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Contractility and Ultrastructure of Cardiac Muscle of Guinea Pigs Treated with Diphtheria Toxin* **

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Summary. The development of tension and the ultrastructure of cardiac papillary muscle of normal guinea pigs were compared to guinea pigs receiving lethal doses of diphtheria toxin. The maximal isometric force of the papillary muscles at various levels of resting tension indicated no significant difference between the control and the diphtheritic animals. Neither did the sarcomere lengths of papillary muscles, fixed at various levels of tension, show any Significant difference between these two groups. Electronmicroscopy in diphtheritic animals showed intact myofilaments but marked dilatation of the *T* and *L* system as well as pathologic fat deposits adjacent to the subsarcolemma and the *T* system. The results, therefore, suggest that diphtheria toxin acts on the *T* and *L* system, but does not affect structure and function of the myofilaments in the acute stage of diphtheria intoxication.

Key words: Diphtheria Toxin — Papillary Muscles — Actomyosin.

Introduction

The concept of a direct myolytic action of diphtheria toxin upon the heart muscle was advanced by Eppinger (1903). It remained unchallenged for nearly half a century until Schmid (1950) demonstrated, that the toxin left the myofibrillar structure unaffected in the acute stage of intoxication between the first and fourth day. However, more recently Pelosi, Meldolesi and Nidiri (1966) demonstrated in an electronmicroscopic study disordered, broken and widely separated myofibrils in the heart muscle of guinea pigs as early as the third day following application of high doses of diphtheria toxin. Similar electronmicroscopic findings were described by Burch and associated (1968) in the heart muscle of a child, that died from diphtheria on the eighth day.

Pelosi *et al.* (1966) postulated an indirect action of diphtheria toxin upon the myofibrils, according to which the formation of stable bonds between actin and myosin filaments leads to the destruction of the myofilaments.

In the course of the present investigation, we examined ultrastructure and contractility of the myocardium of guinea pigs treated with lethal doses of diphtheria toxin. The objective of the study was to determine, whether diphtheria toxin acts upon cardiac myofilaments according to the mechanism proposed by Pelosi *et al.* (1966), by answering the following questions experimentally:

1) Does diphtheria toxin alter the relationship between resting tension and maximal isometric force in heart muscle ?

2) Do tension and myofibrillar damage correlate ?

3) Is the sarcomere length of diphtheritic preparations reduced at high tensions?

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** Dedicated to Prof. W. Doerr on occasion of his 60th birthday.

Material and Methods

Guinea pigs of both sexes ranging in weight from 350–500 g were used. The LDM of diphtheria toxin, as specified by the supplier (Behringwerke A,G. Marburg/Lahn, West Germany), was individually calculated for each of 30 animals,divided into three equal portions and one portion at a time injected intraperitoneally on three consecutive days. Three animals received the injections at the same time. One of them was used for the experiment, which always took place on the fourth day, and the remaining two served as a control to indicate the lethal action of diphtheria toxin. In this group all animals died between the third and fifth day substantiating the lethal effect of the toxin dose.

The guinea pigs were killed by a blow on the head. The entire heart was removed immediately, the great vessels were cut away at their origin and the cardiac cavities freed of blood by washing in cold Krebs—Henseleit solution. The right chamber was opened under a dissecting microscope and papillary muscles 0.5–0.7 mm in diameter were selected. During dissection the hearts were bathed in cold Krebs—Henseleit solution oxygenated with 95% O_2 and 5% CO_2. The muscles were placed on an apparatus (Reiter, 1967) permitting measurement of isotonic tension with a Statham strain gauge transducer (2000 G 10 B) operated through a DC amplifier (Hugo Sachs Electronic). The transducer could be raised or lowered with a micrometer stage allowing the length and tension of the muscle to be changed at will. Resting tension and contractions were displayed on an oscilloscope (Tektronix 565). For constant electrical stimulation with a frequency of 1.5/sec a Grass stimulator was used (SD 5), delivering square wave pulses of 1 msec duration and an intensity 1.5 times above threshold. The organ bath (Reiter, 1967) was filled with Krebs—Henseleit solution oxygenated with 95% O_2 and 5% CO_2; its temperature kept constant at $35 \pm 0.01°$ C. Krebs—Henseleit solution was composed of:

114.9 mM NaCl, 24.9 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 3.2 mM CaCl and 10 mM glucose.

