irreversible inhibition of monoamine oxidase (MAO) by hydrazine derivatives has been explained mainly on the basis of their nucleophilic nature.[1, 2] The electrophilic counterpart of the reactive center of the enzyme was supposed to be an "activated" carbonyl group from a peptide, amide, or ester bond, which would react with the hydrazine moiety analogously to ordinary aldehyde or ketone groups. The weaknesses of this theory are apparent. First, as hydrazone formation is a reversible reaction,[3] restoration of the enzyme activity in *vivo* should occur considerably more rapidly than actually is the case, either by hydrolysis or exchange with highly reactive carbonyl compounds, such as pyruvic acid.[4] Second, the concept of an activated peptide, amide, or ester carbonyl function capable of reacting with a hydrazine group in the way indicated above is entirely hypothetical and does not correspond to any known reaction of these groups. Third, as has been shown by Davison,[5] the irreversible inhibition of MAO by iproniazid and isopropylhydrazine requires the presence of oxygen and is decreased by glutathione, which the above theory does not predict.

Davison pictured the inhibition reaction as a dehydrogenation of the hydrazine taking place at the reactive center with the formation of reaction products which react with the enzyme, producing irreversible changes in its structure. Since we are dealing with an oxidizing enzyme and one of the most characteristic chemical properties of hydrazines, notably mono- and 1,2-disubstituted hydrazines, is their extreme sensitivity to molecular oxygen,[6] we considered it as a natural starting point to investigate the autoxidation of hydrazines as a possible cause of irreversible MAO inhibition or as a model reaction for the events occurring at the enzymic receptor. This paper deals with a preparative and kinetic investigation of the autoxidation of β-phenylisopropylhydrazine (PIH),[7] one of the most potent irreversible MAO inhibitors. The results obtained point to a radical mechanism for the inhibition, the radicals being liberated at or near

(1) J. A. Carbon, W. P. Burkard, and E. A. Zeller, *Helv. Chim. Acta*, **41**, 1883 (1958).
(2) J. Barsky, W. L. Pacha, S. Sarkar, and E. A. Zeller, *J. Biol. Chem.*, **234**, 389 (1959).
(3) J. B. Conant and P. D. Bartlett, *J. Am. Chem. Soc.*, **54**, 2881 (1932).
(4) E. Earl Royals, "Advanced Organic Chemistry," Prentice-Hall, Inc., New York, N. Y., 1954, p. 656.
(5) A. N. Davison, *Biochem. J.*, **67**, 316 (1957).
(6) M. Lesbre, in "Traité de Chimie Organique," Tome XV, V. Grignard, G. Dupont, and R. Locquin, Eds., Masson et Cie, Paris, France, 1948, p. 483.
(7) Pheniprazine, Catron®.

the reactive center of the enzyme and subsequently forming covalent bonds with or oxidizing a functional group within it, presumably a thiol group.

Kinetic Method and Results

The gas stoichiometry of the autoxidation of PIH was first investigated and was found to agree fairly well with the formula

$$\text{PIH} + 1.5\text{O}_2 \longrightarrow \text{products} + \text{N}_2 \qquad (1)$$

when run in a phosphate buffer of pH 7.4 at room temperature with added Cu^{2+} as a catalyst. The copper salt was added to make the reaction go to completion within a reasonable time. From this formula it is evident that the kinetics of the reaction could be followed by a manometric method and a few kinetic series were also run by the Warburg technique in order to study the variation of the rate constant as a function of the oxygen pressure above the solution. However, as the Warburg method is rather time-consuming and the rate constants obtained were poorly reproducible, we elaborated a rapid and reliable method for following the kinetics of the autoxidation. The hydrazine exists partly as the hydrazinium ion, $RNHNH_3^+$ at pH 7.4 and thus the reaction can be followed by measuring the rate of proton liberation.[8] This can be done conveniently by the pH-stat method, and preliminary experiments indicated that the end value of added hydroxide corresponded exactly to the value calculated for the liberation of one proton/molecule of oxidized PIH chloride. The appearance of a typical run is shown in Figure 1. The pseudo first-order rate constant $k_{exp} = (1/t) \ln [a/(a - x)]$ was determined graphically from a plot of log $(a - x)$ against t. Assuming that the free base is the kinetically active species it can be derived easily that k_{exp} is related to the rate constant k for the autoxidation of the free base through the equation (2):

$$\log k = \log k_{exp} + pK_a' - \text{pH} \qquad (2)$$

where pK_a' is the apparent dissociation constant of the equilibrium (3).

