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The Effects of Feeding Different Doses of an Organophosphate Compound (Guthion) on Red Blood Cell and Plasma Cholinesterase in the Horse

By

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THESIS

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Respectfully

dedicated

to my parents

Auditor and Mrs. Modesto C. Llaguno

and to the

Cendaña's Superstars

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	3
MATERIALS AND METHODS	12
RESULTS	17
DISCUSSION	32
CONCLUSION	35
BIBLIOGRAPHY	36

INTRODUCTION

The existence of cholinesterase (ChE), an enzyme capable of hydrolyzing acetylcholine into choline and acetic acid was first suggested in 1914 (Dale, 1914), was established in 1926 (Loewi and Navratil, 1926) and has since been confirmed by many investigators. Evidence indicates that ChE is not a single enzyme, but can be divided into true ChE (acetylcholinesterase, AchE) and pseudo ChE (PchE). These two enzymes essentially differ in their function and distribution (Alles and Hawes, 1940; Mendel and Rudney, 1943; Augustinsson and Nachmansohn, 1949). The true ChE is present in the nervous system, in muscles, and in glands and is bound to the cell membrane of erythrocytes (Croft and Richter, 1943). It has more affinity to acetylcholine than to similar choline esters as butyrylcholine or benzoylcholine. Pseudo ChE occurs in plasma, and also liver, pancreatic and nerve tissue. It can non-specifically hydrolyze choline esters, including acetylcholine (Augustinsson, 1957).

Organophosphorous compounds inhibit ChE, and share certain fundamental characteristics. Their success in pest control depends on selective toxicity. Structurally, they contain the phosphorous radical in a combination that permits them to non-competitively inhibit ChE (Heath, 1961; O'Brien, 1967). Pharmacological effects of organophosphorous compounds result from excessive accumulation of acetylcholine at nerve synapses and neuromuscular end plates (Goodman and Gilman, 1970).

Guthion (Azinphosmethyl) has been used successfully for alfalfa with a tolerance of 5 PPM (Mull, 1972). In the Spring of 1972, however, its use on the first cutting of alfalfa gave rise to levels in hay that were many times the tolerance level. Feeding alfalfa with this great amount

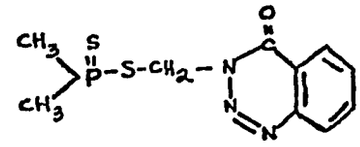
of Guthion to lactating animals would cause excessive residues in the milk and might result in the poisoning of the animal. Feeding this hay to non-food animals such as the horse could be one of the possible uses of this hay.

There have been numerous studies concerning the toxic effect of Guthion in different species of animals, but no comparable studies have been reported in horses. Therefore, the objectives of this investigation were two fold: first, to establish the tolerance level of Guthion in horses by feeding different doses and secondly, to determine the time course for the recovery of the ChE activity of the red blood cell (RBC) and plasma after depressions at the higher doses of Guthion.

LITERATURE REVIEW

Chemistry of Azinphosmethyl (Guthion®, Gusathion M)

O, O-dimethyl S(4-oxo-1,2,3, - benzotriazin-3[4H]-ylmethyl) phosphodithioat



Azinphosmethyl has a molecular formula $C_{10}H_{12}N_3O_3PS_2$. It was develope by W. Lorenz and introduced in 1953 by Bayer Leverkusen under the code numbers "Bayer 17, 147" and "R 1482".

The compound is made by the reaction of N-chloromethyl benzazimide, formed by the reaction of benzazimide with paraldehyde and thionyl chloride with O, O-dimethyl hydrogen phosphorodithiote in the presence of an acid-binding agent such as sodium bicarbonate. It forms white crystals of m.p. 73^0 to 74^0 C; v. p. below 3.8×10^{-4} mu Hg in 20^0 C. Its solubility in water is 1:30,000 at room temperature and it is soluble in most organic solvents. It is unstable at temperatures above 200^0 C and is rapidly hydrolyzed by cold alkali and acid (Martin, 1.72).

Pharmacological Action

The most important effect of insecticidal organophosphate is depression of cholinesterases.

The AchE specifically hydrolyses acetylcholine (Ach). Ach stimulates the parasymphatetic nervous system, and is necessary for the transmission of nerve impulses from preganglionic fibers to autonomic ganglia, from post ganglionic cholinergic nerves to the heart and secretory cells, from motor fibers to straited muscles, and from certain structures within the CNS. Under physiological conditions, Ach is almost immediately hydrolyzed by the AchE. If this enzyme is inhibited, Ach cannot be hydrolyzed and

it will accumulate. A moderate inhibition of AchE results in a non-physiological increase in Ach which stimulates the skeletal muscle function (muscle twitching) and a strong inhibition causes complete dimunition of muscle function (paralysis). Symptoms of poisoning caused by organophosphates thus stem from an increase in endogenous Ach resulting in excessive excitation of the parasymphatetic nervous system (Kimmerie and Lorke, 1968; Goodman and Gilman, 1970).

Phosphorylation of Enzyme

The active center of AchE consists of two parts, an esteratic and an anionic (negative part). The latter binds the quarternary nitrogen of Ach; the esteratic part effects its hydrolysis.

The inhibition of AchE produced by the organophosphate is a kinetic reaction of the first order, therefore, the magnitude of enzymatic inhibition is proportional to the concentration of the inhibitor. The rate of reaction increases with temperature.

Interaction between organophosphate and AchE results in the formation of phosphorylated enzyme complex. In contrast to acetylated enzyme complex the phosphorylated enzyme is resistant to hydrolysis and this causes an irreversible loss of AchE activity (Kimmerie and Lorke, 1968).

Although, the significance of AchE inhibition of red blood cells and pseudo ChE of plasma caused by organophosphates is not yet clearly understood, the fact that these enzymes are inhibited in the same manner as AchE at the neuromuscular and glandular sites, serves as a useful purpose in the sense tht the extent of their inhibition in case of organophosphate poisoning provides a genuine indication of AchE depression at the tissue levels (Freedman et. al., 1949).

Activation process

Organophosphates containing sulfur atoms (P=S), such as Guthion, Parathion, Methylparathion and Malathion are first converted into their oxy-analogs (P=O) before inhibiting the activity of AchE. The microsomal enzymes of liver and also to a lesser extent, of intestinal lumina and kidneys play a major role in bioactivation of these organophosphates (Murphy and Dubois, 1957). In vitro studies carried out by various investigators have provided evidence that the inhibitory effects of oxy-analogs of thiophosphates on AchE is considerably more than that of the parent compounds (O'Brien, 1960; Heath, 1961; Hitchcock and Murphy, 1967).

Breakdown

Another metabolic fate of thiophosphates is their hydrolysis, affected by phophatases. Those phosphatases were previously given specific names, e.g. Sarinase for Sarin; DFP-ase for DFP; Malathionase for Malathion and Guthionase for Guthion (O'Brien, 1960; Heath, 1961).

Distribution and Excretion

Guthion is metabolized in the body. Its various metabolic products are Gutoxon, anthanilic acid, mercaptomethyl benzazimide, methyl benzazimide sulfide. They are rapidly distributed in animal tissues and fats and later excreted largely in urine in the form of conjugates (Everett et. al., 1966).

Toxicity of Guthion in different species of animals

The maximum tolerable dose of Guthion was determined in newborn calves, yearling cattle, sheep and goats. Calves did not manifest any sign of Guthion toxicity at a dose of 0.5 mg/kg body weight and in yearling cattle no toxicity was noted even at a dose of 2.5 mg/kg. The maximum non-toxic dose tested in sheep was 5.0 mg/kg. Goats were found unaffected by Guthion

at a dose of 2.5 mg/kg (McCarthy et. al., 1969).

It was also demonstrated by White et. al. (1968) that an oral daily dose (3.6 mg/kg) of Guthion produced toxic signs in heifers after six days of adminstration.

The oral LD_{50} doses of Guthion (50% wettable powder) in adult male and female rats were 14 mg/kg and 12.5 mg/kg, respectively (Report No. 24933, Chemagro Corp., Supplement No. 1, 1971).

When mature non-pregnant ewes were fed with pelleted alfalfa diet containing 50 PPM Guthion for ten weeks, the activity of AchE decreased gradually. It was suggested by the authors that it took ten times the tolerance dose to alter the AchE activity (Lindahl et. al., 1972).

The oral "no effect" dose of Guthion on ChE activity was investigated in human volunteers. In this study, the activity of ChE of plasma and erythrocytes was measured twice weekly for a period of four weeks. The following doses of Guthion were administered by oral route: 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 6, 8, 9, 10 and 12 mg/kg. There was no depression of ChE activity at any of the above dose levels (Reports Nos. 23886 and 25553, Chemagro Corp., Supplement No. 1, 1971). Similarly, Rider et. al. (1967) also failed to show the effect of Guthion on plasma and red blood cell ChE activity at the lower doses.

Signs of Toxicity

The signs of organophosphate poisoning are accounted for by a depression of AchE in the various parts of the nervous system:

a) signs similar to those caused by muscarine poisoning are due to an excessive accumulation of Ach at the postganglionic cholinergic nerves.

b) signs similar to those of nicotine poisoning are due to the action of Ach on the parasympathetic ganglia and motor end plates.

c) signs of CNS toxicity include excitation and terminal inhibition.

The dose and method of administration determine to a great extent the signs, intensity, onset and duration of organophosphate poisoning.

The initial manifestations of toxic signs are like those caused by muscarine poisoning, such as anorexia, nausea, epigastric and substernal pain, abdominal spasms, increased peristalsis, vomitting, diarrhea, salivation, bradycardia, profuse perspiration, pallor and dyspnea. Signs, similar to nicotine poisoning usually become manifest as soon as the muscarine-like effects are very marked, and consist of muscle twitching and generalized spasms with disappearance of reflexes.

Depression of AchE of the CNS at first results in restlessness, discomfort, giddiness, anxiety, severe headaches, sleeplessness, ataxia, tremor, confusion and ultimately resulting in coma (Kimmerie and Lorke, 1968; Goodman and Gilman, 1970; Goth, 1972).

Respiratory disorders are the primary cause of death. They consist of broncoconstriction accompanied by heavy secretion of bronchial mucus as well as paralysis of the respiratory musculature and the respiratory center (Kimmerie and Lorke, 1968).

The toxic signs of four organophosphate insecticides in newborn calves, yearling cattle, sheep and goats were those usually associated with ChE inhibition. Generally, nervousness was the first manifestation. This was followed by the appearance of slight frothy salivary accumulation on the lips. As toxicity progressed, muscular trembling and excessive salivation with drooling were observed. Uncontrolled muscular trembling was accompanied by convulsive spasm in calves, protrusion of the tongue, abdominal cramps, and diarrhea were also observed. The stools frequently had spots of blood on the surface. As toxicity progressed further,

ataxia became evident, and the test animals were unable to stand. Exhaustion occurred, and then death. At necropsy, the gross pathologic change most consistently observed was pulmonary congestion (McCarthy et. al., 1969).

