replaced by cysteine hydrochloride, 10 mg. per 100 ml., and the vitamin concentrations were changed to the following values (per 100 ml.): thiamine hydrochloride, 50 μg.; pyridoxine hydrochloride, 100 μg.; pyridoxal hydrochloride, 30 μg.; pyridoxamine dihydrochloride, 30 μg.; calcium pantothenate, 50 μg.; nicotinic acid, 100 μg.; riboflavin, 50 μg.; biotin, 0.10 μg.; *p*-aminobenzoic acid, 10 μg.

The techniques used were the same as in the *L. leichmannii* assay, except that the tubes were incubated 20 hours, which incubation time was necessary for obtaining maximal growth in the control tubes.

Inhibition Experiments.—The inhibitors were dissolved and diluted to the required concentrations. One milliliter of each of these dilutions was added to four milliliters of 1.25-fold basal medium, and the inhibitor concentrations were expressed as μg. per (5 ml.) tube.

Reversal Experiments.—The inhibitor was added, in dry form or from stock solution, to the 1.25-fold basal medium. Four milliliters of this medium was added to each assay tube, and one milliliter of a series of dilutions of the reversing agent was added to it.

The turbidity reading corresponding to half-maximal growth was determined from the standard curve. This value was usually between 55 and 65. For every inhibitor concentration in the medium, the turbidity readings were plotted against the concentrations of the reversing agent in the corresponding assay tubes. The concentration of the reversing agent required for half-maximal growth (*i.e.*, half-maximal reversal) was obtained by interpolation to the half-maximal turbidity reading.

These values were tabulated in Tables I, II, IV and V.

CHICAGO 9, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF NEUROLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY]

The Reactivation of Acetylcholinesterase Inhibited by Tetraethyl Pyrophosphate and Diisopropyl Fluorophosphate[1]

BY IRWIN B. WILSON, S. GINSBERG AND E. K. MEISLICH

RECEIVED JANUARY 15, 1955

The reactivation of TEPP and DFP inhibited acetylcholinesterase has been studied with several reactivators and under various conditions with the aim of elucidating the mechanism of the reaction. It is hoped that once the principles are clarified it will be possible to synthesize compounds of practical value in overcoming the toxic effects of these inhibitors. It has been shown that the hydroxamic acids form complexes with the inhibited enzyme analogous to the complexes formed between active enzymes and their substrates. It has been shown that the hydroxamate ions are the reactivators. The shape of the *p*H dependence of reactivation has been interpreted in terms of the theory of inhibition and reactivation which had been previously developed. The reactivation is a combined acid–base attack with the solvent supplying the proton in most cases, but in the case of choline the latter makes both attacks. The anionic site is still functional in TEPP inhibited enzyme, though less so than in the normal enzyme, and serves to promote the activity of certain reactivators. The anionic site does not appear to be functional in DFP inhibited enzyme. The reactivation of DFP inhibited enzyme depends upon the conditions of inhibitions and storage and some of the difficulty in restoring the enzyme can be attributed to secondary effects arising from unfavorable conditions of inhibition and storage. Of the reactivators considered in this paper nicotinhydroxamic acid methiodide is the best for overcoming TEPP inhibition and roughly the same as nicotinhydroxamic acid in overcoming DFP inhibition.

Certain phosphate esters such as tetraalkyl pyrophosphates, dialkyl *p*-nitrophenyl phosphates and dialkylfluorophosphates are potent irreversible inhibitors of acetylcholinesterase and of esterases in general. These compounds are of general interest because the most potent chemical warfare gases and some powerful insecticides belong to this class and owe their lethality to the inhibition of acetylcholinesterase.

The development of the theory of enzymatic hydrolysis[2,3] clarified the mechanism of irreversible inhibition and suggested the means whereby reactivation might be achieved.[4,5] The active site of this enzyme may be conveniently considered as consisting of two subsites: (a) an anionic site which contributes to the catalytic activity by binding and orienting substituted ammonium structures such as that which occurs in acetylcholine; (b) an ester interacting or esteratic site which contains an acidic and a basic group, both of which are necessary for activity. During enzymatic hydrolysis the esteratic site makes a combined acid base attack upon esters and the basic group is thereby acylated. This acyl enzyme reacts rapidly with water to yield a carboxylic acid and free enzyme.

The alkyl phosphates function as inhibitors by interacting with the enzyme in much the same way as substrates to form in this case a dialkylphosphoryl enzyme, but unlike the analogous acyl enzyme the phosphoryl enzyme does not readily react with water and so the free and active enzyme is not rapidly restored.

