The Effects of Glycerol and Urea on the Ultrastructure and Contractility of Fast and Slow Rat Skeletal Muscles

S. A. KROLENKO and D. O. KARPENKO

Laboratory of Cell Physiology, Institute of Cytology, Academy of Sciences of the USSR, 194064 Leningrad, USSR

Abstract. The influence of the influx and efflux of glycerol and urea (400 mmol/l) on the amplitude of isometric twitches and the ultrastructure of isolated fast (EDL) and slow (SOL) muscles of young rats was studied. The influx of non-electrolytes was accompanied by a temporary decrease in the twitch tension. The removal of non-electrolytes resulted in a stable reduction of twitches. Both effects were less pronounced in glycerol experiments on slow muscles. The inhibition of twitches after the removal of non-electrolytes was associated with selective alterations of the T-system: swelling, vacuolation, and lysis of T-tubules. Quantitative analysis of the T-system showed that the extent of these changes may vary for different fibres, and the intensity of morphological alteration of the T-system generally correlated with the degree of twitch inhibition. Reloading of muscles with non-electrolytes tended to improve the T-system structure in some fibres and led to a partial restoration of the amplitude of twitches.

Key words: T-system — Glycerol and urea treatment — Ultrastructure — Contractility — Rat skeletal muscles

Introduction

The efflux of glycerol from frog skeletal muscles and isolated muscle fibres induces a selective destruction of the T-system, namely, vacuolation, lysis and rupture of the T-tubules connections with the surface membrane. As a result, the excitation-contraction coupling between the surface membrane and the muscle contractile apparatus becomes disturbed (Eisenberg and Eisenberg 1968; Howell 1969; Krolenko 1969, 1975*). Under certain conditions functional and morphological changes due to the glycerol removal are partially or completely reversible (Krolenko and Fedorov 1972; Zachar et al. 1972; Dulhunty and Gage

* A complete list of references on the glycerol removal effect up to 1974 is included.

1973; Krolenko 1975; Zacharová et al. 1978). Similar changes may be induced by efflux of urea or some other low-molecular non-electrolytes (Howell 1969; Dulhunty and Gage 1973; Oota and Nagai 1973; Krolenko 1975; Frank and Treffers 1977; Krolenko et al. 1980). The effect of glycerol removal has been described in different types of striated muscles (Nasledov et al. 1972; Weihe et al. 1977; Zacharová and Uhrík 1978; Dulhunty 1979; Davey et al. 1980). The effect of glycerol treatment on mammalian skeletal muscles was studied in detail by Davey et al. (1980). Using bundles of fast twitch red fibres from rat muscles the latter authors could show that, under certain conditions, glycerol removal results in excitation-contraction uncoupling without detubulation.

The aim of this work was to compare morphological changes of the T-system and the contractility of typical fast and slow mammalian muscles during washout of glycerol and urea as well as to study the reversibility of such changes.

Material and Methods

Fast (m. extensor digitorum longus, EDL) and slow (m. soleus, SOL) muscles of young Wistar rats weighing 50—70 g were used. Isolated muscles were immediately transferred to a thermostat (30°C) and their resting length was adjusted to allow maximum twitch tension. Twitches were recorded under isometric conditions using a mechanotron. The maximum sensitivity of the device was 10 mg/mm (about 0.1% of the mean value of the initial twich tension). Twitches were elicited by direct stimulation with 2 msec supramaximal pulses via massive platinum electrodes. Tyrode's solution (T) contained (in mmol/l): NaCl — 135; KCl — 5; $CaCl_2$ — 2; $MgCl_2$ — 1; $NaHCO_3$ — 12; glucose — 5.5; pH 7.2. The non-electrolyte solutions referred to as glycerol solution (T + G), or urea solution (T + U) were prepared with Tyrode's solution. The solutions were continuously saturated with a mixture of 95% O_2 and 5% CO_2.

As shown by the control experiments the twich amplitudes of both muscles reached maxima within 60—70 min after the isolation and remained constant for 4—5 h. Most of our experiments proceeded according to the following protocol. Isolated muscles were first incubated for 60—70 min in Tyrode's solution. The solution was subsequently changed for another one containing 200—600 mmol/l of a non-electrolyte (*loading*). After 1 to 1.5 h of incubation in the non-electrolyte solution the muscles were transferred to a normal Tyrode's for 40—80 min (*removal*). In some experiments after the one-hour removal the muscles were returned to T + G or T + U for another two hours (*re-loading*).

Preparations for electron microscopy were fixed after 1.5 h of loading, 1 h of removal, or 1.5—2 h of re-loading, respectively. With the exception of one series the concentrations of non-electrolyte were 400 mmol/l (Table 2). A detailed study of the glycerol effect on EDL muscle was performed. In all of the experimental series the muscles were fixed approx. 4.5 h after they had been isolated. For this purpose, the incubation time of muscles in non-electrolyte-free Tyrode's solution in loading experiments was increased to 3—3.5 h, and to 2—2.5 h in removal experiments.

Muscles were fixed for 2 h in 2.5% glutaraldehyde in 0.1 mol/l phosphate or Na-cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffers. In loading and re-loading experiments 400 mmol/l urea or glycerol were added to the glutaraldehyde solution. For the first 0.5—1 h muscles as a whole were fixed in glutaraldehyde; pieces of 20 to 100 fibres were then cut and the fixation was further continued. The samples were embedded in Araldite and longitudinal sections were prepared.

