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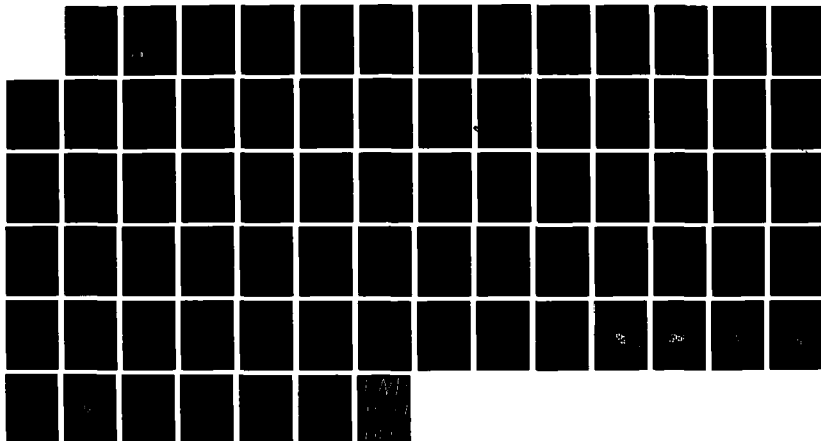
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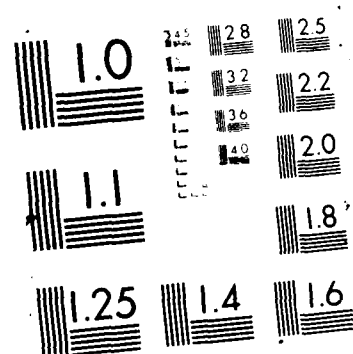
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Quantum Chemical and Physicochemical Studies of Oximes
(Prophylactics Against and Reactivators of Phosphorylated AChE)

ANNUAL REPORT

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Walter S. Koski, Ph.D., Co-Principal Investigator

25 October 1984

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We have carried out high quality three-dimensional ab-initio quantum chemical calculations for these oximes as a function of geometry (CNOH dihedral angles). This gives us calculated conformational energy profiles. All possible conformations within a certain number of kcal (on the order of 12 kcal/mole) from the absolute energy minimum may be possible to achieve in solution or under the influence of an active site, and thus may be significant pharmacologically. The equilibrium geometry of a species is where the total energy is at a minimum. Our results of quantum chemical calculated total energy vs. dihedral angle for syn and anti 2-PA showed that there were multiple maxima and minima in these compounds--thus, many points must be computed. [For these ab-initio quantum chemical calculations, we are using our own fast ab-initio programs which also incorporate optional desirable computational strategies: MODPOT - effective core model potentials which allow calculation of the valence electrons only explicitly, yet accurately; and VRDDO - (variable retention of diatomic differential overlap), an efficient charge conserving integral prescreening evaluation which tells reliably whether an integral will be large enough to be significant or so small that it should not be calculated--which cut down the computer time by at least an order of magnitude even for a compound with one aromatic ring and a side group like 2-PA and considerably more for larger molecules; and MERGE, to save invariant skeletal integrals and reuse them. This MERGE technique is especially effective when the geometry changes and also saves orders of magnitude of computer time when series of related compounds are being studied.

As we calculated the quantum chemical wave functions of those oximes, we generated electrostatic molecular potential contour (EMPC) maps of each molecule.

So far, we have carried out such calculations for syn and anti 2-PA (2-pyridine aldoxime), syn and anti 4-PA (4-pyridine aldoxime), syn and anti 2-PAM (2-pyridinium aldoxime), syn and anti 4-PAM (4-pyridinium aldoxime), HI-6 (a bis-pyridium monooxime) and imidazole, protonated imidazole, and BHH-1 (a methyl imidazolium aldoxime).

We have also carried out similar ab-initio quantum chemical calculations for and generated EMPC maps around deprotonated 2-PAM (the oximate anion suggested as possibly being the active species).

The three dimensional isopotential contour EMPC map for the deprotonated 2-PAM showed vividly a most significant finding. The three dimensional isopotential contour EMPC map of deprotonated 2-PAM indicated clearly that for this species there is a strongly positive region and a strongly negative region. This has significant implications for several aspects of the use of oxime reactivators.

In addition to our theoretical work, we have been carrying out experimental determinations of the lipophilicities (their tendency to partition from aqueous to lipid phases) of these oxime reactivators. We have carried out these lipophilicity determinations, first with our microelectrometric technique. It turns out that neither these quaternary oximes nor their deprotonated species are very lipophilic. However, since there have been reports that some oxime reactivators are effective at low concentrations, we have set up and are also using a more sensitive UV spectrophotometric procedure to establish quantitatively and directly just how much of these compounds is partitioning into the lipid phase.

We have studied the following compounds: Microelectrometric technique: 2-PAM, HI-6, TMB-4, TOXOGONIN, BHH-1, BHH-3, BHH-4, BHH-5, BHH-8; UV spectrophotometric technique: 2-PAM, BHH-5, BHH-13, BHH-14, BHH-15.

SUMMARY

We have carried out high quality three-dimensional ab-initio quantum chemical calculations for these oximes as a function of geometry (CNOH dihedral angles). This gives us calculated conformational energy profiles. All possible conformations within a certain number of kcal (on the order of 12 kcal/mole) from the absolute energy minimum may be possible to achieve in solution or under the influence of an active site, and this may be significant pharmacologically. The equilibrium geometry of a species is where the total energy is at a minimum. Our results of quantum chemical calculated total energy vs. dihedral angle for syn and anti 2-PA showed that there were multiple maxima and minima in these compounds--thus, many points must be computed. [For these ab-initio quantum chemical calculations, we are using our own fast ab-initio programs which also incorporate optional desirable computational strategies: MODPOT - effective core model potentials which allow calculation of the valence electrons only explicitly, yet accurately; and VRDDO - (variable retention of diatomic differential overlap), an efficient charge conserving integral prescreening evaluation which tells reliably whether an integral will be large enough to be significant or so small that it should not be calculated--which cut down the computer time by at least an order of magnitude for a compound with one aromatic ring and a side group like 2-PA; and MERGE, to save invariant skeletal integrals and reuse them. This MERGE technique is especially effective when the geometry changes and also saves orders of magnitude of computer time when series of related compounds are being studied.

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FOREWORD

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I. Statement of the Problem

The ultimate goal of our interdisciplinary research under this Army contract is to delineate the stereoelectronic and physicochemical requisites for oximes to be effective reactivators of acetylcholinesterase (AChE) inhibited by organophosphorus compounds. This is being done by carrying out theoretical high quality three-dimensional ab-initio quantum chemical calculations on the oximes, and from these wave functions generating three-dimensional electrostatic molecular potential contour (EMPC) maps around these oximes as well as experimental determinations of their lipophilicities.

The major objectives of our research as described in the scope of work of this contract are as follows:

- a. To carry out high quality three dimensional quantum chemical calculations and generate electrostatic molecular potential contour maps of the effective oximes 2-PAM*, HI-6* and at least one other oxime (and for various other effective oximes if time and funds permit) to delineate the stereoelectronic requisites necessary for effective action.
- b. To carry out similar quantum chemical and EMPC map calculations for oximes reacting with models of phosphorylated AChE and of the oximes themselves reacting with models of AChE.
- c. To formulate how the quantum chemical and EMPC map information would be used to set up computer based structure-activity prediction program for the efficacy of oximes.
- d. To use the quantum chemical and EMPC map information to set up a program for lead-directed synthesis of new, more effective oximes.
- e. To carry out careful experimental measurements of the pK_a 's of the oximes (including temperature dependence and deconvoluting overlapping pK_a 's). Also, to carry out experimental measurements of the pH dependence of the lipophilicities (drug distribution coefficients) of oxime reactivators (including 2-PAM, HI-6) using contractor's sensitive novel micro-electrometric titration technique to relate this to observed in vivo activity to be carried out elsewhere in accordance with the direction of the COTR; further, to use these experimental measurements as a guide to accurate theoretical prediction of the lipophilic drug distribution coefficients.

* See Figure 1 of this report for sketches of the molecular structure.

II. Background

EMPC maps around a variety of drugs for various different therapies (such as mentioned above in parts a-d) have previously been shown to relate to the effectivities of such drugs. About seven years ago, by invitation, Dr. Kaufman presented lectures at thirteen different pharmaceutical houses both here and abroad, discussing our successes in correlation of quantum chemical indices and EMPC maps with pharmacological activity and in drug design prior to syntheses with this combination of theoretical techniques. A number of pharmaceutical companies have now set up theoretical groups attempting to use these techniques for drug design.

The following discussion indicates briefly how our calculated quantum chemical indices and EMPC maps are relevant to the question of reactivation by oximes of AChE inhibited by organophosphorus compounds.

Previous experimental in-vitro pharmacology studies have shown that effectivity of the reactivation in-vitro can be described by two terms: affinity, K_r , (due to complex formation of the reactivator with the phosphorylated AChE) and chemical reactivity, k_r . The combination of the two terms is $k_r K_r$, a measure which has been shown to correlate with the effectivity in-vivo.¹

This two-component mechanism of reactivation of inhibited AChE has conceptual similarities to the first two steps in the mechanism of inhibition of AChE by phosphorus compounds, namely, formation of the organophosphorus-AChE complex and then a chemical step which involves breaking a P-X bond and phosphorylation of the AChE. We have previously shown that the first step in inhibition of AChE (the complex formation) is related to the EMPC maps around the organophosphorus compounds.² We then showed that the second step in the inhibition of AChE (phosphorylation of the AChE) is related to a theoretical quantum chemical index of the P-X bond.³

Thus, by analogy, for the present problem of reactivation by oximes of inhibited AChE, we likewise expect the first step of complex formation of the oxime reactivator to the phosphorylated AChE to be related to the EMPC map around the oximes. The second step of chemical reactivity is expected to be related to calculated quantum chemical indices and to the EMPC maps. [The oximates--deprotonated oximes--have been suggested by experimentalists probably to be the relevant species.]

There is Army interest in developing oxime reactivators which will be more effective in the central nervous system (CNS) than presently used oximes. The more lipophilic a molecule is (i.e., the greater its propensity to partition from aqueous to lipid phases) the more effective it is in the CNS (under most usual physiological conditions).

III. Approach to the Problem - Theoretical

Thus, the first step of our theoretical research has been systematically to carry out ab-initio quantum chemical calculations around prototype oximes and from wave functions to generate the EMPC maps and other theoretical indices.

A. Pyridinium oximes and bis-pyridinium oximes

Due to a lack of experimental molecular crystal structures for almost all of these oximes, this problem of ab-initio quantum chemical calculations of these oximes is very time consuming. There was only a very old crystal structure for syn 2-PAM⁴, which did not place the H atoms. There had been more recent experimental crystal structure determinations for syn⁵ and anti⁶ 4-PA (4-pyridine aldoxime). Thus, in order to get appropriate starting structures for syn and anti 2-PAM, we first carried out quantum chemical calculations on syn and anti 2-PA (putting the aldoxime side chain either syn or anti in the 2-position of pyridine). We then carried out quantum chemical calculations on syn and anti 2-PA as a function of geometry (CNOH dihedral angle). This gives us a calculated conformational energy profile. All possible conformations within a certain number of kcal (on the order of 12 kcal/mole) from the absolute energy minimum may be possible to achieve in solution or under the influence of an active site, and thus may be significant pharmacologically. We also used differencing techniques to project a minimum which we also checked by calculations. The equilibrium geometry of a species is where the total energy is at a minimum. Our results of quantum chemical calculated total energy vs. dihedral angle for syn and anti 2-PA showed that there were multiple maxima and minima in these compounds--thus, many points must be computed. [For these ab-initio quantum chemical calculations, we are using our own fast ab-initio programs, which also incorporate optional desirable computational strategies:^{7,8,9}

MODPOT - effective core model potentials which allow calculation of the valence electrons only explicitly, but accurately;

VRDDO - (variable retention of diatomic differential overlap), an efficient charge conserving integral prescreening evaluation which tells reliably whether an integral will be large enough to be significant or so small that it should not be calculated

which cut down the computer time by at least an order of magnitude even for a compound with one aromatic ring and a side group like 2-PA and considerably more for larger molecules.

MERGE - to save invariant skeletal integrals and reuse them. (This MERGE technique is especially effective when geometry changes and also saves orders of magnitude of computer time when series of related compounds are being studied).

From these calculated ab-initio MODPOT/VRDDO/MERGE wave functions, we calculate the gross atomic populations on the atoms (the atomic charge distribution in the molecule) and other pertinent theoretical indices.]

Thus, to carry out the quantum chemical calculations on 2-PAM, we meshed together pieces of experimental crystal structures for various fragments and then carried out the quantum chemical calculations as a function of varying the geometry (CNOH dihedral angle) of both the syn and anti 2-PAM. For an initial geometry for quantum chemical calculations on syn and anti 2-PAM, we then ansatzed a CH_3 group onto the pyridine ring of syn or anti 2-PA (using the experimental crystal of a methyl pyridinium as a guide).

We carried out the quantum chemical calculations on syn and anti 2-PAM as a function of geometry (CNOH dihedral angle). Again, our results of total calculated energy vs. dihedral angle showed that syn and anti 2-PAM also have multiple maxima and minima (Figure 2).

(During the course of our investigations, a new crystal structure determination of 2-PAM was reported.¹⁰ We will be running more ab-initio MODPOT/VRDDO/MERGE calculations on 2-PAM using the new crystal structure as a starting point. However, the crystal structure geometries are merely the starting geometries for our quantum chemical calculations. The calculations are then run as a function of various geometry variations.)

