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Pyridine-N-Oxide-Aldoximes

Their Synthesis, and Chemical and Biochemical Properties

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The three isomers of pyridine-N-oxide-aldoximes were synthesised and their properties described, including infrared spectra and dissociation constants. The new aldoximes were incapable of reactivating cholinesterases inhibited by tetraethyl pyrophosphate both *in vitro* and *in vivo*.

Successful treatment by certain oximes of poisoning caused by organophosphorus compounds has recently been reported; especially active are pyridine-2-aldoxime-N-methyl iodide (PAM) and its methane sulphonate analogue (P2S)[1]. The present report describes the synthesis and some chemical and biochemical properties of the corresponding pyridine-N-oxide-2-aldoxime and its two isomers (3- and 4-aldoxime).

EXPERIMENTAL

The N-oxides of pyridine-aldoximes were prepared by a general method described previously by Ochiai[2]. Hydrogen peroxide (30 %) is allowed to act upon the pyridine derivative (Schuchardt, Germany) in glacial acetic acid solution at 70—80°C.

Pyridine-N-oxide-2-aldoxime (*POA 2*). To 2 g (16.4 mmole) of pyridine-2-aldoxime, dissolved in 10 ml of glacial acetic acid, was added 2 ml of hydrogen peroxide (30 %). The mixture was heated for 3 h at 80°C. After cooling 40 ml of ethyl acetate was added to the reaction mixture, resulting in the precipitation of the reaction product. The product was collected by filtration after one hour in the refrigerator, and washed with ethyl acetate. Crude product, 0.5 g. A further 0.4 g of crystallized product was obtained from the filtrate after keeping for 48 h in the refrigerator.

The two fractions of crystals were combined and dissolved in a small amount of boiling water, active charcoal was added, and the mixture filtered while still boiling. The pyridine-N-oxide-2-aldoxime crystallized upon cooling. Recrystallization from water gave 0.78 g (34 % yield) of the pure compound as colourless needles; m.p. 221—222°C with slow decomposition and brown-red colourization. Analysis, see Table 1. This aldoxime was synthesised recently by Jerchel and Heider[3] using another method.

The ethyl acetate solution from which the N-oxide aldoxime was isolated was evaporated to dryness under vacuum, leaving a dark-yellow oil, which dissolved on heating in 10 ml of water to give a clear solution. Upon cooling, a colourless substance crystallized out, which was identified as the original pyridine-2-aldoxime (m.p. 111—113°C). Solid sodium carbonate was added to the filtrate to pH 6.5. After 24 h in the refrigerator another portion of pyridine-2-aldoxime was obtained. Altogether 0.85 g of unreacted aldoxime was isolated in this way.

When a longer reaction period or a greater amount of hydrogen peroxide, was employed, approximately the same amount of N-oxide aldoxime was obtained. The remaining solution contained, however, approximately 1 g of picolinic acid (m.p. 138—139°C with rapid decomposition). The latter procedure was therefore not to be preferred because of difficulties in separating the acid from the oxime.

Pyridine-N-oxide-3-aldoxime (*POA 3*). Pyridine-3-aldoxime, 2 g (16.4 mmole), was dissolved in 20 ml of glacial acetic acid, 6 ml of 30 % hydrogen peroxide was added, and the mixture incubated at 35°C for 48 h.; the same yield was obtained on keeping the mixture for ten days at room temperature. Evaporation under vacuum gave a sticky crystalline product, which was dissolved in 10 ml of absolute ethanol with heating. After cooling at room temperature long, light yellow rods crystallized out; m.p. 212—213°C with decomposition. Recrystallization twice from absolute ethanol gave pure pyridine-N-oxide-3-aldoxime; m.p. 217—218°C. Yield, 1.06 g (46 %).

