JOURNAL OF Pharmaceutical Sciences

September 1964 volume 53, number 9

Review Article

Oximes Antagonistic to Inhibitors of Cholinesterase

Part I

By ROBERT I. ELLIN and J. HENRY WILLS

ORGANOPHOSPHORUS INHIBITORS OF CHOLINESTERASE

Diisopropyl phosphorofluoridate (DFP) and isopropyl methylphosphonofluoridate (sarin) are common prototypes of compounds possessing the general formula $R_1R_2P(O)X$, where R_1 and R_2 are either alcoholic, phenolic, mercaptic, amidic, alkyl, or aryl groups and X may be fluoride, chloride, cyanide, carboxyl, phosphate, pyrophosphate, phenoxy, thiol, or thiophenoxy radicals. These compounds are powerful inhibitors of enzymes which possess carboxylic esterase activity, *i.e.*, true cholinesterase, pseudocholinesterase, ali-esterase, chymotrypsin, trypsin, thrombin, and acetylesterase. However, one should not assume that all esterases are inhibited by organophosphorus compounds; for example, diethyl-*p*-nitrophenylphosphate inhibits only one of the two enzymes in serum capable of hydrolyzing various *p*-nitrophenylesters (1, 2).

CHOLINESTERASES

The existence of an enzyme or enzymes capable of hydrolyzing acetylcholine is well established (3, 4). The true cholinesterase of Mendel and Rudney (5) is found predominantly in nervous tissue and erythrocytes of most species, whereas pseudocholinesterase predominates in the blood plasma (6, 7). Ali-esterase, also present in blood, does not hydrolyze choline esters. The pseudo form does not play a significant part in the neurohumoral transmission of cholinergic impulses, although it can hydrolyze acetylcholine in addition to other esters. If acetylcholine, as suggested by Burn (8), is involved in the spontaneous rhythmic activity of such tissues as heart muscle and cilia, pseudocholinesterase may play an important nonsynaptic role in these tissues or may supplement the action of true cholinesterase wherever high concentrations of acetylcholine occur. Dixon and Webb (9) prefer to use the terms "acetylcholinesterase" and "cholinesterase" for true and pseudocholinesterase. As these names are used interchangeably in the literature, we shall not in this report adhere to a single nomenclature but shall attempt to be as lucid as possible when referring to the individual enzymes.

How biochemical catalysts work is a question germane to the understanding of all biochemical processes. In applying this statement to acetylcholinesterase, one would hope to establish a mechanism for its catalytic action. Many enzymes have been highly purified; in fact, the sequence of amino acids in ribonuclease has been worked out by Hirs *et al.* (10). However, one must modify this optimistic attitude by stating that the stereo-structure of enzymes is presently unknown and that no enzymatic process is completely understood. Considerable progress had been achieved with the esterase enzymes in experiments designed to identify the active site or sites responsible for the catalytic action and en-

Received from the Physiology Division, Directorate of Medical Research, U. S. Army Chemical Research and Development Laboratories, Edgewood Arsenal, Md.

EDITOR'S NOTE: Additional considerations of oximes antagonistic to inhibitors of cholinesterase will be discussed in the concluding portion of this review, Part II, which will appear in the October issue of THIS JOURNAL.

zyme specificity (11). In many instances, the so-called catalytic entities consist of nonproteinoid cofactors, such as pyridoxal phosphate, diphosphopyridine nucleotide (DPN), metal ions, etc. Enzymatic processes are known to be centered around these cofactors, which are relatively simple molecules when compared with the enzyme. One may logically initiate studies by noting the influence of these cofactors on enzymatic reactions. Unfortunately, the problem of elucidating the mechanism of esterase action is much more difficult because the esterases are completely protein in nature and there is no cofactor.

Acetylcholinesterase has not yet been isolated in a completely pure state. Purified enzyme from the electric eel has an activity corresponding to 7×10^3 μmoles of acetylcholine hydrolyzed per minute per milligram of protein at 25° and pH 7.0 (12). The equivalent weight of the purified form has been estimated to be from 2×10^6 to 1.2×10^7 (13, 14). Much of the work on the mechanism of esterase inhibition has been carried out with pure compounds of lower molecular weight, such as crystalline chymotrypsin and trypsin. Classical experiments have shown that, when chymotrypsin is inhibited with DFP, TEPP, or paraoxon, 1 mole of inhibitor reacts with 1 mole of protein and liberates 1 mole of acid. One molecule of phosphorus, with its attached alkyl or alkoxy groups and oxygen atom, is bound to one molecule of enzyme (15–22). A similar conclusion was reached for acetylcholinesterase by calculating the equivalent weight from the amount of phosphorus bound to an enzyme preparation after complete inactivation with DFP and comparing this value with independently derived equivalent weights (17). The inhibition results from a stoichiometric, nonreversible, covalent reaction. The bimolecular rate constant for the reaction of sarin with eel cholinesterase was shown to be as high as 6.3×10^7 L. mole^{-1} minute^{-1} at 25° at pH 7.4 (10). Kinetic data showed also that reactions with the various inhibitors occur at different rates. The phosphorus was so tightly bound to both chymotrypsin and pseudocholinesterase that it could not be removed by treatment with trichloroacetic acid.

A mole-for-mole reaction of esterase with inhibitor indicates that there is one active site per mole of enzyme. A rather ingenious method for determining the concentration of active centers in enzyme preparations was presented by Cohen and Warringa (23). They treated crude ox red cell cholinesterase with butyrylcholine, a strong competitive inhibitor of the active center. The enzyme was then saturated with excess unlabeled DFP. Excess DFP and the reversibly combined butyrylcholine were then removed by dialysis. The activity of the resultant enzyme was determined and found to be 100% of the original activity. The preparation was then treated with radioactive DFP; the reaction product was dialyzed. The amount of radioactive phosphorus bound to the inactivated enzyme was then determined (24) and was used to calculate that there can be only one active center per equivalent of the enzyme.

MECHANISMS OF ACTION OF CHOLINESTERASES

We would do well to examine the fine structures of the active surfaces of cholinesterases before examining the mechanism of ester hydrolysis. Since Adams and Whittaker (25) suggested the presence of an anionic site on the surface of the enzyme, a number of reports on this general subject have been published by Wilson and by Bergmann (26–34). Their conclusions indicate that acetylcholinesterase contains two principal subsites: (*a*) an anionic site which binds and orients the cationic portion of the substrate and (*b*) an esteratic site, HG:, containing an acidic hydrogen atom and a basic group represented by a pair of electrons (Fig. 1*a*). The anionic site is largely concerned with specificity, while the esteratic site is supposedly responsible for the catalytic activity. A theory has been proposed that pseudocholinesterase has one anionic site for each esteratic site, while acetylcholinesterase has

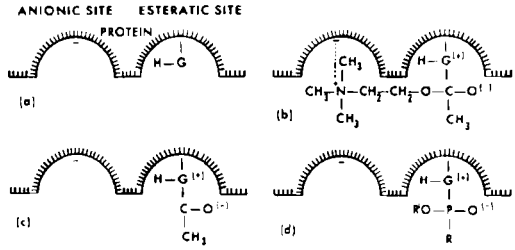

Fig. 1.—(*a*) Diagram of the active sites on the cholinesterase molecule; (*b*) diagram of the interaction between cholinesterase and acetylcholine; (*c*) diagram of acetylated cholinesterase; and (*d*) diagram of phosphorylated cholinesterase (70).

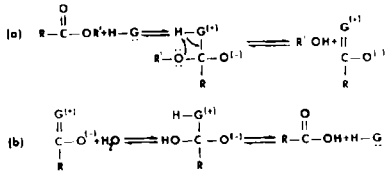

Fig. 2.—Mechanism of acetylcholine hydrolysis (R = CH_3; R′ = $[(CH_3)_3N—CH_2—CH_2]^+$) by cholinesterase (:G—H) (33).

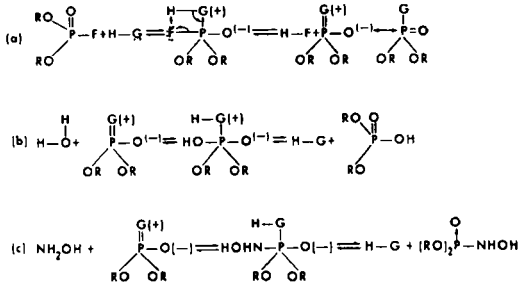

Fig. 3.—(*a*) Reaction of DFP (R = isopropyl) with cholinesterase; (*b*) regeneration of inhibited enzyme by water; and (*c*) regeneration of inhibited enzyme by hydroxylamine (48).

two anionic sites (35). Papers by Friess (36, 37) tend to confirm the two-site theory and in addition suggest that the esteratic site binds to a negative group of the ester rather than to the positive carbonyl carbon. The last concept is not widely accepted.

