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Ca^{2+} HOMEOSTASIS ALTERATIONS INDUCED BY 2,4-DICHLOROPHENOXYACETIC BUTYL ESTER AND 2,4-DICHLOROPHENOXYACETIC ACID ON AVIAN SKELETAL MUSCLE

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Abstract—Fertilized hen eggs were treated externally with 2,4-dichlorophenoxyacetic butyl ester (2,4-D b.e.) (3.1 mg/egg) before the start of the incubation. Actomyosin and sarcoplasmic reticulum adenosine triphosphatase (ATPase) activities from leg and complexus muscles of chicks hatched from treated eggs were measured. No significant variations were detected in the ATPase activities of actomyosin, but the sarcoplasmic reticulum Mg^{2+}-activated ATPase and Ca^{2+}, Mg^{2+}-activated ATPase were inhibited 50 and 38% respectively. $^{45}Ca^{2+}$ uptake into soleus muscle was increased by the 2,4-D b.e. treatment. The compartmental analysis of $^{45}Ca^{2+}$ uptake kinetics showed increases in Ca^{2+} fluxes in sarcolemma and mitochondria and in the mitochondrial calcium pool. Isolated soleus muscles were treated with 2,4-dichlorophenoxyacetic acid (2,4-D) or 2,4-D b.e. [^{14}C]2,4-D reached it highest level in these muscles after 1 hr of treatment. The *in vitro* treatment with 2,4-D or 2,4-D b.e. increased $^{45}Ca^{2+}$ uptake into the muscles. 2,4-D b.e. produced greater alterations than 2,4-D. The compartmental analysis of the $^{45}Ca^{2+}$ uptake kinetics also showed increases of the mitochondrial Ca^{2+} pool and Ca^{2+} fluxes through sarcolemma and mitochondria. These results led to a hypothesis based on Ca^{2+} permeability alterations for explaining the myopathic actions of these phenoxyherbicides.

2,4-Dichlorophenoxyacetic acid (2,4-D)‡, its esters and its salts are widely used herbicides. A number of reports have been published on the effects of 2,4-D on skeletal muscle of adult mammals [1–13]. Acutely, it induces myotonia, an effect that the animals overcome within hours [1–3]. Repetitive treatment with 2,4-D over several days produces biochemical and morphological alterations in skeletal muscles [4–13]. Several investigators have suggested 2,4-D effects on Ca^{2+} homeostasis among other morphological and functional alterations of skeletal muscles [9–11, 13, 14]. Nevertheless, there are neither direct determinations of Ca^{2+} intracellular homeostasis nor evidence that Ca^{2+} disequilibrium would be a primary toxic effect of 2,4-D action *in vivo*.

2,4-Dichlorophenoxyacetic butyl ester (2,4-D b.e.) is one 2,4-D derivative. External treatment of fertilized hen eggs with 2,4-D b.e. at the start of incubation is embryotoxic (lethal $dose_{50}$, 5 mg 2,4-D b.e./egg) [15, 16]. Chicks hatched from 2,4-D b.e. treated eggs show dose-dependent hypomyelination, motor dysfunctions, and postural troubles [15–19]. The muscles of these chicks exhibit weakness, prolonged motor latency, myotonia and edema [20]. Among other biochemical findings, changes in the phospholipid patterns and increases of triglycerides and free fatty acids (FFA) have been detected in these muscles [20].

The purpose of this work was: (i) to examine the effect of 2,4-D b.e. treatment, over the embryonic development, on some biochemical parameters such as actomyosin and sarcoplasmic reticulum ATPases activities and muscle Ca^{2+} fluxes, in order to characterize further the myopathy induced by this drug in this experimental system, and (ii) to study the effects of 2,4-D and 2,4-D b.e. treatment of isolated muscles on Ca^{2+} movements, to clarify whether or not these herbicides have a direct effect on Ca^{2+} homeostasis.

MATERIALS AND METHODS

Fertile hen eggs were obtained from a commercial hatchery, and were treated externally with an ether solution of pure 2,4-D b.e., corresponding to 3.1 mg/egg as described in former studies [15, 16]. Control eggs were treated with ether. After drying, the eggs were placed in an incubator at 38° and 60% relative humidity with two rotations per day. Twenty-four hours after hatching, chicks were decapitated, and the leg and complexus muscles were excised and cleaned of fat and connective tissue.