The ethanol filtrate, from which the reaction product was isolated, was evaporated under vacuum to approximately 3 ml. The remaining yellow oil was stirred, and acetone added until no further precipitation occurred. The collected precipitate was washed with acetone and dried, treated with 25 ml of hot *iso*propanol, and the solution filtered hot; the undissolved matter was then washed afterwards with boiling *iso*propanol. The crystallized product was recrystallized twice from small amounts of water; m.p. 256—257°C. Yield, 0.4 g. The product was identified as nicotinic acid-N-oxide with the same m.p.[4]

Pyridine-N-oxide-4-aldoxime (*POA 4*). Pyridine-4-aldoxime, 2 g (16.4 mmole), dissolved in 10 ml of glacial acetic acid, was treated with 2 ml of hydrogen peroxide (30 %) for 48 h at 35°C. The reaction solution was diluted with 10 ml of water and evaporated under vacuum until crystallization began. The crystalline product was dissolved in 10 ml of methanol by gentle heating, and was then left for crystallization. The yellow prisms were recrystallized twice from methanol. The pyridine-N-oxide-4-aldoxime was obtained as colourless prisms, m.p. 228—229°C; yield 720 mg.

The filtrate was evaporated under vacuum to a yellow oil, which was not possible to crystallize from methanol. The oil, probably consisting of the acetate of the reaction product, was dissolved in 5 ml of water and treated with solid sodium carbonate to pH 6.5, when crystals, a mixture of starting material and the N-oxide were formed. The whole mixture was shaken three times with ethyl acetate. The aqueous phase was left for 24 h at room temperature, the precipitated crystals were collected, washed with a small amount of water and recrystallized from methanol. The product was identified as pyridine-N-oxide-4-aldoxime; yield 400 mg. The total yield was 1.12 g (50 %).

From the ethyl acetate filtrate, 0.35 g of unreacted pyridine-4-aldoxime (m.p. 132—133°C) was isolated. No formation of carboxylic acid was observed.

Infra-red spectrophotometric measurements. The infra-red spectra were recorded on a Perkin-Elmer spectrophotometer, Model 21, equipped with a rock-salt prism, and the following settings were used: resolution 927; response 2:1 170; gain 5.9; suppressions 3; speed about 1 μ/min; scale 5 cm/μ. The potassium bromide technique was employed, and 1 mg of sample was ground mechanically with 300 mg of this salt.

Determination of dissociation constants. The dissociation constants of the three aldoximes were determined at 25°C with an automatic recording titrator as described previously by Larsson and Hansen[5]. The compounds were dissolved in 0.100 M potassium chloride solution and the concentration of the compounds was approximately 5.0 mM. Titration was performed with 0.0991 M sodium hydroxide. The pH-meter was standardized against 0.05 M potassium hydrogen *o*-phthalate (pH = 4.01) and 0.01 M borax (pH = 9.18).

Cholinesterase determinations were performed by the Warburg technique at 25°C with acetylcholine chloride (20 mM) as substrate[6]. Purified cholinesterase preparations from human plasma (butyrocholinesterase, BuChE) and *Torpedo* electric organ (acetyl-

cholinesterase, AChE) were employed, and esterase activity was expressed in b_{30}, *i.e.* μl CO_2 evolved in 30 min from a bicarbonate-CO_2 buffer solution (pH 7.4), corrections being made for spontaneous hydrolysis of acetylcholine.

Reactivation experiments. Tetraethyl pyrophosphate (TEPP) was used as »irreversible» cholinesterase inhibitor and appropriate aqueous solutions of it were prepared immediately before use. The enzyme solutions (in bicarbonate buffer, unless otherwise stated) were incubated with the inhibitor for 45 min in the main compartment of the flask; total volume of the incubation mixture, 1.2 ml. The aldoxime (0.4 ml of an aqueous solution) was then added from one of the ampoules, and after various reaction periods (15 to 60 min) the substrate (0.4 ml of a bicarbonate solution) was added to the mixture from the second ampoule. When other techniques were used, this is stated in the legend to the tables.

Pyridine-2-aldoxime-N-methyl methane sulphonate (P2S), known to be a good reactivator of cholinesterases [1], was used as a reference compound.

RESULTS AND DISCUSSION

Properties of the aldoximes. The 2- and 3-aldoximes were readily soluble in water; the 4-aldoxime was less so, but upon heating it dissolved rapidly. All three compounds dissolved readily in boiling methanol, and were sparingly soluble in benzene, acetone and ethyl acetate. Positive oxime reaction according to Feigl and Demant [7] was given by all three aldoximes. The analytical data for the compounds synthesised are listed in Table 1.