For the quantitative estimation of the T-system state (Eisenberg and Eisenberg 1968; Franzini-

-Armstrong et al. 1973; Krolenko 1975: Davey et al. 1980) sites with triads, or sites where triads are expected to occur in accordance with the structure of the T-system and the sarcoplasmic reticulum of rat skeletal muscle fibre, were counted on electronograms. These sites were divided into 3 categories, or groups (Fig. 2, 3, 5—10). Category A included typical triads with a normal T-system; category B were triads with an altered morphology with the T-tubules present. This group included swollen, vacuolated, collapsed T-tubules, or triads with an undistinct outline. Category C contained triads without the T-system, or with disrupted T-tubules as well as sites with no triads. The number of sites in each category was expressed as percentual value of the total number of the sites counted. Seventy sites per fibre were counted in average (30—160), distributed throughout the cross section. Seven to 36 fibres per muscle were examined. The values given in both the table and text represent the means of all the fibres tested in a given experimental series.

Results

Twitch changes

Loading. During the loading with glycerol and urea the amplitude of twitches first decreased with a subsequent slow recovery exceeding its original level when EDL muscles were incubated in the urea solution (Table 1, Fig. 1). The twitch amplitude was then maintained at a constant level over the entire time of loading (the maximum loading time was 3 h). These changes were generally similar to those observed in similar experiments in isolated frog and crayfish muscle fibres (Caputo 1968; Krolenko and Fedorov 1972; Zacharová and Uhrík 1978; Krolenko et al. 1980). Urea induced a similar decrease in twitch amplitudes in both muscles; in

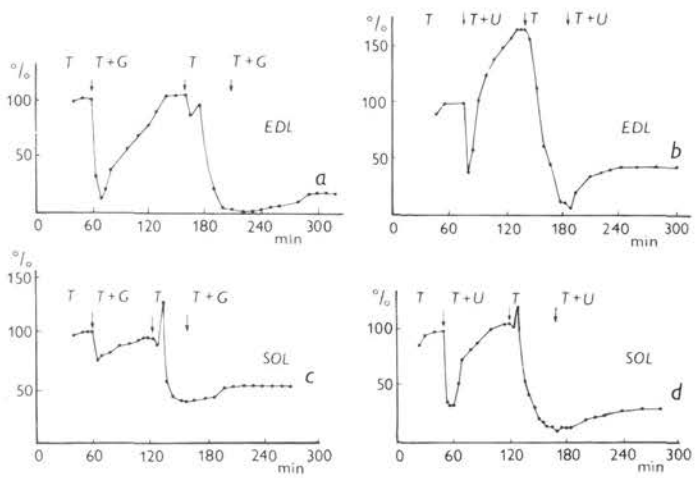

Fig. 1. Changes in the amplitude of isometric twitches of EDL and SOL muscles during loading, removal and re-loading with 400 mmol/l glycerol (*a*, *c*) or urea (*b*, *d*). The zero time corresponds to the transfer of muscles to the chamber. Vertical arrows indicate exchange of solutions. Data derived from individual experiments.

glycerol solutions the reduction and restoration of twitch amplitudes were lesser in SOL compared to EDL (Fig. 1, Table 1). Differences in the behaviour of both muscles could also be detected in hypertonic solutions of sucrose. A $4.0 \pm 1.7\%$ and $17 \pm 1.2\%$ reduction in EDL and SOL twitch amplitude respectively, was observed in solutions containing 100 mmol/l sucrose ($n = 5$). No spontaneous recovery of twitch tension occurred in hypertonic sucrose solutions.

Removal of non-electrolytes. During the first minutes following glycerol or urea removal the time of twitch relaxation increased; a small potentiation of twitches could be observed in SOL muscles. The twitch amplitude gradually decreased reaching minimum values within 40 to 60 min following the initiation of removal (Fig. 1). In the experiments with glycerol the effect was more pronounced in EDL muscle compared to SOL. It should be pointed out that 400 mmol/l of glycerol resulted in a complete disappearance of twitches only in two of 20 EDL muscles tested. The removal of 400 mmol/l urea resulted in a similar twitch decrease in both the EDL and SOL muscles. A prolongation of the time of glycerol removal (to 2—3 h) in some of the experiments revealed that the twitch reduction was constant; only in rare experiments on EDL muscles exposed to 200 mmol/l of glycerol a small (5—10%) restoration of twitches was observed.

