Biochemical Pharmacology, Vol. 25, pp. 835–839. Pergamon Press, 1976. Printed in Great Britain

# EFFECT OF PARATHION AND ITS METABOLITES ON CALCIUM UPTAKE ACTIVITY OF RAT SKELETAL MUSCLE SARCOPLASMIC RETICULUM *IN VITRO*

NEAL BINDER, ERWIN J. LANDON, LYNN WECKER* and WOLF-D. DETTBARN

Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tenn. 37232, U.S.A.

(*Received* 10 *February* 1975; *accepted* 18 *July* 1975)

**Abstract**—The insecticide parathion and its potent anticholinesterase metabolite paraoxon induce skeletal muscle necrosis when administered *in vivo* to rats. In the present study the effects *in vitro* of parathion and its metabolites on microsomal (sarcoplasmic reticular) calcium uptake activity in rat diaphragm skeletal muscle are examined. Parathion (0·05 mM) is a potent inhibitor of this calcium uptake. The inhibition is apparently competitive with calcium in the system. Parathion (0·05 mM) is also shown to inhibit calcium-dependent ATPase activity associated with the microsomal calcium uptake. Paraoxon, the active anticholinesterase metabolite of parathion, and *p*-nitrophenol, a hydrolytic metabolite of paraoxon, have no inhibitory effects at this level. At 1·5 mM levels they do inhibit the skeletal muscle microsomal calcium uptake. Eserine, a chemically unrelated anticholinesterase agent, also has inhibitory effects at 1·5 mM. When these same compounds are incubated with isolated rat hemidiaphragms they antagonize the muscle contraction elicited by direct stimulation of the muscle. The skeletal muscle necrosis caused by parathion and paraoxon appear to relate to the anticholinesterase activity *in vivo*. The relatively potent inhibition of calcium uptake activity of sarcoplasmic reticulum *in vitro* seen with parathion appears to be an independent action and not related to cholinesterase inhibition.

The actions of most organophosphorus agents on the neuromuscular junction have been attributed primarily to their anticholinesterase properties [1, 2]. Highly purified parathion is not a potent inhibitor of cholinesterase but must be oxidized to its corresponding oxygen analog, paraoxon, to exhibit potent cholinesterase inhibition [3–7]. Paraoxon behaves predominantly as a phosphorylating agent whose inhibitory potency is quantitatively predictable by the magnitude of the electron-withdrawing properties of the aryl substituent [8] and rate of release of *p*-nitrophenol [9].

The effects of parathion *in vivo* on cholinesterase may account for some of the skeletal muscle toxicity. Daily injection of parathion or of paraoxon produce a progressive neurally mediated necrosis of skeletal muscle fibers in the rat [10]. A significant increase in frequency of miniature end plate potentials (MEPPs) is also seen in diaphragms from paraoxon-treated rats [11]. This represents an increased release of acetylcholine (ACh) [12]. ACh release in the presence of paraoxon may be plausibly attributed to an increase in entry of $Ca^{2+}$ into the nerve terminal, which in turn accelerates release of the transmitter [13].

The possibility that parathion and paraoxon exert direct effects unrelated to acetylcholine is less well supported. Parathion causes irreversible tonic contraction in crustacean (*Carcinus maenas*) skeletal muscle [14]. In this system the neuromuscular transmission is noncholinergic. The main neurohumoral agents are probably glutamate and $\gamma$-aminobutyric acid [15–18]. Excess acetylcholine is ruled out in this instance as a cause of the tonic contractions that are induced. There is evidence in *Carcinus maenas* that parathion blocks the uptake of calcium *in vitro* by membrane vesicles of sarcoplasmic reticulum, augments the superprecipitation of actomyosin *in vitro* and causes the contracture of skeletal muscle [19]. Skeletal muscle utilizes metabolic energy (ATP) to sequester calcium from the cytoplasm and thereby relaxes the skeletal muscle. This activity may be studied *in vitro* by isolating sarcoplasmic reticular membrane vesicles (skeletal muscle microsomes). A direct effect *in vivo* of parathion or paraoxon on mammalian skeletal muscle is probably obscured by the striking consequences of the cholinesterase inhibition.