The nine muscles of diphtheritic animals as well as the nine muscles of healthy controls were randomly divided into three groups comprising three muscles each. Muscles were fixed in the physiological apparatus after they had been stretched just enough to develop 0.06 g resting tension (group 1), 0.4 g (group 2) or 2.5 g (group 3). Stimulation was stopped for 20 min, then the organ bath was exchanged against another one, cooled with ice water and filled with oxygenated Krebs—Henseleit solution. Subsequently tension was precisely adjusted to the desired levels. Two minutes later the Krebs—Henseleit solution was rapidly replaced by a cold (4° C) fixative solution (3% glutaraldehyde in phosphate buffer, pH = 7.4). Immersion in cold (4° C) Krebs—Henseleit solution for two minutes before fixation was done, because it kept changes in tension during subsequent fixation at a minimum ($< \pm 10\%$). Muscles were removed from their mounting after 30 minutes, further fixed in glutaraldehyde solution for an additional six hours and washed in phosphate buffer for 12 hours. Strips of muscle tissue were postfixed in osmium tetroxide and embedded in araldite. Blocks were oriented carefully to provide longitudinal sections. Ultrathin sections of the middle portion of the papillary muscles were cut with glass knives on a Reichert ultramicrotome OmU_2. Sections were examined under a Zeiss electronmicroscope EM 9. From each muscle preparation 5–10 pictures of unselected areas were taken, to which random numbers were assigned. Five sarcomeres of every muscle were measured from middle to middle of the *Z*-lines on two to three enlarged prints (magnification × 20000), each randomly chosen. An analysis of variance was conducted to evaluate the effects of tension and disease state (diphtheria vs. control) on sarcomere length. Furthermore a rank sum test was done to study the effect of tension on sarcomere length ignoring disease state. A Student *T*-test was performed comparing contraction amplitudes of diphtheritic muscles and controls at various tensions.

Results

Maximal Isometric Force in Relation to Resting Tension in Diphtheritic and Normal Heart Muscle

The relationship between maximal isometric force (± 1 SD) and resting tension in nine papillary muscles of nine healthy controls and nine diphtheritic guinea

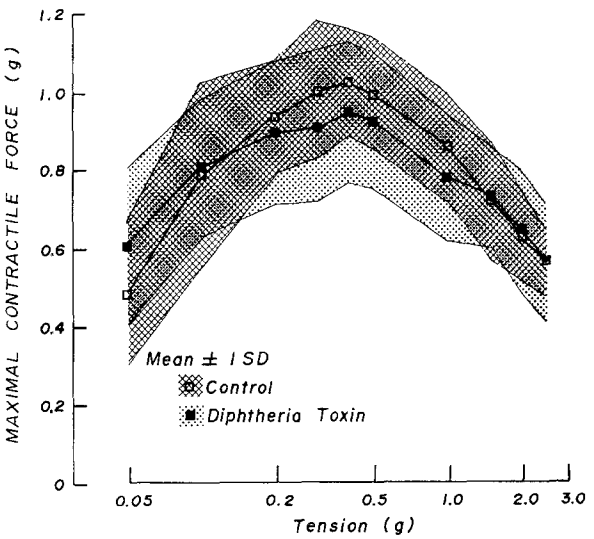


Fig. 1. The effect of increasing tension on the max. contractile force (± 1 SD) of papillary muscles of nine diphtheritic guinea pigs (filled squares) and nine controls (open squares). Isometric contractions electrically stimulated with a frequency of 1.5/sec. Diphtheritic animals had received one injection of 1/3 LDM diphtheria toxin intraperitoneally daily for three consecutive days. Experiments were performed the day following the third injection

pigs is shown in Fig. 1. During these measurements the muscles had been stimulated electrically with a frequency of 1.5/sec. Upon comparison for each tension level, treatment with diphtheria toxin had not altered contractile force significantly As resting tensions rose, insignificant differences became relatively smaller, until, at resting tensions between 1 and 2.5 g, both curves became almost indistinguishable.

