ml. of methylene chloride was heated under reflux for 105 minutes. The resultant yellow solution was concentrated at 40° under reduced pressure; 50 ml. of benzene was added to flush out the thionyl chloride. The residue was taken up in methylene chloride and extracted with a 5% sodium bicarbonate solution. After concentration, the tan resinous product was dissolved in 10 ml. of benzene; after 1 hr. at room temperature, 0.335 g. (23%) of a crystalline substance, m.p. 212–214° dec., was obtained. An analytical sample, obtained by recrystallization from ethanol, had m.p. 214–215° dec. Assignment of structure X (2,2-dimethyl-3-carbomethoxy-5-keto-6-benzylsulfonamido-2,3,4,5-tetrahydro-1,4-thiazepine) was made from its ultraviolet (Fig. 2) and infrared (curve E, Fig. 1) absorption spectra.

Anal. Calcd. for $C_{16}H_{20}N_2O_5S_2$: C, 49.98; H, 5.24; N, 7.29. Found: C, 49.98; H, 5.06; N, 7.61.

The material in the benzene mother liquors from X was passed through a column containing 6.0 g. of Brockman Grade III ethyl acetate neutralized alumina with benzene as eluent. Crystallization of the material in the first 25 ml. of eluate from benzene–ether–petroleum ether afforded 0.625 g. (43%) of crude VIIIα, m.p. 128–131°. Recrystallization from acetone–petroleum ether gave an analytical sample of this β-lactam,[12] m.p. 130–131.5°.

Anal. Calcd. for $C_{16}H_{20}N_2O_5S_2$: C, 49.98; H, 5.24; N, 7.29. Found: C, 50.10; H, 5.30; N, 7.33.

(12) ADDED IN PROOF.—A preliminary *in vivo* assay of VIIIα in male albino mice against *D. pneumoniae* type II using potassium penicillin G as a standard indicated a potency of approximately 7 units/mg. This assay was carried out under the supervision of Dr. J. Lein, Bristol Laboratories, Syracuse, New York.

From a cyclization run involving 0.250 g. (0.57 mmole) of VIIα and 5.0 ml. of thionyl chloride in 15 ml. of methylene chloride, in which the crude reaction products were oxidized with potassium permanganate in 80% acetic acid, there was isolated 0.120 g. (51%) of the sulfone IXα, m.p. 212–213° dec. Recrystallization from acetone–water gave colorless needles, m.p. 214–215° dec.

Anal. Calcd. for $C_{16}H_{20}N_2O_7S_2$: C, 46.14; H, 4.85; N, 6.72. Found: C, 46.18; H, 4.89; N, 6.63.

B. β-Isomer.—A sample of 0.75 g. (0.0017 mole) of VIIβ was treated with 15 ml. of thionyl chloride in 50 ml. of methylene chloride, as described above for the α-isomer. After the preliminary purification of the crude reaction products by extraction with 5% sodium bicarbonate solution, 0.30 g. (46%) of VIIIβ, m.p. 128–130°, was obtained directly by crystallization from benzene. An analytical sample was obtained by recrystallization from acetone–ether–petroleum ether, m.p. 128.5–130°. The mixed m.p. with VIIIα (m.p. 130–131.5°) was depressed (114–124°).

Anal. Calcd. for $C_{16}H_{20}N_2O_5S_2$: C, 49.98; H, 5.24; N, 7.29. Found: C, 50.12; H, 5.22; N, 7.41.

None of the isomeric substance X appeared to have been formed in this reaction.

The sulfone IXβ was prepared by oxidation of the crude reaction products obtained from 0.16 g. (0.36 mmole) of VIIβ and 5 ml. of thionyl chloride in 15 ml. of methylene chloride. The yield of crude sulfone was 86 mg. (59%), m.p. 158–159°. An analytical sample, prepared by recrystallization from acetone–water, had m.p. 159.5–160°.

Anal. Calcd. for $C_{16}H_{20}N_2O_7S_2$. C, 46.14; H, 4.85; N, 6.72. Found: C, 46.11; H, 4.91; N, 6.59.

CAMBRIDGE 39, MASSACHUSETTS

[CONTRIBUTION FROM THE ENZYME CHEMISTRY BRANCH, CHEMICAL WARFARE LABORATORIES]

Chemical Reactions of Nerve Gases in Neutral Solution. I. Reactions with Hydroxylamine[1]

BY BERNARD J. JANDORF

RECEIVED FEBRUARY 14, 1956

Hydroxylamine and certain of its N-substituted derivatives react with organophosphorus anticholinesterases (DFP, GB) stoichiometrically at room temperature and *p*H 7.5. The over-all reaction is $(R)(R'O)P(:O)X + 3NH_2OH \rightarrow (R)(R'O)P(:O)OH + HX + N_2 + NH_3 + 2H_2O$, and is accompanied by loss of anticholinesterase activity. Evidence for several steps in this over-all reaction is presented. Hydroxylamine in 2000-fold excess does not prevent inhibition of cholinesterase by GB and does not reactivate GB-inhibited cholinesterase. An adaptation of the hydroxamic acid reaction to the colorimetric determination of micromole amounts of NH_2OH is described.

