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In Vitro Studies on Human Blood Cholinesterase and its Action Towards Organophosphate Insecticides

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Abstract : Inhibition studies conducted with various concentrations of baytex and malathion indicated that at low levels malathion did not show any inhibitory effect but baytex at low levels and malathion at higher concentrations inhibited the blood pseudocholinesterase activity (ChE). The ChE activities which were inhibited by these insecticides were found to be reactivated slowly by antidote, pyridine-2-aldoxime-N-methiodide (PAM). PAM alone did not produce any inhibitory effect.

The clinical norm of ChE activities for Ceylonese subjects as determined by the Rapid Field Test method is 80% with a SD of 15.3%. The screening of personnel engaged in the handling and spraying of these insecticides for blood ChE activities was carried out by the rapid field test method. Such studies carried out on more than 80 personnel attached to 5 different Filaria units of the Department of Health, indicated that about 25% of them had blood ChE level 50% of the normal. Some of these low values may be due to congenital defects but this aspect was not investigated further.

1. Introduction

In vector control programmes D.D.T. (2,2-bis (p-chlorophenyl)—1,1,1-trichloroethane) and dieldrin (1,2,3,4,10,10—hexachloro 6-7-epoxy—1,4,4a,5,6,7,8,8a—octahydro-1,4-endo-exo 5-8-dimethanonaphthalene), have been in use for several years and no reports of any serious harmful effects have been recorded among the thousands of people who use them daily in malaria and filaria eradication campaigns. It has become necessary to introduce alternative insecticides since vectors have developed resistance to DDT and dieldrin ; such insecticides which are in use in Sri Lanka today are the organophosphates, viz. baytex (0-0-dimethyl-0-4 (methylthio)—m-tolyl-phosphorothioate) and malathion, (S—1,2-bis (ethoxy carbonyl) ethyl 0-0-dimethyl phosphorodithioate).

These organophosphates are generally toxic to animals since they interfere with the mechanism of the transmission of nerve impulse by inhibiting the hydrolysis of acetyl choline. These compounds inhibit the cholinesterase action by phosphorylating the active site of the enzyme.[5] The reactivation of the inactivated enzyme can be accelerated *in vivo* and *in vitro* by oximes, which are used as antidotes for poisoning by organophosphorus compounds.[5]

Considerable interest has developed in recent years in connection with the use of the muscle relaxant suxamethonium (succinyl choline or scoline). This substance is rapidly hydrolysed by pseudocholinesterase (acyl choline-acyl hydrolase, ChE) but

in individuals with low serum ChE, hydrolysis of suxamethonium takes place slowly so that apnoea due to it is considerably prolonged and may threaten life. This occurs in individuals whose ChE activity is low due to pathological conditions such as in liver disease ; gradual poisoning is due to exposure to organophosphates or is due to a congenital defect.

The present investigation was therefore undertaken with a view

1. to determining the inhibitory effects of baytex and malathion on human ChE *in vitro* since such data is not available,

2. to study the antidote effect of PAM (pyridine-2-aldoxime-N-methiodide) on cholinesterase, inactivated by baytex and malathion *in vitro*,

3. to establish the clinical norm of ChE-activity for Ceylonese subjects, and

4. to screen the personnel, who are engaged in the handling of organophosphorus compounds in vector control programmes with a view to determining the inhibitory effect of baytex and malathion on human ChE *in vivo*.

Some of the findings have been reported earlier.[10]

2. Materials and Methods

Baytex and malathion used were commercially available solutions obtained from the Filaria campaign of the Department of Health, Sri Lanka ; they were used without any further purification. Antidote PAM was obtained from Farbenfabriken Bayer A. G., Leverkusen, Germany, and benzoycholine and acetyl choline were obtained from British Drugs House Ltd. Poole, U.K. The blood serum used for the inhibition experiments and for the effect of antidotes was that of one of the authors (S. Sentheshanmuganathan) and was obtained by venepunture. The blood was allowed to clot at 37°C and the clear serum separated by centrifugation. The serum, when stored at –20°C, retained its ChE activity without any change for a month.

