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RATE OF FORMATION *IN VIVO* OF THE UNREACTIVATABLE FORM OF BRAIN CHOLINESTERASE IN CHICKENS GIVEN DDVP OR MALATHION*

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Abstract—The half-time for the conversion of the reversibly inhibited form of the dimethyl phosphorylated cholinesterase to the irreversibly inhibited form of this enzyme in the brains of chickens given DDVP or malathion has been found to be 2 hr. The same half-time is observed *in vitro* in brain homogenates inhibited with DDVP or malaoxon, the active form of malathion.

THE RATE of formation of the irreversibly inhibited form of cholinesterase from the initial reversibly inhibited enzyme has been shown to be dependent on the dialkoxy group which is attached to the phosphorylated enzyme.

Thus, *in vitro*, the rate of the conversion increased in the order: diethyl phosphate < dimethyl phosphate and diisopropyl phosphate.[1–4] *In vivo*, a similar transformation has been noted with the cholinesterases of rabbit erythrocytes,[5] mouse brain and erythrocytes,[6] rat brain and erythrocytes,[7] and sheep erythrocytes.[8] However, the rate of the conversion of the reactivatable to the unreactivatable form of cholinesterase *in vivo* has been measured only for the diethyl and diisopropyl phosphorylated enzyme of mouse brain and erythrocytes. The half-time for the transformation of the diisopropyl phosphate enzyme was 4 hr; that of the diethyl phosphorylated enzyme was 36 hr.[6] It is known that the rate of formation of the irreversible form of the dimethyl phosphorylated enzyme of rat brain and erythrocytes[4,7] and sheep erythrocytes[8] *in vivo* is quite rapid.

However, previous investigators have not been able to measure accurately the rate of transformation of the dimethyl phosphorylated cholinesterase to the unreactivatable form because this process was so fast that significant conversion of the inhibited enzyme took place during the assay period. In addition, the rate of spontaneous reactivation was so rapid that the process of formation of the irreversibly inhibited enzyme could not be separated from the spontaneous reactivation of the inhibited enzyme.[4,7,8]

In previous experiments it was found that the brain cholinesterase of chickens given a subcutaneous dose of either of the dimethyl phosphates, malathion or DDVP, remained 85 to 90 per cent inhibited for one day.[9] This appeared to be sufficient time of inhibition to permit measurement *in vivo* of the rate of the transformation of the

* DDVP is O,O-dimethyl 2,2-dichlorovinyl phosphate; malathion is O,O-dimethyl S-(1,2-dicarbethoxyethyl) phosphorodithioate.

reversible to the irreversible form of cholinesterase uncomplicated by significant spontaneous reactivation of the enzyme. In addition, since malathion was given at 40 times the level of DDVP on a molar basis, it was possible to study the effect of this large difference in dosage on the rate of the transformation *in vivo*.

In these studies it was necessary to use a procedure that permitted a very rapid reactivation *in vitro* so that significant formation of the irreversibly inhibited enzyme did not take place during the reactivation. In preliminary experiments it was found that reactivation could take place very rapidly with an excess of 2-PAM (2-pyridine aldoxime methiodide),[10,11] and this procedure was used in the experiments reported in this paper.

EXPERIMENTAL

Sources of chemicals

The sources of most of the chemicals used and the method and rate of subcutaneous dosing of the chickens are given in another paper.[9] Acetylcholine perchlorate was obtained from K and K Laboratories,* Jamaica, N.Y.; 2-PAM was obtained from the Chemical Procurement Co., New York, N.Y.; malaoxon [O,O-dimethyl S-(1, 2-dicarbethoxyethyl) phosphorothioate] was a highly purified product of the American Cyanamid Co., New York 20, N.Y. With the exceptions listed below, all other methods are given in another publication.[9]

Measurement of cholinesterase activity

The cholinesterase activity of brain was determined with acetylcholine as the substrate in a manner designed to prevent reaction between enzyme and inhibitor during homogenization. This procedure was based on previous observations that the rate of the reaction between organic phosphorus compounds and cholinesterase is temperature dependent[12] and is decreased by the presence of acetylcholine.[12–17]