In the following study *in vitro*, an intracellular activity of skeletal muscle is studied independently of effects deriving from cholinesterase activity. The study examines the calcium uptake *in vitro* of sarcoplasmic reticulum of rat diaphragm muscle as modified by paraoxon, its metabolite *p*-nitrophenol and its parent compound parathion. Parathion, considerably less potent as a cholinesterase inhibitor than paraoxon, is a relatively potent inhibitor of the calcium uptake activity of rat skeletal muscle sarcoplasmic reticulum.

## MATERIALS AND METHODS

Animals employed for this study were male Sprague–Dawley albino rats weighing between 250 and 350 g. Parathion and paraoxon were synthesized in the laboratory of Dr. Robert A. Neal (Dept. of Biochemistry and Center of Toxicology, Vanderbilt Uni-

* Present address: Department of Pharmacology, Northeastern University, Boston, Mass., U.S.A.

versity). Stock solutions of parathion were prepared in absolute methanol (0·5 M) and paraoxon in water (0·0056 M). Eserine $SO_4$ was obtained from Nutritional Biochemical Corp. and *p*-nitrophenol was obtained from Fisher Chemical Co. $^{45}CaCl_2$ was obtained from New England Nuclear Corp.

For muscle contraction studies, animals were sacrificed by decapitation and both hemidiaphragms were removed and bathed in Tyrode solution (NaCl, 8·0; KCl, 0·2; $CaCl_2$, 0·2; $MgCl_2$, 0·1; $NaHCO_3$, 1·0; $NaH_2PO_4$, 0·05; and dextrose, 2·0 g/l.) oxygenated with 95% $O_2$–5% $CO_2$ at 37°. An initial tension of 1·0 g was placed on the muscle which was mounted on a rod containing two electrodes for direct muscle stimulation and attached to an isometric force transducer. The muscles were stimulated with 24 single rectangular pulses/min of 30 msec duration at supramaximal voltage (30–50 V). The preparations were allowed to stabilize for 20 min with constant stimulation. When drugs were added, stimulation was stopped and only elicited every 10 min for 2 min to determine the size of the contractions. The tension developed by contracture of the muscle was recorded on a physiograph (Narco Biosystems, Inc.). One hemidiaphragm from each animal was used for a control and the other was drug-treated.

For the calcium studies, animals were sacrificed by decapitation and the sarcotubular vesicles were prepared from whole rat diaphragm. Each diaphragm was placed in cold (4°) 0·32 M sucrose, cut into small pieces and homogenized with a Potter homogenizer and Teflon pestle. Homogenates were centrifuged at 4° at 1500 *g* for 10 min and the precipitate was discarded. The supernatant was centrifuged at 4° at 34,800 *g* for 30 min and the pellet was discarded. The supernatant was then centrifuged at 4° at 105,000 *g* for 30 min, and the precipitate consisting of light sarcotubular membrane fragments and vesicles was suspended in 0·32 M sucrose. The protein concentration of the preparation was approximately 0·9 mg/ml.

Deionized triple-distilled water was employed for all solutions. This is essentially calcium-free to less than 0·2 μM. Acid-washed glassware was employed where appropriate. Incubations for calcium uptake activity were carried out in the following medium: imidazole-histidine buffer, 30 mM, pH 6·85; KCl, 100 mM; ammonium oxalate, 5 mM; sodium azide, 5 mM; Mg-ATP, 4 or 5 mM; and $^{45}CaCl_2$, 5 μM. The total volume of incubation was 3 ml. Whenever parathion was included in an incubation, an equal amount of methanol was included in the control incubation. The incubations contained 0·3 ml of microsomal protein. The final microsomal protein concentration of the incubation solution was 0·15 to 0·20 mg/ml. The assays were started with the addition of ATP. Incubations were carried out at 8° in order to achieve a linear uptake of calcium over an extended time interval. The light microsomes sedimenting between 35,000 and 105,000 *g* had 40 per cent of the activity of heavy microsomes sedimenting between 12,000 and 35,000 *g*. This was also advantageous in slowing the reaction rate for easier measurements of calcium uptake. At 37° the uptake of calcium is virtually complete in 1 or 2 min.