For the establishment of the clinical norm, capillary blood samples were collected from volunteers of the various grades of officers working at the Medical Research Institute, Colombo, Sri Lanka. Capillary samples of blood for testing were collected from the workers attached to the Filaria units in the country.

2.1. Assay of cholinesterase activity

Spectrophotometric[9] and colorimetric[4] methods were used in the assay of serum and whole blood cholinesterase activities respectively.

2.2. Spectrophotometric assay of ChE

A Unicam SP 500 spectrophotometer was used. All experiments were conducted at 37°C and changes of temperature of the solution during the mixing were kept to a minimum. This was achieved by keeping the reagent tubes in the water bath which was feeding the thermospacers. The cell holder with the absorption cells was always kept in the cell compartment in contact with the thermospacers except for a few seconds for filling.

The decrease of absorbance during the first 3 min was called ΔA_3,[8] if obtained under standard conditions (Serum 1 : 200, Benzoyl choline 5×10^{-5}M 26°C, 10 mm light path, pH = 7·4, wave length 240 mu). Then $0{\cdot}165 \times \Delta A A_3 = 2{\cdot}5 \times 10^{5-}$M. A decrease of absorbance of 0·165 corresponds to a hydrolysis of 0·025 μmoles benzoyl choline per ml. Then $606 \Delta A_3$ = μmoles benzoyl choline hydrolysed by 1·0 ml in 1 h at 26°C. In order to convert the values to 37°C, a factor of 1·74 is used, e.g. from 26 to 37°C it is $1057 \Delta A_3$.[9] Since the experiments were conducted at 37°C, it was not necessary to use a temperature correction factor.

2.3. Colorimetric method

For the colorimetric method of the assay of whole blood ChE, the Lovibond Tintometer developed by the Tintometer Ltd., Waterloo Road, Salisbury, U.K. was used as described by Edson.[4] The standard Lovibond comparator disc 5/30 covers the range 0 - 100% normal activity in steps of 12·5%. The acetic acid liberated from the substrate, acetyl choline, by the cholinesterase lowers the pH of a weak buffer solution and changes the colour of the indicator bromothymol blue. The change in colour is proportional to a degree of enzyme activity, which is matched against the standardised discs of the Lovibond comparator.

3. Results

3.1. Inhibition of cholinesterase activity by baytex and malathion

When the serum was incubated with benzoyl choline, the decrease in absorbancy at 240 mμ at 37°C was 0·225 over the first 3 min of incubation (Figure 1). But when baytex and malathion were incubated singly with the same serum under identical conditions, the corresponding decreases in absorbancies were 0·070 and 0·170 respectively. The final concentration in the mixture was : baytex, 11.25 μM and malathion, 19·23 μM. Baytex thus produced an inhibition of ChE activity by 68% of the total activity as compared to malathion which gave an inhibition of only 24%. The results indicate that baytex is a stronger inhibitor of ChE activity than malathion. The above concentrations of baytex and malathion were chosen since concentrations higher than these amounts produced turbidity which interfered in the spectrophotometric measurements.