Actomyosin was isolated from leg and complexus

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‡ Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-D b.e., 2,4-dichlorophenoxyacetic butyl ester; ATPase, adenosine triphosphatase; Ca^{2+}, Mg^{2+}-ATPase, Ca^{2+}, Mg^{2+}-activated ATPase; Ca^{2+}-ATPase, Ca^{2+}-activated ATPase; Mg^{2+}-ATPase, Mg^{2+}-activated ATPase; FFA, free fatty acids; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; BHM, Birge–Haddad medium, 10% chick serum.

muscles. After dissection, muscles were stored in a Krebs–Ringer solution at 0–4° and processed after rigor mortis. The muscles were homogenized for 90 sec in a Virtis homogenizer using 10 vol. of 0.6 M KCl, 3 mM $NaCO_3H$, pH 7.0 (20°). The homogenates were then centrifuged for 15 min at 10,000 *g* in a refrigerated centrifuge. The resulting supernatants were diluted with 3 vol. of distilled water. After standing for 15 min the actomyosin precipitates were pelleted by centrifugation for 15 min at 10,000 *g*. Supernatants were discarded, and the actomyosin pellets were suspended in 0.6 M KCl, 3 mM $NaCO_3H$, pH 7.0 (20°). The dilution–precipitation procedure was repeated three times. The final sediments were suspended to a protein concentration of 5 mg/mL in 0.6 M KCl, 3 mM $NaCO_3H$, pH 7.0 (20°). All procedures were carried out at 0–4°.

Microsomes were isolated according to the method of Boland *et al.* [21] from leg and complexus muscles. The muscles were kept in a Krebs–Ringer solution (0–4°) until a sufficient amount was collected and then homogenized for 90 sec in a Virtis homogenizer using 4 vol. of 0.1 M KCl, 0.3 M sucrose, 10 mM imidazole, pH 7.4 (20°). The homogenates were then centrifuged for 20 min at 8200 *g* in a refrigerated centrifuge. The pellets were discarded and the supernatants filtered through glass wool. Remaining mitochondria were removed by centrifugation for 30 min at 8200 *g*. Microsomes were sedimented from the supernatants at 50,000 *g* for 1 hr. Microsomal sediments were suspended in 0.6 M KCl, 0.3 M sucrose, 10 mM imidazole, pH 7.4 (20°). After standing for 30 min, the microsomes were pelleted by centrifugation at 50,000 *g* for 1 hr. Sediments were dispersed in 0.3 M sucrose and centrifuged at 58,000 *g* for 30 min. Final sediments were suspended to a protein concentration of 5 mg/mL in 0.3 M sucrose. All procedures were carried out at 0–4°.

The Ca^{2+}, Mg^{2+}-activated adenosine triphosphatase (Ca^{2+}, Mg^{2+}-ATPase) activity of actomyosin was measured using the following medium: 5 mM ATP, 75 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 20 mM histidine, pH 7.0 (20°). For Mg^{2+}-activated ATPase (Mg^{2+}-ATPase) activity determinations $CaCl_2$ was omitted. The reaction was started with the addition of the actomyosin preparation (final concentration of protein 2 mg/mL). After 5 min of incubation at 37°, the assay was stopped by the addition of trichloroacetic acid to a final concentration of 10%. The inorganic phosphate was determined by the method of Fiske and Subbarow [22].

Microsome Ca^{2+}, Mg^{2+}-ATPase activity was determined on the day of microsome preparation in a medium containing 5 mM ATP, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM EGTA, 5 mM potassium oxalate, 10 mM imidazole, pH 7.4 (20°). For Mg^{2+}-ATPase activity determinations $CaCl_2$ was omitted. The microsome protein final concentration was 0.1 to 0.2 mg/mL. The reaction was carried out for 3 min at 25°, and it was stopped by the addition of trichloroacetic acid to a final concentration of 10%. The liberated inorganic phosphate was measured as described above.

Ca^{2+}-activated ATPase (Ca^{2+}-ATPase) activity of actomyosin or microsomes was calculated as the difference between the Ca^{2+}, Mg^{2+}-ATPase and the Mg^{2+}-ATPase activities.