We carried out similar quantum chemical calculations for syn and anti 4-PA and syn and anti 4-PAM as a function of geometry.

For the starting geometries of syn and anti 4-PA, we used the experimentally measured x-ray crystal structures^{5,6} and then also calculated the total energies as a function of CNOH dihedral angle. Again, there were multiple maxima and minima in the energy function of geometry. We carried out the ab-initio MODPOT/VRDDO/MERGE calculations for rotamers of syn and anti 4-PAM. For starting geometries we used the x-ray crystal geometries of syn and anti 4-PA^{5,6} plus a methyl group on the pyridine nitrogen as on the methylpyridinium ion. From the plot of total energy vs. CNOH dihedral angle for syn and anti 4-PAM (Figure 3) it is apparent that again there are multiple maxima and minima.

This aspect of research gave us knowledge about the conformational complexities of mono pyridinealdoxime molecules. As we calculated the quantum chemical wave functions of those oximes, we generated electrostatic molecular potential contour (EMPC) maps in three dimensions around the lowest energy conformation of each molecule. From these EMPC maps we generated three dimensional isopotential EMPC maps around each molecule.

The EMPC maps are calculated point by point for planes through and around the molecules. We customarily calculate the potentials for 1000 points per plane and from 15 to 20 planes per molecule for molecules the size of 2-PAM. From these points we calculate three dimensional isopotential EMPC maps for the molecules. All the three dimensional EMPC points and isopotential EMPC maps are stored in the computer for future matching of one oxime against another.

[Color copies of these three dimensional isopotential EMPC maps were included with our Quarterly Progress Reports (QPR's).] Black and white copies of these three dimensional isopotential EMPC maps are included in this report (Figure 4, syn 2-PAM; Figure 5, anti 2-PAM; Figure 6, syn 4-PAM; Figure 7, anti 4-PAM).

It had been suggested by the developers of the pyridine aldoximes that the deprotonated anions (the oximates) of these compounds were the effective species for the reactivation of the phosphorylated (inhibited) AChE.

Hence we initiated quantum chemical studies of the deprotonated (zwitterionic) forms of 2-PAM (the oximate species). Crystalline 2-PAM had been found to be syn.

However, it had been suggested by Gray that¹¹ in the deprotonated species, the syn-anti distinction is destroyed.

Convergence is strongly dependent on the molecular geometry. We had to carry out quantum chemical calculations at a large number of geometry points for deprotonated 2-PAM.

We had no difficulty obtaining a converged structure for the deprotonated syn 2-PAM anion in the anti-configuration. No converged syn-anions have been obtained to date. In an effort to get a stable structure for the 2-syn anion, we have let the syn structure relax in a number of ways:

From the converged wave functions, deprotonated (zwitterionic) anti 2-PAM we generated the EMPC maps. [A color copy of the three dimensional isopotential EMPC map around deprotonated 2-PAM was included in our QPR 2.] A black and white copy of this three-dimensional isopotential EMPC map is included in this report (Figure 8).

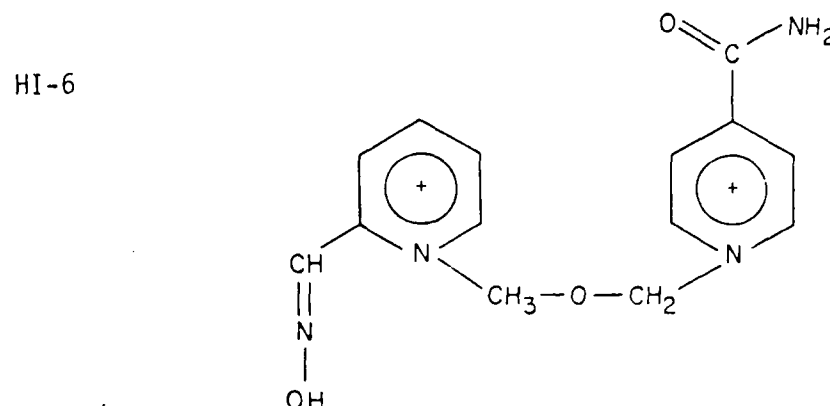
We are continuing to investigate the quantum chemical calculations on deprotonated 2-PAM with other geometry variations (bond lengths, bond angles, dihedral angles, etc.).

* See Figure 1 of this report for sketches of the molecular structures.

We also initiated quantum chemical studies of the deprotonated (zwitterionic) forms of both syn and anti 4-PAM. We have let the structure relax in a number of ways. No converged anions of either of the 4-PAM's have been obtained to date.

It is apparent from our quantum chemical studies on the deprotonated 2-PAM anions (where after many attempts at different geometries we were able finally to get a converged SCF wave function for the deprotonated anti 2-PAM anion) and our quantum chemical studies on the deprotonated 4-PAM anions in the third quarter that the convergence of these species is very strongly dependent on the geometry chosen. A larger number of different geometries must be investigated by explicit quantum chemical calculations. We are continuing to investigate this problem.

While we were continuing our quantum chemical and EMPC studies on 2-PAM and 4-PAM (and their deprotonated anions), we initiated studies on HI-6*, a bis-pyridinium monooxime (which is also an effective oxime reactivator of phosphorylated AChE) to gain insight into the quantum chemical indices and EMPC maps of bis-pyridinium oximes.



For a starting geometry for the left ring system, we used the geometry of 2-PAM as optimized in our previous studies; for the right ring system, we used the geometry from the experimental crystal structure of nicotinic acid,¹² and we used the ether bridge from the crystal structure of dimethyl ether. We are investigating various rotamers with relation to the COCN dihedral angle.

We generated three dimensional isopotential contour EMPC maps around one of the rotamers of HI-6 (a bis-pyridinium monooxime). (A color copy of the isopotential EMPC map around HI-6 was included on our QPR 3. Black and white isopotential EMPC maps for HI-6 are shown in this annual report, Figure 9.)

We will be comparing these quantum chemical indices and isopotential contour EMPC maps of the pertinent species of HI-6 with those of 2-PAM to compare similarities and/or differences. (During the course of our theoretical¹³ investigations, a crystal structure determination of HI-6 was reported. We will be carrying out our theoretical investigations of HI-6 also using this crystal structure. However, we use the experimental crystal structure, if available, only as a starting point. We then carry out the quantum chemical calculations as a function of geometry variations to establish the conformational energy profiles of the molecules.)

We are continuing our quantum chemical and EMPC map calculations on these pyridinium oximes.

* See Figure 1 of this report for sketches of the molecular structures.

B. Imidazolium oximes BHH compounds

In June of 1984, Dr. Lily Tang, the Army Contract Technical Monitor on this project called up and requested by phone that we see if our techniques could shed insight on the relative "protective effects in animals toward organic phosphate poisoning" [i.e. against inhibition of acetylcholinesterase (AChE) by organic phosphates] of certain of the quaternary methylimidazolium oxime derivatives being synthesized by Dr. Scott Bedford at Stanford Research Institute¹⁴ (mentioned in Dr. Tang's letter to us dated June 21, 1984). The compounds listed in Dr. Tang's letter as "protective" when used together with atropine were

Code numbers in Dr. Tang's letter

BK64397
BK64404
BK64413
BK64422
BK64431

Code numbers as assigned to us by Col. Pick our original Army COTR

BHH-0013
BHH-0014
BHH-0015
BHH-0016
BHH-0017

The relative protectivities as confirmed in our subsequent telephone conversation with Dr. Tang were

Compound

Dose (i.m.)

BK64397	BHH-13	1.8 mg/Kg
BK64404	BHH-14	7.2 mg/Kg
BK64413	BHH-15	16.7 mg/Kg
BK64422	BHH-16	21.9 mg/Kg
BK64431	BHH-17	33.3 mg/Kg

Dr. Tang also asked if our technique could set up theoretical stereoelectronic requisites which could help distinguish between BHH compounds which had effectivity as reactivators of inhibited phosphorylated AChE and those which had "protective" effects.

This was a major change in our research plans under this Army contract and also greatly increased the amount of theoretical research to be done.

To be responsive to Dr. Tang's request we turned our attention to considering this problem of the quaternary methylimidazolium oximes. There were several aspects which had to be considered:

1. These particular BHH "protective" compounds were larger and had more conformational flexibility than the oximes which were agreed upon jointly by the Army Medical Research and Development Command and by us in the scope of work of our contract for quantum chemical investigations. These BHH compounds also increased greatly the number of oximes to be investigated theoretically.

To calculate BHH compounds necessitated our decision to devote our immediate attention to expediting our changeover to the new vector supercomputers for quantum chemical calculations. In order to transmit the large data files from the calculations back to our labs at the Johns Hopkins University, we are having to establish and implement asynchronous error detecting, error correcting protocols. Which machine will be used for which particular problems will depend on the core memory (virtual memory, if any) and disc space available at any particular time. This is important when molecules start to get large--of the size of some of the BHH molecules.

2. We initiated ab-initio MODPOT/VRDDO quantum chemical calculations and electrostatic molecular potential contour (EMPC) map generation on the quaternary methylimidazolium oxime derivatives.

Since these quantum chemical calculations must be carried out for several such BHH derivatives and we will be using the MERGE technique, we are carrying out these quantum chemical calculations systematically starting with the parent compounds. To date we have carried out the theoretical calculations for imidazole, imidazolium (protonated imidazole) and BHH-1.

BHH-1 has been reported by Dr. Bedford of SRI to be a good reactivator in-vitro against eel AChE inhibited by EPMP (ethylparanitrophenyl methyl phosphonate) or by GD (soman).¹⁴

For imidazole itself there was a crystal structure geometry to use as a starting point.¹⁵ The quantum chemical and EMPC map calculations on imidazole also have an intrinsic interest with respect to reactivation of phosphorylated AChE because very early experimental reports indicated that the inhibited enzyme can be dephosphorylated and thus reactivated by nucleophilic reagents such as imidazole, pyridine and hydroxylamine^{15,16} (reactivation by these reagents is, however, rather slow, so that high concentrations are required^{16,17}).

We also carried out ab-initio MODPOT/VRDDO calculations for imidazolium (protonated imidazole) and generated the EMPC maps. There was also a crystal structure for imidazolium to use as a starting geometry.¹⁸

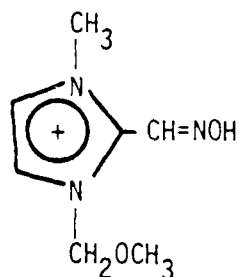
There is no experimental crystal structure for BHH-1. Thus for a starting geometry we constructed a hybrid structure meshing together an N-methyl imidazole fragment (for which there was a crystal structure¹⁹) with a syn aldoxime side chain (from the crystal structure of syn 4-pyridinealdoxime⁵) and with a -CH₂-O-CH₃ side chain on the other N (using the crystal structure parameters from dimethyl ether).

We carried out ab-initio MODPOT/VRDDO calculations for BHH-1.

[See Figure next page]

* See Figure 1 of this report for sketches of the molecular structures.

BHH-1



We are continuing the calculations as a function of geometry and dihedral angles. We generated a three dimensional isopotential EMPC map around one of the rotamers of BHH-1 (Figure 10).

We plan to compare the EMPC maps for 2-PAM and for BHH-1 (and their deprotonated species) to identify similarities [or possibly differences (while since both are good reactivators, these compounds have somewhat different reactivating properties)] between the stereoelectronic characteristics of these two molecules. We plan to continue carrying out calculations for these BHH quaternary methylimidazolium oxime compounds to the extent our Army contract resources permit.

3. Possible mechanisms contributing to protective actions in-vivo of certain of these BHH oximes

At our request, Dr. Tang got Dr. Bedford to send us his technical reports on the BHH quaternary methylimidazolium oximes. We received from him a preprint manuscript.¹⁴ We examined this manuscript carefully. While this manuscript contained data on the reactivation in-vivo of phosphorylated AChE by these BHH compounds there was no mention of the protective in-vivo animal experiments to which Dr. Tang's letter had referred. However, upon reading that preprint manuscript, it appeared that the majority of BHH compounds which were effective reactivators were different from those which were "protective".

Since there was no direct experimental data in Dr. Bedford's report which could shed insight into the mechanistic aspects of the protective effect of the BHH oximes, it was necessary to find out from previous reported experimental results what mechanisms could be contributing to "protective action" of oximes and consider these for the BHH oximes.

* See Figure 1 of this report for sketches of the molecular structures.

We then ascertained, from examining prior experimental research results, that compounds which had prophylactic activity against inhibition of AChE by organophosphorus compounds could do so by several very different mechanisms. (Also prophylactic activity was dependent on the chemical nature of the reactivator and of the particular inhibitor.)

a. Compounds protective against organophosphorus inhibition of AChE can react with the AChE active site (either with the catalytic esteratic site and/or with the neighboring anionic site) and/or with the active site and with the peripheral anionic site, or even just with the peripheral anionic site (if sufficiently bulky).²⁰ Moreover, interaction at the peripheral AChE anionic site can change the conformation of the active AChE site.²⁰

b. Compounds protective against organophosphorus inhibition of AChE can react directly in-vivo with the organophosphorus AChE inhibitor.