Organophosphorus anticholinesterases ("nerve gases") react with susceptible enzymes in a stoichiometric, irreversible fashion. The reaction consists of several steps, the last of which represents an alkylphosphorylation of a serine moiety.[2]

A search has been in progress in this laboratory for compounds which can react rapidly with nerve gases under physiological conditions of *p*H and temperature with the aim of finding substances which may successfully compete in the animal body with cholinesterase (ChE) for the inhibitor. In the first attempt to find such a competitor, naturally occurring amino acids were screened for reactivity with DFP (diisopropyl phosphorofluoridate) and GB (Sarin, isopropylmethyl phosphonofluoridate) under physiological conditions. These experiments were completely unsuccessful, and similar negative results have been reported by others.[3-5] The search was therefore widened to include other representative chemical structures, not necessarily known to exist in protein molecules but potentially able to react with nerve gases.

There exists in many respects a similarity between the reactions of susceptible esterases with their substrates and with nerve gases (leading to acylation and phosphorylation of the enzymes, respectively). With this in mind, one of the first substances to be tested was hydroxylamine which was known to react rapidly with acyl anhydrides and esters,[6] including acetylcholine,[7] with formation of the corresponding acylhydroxamic acid.

As a working hypothesis it was assumed that an

(1) This paper describes experiments which have been carried out during 1950–1951 and formed the basis of a classified report issued at that time.

(2) B. J. Jandorf, H. O. Michel, N. K. Schaffer, R. Egan and W. H. Summerson, *Faraday Soc. Disc.*, in press (1956).

(3) E. F. Jansen, M.-D. F. Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **185**, 209 (1950).

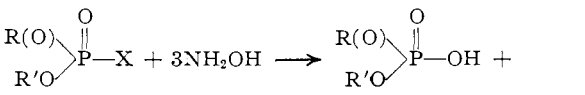
(4) T. Wagner-Jauregg, J. J. O'Neill and W. H. Summerson, THIS JOURNAL, **73**, 5201 (1951).

(5) W. K. Berry, K. P. Fellowes, P. J. Fraser, J. P. Rutland and A. Todrick, *Biochem. J.*, **59**, 1 (1955).

(6) F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **159**, 21 (1945).

(7) S. Hestrin, *ibid.*, **180**, 249 (1949).

analogous reaction might take place between nerve gases (which contain an ester linkage) and hydroxylamine. The results to be presented show that, while hydroxylamine does react with nerve gases at room temperature and neutral *p*H, the mechanism of this reaction is somewhat different from that with acyl esters and may be represented by the equation



$$R(O)(R'O)P(=O)-X + 3NH_2OH \longrightarrow R(O)(R'O)P(=O)-OH + N_2 + NH_3 + HX + 2H_2O \quad (1)$$

The detailed study of this reaction forms the gist of this report.

Results

Mechanism of Reaction between NH_2OH and GB.—The reaction of limiting amounts of GB with hydroxylamine, followed manometrically in bicarbonate buffer (see example under Experimental) leads to the liberation of two moles of gas per mole of GB (Fig. 1).[8] The half-time ($t_{0.5}$) of the reaction is 11–12 minutes at all concentrations of GB employed, indicating that with this large excess of NH_2OH the rate-determining reaction is pseudo first order. This half-time is to be contrasted with an extrapolated $t_{0.5}$ of 7.5 hours for spontaneous hydrolysis of GB (indicated in Fig. 1 by a dotted curve) in this particular experiment. (Other runs which were extended over a considerably longer period of time yielded a $t_{0.5}$ for spontaneous hydrolysis of 9–10 hours under these conditions.)

