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IN VIVO REACTIVATION BY PAM OF BRAIN CHOLINESTERASE INHIBITED BY PARAOXON*

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Abstract—Pyridine-2-aldoxime methiodide (PAM) injected in amounts of from 25 to 150 mg/kg from 0·5 to 1·5 hr after 0·15 mg/kg paraoxon caused a significant increase in the cholinesterase levels of cerebral cortex, pons, cerebellum, region of area postrema in medulla, and the remainder of the medulla of rabbits killed 3 hr after the injection of paraoxon. Reactivation seemed most marked in the pons, region of area postrema and the remainder of the medulla. Similar results were obtained when rabbits were killed 24 hr after paraoxon, and there appeared to be no difference whether the first dose of PAM was given 1 or 5 hr after the inhibitor. A chloroform extraction procedure was used which appears capable of removing at least some of the extracellular paraoxon present in the brain tissue and uncombined with the enzyme at the time of sacrifice of the rabbits. It is concluded that sufficient amounts of PAM are able to penetrate into the brain to cause marked reactivation of inhibited cholinesterase. Possible reasons are discussed why other workers have not observed *in vivo* reactivation of brain cholinesterase by PAM.

INTRODUCTION

SOME organophosphorus compounds are powerful potential chemical warfare agents (the so-called "nerve gases"), others are widely used as insecticides. These compounds are potent irreversible inhibitors of ester splitting enzymes in general, but their lethal action must be attributed to the inhibition of acetylcholinesterase, the enzyme which hydrolyses acetylcholine and is vital for nerve activity. Analysis of the molecular forces at the active site of acetylcholinesterase and the mechanism of the hydrolytic process has revealed the mode of action of these poisons: a nucleophilic group at the active site, acetylated in the normal process, is phosphorylated; this process is not readily reversible and thereby the enzyme is inactivated.[1, 2] It may, however, be reactivated by nucleophilic compounds which in a displacement reaction remove the phosphoryl group from the enzyme. In systematic studies of the forces acting in the enzyme surface, a powerful reactivator of the phosphorylated enzyme has been developed (pyridine-2-aldoxime methiodide-PAM).[3] The compound proved to be an effective antidote against some of the nerve gases and insecticides, especially in combination with atropine.[4–10]

Paralysis of respiration seems to be the main cause of death in all cases of alkylphosphate poisoning tested. Although the respiratory failure produced by some compounds appears to be predominantly the result of a paralysis of the diaphragm, central effects are in many instances an important additional factor. Reactivation of acetylcholinesterase in the diaphragm of mice treated with PAM after exposure to an

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alkylphosphate was demonstrated by Kewitz.[13] Only slight reactivation of enzyme activity by PAM was found in the brain of mice exposed to paraoxon and no reactivation after injection of DFP (di*iso*propyl fluorphosphate).[7] Other observers were unable to find any reactivation in brain.[10–12] It was therefore assumed that PAM, being a quaternary ammonium ion, is unable to pass through the blood-brain barrier in sufficient amounts to produce reactivation of the enzyme. On the other hand, Bovet and Longo found a definite effect of PAM on the electroencephalogram of rabbits exposed to sarin.[14] This observation suggests a central action of the antidote.

It appeared of both practical importance and of theoretical interest to find an unequivocal answer to the problem of whether or not PAM is able to penetrate the blood-brain barrier and repair the chemical lesion in the brain of alkylphosphate poisoned animals by reactivating the enzyme activity either in the entire brain or in some vital areas. This paper records the results of an investigation of this problem.

MATERIALS AND METHODS

All injections were given intravenously into adult male albino rabbits weighing from 2·5 to 3·0 kg. Diethyl-*p*-nitrophenyl phosphate (paraoxon) was used as a representative anticholinesterase and all dilutions for injections were made with saline. PAM was dissolved in distilled water. Sufficient sodium hydroxide was added to give an unbuffered solution of pH 8, which when injected in a dose of 1–2 ml would not alter the blood pH.

