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CHANGES IN THE ACTIVITY OF SARCOPLASMIC RETICULUM FRAGMENTS AND ACTOMYOSIN ISOLATED FROM SKELETAL MUSCLE OF THYROXINE-TREATED CATS

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SUMMARY

1. Sarcoplasmic reticulum fragments (SRF) and actomyosin were isolated from skeletal muscle of cats treated either with thyroxine or with placebo tablets, for 5–16 months.

2. Ca^{2+} uptake and binding by SRF, measured in the presence and absence of oxalate respectively, were reduced by thyroxine treatment.

3. Actomyosin from the thyroxine-treated animals underwent ATP-induced syneresis at a faster rate than did that from the controls.

4. Syneresis of the actomyosin preparations from controls and treated animals was inhibited to the same extent by EGTA when rates of syneresis were made the same by adjustment of the KCl concentration in the assay media. In contrast, at any given KCl concentration, syneresis of actomyosin from thyroxine-treated animals was inhibited to a lesser extent by EGTA.

5. At the end of the isolation procedure, the amount of Ca^{2+} remaining in the actomyosin suspension was similar for both treated and control animals.

6. It was concluded that the effect of thyroxine on skeletal muscle may be the result of an action on both the sarcoplasmic reticulum and the contractile protein complex.

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INTRODUCTION

There is considerable clinical evidence that skeletal muscle can be affected by thyroid hyper- or hypofunction (Grob, 1963). For example, 80% of human patients with thyrotoxicosis were shown by Havard, Campbell, Ross & Spence (1963) to display physical evidence of muscle weakness and electromyographic signs of myopathy. Furthermore, many workers believe that the heart muscle itself may be affected by hyperthyroidism (Werner, 1962; Hoch, 1962). Control of the hyperthyroidism is consistently followed by the patient's complete recovery from the myopathy.

It was once suggested (Plummer, 1926) that some of the symptoms of thyrotoxicosis might be due to the production of a qualitatively abnormal hormone; but there is no evidence that any circulating thyroid hormone – other than L-thyroxine and L-triiodothyronine – is produced in this disorder (Dingledine, Pitt-Rivers & Stanbury, 1955; Maclagan, Bowden & Wilkinson, 1957). And indeed, the chronic administration of L-thyroxine to animals gives rise to abnormalities in both skeletal and heart muscle (Zaimis, Metaxas, Havard & Campbell, 1965; Zaimis, Papadaki, Ash, Larbi, Kakari, Matthew & Paradelis, 1969).

These animal studies provided evidence that the changes in skeletal muscle induced by thyroxine occurred in the muscle fibre itself, and not at the neuromuscular junction (Zaimis *et al.* 1965). It was therefore decided to examine both (*a*) SRF and (*b*) actomyosin isolated from skeletal muscle of thyroxine-treated cats.

The coupling of the electrical impulse with the contraction of muscle is thought to be initiated by the release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Weber, 1966; Ebashi & Endo, 1968). During repolarization, Ca^{2+} is presumably actively reaccumulated by the SR, and the muscle relaxes. The SR was originally isolated from skeletal muscle homogenates as a light particulate fraction consisting mainly of fragmented membranes in vesicular form (Ebashi & Lipmann, 1962; Martonosi, 1968; Deamer & Baskin, 1969; Greaser, Cassens, Hoekstra & Briskey, 1969). In the presence of ATP and Mg^{2+}, the SRF accumulate Ca^{2+} from the surrounding medium, thereby providing a model of the relaxation process (Nagai, Makinose & Hasselbach, 1960; Ebashi, 1961; Muscatello, Andersson-Cedergren & Azzone, 1962; Weber, Herz & Reiss, 1963). In the present experiments the contractile protein complex was isolated as an actomyosin gel which, in dilute solution, forms a network of randomly orientated threads. The addition of ATP induces an isodimensional contraction, or shrinkage: a process known as syneresis, or superprecipitation (Szent-Györgyi, 1951). Although the random

orientation of isolated actomyosin threads contrasts with the highly ordered state of the myofilaments *in vivo*, syneresis provides a convenient means of examining some of the contractile properties of actomyosin.

METHODS

Treatment of animals

Cats of either sex, whose body weights ranged between 2 and 2·8 kg, were treated orally with L-thyroxine (0·3 mg/day; eight cats) or with placebo tablets (eight cats) for 5–16 months. Subsequently, L-thyroxine will be referred to as thyroxine. Under treatment, both groups of animals gained weight and appeared healthy: the mean increase in weight for the thyroxine-treated cats was 48 % and for the controls 52 %. For each experiment we selected a pair of cats, one thyroxine-treated, one control; both of which had undergone identical periods of treatment.

It is noteworthy that, although the treatment with thyroxine ranged between 5 and 16 months, the changes in SRF and actomyosin had appeared by the end of the shorter period and prolongation of treatment produced no further qualitative of quantitative change.

Sarcoplasmic reticulum fragments (SRF)

Isolation. Hind-limb muscles were removed under chloralose anaesthesia (80 mg/kg I.V.) and immediately immersed in ice-cold 0·9 % (w/v) NaCl solution. SRF were subsequently isolated at 0–4° C by a method similar to that of Harigaya & Schwartz (1969). After the removal of loose fat and connective tissue about 15 g of muscle was cut into small pieces and dropped into 60 ml. of a solution containing 10 mM-$NaHCO_3$ and 5 mM-Na azide. The tissue suspension was homogenized in four aliquots with an Ultra-Turrax processor (2 × 5 sec at 20,000 rev/min) and the homogenate centrifuged at 7000 *g* for 20 min. The pellet, stored in ice, was set aside for the preparation of actomyosin. The supernatant was centrifuged at 10,000 *g* for 20 min, the pellet discarded and the supernatant then centrifuged at 37,000 *g* for 30 min. The resulting pellet was suspended in 0·6 M-KCl solution to solubilize any residual actomyosin; and the suspension was recentrifuged at 37,000 *g* for a further 30 min. The final pellet was suspended in a solution containing 50 mM-KCl and 20 mM-Tris-maleate buffer (pH 6·8). This fraction mainly consists of fragmented SR in vesicular form (Ebashi & Lipmann, 1962; Martonosi, 1968; Greaser *et al.* 1969). Protein was determined by a biuret method (Gornall, Bardawill & David, 1949) with the addition of 1 % Na deoxycholate to solubilize the protein.

Assay of Ca^{2+} accumulation by SRF. The term 'Ca^{2+} accumulation' has been used to refer either to Ca^{2+} binding or to Ca^{2+} uptake, or to both (Carvalho, 1968; Fuchs, Gertz & Briggs, 1968; De Meis, Rubin-Altschul & Machado, 1970). In this paper, Ca^{2+} *binding* is defined as the ATP-dependent accumulation of Ca^{2+} by skeletal muscle SRF when the initial Ca^{2+} concentration of the medium is about 10^{-5} M. At 25° C, Ca^{2+} binding increases rapidly, reaches a peak during the first 2 min of incubation, and then decreases with the progressive release of Ca^{2+}. Ca^{2+} *uptake* is defined as the ATP-dependent accumulation of Ca^{2+} by SRF in the presence of oxalate, at an initial Ca^{2+} concentration of $1·1 \times 10^{-4}$ M. Because the SR vesicles are freely permeable to oxalate the intravesicular concentration of oxalate is, according to Weber, Herz & Reiss (1966), the same as that of the assay medium. When Ca^{2+} is actively transported by the SRF, the concentration of Ca^{2+} within the vesicles increases and calcium oxalate precipitates. The formation of Ca oxalate

limits the rate of Ca^{2+} efflux from the vesicles, thus rendering Ca^{2+} uptake virtually irreversible.

