*Biochemical Pharmacology*, Vol. 37, No. 17, pp. 3251–3256, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00


# EFFECT OF PARATHION AND METHYLPARATHION ON PROTEIN CONTENT OF CHICKEN EMBRYO MUSCLE *IN VIVO*

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(*Received* 2 *December* 1987; *accepted* 22 *February* 1988)

**Abstract**—Chicken eggs were treated with 0.4 per cent solutions of parathion or methylparathion for four or eight days, and the two-dimensional gel electrophoretic protein pattern of cervical muscles of eighteen days old embryos was analyzed. Both compounds significantly decreased the content of α-actinin, α-tubulin and β-tubulin after four days treatment, and, in addition, that of three other related proteins (γ-proteins) after eight days treatment. Under *in vitro* phosphorylating conditions, both methylparathion and parathion specifically inhibited the phosphorylation of one isoform of β-tubulin. Data suggest that the muscle-damaging effects of organophosphorous insecticides, such as parathion and its derivates, may be related to the decrease of tissue content of certain cytoskeletal proteins.

Acute treatment of experimental animals with high doses of organophosphates, potent inhibitors of acetylcholinesterase and other esterases, have been shown to lead to necrosis of skeletal muscle fibers along with symptoms of excessive cholinergic activity [1–7]. Recently, chronic exposure to low concentrations of organophosphates, which do not produce parasympathomimetic effects, was shown to cause similar deleterious effects in skeletal muscle [8]. Biochemical effects of organophosphates, unrelated to the inhibition of cholinergic system, were found in several laboratories [9–11]. Understanding of the biochemical basis of muscle damage is important since many agricultural workers are exposed to low, acutely non-symptom producing doses of organophosphate pesticides, such as parathion or methylparathion. In addition, above a certain level, pesticides are also harmful to warm-blooded animals.

Parathion [12], and presumably other closely related compounds as well, accumulates in biological membranes; therefore, one would expect that membrane-bound enzymes, other than acetylcholinesterase, will also be targets for their actions. Recently we showed significant effects of methylparathion on adenylate cyclase activity in homogenates of cervical muscles of chicken embryo [13]. Malformation of this muscle in response to insecticide treatment was previously reported [7]. In the present study we further investigated the possible biochemical basis for insecticide-related muscle damage. Since abnormal cell morphology reflects an altered organization of cytoskeleton [ref. 14, and additional references therein], possible quantitative changes of cytoskeletal proteins were given special attention. Phosphorylation of several cytoskeletal or cytoskeleton-associated proteins, accompanied by changes in protein function, have been reported [15–22]. For this reason we also studied the possible effect of insecticides on protein phosphorylation. Here we show that treatment of eggs with parathion or methylparathion decreased the amount of several cytoskeletal proteins.

## MATERIALS AND METHODS

*Materials.* Parathione, methylparathione and *Staphylococcus aureus* V8 protease were bought from Sigma. Ampholines (pH ranges of 3.5–10 and 5–7) were the product of LKB. [$\gamma$-$^{32}$P]ATP (1000 Ci/mmol) was prepared by the Isotope Institute of Biological Research Center (Szeged). All reagents for gel electrophoresis were from Serva. Chicken eggs of the Shaver Starcross 288 strain were used.

*Methods.* (i) Treatment of eggs. Eggs were incubated in a Ragus automatic incubator. On various days of incubation eggs were opened, 0.5 ml of 0.4% solutions of parathion or methylparathion were injected, and then closed with paraffin. The musculus complexus major (the major cervical muscle) was excised from the 18 days old embryos, homogenized in 10 vol of ice-cold 20 mM Tris–HCl buffer, pH 7.6, and proteins were precipitated by ice-cold acetone. Identical protein patterns were obtained if precipitation was performed with 10% trichloracetic acid; however, complete removal of the acid was tedious. (ii) Two-dimensional polyacrylamide gel electrophoresis. This procedure was performed essentially as described by O'Farrell [23]. Precipitated proteins were dissolved in 50 μl lysis buffer [24] and equal amounts of proteins (80 μg) were applied onto the first-dimension isoelectrofocusing gel (4.5% ampholine, pH 5–7, and 1.5% ampholine, pH 3.5–10). Second-dimension SDS gels were 10% in polyacrylamide. Proteins were stained with silver [25] or Coomassie blue. To quantitate the relative

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amounts of silver-stained proteins, gels were dried between two cellophane sheets and the appropriate proteins were scanned on a scanning densitometer (RFT Scanning Densitometer, Model 2950; Biomed Instruments Inc. Chicago, IL.).

