# THE METABOLISM OF THE ALKYLPHOSPHATE ANTAGONISTS AND ITS PHARMACOLOGIC IMPLICATIONS[1,2]

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Studies on the metabolic disposition of chemical compounds have so accelerated in the past few years that if a general review of the subject for the last year were attempted, it would be possible to give no more than a sketchy summary of the voluminous literature in the space allocated. More comprehensive reviews on the general topic of drug metabolism are available as monographs (1–3). The present review, like many previous ones appearing in this series on drug metabolism (4–6), is restricted to selected topics, and is primarily concerned with a descriptive and interpretive appraisal of the status of the alkylphosphate antagonists, also known as the cholinesterase reactivators. These compounds are of interest because of their ability to antagonize the toxic effects of the organophosphorous cholinesterase inhibitors that are used as insecticides and war gases.

All compounds in the alkylphosphate antagonist series are nucleophilic agents and are mainly oximes. Although the organic oximes have been employed by chemists for a number of years, very little is really known concerning the biotransformation of this functional group. Yamafugi and coworkers (7–9) have investigated the metabolism of organic oximes in plants, silkworms, and other animals. Mahadevan (10) has reported on the metabolism of 3-indoleacetaldoxime from various plants and fungi. However, the impetus to investigate the biotransformation of oximes can be attributed primarily to the work of Wilson and co-workers (11–14), and Childs et al. (15), who have made important contributions in the development of the cholinesterase reactivation properties of these compounds.

Initially, it was believed that the alkylphosphates were such potent inhibitors of acetylcholinesterase as well as other esterases, that they produced an "irreversible" inhibition of this enzyme. Subsequent work, how-

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ever, indicated that this is not altogether true. The elucidation of the mechanism of enzyme action and inhibition of enzyme action provided a rational basis for development of effective reactivators of inhibited acetylcholinesterase. Reactivation of inhibited acetylcholinesterase was greatly enhanced by the presence of simple nucleophilic substances, such as hydroxylamine. The reactivation can be inhibited by quaternary amines which indicates that the anionic site is still functional in the inhibited enzyme (16). Theoretical considerations related to binding of reactivators to the anionic site led to the development of compounds which contained a quaternary nitrogen as well as a nucleophilic oxygen atom. Various nucleophilic agents have been studied and the most successful agents discovered for reactivating the alkylphosphate inhibited cholinesterase have been oxime derivatives. The most potent oxime developed at that time was pralidoxime (1-methyl-2-aldoximinopyridinium iodide), or 2-PAM (11, 15).

Although Poziomek and co-workers (17–19) have pointed out several objections to the theories of molecular complimentarity upon which 2-PAM was developed and to the proposed mechanism of reactivation, the fact remains that 2-PAM is effective as an acetylcholinesterase reactivator, both *in vitro* (20–22) and *in vivo* (20, 23). Furthermore, many studies of prophylactic and therapeutic treatment in animals and therapeutic treatment in man have indicated the efficacy of 2-PAM in the treatment of alkylphosphate intoxication (24–33).

There have been many general books and reviews on the subject of the mechanism of action of the anticholinesterase alkylphosphates as well as the alkylphosphate antagonists (34–39). Absorption, distribution, and excretion of the alkylphosphate antagonists have also been considered (34, 37), but a review in depth on the biotransformation of oximes, including the alkylphosphate antagonists, has not yet been published.

Most of the metabolic studies on the alkylphosphate antagonists have been carried out on 2-PAM. The paucity of data on other agents in this group, however, should not deter a general consideration of the metabolism of the oximes, since 2-PAM can serve as a model for drawing conclusions concerning the disposition of related compounds. Since the alkylphosphate antagonists possess a common functional group, the basic mechanism involved in oxime biotransformation should be similar for most, if not all, the compounds in this series. Furthermore, some recent significant studies on the chemical attributes of these agents appear to have important implications with respect to biologic mechanisms concerned with their biotransformation and their biochemorphology. Consequently, the chemical properties of these compounds that may relate to drug metabolic aspects and to the development of newer, more sensitive and convenient methods for detecting and estimating these compounds and their metabolites in biologic tissues will also be considered. Finally, some pharmacologic implications of the metabolic findings with respect to the management of intoxication resulting from cyanide liberated by the alkylphosphate antagonists are discussed.

## Methodology

*Detection and estimation.*—Most of the early procedures for the alkylphosphate antagonists in biological tissues employed the bound oxime method of Hahn & Jaeger (40) and Blom (41) as modified by Csaky (42). This method is based on the hydrolysis of the oxime to liberate hydroxylamine, which is subsequently oxidized by iodine to form nitrous acid. The latter compound is used to form a diazonium salt with sulfanilic acid which is then coupled with N-(1-naphthyl) ethylenediamine to produce a blue color. This general procedure is applicable for measuring 2-PAM. Although it is a sensitive method for 2-PAM assay, to obtain quantitative oxime hydrolysis, precautions must be taken to insure sufficient hydrogen ion concentration (37). Highly reproducible results can be obtained by analysis of

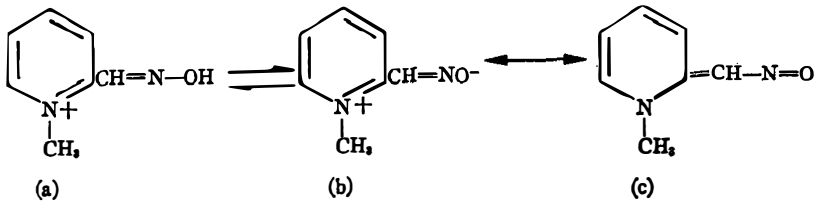



Fig. 1. Chemical species of 2-PAM.

the nitrous acid produced from 2-PAM in pure aqueous solutions, but there is some question concerning the specificity of the method for nitrous acid in biological milieu. The method appears to be satisfactory for estimations of 2-PAM in liver perfusates and blood, but can lead to erroneous interpretations when applied to liver homogenates or subcellular fractions (43).

A more convenient and rapid method of measuring 2-PAM is based on the observation that 2-PAM exhibits an absorption maximum at 333 mμ in alkaline solution (44–46). Not only is this an ideal region to measure the molar absorption of a compound due to the minimal interference by the absorbance of other biological compounds, but at this wavelength the molar absorption of 2-PAM in alkaline solution is greatly enhanced.

In addition to an absorption peak in alkaline solution at 333 mμ, 2-PAM exhibits an absorption maximum at 292 mμ in acid solution. The molecular basis for this bathochromic displacement was investigated by Way (46) and was attributed to the existence of 2-PAM in three chemical species (Fig. 1): (*a*) the enolic form; (*b*) the zwitterionic form; (*c*) and its resonance stabilized form. The *syn* and *anti* configurations of 2-PAM have not yet been clearly established.

The measurement of increased molar absorption of 2-PAM in dilute alkaline solution presents a highly specific method for measuring the 2-PAM, for if the aldoximino functional group, which represents the auxochromic group, is altered in any way by presently known metabolic reactions, the bathochromic displacement of 2-PAM from 292 to 333 mμ would be altered.

Creasey & Green (44) and Way and co-workers (45, 46) have independently employed this enhanced molar absorptivity of 2-PAM in alkaline solution to measure the 2-PAM content in various biologic tissues. The ultraviolet procedure has proved to be a more specific method for measuring 2-PAM than the conventional colorimetric bound oxime method, and a far more convenient one in application as well.

A fluorometric method recently has been developed by Gibbon & Way (47) to measure 2-PAM in biological fluids including plasma levels of 2-PAM in dogs. This method is based on the liberation of 1-methyl-2-pyridone (48) upon the alkaline hydrolysis of 2-PAM at elevated temperatures (49). The highly fluorescent end product which is formed is then extracted with an organic solvent and measured. The characterization of the fluorescent product has been described by Miranda & Way (50).

One of the primary advantages in the fluorometric method is that it enables measurements of low concentration of 2-PAM in biologic fluids. It is approximately ten times more sensitive than the previously described ultraviolet procedure (47); however, the ultraviolet method has a greater specificity for 2-PAM and is usually adequate for organ concentration of 2-PAM as low as $1.0 \times 10^{-5}$ *M*. It should be emphasized that under the conditions described (47), the fluorometric method will measure not only 2-PAM but almost all of its metabolic products. However, by minor modifications of the procedure and careful control of the alkaline concentration, it should be possible to estimate 2-PAM and each established metabolite of 2-PAM, i.e., 1-methyl-2-pyridone, 1-methyl-2-O-conjugate pyridinium ion, and 1-methyl-2-cyanopyridinium ion separately. The method should also be adaptable for the estimation of any aldoximinopyridinium alkylphosphate antagonist.

*Isolation.*—Investigation of the metabolism of 2-PAM and other alkylphosphate antagonists is complicated by the polarity of these quaternary ammonium compounds. The separation of these quaternary ammonium compounds and their metabolites from other inorganic and polar organic constituents is not always easy because 2-PAM and many of its metabolites have a net charge of approximately +1. To effect their separation, some of the earlier investigators relied heavily on paper chromatographic techniques. Way and co-workers (45, 46, 51, 52) and others (53) have described various chromatographic systems for separating 2-PAM from some of its potential chemical degradation products.

Most of the early investigative efforts did not extend beyond the comparison of Rf values of the separated unidentified spots on paper chromatograms with those of known synthetic compounds. Unfortunately, with 2-PAM and its metabolites, almost any Rf value can be attained. It has been demonstrated that 2-PAM and its major metabolic product, the 1-methyl-2-cyanopyridinium ion, and probably other pyridinium ions, can exhibit paper chromatographic multiple spot phenomena (54). This is really not surprising as various other quaternary ammonium compounds (55) behave similarly, and in some instances, these spots probably have been erroneously interpreted to be new metabolites. This may well explain the reports of the resolu-

tion by paper chromatographic systems of "urinary metabolites" of 2-PAM into six or as many as eleven chromatographic bands (37, 56). The appearance of multiple spot phenomena can be greatly minimized by the conversion of all ionized compounds to a single common anion (54). Such precautions would greatly minimize artifacts and fallacious interpretations with respect to paper chromatographic findings.

Disposition studies on alkylphosphate antagonists may be complicated further by the formation of unstable intermediate products. With 2-PAM, for example, an extremely labile intermediate, 1-methyl-2-cyanopyridinium ion, appears early in the metabolic scheme (45, 52). Although the synthesis of this intermediate was described earlier (49, 57), it was not until the elegant studies of Kosower & Patton (58, 59) that the extreme lability of this 2-cyanopyridinium ion became apparent. Since the early workers on 2-PAM metabolism were not cognizant of the lability of the 2-cyanopyridinium ion, several new metabolites probably were created by isolation artifacts. In order to eliminate experimental artifacts in any study of 2-PAM metabolism, therefore, it is essential that proper control be carried out with 1-methyl-2-cyanopyridinium ion as well as with 2-PAM.