A diagram representing the interaction of acetylcholine with cholinesterase is shown in Fig. 1*b*. The combination of acetylcholine with the subsite of the enzyme is the rate-controlling step of the reaction. Both groups of the esteratic site are required for catalytic activity, but only the basic group is required for binding. Hydrolysis then occurs at the esteratic site, resulting in the unstable acetylated enzyme shown in Fig. 1*c* after diffusion away of the choline liberated in the reaction. Acetic acid promptly splits from this site, restoring the enzyme to its original state (Fig. 1*a*). As illustrated in Fig. 2, an incipient hydrogen bond sets off an electronic cycle which results in the splitting off of choline (if acetylcholine is the substrate), with the subsequent formation of an acyl enzyme. The acyl enzyme complex reacts with water within a few microseconds to produce regenerated enzyme and acid. The reaction with water apparently involves exchange of oxygen between carboxylate ions and water (30).

MECHANISMS OF ACTION OF CHOLINESTERASE INHIBITORS

Organophosphorus compounds also phosphorylate the enzyme at the esteratic site (Fig. 1*d*). The mechanism of inhibition of acetylcholinesterase parallels the reaction of the enzyme with its normal substrate. When DFP reacts with acetylcholinesterase, a reversible enzyme-inhibitor complex involving a covalent bond between the basic group of the esteratic site and the electrophylic phosphorus atom of DFP is formed. A transfer of a proton from the acid group to the halide atom follows, resulting in the splitting out of a halogen acid and the formation of phosphorylated enzyme (Fig. 3*a*). The reaction of the latter with water is now rate determining. Reactivation of cholinesterase now occurs rather slowly (Fig. 3*b*). The rate of hydrolysis would depend also on the nature of the alkyl group or groups within the phosphorylating agent.

Studies carried out with DFP-inhibited chymotrypsin, trypsin, acetylcholinesterase, and aliesterase (24, 38, 39) indicate that in each enzyme the phosphoryl group is attached to a hydroxy group of serine. Examination of the pH profile of acetylcholine hydrolysis, catalyzed by cholinesterase, indicates the existence of a basic group at an esteratic site which has a pKa value of about 6.5 (40, 41). Wagner-Jauregg and Hackley (42) pointed out that histidine is the only naturally occurring amino acid which (*a*) has a pKa value within this region and (*b*) can react with DFP in aqueous solution at neutral pH. Photochemical oxidation (43) of chymotrypsin in the presence of methylene blue led to the loss of one histidine residue and a corresponding loss of enzyme activity. There are, however, two amino groups in cystidyl diglycine which have pKa's of 6.36 and 6.96. Porter *et al.* (44) suggest that the serine molecule at the esteratic site is present in the form of a cyclic anhydride, a Δ2-oxazoline. The structure of the latter would be basic enough to react with DFP at a pKa value of 5. Bergmann (40) also does not believe that the serine molecule is representative of the esteratic site because it does not contain a group responsible for the measured pKa value and does not provide an explanation for the spontaneous hydrolysis of the intermediate acyl enzymes. Evidence, however, does favor the roles of the imidazole group of histidine and the hydroxyl group of serine as coparticipants in the active center.

Many researchers believe that acylation of the imidazole ring of histidine occurs first; they hold that a secondary reaction takes place during subsequent hydrolysis to produce phosphorylated serine. Peptides thus far isolated from inhibited enzyme indicate that histidine is a good distance from the serine in the peptide chain. However, it is reasonable to suppose that the peptide chains are flexible and that these two amino acids can approach one another. The hypothesis concerning migration of a phosphoryl group fits the results of work on the "aging" phenomenon, in which resistance to reactivation develops following initial inactivation of the enzyme (17, 45). A group in Holland (46) feels, however, that aging is not caused by transfer of the phosphoryl group, but rather by changes in structure of that group on the inhibited enzyme. They were able to show with C^{14}- and P^{32}-labeled DFP that in-

hibited pseudocholinesterase is spontaneously converted from a reactivatable diisopropyl phosphorylated enzyme into a nonreactivatable monoisopropyl enzyme. In both cases, the phosphorus was bound to a hydroxyl group in serine.

DEVELOPMENT OF OXIMES AS ANTIDOTES TO ORGANOPHOSPHATES

The value of oximes for treating organophosphate poisoning was suggested initially by experiments involving hydroxylamine. Hestrin (47) in 1949 found that acetylcholinesterase catalyzed the formation of acetohydroxamic acid when the enzyme was incubated with hydroxylamine and acetate. An analysis of this reaction suggests that the latter is bound to the enzyme, the resulting product then being attacked by hydroxylamine to release intact enzyme and hydroxamate. Wilson and his collaborators (27, 33, 48) subsequently demonstrated that cholinesterase inhibited by tetraethyl pyrophosphate (TEPP) can be reactivated rapidly by hydroxylamine and more slowly by choline. Reactivation with hydroxylamine is similar in mechanism to the reaction of the acetylated enzyme with water. The nitrogen atom reacts with the electrophylic center of the inhibited enzyme to produce a phosphorylated hydroxamate and reactivation of the enzyme (Fig. 3*c*).

Jandorf (49) showed that hydroxylamine reacts with sarin in a stoichiometric manner at neutral pH and room temperature. The mechanism indicated a mole-for-mole reaction between the two, followed, in excess hydroxylamine, by additional decomposition. Mole ratios between sarin and hydroxylamine greater than three increase markedly the rate of destruction of sarin above that by hydrolysis. This reaction is not the same as the reaction with acyl esters. Instead of the ester linkage's splitting, the P-F bond splits and 1 mole each of nitrogen and of ammonia are liberated (Fig. 4). Choline is, at best, a weak nucleophilic agent. Its activity is attributed to the electrostatic attraction of its quaternary ammonium center to the anionic site of the enzyme. Yet choline is almost completely inactive toward DFP-inhibited enzyme. It then appears that the anionic site is functional in TEPP-inhibited enzyme but not in that inhibited by DFP. Similarly, hydroxylamine partially reactivates diethyl phosphorylated chymotrypsin (50) but reactivates DFP-inhibited acetylcholinesterase somewhat less. A rational explanation for these differences in molecular behavior may be found in steric shielding of the anionic site by the large isopropyl radicals of DFP.

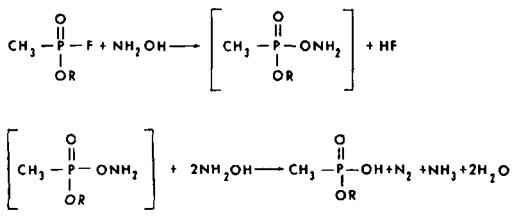

Fig. 4.—Reaction of hydroxylamine with sarin (R = isopropyl) (49).

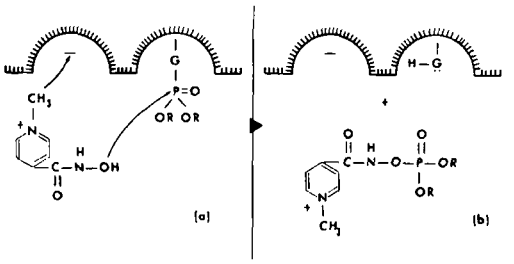

Fig. 5.—Diagrammatic illustration of the reactivation of DFP-inhibited enzyme by isonicotinic acid methiodide (52).

In a search for a more potent nucleophylic compound than hydroxylamine, nicotinehydroxamic acid (51) and the corresponding methiodide (the latter containing both a reacting nucleus and a suitably positioned cationic center) were prepared. Subsequent tests showed that these compounds were better reactivators than hydroxylamine. Isonicotinehydroxamic acid, picolinehydroxamic acid, and their corresponding methiodides were also prepared. Kinetic studies were consistent with the idea that the hydroxamate ions were the reactivating species (52). Reactivation of cholinesterase by picolinehydroxamic acid methiodide probably takes place *via* a nucleophilic attack on the phosphorus atom of the inhibited enzyme, followed by subsequent regeneration of the enzyme in a bimolecular displacement. Here, the hydroxamate may be phosphorylated by the mechanism proposed in Fig. 5. In all but one instance (53), quaternary hydroxamic acids were better than their tertiary analogs as reactivators of TEPP-inhibited enzyme and poorer for DFP-inhibited enzyme. Picolinehydroxamic acid was the exception in that it was more active than its quaternary derivative in reactivating cholinesterase inhibited by either TEPP or DFP. Wilson (53) explains this phenomenon as due, in part, to the low basicity of the zwitterion, which decreases the affinity of the hydroxamate for the phosphorus atom in the inhibited enzyme.