HS-6 has been reported to react with soman under physiological conditions by accelerating the hydrolysis of soman before it is able to react with AChE.²¹ HS-6 shows protective effects in-vivo.

c. Certain oxime reactivators, such as 2-PAM, do not prevent an increase in brain AChE after exposure to organophosphorus AChE inhibitors (2-PAM itself prophylactically actually causes a rise in brain AChE) but are protective against symptoms. This has been suggested as being due to the cholinergic receptors becoming tolerant to increased levels of ACh.²²

To date we have not yet received any other information from Dr. Tang or Dr. Bedford on experimental results which could clarify these points concerning the mechanism of protective effects of the BHH compound.

From our analysis there are several specific other experiments which might shed light on some of these points. Thus, in the absence of experimental mechanistic data we plan to look for differences in our calculated quantum chemical EMPC map results between the BHH quaternary methylimidazolium oximes which are effective as reactivators and those which are effective as "protective" agents.

C. Possible steps in mechanism of reactivation of phosphorylated AChE

One of the objectives of our original scope of work was:

- b. To carry out similar quantum chemical and EMPC map calculations for oximes reacting with models of phosphorylated AChE and of the oximes themselves reacting with models of AChE.

Thus, we began to examine what might be appropriate approaches for understanding the mechanism of reactivation of phosphorylated AChE to derive appropriate quantum chemical and other indices.

It had been shown that effectivity of the reactivation in-vitro can be described by two terms: affinity, K_r , (due to complex formation of the reactivator with the phosphorylated AChE) and chemical reactivity, k_r . The

combination of the two terms is a measure which has been shown to correlate with the effectivity in-vivo.¹

The Bedford manuscript did contain K_i and k_r results for the reactivation of EPMP-inhibited eel AChE by the BHH compounds, but the aging of soman-inhibited AChE was sufficiently rapid to preclude reliable measurement of K_i and k_r , so the maximum reactivation of AChE was used by them as a measure of reactivating efficacy.

We plan to examine reactivation of phosphorylated AChE from the above point of view of affinity and of chemical reactivity since our prior experience on quantum chemical calculations and EMPC map generation for the inhibition of AChE by organophosphorus compounds indicated that each of the three separate steps in the inhibition mechanism of AChE was governed by a different theoretical index.

As a measure of the affinity factor, we are examining the size and shape of the positive and negative EMPC contours around the deprotonated 2-PAM (color copies of which were included in our QPR2; black and white copies are included in this annual report, Figure 8) with respect to the inferences that have been made previously for the spatial relation between the esteratic and anionic subsites of the AChE active site.

IV. Results and Discussion of the Results - Theoretical

The three dimensional isopotential contour EMPC map for the deprotonated 2-PAM showed vividly a most significant finding. [A color copy of the isopotential EMPC map around deprotonated 2-PAM was included with our Quarterly Progress Report 2 (QPR 2). A black and white isopotential EMPC map for deprotonated 2-PAM is shown in this Annual Report as Figure 8.] The shape and disposition of isopotential contours of this map indicates vividly that although the deprotonated 2-PAM is an overall neutral species, [since there is a unit (+) charge from the quaternization of the nitrogen and a unit (-) charge from the deprotonation of the oxime H], the isopotential EMPC map indicates this species to be a dipolar ion. The three dimensional isopotential EMPC map of deprotonated 2-PAM indicated clearly that there is a strongly positive region and a strongly negative region. This has significant implications for several aspects of the use of oxime reactivators. First, the size and shape and relative spatial relationship of the positive and negative isopotential portions of these EMPC maps should have a complementary relation to the spatial relationship and electronic characteristics of esteratic and anionic subsites of the active site of AChE. This is particularly important in view of the complete lack of molecular crystal structural information on any AChE. Our isopotential EMPC maps of deprotonated 2-PAM can help shed insight onto the size, shape and stereoelectronic characteristics of the AChE active sites--quantities at present not yet obtained, since so far it has apparently not been possible to crystallize AChE to determine its crystal structure.

Second, the isopotential EMPC map of deprotonated 2-PAM is as unique as a fingerprint in identifying stereoelectronic characteristics that have the proper stereoelectronic complementarity and are favorable for effective reactivation of AChE inhibited by organophosphorus compounds. The corresponding calculated isopotential contour EMPC maps of the oximates of proposed hypothetical new oxime reactivators can be computer-compared to those of deprotonated 2-PAM prior to the synthesis of the new compounds to see if these new compounds have the proper stereoelectronic characteristics to be effective new reactivators.

Third, our three-dimensional isopotential EMPC maps around deprotonated 2-PAM also shed insight into another important aspect of the properties of 2-PAM and deprotonated 2-PAM. 2-PAM itself is a positively charged quaternary species. As such, it is not expected to be, nor is it, very lipophilic. 2-PAM has little tendency to partition from aqueous to lipid.

It had been hoped that when 2-PAM was deprotonated and was then an overall neutral species, that it would have a much greater tendency to partition into lipid.

Our experimental determination of the lipophilicity of 2-PAM as a function of pH indicated that even when the positively charged quaternary 2-PAM was deprotonated, the resulting species was still only very slightly lipophilic. This behavior is commensurate with our EMPC map results, namely, that deprotonated 2-PAM (while overall neutral) has the electronic characteristics of a strongly dipolar ion.

* See Figure 1 of this report for sketches of the molecular structures.

Because 2-PAM would have little tendency to partition through the lipophilic blood-brain barrier under usual physiological conditions, 2-PAM is not an effective reactivator in the CNS of AChE inhibited by organophosphorus compounds.

Our EMPC map of deprotonated 2-PAM indicated that while this species is neutral overall, it appears to be a strongly dipolar ion and this would not be expected to have a significant tendency to partition into lipids. One of the greatest values of quantum chemical calculations and EMPC maps is that these can be calculated for an hypothetical molecule **prior** to its synthesis. If the calculated quantum chemical indices and the EMPC maps indicate that a particular new oxime (or other proposed reactivator) has favorable stereoelectronic characteristics, then it may be desirable to pursue its synthesis and testing. If, on the other hand, the quantum chemical indices and EMPC maps indicate that a molecule does not seem to be favorable (i.e., stereoelectronic characteristics of its EMPC maps indicate that it will not be complementary to the AChE active site or, for CNS reactivation, that its oximate may be a strongly dipolar ion with little tendency to partition into the CNS under specified blood-brain barrier conditions), then the synthesis and testing of the molecule would be less desirable as a candidate for an effective CNS reactivator.

* See Figure 1 of this report for sketches of the molecular structures.

V. Approach to the Problem - Experimental

In addition to our theoretical work, we have been carrying out experimental determinations of the lipophilicities of these oxime reactivators. We have carried out these lipophilicity determinations, first with our microelectrometric technique.²³ It turns out that neither these quaternary oximes nor their deprotonated species are very lipophilic. However, since there have been reports that some oxime reactivators are effective at low concentrations, we have set up and are also using a more sensitive UV spectrophotometric procedure to establish quantitatively and directly just how much of these compounds is partitioning into the lipid phase.

A. Microelectrometric titration technique

This microelectrometric titration technique enables one to determine not only the dissociation constant of compounds which may have more than one pK_a (by deconvolution the overlapping curves) but also the solubilities and oil-water (n-octanol/water) distribution of compounds as a function of pH.

The octanol/water partition coefficients (P) of the unionized compound were determined by microelectrometric titration by measuring the apparent shift in the pK_a value in the presence of octanol. In the aqueous phase, the compound dissociates into its respective ions according to its K_a value:

$$K_a = [A] [H^+] / [HA]$$

Where the dissolved compound is titrated in the presence of finely dispersed octanol, the protonated, undissociated compound distributes between the octanol and water. The removal of HA into the octanol results in a shift in the apparent pK_a , by an amount which depends on the volume (V_o) of octanol and the volume (V_w) of water. From this shift, the partition coefficient (P) can be calculated:

$$P = \frac{V_w}{V_o} [10^{\Delta pK_a} - 1]$$

The partition coefficient (P) is defined as the partitioning of precisely the same species between oil and water, i.e.

$$\frac{[AH]_{oil}}{[AH]_{water}}$$

However, the total drug distribution coefficient (D) at a certain pH, which is pertinent to an understanding of the differences in pharmacological potency, is the ratio:

$$\frac{[AH + A^-]_{oil}}{[AH + A^-]_{water}} = \frac{[AH]_{oil}}{[AH + A^-]_{water}}$$

[Customarily, we determine the lipophilicities of drugs at 37°C (which is physiological body temperature) and sometimes at 20°C which is a fairly standard temperature at which the lipophilicities of various chemicals have been reported. At the request of Dr. Lily Tang, the Army Contract Monitor on this project, we also initiated studies at 30°C for these oximes. However, after initial studies at 30°C on several oximes, fir the oximes were not lipophilic at 37°C, we did not carry out further lipophilicity determinations at 30°C.]

B. UV spectrophotometric technique

The octanol/water distribution coefficients can be determined from the electrometric titration data if the titration is carried out in a water/octanol mixture as described above.

However, this electrometric titration method is not a suitable one if the distribution coefficient is very small. Since this has turned out to be the case with many of the oximes we have under study, we developed the following, more sensitive, spectrophotometric approach using ultraviolet absorption spectroscopy. The concentrations of the oximes in both aqueous and octanol phases were determined by UV absorption. Frequently, in using this technique, the concentration in the aqueous phase is measured and the concentration in the oil phase is determined by difference since the total amount of material used is known. This latter approach was deemed not completely suitable for the oximes since the amount of material in the octanol phase is very small and large errors can result.

The concentrations of the oxime in both phases were determined from UV absorption from the equation

$$A = \epsilon tc$$

where A = optical absorbance, ϵ = molar absorptivity of the solute, t is the path length in cm and c is the molar concentration of the solute.

For each of the compounds studied, the absorption maximum ($\lambda_{\max}$) and ϵ were determined for the different ionic species in aqueous solution at a pH = 1.0 (0% oxime anion), at a pH = 13 (100% oxime anion) and at physiological pH = 7.4. A calibration curve (absorbance vs concentration) was also determined for the octanol solution to establish that Beer's Law is obeyed in the working concentration range. Finally, checks were made of the hydrolytic stability of the oximes at 37°C and pH = 7.4. The hydrolysis of the imidazolium compounds was found to be negligible for times of up to six hours.

The samples were mixed and put into a shaker at 37°C for an appropriate period of time. The two phases were separated by centrifugation and the amount of material in each phase was determined from the calibration curves. In each case, good material balance was obtained and all determinations were carried out in triplicate. The distribution coefficient (D) was determined from the relation

$$D = \frac{C_1}{V_1} \frac{V_2}{C_2}$$

where C_1 = concentration in octanol phase, V_1 = volume of octanol, C_2 = concentration in buffer phase and V_2 = volume of buffer. The partition coefficient was calculated from the relation

$$P = D [1 + 10^{(pH - pK_a)}]$$

where P is partition coefficient and D is drug distribution coefficient.

The procedure is time consuming; however, when small lipophilicities are involved, it is a method of necessity.

C. Oximes investigated

To date we have carried out microelectrometric lipophilicity determinations on:

2-PAM	BHH-1
TMB-4	BHH-3
HI-6	BHH-4
TOXOGONIN	BHH-5
	BHH-3

and UV spectrophotometric lipophilicity determinations on:

2-PAM
BHH-13
BHH-14
BHH-15.

The detailed results have been reported on our QPR's and are included in the next section of this report.

* See Figure 1 of this report for sketches of the molecular structures.

VI. Results and Discussion - Experimental

A. Lipophilicity determinations

1. 2-PAM

a. Microelectrometric technique

The ionization constants and the octanol/water partition coefficients were determined for 2-pyridine aldoxime chloride (2-PAM) at 20°C, 30°, and 37°C.

The following observations were made:

The titration curves (after the subtraction of blank), show only one titratable group, i.e., the oxime moiety.

The aqueous solution of 2-PAM in water is colorless. During the titration of the sample with NaOH, the color of the solution turns yellow. The sample with HCl follows the same course in reverse, indicating that the formation of the yellow colored derivative is an entirely reversible process. At the end of titration (pH $\approx$ 5.5), the solution is colorless again.

Below is the summary of the results. The pK_a values represent the average of at least 4 determinations.

<u>TEMP</u>	<u>CONDITIONS</u>	<u>pK_a</u>
20° C	Aqueous	7.904 ± 0.01
	n-octanol/water	7.895 ± 0.01
30° C	Aqueous	7.785 ± 0.01
	n-octanol/water	7.773 ± 0.01
37° C	Aqueous	7.675 ± 0.01
	n-octanol/water	7.674 ± 0.01

Since the difference in the pK_a 's determined in aqueous or n-octanol/water solution is within experimental error, we conclude from our microelectrometric determinations that to within experimental error there is virtually no partitioning of any of the species of 2-PAM from water to oil at any pH in the range from 20° C to 37° C.

Hence, neither 2-PAM (which is a positively charged ion and does not appear from these microelectrometric experiments to partition) nor the deprotonated anion of 2-PAM (which while formally neutral, still behaves as a dipolar ion and does not partition from water to octanol) appear to have much tendency to pass through any lipophilic barriers including the blood/brain barrier.

A brief survey of the literature revealed the following pK_a values assigned for 2-PAM.

* See Figure 1 of this report for sketches of the molecular structures.

Ginsburg and Wilson²⁴ determined a pK_a at 25° C by measuring the pH of a half neutralized solution; Mason²⁵ reports a value of 8.00 ± 0.01 at 20°C measured by potentiometric titration; Hagedorn et al.²⁶ report a value of 7.68 ± 0.03 by potentiometric titration (no temperature stated).

* See Figure 1 of this report for sketches of the molecular structures.