In summary, the investigation of the biotransformation of alkylphosphate antagonists has many pitfalls. Misleading data on these compounds may be obtained unless proper measures are taken to insure the specificity of the method for estimating this compound in biological tissues. Not only does the polarity of the alkylphosphate antagonists complicate isolation procedures, but extreme caution must be taken in the interpretation of any experimental data, since artifacts can arise from the formation of multiple spots from one of the pyridinium ions and from chemical degradation of some of the early labile intermediates even with fairly gentle isolation procedures.

## METABOLISM

Since the absorption, distribution, and excretion of 2-PAM and other alkylphosphate antagonists have received considerable attention (34, 37), we will confine our discussion largely to the metabolic aspects and the mechanisms that relate particularly to the pharmacologic activity of the compounds. As an example, we cite the discrepancy that exists between the *in vitro* and *in vivo* efficacy of 2-PAM. The order of magnitude of the protection conferred by 2-PAM *in vivo* is only a small fraction of that expected from studies on the *in vitro* reactivation of acetylcholinesterase (24, 60). This difference can be explained largely in terms of physiologic disposition characteristics that are related to the polarity of the compound.

The theoretical considerations which led to the development of 2-PAM dictated a highly polar, water soluble compound, while most of the alkylphosphates are relatively nonpolar and highly lipid soluble. It has been well documented that great differences exist in the absorption, distribution, and excretion of polar and nonpolar compounds (61–66). Some of the factors which probably are responsible for the disparity between *in vivo* and *in*

*vitro* results are those differences between the biological disposition of 2-PAM and the alkylphosphates. Thus, while most of the alkylphosphates would readily distribute to the central nervous system, the polarity of 2-PAM limits its penetration into the CNS. The reports are conflicting with respect to whether the compound is or is not detectable in the brain, and whether the amounts found there are sufficient to contribute to their antidotal effects (20, 23, 26, 56, 67–77). In large part, these contradictions merely reflect different dosages and degrees of sensitivity of the procedures for 2-PAM. Since the therapeutic efficacy of 2-PAM against alkylphosphate poisoning can be strikingly enhanced by injecting 2-PAM intracerebrally (78, 79), it appears to us that even though only small quantities of 2-PAM gain access into the CNS with systemic administration, enough must be there to elicit pharmacologic effects. Although brain uptake of 2-PAM is restricted by its polarity, this property greatly facilitates its renal excretion via the organic base excretory mechanism (67, 80, 81), whereas the lipid-soluble organophosphates are excreted in negligible amounts. An early study reported that the major substance excreted in the urine in the first 30 minutes was not 2-PAM but an altered derivative, perhaps a pyridine-2-aldehyde derivative (67). However, this conclusion was based solely on paper chromatographic evidence which, as we have pointed out, can be misleading. Subsequent studies have indicated, however, that 2-PAM appears predominantly in the urine unchanged and only in small amounts as biotransformation products (81–84).

The isolation and elucidation of the structures of these trace metabolites of 2-PAM pose many technical problems with respect to the small amounts, isolation artifacts, and labile intermediary products. Successful identification of certain biotransformation products of 2-PAM was greatly facilitated by the use of isolated liver perfusion (45, 46, 50–52) and special chromatographic techniques designed for such purposes (85).

Early studies *in vitro* on the metabolism of 2-PAM have yielded conflicting and confusing data with respect to its metabolism. In part the term, *in vitro*, itself, has led to some misunderstanding concerning the metabolic reaction of 2-PAM. The problem arises chiefly because the *in vitro* mechanisms ascribed to purely chemical reactions of 2-PAM have on occasions been implied to be similar to metabolic mechanisms (37). This has been very misleading to the biologist. In this review, *in vitro* refers only to those reactions of 2-PAM or its metabolic products which have been carried out in the presence of biological material. Even when only the biologic *in vitro* findings are considered, one finds much conflicting data that should be reconciled.

Most of the early *in vivo* metabolic investigations of 2-PAM involved some oral administration despite the fact that the antagonist usually is administered by the parenteral route for maximal efficacy (82, 86–88). Such studies raise the question of whether the reported metabolism of 2-PAM can be attributed to the microbial flora in the intestinal tract prior to its absorption from the intestinal lumen.

*Biotransformation site.*—Experiments on various tissue preparations indicate that the liver is the primary site for the metabolism of the alkylphosphate antagonists. Incubation of diacetylmonoxime (DAM), with rat liver slices in the presence of oxygen, resulted in the disappearance of oxime and when carbon dioxide or nitrogen was employed as the complete atmosphere, no metabolism occurred (89). Jager et al. (67), employing rat liver homogenates rather than liver slices, found that aerobic incubation of 2-PAM or diacetylmonoxime with liver homogenates also resulted in the complete disappearance of the oxime content, while anaerobic conditions produced a complete inhibition in metabolism of the oxime group. Tong et al. (90) subsequently confirmed these results employing rat liver homogenates and partially purified liver enzymes. However, attempts to isolate a metabolic product from the incubation mixture were unsuccessful (91).

It should be pointed out that all of the above studies with rat liver slices, homogenates and partially purified enzymes were carried out by the measurement of the alkylphosphate antagonists by the classical colorimetric bound oxime method. Attempts to confirm the liver homogenate and subcellular liver preparation findings with the more specific ultraviolet procedure produced conflicting results, although the correlation between the two procedures was satisfactory for estimation of 2-PAM in liver perfusates and in blood (46, 47). With homogenates or subcellular preparations of liver, however, the colorimetric method indicated considerable disappearance of 2-PAM from the incubation media, while the ultraviolet spectral method showed little or none (91). Thus, a factor in the liver may be liberated or synthesized following its disruption that interferes with the colorimetric determination of 2-PAM. Creasey & Green (44) reported a very rapid metabolism of 2-PAM by liver homogenates with their ultraviolet spectral method, but others (91) have not been able to confirm their data.

*1-Methyl-2-cyanopyridinium ion.*—The formation of 1-methyl-2-cyanopyridinium ion as a metabolite of 2-PAM metabolism was first described by Way et al. (45, 52), and this may represent a new type of metabolic reaction for the disposition of drugs. As would be expected, the 2-cyanopyridinium ion exists in a very small amount as an urinary metabolite when compared with the large amount of unchanged 2-PAM which is excreted. This would be expected from the renal mechanisms reported by Peters (80), that quaternary ammonium compounds are excreted by glomerular filtration and active tubular secretion.

This metabolite was isolated from the liver perfusate by ethanol extraction and a series of column chromatographic procedures employing activated charcoal, ion exchange resins and, in some cases, followed by paper chromatography and paper electrophoresis. This metabolite also has been isolated from the urine of rats and humans (83, 84, 87). In the *in vivo* studies, the 2-PAM was administered by the parenteral route usually in divided doses or by continuous infusion and collected over dry ice and ethanol mixture. Isolation of the metabolite was conducted by the unorthodox column chromatographic procedure developed by Gibbon & Way (85). This procedure

permitted the rapid removal of 2-PAM from other quaternary compounds under very mild conditions. The metabolite was isolated in sufficient quantities by these procedures so that adequate characterization was possible by comparing its chemical, radioactive, spectral, chromatographic, and electrophoretic properties with that of authentic 1-methyl-2-cyanopyridinium ion (45, 52). Also, alkaline degradation of the metabolite yielded cyanide ion, 1-methyl-2-carbamidopyridinium ion and 1-methyl-2-pyridone, the expected alkaline hydrolytic products of 1-methyl-2-cyanopyridinium ion in the stoichiometric amounts as described by Kosower & Patton (58, 59).

Enander et al. (86) reported that the amount of cyanide in the urine of rats and humans could be greatly enhanced after giving 2-PAM as the methane sulfonate salt and also indicated that 1-methyl-2-cyanopyridinium ion (87) could be a 2-PAM metabolite. However, it would be difficult to explain why a pyridinium ion would not adsorb on a strong cation column, and yet be completely adsorbed on a strong anion exchange column. The opposite results would be expected if the compound isolated were the 1-methyl-2-cyanopyridinium ion. Also, if the strong anion exchange was in the hydroxide form, one would expect 1-methyl-2-cyanopyridinium ion to be very rapidly hydrolyzed to form 1-methyl-2-pyridone, cyanide ion and the 1-methyl-2-carbamidopyridinium and possibly homarine (1-methyl betaine of picolinic acid). Unfortunately, since the exact resins employed in these experiments were not described, a test of these objections cannot be carried out.

Identification of 1-methyl-2-cyanopyridinium ion as a metabolite of 2-PAM raises the question with respect to the molecular mechanism which is concerned with its formation under biological conditions. The chemical decomposition of 2-PAM to form 1-methyl-2-pyridone, presumably via 1-methyl-2-cyanopyridinium ion, has been described by Ellin and coworkers (49, 92). The chemical conversion of 2-PAM to a 2-cyanopyridinium was interpreted mechanistically as a base attack resulting from elimination of the aldehydic hydrogen with the hydroxyl group as the leaving group. The hydroxyl group, however, is not considered generally to be a good leaving group. Either a higher hydroxyl ion concentration or a higher temperature would be required to form the 2-cyanopyridinium ion from 2-PAM at a rate comparable to that occurring biologically. It is unlikely, therefore, that such a mechanism is operative under physiologic conditions.

It is conceivable, however, that the biotransformation of an organic aldoxime to a cyano derivative could involve an intermediate form of a known biochemical reaction for drug biotransformation. Such an intermediate could be an acetylated or phosphorylated derivative of an aldoxime which would then be a superior leaving group for forming the nitrile. This proposal would be consistent with the chemical literature described for oximes (93–113). To test the above proposal, it would be necessary to have the acetylated aldoxime of 2-PAM which is not available at present. However, the 3- or 4-substituted pyridinium aldoximes have been synthesized (110, 111), and studies have been carried out on the acetylation of these two substances.

Others have proposed that the aldoximes may interfere with the biological acetylation of substrates. Wagner-Jauregg & Saner (114) have shown that

the oximes can interfere with sulfonamide acetylation of liver enzyme preparations. Also, O'Neill et al. (115), on the basis of kinetic and equilibrium studies, have indicated that a reaction between oximes and thioesters may be expected to take place under physiological conditions, and have suggested that the oxime of 4-PAM may compete with choline for acetyl coA and thus inhibit acetylation of choline.