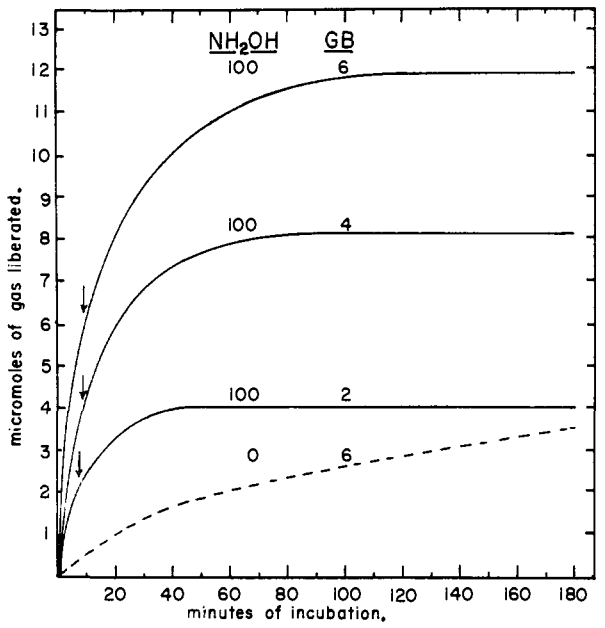


Fig. 1.—Reaction of GB with excess hydroxylamine in bicarbonate–CO_2 buffer, *p*H 7.54, *t* 25°. Figures on curves represent micromoles of reactants in 2.2 ml. Arrows = $t_{0.5}$.

Substitution of phosphate for bicarbonate buffer in an atmosphere of nitrogen (Table I) results in the liberation of one mole of gas per mole of added GB. This gas is absorbed by neither acid nor alkali, and has been presumptively identified as nitrogen. The $t_{0.5}$ of its evolution is the same as that found in bicarbonate. Determination of the *p*H of the vessel contents after completion of the reaction indicates the simultaneous production of acid. These results therefore indicate that the action of NH_2OH on GB is not that of a catalyst of spontaneous hydrolysis (which would also result in the production of two moles of gas in bicarbonate per mole of GB, both of which would however be CO_2) since, if the latter were true, there should have occurred only acid production without gas evolution in phosphate buffer.

TABLE I

GAS EVOLUTION IN THE REACTION BETWEEN GB AND EXCESS (100 μMOLES) HYDROXYLAMINE IN 0.025 *M* PHOSPHATE BUFFER, *p*H 7.4 AT 25°

GB added (μmoles)	0	4	0	6	0	6
Absorber			NaOH		HCl	
Gas evolved (μl.)	49	137	32	178	47.5	199
Net gas evolved due to reaction between GB and NH_2OH (μmoles)		3.93		6.50		6.76
Gas evolved per mole GB (moles)		0.98		1.08		1.13
$t_{0.5}$ (min.)		13		12		13
Final *p*H of reaction mixture			7.21	7.00	7.22	6.98

Further evidence against the catalytic nature of NH_2OH in this reaction is provided by the data in Fig. 2 which show a rapid disappearance of NH_2OH

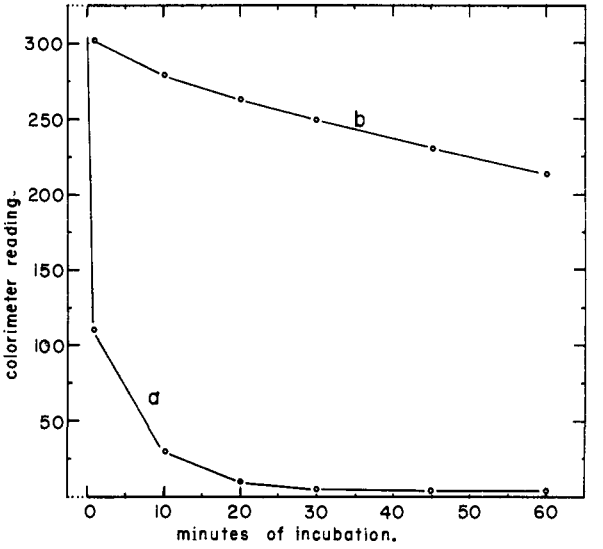


Fig. 2.—Disappearance of hydroxylamine in reaction with GB. NH_2OH (5×10^{-3} *M*) with (curve a) and without (curve b) 0.1 *M* GB was incubated in 0.1 *M* phosphate, *p*H 7.2 at room temperature. Aliquots were removed at intervals and tested for remaining NH_2OH by the colorimetric procedure (see Experimental).

in the presence of excess GB. A balance experiment was therefore run in which, in addition to the measurement of gas evolution due to decomposition of GB and of NH_2OH disappearance, production of ammonia was measured also, since it was suspected that the *net* production of one mole of acid in reality constituted the liberation of 2 moles of acid with

(8) Data in Fig. 1 have been corrected for a small amount of gas production in a control containing 100 μmoles NH_2OH but no GB. This blank, amounting to about 30 μl. of gas over the 180-minute period, probably represents a slow decomposition of NH_2OH in neutral solution.

TABLE II

DISAPPEARANCE OF NH_2OH, AND FORMATION OF NH_3, IN THE REACTION BETWEEN NH_2OH AND GB

Reaction medium: 0.025 *M* phosphate, *p*H 7.4. $t = 25°$. Gas phase: nitrogen. After 90 minutes[a] MnO_2 was tipped in, the remaining NH_2OH was determined manometrically, the vessel contents were filtered and used for NH_3 determinations. All figures = μmoles of compound per vessel.

