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BIOCHEMICAL ALTERATIONS IN SKELETAL MUSCLE INDUCED BY 2,4-DICHLOROPHENOXYACETIC BUTYL ESTER DURING CHICK EMBRYONIC DEVELOPMENT

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Abstract—2,4-Dichlorophenoxyacetic butyl ester (2,4-D b.e.) (3.1 mg/egg) was applied on fertile hen eggs before starting the incubation. Chicks hatched from treated eggs showed motor dysfunctions, postural troubles and edematous muscles. The electromyography revealed muscular weakness, prolonged motor distal latency, and myotonia. The biochemical composition of leg and complexus muscles from 1-day-old chicks was determined. A significant diminution (24%) in the glycogen level of leg muscles was produced by the treatment. There was a small increase (15%) in sarcoplasmic proteins from leg muscles and an increase of a 20 kD protein in the myofibrillar proteins from complexus muscles. Even though total lipid content was not changed, 2,4-D b.e. treatment produced a diminution of sterol esters (20%) and phosphatidylcholine (11%) and an increase of phosphatidylserine (61%), triglycerides (37%) and free fatty acids (FFA) (448%) in leg muscles. Increases of phosphatidylethanolamine (16%), sterols (58%) and FFA (267%) were detected in complexus muscles. A remarkable increase (700–1500%) of unsaturated FFA, e.g. oleic, linoleic and arachidonic acids, was observed. Considering the avian embryo lipid metabolism, it is proposed that FFA and triglycerides were accumulated because they could not be metabolized in the mitochondria. Since FFA are potent cytotoxic compounds, their increase may be a key factor in the 2,4-D b.e. toxic action in muscle and other tissues during embryonic development.

2,4-Dichlorophenoxyacetic acid (2,4-D‡), its esters and its salts constitute a widely used family of herbicides. 2,4-Dichlorophenoxyacetic butyl ester (2,4-D b.e.) is another weed-killer of this group; it is used more than 2,4-D in agriculture, and they have very different chemical properties [1]. 2,4-D, as other substituted aromatic acids, produces myotonia in laboratory animals within a very short time after the treatment [2–4]. Chronic treatment of adult mammals with high 2,4-D doses results in a severe myopathy accompanied by significant biochemical changes [5–14].

Previous studies from our laboratory have indicated that external application of 2,4-D b.e. on fertile hen eggs before starting incubation is fetotoxic, producing a decrease in hatching in a dose-dependent manner [15, 16]. A lethal $dose_{50}$ of 5 mg 2,4-D b.e./egg was determined in this experimental system. 2,4-D b.e. has been detected in these embryos since day 10 of incubation (17.6 ppm after the application of 3.1 mg/egg) [17]. It was also found in different tissues, including muscle (leg muscles: 28 ± 8 μg/g wet weight; complexus muscles: 51 ± 21 μg/g wet weight), of 1-day-old chicks hatched from treated eggs [17]. 2,4-D, as a metabolic product of 2,4-D b.e., was not detected in embryos at any time during the incubation period or in 1-day-old chicks [17]. Chicks hatched from 2,4-D b.e. treated eggs show dose-dependent hypomyelination, motor dysfunctions and postural troubles [15, 16, 18–20]. These chicks also had trouble in standing upright [15, 16], and they could not pass the "flapping test." This test examines the ability of chicks to right themselves on a flat surface when laid on their backs. These facts suggested that 2,4-D b.e. may produce muscular alterations during embryonic development.

Studies concerning the action of 2,4-D b.e. on skeletal muscle or reports about the effects of phenoxyherbicides on skeletal muscles during embryonic development have not been published. The purpose of this work was to characterize some of the biochemical changes in the chick muscle induced by 2,4-D b.e. during embryonic development, looking for clues that help to clarify the onset of the probable myopathy and the 2,4-D b.e. action mechanism. Preliminary electromyographic studies were also performed to verify the functional alteration of the muscle.