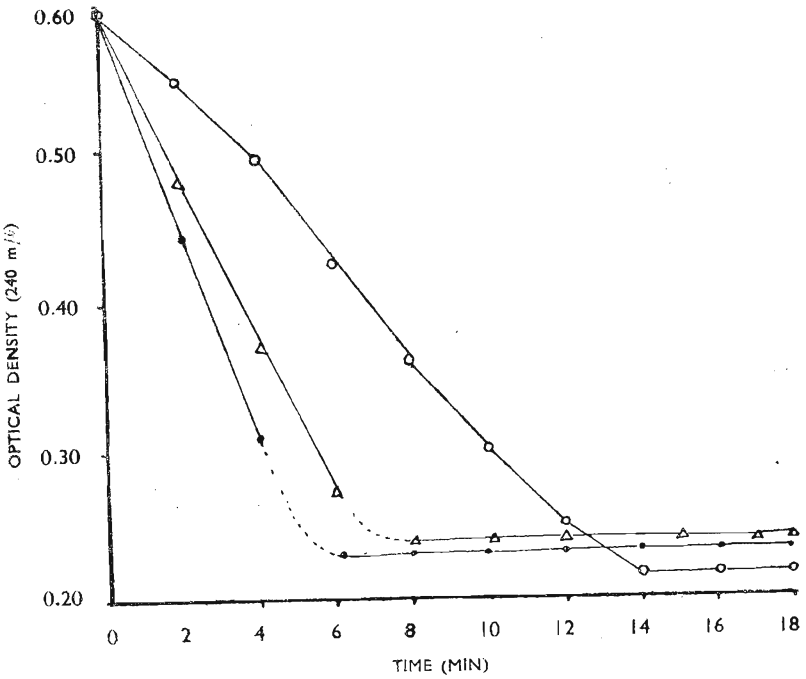

FIG. 1. Inhibition of cholinesterase activity by baytex and malathion.

The enzyme activity was followed by observing the decrease in absorbancy at 240 mμ. The incubation mixture (4.0 ml) contained serum (2.0 ml of 1 : 100 dilution in phosphate buffer pH. 7.4, 2.0 ml benzoyl choline in phosphate buffer, conc. 5×10^{-5} M), organophosphate (0.1 ml = 11.25 μm baytex or 19.23 μm, malathion) Incubation temp., 37°

•—• No inhibitor
o—o Baytex
Δ–Δ Malathion

3.2. Effect of insecticide concentration on ChE activity

In this experiment, the concentration of the substrate and enzyme (serum) was kept constant while the amounts of baytex and malathion were increased. The conditions of assay used were as described (Figure 1). This was studied over the concentration range of 0.3 to 3 μM. The percentage inhibition of ChE activity by malathion increased linearly with increasing concentration of the organophosphate up to a final concentration of 0·75 μM while that of baytex increased to 0·45 μM (Figure 2). At these two concentrations of organophosphates the inhibitions observed were 55% and 45% respectively. Thereafter, increasing the concentration of either baytex or malathion did not show the same linearity. At the final concentration of 3.0 μM of the organophosphate, baytex produced an inhibition of 83% of the total activity while malathion showed an inhibition of 63%.

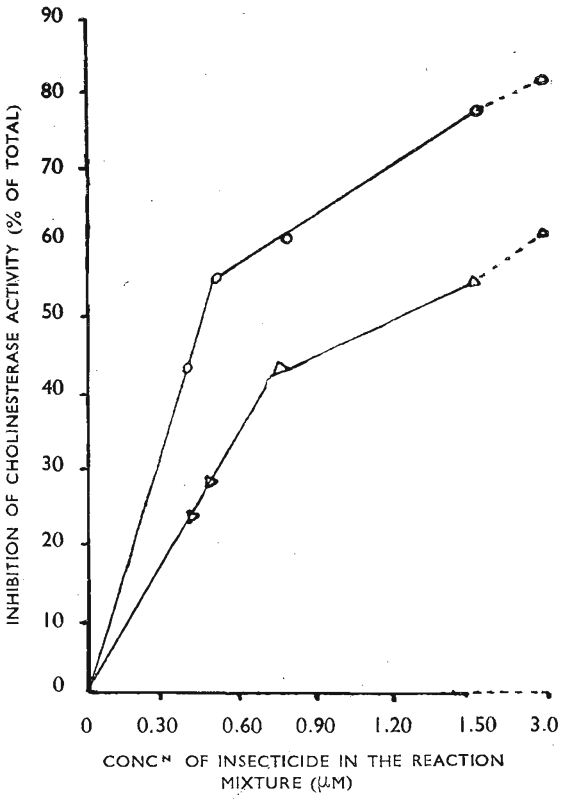

FIG. 2. Inhibition of serum cholinesterase activity by increasing amounts of baytex and malathion.

The experimental conditions were as described under Fig I.

o—o Baytex

Δ–Δ Malathion

3.3. Antidote effect of PAM on the inactivated serum cholinesterase by baytex and malathion, *in vitro*

In order to establish that the antidote PAM could reactivate the ChE activity inhibited by baytex or malathion, it was thought desirable to carry out this study in the following manner :—

(1) Enzyme (serum) was incubated simultaneously with the organophosphate, antidote PAM and the substrate, benzoyl choline.