Reaction mixture	NH_2OH:	30	30	30	30	30	
	GB:	0	2	4	6	8	
(1) Reaction between NH_2OH and GB							
Gas produced in 90 min.		0.42	1.88	3.04	4.31	5.45	
Net gas produced = GB reacted			1.46	2.62	3.89	5.03	
(2) NH_2OH disappearance							
N_2O produced by MnO_2		12.64	10.80	8.16	7.24	5.98	
NH_2OH disappeared			3.68	8.96	10.80	13.32	
(3) Ammonia formation							
NH_3 present		1.77	3.62	4.06	5.28	6.17	
Net NH_3 produced			1.85	2.29	3.51	4.40	
(4) Balance sheet							
NH_2OH disappeared per μmole GB reacted			2.25	3.42	2.78	2.64	Av. 2.84
NH_3 formed per μmole GB reacted			1.26	0.88	0.91	0.88	Av. 0.98

[a] The reaction was stopped deliberately before being complete to minimize spontaneous decomposition of NH_2OH.

simultaneous liberation of one mole of base. The results of such a balance study are shown in Table II. Though the results are not as precise as might be desired (probably due in part to the fact that addition of MnO_2 did not stop the reaction between GB and NH_2OH instantaneously) they nevertheless indicate the disappearance of 3 moles of NH_2OH and the production of 1 mole of NH_3 per mole of GB destroyed.

When a limiting amount of NH_2OH is allowed to react with excess of GB there is a net evolution of one mole of gas per mole of GB in bicarbonate, and no gas production in phosphate buffer (Table III).[9]

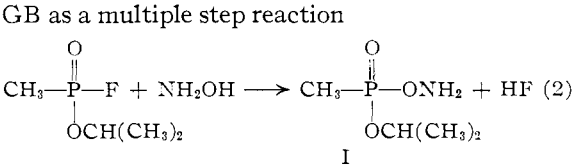
TABLE III

REACTION BETWEEN EXCESS GB AND LIMITING AMOUNTS OF NH_2OH

(For experimental details, see text)

NH_2OH added (μmoles)	0	1	2	4	6
GB added (μmoles)	100	100	100	100	100
(1) In 0.025 *M* $NaHCO_3$-CO_2 buffer, *p*H 7.4					
Gas evolved at zero time (extrap.) (μl.)	44	68	84	135	177
Net zero time evolution (μl.)		24	40	91	133
Net zero time evolution (μmoles)		1.07	1.79	4.06	5.93
Gas evolved per mole NH_2OH (moles)		1.07	0.90	1.02	0.99
(2) In 0.025 *M* phosphate buffer, *p*H 7.4					
Gas evolved at zero time (extrap.) (μl.)	12		16	14	14
Net zero time evolution (μl.)			4	2	2

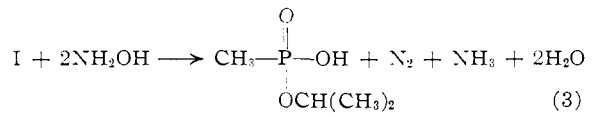
The above results, taken together, lead to the formulation of the reaction between NH_2OH and GB as a multiple step reaction



(2)

I

(9) In this experiment the rates of gas production after the initial rapid evolution, which were high in all cases due to the superimposed spontaneous hydrolysis of excess GB, were extrapolated back to time of mixing of the reactants, and the amount of gas production due to the presence of NH_2OH was thus determined.



(3)

which, in more general terms, yields equation 1 as the summation. The reason for representation of the intermediary (I) as pictured above, rather than as the hydroxamic acid CH_3–P(:O) [$OCH(CH_3)_2$]–NHOH will be presented below. The results of Table III indicate, as might be expected, that in the presence of a limiting amount of NH_2OH only reaction (2) occurs which results in the production of one mole of acid but no other gas.

In the complete reaction, which takes place with excess NH_2OH, the rate-determining step appears to be the first (equation 2) since the values of $t_{0.5}$ for acid production and nitrogen liberation are the same. When the initial course of reaction between a fixed amount of GB (3 μmoles) and a varying stoichiometric excess of NH_2OH (10, 25, 50 μmoles) in bicarbonate buffer is plotted as log $[a/(a - x)]$ (where a = maximal gas evolution = 6 μmoles and x = gas evolution at time t) *vs.* time, straight lines are obtained (Fig. 3a) indicating that for each concentration of NH_2OH the reaction shows the characteristics of a first-order reaction, a conclusion already previously drawn from other evidence. A plot of $t_{0.5}$ against the molar ratio of GB:NH_2OH also yields a straight line (Fig. 3b), which leads to the bimolecular rate constant

$$K_2 = \frac{1}{t(NH_2OH)} \ln \frac{a}{a - x} = 2.45 \text{ l. mole}^{-1} \text{ min.}^{-1}$$