Ca^{2+} uptake determinations were made according to the method of Borle [23]. Soleus muscles from chicks hatched from 2,4-D b.e. treated eggs were suspended in a Krebs–Henseleit solution,* pH 7.4 (20°), and preincubated for 1 hr at 37° under a 95% O_2 and 5% CO_2 atmosphere in a shaker bath in order to reach steady-state conditions. At zero time, $^{45}Ca^{2+}$ was added to the medium (0.5 μCi/mL final concentration). Muscles were taken at different times, rinsed with ice-cold medium, placed in tubes containing 0.5 M NaOH, and solubilized by heating at 100° for 15 min. Aliquots were taken from each sample for protein content and radioactivity determinations. Radioactivity was measured by scintillation spectrophotometry using Triton X-100: toluene (1:3), 0.02% 1,4-bis (5-phenyl-2-oxazyl) benzene (POPOP), 0.4% 2,5-diphenyl oxazole (PPO) as scintillation medium.

In herbicide uptake experiments, soleus muscles from untreated 1-day-old chicks were preincubated in a Birge–Haddad medium, 10% chick serum (BHM) [24], at 37° under a 95% O_2 and 5% CO_2 atmosphere in a shaker bath, for 30 min. At zero time, 0.02 μCi/mL [^{14}C]2,4-D, plus the required concentration of unlabeled 2,4-D, were added to the medium. Samples were taken at different times, rinsed in ice-cold Krebs–Ringer solution, placed in tubes containing 0.5 M NaOH, and solubilized by heating at 100° for 15 min. Aliquots were taken from each sample for protein content and radioactivity determinations as described above. In the 2,4-D efflux assay, the soleus muscles were preincubated, for 1 hr, in BHM with 0.02 μCi/mL [^{14}C]2,4-D plus the required concentration of unlabeled, 2,4-D. At different times samples were taken and the BHM was changed by the addition of 2,4-D free BHM. The samples were then processed as described above.

The effects of 2,4-D or 2,4-D b.e. treatment of isolated muscles on Ca^{2+} uptake were determined in soleus muscles from untreated chicks. The muscles were preincubated in BHM with the desired concentration of 2,4-D or 2,4-D b.e., for 1 or 3 hr. Controls were processed in the same way but in a 2,4-D and 2,4-D b.e. free BHM. Then BHM was changed for a Krebs–Henseleit solution, pH 7.4 (20°), and the muscles were preincubated for 30 min at 37° under a 95% O_2 and 5% CO_2 atmosphere in a shaker bath in order to reach steady-state conditions. At zero time, 0.5 μCi/mL $^{45}Ca^{2+}$ was added to the medium; samples were taken at different times and processed as described above.

Kinetic analyses of Ca^{2+} uptake curves were performed as reported by Borle [23, 25], assuming the calcium distribution in a three-compartment parallel closed system. From the uptake curves the time derivative of each point was derived and plotted on semilog paper. The parameters of the two exponentials were obtained by graphical analysis using a computer. For expressing the results of these analyses the symbols used were defined as follows:

* Krebs–Henseleit solution was 1 mM $CaCl_2$.

Table 1. Mg^{2+}-, Ca^{2+}-, and Ca^{2+}, Mg^{2+}-ATPase activities of sarcoplasmic reticulum and actomyosin from muscles of 1-day-old chicks hatched from 2,4-D b.e. treated eggs

	P_i (μmol/min/mg protein)			
	Leg muscle		Complexus muscle	
	Control	2,4-D b.e.	Control	2,4-D b.e.
Sarcoplasmic reticulum				
Mg^{2+}-ATPase	1.16 ± 0.08*	0.88 ± 0.03†	0.92 ± 0.14	0.46 ± 0.07†
Ca^{2+}, Mg^{2+}-ATPase	1.70 ± 0.12	1.40 ± 0.05	1.85 ± 0.14	1.15 ± 0.11‡
Ca^{2+}-ATPase	0.54 ± 0.05	0.52 ± 0.02	0.86 ± 0.06	0.69 ± 0.12
Actomyosin				
Mg^{2+}-ATPase	0.25 ± 0.01	0.27 ± 0.01	0.29 ± 0.04	0.25 ± 0.09
Ca^{2+}, Mg^{2+}-ATPase	0.55 ± 0.04	0.60 ± 0.02	0.47 ± 0.03	0.49 ± 0.10
Ca^{2+}-ATPase	0.30 ± 0.05	0.33 ± 0.03	0.18 ± 0.01	0.24 ± 0.08

* Mean ± SE, N = 5.
†, ‡ P values: † $P < 0.05$; and ‡ $P < 0.005$.

Compartment 1: Extracellular medium.

Compartment 2: Fast exchange compartment (sarcoplasm).

Compartment 3: Slow exchange compartment (mitochondria and sarcoplasmic reticulum).