In the present experiments, both Ca^{2+} binding and Ca^{2+} uptake were used to assess the ability of SRF to accumulate Ca^{2+}. The assay medium contained (mM): KCl 100; $MgCl_2$ 10; Na_2ATP 2; Tris-maleate buffer (pH 6·8) 20; in a final volume of 4 ml. For the Ca^{2+} binding experiments the medium also contained 24 μM-[^{45}Ca]$CaCl_2$ and 50 μg SRF protein/ml.; for Ca^{2+} uptake, the medium contained 111 μM-[^{45}Ca]$CaCl_2$, 10 μg SRF protein/ml. and 5 mM Na oxalate. After temperature equilibration (25° C, binding; 37° C, uptake), ATP was added. All preparations were also incubated without ATP; ATP-independent Ca^{2+} accumulation was usually less than 10% of the total. Following 0·5–30 min incubation, aliquots (0·5 ml.) were withdrawn and filtered through Millipore filters of 0·45 μm pore diameter (Martonosi & Feretos, 1964).

The calcium retained by SRF was estimated by counting (by means of a Nuclear Chicago liquid scintillation spectrometer) the radioactivity of both the filter and filtrate in a dioxane-based scintillation fluid containing 2,5-diphenyloxazole, 6 g/l.; 1,4-di-[2-(5-phenyloxazolyl)]-benzene, 50 mg/l.; and 2-ethoxyethanol, 20 g/l. Sufficient counts were taken to ensure < 3% probable error.

Adenosine triphosphatase (ATPase) activity of SRF. The Ca^{2+}-activated ATPase was determined by measuring the release of inorganic orthophosphate (P_i) from ATP at 25° C. P_i was measured by the method of Fiske & Subbarow (1925). The reaction medium, containing 50 μg SRF protein/ml., was the same as that used for measurement of Ca^{2+} uptake, except that alternate tubes contained 0·1 mM-ethylene-glycol-*bis*-(β-aminoethylether)-N,N′-tetraacetic acid (EGTA).

Actomyosin

Isolation. Actomyosin was prepared from the same muscle samples by a method adapted from those of Ebashi & Ebashi (1964) and Endo (1964). Centrifuge tubes and glassware were pre-cooled in ice, and the entire preparation was carried out at 0–4° C. Double glass distilled water was used throughout and great care was taken to minimize Ca^{2+} contamination.

The actomyosin-containing pellets from the first 7000 *g* centrifugation of the SRF preparation (see above) were suspended in distilled water, to give a total volume of 40 ml.; to this suspension 1 M-KCl was added to bring the final KCl concentration to 0·6 M. After 20 hr at 0° C the viscous suspension was centrifuged at 30,000 *g* for 20 min. The supernatant was gently decanted; diluted slowly (with 2 volumes of 0·3 mM-$NaHCO_3$) to give a final KCl concentration of 0·2 M; and then centrifuged at 6000 *g* for 20 min. After aspiration of the supernatant, the soft pellet was stirred with 2 volumes of 0·8 M-KCl and centrifuged at 37,000 *g* for 30 min. Three volumes of 0·3 mM-$NaHCO_3$ were then added slowly to the supernatant over a period of 20 min, while mixing with a magnetic stirrer. The large volume and the slow addition ensured uniform flocculation of the protein. The actomyosin was then collected by centrifugation at 5750 *g* for 20 min and was suspended, by stirring, in approximately 10 ml. of 1 mM-dithiothreitol. Air bubbles were removed by aspiration from the surface of the suspension, after centrifugation at 500 *g* for 5 min. The protein concentration was usually within the range of 10–15 mg/ml. All assays were carried out within 4 hr of isolation.

Syneresis. An actomyosin gel, prepared by the above method, contains Ca^{2+} and undergoes shrinkage, or syneresis, when ATP is added (Szent-Györgyi, 1951). In the present experiments syneresis has been measured by continuously recording, at 25° C, the changes in extinction at 660 nm (Ebashi, 1961). The assay medium contained 0·5–0·8 mg actomyosin/ml. and the following salts (mM): KCl 35–65; $MgCl_2$ 1; Tris-maleate buffer (pH 7·0) 20; Na_2 ATP 1; and EGTA 0·01–0·03. Control

measurements were made in the absence of EGTA. Syneresis, initiated by the sequential addition of ATP to each cuvette at 1 min intervals, was recorded with a Unicam SP 500 Series II spectrophotometer.

To estimate the inhibition by EGTA of syneresis of actomyosin, isolated from control and thyroxine-treated animals, the time required for maximum syneresis was measured in the presence and absence of EGTA. The extinction recorded at half this time ($t_{\frac{1}{2}}$) was then calculated in each case. The inhibition in the presence of EGTA at $t_{\frac{1}{2}}$ was then expressed as a percentage of the inhibition in the absence of EGTA.

In experiments where SRF were used to lower free Ca^{2+} concentrations, the extinction due to the SRF *per se* was subtracted from each measurement of the total extinction of the suspension.

In order to assess the amount of Ca^{2+} present in the actomyosin preparations, the suspension was buffered at 0·3, 1·0 or 2·5 μM-Ca^{2+}. The estimated Ca^{2+} concentrations were based on a Ca^{2+} binding constant of EGTA of 5×10^5 M^{-1} at pH 6·8 (Ebashi & Endo, 1968; Ogawa, 1968).

Statistics

Regression analyses and correlation coefficients (r) were calculated by the method of least squares. The significance of the differences between the slopes of the lines of best fit and between means were tested by Student's t-test. Vertical distances between parallel lines were calculated by analysis of covariance. An Olivetti Programma 101 computer was used for the calculations.

RESULTS

Sarcoplasmic reticulum fragments (SRF)

Accumulation of Ca^{2+}. ATP-dependent Ca^{2+} binding and uptake were studied in SRF isolated from skeletal muscle of cats treated with either thyroxine or placebo tablets. After the addition of ATP, *Ca^{2+} binding* developed rapidly over the first $\frac{1}{2}$ min and then increased more slowly until a peak was reached at about 2 min (Fig. 1*a*). The amount of bound Ca^{2+} then declined as Ca^{2+} was progressively released. The over-all Ca^{2+} binding by SRF isolated from the control cats was always greater than that by SRF from the thyroxine-treated animals. The differences were, however, only significant after peak Ca^{2+} binding levels had been reached. For example, 8 min after the addition of ATP, during the release phase, the Ca^{2+} levels were (control) 91 ± 13 and (thyroxine) 57 ± 11 n-mole/mg protein ($P < 0{\cdot}01$). Although the initial Ca^{2+} release (at 2–4 min) by SRF from the thyroxine-treated animals appears greater than that of the controls (Fig. 1*a*), the over-all release rates were shown to be similar by the regression equations of the exponential time course of Ca^{2+} release ($r > 0{\cdot}9$) (Fig. 1*b*). The calculated lines did not vary significantly from parallel. The total amount of Ca^{2+} bound by the SRF from thyroxine-treated cats was lower than in controls during this release phase, as is illustrated by the significantly different intercepts with the ordinate ($P < 0{\cdot}01$).

