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# Intracellular Localization of the Skeletal-Muscle Relaxing Factor

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The relaxing factor, originally described by Marsh (1951) in the crude extract of blended skeletal muscle, has been shown to be associated with minute particles which can only be centrifuged out of suspension at high centrifugal forces (Kumagai, Ebashi & Takeda, 1955; Portzehl, 1957). Portzehl (1957) observed that, after centrifuging at 19 000*g* for 1 hr., the supernatant solution still contained relaxing factor as assayed by inhibition of myofibril adenosine triphosphatase activity, whereas centrifuging at 35 000*g* for over 1 hr. brought down all the active granules. On the basis of these characteristics she postulated that the granular fraction responsible for relaxing activity consisted of microsomes.

With passage of time alone this postulate has become an assumption (Lorand, Mulnar & Moos, 1957; Briggs & Fuchs, 1960). However, support for this assumption was presented by Nagai, Makinose & Hasselbach (1960), who showed that the granules which centrifuged down at 25 000*g* in 1 hr. were vesicular and had an average diameter of about 1000 Å. The electron micrograph of these muscle granules is quite similar in appearance to the microsome pellet of liver (Palade & Siekevitz, 1956).

The present work was undertaken to determine, by means of enzyme markers, the nature of the skeletal-muscle granules which possess the relaxing activity.

## METHODS

*Myofibrils.* Rabbit leg and back muscles were chilled, ground, and blended for 2 min. in a Waring Blendor with 5 vol. of 0·1 M-KCl–0·01 M-$KHCO_3$ soln. The homogenate was centrifuged at 2000*g* for 10 min., the supernatant discarded, and the sedimented fibrils blended for 30 sec. with 5 vol. of 0·1 M-KCl. After sedimenting the fibrils, the blending for 30 sec. with 5 vol. of 0·1 M-KCl was repeated eight times in all; this broke up the fibrils into small segments and washed them free of relaxing factor. All steps were carried out at 0–4°.

After the final blending, the myofibrils were filtered through four thicknesses of gauze to remove lumps of fibrous tissue, and centrifuged for 10 min. To the precipitate was added cold glycerol (1 vol.) and sufficient 3·0 M-KCl to give a final KCl concn. of 0·1 M. The pH was adjusted to 7·0 by the addition of 0·1 N-KOH. This mixture was then blended for 15 sec. to yield a uniform suspension and was stored at −10°. On the day of an experiment a portion of the glycerol suspension was centrifuged, and the myofibril precipitate washed once with 0·1 M-KCl and recentrifuged. It was then suspended in 0·1 M-KCl at pH 7·0 to give a protein concentration of about 5 mg./ml. Essentially no change in adenosine triphosphatase activity occurred in the 7–8 months that the myofibril preparation was used.

*Muscle-granule preparation.* Diced muscle (100 g.) from the hind legs of a freshly killed rabbit was chilled, ground and blended at high speed with 2 vol. of a cold 0·1 M-KCl–0·02 M-$KHCO_3$–0·005 M-potassium oxalate soln. for 1 min. The blended muscle (fraction W.H.) was then centrifuged at 600*g* for 4 min., the precipitate discarded, and the supernatant (fraction $S_1$) centrifuged at 3500*g* for 10 min. The precipitate was gently homogenized by hand with 10–15 ml. of 0·1 M-KCl–0·001 M-potassium oxalate–0·02 M-histidine soln. at pH 7·0 (histidine mixture), recentrifuged for 10 min. at 3500*g* and the supernatant added to that of the first 3500*g* centrifuging. The precipitate was taken up in a small volume of the histidine mixture and homogenized by hand to yield an even suspension. This particulate fraction is referred to below as the 'mitochondrial 1' or '$M_1$' fraction. The combined supernatants were then centrifuged at 12 500*g* for 20 min. The sediment was washed and suspended as described for the $M_1$ fraction above, recentrifuged at 12 500*g* for 20 min. and suspended

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in a small volume of histidine mixture. This fraction is referred to below as the 'mitochondrial 2' or '$M_2$' fraction. The supernatants from the 12 500$g$ centrifugings were combined and centrifuged in the Spinco preparative ultracentrifuge at 105 000$g$ for 30 min. The precipitate was washed as described above for the mitochondrial fractions and suspended in histidine mixture. This final precipitate is referred to below as the 'microsomal' or 'Mic' fraction. The final supernatant, including the wash of the microsomal fraction, is referred to below as the 'supernatant' or '$S_2$' fraction. Granule preparations were carried out at 0–4° and final suspensions were made to yield a protein concentration of approximately 10 mg./ml. All $g$ values are those at the middle of the tubes.