$$RNHNH_3^+ + H_2O \rightleftarrows RNHNH_2 + H_3O^+ \qquad (3)$$

(8) It is assumed that the rate of complex formation between cupric ion and the hydrazine compound is extremely rapid compared to the rate of autoxidation in the pH range studied. This could be shown semiquantitatively in the following way: A small volume of cupric sulfate solution was added as fast as possible to a well stirred, nitrogen flushed solution of PIH maintained at a fixed pH by the pH-stat. Even when the pH-stat was operated at a high speed (0.3 ml. of 0.1 *M* sodium hydroxide/min.) the rate of proton liberation due to the reaction

$$Cu^{2+} + RNHNH_3^+ \longrightarrow RNHNH_2 \ldots Cu^{2+} + H^+$$

was too rapid to be followed. With [PIH] = 10^{-3} *M* and [Cu^{2+}] = 7.5×10^{-5} *M* this corresponds to a rate constant for complex formation >400 l. mole^{-1} min.$^{-1}$.

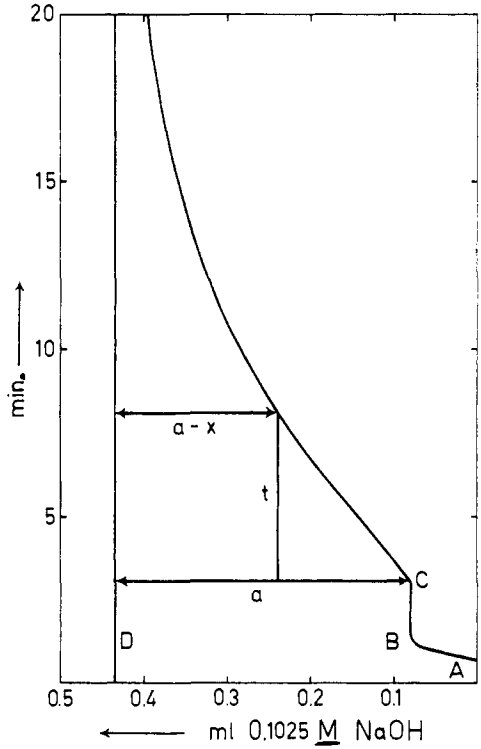


Fig. 1.—Appearance of a typical primary curve obtained by the pH-stat method. The straight line D represents the end value of added hydroxide, calculated on the basis that one proton/molecule of PIH chloride is liberated; $[PIH_{total}] = 1.71 \times 10^{-3} M$, $[Cu^{2+}] = 1.54 \times 10^{-5} M$. For details, *cf.* Experimental.

The pH-stat method was used for studying the kinetics of the autoxidation of PIH in aqueous solution in the presence of catalytic amounts of cupric ion and was found to give reproducible first-order rate constant k_{exp} provided these conditions were fulfilled: (1) The temperature was controlled within $\pm 0.1°$ by the use of a thermostated reaction vessel. (2) The reaction was run in 0.15 M sodium perchlorate in order to maintain a constant ionic strength during the run. (3) The oxygen concentration of the solution was kept constant by blowing in oxygen slowly through a fritted glass disk. (4) The pH was kept at 6.50 instead of the physiological value 7.4, as the accuracy of the rate constant calculations was improved if the experimental curve in Fig. 1 was run over a large interval (a in Fig. 1).

(5) The autoxidation is specifically catalyzed by cupric ion. There occurs a slow autoxidation ($k \sim 0.02$ min.$^{-1}$) in the absence of added cupric ion, which probably is due to trace impurities of cupric ion in the water and chemicals used for preparing the solutions. To obtain reasonable rates of autoxidation catalytic amounts of cupric ion must be added and after some preliminary experiments the cupric ion concentration was fixed at 7.70×10^{-6} M and the total concentration of PIH at 1.76×10^{-3} M. In all runs the ratio $[Cu^{2+}]/[\text{Hydrazine}_{\text{total}}]$ was kept constant except in the case when the dependence of $[Cu^{2+}]$ was studied.

The reproducibility of the method is shown in Table I, which gives the values of k_{exp} and k from six runs at 37.0° and pH 6.50 in 0.15 M aqueous sodium perchlorate. Another demonstration of the accuracy is shown in Table II and Fig. 2, which give the results of measurements carried out at different pH values. The equation of the straight line in Fig. 2 was calculated by means of the least squares method and found to be

TABLE I

RATE CONSTANTS FOR THE AUTOXIDATION OF PIH AT 37.0° AND PH 6.50 IN 0.15 M $NaClO_4$; $[PIH_{\text{total}}] = 1.76 \times 10^{-3}$ M, $[Cu^{2+}] = 7.70 \times 10^{-6}$ M

Number of run	k_{exp} 10^2 in min.$^{-1}$	k in min.$^{-1}$ calcd. from formula (2)[a]
1	5.50	0.24
2	6.19	0.27
3	6.11	0.27
4	5.66	0.25
5	6.46	0.28
6	5.55	0.24
	Mean value	0.26
	Standard deviation	±0.02

[a] The value 7.14 was used for pK_a' (see Table V).

$$\log k_{\text{exp}} = -7.63 + 0.99\text{pH}$$

which was in good agreement with that calculated from formula (2) using the mean value of k in Tables I and II, 0.26 min.$^{-1}$, and $pK_a' = 7.14$

$$\log k_{\text{exp}} = -7.72 + \text{pH}$$

Thus the free base must be the kinetically active species in the reaction.

