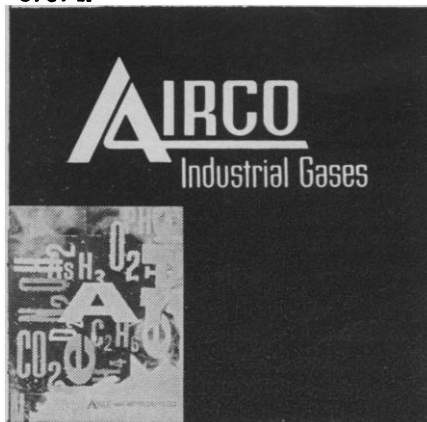


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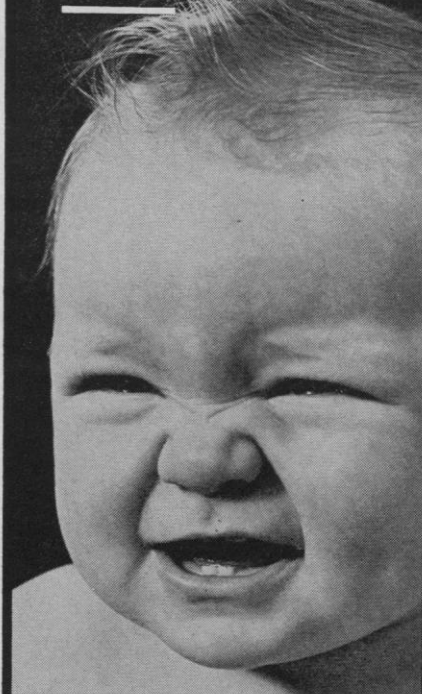
MEETINGS

Biochemical Toxicology of Insecticide Action

The last year has seen the tide of public interest and legislative action concerning the question of pesticides rise to a new height. A recurrent theme of the discussions is the inadequacy of available information to evaluate the effects of the use of pesticides on health and on the environment. The pesticides of maximum concern are the insecticides. The United States and Japan account for the largest share of the fundamental research that has been done in the area of insecticide metabolism and action. This was the background for the fifth meeting of the U.S.-Japan Cooperative Science Program, which met in Tokyo, 16-20 June 1969, under the joint sponsorship of the Japan Society for the Promotion of Science and the U.S. National Science Foundation.

Fundamental to the background of understanding needed to design new compounds is a way of dealing with the interaction of multiple factors which can affect toxicity. C. Hansch (Pomona College, California) discussed the application of his sigma-rho-pi analysis to the problem of accounting for the variations in anticholinesterase activity of organophosphates and carbamates. He analyzed the contributions of hydrophobic, electronic, and steric factors to the variations which had been described in the publications of Metcalf and Fukuto; in one series of aromatic phosphonates, hydrophobic character had negligible importance and one could account for virtually all the variation in terms of steric factors only. By contrast, in substituted phenyl carbamates, hydrophobic character was the single most important variable, and the electronic contribution was relatively small. Finally, in diethyl phenyl phosphates, one could account for the variation in the potency of the para derivatives using electronic factors alone; for the meta derivatives, extremely good accounting for the effects could be obtained if one included the steric parameter, and once more the hydrophobic character was important. Still better correlation was obtained if one included a "position term" to account for the consistent difference between meta and para compounds. In the discussion of this paper, the importance of the free radical character in some particular enzyme-inhibitor interactions

