*J. Pharm. Pharmac.*, 1968, **20**, *Suppl.*, 135S–141S Received May 1, 1968

# Micro-colorimetric determination of adenosinetriphosphatase activity in freeze-dried sections of rat diaphragm muscle

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A technique is described for determination of ATPase activity in 3–30 μg samples dissected from freeze-dried sections of rat diaphragm muscle. Tissue samples are incubated at room temperature with optimal concentrations of ATP (10 mM) and $MgSO_4$ (5 mM) in 0·1 M tris/HCl buffer at pH 7·4. Inorganic phosphate is measured spectrophotometrically. Mean activity for 20 rats was 0·22 ± 0·06 (s.d.) mole-Pi/kg/wet tissue/15 min. Results did not depend on plane of section or section thickness. 5 mM $Mg^{2+}$, 10 mM $Ca^{2+}$ and 1 mM 2,4-dinitrophenol produced maximal ATPase activation and inhibition was obtained with *p*-chloromercuribenzoic acid ($pI_{50}$3·9) but not chlorpromazine (0·01–0·1 mM). There was no significant evidence of $Na^+$ plus $K^+$ activated ATPase or inhibition with ouabain (5 mM). ATPase activity was uncharacterized and relative contributions of specified muscle ATPase systems were unknown. As an insoluble enzyme system was involved and photomicrographs showed characteristic muscle features in freeze-dried sections, ATPase activity appeared due to enzymic function *in situ*. Results are discussed in relation to mutual availability of enzymic sites and reagents.

THE importance of studying enzymes in the milieu in which they normally have their function was emphasized by Linderstrøm-Lang & Holter (1931). However, this cannot be achieved with any degree of certainty in the case of adenosinetriphosphatase (ATPase) systems in muscle due to limitations of the techniques available. For example, homogenization and differential centrifugation are far from ideal because the enzymes are removed from their cellular sites. With histochemical staining procedures the problems are enzyme diffusion and quantitation. To minimize experimental artefacts, the quantitative histochemical procedure described by Lowry, Roberts & others (1954) for determination of ATPase activity in freeze-dried sections of central nervous tissue was applied to muscle. Modifications included technical refinements, different incubation conditions, and the use of cupric ions to catalyse the formation of reduced phosphomolybdate during the colorimetric determination of inorganic phosphate (Peel & Loughman, 1957). The technique was developed as part of an investigation on the pharmacological significance of enzymes in voluntary muscle (Buckley & Nowell, 1966).

## Experimental

*Buffer solution.* N hydrochloric acid (125 ml) was mixed with 1·2 M tris base [2-amino-2-(hydroxy-methyl)-propane-1,3-diol, BDH] (125 ml) and the required volume of stock magnesium sulphate solution added. The pH was adjusted to 7·4 with hydrochloric acid and the volume made up to 500 ml with distilled water to give a 0·3 M tris pH 7·4 buffer/15 mM $MgSO_4$ mixture. When required, other cations were added as chloride

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salts. Calcium chloride solution was prepared by dissolving weighed calcium carbonate in N hydrochloric acid.

*ATP solution.* A 30 mM solution of adenosine 5-triphosphate, disodium salt (Sigma) in 0·045 N sodium hydroxide.

*Colour reagent.* Prepared by mixing 1 ml 1% ammonium molybdate 1 ml 1% ascorbic acid, 1 ml 0·3 mM cupric sulphate and 22 ml 0·1 M acetic acid/0·065 M sodium acetate buffer.

*Activators and inhibitors.* Solutions of 2,4-dinitrophenol, sodium salt (BDH), *p*-chloromercuribenzoic acid (Sigma), chlorpromazine hydrochloride ("Largactil", May and Baker) and ouabain (stropanthin-G, BDH) were prepared in buffer solution at the concentrations required.