Since some authors indicate that the reactivation efficiency of AChE is related to the concentration of the oxime anion at physiological pH, we calculated the function of the anion at the range 7.10 - 7.80 from the relationship:

$$\alpha = 1 - \frac{\text{antilog}(pK_a - \text{pH})}{\text{antilog}(pK_a - \text{pH}) + 1} = \quad (\text{for 2-PAM at } 37^\circ)$$

pH	% oxime anion
6.80	11.8
7.10	21.0
7.20	25.1
7.35	32.1
7.40	34.7
7.45	37.3
7.50	40.1
7.60	45.7
7.675	50.0
7.70	51.4

This might provide especially useful in the case of dioximes, where the true proportion of the oxime anion to molecules at physiological pH can be calculated knowing the two ionization constants.

* See Figure 1 of this report for sketches of the molecular structures.

b. UV spectrophotometric technique

2-PAM was one of the oximes for which the electrometric measurements indicated a very small distribution coefficient between octanol and water.

We began by first investigating the UV spectra of 2-PAM in HCl, NaOH and buffer at pH = 7.4, in order to establish the molar extinction, at $\lambda = 293$ nm and from there a calibration curve for different concentrations.

$$\epsilon = 1.04 \times 10^4 \text{ at } \lambda = 293 \text{ nm}$$

which means that the lower limit of the detectable amount of 2-PAM in octanol is 2×10^{-6} g/l.

We determined the solubility of 2-PAM in octanol, which is 2×10^5 g/l (1.2×10^{-4} M).

The following characteristic constants were determined for 2-PAM from the optical measurements:

SOLUTION	pH	% OXIME ANION	λ_{max} (nm)	ϵ
0.1N HCl	1	0%	293	1.219×10^4
0.1N NaOH	13	100%	336	1.810×10^4
Phosphate Buffer	7.4	34.7%	295	1.064×10^4

The distribution coefficient at 37° C was found to be 3.6×10^{-3} .

For all the following microelectrometric studies, the determinations were carried out at 37°C by titration in a 10% oil/water solution. Since the experimentally measured pK_a values at 37°C were the same to within experimental error in aqueous solution or in 10% oil/water solution, the lipophilicities of these compounds were all very small at 37°C and were expected to be very small at any lower temperature. Hence no further lipophilicity determinations were made on such compounds at lower temperatures.

2. HI-6

Microelectrometric technique

The results are the following:

Temperature	Conditions	pK_a
37°C	Aqueous	7.082 ± 0.01
37°C	10% oil/water	7.081 ± 0.01
20°C	Aqueous	7.302 ± 0.014

The following observations were made:

- The titration curve (after the subtraction of the blank curve) shows only one titrable group, i.e. the oxime moiety.
- The aqueous solution of HI-6 (conc. 8×10^{-3} M) is pale yellow even at pH = 5. The yellow color intensifies gradually with the increase of pH.

At 37°C the compound starts to hydrolyze at pH values above 9.75. The retitration curve (with HCl) does not retrace the original titration curve and the final solution has a deep yellow color even at pH = 2.50. The compound hydrolyzes 40-45% in ca 15 minutes at 37°C at pH > 10.00.

At 20°C, the hydrolysis process (starting at higher pH) does not

* See Figure 1 of this report for sketches of the molecular structures.

interfere with the pK_a determination of the oxime group.

- The pK_a of the amide group lies well outside the range of potentiometric determination and physiological pH.

- The value reported in literature is $pK_a = 7.28$, no temperature stated²⁶.

Since it has been postulated that the anion is the effective reactivating species, we calculated the fraction of oxime anion in the physiological pH range from pH = 6.80 to pH = 7.70, from the relationship

$$\alpha = .1 - \frac{\text{antilog}(pK_a - \text{pH})}{\text{antilog}(pK_a - \text{pH}) + 1}$$

pH	% Oxime anion	
	20° $pK_a = 7.30$	37° $pK_a = 7.08$
6.80	24.0	34.4
7.10	38.7	51.2
7.20	44.3	56.9
7.30	50.0	62.4
7.35	52.9	65.1
7.40	55.7	67.6
7.45	58.5	70.1
7.50	61.3	72.5
7.60	66.6	76.8
7.70	71.5	80.7
7.80	76.0	84.0

As one can see from the table, the temperature makes a difference. At pH = 7.40, the normal physiological pH, the percentage of the oxime ion increases 21% from 20°C to 37°C.

-HI-6 did not partition between oil/water at 37°, thus no determinations were made at 20°C in oil/water solution since it is not expected that the compound will partition into the oil phase at the lower temperature.

* See Figure 1 of this report for sketches of the molecular structures.

3. TMB-4

Microelectrometric technique

The determinations were made at 37°C and 20°C. The titration curve, after subtraction of the blank curve, shows two titrable groups, i.e. the two oxime moieties.

The results are as follows:

Temperature	Conditions	pK_{a1}	pK_{a2}
37°C.	Aqueous	7.61 ± 0.02	8.33 ± 0.02
37°C	10% oil/water	7.60 ± 0.02	8.35 ± 0.02
20°C	Aqueous	7.82 ± 0.03	8.59 ± 0.03

Our previously proven technique²³ of graphically deconvoluting overlapping pK_a 's was applied for the determination of the two pK_a 's.

From graphical deconvolution the two pK_a 's are only 0.72 pH units apart. That means that the ionization of the second oxime group starts even before half of the first oxime group is ionized.

The literature value for TMB-4 is $pK_{a1} = 7.78 \pm 0.01$, $pK_{a2} = 8.61 \pm 0.03$; no temperature given.^{27,28}

The fraction of the oxime anion in the physiological significant range is the sum of the mono aldoxime and bispyridiniumaldoxime anions.

The compound does not hydrolyze readily at pH up to 11.50 in 15 min., even at 37°C -- the retitration curve with HCl retraces the original titration curve.

* See Figure 1 of this report for sketches of the molecular structures.

Since the two pK_a values lie again quite close to one another--less than one pK unit apart--our technique of graphical deconvolution of overlapping pK_a 's may be less accurate than would be desirable.

Therefore, we set up a computer program²⁹ for calculating pK_a values based on an equation developed by Speakman.

However, for the separate pK_a 's, our graphical results and the results from the computer program were virtually identical.

* See Figure 1 of this report for sketches of the molecular structures.

TMB-4

pH	% OXIME ANION					
	20°			37°		
	MONOXIME	BISOXIME	SUM	MONOXIME	BISOXIME	SUM
6.80	8.7	1.6	10.3	13.4	2.9	16.3
7.10	16.0	3.1	19.1	23.6	5.6	29.2
7.20	19.3	3.9	23.2	28.0	6.9	34.9
7.30	23.2	4.9	28.1	32.9	8.5	41.4
7.35	25.3	5.4	30.7	35.5	9.5	45.0
7.40	27.6	6.1	33.7	38.1	10.5	48.6
7.45	29.9	6.8	36.7	40.9	11.6	52.5
7.50	32.2	7.5	39.7	43.7	12.9	56.6
7.60	37.6	9.3	46.9	49.4	15.7	65.1
7.70	43.1	11.4	54.5	55.2	19.0	74.2
7.80	48.8	14.0	62.8	60.8	22.8	83.6

The pK_a was also determined at 30 °C.

Temperature	Conditions	pK_a			
		Graphical Method		Computer Method	
		pK_1^M	pK_2^M	pK_1^M	pK_2^M
30 °C	Aqueous	7.67 ± .015	8.42 ± .04	7.685 ± .015	8.418 ± .015

* See Figure 1 of this report for sketches of the molecular structures.

4. TOXOGONIN

The determinations were made at 37°C and 20°C. The results are the following.

Temperature	Conditions	pK_{a1}	pK_{a2}
37°C	Aqueous	7.30 ± 0.02	8.14 ± 0.02
	10% oil / water	7.30 ± 0.04	8.17 ± 0.01
20°C	Aqueous	7.55 ± 0.02	8.32 ± 0.04

All the observations made for TMB-4 apply to TOXOGONIN also. We used the graphic method for deconvoluting the two overlapping pK_a 's.

The compound does not hydrolyze at 37°C in about 15 minutes at pH up to 11.50. The retitration curve with HCl retraces the original titration curve.

The literature values are 7.54 ± 0.01 and 8.24 ± 0.2 - no temperature given.²⁹

Hagedorn²⁶ lists a value of 7.80, which she derived from $pK_{a1} = 7.54$ and $pK_{a2} = 8.12$, stating that "the two oxime groups of the symmetrical-ly structured compound are identical in chemical behavior and the nucleophilicity of their anions correspond to that of a monoxime with $pK_a = 7.80$."

The fraction of the oxime anion in the physiological pH range is:

* See Figure 1 of this report for sketches of the molecular structures.

TOXOGONIN

pH	% OXIME ANION					
	20°			37°		
	MONOXIME	BISOXIME	SUM	MONOXIME	BISOXIME	SUM
6.80	15.1	3.1	18.2	24.0	4.4	28.4
7.10	26.2	5.7	31.9	38.7	8.4	47.1
7.20	30.9	7.1	38.0	44.3	10.3	54.6
7.30	36.0	8.7	44.7	50.0	12.6	62.6
7.35	38.7	9.7	48.4	52.9	14.0	66.9
7.40	41.5	10.7	52.2	55.7	15.4	71.1
7.45	44.3	11.9	56.2	57.4	17.0	74.4
7.50	47.1	13.1	60.2	61.3	18.6	79.9
7.60	52.9	16.0	68.9	66.6	22.4	89.0
7.70	58.5	19.3	77.8	71.5	26.6	98.1
7.80	64.0	23.2	87.2	76.0	31.4	107.4

There had been a report^{30,31} that TOXOGONIN in high doses was capable of overcoming the blood/brain barrier threshold applicable to other quaternary oximes.

In view of the above report³¹ that in high doses TOXOGONIN is capable of overcoming the blood/brain threshold, we plan some further lipophilicity experimentation on TOXOGONIN.

However, our present lipophilicity experiments reported here were carried out under our standard concentration conditions, 8×10^{-3} molar, and 10% oil/water solution. These drug concentrations and a 10% oil/water solution have proven a good model for blood/brain partitioning of drugs. These standard conditions have proven reliable to allow us to measure the lipophilicities of more than 50 other diverse compounds. Our standard conditions have allowed us to measure lipophilicities as low as that of morphine over the range from pH 2 to pH 11, including the physiologic pH range.

<u>Drug Distribution Coefficient</u>				
Morphine Sulfate	<u>Temp.</u>	<u>D_{7.10}</u>	<u>D_{7.40}</u>	<u>D_{7.70}</u>
	37°C	0.80	1.42	2.30

* See Figure 1 of this report for sketches of the molecular structures.

As a reliable model for penetration of a drug through the lipid blood/brain barrier, the reliability of our measured morphine lipophilicities was verified by administering morphine i.v. to rats where the physiologic pH's of the rats were varied from 7.14 to 7.70.^{32,33} Both the relative ED₅₀'s at the various physiologic pH's and the relative concentrations of radioactive morphine in various portions of the brain as a function of physiologic pH were correctly predicted by our experimental lipophilicity values.

The ionization constants of TOXOGONIN were also determined at 30°C.

TOXOGONIN does not seem to hydrolyze. Retitration of the compound with HCl retraces exactly the titration curve, so it seems that the oxime anion in alkaline media, up to a pH of 11.50 and over a time interval of ~20 minutes, does not undergo an irreversible reaction.

Since there are also two overlapping pK_a's in TOXOGONIN, the computer program was used for the calculation of the two pK_a's. The results at 30° are as follows:

* See Figure 1 of this report for sketches of the molecular structures.

Temperature	Condition	pK_a			
		Graphical Method		Computer Method	
		pK_1^M	pK_2^M	pK_1^M	pK_2^M
30°C	Aqueous	7.39±.01	8.18±.01	7.385±.01	8.216±.01

The percentages of mono and bisoxime anions at physiological pH are:

pH	Monoxime Anion	Bisoxime Anion	Total Oxime Anion
6.80	20.64	3.69	24.33
7.10	34.16	7.11	41.27
7.20	39.51	8.79	48.30
7.30	45.12	10.82	55.94
7.35	47.99	11.98	59.97
7.40	50.86	13.25	64.11
7.45	53.73	14.63	68.36
7.50	56.58	16.13	72.71
7.60	62.13	19.49	81.62
7.70	67.38	23.35	90.74
7.80	72.22	27.73	99.95

As we finished our pK_a measurements for the following compounds: 2-PAM, HI-6, TMB-4 and Toxogonin,^a at three different temperatures (20°C, 30°C and 37°C), we calculated the temperature dependences of their pK_a 's. The pK_a of many non-carboxylic organic acids are temperature sensitive (for example, phenol becomes stronger by 0.012 units pK_a for each degree rise in temperature).

The results are the following:

Compound	$-dpK_{a1}/dt$	$-dpK_{a2}/dt$
2-PAM	0.0128	—
HI-6	0.0131	—
TMB-4	0.0125	0.0154
TOXOGONIN	0.0148	0.0103

* See Figure 1 of this report for sketches of the molecular structures.

We then began systematically to study the class of methylimidazolium oxime derivatives, the BHH compounds; in numerical order

5. BHH-1

Microelectrometric technique

The ionization constants determinations were made at 37°C. The compound has only one titratable group.

The compound did not hydrolyse subjected to our standard conditions during titration and retitration. The results are the following:

No partitioning occurs in the oil phase.