S_i: Amount of exchangeable calcium in compartment i.

J_{ik}: Rate of calcium transport from compartment i to compartment k. $J_{ik} = -J_{ki}$.

K_{ik}: Rate constant of calcium transport from compartment i to compartment k. Fraction of S_i transferred to compartment k in unit time. $K_{ik} = J_{ik}/S_i$.

All protein determinations were carried out by the method of Bradford [26]. Data were evaluated for statistical differences by Student's t-test.

The standards were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. $^{45}Ca^{2+}$ was purchased from New England Nuclear, Boston, MA, U.S.A., as an $CaCl_2$ aqueous solution. 2,4-D b.e., containing 96% active drug, was obtained from Atanor, Buenos Aires, Argentina, and its purity was checked by GLC [27]. Other chemicals were analytical grade.

RESULTS

Effects of 2,4-*D b.e.* in ovo *treatment on the actomyosin and sarcoplasmic reticulum ATPase activities and* Ca^{2+} *movements.* ATPase activities of actomyosin and sarcoplasmic reticulum are two key enzymatic activities in skeletal muscle [28–30]. The effects of 2,4-D b.e. treatment during embryonic development on the ATPases from leg and complexus muscles are shown in Table 1. Sarcoplasmic reticulum is the major component of the muscle microsome fraction, and it is possible to follow changes in the Ca^{2+}, Mg^{2+}-ATPase activity of the sarcoplasmic reticulum, determining this ATPase activity in the microsomal fraction [21]. Considering this fact, microsomes were used for studying the sarcoplasmic reticulum ATPase activities. Mg^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase were inhibited 24 and 18%, respectively, in leg muscles of treated chicks. Sarcoplasmic reticulum from complexus muscles was also affected: Mg^{2+}-ATPase was inhibited 50%, while Ca^{2+}, Mg^{2+}-ATPase decreased 38%. In spite of these changes, the Ca^{2+}-ATPase activity did not show significant alterations. On the other hand, there were no differences between actomyosin ATPase activities in control and 2,4-D b.e. treated groups (Table 1).

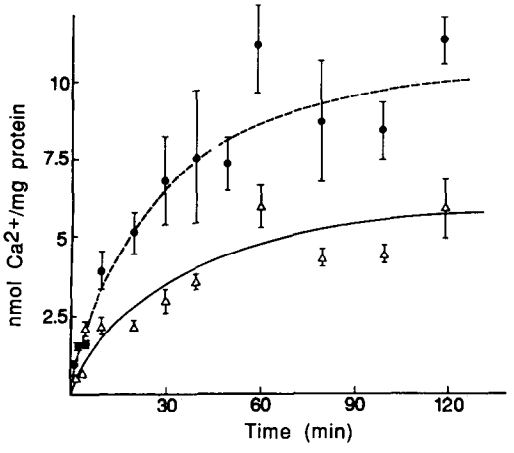

Fig. 1. Ca^{2+} uptake curves of soleus muscles of 1-day-old chicks hatched from 2,4-D b.e. treated and untreated eggs (control). Values are means ± SE, N = 4. Each point of the 2,4-D b.e. treated group is statistically different ($P < 0.005$) from the control (Student's t-test). Key: (△) control, and (●) 2,4-D b.e. treated group.

From the group of leg muscles, the soleus was chosen to perform $^{45}Ca^{2+}$ uptake studies. Figure 1 shows $^{45}Ca^{2+}$ uptake kinetics in control muscles and in muscles of chicks hatched from eggs treated with

Table 2. Ca^{2+} fluxes, Ca^{2+} compartment size, and rate constants in soleus muscles of 1-day-old chicks hatched from 2,4-D b.e. treated eggs

	Control	2,4-D b.e.
S_1*†	100,000	100,000
J_{21}	566 ± 210‡	907 ± 207§
K_{21}	0.46 ± 0.04	0.70 ± 0.16
K_{12}	0.0056 ± 0.0021	0.0091 ± 0.0021§
S_2	1518 ± 246	1208 ± 37
J_{23}	127 ± 22	202 ± 10‖
K_{32}	0.027 ± 0.001	0.015 ± 0.002¶
K_{23}	0.084 ± 0.016	0.144 ± 0.008¶
S_3	5237 ± 614	12,891 ± 650¶

* Units: S (pmol Ca^{2+}/mg protein); J (pmol Ca^{2+}/mg protein/min); and K (1/min).