Infra-red spectra. The following group frequency assignements can be made from the infra-red spectra (Fig. 1):

The 3 000 cm^{-1} region. The three absorption bands at 3 175—2 958 cm^{-1} can be assigned to CH stretchning frequencies [8], and the unresolved bands at 2 874—2 597 cm^{-1} can probably be ascribed to associated OH stretching frequencies.

The 1 650—1 400 cm^{-1} region. The C=N stretching of the oxime group is expected to occur at about 1 650 cm^{-1}, and the weak absorption band at 1 650—1 639 mc^{-1} has thus been attributed to this group [9]. The remaining five bands in this region may be ascribed to vibrations of the pyridine nucleus [8,10,11]. The origin of the absorption band at 1 508—1 504 cm^{-1} is, however, ambiguous.

The 1 325—1 290 cm^{-1} region. The two absorption bands appearing in this region may be possibly be assigned to the OH bending frequencies [9].

Table 1. Analytical data and thermodynamic dissociation constants of pyridine-N-oxide-X-aldoximes ($C_6H_6N_2O_2$; M = 138.1) and dissociation constants of the corresponding N-methyl-derivatives [13].

X	% C		% H		% N		pK_a	
	Calc.	Found	Calc.	Found	Calc.	Found	N-oxides	N-methyl deriv.
2	52.2	52.0	4.4	4.2	20.3	19.7	9.23	7.82
3	52.2	52.2	4.4	4.3	20.3	19.5	9.68	9.10
4	52.2	52.3	4.4	4.5	20.3	20.1	9.57	8.23

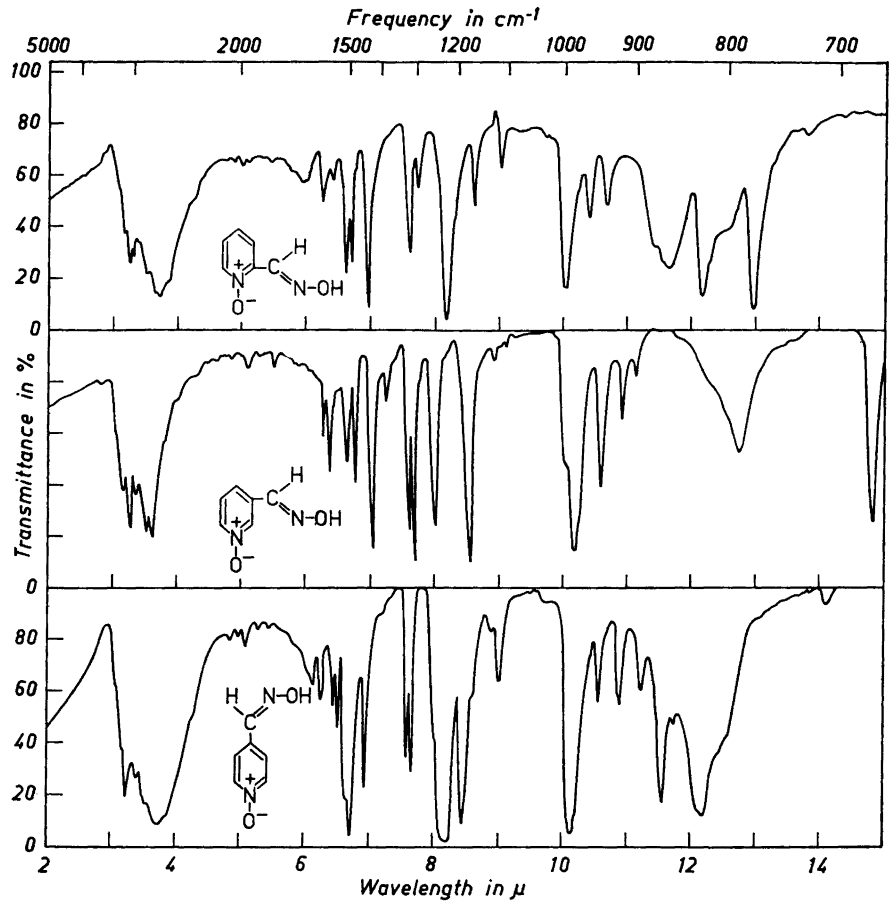


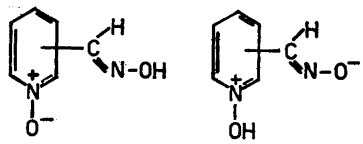
Fig. 1. The infra-red spectra of pyridine-N-oxide-aldoximes.