Chemical findings on the alkaline decomposition of the acetylated aldoximes are also consistent with an acetylated biological derivative having a role in the formation of the 1-methyl-2-cyanopyridinium ion. Classic chemical studies on oxime configuration involve acylation of the oximes and analy-

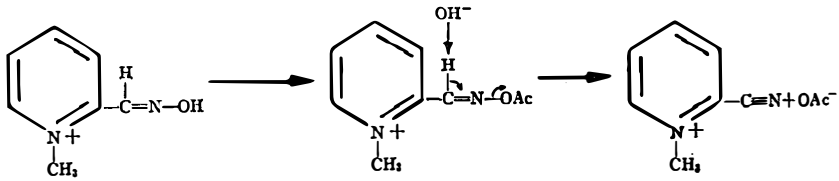



FIG. 2. Proposed biological mechanism for the conversion of 2-PAM to the 1-methyl-2-cyanopyridinium ion.

sis of the alkaline degradation products found. Alkaline degradation of acetylated aldoximes of the *anti* configuration (98, 102) usually proceeds preferentially by the elimination of the carbonyl hydrogen atom and results predominantly in the formation of the nitrile (Fig. 2). On the other hand, an attack by the hydroxyl ion on the *syn* configuration would effect an hydrolysis of the acyl carbon atom leading preferentially to the formation of an oxime again. However, more recent studies indicate that this method of assigning oxime configuration is not absolute. Some *syn* acylated aldoximes, on some occasions, are converted principally to nitriles (104). The studies of the O-acetates of 3- and 4-PAM, *syn* configuration, by Blanch & Onsager (110, 111) showed that the alkaline hydrolysis of these two acylated aldoxime derivatives give a mixture of aldoximes and nitriles as the end products. In aqueous solution the O-(isopropylmethylphosphonyl)-4-formyl-1-methylpyridinium iodide oxime decomposes to form the 1-methyl-4-cyanopyridinium ion rather than 4-PAM (113). Under biological conditions, it would seem that the reaction involving an attack by a base at the aldehydic hydrogen atom to give a Beckman elimination with nitrile formation would be favored.

Several studies have been made to establish a biological acylation reaction for oximes (115, 116). Tong et al. (116) and Miranda (117) prepared the O-acetate of 4-PAM and showed that the rate of degradation of this compound at pH 7.4 would be consistent with its being the intermediate. Chemical studies by Blanch & Onsager (110, 111) on the stability of N-heterocyclic oxime derivatives further support such a mechanism. Miranda (117) perfused 4-PAM through an isolated rat liver and simultaneously infused radioactive acetate. The radioactive material isolated from the perfusate was co-crystallized with an authentic sample of the 4-O-acetate of 4-PAM to a constant isotopic content which would suggest that the O-acetate

of 4-PAM probably was formed in the liver perfusate. However, the extreme lability of this acetylated aldoximino derivative has not permitted a definitive isolation and characterization at the present time. To elucidate these points, the use of mixed multiple labelled compounds and superior isolation procedures would be necessary.

As a possible alternative to oxime acetylation, phosphorylation of the alkylphosphate antagonists would be a logical biological mechanism leading to the formation of 1-methyl-2-cyanopyridinium ion. This mechanism would be analogous to the drug interaction of the oximes and the phosphonates (118–121). The phosphonylated aldoxime would be much more susceptible to decomposition under biologic conditions (113, 120, 121). Steinberg & Solomon (113) have shown that in aqueous solution at pH 7.6 the phosphonylated derivative of 4-PAM can decompose to form the corresponding nitrile, 1-methyl-4-cyanopyridinium ion rather than 1-methyl-4-aldoximino-pyridinium ion.

*Homarine (1-methyl betaine of picolinic acid).*—This compound was reported to be a urinary metabolite of 2-PAM methane sulfonate by Enander et al. (82). However, the conclusion was based solely on paper chromatographic evidence and, as we have pointed out earlier, pyridinium ions can exhibit multiple spot phenomena. Another artifact can arise from the degradation of the 1-methyl-2-cyanopyridinium ion which is present in the urine as a 2-PAM metabolite. Under certain isolation conditions the 2-cyanopyridinium ion may form the amide which can be hydrolyzed to homarine.

Kramer isolated an *in vivo* metabolite of 2-PAM iodide and on the basis of ultraviolet and infrared evidence tentatively proposed it to be a derivative of homarine (88). However, the ultraviolet spectrum of the material he isolated appears to be more compatible with that for the 1-methyl-2-cyanopyridinium ion (contaminated possibly with some degradation products). The absorption maxima of the metabolite are similar to those of the 2-cyanopyridinium ion, being in the region of 270 mμ and 279 mμ in acid, and 288 mμ in alkaline solutions. In the case of 2-cyanopyridiniumion, degradation occurs in alkali so that reversion of the spectral curve does not occur upon neutralization. Also the peak in alkali shifts with the ratio of the degradation products formed and this ratio is pH dependent. Unfortunately, Kramer (88) did not describe any attempts to observe whether the absorption spectrum of the metabolite in a more dilute alkaline solution would revert to the original spectral curve in acid, which should occur if the metabolite were homarine. His characterization of the metabolite as a carbonyl compound on the basis of infrared data may be attributed to some homarine formed via hydrolysis of the 1-methyl-2-carbamidopyridinium ion which can be formed from the hydrolysis of the 1-methyl-2-cyanopyridinium ion. Thus, the evidence in support of homarine as a metabolite of 2-PAM is not convincing, although the arguments marshalled to the contrary do not completely exclude such a possibility.

*1-Methyl-2-pyridone.*—This compound has been isolated from liver perfu-

sates and urine of rats receiving 2-PAM and characterized by paper and gas chromatography, paper electrophoresis, countercurrent distribution, ultraviolet, infrared, and fluorescent emission spectroscopy (50, 122, 123). In all these studies not only 2-PAM, but also the labile 1-methyl-2-cyanopyridinium ion, was included in the control experiment in order to establish unequivocally that the isolated 1-methyl-2-pyridone was a biotransformation product rather than an isolation artifact. Earlier studies reported 1-methyl-2-pyridone as a urinary metabolite of 2-PAM (82); however, the evidence in support of 1-methyl-2-pyridone as a 2-PAM metabolite is similar to that of homarine. Furthermore, the volatile properties reported for 1-methyl-2-pyridone during paper chromatographic procedures (82) are not consistent with the compound isolated by others (50, 59, 122–124).

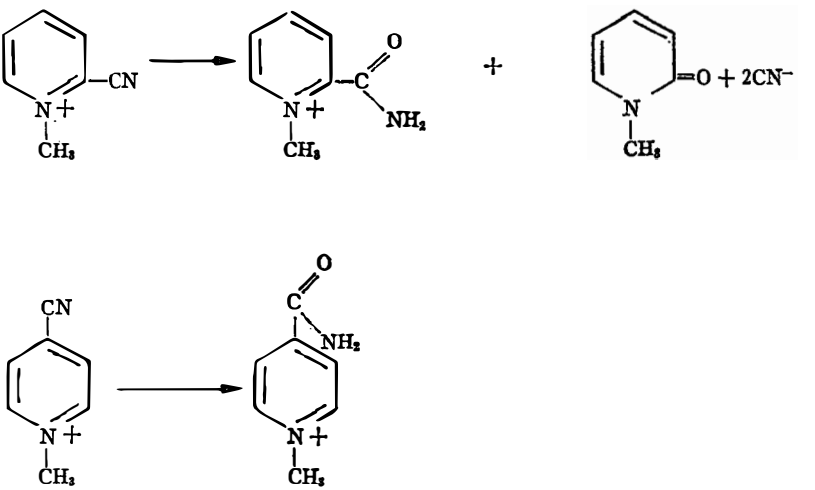



FIG. 3. Chemical hydrolysis of the 1-methyl-2-cyano and 4-cyanopyridinium ion at pH 7.0.

A possible molecular mechanism for the formation of 1-methyl-2-pyridone from 2-PAM (Fig. 3) might involve the 1-methyl-2-cyanopyridinium ion as the intermediate. Chemical studies (49) indicate that when 2-PAM is treated under relatively vigorous conditions, 1-methyl-2-pyridone can be isolated as one of the degradation products, presumably formed from the labile intermediate 1-methyl-2-cyanopyridinium ion. The alkaline hydrolysis of the 2-cyanopyridinium ion would then occur by a bimolecular nucleophilic aromatic substitution mechanism with hydroxyl ion adding to the ring to form the 1-methyl-2-pyridone cyanohydrin as the intermediate and the subsequent elimination of cyanide ion to form 1-methyl-2-pyridone (58, 59). Since these studies indicate that the $pK_a$ of 1-methyl-2-pyridone cyanohydrin is approximately 10.3 (124), the reactive species under biological conditions then would probably be in the form of a cyanohydrin rather

than the anion of the cyanohydrin. However, before such a mechanism can be accepted as an occurrence under biologic conditions, certain data need to be reconciled.

Kosower & Patton (58, 59) have reported that the alkaline hydrolysis of the 2-cyanopyridinium ion leads to the formation of 1-methyl-2-carbamidopyridinium ion as well as 1-methyl-2-pyridone and cyanide ion (Fig. 3), but as yet, it still has not been possible to establish 1-methyl-2-carbamidopyridinium ion as a metabolite of 2-PAM (125). These results are a little surprising in view of the chemical data. If the cyanopyridinium ion formed from 2-PAM were hydrolyzed in biological fluids solely by a chemical mechanism, approximately 1 part of the 2-carbamidopyridinium ion should have been formed for every 2.5 parts of 1-methyl-2-pyridone and cyanide ion detected (59). Since the isolation procedures employed (125) would most certainly have detected 1-methyl-2-carbamidopyridinium ion even in very small quantities, an enzymatic mechanism appears to be involved. Perhaps the interaction of the 2-cyanopyridinium ion with a receptor site may be necessary to link what is presently known concerning the chemical reaction mechanism with biochemical processes. At the relatively low pH value of biological fluids, the water rate of hydrolysis of the 2-cyanopyridinium ion could also play an important role (124).

*1-Methyl-2-O-conjugate pyridinium ion.*—This was the first 2-PAM metabolite to be isolated by liver perfusion studies (51). It was isolated by charcoal adsorption and ion exchange column chromatography, and subsequently characterized by ultraviolet spectrophotometry and chemical degradation. Liberation of 1-methyl-2-pyridone from alkaline degradation studies of this conjugated metabolite subsequently formed the basis for its isolation and characterization.