At the end of the experiments the resting tension of 2.5 g was reduced to 0.4 g and all preparations developed the same maximal force that had been measured before with this tension. Thus, the decline in contractile force with higher tension was found to be fully reversible in diphtheritic and normal muscles.

Observations on Sarcomere Lengths in Papillary Muscles of Normal and Diphtheritic Guinea Pigs Fixed at Different Levels of Tension

The normal and diphtheritic papillary muscles from which the data shown in Fig. 1 have been obtained were divided into three groups comprising three muscles each. Muscles from group 1 were fixed with 0.06 g tension, from group 2 with 0.4 g and from group 3 with 2.5 g. In electronmicrographs five sarcomeres were measured in each muscle. The result of five measurements in each preparation are shown in Table 1. To evalute the effects of tension and disease state (diphtheria vs. control) on sarcomere length an analysis of variance was conducted. There was clearly no difference in sarcomere length between diphtheritic muscles and controls. The effect of tension on length was also not statistically significant ($p = 0.06$). Additionally, tension effects were studied using a rank sum test ignoring disease state. The result of this test show, that while length was not significantly greater

Table 1. Sarcomere length (μ)

Passive tension (g)	Observation	Control Replicate				Diphtheria Replicate			
		1	2	3	mean	1	2	3	mean
0.06	1	2.45	2.30	1.95	2.20	2.35	1.85	1.80	2.00
	2	2.30	2.35	1.95		2.50	1.80	1.80	
	3	2.35	2.30	2.00		2.40	1.80	1.80	
	4	2.25	2.35	1.95		2.40	1.75	1.75	
	5	2.30	2.20	1.95		2.50	1.80	1.80	
	mean	2.33	2.30	1.96		2.43	1.80	1.79	
0.4	1	2.00	2.35	2.35	2.16	2.30	2.35	2.10	2.22
	2	1.95	2.25	2.30		2.35	2.10	2.10	
	3	2.00	2.30	2.30		2.40	2.10	2.10	
	4	2.00	2.20	2.30		2.35	2.05	2.15	
	5	2.00	1.80	2.30		2.50	2.25	2.15	
	mean	1.99	2.18	2.31		2.38	2.17	2.12	
2.5	1	2.25	2.55	2.55	2.51	2.50	2.25	2.30	2.33
	2	2.20	2.55	2.50		2.50	2.30	2.10	
	3	2.65	2.55	2.45		2.50	2.05	2.45	
	4	2.50	2.55	2.70		2.50	2.05	2.35	
	5	2.45	2.55	2.65		2.45	2.10	2.50	
	mean	2.41	2.55	2.57		2.49	2.15	2.34	

Anova table

Source	SS	d.f.	M.S.	F	P
Disease state	0.048050	1	0.048050	1.116432	NS
Tension	0.319511	2	0.159756	3.711888	≈ 0.06
Disease state × tension	0.062533	2	0.031267	0.726475	NS
Error	0.516467	12	0.043039		
Total	0.946561	17	0.055680		

at 0.4 g than at 0.06 g, the length at 2.5 g was significantly greater than that observed at 0.06 g and at 0.4 g ($p = 0.05$).

Morphological Observations

On inspection diphtheritic hearts were easily distinguishable from normal hearts by their pale and yellowish color. They also seemed to be moderately enlarged. Subepicardial bleedings next to coronary vessels and subendocardial bleedings were seen frequently, mural thrombi occasionally. Electronmicroscopic examination of papillary muscles showed little influence of prolonged work in vitro on normal ultrastructure. In all preparations, controls and diphtheritic alike, arrangement of actin and myosin filaments, *Z*-lines, *A*- and *I*-bands were found