MATERIALS AND METHODS

Fertile hen eggs were obtained from a commercial hatchery. They were treated externally with an ether solution of pure 2,4-D b.e. corresponding to 3.1 mg/egg as previously described [15, 16]. Control eggs were treated with ether. After drying, the eggs were

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‡ Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-D b.e., 2,4-dichlorophenoxyacetic butyl ester; EMG, electromyography; FFA, free fatty acids; 14:0, miristic acid; 16:0, palmitic acid; 16:1, palmitolcic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:4, arachidonic acid; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

placed in an incubator at 38° and 60% relative humidity. They were rotated twice a day.

To study the electromyographic activity, unanesthetized 1-day-old chicks were restrained with tape, and a ground wire was attached to a leg. A concentric needle electrode was inserted into the test muscle: ilio tibial lateral or complexus muscles. Electromyography (EMG) output was connected to an audio speaker as well as to an oscilloscope, and data were recorded on tape for later review. Myotonic discharges were recorded in several sites after needle movements or percussion of muscle tendon.

Twenty-four hours after hatching, chicks were decapitated, and the leg and complexus muscles were excised, cleaned of fat and connective tissue, and weighed. The muscles were homogenized in a Virtis homogenizer with 5 vol. of distilled water at 0–2° and lyophilized; next dry weight was determined.

Part of the tissue was immediately frozen in liquid nitrogen for glycogen and lipid determinations. Glycogen was determined by the method of Hassid and Abraham [21]. Lipids were extracted from lyophilized muscles with 20 vol. (over wet weight) of chloroform–methanol–water (60:30:4.5; by vol.). The extracts were filtered and partitioned with 0.2 vol. of distilled water as described by Folch-Pi *et al.* [22]. The chloroform phases were dried under a stream of nitrogen and weighed. These total lipid extracts were resuspended in chloroform–methanol (2:1; v/v), and aliquots were taken for the different lipid determinations.

Phospholipid phosphorus was determined according to the method Dodge and Phillips [23]. Phospholipid classes were isolated by two-dimensional thin-layer chromatography on silica gel H/Florisil (9:1; w/w). Solvent systems were chloroform–methanol–concentrated NH_4OH (70:25:5; by vol.) and chloroform–methanol–glacial acetic acid–water (80:10:2:0.75; by vol.). Spots were visualized with iodine vapors and isolated by scraping off the silica; then phosphorus was measured as described above.

Phospholipids, free sterols, free fatty acids (FFA), triglycerides and sterol esters were separated by one-dimensional thin-layer chromatography on silica gel G. The developing solvent system was *n*-hexane–diethyl ether–glacial acetic acid (80:20:1, by vol.). A mixture of standards was run in each plate, and their positions were detected by exposure to iodine vapors. Free sterols and sterol esters were determined by the method of Searcy *et al.* [24]. Triglycerides were measured by a modification of the method of Flecker [25], using 0.2 M $NaAsO_3$ to eliminate the oxidant excess.

Fatty acids were determined by gas–liquid chromatography (GLC). Total lipid extracts were separated by one-dimensional thin-layer chromatography as described above. The standards were detected by iodine vapors while the samples were coated with a glass in order to protect the unsaturated fatty acids. Then, after comparing the samples with the standards, they were scraped from the plates into ampoules. Five milliliters of 5% H_2SO_4-95% methanol was added to the samples for transesterification of the fatty acids. The ampoules were closed under N_2 and placed in a 75° water bath for 12 hr [26]. The resulting fatty acid methyl esters were extracted with *n*-hexane–water (2:1; v/v) and concentrated. GLC was performed on a Varian 2100 glass chromatographer, equipped with a dual flame ionization detector, using a glass column packed with 15% diethylene glycol succinate polymers on a 100/200 mesh Gas-Chrom P solid support, and it was operated under 4.5 kg/cm^2 of N_2 pressure. Temperatures used were: injector, 220°; column oven, 195°; detector, 220°. The fatty acid methyl esters were identified by comparison of their retention times with those of standards. Other fatty acid methyl esters were identified by extrapolation from the standards assuming a logarithmic relationship between retention time and fatty acid chain length for homologous series. Quantitative determinations of fatty acids were performed using heneicosanoic (21:0) fatty acid methyl ester as internal standard and the areas evaluated by half-height analysis of the peaks of gas chromatograms.