Effect of *p*H.—No systematic study of this aspect was carried out. Table IV presents first-order constants, obtained as described in a previous section, at various values of *p*H, for the reaction between 4 μmoles of GB and 100 μmoles of NH_2OH. From these values second-order constants have been calculated, on the basis of *total* initial hydroxylamine concentration (C_t = 100 μmoles in 2.2 ml. = 4.54×10^{-2} *M*), as well as on the basis of undissociated hydroxylamine $C_u = K'C_t/(K' + H^+)$

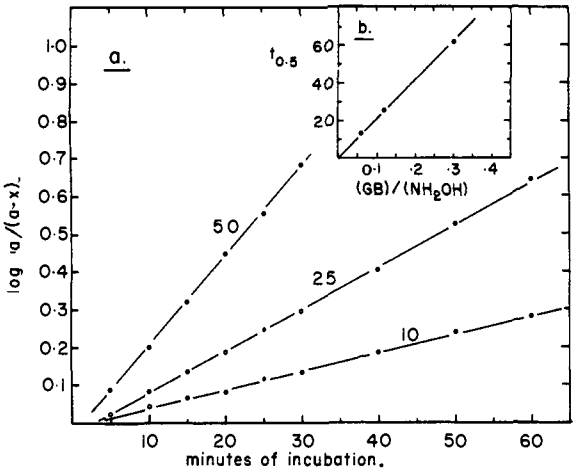


Fig. 3.—A, reaction between 3 μmoles of GB and 10, 25 and 50 μmoles of NH_2OH in bicarbonate–CO_2 buffer, *p*H 7.54, *t* 25°. For method of plotting see text. B, plot of $t_{0.5}$ against ratio of reactants (data derived from Fig. 3A).

where K' is the apparent dissociation constant of the reaction $N^+H_3OH \rightleftarrows NH_2OH + H^+$. Titration of 4.5×10^{-2} *M* $NH_2OH \cdot HCl$, containing 0.025 *M* NaCl, yielded a $pK' = 6.03$ for this reaction. While the data are not sufficiently numerous for an unequivocal conclusion to be drawn, they nevertheless indicate that NH_2OH is the species involved in the reaction with GB.

TABLE IV

EFFECT OF *p*H ON REACTIVITY BETWEEN GB (4 μMOLES) AND NH_2OH (100 μMOLES)

The *p*H of bicarbonate–CO_2 mixtures was calculated, that for other buffers determined by glass electrode; $t = 25°$.

Buffer	Gas phase	*p*H	First-order constant (min.$^{-1}$)	Second-order const. based on NH_2OH Total	Undissc.
K-H phthalate, 0.05 *M*	N_2	4.62	No reaction		
$NaHCO_3$, 0.025 *M*	CO_2	6.14	0.0273	0.60	1.07
Na–K phosphate, 0.05 *M*	N_2	6.95	.0539	1.17	1.31
$NaHCO_3$, 0.025 *M*	5% CO_2, 95% N_2	7.54	.0607	1.34	1.37
$NaHCO_3$, 0.10 *M*	5% CO_2, 95% N_2	8.04	.0606	1.33	1.34

Effect of Oxygen.—A few experiments were run in bicarbonate buffer with 5% CO_2 in oxygen (instead of nitrogen), and in phosphate buffer with air as the gas phase. In both cases there was a rather high rate of evolution of gas (not further identified) in vessels containing NH_2OH only. When GB was also present under these conditions the *net* gas evolution (*i.e.*, the increase over that due to NH_2OH alone) appeared to occur at about the same rate and to the same extent as under anaerobic conditions.

Reactivity of Hydroxylamine with DFP and Tabun.—Hydroxylamine reacts with DFP in the same way as with GB, but its reactivity is much lower (Fig. 4), the $t_{0.5}$ for this compound being approximately 90 minutes. Comparison with the rate of spontaneous hydrolysis of DFP at equal concentration (dotted curve) shows that a reaction does occur, and the total gas evolution approaches the expected 2 moles per mole of DFP.