Three observations showed that the method employed was an assay of brain true cholinesterase. First of all, the hydrolysis of this choline ester was decreased when the concentration was raised above 0·01 M. Second, the rate of hydrolysis of butyrylcholine, a substrate for pseudocholinesterase of chicken brain,[18,19] was only 5 per cent that of acetylcholine. Third, acetyl-β-methylcholine was hydrolysed at 37·5 per cent the rate of acetylcholine. The ratio of the rates of hydrolysis of these two substrates is similar to that observed with other true cholinesterases.[20]

The assay of brain true cholinesterase was carried out in the following manner. The whole brain of a chicken was quickly removed, chilled, and homogenized at 0° with a motor-driven, all-glass homogenizer[21] in 4 volumes of solution which contained 0·02 M acetylcholine perchlorate, 0·095 M NaCl, 0·004 M KCl, 0·001 M KH_2PO_4, 0·001 M $MgCl_2$, and 0·02 M $NaHCO_3$. This solution was gassed 5 min with nitrogen–carbon dioxide (95/5) and kept in a stoppered bottle until it was used for the preparation of brain homogenate. The homogenate had a pH of 7·45. No more than 45 min elapsed between the time the chicken was killed and the time the assay of the homogenate was begun. A 0·1-ml portion of the ice-cold homogenate was placed in the main compartment of the Warburg flask that was chilled in an ice bath at 0° and

* Throughout this paper, the use of trade names and the names of suppliers is for identification only and does not constitute endorsement by the Public Health Service.

that contained 3 ml of ice-cold 0·02 M sodium bicarbonate, 0·001 M magnesium chloride, 0·095 M sodium chloride, 0·004 M potassium chloride, 0·001 M potassium acid phosphate, and 0·01 M acetylcholine perchlorate. The sodium bicarbonate solution had been pregassed with N_2/CO_2, 95/5, v/v. The flask was gassed with N_2/CO_2, 95/5, v/v on the manometer at 0° for 6 min, was transferred to the water bath at 25°, and readings were begun after 5 min. The manometer readings were taken at 5-min intervals for 30 min.

It was shown by incubation of 10% brain homogenate with inhibitor that the procedure just described would prevent reaction between brain cholinesterase and 33 and 80 times the I_{50} concentration of DDVP and of malaoxon, respectively, for 2 hr but not for 18 hr at 0°. Malaoxon is the active metabolite of malathion[22] and is responsible for the inhibition observed in homogenates of the brains of chickens given malathion. This procedure afforded 85 per cent protection against these concentrations of inhibitor which were in great excess over those required to produce 95 per cent inhibition of brain cholinesterase at 25° under conditions in which the I_{50} concentration was measured.

Reactivation of brain homogenates with 2-PAM

The following procedure was used in studies of reactivation *in vitro* of brain cholinesterase in (a) homogenates inhibited *in vitro* with DDVP or malaoxon, or (b) chickens given DDVP or malathion. One-tenth ml aliquots of ice-cold homogenate, prepared as described in the previous section with acetycholine at 0°, were added to duplicate ice-cold flasks. One flask (A) contained 0·01 M acetylcholine and the other reagents given in the previous section. The other flask (B) contained 0·01 M acetylcholine, the other reagents, and also 0·0018 M 2-PAM in the main compartment. The flasks were ice-cold when the homogenate was added, were kept in ice during the 6-min gassing period, and were then brought to 25° within 4 to 5 min in order to start the enzymatic hydrolysis of acetylcholine. Reactivation occurred during this time, since no greater extent of reactivation was observed in incubating the homogenates at 25° with 0·02 M 2-PAM for 1 hr. More consistent results were obtained with the addition of the homogenate to the main compartment of the flask at 0° than by incubation in the side arm at 25°. At the concentration of acetylcholine and 2-PAM employed, 2-PAM inhibited the cholinesterase of homogenates of normal chicken brain an average of 7 per cent.