In regard to the nature of the conjugate, various possibilities may be considered. First, phenolic hydroxyl groups are readily converted to ethereal sulfates. Since the 2-O-conjugate pyridinium ion is relatively unstable in alkaline solution, the possibility of an ethereal sulfate conjugation seems less likely, as the negative charge of the sulfate anion probably would make it less susceptible to a base attack. Consistent with these expectations, when the metabolite was incubated with phenol sulfatase, no evidence was noted that an ethereal sulfate bond was cleaved. Second, the phenolic hydroxyl group can be converted to an O-glucuronide. This conjugate would be stable in acidic solution and relatively labile in basic solution; however, the enzyme $\beta$ glucuronidase did not cleave the 2-O-conjugate. While the inability of $\beta$ glucuronidase to cleave the conjugate does not necessarily rule out this addition product, it certainly makes it much less likely. Third, the chemical properties of the 2-O-conjugate pyridinium also would be compatible with those of a 1-methyl-2-methoxypyridinium ion. Preliminary ultraviolet spectral and chromatographic data support this view (126), but degradation studies are necessary to establish unequivocally that the 2-O-conjugate is indeed a 2-methoxy derivative.

If 1-methyl-2-methoxypyridinium ion is tentatively assumed to be a

metabolite of 2-PAM, two considerations arise concerning its mechanism of formation. One possibility is that the 2-O-conjugate pyridinium ion can be formed directly from 1-methyl-2-pyridone. However, as shown by Mason (127, 128), compounds such as 1-methyl-2-pyridone exist in aqueous solution in equilibrium as an amide or as an $\alpha$ hydroxy form, normally with the amide form predominating. The equilibrium constant of 1-methyl-2-pyridone as shown by Albert & Phillips (129) has a $pK_a$ value of 0.32. This means a strongly acidic solution is necessary to protonate the oxygen atom to the hydroxy form. This change would be consistent with the hypsochromic shift shown by 1-methyl-2-pyridone in going from an alkaline or neutral medium to a strongly acidic medium (51). Similar hypsochromic shifts have been shown to occur with other pyridone derivatives (130). With such a low $pK_a$ for the oxygen atom of 1-methyl-2-pyridone, it would seem very unlikely that the biological mechanism for the formation of 2-methoxypyridinium ion would involve 1-methyl-2-pyridone as a precursor, especially since Way et al. (125) have indicated that 1-methyl-2-pyridone does not form 1-methyl-2-O-conjugate pyridinium in liver perfusion studies.

A second possibility is that the 1-methyl-2-methoxypyridinium ion could be formed from the intermediate 1-methyl-2-pyridone cyanohydrin which is formed in the alkaline hydrolysis of the 1-methyl-2-cyanopyridinium ion. Chemical studies (124) indicate that the $pK_a$ of 1-methyl-2-pyridone cyanohydrin is approximately 10.3. Therefore, under biological conditions the cyanohydrin would exist primarily in the unionized form rather than as its anion. The reaction mechanism proposed would be a little unusual in that it would involve direct methylation of an hydroxyl group in 1-methyl-2-pyridone cyanohydrin rather than the 1-methyl-2-pyridone cyanohydrin anion. Although most methylation reactions involve the methylation of an oxygen atom as an anion, direct methylation of an hydroxy group has been proposed by Barber et al. (131) for spirilloxanthin, a compound containing two tertiary methoxy groups, which is supposedly formed from an alkene through the addition of water.

Attempts have been made to obtain some direct evidence to indicate that the 1-methyl-2-cyanopyridinium ion is the intermediate for the formation of the 1-methyl-2-O-conjugate pyridinium ion from 2-PAM (132) in the isolated perfused liver system, employing radioactive labeled 2-PAM, 1-methyl-2-cyanopyridinium ion and 1-methyl-2-pyridone. These isotopic studies indicate that not only will 2-PAM form 2-cyanopyridinium ion, 1-methyl-2-pyridone ion and the 2-O-conjugate pyridinium ion, but also that the 2-cyanopyridinium ion will form the 2-O-conjugate pyridinium ion (132). Furthermore, it was shown that 1-methyl-2-pyridone could not serve as a precursor to 1-methyl-2-O-conjugate pyridinium ion. Although one might suspect that the 2-cyanopyridinium ion then would be the logical precursor to the formation of the 2-O-conjugate pyridinium ion, it is still necessary to establish that 2-cyanopyridinium ion can form the 2-O-conjugate pyridinium ion at a rate which is equal to or faster than 2-PAM. Kinetic studies by Way et al. (132), employing equimolar amounts of radioactive 2-PAM and carrier

1-methyl-2-cyanopyridinium ion and vice versa, indicate that the biological reaction for the conversion of the 1-methyl-2-cyanopyridinium ion to the 1-methyl-2-O-conjugate pyridinium ion occurs at a rate which is five to seven times faster than that of 2-PAM to the 2-O-conjugate pyridinium ion. In the conversion of 2-PAM to the 1-methyl-2-O-conjugate pyridinium ion, therefore, the rate limiting step is the formation of the 1-methyl-2-cyanopyridinium ion or its precursors.

*Cyanide ion.*—Cyanide ion and its metabolic product thiocyanate ion were found as urinary metabolites of 2-PAM (86). Also, Way & Pfeiffer (133) found that if 2-PAM is administered to nephrectomized rats, an elevation of blood cyanide level results. Chemical studies by Ellin (49) indicate that 1-methyl-2-pyridone and cyanide ion are formed upon alkaline degradation of 2-PAM via the intermediate 1-methyl-2-cyanopyridinium ion. Liberation of cyanide ion by biological systems (134) has been reported also for other alkylphosphate antagonists, i.e. monoisonitrosoacetone (MINA) and diisonitrosoacetone (DINA). On the other hand, other antagonists including 4-PAM, 1,1′-trimethylene bis (4-aldoximinopyridinium) dibromide (TMB-4) (133) and diacetylmonoxime (DAM) (89) do not liberate cyanide as a biotransformation product. The formation of cyanide by the alkylphosphate antagonists can be a complicating factor with respect to the toxicity of these agents, and these aspects will be reserved for later discussion.

*1-Methyl-4-pyridone-2-carboxamide.*—This compound was found in the chloroform extract of rat urine. The metabolite was isolated by adsorption charcoal column chromatography and ion exchange column chromatography by Zampaglione & Way (135). Authentic 1-methyl-4-pyridone-2-carboxamide was synthesized by these investigations (135), and compared with the isolated radioactive metabolite. Evidence by nuclear magnetic resonance spectroscopy, ultraviolet and infrared spectrophotometry, paper and thin layer chromatography, and paper electrophoresis studies indicate that this new isolated radioactive metabolite is 1-methyl-4-pyridone-2-carboxamide (135). Other carbamido substituted pyridone derivatives have been described: 1-methyl-2-pyridone-5-carboxamide (136–140) and 1-methyl-4-pyridone-3-carboxamide (130, 141, 142) are reported as metabolites of nicotinamide and nicotinic acid. Also, Kramer et al. (143) has isolated 1-methyl-2-pyridone-5-carboxamide from uremic patients.

There are some interesting possibilities concerning the mechanism of the formation of 1-methyl-4-pyridone-2-carboxamide. As discussed earlier, although the 1-methyl-2-carbamidopyridinium ion is readily formed by the alkaline hydrolysis of the 1-methyl-2-cyanopyridinium ion (58, 59), this pyridinium ion has never been isolated as a metabolite of 2-PAM. While the 2-cyanopyridinium ion is formed fairly rapidly as a metabolite of 2-PAM in the urine, there is a considerable delay in the formation of 1-methyl-4-pyridone-2-carboxamide (135), This delay is consistent with findings of Wu Chang & Johnson (130), who have shown that the rate of appearance of pyridones as urinary metabolites can be extremely slow. This can be partly attributed to the fact that the pyridinium ions are more polar and are

excreted by an active transport mechanism (80), whereas the excretion of relatively nonpolar compounds like the pyridones would be expected to be considerably slower.

The possible modes of formation of the 1-methyl-4-pyridone-2-carboxamide are shown in Figure 4. The metabolite could be formed by an hydroxylation of 1-methyl-2-carbamidopyridinium ion, although this would seem unlikely, as there is no evidence that 1-methyl-2-carbamidopyridinium ion is formed as a metabolite of 2-PAM. This does not rule out the possibility that the hydroxylation of 1-methyl-2-carbamidopyridinium ion to 1-methyl-4-pyridone-2-carboxamide could be the fast reaction, while the formation of 1-methyl-2-carbamidopyridinium ion may be the rate-limiting reaction. However, Chaykin et al. (142) reported that with nicotinamide, most of the compound was excreted unchanged or as the N-oxide derivative, while only small amounts of 1-methyl-2-pyridone-5-carboxamide and 1-methyl-4-pyridone-3-carboxamide were formed. A second possible mode of 1-methyl-4-pyridone-2-carboxamide formation could be by hydroxylation of the 2-cyanopyridinium ion or one of its precursors. This proposal would have a rational chemical basis for the formation of the carbamido derivative, as the reaction would be analogous to the preferential formation of the carbamido derivative in the hydrolysis of the 3- and 4-cyanopyridinium ions (57, 58). There is a third possibility that the 4-pyridone compound is formed by a transamination reaction following the formation of homarine (1-methyl betaine of picolinic acid). This possibility is favored by the fact that homarine resembles an alpha amino acid and by the fact that a high concentration of the pyridinium ion exists in the kidneys. Furthermore, when nicotinic acid is administered, the amide derivative is found in the urine (139). However, the evidence in support of homarine as a 2-PAM metabolite, as was pointed out earlier, is rather tenuous. Of the three possibilities, therefore, it would appear that the most likely pathway for the formation of 1-methyl-4-pyridone-2-carboxamide is via hydroxylation of the 2-cyanopyridinium ion or its precursors. While the site of the hydroxylation of many foreign compounds is in the liver, this metabolite was discovered by *in vivo* studies rather than by *in vitro* studies employing isolated perfused liver systems. This implies that this metabolite is formed either at an extrahepatic site or that the enzymes to form this metabolite may have been induced by the prolonged administration of 2-PAM in the *in vivo* studies. It has been reported, however, that the conversion of 1-methyl-3-carbamidopyridinium ion to 1-methyl-4-pyridone-3-carboxamide is catalyzed by soluble rat liver enzymes (144).

