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Effects of Calcium Ions and Adenosine Diphosphate on the Activities of NAD^+-Linked Isocitrate Dehydrogenase from the Radular Muscles of the Whelk and Flight Muscles of Insects

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1. The activity of NAD^+-linked isocitrate dehydrogenase from the radular muscle of the whelk is higher than those in many vertebrate muscles and only slightly lower than in the flight muscles of insects. The enzyme activity from the whelk (*Buccinum undatum*) is stable for several hours after homogenization of the radular muscle, whereas that from insect flight muscle is very unstable. Consequently, the enzyme from the whelk muscle is suitable for a systematic investigation of the effects of Ca^{2+} and ADP. 2. The sigmoid response of the enzyme activity to isocitrate concentration is markedly increased by raising the Ca^{2+} concentration from 0.001 to 10μM, but it is decreased by ADP. The inhibitory effect of Ca^{2+} is most pronounced at pH7.1; it is not observed at pH6.5. Similar effects are observed for the enzyme from the flight muscle of the locust (*Schistocerca gregaria*) and the water bug (*Lethocerus cordofanus*). The percentage activation by ADP of the enzyme from either the whelk or the insects is greater at 10μM-Ca^{2+}, and 50% of the maximum activation is obtained at 0.10 and 0.16mM-ADP for the enzyme from whelk and locust respectively at this Ca^{2+} concentration. At 10μM-Ca^{2+} in the absence of added ADP, the apparent K_m for isocitrate is markedly higher than in other conditions. Ca^{2+} concentrations of 0.01, 0.1 and 0.2μM cause 50% inhibition of maximum activity of the enzyme from the muscles of the whelk, locust and water bug respectively. 3. Recent work has indicated that mitochondria may play a complementary role to the sarcoplasmic reticulum in the control of the distribution of Ca^{2+} in muscle. The opposite effects of Ca^{2+} on the activities of isocitrate dehydrogenase and mitochondrial glycerol phosphate dehydrogenase from muscle tissue are consistent with the hypothesis that changes in the intracellular distribution of Ca^{2+} control the activities of these two enzymes in order to stimulate energy production for the contraction process in the muscle. Although both enzymes are mitochondrial, glycerol phosphate dehydrogenase resides on the outer surface of the inner membrane and responds to sarcoplasmic changes in Ca^{2+} concentration (i.e. an increase during contraction), whereas the isocitrate dehydrogenase resides in the matrix of the mitochondria and responds to intramitochondrial concentrations of Ca^{2+} (i.e. a decrease during contraction). It is suggested that changes in intramitochondrial Ca^{2+} concentrations are primarily responsible for regulation of the activity of NAD^+-isocitrate dehydrogenase in order to control energy formation for the contractile process. However, when the muscle is at rest, changes in intramitochondrial concentrations of ADP may regulate energy formation for non-contractile processes.

There is considerable evidence that changes in the sarcoplasmic Ca^{2+} concentration (from 0.01 to 1μM) during the contraction cycle in muscle control the changes in activities of myofibrillar ATPase† and some key metabolic enzymes, including phosphorylase and mitochondrial glycerol phosphate dehydrogenase (EC 1.1.99.5) (see Ebashi & Endo, 1968; Ashley, 1971; Ozawa *et al.*, 1967; Brostrom *et al.*, 1971; Hansford & Chappell, 1967). The latter enzyme resides on the outer surface of the inner membrane of the mitochondria (Klingenberg & Buchholz, 1970; Donnellan *et al.*, 1970) and responds to extramitochondrial changes in concentration of Ca^{2+}. Similar changes in Ca^{2+} concentration modify the activities of the intramitochondrial enzymes pyruvate dehydrogenase [via changes in the activity of the interconverting enzyme, pyruvate

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† Abbreviation: ATPase, adenosine triphosphatase.

dehydrogenase phosphatase (Randle *et al.*, 1974)] and NAD^+-linked isocitrate dehydrogenase (EC 1.1.1.41) (Vaughan & Newsholme, 1969). However, an increase in Ca^{2+} concentration decreases the activity of the latter enzyme but it increases that of pyruvate dehydrogenase. If these properties are important *in vivo*, they would cause opposite changes in the activities of two enzymes that are important in the oxidation of glucose via the tricarboxylic acid cycle. This seems unlikely. One difficulty in studying the properties of NAD^+–isocitrate dehydrogenase is the lack of stability of the enzyme in the absence of ADP (Goebell & Klingenberg, 1964). It is possible that reported effects of Ca^{2+} on this enzyme could be due to effects on stability. The discovery that the radular muscles of prosobranch molluscs possessed high activities of this enzyme, which are stable in the absence of either ADP or isocitrate, has provided the basis for a systematic investigation of the effects of Ca^{2+}, ADP and other substances on this enzyme in crude extracts of muscle.

Recent work has indicated that mitochondria may play a complementary role to the sarcoplasmic reticulum in control of changes in the intracellular Ca^{2+} concentrations in certain muscles (see Carafoli, 1973, for review). Since these changes in mitochondrial concentrations of Ca^{2+} are considered to control the activities of enzymes involved in energy production for the contraction process, it is unlikely that mitochondrial enzymes from non-muscle tissues respond to these changes in Ca^{2+} concentration. Consequently, the effects of Ca^{2+} on the activities of mitochondrial glycerol phosphate dehydrogenase and NAD^+–isocitrate dehydrogenase from muscle and other tissues were investigated, and the results are also reported in this paper.