Ca^{2+} uptake by SRF, measured for a period of 30 min in the presence of 5 mM-Na oxalate, showed that thyroxine treatment results not only in a slower rate of Ca^{2+} accumulation but also in a decrease in the ability of SRF to sequester Ca^{2+} (Fig. 2). In both cases, Ca^{2+} uptake over the first 8 min followed an exponential course ($r > 0{\cdot}98$), the difference in the slopes of the calculated regression lines being highly significant ($P < 0{\cdot}001$). Eight min after the addition of ATP, there was a 57 % difference between Ca^{2+} uptake by SRF from the control and thyroxine-treated animals ($8{\cdot}5 \pm 0{\cdot}6$ and $3{\cdot}7 \pm 0{\cdot}6$ μmole Ca^{2+}/mg protein respectively; $P < 0{\cdot}001$) which subsequently remained almost unchanged.

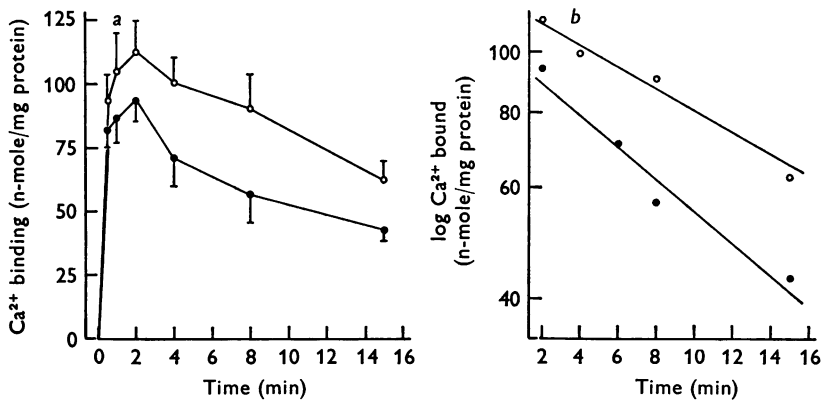

Fig. 1. Ca^{2+} binding by SRF isolated from skeletal muscle of control (○) and thyroxine-treated (●) cats, at 25° C. The assay medium contained 50 μg SRF protein/ml. and (mM): [^{45}Ca]$CaCl_2$ 0·024; KCl 100; $MgCl_2$ 10; Na_2ATP 2; Tris-maleate buffer (pH 6·8) 20. In (*a*) mean values and S.E. of mean are shown at time intervals measured from the addition of ATP. Differences are significant ($P < 0{\cdot}5$) after 2 min. In (*b*) the data from the release phase are plotted on a log scale. The calculated regression lines ($r > 0{\cdot}9$) indicate an over-all similarity in rates of Ca^{2+} release and a significant difference in the average amount of Ca^{2+} remaining bound ($P \leqslant 0{\cdot}01$).

Both binding and uptake of Ca^{2+} were therefore reduced by the thyroxine treatment.

Ca^{2+}-ATPase activity of SRF. Ca^{2+}-stimulated ATPase activity (on which Ca^{2+} accumulation depends) was not significantly different in SRF from control and thyroxine-treated animals ($5{\cdot}0 \pm 0{\cdot}8$ and $4{\cdot}6 \pm 0{\cdot}7$ μmole P_i/mg.hr) (Table 1). In the presence of 0·1 mM-Ca^{2+} the enzyme is maximally stimulated, while 0·1 mM-EGTA reduces the enzyme activity to its basal Mg^{2+}-stimulated level. The Ca^{2+}-ATPase activity is then expressed as the difference between the activity in the presence of Ca^{2+} and that in the presence of the chelating agent.

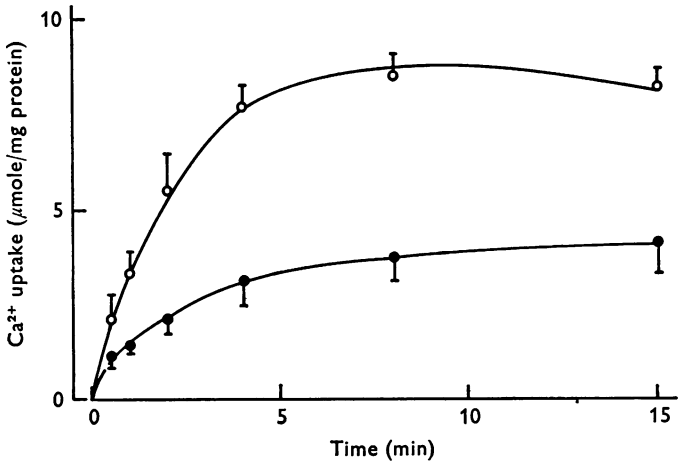

Fig. 2. Ca^{2+} uptake by SRF isolated from skeletal muscle of control (○) and thyroxine-treated (●) cats, at 37° C. The assay medium contained 10 μg SRF protein/ml. and (mM): [^{45}Ca]$CaCl_2$ 0·11; Na oxalate 5; other salts as in Fig. 1. Means and S.E. of mean are significantly different ($P \leqslant 0{\cdot}1$) at incubation times longer than 0·5 min.

TABLE 1. Mean values and S.E. of mean of ATPase activities of SRF

	SRF		
	(*a*) Total	(*b*) + EGTA	(*a* − *b*) Ca^{2+} stimulation
Control (8 cats)	11·4 ± 1·2	6·5 ± 0·8	5·0 ± 0·8
Thyroxine-treated (8 cats)	12·3 ± 1·1	7·6 ± 0·8	4·6 ± 0·7

ATPase activities are expressed as μmole P_i/mg per hour measured in the presence of Ca^{2+} and Mg^{2+} (*a*) without and (*b*) with EGTA. An estimate of Ca^{2+} stimulation was obtained by subtraction (*a* − *b*).

Skeletal muscle actomyosin

Syneresis at 55 mM-KCl. It is well known that immediately after the addition of ATP to actomyosin suspensions, syneresis increases rapidly. We were unable to record the changes in extinction during the first 5 sec, because this time was required for mixing ATP with the suspension. After 5 sec, however, the increase in extinction of actomyosin isolated from the thyroxine-treated animals (ΔE, 0–6 sec: 0·395) was much greater than that from the controls (ΔE, 0–6 sec: 0·205; $P < 0{\cdot}001$) (Fig. 3). Thereafter, the two actomyosin preparations underwent syneresis at similar rates until a maximum degree of syneresis was reached. Because of the initial differences in rate, the degree of syneresis in actomyosin from

the treated cats was consistently greater than that of actomyosin from the control animals. For example, there was a 27 % difference in extinction between the two actomyosin samples at 14 min, the half-time required for maximal syneresis in the controls ($t_{\frac{1}{2}}$). The final level of syneresis was the same for both preparations, but it was reached approximately 8 min earlier in the actomyosin which had been isolated from the treated cats.