The equation for the inhibitory reaction (here illustrated with a fluorophosphate) is

$$\text{H—}\ddot{\text{G}} + (\text{RO})_2\text{—}\overset{\overset{\text{O}}{\|}}{\text{P}}\text{—F} \longrightarrow \text{G—}\overset{\overset{\text{O}}{\|}}{\text{P}}(\text{OR})_2 + \text{HF}$$

Here H-G is the esteratic site of the enzyme: H represents the acidic group and the electron pair (. .) represents the basic group.

It would appear from the theory that the enzyme could be dephosphorylated by means of bimolecular nucleophilic displacement reactions and its activity thus be restored. And in fact, when R = ethyl (inhibitor = tetraethyl pyrophosphate (TEPP) or diethyl fluorophosphate) reactivation is readily accomplished with nucleophilic reagents such as hydroxylamine, but when R = isopropyl (inhibi-

(1) This work was supported in part by the Medical Research and Development Board, Department of the Army, Office of the Surgeon General, Contract No. DA-49-007-MD-37, and in part by the Division of Research Grants and Fellowships of the National Institutes of Health, Grant No. B-573, United States Public Health Service.

(2) (a) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **185**, 479 (1950); (b) I. B. Wilson, F. Bergmann and D. Nachmansohn, *ibid.*, **186**, 781 (1950).

(3) I. B. Wilson, in "The Mechanism of Enzyme Action," Ed. by W. D. McElroy and B. Glass, The John Hopkins Press, Baltimore, Md., 1954, p. 642.

(4) I. B. Wilson, *J. Biol. Chem.*, **190**, 111 (1951).

(5) I. B. Wilson, *ibid.*, **199**, 113 (1952).

for = diisopropyl fluorophosphate (DFP)) reactivation may be very difficult.

Experiments indicated that the anionic site of the phosphorylated enzyme is still capable of binding ammonium structures although with an affinity only one-sixtieth that in the normal enzyme.[4] Choline could hardly be considered an intrinsically active nucleophilic agent; that it is, nevertheless, a good reactivator for TEPP inhibition can be explained only if we assume that its activity is promoted through interaction with the anionic site. Consequently it appeared desirable to synthesize a compound which combined in the same molecule a quaternary nitrogen structure and an intrinsically good reactivating group. Nicotinhydroxamic acid methiodide is such a compound.

This paper deals with the reactivation of TEPP and DFP inhibited enzyme by hydroxylamine, nicotinhydroxamic acid methiodide (NMI), nicotinhydroxamic acid (NH), acethydroxamic acid, pyridine and choline. Large and even complete reactivations may be obtained with the hydroxamic acids.

Method

The enzyme solution was a partially purified preparation from electric tissue of *Electrophorus electricus*, having an activity of 2400 μmole/min. per ml. and a specific activity of 330 μmole/min. per mg. of protein. Enzyme activity was assayed at 25° manometrically in a medium containing 0.1 *M* NaCl, 0.025 *M* $NaHCO_3$, 0.05% gelatin, as substrate 0.004 *M* acetylcholine bromide, and gassed with 95% N_2–5% CO_2 (*p*H 7.4).

Inhibited enzyme was prepared by adding 0.010 ml. of inhibitor solution to 0.200 ml. of enzyme so that the final concentration of inhibitor was generally 1 γ/ml. After two hours in the cold in the case of TEPP, the solution was diluted to 4.0 ml. with 0.95 *M* NaCl, 0.05% gelatine. This stock was stored in the cold and used for the two or three days. To 0.200 ml. were added for each reactivation 0.2 ml. of 0.1 *M* reactivator in 0.03 *M* EDTA (to guard against contamination by heavy metals) and 0.06 *M* phosphate buffer adjusted to the desired *p*H. Reactivation was carried out at 25.0° for a suitable time and then diluted to from 10 to 50 ml. depending upon the anticipated activity. Controls without reactivator were usually diluted to 5 ml. and controls using uninhibited enzyme were diluted to 50 ml. In the case of DFP, inhibition was carried out from 1 to 2 hours and either in the cold or at 25°. Reactivation was also carried out at both temperatures.