† The parameters were calculated from the Ca^{2+} uptake curves (Fig. 1).

‡ Mean ± SE, N = 4.

§–¶ P values: § $P < 0.05$, ‖ $P < 0.025$, and ¶ $P < 0.001$.

2,4-D b.e. The 2,4-D b.e. treated group had higher Ca^{2+} uptake, revealing a clear alteration in the Ca^{2+} fluxes. These curves were then analyzed by the method of Borle [23]. Table 2 shows important increases in the J_{21} (extracellular medium-sarcoplasm) and J_{23} (sarcoplasm-mitochondria, sarcoplasmic reticulum) fluxes. Moreover, there was an enlargement (146%) of the compartment size of the mitochondrial-sarcoplasmic reticulum calcium pool (S_3). As a consequence of these increases, the rate constants K_{12}, K_{23} and K_{32} were also affected. These results pointed out an alteration in the calcium homeostasis with increases in Ca^{2+} fluxes and mitochondrial-sarcoplasmic reticulum Ca^{2+} pools. This anomaly would lead to an enlargement in the total intracellular Ca^{2+} [23].

Effects of in vitro *treatment of soleus muscles with 2,4-D and 2,4-D b.e. on* Ca^{2+} *movements.* Before starting to study the effects of 2,4-D treatment of isolated muscles, the kinetics of 2,4-D influx and efflux in soleus muscles were studied. Figure 2 shows that 2,4-D uptake reached steady-state conditions after 1 hr of incubation at both concentrations studied, 1 mM and 0.01 mM. For determination of the 2,4-D efflux curves, soleus muscles were preincubated with 1 mM or 0.01 mM [^{14}C]2,4-D and then placed in a 2,4-D free medium. A rapid initial efflux was observed; 75% of the initial radioactivity was lost within 45 min. This initial phase was followed by a slow one (Fig. 3). In spite of the successive wash out, 10–15% of initial radioactivity remained in the muscles after 5 hr of efflux. Taking into account these results we decided to treat the muscles for at least 1 hr and let them reach steady-state conditions for at least 30 min before initiating the Ca^{2+} experiments.

Table 3 shows that treatment of isolated muscles with 2,4-D or 2,4-D b.e. affected the Ca^{2+} uptake. 2,4-D b.c. produced higher increases of Ca^{2+} uptake than 2,4-D. Likewise, it is remarkable that 1 hr of treatment with 0.01 mM 2,4-D b.e. or 2,4-D was enough to increase Ca^{2+} influx.

Figure 4 shows $^{45}Ca^{2+}$ uptake kinetics after 1 hr and 3 hr of 1 mM 2,4-D treatment. There were important differences between the control and the 2,4-D treated group which were observed more clearly when the curves were analyzed by the method of Borle [23, 25] (Table 4). After 1 or 3 hr of 1 mM 2,4-D treatment, there were significant increases of the K_{21} rate constant and the slow exchange Ca^{2+} pool (S_3; mitochondrial-sarcoplasmic reticulum) with a consequent variation of the K_{32} rate constant. In addition, 3 hr of treatment also increased J_{21} and J_{32}. Good agreement was found when the results obtained after 3 hr of *in vitro* treatment were compared with those observed after *in ovo* 2,4-D b.e. treatment; the main alterations (increases of J_{21}, J_{23} and S_3), were similar (Tables 2 and 4).

DISCUSSION

In ovo *effects of 2,4-D b.e.* We have shown previously that muscles of chicks hatched from 2,4-D b.e. treated eggs exhibit a myopathy characterized

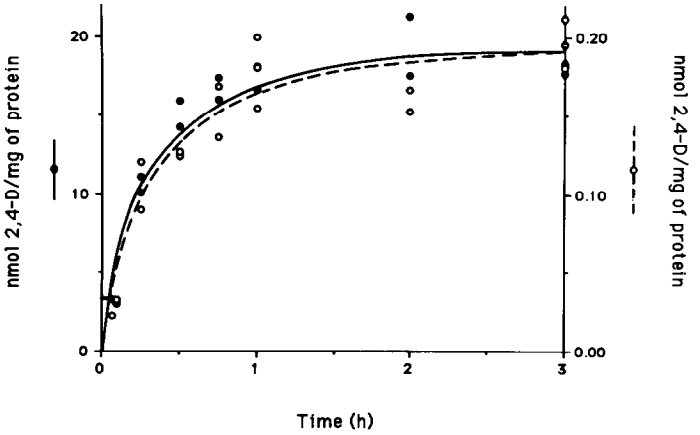

Fig. 2. 2,4-D uptake curves of soleus muscles of 1-day-old chicks. Muscles were incubated with (—●—) 1 mM [^{14}C]2,4-D or (—○—) 0.01 mM [^{14}C]2,4-D, and the incorporation of the drug was determined at the indicated times.