The procedure employed for preincubation of microsomes with the tested compound was as follows. Microsomes were added to the incubation medium with ATP absent. The incubation was held at room temperature for 10 min and at 35° for 30 min. The temperature was then returned to 8° and the calcium uptake reaction was started by the addition of ATP. Controls were preincubated in similar fashion in the absence of the tested compound.

Calcium uptake was determined by taking 0·5-ml aliquots of the incubation and filtering through 0·45-μm membrane filters (Millipore Corp.). Measurements were made at 5, 10, 15 and 25 min.

Filters were prepared with a wash of 0·25 M KCl (2 ml) followed by water (10 ml). Samples were filtered with the aid of a vacuum apparatus and were washed with 0·25 M sucrose (2 ml). The filtration in general followed the procedure described by Palmer and Posey [20]. Filters were dried and radioactive calcium was determined by liquid scintillation spectrophometry in 2,5-diphenyloxazole (6 g/l.) in toluene. Less than 200 cpm appeared on the filters in the absence of microsomes as filter blanks. Microsomal calcium uptake in the presence of ATP typically reached 70,000 cpm.

Calcium-stimulated ATPase activity was assayed in an incubation medium identical to that employed for measuring calcium uptake except for the omission of oxalate. Baseline activity for the absence of calcium was measured with the addition of EGTA (0·1 mM). The reaction was carried out at 32° for 20 min. The incubation reaction was terminated with an equal volume of cold 5% TCA, and the liberated inorganic phosphate was measured by the procedure of Lowry and Lopez [21]. Protein content was measured by the procedure of Sutherland *et al.* [22] for all experiments.

## RESULTS

Figure 1 shows the calcium uptake of rat diaphragm microsomes in the presence of 5 mM Mg-ATP and oxalate as a trapping agent at 8°. There is a progressive, easily measured ATP-dependent, oxalate-dependent calcium uptake/mg of protein over

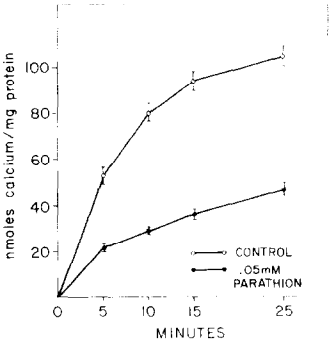


Fig. 1. Effect of parathion (0·05 mM) on ATP-dependent calcium uptake of rat skeletal muscle microsomes. The incubation mixture contained: imidazole histidine buffer (pH 6·85), 50 mM; KCl, 100 mM; ammonium oxalate, 5 mM; sodium azide, 5 mM; Mg-ATP, 5 mM; and $^{45}CaCl_2$, 5 μM. The incubation volume was 3 ml. Incubations were started with the addition of ATP. Results are mean ± S.E. for nine incubations. Control (○ ○); 0·05 mM parathion (● ●).

Table 1. Effect of parathion on ATP-dependent calcium uptake of rat skeletal muscle microsomes*

| Parathion (mM) | (nmoles/5 min/mg protein) | Per cent of control |
|---|---|---|
| 0·0 | 63·8 ± 3·7 | |
| 0·01 | 56·0 ± 2·2 | 87 |
| 0·02 | 31·3 ± 2·0 | 55 |
| 0·03 | 25·3 ± 1·4 | 40 |
| 0·04 | 20·1 ± 0·6 | 31 |
| 0·05 | 20·5 ± 1·0 | 32 |
| 0·15 | 21·5 ± 1·5 | 34 |

* The incubation is described in Table 1. The data represent mean ± S. E. for six experiments.

a 25-min period. In the absence of ATP, only 1 nmole calcium is taken up per mg of protein. In the absence of oxalate as a trapping agent, only 5 nmoles calcium/mg of protein is taken up. The addition of 0·05 mM parathion at the start of the incubation inhibits the calcium uptake approximately 60 per cent. The effect of increasing levels of parathion on the first 5 min of calcium uptake by rat diaphragm microsomes is shown in Table 1. There is increasing inhibition between 0·01 and 0·04 mM parathion. A maximal inhibition of more than 60 per cent is reached at 0·04 mM parathion. A further increase in the inhibitor level up to 0·15 mM failed to increase the inhibition. If the parathion is preincubated with the microsomes prior to addition of ATP, the inhibition of calcium uptake is further increased to 80 per cent; this is illustrated in Fig. 2. This level of inhibition is seen following preincubation with 0·05 mM parathion and with 1·5 mM parathion.