The predominant signs of mild to moderate organophosphate poisoning in horses were of gastro-intestinal origin, including colic and diarrhea. Signs of toxicity in horses differ from those of ruminants. Toxic signs in the latter are usually characterized by excessive salivation, dyspnea, stiff-legged walking, weakness and diarrhea. In horses, the signs are usually colic, digestive disturbances, muscular weakness, salivation and dyspnea (Younger, 1965).

Cholinesterase in RBC and plasma

Alles and Hawes (1940) demonstrated two types of ChE's in blood, Pseudo ChE and true or AchE. The former was associated with serum and the latter with red blood cells. It was also shown that serum contains a mixture of PchE and AchE (Mendel, Mundell and Rudney, 1943; Augustinsson, 1948) with PchE predominating in man and horses, while AchE in the rabbit. The sera of ruminants, birds and fishes have almost no cholinesterase. AchE is deficient, or absent from the erythrocytes of birds, fish and cats, and this is compensated for by the presence of a comparatively larger amount of AchE in the plasma of these animals (Mendel, Mundell, Rudney, 1943

Whittaker (1951, 1953; Mounter and Whittaker, 1950) have reported that cholinesterases of erythrocytes and brain are most active towards cholines which have a chemical structure similar to that of acetylcholine. Plasma ChE's exhibit highest activity towards those cholines which resemble butyrylcholine in chemical structure (Struge and Whittaker, 1950). The presence of a butyryl ChE is a characteristic of man, dog, cat, horse,

and guinea pig plasma (Augustinsson, 1959).

Studies on the specificity of horse plasma and erythrocyte cholinesterases (Struge and Whittaker, 1950; Mounter and Whittaker, 1950) reveal that cholinesterase of horse plasma has been found to resemble human plasma, in so far as its capacity to hydrolyse large numbers of aliphatic esters. These results were consistent with the hypothesis that the closer the structural configuration to butyrylcholine the greater the rate of hydrolysis. ChE of horse erythrocyte thus closely resembles ChE of human erythrocytes with respect to specificity for substrates and physical properties.

It has also been reported that ChE activity of horse erythrocytes was approximately one-fifth that of human erythrocytes (Mendel and Rudney, 1943). Activity of acetylcholinesterase seems to vary in different species of animals. For example, it decreases in mammals in the following order: man, cow, guinea pig, horse, dog, sheep, rabbit, rat and cat (Augustinsson, 1950; Zajicek and Datta, 1953).

The presence of AchE in blood suggests that this enzyme performs an inportant physiological function in the body. Possibly by regulating the amount of acetylcholine in the circulation (Mendel, 1943; Gerebtzoff, 1959). In an attempt to explain the role of erythrocytes AchE, it was pointed out that this enzyme was indispensable to the integrity of red corpuscles. Holland and Greig (1950) concluded that AchE is essential to permeability to Na^+ and K^+ ions. Hajdukovic (1955) showed that AchE offers a certain degree of resistance to irradiation and this may have some importance regarding its physiological role in the body. Pseudo ChE being absent from the blood of certain species, is apparently not indispensable. It could nevertheless play a minor role in controlling the level of acetylcholine in the blood (Mendel, 1943).

Inhibition and Recovery of Cholinesterase Activity

Evidence has been accumulated to support the view that inhibition of esterase activity by organophophorous compounds is the result of phosphorylation of the center of the enzyme (Aldridge, 1953; Aldridge and Davison, 1953). The kinetics of inhibition, the reversal of inhibition and the apparent energies of activation provide good evidence that true cholinesterase is readily phosphorylated by organophosphorous compounds. The phosphorylation of pseudo cholinesterase by this group of compounds is less complete. Davison (1955) has presented evidence indicating that PChE, like true ChE, is phosphorylated by organophosphorous compounds and that the stability of the phosphorylated enzyme depends upon the type of enzyme inhibited and the organophosphate used.

It has been shown that PchE can be almost completely inactivated without the development of any significant signs of poisoning (Mazur and Bodansky, 1946; Hawkins and Gunter, 1946; Hawkins and Mendel, 1949) while a complete inhibition of true ChE is invariably fatal.

Several mechanisms are responsible for the recovery of ChE activity following poisoning by organophosphate compounds (Blaber and Creasey, 1960; Davison, 1955). If ChE inhibition is irreversible, recovery of ChE results from de novo synthesis of enzyme. If the inhibitor-enzyme complex is unstable, return of ChE activity may follow hydrolysis of the phosphorylated enzyme. Recovery of the ChE activity may also result from a combination of both processes.

If the freshly inhibited and completely reactivatable ChE is stored, it gradually changes into a form which can no longer be reactivated by oximes (Davies and Green, 1956; Wilson et. al., 1955; Hobbiger, 1956). This may be due either to the migration of the phosphoryl group to a more stable position in the inhibited enzyme (Wilson et. al., 1955; Hobbiger,

1956), or to loss of one of the substituents on the phosphorous atom which would then acquire a negative charge and consequently become resistant to nucleophillic attack (Oosterbaan et. al., 1958). The change which occurs during storage is referred to as "aging" although some authors have in fact referred to it as a transphosphorylation process (Hobbiger, 1956; Jandorf et. al., 1955).

Davison (1955) postulated that there are two forms of inhibited cholinesterase with different stabilities: the freshly inhibited enzyme, which can undergo spontaneous reactivation, and the aged which cannot. The first form may undergo spontaneous and complete conversion into the second form.

Blaber and Creasey (1960) reported that the recovery of sheep erythrocyte occurred in two stages, a rapid partial recovery being followed by a slower linear return to normal activity. During the first stage of the recovery, spontaneous hydrolysis and aging of the cholinesterase occurred simultaneously. The characteristics of this stage depended on the inhibitor used. The second stage of the recovery was correlated with new red blood cell formation and was independent of the inhibitor used.

MATERIALS AND METHODS

Horses fed with 5 and 15 PPM Guthion for 30 days

Nine adult horses, free from disease were obtained from the University of California, Veterinary Teaching Hospital, from owners and dealers. Physical condition of the horses varied from good to fair, but most of them were in good condition. The horses were weighed and each was kept in a separate stable throughout the length of the experiment. The weights of the horses are shown in Table I. They were carefully examined by a Veterinarian at the beginning and at intervals during the experiment for characteristic signs of Guthion poisoning. The horses were divided into three groups: control, 5 PPM and 15 PPM.

Alfalfa hay from a single lot was used for the experiment. Hay samples were analyzed for Guthion by gas chromatography. No Guthion was detected in any of the samples.

The daily feed intake for each horse was taken as the difference between the weight of the hay offered in the morning and the weight which was left uneaten at night. The average feed intake for each animal was determined from two consecutive daily feedings. This value was then assumed to be constant over the feeding period and formed the basis for calculating the amount of Guthion fed per day. These values are shown in Table I.

Administration of the drug

The Guthion was furnished by Chemagro Company as Guthion Wettable Dry Powder containing 54% of active azinphosmethyl.

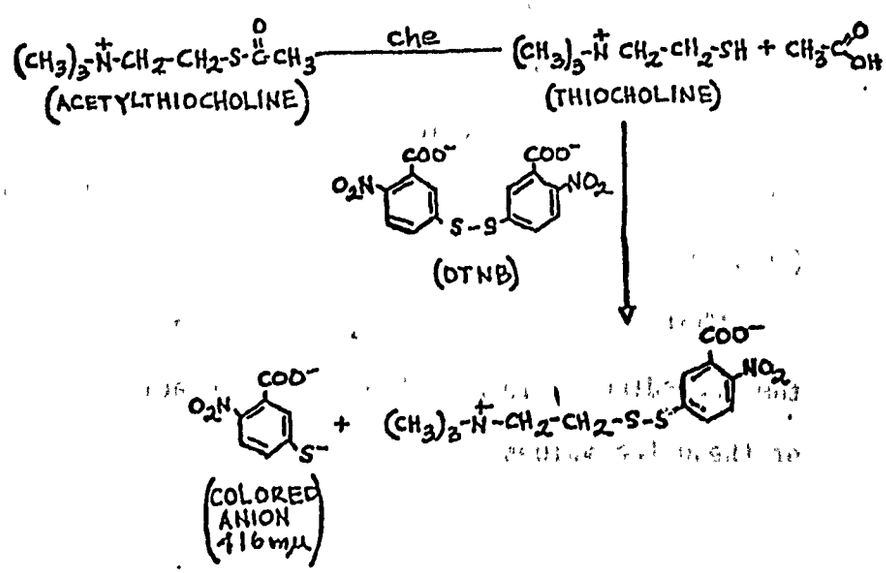
The compound dissolved in acetone was mixed with Omalene® (Purina horse chow) and fed in an aluminum bucket once a day for thirty days.

Since Guthion decomposes easily when put into solution, fresh doses were weighed daily. The horses appeared to enjoy the mixture and quickly consumed the daily doses. It was necessary that the acetone volatilize first. Control horses received Omalene® without Guthion. Alfalfa hay and water were offered ad libitum to all animals.

Principle of Spectrophotometric method for Cholinesterase assay:

Cholinesterase activity was measured by the procedure developed by Ellman et. al. (1961). Acetylthiocholine chloride was used as a substrate and Dithiobisnitrobenzoic acid (DTNB) as the complexing reagent which reacts with thiocholine and produces yellow color. The rate of enzymatic hydrolysis of acetylcholine was measured by the increase in absorbancy at 416 mu due to the yellow color formed with thiocholine and DTNB. All determinations were carried out at room temperature using a Beckman DU Spectrophotometer equipped with Gilford recording Photometer and Minneapolis-Honeywell recorder. The enzyme activity was linear for a period of 15 minutes and the activity was calculated as a change in the optical density for the initial 5 minutes per 2.95 ml of diluted whole blood or plasma.

Chemical Reactions involved:



Working Procedure:

A. Animal bleeding

Horse blood was collected from the jugular vein in heparinized vacutainers. Blood samples were taken on 3 consecutive days prior to the administration of Guthion in order to determine control values of cholinesterase activity in whole blood and plasma. During the experiment, samples of blood were collected and analyzed for cholinesterase activity 3, 7, 14, 21, and 30 days after feeding Guthion.

B. Measurement of cholinesterase activity of whole blood, plasma and RBC.

The whole blood was centrifuged for ten minutes of 500 g in a refrigerated Sorvoll RC-2 type Centrifuge at 0.4°C. The plasma was carefully aspirated and diluted to the original volume of the whole blood with phosphate buffer (pH 8.0, 0.1M) in a ratio of 1:600 (e.g., 20 ul. of whole blood or plasma + 11.98 ml. PO_4 buffer). 2.95 ml. of the diluted sample were pippetted into a cuvette. To this, 25 ul. of DTNB (0.01M) was added and the cuvette was placed in the spectrophotometer chamber. The slit of the spectrophotometer was adjusted so that the absorbance in the cuvette was zero at 416 mu. Twenty-five ul. of the substrate (0.075M) was then added to this cuvette. Thereafter, an increase in absorbance was continuously monitored for at least ten minutes. Cholinesterase activity was expressed as a change in optical density for the initial five minutes (ΔOD/5 min.).