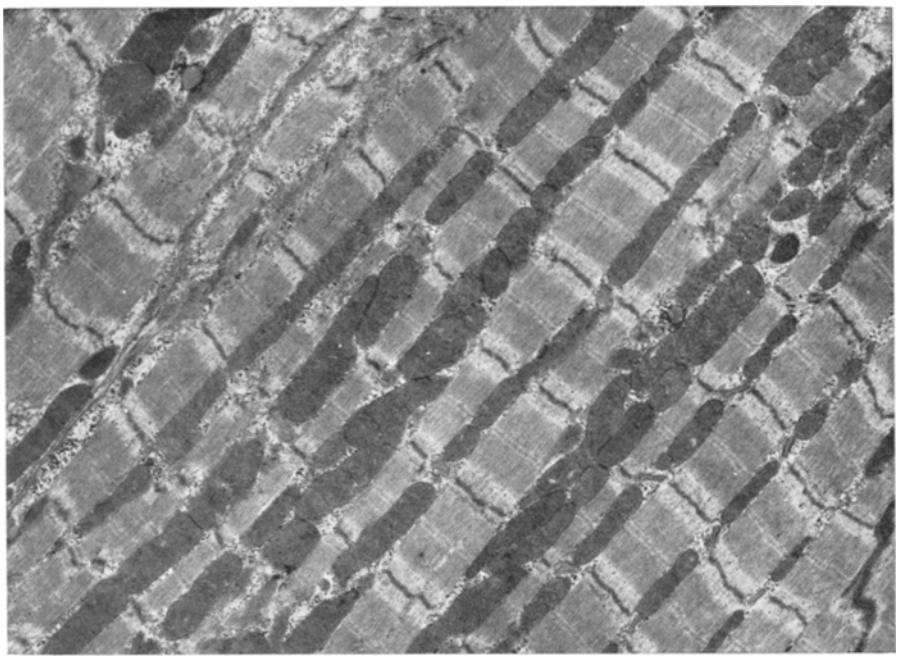


Fig. 2

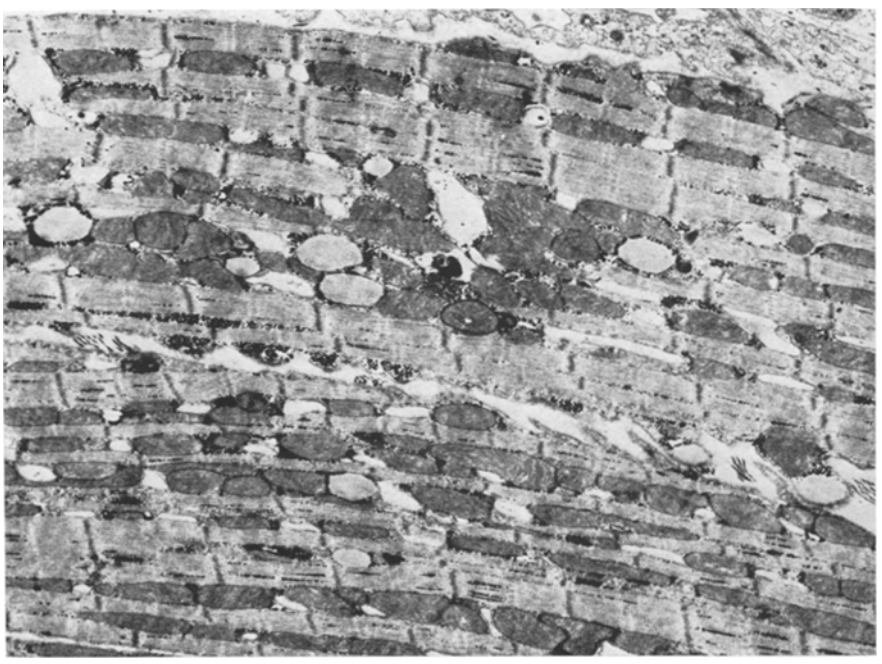
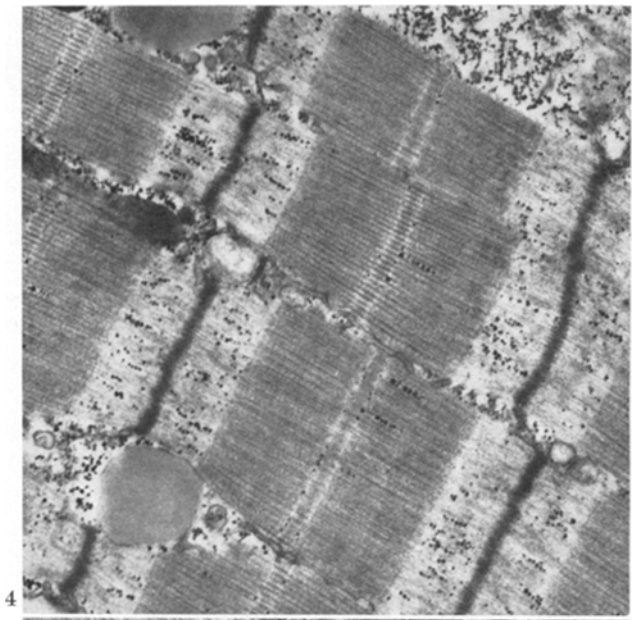