At specified intervals of time after poisoning with paraoxon, the rabbits were sacrificed by the intravenous injection of air, their brains removed and dissected into the areas used for determination of cholinesterase activity. All brain areas unless otherwise specified were extracted with chloroform by a procedure modified from that described by Kewitz.[13] The samples were sliced by hand, weighed and frozen in dry ice. They were then lyophilized and each sample placed in 15 ml of — 60 °C chloroform for 0·5 hr during which time they were stirred, and the extractant replaced by fresh chloroform. They were filtered in funnels surrounded by dry ice, rinsed with ether and dried. The tissues were then homogenized in Krebs phosphate media;[15] the suspension containing 5–10 mg wet wt/ml, depending on the activity expected, except for samples from the region of the area postrema where due to the small amount of tissue obtainable the final concentration was 2·5–3·5 mg/ml. Acetylcholine chloride at a concentration of 5×10^{-3} M was used as the substrate, and all tissues were incubated while shaking in a water bath, at 38 ° for 2 hr. Acetylcholine was employed as the substrate instead of substrates more specific for different types of cholinesterase, since it is known that the great majority of cholinesterase in the brain is of the specific type.[16] In addition, low concentrations of PAM reactivate almost exclusively acetylcholinesterase due to molecular complementarity and not the non-specific esterases. Since probably only small amounts of PAM will penetrate into the brain, it is highly unlikely that any non-specific esterases would be reactivated. Cholinesterase activity was determined at the 1 and 2 hr periods of incubation by the Hestrin[17] colorimetric procedure.

RESULTS

A procedure often used for determining cholinesterase activity of tissues from animals previously exposed to alkylphosphates or other inhibitors of cholinesterase

has been that of preparing a homogenized suspension immediately after removal of the tissue. This may not, however, indicate the true cholinesterase activity of the cells at the time of death or sacrifice of the animals, since a portion of the alkylphosphate may have been localized outside of the cell, and excess inhibition may thereby be produced during homogenization. This problem was pointed out by Nachmansohn and Feld.[18] Recently Kewitz and Nachmansohn have attempted to avoid these difficulties through a procedure by which most of the alkylphosphate in the extracellular spaces seemed to have been removed.[7, 13]

Before determining whether PAM can reactivate inhibited brain cholinesterase it was necesssry to devise an extraction procedure which would remove with the greatest possible efficiency the excess alkylphosphate uncombined with cellular cholinesterase. The precedure found most satisfactory is described in the methods section. Other techniques employing acetone, ether and methanol as extractants for varied lengths of time were tried. None of these procedures were found to be satisfactory either because the loss of enzyme activity compared with the control was too great or because the extraction of uncombined alkylphosphate was inefficient. It was found that control cerebral cortical tissue extracted with chloroform had 75 $\pm$ 2·7 per cent of the control unextracted values. This 25 per cent decrease in activity is probably partly due to denaturation and partly to unavoidable losses involved in the transfer of the tissue slices. Unextracted cerebral cortical tissue from rabbits treated with 0·2 mg/kg paraoxon and sacrificed 2 hr later had 5·6 $\pm$ 0·12 per cent of the normal cholinesterase levels. When the tissue was extracted with chloroform, all other conditions being equal, the activity was 11·5 $\pm$ 0·41 per cent uncorrected for the 25 per cent loss. Each of these above values are the means $\pm$ standard error based upon determinations on three rabbits. No correction for loss in control activity was applied to the data to be presented, since the extraction procedure was uniformly used in all experiments including the controls. The approximately twofold increase of cerebral cortical enzyme levels due to chloroform extraction is even more significant when one considers the loss due to extraction. It was observed that when rabbits were killed 24 hr after paraoxon, the extraction procedure caused no increase in the cholinesterase values. Apparently by this time all free paraoxon had been inactivated or removed. For uniformity, however, the tissues obtained from rabbits killed 24 hr after paraoxon were extracted in the usual manner.

Before testing the enzyme activity in brain tissues of paraoxon treated rabbits, the cholinesterase levels in various areas of the brain of normal animals were determined. The mean activities based upon determinations from three rabbits are as follows: cerebral cortex 265 $\pm$ 5·0, medulla 407 $\pm$ 17·6, pons 460 $\pm$ 25·2, cerebellum 403 $\pm$ 43·4 and region of the area postrema 530 $\pm$ 5·0 μmoles acetylcholine hydrolysed per g per hr. In order to test whether PAM reactivates brain cholinesterase, rabbits were killed either 3 or 24 hr after paraoxon or paraoxon plus PAM. The dose of paraoxon used (0·15 mg/kg) killed about 20 per cent of the rabbits; on these no determinations were made. In the 3 hr experiments PAM was injected either in a single dose of 100 mg/kg 1 hr after paraoxon, or in three doses of 40 mg/kg each at 0·5, 1 and 2 hr after paraoxon. Since there was no significant difference between these two procedures the results were combined. In Fig. 1 the results are presented in per cent of the mean control values based upon determinations from three or four rabbits for each brain area. In the 24 hr experiments PAM was injected in five doses of

50 mg/kg each over a period from 1 to 8 hr after injection of paraoxon. Fig. 2 presents the results with each percentage being based on determinations from four or five rabbits.