Whole blood and plasma cholinesterase values were read directly from the recording while RBC cholinesterase activity was taken as the difference of these two values.

Rats fed with 5, 15 and 25 mg Guthion/kg.

After the 5 PPM and 15 PPM experiment in horses, the potency of the Guthion used was in question. Oral toxicity of Guthion in rats was therefore tested.

Male Sprague-Dawley rats weighing 350 - 400 grams were used. The rats were divided into four groups: control, 5, 15 and 25 mg Guthion/kg. The Guthion, dissolved in propylene glycol, was administered orally with feeding needles. As soon as the animals showed symptoms of organophosphorous poisoning, like fasciculation and dyspnea, they were decapitated and their blood was collected in heparinized test tubes. The surviving animals were observed for six more hours and then decapitated.

Cholinesterase activity of the rat blood was determined by the method described above.

Horses fed with 25, 50, 75 and 100 PPM Guthion for 7 days

Twelve horses were obtained from the University of California, at Davis, School of Veterinary Medicine. They were placed into four different groups. Each of the three horses in group one were fed with 25 PPM Guthion, group two 50 PPM, group three 75 PPM, and in group four 100 PPM as described previously. In order to determine the ChE activity in these horses, blood samples were taken after 2, 5 and 7 days of feeding Guthion. Recovery of ChE activity was determined after 2, 7, and 10 days post-treatment.

Table 1. Animal Data: Animal no.; age; weight; hay consumption; Dose (PPM), feed consumption basis; dose, mg/kg body weight.

Dose (PPM) (hay consumption basis)	Animal Number	Age (Year)	Animal Weight (Kg)	Average Animal wt. (Kg)	Average Hay Consumption (Kg/day/horse)	Average Hay Consumption (Kg/day/group)	Total daily Dose of Guthion (mg)	Daily Dose (mg/kg b.w.)
0	1	15	468		9.0		0	
0	2	16	430	447	9.0	9.16	0	0
0	3	4	443		9.5		0	
5	4	12	428		8.5		85	
5	5	20	496	452	9.0	9.00	85	0.118
5	6	5	432		9.5		85	
15	7	9	457		9.0		255	
15	8	12	439	451	9.0	9.00	255	0.565
15	9	16	432		9.0		255	
25	10	9	435		9.0		425	
25	11	8	400	426	9.5	9.16	425	1.00
25	12	6	442		9.0		425	
50	13	10	482		9.5		850	
50	14	4	404	432	8.5	9.16	850	1.97
50	15	7	432		9.5		850	
75	16	6	432		9.5		1275	
75	17	12	432	424	9.0	9.00	1275	3.00
75	18	6	409		8.5		1275	
100	19	16	459		8.5		1700	
100	20	7	409	430	9.0	9.00	1700	3.97
					9.5		1700	

RESULTS

Experiment on horses fed 5 and 15 PPM Guthion

It was found that although there was a considerable difference in cholinesterase activity among the three groups of horses, there was nevertheless a remarkable constancy of both the red blood cell and plasma cholinesterase values within each group of animals. This demonstrates the non-toxic effects of Guthion at these doses. It was also found that none of the horses exhibited any physical signs of toxicity during the 30 day feeding trial period.

The effects of 5 and 15 PPM Guthion on red blood cell and plasma cholinesterase activity of individual horses are shown in Figures 1 and 2. Table II shows the composite data of the control and the Guthion fed horses at the above doses.

Experiment on oral toxicity of Guthion in adult male rats

At the 5 mg/kg dose, no physical signs of poisoning were observed, although there was almost a 20 percent decrease in RBC and plasma cholinesterase activity. The 15 mg/kg dose induced signs of poisoning in 50 percent of the rats and a corresponding 50 percent decrease in cholinesterase activity. At the highest dose (25 mg/kg) of Guthion, all the rats exhibited physical signs of poisoning. A 75 and 70 percent decrease in RBC and plasma ChE activity, respectively, was observed in these rats. The symptoms of toxicity observed were: fasciculation, lacrimation, defecation, urination, difficulty in breathing and death.

The effects of 5, 15 and 25 mg/kg Guthion are shown in Table III.

This experiment provided evidence that the Guthion used during the first experiment in horses was as potent as claimed by the supplier.

Experiment on horses fed with 25, 50, 75 and 100 PPM Guthion

It was observed that 25 PPM Guthion after five days of feeding caused a 5 percent depression of RBC ChE activity and stayed almost at the same level at the end of the experiment. However, at this dose level, no depression of the plasma ChE activity was observed. It was found that 50, 75, and 100 PPM Guthion caused a decrease in ChE activity of both the RBC and plasma. The activity of the former was more depressed than that of the latter. The RBC ChE activity after seven days of Guthion feeding dropped to 61, 53 and 50 percent of pretreatment control at 50, 75 and 100 PPM, respectively. The plasma ChE activity, on the other hand, dropped to 75, 58 and 53 percent at the corresponding level of Guthion feeding.

At no time during the seven day feeding period did any horse exhibit physical signs of organophosphate poisoning. No attempts were made to induce physical signs of toxicity in the animals by continued feeding of Guthion.

The effects of 25, 50, 75 and 100 PPM Guthion on the RBC and plasma ChE activity of individual horses are shown in Fugures 3, 4, 5 and 6, respectively. Table IV shows the composite data of the four groups of horses.

All plasma ChE activities recovered after seven days post-treatment, except for 100 PPM, which still had 8 percent depression. Only the RBC ChE activity at 50 PPM have recovered completely after 10 days post-treatment, 75 and 100 PPM still showed 7 and 16 percent depressions, respectively.

Figures 4, 5 and 6 show the post-treatment recovery of RBC and plasma ChE activity of individual horses after feeding 50, 75 and 100 PPM Guthion. A composite data for these three groups of horses is shown in Table V. It can be seen that first, there is a rapid partial recovery of both RBC

and plasma ChE activity, followed by a slower return to control values.

Summary graphs of RBC and plasma ChE activity for all doses of Guthion feeding are shown in Figure 7. Recovery of ChE activity at 50, 75 and 100 PPM of Guthion feeding are also represented in the above figure.

On the body weight basis (Table I), doses of Guthion at 0.12, 0.57 and 1.00 mg/kg neither caused any physical signs of toxicity nor any depression of cholinesterase activity in any of the horses. Guthion at 2, 3 and 4 mg/kg was however associated with a considerable depression of ChE activity but no physical signs of toxicity.

Table II. Red blood cell and plasma cholinesterase activity after 3, 7, 14, 21 and 30 days of feeding Guthion at 5 and 15 PPM.

Dose Guthion parts per Million (PPM)	Cholinesterase Activity of RBC and Plasma Expressed as ΔOD/5 min.[a]			
	RBC	% of Control[b]	Plasma	% of Control[b]
	Avg ± SE(3)[c]		Avg ± SE(3)[c]	
Control				
0 day	0.134 ± 0.025	100.00	0.110 ± 0.031	100.00
3 days	0.132 ± 0.026	98.50	0.110 ± 0.029	100.00
7 days	0.134 ± 0.024	100.00	0.111 ± 0.033	100.90
14 days	0.134 ± 0.024	100.00	0.110 ± 0.029	100.00
21 days	0.133 ± 0.020	99.40	0.108 ± 0.029	98.18
30 days	0.136 ± 0.015	101.42	0.108 ± 0.032	98.18
5 PPM				
0 day	0.110 ± 0.012	100.00	0.120 ± 0.026	100.00
3 days	0.111 ± 0.009	100.90	0.120 ± 0.027	100.00
7 days	0.109 ± 0.012	99.99	0.120 ± 0.026	100.00
14 days	0.112 ± 0.010	101.80	0.121 ± 0.026	100.83
21 days	0.109 ± 0.013	99.99	0.119 ± 0.024	99.17
30 days	0.111 ± 0.014	100.90	0.118 ± 0.023	98.33
15 PPM				
0 day	0.116 ± 0.008	100.00	0.083 ± 0.005	100.00
3 days	0.114 ± 0.013	98.27	0.086 ± 0.003	103.60
7 days	0.114 ± 0.015	98.27	0.085 ± 0.002	102.41
14 days	0.114 ± 0.014	98.27	0.084 ± 0.005	101.20
21 days	0.113 ± 0.015	97.33	0.085 ± 0.002	102.41
30 days	0.115 ± 0.010	99.33	0.085 ± 0.004	102.41

a. See the method section for details.

b. In each group ChE activity on Zero day was the control value.

c. Figure in parenthesis indicates number of separate determinations.

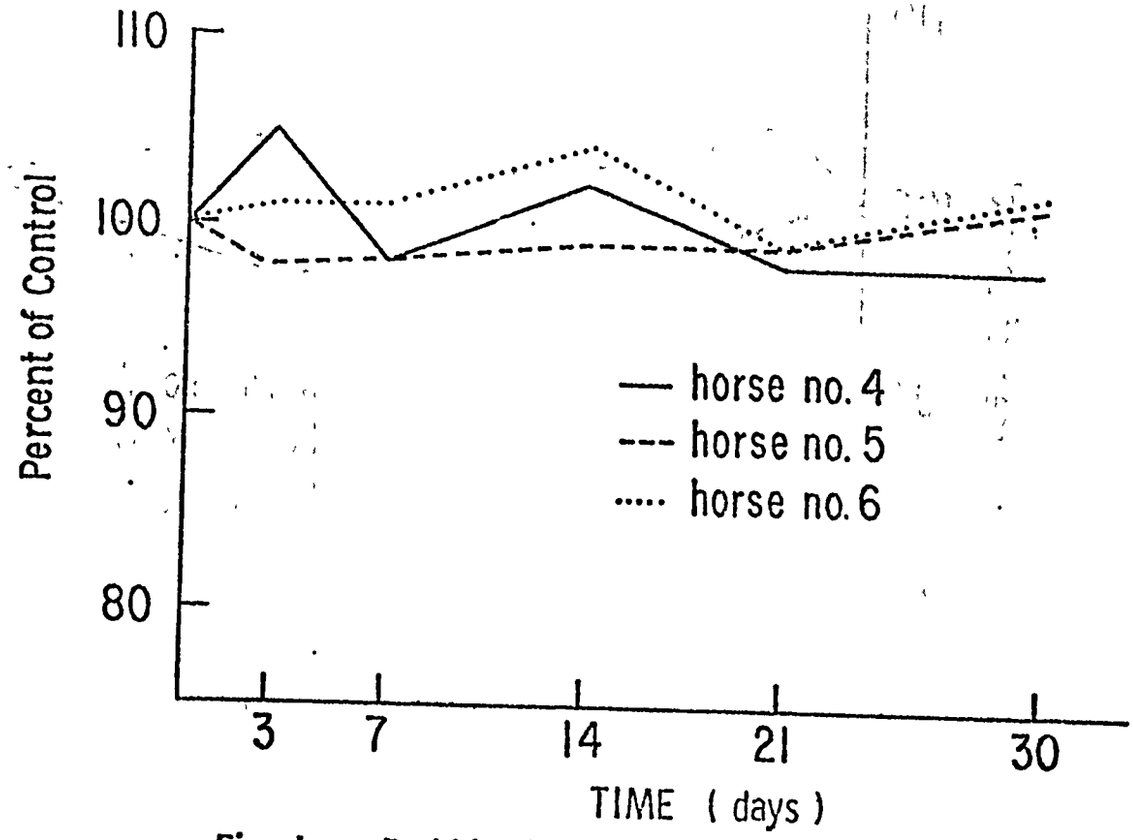


Fig. 1 a: Red blood cell cholinesterase activity of horses (no's. 4, 5 and 6) dosed with 5 PPM Guthion.