Fig. 3

Figs. 2 and 3. Examples of micrographs of longitudinal sections of guinea pig papillary muscles after incubation and stimulation in an experimental chamber for six hours. Healthy control (2) demonstrating good preservation of ultrastructure as evidenced by well defined mitochondrial christae. Sarcoplasmic reticulum and transverse tubules undilated and of normal size. Diphtheritic papillary muscle (3) presenting numerous lipid droplets, dilated T-tubules and normal arrangement of myofibrils. (Magnification $\times$ 6800)



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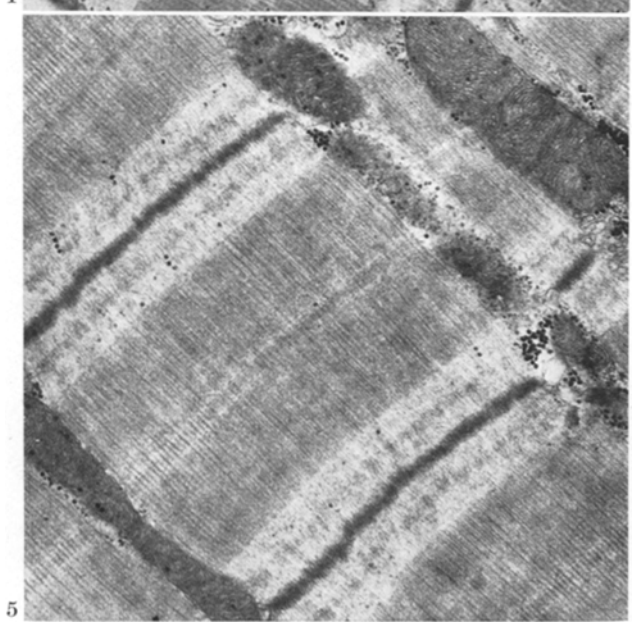

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Figs. 4 and 5. High magnification micrographs of diphtheritic (4) and normal (5) papillary muscles fixed with 2.5 g tension. Sarcomere lengths in both preparations approximate 2.6 μ. Myofibrils, Z-lines, A- and I-bands of normal appearance. (Magnification $\times$ 20000)

to be normal, irrespective of tensions applied during experiments and fixation (Figs. 2, 3, 4, 5). In diphtheritic preparations excessive dilatations of both the T- and L-system were seen (Fig. 3). Frequently it was not possible to decide whether these clearly dilated structures belonged to the T- or L-system. Clearly

the T- and L-system displayed the most significant changes in the acute stage of diphtheria toxin poisoning as described earlier by other investigators (Burch *et al.*, 1968; Pelosi *et al.*, 1966). Diphtheritic specimen contained numerous small lipid droplets, usually located near the T-system, close to Z-lines, to mitochondriae or subsarcolemmal. Mitochondriae were found intact in normal and most diphtheritic preparations.