The myofibrillar, sarcoplasmic and insoluble protein fractions were separated for quantification. Muscles were homogenized with 10 vol. of distilled water for 90 sec in a Virtis homogenizer and the homogenates centrifuged for 15 min at 10,000 *g* in a refrigerated centrifuge. Sarcoplasmic (water soluble) proteins were measured in aliquots taken from the supernatants. Pellets were extracted with 10 vol. of 0.6 M KCl, 3 mM $NaCO_3H$, pH 7.0 (20°), and the extracts centrifuged for 15 min at 10,000 *g*. Myofibrillar proteins were measured in the supernatants, while insoluble proteins were determined in the extracts obtained by solubilizing the pellets with 10 vol. of 0.5 M NaOH at 100° for 15 min. Whole muscles were solubilized in 0.5 M NaOH at 100° for 15 min for total protein determinations. All protein determinations were carried out by the method of Lowry *et al.* [27]. SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) was performed as described by Laemmli [28].

Data were evaluated for statistical differences by Student's *t*-test.

Standards were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Standards used in thin-layer chromatography were a mixture of cholesterol, methyl oleate, oleic acid (18:1) and triolein. Standards used in GLC were the methyl esters of palmitic (16:0), stearic (18:0), oleic (18:1), linolenic (18:3) and arachidonic (20:4) acids. 2,4-D b.e. (96%) was obtained from Atanor, Buenos Aires, Argentina, and its purity was checked by GLC [17]. Other chemicals used were analytical grade.

RESULTS

Ilio tibial lateral and complexus muscles of chicks hatched from 2,4-D b.e. treated eggs showed similar EMG alterations. Figure 1 shows the EMG data obtained from ilio tibial lateral muscle in a typical experiment; the same results were found in tests performed in five other independent cases. A decrease in the motor potential amplitude of spontaneous activity was observed in the 2,4-D b.e. treated group (Fig. 1B), suggesting muscular weakness. A prolonged distal motor latency was also detected (Fig. 1D); this indicates a slow response of

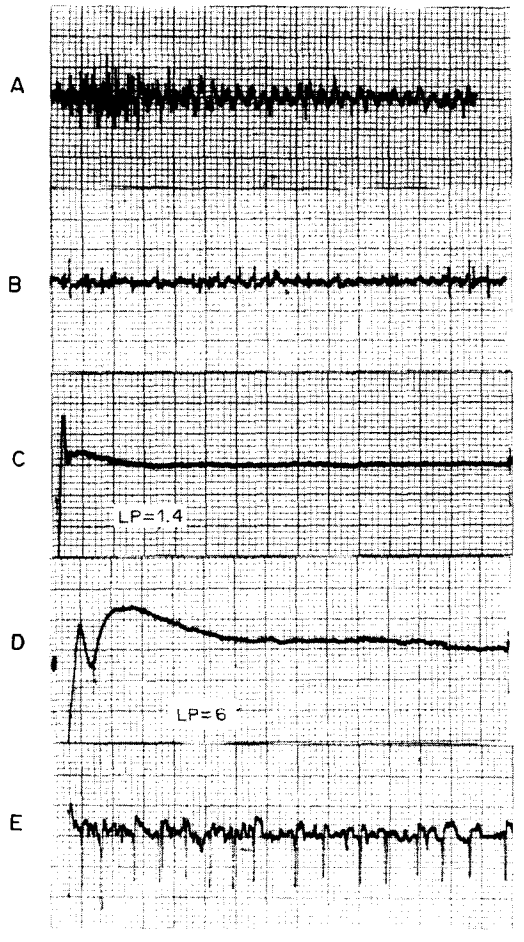

Fig. 1. EMG recorded from ilio tibial lateral muscles of 1-day-old chicks hatched from 2,4-dichlorophenoxyacetic butyl ester (2,4-D b.e.) treated eggs. Spontaneous activity of control (A) and 2,4-D b.e. treated chick (B) (calibration: 1 mV, 200 msec). Distal motor latency of control (1.4 msec) (C) and 2,4-D b.e. treated chick (6 msec) (D). Myotonic discharges after tendon percussion of a 2,4-D b.e. treated chick (E).