At the same time, Wagner-Jauregg (54), seeking compounds of low toxicity which would react

rapidly with sarin, synthesized a number of hydroxamates. A study of the chemical reaction between a hydroxamic acid and sarin showed that the first step is a reaction between the organophosphorus compound and the hydroxamate in which a phosphorylated hydroxamate and HF are formed. The phosphorylated hydroxamate undergoes a rearrangement in which hydrolyzed organophosphorus compound and isocyanate are formed. The isocyanate then reacts with another molecule of the hydroxamic acid to form *N*-phenylcarbamyl benzhydroxamate. Hurd and Bauer (55) had reported a similar mechanism when they reacted benzhydroxamic acid with benzenesulfonylchloride.

Wilson and Meislich (51) showed that DFP-inhibited cholinesterase could be reactivated *in vitro* by hydroxamates; however, there was a question whether hydroxamates would be effective on sarin-inhibited enzyme; although there are many similarities between the *in vitro* reactions of DFP and sarin, there are significant differences between their biochemical reactivities. Nicotinehydroxamic acid methiodide proved to be effective on sarin-inhibited enzyme also.

Hydroxamic acids having pKa values of 7.5 and higher accelerated the decomposition of DFP and sarin. Hackley *et al.* (56) determined the pKa's of 15 hydroxamic acids and reported that those with values between 7.8 and 9.3 reacted rapidly with sarin. Stolberg and Mosher (57) studied the effects of substituents in the *ortho* position of hydroxamates, using the rationale that the reaction rates of certain *ortho*-substituted phenyl derivatives were more than 100 times greater than would normally be expected. They found, however, that a vicinal hydroxamic group did not produce a marked increase in the rate of reaction of benzhydroxamic acids with sarin. *Cis*-hexahydrophthalohydroxamic acid, with a pKa of 9.75, was the most active of the series. Their

TABLE I.—REACTIVATION OF INHIBITED CHOLINESTERASE BY VARIOUS ISONITROSO COMPOUNDS (59)

Agent	Concn.	Reactivation (%) of Cholinesterase Inhibited by TEPP[a]	Sarin[b]	DFP[c]
Diisonitrosoacetone (DINA)	0.01	78	100	32
Monoisonitrosoacetone (MINA)	0.01	97	100	15
Isonitrosoacetophenone (INAP)	0.01	83	65	45
Isonitrosoacetylacetone (INAA)	0.01	24	33	4
Diacetylmonoxime (DAM)	0.01	11	5	0

[a] Inhibitor, 10^{-6} *M* for 10 minutes at 25°. [b] Inhibitor, 10^{-7} *M* for 10 minutes at 25°. [c] Inhibitor, 10^{-6} *M* for 10 minutes at 25°.

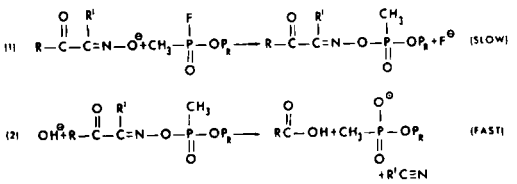

Fig. 6.—Reaction of MINA (R = CH_3; R' = H) with sarin (60).

measurements also indicated that the reactive form was the hydroxamate species, and that the increased activity was dependent only on the ionization constant of the hydroxamic acid. The reaction of sarin with hydroxamic acids followed a linear free energy Brönsted type of relationship when the reaction rates were plotted against the ionization constants. Steinberg *et al.* (58) indicated that in a series of hydroxamates an optimal ionization constant can be predicted for reactivation of inhibited enzyme.

With modified concepts of nucleophilicity and acidity in mind, a series of isonitroso compounds were prepared and tested (59) for their ability to reactivate inhibited cholinesterase; diisonitrosoacetone (DINA), monoisonitrosoacetone (MINA), isonitrosoacetophenone (INAP), isonitrosoacetylacetone (INAA), and diacetylmonoxime (DAM) were a few of the compounds investigated. The extents to which several of these isonitroso compounds reactivate acetylcholinesterase inhibited by TEPP, DFP, or sarin during a 10-minute reactivation period are given in Table I.

MECHANISMS OF ACTION OF OXIMES

Green and Saville (60) reacted monoisonitrosoacetone (MINA) with sarin and reported that 1 mole of oxime reacted for each mole of sarin decomposed. The reaction was accompanied by the liberation of 3 moles of acid—an aliphatic acid, hydrogen fluoride, and a phosphorus acid (Fig. 6). Second-order rate constants, determined from the rate of acid liberation, indicate that the rate-determining step is the initial reaction between the oxime and sarin.

A large variety of oximes, with pKa's ranging from 5 to 12, were subsequently examined. Working on the hypothesis that the anionic site survives inhibition of acetylcholinesterase by organophosphorus compounds, Wilson and Ginsburg (61) decided to combine a good reactivating group, such as an oxime, with a suitably placed quaternary nitrogen atom. Pyridine-2-aldoxime methiodide (Fig. 7*a*), better known as 2-PAM, P-2-AM, or 2-formyl, *N*-methylpyridinium oxime iodide (2FMPOI) resulted from these efforts.

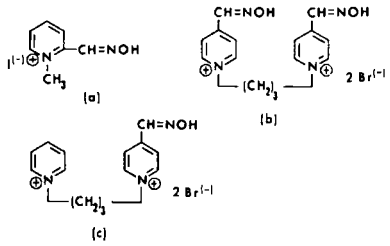

Fig. 7.—Structures of pyridinium aldoximes used in the therapy of nerve gas poisoning: (*a*) pyridine-2-aldoxime methiodide, 2-PAM or 2FMPOI; (*b*) 1,1′-trimethylene bis(4-formylpyridinium bromide) dioxime, TMB-4 or B4FPBOBr$_2$; (*c*) 1,1′-trimethylene bispyridinium-4-formyloxime dibromide or 4FPPOBr$_2$.

2-PAM was effective in reactivating enzymes inhibited by either TEPP or DEP; *i.e.*, this quaternary salt was 50,000 times faster than picolinehydroxamic acid and almost 10^6 times as active as NH_2OH when tested against TEPP-inhibited enzyme. At about the same time, Davies and Green (62) using a somewhat different train of reasoning (63), also found that 2-PAM *in vitro* is an effective reactivator of cholinesterase inhibited by organophosphorus compounds. Childs, Davies, *et al.* (59) reported work on a series of oximes, the most potent in reactivating cholinesterase inhibited by TEPP, DFP, or sarin being 2-PAM.

A and *B* configurations of the quaternary pyridinium aldoximes were prepared (64) and their physical properties listed. The *A* series was made by treating pyridine-2-carboxaldehyde methiodide (PCAM) with hydroxylamine at −5° while the *B* series was prepared by refluxing pyridine-2-aldoxime (P2A) with methyl iodide. The *A* form (colorless) was stable at low temperature but reverted to the *B* (yellow) form on standing at room temperature. The following facts led Wilson and Ginsburg to the postulation that the *B* series was of the anti configuration: (*a*) furan-2-aldehyde and pyrrol-2-aldehyde, when warmed with hydroxylamine, yield the anti configuration, while the syn configuration forms in the cold (65–68), (*b*) members of the *B* series have the higher acidities and therefore are anti because this configuration brings the hydroxyl group closer to the positive center and to the ring (69), and (*c*) the *A* series, having lower melting points and greater solubilities, corresponds to the syn series of benzene and pyrrole oximes.

Wilson (70) in 1959 advanced an explanation for the action of 2-PAM on the basis of molecular complementarity. From a series of experiments he determined that the esteratic site of cholinesterase is occupied by the organophosphorus moiety and that the anionic site is still functional. He then determined that: (*a*) an ideal reactivator should be bound to the organophosphorus-inhibited enzyme in such a way that a nucleophylic grouping, such as the oxygen in the anti configuration of 2-PAM, should fall one bond length from the phosphorus atom, (*b*) that the anti configuration of the 4-PAM molecule would also be satisfactorily oriented, and (*c*) that 3-PAM would not have the proper spatial requirements and would be devoid of enzyme reactivating activity. Experiments confirmed the inactivity of 3-PAM. The 2-PAM anti was 40 times more active than the 4-PAM anti, and the syn configuration of 2-PAM was inactive in reactivating inhibited enzyme. This report appeared to foreshadow the time when one could tailor therapeutic compounds on the basis of attainable physicochemical data.