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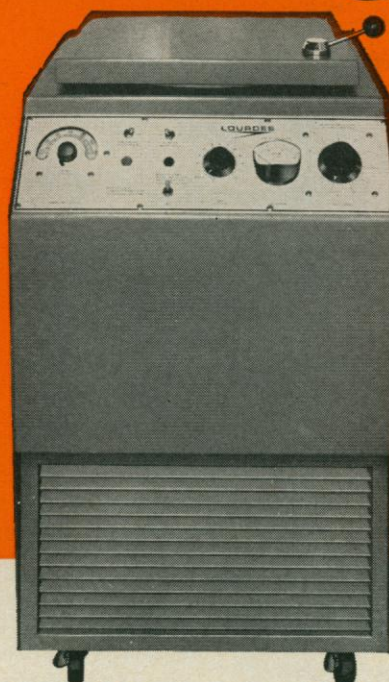
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was stressed by Hansch. It was also pointed out that, in alkyl diethyl phosphates, the hydrophobic character of the alkyl group could be all-important, quite unlike the two aromatic series described previously. T. Fujita (Kyoto University) described collaborative work with I. Yamamoto and M. Nakajima on the analysis of activity in nicotine analogs. The role of electronic, hydrophobic, and hydrogen bonding factors was considered and also the distance between the pyridyl and pyrrolidyl nitrogens. Although nicotine probably acts on the acetylcholine receptor in insect ganglia, there were similarities between the effect of factors upon acetylcholinesterase and insecticide activity. Both were favored by substituents of low electron affinity and marked hydrophobic character. The results lead to a modification of the simple model which stressed the importance of coulombic interaction between cationic nitrogen and the anionic site of the receptor or the enzyme.

The metabolism of insecticides is commonly of major importance in determining toxicity, selectivity, synergism, and biodegradability. The role of microsomal systems, both in insects and vertebrates, in metabolizing virtually all insecticides, has been a subject of intensive interest in recent years. R. Kato (National Institute of Hygienic Sciences, Tokyo) investigated various factors affecting drug metabolism by liver microsomes of the male rat. Drug-oxidizing activities were influenced by hormones and abnormal physiological states. However, the effect appeared in different ways for different substrates (inducers). For example, the magnitude of spectral change induced by hexobarbital was decreased by methylcholanthrene, but increased by administration of androgen, to the castrated rat. However, the change induced by aniline was increased by methylcholanthrene; androgen did not increase the change. The difference in the androgen dependence for the substrate interaction with cytochrome P-450 may be a factor responsible for the difference in the alterations of the spectral change between aniline and hexobarbital under the abnormal physiological states. It was suggested that the magnitude of substrate-binding with P-450 is one of the rate-limiting steps in the drug oxidation. D. J. Hennessy (Fordham University) reviewed the effectiveness and selectivity of several types of carbamate synergists; he argued that the potential of some of these for practical utility

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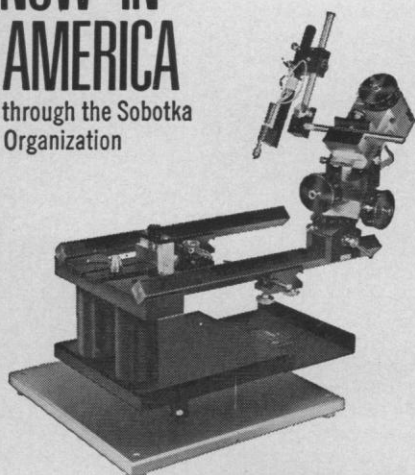
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was good, even though not presently realized. He pointed out that the target of the synergists was the insect microsomal oxygenase system, and discussed the mechanism by which the synergist attacked this target. Replacement of hydrogen in the methylene group of methylenedioxybenzenes by deuterium substantially reduced the synergistic ability, suggesting that the methylene group was involved in the attack. He suggested that the current debate as to whether the active attacking species was a benzodioxolium ion or a free radical could be resolved if one argued that the parent compound was converted through a free radical mechanism to the hydroxymethylene-dioxy derivative and the latter was in equilibrium with the diheterolium ion, of which it was a pseudo-base. He pointed out the plausibility of the diheterolium ion, which is an aromatic species, forming π -complexes with iron or copper in the microsomal oxygenase and suggested similarity in mode of action with organic thiocyanates, propynyl ethers, and aryloxyalkylamines. S. Kuwatsuka (Institute of Physical and Chemical Research, Saitama) examined the mode of action of methylenedioxyphenyl synergists. These synergists inhibited the microsomal hydroxylation of various insecticides, and themselves were degraded through hydroxylation by the same microsomal system. There were no essential differences between demethylenation, demethylation, and aniline ring hydroxylation. His study on various cases suggested that though the methylenedioxyphenyl compounds may serve as alternate substrates or competitive inhibitors for the microsomal hydroxylation, other factors may modify this basic mechanism in various degrees. The possibility that formate released from the methylenedioxy moiety reacts with microsomal enzyme was denied; but catechol released may act as an inhibitor. Possibly an allosteric effect on the microsomal components may be produced by the synergist.