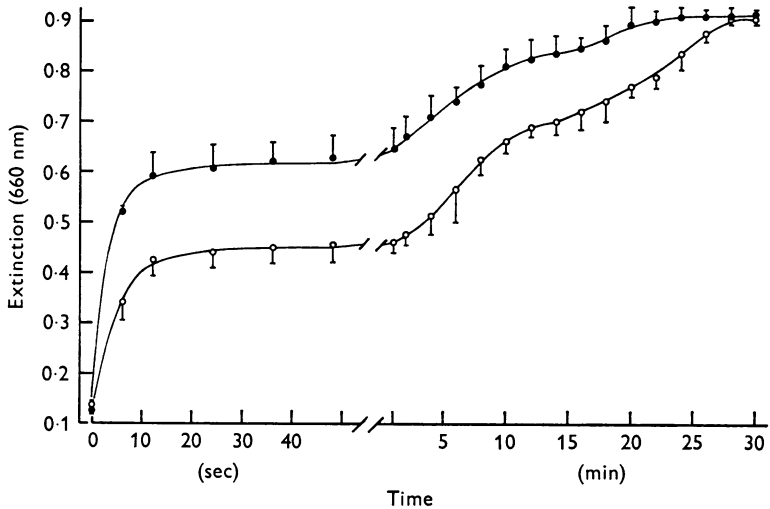

Fig. 3. Syneresis of actomyosin, isolated from skeletal muscle of control (○) and thyroxine-treated (●) cats, at 55 mM-KCl. The assay medium contained 0·5–0·8 mg actomyosin/ml. and (mM): KCl 55; $MgCl_2$ 1; Na_2ATP 1; Tris-maleate buffer (pH 7·0) 20. Incubation temperature = 25° C. In the left-hand panel the time scale is expanded to show more clearly changes in extinction during the first minute. Means and S.E. of mean are significantly different ($P \leqslant 0{\cdot}1$) during the first 20 min.

Syneresis at various concentrations of KCl. By varying the KCl concentration of the medium – and thus the ionic strength – it is possible to alter the rate of syneresis of actomyosin (Weber & Herz, 1963). In our experiments, an increase in the ionic strength of the assay medium slowed down the initial fast rate of syneresis, but accelerated the subsequent slow phase occurring after 1 min (Fig. 4). The over-all effect was that actomyosin approached maximal syneresis more slowly when the ionic strength was higher. This was particularly noticeable when the KCl concentration was increased from 40 to 55 mM in the control actomyosin and from 50 to 60 mM in that prepared from the thyroxine-treated cats. At 65 mM-KCl the initial fast phase of syneresis of actomyosin from the treated animals was completely suppressed and the onset of syneresis delayed. Despite the fact that syneresis of actomyosin from both control

and thyroxine-treated animals was similarly affected by variation of ionic strength, the extent of syneresis, at the same concentration of KCl (60 mM) was greater in actomyosin from the thyroxine-treated cats. This is also supported by the results illustrated in Figs. 3 and 7.

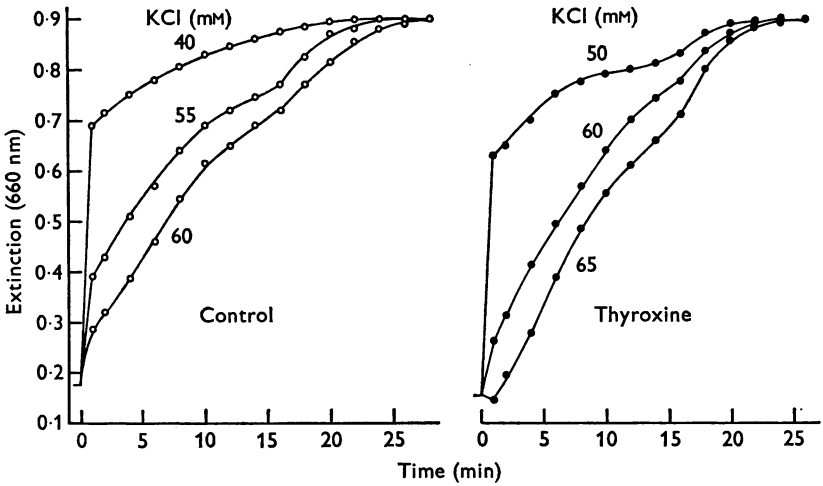

Fig. 4. Effect of KCl concentration on syneresis of actomyosin; syneresis developed more slowly when the KCl concentration was increased. Experimental conditions, except for KCl concentration, were as described in Fig. 3.

Inhibition of syneresis by SRF. Syneresis of actomyosin can be inhibited by lowering the free Ca^{2+} concentration of the assay medium (Ebashi & Endo, 1968). In preliminary experiments, Ca^{2+} concentrations were reduced by adding SRF to the actomyosin suspensions immediately before ATP was added. Two concentrations of SRF were used to measure the inhibition of actomyosin syneresis. At 125 μg SRF protein/ml., syneresis was completely inhibited for 10 min after a short-lasting initial rapid increase in extinction. At a concentration of 250 μg SRF protein/ml., after the initial burst of activity, the inhibition lasted for 15 min. Although SRF always inhibited syneresis, there was considerable variation in the degree of inhibition produced by different preparations. This variability was probably associated with fluctuations in the free Ca^{2+} concentration. In the presence of SRF, the free Ca^{2+} concentration falls at first, as the vesicles bind Ca^{2+}, and then slowly increases as Ca^{2+} is released (cf. Fig. 1*a*). Because of this lack of uniformity, levels of Ca^{2+} were subsequently varied by the use of EGTA only.

Inhibition of syneresis by EGTA at various concentrations of KCl. Syneresis of actomyosin from control and treated cats was studied at

three different concentrations of EGTA: 10, 20, and 30 μM (Table 2). At the same time, KCl concentrations were varied between 35 and 65 mM. In the absence of EGTA, the half-time ($t_{\frac{1}{2}}$) required for maximum syneresis differs from preparation to preparation. Although this difference was small, in each experiment the inhibition of syneresis by various concentrations of EGTA was measured at the $t_{\frac{1}{2}}$ of that particular preparation.