Table III shows the variation of the rate constant as a function of the cupric ion concentration. Within the limits of error the rate constant is directly proportional to $[Cu^{2+}]$ in the concentration interval

TABLE II

RATE CONSTANTS FOR THE AUTOXIDATION OF PIH AS A FUNCTION OF pH AT 37.0° IN 0.15 M $NaClO_4$; $[PIH_{total}] = 1.76 \times 10^{-3} M$, $[Cu^{2+}] = 7.70 \times 10^{-6} M$

pH	k_{exp} 10^2 in min.$^{-1}$	log k + 1 calcd. from formula (2)[a]	k in min.$^{-1}$
5.50	0.66	0.46	0.29
5.75	0.98	0.38	0.24
6.00	1.98	0.44	0.27
6.25	3.23	0.40	0.25
6.50	5.9	0.41	0.26
6.75	11.2	0.44	0.27
7.00	21	0.46	0.29
7.25	34	0.42	0.26
7.50	52	0.36	0.23
		Mean value	0.26
		Standard deviation	±0.02

[a] The value 7.14 was used for pK_a' (see Table V).

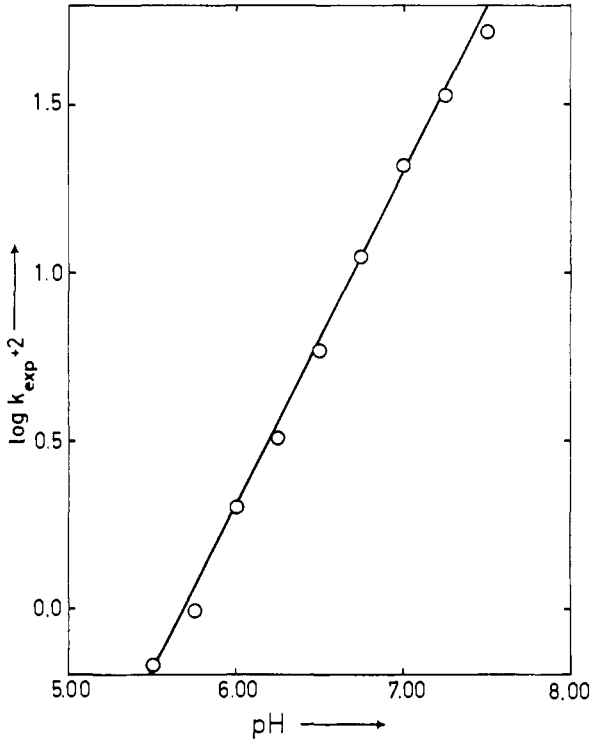


Fig. 2.—Representation of log k_{exp} against pH (see Table II). The equation of the straight line is log k_{exp} = −7.63 + 0.99pH.

studied. The catalytic potency of some other heavy metal ions was also investigated, but none of them (Fe^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}) had any appreciable influence on the autoxidation rate even in considerably higher concentrations.[9]

TABLE III

RATE CONSTANTS FOR THE AUTOXIDATION OF PIH AS A FUNCTION OF $[Cu^{2+}]$ AT 37.0° AND pH 6.50 IN 0.15 M $NaClO_4$

$[Cu^{2+}] \times 10^6$ M	$[PIH_{total}] \times 10^3$ M	$k_{exp} \times 10^2$ in min.$^{-1}$	k in min.$^{-1}$
1.54	1.73	1.30	0.057
3.08	1.83	2.67	0.12
4.62	1.80	3.7	0.16
6.15	1.76	5.2	0.23
7.70	1.76	5.9	0.26
9.23	1.69	6.5	0.28
10.8	1.71	8.3	0.36
12.3	1.72	9.5	0.42
13.9	1.79	11.2	0.49
15.4	1.71	12.9	0.56

If substances which can form very stable complexes with cupric ion were added the autoxidation was strongly inhibited, as is shown in Table IV, which gives the rate constants of the cupric ion-catalyzed reaction in the presence of propyl gallate.