P. A. Dahm (Iowa State University) discussed the work performed in his laboratories on the degradation of phosphorothionate insecticides by a microsomal system requiring oxygen and NADPH₂. It has been shown that such a system splits aromatic phosphorothionates (P=S compounds) at the aryl phosphate bond; phosphates (P=O compounds) are not usually split in this way, an exception being *n*-propyl paraoxon. The data covered a number of parathion analogs and also the phos-

phonate EPN. Comparisons were made with parathion labeled with P³² and S³⁵. Cleavage at the aryl group was important, as well as the expected activation to paraoxon (with S³⁵ going to S³⁵-sulfate) and its subsequent degradation. Parallel studies performed with diazinon suggested that in this case also, two-thirds of the metabolism by isolated microsomes occurs through cleavage of the "leaving group" by an oxidative process. Similarly in the housefly, there was substantial cleavage of the aryl group of parathion, and the evidence was that this was again by a microsomal oxidase rather than by a hydrolase. In two species of *Rhizobium* bacteria, by contrast, most of the metabolism was to aminoparathion, although about 10 percent of the compound was degraded through aryl cleavage. R. M. Hollingworth (Purdue University) reviewed the cleavage of organophosphorus triesters by liver enzymes, paying special attention to the soluble *O*-dealkylation system which has glutathione as a cofactor, and to the oxidative dealkylation pathway catalyzed by microsomes. Studies of the metabolism of C¹⁴-methyl-labeled methyl paraoxon in mouse liver homogenates showed that most activity was in the supernatant fraction, and that in this fraction there was a dependence on glutathione concentration. This system showed a pronounced preference for *O*-methyl phosphates. Studies in the whole mouse showed that when the labeled methyl group is removed, it is catabolized to volatile compounds which can be recovered in the respired air. Supplementary evidence on the importance of glutathione in *in vivo* metabolism was the finding that liver glutathione was substantially decreased when the phosphates Sumithion or Sumioxon were administered; Sumithion administration reduced the liver glutathione level by almost 50 percent. In other experiments, methyl iodide (135 mg/kg) was administered to mice and caused no overt symptoms, yet reduced the liver glutathione level to about one-third in an hour; such treated mice were ten times more susceptible to Sumithion poisoning. It is clear that glutathione-dependent degradation is an extremely important pathway in the metabolism of *O*-methyl organophosphates.

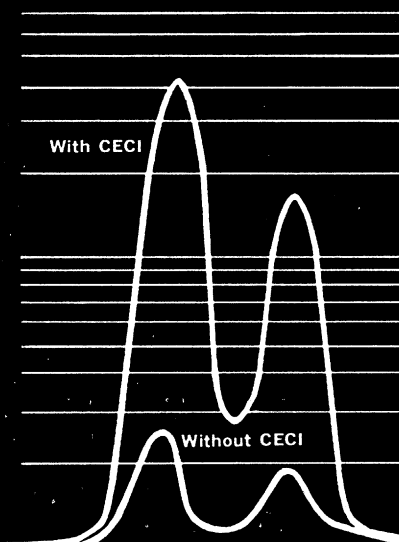
In the glutathione reaction just discussed, organophosphates are acting as alkylating agents rather than phosphorylating agents. M. Eto and H. Ohkawa (Kyushu University) have shown that