Of the reactivators, nicotinhydroxamic acid methiodide is a new compound. It was prepared in the following manner. Ethyl nicotinate (0.2 mole) was methylated in 25 ml. of methanol solution, at room temperature, with 10% excess of methyl iodide. Hydroxylamine hydrochloride (0.3 mole) was neutralized with 20% excess of KOH in methanol. The KCl was filtered off and the solution added to the ethyl nicotinate methyl iodide. KOH in CH_3OH was added until the mixture was just alkaline to brom thymol blue and the mixture was left overnight at room temperature. After cooling to 0°, crystallization was started by scratching the sides of the flask and the mixture was left standing in the refrigerator for several days. The crystals were recrystallized from methanol, yield 15%, m.p. 182–184° dec.

Anal. Calcd.: C, 30.0; H, 3.24; I, 45.3. Found: C, 30.2; H, 3.18; I, 45.1. The preparation of nicotinhydroxamic acid hydrochloride from hydroxylamine and ethyl nicotinate has been described.[6] But we preferred to use the same method as for nicotinhydroxamic methiodide except, of course, that the methylation step was omitted. We obtained nicotinhydroxamic acid; yield 40%, m.p. 157–158°.

Results

Reactivation of TEPP Inhibition.—Using nicotinhydroxamic acid and nicotinhydroxamic acid methiodide as reactivators, the time course of the reactivation was found to follow first-order kinetics, but the first-order constant was not linear with the concentration of reactivator. A similar behavior was observed in the reactivation with hydroxylamine of α-chymotrypsin inhibited by diethyl-*p*-nitrophenyl phosphate.[7]

The constants (k) appear to approach a constant value as the reactivator concentration (R) is increased. This is illustrated with nicotinhydroxamic acid at *p*H 7.2 in Fig. 1. Also included is a plot of k^{-1} against R^{-1}. The linearity of this graph indicates a function of the form

$$k = \frac{k_m R}{K_R + R}$$

where k_m is the maximum reaction rate constant and K_R is a constant and in this case has the value 0.034 *M*. This relationship is the same as exists for enzymes and substrates, and the same kind of interpretation, namely, that a reactivator-inhibited enzyme complex is formed, appears reasonable.

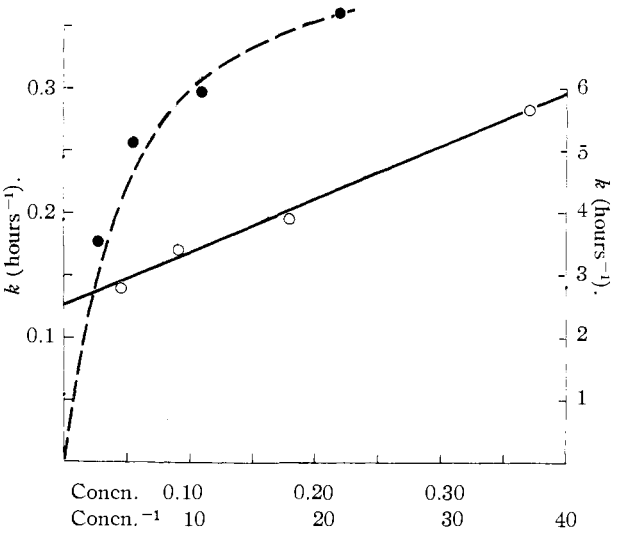


Fig. 1.—Nicotinhydroxamic acid reactivator of TEPP inhibited enzyme. The first-order constants at 25° and *p*H 8.0 are given as a function of the concentration of the reactivator ●. Also shown is a plot of the reciprocals ○.

To test the hypothesis of complex formation between the reactivator and diethyl phosphorylated enzyme the following experiment was run: a portion of TEPP inhibited enzyme was reactivated at *p*H 8 with 0.05 *M* nicotinhydroxamic acid, another portion with 0.05 *M* nicotinhydroxamic acid methiodide, and finally a portion with both reactivators together, each at 0.05 *M*. Since the medium in which both reactivators are together has a somewhat higher ionic strength than would be the case when run individually, the latter solutions were brought up to the same ionic strength with NaCl. The reactivation with NMI is considerably greater than with NH. If no complex is formed, we should expect the reactivation with both reagents together to be greater than with NMI alone. If, on the other hand, NH forms a complex, it should inhibit

(6) T. S. Gardener, E. Wenis and F. A. Smith, THIS JOURNAL, **73**, 5455 (1951).

(7) L. W. Cunningham, *J. Biol. Chem.*, **207**, 443 (1953).

the reactivation by NMI and the results should fall below that of NMI alone but above that of NH alone. The results of Table I evidently support the hypothesis that complexes are formed between these reactivators and the phosphorylated enzyme.