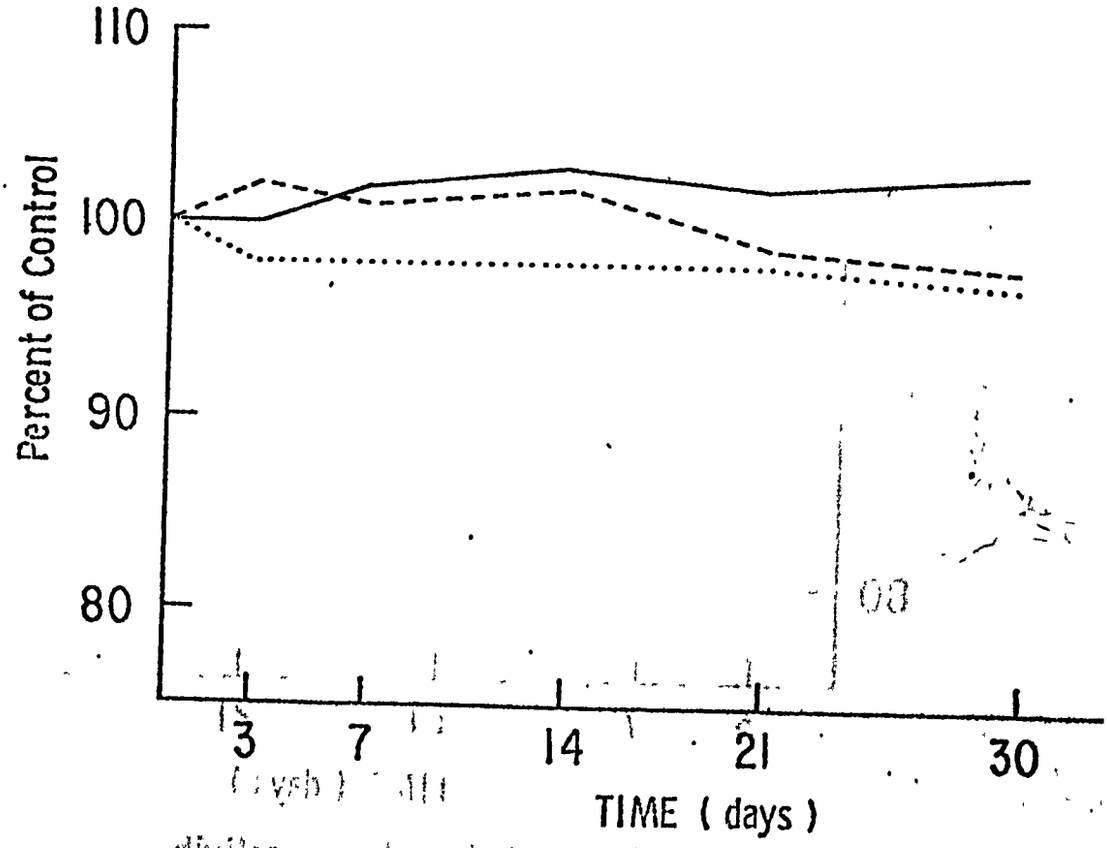


Fig. 1 b: Plasma cholinesterase activity of horses (no's. 4, 5 and 6) dosed with 5 PPM Guthion.

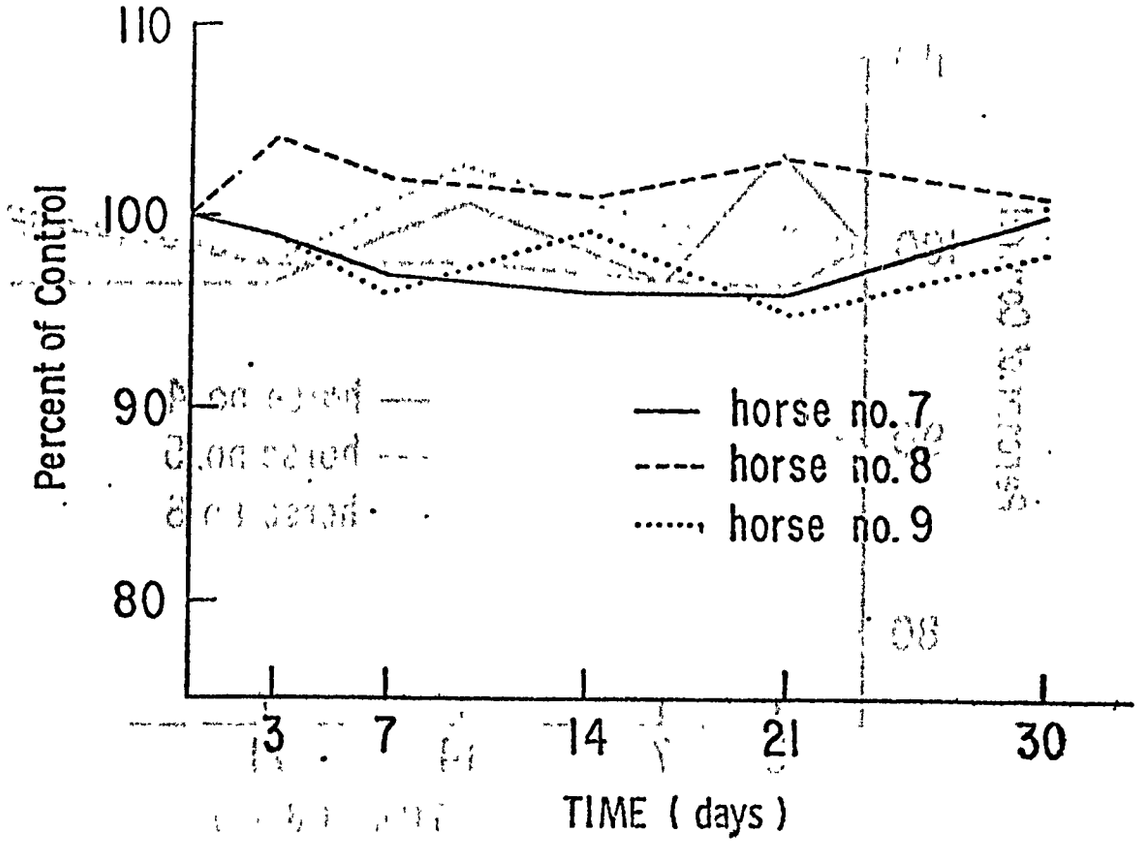


Fig. 2 a. Red blood cell cholinesterase activity of horses (no's. 7, 8 and 9) dosed with 15 PPM Guthion.

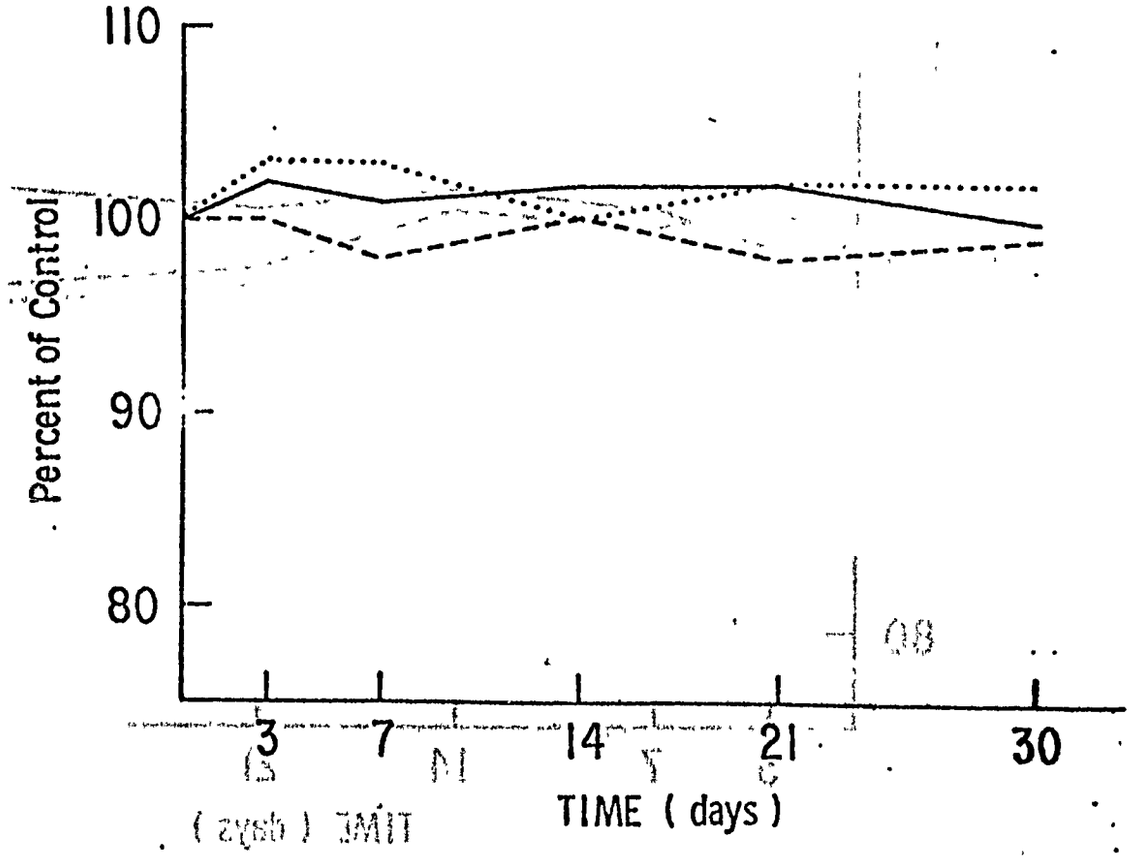


Fig. 2 b. Plasma cholinesterase activity of horses (no's. 7, 8 and 9) dosed with 15 PPM Guthion.