Temperature	Conditions	pK _a
37°C	Aqueous	7.76±0.01
37°C	10% oil/water	7.76±0.006

We calculated the percent oxime anion at various pH's.

pH	% Oxime anion
6.80	9.88
7.10	17.95
7.20	21.59
7.30	25.75
7.35	28.00
7.40	30.39
7.45	32.87
7.50	35.46
7.60	40.89
7.70	46.55
7.80	52.30

* See Figure 1 of this report for sketches of the molecular structures.

6. BHH-3

Microelectrometric technique

The shape of the titration curve indicates that the compound has only one titratable group. The retitration curve is identical to the titration one, indicating that in the conditions used ($C = 8 \times 10^{-3} M$, temp. = $37^\circ C$ and time ~ 20 minutes, at pH values up to 11.70), the compound does not undergo hydrolysis and the formation of the oxime anion is a reversible process. The compound does not partition in the oil phase under our standard conditions. The results are the following:

Temperature	Conditions	pK_a
$37^\circ C$	Aqueous	7.71 ± 0.01
$37^\circ C$	10% Oil/water	7.70 ± 0.01

pH	% Oxime anion
6.80	10.95
7.10	19.71
7.20	23.61
7.30	28.00
7.35	30.39
7.40	32.87
7.45	35.46
7.50	38.14
7.60	43.70
7.70	49.42
7.80	55.16

* See Figure 1 of this report for sketches of the molecular structures.

7. BHH-4

Microelectrometric technique

The same remarks as for BHH-3 apply here. The results are as follows:

Temperature	Conditions	pK_a
37°C	Aqueous	7.82 ± 0.01
Preliminary measurements in the 10% oil/water solution for the pK_a shift was negligible.		

pH	% Oxime anion
6.80	8.72
7.10	16.00
7.20	19.35
7.30	23.19
7.35	25.31
7.40	27.55
7.45	29.90
7.50	32.37
7.60	37.60
7.70	43.13

* See Figure 1 of this report for sketches of the molecular structures.

8. BHH-5

Microelectrometric technique

This compound has one titratable group. It appears that this compound, due probably to the long aliphatic side chain, does have a tendency to partition into the oil phase. Our preliminary measurements indicate that the pK_a shift (between aqueous and oil/water titrations) is still quite small, in the range of experimental error. Thus, this compound is a prime candidate for a spectrophotometric measurement of partitioning.

Temperature	Conditions	pK_a
37°C	Aqueous	7.88±0.006

The percent of oxime anion is:

pH	% Oxime Anion
6.80	7.68
7.10	14.23
7.20	17.28
7.30	20.83
7.35	22.79
7.40	24.87
7.45	27.09
7.50	29.42
7.60	34.41
7.70	39.78
7.80	45.41

* See Figure 1 of this report for sketches of the molecular structures.

This is an interesting compound since it is the only oxime that we have studied to date that has a large partition coefficient. This was determined from the pK_a shift (ΔpK_a) by titration in aqueous solution and in an octanol/water mixture. ^aThe values for three determinations are as follows:

<u>SAMPLE NO.</u>	<u>ΔpK_a</u>	<u>P</u>	<u>log P</u>
1	0.544	25.0	1.398
2	0.555	25.9	1.413
3	0.527	23.7	1.374
Average Value	0.542 ± 0.014	24.8 ± 1.1	1.395

It should be noted that we are reporting a partition coefficient (P) rather than a distribution coefficient (D). This stems from the fact that to derive D from P we must have some information on the identity of the species distributed in the octanol.

A brief comment about the high value of P seems in order. This high value probably arises from the fact that we are partitioning into octanol and the long tail attached to the oxygen is an octyl group. The close structural similarity between the model lipid (octanol) and the octyl tail probably accounts for the high P. In the next quarter, experiments using other solvents should help establish whether this is the reason for the high P or whether other factors are involved.

* See Figure 1 of this report for sketches of the molecular structures.

9. BHH-7

Microelectrometric technique

This compound is a bis pyridinium derivative (not an imidazilium one) with two titratable groups. Color changes during titration from colorless to deep yellow. It is reversible upon retitration.

The pK_a 's are the following:

Temperature	Conditions	pK_a			
		Graphical Method		Computer Method	
		pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}
30°C	Aqueous	7.54±0.02	8.27±0.03	7.511±0.01	8.344±0.01
30°C	Oil/water	7.48	8.28	---	---

pH	% Monoxime Anion	% Bisoxime Anion	% Total Oxime Anion
6.80	16.23	2.78	19.06
7.10	27.96	5.39	33.35
7.20	32.32	6.69	39.52
7.30	38.09	8.29	46.38
7.35	40.34	9.21	50.05
7.40	43.65	10.21	53.36
7.45	46.49	11.32	57.81
7.50	49.37	12.53	61.90
7.60	55.11	15.23	70.39
7.70	60.71	18.50	79.21
7.80	66.05	22.22	88.27

* See Figure 1 of this report for sketches of the molecular structures.

10. BHH-8

This compound has two titratable groups: one attached to the pyridinium ring, the other to the imidazolium ring. During titration the color of the solution turns from colorless to deep yellow. The retitration curve retraces the original titration curve, so no hydrolysis occurs under these conditions.

Temperature	Conditions	pK_a			
		Graphical Method		Computer Method	
		pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}
37°C	Aqueous	6.87 ± 0.01	7.79 ± 0.005	6.87 ± 0.003	7.85 ± 0.003
37°C	Oil/water	6.85	7.79	---	---

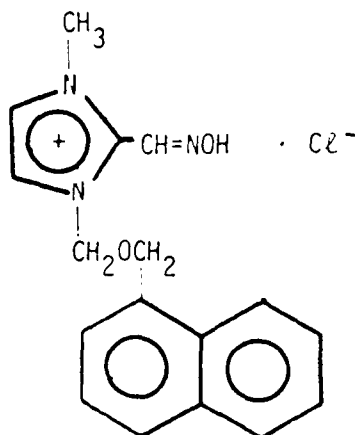
The interesting point is that the first pK_{a1} has a very unusually low value. All the derivatives studied up till now had a $pK_{a1} > 7.00$ and $pK_{a2} > 8.10$.

It would be interesting to establish which pK_a corresponds to which ring. Single oxime imidazolium ions had a pK_a around 7.70 - 7.80. One tends to assume therefore that the $pK_a = 6.87$ can be attributed to the oxime group attached to the pyridinium ring. Although unsubstituted 2-PAM has a pK_a of 7.68 versus the first pK_a BHH-8 has, which is such a low value, the concentration of oxime anions of BHH-8 is very high at physiological pH levels:

pH	Monoxime Anion	Bisoxime Anion	Total Oxime Anion
6.80	46.09	8.25	54.34
7.10	63.04	15.22	78.25
7.20	68.23	18.43	86.66
7.30	73.00	22.15	95.15
7.35	75.21	24.19	99.40
7.40	77.29	26.37	103.66
7.45	79.25	28.66	107.91
7.50	81.08	31.07	112.15
7.60	84.36	36.21	120.57
7.70	87.19	41.67	128.84
7.80	89.53	47.35	136.88

* See Figure 1 of this report for sketches of the molecular structures.

11. BHH-13



a. Microelectrometric technique

This compound has also one titratable group--no hydrolysis occurs during titration.

Conditions	pK_{a1}
Aqueous	$7.83 \pm .003$

pH	% Oxime Anion
6.80	8.53
7.10	15.70
7.20	18.99
7.30	22.79
7.35	24.88
7.40	27.09
7.45	29.42
7.50	31.87
7.60	37.06
7.70	42.57
7.80	48.27

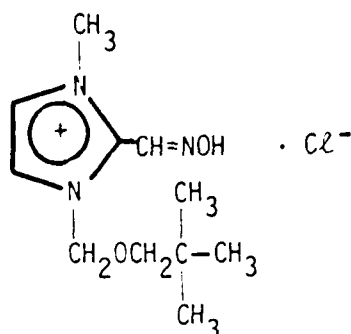
b. UV spectrophotometric technique

The characteristic optical constants for this compound are:

SOLUTION	pH	% OXIME ANION	λ_{max} (nm)	ϵ
0.1N HCl	1	0	272	1.434×10^4
0.1N NaOH	13	100	292	1.851×10^4
Phosphate Buffer	7.4	27.1	278	1.414×10^4

The distribution coefficient $D = 0.14 \pm 0.005$. This distribution coefficient is based on the concentration of the oxime in both the aqueous and in octanol phases. If we had only used the measured aqueous concentration of oxime and used the difference between this value and the total amount of oxime used as the octanol concentration, the distribution coefficient would have been reported as 0.24 ± 0.02 .

12. BHH-14



a. Microelectrometric technique

The ionization constant of this compound as determined by electrometric titration is

$$pK_a = 7.81 \pm 0.01 \text{ at } 37^\circ\text{C.}$$

b. UV spectrophotometric technique

The percentage of oxime anions in the physiological pH range is as follows:

pH	% OXIME ANION
6.80	8.85
7.10	16.22
7.15	17.85
7.20	19.56
7.25	21.48
7.30	23.48
7.35	25.61
7.40	28.01
7.45	30.24
7.50	32.72
7.60	37.98
7.70	43.53
7.80	49.25

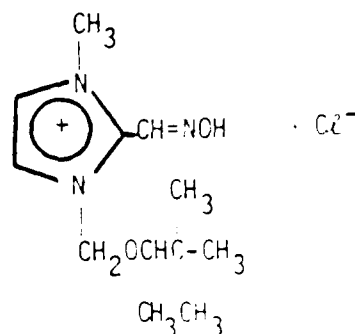
The distribution coefficient of this compound was determined by UV spectrophotometry since the partitioning was too small to determine electrometrically. The characteristic optical constants are:

SOLUTION	pH	% OXIME ANION	λ_{max} (nm)	Σ
0.1N HCl	1	0	268	1.190×10^4
0.1N NaOH	13	100	292	1.473×10^4
Phosphate Buffer	7.4	28	274	1.130×10^4

The distribution coefficient, $D = 0.012 \pm 0.008$.

* See Figure 1 of this report for sketches of molecular structures.

13. BHH-15



a. Microelectrometric technique

The pK_a value as determined by electrometric titration is 7.78 ± 0.02 at 37°C .

The percentage of oxime anion in the physiological range is as follows:

pH	% OXIME ANION
6.80	9.48
7.10	17.28
7.15	18.99
7.20	20.83
7.25	22.79
7.30	24.88
7.35	27.09
7.40	29.42
7.45	31.87
7.50	34.42
7.60	39.78
7.70	45.41
7.80	50.50

b. UV spectrophotometric technique

The distribution coefficient $D = 0.18 \pm 0.02$, determined spectrophotometrically only. The optical constants are:

SOLUTION	pH	% OXIME ANIONS	λ_{max} (nm)	ϵ
0.1N HCl	1	0	269	1.18×10^4
0.1N NaOH	13	100	293	1.47×10^4
Phosphate Buffer	7.4	29	275	1.13×10^4

* See Figure 1 of this report for sketches of molecular structures.

VII. Conclusions

While it is too early to have firm conclusions after only the first year of a several-year new research project, we have already made some significant findings which lead to insight into the characterization of the oximes as they may relate to their in-vivo efficacy as reactivators of AChE inhibited by organophosphorus compounds.

A. General conclusions

Our theoretical and experimental results to date indicate that we are pursuing fruitful lines of investigation. Our results to date already shed considerable insight into:

1. Theoretical stereoelectronic requisites for an effective oxime reactivator of inhibited AChE (using the EMPC map of deprotonated 2-PAM as a model);
2. The fundamental underlying theoretical basis for the lack of lipophilicity of the deprotonated oximes (even though they are formally overall neutral species), since the EMPC maps indicate these species to be strongly dipolar ions;
3. Theoretical techniques to predict, prior to synthesis of a hypothetical new oxime, whether or not it will have the correct stereoelectronic properties to reactivate AChE and whether the deprotonated species will have much tendency to be lipophilic and partition into the CNS to be an effective reactivator of inhibited AChE in the CNS;
4. A strategy using the principle of complementarity of deprotonated oximes to map out the possible stereoelectronic characteristics of the AChE active site (esteratic and anionic). This is significant because it has not yet been possible to crystallize AChE to determine experimentally the structure and characterization of the AChE sites;
5. The physicochemical reason for the non-effectivity of the oxime reactivators in the CNS (since neither the quaternary nor the deprotonated species are very lipophilic and thus will not have much tendency to partition through the CNS blood-brain barrier under most physiological conditions).

B. Specific conclusions

Deprotonated 2-PAM (the oximate that has been suggested to be the pertinent species) is formally an overall neutral species [with a formal + charge from the quaternization and a formal - charge from the deprotonation of the oxime].

However, the three dimensional isopotential EMPC map around deprotonated (zwitterionic) 2-PAM (generated from our calculated quantum chemical wave function of deprotonated 2-PAM) indicated that clearly there

is a strongly positive region and a strongly negative region. Thus, this species appears to be a strongly dipolar ion.

This has significant implications for several aspects of the use of oxime reactivators. First, the size and shape and relative spatial relationship of the positive and negative isopotential portions of these EMPC maps should have a complementary relation to the spatial relationship and electronic characteristics of esteratic and anionic subsites of the active site of AChE. This is particularly important in view of the complete lack of molecular crystal structural information on any AChE. Our isopotential EMPC maps of deprotonated 2-PAM can help shed insight onto the size, shape and stereoelectronic characteristics of the AChE active sites--quantities at present not yet obtained, since so far it has apparently not been possible to crystallize AChE to determine its crystal structure.