TABLE I

PERCENTAGE REACTIVATION OF TEPP INHIBITED ENZYME AT *p*H 8

	0.25 hr.	0.50 hr.	k (hour^{-1})
NMI	43	66	2.20
NMI + NH	33	51	1.50
NH	10	18	0.41

The *p*H dependence of the reactivation at 25.0° with hydroxylamine (0.05 *M*) for 15 minutes, nicotinhydroxamic acid methiodide (0.05 *M*) for 15 minutes, nicotinhydroxamic acid (0.05 *M*) for three hours, and acethydroxamic acid (0.20 *M*) for three hours are presented in Fig. 2. The values given are

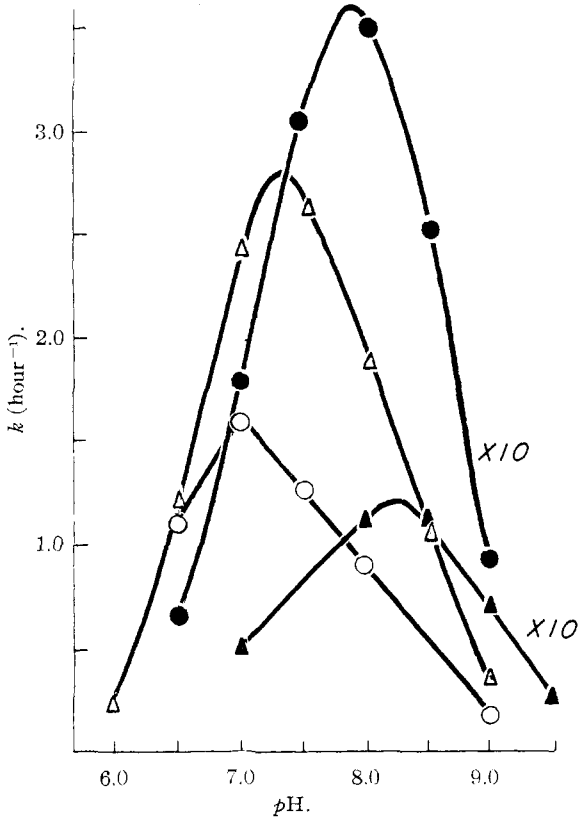


Fig. 2.—Reactivation of TEPP inhibited enzyme at 25° as a function of *p*H: △, NMI, 0.05 *M*; ●, NH × 10, 0.05 *M*; ○, hydroxylamine, 0.05 *M*; ▲, acethydroxamic acid × 10, 0.20 *M*.

the first-order rate constants calculated from the equation

$$k = -\frac{2.30}{t} \log \text{EP}$$

where EP is the fraction of inhibited enzyme at time t (hours). On this scale $k = 0.7$ corresponds to 50% reactivation in one hour. The acethydroxamic acid concentration was made higher than the others in order to obtain sufficiently high reactivations. The values for NH and acethydroxamic acid are multiplied by 10 so as to appear on the same plot. The *p*H optima follow the order of the pK_a's of the reactivators (Table II).

TABLE II

	*p*Ka	*p*H optimum
Hydroxylamine	6.22	7.0
NMI	6.48	7.3
NH	8.26	7.8
Acethydroxamic acid	9.43	8.3

The values presented as the pK_a's for the acids are the *p*H values of half neutralized 0.01 *M* solutions as measured with a Beckman model G glass electrode *p*H meter at 25°. They are probably within a few hundredths of a *p*H unit of the correct values. The pK_a for acethydroxamic acid has been previously reported as 7.55[8] which differs very much from our value.

The *p*H dependence of reactivation by pyridine (3 hours, 0.05 *M*) and choline (3 hours, 0.3 *M*) are given in Fig. 3 for comparison.