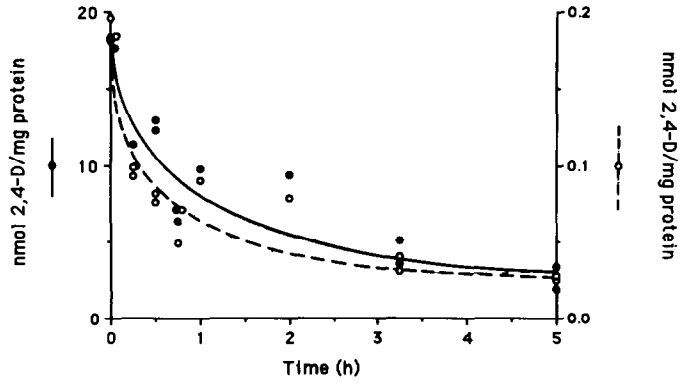

Fig. 3. 2,4-D efflux curves from soleus muscles of 1-day-old chicks. The muscles were treated previously with 1 or 0.01 mM [^{14}C]2,4-D for 1 hr; at zero time the drug was removed, and the efflux of 2,4-D was measured at the indicated times. Key: (—●—) 1 mM 2,4-D; and (- -○- -) 0.01 mM 2,4-D.

Table 3. Effect of 2,4-D or 2,4-D b.e. on Ca^{2+} uptake into soleus muscles from 1-day-old chicks: Influence of treatment time and 2,4-D concentration

		Ca^{2+} (nmol/mg protein/120 min) Concentration of phenoxyherbicide		
Treatment	Control	1 mM	0.1 mM	0.01 mM
2,4-D b.e.				
1 hr	5.99 ± 0.72*	22.67 ± 4.74†	15.24 ± 3.78‡	13.84 ± 0.17†
3 hr	6.30 ± 0.89	15.07 ± 4.24‡	10.48 ± 1.64§	9.44 ± 1.41§
2,4-D				
1 hr	5.99 ± 0.72	11.63 ± 0.99†	8.49 ± 1.66	10.87 ± 2.52§
3 hr	6.30 ± 0.89	10.33 ± 0.77†	8.70 ± 0.84	11.19 ± 1.22†

* Mean ± SE, N = 4.
†–§ P values: † $P < 0.001$, ‡ $P < 0.01$, and § $P < 0.05$.

by weakness, prolonged motor latency, myotonia, edema and important biochemical alterations [20]. The lipid composition of these muscles showed several changes in the phospholipid and fatty acid patterns and increases of triglycerides and FFA [20]. To further characterize this myopathy, we have determined the effects of this treatment on the actomyosin and sarcoplasmic reticulum ATPase activities and Ca^{2+} movements. It should be kept in mind that the concentrations of 2,4-D b.e. in these muscles were 28 ± 8 μg/g wet weight (leg muscle) and 51 ± 21 μg/g wet weight (complexus muscles) [27]; these levels produce tissue concentrations of roughly 0.14 and 0.25 mM respectively. These concentrations are similar to those determined or expected in other systems in which phenoxyherbicides produce different toxicological effects [3–14]. It is also important to mention that 2,4-D (the acid form of this herbicide) was not detected in the embryos or in 1-day-old chicks after 2,4-D b.e. treatment [27].

The study of myofibrillar protein composition and the associated Ca^{2+}, Mg^{2+}-ATPase activity is important in the understanding of different myopathies [31]. The electrophoretic pattern of myofibrillar proteins from chicks hatched from 2,4-D b.e. treated eggs does not show deep changes [20]. No variations were detected in the ATPase activities of actomyosin isolated from the 2,4-D b.e. treated group (Table I). Therefore, these results suggest that 2,4-D b.e. does not affect either the active site of ATPase or the role of the regulatory proteins, tropomyosin and troponin complex. This does not mean that the herbicide treatment would not alter the myofibril, for instance, affecting some of the interactions between the contractile proteins in the myofilaments. In fact, some alteration has been observed in the myofibrillar structure of these muscles.*