Second, the isopotential EMPC map of deprotonated 2-PAM is as unique as a fingerprint in identifying stereoelectronic characteristics that have the proper stereoelectronic complementarity and are favorable for effective reactivation of AChE inhibited by organophosphorus compounds. The corresponding calculated isopotential EMPC maps of the oximates of proposed hypothetical new oxime reactivators can be computer-compared to those of deprotonated 2-PAM prior to the synthesis of the new compounds to see if these new compounds have the proper stereoelectronic characteristics to be effective new reactivators.

Third, our three-dimensional isopotential EMPC maps around deprotonated 2-PAM also shed insight into another important aspect of the properties of 2-PAM and deprotonated 2-PAM. 2-PAM itself is a positively charged quaternary species. As such, it is not expected to be, nor is it, very lipophilic. 2-PAM has little tendency to partition from aqueous to lipid.

It had been hoped that when 2-PAM was deprotonated and was then an overall neutral species, that it would have a much greater tendency to partition into lipid.

Our experimental determination of the lipophilicity of 2-PAM as a function of pH indicated that even when the positively charged quaternary 2-PAM was deprotonated, the resulting species was still only very slightly lipophilic. This behavior is commensurate with our EMPC map results, namely, that deprotonated 2-PAM (while overall neutral) has the electronic characteristics of a strongly dipolar ion.

Because 2-PAM would have little tendency to partition through the lipophilic blood-brain barrier under usual physiological conditions, 2-PAM is not an effective reactivator in the CNS of AChE inhibited by organophosphorus compounds.

Our EMPC map of deprotonated 2-PAM indicated that while this species is neutral overall, it appears to be a strongly dipolar ion and thus would not be expected to have a significant tendency to partition into lipids.

Thus, our theoretical finding that the EMPC map of deprotonated 2-PAM indicates it to be a strongly dipolar ion has concomitantly been validated by our experimental lipophilicity determination of 2-PAM as a function of

pH. Neither the quaternary 2-PAM nor the deprotonated 2-PAM has much tendency to partition from aqueous to lipid. Thus, under most physiological circumstances, 2-PAM would not be expected to partition into the CNS.

One of the Army's interests is in finding and developing new oxime reactivators that would be effective in the CNS. Oximes that were lipophilic would have a better chance to be effective reactivators in the CNS.

If the EMPC map of the deprotonated species of a proposed new oxime reactivator indicated that, like deprotonated 2-PAM, it was a strongly dipolar ion, then this oxime would not appear to be a good candidate for an effective reactivator of inhibited AChE in the CNS. (While there have been reports that the CNS blood-brain barrier loses some of its integrity after exposure to organophosphorus AChE inhibitors, sufficient blood-brain barrier integrity must still remain, because 2-PAM has been shown not to be an effective AChE reactivator in the CNS.)

We are continuing our theoretical and experimental investigations of other oxime reactivators.

Our experimental microelectrometric results on the pH dependence of the lipophilicities of the quaternary oximes and their deprotonated species indicate that neither the positively charged quaternary nor the overall neutral deprotonated species are very lipophilic. However, in view of the fact that inhibited AChE can be reactivated by low concentrations of the oximes, we set up and are using a more sensitive UV spectrophotometric method in which the two phases are separated by centrifugation and the amount of material in each phase is determined from calibration curves.

VIII. Recommendations

A. General

To continue to mesh together our theoretical quantum chemical and EMPC map results with our experimental lipophilicity results to get a better understanding of the efficacy of the oxime reactivators.

B. Theoretical

Our recommendations are:

1. Pyridinium oximes and bis-pyridinium oximes

To carry out in depth further quantum chemical calculations for the conformational energy profile of 2-PAM as a function of various geometry variations based both on the new experimental crystal structure of 2-PAM and on our ansatz structure, and to compare the results carefully. Since there are still very few experimental crystal structures of these quaternary oxime reactivators, this is an important point. There are no experimental crystal structures for most of the oximes of interest, including the BHH compounds. Thus, for quantum chemical calculations on the newer oximes, the structures will have to be ansatzed as a starting point for further quantum chemical calculations.

To carry out further in depth quantum chemical calculations on deprotonated 2-PAM (the oximate) using the new crystal structure of 2-PAM as a starting point and making extensive geometry variations, including bond lengths and bond angles, to calculate the conformational energy profile. The convergence of the quantum chemical wave function of this species has proven to be sensitively dependent on the geometry. The oximate of 2-PAM is the model for all other effective oxime reactivators. Thus, a thorough understanding of its wave functions and behavior will be an important guide for the oximates of the other oximes.

For both 2-PAM and its oximates, the EMPC maps will be generated for various conformations to assess their sensitivity to geometry.

To carry out in depth quantum chemical investigations of the conformational energy profile of HI-6 as a function of various geometry variations, to calculate conformational energy profiles, based both on the new experimental crystal structure and on our ansatz geometry, and to compare the results carefully. HI-6 is the prototype of the bis-pyridinium oximes which have many more conformational degrees of freedom than the monopyridinium compounds.

To carry out similar quantum chemical calculations for the conformational energy profiles of deprotonated HI-6 (the oximate) as a function of geometry variations based both on the new crystal structure and the ansatz structure, and to compare the results carefully. These are models for the bis-pyridinium compounds which have many more degrees of conformational freedom than the monopyridinium compounds.

Then, to generate EMPC maps around various conformations of HI-6 and its oximates and to study the variations of the EMPC maps as a function of conformation.

2. Interactions with AChE

To use the principle of complementarity of the EMPC maps around deprotonated 2-PAM (the oximate) to infer stereoelectronic characteristics of the AChE active site (esteratic and anionic) which have not yet proven amenable to experiment.

To carry out quantum chemical calculations and intermolecular interaction calculations of deprotonated 2-PAM interacting with a model of the AChE active site.

To carry out similar calculations of deprotonated 2-PAM interacting with inhibiting AChE.

3. To formulate how the quantum chemical and EMPC map information would be used to set up computer based structure-activity prediction program for the efficacy of oximes.

This will be done based on EMPC maps as we get theoretical results on more molecules.

4. To use the quantum chemical and EMPC map information to set up a program for lead-directed synthesis of new, more effective oximes.

We will also be doing this based on the EMPC maps of already effective oximes and how they vary with reactivating effectivities. Since quantum chemical calculations can be run and EMPC maps generated for proposed hypothetical oximes prior to their synthesis, this theoretical approach is a very effective predictive tool.

5. BHH Compounds - quaternary methyl imidazolium oximes

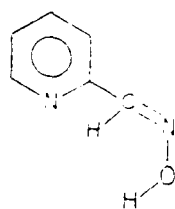
To carry out the quantum chemical calculations and EMPC map generation on these compounds as time and resources permit. In particular, to compare these to 2-PAM and its oximate as a model effective oxime reactivator and to compare the BHH effective reactivators against those which are "protective".

C. Experimental

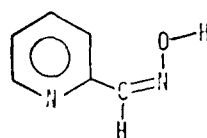
To continue to carry out the determinations of lipophilicity of oximes by both the microelectrometric and the UV spectrophotometric techniques as a function of pH.

Further, for the one species to date, BHH-5, that had a reasonable lipophilicity in octanol, to attempt to identify the specific species which have partitioned into octanol using NMR technique.

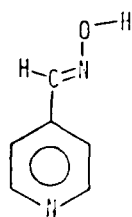
IX. Figures

Sketches of Oximes Mentioned in this Report

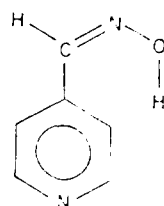
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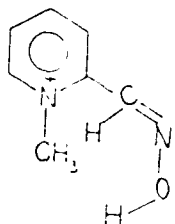
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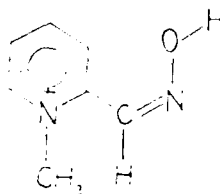
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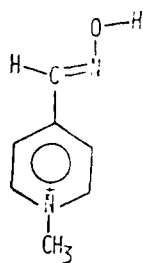
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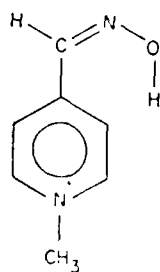
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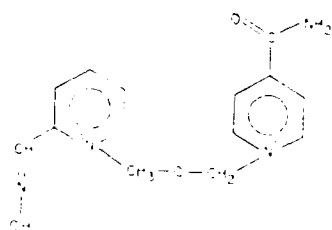
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syn 4-PAM

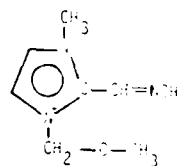


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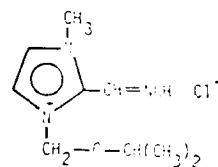


H1-6

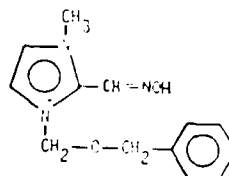
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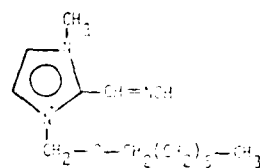
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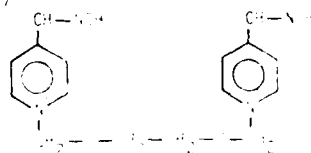
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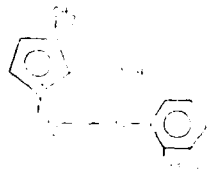
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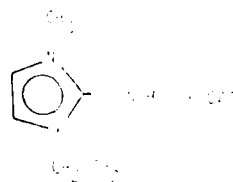
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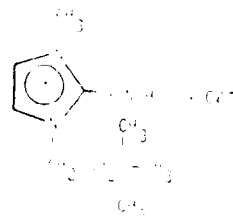
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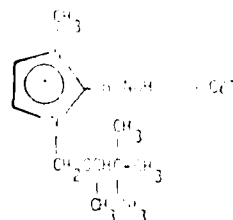
BH-9