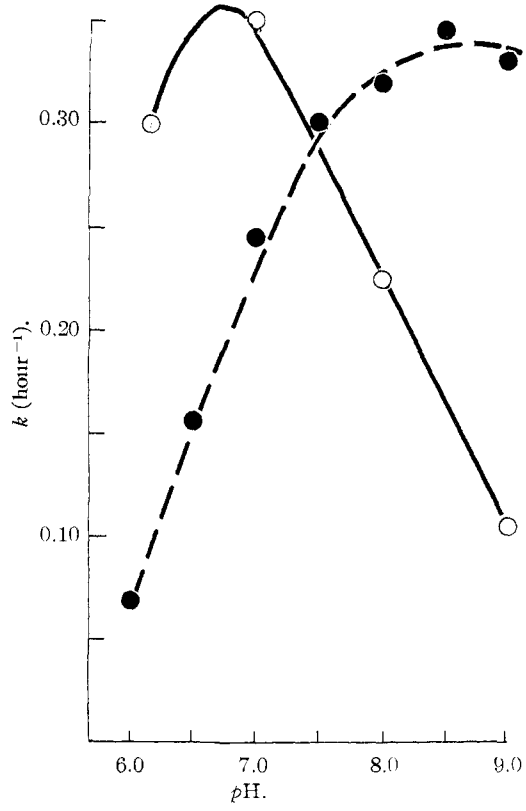


Fig. 3.—Reactivation of TEPP inhibited enzyme at 25° as a function of *p*H: ○, pyridine, 0.05 *M*; ●, choline, 0.30 *M*.

Reactivation of DFP Inhibition.—The rate and maximum extent of reactivation attained when DFP is inhibitor is highly dependent upon the conditions which prevail during the inhibition and during storage prior to the reactivation. The reactivation does not always follow first-order kinetics and complete reactivation can be obtained only under special conditions. The studies have not been extensive enough to elucidate the nature

(8) T. W. Taylor and W. Baker, in "Sidgwick's Organic Chemistry of Nitrogen," Oxford at the Clarendon Press, 1949.

of the deviation from simple inhibition, but it has been observed that the highest rates of reactivation and the highest extents of reactivation occur when the temperatures during inhibition and prior to reactivation are very low. Storage before reactivation, especially if not under refrigeration, greatly diminishes the reactivations.

The results of an experiment in which inhibition with 2 γ DFP/ml. occurred in one hour at room temperature is given in Table III. The results are fairly characteristic. The *p*H optima are evidently about the same as found in TEPP reactivation. Nicotinhydroxamic acid and N-methylnicotinhydroxamic acid at their *p*H optima (*p*H 8 and *p*H 7, respectively) are of comparable activity. In this respect DFP reactivation differs markedly from TEPP reactivation. That higher ultimate reactivations are more easily obtained with the tertiary hydroxamic acid is quite typical. If the incubated stock is first stored for 24 hours at 25° and then treated with nicotinhydroxamic acid methiodide for 4 hours, no reactivation is obtained. Storage at 5° for 24 hours is less unfavorable but still only 12% reactivation is obtained which is only about half of that found when the reactivator is added immediately. It was consistently observed that the reactivation potential declines on storage.

TABLE III

REACTIVATION OF DFP INHIBITED ENZYME AS A FUNCTION OF *p*H

	Hours	6.5	7.0	*p*H 7.5	8.0	8.5
NMI	4	14	21	23	18	
	24		35			
	96		33			
NH	4		12	20	25	20
	24				72	
	96				48	

In another case 0.2 ml. of enzyme was inhibited at room temperature as before, but with 1 γ DFP/ml., and then brought to 0.4 ml. with 0.2 *M* nicotinhydroxamic acid and kept at room temperature for one hour to destroy much of the DFP by direct reaction with the hydroxamic acid (unpublished observations). Finally this solution was diluted 20 times and used as stock. The inhibition was 83% and did not change after 24 hours of storage, indicating that DFP was reduced to an inactive level, but the reactivating potential as measured by 4 hours reactivation with nicotinhydroxamic acid methiodide still went down greatly on storage. The "four hour" reactivations obtained immediately were higher than without the removal of excess DFP, 28 and 30% with NMI at *p*H 7 and NH at *p*H 8, respectively.

In another experiment (Table IV) the enzyme was inhibited at about 5° with 2 γ DFP/ml. for one hour and then diluted 20-fold. The 4-hour reactivations were still higher. The DFP concentration again was relatively high in the inhibited stock and both the activity and reactivating potential of the inhibited stock declined on storage at 5° and at 25°. In order to lower the DFP concentration further, enzyme was inhibited as above but diluted 200 times before reactivation. Even at this high dilution DFP was active and inhibition continued on storage. High reactivations were obtained both at 5° and at 25° (Table V). The reactivation kinetics at 5° is clearly very different from 1st order.