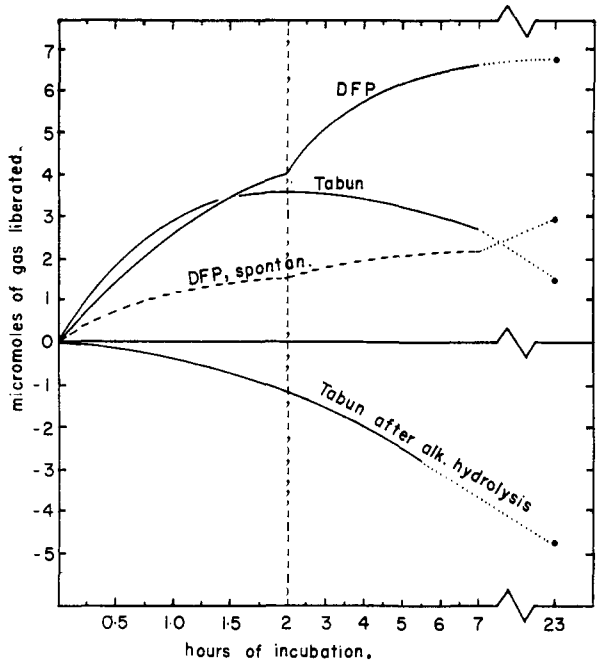


Fig. 4.—Reaction of DFP (4 μmoles) and Tabun (4 μmoles) with hydroxylamine (100 μmoles) in bicarbonate–CO_2 buffer. For other experimental details and controls see text.

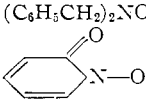
Tabun (ethyl N,N-dimethylphosphoramidocyanidate) presents a slightly different behavior. It was expected that, if Tabun reacts with NH_2OH by a mechanism analogous to that of GB, one mole of gas (nitrogen) should be liberated in bicarbonate (or in phosphate) buffer but there should be no net acid production since HCN is too weak an acid to liberate CO_2 from bicarbonate buffer. The results of Fig. 4 show for this compound a gas evolution which approaches one mole per mole of Tabun added, with $t_{0.5}$ of approximately 30 minutes for this phase of the reaction. This evolution is followed by a gas absorption: at the end of 23 hours the net gas production amounts to less than 0.4 moles per mole of Tabun. A possible explanation for this phenomenon may be a slow reaction of NH_2OH with the N–P linkage of Tabun, resulting in liberation of dimethylamine which is a strong enough base to cause CO_2 absorption from the gas phase. That this explanation may be the correct one is shown by the fact that Tabun, previously hydrolyzed with alkali at the P–CN bond and neutralized, exhibits only gas absorption when treated with NH_2OH under these conditions. Alkali-hydrolyzed GB shows neither gas production nor absorption on subsequent treatment with NH_2OH. No inorganic phosphate is liberated when Tabun or DFP is treated with hydroxylamine for 24 hours.

Reactivity of GB with Derivatives of Hydroxylamine.—The reactivity of some substituted hydroxylamines against GB was compared on an equimolar basis with that of the parent compound (Table V). The results show that substitution in the hydroxyl group of hydroxylamine greatly lowers or abolishes reactivity. Monobenzylation of the amino group lowers the reactivity to approximately 50% that of NH_2OH; the data con-

TABLE V

REACTIVITY OF HYDROXYLAMINE DERIVATIVES (100 μMOLES) WITH GB (4 μMOLES)

(Bicarbonate–CO_2 buffer, pH 7.5; t = 25°)

Compound Name	Structure	Source	$t_{0.5}$	Remarks
Hydroxylamine	NH_2OH		13	
Aminoöxyacetic acid	NH_2OCH_2COOH	*a*	*c*	
Ethyl aminoöxyacetate	$NH_2OCH_2COOC_2H_5$	*a*	*c*	
O-Benzylhydroxylamine	$NH_2OCH_2C_6H_5$	*b*	Approx. 200	
N-Benzylhydroxylamine	$C_6H_5CH_2NHOH$	*b*	25	Liberates 1 mole of acid in bicarbonate, no gas in phosphate
N,N-Dibenzylhydroxylamine	$(C_6H_5CH_2)_2NOH$	*b*	*c*	Very insoluble
N-Hydroxyl-α-pyridone		*a*	35	
Acetoxime	$(CH_3)_2C{=}NOH$		*c*	

[a] Obtained through the Chemical and Biological Coordination Center. [b] Locally synthesized. [c] Denotes a rate of gas liberation equal to, or less than, that due to spontaneous hydrolysis of GB.

cerning gas evolution with this compound might indicate a reaction proceeding only through the first step of decomposition (equation 2). Results with the dibenzylated compound are equivocal since the material was present in suspension rather than in solution, and its true concentration in the reaction mixture was probably much lower than indicated. N-Hydroxy-α-pyridone may be regarded as a substituted hydroxamic acid (RCO–N(R′)OH) and its reactivity, albeit smaller than that of NH_2OH, confirms the necessity of a free OH group in the NH_2OH molecule.[10]

The System NH_2OH : GB : Cholinesterase.—In connection with the general purpose of these investigations, as outlined in the Introduction, information was desired on the following points: (1) does the reaction between NH_2OH and GB result in products which no longer show anticholinesterase activity? (2) will addition of NH_2OH to cholinesterase (ChE) result in their competition for GB, with a resultant lowered anticholinesterase activity of GB? (3) does NH_2OH serve as a reactivator of GB-inhibited ChE?