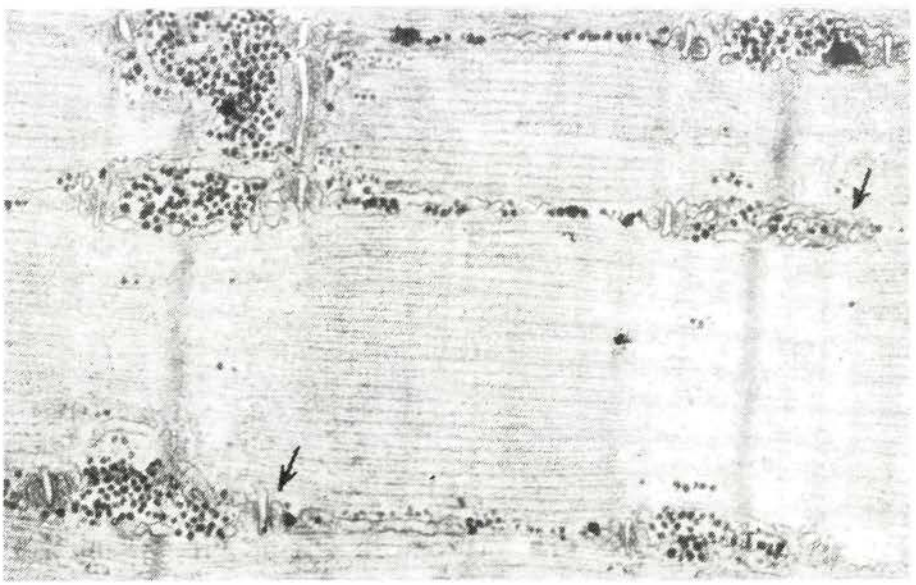

Fig. 2. EDL fibre 1.5 h after loading with 400mmol/l glycerol. Most of the triads have a normal structure (category A). The lower arrow indicates a slightly altered triad (category B). The upper arrow points to a site where the T-tubule is not distinguishable. × 35,000.

Table 1. Effect of glycerol and urea on twitch tension of EDL and SOL muscles

Muscle	Non-electrolyte concentration mmol/l	Minimal twitch tension during loading*	Restored twitch tension during loading*	Twitch tension 1 h after removal*	Twitch tension after 2 h of re-loading*
EDL	200 glycerol	69.9 ± 4.05 (6)**	105.0 ± 2.3 (4)	49.3 ± 5.7 (11)	81.2 ± 4.0 (6)
EDL	400 glycerol	19.3 ± 3.5 (13)	101.3 ± 4.5 (12)	6.2 ± 0.6 (20)	16.5 ± 1.6 (8)
SOL	400 glycerol	80.3 ± 4.2 (5)	90.4 ± 2.8 (5)	44.6 ± 3.3 (5)	52.1 ± 4.9 (5)
SOL	600 glycerol	66.5 ± 6.0 (3)	74.0 ± 9.3 (3)	19.2 ± 1.2 (3)	31.5 (2)
EDL	400 urea	41.4 ± 2.7 (16)	164.3 ± 3.1 (16)	11.0 ± 1.5 (14)	43.5 ± 3.8 (10)
SOL	400 urea	46.6 ± 3.7 (12)	105.9 ± 5.4 (12)	14.8 ± 2.3 (12)	33.9 ± 2.8 (10)

* Changes of twitch tension are presented as percentual value of twitch tension prior to the start of loading
** Mean ± SEM are given. Figures in the parentheses indicate the number of experiments.

Table 2. Effect of glycerol and urea on the T-system of EDL and SOL fibres

Muscle	Non-electrolyte concentration (mmol/l)	Type of experiment	Number of muscles	Total number of fibres	Group A: normal T-tubules	Group B: altered T-tubules	Group C: without T-tubules
					(percent of the total sites counted)		
EDL	400 glycerol	loading	9	131	56 ± 1.2*	19 ± 1.0	25 ± 0.7
EDL	400 glycerol	removal	8	140	8 ± 0.7	37 ± 1.1	55 ± 1.4
EDL	400 glycerol	re-loading	8	156	17 ± 1.4	32 ± 0.9	51 ± 1.5
EDL	500 glycerol	removal	3	47	9 ± 1.9	19 ± 1.3	72 ± 2.3
EDL	400 urea	removal	2	31	12 ± 2.2	39 ± 1.8	49 ± 2.7
SOL		in vivo	2	20	31 ± 1.9	45 ± 1.9	24 ± 1.2
SOL	400 urea	loading	3	22	33 ± 3.3	40 ± 2.0	27 ± 1.2
SOL	400 urea	removal	2	26	7 ± 1.2	47 ± 2.3	46 ± 2.8
SOL	400 urea	re-loading	3	24	17 ± 1.7	44 ± 2.1	39 ± 2.4
SOL	400 glycerol	removal	2	49	8 ± 0.8	52 ± 2.2	40 ± 2.2

* Each value is the mean and SEM of all fibres used in the given type of experiment

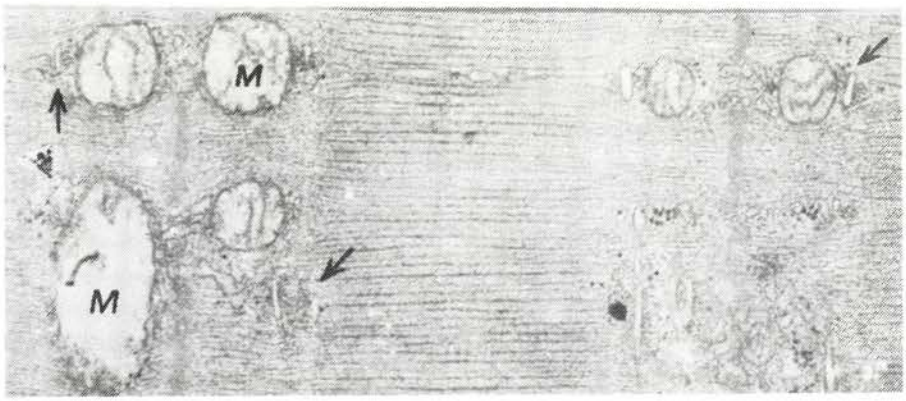

Fig. 3. SOL fibre immediately after the isolation of the muscle. Most of the triads belong to category A. Arrows indicate normal triads and the arrow-head points to a site of C-category. Some mitochondria (M) are swollen and have some of their cristae lost. × 30,000.