## Metabolic Scheme and Reaction Mechanisms

The general metabolic pathways for the disposition of 2-PAM are shown in Figure 5-I. This scheme shows only those metabolites whose formation from 2-PAM either in *in vitro* or *in vivo* systems has been clearly established. The formation of 1-methyl-2-cyanopyridinium ion (Fig. 5-II), early in the metabolic scheme is of importance, as the metabolic mechanisms proposed

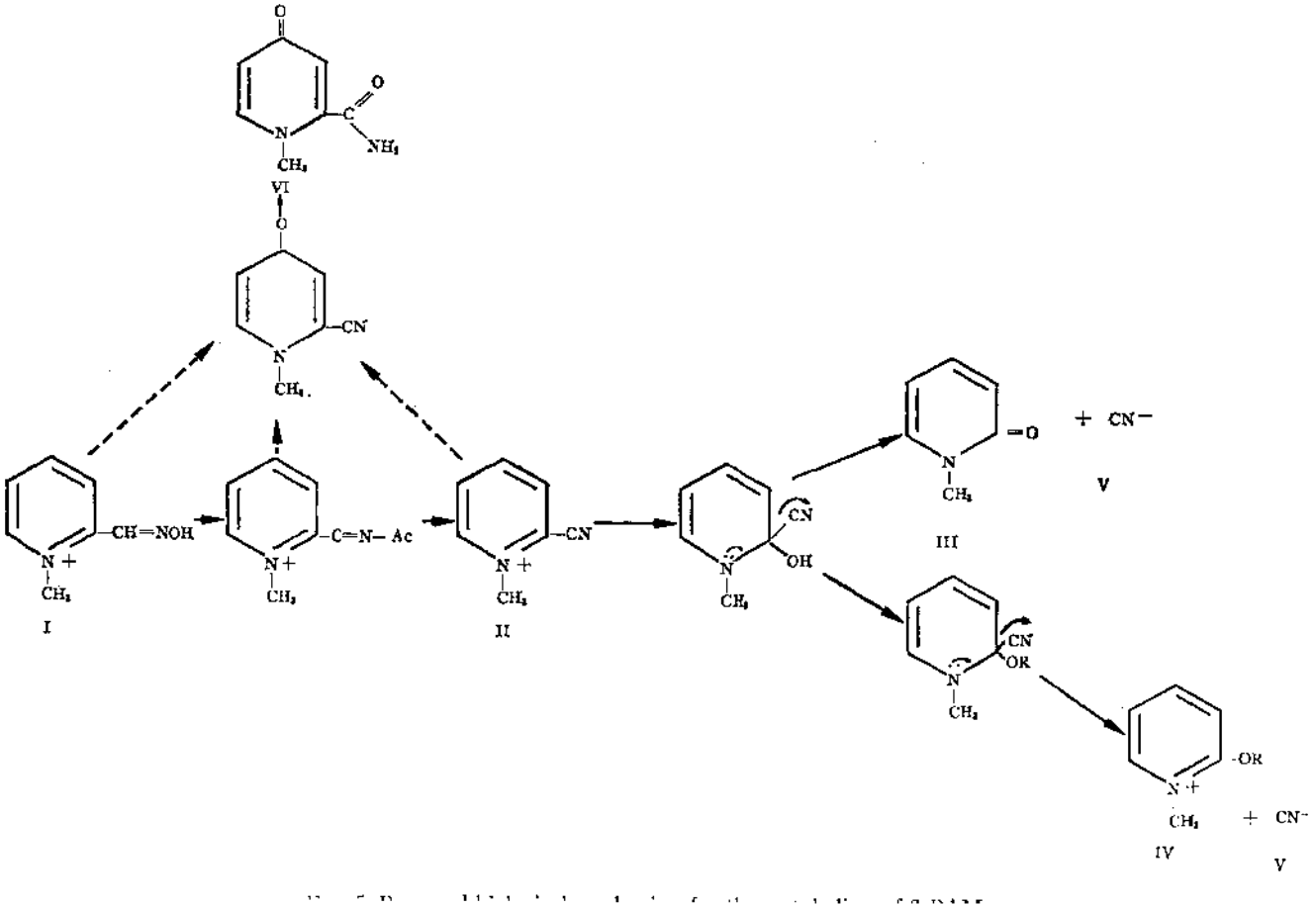
FIG. 4. Possible mechanisms for the formation of 1-methyl-4-pyridone-2-carbamide.

revolve around the formation of the labile intermediate, 1-methyl-2-cyanopyridinium ion (Fig. 5-II) or its derivatives. Four metabolic products could be formed from the 1-methyl-2-cyanopyridinium ion, e.g. 1-methyl-2-pyridone (Fig. 5-III), 1-methyl-2-O-conjugate pyridinium ion (Fig. 5-IV), and 1-methyl-4-pyridone-2-carboxamide (Fig. 5-VI), and cyanide ion (Fig. 5-V). It should be emphasized that the predominant products formed from the 1-methyl-2-cyanopyridinium ion are 1-methyl-2-pyridone, cyanide ion, and the 1-methyl-2-O-conjugate pyridinium ion, while the formation of 1-methyl-4-pyridone-2-carboxamide is delayed in onset but is formed in increasing amounts on prolonged administration of 2-PAM.

Studies on the chemical hydrolysis of 1-methyl-2-cyanopyridinium ions (58, 59) indicate that the 1-methyl-2-cyanopyridinium ion is very reactive in alkaline solution and the rate of reaction is dependent on the position of the nitrile group on the pyridinium ring. Hydrolysis of the 1-methyl-2-cyanopyridinium ion can lead to the formation of a 1-methyl-2-pyridone, cyanide ion and 1-methyl-2-carbamidopyridinium ion (Fig. 5). The ratio of the formation of pyridone (or cyanide ion) to amide is pH dependent. At biologic pH, 1-methyl-2-cyanopyridinium ion should liberate approximately 2.5 parts of 1-methyl-2-pyridone and cyanide and one part of 1-methyl-2-carbamidopyridinium ion.

The reaction mechanism proposed by Kosower & Patton (59) for the hydrolysis of the 1-methyl-2-cyanopyridinium ion and the formation of 1-methyl-2-carbamidopyridinium ion would involve an attack by hydroxyl ion directly on the cyano group. Similarly, an attack by hydroxide ion directly on the ring would form the 1-methyl-2-pyridone cyanohydrin which is in equilibrium with its corresponding anion, and both would form the 1-methyl-pyridone. The possibility that these mechanisms also may occur under biologic conditions is favored by the fact that 1-methyl-2-pyridone and cyanide ion have been identified as biotransformation products of 2-PAM. The 1-methyl-2-cyanopyridinium ion was established as an early intermediate in the metabolic disposition of 2-PAM, both *in vitro* (45, 52) and *in vivo* (83, 84, 87), and its presence would account for the formation of 1-methyl-2-pyridone and cyanide ion (50, 82, 86, 122, 123).

There are some biologic findings, however, which cannot be completely reconciled with the chemical data. The chemical hydrolysis of the 1-methyl-2-cyanopyridinium ion at pH 7.4 results in the formation of appreciable amounts of 1-methyl-2-carbamidopyridinium ion. Although this latter pyridinium ion can be readily isolated, there has been no convincing evidence to indicate that the 1-methyl-2-carbamidopyridinium ion is actually a metabolite of 2-PAM (125). This suggests that the formation of products such as 1-methyl-2-pyridone and cyanide ion may not be due solely to chemical hydrolysis of the labile 1-methyl-2-cyanopyridinium ion, since if this were the case, one would expect approximately one-third of the product to be the 1-methyl-2-carbamidopyridinium ion. Furthermore, in liver perfusion studies, the 2-PAM metabolites isolated were 1-methyl-2-pyridone, 1-methyl-2-O-conjugate pyridinium ion and cyanide ion, as well as 1-methyl-



2-cyanopyridinium ion. However, when 1-methyl-2-cyanopyridinium ion was placed in the same system, in addition to the above products, the 1-methyl-2-carbamidopyridinium ion was also isolated (132). These experiments suggest that 2-PAM is slowly converted to the 1-methyl-2-cyanopyridinium ion and this rate-limiting product then forms predominantly the pyridone derivatives and cyanide. On the other hand, when 1-methyl-2-cyanopyridinium ion is present in excess, as it is when administered directly into a biological system, it is also hydrolyzed chemically to yield an additional product, the 1-methyl-2-carbamidopyridinium ion.

### Implications of Cyanide Liberation

The liberation of cyanide by alkylphosphate antagonists has important pharmacologic implications. One of the most effective early antagonists, monoisonitrosoacetone (MINA), was found to yield cyanide very rapidly (134). Subsequently, it was reported that 2-PAM administered orally or parenterally (86) would also liberate cyanide ion. The possibility of cyanide intoxication, therefore, is a factor to be considered when alkylphosphate antagonists are administered. Any toxic effects of cyanide can be treated by the classic antidotes, sodium thiosulfate and sodium nitrite. This combination has been reported to protect against the toxic manifestation of MINA (134), and to a lesser extent against 2-PAM (86). The less sucessful results of such treatment in antagonizing 2-PAM intoxication (86) can be attributed to the fact that 2-PAM is excreted so rapidly (67, 81) that cyanide ion formation would be relatively negligible. However, if the renal excretion of 2-PAM is reduced, cyanide formation would be increased (133) and may become a complicating toxic factor. Before considering this aspect, it appears feasible to discuss first more recent concepts of cyanide toxicity with respect to the basic mechanisms involved.

In cyanide intoxication, inhibition of cytochrome oxidase is believed to be the primary cause of the toxic effect (145, 146). Concentrations as low as $3 \times 10^{-8}$ moles/ml have been shown to produce complete inhibition of this enzyme (147), and lethal doses of cyanide have been reported to inhibit this respiratory enzyme *in vivo* (148). Since only oxygen utilization is inhibited, oxygen transport and oxygen tension are generally considered to be adequate, at least prior to the onset of respiratory and cardiovascular collapse. Based on this reasoning, the administration of oxygen theoretically would serve no useful purpose in antagonizing cyanide intoxication.

The classical treatment of cyanide poisoning includes the use of amyl nitrite or sodium nitrite in combination with sodium thiosulfate (149–151). The nitrites are employed to form methemoglobin, which can bind cyanide ions and therefore compete with cytochrome oxidase for these ions (152). Sodium thiosulfate is employed as a sulfur donor for the enzyme rhodanese, which detoxifies cyanide by its conversion to thiocyanate (153–156).