The 1 255—1 220 cm^{-1} region. The very strong absorption band occurring in this region has been attributed to the $N^+—O^-$ stretching frequency [10,11].

The 1 185—975 cm^{-1} region. The two absorption bands at 1 185—1 159 cm^{-1} and 1 119—1 105 cm^{-1} have been interpreted as CH deformation bands [10,11]. The strong and broad absorption at 996—978 cm^{-1} has been given the same assignment [10,11], but it is probably overlapped by a band caused by stretching of the N-OH linkage [9,12].

The infra-red data obtained are thus in agreement with structures proposed from the synthesis of the compounds, and they indicate that the compounds exist as N-oxides (I) and not in the tautomeric form (II).

An impurity which can be expected to be present in the samples is the carboxylic acid corresponding to the oxime. Owing to the weak overtone

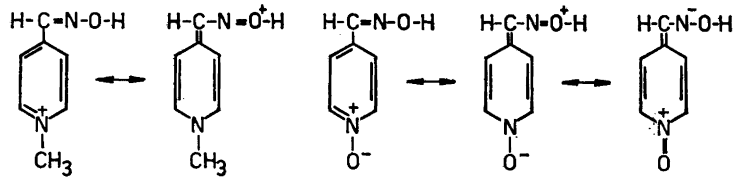


I II

bands of the pyridine nucleus occurring in the region of the carbonyl absorption band (1 700 cm^{-1}) it is, however, not possible to decide from the spectra obtained whether traces of carboxylic acid may be present or not.

From the titration curves of the N-oxide of the pyridine aldoximes it was found that the deviation between the amount of titrand added to the equivalence point, and the amount calculated from the quantity weighed out in no case exceeded 1.1 %.

The thermodynamic dissociation constants of the compounds are given in Table 1, where the constants of the N-methyl pyridine-aldoximes [13] have also been included. A comparison between the constants of the two series shows that the substitution of an oxide group for a methyl group at the nitrogen atom causes an increase in the pK_a-value. Primarily, this increase may be ascribed to the reduction of the strong inductive effect of the positively charged nitrogen atom when the substituent is changed from methyl to oxygen. This increase is expected to be greatest in the 2-aldoxime and least in the 4-aldoxime. As appears from Table 1, the increase is especially pronounced in the 2- and 4-aldoximes which probably depends upon a mesomeric effect. The pyridine-4-aldoxime-N-methyl can be described as a resonance hybrid of structures III and IV, and the N-oxide as a hybrid of the analogous structures V and VI and moreover structure VII [14]. The large difference between the pK_a-value of the N-methyl-derivative and of the oxide indicates that the contribution of structure VII may more or less balance structure VI. This will thus serve as a partial explanation of the lower acidity, *i.e.* higher pK_a-value, of the pyridine-N-oxide-4-aldoxime. Analogous structures and arguments are valid for the 2-aldoxime but not for the 3-aldoxime.



III IV V VI VII

On the presumption that the aldoxime group in the pyridine-N-oxide-2-aldoxime exists in the anti-form, the formation of a weak intramolecular hydrogen bond is conceivable, and such a bond would furthermore increase its pK_a-value. It is, however, not possible to decide from an infra-red spectrum

Table 2. Reactivation of cholinesterases inhibited by tetraethylpyrophosphate (TEPP). The solutions of esterase preparations (E) were incubated with the inhibitor (I) for 45 min before the addition of aldoxime (R). After various periods of time (»reactivation») the substrate (S) was added to the reaction mixture. BuChE, human serum butyrocholinesterase; AChE, electric organ acetylcholinesterase, P2S, pyridine-2-aldoxime-N-methyl methane sulphonate, 0.125 mM; POA 2, POA 3 and POA 4, pyridine-N-oxide-X-aldoximes, 1.25 mM (concentration values for the aldoximes are those during reactivation).