Re-loading. When muscles were repeatedly transferred to T + G or T + U one hour after the start of the removal of non-electrolytes a slight decrease followed by a slow increase in twitches could be observed (Fig. 1, Table 1). Within 2 hours of re-loading the twitch amplitude generally increased 1.5 to 4 fold compared to the minimal level after the removal. A complete restoration of twitches to the initial level has never been observed in experiments with 400 mmol/l glycerol or urea.

Miscellaneous. In 4 experiments with 400 mmol/l glycerol short tetanic stimulation was applied to EDL muscles. During the loading and removal the amplitude changed similarly as that of the twitch tension. Several experiments were performed on SOL and EDL muscles with methylurea, dimethylurea, acetamide (400 mmol/l) and ethylenglycole (600—800 mmol/l). The loading with these substances and their removal induced no considerable alteration of the twitch amplitude.

Ultrastructure changes

Control. The ultrastructure of muscles fixed immediatly after isolation (SOL), or incubated after the isolation in T and subsequently in T + 400 mmol/l glycerol (EDL), or T + 400 mmol/l urea (SOL) (Fig. 2,3) did not differ from the norm. Table 2 and Figure 4 show that the T-system is absent in about a quarter of all the sites in both the SOL and EDL fibres. It should be noted that this value may be overestimated since an exact identification of the T-tubules on pure anatomical ground without the use of markers as ferritin or peroxidase is difficult. Normal triads (group A, Table 2) were almost twice as numerous in EDL fibres, as in SOL fibres. In EDL and SOL fibres approximately 50% of the sites classified as group

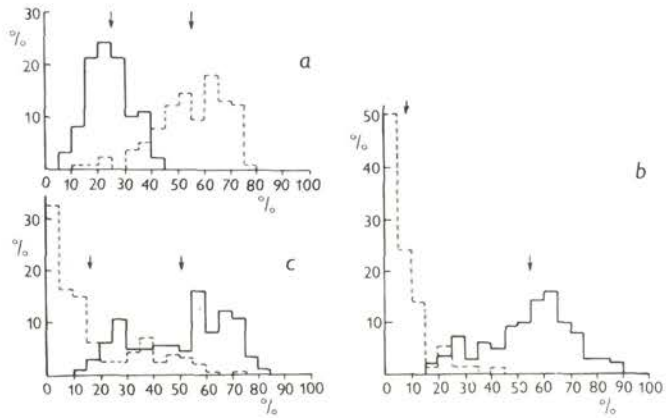

Fig. 4. Distribution of EDL fibres according to the percentage of different group sites in a fibre. The dashed line indicates normal triads (group A), the solid one indicates sites without T-tubules (group C). Vertical arrows point to mean values. *a*) Loading with 400 mmol/l glycerol, *b*) its removal, *c*) re-loading with glycerol. The total number of fibres is given in Table 2.

B (altered T-system) was occupied by swollen T-tubules. They were mostly localized in the surface layer of the fibre. The swelling of the T-tubules was usually local, affecting small regions of the T-system. However, 2—3% of the control fibres were characterized by a total swelling of the T-system. Single small vacoules (up to 1 μm in diameter) could be found in 10—15% of the fibres. They may have been a result of a serious swelling of the T-tubules or originate from mitochondria. It should be noted that single or multiple defects of mitochondria were observed in several preparations: swelling, destruction of some cristae, formation of empty spaces, sometimes filled with membranous profiles (Fig. 3, 7, 8). The extent of the above changes did not correlate with the type of the muscle or with experimental conditions.

Removal. The removal of glycerol and urea from EDL and SOL muscles was accompanied by structural changes, primarily of the T-system, including a serious swelling of the T-tubules leading to the formation of vacuoles and to lysis of the T-system (Fig. 5—9). Table 2 shows that the number of normal triads (group A) was reduced 4 to 7-fold after the removal of non-electrolytes.

After the removal of non-electrolytes more than 70% of the fibres were found to contain numerous vacuoles localized mainly at the boundary of A and I bands (Fig. 5—9). In many cases the vacuoles clearly originated from the T-system. It was sometimes hard to identify the origin of the vacuoles. The possibility that some of them originated from mitochondria could not be ruled out.