(iii) Peptide mapping. Proteins, stained with Coomassie blue, were cut out from dried gels, placed on top of a second (15% polyacrylamide) gel and treated with *Staphylococcus aureus* V8 protease as described before [26] with slight modification of Cleveland's method [27]. Peptides were stained with silver.

(iv) Phosphorylation of muscle proteins *in vitro*. Phosphorylation of proteins was performed with [$\gamma$-$^{32}$P]ATP (50 $\mu$Ci/sample; 100 $\mu$M) essentially as described earlier [28]. The incubation mixture (0.1 ml) contained, in addition to ATP, 10 mM $MgCl_2$, 25 mM Tris–HCl, pH 7.6, and 80 $\mu$g muscle homogenate proteins. Incubations were performed for 5 min (during this period the ATP concentration fell below 10 $\mu$M), then terminated by adding 1 ml ice-cold acetone (the mixtures were kept at 0° for 10 min) and the samples were prepared for gel electrophoresis as described above. Phosphorylated proteins were detected by autoradiography as described previously [26, 28].

(v) Preparation of standard proteins. Skeletal $\alpha$-actinin was prepared by the method of Masaki and Takaiti [29], skeletal actin was prepared as described by Spudich and Watt [30], and tubulins were purified from brain [31].

(vi) Identification of muscle proteins. Proteins were identified by corunning muscle samples with the individual proteins and by comparing their peptide maps generated by V8 protease. Actinin, tubulins and actin from the embryonic muscle (present work) are clearly in very similar positions on the two-dimensional gels as those in other muscles [32], kidney cells [33] or granulosa cells [34]. In all cases, protein was determined by the method of Lowry *et al.* [35].

Statistical significance of data was calculated by Student's *t*-test.

## RESULTS

As shown in Fig. 1(a) $\alpha$-actinin, tubulins, actin (indicated as "A") and three presently unidentified proteins, designated as $\gamma_1$, $\gamma_2$ and $\gamma_3$ are among the major proteins of cervical muscle of untreated embryo. Both $\alpha$-actinin [32, 34, 36] and $\beta$-tubulin