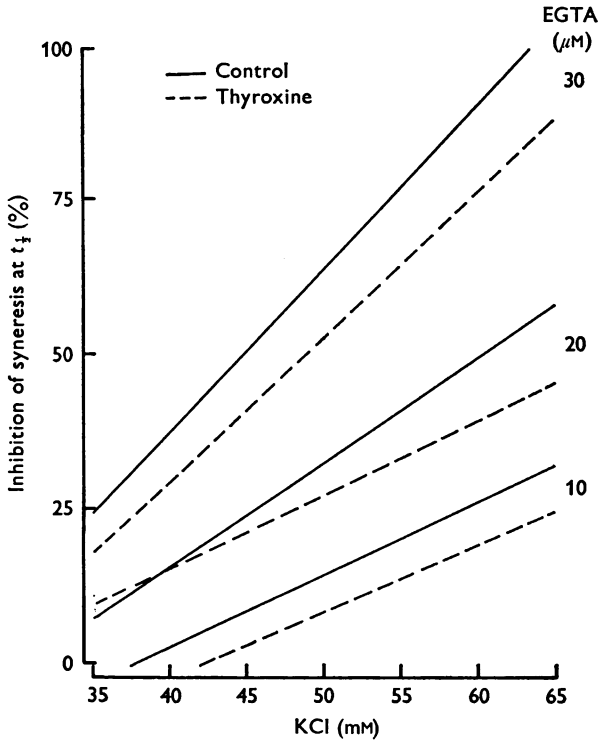

Fig. 5. Inhibition of syneresis of actomyosin, isolated from control (continuous lines) and thyroxine-treated (interrupted lines) cats, by EGTA (10, 20 and 30 μM) compared at various concentrations of KCl. The regression lines, calculated from the data in Table 2, show that, under equivalent conditions (see text), actomyosin from thyroxine-treated animals was less inhibited by EGTA than that from controls ($P \leqslant 0{\cdot}001$). For the estimation of inhibition of syneresis see text, p. 5.

The degree of inhibition of syneresis increased with increasing concentrations of both EGTA and KCl ($P \leqslant 0{\cdot}001$) (Fig. 5). Variation in KCl concentration had a similar effect on the EGTA inhibition of syneresis of actomyosin from both control and thyroxine-treated cats; the regression lines showed no significant deviation from parallelism. At each concentration of EGTA, however, and throughout the range of KCl concentrations, syneresis of actomyosin isolated from thyroxine-treated animals was

inhibited less than was that of the controls ($P \leqslant 0{\cdot}001$). The possibility that the diminished inhibition was a direct consequence of faster syneresis was tested by altering the ionic strength of the medium, to induce approximately equal rates of syneresis. The two actomyosin preparations were then inhibited to a similar degree by EGTA (Fig. 6). It was concluded that under these conditions there was little difference in the Ca^{2+} sensitivity of the two preparations.

TABLE 2. Statistical data of evidence for inhibition of syneresis by EGTA at different concentrations of KCl in control and thyroxine-treated cats

[EGTA] (μM)	[KCl] (mM)	Inhibition at $t_{\frac{1}{2}}$ (%)	
		Control	Thyroxine
10	35	1·0	—
	40	4·1, 5·7, 1·7, 3·6, 5·9,	1·5
	55	5·2, 12·0, 16·8, 17·4,	13·7, 8·1, 13·2,
		25·8, 14·8	13·1, 15·9, 13·6,
	60	38·5	23·0, 22·1, 19·6,
	65	41·5	29·0
Equation of regression line		$y = 1{\cdot}16x - 43{\cdot}21$	$y = 1{\cdot}11x - 46{\cdot}35$
20	35	3·0	—
	40	11·9, 14·4, 24·3, 25·7,	19·1
		12·9	—
	55	55·6, 61·1, 23·4, 18·9,	50·4, 22·1, 18·9
		35·1,	26·4
	60	45·2, 49·3, 63·0,	48·0, 41·1, 26·0,
			47·0, 38·5,
	65	—	51·0
Equation of regression line		$y = 1{\cdot}69x - 51{\cdot}34$	$y = 1{\cdot}26x - 36{\cdot}13$
30	35	6·7	—
	40	39·0, 38·8, 38·3, 47·7,	30·3
		40·0,	
	55	72·7, 73·0, 83·1,	70·6, 71·4, 64·6,
		84·1, 78·5, 83·3,	64·0, 57·1, 56·0,
	60	78·5, 93·6, 82·6,	69·0, 86·3, 71·0,
	65	—	76·2, 98·0,
Equation of regression line		$y = 2{\cdot}64x - 68{\cdot}36$	$y = 2{\cdot}28x - 61{\cdot}02$

In all the experiments described so far, syneresis was induced without further addition of Ca^{2+} to that already present in the actomyosin suspension. The faster syneresis of actomyosin from the thyroxine-treated cats could therefore be due to a greater amount of Ca^{2+} remaining associated with the actomyosin during the isolation procedure. An indirect estimate of this Ca^{2+} concentration was made by comparing the rates of syneresis occurring with and without a Ca^{2+}-EGTA buffer. In actomyosin

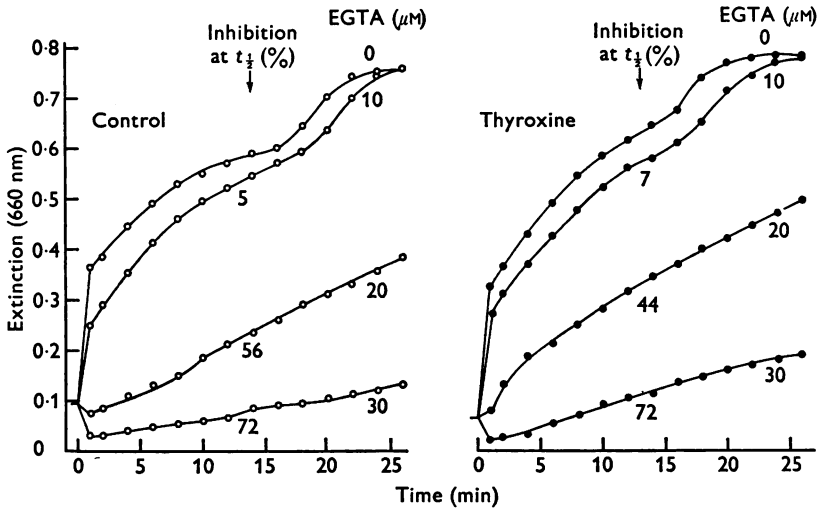

Fig. 6. The effect of different concentrations of EGTA on syneresis when the rates of syneresis of actomyosin from both groups of animals have been made similar by adjustment of the KCl concentration (control, 55 mM; thyroxine, 60 mM). Under these conditions, the % inhibitions of both preparations, indicated at $t_{\frac{1}{2}}$ (see text), are of a similar order.