The experiments in Table VI provide answers to these questions and have been grouped as corresponding Parts 1-3. The following conclusions may be drawn: (1) Incubation of GB with a twenty-fold excess of NH_2OH results in loss of the inhibitory power of GB; this was to be expected and conforms with the proposed mechanism of the reaction between GB and NH_2OH. The concentration of GB in this experiment was at least ten times higher than necessary to produce complete inactivation of ChE. (2) A 2,000-fold excess of NH_2OH cannot protect ChE against subsequently added GB. As an incidental result, it is also found that the enzymatic activity of ChE (in the form of a brain homogenate) is unaffected by 2.5×10^{-3} *M* NH_2OH. (3) The same excess of NH_2OH is unable to reactivate GB-inactivated ChE.

TABLE VI

THE SYSTEM NH_2OH : GB : CHOLINESTERASE

Procedure.—The mixtures shown below (total vol. = 2.0 ml.) were incubated for 2 hours at 20° (Part 1) or 30 min. at 38° (Parts 2, 3). After a 1:500 dilution (for Part 1 only) the second addition was made, and the mixtures were incubated further (30 min., 38°). Water was added where necessary to equalize the volume. At the end of the second incubation aliquots were tested for ChE activity.

Part	Title	First incubation	Second incubation	ChE activ. (% of control)
1	Test of reaction products	NH_2OH (100)[a]	ChE	105
		GB (5)	ChE	1
		NH_2OH (100) + GB (5)	ChE	98
2	Test of competition	ChE + GB (0.005)		0
		ChE + NH_2OH (10)		101
		ChE + NH_2OH (10), GB (0.005) 5 min. later		0
3	Test of reversal	ChE	NH_2OH (10)	100
		ChE + GB (0.005)	NH_2OH (10)	0

[a] Figures in parentheses: μmoles of reagent in incubation mixture. ChE: 10% rabbit brain homogenate in water, added to equal volume of reaction mixture.

Discussion

This paper has described what so far as we know was the first instance of a compound capable of reacting with nerve gases and related anticholinesterases under physiological conditions of *p*H and temperature and within reasonably short periods of time, in such a fashion as to abolish their enzyme-inactivating properties. The proposed mechanism for the reaction between NH_2OH and GB is supported by the experimental data here presented.

Hydroxylamine, even in relatively large excess, neither prevents inhibition of ChE by nerve gases nor does it reactivate inhibited ChE. In the light of later findings in our laboratory as well as in others these results are not unexpected; the bimolecular rate constant of GB with NH_2OH is of the order of unity, while that with cholinesterases of the type found in brain tissue[2] is of the order of 10^7 l. mole^{-1} min.$^{-1}$; likewise, the relative resistance to reactivation of ChE which has been inhibited with DFP[11] and GB,[12] as compared with TEPP, has been subsequently recognized. These studies did however point the way toward the more effective hydroxamic acids as reactants with anticholinesterases and as potent reactivators.

(10) The above results led to the testing, at the time this work was in progress, of other hydroxamic acids for ability to react with nerve gases by Wagner-Jauregg, *et al.*, in the Medicinal Chemistry Branch of these laboratories; their experiments were successful but could not be published until recently; *cf.*, B. E. Hackley, Jr., R. Plapinger, M. Stolberg and T. Wagner-Jauregg, THIS JOURNAL, **77**, 3651 (1955).

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(12) B. J. Jandorf, E. A. Crowell and A. P. Levin, *Federation Proc.*, **14**, 231 (1955).

Experimental

Manometric Experiments.—As a representative example, the details of an experiment on the reaction between 100 μmoles NH_2OH and 4 μmoles GB in 0.025 *M* bicarbonate are described. Suitable variations were introduced in this standard technique as needed.

$NH_2OH \cdot HCl$ (69.5 mg., 1 mmole) was dissolved in 5 ml. of H_2O, neutralized to phenol red and diluted to 10 ml. One ml. was pipetted into a standard Warburg conical flask equipped with one side arm and containing 1.0 ml. 0.05 *M* $NaHCO_3$ solution which had previously been equilibrated for 10 minutes with a rapid stream of 5% CO_2:95% N_2. The vessel, with the side-arm left unstoppered, was attached to its manometer and gassed for 10 minutes with the above gas mixture with occasional shaking. Shortly before the end of the gassing period, 0.128 ml. (140 mg., 1 mmole) of GB was dissolved to 25 ml. in water, 25 ml. of 0.05 *M* $NaHCO_3$ was added, and 0.2 ml. of this mixture was pipetted into the side arm. Immediately the side arm was stoppered and the manometer stopcock was turned off simultaneously. The manometer was transferred to the Warburg bath at 25° where the rate of gas evolution was read at intervals according to usual procedures.