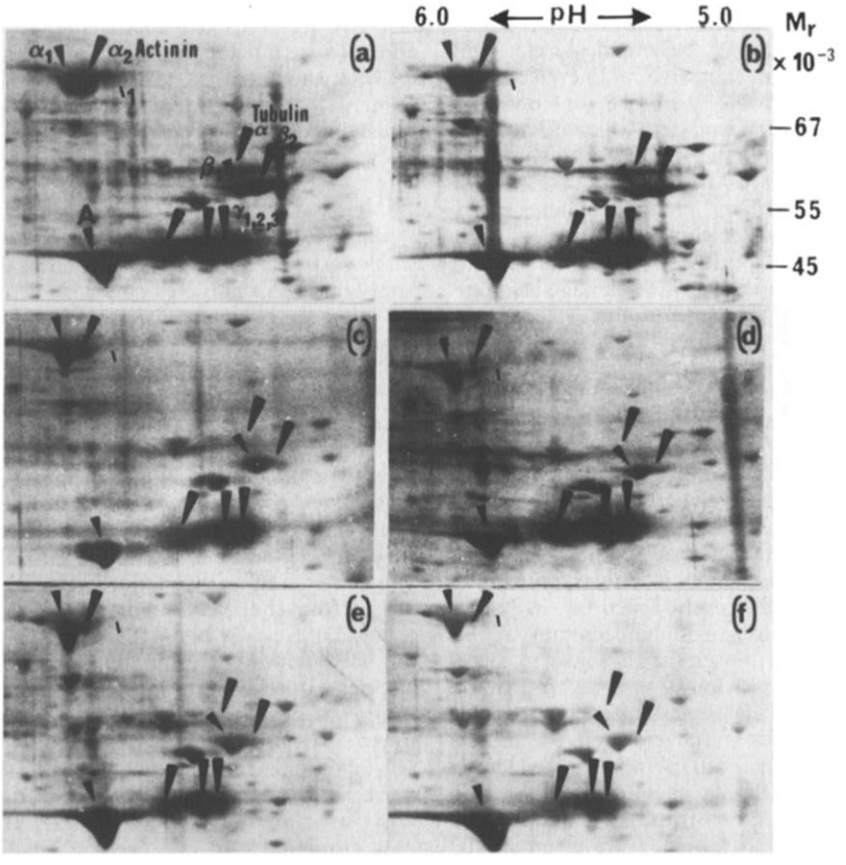


Fig. 1. Effects of parathion and methylparathion on protein content of cervical muscle. Proteins were derived from m. complexus major of 18-day old embryos. For each determination, muscle samples were pooled from three embryos. Eggs were untreated (a) or treated with 0.5 ml water for 8 days (b), or with 0.5 ml of 0.4% solution of methylparathion for 4 (c) or 8 days (e), or with 0.5 ml of 0.4% solution of parathion for 4 (d) or 8 days (f). Similar results were obtained in two other experiments. A is actin.

[37–42] show microheterogeneity in various muscle tissues. When less than 40 μg cervical muscle proteins were applied on gels, both α-actinin and β-tubulin were clearly resolved into two spots. The less acidic spots were designated as $\alpha_1$-actinin or $\beta_1$-tubulin while the more acidic spots were designated as $\alpha_2$-actinin or $\beta_2$-tubulin, respectively, merely to indicate the heterogeneity of these proteins. The peptide maps of $\alpha_1$- and $\alpha_2$-actinins, generated with V8 protease, were virtually identical (Fig. 2). Similarly, the peptide maps of $\beta_1$- and $\beta_2$-tubulins were very similar and showed only partial overlap with that of α-tubulin. Each γ protein contained the same major peptides, indicating their relatedness. The peptide maps of γ proteins, however, were different from that of actin (Fig. 2).

When compared to untreated embryos [Fig. 1(a)], treatment of eggs with water alone for eight days [Fig. 1(b)] did not cause detectable changes in muscle protein pattern. Compared to either of these controls, treatment of eggs with 0.4% solutions of methylparathion [Fig. 1(c)] or parathion [Fig. 1(d)] during the last four days of the incubation (eighteen days) period caused significant reduction in the cellular content of α-actinin (32–41%), α-tubulin (90–95%) and β-tubulin (42–65%), as determined by densitometry in three different experiments. It is noteworthy that the amount of actin remained unaltered after the above treatments. For this reason, actin was used as an internal standard to verify the above changes and those which follow. When treatment of eggs with methylparathion [Fig. 1(e)] or parathion [Fig. 1(f)] was extended for the last eight days of incubation, we observed significant reduction in the content of muscular γ proteins as well, while the amount of α-actinin and tubulins remained low compared to control embryonal tissue (data are summarized in Table 1). We should note here that the relatively poor resolution of various forms of α-actinin, β-tubulin and γ proteins did not allow their separate quantitation. However, visual comparison of various gels, guided by surrounding reference proteins with constant position, suggests that mostly $\alpha_2$-actinin and $\beta_2$-tubulin were decreased in response to insecticide treatment. We are presently investigating this possibility.