The cholinesterase activity of all brain areas tested, from the 3 and 24 hr experiments, are significantly ($P < 0{\cdot}01$ in all cases) greater in the rabbits that received PAM than in those which did not receive PAM. Examination of the data also indicates that the pons, the region of area postrema and the remainder of the medulla show greater reactivation by PAM than do the cerebral cortex and cerebellum, especially at the 3 hr interval of time. A single experiment with the entire brain minus the five areas

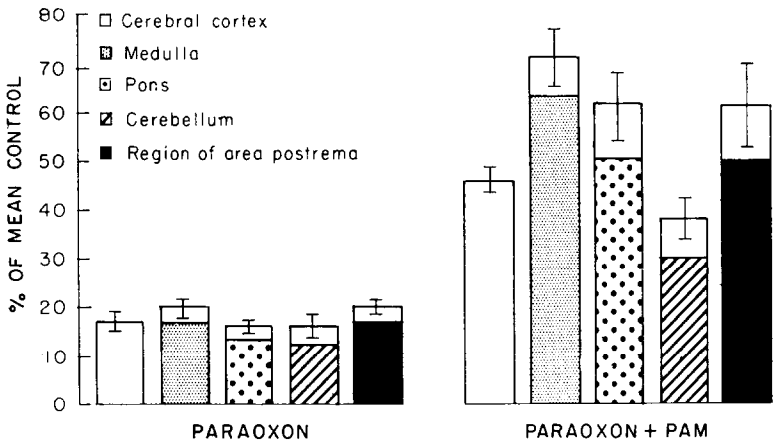

FIG. 1. Reactivation of inhibited cholinesterase by PAM. All rabbits received 0·15 mg/kg paraoxon 3 hr before sacrifice. PAM 100–150 mg/kg injected 0·5–2 hr after paraoxon. Lines at top of bars indicate ± 1 standard error.

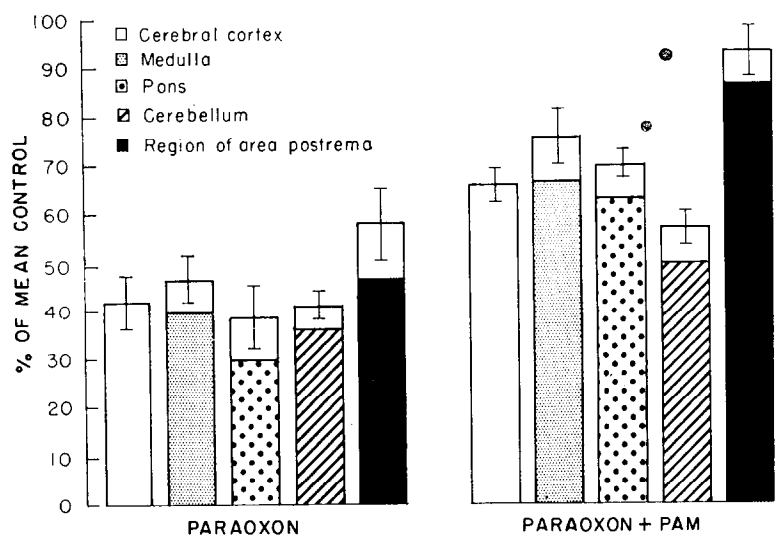

FIG. 2. Reactivation of inhibited cholinesterase by PAM. All rabbits received 0·15 mg/kg paraoxon 24 hr before sacrifice. Pam 250 mg/kg injected in five divided doses 1–8 hr after paraoxon. Lines at top of bars indicate ± 1 standard error.

listed in Fig. 1 indicates that PAM reactivates this cholinesterase to about the same extent as in cerebral cortex and cerebellum.