Discussion

The results of this study show, that despite marked changes in cell ultrastructure, characteristic of the acute stage of diphtheria intoxication, contractility of the heart muscle is unimpaired. This is in keeping with clinical experiences. For example digitalis is of no value in the treatment of the early stage of diphtheria (Stroeder and Niggemeyer, 1963). Differences in sarcomere lengths between diphtheritic and normal papillary muscles compared at various levels of tension were insignificant. It may be argued, that the effect of tension on sarcomere length failed to be significant too. However, this might be due to the fragmentation of the total sample into smaller subsamples in order to evaluate tension effects, thereby reducing the sensitivity of the test. Furthermore, there is a lack of homogeneity of the variations of sarcomere lengths between muscles studied at different levels of tension (i.e. from a physiological viewpoint one would expect the variations to be greater at lower levels of tension). Damaged myofibrillar structures have not been observed, even in overstretched preparations.

Discrepancies with the findings of Pelosi *et al.* (1966) may be attributed to differences in biopsy sites, toxin dosages and time elapsed until the specimen were taken. Gukelberger (1935) demonstrated, that in guinea pigs, treated with diphtheria toxin pathological changes occur first and most severe in the apex of the heart. And tissue from the apex was used by Pelosi and associates (1966). When myofibrillar damage was observed on the fourth day, daily administered toxin doses (2 LD_{50}/kg i.p.) had been much bigger than the doses given by us. Consequently with smaller doses (0.25 LD_{50}/kg i.p.) myofibrillar damage was not regularly seen until the seventh day. Since the viability of cells with myofibrillar damage has never been demonstrated, the possibility, that myofibrillar damage occurs as a consequence of cell death, cannot be excluded.

Pelosi and his coworkers (1966) postulated, that non-utilization of ATP by the myofibrils causes the formation of stable bonds between the bridges stretching from the myosin filaments to those of actin and then their breakdown for mechanical reasons. Our observations render the existence of such a mechanism highly unlikely. The assumptions of Pelosi and his colleagues (1966) are also in disagreement with the findings of Hasselbach (1974), who saw only small changes in the affinity between actin and myosin under the conditions of contraction in the presence or absence of ATP.

Our results indicate, that in the acute stage of diphtheria intoxication diphtheria toxin in lethal doses does not act directly or indirectly upon myofilaments. We can confirm the findings of Schmid (1950), that in the acute stage of diphtheria the myofilaments are undamaged. We are also able to verify his conclusion, that in this stage "the preservation of contractility can be assumed beyond doubt".

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References

Burch, G. E., Sun, S. C., Sohal, R. S., Chu, K., Colcolough, H. L.: Diphtheritic myocarditis. A histochemical and electronmicroscopic study. Amer. J. Cardiol. **21**, 261–268 (1968)

Eppinger, H.: Die toxische Myolyse des Herzens bei Diphtheritis. Dtsch. med. Wschr. **29**, 257–259 (1903)

Gukelberger, M.: Neue experimentelle Arbeiten über Beginn und Ausbreitung der diphtheritischen Schädigungen des Herzmuskels. Z. ges. exp. Med. **97**, 749–755 (1935)

Hasselbach, W.: Die Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V., Tätigkeitsbericht. Naturwissenschaften **61**, 645 (1974)

Pelosi, G., Meldolesi, J., Nidiri, J.: Ultrastructural and biochemical aspects of the myocardium in guinea pigs treated with diphtheria toxin. Med. Pharmacol. exp. **14**, 537–569 (1966)

Reiter, M.: Die Wertbestimmung inotrop wirkender Arzneimittel am isolierten Papillarmuskel. Arzneimittel-Forsch. **17**, 1249–1253 (1967)

Schmid, F.: Myosin und Diphtherietoxin. Virchows Arch. path. Anat. **319**, 331–338 (1950)

Stroeder, J., Niggemeyer, H.: Die Diphtherie. In: Handbuch der Kinderheilkunde, Bd. V, 3, S. 325. Berlin-Göttingen-Heidelberg: Springer 1963

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