TABLE IV

RATE CONSTANTS FOR THE AUTOXIDATION OF PIH IN THE PRESENCE OF PROPYL GALLATE AT 37.0° AND pH 6.50 IN 0.15 M $NaClO_4$; $[PIH_{total}] = 1.76 \times 10^{-3}$ M, $[Cu^{2+}] = 7.70 \times 10^{-6}$ M

[Propyl gallate] $\times 10^6$ M	k_{exp} 10^2 in min.$^{-1}$	k in min.$^{-1}$
0	5.9	0.26
1.75	4.5	0.20
3.50	3.6	0.16
5.25	1.97	0.086

Table V gives the rate constants k_{exp} and k and pK_a' as a function of the temperature. From these values the energy of activation was calculated to be 17 kcal./mole by the method of least squares.

Addition of chloride and bromide had a very strong influence on the rate of autoxidation, presumably by altering the oxidation-reduction potential of the Cu^{2+}/Cu^{+} system.[10] Thus, in 0.15 M aqueous sodium chloride or bromide the reaction rate was too high to be meas-

(9) *Cf.* L. F. Audrieth and P. M. Mohr, *Ind. and Eng. Chem.*, **43**, 1774 (1951).

(10) W. M. Latimer, "Oxidation Potentials," Prentice-Hall, Inc., Englewood Cliffs, N. J., 1952, p. 186.

TABLE V

RATE CONSTANTS FOR THE AUTOXIDATION OF PIH AS A FUNCTION OF TEMPERATURE AT pH 6.50 IN 0.15 M $NaClO_4$; $[PIH_{total}] = 1.76 \times 10^{-3}$ M, $[Cu^{2+}] = 7.70 \times 10^{-6}$ M

T, °K.	$(1/T)$ 10^3	pK_a'	k_{exp} 10^2 in min.$^{-1}$	log k	k in min.$^{-1}$
293.16	3.411	7.31	0.76	0.69–2	0.049
298.16	3.354	7.26	1.39	0.90–2	0.080
303.16	3.299	7.21	2.5	0.11–1	0.13
308.16	3.245	7.16	4.8	0.34–1	0.22
310.16	3.224	7.14	5.9	0.41–1	0.26
313.16	3.193	7.08	7.9	0.48–1	0.30

TABLE VI

RATE CONSTANTS FOR THE AUTOXIDATION OF PIH AS A FUNCTION OF CHLORIDE ION CONCENTRATION AT 37.0° AND pH 6.50; $[PIH_{total}] = 1.76 \times 10^{-3}$ M, $[Cu^{2+}] = 7.70 \times 10^{-6}$ M; NO SODIUM PERCHLORATE ADDED

$[Cl^-]$, M	k_{exp} 10^2 in min.$^{-1}$	k in min.$^{-1}$ [a]
0.00176	9.9	0.31
0.00226	13.2	0.42
0.00276	17.2	0.54
0.00325	17.8	0.56
0.00426	19.1	0.60
0.00676	24.6	0.78

[a] At these salt concentrations a pK_a' of 7.00 was used.

ured by the pH-stat method, as the rate of hydroxide addition could not be increased enough. The rate constants at low chloride concentrations are shown in Table VI.

The rate dependence of the oxygen pressure above the solution was determined by the Warburg method and the results are shown in Fig. 3, where the rate constants k for autoxidations in air and oxygen are represented against $[Cu^{2+}]$. The straight lines in Fig. 3 are identical within the limits of the experimental error, indicating that the rate is independent of the oxygen concentration. This could also be shown by the pH-stat method, if air was blown into the solution instead of oxygen, in which case the rate constant (mean value from three runs) was 0.23 min.$^{-1}$. This value agrees fairly well with that obtained with oxygen, 0.26 min.$^{-1}$. Unfortunately the rate constants determined by the Warburg method are not comparable with those determined by the pH-stat method, as in the manometric experiments the reaction had to be run in a phosphate buffer.

Finally the rate of autoxidation and pK_a' of a number of other substituted hydrazines have been determined (Table VII). These

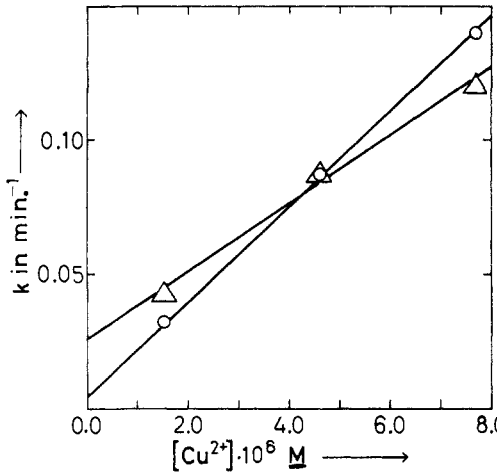


Fig. 3.—Representation of k against $[Cu^{2+}]$ for autoxidations in air and oxygen (see Table VII): O in air, △ in oxygen; medium was 0.067 M phosphate buffer of pH 6.50; temp. 37.0°; [PIH] = 1.65×10^{-3} M.