Poziomek *et al.* (71) reinvestigated the reported configuration of syn and anti forms of the oximes and phenylhydrazones derived from pyridine-2-carboxaldehyde methiodide (PCAM) and found that there are differences of at least 29 mμ between the wavelengths of maximum absorption for the *A* and *B* series. Previous studies with isomeric benzaldehyde oximes (72, 73), furfuraldehyde oximes (74), and picolinaldehyde phenylhydrazones (75) had shown that there are only small differences between the absorption maxima. In addition, the group reported that PCAM forms hydroxaminohydroxymethyl, hydrazinehydroxymethyl, and hydroxyphenylhydrazomethyl derivatives with hydroxylamine, hydrazine, and phenylhydrazine, respectively; these various findings led to the proposal of carbinolamine structures—rather than oxime ones—for the *A* series. These results would cloud the configuration previously assigned to the syn configuration of 2-PAM and leave in doubt the configuration of anti-2-PAM. Data from our laboratories (76) suggest that the previously mentioned *A*-series 2-PAM is not a pure compound but a mixture of a carbinolamine and a geometric isomer (x-isomer) of 2-PAM. The *B* series, or the reputed *trans* 2-PAM, degrades in alkaline solution *via* a bimolecular (E2) elimination mechanism in which a β-hydrogen is removed and simultaneously a multiple covalent bond is formed. This type of mechanism is reported to possess stereospecificity (77–79) such that groups in the *trans* position are eliminated more rapidly than similar groups in the *cis* position. The rate of degradation of the x-isomer of 2-PAM in alkaline solution was about 75 times as rapid as that of the *B*-series 2-PAM. The x-isomer, then, should be the

trans configuration; consequently, the *B*-series 2-PAM would be the syn isomer. These results reverse present concepts concerning the configurations of the *A* and *B* series of 2-PAM. In addition, the rate of reactivation by syn-4-PAM of eel acetylcholinesterase inhibited by sarin was found to be nearly three times that by the corresponding anti-isomer (80). The molecular complementarity theory of Wilson predicts that anti-4-PAM would be the more active reactivator.

Irrespective of oxime configuration or structural requirements, the oxime with the lower pKa of a geometrically isomeric pair was shown to be the more efficient reactivator of inhibited acetylcholinesterase (81). There may be an inherent fallacy in generalizing the conclusion, however. It is generally accepted that the dissociated species of the oxime is responsible for reactivation of inhibited enzyme. In experiments performed at or near physiological pH, the oxime with the lower pKa will contain a greater fraction of the ionized form than that with the higher pKa value. The latter would not appear, then, to be as effective a reactivator as the former. On the other hand, a weakly nucleophilic oxime, even though completely dissociated, may be ineffective as a reactivator.

In vivo experiments also proved that 2-PAM was an effective antidote, especially when administered with atropine, against many anticholinesterase agents. Kewitz pointed out early that 2-PAM, being a quaternary salt, would be poorly soluble in lipids, so that its physiological actions would tend to be limited to those on peripheral effectors (81) and not to extend to the central nervous system (82). Wilson subsequently proposed (83) a quaternary pyridinium aldoxime in which the methyl group of 2-PAM would be replaced by an alkyl group of high molecular weight to increase its lipid solubility. Pyridine-2-aldoxime dodecyliodide (2-PAD) was synthesized and found to be 40,000 times more soluble in chloroform than 2-PAM and 200 times less soluble in water. The addition of 2-PAD to a mixture of 2-PAM and atropine, used as an antagonist to sarin in the mouse, raised the LD_{50} dose of sarin to nearly double that in mice given only the mixture of 2-PAM and atropine as antagonist. However, 2-PAD was far less effective than 2-PAM in reactivating a fly-brain cholinesterase preparation inhibited by DFP (84).

Another attempt to enhance the activity of the pyridinium aldoxime molecule was carried out by Poziomek *et al.* (85). They thought that, because compounds possessing two quaternary nitrogen atoms are more effective reversible inhibitors of acetylcholinesterase than those having a single charged group, a bis-quaternary oximino compound should form a stronger association complex with inhibited enzyme and thereby enhance the reactivation process. A series of 1,1′-polymethylene-bis-(4-formylpyridinium bromide) dioximes was prepared (Fig. 7*b*), in which the number of methylene groups between the pyridine rings varied from 2 to 10. Some of these compounds were more rapid reactivators of sarin-inhibited eel cholinesterase than 2-PAM. The most effective in treating poisoned animals was 1,1′-trimethylene-bis-(4-formylpyridinium bromide) dioxime, abbreviated TMB-4. Hobbiger *et al.* (86) independently and simultaneously, reached a similar conclusion. They prepared a number of monoximes and dioximes of bis-pyridinium ions (Fig. 7*b,c*) and studied the affinities of these compounds for cholinesterase, their interactions with TEPP, and their abilities to reactivate TEPP-inhibited cholinesterase. They reported that every compound in this series is a more potent reactivator than 2-PAM, that TMB-4 has the greatest therapeutic potential and indicated that, should TMB-4 be applicable to species other than mice, 2-PAM would become obsolete as an antidote for TEPP poisoning. Recently Loomis (87) synthesized a series of bis-quaternary bis-aldoximes and a tris-quaternary tris-oxime containing the benzene ring in the chain connecting the quaternary nitrogens. The bis-oximes with the lowest *in vitro* activities had the highest toxicities *in vivo*. The tris-oxime was a better reactivator of sarin-inhibited acetylcholinesterase than 2-PAM but was 26 times as toxic.

The reaction mechanism for the reactivation of sarin-inhibited enzyme by 2-PAM is postulated as a nucleophilic substitution by oximate ions on the phosphorus atom, giving rise to the product *O*-(isopropyl methylphosphono)-1-methyl-2-formylpyridinium iodide oxime, $(CH_3)_2-CH-O-P(O)(CH_3)-O-N=CH-C-(CH)_4-N^+-CH_3$.

Hackley and Owens (88) unsuccessfully attempted to prepare and isolate phosphorylated 2-PAM. However, the phosphorylated 4-PAM was synthesized. The compound decomposes at room temperature, but may be stored in a desiccator at 0° for several months with little decomposition. It was a potent inhibitor of eel acetylcholinesterase and quite toxic to mice, with an i.v. LD_{50} of 0.2 mg./Kg. If phosphorylated 2-PAM (P-2-PAM) is formed when inhibited enzyme is reactivated, one would expect

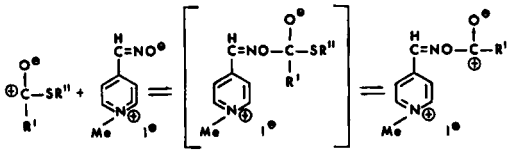

Fig. 8.—Reaction of a thioester with 4-PAM (90).

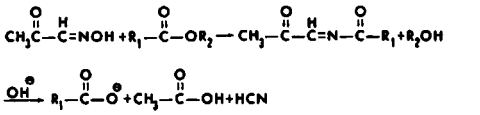

Fig. 9.—Reaction of MINA with an acylating agent in the body (99).

it also to be toxic. One supposes, because of the low toxicity of 2-PAM even in the presence of sarin (89), that P-2-PAM, upon forming in the body, is rapidly hydrolyzed to the corresponding nontoxic phosphorus-containing acid and some salt of intact oxime.

O'Neil *et al.* (90) proposed that part of the therapeutic effect of oximes may be due to their interference with acetylcholine synthesis. Their suggested mechanism (Fig. 8) is that oximes compete with choline for acetyl CoA (a thioester) and thereby prevent acetylation of choline. A mechanism which decreases the production of acetylcholine would have, of course, effects resembling to some extent those of reactivation of acetylcholinesterase. This type of reaction may be unimportant practically (91); however, we believe that interference with production of acetylcholine may be a useful therapeutic procedure in organophosphorus poisoning if sufficiently active, nontoxic compounds with this action can be found.

A study was recently conducted on the ability of pyridine-2-aldoxime to form chelates with metal ions (92), the rationale being that these compounds could be of value in treating poisoning by organophosphorus anticholinesterases because the hydrolysis of DFP is known (93, 94) to be accelerated by the presence of the cupric chelate of α,α'-dipyridyl, a compound related structurally to P2A. Courtney *et al.* (95) described a number of metal chelates which catalyze hydrolyses of DFP and sarin. A number of stable chelates of P2A are being tested for their physiological activities in antagonizing poisoning by organophosphorus compounds.