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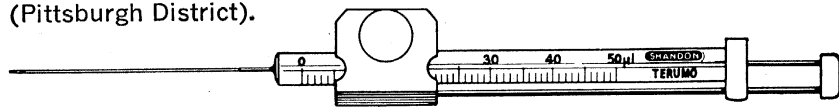
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saligenin cyclic phosphates are particularly good alkylating agents. They form S-salicyl glutathione in the above liver system. Furthermore, because they salicylate oximes quite readily, oximes cannot be used to reactivate cholinesterase which has been inhibited by these phosphates. One finds that the oxime, instead of dephosphorylating the inhibited cholinesterase in the usual way, removes the salicyl group and so yields an "aged" enzyme, whose esteratic site now has an ionic phosphate group bonded to it. Saligenin cyclic phosphates are fairly readily hydrolyzed to salicyl phosphates, which then can be converted to an *O*-hydrobenzyl cation, which in turn readily alkylates amines and mercaptans. Consequently, SH-enzymes can be inhibited by this route, and the fungicidal activity of saligenin cyclic phosphates appears to be due to this SH-alkylation.

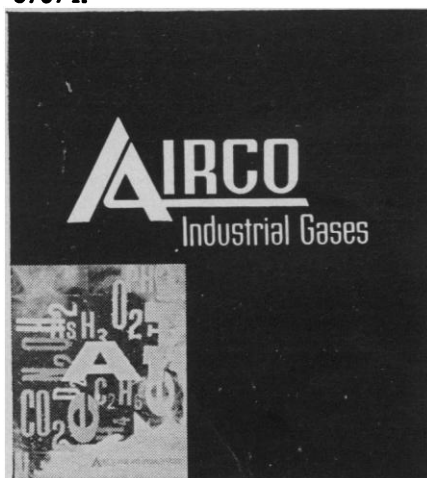
J. Miyamoto (Sumitomo Chemical Co., Osaka) described studies on the metabolic fate in rats of Meobal, 3,4-dimethylphenyl *N*-methylcarbamate, using a 4-methyl- ^{14}C -labeled sample. When orally administered, it was easily absorbed and distributed into several tissues within 15 minutes and then excreted rapidly and almost completely within 4 hours, mostly into urine and to a small extent into feces. At least 28 metabolites were shown in urine, while the unchanged carbamate was less than 0.5 percent after 48 hours. A thorough examination of the chemical structures of the metabolites both in vitro and in vivo revealed that the major metabolic pathway was the hydroxylation of either ring methyl group followed by further oxidation or conjugation as glucuronides. Hydroxylation of the *N*-methyl group and hydrolysis at the carbamoyl moiety were minor routes.

Metabolism is one of the important factors involved in resistance to insecticides, a phenomenon which has in some cases confounded attempts at insect control. F. W. Plapp, Jr. (Texas A & M University) presented a review of the status of our information on resistance mechanisms in houseflies. The numerous kinds of resistance appeared to be the expression of seven major genes, although additional genes might make minor contributions. He stressed the multiplicative nature of resistance which occurred when two genes were involved with a single compound, for instance, one gene controlling penetration and another controlling metabolism. There appeared to



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be only two kinds of resistance. One involved semi-dominant genes acting through a detoxifying mechanism. The other involved recessive genes conferring resistance by various other mechanisms. Only the former type appeared to be capable of being overcome, for instance by the use of synergists; the latter type appeared to be intractable. Plapp pointed to parallels between resistance and the induction of enzymes by their substrates, a phenomenon thoroughly explored in microorganisms. He had been able to induce microsomal oxidizing enzymes in insects by treating with DDT. The possibility therefore exists that the relatively simple view of resistance as involving Mendelian selection among randomly fluctuating levels of detoxifying enzymes (for instance) might need to be replaced by a model in which the selecting agent actively increased the level of detoxifying enzyme. A study of the effects of insecticides at the nuclear level was clearly called for.