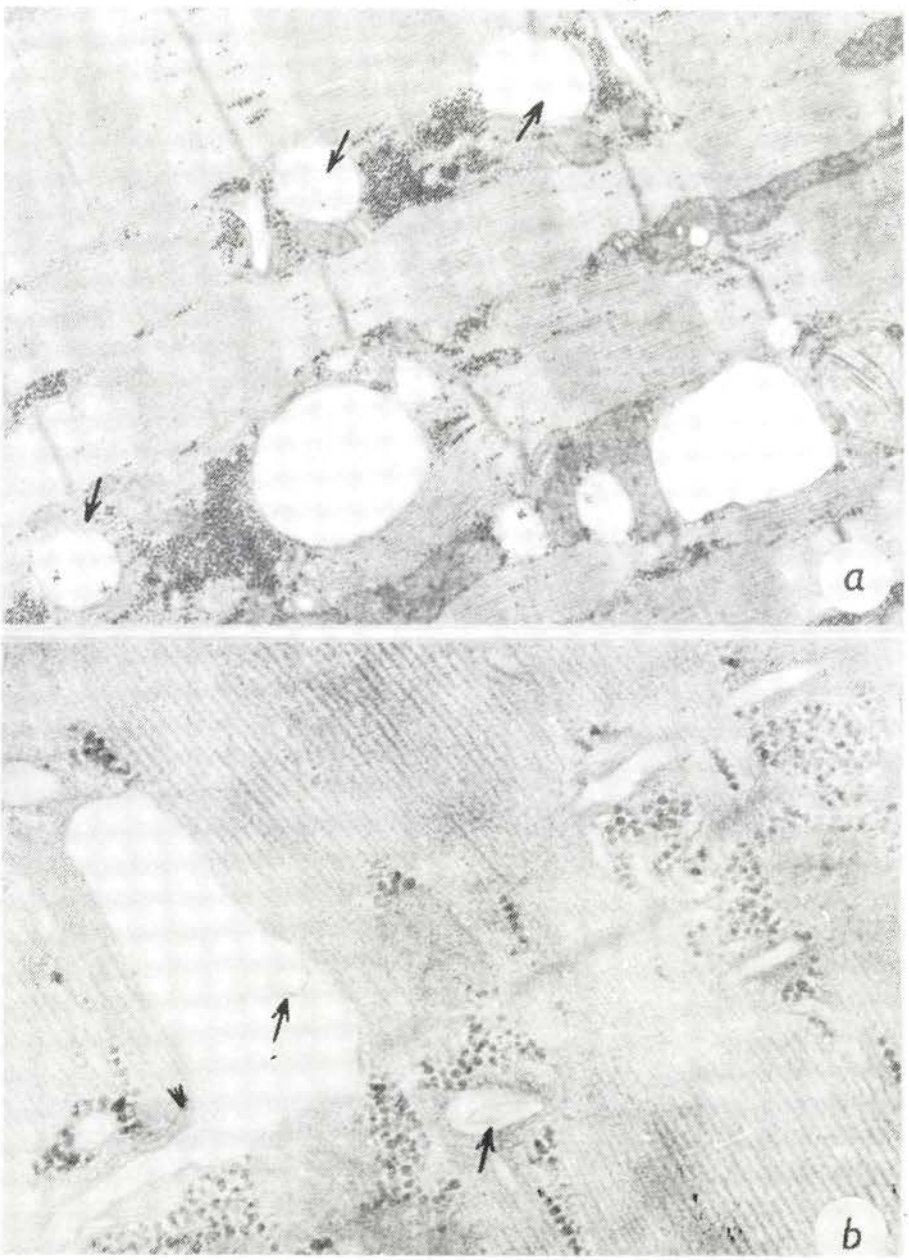

Fig. 5*a*, *b*. EDL fibres 60 min after removal of 400 mmol/l glycerol. Most of the T-tubules are swollen or vacuolated. Some vacuoles contain membranous structures and their walls are broken (arrows). The large vacuole in *b* maintains a direct connection with the slightly swollen T-tubule. *a*) × 22,000: *b*) × 50,000.

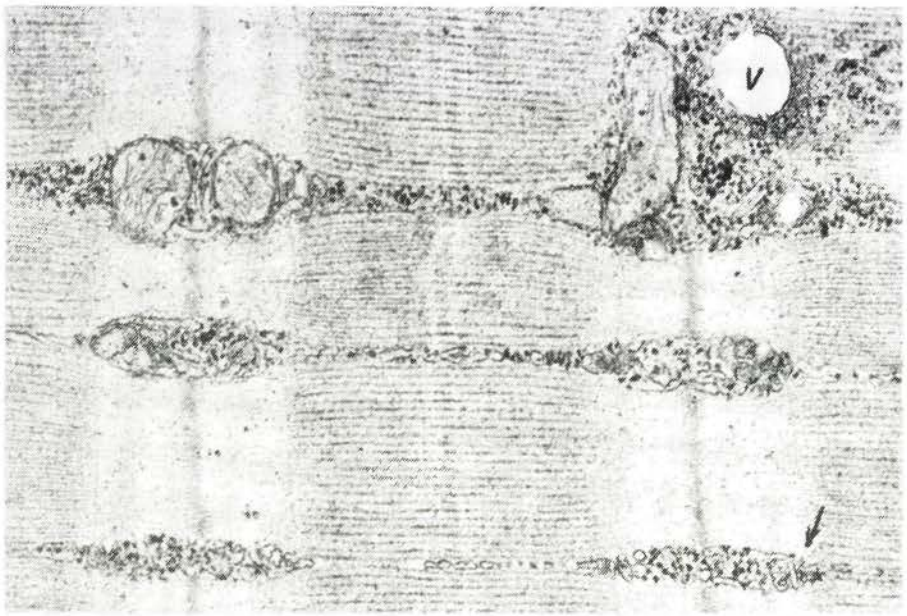

Fig. 6. EDL fibre 70 min after removal of 400 mmol/l glycerol. Most of the sites where the T-system is expected have no tubules. The arrow indicates a normal triad. Swollen T-tubules (V) partly maintain a normal junction with the sarcoplasmic reticulum. × 33,000.

Most of the vacuoles were surrounded by a membrane, their content was structureless and had a low electron density. Vacuoles sometimes contained some membraneous structures (Fig. 5, 7, 8). They seemed to arise either from invaginations of vacuolar wall or from its collapse.

The lysis of the T-system was commonly displayed as a thinning of the T-tubule profiles, their collapse and disruption or a complete disappearance of T-tubules between relatively intact terminal cisternae (Fig. 6—9). Some of the T-tubule profiles had arched or even circular forms. In all experiments with non-electrolyte removal the numbers of triads of category C increased 1.6—2.2 fold (Table 2).