DISTRIBUTION AND METABOLISM OF OXIMES

As would be expected, the oximes have definite toxic actions. Hydroxylamine reacts with many substances normally found in the body and is appreciably toxic. Riemann (96) reported that the feeding of hydroxylamine hydrochloride to rats resulted in a considerable enlargement of the spleen and a marked reduction in the size of the thyroid gland. Hydroxylamine affects a number of enzyme systems; for example, it inhibits tryptophan pyrrolase, both *in vivo* and *in vitro* (97), glutamic-oxalacetic transaminase in liver and brain, and glutamic-pyruvic transaminase in liver.

Although many studies have been performed on the reaction *in vitro* of hydroxamic acids with organophosphorus compounds, relatively little work has been done *in vivo*. Reports concerning the distribution and fate of hydroxamates in animals or humans have not been published. Epstein and Freeman (98) studied the prophylactic and therapeutic efficacies of a number of selected hydroxamic acid derivatives in protecting against sarin in mice and stated that the large doses required for protective action would limit the usefulness of these compounds in humans. Of the aliphatic isonitroso derivatives, DINA was the most toxic, followed by MINA and DAM, successively.

The breakdown of DINA is accompanied by the production of 2 moles of hydrogen cyanide (HCN). The breakdown of MINA (Fig. 9) gives rise to 1 mole of HCN, but there is no production of HCN during the biotransformation of DAM (99, 100). It was felt that sufficient amounts of HCN could build up from doses of DINA and MINA to account for their toxicity; however, the acute toxicity of DAM would be due to the compound itself. Sodium thiosulfate and sodium nitrite were subsequently shown to protect rats against poisoning by MINA. A small increase in urinary excretion of thiocyanate has been observed after administration of large doses of 2-PAM to rats (101), but the amount of cyanide formed in the body during metabolism of this oxime was considered to be too small to contribute to the compound's toxicity.

In experiments with MINA in dogs and rabbits Askew *et al.* (99) showed that there is an inverse relationship between the concentrations of cyanide in plasma and in whole blood; that is, samplings showed a steady decrease in concentration of oxime accompanied by a corresponding rise in the concentration of cyanide in plasma. Maximum concentrations of MINA and DAM in blood were found within 10 minutes after i.p. injections of the oximes. Rutland (102), in studying the disappearance of MINA and DAM from the blood of male albino rats

after i.p. injections of the oximes, found that maximum concentrations of oxime in blood were reached within an average of 12 minutes. Dultz *et al.* (100) showed that in rats DAM enters rapidly into most tissues, but somewhat slower into skeletal muscle and fat. Studies were also performed on the disappearance of DAM in the presence of tissue slices. Incubation of DAM with slices of rat liver resulted in a 25% reduction of the concentration of this oxime in the reaction vessel during the first hour; brain and kidney produced only a 5% decrease in oxime concentration, while other tissues caused even smaller decreases.

Jager *et al.* (103) studied the distribution, destruction, and elimination of DAM in humans. They found that the time required for the concentration of DAM in serum to fall 50% from its initial level after i.v. injection of the oxime was more than 7 hours. Dultz (100) reported times of 4.5 hours for dogs and approximately 2 hours for rats. The rather low rate of elimination of DAM from plasma could be significant in determining the potential usefulness of this oxime as a prophylactic agent against poisoning by organophosphorus anticholinesterases. After 6 hours, less than 10% of the DAM injected was found in the urine. DAM was found within 1 hour after its injection in the cerebrospinal fluid, indicating that the so-called blood-brain barrier is relatively permeable to this oxime.

If a choice of a therapeutic agent for poisoning by sarin, DFP, etc., had to be made today, the most likely candidate would be some salt of 2-PAM. Though other compounds may be found at some future date to be of greater value, this oxime is effective and, in addition, there is a comparatively large amount of information about its toxicity, pharmacology, stability, and metabolism. Table II gives recoveries of oxime from pooled rabbit tissues and fluids after 50 mg./Kg. of 2-PAM was injected intravenously during a 1-hour period. The greatest concentration of 2-PAM was found in the kidney.

In a study undertaken to determine the distribution of 2-PAM within experimental animals, 2-PAM was synthesized with a C^{14} label in the methyl group (112). Though only 64% of the labeled compound injected was recovered, the distribution of the label among the tissues of injected mice was measured. The liver, duodenum, jejunum, ileum, kidney, caecum, colon, and blood contained about 20% of the activity after 1 hour; the urine contained 44%. Only trace amounts of the label were found in the brain. A labeled metabolite identified tentatively as *N*-methyl-2-pyridone was an important excretory product. Ten minutes after i.v. injection of the labeled oxime into a cat, the blood was estimated to contain only about 5% of the label; after 2.5 hours, only 0.3% remained in the blood.

TABLE II.—CONCENTRATION OF 2-PAM IN RABBIT SERUM AND TISSUES AFTER INTRAVENOUS INJECTION OF 50 mg./Kg. (103)

Tissue	Concn.[a] Animal No. 1	Animal No. 2
Serum	1.5	0.7
Spleen	2.0	1.5
Liver	2.9	1.3
Kidney	10.3	4.5
Brain	0.6	0.1[b]

[a] Concentration expressed as milligrams of PAM per 100 ml. of serum or per 100 Gm. (wet weight) of tissue. [b] Less than 0.6 mg. PAM cannot be measured accurately with the technique employed for tissues.

Two good analytical methods are available for determining 2-PAM in biological material. The method of Blom (104), later modified by Csaky (105), can be applied to any oxime. After deproteinization of a sample, the supernatant is hydrolyzed with acid on a boiling water bath. The resulting hydroxylamine is oxidized by iodine to nitrous acid, which is used to diazotize sulfanilic acid. The resulting diazonium salt is coupled with *N*-(1-naphthyl) ethylenediamine dihydrochloride to produce a violet color. The method is applicable to most experiments, but there were several pitfalls. One must be certain that the pH of the acid solution used in the hydrolysis is effective in producing quantitative splitting. We found that various conditions of equilibrium tend to cause incomplete reactions.

A much simpler method may be used. In acid solution, 2-PAM exhibits an absorption peak at 292 mμ; in basic solution, 2-PAM shows a significant bathochromic shift at 335 mμ, which is attributed to the oximate anion (106). This phenomenon is quite advantageous, for at this wavelength the likelihood of interference from most biological materials is greatly reduced. The method is about 100 times more sensitive than the colorimetric assay and has been adapted for the analysis of 2-PAM in biological tissues and fluids by several authors (107–109). Creasey and Green (107) report reproducible determination of 2-PAM in skeletal muscle, blood, liver, kidney, urine, and feces of the rat.

When 2-PAM is incubated aerobically with rat liver, it is completely metabolized. Unlike DAM, 2-PAM is removed from the human body primarily by renal excretion. The half-life in man of 2-PAM, given in an i.v. dose of 15 mg./Kg., was found to be 0.8 hours (103). Approximately 80% of the oxime was excreted within 6 hours, most of the unchanged oxime being found

in the urine within the first 30 minutes. No appreciable binding of 2-PAM to human serum protein was observed, and the oxime did not enter the erythrocytes. The excretion of 2-PAM in urine is, therefore, intrinsically rapid. Studies of the prophylactic activity of the oxime *in vivo* (110, 110*a*) indicated the need for appreciable concentrations in the blood. The persistence of effective concentrations of oxime in the blood after administration of a dose of a salt of 2-PAM should be prolonged by preventing rapid renal elimination of the oxime. Berglund *et al.* (109) demonstrated that the renal elimination of 2-PAM is markedly reduced in dogs which receive an intravenous infusion of sodium bicarbonate.

Sundwall (111) investigated the rate of absorption of 2-PAM in human volunteers after intramuscular or oral administration. After intramuscular injection into 10 subjects (in a dosage of 30 mg./Kg.), a maximum concentration in plasma averaging about 15 mcg. per ml. was found after approximately 20 minutes. A progressive fall in the concentration of oxime ensued. After 90 minutes the concentration was about 9 mcg. per ml. A plot of individual results indicated that there were two rates of absorption from intramuscular injections: one fairly rapid, with the plasma concentration rising to about 20 mcg. per ml. after 5 minutes, and a slower one, with the plasma concentration rising to only 10 mcg. per ml. after 20 minutes. After oral administration, a slow rise in plasma concentration occurred, reaching a maximum in about 2.5 hours. Twenty-three per cent of the oral dose was excreted within 4 hours. Later Sundwall (111) designed experiments to determine the effectiveness of various concentrations of P2S (pyridine-2-aldoxime methylmethanesulfonate) in antagonizing the neuromuscular block produced by organophosphorus cholinesterase inhibitors. Anesthetized cats were given approximately one LD_{50} of inhibitor and then an intramuscular injection of oxime. Plasma levels of P2S above 4 mcg. per ml. counteracted neuromuscular block, bradycardia, hypotension, and respiratory failure.