(2) Enzyme was first preincubated with the organophosphate for 20 min to inactivate the enzyme. Then the substrate, benzoyl choline was added and the hydrolysis followed until it ceased. Subsequently, PAM was added and the hydrolysis was followed again until the reaction reached a stationary stage.

(3) Enzyme was preincubated with the organophosphate for 20 min to inactivate the enzyme. At the end of this period, PAM was added to reactivate the enzyme by incubating for a further 20 min. Finally the substrate, benzoyl choline was added and the activity followed as described in Section 2.

When the serum was incubated with the substrate, benzoyl choline with or without the addition of the antidote PAM, the rate of hydrolysis remained unchanged. Similar observations were made when the enzyme and PAM were incubated first before the addition of the substrate. These results therefore indicate that PAM has no action on the enzyme activity (Figure 3). In the first experiment, enzyme and substrate were incubated simultaneously with either baytex or malathion. Such studies showed that though the rate of hydrolysis as measured within the first 3 min of incubation was low, (0·175 to 0·05) yet complete hydrolysis was achieved in 16 min with baytex and malathion as compared to 5 min in the absence of inhibitor (Figure 3).

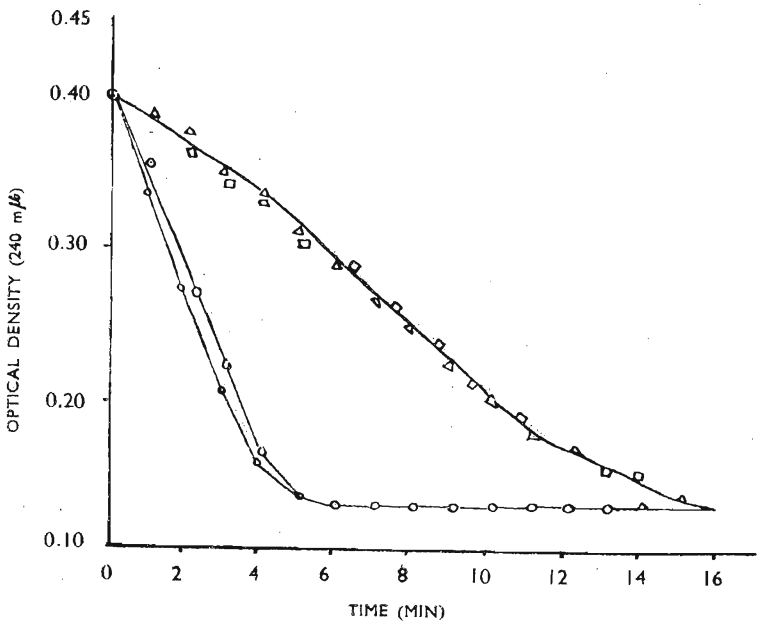

FIG. 3. Inhibition of serum cholinesterase activity by baytex and malathion and the subsequent effects by PAM (Pyridine-2-aldoxime-N-methyl iodide).

The experimental conditions were as described under Fig. 1. PAM and baytex or malathion were added in equimolecular amounts.

•—• Serum + Benzoyl choline.

o—o Serum was preincubated with PAM for 20 min then the substrate, benzoyl choline was added.

Δ–Δ Serum + malathion or baytex + benzoyl choline were added simultaneously.

□—□ Serum preincubated with baytex or malathion for 20 min then PAM and benzoyl choline were added simultaneously.

In the second series of experiments, when the substrate was incubated with the malathion inactivated enzyme, a slight change in the absorption was observed (from 0·425 to 0.413) but when PAM was added, the hydrolysis proceeded gradually reaching a value of 0.30 in 20 min which is equivalent to a hydrolysis of 40% of the total (Figure 4). With baytex, however, a hydrolysis of 60% was achieved over the same period. In all experiments, PAM and malathion or baytex were present in equimolecular amounts in the incubation mixture.