TABLE VII

RATE CONSTANTS FOR THE AUTOXIDATION OF SUBSTITUTED HYDRAZINES $RNHNH_2$ AT 37.0° AND pH 6.50 IN 0.15 M $NaClO_4$; $[RNHNH_{2\ total}] = 1.76 \times 10^{-3}$ M, $[Cu^{2+}] = 7.70 \times 10^{-6}$ M

R	pK_a'	k_{exp} 10^2 in min.$^{-1}$	k in min.$^{-1}$
Isopropyl	7.48	0.93	0.09
β-Cyclohexylisopropyl	7.78	2.6	0.49
Phenyl at pH 4.50	5.14	15.1	0.66
Benzyl	6.81	6.8	0.14
Phenylethyl	7.12	9.8	0.41
β-Phenylisopropyl	7.14	5.9	0.26
β-(2-Methylphenyl)-isopropyl	7.08	5.3	0.20
β-(4-Methoxyphenyl)-isopropyl	7.17	5.2	0.24
β-(3,4-Methylenedioxyphenyl)-isopropyl	7.09	3.5	0.14
β-(3,4,5-Trimethoxyphenyl)-isopropyl	7.03	6.2	0.21

include the therapeutically employed isopropyl-,[11] benzyl-,[12] and phenylethylhydrazine,[13] and it is obvious that these compounds undergo autoxidation at rates comparable with that of PIH. A semi-quantitative study on iproniazid revealed that it was also autoxidized under the conditions employed in this investigation and that the

(11) The hydrazine part of N^1-isonicotinoyl-N^2-isopropylhydrazine, iproniazid.

(12) The hydrazine part of N^1-benzyl-N^2-(5-methyl-3-isoxazolylcarbonyl)-hydrazine, isocarboxazide.

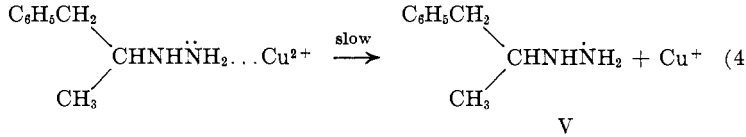
(13) Phenelzine.

reaction displayed the same characteristics, *i.e.*, it was strongly dependent on cupric and chloride ion concentration.[14] Hydrazine itself was found to undergo rapid autoxidation under the conditions employed, but the kinetics deviated considerably from first-order behavior.[9]

Preparative Results.—The reaction products from the autoxidation of a large sample of PIH run in a phosphate buffer of pH 7.4 at room temperature in the presence of catalytic amounts of cupric ion were isolated and crudely separated by fractional distillation. The fractions were analyzed by the infrared and gas chromatographic technique and found to consist of *n*-propylbenzene (I), propenylbenzene (II), phenylacetone (III), and 1-phenyl-2-propanol (β-phenyl-2-propanol) (IV). The distillation residue was a black tar, from which no defined compound could be isolated. The amounts of I, II, III, and IV corresponded approximately to yields of 10, 10, 15, and 5% respectively, whereas the residue accounted for 30–40%.

In order to obtain more evidence for the radical nature of the autoxidation, methyl methacrylate in large excess was treated with PIH and cupric ion in aqueous ethanolic solution in the presence of oxygen. The methyl methacrylate quantitatively polymerized to a solid polymer, whereas no polymerization occurred even after storage for several weeks of a solution containing the same components except PIH. In a semiquantitative kinetic experiment it was shown that peroxidic material, presumably hydrogen peroxide, accumulated during the autoxidation process.

Discussion of Results.—It is well known that many autoxidations proceed according to a chain mechanism involving free radicals,[15–17] and therefore it is not surprising that the autoxidation of PIH follows the pattern of a radical reaction. The data presented in this paper do not allow a complete mechanism to be elucidated but it probably involves these steps: (1) A complex between PIH and Cu^{2+} is formed, which decomposes according to



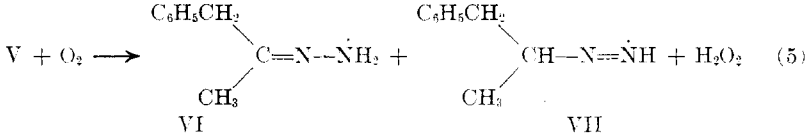
(14) B. Koechlin and V. Iliev, *Ann. N. Y. Acad. Sci.*, **80**, 868 (1959).

(15) C. Walling, "Free Radicals in Solution," John Wiley & Sons, Inc., New York, N. Y., 1957, p. 397.