METHOD

Male Wistar rats (100–250 g) were decapitated and the diaphragms removed. Pieces of diaphragm muscle, approximately 4 × 4 × 1 mm, were snap frozen on to a microtome chuck using liquid nitrogen. 17·5 μ sections were cut using a refrigerated microtome ("Slee" cryostat) and freeze-dried at −18° (Lowry, 1953) with the aid of a liquid nitrogen cold finger fitted in the vacuum line (1 cm bore tubing). The sections still under vacuum were warmed to room temperature and then removed from the bottle. Approximately 1 $mm^2$ samples of freeze-dried muscle were dissected from the sections under the dissecting microscope, at 32 × magnification, taking care to exclude tendon, fat, connective tissue and blood vessels. The tissue samples were weighed on a quartz "fish pole" balance (Lowry, 1953), transferred with a hair point to tapered "Pyrex" reaction tubes (30 mm × 4 mm) and pressed to the bottom by means of a glass rod with a round tip. Such fixation was necessary to prevent the samples rising up the sides of the tubes when solutions were added. Buffer solution (20 μl) and distilled water (20 μl) were added to each tube followed by ATP solution (20 μl) 20 min later. Blanks (20 μl buffer, 20 μl $H_2O$, 20 μl ATP) and standards (20 μl buffer, 20 μl 1 mM $KH_2PO_4$, 20 μl ATP) were prepared similarly. Activators and/or inhibitors were added in the buffer solution, a Hamilton micro-syringe being used for all additions. The tubes were incubated (15 min) at room temperature and the reaction stopped by the addition of 0·5 ml colour reagent. The tubes were then inverted twice and 30 min later the absorbance at 870 mμ determined in a 1 cm microcuvette. Results were obtained from not less than five tissue samples taken from each diaphragm. A previously determined factor of five was used for the ratio of wet weight/dry weight of tissue. Since the rate of inorganic phosphate release from ATP is linear with time over the period of determination, ATPase activities were calculated for each tissue sample as moles of inorganic phosphate (Pi) produced per kg of wet tissue per 15 min (mole Pi/kg/15 min).

## Results

The mean ATPase activity of 3–30 μg samples of freeze-dried diaphragm muscle taken from twenty rats was 0·22 ± 0·06 (s.d.) mole-Pi/kg/15 min. Incubation of tissue samples in buffer for 35 min at room temperature

produced no demonstable activity in the supernatants; all the activity remained in the tissue samples. This implied that the enzyme system was insoluble. Further results were obtained under varying conditions; each point and bar shown on the graphs represent the mean result from at least six animals ± the standard deviation of the mean.

### SUBSTRATE CONCENTRATION

Maximal activity was observed with 9–12 mM ATP (Fig. 1). When 10 mM ATP was used there was a reasonable correlation between phosphate

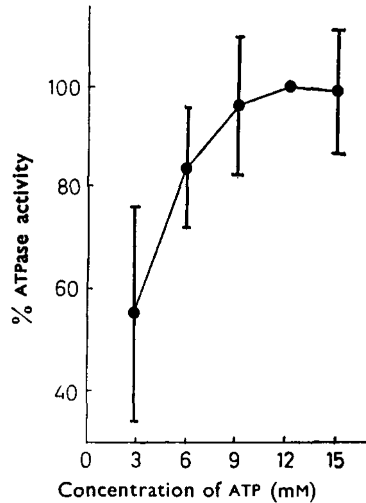


FIG. 1. A plot of ATPase activity against subtrate concentration for freeze-dried muscle sections.

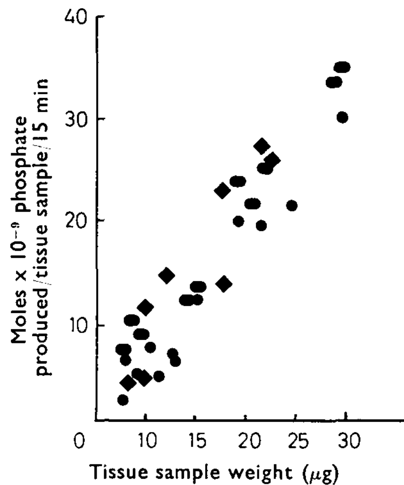


FIG. 2. Relation between tissue sample weight and phosphate measured in determination of ATPase activity of freeze-dried muscle sections. Rat 1 ◆. Rat 2 ⬬. Rat 3 ●.

determined and tissue sample weight (Fig. 2). Optimal working conditions were thus obtained.