Table III. Effects of oral administration of Guthion on the Red blood cell and plasma cholinesterase activity in adult male rats.

Dose mg/kg	Cholinesterase activity of RBC and plasma expressed as ΔOD/5 mins.[a]				% of Rats showing toxic signs
	RBC	% of control[b]	Plasma	% of control[b]	
	Avg ± SE(4)[c]		Avg ± SE(4)[c]		
Control	0.094 ± 0.002	100.00	0.031 ±	100.00	0
5 mg/kg	0.075 ± 0.005	79.78	0.026 ±	83.87	0
15 mg/kg	0.045 ± 0.008	47.87	0.017 ±	54.84	50
25 mg/kg	0.024 ± 0.003	25.53	0.009 ±	31.60	100

a. See the method section for details.

b. In each group ChE activity on Zero day was the control value.

c. Figure in parenthesis indicates number of separate determinations.

Table IV. Red blood cell and plasma cholinesterase activity after 2, 5, and 7 days of feeding Guthion at 25 PPM, 50 PPM, 75 PPM and 100 PPM.

Dose Guthion parts per Million (PPM)	Cholinesterase Activity of RBC and Plasma Expressed as ΔOD/5 min.[a]			
	RBC	% of Control[b]	Plasma	% of Control[b]
	Avg ± SE(3)[c]		Avg ± SE(3)[c]	
25 PPM				
0 day	0.117 ± 0.015	100.00	0.092 ± 0.018	100.00
2 days	0.117 ± 0.017	100.00	0.093 ± 0.019	101.08
5 days	0.111 ± 0.015	94.90	0.090 ± 0.017	98.00
7 days	0.113 ± 0.016	96.58	0.092 ± 0.015	100.00
50 PPM				
0 day	0.135 ± 0.016	100.00	0.083 ± 0.013	100.00
2 days	0.125 ± 0.015	92.60	0.078 ± 0.012	94.00
5 days	0.101 ± 0.012	74.81	0.066 ± 0.010	80.00
7 days	0.083 ± 0.010	61.47	0.062 ± 0.009	74.70
75 PPM				
0 day	0.113 ± 0.008	100.00	0.094 ± 0.007	100.00
2 days	0.101 ± 0.011	83.20	0.070 ± 0.017	84.04
5 days	0.068 ± 0.005	60.17	0.064 ± 0.005	68.00
7 days	0.060 ± 0.004	53.10	0.055 ± 0.004	58.50
100 PPM				
0 day	0.127 ± 0.013	100.00	0.088 ± 0.021	100.00
2 days	0.101 ± 0.011	80.00	0.070 ± 0.017	79.93
5 days	0.069 ± 0.007	55.00	0.053 ± 0.012	60.24
7 days	0.063 ± 0.006	49.66	0.047 ± 0.010	53.40

a. See the method section for details.

b. In each group ChE activity on Zero day was the control value.

c. Figure in parenthesis indicates number of separate determinations.

Table V. Post-treatment recovery of Red blood cell and plasma cholinesterase activity after 2, 7, and 10 days.

Dose Guthion parts per Million (PPM)	Cholinesterase Activity of RBC and plasma as ΔOD/5 min.[a]		
	RBC	% of Control[b]	Plasma
	Avg ± SE(3)[c]		Avg ± SE(3
50 PPM			
2 days	0.121 ± 0.014	89.63	0.077 ± 0.01[illegible]
7 days	0.134 ± 0.015	99.26	0.082 ± 0.01[illegible]
10 days	0.137 ± 0.015	101.45	0.085 ± 0.015
75 PPM			
2 days	0.090 ± 0.006	79.64	0.082 ± 0.[illegible]
7 days	0.096 ± 0.007	84.95	0.092 ± 0.006
10 days	0.105 ± 0.014	93.00	0.093 ± [illegible]
100 PPM			
2 days	0.087 ± 0.009	68.42	0.069 ± 0.01[illegible]
7 days	0.096 ± 0.010	75.58	0.081 ± 0.020
10 days	0.106 ± 0.011	83.46	0.085 ± 0.021

a. See the method section for details.

b. In each group ChE activity on zero day was the control value.

c. Figure in parenthesis indicates number of separate determinations.

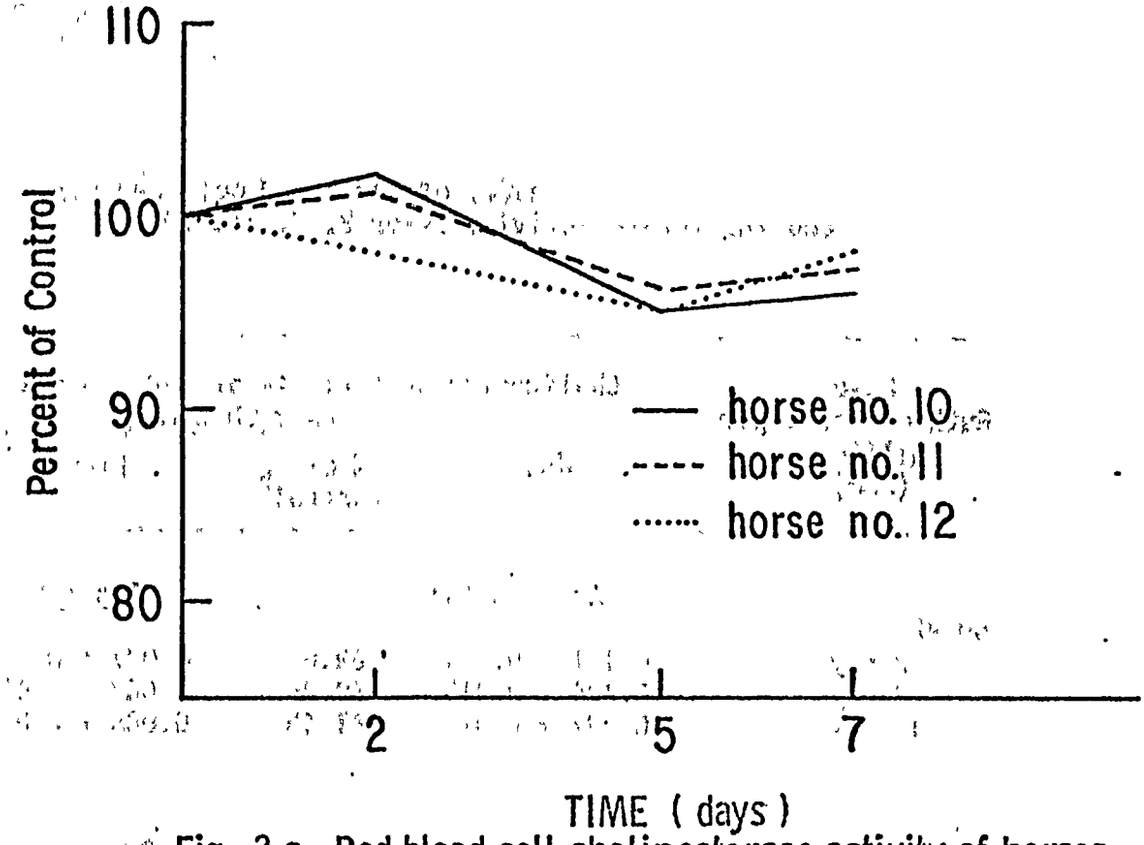


Fig. 3 a. Red blood cell cholinesterase activity of horses (no's. 10, 11 and 12) dosed with 25 PPM Guthion.

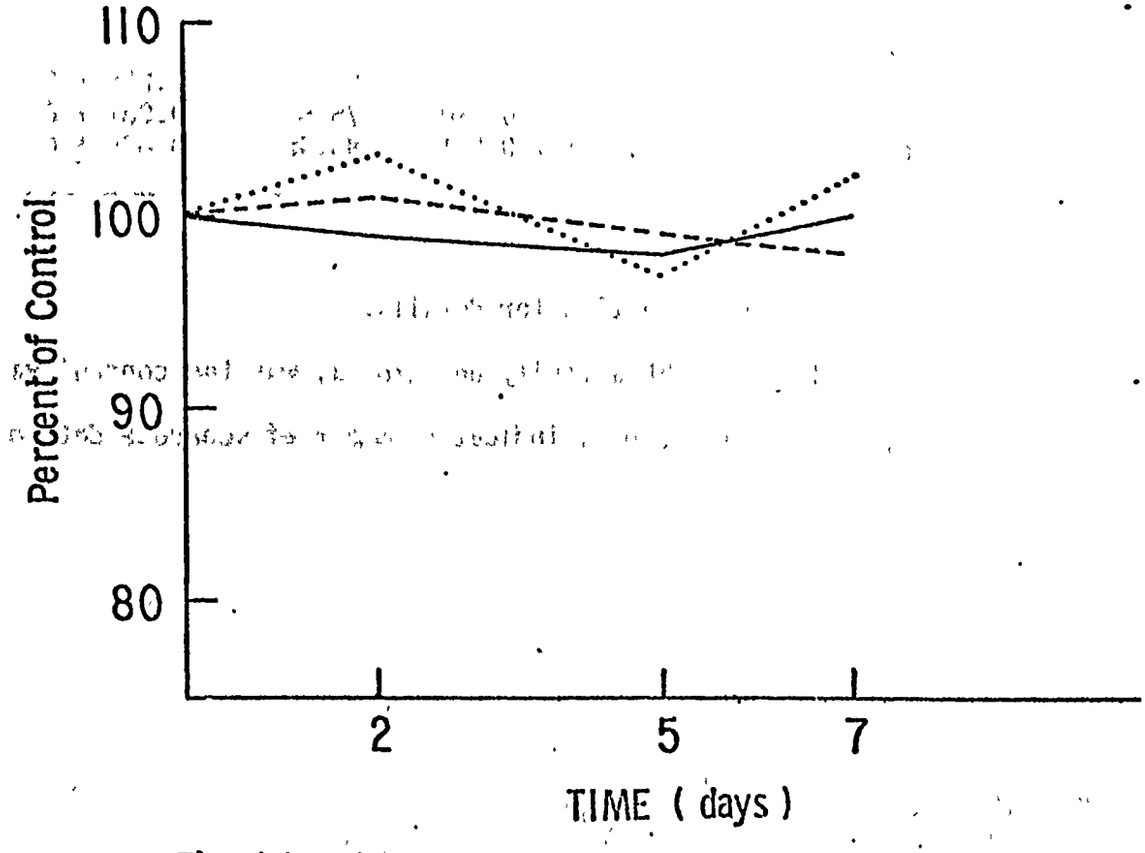


Fig. 3 b. Plasma cholinesterase activity of horses (no's. 10, 11 and 12) dosed with 25 PPM Guthion.