BH-10



BH-11



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Figure 2

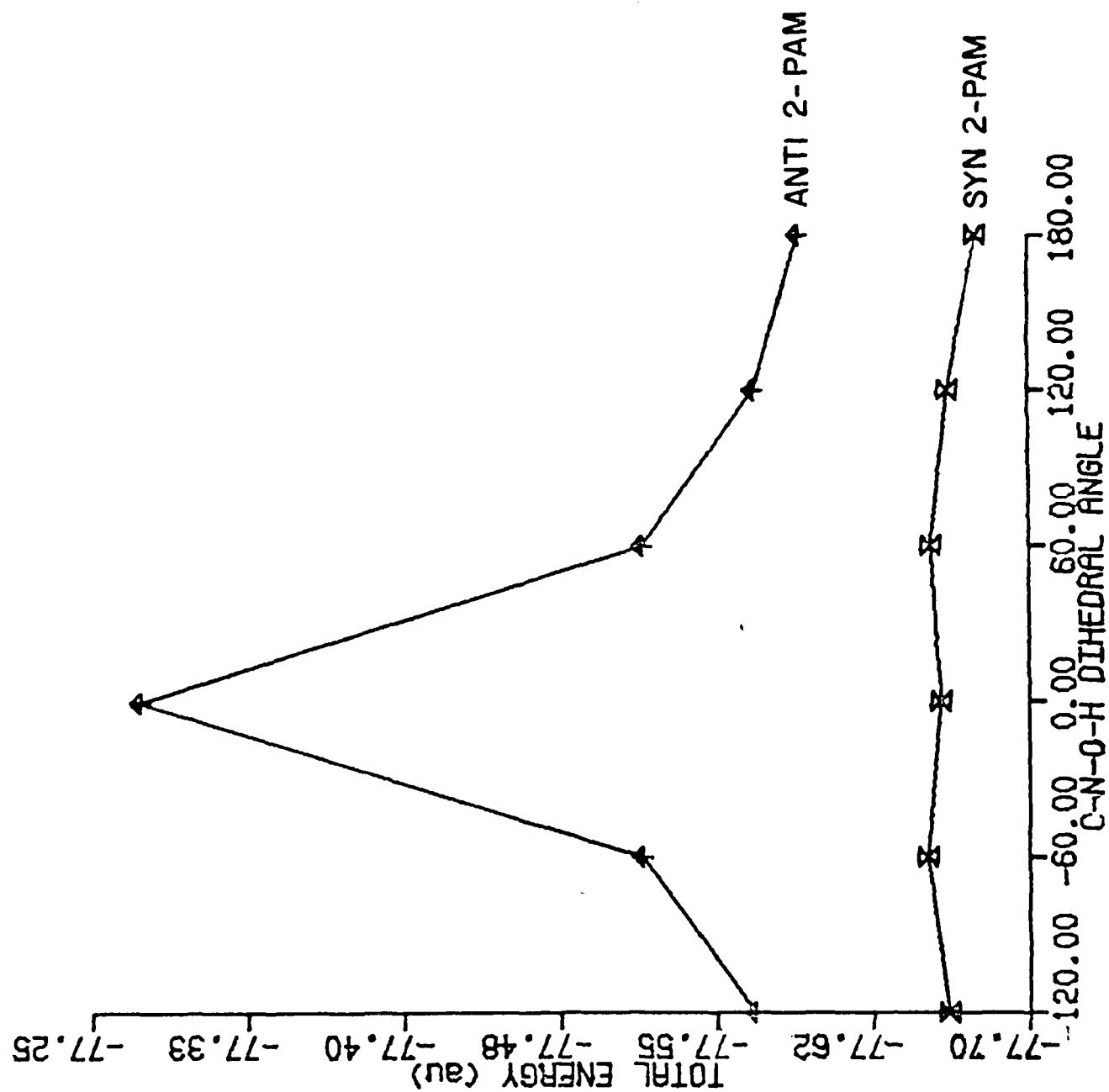


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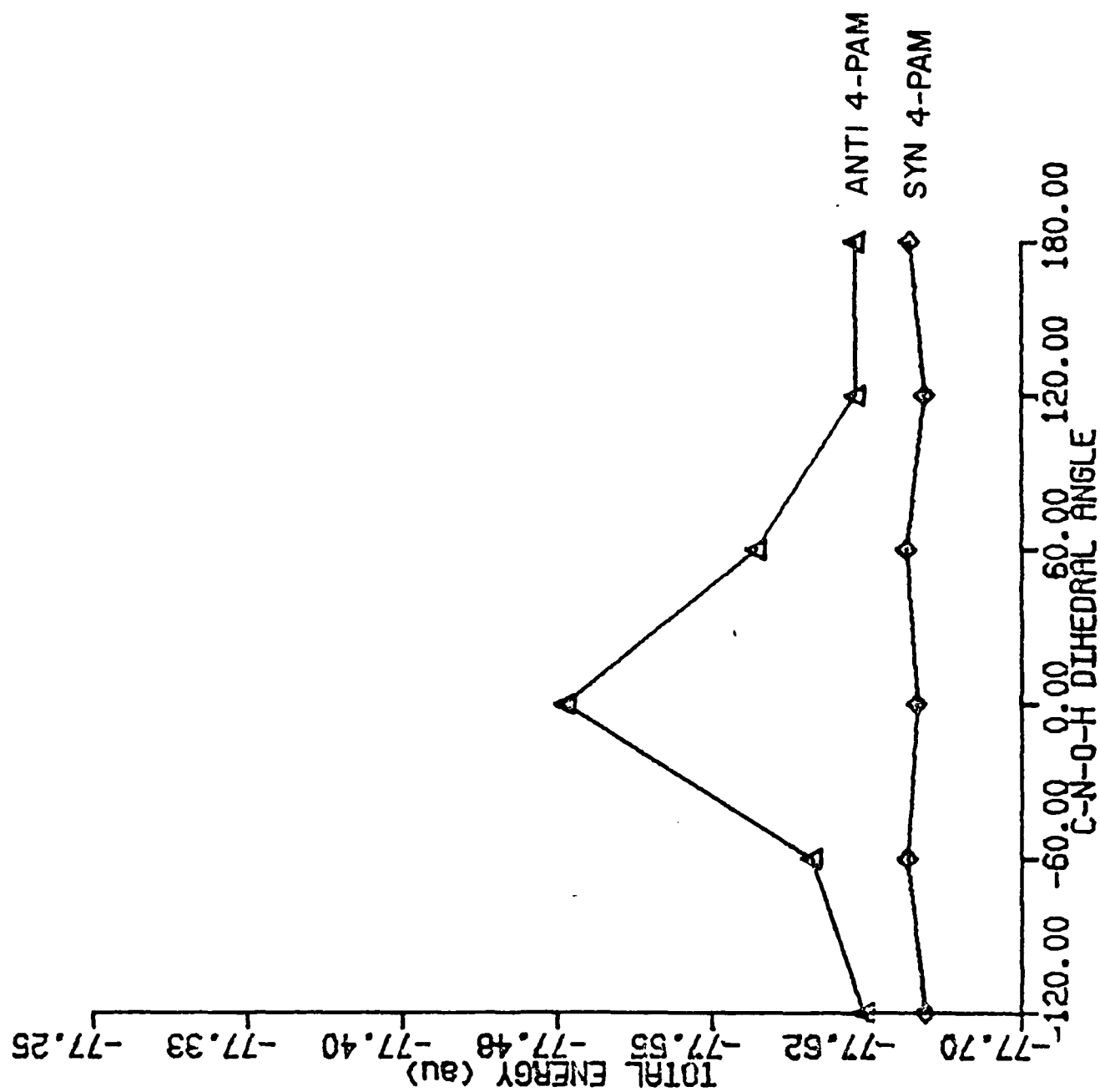
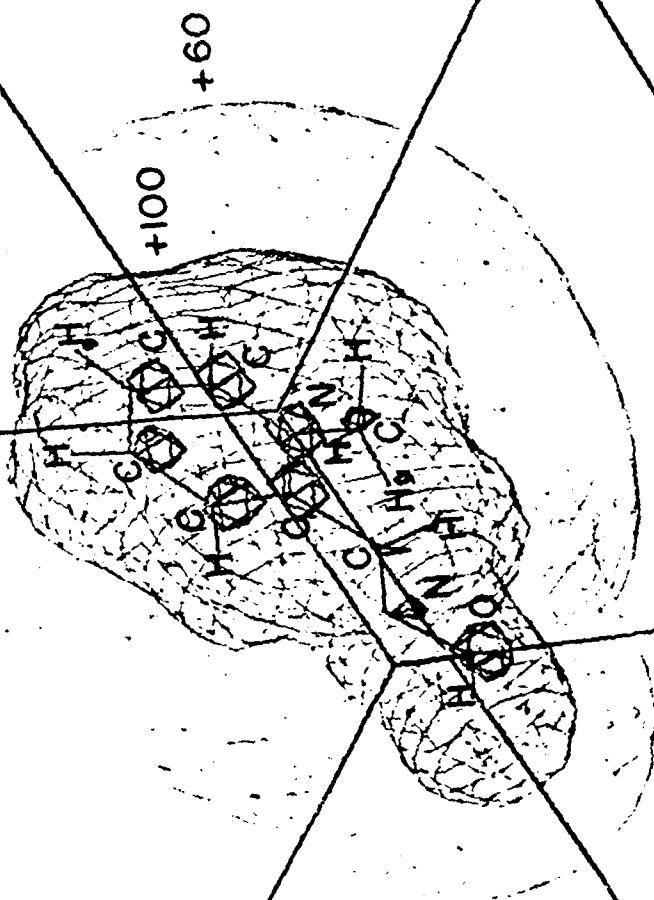


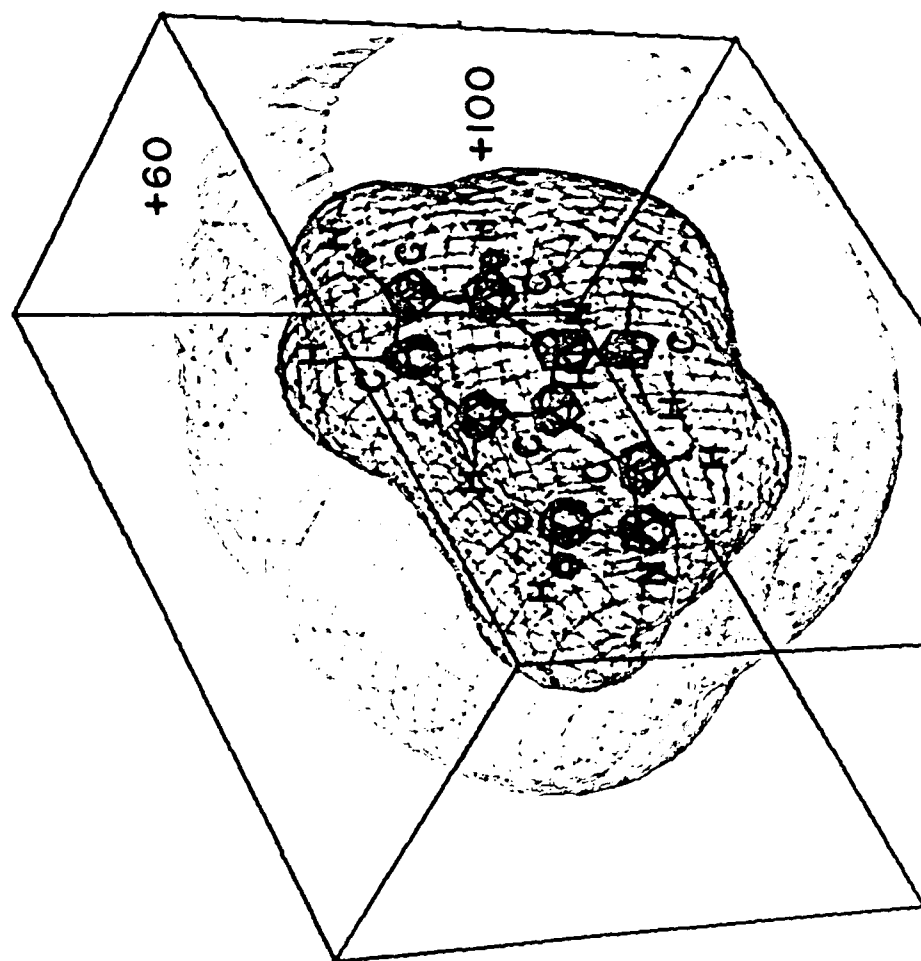
Figure 4



Red=50.0, Black=2000.0, Kcal/mol
Blue=100.0

SYN 2-PAM

Figure 5



Red=60.0, Black=2000.0, Kcal/mol
Blue=100.0

ANTI 2-PAM

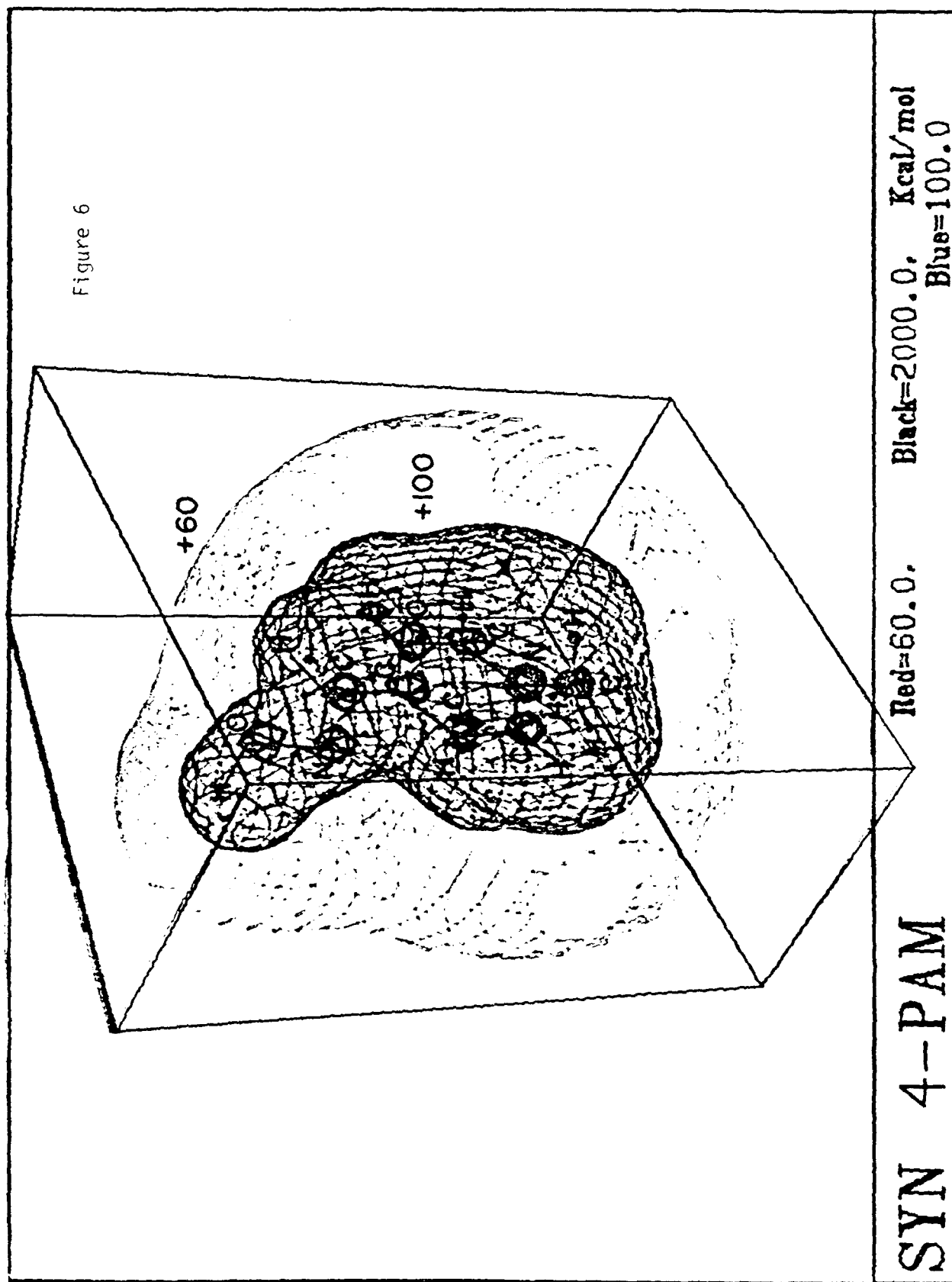
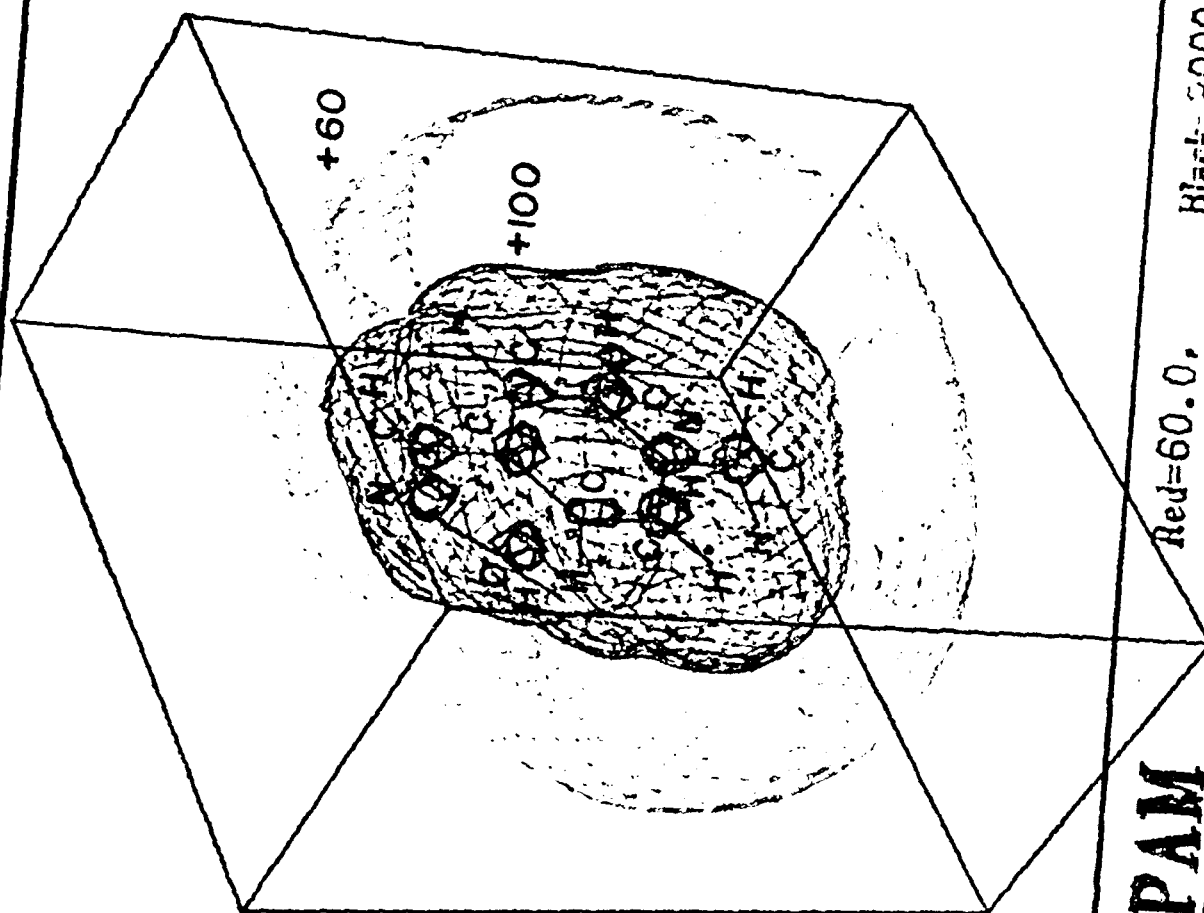