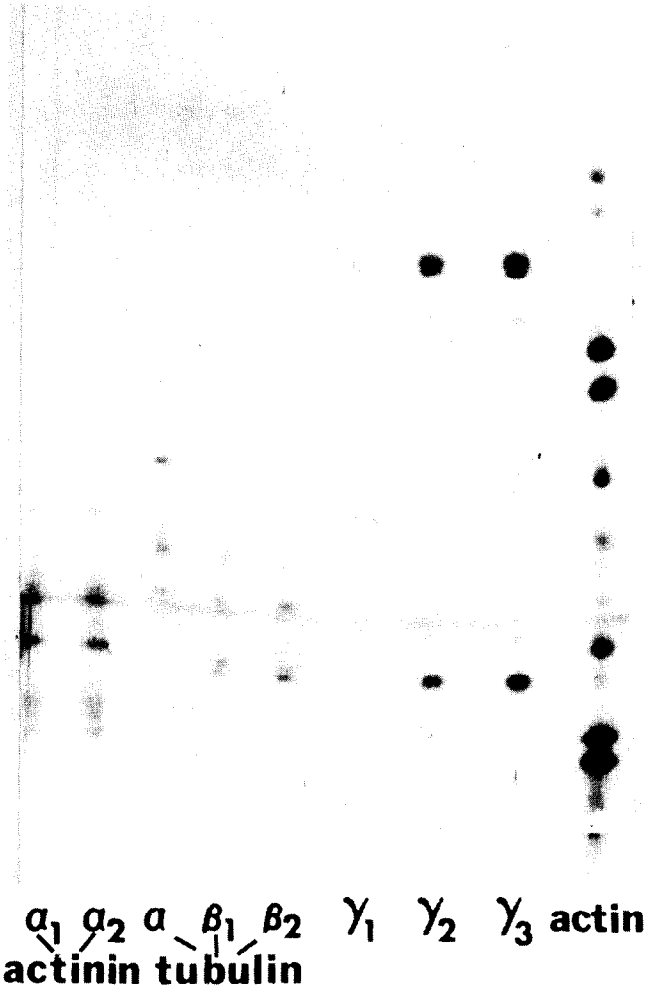


Fig. 2. Peptide map of muscle proteins. Excised gel pieces, placed on top of a second gel, were overlayed with 1 ml 0.9% (w/v) agarose containing 5 μg *Staphylococcus aureus* V8 proteinase. The time left for digestion in stacking gel (4% acrylamide) was 30 min.

Table 1. Effect of parathion and methylparathion on protein content of chicken embryo muscle

| Treatment of eggs | $\alpha$-actinin | $\alpha$-tubulin | $\beta$-tubulin | $\gamma$-proteins |
|---|---|---|---|---|
| | | (% of control) | | |
| Untreated (control) | 100 ± 6 | 100 ± 11 | 100 ± 8 | 100 ± 13 |
| Parathion | 52 ± 9 | <5 | 32 ± 3 | 47 ± 10 |
| Methylparathion | 63 ± 4 | <5 | 49 ± 11 | 35 ± 6 |

Eggs were treated with 0.5 ml of 0.4% solutions of parathion and methylparathion for the last eight days of incubation. Muscle proteins were separated by two-dimenstional gel electrophoresis and their relative amounts determined by densitometry as described in Methods. Changes in protein content are expressed as % of control values (100%). Results are mean ± SE of three experiments. All changes shown were significant ($P < 0.05$).

As it is shown in Fig. 3(a), $\beta_1$ and $\beta_2$-tubulins were phosphorylated under *in-vitro* phosphorylating conditions which is in accordance with data in the literature that they are phosphoproteins [43–45]. Phosphorylation of $\beta_2$-tubulin was significantly inhibited (44%) while that of $\beta_1$-tubulin was unaffected by 0.1 mM methylparathion [Fig. 3(b)], added to the protein phosphorylation assay. $\gamma$ proteins were also well labelled with [$^{33}$P]; their phosphorylation, however, was not affected by methylparathion. Finally, $\alpha$-actinin was not labelled with [$^{32}$P]. Methylparathion, beside inhibiting the phosphorylation of $\beta_2$-tubulin, also inhibited the [$^{32}$P]labelling of protein 2, and stimulated that of