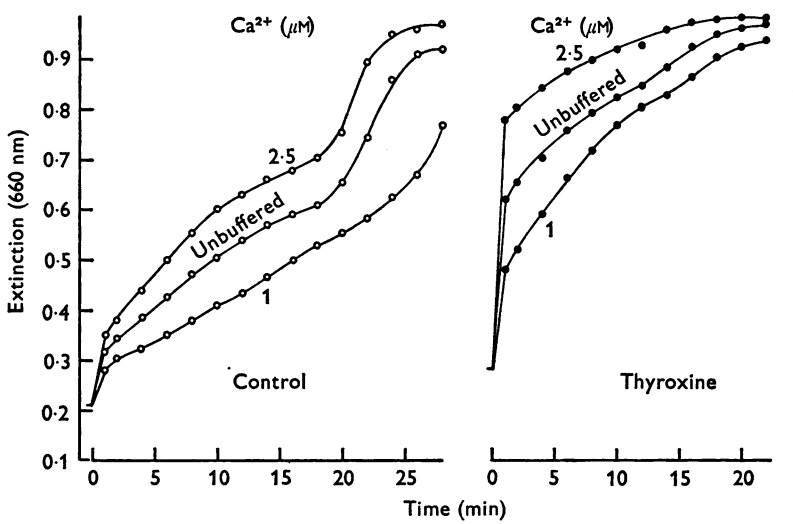

Fig. 7. Syneresis of actomyosin with two concentrations of Ca^{2+} buffered by EGTA. The curves labelled 'unbuffered' represent syneresis in the presence of the Ca^{2+} which remains associated with the actomyosin at the end of the isolation procedure (assay conditions as in Fig. 3.)

isolated from both control and treated cats, the rate of syneresis without the addition of Ca^{2+}-EGTA fell between the rates induced in the presence of 1 and 2·5 μM-Ca^{2+} (Fig. 7). Thus, the free Ca^{2+} concentrations in both actomyosin preparations were similar and of the order of 2 μM in the suspension, which is equivalent to approximately 3·0 μmole Ca^{2+}/g muscle.

DISCUSSION

The Ca^{2+} sequestering activity of skeletal muscle SR was assessed by measuring both Ca^{2+} binding and Ca^{2+} uptake by SRF isolated from control and thyroxine-treated cats. The SRF isolated from the thyroxine-treated cats bound Ca^{2+} less effectively than did SRF from the controls. This could be the result either of a slower accumulation of Ca^{2+} or of a faster release. In the binding assay the net Ca^{2+} accumulation depends on both the rate of Ca^{2+} binding and the rate of Ca^{2+} release, since both processes occur simultaneously (Martonosi & Feretos, 1964). Although, in the 2 min immediately following peak binding, SRF from the thyroxine-treated animals appeared to release Ca^{2+} more rapidly than that of the controls, measurements of Ca^{2+} uptake in the presence of oxalate indicated that a faster initial rate of release was unlikely, on its own, to explain the reduction in binding. It is known that in the presence of oxalate, Ca^{2+} accumulation by SRF is accelerated because a high proportion of the intravesicular Ca^{2+} is precipitated as calcium oxalate (Baird & Perry, 1960; Makinose & Hasselbach, 1965; Weber *et al.* 1966); and that the efflux of Ca^{2+} is reduced to a low level (Martonosi & Feretos, 1964). In the present study, SRF from thyroxine-treated cats accumulated Ca^{2+} more slowly and to a lesser extent than did SRF from the control animals; the maximum amount of accumulated Ca^{2+} was reduced by 75%. Thyroxine therefore reduces both binding and uptake of Ca^{2+} by SRF, which suggests that the hormone has some specific effect on the Ca^{2+} transport system of the SR.

In the absence of ATP, SRF bind Mg^{2+} more effectively than Ca^{2+}, but ATP reverses the relationship; the binding of Ca^{2+} is increased and that of Mg^{2+} reduced. According to Carvalho & Leo (1967), the preferential affinity for Ca^{2+} may be associated with a change in the conformation of the binding site. Since ATP is required for optimum binding of Ca^{2+} by SRF, measurement of the associated ATPase activity provides an additional assessment of the sarcotubular membrane function. Our results indicated, however, that this enzyme was not affected by thyroxine. Normally, Ca^{2+} binding and the Ca^{2+}-ATPase activity of SR are linked, the ratio Ca^{2+} accumulated/ATP hydrolysed varying from 1 to 2 according to the concentration of free Ca^{2+} in the assay medium (Hasselbach, 1966),

although at low concentrations of ATP this value may be as high as 10 (Ebashi & Yamanouchi, 1964). The lower binding of Ca^{2+} was not associated with a reduction in Ca^{2+}-ATPase activity, which suggests that thyroxine has partially dissociated these two processes.

If the reduced Ca^{2+} binding by SRF after thyroxine treatment reflects a change which occurs *in vivo*, the intracellular concentration of free Ca^{2+} in the intact skeletal muscle will tend to increase. Liu & Overman (1964) found increased intracellular levels of Ca^{2+}, Na^+ and Cl^- in skeletal muscle of thyroxine-treated rats associated with decreased levels of K^+. Similar changes in Na^+ and K^+ distribution, together with an increase in excitability, were produced in rat brain by thyroxine treatment (Timiras & Woodbury, 1956). A Ca^{2+} influx component, which is dependent on intracellular Na^+, has been demonstrated in crab nerve, squid axon and guinea-pig atria (Baker & Blaustein, 1968; Baker, Blaustein, Hodgkin & Steinhardt, 1969; Glitsch, Reuter & Scholz, 1970). Thus, an increase in intracellular Na^+ would, in itself, be expected to lead to an increase in intracellular Ca^{2+}. The decrease in rate of recovery from a depolarizing neuromuscular block accompanied by a parallel slowing of the rate of repolarization in thyroxine-treated cats (Zaimis *et al.* 1965) is in accord with such a redistribution of Na^+ and Ca^{2+}. Smith & Samuel (1970) suggested that erythrocyte Na^+,K^+-ATPase is inhibited in patients with hyperthyroidism, since, in their experiments, a high intracellular Na^+ concentration was associated with a diminished rate of Na^+ efflux. The possibility of a change in Na^+,K^+-ATPase activity in the skeletal muscle of thyroxine-treated animals is being investigated.

In parallel with the studies of the accumulation of Ca^{2+} by SRF, the effects of thyroxine on the contractile proteins were assessed by studying the syneresis and the Ca^{2+}-sensitivity of isolated actomyosin. Two phases of syneresis can generally be distinguished with the turbidometric procedure: an initial rapid phase lasting 5–10 sec gives way to a slower phase which persists until the maximum level of syneresis is reached 20–25 min after the addition of ATP. Under identical assay conditions, the actomyosin from the thyroxine-treated cats underwent syneresis at a faster rate than did that from the controls during the first 10 sec after the addition of ATP. The subsequent rates of syneresis and the maximum levels reached were, however, unaffected by thyroxine. Since the rate of syneresis depended on the ionic strength of the medium, a series of assays was done at different concentrations of KCl. Each variation in KCl concentration produced a change in syneresis rate which was similar in the two actomyosin preparations. The lower the ionic strength, the faster was the initial phase of syneresis. Thyroxine therefore altered the threshold for syneresis, but not the pattern of response to ionic strength; at each ionic

concentration the initial rate of syneresis was always faster with actomyosin from the thyroxine-treated animals.