Since antidotal effects of PAM have been obtained with smaller doses than 100 mg/kg the reactivation of brain cholinesterase by 25 mg/kg PAM was compared in one experiment to that by 100 mg/kg PAM. The results are presented in Fig. 3. The

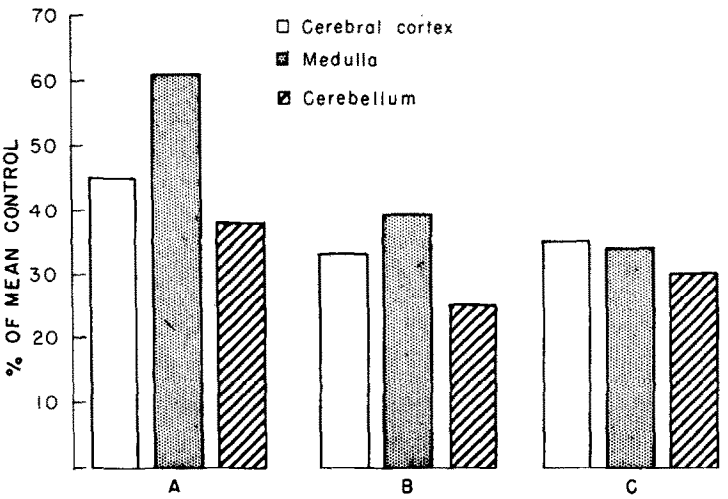

FIG. 3. Effect of alteration of dosage and time of administration of PAM upon reactivation of inhibited cholinesterase. All rabbits received 0·15 mg/kg paraoxon 3 hr before sacrifice. A, PAM 100 mg/kg njected 1 hr after paraoxon. B, PAM 25 mg/kg injected 1 hr after paraoxon. C, PAM 100 mg/kg injected 2 hr 45 min after paraoxon (15 min prior to sacrifice).

enzyme levels are not increased as much as with 100 mg/kg PAM but still appear significantly greater than with paraoxon alone (Fig. 1).

The question may be raised whether the reactivation obtained was an artifact due to *in vitro* reactivation of inhibited cholinesterase by free PAM present in the blood vessels of the brain at the time of homogenization. The 1 to 2 hr interval between the administration of PAM and the sacrifice of the animal in the 3 hr experiments was, however, probably sufficient for removal of most of the excess PAM. In the 24 hr experiments about a 16 hr interval was present between the last dose of PAM and sacrifice of the animal, which was undoubtedly sufficient for removal of uncombined PAM. To investigate this problem further an experiment was performed in which 100 mg/kg PAM was injected 15 min before the sacrifice of rabbits which had received paraoxon. These conditions would be expected to be favourable for any *in vitro* reactivation. As seen in Fig. 3 however the reactivation was actually less than when PAM was given 2 hr before death. The reason may be that PAM had not had sufficient time to penetrate into the brain to its maximum extent.

In order to minimize the possibility of a direct reaction between PAM and paraoxon in peripheral tissues, thereby decreasing the concentration of inhibitor which penetrates into the brain, PAM was injected in five doses of 50 mg/kg each over a time interval from 5 to 12 hr after the injection of paraoxon. The rabbits were sacrificed 24 hr after the injection of paraoxon. Each bar in Fig. 4 is the mean determination

from two rabbits. Under these conditions little free paraoxon would be expected to be present peripherally at the time when the first dose of PAM was given. The reactivation obtained was very similar to that observed in Fig. 2 where PAM was injected from 1 to 8 hr after paraoxon.

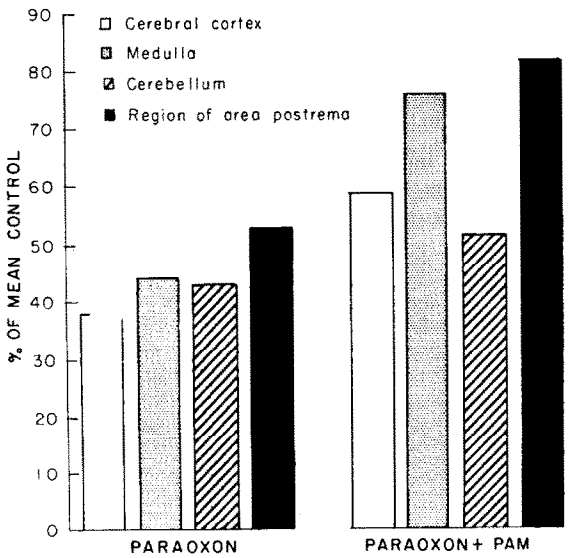

FIG. 4. Effect of delayed administration of PAM upon reactivation of inhibited cholinesterase. All rabbits received 0·15 mg/kg paraoxon 24 hr before sacrifice. PAM 250 mg/kg injected in 5 divided doses 5–12 hr after paraoxon.