the muscle to a stimulus. It is known that phenoxycarboxylic acids produce myotonia in adult rats [2–4]. The repetitive spiking characteristic of myotonia was also observed in this system. Figure 1E shows the myotonic discharges detected in the ilio tibial lateral muscles of the 2,4-D b.e. treated group after tendon percussion. All of these EMG effects indicate that the 2,4-D b.e. produces, during embryonic development, functional alterations in skeletal muscle.

Muscles from chicks hatched from 2,4-D b.e. treated eggs appeared edematous (mainly the complexus muscles). The external edema was removed for all the determinations. Increases in the wet weights of both complexus and leg muscles were observed (Table 1). Furthermore, dry weight was also increased in complexus muscles from the 2,4-D b.e. treated group. No alterations were found in total protein or in total lipid contents, but a significant difference (−24%) was detected in the glycogen level in leg muscles of the 2,4-D b.e. treated group (Table 1). To study the muscular protein composition, three fractions were extracted: myofibrillar, sarcoplasmic and insoluble proteins. Table 1 shows that myofibrillar and insoluble protein contents remained unchanged in both of the muscles studied, while sarcoplasmic protein content had increased 15% in leg muscles. Myofibrillar and sarcoplasmic proteins were analyzed by SDS–PAGE. Figure 2 shows that there were no important variations in the myofibrillar protein pattern from leg muscles. However, the increase of a 20,000 dalton band in complexus muscles of the 2,4-D b.e. treated group was observed. No alterations were found in sarcoplasmic protein SDS–PAGE (not shown).

Even though total lipids were not altered, the lipid composition of muscles from chicks hatched from 2,4-D b.e. treated eggs revealed important changes (Table 2). There was an increase of triglycerides (37%) and a diminution of sterol esters (20%) in leg muscles. We realize that this increase in triglycerides should be compensated for by an equivalent diminution in other lipids. However, we have not been able to detect such a diminution. This discrepancy may be due to the accumulative error in the separations and determinations of each lipid class. However, the total lipid recovery (assuming the following average molecular weights: phospholipids: 750; sterol esters: 650; sterols: 380) did not indicate significant differences between the control ($79 \pm 14\%$) and the treated ($98 \pm 10\%$) set of leg muscle samples. In complexus muscles, the content of free sterols was augmented significantly (58%). Consequently, the ratio of sterols/phospholipids was increased in the complexus muscles (1.37 ± 0.03 in control vs 1.67 ± 0.07 in the 2,4-D b.e. treated group; $P < 0.0025$). The reference to sterol, instead of cholesterol, is due to the fact that 2,4-D inhibits the synthesis of cholesterol in fibroblasts inducing an accumulation of demosterol [29]. The method used in this work [24] measures sterol, and no determinations were performed to determine if cholesterol synthesis was inhibited in this experimental system. The biggest change in both complexus and leg muscles was the increase in FFA contents, 267 and 448% respectively. These increases acquire relevance not only because of their magnitude, but also because of the deleterious actions that FFA have on the cell structure [30–34].

The pattern of phospholipids, expressed as a percentage of total lipid phosphorus, showed a 61% increase of phosphatidylserine accompanied by a slight decrease of phosphatidylcholine (11%) in leg muscle of treated chicks (Table 3). Likewise, phosphatidylethanolamine from complexus muscles was increased 16%. No important differences were observed in the fatty acid pattern of total phospholipids (not shown).