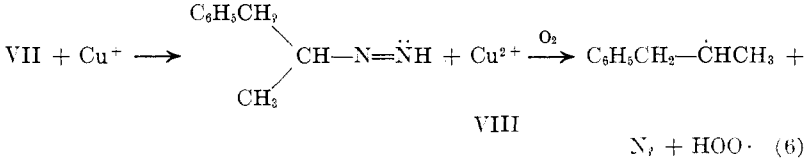
(16) W. A. Waters, "The Chemistry of Free Radicals," Oxford University Press, London, 1948, p. 232.

(17) W. A. Waters, in "Progress in Organic Chemistry," Part 5, J. W. Cook and W. Carruthers, Eds., Butterworth & Co. Ltd., London, 1961, p. 17.

(2) The radical V is oxidized by oxygen with the formation of hydrogen peroxide and the radicals VI and VII.



(3) VI then gives rise to phenylacetone (III) and a hydrazine radical by rapid hydrolysis whereas VII may capture an electron from a cuprous ion and become further oxidized by oxygen according to



The radical VIII may react in several ways, dependent on the concentration of the hydrazine compound. At relatively high concentrations, as in the preparative experiments, coupling or disproportionation will dominate, while in the concentrations employed in the kinetic experiments or under biological conditions reaction with solvent molecules or other substances present is more likely to occur.

This reaction scheme is supported by the following facts: Cupric ion is known to form complexes with hydrazine, but unfortunately no complex constants have been reported for such complexes.[18,19] However, a comparison between the ammonia and hydrazine complexes of Cd^{2+}, Zn^{2+} and Ni^{2+} shows that the stability constants K_1 and K_2 for the first two complexes are approximately equal.[18] It seems reasonable to assume that log K_1 and log K_2 for the complexes between cupric ion and hydrazine or monoalkylhydrazine are of the same order of magnitude as those of the ammonia complexes, 4.3 and 3.7. Using the values log K_1 = log K_2 = 4 it can be calculated that complexes between cupric ion and PIH should dominate over free cupric ion in the concentration interval used in this investigation.

The proposal that the decomposition of the complex according to reaction (4) is the rate determining step is consistent with the kinetic results, *i.e.*, the rate constant is approximately proportional to the concentration of cupric ion and independent of the oxygen concentra-

(18) J. Bjerrum, G. Schwarzenbach, and L. G. Sillén, "Stability Constants of Metal-ion Complexes, Part II (Inorganic Ligands and Solubility Products)," Chem. Soc. London, Burlington House, 1958.

(19) L. F. Audrieth and B. A. Ogg, "The Chemistry of Hydrazine," John Wiley & Sons, Inc., New York, N. Y., 1951, p. 190.

tion. Furthermore, the autoxidation is inhibited by reagents which form strong complexes with cupric ion and accelerated by anions which stabilize the cuprous state, such as chloride and bromide ion (Table VI).[10] An analogous initial step has been postulated in the autoxidation of dialkylhydroxylamines in alkaline solution in the presence of cupric ion.[20,21] Once formed, the radical V is very reactive toward oxygen and undergoes the reaction steps outlined in the equations (5) and (6), which in a rough manner account for the products isolated and identified. The radical nature of the autoxidation is also shown by its capability to initiate the polymerization of a large excess of methyl methacrylate, which is rather insensitive to ionic polymerization mechanisms. Radical mechanisms have been proposed for a number of oxidative processes in which hydrazine derivatives or related compounds take part, *e.g.*, in the autoxidation of hydrazones,[22] in the oxidation of hydrazine by ferric ion,[23,24] and in the oxidation of phenylhydrazine by silver oxide.[25] Likewise, the oxidation of hydrazones in the presence of cupric ion has been found to initiate polymerization of several ethylenically unsaturated compounds.[26]

As it can be shown that PIH is oxidized in the presence of oxygen also in biological systems,[27] *e.g.*, blood, the formation of radicals during this process has some important biochemical implications. Radicals are highly reactive and react rather unspecifically in a number of ways with biological structures, giving rise to irreversible changes by virtue of their ability to act as oxidizing agents, to form covalent bonds or to initiate chain reactions. There are several indications that this is what happens when hydrazine derivatives interact with biological systems. When phenylhydrazine is treated with oxygen in the presence of oxyhemoglobin benzene and nitrogen are formed, while the oxyhemoglobin is irreversibly changed.[28] These findings point to an oxidation–reduction system between the oxyhemoglobin and phenylhydrazine, with hydrogen transfer from the latter to molecular oxygen. Hemin has been found to oxidize isoniazid to 1,2-diisonicotinoylhydrazine and isonicotinic acid in air at