The stimulating effect of increasing levels of ATP on the initial calcium uptake rate of rat diaphragm microsomes is shown in Fig. 3. Parathion inhibition appears to be unchanged at all ATP levels tested. The stimulatory effect of increasing levels of calcium

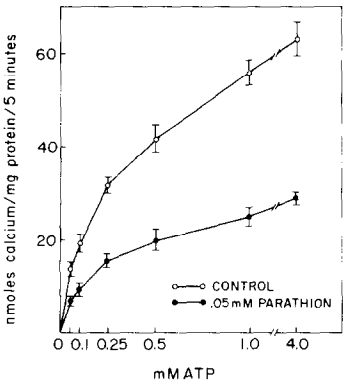


Fig. 3. Effect of increasing levels of ATP on parathion inhibition of ATP-dependent calcium uptake by rat skeletal muscle microsomes. The incubation is as described in Fig. 1 except for the ATP levels, which are indicated on the chart. Results are mean ± S. E. for six experiments. Control (∅—∅); 0·05 mM parathion (●—●).

on the initial rate of calcium uptake of rat diaphragm microsomes is illustrated in Fig. 4. At high calcium levels the parathion inhibition diminishes markedly. When these data are plotted in accordance with Michaelis–Menten kinetics, the parathion inhibition appears to be directly competitive with calcium.

ATP-dependent calcium uptake of skeletal muscle microsomes is associated with an extra splitting or hydrolysis of ATP in the presence of calcium. The effect of parathion on the calcium-induced ATP hydrolysis is presented in Table 2. In the presence of 20 $\mu$M calcium both at 32° and at 8°, there is a 70 per cent inhibition of the ATP splitting. In the presence of a high level of calcium (100 $\mu$M) this inhibition was markedly reduced just as the parathion effect on calcium uptake was reduced at high calcium levels.

Paraoxon and *p*-nitrophenol at levels of 0·05 mM had no detectable inhibitory effects on calcium uptake of rat diaphragm microsomes. At 1·5 mM, paraoxon

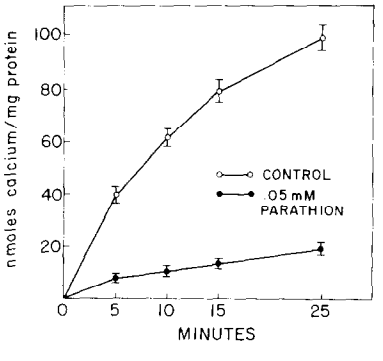


Fig. 2. Effect of preincubating parathion with microsomal protein on the ATP-dependent calcium uptake of rat skeletal muscle microsomes. Microsomes were added to the incubation medium (legend Fig. 1) with ATP absent. The incubation was held at room temperature for 10 min and at 35° for 30 min. The temperature was then returned to 8° and the reactions were started by the addition of ATP. Control microsomes were preincubated without the addition of parathion. The final concentration of parathion is 0·05 mM. Results are mean ± S. E. for nine experiments. Control (○—○); 0·05 mM parathion (●—●).

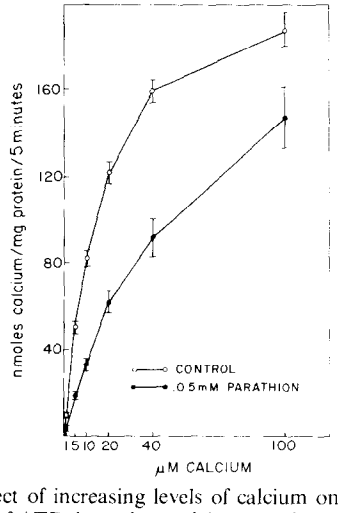


Fig. 4. Effect of increasing levels of calcium on parathion inhibition of ATP-dependent calcium uptake by rat skeletal muscle microsomes. The incubation is as described in Fig. 1 except for the calcium levels, which are indicated on the chart. Results are mean ± S. E. for six experiments. Control (○—○); 0·05 mM parathion (●—●).