Triglyceride fatty acids represented the largest pool of fatty acids. In complexus muscles 18:1 fatty acid was augmented 71% and in leg muscles 16:0, 18:0 and 18:1 fatty acids increased 71, 52 and 53% respectively (Table 4). These results are in accord and support the data obtained by the spectrophotometric determination of triglycerides (Table 2). On the other hand, complexus muscles had significant increases in both chain length longer than

Table 1. Wet weight, dry weight, glycogen, total lipid and protein determinations in muscles from 1-day-old chicks hatched from 2,4-D b.e. treated eggs*

	Leg muscle		Complexus muscle	
	Control	2,4-D b.e.	Control	2,4-D b.e.
Wet weight	0.82 ± 0.01†	0.88 ± 0.01‡	0.35 ± 0.01	0.42 ± 0.02§
Dry weight	0.156 ± 0.004	0.168 ± 0.005	0.046 ± 0.002	0.055 ± 0.002‖
Glycogen	0.68 ± 0.04	0.52 ± 0.04‡	0.15 ± 0.03	0.16 ± 0.03
Total lipid	29 ± 2	31 ± 1	21 ± 2	16 ± 3
Total proteins	126 ± 6	127 ± 4	119 ± 4	126 ± 7
Myofibrillar	36 ± 2	36 ± 2	29 ± 1	32 ± 2
Sarcoplasmic	26 ± 2	30 ± 1‡	26 ± 1	28 ± 1
Insoluble	63 ± 2	62 ± 2	63 ± 4	67 ± 5

* Units for variables are as follows: wet and dry weight, g; all other determinations are given in mg/g wet weight.

† Mean ± SE, N = 15. Abbreviation: 2,4-D b.e. = 2,4-dichlorophenoxyacetic butyl ester.

‡–‖ P values: ‡$P < 0.05$, §$P < 0.025$, and ‖$P < 0.0025$.

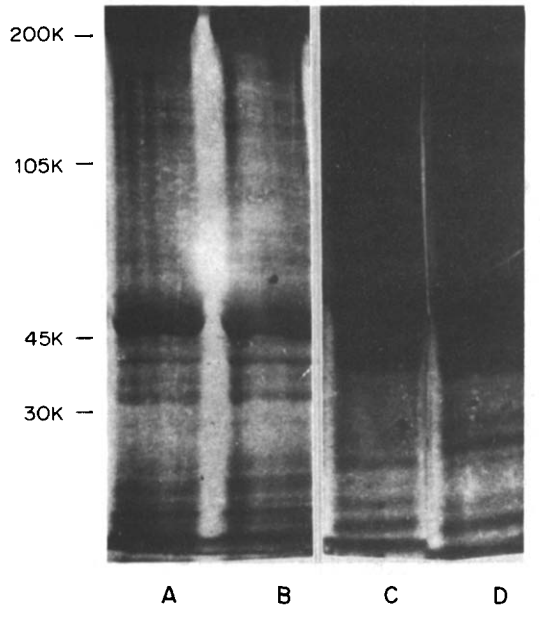

Fig. 2. SDS–PAGE of myofibrillar proteins from muscles of 1-day-old chicks hatched from 2,4-D b.e. treated eggs. Leg muscles of control (A) and 2,4-D b.e. treated chick (B). Complexus muscles of control (C) and 2,4-D b.e. treated chick (D). A higher amount of protein (150 μg) was run in lanes C and D in order to point out the increase of a 20 kD band.

18 C atoms/shorter than 18 C atoms and unsaturated/saturated ratios, whereas no variations were observed in these ratios in leg muscles.

High increases were found in almost all FFA in the two muscles studied (Table 5). The increases were higher in 18:1, linoleic (18:2) and 20:4 fatty acids; these long unsaturated fatty acids were augmented between 700 and 1500%, whereas total FFA increased 260–450% (Tables 2 and 5). Consequently, chain length longer than 18 C atoms/shorter than 18 C atoms and unsaturated/saturated ratios were enlarged.

DISCUSSION

Results from the present study demonstrate that 2,4-D b.e., applied on fertile hen eggs before incubation, produces important biochemical and electrophysiological alterations in skeletal muscles of chicks hatched from treated eggs.