Recently Barkman (113) determined plasma levels of P2S in human volunteers following the self-administration of 1.5 ml. of a 50% concentration of oxime (a dose of about 10 mg./Kg.) contained in an automatic injector. Within 6 minutes, plasma concentrations of over 4 mcg./ml. were reached; they were maintained for about 90 minutes. After a dose of 20 mg./Kg., the plasma concentration of oxime was maintained above 4 mcg./ml. for about 170 minutes.

One significant and controversial point still exists: 2-PAM is essentially an ionic compound and thus quite polar. It would not be expected to cross the blood brain barrier. Jager *et al.* (103) were not able to demonstrate 2-PAM in cerebrospinal fluid 70 minutes after it had been administered intravenously. Recently Clark and Roth (114) reported the synthesis of 2-PAM with a C^{14} atom in the carbonyl position of the side chain. Autoradiography of the rat brain following i.v. injection of 20 mg./Kg. of labeled 2-PAM showed (115) that unmetabolized 2-PAM entered all anatomical areas of the brain in concentrations shown previously to be adequate for reactivation of inhibited acetylcholinesterase. For example, 1.2 mcg. of oxime per gram of wet tissue was found in cerebral cortex 10 minutes after injection of the oxime. The conclusion of Roth's group that a quaternary compound can pass the blood-brain barrier readily is contrary to the generally accepted view that quaternary amines enter the CNS much more slowly than the corresponding tertiary amines.

The question of whether 2-PAM penetrates brain is one on which there are conflicting opinions and inconsistent evidence. Although therapeutic doses of 2-PAM will reactivate *in vitro* cholinesterase of brain inhibited by organophosphorus compounds and will reactivate *in vivo* cholinesterases of muscle, blood, and parotid gland, they do not reactivate *in vivo* cholinesterase of brain (81, 82, 102, 116–120). Kewitz (81), and Kewitz and Nachmansohn (82), and Rosenberg (121) found that use of repeated or enlarged doses of 2-PAM is capable of reactivating cholinesterase in brain, but Rutland (102) found that an i.p. dose of 100 mg./Kg. of 2-PAM reactivated only 4% of the cholinesterase of the brain of the rat after intoxication *in vivo* by sarin.

Sakai *et al.* (122) reported that 2-PAM is unable to overcome the paralysis of respiratory centers induced by parathion or paraoxon. On the other hand, Wislicki (123) found that 2-PAM, injected i.v. into the anesthetized (sodium pentobarbital) cat, promptly slows the rate of repetition of phrenic volleys, with the appearance of a relative inspiratory apneusis, and Brown *et al.* (124) reported rapid improvement in ventilation in animals poisoned with tabun or sarin and then treated first with atropine and later with 2-PAM. They believe that the improvement is too abrupt to be explained solely by reactivation of cholinesterase. Longo *et al.*

(125) found that, although i.v. injection of 2-PAM has no effect on the EEG of the rabbit, administration of 2-PAM before i.v. injection of sarin doubles or triples the dose of Sarin required to establish a "grand mal" pattern; prophylaxis with 2-PAM was said to abolish entirely the desynchronization of the EEG pattern induced by sarin prior to the appearance of the "grand mal" pattern. Brown (126) reported that P2S, even when injected intracisternally, had no effect on the respiratory paralysis and cardiovascular actions of intracisternal injections of sarin. Edery reported (127), however, that cerebral intraventricular injections of oximes, including 2-PAM, do have some effectiveness in overcoming the toxic effects from cerebral intraventricular injections of ethyl pyrophosphate. Tong and Way (128) found that cerebral intraventricular injection of 2-PAM is capable of modifying the lethal effects of injections of paraoxon, TEPP, or DFP following i.p. injections of atropine and 2-PAM, protection against the lethal actions of paraoxon and TEPP being especially marked.

In addition to the evidences of some central action of 2-PAM cited previously, this oxime has been noted to induce rapid recovery of consciousness in patients poisoned by parathion (129–132) and to have an anticonvulsant action in the same type of patient (130, 132). One is forced to conclude, therefore, that 2-PAM has some ability to penetrate the blood-brain barrier despite its quaternary character and to induce effects dependent upon the presence of the oxime within the brain. The work of Firemark and his collaborators (115) seems to show that significant crossing of the blood-brain barrier by 2-PAM can occur within 10 minutes in the rat.

Studies of the metabolism of 2-PAM are incomplete and are presently proceeding within a number of laboratories. *In vitro* studies indicate that 2-PAM forms the following products in aqueous solutions at various pH values: 2-cyanopyridine methiodide, pyridine-2-carboxaldehyde methiodide, pyridine-2-carboxylic acid methiodide, 2-carbamidopyridine methiodide, and *N*-methyl-α-pyridone (133–135). A graphic representation of the reactions involved in the degradation of 2-PAM is shown in Fig. 10. The products of decomposition do not interfere with the ultraviolet analysis of 2-PAM in basic media and are readily separated and identified by paper chromatographic techniques (136).

Kramer (137) analyzed human urine following oral administration of 2-PAM. His report describes the isolation of both the intact drug and a fluorescent metabolite. The latter is described as highly polar and having both a carboxyl function and a weakly acidic group. A number of procedures used to characterize the metabolite indicated that it was a derivative of *N*-methyl-picolinic acid.

Since the liver had been implicated as the major biotransformation site of several oximes, Way *et al.* (138–140) studied the metabolism of C^{14} radiolabeled 2-PAM by perfusing the oxime through isolated rat liver. They eventually separated two radioactive fractions: one had ultraviolet spectral characteristics similar to those of 2-PAM, and the other was thought to be an *O*-conjugated form of *N*-methyl-α-pyridone. Later, Way (141) described the isolation and characterization of a second metabolite, methyl-2-cyanopyridinium ion, by procedures involving adsorption on charcoal, paper electrophoresis, ion-exchange chromatography, paper chromatography, and ultraviolet analysis.

Kalser (112) found at least six chromatographically separable metabolites in the urines of mice as early as 20 minutes after injection of 2-PAM. She indicated that one metabolite was

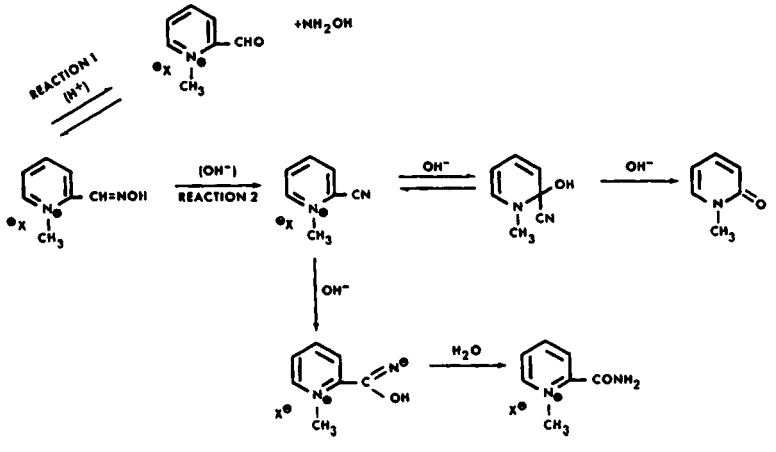

Fig. 10.—Mechanism of the decomposition of 2-PAM in aqueous solution (133–135).

probably *N*-methyl-α-pyridone. Firemark *et al.* (115) reported that rat urine contained as many as 11 distinct chromatographic bands. The largest band was identified as unmetabolized 2-PAM. No attempt was made to identify the other products.

Since 2-PAM is known to produce cyanide ion *in vitro* at physiological pH, experiments were carried out by Enander *et al.* (101, 142, 143) with the methanesulfonate salt (P2S) to determine whether formation of cyanide contributes to the toxicity of the oxime. The increase in excretion of thiocyanate, the product of detoxication of cyanide in the body, in the urines of humans given P2S orally was statistically insignificant. Another compound, metabolite *X*, that is easily converted to cyanide but is not identical with thiocyanate, was found also. The same investigations subsequently detected 2-cyanopyridinium ion (PCNM) in the urines of rats and humans given P2S by mouth. The amount in rat urine during the first day corresponds to about 8% of the oxime administered, opposed to 0.3% in the urines of human volunteers. Thiocyanate and a compound with properties similar to those of metabolite *X* were subsequently found in the urines of rats injected with PCNM. P2S appears, therefore, to be converted to PCNM, which then is degraded to metabolite *X* and thiocyanate.