Figure 7

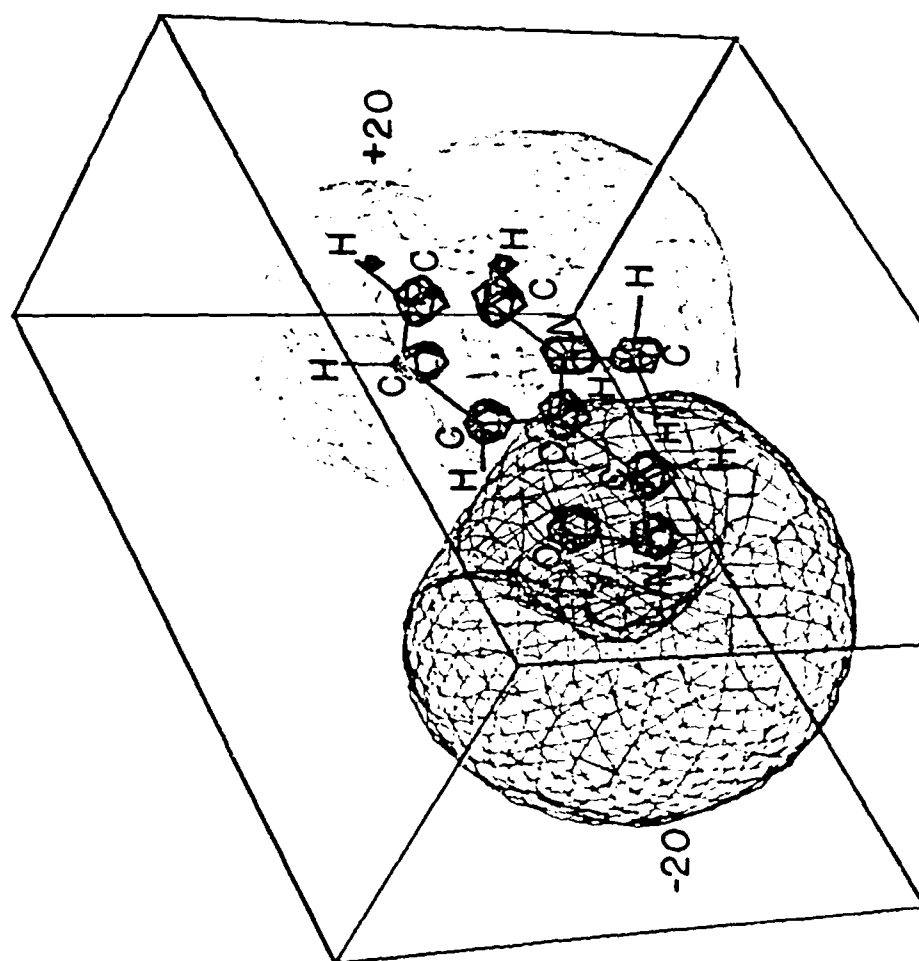


Red=60.0,

Black=2000.0, Kcal/mol

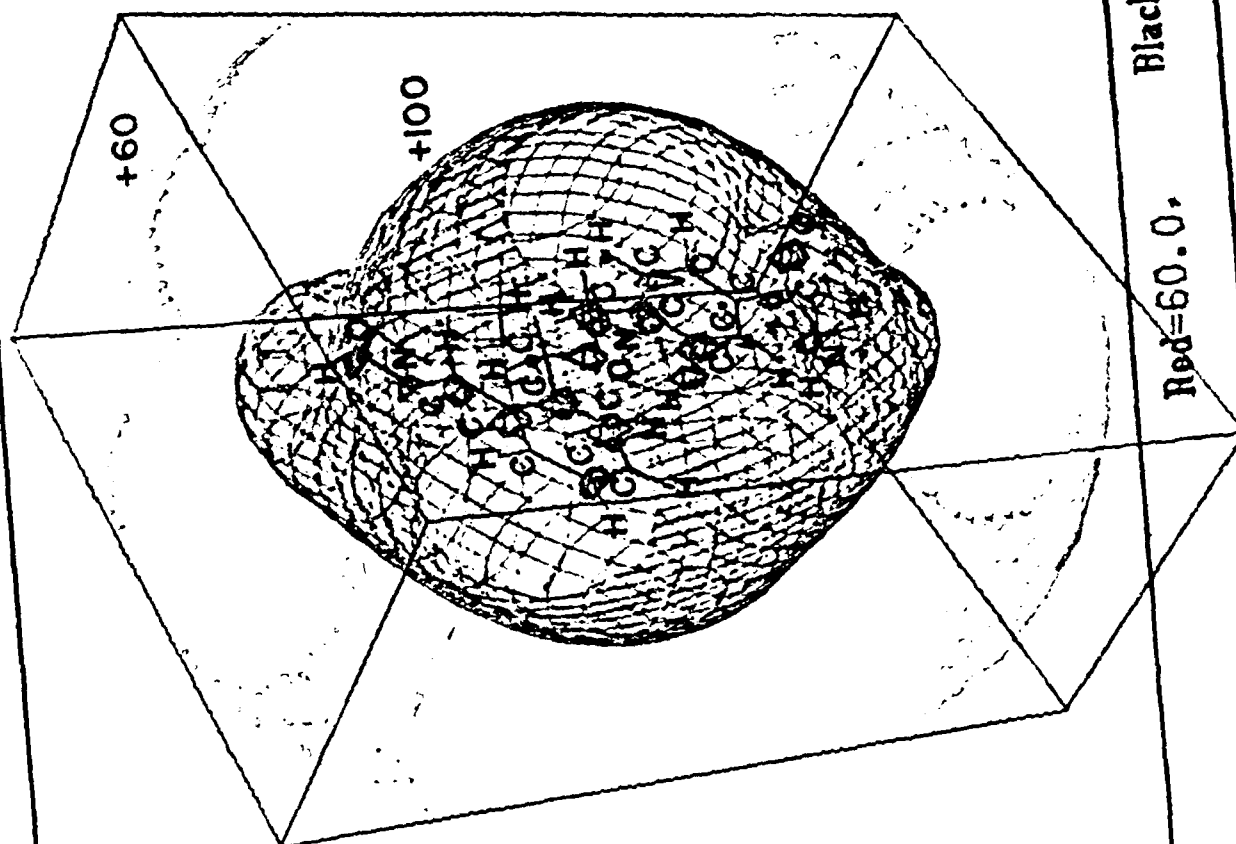
Blue=100.0

Figure 8



A2PAM ZWITTERION		Kcal/mol
Black=2000.0.	Red=20.0.	
Blue=-20.0.		

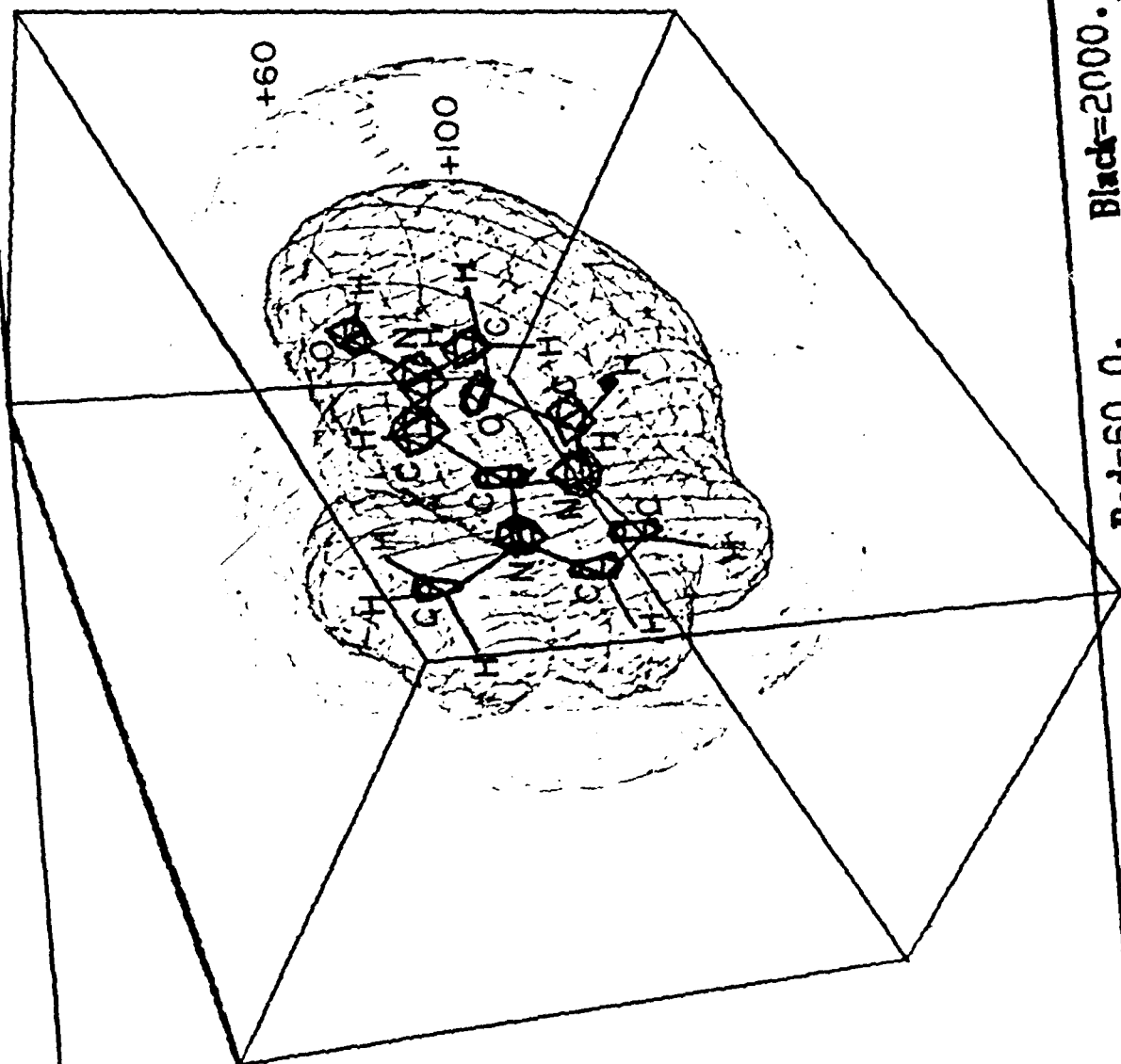
Figure 9



Black=2000.0, Kcal/mol
Blue=100.0

5-11

Figure 10



Red=60.0, Black=2000.0, Kcal/mol
Blue=100.0

BHH0001

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