Table 2. Effect of parathion on calcium-stimulated ATPase activity of rat skeletal muscle microsomes*

| Calcium ($\mu$M) | Temperature | Control ($\mu$moles Pi min mg protein) | $5 \times 10^{-5}$ M parathion | No. of experiments | Per cent inhibition |
|---|---|---|---|---|---|
| 20 | 8 | 0·014 ± 0·0007 | 0·004 ± 0·0006 | 8 | 70 |
| 20 | 32 | 0·14 ± 0·008 | 0·044 ± 0·016 | 6 | 68 |
| 100 | 32 | 0·21 ± 0·011 | 0·18 ± 0·01 | 5 | 14 |

* The incubation medium is the same as that in Fig. 1, except that oxalate is omitted and calcium levels are indicated in the table. Results are mean ± S. E.

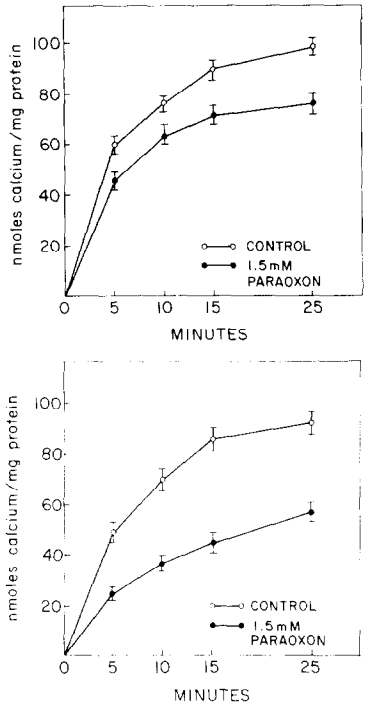


Fig. 5. Effect of paraoxon (1·5 mM) on ATP-dependent calcium uptake of rat skeletal muscle microsomes. The incubation is described in legend of Fig. 1, except that ATP is 4 mM. (A) Without preincubation of enzyme and paraoxon (11 experiments); (B) paraoxon preincubated with microsomes as described in legend of Fig. 2 (13 experiments). Results are mean ± S. E. Control (○ ○); paraoxon, 1·5 mM (● ●).

inhibited the ATP-dependent calcium uptake about 20 per cent as shown in Fig. 5A. When the paraoxon was preincubated with the microsomal vesicles for 30 min, the inhibition was markedly enhanced. The activity following paraoxon preincubation is 45 per cent of the control (Fig. 5B). *p*-Nitrophenol, 1·5 mM, is like paraoxon. This inhibition, however, is not further enhanced with additional preincubation of the microsomes and the compound.

A cholinesterase inhibitor (eserine) chemically unrelated to paraoxon was tested in the calcium uptake system. Addition of 1·5 mM eserine inhibits activity 15 per cent. If eserine and the microsomes are preincubated for 30 min, the inhibition of the calcium uptake is enhanced to 42 per cent. This is similar to the results obtained with paraoxon. Eserine, like paraoxon, requires a higher concentration for inhibition when compared to parathion.

Rat diaphragms were incubated in the presence of these agents and the effect on direct electrical stimulation of contraction was measured. A different property of the compounds was noted in these experiments. A time-dependent inhibition of muscle contraction elicited by direct stimulation of the rat diaphragm could be obtained with all the agents. Table 3 reveals that after 30 min paraoxon, *p*-nitrophenol and physostigmine inhibited the muscle contraction more than 50 per cent. Parathion was, however, relatively weak as an inhibitor. It only elicited 19 per cent inhibition. The apparent inhibition of contractions is tentatively interpreted as an effect similar to that of local anesthetics on ion fluxes across the cell membrane.