DISCUSSION

Some reports indicate that *in vivo* PAM does not reactivate brain cholinesterase inhibited by alkylphosphates.[10–12] Since PAM *in vitro* can dephosphorylate brain cholinesterase inhibited by alkylphosphates[7, 11, 19] the lack of *in vivo* effect was explained as being due to the inability of PAM to penetrate into the brain. The reasoning was based on the fact that PAM is a quaternary ammonium ion; this type of compound is thought to penetrate the blood–brain barrier only slightly.[20, 21] The brain, however, cannot be considered as a single compartment through which drugs distribute themselves uniformly. It has been shown that certain areas of the brain such as the neurohypophysis, intercolumnar tubercles and area postrema apparently lack a well-developed blood–brain battier.[22, 23] This was one of the reasons for selecting among other areas the region of the area postrema in the present study. Differences in the penetration of PAM across the blood–brain barrier may explain why the pons, region of area postrema, and remainder of medulla showed greater reactivation of cholinesterase than did the cerebral cortex and cerebellum. Since many of the most vital central control centres, such as the respiratory centre are located in the medulla, near the area postrema, it is perhaps very important that the greatest reactivation was also obtained in these areas. The lack of reactivation reported in other investigations[10, 12] may be due to the omission of efficient extraction procedures. Since these workers used inhibitors which *in vitro* are reactivated by PAM, it does not appear likely that the reactivation observed here is due to the use of a different

inhibitor. Certain drugs may have different actions dependent upon the species as was shown for the metabolism of hexobarbital,[24] and since the other workers used rats and mice whereas we used rabbits, it is possible that there is a species difference in the penetration of PAM into the brain.

Our extraction procedure is probably far from 100 per cent effective in extracting uncombined inhibitor, as indicated by the fact that if rabbits were sacrificed 0·5 or 1 hr after high doses of paraoxon the cholinesterase values were lower than those in animals sacrificed after 3 hr and there was in the former experiments little difference between extracted or unextracted tissues (unpublished observations). The excess of uncombined paraoxon was apparently so great that the chloroform extraction was unable to remove enough paraoxon to prevent the inactivation of cholinesterase during homogenization.

In rabbits that received paraoxon alone there is a significant difference in the enzyme levels between those sacrificed at 3 hr and those sacrificed at 24 hr. This is probably because the extraction procedure is not perfect, and therefore the 3 hr values too low. Some spontaneous reactivation of inhibited cholinesterase and some synthesis of new enzyme also may occur *in vivo* in the course of 24 hr, but based upon the report of Oberst and Christensen[25] on the time required for spontaneous recovery of cerebral cholinesterase it appears unlikely that these two processes are adequate to explain the entire increase in cholinesterase activity.

When the first dose of PAM was given 5 hr after paraoxon it still was effective in causing an increase in brain cholinesterase levels although most of the extracellular paraoxon must have been removed by that time. It is also known that *in vitro* PAM reacts very slowly with paraoxon.[5] Based upon these two facts it appears safe to assume that the greater activity of brain cholinesterase observed when PAM is administered is not due to a direct reaction of PAM with paraoxon peripherally thereby decreasing the concentration of paraoxon which penetrates into the brain.

The brains were removed as fast as possible after death in order to avoid the breakdown of the blood–brain barrier. At most from 4 to 5 min were required to remove the brain. It is doubtful that in such a short period of time there is a sufficient breakdown of the blood–brain barrier to allow enough PAM to penetrate and in addition to reactivate the enzyme. Although the possibility of some breakdown of the barrier cannot be ruled out, it cannot be the only or most important factor responsible for the observed action of PAM, as indicated by Fig. 3. PAM, injected 2 hr and 45 min after paraoxon but 15 min prior to sacrifice, resulted in less reactivation than PAM, injected 1 hr after paraoxon but 2 hr prior to sacrifice. If there was a breakdown of the blood–brain barrier after death, we would expect greater reactivation since more PAM is present in the blood vessels of the brain when the rabbits are killed 15 min after the injection of PAM than when killed 2 hr after its injection.