The per cent reactivation (%R), or the per cent of the enzyme in the reactivatable form, was calculated from the following formula, proposed by Hobbiger:[5]

$$\%R = 100\left(\frac{\text{rate of reactivated flask B} - \text{rate of inhibited flask A}}{\text{rate of control C} - \text{rate of inhibited flask A}}\right)$$

In experiments in which the per cent reactivation was measured in homogenates of the brains of poisoned chickens, the control rate C was the mean cholinesterase activity of twelve chickens corrected for 7 per cent inhibition by 2-PAM. In studies in which homogenates were inhibited *in vitro*, the control rate C was the rate of hydrolysis of acetylcholine in the presence of 2-PAM by the uninhibited homogenate from the same chicken.

This procedure was capable of reactivating 80 to 85 per cent of the cholinesterase in brain homogenates that had been inhibited by incubation at 25° for 1 hr with as high

as 1,000 times the I_{50} concentrations of DDVP or malaoxon. In these experiments *in vitro*, acetylcholine was omitted from the homogenization medium in order to facilitate the inhibition of the enzyme by malaoxon or DDVP. Reactivation of the inhibited cholinesterase by 2-PAM under the conditions of these experiments was not influenced by the presence of acetylcholine, and homogenates prepared from the same brain of a normal chicken had the same enzymatic activity in the absence or presence of acetylcholine in the homogenizing medium.

Measurement of pI_{50} *value*

The pI_{50} value of malaoxon or DDVP in chicken brain homogenates was determined in the following manner. A 0·2-ml portion of a 10% homogenate was incubated in the side arm of the Warburg flask at 25° in a N_2/CO_2, 95/5, v/v atmosphere with a solution containing 0·02 M sodium chloride and the other salts mentioned in the description of the cholinesterase assay except the acetylcholine which was also omitted from the solution used for homogenization. After 1 hr the contents of the side arm were dumped into the main compartment, and cholinesterase activity was measured with 0·01 M acetylcholine as previously described. Preliminary experiments indicated that 45 min at 25° was sufficient time to permit complete reaction between inhibitor and enzyme. The pI_{50} value was then calculated in the usual manner from a plot of pI versus per cent inhibition of the homogenate.

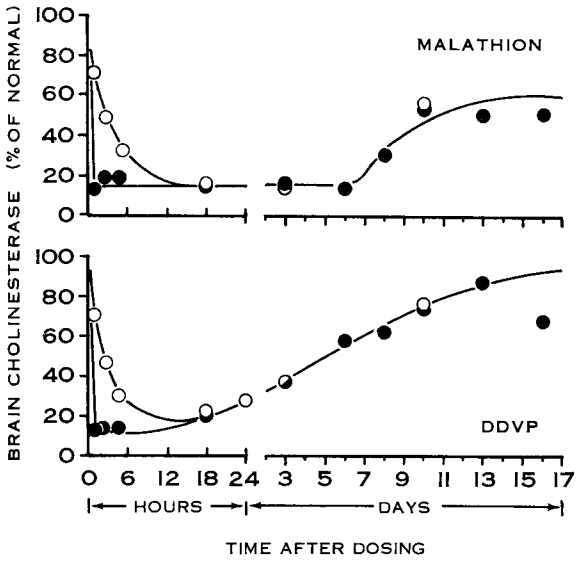

FIG. 1. Reactivation *in vitro* with 2-PAM of brain cholinesterase of chickens dosed with DDVP or malathion. In this experiment one group of chickens was dosed with malathion and the other with DDVP. At various time intervals fowls were sacrificed, and the brain true cholinesterase was determined as described in the experimental section. The ability of the inhibited enzyme to be reactivated was estimated in a duplicate flask to which 2-PAM was added according to the procedure described. Each point represents the average of the analysis of the brains of two chickens; ● represents per cent activity in the absence of 2-PAM; ○ represents the activity of the corresponding homogenates to which 2-PAM was added.