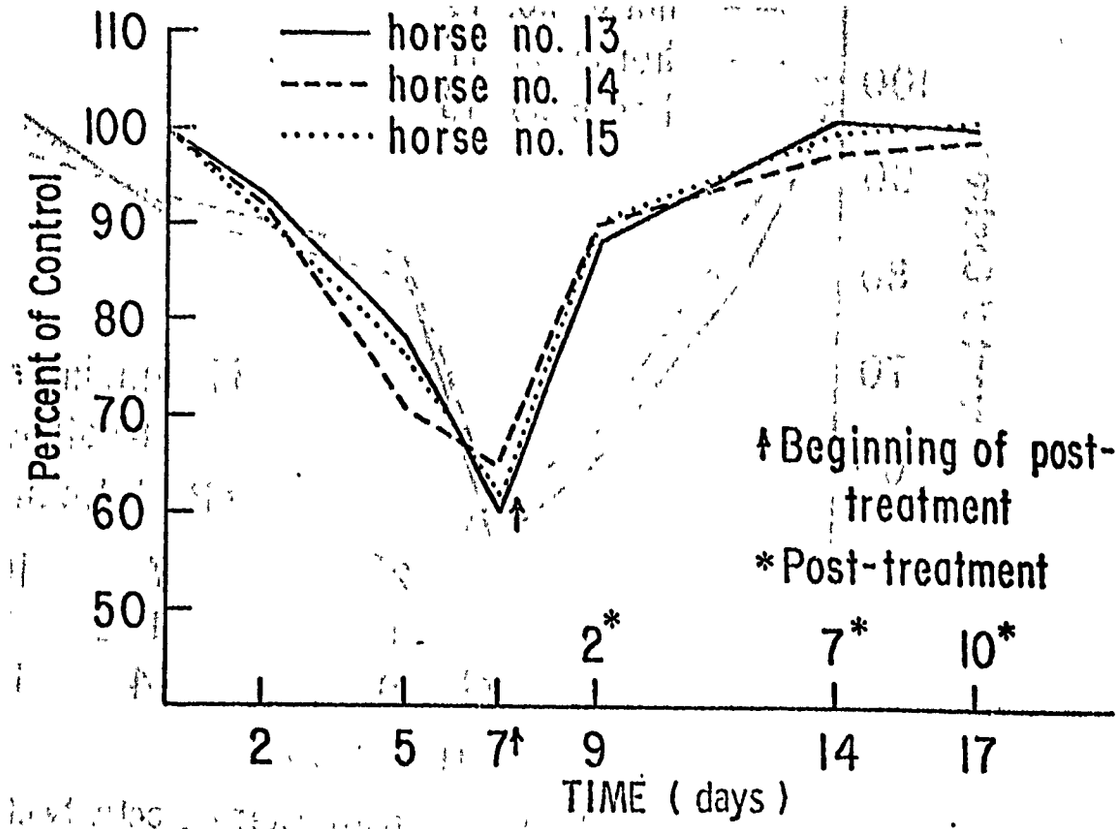


Fig. 4 a. Red blood cell cholinesterase activity of horses (no's. 13, 14 and 15) dosed with 50 PPM Guthion.

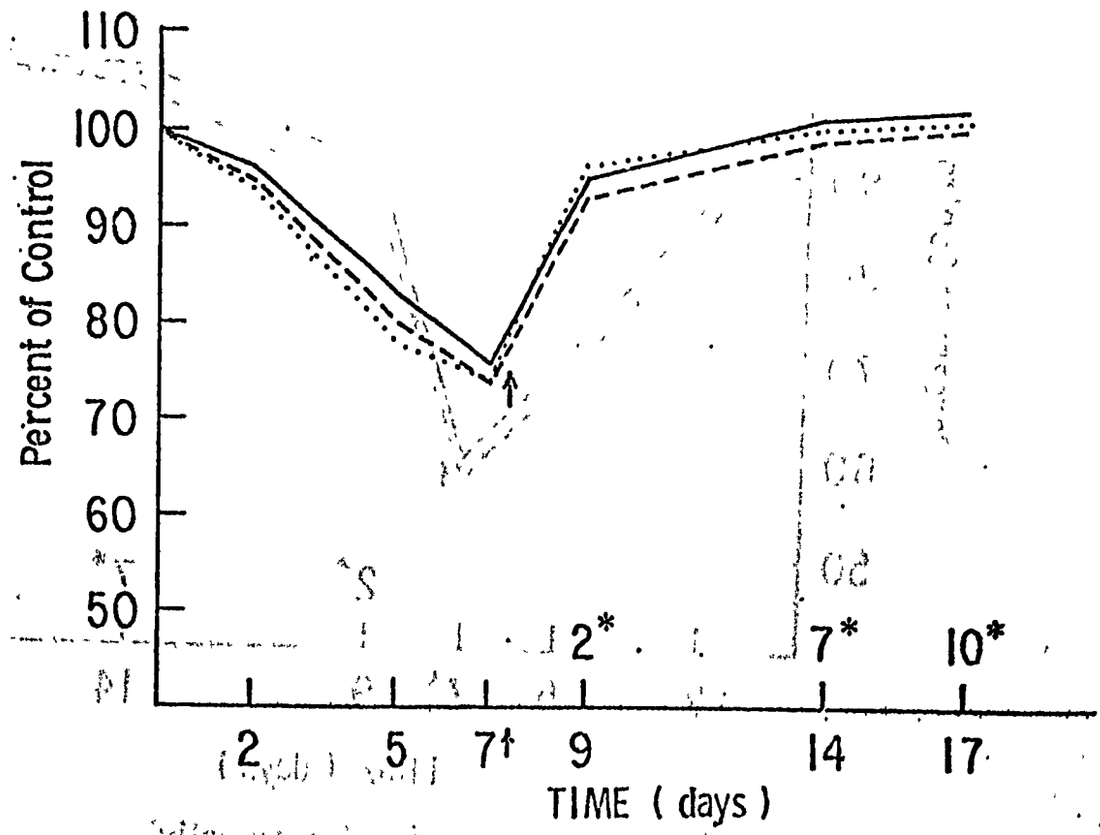


Fig. 4 b. Plasma cholinesterase activity of horses (no's. 13, 14 and 15) dosed with 50 PPM Guthion.

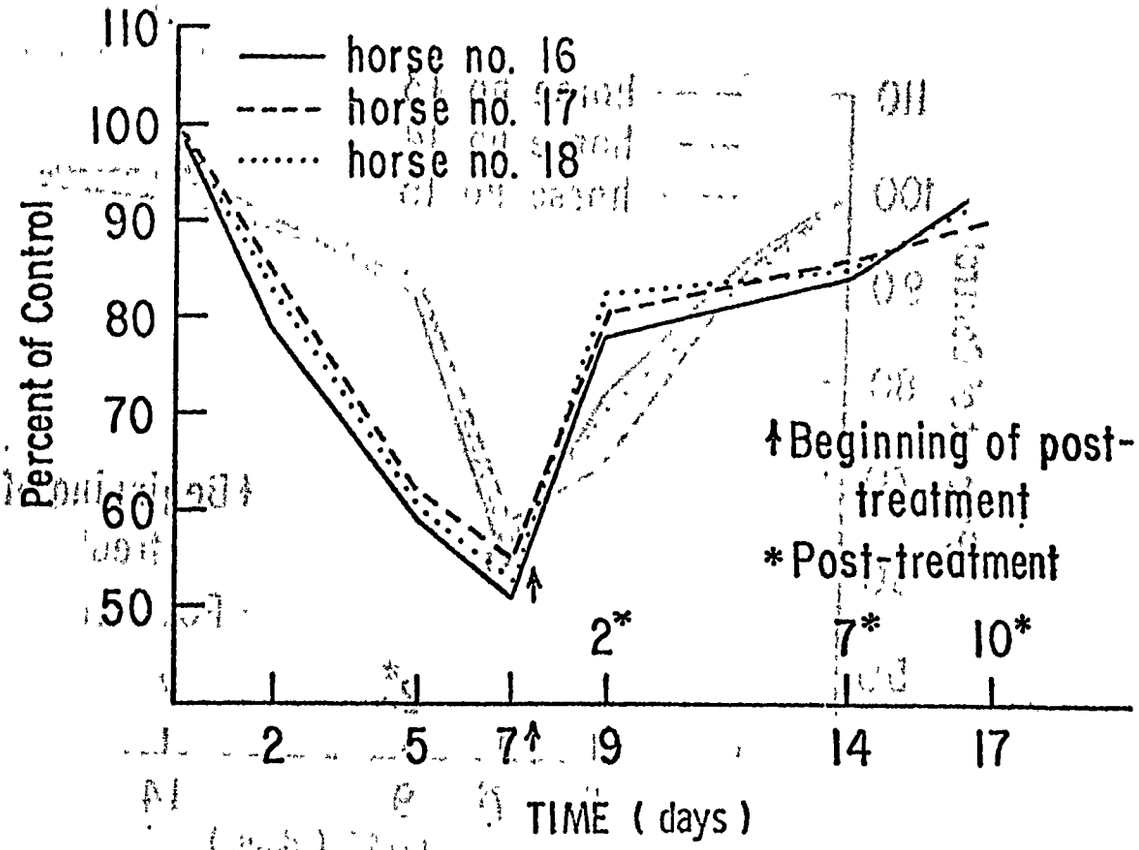


Fig. 5 a. Red blood cell cholinesterase activity of horses (no's. 16, 17 and 18) dosed with 75 PPM Guthion.