All experiments included controls for spontaneous hydrolysis of GB, decomposition of NH_2OH, etc. Experiments in phosphate buffer were run with nitrogen as the gas phase unless otherwise noted. In some experiments NaOH or HCl (2.5 *N*, 0.2 ml.) were added to the center well of the vessel with a roll of filter paper to facilitate any gas absorption.

Reactivity was evaluated from the value of $t_{0.5}$, *i.e.*, the time necessary under stated conditions for 50% of the measured reaction to take place. This "half-time" is inversely proportional to reactivity.

Determination of NH_2OH. a. **Colorimetrically.**—A modification of Hestrin's procedure[7] was used, employing an excess of acetylcholine and limiting amounts of NH_2OH. An aliquot of the solution to be tested, containing no more than 5 μmoles of NH_2OH was added to a test-tube or calibrated colorimeter tube containing 1.0 ml. of acetylcholine bromide (0.5 *M*, kept cold when not in use) and water to yield a total volume of 3.0 ml. Exactly 30 seconds after introduction of the sample, 1.0 ml. of 1.5 *N* NaOH was added, the contents were mixed and allowed to stand for at least one minute (standing up to 1 hour has no effect). Then 1.0 ml. of 2 *N* HCl was added, mixing was repeated (standing up to 20 minutes has no effect) and 1.0 ml. of color reagent (10% $FeCl_3 \cdot 6H_2O$ in 0.1 *N* HCl) was added from a buret with shaking. The red color, which is stable for about 15 minutes, was read in the Klett–Summerson photoelectric colorimeter with filter 54. The linear range is from 0–6 μmoles NH_2OH per sample, and the sensitivity approximately 80 Klett units per μmole.

b. **Manometric.**—At the time this work was in progress, Colter and Quastel[13] described the reaction: $2NH_2OH + 2MnO_2 \rightarrow N_2O + 2MnO + 3H_2O$, which permits determination of NH_2OH by the release of 0.5 mole of N_2O for each mole NH_2OH present. This reaction was carried out in phosphate buffer with N_2 in the gas phase. The side arm contained 0.2 ml. of a 10% suspension of MnO_2 in buffer. Destruction of NH_2OH was essentially complete within 15–20 minutes at 25°.

Determination of Ammonia.—This was carried out on reaction mixtures which had previously been freed of excess NH_2OH as described above. The vessel contents were filtered through Whatman #42 filter paper, diluted and analyzed by direct Nesslerization.[14]

Determination of Cholinesterase Activity.—The standard manometric procedure, with 0.015 *M* acetylcholine as substrate at 38° in bicarbonate buffer, was used. This method is sufficiently well known (*e.g.*, ref. 15) that it need not be described in detail.

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(14) The presence of NH_2OH was found to interfere with NH_3 determinations by direct Nesslerization or by microdistillation using the Conway dish technique; prior destruction of NH_2OH in the Conway dish with acetylcholine or triacetin was also unsuccessful. Kjeldahl procedures could not be applied owing to the small quantities of NH_3 involved.

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[CONTRIBUTION FROM DANIEL SIEFF RESEARCH INSTITUTE, WEIZMANN INSTITUTE OF SCIENCE]

Conformational Analysis of Certain Morphine Derivatives[1]

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The conformations of the epimeric sets of alcohols—dihydrocodeine (I), dihydroisocodeine (II), and dihydropseudocodeine (IIIa), dihydroallopseudocodeine (IVa), are discussed in the light of previous work. The rates of saponification of the respective acetates support the formulation of I and IVa as the axial isomers and II and IIIa as the equatorial isomers.

The relative rates of saponification of dihydrocodeine (I) and dihydroisocodeine (II), of dihydropseudocodeine (IIIa) and dihydroallopseudocodeine (IVa), and of dihydrothebainol A (IIIb) and dihydrothebainol B (IVb) were studied in order to provide additional evidence for the conformations of these epimers.

Previous work has shown that conformational considerations apply equally well in heterocyclic systems as in alicyclic systems.[3–5]

Catalytic reduction of dihydrocodeinone gives dihydrocodeine,[6] whereas aluminum isopropoxide reduction yields dihydroisocodeine.[7]

Results of oxidation experiments on the epimeric alcohols I, II, IIIa and IVa[8] and Rapoport's stereochemical correlation of the various asymmetric centers of the morphine molecule,[9] indicate that I and IVa are the axial isomers while II and IIIa are the equatorial isomers.

The relative rates of saponification of the acetates of these alcohols reported in the Experimental section further support these formulations.

Two epimeric dihydrothebainols have been re-

(1) Presented at XIVth Congress of Pure and Applied Chemistry, Zurich, July, 1955.

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