*Estimation of the relaxing factor in the particulate fractions.* Inhibition of myofibril adenosine triphosphatase activity by the particulate fractions served as an index of relaxing-factor activity. Ebashi (1958) has shown good correlation between the inhibition of adenosine triphosphatase and relaxation of single glycerol-extracted fibres, and control experiments in the present study with myofibrils and actomyosin preparations confirmed this observation. The assay for relaxing factor was essentially that of Nagai *et al.* (1960). The reaction mixture contained KCl (50 mM), $MgCl_2$ (5 mM), potassium oxalate (5 mM), histidine buffer (20 mM, pH 7·0), ATP (5 mM), myofibrils (approx. 0·5 mg. of protein/ml.) and muscle granules (from 1 to 100 $\mu$g. of protein/ml.) in a volume of 3·0 ml. Incubation with shaking was at room temperature for 12 min., and 1 ml. of reaction mixture was added to 2 ml. of 7·5% (w/v) trichloracetic acid 2 and 12 min. after the addition of ATP. Myofibril adenosine triphosphatase activity was determined from the difference in inorganic phosphate content of the reaction mixture between the 2nd and 12th minutes, and inhibition of adenosine triphosphatase activity was expressed as a percentage of the ATP hydrolysed in the absence of granules. Corrections were made for the adenosine triphosphatase activity of the granules alone.

*Cytochrome oxidase.* The activity of this enzyme in the muscle fractions was determined with differential manometers at 25°. The reaction mixture consisted of potassium phosphate buffer (50 mM, pH 7·4), EDTA (0·3 mM), cytochrome $c$ (0·06 mM), potassium ascorbate (30 mM, pH 7·4) and a portion of a muscle fraction. After temperature equilibration was reached, the reaction was started by tipping the ascorbate from the side arm into the main vessel. Oxygen consumption was linear with time and proportional to enzyme concentration in the range employed. The activity was expressed as $q_{O_2}$ ($\mu$l. of $O_2$/mg. of protein/hr.).

*Esterase.* The esterase activity of the muscle fractions was measured with differential manometers. The substrate, $p$-nitrophenyl acetate, was ground in a mortar with water and gum arabic to give a 10% suspension in 5% gum arabic. A sample (0·02 ml.) of this suspension was placed in the side arm of each flask. The tissue fraction was placed in the main vessel in 23 mM-$NaHCO_3$ equilibrated with $N_2+CO_2$ (95:5). The total volume of the vessel was 1·0 ml. and the pH 7·6. After the vessel had been flushed with $N_2+CO_2$ (95:5) for 10 min. and temperature equilibration at 37° had been reached, the reaction was started by tipping the $p$-nitrophenyl acetate into the main vessel and measuring $CO_2$ production for the next 10 min. The activity was expressed as $q_{O_2}$ ($\mu$l. of $CO_2$/mg. of protein/hr.).

Inorganic phosphate was determined by the method of Fiske & Subbarow as modified by Sumner (1944), and protein by the biuret method of Gornall, Bardawill & David (1949) as modified by Cleland & Slater (1953), or by the Kjeldahl method.

## RESULTS

The inhibition of the myofibril adenosine triphosphatase activity of the three particulate fractions is shown in Fig. 1. The amount of inorganic phosphate split by myofibrils from ATP in 10 min. is unaffected by the presence of the $M_1$ fraction in concentrations as high as 36 $\mu$g. of protein/ml. of reaction mixture. In contrast, the $M_2$ and the Mic fractions showed considerable inhibition with 5–10 $\mu$g. of protein/ml. of reaction mixture and maximal inhibition below 30 $\mu$g./ml. Further, there is no significant difference in relaxing-factor activity of these two particulate fractions.