To study the effects of 2,4-D b.e. on skeletal muscle, we chose a dose of 3.1 mg/egg. This dose [which is under the lethal $dose_{50}$ (5 mg/egg) [16]] produces significant motor disfunctions in the system under study. Furthermore, at this dose, the levels of 2,4-D b.e. in the muscles of 1-day-old chicks (leg muscles: 28 ± 8 μg/g wet weight; complexus muscles: 51 ± 21 μg/g wet weight) are similar to those detected in other systems in which phenoxyherbicides (2,4-D or 2,4-D b.e.) [17] produce different toxicological effects [5–14]. For instance Danon *et al.* [12] detected 40 μg 2,4-D/g wet weight in rat muscles after doses of 200 mg/kg/day. Comparable values would be expected in most of the studies with phenoxyherbicides which use similar doses and systems (although they did not perform 2,4-D determinations) [5–14]. The indicated 2,4-D b.e. levels in our experimental system would give tissue concentrations of roughly 0.14 to 0.25 mM, which are also in the same range as the concentrations used in most of the *in vitro* studies performed with phenoxyherbicides [3, 4, 35–38]. Consequently, the use of this dose allows the comparison of our studies with others in the field.

The muscular weakness, prolonged motor distal latency, and myotonia (Fig. 1) observed in the 2,4-D b.e. treated group indicate functional alterations after muscle development in the presence of 2,4-D b.e. The myotonic syndrome may be produced by different causes. It is known that myotonia induced by phenoxycarboxylic acid (i.e. 2,4-D), or other substituted aromatic acids, is a short-time consequence of the presence of these compounds in the sarcolemma [3, 4]. However, myotonia may also be produced by a specific biochemical change in sarcolemma composition such as that induced by 20,25-diazocholesterol [39]. As 2,4-D was not detected in these chicks [17], it would seem clear that it is not

Table 2. Lipid composition of muscles from 1-day-old chicks hatched from 2,4-D b.e. treated eggs

	Leg muscle		Complexus muscle	
	Control	2,4-D b.e.	Control	2,4-D b.e.
Triglycerides (mg/g lipids)	454 ± 91*	622 ± 72†	241 ± 28	287 ± 31
Free sterols (μmol/g lipids)	123 ± 2	147 ± 12	181 ± 32	287 ± 25†
Sterol esters (μmol/g lipids)	84 ± 2	67 ± 4‡	161 ± 25	190 ± 16
Phospholipids (μmol/g lipids)	292 ± 26	312 ± 20	313 ± 29	315 ± 40
Free fatty acids (mg/g lipids)	7.1 ± 1.0	38.9 ± 4.1§	11.4 ± 2.8	41.9 ± 4.9§

* Mean ± SE, N = 5.
†–§ P values: †$P < 0.05$; ‡$P < 0.025$, and §$P < 0.0005$.

Table 3. Phospholipid composition of muscles from 1-day-old chicks hatched from 2.4-D b.e. treated eggs

	Percentage of total lipid phosphorus			
	Leg muscle		Complexus muscle	
	Control	2,4-D b.e.	Control	2,4-D b.e.
Phosphatidylserine	8.9 ± 0.4*	14.3 ± 3.0†	7.9 ± 1.4	6.7 ± 1.0
Phosphatidylinositol + sphingomyelin	11.9 ± 15.5	14.0 ± 1.8	16.3 ± 3.0	15.4 ± 0.9
Phosphatidylcholine	45.8 ± 1.4	40.7 ± 1.9‡	53.4 ± 2.5	50.7 ± 0.4
Phosphatidylethanolamine	24.1 ± 1.0	29.6 ± 3.0	22.1 ± 1.1	25.7 ± 1.3‡
Unidentified	3.1 ± 0.6	1.2 ± 0.7	0.8 ± 0.4	ND§

* Mean ± SE, N = 4.
†, ‡ P values: †$P < 0.025$, and ‡$P < 0.05$.
§ ND: not detected.