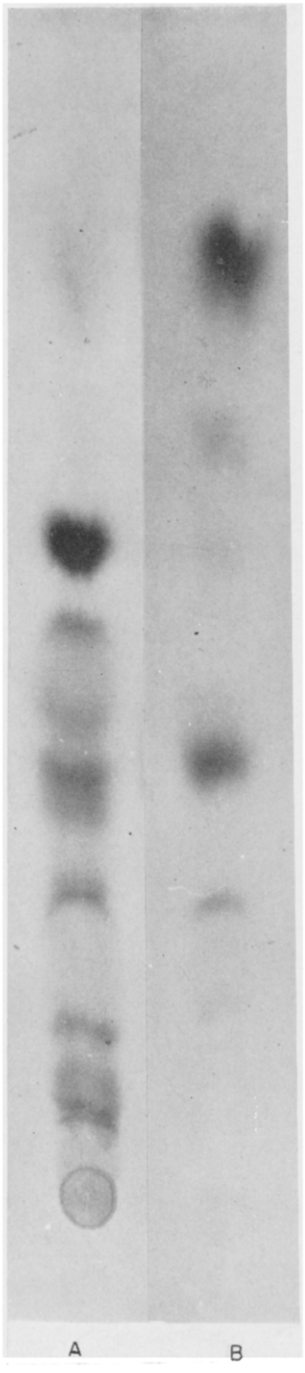

FIG. 1. An autoradiograph of an ascending chromatogram (Solvent 1) of urine obtained during the first 24 hr after i.p. administration of NN-di-2-chloroethyl-^{14}C-aniline A, and NN-di-2-hydroxyethyl-^{14}C-aniline, B.

facing page 1324

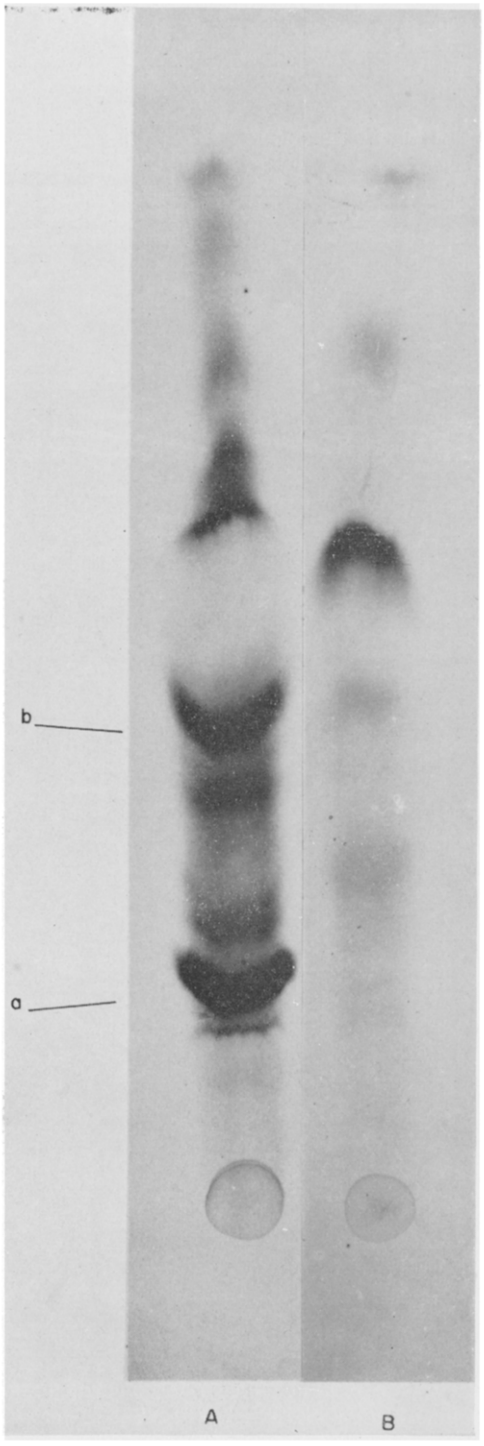

FIG. 2. An autoradiograph of an ascending chromatogram (Solvent 1) of urine obtained during the first 24 hr after i.p. administration of N-2-chloro-1:2-^{14}C-ethyl-N-ethylaniline, A, and N-2-hydroxy-1:2-^{14}C-ethyl-N-ethylaniline, B.