At this stage it seemed possible that the Ca^{2+} sensitivity of the actomyosin might have been affected by thyroxine. Syneresis of native actomyosin, i.e. actomyosin containing the regulatory proteins troponin and tropomyosin, is a Ca^{2+}-dependent process (Ebashi & Ebashi, 1964; Perry, 1967; Arai & Watanabe, 1968; Ebashi, Kodama & Ebashi, 1968). Ca^{2+} is normally present in isolated actomyosin in sufficient concentration to promote syneresis when Mg^{2+} and ATP are added (Weber & Winicur, 1961). Stewart & Levy (1970) suggested that relaxation of actomyosin is caused by the binding of Mg^{2+}-ATP to an inhibitory site and that the troponin–tropomyosin complex facilitates this binding. The inhibition is relieved by the formation of a calcium–troponin–tropomyosin complex which interferes with the binding of Mg^{2+}-ATP and therefore promotes contraction. Thus an increase in the Ca^{2+} affinity of troponin would result in faster rates of syneresis. An indirect measurement of Ca^{2+} affinity was made by varying the amount of free Ca^{2+} in the assay system by adding either SRF or EGTA. In principle, it would be preferable to use SRF, the natural Ca^{2+}-sequestering membranes, because the assay would then more closely resemble the physiological process of excitation–contraction coupling. However, the wide variations in the inhibitory activities of SRF preparations, probably associated with the reversibility of Ca^{2+} binding, precluded their use. Ca^{2+} concentrations were therefore varied by adding EGTA, a chelating agent with a high affinity for Ca^{2+} and a low affinity for Mg^{2+} (Schwarzenbach, Senn & Anderegg, 1957). Selective chelation was essential for these assays, since the troponin-tropomyosin complex only inhibits syneresis in the presence of Mg^{2+} (Kaminer, 1968; Weber, Herz & Reiss, 1969). The degree of inhibition produced by EGTA depended not only on the concentration of the chelating agent but also on the rate of syneresis, and therefore on the concentration of KCl. In assay media of low ionic strength, i.e. at fast rates of syneresis, EGTA produced little inhibition. Under these conditions, the Ca^{2+} affinity of native actomyosin is high (Weber & Herz, 1963); troponin therefore competes for Ca^{2+} more effectively. Increases in the concentration of either EGTA or KCl produced an increasing inhibition of syneresis as more Ca^{2+} was removed from troponin, either as a result of increased chelation by EGTA, or as a result of diminished binding of Ca^{2+} to troponin in the presence of high ionic concentrations.

When the inhibition of syneresis by EGTA was measured at several concentrations of KCl in actomyosin preparations from control and thyroxine-treated cats, the inhibition was in each case greater with actomyosin from the treated animals. This suggested that an alteration

in the Ca^{2+} sensitivity of the contractile proteins had occurred. However, when syneresis rates of actomyosin from the two groups of animals were made equal by altering KCl concentrations, there was no appreciable difference in the degree of inhibition produced by EGTA and, therefore, no evidence of an alternation in Ca^{2+} sensitivity. The increased inhibition observed when syneresis was compared at the same ionic strengths was presumably a consequence of the faster rates of syneresis in actomyosin from the thyroxine-treated cats.

Finally, the possibility was explored that increased intracellular concentrations of Ca^{2+} after thyroxine treatment might have led to an increase in the Ca^{2+} content of actomyosin and therefore an increased rate of syneresis. However, when free Ca^{2+} concentrations in suspensions of actomyosin isolated from control and thyroxine-treated cats were compared by measuring syneresis in the presence and absence of Ca^{2+}-EGTA buffers, both actomyosin preparations underwent syneresis at rates falling between those obtained in the presence of 1·0 and 2·5 μM free Ca^{2+}. This indicated that the free Ca^{2+} concentrations were similar in the two preparations, and about 2·0 μM. This is equivalent to approximately 3·0 μmole Ca^{2+}/g muscle, a value similar to the average Ca^{2+} content of skeletal muscle myofibrils (Weber & Herz, 1963).

It may therefore be concluded that thyroxine has modified the properties of both the sarcoplasmic reticulum and the contractile proteins. The effects on actomyosin syneresis show that the contractile proteins are capable of an exaggerated response to ATP; but the behaviour of the muscle *in vivo* may largely reflect the reduced accumulation of Ca^{2+} by the SR and other changes in cation distribution. It is also conceivable that an altered ionic milieu has itself induced compensatory changes in the relationship between the regulatory and the contractile proteins.

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REFERENCES

ARAI, K.-I. & WATANABE, S. (1968). A study of troponin, a myofibrillar protein from rabbit skeletal muscle. *J. biol. Chem.* **243**, 5670–5678.

BAIRD, G. D. & PERRY, S. V. (1960). The inhibitory action of relaxing-factor preparations on the myofibrillar adenosine triphosphatase. *Biochem. J.* **77**, 262–271.

BAKER, P. F. & BLAUSTEIN, M. P. (1968). Sodium-dependent uptake of calcium by crab nerve. *Biochim. biophys. Acta* **150**, 167–170.

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L. & STEINHARDT, R. A. (1969). The influence of calcium on sodium efflux in squid axons. *J. Physiol.* **200**, 431–458.

CARVALHO, A. P. (1968). Effects of potentiators of muscular contraction on binding of calcium by sarcoplasmic reticulum. *J. gen. Physiol.* **51**, 427–442.

CARVALHO, A. P. & LEO, B. (1967). Effects of ATP on the interaction of Ca^{++}, Mg^{++} and K^{+} with fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle. *J. gen. Physiol.* **50**, 1327–1352.

DEAMER, D. W. & BASKIN, R. J. (1969). Ultrastructure of sarcoplasmic reticulum preparations. *J. cell Biol.* **42**, 296–307.

DE MEIS, L., RUBIN-ALTSCHUL, B. M. & MACHADO, R. D. (1970). Comparative data of Ca^{2+} transport in brain and skeletal muscle microsomes. *J. biol. Chem.* **245**, 1883–1889.

DINGLEDINE, W. S., PITT-RIVERS, R. & STANBURY, J. B. (1955). Nature and transport of the iodinated substances of the blood of normal subjects and of patients with thyroid disease. *J. clin. Endocr. Metab.* **15**, 724–731.

EBASHI, S. (1961). Calcium binding activity of vesicular relaxing factor. *J. Biochem., Tokyo* **50**, 236–244.

EBASHI, S. & EBASHI, F. (1964). A new protein component participating in the superprecipitation of myosin B. *J. Biochem., Tokyo* **55**, 604–613.

EBASHI, S. & ENDO, M. (1968). Calcium ion and muscle contraction. In *Progress in Biophysics and Molecular Biology*, vol. 18, pp. 125–183, ed. BUTLER, J. A. V. & NOBLE, D. Oxford: Pergamon Press.

EBASHI, S., KODAMA, A. & EBASHI, F. (1968). Troponin. I. Preparation and physiological function. *J. Biochem., Tokyo* **64**, 465–477.

EBASHI, S. & LIPMANN, F. (1962). Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J. cell Biol.* **14**, 389–400.

EBASHI, F. & YAMANOUCHI, I. (1964). Calcium accumulation and adenosine triphosphatase activity of relaxing factor. *J. Biochem., Tokyo* **55**, 504–509.

ENDO, M. (1964). The superprecipitation of actomyosin and its ATPase activity in the presence of low concentrations of ATP. *J. Biochem., Tokyo* **55**, 614–622.