Since adenosine triphosphatase inhibition is dependent on the granule:myofibril protein ratio (Nagai *et al.* 1960) and the myofibril-protein content is not exactly the same in all experiments, it is necessary to plot results from several experiments (cf. Nagai *et al.* 1960). This has been done in Fig. 2 in which the points represent the means of 11 experiments. It is evident that the $M_1$ fraction is practically devoid of any relaxing-factor activity whereas the $M_2$ and Mic fractions possess potent and approximately equal activity.

A comparison of cytochrome-oxidase and esterase activities with relaxing-factor activity of the particulate fractions is presented in Table 1. The specific activity of the cytochrome oxidase in the $M_1$ fraction is almost 15-fold, and in the $M_2$ fraction 12-fold, greater than that in the Mic fraction. In

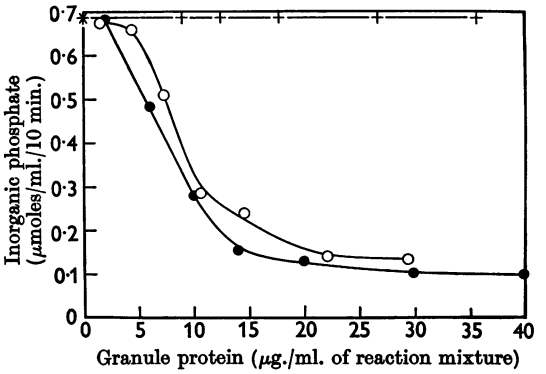


Fig. 1. Effect of different concentrations of granules on the inorganic phosphate split from ATP during incubation for 10 min. at 25°. +, Mitochondrial 1 fraction; ○, mitochondrial 2 fraction; ●, microsomal fraction; ✳, myofibril adenosine triphosphatase activity in the absence of granules.

contrast, the specific activity of the esterase is greatest in the Mic fraction. At a granule protein:myofibril protein ratio of 0·03 the heaviest particulate fraction ($M_1$) fails to inhibit myofibril adenosine triphosphatase, whereas the two lighter fractions induce marked adenosine triphosphatase inhibition (Table 1).

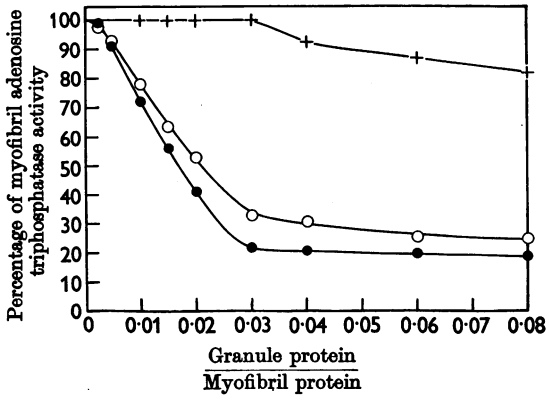


Fig. 2. Myofibril adenosine triphosphatase activity at different granule protein:myofibril protein ratios (mean of 11 experiments). Adenosine triphosphatase activity is expressed as a percentage of that obtained in the absence of granules. +, Mitochondrial 1 fraction; ○, mitochondrial 2 fraction; ●, microsomal fraction.

In two experiments the muscle was blended for only 15 and 30 sec. respectively instead of the normal 1 min. In each case both the esterase specific activity and the relaxing-factor activity were several-fold greater in the $M_2$ than in the Mic fraction, in contrast with the results observed after blending for 1 min.; further, the cytochrome-oxidase activity was about three times higher in the mitochondrial fractions of muscle blended for 15–30 sec. than in muscle blended for 1 min.

In an attempt to achieve greater separation of enzyme and relaxing-factor activity, four particulate fractions were made from rabbit skeletal muscle, and the means of the results of three such experiments are given in Table 2. After centrifuging the myofibrils and nuclei (600*g* for 4 min.), the supernatant ($S_1$) was successively centrifuged at 3500*g* for 10 min., 8000*g* for 15 min., 19 000*g* for 20 min. and 105 000*g* for 30 min. to give mitochondrial fractions 1, 2 and 3 ($M_1$, $M_2$, $M_3$) and microsomes (Mic) respectively (Table 2). The supernatant from the last centrifuging is the $S_2$ fraction. The highest specific activity and the bulk of the

Table 1. *Cytochrome oxidase, esterase and relaxing-factor activity in skeletal-muscle fractions*