TABLE IV

REACTIVATION OF DFP INHIBITED ENZYME AT 25°

	*p*H	4 hr.	24 hr.
NMI	7	34	45
NH	8	41	62
Hydroxylamine	7	14	10

TABLE V

REACTIVATION OF DFP INHIBITED ENZYME

	Temp., °C.	Hours 4	24	48	288	480
NMI	5	0	26	21	56	50
	25	37	45	53	32	
NH	5	0	50	53	90	100
	25	34	90	100		

In yet another technique the enzyme was diluted 5-fold with gelatine–phosphate buffer and then inhibited for 1 hour at 5° with 1 γ/ml DFP. This solution (inhibition 80%) was reactivated without dilution. After 4 hours at 25° 38% reactivation was obtained with nicotinhydroxamic acid methiodide and 96% after 24 hours. These results were reported in a "Communication to the Editor,"[9] but an error was made in reporting 50% reactivation instead of 38% after 4 hours.

In all cases the percentage reactivation was computed by subtracting the activity of the inhibited stock, at the time the reactivators were added, from the activity of the uninhibited enzyme and from the reactivated enzyme. In the error referred to above, the activity of the inhibited enzyme at 4 hours (0%) was used in the computation instead of the value after one hour (20%), the time when the reactivator was added. There is in fact a question which value should be used when there are large differences between the two, but since the reactivators can react directly with DFP and some probably can also protect the unphosphorylated active sites, it is probably best to use the value at the time the reactivators are added.

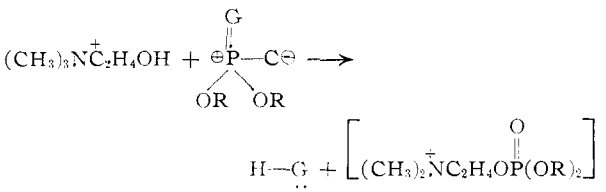
The various results which are obtained under the different conditions described indicate that the inhibition is complicated by secondary effects and that therefore the comparative figures for different substrates and for different *p*H are probably only of a semi-quantitative nature when considered as a measure of the dephosphorylation of the active site. For example, one might argue that the percentage reactivation should be based upon the maximum reactivation ultimately attained rather than upon 100%.[7] On this basis NMI would appear to be rather faster than NH. These complications are being studied with a view to understanding their molecular basis and toward finding experimental conditions under which the DFP inhibition is of a simple type.

Discussion

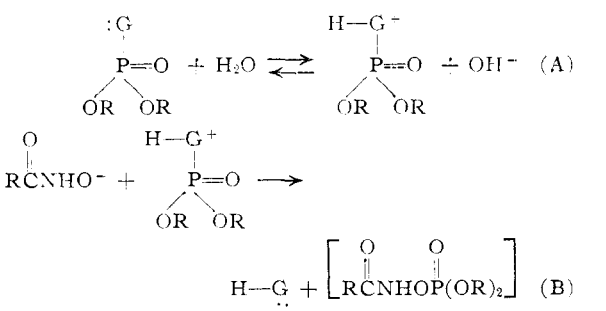
The *p*H dependence of reactivation is fairly complicated, containing contributions from a num-

(9) I. B. Wilson and E. K. Meislich, THIS JOURNAL, **75**, 4628 (1953).

ber of sources, some of which pertain to the enzyme and others to the reactivators. Those of prominence arising from the *enzyme* are (1) the discharging of the anionic site,[10] which is most pronounced below *p*H 7 and affects only those compounds which interact electrostatically with this site, *i.e.*, cations. Interaction with neutral molecules declines much more slowly in acid media. This is well established from studies with the normal enzyme. As one illustration we might note that while the (competitive) inhibition of the normal enzyme by the ammonium ions of dimethylaminoethanol declines in acid media, that of the uncharged analog isoamyl alcohol remains substantially the same (unpublished observations of I.B.W.). (2) The dissociation of the acidic group in the esteratic site in alkaline *p*H. This effect might also have been anticipated from the enzyme theory since a proton is lost from the acidic group during the phosphorylation of the basic group (equation 1). But we had formulated the reactivation with choline, for example, as



which involves a combined acid–base attack by the H and O atoms, respectively, of choline. In this formulation the acid group of the esteratic site was restored by a hydrogen atom from the reactivator. Hydroxylamine reactivation was similarly depicted. In the case of pyridine reactivation (Fig. 3), the pyridine evidently could not supply the proton, and so it was necessary to assume that it was supplied by the solvent. Only in such a case then was it expected that the reactivation would fall at higher *p*H. Evidently from the data of Fig. 2, hydroxylamine and the hydroxamic acids behave similarly and it appears that the proton is obtained from the solvent. The reactivations should then be written for the hydroxamic acids as follows (the hydroxamate ion is the reactivator)