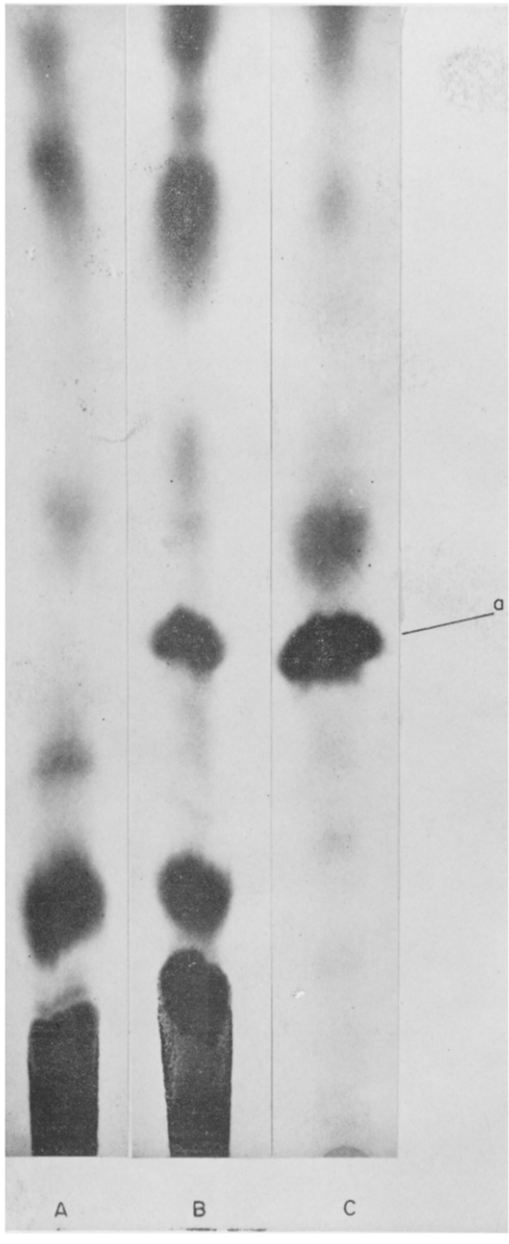

FIG. 3. An autoradiograph of an ascending chromatogram (Solvent III) of urine obtained during the first 24 hr after administration of ^{35}S-L-cystine, A, ^{35}S-L-cystine followed after 5 min by injection of N-2-chloroethyl-N-ethylaniline B, and N-2-chloro-1:2-^{14}C-ethyl-N-ethylaniline, C.