Many quaternary ammonium ions are toxic and, *in vivo*, can only be used in small doses. Thus, when small doses of such compounds are administered, the barrier to their penetration into the brain usually effectively prevents entry, with the result that only very small amounts or none are found in the brain. Because of the relatively low toxicity of PAM high doses can be used. With a dose of 100 mg/kg intravenously one would expect, assuming uniform distribution and neglecting inactivation, a concentration of about 10^{-4} M in the tissues including the blood vessels which initially will probably have a higher concentration. Even assuming a blood–brain ratio for

PAM of 100 to 1 this would still provide a concentration inside the brain tissue of 10^{-6} M which is probably sufficient for reactivation of a significant percentage of inhibited cholinesterase.[3, 26] With doses of PAM lower than 25 mg/kg, which was the lowest dose used in these experiments, it is possible that no *in vivo* reactivation of cholinesterase would be observed, although undoubtedly even with small doses some PAM does penetrate into the central nervous system. It appears that central reactivation of cholinesterase, especially in the vital areas, such as the respiratory centres is an important factor in the antidotal effect which PAM exerts against paraoxon and possibly other anticholinesterases.

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REFERENCES

1. I. B. Wilson, *J. Biol. Chem.* **190**, 111 (1951).
2. I. B. Wilson, *The Mechanism of Enzyme Action* (Edited by W. D. McElroy and B. Glass) p. 642. Johns Hopkins Press, Baltimore (1954).
3. I. B. Wilson and S. Ginsburg, *Biochim. Biophys. Acta* **18**, 168 (1955).
4. H. Kewitz and I. B. Wilson, *Arch. Biochem. Biophys.* **60**, 261 (1956).
5. H. Kewitz, I. B. Wilson and D. Nachmansohn, *Arch. Biochem. Biophys.* **64**, 456 (1956).
6. I. B. Wilson and F. Sondheimer, *Arch. Biochem. Biophys.* **69**, 468 (1957).
7. H. Kewitz and D. Nachmansohn, *Arch. Biochem. Biophys.* **66**, 271 (1957).
8. T. Namba and K. Hiraki, *J. Amer. Med. Assoc.* **166**, 1834 (1958).
9. J. H. Wills, A. M. Kunkel, R. V. Brown and G. E. Groblewski, *Science* **125**, 743 (1957).
10. J. P. Rutland, *Brit. J. Pharmacol.* **13**, 399 (1958).
11. F. Hobbiger, *Brit. J. Pharmacol.* **12**, 438 (1957).
12. J. F. O'Leary, B. Harrison, G. Groblewski and J. H. Wills, *Fed. Proc.* **18**, 430 (1959).
13. H. Kewitz, *Arch. Biochem. Biophys.* **66**, 263 (1957).
14. D. Bovet and V. G. Longo. Unpublished observations.
15. W. W. Umbreit, R. H. Burris and J. F. Stauffer, *Manometric Techniques* (3rd Ed.) p. 149. Burgess, Minneapolis (1957).
16. M. G. Ord and R. H. S. Thompson, *Biochem. J.* **46**, 346 (1950).
17. S. Hestrin, *J. Biol. Chem.* **180**, 249 (1949).
18. D. Nachmansohn and E. A. Feld, *J. Biol. Chem.* **171**, 715 (1947).
19. A. F. Childs, D. R. Davies, A. L. Green and J. P. Rutland, *Brit. J. Pharmacol.* **10**, 462 (1955).
20. B. B. Brodie and C. A. M. Hogben, *J. Pharm., London* **9**, 345 (1957).
21. S. E. Mayer and J. A. Bain, *J. Pharmacol.* **118**, 17 (1956).
22. E. W. Dempsey and G. B. Wislocki, *J. Biophys. Biochem. Cytol.* **1**, 245 (1955).
23. J. B. Brierley, *Metabolism of the Nervous System* (Edited by D. Richter) p. 121. Pergamon Press, New York (1957).
24. J. P. Quinn, J. Axelrod and B. B. Brodie, *Biochem. Pharmacol.* **1**, 152 (1958).
25. F. W. Oberst and M. K. Christensen, *J. Pharmacol.* **116**, 216 (1956).
26. I. B. Wilson, *Biochim. Biophys. Acta* **27**, 196 (1958).