ACTIVATORS AND INHIBITORS

There was negligible activity in the absence of added magnesium or calcium ions and similar maximal activities were produced by approximately 5 mM Mg $SO_4$ and 10 mM $CaCl_2$ (Fig. 3). Sodium and potassium ions in various concentrations caused no significant activation in the presence or absence of magnesium ions using disodium or tris ATP.

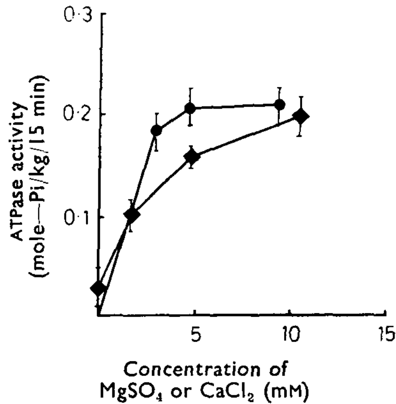


FIG. 3. Variations of ATPase activity of freeze-dried muscle sections with magnesium and calcium ion concentration. ● $MgSO_4$. ◆ $CaCl_2$.

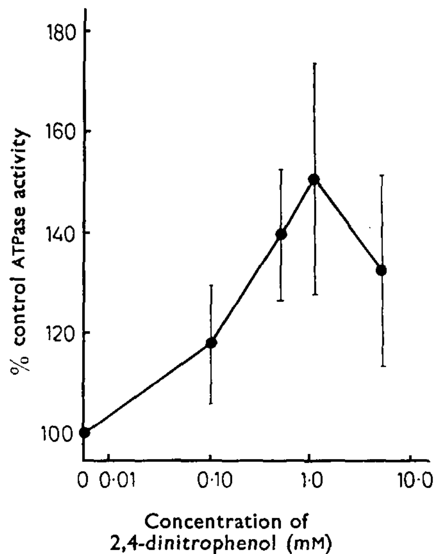


FIG. 4. Plot of 2,4-dinitrophenol activated ATPase activity of freeze-dried muscle sections. Log scale abscissa.

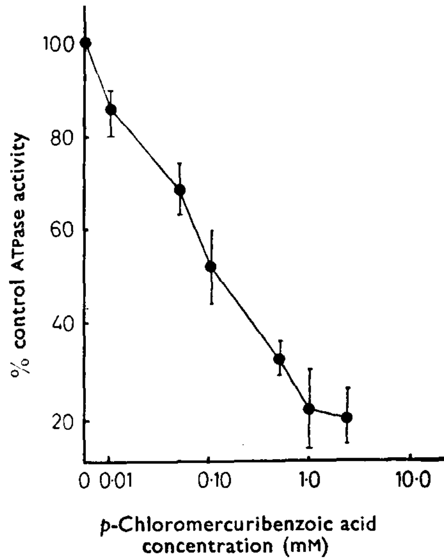


FIG. 5. Plot of *p*-chloromercuribenzoic acid inhibition of ATPase activity of freeze-dried muscle sections. Log scale abscissa.

A peak of 50% activation (Fig. 4) was shown with 1 mM 2,4-dinitrophenol whereas *p*-chloromercuribenzoic acid produced inhibition ($pI_{50}$3·9) (Fig. 5). No significant effects were observed with 0·01–0·1 mM chlorpromazine (higher concentrations of chlorpromazine could not be used because of its low solubility). Ouabain in concentrations up to 5 mM, in the presence or absence of sodium and potassium ions, also had no significant effect.

HISTOLOGICAL FACTORS

Photomicrographs of frozen and freeze-dried sections showed striations and nuclei which were characteristic of normal muscle.

When ten carefully selected tissue samples were prepared from longitudinal and transverse muscle sections, taken from each of five animals, the activities were 0·19 ± 0·04 (s.d.) and 0·25 ± 0·05 (s.d.) mole-Pi/kg/15 min respectively. This suggested that any areas of transverse section which were present in the longitudinal sections generally used (due to curling of the tissue during freezing) did not influence activity. When the section thickness was varied from 5·6–35·5 $\mu$ no corresponding effects on activity were obtained (Table 1). Thus any variation in results was not due to occasional inconsistencies in section width experienced with the cryostat.