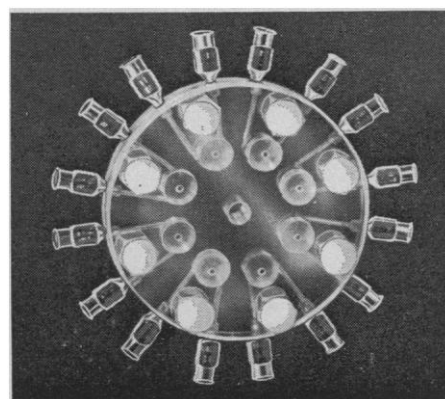
Certain aspects of the toxicity of organophosphate insecticides were discussed. T. Saito, T. Miyata, and K. Iyatomi (Nagoya University) described the effects of chronic poisoning by several such compounds. By frequent exposure to subacute doses of dimethoate and Sumithion contained in food (300 and 1000 ppm, respectively), an accumulation of physiological effects were observed in mice, such as the occurrence of typical organophosphorus toxicity symptoms after several days, reduced growth rate, and marked inhibition of cholinesterase and aliesterase activities of brain, blood, and liver. However, such effects were very small or absent with trichlorophon. The metabolic pattern as observed with the use of labeled insecticides was altered by continuous administration. Of interest was that such insecticides did not accumulate in the tissues, although giving marked chronic effects.

There has been a resurgence of interest in botanically derived insecticides, which will undoubtedly increase as the chlorinated hydrocarbons are phased out by legislative action. J. Fukami, T. Mitsui, T. Shishido, and K. Fukunaga (Institute of Physical and Chemical Research, Saitama, and National Institute of Agriculture Science, Tokyo) discussed the causes of the selective toxicity of rotenone insecticide between mammals, fish, and insects from a biochemical point of view. Vitamin K₃ played a part in restoring the mitochondrial respiration inhibited

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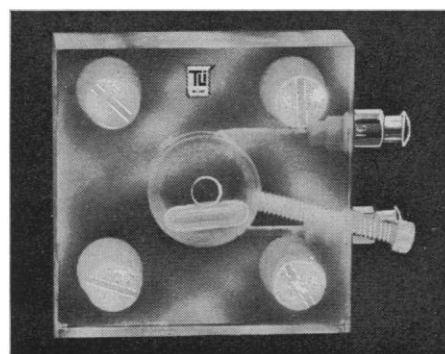
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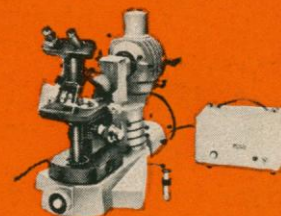
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by rotenone or piericidin A in the case of rat liver, but not in the case of insect tissue. This may be due to the reduced DT-diaphorase activity in insects. For metabolic degradation of rotenone, qualitative and quantitative differences in the soluble fractions derived from various organisms were also an important factor; one derived from mammals and fish enhanced the production of water soluble metabolites, while one from insects did not. Of interest was the probable presence in the soluble fraction derived from insects of a natural inhibitor, which was found to be a protein of molecular weight of 6,000 to 15,000. I. Yamamoto (Tokyo University of Agriculture) reviewed the problems of pyrethroid insecticides, which interact with the nerve axon. Recent electrophysiological studies disclosed the mechanism of action at the cellular level: effects on the negative after-potential are the cause of abnormal excitation and convulsions, and the block of both sodium and potassium conductances is the cause of paralysis. However, many problems, such as the primary site of action, central or peripheral; the mechanistic difference for knockdown and kill; the molecular aspects of the interaction with the nerve components; the cause of the effect on the negative after-potential; and the chemistry of the neurotoxin released by intoxication, remained to be solved. On the other hand, our knowledge of the metabolic pathway of pyrethroids in insects and mammals has greatly progressed. Oxidation is a major mechanism. While the structure-activity relation has remained empirical, the essentiality of *gem*-dimethyl group on the acid moiety was recognized and the introduction of new alcohol moieties provided several potent new pyrethroids.