Table 4. Fatty acid composition of triglycerides of muscles from 1-day-old chicks hatched from 2,4-D b.e. treated eggs

	Fatty acids (mg/g total lipids)			
	Leg muscle		Complexus muscle	
Fatty acid	Control	2,4-D b.e.	Control	2,4-D b.e.
14:0	1.7 ± 0.5*	2.3 ± 0.3	3.9 ± 0.7	1.6 ± 1.0
16:0	91.9 ± 8.9	156.9 ± 12.7†	66.8 ± 11.8	84.1 ± 20.3
16:1	17.3 ± 6.5	15.8 ± 1.7	7.1 ± 1.8	8.4 ± 2.1
18:0	20.8 ± 2.4	31.5 ± 5.3‡	24.1 ± 6.0	20.9 ± 5.9
18:1	134.0 ± 11.6	205.6 ± 31.5‡	74.9 ± 13.0	127.9 ± 28.6‡
18:2	51.7 ± 9.1	56.3 ± 2.9	35.8 ± 11.3	23.7 ± 5.3
?	0.2 ± 0.1	ND§	1.8 ± 1.1	0.6 ± 0.5
20:4	0.1 ± 0.1	ND	1.1 ± 0.7	ND
Ratio				
<18C/>18C	1.8 ± 0.2	1.7 ± 0.1	1.6 ± 0.1	1.8 ± 0.1‡
Unsat/Sat	1.6 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.6 ± 0.1‡

* Mean ± SE, N = 4.
†, ‡ P values: †$P < 0.01$, and ‡$P < 0.05$.
§ ND: not detected.

Table 5. Composition of free fatty acids of muscles from 1-day-old chicks hatched from 2,4-D b.e. treated eggs

Fatty acid	Fatty acids (mg/g total lipids)			
	Leg muscle		Complexus muscle	
	Control	2,4-D b.e.	Control	2,4-D b.e.
14:0	0.3 ± 0.1*	0.3 ± 0.1	0.4 ± 0.1	2.2 ± 0.7†
16:0	2.6 ± 0.2	5.4 ± 0.7‡	4.0 ± 0.9	11.2 ± 1.4‡
16:1	0.5 ± 0.2	1.0 ± 0.2	0.2 ± 0.1	1.7 ± 0.8
18:0	1.2 ± 0.1	2.8 ± 0.5‡	2.6 ± 0.8	10.4 ± 2.5†
18:1	2.3 ± 0.5	16.9 ± 3.3‡	2.8 ± 0.8	12.4 ± 1.6‡
18:2	0.4 ± 0.1	6.1 ± 1.2§	0.3 ± 0.1	3.8 ± 1.0†
20:4	0.08 ± 0.04	1.2 ± 0.1§	ND‖	2.1 ± 0.7†
Ratio				
<18C/>18C	0.9 ± 0.1	4.3 ± 0.2§	1.4 ± 0.1	1.6 ± 0.2
Unsat/Sat	0.4 ± 0.1	1.3 ± 0.1§	0.5 ± 0.1	0.7 ± 0.1

* Mean ± SE, N = 4.
†–§ P values: †$P < 0.05$, ‡$P < 0.005$, and §$P < 0.0005$.
‖ ND: not detected.

the presence of a substituted aromatic acid which produces the myotonia. The causes of the myotonia in these chicks may be different from those that produce it in adult mammals. Clarification of this point would require further electrophysiological studies that are beyond the scope of this work.

Biochemical studies were performed in leg and complexus muscles. Complexus muscles are located in the dorsal surface of the head and neck, and they undergo an accelerated growth and hypertrophy prior to hatching. It seems that their primary function is to provide the power for breaking the shell during the hatching [40]. A large number of embryos that died while pipping the shell were found at the end of each incubation [15, 16]. This suggested that complexus muscles in particular could be affected by 2,4-D b.e., not providing the power for hatching. Leg and complexus muscles presented different biochemical responses to the action of 2,4-D b.e. (Tables 1–5). Since no significant differences were observed in the 2,4-D b.e. level in these muscles [17], the dissimilar answers may be related to distinct muscle composition and metabolism [40]. Distinct effects of 2,4-D have also been observed in muscles with different fiber composition [5–7, 12–14].