Although there have been many reports indicating that oxygen might be of value in cyanide poisoning (157–159), most pharmacology and toxi-

cology texts are either noncommittal on its use, or advise it as an adjunct to the usual nitrite-thiosulfate therapy rather than as an integral part of therapy. The lack of enthusiasm for the use of oxygen undoubtedly is based in part on the work of Stotz et al. (160), who noted that cytochrome oxidase has its maximal activity at 70 mm Hg or greater oxygen tensions. Theoretical considerations led, therefore, to the conclusion that since the utilization of oxygen was decreased, adequate tissue oxygen must be present to allow maximal rates of reaction of any uninhibited cytochrome oxidase. The arguments were supported by the fact that the protection against cyanide by oxygen was considerably less than that obtained with either sodium nitrite or sodium thiosulfate and appeared trivial when compared to that obtained with combined nitrite-thiosulfate treatment (149–151, 161, 162). However, the efficacy of oxygen in combination with the other known antagonists was not assessed, although it was known that greater than additive effects could be obtained by combined therapy with other antidotal agents (149–151).

No study of the effects of oxygen in combination with the accepted therapeutic agents utilized in cyanide toxicity were attempted until the study of Way et al. (161, 162). These investigations clearly established the efficacy of oxygen in cyanide toxicity, especially when it is combined with sodium nitrite and sodium thiosulfate.

There are at least four possible mechanisms by which oxygen may reverse cyanide toxicity. One possibility could result from an increased respiratory excretion of cyanide or its volatile metabolites (163). Preliminary results by Gibbon & Way (164) suggest oxygen may enhance the respiratory excretion of radioactive material after $C^{14}$-NaCN administration. The second possible mechanism is a partial reversal of the inhibition of the cytochrome C-oxidase reaction. Utilizing the method of Albaum et al. (148) which measures the rate of cytochrome C oxidation, an increase in the rate of oxidation in the presence of high oxygen concentrations occurs (164). This increase in cytochrome C oxidation has been noted with crude rat brain homogenates, and with a purified cytochrome oxidase preparation, Complex IV (164, 165). Third, other oxidative processes also might be stimulated by oxygen which are independent of the effect on cytochrome oxidase. Fourth, oxygen may accelerate the biotransformation of cyanide, although it should be pointed out that the cyanide detoxifying enzyme, rhodanese, does not require oxygen. However, an increased oxygen tension may still enhance cyanide detoxication by removal of sulfite ion, an end product of the rhodanese reaction and an inhibitor of this enzyme (166–168). Therefore, an increased rate of oxidation of sulfite to sulfate by oxidative enzymes in the presence of oxygen would result in a less likelihood of product inhibition occurring.

Although the above mechanisms may explain the prophylactic and antidotal effect of oxygen (161, 162, 164, 169), they are not completely compatible with the inability of oxygen to augment the effect of sodium nitrite, while enhancing that of sodium thiosulfate and the combined sodium thio-

sulfate-nitrite treatment. Since the methemoglobin produced by sodium nitrite treatment decreases the oxygen transporting ability of blood, theoretical considerations would indicate that oxygen therapy would be of greatest value in those experiments in which sodium nitrite is utilized. However, the experimental evidence shows that oxygen has little or no effect on sodium nitrite, while strikingly increasing the protective effect seen with combined nitrite-thiosulfate therapy (162). A mechanism proposed by Gibbon (165) to explain these findings is associated with the various complex equilibrium processes occurring during treatment and their relationship to the impaired ability to transport oxygen caused by the nitrite induced methemoglobinemia.

The fact that the cyanide antidotes, sodium nitrite and sodium thiosulfate (149–151) antagonize 2-PAM toxicity only to a minor extent (86) suggests that the small amount of cyanide liberated after 2-PAM administration does not play a major role in 2-PAM toxicity. However, should alkylphosphate poisoning be complicated by impaired renal function, this would be another matter.

Under war time conditions, the use of 2-PAM either prophylactically or therapeutically for the antagonism of war gases, such as sarin and tabun may pose a very different problem. It would be quite realistic to anticipate that a segment of the population might have poor renal function resulting from shock due to trauma and hemorrhage. Should these conditions accompany war gas poisoning, the administration of 2-PAM might be hazardous, as the primary mechanism for the excretion of 2-PAM would be greatly impaired. However, the metabolism of 2-PAM and, therefore, the formation of cyanide ion, would be relatively enhanced, providing liver function was not too greatly impaired. On the other hand, this should not be a factor with 4-aldoximino pyridinium substituted antagonists.

Studies on the basic chemical mechanism of the hydrolysis of the 1-methyl-2-cyano- and 4-cyanopyridinium ions by Kosower & Patton (58, 59) indicate that the ratio of the formation of cyanide (or pyridone)/amide from the hydrolysis of either cyanopyridinium ion is pH dependent and a higher ratio is attained with increasing pH; however, irrespective of pH, the cyanide (or pyridone)/amide ratio of the 4-cyanopyridinium ion has always been much less than that of the 2-cyanopyridinium ion. More important, at a pH which would be comparable to that in biological systems, the 2-cyanopyridinium ion would continue to form cyanide as well as 1-methyl-2-pyridone and 1-methyl-2-carbamidopyridinium ion, while the 4-cyanopyridinium ion would form only one product, the 1-methyl-4-carbamidopyridinium ion (Fig. 3).

The water hydrolysis of the cyanopyridinium ion may be of importance (124) and in this respect with 1-methyl-2-cyanopyridinium ion, the formation of cyanide ion (and 1-methyl-2-pyridone) would be favored because in amide formation, a partial positive charge would probably be formed in the transition state, and the partial positive charge would be close to the positive hetero-nitrogen. Conversely, with 4-cyanopyridinium ion, the hydrolysis

of the amide by water would be favored, as the resulting partial positive charge in the transition state is further from the positive hetero-nitrogen. These chemical studies suggest the possibility that if both the 2- and 4-substituted aldoximinopyridinium ions were converted in the body to their respective cyanopyridinium ions, 2-PAM would liberate predominantly cyanide ions, while the 4-substituted derivative would not. Hence the 4-substituted derivative might have advantages as an antidote for alkylphosphate antagonist poisoning (59). The biologic findings are in accordance with these predictions. Preliminary studies in nephrectomized rats which received 2-PAM and two 4-substituted aldoximinopyridinium compounds (4-PAM and TMB-4) by the intravenous route indicate that while appreciable levels of blood cyanide are attained with 2-PAM (133), no increase occurs with 4-PAM or TMB-4 (133).



## 4-Aldoximino Pyridinium Substituted Antagonists

Since metabolic studies on the biotransformation of 2-PAM indicate that one of the end products formed is the toxic cyanide ion (86, 133), this raised the possibility that the 4-aldoximino substituted pyridinium ions may be more desirable in the treatment of alkylphosphate intoxication. Although 1-methyl-4-aldoximinopyridinium ion (4-PAM) was known to reactivate alkylphosphate inhibited cholinesterase, its activity was considerably less than that of 2-PAM (170, 171). Various investigators have attempted to develop a more effective 4-aldoximino substituted antagonist. Poziomek et al. (17), Hobbiger et al. (172), Loomis et al. (173) and Lüttringhaus & Hagedorn (174) described the preparation of a series of bisquaternary derivatives. These compounds were synthesized on the assumption that if strong associations between the organic oximes and the inhibited enzymes were an important factor in the reactivation process, it would be feasible to increase the rate of reactivation by combining the reactive aldoximinopyridinium moieties with functional groups, which are known to associate strongly with the enzymes, such as the bisquaternary groups. A few of these compounds were found to be not only more rapid reactivators of alkylphosphate inhibited cholinesterase than 2-PAM (170, 175), but also more effective in the treatment of animals poisoned with some of these alkylphosphates (176). The most effective aldoximinobispyridiniums have been 1,1′-trimethylene bis(4-aldoximinopyridinium) (TMB-4) (17, 172) and bis(4-hydroxyiminomethyl pyridinium-(1)-methyl)-ether (LüH6) (174) dibromides. These two bisquaternary compounds appear to be quite similar as alkylphosphate antagonists (177), however, Erdman and co-workers (178, 179) feel that LüH6 is less toxic and distributes in higher concentrations to the CNS than TMB-4.

The efficacy and limitations of TMB-4 in the treatment of alkylphosphate intoxication have been studied more extensively than LüH6. Bay et al. (176) investigated the effects of TMB-4 on sarin and this bisquaternary compound was reported to be more potent than 2-PAM. Oberst et al. (180) Hobbiger and co-workers (172) and Fleisher et al. (181) also indicate that

TMB-4 was shown to be therapeutically more effective than 2-PAM in antagonizing DFP, TEPP, sarin and dimethylamidomethoxyphosphorylcyanide (tabun) intoxication. Although the acute and subacute toxicity of TMB-4 is greater than that of 2-PAM, it is also a more effective and more potent antagonist than 2-PAM (182, 183). This should allow the use of a lower dose schedule of TMB-4 in the treatment of alkylphosphate poisoning than is presently employed for 2-PAM. The predominant pharmacological action produced by TMB-4 is referable to the cardiovascular system producing tachycardia and hypotension (184). The hypotensive effect of TMB-4 has been shown by Bay (185) to be attributed mainly to its ganglionic blocking properties. This effect on the cardiovascular system was thought to be an undesirable effect for treating alkylphosphate poisoning. However, under certain conditions the ganglionic blocking properties may be beneficial rather than harmful, e.g. in the treatment of shock (186, 187). Finally, preliminary reports by Way et al. (188) indicate that the TMB-4 is metabolized to a 4-cyano derivative, trimethylene-1-(4-aldoximinopyridinium)-1′-(4-cyanopyridinium) ion, which should not hydrolyze to form cyanide. This assumption is supported by the fact that unlike 2-PAM, TMB-4 did not elevate cyanide levels when administered to nephrectomized animals (133). These factors certainly warrant further investigation and evaluation of TMB-4 and LüH6 in the treatment of alkylphosphate poisoning.