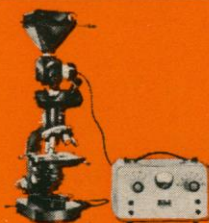
The relation between insecticides and the nervous system was further explored. M. Sakai (Takeda Chemical Industries, Kyoto) reported the mode of insecticidal action of nereistoxin, a poison from a marine annelid, *Lumbrineris heteropoda*, and having the structure of 4-*N,N*-dimethylamino-1,2-dithiolane. Electrophysiological experiments gave evidence that it competitively blocked the acetylcholine receptor of the cockroach central nervous system by depressing excitatory postsynaptic potentials without potentiating the postsynaptic membrane potentials, in contrast to nicotine and eserine. The ganglionic blocking activity of the derivatives was nearly pro-



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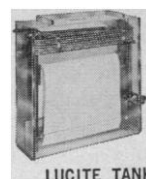
portional to their insecticidal activity. Cartap, which is 1,3-bis(carbamoylthio)-2-(*N,N*-dimethylamino) propane hydrochloride, was developed from nereistoxin and used in practical insect control, particularly for lepidopterous insects. It probably acts by being converted metabolically into nereistoxin or its dihydro form. R. D. O'Brien (Cornell University) described the first successful attempts to study the acetylcholine receptor in broken cell preparations. Evidence was provided that preparations from the electroplax of the electric skate *Torpedo*, and from housefly head, contained receptor activity, which could be followed by the binding of tritiated muscarone in concentrations of the order of $10^{-6}M$. The evidence included the suitability of the location, the high affinity and reversibility of the binding, the appropriate amount, and above all the appropriate response to selected blocking agents. Evidence was provided that the plax receptor was a phospholipoprotein. The effect of numerous blocking agents suggested that the plax receptor was of the classic neuromuscular type, as is generally believed, but that the housefly receptor was of a mixed muscarinic and nicotinic type. In addition, the housefly receptor was unexpectedly blocked by compounds such as tyramine and hordenine. The findings gave promise that the receptor in the insect might be quite unlike that in the vertebrate and might offer the possibility of designing new kinds of insecticidal compound.

Finally, some new techniques of interest to students of insecticide action were described. N. Kurihara (Kyoto University) and E. Nakajima and H. Shindo (Sankyo Company) have developed a new frozen technique for whole insect body autoradiography. With the use of labeled compounds on the American cockroach, it was shown that γ -BHC penetrated much faster than the β -isomer and reached almost all parts of the central nervous system, crop, and gizzard within 15 minutes, but very little if any reached the area of the fat body. Of interest was the striking difference in the penetration pattern of γ -BHC and nicotine into the central nervous system: γ -BHC accumulated at the peripheral region of the brain, ganglia, and other parts of the central nervous system, but not inside, while nicotine penetrated into the central nervous system very easily, and the concentration difference between inside and outside the central nervous

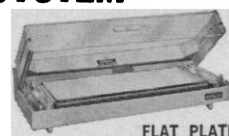
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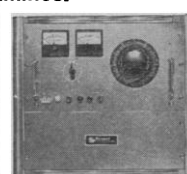
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system was very small. These studies were consistent with the known behavior of nicotine and may throw a light on the mode of action of BHC. Y. P. Sun (Shell Chemical Company, Modesto, California) discussed the complicated interplay of events which follows the treatment of an insect with an insecticide. He described a method by which a given dose of insecticide could be injected into a housefly over a time period varying from a few seconds up to many hours. In general, the toxicity of any insecticide was reduced by giving the dose over a long period. It was of special interest that dieldrin, which is not metabolized by houseflies, was of much less toxicity if administered over 30 minutes. The implication is that slow delivery permits the operation of other mechanisms (such as storage) quite apart from the anticipated increased role of metabolism which occurs in compounds such as organophosphates. It was also observed that, in a series of vinyl phosphate analogs of Azodrin®, the toxicities by topical application varied greatly, but by injection of flies pretreated with a synergist the toxicities varied very little. The implication was that the compounds, which differed only in their *N*-alkyl substituents, showed a great variation in topical toxicity only because of variations in their penetration and detoxication rates in the organism.

The above papers are scheduled for publication in the near future by Academic Press. Another outcome of the conference is an attempt to provide information through a "clearing house" mechanism to Japanese and U.S. scientists about the opportunities for research by visitors in the two countries. Scientists working in insecticide action or metabolism who would be interested in such information, should communicate with either one of the undersigned.

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