Incubation mixture	Reactivation min	P2S		POA 2		POA 3		POA 4	
		b_{30}	% inhib.	b_{30}	% inhib.	b_{30}	% inhib.	b_{30}	% inhib.
BuChE:									
E		133		135		138		138	
E + I		13	90	71	47	64	54	65	53
E + I + R	0	32	76	71	47	64	54	59	57
—»—	15	32	76	71	47	—	—	59	57
—»—	30	40	70	73	45	64	54	59	57
—»—	60	—	—	81	39	61	56	58	58
AChE:									
E		140		130		112		112	
E + I		34	76	34	74	26	77	26	77
E + I + R	0	139	0	39	70	24	79	25	78
—»—	15	137	2	29	78	19	83	19	83
—»—	30	141	0	23	82	15	87	—	—
—»—	60	137	2	17	87	12	89	11	92

recorded on a solid sample whether there is an intra- or intermolecular hydrogen bond.

Cholinesterase inhibition. None of the three pyridine-N-oxide-aldoximes in 10^{-3}M concentration inhibited acetyl- and butyrocholinesterase. Pyridine-2-aldoxime-N-methyl methane sulphonate, used in the same concentration, inhibited these esterases 12 and 17 % respectively.

Reactivation of cholinesterases inhibited by an organophosphorus compound. In 1.25×10^{-3}M concentration the three pyridine-N-oxide aldoximes were incapable of reactivating both butyro- and acetylcholinesterase inhibited by tetraethyl pyrophosphate (TEPP) (Table 2). For comparison, pyridine-2-aldoxime-N-methyl methane sulphonate used in ten times lower concentration (1.25×10^{-4} M) was tested in the same series of experiments. The results confirmed previous observations [1] that this derivative could reactivate phosphorylated cholinesterases, particularly acetylcholinesterase. It was observed that during incubation of the inhibited acetylcholinesterase with the pyridine-N-oxide-aldoximes, the activity of the partly inhibited esterase was reduced progressively with time. It was concluded that the phosphorylated aldoximes, formed during the incubation with the reaction mixture, containing phosphorylated esterase and unreacted TEPP, are more active esterase inhibitors than TEPP. This further decrease in enzyme activity was not due to TEPP alone, as could be demonstrated in control experiments.

Table 3. Reactivation of inhibited butyrocholinesterase by pyridine-N-oxide-2-aldoxime. Details as in Table 2, except for the concentration of the aldoxime which was seven times higher (8.75 mM). Reactivation period, 60 min.

Incubation mixture	Buffer soln. during incubation with TEPP and reactivations with POA 2			
	Bicarbonate, pH 7.4		Phosphate, pH 8.0	
	b_{30}	% inhib.	b_{30}	% inhib.
E	124		108	
E + I	0	100	0	100
E + I + R	30	75	40	63

It is reasonable to assume that in the reactivation of the phosphorylated enzyme the oximes react in their anionic forms [15]. Owing to the higher pK_a-values of the pyridine-N-oxide-aldoximes it might be expected that they are more nucleophilic and thus better reactivators than the N-methylderivatives, but the higher nucleophility is counteracted by the comparably lower concentration of the anions of the N-oxides at a fixed pH-value. One probable reason of the low reactivation power of the N-oxides may thus be that their pK_a-values are too high to give maximum reactivation at the pH-value in question [16]. According to these hypotheses, the N-oxide-aldoximes are expected to give better reactivation when the pH is increased. Preliminary experiments with pyridine-N-oxide-2-aldoxime used in higher concentration (8.75×10^{-3}M) and in a buffer solution of higher pH (8.0) revealed that butyrocholinesterase inhibited by TEPP could be partly reactivated under such experimental conditions (Table 3). A more decisive reason for the low reactivation power of the N-oxides compared with the N-methyl-derivatives is that the orientating effect of the positively charged nitrogen atom is cancelled by the adjacent, negatively charged oxygen atom [17,18].

Effect in vivo. The pyridine-N-oxide-aldoximes were less toxic ($LD_{50} >$ 200 mg/kg body weight, white mice, i.p.) than P2S. Mice injected intraperitoneally with 200 mg per kg body weight showed no toxic symptoms. Preliminary experiments *in vivo,* revealed that the new aldoximes were inactive in protecting mice from fatal intoxication with ω-dimethylaminoethylthio-*iso*propoxy-methyl-phosphine oxide [19].

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