## LITERATURE CITED


1. Williams, R. T., *Detoxication Mechanisms* (John Wiley & Sons, Inc., New York, 1959)
2. Fishman, W. H., *Chemistry of Drug Metabolism* (C. C Thomas, Springfield, Ill., 1961)
3. Gillette, J. R., *Advan. Pharmacol.*, **4**, 219–61 (1966)
4. Maynert, E. W., *Ann. Rev. Pharmacol.*, **1**, 45–64 (1961)
5. Hayes, W. J., *Ann. Rev. Pharmacol.*, **5**, 27–52 (1965)
6. Bush, M., Sanders, E., *Ann. Rev. Pharmacol.*, **7**, 57–76 (1967)
7. Yamafugi, K., Aoki, M., Omura, H., *Enzymologia*, **17**, 371–77 (1956)
8. Yamafugi, K., Omura, H., Yoshihara, F., Yoshitake, M., Oyama, H., *Enzymologia*, **19**, 180–85 (1958)
9. Yamafugi, K., Osajima, Y., Omura, H., Yoshida, T., *Enzymologia*, **21**, 37–42 (1960)
10. Mahadevan, S., *Arch. Biochem. Biophys.*, **100**, 557–58 (1963)
11. Wilson, I. B., Ginsburg, S., *Biochim. Biophys. Acta*, **18**, 168–70 (1955)
12. Ginsburg, S., Wilson, I. B., *J. Am. Chem. Soc.*, **79**, 481–85 (1957)
13. Wilson, I. B., Quan, C., *Arch. Biochem. Biophys.*, **73**, 131–43 (1958)
14. Wilson, I. B., *Federation Proc.*, **18**, 752–58 (1959)
15. Childs, A. F., Davies, D. R., Green, A. L., Rutland, J. P., *Brit. J. Pharmacol.*, **10**, 462–65 (1955)
16. Wilson, I. B., *J. Biol. Chem.*, **199**, 113–20 (1952)
17. Poziomek, E. J., Hackley, B. E., Jr., Steinberg, G. M., *J. Org. Chem.*, **23**, 714–17 (1958)
18. Poziomek, E. J., Kramer, D. N., Fromm, B. W., Mosher, W. A., *J. Org. Chem.*, 26, 423–27 (1961)
19. Poziomek, E. J., Kramer, D. N., Mosher, W. A., Michel, H. O., *J. Am. Chem. Soc.*, **83**, 3916–17 (1961)
20. Kewitz, H., *Arch. Biochem. Biophys.*, **66**, 263–70 (1957)
21. Green, A. L., Smith, H. J., *Biochem. J.*, **68**, 28–31 (1958)
22. Green, A. L., Smith, H. J., *Biochem. J.*, **68**, 32–35 (1958)
23. Hobbiger, F., *Brit. J. Pharmacol.*, **12**, 438–46 (1957)
24. Kewitz, H., Wilson, I. B., *Arch. Biochem. Biophys.*, **60**, 261–63 (1956)
25. Kewitz, H., Wilson, I. B., Nachmansohn, D., *Arch. Biochem. Biophys.*, **64**, 456–65 (1956)
26. Brown, R. V., Kunkel, A. M., Somers,

L. M., Wills, J. H., *J. Pharmacol.*, **120**, 276–84 (1957)
27. Askew, B. M., *Brit. J. Pharmacol.*, **12**, 340–43 (1957)
28. Grob, D., Johns, R., *J. Am. Med. Assoc.*, **166**, 1855–58 (1958)
29. Namba, T., Hiraki, K., *J. Am. Med. Assoc.*, **166**, 1834–39 (1958)
30. Davies, D. R., Green, A. L., *Brit. J. Ind. Med.*, **16**, 128–34 (1959)
31. Cohen, E. M., Wiersinga, H., *Acta Physiol. Pharmacol. Neerl.*, **8**, 40–51 (1959)
32. Sanderson, D. M., *J. Pharm. Pharmacol.*, **13**, 435–42 (1961)
33. Loomis, T. A., *Toxicol. Appl. Pharmacol.*, **5**, 489–99 (1963)
34. Holmstedt, B., *Pharmacol. Rev.*, **11**, 567–688 (1959)
35. O'Brien, R. D., *Toxic Phosphorus Esters* (Academic Press, New York, 1960)
36. Heath, D. F., *Organophosphorus Poisons* (Pergamon Press, New York, 1961)
37. Ellin, R. I., Wills, J. H., *J. Pharm. Sci.*, **53**, 995–1007 (1964)
38. Ellin, R. I., Wills, J. H., *J. Pharm. Sci.*, **53**, 1143–49 (1964)
39. Koelle, G. B., *Cholinesterase and Anticholinesterase Agents* (Springer-Verlag, Berlin, 1963)
40. Hahn, F. L., Jaeger, G., *Ber. Deut. Chem. Ges.*, **58**, 2335–40 (1925)
41. Blom, J., *Ber. Deut. Chem. Ges.*, **59**, 121–25 (1926)
42. Csaky, T. Z., *Acta Chem. Scand.*, **2**, 450–54 (1948)
43. Masterson, P., Tong, H. W., Way, J. L. (Unpublished)
44. Creasey, N. H., Green, A. L., *J. Pharm. Pharmacol.*, **11**, 485–90 (1959)
45. Way, J. L., Tong, H., Rabideau, R., *Federation Proc.*, **19**, 276 (1960)
46. Way, J. L., *J. Pharmacol.*, **138**, 258–63 (1962)
47. Gibbon, S. L., Way, J. L., *Biochem. Pharmacol.*, **15**, 1659–64 (1966)
48. Bradlow, L. H., Vanderwerf, C. A., *J. Org. Chem.*, **16**, 73–83 (1951)
49. Ellin, R. I., *J. Am. Chem. Soc.*, **80**, 6588–89 (1958)
50. Miranda, P. M. S., Way, J. L., *Mol. Pharmacol.*, **2**, 117–24 (1966)
51. Way, J. L., *J. Pharmacol.*, **138**, 264–68 (1962)
52. Way, J. L., Masterson, P. B., Beres, J. A., *J. Pharmacol.*, **140**, 117–24 (1963)
53. Ellin, R. I., Easterday, D. E., *J. Pharm. Pharmacol.*, **13**, 370–73 (1961)
54. Way, J. L., Brady, W., Tong, H., *J. Chromatog.*, **21**, 280–85 (1966)
55. Crocker, C., *J. Chromatog.*, **2**, 115–16 (1959)
56. Firemark, H., Barlow, C., Roth, L., *J. Pharmacol.*, **145**, 252–65 (1964)
57. Kosower, E. M., Skorcz, J. A., Schwarz, W. M., Patton, J. W., *J. Am. Chem. Soc.*, **82**, 2188–91 (1960)
58. Kosower, E. M., *Molecular Biochemistry* (McGraw-Hill Co., New York, 1962)
59. Kosower, E. M., Patton, J. W., *Tetrahedron*, **22**, 2081–98 (1966)
60. Hobbiger, F., Sadler, P. W., *Brit. J. Pharmacol.*, **14**, 192–201 (1959)
61. Burgen, A. S. V., Chipman, L. M., *Quart. J. Exptl. Physiol.*, **37**, 61–74 (1952)
62. Rodriquez, L. A., *J. Comp. Neurol.*, **102**, 27–46 (1955)
63. Mayer, S. E., Bain, J. A., *J. Pharmacol.*, **118**, 17–25 (1956)
64. Brodie, B. B., Kurz, H., Schanker, L. S., *J. Pharmacol.*, **130**, 20–25 (1960)
65. Schanker, L. S., *Ann. Rev. Pharmacol.*, **1**, 29–44 (1961)
66. Schanker, L. S., *Pharmacol. Rev.*, **14**, 501–30 (1962)
67. Jager, B. V., Stagg, G. N., Green, N., Jager, L., *Bull. Johns Hopkins Hosp.*, **102**, 225–34 (1958)
68. Kewitz, H., Nachmansohn, D., *Arch. Biochem. Biophys.*, **66**, 271–83 (1957)
69. Edery, H., Schatzberg-Porath, G., *Science*, **128**, 1137–38 (1958)
70. Sakai, F., Ri, H., Erdmann, W. D., Schmidt, G., *Arch. Exptl. Pathol. Pharmacol.*, **234**, 210–19 (1958)
71. Rutland, J. P., *Brit. J. Pharmacol.*, **13**, 399–403 (1958)
72. Cohen, E. M., Wiersinga, H., *Acta Physiol. Pharmacol. Neerl.*, **9**, 276–302 (1960)
73. Rosenberg, P., *Biochem. Pharmacol.*, **3**, 212–19 (1960)
74. Longo, V. G., Nachmansohn, D., Bovet, D., *Arch. Intern. Pharmacodyn.*, **123**, 282–90 (1960)
75. Wislicki, L., *Arch. Intern. Pharmacodyn.*, **129**, 1–19 (1960)
76. Brown, R. V., *Brit. J. Pharmacol.*, **15**, 170–74 (1960)
77. Edery, H., *Brit. J. Pharmacol.*, **18**, 19–28 (1962)
78. Tong, H., Way, J. L., *Federation Proc.*, **19**, 276 (1960)
79. Tong, H. S., Way, J. L., *J. Pharmacol.*, **138**, 218–23 (1962)
80. Peters, L., *Pharmacol. Rev.*, **12**, 1–35 (1960)