Glycogen accumulation has been described in red fiber muscles of 2,4-D treated rats [5, 6, 12]. The glycogen determinations in the 2,4-D b.e. treated group suggest that this would not occur in these systems; in fact, a small decrease was detected in leg muscles (Table 1). This was not unexpected since the chick embryo does not use glycogen as a source of energy [41, 42], and the enzymes of glycogen metabolism are induced after hatching [43].

In a pathological state, proteolysis of some myofibrillar protein could be expected [44, 45]; the protein determinations and electrophoretic examinations did not indicate protein degradation. The electrophoretic pattern of myofibrillar proteins from the 2,4-D b.e. treated group was very similar to that of the control, except for the increase of a 20 kD band in the complexus muscles. Considering the molecular weight of this peptide, it could be a slow myosin light chain isoprotein, the $LC1_s$, but to clarify this point would require further specific studies [46].

Small lipid droplets have been observed in mammal skeletal muscle after 2,4-D treatment [12]. Kolberg *et al.* [37, 38] have also detected lipid droplets in L cells exposed to 2,4-D and they have associated this finding with an important increase of triglycerides. These authors observed that the fatty acids of the accumulated triglycerides are, to a large extent, obtained from the cell culture medium. They concluded that 2,4-D may affect the permeability of the plasma membrane, increasing fatty acid influx into the cells [37, 38]. Variations in phospholipid and fatty acid patterns and increases of triglycerides and FFA contents were detected in muscles from chicks hatched from 2,4-D b.e. treated eggs. The changes observed in phospholipid and fatty acid patterns would be relevant since they may influence permeability [47] and fluidity of membranes [48]. However, the more important finding was the increase of FFA levels because of their cytotoxic actions. FFA have detergent and ionophoric properties; they are able to generate free radicals and to induce structural perturbations in the lipid bilayer [30, 31]. FFA are inhibitors of oxidative phosphorylation and inorganic phosphate-ATP exchange; they activate the mitochondrial Mg^{2+}-activated adenosine triphosphatase and induce mitochondrial swelling [32, 33]. Moreover, they inhibit Ca^{2+} uptake and efflux in sarcoplasmic reticulum [34].

In contrast with the mammal fetus, which is primarily dependent on glucose, the avian embryo is dependent on the lipid filled yolk as an energy source [41, 42]. Consequently, the avian embryo has a high capacity to carry out fatty acid oxidation [49] which is localized in mitochondria [50]. Mitochondria in these muscles have been observed to be morphologically altered,* and they have a high Ca^{2+}

* Argüello JM, Hliba R, Evangelista de Duffard AM and Duffard RO, unpublished results.

level [51]. Such an increase in the mitochondrial Ca^{2+} level would inhibit the β-oxidation [52]. Considering these facts, a probable hypothesis for explaining the increase in FFA might be that mitochondria could not carry out fatty acid β-oxidation efficiently. During hatching, a high amount of energy is required and the FFA pool of blood should be exhausted in 1-day-old chicks. However, a high FFA level was detected in blood of chicks hatched from 2,4-D b.e. treated eggs (control 0.17 ± 0.02 mg/mL; 2,4-D b.e. treated group 0.32 ± 0.07 mg/mL; $P < 0.01$). This result would also suggest that these chicks may not be able to metabolize the FFA supplied by the egg yolk. However, more specific data are required to prove this hypothesis; for instance, to determine: (i) the capacity of these 2,4-D b.e. treated muscles to β-oxidate fatty acid, and (ii) the probable correlation between altered Ca^{2+} homeostasis [51] and mitochondrial disfunction in these muscles.

In conclusion, the data in this report support the concept that 2,4-D b.e., during embryonic development, induces important biochemical alterations in skeletal muscle. These muscles showed muscular weakness, edema and myotonia. Increases of muscular triglycerides and FFA levels were observed. These FFA would play an important role in the 2,4-D b.e. toxic mechanism in this experimental system.

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