For experimental details, see text. W.H., Whole homogenate; $S_1$, first supernatant; $S_2$, final supernatant; $M_1$, mitochondrial 1 fraction; $M_2$, mitochondrial 2 fraction; Mic, microsomal fraction. Values represent the mean of five experiments.

| | | Cytochrome oxidase activity | | Esterase activity | | |
|---|---|---|---|---|---|---|
| Fraction | Protein (g./100 g. of wet muscle) | $Q_{O_2}$ | Percentage of total of granules | $Q_{CO_2}$ | Percentage of total of granules | Percentage inhibition of myofibril ATPase* |
| W.H. | 13·77 | — | — | — | — | — |
| $S_1$ | 1·62 | 60 | — | 118 | — | — |
| $M_1$ | 0·06 | 554 | 50 | 100 | 18 | 0 |
| $M_2$ | 0·06 | 470 | 44 | 107 | 19 | 67 |
| Mic | 0·11 | 38 | 6 | 188 | 63 | 81 |
| $S_2$ | 1·08 | 0 | — | 120 | — | 0 |

* At (granule protein)/(myofibril protein) = 0·03.

Table 2. *Cytochrome oxidase, esterase and relaxing-factor activity in skeletal-muscle fractions*

For experimental details and definition of fractions, see text. Values represent the mean of three experiments.

| | | Cytochrome oxidase activity | | Esterase activity | | |
|---|---|---|---|---|---|---|
| Fraction | Protein (g./100 g. of wet muscle) | $Q_{O_2}$ | Percentage of total of granules | $Q_{CO_2}$ | Percentage of total of granules | Percentage inhibition of myofibril ATPase* |
| W.H. | 13·86 | — | — | — | — | — |
| $S_1$ | 1·50 | 72 | — | 194 | — | 0 |
| $M_1$ | 0·07 | 489 | 52 | 109 | 19 | 0 |
| $M_2$ | 0·04 | 545 | 33 | 183 | 18 | 18 |
| $M_3$ | 0·04 | 179 | 11 | 193 | 20 | 73 |
| Mic | 0·10 | 26 | 4 | 168 | 43 | 65 |
| $S_2$ | 1·01 | 5 | — | 244 | — | 0 |

* At (granule protein)/(myofibril protein) = 0·06.

cytochrome oxidase were found in the first two granular fractions ($M_1$ and $M_2$). The specific activity of the esterase was lowest in the $M_1$ fraction but was approximately equal in the $M_2$, $M_3$ and Mic fractions. However, 63 % of the total granule esterase activity was located in the $M_3$ and Mic fractions. The relaxing-factor activity, at a granule protein:myofibril protein ratio of 0·06, was highest in the $M_3$ and Mic fractions, low in the $M_2$ fraction, and absent in the $M_1$ fraction.

## DISCUSSION

By differential centrifuging, in which cellular particles other than nuclei and myofibrils were separated into three or four fractions, it has been demonstrated that relaxing-factor activity resides in the lighter granules. This confirms the work of all previous investigators. One possible difference revealed by the present study is that the intermediate fractions ($M_2$, Table 1; and $M_3$, Table 2) possess relaxing-factor activity equal to that observed with the lightest fractions.

Cytochrome oxidase has been shown to be an appropriate marker for mitochondria in liver (Hogeboom, Claude & Hotchkiss, 1946) and heart (Cleland & Slater, 1953). Assuming that this enzyme is an equally effective marker for rabbit skeletal-muscle mitochondria, the results in Tables 1 and 2 show that the relaxing factor is not associated with the mitochondria. This observation is in contrast with the results reported by Baird & Perry (1960), who found the relaxing factor to be associated with the heavier granules of rabbit-skeletal muscle but with the lighter granules of pigeon-breast muscle. The reason for these conflicting results is not clear, but Baird & Perry (1960) suggested that their mitochondrial fraction may have been contaminated with elements containing the relaxing factor. Since our results clearly indicate that the relaxing factor can be separated from muscle mitochondria, it is quite possible that in the experiments of Baird & Perry (1960) the lighter granules containing relaxing factor may have sedimented in clumps with the mitochondria.