81. Berglund, F., Elwin, C., Sundwall, A., *Biochem. Pharmacol.*, **11**, 383–88 (1962)
82. Enander, I., Sundwall, A., Sörbo, B., *Biochem. Pharmacol.*, **11**, 377–82 (1962)
83. Way, J. L., Tong, H., *Federation Proc.*, **22**, 366 (1963)
84. Way, J. L., *Federation Proc.*, **23**, 279 (1964)
85. Gibbon, S. L., Way, J. L., *J. Chromatog.*, **26**, 202–7 (1967)
86. Enander, I., Sundwall, A., Sörbo, B., *Biochem. Pharmacol.*, **7**, 226–31 (1961)
87. Enander, I., Sundwall, A., Sörbo, B., *Biochem. Pharmacol.*, **7**, 232–36 (1961)
88. Kramer, B., *Biochem. Pharmacol.*, **11**, 299–305 (1962)
89. Dultz, L., Epstein, M. A., Freeman, G., Gray, E. H., Weil, W. B., *J. Pharmacol.*, **119**, 522–31 (1957)
90. Tong, J., Masterson, P., Way, J. L., *Federation Proc.*, **20**, 172 (1961)
91. Way, J. L., Tong, H. W., Masterson, P. (Unpublished)
92. Ellin, R. I., Carlese, J. S., Kondritzer, A. A., *J. Pharm. Sci.*, **51**, 141–46 (1962)
93. Hantzsch, A., *Ber. Deut. Chem. Ges.*, **24**, 37–51 (1891)
94. Hantzsch, A., *Ber. Deut. Chem. Ges.*, **27**, 702–3 (1894)
95. Hantzsch, A., Lucas, A., *Ber. Deut. Chem. Ges.*, **28**, 744–53 (1895)
96. Brady, O. L., Goldstein, R. F., *J. Chem. Soc.*, 1918–24 (1926)
97. Hauser, C. R., Sullivan, C. T., *J. Am. Chem. Soc.*, **55**, 4611–13 (1933)
98. Hauser, C. R., Jordan, E., *J. Am. Chem. Soc.*, **57**, 2450–56 (1935)
99. Hauser, C. R., Jordan, E., O'Connor, R., *J. Am. Chem. Soc.*, **57**, 2456–58 (1935)
100. Jordan, E., Hauser, C. R., *J. Am. Chem. Soc.*, **58**, 1304–5 (1936)
101. Hauser, C. R., Jordan, E., *J. Am. Chem. Soc.*, **58**, 1419–22 (1936)
102. Hauser, C. R., Jordan, E., *J. Am. Chem. Soc.*, **58**, 1772–76 (1936)
103. Vermillion, G., Hauser, C. R., *J. Am. Chem. Soc.*, **62**, 2939–42 (1940)
104. Hauser, C. R., Vermillion, G., *J. Am. Chem. Soc.*, **63**, 1224–29 (1941)
105. Benger, M., Brady, O. L., *J. Chem. Soc.*, 1220–27 (1950)
106. Brady, O. L., Jarrett, S. G., *J. Chem. Soc.*, 1227–32 (1950)
107. Brady, O. L., Jarrett, S. G., *J. Chem. Soc.*, 1232–33 (1950)
108. Ambrose, D., Brady, O. L., *J. Chem. Soc.*, 1243–49 (1950)
109. Lamb, J. C., Steinberg, G. M., *Biochim. Biophys. Acta*, **89**, 171–74 (1964)
110. Blanch, J. H., Onsager, O. T., *J. Chem. Soc.*, 3729–34 (1965)
111. Blanch, J. H., Onsager, O. T., *J. Chem. Soc.*, 3734–38 (1965)
112. Lamb, J. C., Steinberg, G. M., Hackley, B. E., Jr., Solomon, S., *Biochemistry*, **4**, 2475–89 (1965)
113. Steinberg, G., Solomon, S., *Biochemistry*, **5**, 3142–50 (1966)
114. Wagner-Jauregg, T., Saner, H., *Arzneimittel-Forsch.*, **9**, 579–81 (1959)
115. O'Neill, J. J., Kohl, H., Epstein, H., *Biochem. Pharmacol.*, **8**, 399–407 (1961)
116. Tong, H. W., Miranda, P. M., Way, J. L. (Unpublished)
117. Miranda, P. M., Doctoral Dissertation (Marquette Univ., 1966)
118. Hackley, B. E., Jr., Doctoral Dissertation (Univ. Delaware, 1958)
119. Green, A. L., Sainsbury, G. L., Saville, B., Stansfield, M., *J. Chem. Soc.*, 1583–88 (1958)
120. Hackley, B. E., Jr., Lamb, J. C., Steinberg, G. M., *Arch. Biochem. Biophys.*, **80**, 211–14 (1959)
121. Lamb, J. C., Steinberg, G. M., Solomon, S., Hackley, B. E., Jr., *Biochemistry*, **4**, 2475–84 (1965)
122. Miranda, P. M., Way, J. L., *Pharmacologist*, **5**, 241 (1963)
123. Miranda, P. M., Zampaglione, N. G., Way, J. L., *Pharmacologist*, **8**, 180 (1966)
124. Patton, J. W., Doctoral Dissertation (Univ. Wisconsin, 1961)
125. Way, J. L., Masterson, P. M., Tong, H. W. (Unpublished)
126. Way, J. L., Uszler, M., Poskie, J. (Unpublished)
127. Mason, S. F., *J. Chem. Soc.*, 4874–80 (1957)
128. Mason, S. F., *J. Chem. Soc.*, 5010–17 (1957)
129. Albert, A., Phillips, J. N., *J. Chem. Soc.*, 1294–1304 (1951)
130. Wu Chang, M. L., Johnson, B. C., *J. Biol. Chem.*, **234**, 1817–21 (1959)
131. Barber, M. S., Jackman, L. M., Weedon, B. C. L., *Proc. Chem. Soc.*, 96–97 (1959)
132. Way, J. L., Masterson, P. M., Beres, J. A. (Unpublished)
133. Way, J. L., Pfeiffer, W. (Unpublished)
134. Askew, B. M., Davies, D. R., Green, A. L., Holmes, R., *Brit. J. Pharmacol.*, **11**, 424–27 (1951)
135. Zampaglione, N. G., Way, J. L., *Federation Proc.*, **26**, 685 (1967)

136. Knox, W. E., Grossman, W. I., *J. Biol. Chem.*, **166,** 391–92 (1946)
137. Knox, W. E., Grossman, W. I., *J. Biol. Chem.*, **168,** 363–66 (1947)
138. Holman, W., DeLange, D. J., *J. Biol. Chem.*, **45,** 559–63 (1949)
139. Leifer, E., Roth, L., Hogness, D., Corson, M., *J. Biol. Chem.*, **190,** 595–602 (1951)
140. Lin, P., Johnson, B., *J. Am. Chem. Soc.*, **75,** 2974–77 (1953)
141. Wu Chang, M., Johnson, B., *J. Biol. Chem.*, **236,** 2096–98 (1961)
142. Chaykin, S., Dagani, M., Johnson, L., Samli, M., *J. Biol. Chem.*, **240,** 932–38 (1965)
143. Kramer, B., Seligson, H., Seligson, D., Baltrush, H., *Clin. Chim. Acta*, **10,** 447–52 (1964)
144. Quinn, G., Greengard, P., *Arch. Biochem. Biophys.*, **115,** 146–52 (1966)
145. Keilin, D., *Proc. Roy. Soc. (London), Ser. B*, **106,** 418–44 (1930)
146. Warburg, O., *Biochem. Z.*, **231,** 493–97 (1931)
147. Hogness, T. R., *Symp. Quant. Biol.*, **7,** 121–27 (1939)
148. Albaum, H. G., Tepperman, J., Bodansky, O., *J. Biol. Chem.*, **163,** 641–47 (1946)
149. Chen, K. K., Rose, C. L., *J. Am. Med. Assoc.*, **149,** 113–19 (1952)
150. Hug, E., *Rev. Soc. Arg. Biol.*, **9,** 91–97 (1933)
151. Chen, K. K., Rose, C. L., Clowes, G. H. A., *Proc. Soc. Exptl. Biol. Med.*, **31,** 250–52 (1933)
152. Albaum, H. G., Tepperman, J., Bodansky, O., *J. Biol. Chem.*, **164,** 45–51 (1946)
153. Lang, K., *Biochem. Z.*, **259,** 243–56 (1932)
154. Himwich, W. A., Saunder, J. P., *Am. J. Physiol.*, **153,** 348–54 (1948)
155. Sörbo, B., *Acta Chem. Scand.*, **7,** 1129–36 (1953)
156. Westley, J., Nakamoto, T., *J. Biol. Chem.*, **237,** 547–49 (1962)
157. Gordh, T., Norberg, B., *Acta Physiol. Scand.*, **13,** 26–34 (1947)
158. Levine, S., *J. Am. Med. Assoc.*, **170,** 1585 (1959)
159. Cope, C., *J. Am. Med. Assoc.*, **175,** 1061–64 (1961)
160. Stotz, E., Altschul, A. M., Hogness, T. R., *J. Biol. Chem.*, **124,** 745–54 (1938)
161. Way, J. L., Gibbon, S. L., Sheehy, M., *Science*, **152,** 210–11 (1966)
162. Way, J. L., Gibbon, S. L., Sheehy, M., *J. Pharmacol.*, **153,** 381–85 (1966)
163. Boxer, G. E., Rickards, J. C., *Arch. Biochem. Biophys.*, **39,** 7–26 (1952)
164. Gibbon, S. L., Way, J. L. (Unpublished)
165. Gibbon, S. L., Doctoral Dissertation (Marquette Univ., 1967)
166. Sörbo, B., *Acta Chem. Scand.*, **5,** 724–34 (1951)
167. Green, J. R., Westley, J., *J. Biol. Chem.*, **236,** 3047–50 (1961)
168. Sörbo, B., *Acta Chem. Scand.*, **16,** 2455–56 (1962)
169. Way, J. L., Sheehy, M., Gibbon, S. L., *J. Pharmacol.* (Submitted for publication)
170. Wilson, I. B., Ginsburg, S., *Biochem. Pharmacol.*, **1,** 200–6 (1958)
171. Kitz, R. J., Ginsburg, S., Wilson, I. B., *Biochem. Pharmacol.*, **14,** 1471–77 (1965)
172. Hobbiger, F., O'Sullivan, D. G., Sadler, P. W., *Nature*, **182,** 1498–99 (1958)
173. Loomis, T. A., Welsh, M. J., Jr., Miller, G. T., *Toxicol. Appl. Pharmacol.*, **5,** 588–98 (1963)
174. Lüttringhaus, A., Hagedorn, J., *Arzneimittel-Forsch.*, **14,** 1–5 (1964)
175. Berry, W. K., Davies, D. R., Green, A. L., *Brit. J. Pharmacol.*, **14,** 186–91 (1959)
176. Bay, E., Krop, S., Yates, L. F., *Proc. Soc., Exptl. Biol. Med.*, **98,** 107–10 (1958)
177. Heilbronn, E., Tolagen, B., *Biochem. Pharmacol.*, **14,** 73–77 (1965)
178. Erdmann, W. D., Eugelhard, H., *Arzneimittel-Forsch.*, **14,** 5–11 (1964)
179. Erdman, W. D., *Arzneimittel-Forsch.*, **15,** 135–39 (1965)
180. Oberst, F., Crook, J., Koon, W., *J. Pharmacol.* **136,** 393–96 (1962)
181. Fleisher, J. H., Michel, H. O., Yates, L., Harrison, C. S., *J. Pharmacol.*, **129,** 31–35 (1960)
182. O'Leary, J. F., Kunkel, A. M., Jones, A. H., *J. Pharmacol.*, **132,** 50–57 (1961)
183. Albanus, L., Jarlip, B., Sundwall, A., *Brit. J. Exptl. Pathol.*, **45,** 120–27 (1964)
184. Wills, J. H., *Federation Proc.*, **18,** 1020–25 (1959)
185. Bay, E., *Federation Proc.*, **18,** 366 (1959)
186. Hopkins, R. W., Simeone, F. A., *Arch. Surg.*, **89,** 365–72 (1964)
187. Bloch, J. H., Pierce, C. H., Lillehei, R. C., *Ann. Rev. Med.*, **17,** 483–508 (1966)
188. Way, J. L., Feitknecht, U. F., Corder, C. N., Miranda, P. M., *Biochem. Biophys. Acta*, **121,** 432–34 (1966)

# CONTENTS