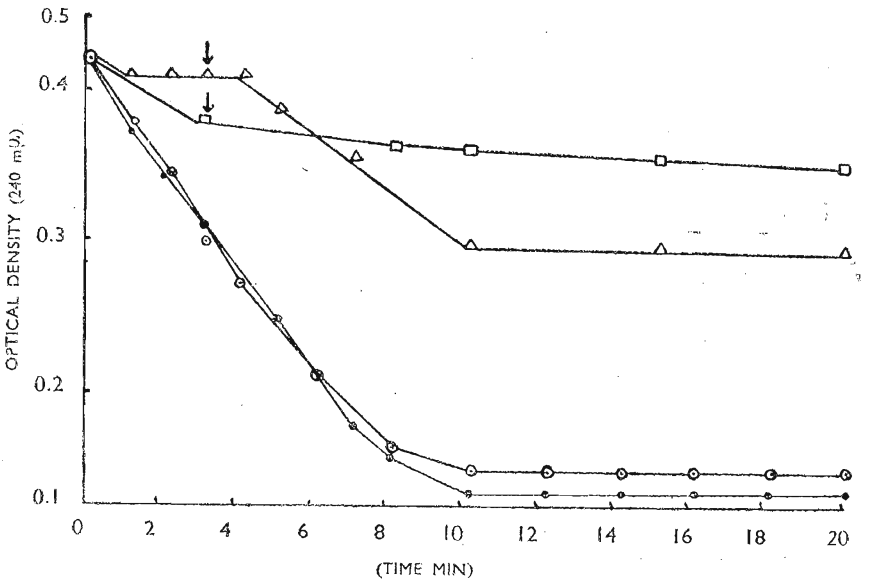

FIG. 4. Inhibition of serum cholinesterase activity by malathion and reactivation of the inactivated enzyme by PAM.

The experimental conditions were as described under Fig 1. Arrows indicate time of addition of either PAM or Benzoyl choline (BC).

•—• Serum + Benzoyl choline

o—o Serum + Benzoyl choline + PAM.

Δ–Δ Serum was incubated with malathion (for 20 min), then benzoyl choline was added and the reaction was followed until there was no change in the optical density at 240 mμ,

PAM was added and the reaction was followed again.

▫—▫ Serum + malathion were incubated (20 min). PAM was added and incubated further for 20 min Benzoyl choline was added and the reaction was followed again.

(PAM and malathion were added in equimolecular amounts, 76.9 μm.).

In the third series of investigations, when the substrate was added after reactivating the inhibited enzyme by PAM, the amounts of hydrolysis achieved with malathion was 16% of the total as compared to 80% with baytex (Figures 4 & 5).

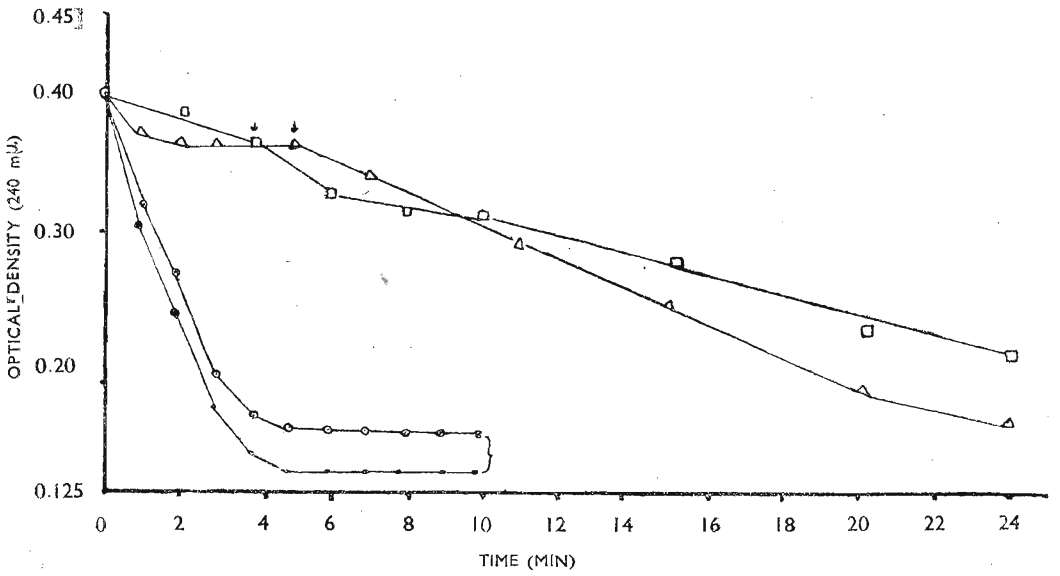

FIG. 5. Inhibition of serum cholinesterase activity by baytex and reactivation of the inactivated enzyme by PAM.