(20) D. H. Johnson, M. A. Thorold Rogers, and G. Trappe, *J. Chem. Soc.*, 1093 (1956).
(21) Ref. 14, p. 416.
(22) K. H. Pausacker, *J. Chem. Soc.*, 3479 (1950).
(23) W. C. E. Higginson and P. Wright, *J. Chem. Soc.*, 1551 (1955), and preceding papers of the series.
(24) J. W. Cahn and R. E. Powell, *J. Am. Chem. Soc.*, **76**, 2568 (1954).
(25) R. L. Hardie and R. H. Thomson, *J. Chem. Soc.*, 2512 (1957).
(26) U. S. Patent 2,686,775; *cf. C. A.*, **48**, 14294i (1954).
(27) L. E. Eberson and K. Persson, to be published.
(28) G. H. Beaven and J. C. White, *Nature*, **173**, 389 (1954).

pH 7.5, presumably *via* radical intermediates.[29] Phenylhydrazine, C^{14}-labelled in the ring, is excreted only to 60% after 10 days when administered orally to rabbits,[30] suggesting that the rest has been irreversibly attached to various sites in the body.

The irreversible inhibition of MAO by hydrazine derivatives is analogous to their copper-catalyzed autoxidation in some important ways. First, in view of their similarity to the natural substrates of MAO, the hydrazine-type MAO-inhibitors have an affinity for the reactive center of the enzyme, which may be considered analogous to their ability to form complexes with cupric ion. MAO has an oxidation–reduction potential which lies between -0.05 and $+0.195$ v.,[31] whereas the system Cu^{2+}/Cu^{+} has the potential $+0.153$ v. By transfer of one electron from the hydrazine nitrogen to MAO analogous to the transfer of an electron within the cupric ion–hydrazine complex, the radical V is formed. By reaction of V with molecular oxygen a reaction sequence similar to (5) and (6) involving radical intermediates is possible, and as the radicals are liberated at the reactive center of the enzyme, they dominantly react with structures within it. Since MAO has been shown to be a thiol enzyme, it seems likely that this group is involved in the inhibition reaction, as thiol groups are easily attacked by radicals. This mechanism for irreversible MAO inhibition is in agreement with Davison's findings,[5] but it cannot be excluded that the autoxidation itself is responsible for the inhibition. In this case the radicals would be liberated in the solvent bulk outside the enzyme and react with it in an unspecific manner. However, we favor the first mechanism, as this offers an explanation for the stereospecificity found for example in the inhibition of MAO with D- and L-forms of PIH.[32]

It may be of interest to mention that the amine oxidase of pea-seedlings contains cupric ion which appears to be important for the enzymic activity.[33]

Experimental

Materials.—The hydrazines were kindly supplied by Dr. John Biel, Lakeside Laboratories, Milwaukee, Wis., and used as hydrochlorides. All other chemicals were of analytical grade and the water used was distilled and passed through a demineralizing apparatus.

(29) A. Albert and C. W. Rees, *Biochem. J.*, **61**, 128 (1955).
(30) W. M. McIsaac, D. V. Parke, and R. T. Williams, *Biochem. J.*, **70**, 688 (1958).
(31) A. Pletscher, K. F. Gey, and P. Zeller in "Progress in Drug Research," Part 2, E. Jucker, Birkhäuser Verlag, Basel und Stuttgart, 1960, p. 427.
(32) J. Bernstein, K. A. Losee, C. Smith, and B. Rubin, *J. Am. Chem. Soc.*, **81**, 4433 (1959).
(33) P. J. G. Mann, *Biochem. J.*, **79**, 623 (1961).

Determination of the Gas Stoichiometry.[34]—The gas stoichiometry was determined by conventional gas-buret technique, and a ratio between absorbed oxygen and evolved nitrogen of 1.49 was obtained.

Kinetics.—The pH-stat was the type SBR2c Titrigraph combined with the type SBUla syringe buret, the type TTTlc Titrator and the type TTA2 titration assembly from Radiometer, Copenhagen, Denmark. The reaction vessel was equipped with a jacket, through which thermostated water from an ultrathermostat was circulated at a speed of about 10 l./min. The temperature constancy was better than ±0.1°. The substituted hydrazine hydrochloride (4.58×10^{-5} mole) was dissolved in 26.0 ml. of 0.15 *M* aqueous sodium perchlorate in the reaction vessel and the mixture was allowed to reach thermal equilibrium (point A in Fig. 1). Then the pH-stat was started and sodium hydroxide (0.1025 *M*) was fed into the solution until the desired pH was obtained (point B in Fig. 1). At B the oxygen inlet was introduced into the reaction vessel and at C the reaction was started by adding the appropriate amount of 2.00 m*M* cupric sulfate solution by means of a constriction micropipet. The end value of added sodium hydroxide (line D in Fig. 1) always agreed very well with that calculated for the liberation of one proton/molecule of substituted hydrazine chloride. The rate constant, k_{exp}, was calculated by means of the usual formula $k_{exp} = (1/t) \ln [a/(a - x)]$ where a and $(a - x)$ had the meaning which is indicated in Fig. 1. All rate constants given in Tables II–VIII are the mean values obtained from at least two runs. As is seen from Tables I and II the relative standard error is about ±7%.