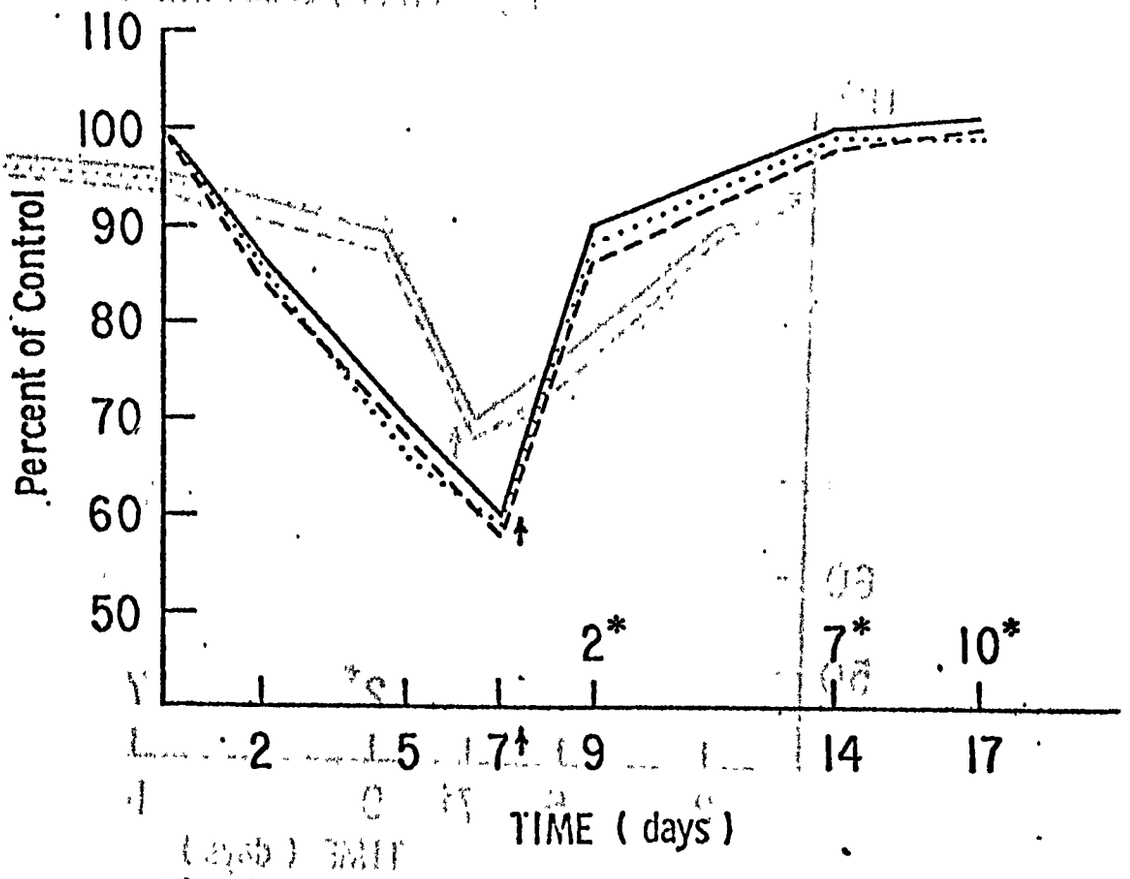


Fig. 5 b. Plasma cholinesterase activity of horses (no's. 16, 17 and 18) dosed with 75 PPM Guthion.

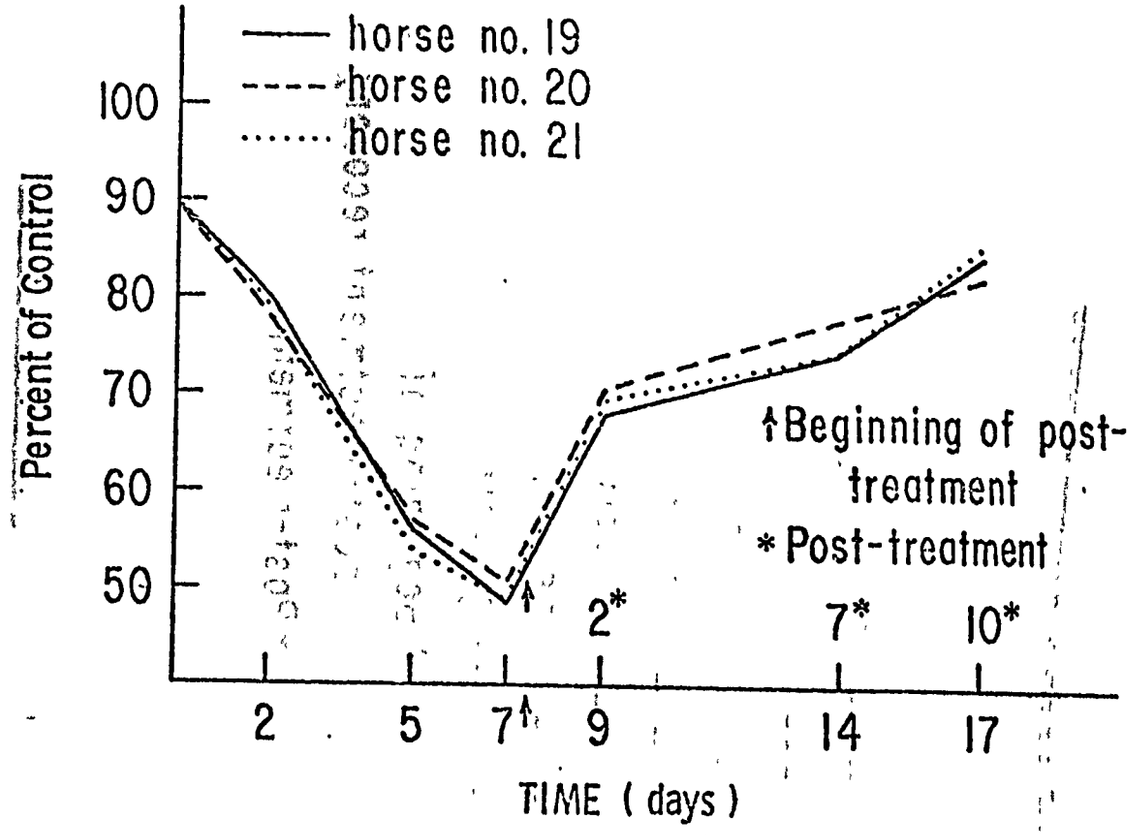


Fig. 6 a. Red blood cell cholinesterase activity of horses (no's. 19, 20 and 21) dosed with 100 PPM Guthion.

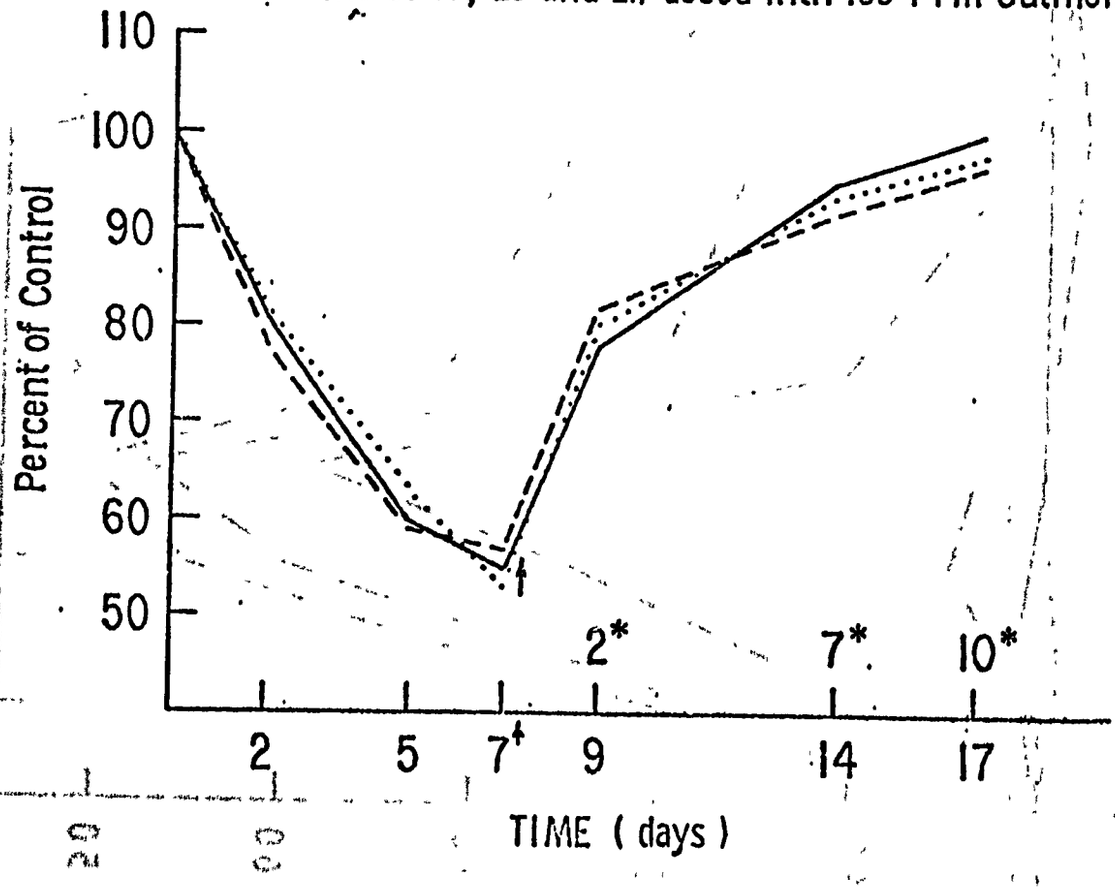


Fig. 6 b. Plasma cholinesterase activity of horses (no's. 19, 20 and 21) dosed with 100 PPM Guthion.