The experimental conditions were as described under Fig. 1. Arrows indicate time of addition of either PAM or Benzoyl choline (BC).

•—• Serum + Benzoyl choline

o—o Serum + Benzoyl choline + PAM

Δ–Δ Serum was incubated with baytex (20 min) Benzoyl choline was added and the reaction was followed until there was no change in the optical density at 240 mμ. PAM was added and the OD was measured.

□—□ Serum + Baytex were incubated (20 min.) : PAM was added and incubated further for 20 min Benzoyl choline was added and the reaction was followed again.

(PAM & Baytex were present in equimolecular quantities, 45 μ-moles).

3.4. Clinical norm for Ceylonese subjects by the Rapid Field Test (Colorimetric)

More than 100 samples of capillary blood collected from normal subjects have been estimated for cholinesterase activity (Table 1). The clinical norm for Ceylonese subjects by this method was 80 ± 15·28 with a range of 50 to 100%. 101 workers attached to 6 Filaria units in the country, who had been handling baytex and malathion for about 3 years were screened for their ChE levels (Table 1). This survey revealed that 25% of the workers had their ChE levels lowered by 50% of the normal (95%).

TABLE 1. Blood cholinesterase levels of normal subjects and personnel, involved in the spraying of insecticides, attached to the various Filaria Units of the Department of Health, Sri Lanka.

Subjects	No. of Subjects	Blood cholinesterase levels 0	12.5	25	37.5	50	62.5	75	87.5	100
Volunteers (MRI Staff)	101	00	00	00	00	00	18	36	17	24
Filaria Units										
1. Dehiwela	40	00	00	01	06	06	07	13	05	02
2. Kolonnawa	12	00	00	00	01	00	01	03	05	02
3. Kolonnawa Installation ...	11	00	00	00	00	01	02	02	05	01
4. Kotte	19	00	00	00	01	03	02	09	03	01
5. Peliyagoda	12	00	00	00	01	01	04	04	01	01
6. Wattala	07	00	00	00	00	01	03	02	00	01

The cholinesterase levels were determined by the Rapid Field Test method.[2]
Clinical norms = mean = 80% ; SD = ± 15.3%.

In another study, 4 workers attached to the blending plant of the Ceylon Petroleum Corporation (CPC) were tested for their ChE levels at the time of recruitment for employment (pre-exposure level) and subsequently at monthly intervals for 12 months. None of the 4 showed any change in the ChE levels (Table 2 a) over the 12 months of handling the organophosphates. Seven other workers of the same plant were screened before coming into contact (pre exposure level) with the organophosphates and subsequently for another 2 months of handling the insecticides. Of the 7, 1 had a drop of 50% (87·5 to 37·5%) and another worker of 25% (62·5 to 37·5% Table 2 b) ; while the remaining 5 showed no change.

TABLE 2. (a) Blood cholinesterase levels of workers attached to the Blending Plant, Ceylon Petroleum Corporation. (Batch No. 1 : 4 workers).

Dates on which tests were carried out	(1)	(2)	(3)	(4)
21.09.71* ...	100	87.5	100	87.5
09.11.71 ...	100	87.5	100	87.5
09.12.71 ...	100	87.5	100	87.5
11.01.72 ...	100	100	100	100
16.02.72 ...	100	100	100	87.5
23.05.72 ...	100	100	100	100
31.07.72 ...	—	—	—	100
29.09.72 ...	—	—	—	87.5

*Pre-exposure values.

Blood cholinesterase levels were estimated by the Rapid Field Test method.[4]

TABLE 2(b). Blood cholinesterase levels of workers attached to the Blending Plant, Ceylon Petroleum Corporation. (Batch No. 2 : 7 workers).

Dates on which tests were carried out	(1)	(2)	(3)	(4)	(5)	(6)	(7)
31.07.72*	100	87.5	75	87.5	87.5	62.5	62.5
29.09.72	75	37.5	62.5	100	75	37.5	62.5

*Pre-exposure values.