This group of investigators (143) also found that 80 to 90% of C^{14}-labeled 2-PAM injected into rats was excreted in the urine within 24 hours. A radiochromatograph of the urine demonstrated the presence of five compounds. The major component was unchanged oxime. A second component was identified as PCNM. A third component had an R_f value identical with that of *N*-methyl-pyridinium-carboxylic acid. Another peak had the same R_f value as metabolite *X*. About 5% of the injected radioactivity was recovered as *N*-methyl-α-pyridone picrate from the urines of rats; the same metabolite was also demonstrated in mice.

REFERENCES

(1) Aldridge, W. N., *Biochem. J.*, **53**, 110(1953).
(2) Goutier, R., *Biochim. Biophys. Acta*, **19**, 524(1956).
(3) Dale, H. H., *J. Pharmacol.*, **6**, 147(1914).
(4) Loewi, O., and Navratil, E., *Arch. Ges. Physiol.*, **214**, 678(1926).
(5) Mendel, B., and Rudney, H., *Biochem. J.*, **37**, 59 (1943).
(6) Ord, M. J., and Thompson, R. H. S., *ibid.*, **46**, 347 (1950).
(7) Augustinsson, K., *Acta Physiol. Scand.*, **15** (Suppl. 52), 158(1948).
(8) Burn, J. H., *Pharmacol. Rev.*, **6**, 107(1954).
(9) Dixon, M., and Webb, E. C., "Enzymes," Academic Press, New York, N. Y., 1958.
(10) Hirs, C. H., Moore, S., and Stein, W. A., *J. Biol. Chem.*, **235**, 633(1960).
(11) Jandorf, B. J., *J. Agr. Food Chem.*, **4**, 853(1956).
(12) Wilson, I. B., in Boyer, P. D., Lardy, H., and Myrbäck, K., "The Enzymes," Vol. 4, Academic Press, New York, N. Y., 1960, p. 501.
(13) Rothenberg, M. A., and Nachmansohn, D., *J. Biol. Chem.*, **168**, 223(1947).
(14) Lawler, H. C., *Federation Proc.*, **19**, 337(1960).
(15) Balls, A. K., and Jansen, E. F., *Advan. Enzymol.*, **13**, 321(1952).
(16) Hartley, G. S., and Kilby, B. A., *Nature*, **166**, 784 (1950).
(17) Jandorf, B. J., Michel, H. O., Schaffer, N. K., Egan, R., and Summerson, W. H., *Discussions Faraday Soc.*, **20**, 134 (1955).
(18) Jansen, E. F., and Balls, A. K., *J. Biol. Chem.*, **194**, 721(1952).
(19) Jansen, E. F., Fellows-Nutting, M. D., Jang, R., and Balls, A. K., *ibid.*, **185**, 209(1950).
(20) *Ibid.*, **179**, 201(1949).
(21) Kilby, B. A., and Yowatt, G., *Biochim. Biophys. Acta*, **8**, 112(1952).
(22) Boursnell, J. C., and Webb, E. C., *Nature*, **164**, 875 (1949).
(23) Cohen, J. A., and Warringa, M. G. P. J., *Biochim. Biophys. Acta*, **11**, 52(1953).
(24) Cohen, J. A., Oosterbaan, R. A., Warringa, M. G. P. J., and Jansz, H. S., *Discussions Faraday Soc.*, **20**, 144 (1958).
(25) Adams, D. H., and Whittaker, V. P., *Biochim. Biophys. Acta*, **4**, 543(1950).
(26) Bergmann, F., and Shimoni, E., *Biochem. J.*, **55**, 50 (1953).
(27) Wilson, I. B., and Bergmann, F., *J. Biol. Chem.*, **185**, 479(1950).
(28) *Ibid.*, **186**, 683(1950).
(29) Wilson, I. B., *ibid.*, **197**, 215(1952).
(30) Wilson, I. B., *Biochim. Biophys. Acta*, **7**, 520(1951).
(31) Bergmann, F., Wilson, I. B., and Nachmansohn, D. N., *J. Biol. Chem.*, **186**, 693(1950).
(32) Wilson, I. B., *Discussions Faraday Soc.*, **20**, 119 (1955).
(33) Wilson, I. B., Bergmann, F., and Nachmansohn, D., *J. Biol. Chem.*, **186**, 781(1950).
(34) Wilson, I. B., *ibid.*, **199**, 113(1952).
(35) Bergmann, F., *Discussions Faraday Soc.*, **20**, 132 (1955).
(36) Friess, S. L., and Baldridge, H. D., *J. Am. Chem. Soc.*, **78**, 966(1956).
(37) Friess, S. L., and McCarville, W. J., *ibid.*, **76**, 1363, 2260(1954).
(38) Schaffer, N. K., May, S. C., and Summerson, W. H., *J. Biol. Chem.*, **202**, 67(1953).
(39) *Ibid.*, **206**, 201(1954).
(40) Bergmann, F., *Discussions Faraday Soc.*, **20**, 126 (1955).
(41) Mounter, L. A., Alexander, H. C., Tuck, K. D., and Dien, L. T. H., *J. Biol. Chem.*, **226**, 867(1957).
(42) Wagner-Jauregg, T., and Hackley, B. E., *J. Am. Chem. Soc.*, **75**, 2125(1953).
(43) Weil, L., James, S., and Buchert, A. R., *Arch. Biochem. Biophys.*, **46**, 266(1953).
(44) Porter, G. R., Rydon, H. N., and Scholfield, J. A., *Nature*, **182**, 927(1958).
(45) Hobbiger, F., *Brit. J. Pharmacol.*, **10**, 356(1955).
(46) Jansz, H. S., Berends, F., and Oosterbaan, R. A., *Rec. Trav. Chim.*, **78**, 876(1959).
(47) Hestrin, S., *J. Biol. Chem.*, **180**, 879(1949).
(48) Wilson, I. B., *ibid.*, **190**, 111(1951).
(49) Jandorf, B. J., *J. Am. Chem. Soc.*, **78**, 3686(1956).
(50) Cunningham, L. W., *J. Biol. Chem.*, **207**, 443 (1954).
(51) Wilson, I. B., and Meislich, E. K., *J. Am. Chem. Soc.*, **75**, 4629(1953).
(52) Wilson, I. B., *ibid.*, **77**, 2385(1955).
(53) Wilson, I. B., and Ginsburg, S., *Arch. Biochem. Biophys.*, **54**, 570(1955).
(54) Wagner-Jauregg, T., *Arzneimittel-Forsch.*, **6**, 194 (1956).
(55) Hurd, C. J., and Bauer, L., *J. Am. Chem. Soc.*, **76**, 2791(1954).
(56) Hackley, B. E., Jr., Plappinger, R., Stolberg, M., and Wagner-Jauregg, T., *ibid.*, **77**, 3651(1955).
(57) Stolberg, M. A., and Mosher, W. A., *ibid.*, **79**, 2618 (1957).
(58) Steinberg, G. M., Swidler, R., and Seltzer, S., *Science*, **125**, 336(1957).
(59) Childs, A. F., Davies, D. R., Green, A. L., and Rutland, J. P., *Brit. J. Pharmacol.*, **10**, 462(1955).
(60) Green, A. L., and Saville, B., *J. Chem. Soc.*, **1956**, 3887.
(61) Wilson, I. B., and Ginsburg, S., *Biochim. Biophys. Acta*, **18**, 168(1955).
(62) Davies, D. R., and Green, A. L., *Discussions Faraday Soc.*, **20**, 269(1955).
(63) Davies, D. R., and Green, A. L., *Brit. J. Ind. Med.*, **16**, 128(1959).
(64) Wilson, I. B., and Ginsburg, S., *J. Am. Chem. Soc.*, **79**, 481(1957).
(65) Wentworth, V., and Brady, O. L., *J. Chem. Soc.*, **117**, 1040(1920).
(66) Brady, O. L., and Grayson, H. J., *ibid.*, **125**, 1418 (1924).
(67) Brady, O. L., and Goldstein, R. F., *ibid.*, **1927**, 1959.
(68) Teretev, A. P., and Markova, A. N., *J. Gen. Chem., USSR*, **21**, 270(1951).