In addition to changes in the T-system, swelling of cisternae of the sarcoplasmic reticulum could be observed in some fibres, most often after the removal of 500 mmol/l glycerol from EDL (50% of the fibres), or 400 mmol/l urea from SOL (23% of the fibres). In the remaining experimental series such fibres constituted less than 10%. There is evidence that the swelling of the sarcoplasmic reticulum is one of the first symptoms of Zenker's degeneration (Krolenko 1975; Krolenko and Rizhamadze 1979). Single necrotic fibres could be observed on the muscle surface after the removal of glycerol or urea even under the dissection microscope. Their

Fig. 7. EDL fibre 60 min after removal of 400 mmol/l urea. Large vacuoles (V) contain numerous membrane profiles. Most of the T-tubules are destroyed. ×29,500.

ultrastructure was characterized either by a strongly contracted sarcomere or by a complete disorganization of the contractile material. These fibres were excluded from the quantitative estimation of changes in the T-system.

Structural changes of the T-system show a high heterogeneity in various fibres of the same muscle after the removal of glycerol or urea (Fig. 4). Very tentatively we should speak about fibres with predominant swelling and vacuolation of T-tubules, and fibres where lysis of the T-system prevails. There are smooth transitions between these two extreme forms of the T-system alteration.

A comparison of the state of T-tubules in EDL muscles after loading them with glycerol and after its removal (Table 2, Fig. 4) shows that the latter involved some degree of alteration of T-tubules in most of the fibres, despite the fact that single fibres with a swollen T-system in the control generally did not differ from fibres with a best preserved T-system after the removal of glycerol. The portion of the fibres in which triads of category A occupied more than 40% of all the sites made up 88% after glycerol loading (400 mmol/l) and only 1.5% after glycerol removal. Sampling of the "best" fibres ($n = 22$) from the experiments with glycerol removal (400 mmol/l) which have the same percentage of C sites (from 5% to 45%) as in experiments with glycerol loading yields the following pattern of triad distribution: Category A — 21%, B — 49%, C — 30%. A comparison of these

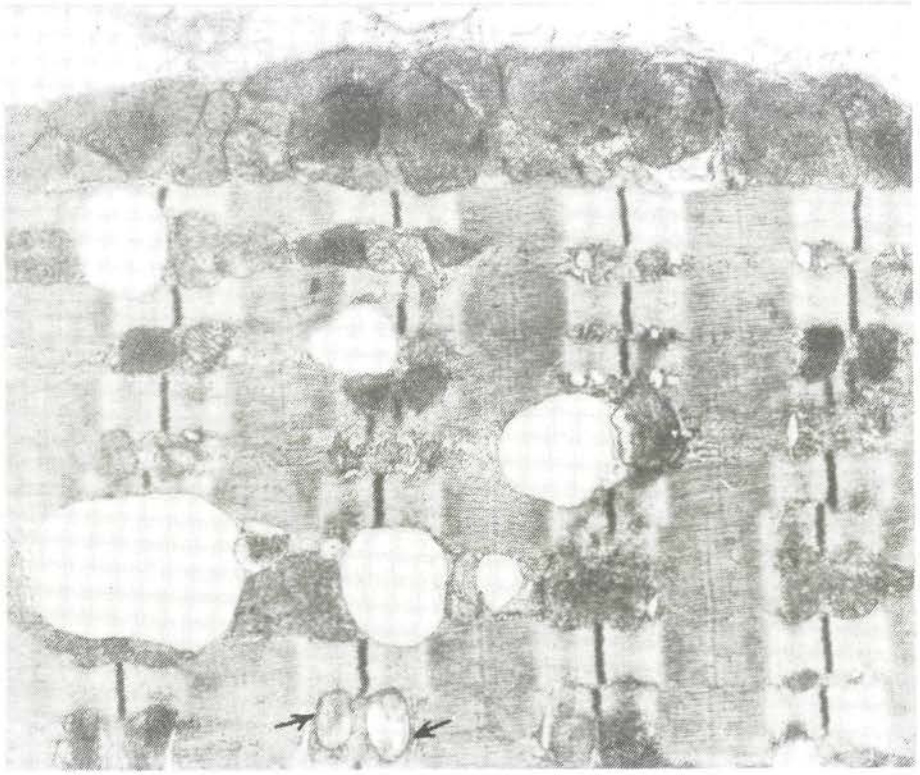

Fig. 8. SOL fibre 60 min after removal of 400 mmol/l urea. Vacuolation and swelling of the T-system. Single mitochondria are swollen (arrows). × 11,500.

values with the first line in Table 2 shows that even in this group of fibres the decrease in the number of normal triads is conspicuous.