Blood cholinesterase levels were estimated by the Rapid Field Test method.[4]

4. Discussion

There are 2 types of cholinesterases present in man. One of them is true cholinesterase (acetylcholine-acyl hydrolase) which is specific for acetylcholine and present mainly in erythrocytes and in the region of cholinergic nerve endings. In various tissues especially plasma, there are other cholinesterase which are non specific for acetylcholine, called pseudocholinesterase (ChE). It is the latter type which we were interested in, since they are inhibited by organophosphates, eg. baytex, malathion, fenthion and sumithion.

Most of the data available on the inhibitory effect on blood ChE have been obtained with rats, rabbits and insect blood.[6] The only data available on human blood have been derived with the ChE obtained from human erythrocytes and serum using Parathion, Systex, Systox Thinoisomer and Dithiono-pyrophosphate *in vivo* and *in vitro*.[6] In view of the use of baytex and malathion in Sri Lanka in vector control programmes and the non availability of data for anti-ChE activity in human blood, we investigated the effect of these compounds on human serum.

The present investigation has shown that both baytex and malathion are inhibitors of pseudocholinesterase activity of human blood serum. In this study we followed the enzyme activity using benzoyl choline as substrate as described.[9] The rate of hydrolysis was observed by following the decrease in absorbance at 240 mμ over the first 3 min of adding the substrate. It is seen from Figure I, that the rate of hydrolysis of benzoyl choline by the serum corresponds to a decrease in absorbance of 0·225 ; when baytex (11·25 μm) was also added to a system, the decrease in absorbancy was 0·07 while with malathion (19·23 μm), it was 0·170. Thus baytex and malathion inhibited the enzymic activities by 68% and 24% of the original activity respectively. These findings indicate that baytex is a stronger inhibitor of ChE activity than malathion. It has been reported[11] that malathion has the lowest toxicity of all organophosphorus compounds. Our findings are in agreement. The WHO report, however, states that in practice malathion at lower concentration does not depress the cholinesterase level. In the presence of baytex and malathion, a given amount of enzyme will hydrolyse the same amount of substrate in 14 and 8 min respectively,

while without any inhibitor it would take 6 min to bring about an equal degree of hydrolysis. When the concentration of baytex was increased from 0 to 0·45 μm, the percentage inhibition of ChE activity also increased in a linear manner and above that the inhibition attained a maximum value of 78% at a concentration of 1·5 μm ; with malathion a similar pattern was obtained. Malathion produced a linear relationship up to a concentration of 0·75 μm of the inhibitor and produced a maximum inhibition value of 55% at a concentration of 1·5 μm. Above a concentration of 1·5 μm of either of the organophosphates, the inhibitions produced were not very marked. Increasing the concentration 10 fold (3·0 μm) increased the inhibition by only 5% and 9% (Figure 2). We may therefore assume that at these concentrations, the active sites of the enzymes are saturated by phosphorylation and this prevents the entry of the substrate for action. It could be inferred from Figure 2, that baytex is a stronger inhibitor of ChE activity than malathion. The high toxicity of the organophosphates, which have been developed as insecticides for agricultural use, depends largely upon the blockage of esterases such as the cholinesterases. These enzymes are phosphorylated and the inhibition that results is practically irreversible.[1,2,3] Recovery from such poisons depends on the formation of fresh enzyme which takes weeks. The usual symptomatic treatment for the endogenous acetylcholine intoxication that is produced by these drugs has hitherto consisted of the administration of high doses of atropine, artificial ventilation of the lung and correction of dehydration. It has now become possible to supplement these measures with the use of specific antidotes, *viz.* oximes whose chemical properties enable them to displace the phosphate radical from the phosphorylated esterases and thus restore the activity of the enzyme. The most widely used oxime is PAM (Pyridine-2-aldoxime methiodide). Several cases of parathion poisoning treated successfully with antidote PAM have been reported.[5] Though the poisoning by parathion and its successful management have been reported,[5] there are no reports of successful management of either baytex or malathion poisoning. In view of this, we studied the effect of antidote PAM on the enzyme inhibited by baytex and malathion. Such studies showed that PAM alone has no anticholinesterase activity on human serum (Figure 4). When PAM was added to a serum sample inhibited by either baytex or malathion and incubated further, there was a release of the phosphorylated esterase, which hydrolysed benzoyl choline gradually. Even though the amount of PAM used in the present study was in equimolecular amounts to that of baytex or malathion, yet it could not release all of the activity originally present. With malathion only 16% of the total activity was restored. This may have been due to some denaturation caused during the long period of incubation or of some other factor ; this aspect was not investigated. With baytex, however, 60% of the original activity was restored in 20 min. Even though baytex is a stronger inhibitor of ChE activity than malathion (Figures 2 & 3), yet the inactivation caused by baytex seems to be of a milder or weaker type than that of malathion. It might be that the affinity of the esterase is more towards PAM than baytex, or that the bond which operates in the phosphorylated intermediate