The sarcoplasmic reticulum Ca^{2+}, Mg^{2+}-ATPase plays a key role in the muscular relaxation mechanism [28, 29] and to know its functionality is relevant in the study of a muscular disorder. Besides, the report by Kuhn and Stein [14], describing the activation of isolated sarcoplasmic reticulum Ca^{2+}, Mg^{2+}-ATPase by 1 mM 2,4-D, suggested some interaction between phenoxyherbicides and this enzyme. Sarcoplasmic reticulum ATPase activities were inhibited in muscles of the 2,4-D b.e. treated group. These inhibitions may be mediated by the 2,4-D b.e. *per se* or by changes in the lipid environment [32, 33]. The variations in the phospholipid composition and

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

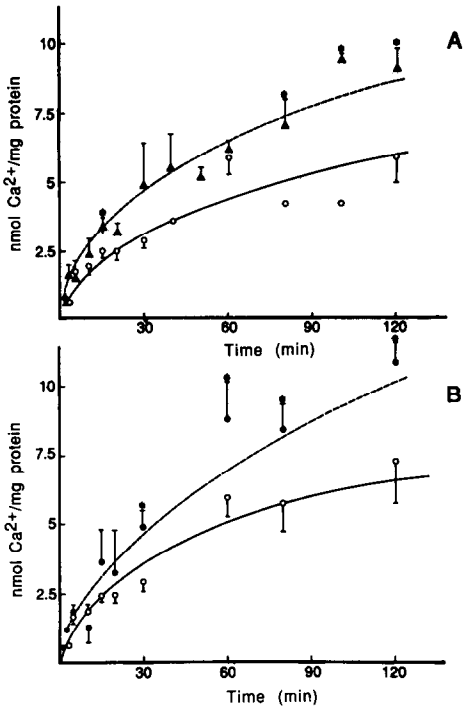

Fig. 4. 2,4-D effect on Ca^{2+} uptake curves of soleus muscles of 1-day-old chicks. The muscles were treated with 1 mM 2,4-D for 1 h (A) or 3 hr (B) and then preincubated for 30 min in order to reach steady-state. Values are means ± SE, N = 4. The asterisks (*) indicate a significant difference from the control groups, $P < 0.005$. Key: (○) control; and (●, ▲) 2,4-D b.e. treated groups.

the increase of FFA level detected in these muscles [20] support the second possibility. On the other hand, the disagreement with the results of Kuhn and Stein [14] could be due to the distinct experimental system and the different phenoxyherbicide tested.

Intracellular Ca^{2+} levels are very important since this ion mediates many cellular processes [34–36]. Ca^{2+} controls the contraction–relaxation mechanism in skeletal muscles in particular [28, 29]. It is also known that Ca^{2+} disequilibrium leads to cellular alterations and ultimately necrosis [37–39]. Important changes in the muscular Ca^{2+} homeostasis were found in chicks hatched from 2,4-D b.e. treated eggs (Table 3). Besides the sacrolemmal Ca^{2+} pump, there are two organelles that regulate sarcoplasmic calcium concentration in adult skeletal muscles, mitochondria and sarcoplasmic reticulum [28, 29, 35, 36, 40]. But at 1 day post-hatching, when these experiments were performed, it is the mitochondrial calcium deposit which regulates the sarcoplasmic Ca^{2+} level [41]. The parameters determined would indicate increases in Ca^{2+} fluxes through the sarcolemma ($J_{12} = -J_{21}$), the mitochondrial calcium pool (S_3) and mitochondrial Ca^{2+} fluxes ($J_{23} = -J_{32}$). The increases in these fluxes would imply a higher Ca^{2+} cycling in sarcolemma and mitochondria, whereas the mitochondrial calcium overload would involve a net rise in the total intracellular Ca^{2+} and transiently a net influx of Ca^{2+} [23]. On the other hand, these results are also relevant because they could be related to the lipid accumulation observed in these muscles [20]. Increases of FFA and triglycerides were detected, and it was proposed that they would accumulate because they could not be metabolized in the mitochondria [20]. The observed mitochondrial calcium overload would support this hypothesis, since high mitochondrial Ca^{2+} levels inhibit β-oxidation [42].