FISKE, C. H. & SUBBAROW, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375–400.

FUCHS, F., GERTZ, E. W. & BRIGGS, F. N. (1968). The effect of quinidine on calcium accumulation by isolated sarcoplasmic reticulum of skeletal and cardiac muscle. *J. gen. Physiol.* **52**, 955–968.

GLITSCH, H. G., REUTER, H. & SCHOLZ, H. (1970). The effect of the internal sodium concentration on calcium fluxes in isolated guinea-pig auricles. *J. Physiol.* **209**, 25–43.

GORNALL, A. G., BARDAWILL, C. J. & DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.* **177**, 751–766.

GREASER, M. L., CASSENS, R. G., HOEKSTRA, W. G. & BRISKEY, E. J. (1969). Purification and ultrastructural properties of the calcium accumulating membranes in isolated sarcoplasmic reticulum preparations from skeletal muscle. *J. cell. comp. Physiol.* **74**, 37–50.

GROB, D. (1963). Myopathies and their relation to thyroid disease. *N.Y. St. J. Med.* **63**, 218–227.

HARIGAYA, S. & SCHWARTZ, A. (1969). Rate of calcium binding and uptake in normal animal and failing human cardiac muscle. *Circulation Res.* **25**, 781–794.

HASSELBACH, W. (1966). Structural and enzymatic properties of the calcium transporting membranes of the sarcoplasmic reticulum. *Ann. N.Y. Acad. Sci.* **137**, 1041–1048.

HAVARD, C. W. H., CAMPBELL, E. D. R., ROSS, M. B. & SPENCE, A. W. (1963). Electromyographic and histological findings in the muscles of patients with thyrotoxicosis. *Q. Jl Med.* **32**, 143–163.

HOCH, F. L. (1962). Biochemical actions of thyroid hormone. *Physiol. Rev.* **42**, 605–673.

KAMINER, B. (1968). Magnesium and the regulatory role of 'calcium-sensitizing factor' in muscle. *Biochem. biophys. Res. Commun.* **33**, 542–545.

LIU, CH.-T. & OVERMAN, R. R. (1964). Effect of toxic doses of L-thyroxine on tissue water, electrolytes, and plasma protein in rats. *Proc. Soc. exp. Biol. Med.* **117**, 232–236.

MACLAGAN, N. F., BOWDEN, C. H. & WILKINSON, J. H. (1957). The metabolism of thyroid hormones. 2. Detection of thyroxine and tri-iodothyronine in human plasma. *Biochem. J.* **67**, 5–11.

MAKINOSE, M. & HASSELBACH, W. (1965). Der Einfluss von Oxalat auf den Calcium-Transport isolierter Vesikel des sarkoplasmatischen Reticulum. *Biochem. Z.* **343**, 360–382.

MARTONOSI, A. (1968). Sarcoplasmic reticulum. V. The structure of sarcoplasmic reticulum membranes. *Biochim. biophys. Acta* **150**, 694–704.

MARTONOSI, A. & FERETOS, R. (1964). Sarcoplasmic reticulum. I. The uptake of Ca^{++} by sarcoplasmic reticulum fragments. *J. biol. Chem.* **239**, 648–657.

MUSCATELLO, U., ANDERSSON-CEDERGREN, E. & AZZONE, G. F. (1962). The mechanism of muscle-fiber relaxation, adenosine triphosphatase and relaxing activity of the sarcotubular system. *Biochim. biophys. Acta* **63**, 55–74.

NAGAI, T., MAKINOSE, M. & HASSELBACH, W. (1960). Der Physiologische Erschlaffungsfaktor und die Muskelgrana. *Biochim. biophys. Acta* **43**, 223–238.

OGAWA, Y. (1968). An apparent binding constant of glycoletherdiaminetetraacetic acid for calcium at neutral pH. *J. Biochem., Tokyo* **64**, 255–257.

PERRY, S. V. (1967). The structure and interactions of myosin. In *Progress in Biophysics and Molecular Biology*, vol. 17, pp. 325–381, ed. BUTLER, J. A. V. & HUXLEY, H. E. Oxford: Pergamon Press.

PLUMMER, H. S. (1926). The function of the thyroid gland. In MAYO, C. H. & PLUMMER, H. S., *The Thyroid Gland*. Baumont Federation Lecture Series No. 4, London.

SCHWARZENBACH, G., SENN, H. & ANDEREGG, G. (1957). Komplexone. XXIX. Ein grosser Chelateffekt besonderer Art. *Helv. chim. Acta* **40**, 1886–1900.

SMITH, E. K. M. & SAMUEL, P. D. (1970). Abnormalities in the sodium pump of erythrocytes from patients with hyperthyroidism. *Clin. Sci.* **38**, 49–61.

STEWART, J. M. & LEVY, H. M. (1970). The role of the calcium–troponin–tropomyosin complex in the activation of contraction. *J. biol. Chem.* **245**, 5764–5772.

SZENT-GYÖRGYI, A. (1951). *Chemistry of Muscular Contraction*, 2nd edn., pp. 28–37. New York: Academic Press.

TIMIRAS, P. S. & WOODBURY, D. M. (1956). Effect of thyroid activity on brain function and brain electrolyte distribution in rats. *Endocrinology* **58**, 181–192.

WEBER, A. (1966). Energized calcium transport and relaxing factors. In *Current Topics in Bioenergetics*, vol. 1, pp. 203–254, ed. SANADI, D. R. New York and London: Academic Press.

WEBER, A. & HERZ, R. (1963). The binding of calcium to actomyosin systems in relation to their biological activity. *J. biol. Chem.* **238**, 599–605.

WEBER, A., HERZ, R. & REISS, I. (1963). On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum. *J. gen. Physiol.* **46**, 679–702.

WEBER, A., HERZ, R. & REISS, I. (1966). Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. *Biochem. Z.* **345**, 329–369.

WEBER, A., HERZ, R. & REISS, I. (1969). The role of magnesium in the relaxation of myofibrils. *Biochemistry, N.Y.* **8**, 2266–2270.

WEBER, A. & WINICUR, S. (1961). The role of calcium in the superprecipitation of actomyosin. *J. biol. Chem.* **236**, 3198–3202.

WERNER, S. C. (1962). *The Thyroid. A Fundamental and Clinical Text*, 2nd edn. New York: Harper and Row.

ZAIMIS, E., METAXAS, N., HAVARD, C. W. N. & CAMPBELL, E. D. R. (1965). Cardiovascular and skeletal muscle changes in cats treated with thyroxine. In *Research in Muscular Dystrophy*, Proc. 3rd Symposium of the Muscular Dystrophy Group, pp. 301–311, ed. Members of the Research Committee of the Muscular Dystrophy Group. London: Pittman Medical Publishing Co.

ZAIMIS, E., PAPADAKI, L., ASH, A. S. F., LARBI, E., KAKARI, S., MATTHEW, M. & PARADELIS, A. (1969). Cardiovascular effects of thyroxine. *Cardiovasc. Res.* **3**, 118–133.