The pK_a for reaction (A) appears to be around 8.2—this is interpreted as the acidity constant for the acid group of the esteratic site when the basic group is phosphorylated. The pK_a for this group is about 10.5 when the enzyme is in the form of a complex with acetylcholine and probably somewhat higher in the free enzyme. The large increase in acidity is appropriate for the phosphorylation of a neighboring group (G is not a single atom but a structure containing several atoms) with which the acidic group is probably in electronic communication, as for example in imidazole, and can be considered as evidence of electronic fluidity between the acidic and basic groups.

Interestingly enough choline apparently does supply its own proton for the rate of reactivation does not decline in an alkaline medium (Fig. 3). The decline in the acid direction is caused by discharge of the anionic site. Pyridine which cannot interact electrostatically with the anionic site shows practically no decline toward the acid direction.

In comparing the quaternary hydroxamic acid—nicotinhydroxamic acid methiodide, with the tertiary nicotinhydroxamic acid with regard to TEPP reactivation it is apparent that even in alkaline *p*H the quaternary is some five times more active. Now the nucleophilicity of the anions toward hydrogen is of course given by their basicity. The nucleophilicity toward phosphorus might be expected to follow the same order and we might therefore on this basis have expected the tertiary compound to be superior. Studies on the direct reaction of DFP with these compounds showed that NH reacted almost twice as fast as NMI. That despite this consideration the quaternary is far more active in reactivating the enzyme demonstrates the importance of the anionic site in promoting the reactivation. Evidently as anticipated, the properties of the anionic site in the catalytic mechanism, albeit weaker, carry over in the diethylphosphoryl enzyme and can serve to promote the activity of a reactivator. In the present case, the two hydroxamic acids differ in electrical charge; the tertiary compound bears no cationic charge in the *p*H range considered. It would be expected, therefore, that there would be little or no interaction of the tertiary compound with the anionic site.

The distribution of the reactivator among acid and base forms contributes to the reactions since the acidity constants of the reactivators fall within the *p*H range studied. In the case of hydroxylamine it is of course anticipated that the electrically neutral molecule rather than the conjugate acid is the reacting species. Similarly, one might anticipate that the hydroxamate ions (dissociated forms of the hydroxamic acids) would be the more active forms. It will be noted that the activity of these compounds rises to a maximum value as the solution is made more alkaline and that the optimum *p*H shifts with the pK_a of the acid.

The decline in stronger alkali has already been discussed. From what has been said it is apparent that the precise form of these curves is governed by a number of factors including the observation that the value of k is not directly proportional to the concentration of reactivator. Consequently we have not attempted to fit the data with a theoretical curve, but it is nevertheless apparent that the base forms of the reactivators are more active than their conjugate acids.

When we turn now to consider the reactivation of DFP inhibited enzyme, it is apparent that the data

(10) F. Bergmann and A. Shimoni, *Biochim. et Biophys. Acta*, **9**, 473 (1952).

do not support any view that the activity of NMI is promoted by the anionic site. The initial rates of reactivation by NMI and NH appear to be about equal at their optimum *p*H but slightly greater for NH if both are compared at *p*H 8.0. The rate of reactivation of DFP inhibited cholinesterase by NH at 3 hours is only about 30% of TEPP reactivation which can be readily understood in terms of shielding of the phosphorus atom by the more extensive isopropyl groups or in terms of small "chemical" differences. At any rate, the change is quite characteristic of chemical reactions involving isopropyl and ethyl derivatives.[11]

If we accept the comparative data as being essentially correct, is it plausible to conclude that the anionic site is functional toward an N-methylpyridine structure when the esteratic site bears a diethylphosphoryl group, but practically non-functioning when the esteratic site bears a diisopropyl phosphoryl group? Such a conclusion would not seem too difficult if we bear in mind that in the diethylphosphoryl enzyme the affinity of the anionic site toward methylated ammonium ions has been diminished some sixty-fold as compared to the active enzyme and toward the larger pyridinium ions the diminution might be even greater. Evidently, this effect arises from the geometrical closeness of the esteratic and anionic sites whereby the ethyl groups in some configurations physically interfere with the close approach of the substituted ammonium ions and it should not, therefore, be unrealistic to assume that the larger isopropyl groups could further diminish the effectiveness of the anionic site. However, because of the uncertainty in the experimental interpretation of the data, as mentioned before, it is not clear whether there may not be some small promoting effect.