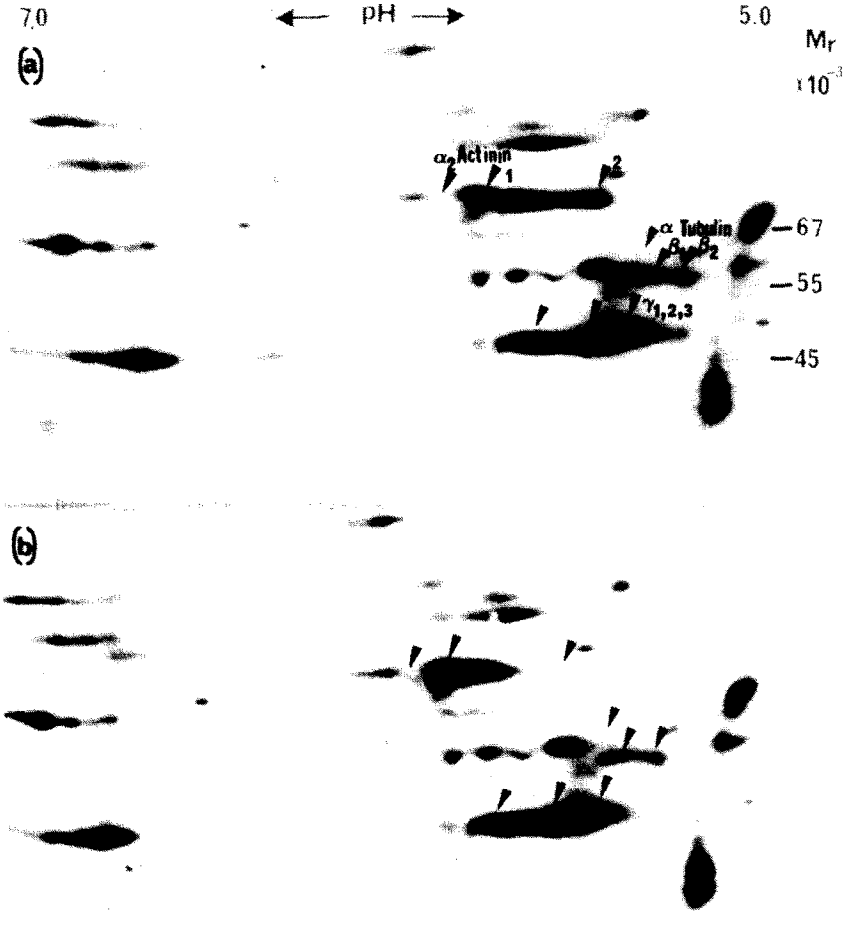


Fig. 3. Effect of methylparathion on the phosphorylation of muscle proteins. Proteins of cervical muscle homogenates were phosphorylated as described in the Methods in the absence (a) or presence of 0.1 mM methylparathion (b). Phosphoproteins ($2.3 \times 10^5$ acid-precipitable cpm were applied) were subjected to two-dimensional gel electrophoresis and, after staining with silver, gels were dried and exposed for autoradiography for 6 days.

protein 1. It remains to be established how proteins 1 and 2 are related to each other and to $\alpha$-actinin. Parathion mimicked the effect of methylparathion on protein phosphorylation in every respect (data not shown). It is noteworthy that only protein 1, but not protein 2, could be detected (in close vicinity of $\alpha_2$-actinin) by silver staining in the embryonal muscle [Fig. 1(a)]. Treatment of eggs with the above insecticides resulted in the disappearance of protein 1 [Figs 1(c)–(f)].

## DISCUSSION

Our experiments demonstrated that prolonged treatment of eggs with organophosphorous insecticides caused substantial decrease in the amount of several muscle proteins. Taking into account the important roles of $\alpha$-actinin and tubulins in the physiology of muscle, it is reasonable to assume that the presently described phenomenon may contribute to the deleterious effects of parathion and related compounds. The mechanism(s) of effect of insecticides on protein metabolism and its possible relationship to the activation of cholinergic system remains to be established. An inhibition of protein synthesis is unlikely, since it would equally affect the different forms of actinin and $\beta$-tubulin. A selective activation of protein degradation appears more likely. Post-translational modifications, which are probably responsible for the apparent microheterogeneity of these proteins, may render the molecules more susceptible for proteolytic attack. Such possibility is indicated by recent data in the literature [46–48] that active oxygen species, which induce charge changes in protein molecule, can potentiate proteolysis. Phosphorylation, a widely occurring post-translational regulatory process, causes similar charge changes in proteins. We are presently investigating the possibility that the phosphorylation state of $\beta$-tubulin and protein 1, apparently modified by insecticides, may affect the rate of their proteolytic degradation.

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