between baytex and enzyme is weaker than that which operates between the malathion enzyme complex. This is the only explanation we could provide with the existing data. It has been observed[5] that PAM should be given by injection within 24 to 48 h of parathion, poisoning since after this period, intravenous injection of PAM no longer leads to a reactivation of the enzyme. This is also true *in vitro*, when PAM was added to a sample of blood from the patient poisoned with parathion;[5] at this time PAM even showed anticholinesterase activity of its own. From the present studies it could be inferred that the failure of PAM to liberate the lost activity may be a phenomenon similar to that observed with parathion, suggesting that poisoning by malathion management may need a different approach altogether. With baytex poisoning, the treatment with PAM could yield rapid results.

The clinical norm of ChE activities for Ceylonese subjects was determined by the Rapid Field Test method[4]. This method was chosen so that it could be used by any trained technician in the field, factory or any hospital with the use of simple portable equipment. More than 100 samples of capillary blood collected from normal subjects have been estimated for ChE activities by this method (Table 1). The clinical norm for Ceylon subjects is 80% with a standard deviation of 15·3% thus giving a range of 64·7 to 95.3%. For the western countries, the normal value was found to be 100%.[11] Diagnosis of poisoning by organophosphorus compounds can be confirmed by demonstrating a much reduced ChE activity in the whole blood, plasma or serum. A reduced level of ChE activity in the blood indicates that an anti-cholinesterase chemical has been absorbed, or the individual is having some pathological conditions affecting the liver or a congenital defect. The latter can be confirmed by determination of the dibucaine and fluoride numbers.[7]

If the depression of ChE is marked, the patient will be more susceptible to the effects of further exposure to an organophosphate. Inhibition of cholinesterase only causes inequivocal symptoms when the enzyme activity has fallen to less than 25 to 30% of the pre-exposure value of that individual.[11] When this degree of inhibition is reached the symptoms of poisoning may progress so rapidly as to threaten life ; immediate treatment then becomes imperative. In view of the above observations made[11] and the normal values established by us, we suggest that any person who has a ChE value of 60% or less should not be employed in the handling of organophosphorous compounds either in a blending plant, in vector control programmes or in agriculture. All personnel handling these insecticides are therefore advised to have their ChE levels examined every 7 to 14 days. If the ChE value is found to be less than 60%, the individual should not be allowed to return to work, or to come into contact with organophosphates until his ChE has returned to at least 70% of the normal.[11]

The ChE levels of 4 workers were tested before assigning them to the Ceylon Petroleum Corporation blending plant of organophosphorus compounds. These were taken as their pre-exposure values. They were subsequently screened for ChE levels at regular intervals for 12 months (Table 2 a). In all the 4 cases tested, pre-exposure values were 87.5 to 100%. None of them showed any change in their ChE levels. In another study, with 7 other workers over a period of 2 months, one had a decrease in the ChE value of 50% (87.5 to 37.5%) and another one by 25% (62.5 to 37.5%); the value of 37.5 is very much lower than the limiting value of 60%. Even with this value of 37.5% both the workers did not show any positive symptoms of organophosphorous compounds poisoning. This is expalained in the earlier findings[5] in which it has been established that symptoms appear only if the value falls below 25% of the pre-exposure value which in these 2 cases is 16.8%.

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