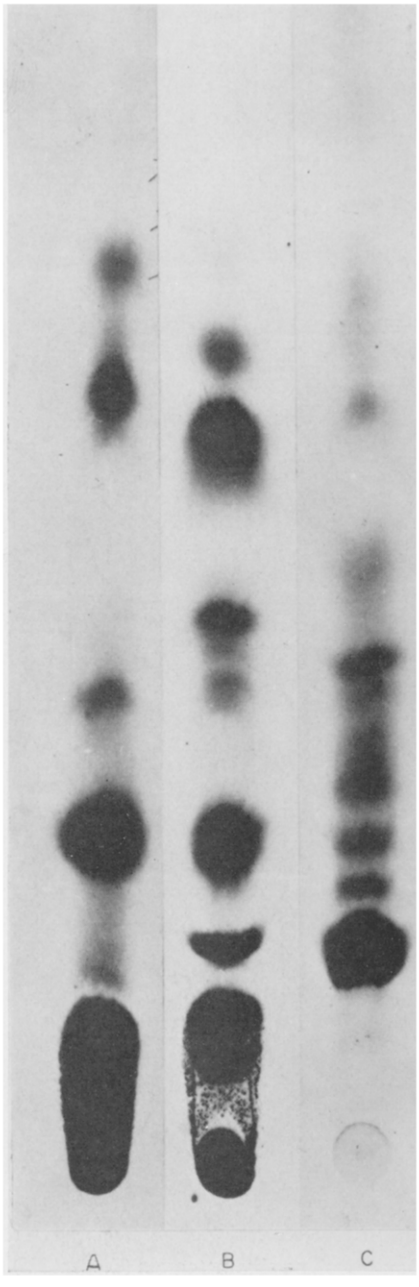

FIG. 4. An autoradiograph of a unidimentional ascending chromatogram (Solvent I) of urine obtained during the first 24 hr after administration of ^{35}S-L-cystine, A, ^{35}S-L-cystine followed after 5 min by injection of N-2-chloro-1:2-ethyl-N-ethylaniline, B, and N-2-chloro-1:2-^{14}C-ethyl-N-ethylaniline, C.

RESULTS AND DISCUSSION

The results of the studies of the rate of formation of the irreversible form of brain true cholinesterase in chickens dosed subcutaneously with 1,000 mg of malathion or 16 mg of DDVP/kg are presented in Figs. 1 and 2. It can be seen that the brain cholinesterase of chickens poisoned with either of these compounds was depressed to minimal value, 16 to 17 per cent of the control level, and remained inhibited for 18 hr. After that time, in confirmation of previous results,[9] the enzyme activity began to recover with DDVP but remained inhibited for 6 days with malathion. As shown in another paper,[9] the relatively rapid recovery of brain cholinesterase after DDVP is due to synthesis of new enzyme; retention of inhibitor in the body accounts, in part at least, for the slow recovery of brain cholinesterase after dosing with malathion.

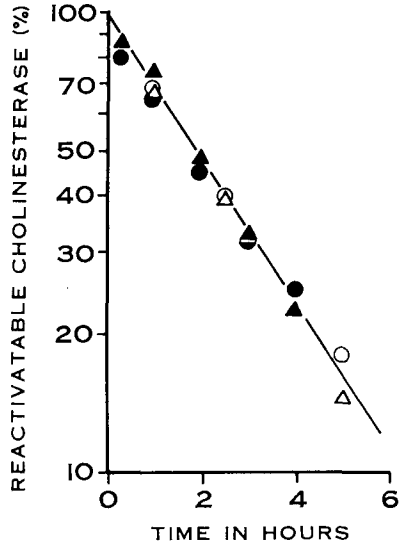

FIG. 2. The rate of disappearance of reactivatable brain cholinesterase *in vivo* was determined from the data on which the curves in Fig. 1 are based. The per cent reactivatable enzyme was calculated with these data according to the formula in the experimental section.

The rate of disappearance of reactivatable brain cholinesterase was measured *in vitro* in the following way. The homogenate containing no acetylcholine but in bicarbonate buffer (pH 7·4) in a N_2/CO_2 atmosphere was equilibrated at 40° for 10 min, and then sufficient inhibitor was added to bring its concentration to 1,000 times the I_{50} level. A control homogenate diluted with water also was run. At various intervals, 0·1-ml aliquots of the homogenates were assayed for cholinesterase activity in the presence and absence of 2-PAM. The details of the experimental procedure and the calculation of the per cent reactivatable enzyme are presented in the experimental section. The enzyme was inhibited 95 per cent with the concentration of inhibitor used and in the absence of 2-PAM did not increase in activity more than 5 per cent during the 4-hr incubation at 40°. ○, DDVP *in vivo*; ●, DDVP *in vitro*; △, malathion *in vivo*; ▲, malaoxon *in vitro*.

The ability of brain homogenates from chickens given DDVP or malathion to be reactivated with 2-PAM rapidly disappeared. Only 5 per cent of the enzyme could be reactivated after 18 hr. A plot of the logarithm of per cent reactivation with 2-PAM against time (Fig. 2) showed that the half-time for the disappearance of the reactivatable dimethyl phosphorylated cholinesterase was 2 hr for chickens dosed with either DDVP or malathion, despite the large difference in dosage between the two compounds. The

same value of 2 hr was found for the half-time of disappearance of the reactivatable form of brain cholinesterase in homogenates inhibited with 1,000 times the I_{50} concentration of DDVP or malaoxon, respectively. The I_{50} concentrations of these inhibitors were $1{\cdot}25 \times 10^{-7}$ M and $1{\cdot}8 \times 10^{-8}$ M for DDVP and malaoxon, respectively.