Effects of 2,4-D and 2,4-D b.e. on Ca^{2+} movements after in vitro *treatment.* Several reports have proposed (but not directly determined) probable 2,4-D effects on Ca^{2+} homeostasis [9–11, 13, 14]. Danon *et al.* [11] proposed this possibility considering morphological alterations (osmophilic deposits) in mitochondrias of rats treated with 2,4-D (200 mg/kg/day, 40 μg 2,4-D/g wet weight or 0.18 mM 2,4-D in muscle after 5 days of treatment). Dux *et al.* [9, 10] have observed Ca^{2+} pyroantimonate precipitates between

Table 4. Effects of 1 mM 2,4-D treatment on Ca^{2+} fluxes and subcellular distribution in soleus muscles from 1-day-old chicks

	Control	2,4-D	
		1 hr	3 hr
S_1*,†	100,000	100,000	100,000
J_{21}	566 ± 210‡	221 ± 46	1173 ± 199§
K_{21}	0.46 ± 0.04	0.192 ± 0.049‖	1.44 ± 0.65§
K_{12}	0.0056 ± 0.0021	0.0022 ± 0.0005	0.0045 ± 0.0020
S_2	1518 ± 246	1180 ± 64	1673 ± 143
J_{32}	127 ± 22	122 ± 19	238 ± 86
K_{32}	0.027 ± 0.001	0.013 ± 0.002¶	0.013 ± 0.002¶
K_{23}	0.084 ± 0.016	0.105 ± 0.023	0.129 ± 0.035
S_3	5237 ± 614	10,438 ± 490¶	11,738 ± 305¶

* Units: S (pmol Ca^{2+}/mg protein); J (pmol Ca^{2+}/mg protein/min); and K (1/min).

† The parameters were calculated from the Ca^{2+} uptake curves (Fig. 4).

‡ Mean ± SE, of 4 determinations.

§–¶ P values: § $P < 0.05$, ‖ $P < 0.025$, and ¶ $P < 0.001$.

the myofibrils in rats treated with 2,4-D (50 mg/kg/day) for 3 weeks. Since these Ca^{2+} precipitates were found after a long treatment time and together with other morphological alterations, it is difficult to associate the Ca^{2+} subcellular distribution changes with a primary effect of the drug. Likewise, it is not clear from our experiments *in ovo*, in which the drug was affecting the muscles during a long time, whether these Ca^{2+} disequilibriums are a primary effect of 2,4-D b.e. or are part of a sequence of events initiated elsewhere. To clarify these points, the effects of 2,4-D and 2,4-D b.e. on isolated muscles were studied, determining Ca^{2+} uptake. To work under conditions similar to those discussed above [9–11], to *in vitro* studies with 2,4-D [2, 8, 14, 43] and to our own conditions during *in ovo* treatment [27], three concentrations were chosen to perform these experiments: 1, 0.1 and 0.01 mM. It was found that 2,4-D b.e. and 2,4-D increased the Ca^{2+} uptake in soleus muscles. It is remarkable that only a 1-hr treatment with 0.01 mM herbicide was needed to increase Ca^{2+} uptake. This treatment time was short, as judged by the slow 2,4-D diffusion into the muscles (Fig. 2). On the other hand, the compartmental analysis of Ca^{2+} uptake curves revealed that 2,4-D affected Ca^{2+} fluxes and distribution in ways very similar to those observed after 2,4-D b.e. treatment *in ovo*. The 2,4-D treatment for 1 hr or 3 hr produced increases of Ca^{2+} fluxes through sarcolemma (J_{12}) and mitochondrial membranes (J_{32}) and the mitochondrial Ca^{2+} pool (Table 4).

The fact that 2,4-D b.e. *in vitro* treatment increased Ca^{2+} levels would indicate a close relationship between phenoxyherbicide myopathic actions and Ca^{2+} homeostasis alterations. On the other hand, 2,4-D also affected Ca^{2+} homeostasis. Previous studies have described cellular alterations by 2,4-D, only after longer periods of treatment and/or higher 2,4-D doses [1–14, 20, 43]. Consequently, in a hypothetical sequence of events produced by the drug, these effects on Ca^{2+} movements would be the earliest in such a sequence as has been described. Furthermore, considering the 2,4-D or 2,4-D b.e. concentrations, in particular 0.01 mM, no other effect was observed at this low concentration, except the alterations described herein.

To conclude, these results, together with those of a previous study [20], show that the butyl ester form of 2,4-D produces important biochemical alterations in skeletal muscle during embryonic development. The present report indicates that 2,4-D and 2,4-D b.e. affect Ca^{2+} homeostasis in a short time, suggesting that their primary effect could be an alteration of Ca^{2+} permeability.

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