(11) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., New York, N. Y., 1949.

Acknowledgments.—The authors wish to acknowledge the assistance of Miss Carole Quan and to thank Dr. David Nachmansohn for his continuous interest.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF WAYNE UNIVERSITY]

Constitution and Stereochemistry of Samogenin, Markogenin and Mexogenin[1]

BY CARL DJERASSI AND JACK FISHMAN

RECEIVED JANUARY 31, 1955

Employing both degradative and synthetic evidence, samogenin has been shown to be 22a,25a-spirostane-2β,3β-diol (Ia). In view of earlier interrelations, markogenin is now established as the 25b-epimer of samogenin while mexogenin is best defined as 12-ketosamogenin. These three sapogenins are the first examples in the steroid series of naturally occurring *cis*-glycols and mention is made of the consequences of this observation on the current thoughts on the biogenesis of steroidal sapogenins. Several 3,4-dihydroxysapogenins and both 3,4-*seco*-acids of the 5α- and 5β-series have been synthesized and attention is called to the observation that in the 5β-series, attack by peracid or osmium tetroxide on ring A olefins (Δ^2 or Δ^3) proceeds from the front side (β) while rearward approach always is observed with 5α-isomers. A modification of the current sapogenin nomenclature is suggested which defines the configuration at C-25.

The large majority of the known steroidal sapogenins[2] either possess a double bond in the 5,6-position or belong to the 5α(*allo*) series. In fact, only two sapogenins, sarsasapogenin and smilagenin have been shown unequivocally to belong to the 5β(*normal*) series by degradation[3] to pregnane derivatives of established stereochemistry. In 1947, Marker and collaborators[4] recorded the isolation of three new sapogenins, samogenin, texogenin (markogenin[5]) and mexogenin and assigned them to the 5β-series on insufficient evidence.[6] The present paper is concerned with the elucidation of the structure and stereochemistry of these three sapogenins.

Samogenin, first isolated[4] from *Samuela carnerosana* Trel., is the most abundant one and since markogenin (a side chain epimer of samogenin) and mexogenin (x-ketosamogenin) have been interrelated with it,[4,5] most of the structural arguments given below can be applied *ipso facto* to the other two sapogenins. The fallacies in the earlier[4] structure assignment already have been reviewed[1,6] and in particular it has been pointed out that no unequivocal correlation between samogenin and a sapogenin of known structure has been accomplished. A supply of samogenin[7] from the original Marker collection has enabled us to settle most of the outstanding points in the chemistry of these three sapogenins.

Samogenin (Ia) has been shown[4] earlier to be a vicinal dihydroxy sapogenin by oxidation to a dibasic acid V. Conversion of the sapogenin to the dimesylate Ic and treatment with sodium iodide in acetone solution[8] now has furnished an olefin (subsequently proved to possess structure II) which

(1) A preliminary note covering part of this material already has been published (C. Djerassi, J. Fishman and J. A. Moore, *Chemistry & Industry*, 1320 (1954)).

(2) L. F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene," Reinhold Publ. Corp., New York, N. Y., 1949, 3rd Edit., Chapter VIII.

(3) *Inter al.*, R. E. Marker, THIS JOURNAL, **62**, 3350 (1940).

(4) R. E. Marker, R. B. Wagner, P. R. Ulshafer, E. L. Wittbecker, D. P. J. Goldsmith and C. H. Ruof, *ibid.*, **69**, 2167 (1947).

(5) M. E. Wall, C. R. Eddy, S. Serota and R. F. Mininger (*ibid.*, **75**, 4437 (1953)) isolated a dihydroxysapogenin with presumably the same structure as "texogenin" but with completely different physical constants. The substance was renamed "markogenin" and this term is being used throughout the present paper.

(6) *Cf.* C. Djerassi and R. Ehrlich, *J. Org. Chem.*, **19**, 1351 (1954).

(7) We are greatly indebted to Dr. J. A. Moore of Parke Davis and Co., and to Dr. R. B. Wagner, formerly of the Pennsylvania State College, for donating this material.

(8) This procedure first was introduced into sapogenin chemistry by N. L. Wendler, H. L. Slates and M. Tishler (THIS JOURNAL, **74**, 4894 (1952)) in the case of manogenin.