## DISCUSSION

Irreversible inhibitors of ChE such as parathion and paraoxon are known to produce pathologic changes in muscle [10, 23, 24]. The muscle directly beneath the subsynaptic fold has lost its cytoarchitectural organization and often contains large vacuoles, whereas the area away from the motor end-plate is relatively unaffected. The number of lesions produced is greatly reduced by prior nerve section or pretreatment with hemicholinium [10]. In addition to its inhibition of AChE, paraoxon produces a transient in-

Table 3. Inhibition of muscle contraction by parathion, paraoxon, *p*-nitrophenol and physostigmine

| Inhibitor | Time after drug addition (min) | | | | | |
|---|---|---|---|---|---|---|
| | 10 | 20 | 30 | 40 | 50 | 60 |
| Parathion ($10^{-3}$ M) | 7·99 ± 4·37* | 12·4 ± 5·30 | 18·7 ± 4·99 | 25·2 ± 6·38 | 28·4 ± 6·17 | 31·7 ± 6·71 |
| Paraoxon ($10^{-3}$ M) | 32·9 ± 5·33 | 49·0 ± 6·54 | 57·3 ± 5·88 | 65·1 ± 7·17 | 70·3 ± 5·72 | 73·7 ± 7·00 |
| Paranitrophenol ($5 \times 10^{-5}$ M) | 13·8 ± 2·34 | 35·0 ± 5·02 | 58·7 ± 3·70 | 75·5 ± 6·32 | 83·8 ± 5·75 | 86·8 ± 6·28 |
| Physostigmine $SO_4^{=}$ ($3 \times 10^{-4}$ M) | 30·4 ± 3·06 | 47·1 ± 2·29 | 54·3 ± 1·80 | 59·0 ± 5·23 | 62·6 ± 5·23 | 64·7 ± 4·68 |

* Per cent inhibition of muscle contraction. Values represent the mean of N = 5 ± S. E.

crease in ACh release from motor nerve terminals [11].

The present investigation relates to possible direct effects of parathion on rat skeletal muscle independent of the anticholinesterase action. Parathion *in vitro* inhibits the calcium uptake activity of rat diaphragm sarcoplasmic reticulum and the associated calcium-dependent ATPase activity. The inhibition of calcium uptake increases with the parathion concentration and becomes maximal with 0·04 mM parathion, resulting in a 60–70 per cent inhibition. Preincubating parathion and the sarcoplasmic reticular (microsomal) fraction increases the inhibition to 80 per cent of the original activity. The inhibition of the calcium pump and of calcium-dependent ATPase activity appears to be reversible with high levels of calcium. Paraoxon, the active metabolite of parathion, is a weaker inhibitor of the skeletal muscle sarcoplasmic reticular calcium uptake. At 1·5 mM it inhibits 24 per cent. Similar findings were obtained with the paraoxon metabolite *p*-nitrophenol and with the reversible cholinesterase inhibitor eserine. Eserine has been shown previously to inhibit calcium uptake of sarcoplasmic reticulum in lobster skeletal muscle [25]. Preincubation of rat skeletal muscle microsomes with 1·5 mM eserine or 1·5 mM paraoxon increases the inhibition to about 50 per cent.

In the British shore crab *Carcinus maenas*, inhibition of sarcoplasmic reticular calcium uptake by parathion may be the cause of a tonic contracture. This crustacean has noncholinergic neuromuscular transmission so that one can dissociate the anticholinesterase activity from other effects of parathion and paraoxon in the skeletal muscle. Within seconds of direct perfusion (0·05 mM parathion) of the claw closure muscle in *Carcinus* there is a strong irreversible tonic contracture [19]. This occurs even in the absence of potassium. The depolarizing effect of parathion, like that of quinine, is much slower and occurs over a period of 8–10 min. Parathion *in vitro* inhibits calcium uptake by sarcoplasmic reticulum of *Carcinus* [19]. It also potentiates superprecipitation of actomyosin [19]. These effects could well play a role in the observed contracture.

Caffeine is an inhibitor of calcium uptake *in vitro* in mammalian skeletal sarcoplasmic reticulum [26]. It has been shown to induce contracture in frog skeletal muscle but not in rat skeletal muscle unless it is first denervated [26, 27]. Parathion, the potent inhibitor of sarcoplasmic reticulum *in vitro*, also does not induce a contracture in the isolated rat diaphragm.

Parathion and its metabolites have an apparent local anesthetic-type action on electrically stimulated contraction of the isolated muscle and reduce the contractile response. The relatively weaker activity of parathion in this respect may reflect an additional action on the sarcoplasmic reticulum calcium uptake system. These results may not be totally in contradiction. Parathion is a potent calcium antagonist in the isolated sarcoplasmic reticulum. Parathion and its metabolites may, in blocking the contractile response of the electrically stimulated skeletal muscle, antagonize calcium channels of the excitable membrane.