A comparison of results for twelve tissue samples fixed to the reaction tubes, with twelve not fixed showed no significant difference in the respective activities (0·20 ± 0·04 (s.d.) and 0·18 ± 0·07 (s.d.) mole-Pi/kg/15 min). Thus the procedure did not cause an irregular liberation of activity appreciable enough to significantly influence the results.

TABLE 1. THE EFFECT OF SECTION THICKNESS OF ATPASE ACTIVITY OF FREEZE-DRIED MUSCLE

| Rat | Section thickness (μ) | Mean ATPase activity* (mole-Pi/kg/15 min) | Standard deviation |
|---|---|---|---|
| 1 | 5·6 | 0·22 | 0·04 |
| 1 | 9·4 | 0·18 | 0·07 |
| 1 | 16·1 | 0·18 | 0·02 |
| 1 | 17·5 | 0·18 | 0·02 |
| 2 | 17·5 | 0·20 | 0·04 |
| 1 | 18·9 | 0·20 | 0·02 |
| 1 | 21·7 | 0·20 | 0·02 |
| 2 | 22·0 | 0·18 | 0·02 |
| 2 | 26·5 | 0·18 | 0·02 |
| 2 | 31·0 | 0·20 | 0·02 |
| 2 | 35·5 | 0·18 | 0·02 |

* 10 determinations; respective serial sections from same muscle block.

## Discussion

Measurements represent net phosphate release from ATP rather than true ATPase activity, since no steps were taken to eliminate myokinase, pyrophosphatase and apyrase. Many muscle enzymes behave as ATPases and the results refer to uncharacterized ATPase activity in the presence of magnesium ions at pH 7·4. Thus no conclusions can be drawn about the relative contributions of ATPase systems in the myofibrils, mitochondria, sarcoplasm, nuclei, sarcolemma and microsomes. The last two mentioned are probably of minor importance because there was little evidence of inhibition with ouabain or $Na^+$ plus $K^+$ activated ATPase activity which is generally associated with ion transport systems in cell membranes and cytomembranes (Albers, 1967). Such a view would agree with the reported that the $Na^+$ plus $K^+$-activated ATPase activity of muscle (ouabain sensitive) is low compared with other tissues (Bonting, Simon & Hawkins, 1961).

Since the freeze-dried tissue used was reasonably intact it is suggested that the results observed depend on enzymic function *in situ*: this is supported by the observation that no ATPase leaked into the incubation medium. An increase in activity was obtained when freeze-dried tissue was homogenized, thus the activity of the enzyme system in the tissue may have been limited by the availability of substrate or of enzymic sites. But homogenates of fresh tissue were five times more active than freeze-dried sections and the respective activity/substrate curves were similar, so the availability of substrate was concluded not to be a major problem (Hopsu & McMillan, 1964). This is supported by the observation that activity was not influenced by section thickness.

The effects of inhibitors were smaller in freeze-dried sections than in homogenates of fresh tissue. Chlorpromazine for example although inactive in freeze-dried sections, was an effective inhibitor in homogenates. *p*-Chloromercuribenzoic acid was 2–3 times more potent an inhibitor in homogenates than in freeze-dried sections. In these cases there was no evidence whether the small effects in freeze-dried sections were due to lack of access of the inhibitor to the enzyme sites or to a lack of the sites themselves. Although concentrations of inhibitor lower than those

of substrate were used, the inhibitors have higher lipid solubility so that presumably they may penetrate the tissue relatively easily.

*Acknowledgements.* This work was supported financially by the Ministry of Defence. Pure chlorpromazine hydrochloride was obtained by the courtesy of Mr. A. W. Nineham, Products Research Group, May & Baker Ltd., Dagenham, Essex. We are also grateful to Professor Andrew Wilson, Head of the Department of Pharmacology University of Liverpool, for helpful advice and encouragement throughout the work which was part of a project on the development of ultramicrobiochemical techniques for exploring the mode of action of drugs.

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