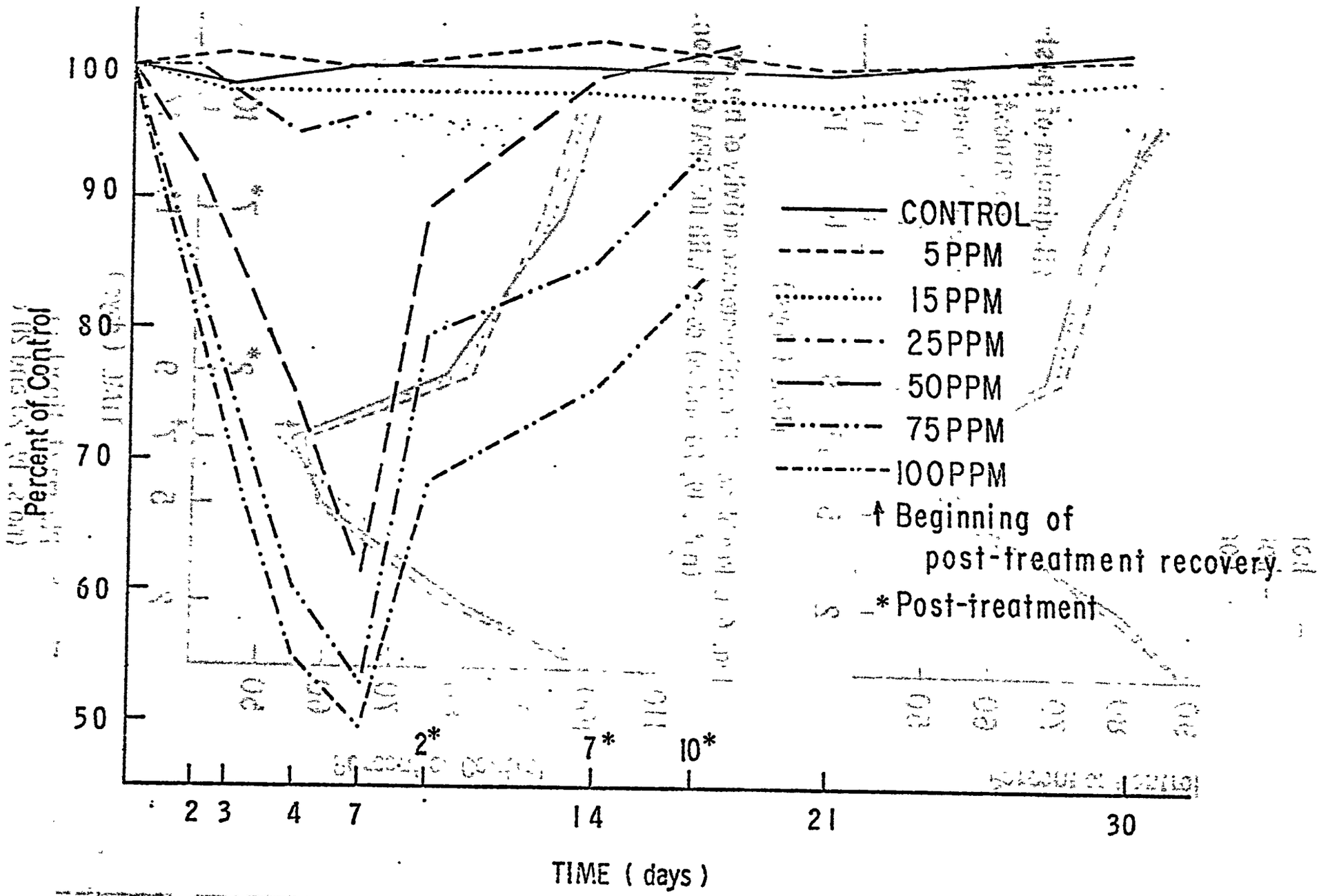


Fig. 7 a. Summary graph· Red blood cell cholinesterase activity of horses fed

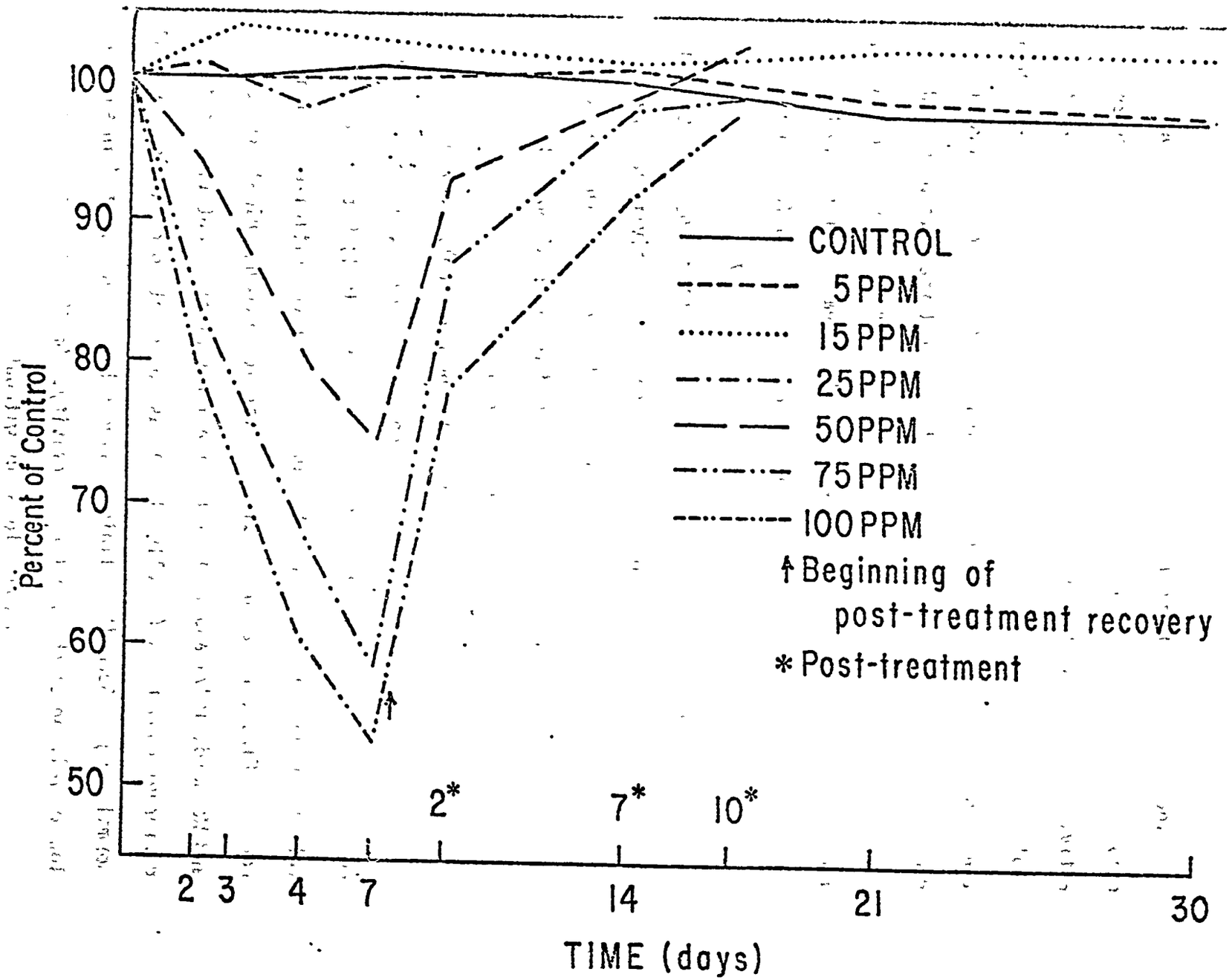


Fig. 7 b. Summary graph: Plasma cholinesterase activity of horses fed with 5, 15, 25, 50, 75 and 100 PPM Guthion and post-treatment recovery

DISCUSSION

The maximum permitted residual levels of an organophosphorous compound on a food crop are based primarily on the anticholinesterase activity of the insecticide. A study of the potency of Guthion as a cholinesterase inhibitor is important from the standpoint of ascertaining the adequacy of cholinesterase inhibition as a measure of permissible dietary levels. Those data in Table II show that there is no marked change in blood ChE activity when horses were fed 5 PPM and 15 PPM Guthion. The dose of 25 PPM seemed to cause a slight decrease. It should be noted that this dose might cause a significant change when fed for a longer period of time (Table IV). Table IV shows that 50, 75 and 100 PPM caused a very marked decrease in ChE activity without manifesting any physical sign of poisoning. Therefore, decreased blood ChE activity was used primarily as an index of Guthions biological effect. In this study, the maximum residual level that the horse could tolerate without showing any signs of toxicity would be between 25 and 50 PPM.

Depression of blood ChE activity has been commonly used as a reliable indication of exposure to organophosphorous pesticides, particularly in human medicine (Metcalf, 1951; Callaway et. al., 1951) but its diagnostic validity when applied to domestic animals has been criticized on two counts. First, it has been said that variation among animals (Radeleff and Woodard, 1957) and in individual animals at different times and under different conditions (O'Kelly, 1965) was too great to establish a useful normal range. Secondly, clinical effects have been reported in spite of low inhibition of cholinesterase activity. On the other hand, complete inactivation has been found to occur without symptoms (Radeleff and

Woodard, 1957). Some authors (Radeleff et. al, 1963) have observed clinical toxic signs that are accompanied by a very marked fall in blood cholinesterase activity.

In this study, depression of ChE activity occurred as high as 50% without manifestation of any overt toxic signs. This is consistent with the observation made by Anderson (1969) that calves would tolerate an inhibition of their ChE activity by as much as 64% without exhibiting any physical signs of toxicity. In sheep, signs of organophosphate toxicity would be seen only after inhibition of at least 70% of the cholinesterase activity. In rats, on the other hand, 50% inhibition resulted in toxic signs in 50% of the animals and 75% inhibition caused clinical signs in all animals. It is evident, therefore, that toxic effects do not appear without a severe depression of ChE activity in cattle, sheep and horse. The presence of overt toxic signs, a depression of ChE activity can be of some help in confirming the organophosphate poisoning in certain species of animals but not in others.

RBC ChE activity as shown in Tables II, III, and IV, had higher activity than plasma. It appears that there is more cholinesterase in the RBC, except that of the animals nos. 5 and 6 fed Euthion 5 PPM. Secondly, as there is more AchE in RBC than in plasma, the substrate (acetylcholine) used in this study is hydrolyzed faster by AchE than by PchE (Struge and Whittaker, 1950; Mounter and Whittaker, 1950), this may account, therefore, for a greater activity of AchE associated with the red blood cells than with the plasma.

Recovery of RBC ChE has been reported to occur in two stages, a rapid partial recovery being followed by a slower linear return to normal (Blaber and Creasey, 1960). In this study, the data suggests a similar pattern of recovery for RBC ChE. During the first stage the inhibited enzyme undergoes two simultaneous reactions: spontaneous reactivation by hydrolysis,

and aging in which the inhibited enzyme is converted into a form which would no longer be reactivated spontaneously or be reactivated by oxime treatment. When all the inhibited enzymes are present in the aged form no further recovery of the blood ChE will occur. The second stage of recovery is that in which the rate of blood ChE recovery becomes equal to the rate of de novo enzyme synthesis and this in turn replaces the inhibited enzymes.

The second stage of the red blood cell ChE recovery was associated with the formation of new cells (Blaber and Creasey, 1960). Although no attempt was made to determine the hematocrit of horses in the present study, a slight increase in the packed red blood cell volume seemed to exist at the end of the ten day recovery period.

In general, the rate of recovery of inhibited PchE activity is much faster than that of the true ChE inhibited by the same compound (Davison, 1955). The mode of recovery of plasma ChE was similar to that of RBC, but it appeared to recover well ahead of RBC. This is probably due to two reasons: first, a less stable enzyme-inhibitor complex (Aldridge, 1953; Fulton and Mogey, 1954) which undergoes spontaneous hydrolysis at a much faster rate and secondly, de novo synthesis of PchE by the liver.

It was frequently noticed that the activity of both plasma and red blood cell ChE's was higher during the post-treatment period than the pre-treatment. This could be attributed to induction of new enzyme β synthesis after the termination of Guthion treatment as suggested by Conney (1959).

CONCLUSION

Guthion fed to horses at levels 5 and 15 PPM for thirty days and 25 PPM for seven days failed to produce any signs of organophosphorous poisoning. No detectable physical signs of toxicity or any depression of blood ChE activity was observed at these levels. On the contrary, when Guthion was fed at higher dose levels, such as 50, 75 and 100 PPM for seven days then a marked decrease in blood ChE activity was obtained. The red blood cell ChE activity at the above dose levels dropped to 61, 53 and 50% of the pre-treatment control, respectively. The PchE activity at the corresponding doses was reduced to 76, 58 and 53 percent of the control. Inspite of a striking decrease in the blood ChEs activity no physical signs of toxicity were observed at any time. It was therefore concluded by this investigation that the maximum tolerable dose of Guthion in horses would fall between 25 and 50 PPM.

A marked decrease in blood ChE activity after seven days of Guthion feeding at 50, 75 or 100 PPM was followed by a rapid recovery in enzymatic activity. Consequently, no sign of ChE inhibition was demonstrable at the first two dose levels after ten days post-treatment except for a slight inhibition (7%) in RBC ChE activity at 75 PPM Guthion level. A slightly higher inhibition (17%) in RBC ChE activity was however present after that time only at 100 PPM Guthion.

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