It may be concluded that parathion has direct effects on calcium uptake of sarcoplasmic reticulum in rat skeletal muscle and in crab skeletal muscle that are unrelated to anticholinesterase activity. The magnitude of the effect on the sarcoplasmic reticulum *in vitro* is sizable and of potential significance even if complicated by anticholinesterase activity. The calcium uptake effect is much weaker with the parathion metabolite paraoxon. The anticholinesterase activity of paraoxon in turn is much greater. The present evidence would link the myopathy seen with parathion or paraoxon to the anticholinesterase activity rather than to its action on $Ca^{2+}$ uptake.

*Acknowledgements*—This study was supported by United States Public Health Service grants ES 00169 TOX. NS 10175 and HL 14681, and by a grant from the Muscular Dystrophy Association of America.

## REFERENCES

1. R. D. O'Brien, *Toxic Phosphorus Esters*, Vol. 1, p. 434. Academic Press, New York (1960).
2. G. B. Koelle, *Handbuch der Experimentellen Pharmakologie*, Vol. 15, p. 1220. Springer-Verlag, Berlin (1963).
3. J. C. Gage, *Biochem. J.* **54**, 426 (1953).
4. A. N. Davison, *Biochem. J.* **60**, 339 (1955).
5. K. P. DuBois, D. R. Thursh and S. D. Murphy, *J. Pharmac. exp. Ther.* **119**, 208 (1957).
6. J. L. Potter and R. D. O'Brien, *Science, N.Y.* **144**, 55 (1964).
7. R. B. March, T. R. Fukuto, R. L. Metcalf and M. G. Mason, *J. Econ. Ent.* **49**, 186 (1956).
8. T. R. Fukuto and R. L. Metcalf, *J. Agric. Fd Chem.* **4**, 930 (1956).
9. R. A. Neal, *Biochem. J.* **103**, 183 (1967).
10. G. M. Fenichel, W. B. Kibler, W. H. Olson and W-D. Dettbarn, *Archs Neurol.* **22**, 1026 (1972).
11. M. Laskowski and W-D. Dettbarn, *J. Pharmac. exp. Ther.*, **194**, 351 (1975).
12. F. M. Glasgoleva, E. A. Liberman and Z-Kh.-M. Khashaev, *Biofisika* **15**, 76 (1970).
13. J. F. Hubbard, *Physiol. Rev.* **53**, 674 (1973).
14. S. J. Bradbury, *Comp. Biochem. Physiol.* **44A**, 1021 (1973).
15. H. L. Atwood, *Am. Zoologist* **7**, 527 (1967).
16. A. Takeuchi and N. Takeuchi, *J. Physiol., Lond.* **183**, 418 (1966).
17. W. A. Morin and H. L. Atwood, *Comp. Biochem. Physiol.* **30**, 577 (1969).
18. R. Epstein and H. Grundfest, *J. gen. Physiol.* **56**, 33 (1970).
19. S. J. Bradbury, *Comp. Biochem. Physiol.* **44A**, 1033 (1973).
20. R. F. Palmer and V. A. Posey, *J. gen. Physiol.* **55**, 89 (1970).
21. O. H. Lowry and J. A. Lopez, *J. biol. Chem.* **162**, 421 (1946).
22. E. Sutherland, C. F. Cori, R. Haynes and N. Olsen, *J. biol. Chem.* **180**, 825 (1949).
23. A. Th. Ariens, E. Meeter, O. C. Walthuis and R. M. J. Van Bentham, *Experientia* **25**, 57 (1969).
24. M. Laskowski, W. Olson and W-D. Dettbarn, *Expl. Neurol.* **47**, 290 (1975).
25. W. G. Van der Kloot, *Comp. Biochem. Physiol.* **17**, 75 (1966).
26. A. Weber and R. Herz, *J. gen. Physiol.* **52**, 750 (1968).
27. E. Gutmann and A. Sandow, *Life Sci.* **4**, 1149 (1965).