The Warburg experiments were made under the same conditions as the pH-stat experiments, except that the medium was a phosphate buffer. The rate constants were calculated by the formula $k_{exp} \times t = \ln [V_\infty/(V_\infty - V_t)]$ and transformed into the rate constants k for the autoxidation of the free base.

The apparent dissociation constants pK_a' for the equilibrium (3) were determined from the half-neutralization points of the titration curves, and each pK_a' was the mean value from four determinations.

Autoxidation of PIH.—PIH chloride, 18.7 g. (0.1 mole), was dissolved in 600 ml. of 0.6 *M* aqueous phosphate buffer of pH 7.4. The solution was poured into a 5-l. round-bottomed flask, which previously had been flushed with oxygen. A solution of 0.1 g. of cupric sulfate in a small volume of water was added, the flask was stoppered and stirred by means of a magnetic stirrer for 24 hr. at room temperature. The reaction mixture was acidified with concentrated hydrochloric acid and extracted with three portions of ether. The ether extracts were combined and washed with sodium carbonate solution and water and finally dried with anhydrous calcium sulfate. The sodium carbonate solution was acidified but no acidic products were obtained.

The ether solution was distilled through a semi-micro distillation column of about 8 theoretical plates, and these fractions were collected: I, 1.1 g., b.p. 50–60° (15 mm.); II, 1.4 g., b.p. 60–98° (15 mm.); III, 2.3 g., b.p. 98–99° (15 mm.); IV, 0.6 g., b.p. 75–100° (0.1 mm.). The distillation residue was a black tar (5.1 g.) from which no defined compound could be isolated. By means of infrared spectra and gas chromatograms[35] it was found that I was mainly *n*-propyl-

(34) The authors are indebted to Dr. E. Bladh, Department of Analytical Chemistry, University of Lund, for this investigation.

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benzene, II a mixture of *n*-propylbenzene and propenylbenzene, III phenylacetone and IV 1-phenyl-2-propanol. Phenylacetone was also identified by conversion into its dinitrophenylhydrazone and 1-phenyl-2-propanol by conversion into its 3,5-dinitrobenzoate. Minor amounts of other compounds were also present but no attempts to identify them were made.

The acidic solution from the ether extraction was made alkaline and continuously extracted with ether for 12 hr. The ether solution was washed with a small volume of water, dried with anhydrous potassium carbonate and distilled. PIH, 1.1 g., b.p. 70–75° (0.02 mm.) was recovered, identified by conversion into the hydrochloride.

Polymerization of Methyl Methacrylate.—Methyl methacrylate (8.0 g., 0.08 mole), PIH (0.25 g., 0.0013 mole) and cupric chloride (0.2 mg., 1.2×10^{-6} mole) were dissolved in a mixture of water (40 g.) and ethanol (55 g.). The solution was shaken overnight after which time a solid polymer had precipitated, which was collected, washed with water and dried. It weighed 8.0 g. (100% yield). A control solution, which had the same composition except that PIH was omitted, had not polymerized after standing for 3 weeks.

Pyrrolidines. VII. 3-Hydroxy-1-Pyrrolidinecarboxylic Acid Esters

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N-Alkoxycarbonylamino acid esters were condensed with ethyl esters of α,β-unsaturated carboxylic acids in the presence of sodium hydride and dry benzene to form alkyl ethyl 4-oxo-1,3-pyrrolidinedicarboxylates, which were hydrolyzed and decarboxylated to the corresponding 3-oxo-1-pyrrolidinecarboxylic acid esters. The carbonyl groups at the 3-position of these esters were reacted selectively with appropriate Grignard reagents to yield various 3-substituted 3-hydroxy-1-pyrrolidinecarboxylic acid esters, which were submitted for screening as hypnotic agents.

For a drug producing central activity an adequate lipid-water solubility ratio is important in order to enable it to reach the site of its action.[1,2] With this hypothesis in mind we prepared a series of compounds of structure (I) for screening as hypnotic agents,

(1) T. Sollmann, "A Manual of Pharmacology and Its Application to Therapeutics and Toxicology," W. B. Saunders Company, Philadelphia, Pa., 1957, p. 846.

(2) R. B. Barlow, "Introduction to Chemical Pharmacology," John Wiley & Sons, Inc., New York, N.Y., 1955, p. 29.