The rate of regeneration of brain cholinesterase *in vivo* is governed by several opposing factors. Inhibition with the active form of the inhibitor and the formation of the irreversible form of cholinesterase from the reversible form act to keep the enzyme inhibited. On the other hand, spontaneous reactivation of the inhibited cholinesterase and the synthesis of new cholinesterase molecules tend to reactivate the enzyme. In the present experiments over the period of 18 hr, synthesis *de novo* of new enzyme could not contribute significantly to the rate of regeneration of cholinesterase.

The same factors also govern the rate of regeneration of cholinesterase *in vitro*. Vandekar and Heath[7] have found *in vitro* that the rate of spontaneous reactivation of the dimethyl phosphorylated brain cholinesterase, such as that formed from malaoxon or DDVP, is quite fast; the half-time for this reaction is 1·3 hr at 37°. In the present studies *in vitro*, the brain cholinestersae remained inhibited during the experiment since, as is shown in the legend of Fig. 2, a large excess of inhibitor was present, and less than 5 per cent of the enzyme was spontaneously reactivated. Therefore, in these experiments the rate of inhibition *in vitro* of brain cholinesterase must have been greater than the rate of spontaneous reactivation of the dimethyl phosphorylated enzyme. Since the latter reaction, according to the results of Vandekar and Heath[7] cited above, is faster than the rate of disappearance of the reactivatable enzyme as determined *in vitro* in the present experiments (i.e., half-time 2 hr), the rate of conversion of the reversible form of cholinesterase to the irreversible form of the dimethyl phosphorylated enzyme must be the rate-limiting step in the disappearance of the reactivatable enzyme. Thus, the rate of disappearance of the reactivatable form of the inhibited cholinesterase measured in the present experiments is the same as the rate of formation of the irreversible form of the dimethyl phosphorylated enzyme from the reversible form of the inhibited cholinesterase.

The brain cholinesterase of chickens poisoned with DDVP or malathion was inhibited to rates 16 or 17 per cent of normal within 1 hr of dosing and remained inhibited for at least 18 hr. Thus, the rate of inhibition *in vivo* must have been greater than the rate of spontaneous reactivation of the dimethyl phosphorylated enzyme.

The rate of disappearance of the reactivatable form of the dimethyl phosphorylated enzyme was independent of the dosage of the inhibitor administered over a 40-fold range and was the same *in vivo* as *in vitro*. It therefore seems highly probably that the rate-limiting step in the disappearance of the reactivatable form of the dimethyl phosphorylated enzyme *in vivo* also is the rate of conversion of the reactivatable form of the inhibited cholinesterase to the unreactivatable form of this enzyme. Thus the rate of disappearance of the reactivatable form of the inhibited cholinesterase *in vivo* also is the same as the rate of formation of the irreversible form of the dimethyl phosphorylated enzyme from the reversible form of the inhibited cholinesterase.

As far as we are aware, this is the first time that the rate of formation of the irreversible form from the reversibly inhibited dimethyl phosphorylated enzyme has been measured.

The results of the present experiments, when combined with those of Hobbiger,[6] indicate that the rate of formation of the irreversibly inhibited dialkyl phosphorylated enzymes of brain increases in the order: diethyl phosphate < diisopropyl phosphate < dimethyl phosphate.

The methods employed for the protection of the cholinesterase in the homogenate from the reaction with inhibitor previously separated spatially from the enzyme, and for the reactivation of the enzyme with 2-PAM, should be of value to others who wish to study the inhibition and reactivation of cholinesterase *in vivo*. However, in other applications of these procedures, it will be necessary to prove that they will work with each new source of cholinesterase and with other organic phosphorus compounds.

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