Evidence has been presented that certain esterases are localized in the microsomes of liver (Underhay, Holt, Beaufay & de Duve, 1956) and of heart (Hulsmans, 1961). *p*-Nitrophenyl acetate, a substrate for ali-esterases and aromatic esterase (Myers, 1954), is hydrolysed by both the microsomes and the supernatant fraction in homogenates of heart muscle (Hulsmans, 1961) and skeletal muscle (Tables 1 and 2), and cannot therefore be used to distinguish between particulate and supernatant fractions in these tissues. Nevertheless, this substrate may be used to compare the esterase activity of the particulate fractions. In rabbit skeletal muscle, the esterase activity is highest in the lighter particulate fractions. However, skeletal muscle differs from heart in that specific activity differences between the particulate fractions are smaller, in that there is considerably less total esterase activity, and in that there is poor correlation of esterase and relaxing-factor activities in the fractions. This could mean that esterase activity, as determined with *p*-nitrophenyl acetate as substrate, is not chiefly localized in the microsomal fraction of skeletal muscle, in contrast with liver and heart. However, the possibilities of microsomal contamination of the mitochondrial fractions or of contamination of the microsomal fraction with esterase-inert particulate protein material cannot be excluded. It is conceivable that larger pieces of sarcoplasmic reticulum or clumps of microsomes are sedimented with the larger mitochondria. This idea is supported by the observation that with blending for only 15 or 30 sec. the esterase and relaxing-factor activities were several-fold greater in the $M_2$ than in the Mic fractions. It is of interest that, although poor microsome separation was obtained with short periods of blending, the mitochondria were better preserved, as shown by a specific activity for cytochrome oxidase three times that of muscle blended for 1 min. No preparative procedure which will provide complete particulate separation with granules showing very high enzyme activities has yet been found. The fact that the Mic fraction contains most of the esterase activity, yet has a relatively low specific activity of this enzyme, is compatible with contamination of this fraction with esterase-inert protein.

Enzyme marker studies have shown that the relaxing factor is definitely not associated with the mitochondria, but have not conclusively established its association with microsomes. The bulk of evidence, including electron micrographs of microsome pellets (Nagai *et al.* 1960), suggests a close association of the relaxing factor with muscle microsomes. However, the possibility that the relaxing factor is located in granules other than microsomes remains, in view of the high relaxing-factor activity of the 'particles of intermediate density' ($M_2$, Table 1; and $M_3$, Table 2). In this regard it is tempting to speculate that the vesicles of the muscle triads (Porter & Palade, 1957), which may play a role in muscle contraction and relaxation, elaborate the relaxing factor.

## SUMMARY

1. A comparison of the cytochrome-oxidase and esterase activities with the relaxing-factor activity of the particulate fractions of rabbit skeletal muscle indicates that the relaxing factor is not

associated with the mitochondria and is most probably associated with the microsomes.

2. The possibility of the presence of the relaxing factor in a third particle of intermediate density cannot be excluded by these studies.

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# The Oxidation of Reduced Flavin Mononucleotide by Molecular Oxygen

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Reduced flavin mononucleotide is active as a 'luciferin' in the bacterial bioluminescent system (Hastings & McElroy, 1955; McElroy & Green, 1955).

The components required for light-production are luciferin, luciferase, a long-chain aliphatic aldehyde and oxygen. The work described in this paper was undertaken as a preliminary to a study of the kinetics of the bacterial luminescent system.

The reaction of reduced flavin mononucleotide with oxygen has been studied by Gutfreund (1960) who interpreted his kinetic findings in terms of the formation of a flavin–oxygen complex whose breakdown was rate-limiting. He found no spectroscopic evidence for the transient existence of intermediate species, either the reduced flavin–oxygen complex or the species observed by Beinert (1956), in equilibrium mixtures of both oxidized and reduced riboflavin and oxidized and reduced flavin mononucleotide.

Although our kinetic observations on the reaction with oxygen generally agree well with those of Gutfreund (1960), we have been able to observe intermediate compounds similar to those seen by Beinert (1956), and have also found that the reaction is autocatalytic. Some additional observations on the part played by hydrogen peroxide and on the coupling of flavin oxidation to luminol chemiluminescence are presented.

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## EXPERIMENTAL

### *Materials*

Flavin mononucleotide (FMN) was obtained from Sigma Chemical Co., and 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) from Eastman Organic Chemicals, Rochester 3, N.Y., U.S.A. Other reagents were of analytical