Re-loading. The ultrastructure of the fibres after re-loading of muscles in a glycerol or urea solution did not differ qualitatively from that after the removal (Fig. 10). The numbers of vacuolized fibres decreased after glycerol re-loading from 76% to 57% (compared to the removal series) and after urea from 73% to 37%. The intensity of vacuolation was also reduced. Table 2 and Fig. 4 show that re-loading led mainly to an increase of the group A (normal triads). Also shifts within group B in experiments with EDL muscles proved to be statistically significant. The distribution of fibres according to the percentage of both the A and C category tends to fall into two groups: fibres with a predominant lysis of the T-system and fibres with a relatively normal structure of the T-system (Fig. 4,10). The percentage of fibres in which normal triads occupy more than 40% of all the sites

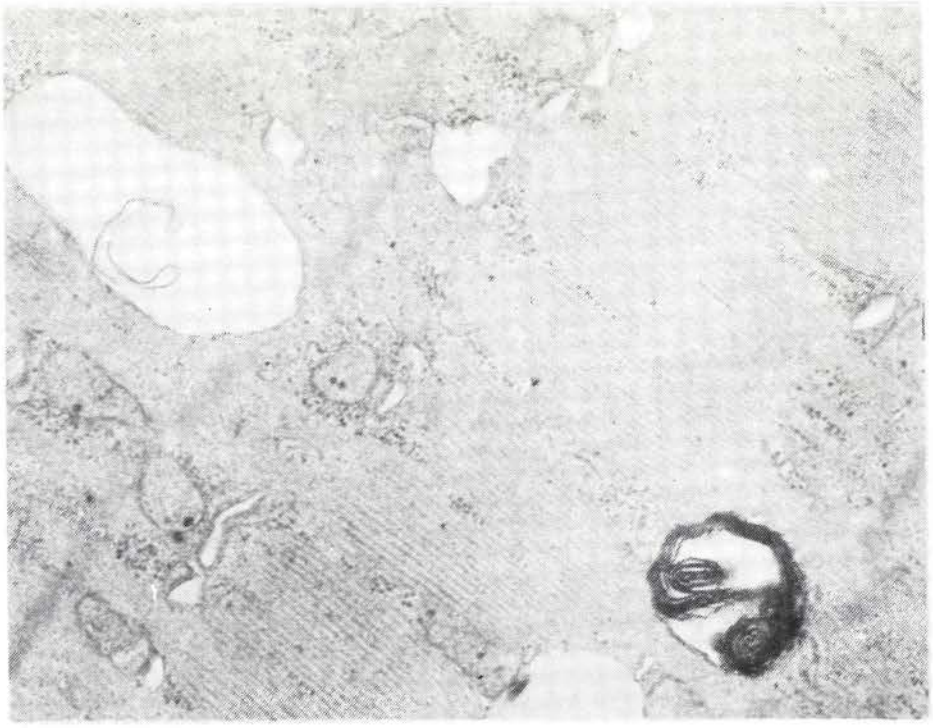

Fig. 9. SOL fibre 60 min after removal of 400 mmol/l glycerol. Vacuolation and swelling of the T-system. × 35,000.

increases from 1.5% to 14% after glycerol re-loading. This indicates that re-loading tends to normalize the T-system structure in a relatively small portion of fibres. Alteration of T-tubules may be even more intense in the majority of fibres.

Discussion

In general, the results of our investigation agree with the data of similar experiments by other authors. It was shown that the efflux of glycerol or urea may induce alternation in the T-system and inhibit the contractility of fast and slow muscle fibres of the rat.

Many authors who had studied the glycerol removal effect (Franzini–Armstrong et al. 1973; Krolenko 1975; Zacharová et al. 1978; Dulhunty 1979; Davey et al. 1980) reported a significant variability of morphological and functional changes even under standard experimental conditions and with single fibres. In our experiments changes of the T-system ranged from slight swelling of the T-tubules to complete lysis. Structural variations between separate fibres were found to be