(69) Potwardham, N. K., and Deshpande, S. S., *J. Indian Chem. Soc.*, **21**, 135(1944); through *Chem. Abstr.*, **39**, 1390 (1945).
(70) Wilson, I. B., *Federation Proc.*, **18**, 752(1959).
(71) Poziomek, E. J., Kramer, D. N., Fromm, B. W., and Mosher, W. A., *J. Org. Chem.*, **26**, 423(1961).
(72) Meisenheimer, J., and Dorner, O., *Ann.*, **502**, 156 (1933).
(73) Rekker, R. F., and Veenland, J. U., *Rec. Trav. Chim.*, **78**, 739(1959).
(74) Raffauf, R. F., *J. Am. Chem. Soc.*, **68**, 1765(1946).
(75) Schulte-Fröhlinde, D., Kuhn, R., Münzing, W., and Otting, W., *Ann.*, **622**, 43(1959).
(76) Ellin, R. I., unpublished data, 1963.
(77) Michael, A., *J. Pract. Chem.*, **52**, 308(1895).
(78) Cristol, S. J., and Begoon, A., *J. Am. Chem. Soc.*, **74**, 5025(1952).
(79) Miller, S. I., and Noyes, J., *ibid.*, **74**, 629(1952).
(80) Poziomek, E. J., Kramer, D. N., Mosher, W. A., and Michel, H. O., *ibid.*, **83**, 3916(1961).
(81) Kewitz, H., *Arch. Biochem. Biophys.*, **66**, 263(1957).
(82) Kewitz, H., and Nachmansohn, D., *ibid.*, **66**, 271 1957).
(83) Wilson, I. B., *Biochim. Biophys. Acta*, **27**, 196(1958).
(84) Mengle, D. C., and O'Brien, R. D., *Biochem. J.*, **75**, 201(1960).
(85) Poziomek, E. J., Hackley, B. E., Jr., and Steinberg, G. M., *J. Org. Chem.*, **23**, 714(1957).
(86) Hobbiger, F., O'Sullivan, D. G., and Sadler, P. W., *Nature* **182**, 1498(1958).
(87) Loomis, T. A., Welsh, M. J., Jr., and Miller, G. T., *Toxicol. Appl. Pharmacol.*, **5**, 588(1963).
(88) Hackley, B. E., Jr., and Owens, O. O., *J. Org. Chem.*, **24**, 1120(1959).
(89) Oberst, F. W., Crook, J. W., and Koon, W. S., *J. Pharmacol. Exptl. Therap.*, **136**, 393(1962).
(90) O'Neil, J. J., Kohl, H., and Epstein, J., *Biochem. Pharmacol.*, **8**, 399(1961).
(91) Holmstedt, B., *Pharmacol. Rev.*, **11**, 592(1959).
(92) Bolton, S., and Ellin, R. I., THIS JOURNAL, **51**, 533 (1962).
(93) Wagner-Jauregg, T., Hackley, B. E., Jr., Lies, T. A., Owens, O. O., and Proper, R., *J. Am. Chem. Soc.*, **77**, 922 (1955).
(94) Mounter, L. A., Floyd, C. S., and Chanutin, A., *J. Biol. Chem.*, **204**, 221(1953).
(95) Courtney, R. C., Gustafson, R. L., Westerback, S. J., Hyytiainen, H., Chaberek, S. C., Jr., and Martell, A. E., *J. Am. Chem. Soc.*, **79**, 3030(1957).
(96) Riemann, H., *Acta Pharmacol. Toxicol.*, **6**, 285(1950).
(97) Choi, S. H., *New Med. J. (Seoul, Korea)*, **3**, 69(1960); through *Chem. Abstr.*, **55**, 5783(1960).
(98) Epstein, N. A., and Freeman, G., *Proc. Soc. Exptl. Biol. Med.*, **92**, 660(1956).
(99) Askew, B. M., Davies, D. R., Green, A. L., and Holmes, R., *Brit. J. Pharmacol.*, **11**, 424(1956).
(100) Dultz, L., Epstein, M. A., Freeman, G., Gray, E. H., and Weil, W. B., *J. Pharmacol. Exptl. Therap.*, **119**, 522 (1957).
(101) Enander, I., Sundwall, A., and Sörbo, B., *Biochem. Pharmacol.*, **7**, 228(1961).
(102) Rutland, J. P., *Brit. J. Pharmacol.*, **13**, 399(1958).
(103) Jager, B. V., Stagg, G. N., Green, N., and Jager, L., *Bull. Johns Hopkins Hosp.*, **102**, 225(1958).
(104) Blom, J., *Ber. Deut. Chem. Ges.*, **59**, 121(1926).
(105) Csaky, T. Z., *Acta Chem. Scand.*, **2**, 450(1948).
(106) Ellin, R. I., and Kondritzer, A. A., *Anal. Chem.*, **31**, 200(1959).
(107) Creasey, N. H., and Green, A. L., *J. Pharm. Pharmacol.*, **11**, 485(1959).
(108) Zvirblis, P., and Kondritzer, A. A., unpublished data, 1959.
(109) Berglund, F., Elwin, C., and Sundwall, A., *Biochem. Pharmacol.*, **11**, 383(1962).
(110) Sundwall, A., *ibid.*, **5**, 225(1960).
(110a) Crook, J. W., *et al.*, *J. Pharmacol. Exptl. Therap.*, **136**, 397(1962).
(111) *Ibid.*, **8**, 413(1961).
(112) Kaiser, S. C., unpublished data, 1959.
(113) Barkman, R., Edgren, B., and Sundwall, A., *J. Pharm. Pharmacol.*, **15**, 671(1963).
(114) Clark, L., and Roth, L. J., THIS JOURNAL, **52**, 961 (1963).
(115) Firemark, H., Roth, L. J., and Barlow, C. F., *Pharmacologist*, **4**, 174(1962).
(116) Kewitz, H., *Klin. Wochschr.*, **35**, 521(1957).
(117) Hobbiger, F., *Brit. J. Pharmacol.*, **12**, 438(1957).
(118) Edery, H., and Schatzberg-Porath, G., *Science*, **128**, 1137(1958).
(119) Wills, J. H., *Federation Proc.*, **18**, 1020(1959).
(120) Cohen, E. M., and Wiersinga, H., *Acta Physiol. Pharmacol. Neerl.*, **9**, 276(1960).
(121) Rosenberg, P., *Biochem. Pharmacol.*, **3**, 212(1960).
(122) Sakai, F., dal Ri, H., Erdmann, W. D., and Schmidt, G., *Arch. Exptl. Pathol. Pharmakol.*, **234**, 210(1958).
(123) Wislicki, L., *Arch. Intern. Pharmacodyn.*, **129**, 1 (1960).
(124) Brown, R. V., Kunkel, A. M., Somers, L. M., and Wills, J. H., *J. Pharmacol.*, **120**, 276(1957).
(125) Longo, V. G., Nachmansohn, D., and Bovet, D., *Arch. Intern. Pharmacodyn.*, **123**, 282(1960).
(126) Brown, R. V., *Brit. J. Pharmacol.*, **15**, 170(1960).
(127) Edery, H., *ibid.*, **18**, 19(1962).
(128) Tong, H. S., and Way, J. L., *J. Pharmacol. Exptl. Therap.*, **138**, 218(1962).
(129) Namba, T., and Hiraki, K., *J. Am. Med. Assoc.*, **166**, 1834(1958).
(130) Karlog, O., Nimb, M., and Poulsen, E., *Ugeskrift Laeger*, **120**, 177(1958).
(131) Schuchter, A., Kawel, H. G., and Schneider, J. A., *Arzneimittel-Forsch.*, **10**, 399(1960).
(132) Funckes, A. J., *Arch. Environ. Health*, **1**, 404(1960).
(133) Ellin, R. I., *J. Am. Chem. Soc.*, **80**, 6588(1958).
(134) Ellin, R. I., Carlese, J. S., and Kondritzer, A. A., THIS JOURNAL, **51**, 141(1962).
(135) Patton, J. W., Doctoral Dissertation, University of Wisconsin, Madison, 1961.
(136) Ellin, R. I., and Easterday, D. E., *J. Pharm. Pharmacol.*, **13**, 370(1961).
(137) Kramer, B., *Biochem. Pharmacol.*, **11**, 299(1962).
(138) Way, J. L., Tong, H., and Rabideau, R., *Federation Proc.*, **19**, 276(1961).
(139) Way, J. L., *J. Pharmacol. Exptl. Therap.*, **138**, 358 (1962).
(140) *Ibid.*, **138**, 264(1962).
(141) Way, J. L., Maserson, P. B., and Beres, J. A., *ibid.*, **140**, 117(1963).
(142) Enander, I., Sundwall, A., and Sörbo, B., *Biochem. Pharmacol.*, **7**, 232(1961).
(143) *Ibid.*, **11**, 377(1962).