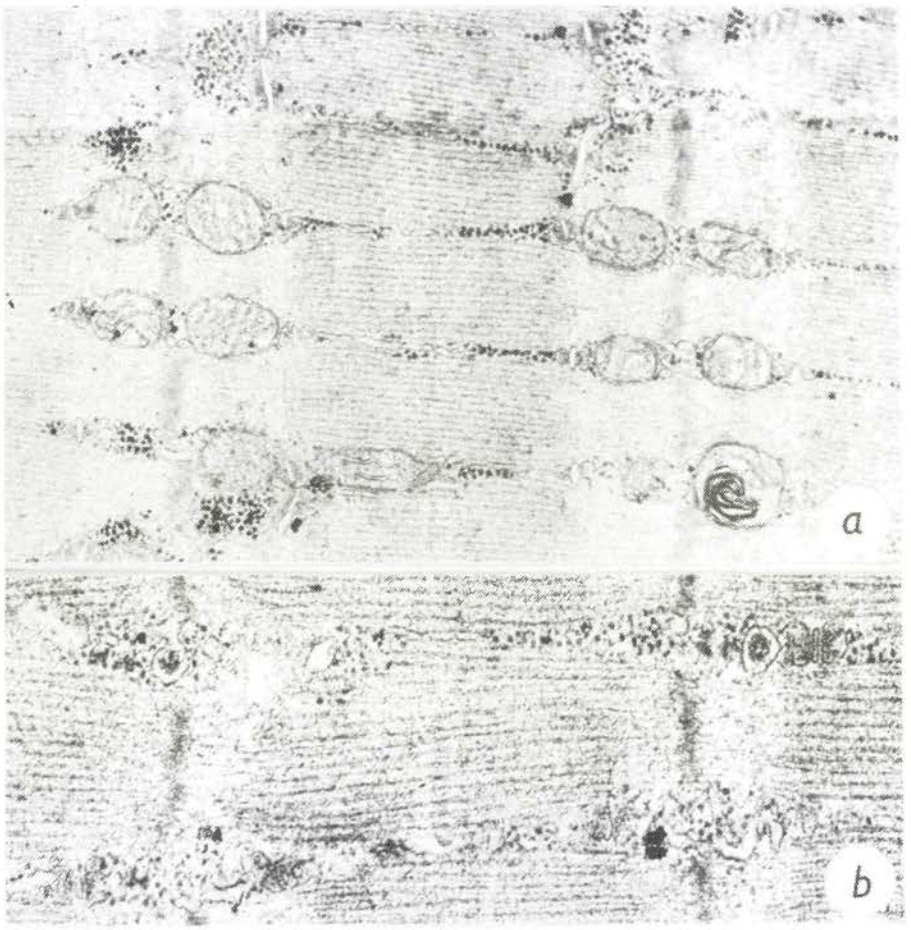

Fig. 10. EDL fibres after 1.5 h of re-loading with 400 mmol/l glycerol. *a*) A fibre with predominantly normal triads. × 32,000. *b*) A fibre with predominant lysis and alteration of the T-system. × 45,000.

much greater than within one fibre. Due to this the condition of the T-system in many muscle fibres was examined in comparing morphological and functional changes. Despite a semiquantitative rough estimation of changes in the T-system a good correlation between the T-system state and the contractility both during removal of non-electrolytes and after re-loading was obtainted (Table 1,2). The number of sites without T-tubules and that of normal triads were found to be the most sensitive indices for the removal and re-loading experiments, respectively. Based on the T-system alteration and inhibition of twitches the sequence of experiments with removal of 400 mmol/l glycerol and urea is as follows: EDL, glycerol > EDL, urea > SOL, urea > SOL, glycerol. If differences between the first three members

of the row are small removal of glycerol from a slow muscle produces a much weaker effect. Even an increase in glycerol concentration to 600 mmol/l does not level the difference. The low sensitivity of SOL muscles to glycerol removal may likely be ascribed to a greater permeability of the slow muscle fibres to this substance. As demonstrated on frog muscles, the greater the fibre permeability to non-electrolyte the lesser the effect of its removal on excitation-contraction coupling (Krolenko 1975). We failed to observe twitch inhibition after removal of ethylenglycol, acetamide or methylated urea. As judged by the size of their molecules and the degree of their lipid solubility these substances must penetrate the plasma membrane faster than do glycerol and urea. As has been recently reported slow fibres of rodents are more permeable to sugars than the fast ones (Bonen et al. 1981). The pattern of twitch changes during muscle loading with glycerol (Fig. 1) may also indicate a higher permeability of the slow fibres. Keeping in mind our own data and findings of other authors (Kovács et al. 1970; Clausen et al. 1979) that twitches of slow muscles are less sensitive to hypertonic solution than fast muscles such kind of evidence should however be interpreted with care.

A most detailed study on the effect of glycerol removal in mammals was made by Davey et al. (1980). They used bundles of fast-twitch red fibres from rat sternomastoid muscles. Despite the unusual type of fibres described in this muscle (Dulhunty and Dlutowski 1979) and the essential differences in the experimental conditions (whole muscle vs. isolated bundle, animal age, temperature, type of stimulation) our data on removal of 400 mmol/l urea from SOL and 400 mmol/l glycerol and urea from EDL were similar to the results of the above authors. The percentage of normal triads was reduced to 15% of the control values in the experiments with removal of 400 mmol/l glycerol from EDL muscle and 350 mmol/l glycerol from sternomastoid red bundle. However we cannot agree with the conclusion of Davey et al. (1980) who suggest a greater sensitivity of mammalian fast muscles to glycerol removal as compared to amphibian muscles. Under similar experimental conditions alterations of the T-system of EDL fibres after removal of 400 mmol/l glycerol were found to be similar to those described for frog sartorius fibres (Franzini–Armstrong et al. 1973). The number of sites without T-tubules increases 2.2 and 3.2 times and the percentage of normal triads is reduced to 14 and 33 respectively. It should be noted that a complete twitch inhibition occurs in isolated frog fibres during removal of approximately 100 mmol/l glycerol (Zachar et al. 1972; Krolenko 1975). In rat fibre bundles the weakest glycerol concentration used (200 mmol/l) did not always induce a complete excitation-contraction uncoupling (Davey et al. 1980). Some morphological peculiarities of the T-system alterations during glycerol removal (good preservation of local widening of T-tubules, direct communication of large vacuoles with normal tubules) are likely to indicate a greater resistance of the mammalian T-system to osmotic shock as compared with amphibians.

It is difficult to conclude from the above experiments with whole muscles which of the alterations of the T-system are responsible for the excitation-contraction uncoupling. As suggested earlier it may be either the destruction of T-tubules or the breake in their contacts with the surface membrane (Krolenko 1975). Davey et al. (1980) support the idea that, during removal of comparatively small glycerol concentrations, inhibition of isometric contractions is not necessarily accompanied by changes in the muscle fibre capacity and that the T-system remains accessible to horseradish peroxidase. In our experiments on glycerol removal a certain degree of the T-system disintegration was typical for even the "best" fibres. A partial restoration of twitches after re-loading with glycerol is followed by an increase in the number of fibres with a high percentage of normal triads. In our opinion, these data stress the role of the T-system integrity in maintaining excitation-contraction coupling. As for the swelling and vacuolation observed in sarcoplasmic reticulum of some fibres during glycerol removal (Davey et al. 1980) we tend to regard these phenomena as manifestations of Zenker's necrosis resulting from fibre injury, and not as a specific feature of a non-electrolyte removal.

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