Materials and Methods

Chemicals

All chemicals and enzymes were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., except for the following: Pipes [piperazine-*NN'*-bis-(2-ethanesulphonic acid)] and DL-isocitrate were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; 2-mercaptoethanol was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; EDTA, EGTA [ethanedioxybis(ethylamine)tetra-acetate], 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride and all inorganic chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.

Sources of animals

Whelks (*Buccinum undatum*) were purchased from the Marine Biological Association, Plymouth. They were kept in tanks of running sea water and were used within 1 week of delivery. Other animals (locust, *Schistocerca gregaria*; waterbug, *Lethocerus cordofanus*; and laboratory rat) were obtained from the sources given by Sugden & Newsholme (1973).

Preparation of homogenates

Animals were killed and the muscles removed as rapidly as possible. They were homogenized in an all-glass homogenizer with 10–20vol. of extraction medium at 0°C. The latter consisted of 50mM-triethanolamine, 1mM-EDTA, 2mM-$MgCl_2$, 30mM-mercaptoethanol adjusted with HCl to pH7.4. Homogenates were sonicated for 2×15s periods at 0°C in an MSE 100W ultrasonic disintegrator. Higher enzyme activities were obtained by sonication than by homogenization in ground-glass homogenizers, but other methods of mitochondrial rupture were not tested.

Assay of enzyme activities

NAD^+–isocitrate dehydrogenase was assayed by following the change in E_{340} in a Gilford recording spectrophotometer (model 240) at 25°C. The assay medium consisted of 40mM-Pipes, 2mM-NAD^+, 5mM-$MnCl_2$, 5μg of antimycin A [added as 5μl of a 98% (w/v) ethanolic solution] and various concentrations of D-isocitrate and citrate (concentration ratio 1:7.5) at a pH of 7.1 in a total volume of 2.0ml (including 10–20μl of homogenate). The citrate/D-isocitrate ratio of 7.5:1 was used to buffer the D-isocitrate concentration during the assay (see Krebs, 1953). It was assumed that aconitase was sufficiently active to establish equilibrium between citrate and isocitrate. Preliminary experiments indicated that a given concentration of D-isocitrate had the same effect on the activity of NAD^+–isocitrate dehydrogenase as that concentration of DL-isocitrate that would contain a corresponding amount of D-isocitrate. Consequently, DL-isocitrate was used in many experiments to lessen the financial cost of each assay. The concentration of ADP was varied (see Figure legends). The concentration of free Ca^{2+} was maintained by the use of a Ca^{2+}/EGTA buffer (see below).

Mitochondrial glycerol phosphate dehydrogenase was assayed by a modification of the method described by Pennington (1961) (see Crabtree & Newsholme, 1972). The assay medium contained 50mM-P_i (a mixture of Na_2HPO_4 and NaH_2PO_4 was used to give a pH of 7.1), 1mM-KCN, 1.7mM-acetaldehyde, 200μg of yeast alcohol dehydrogenase, 0.0005% 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, various concentrations of DL-glycerol phosphate and 50–100μl of homogenate. Controls omitting substrate were run concurrently. The final volume was 1.5ml, contained

in a stoppered glass tube (10ml capacity). The tubes were incubated at 25°C in the absence of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride for 5min and the reaction was started by addition of a 15μl volume of 0.05% (w/v) solution of the dye. The tubes were stoppered and incubated for periods of up to 30min at 25°C. The reactions were terminated by the addition of 1.5ml of 10% (w/v) $HClO_4$, after which the tubes were placed on ice. The reduced dye was extracted by the addition of 4ml of ethyl acetate to each tube. The contents were shaken for 30s and the tubes centrifuged at 300*g* for 1min to achieve complete separation of the two phases. The top (ethyl acetate) layer was removed and its E_{490} was measured against that of the ethyl acetate layer of the control tube in a Zeiss spectrophotometer (model M4Q. III).

Control of free Ca^{2+} concentration

Solutions used in preparation of the extraction and assay media were made up by using water that had been passed through an Elgastat portable deionizer to remove traces of metal ions. The free Ca^{2+} in the assay media was controlled between concentrations of 0.001 and 10μM by using Ca^{2+}/EGTA buffers as described by Portzehl *et al.* (1964). Two stock solutions were used, 0.1M-EGTA and 0.1M-$CaCl_2$, that were adjusted to pH7.1 with KOH, and the proportions in the assay media were varied to give the required free Ca^{2+} concentration. The final concentration of EGTA was always 1mM. The free concentration of Ca^{2+} is not significantly modified by other cations present in this work or the presence of ADP or citrate. However, variations in pH will modify the Ca^{2+} concentration: a decrease in pH below 7.1 will raise the free Ca^{2+} concentration by approximately 1pCa^{2+} unit for 1pH unit.

Expression of results

Enzyme activities are expressed in terms of μmol of product formed/min per g fresh wt. of tissue at 25°C. The results of each experiment presented are representative of at least three similar experiments. The biological variation in the activity of an enzyme from the same tissue, but from different individual animals, at specific concentrations of substrate and Ca^{2+} was less than 15% in all results reported in this paper. Results of representative experiments are reported rather than mean values of activities from several experiments with ranges or S.E.M. values, since it was considered that the former method of presentation would produce more comprehensible Figures and Tables. Further, it is not the purpose of this paper to deduce precise quantitative correlations but rather to demonstrate the effects of Ca^{2+} on NAD+-isocitrate dehydrogenase from several muscle and non-muscle tissues and to discuss the physiological importance of these effects in control of the tricarboxylic acid cycle in muscle.

Results

The radular muscles of the prosobranchs possess activities of NAD+-isocitrate dehydrogenase that are slightly lower than those of insect flight muscles, but they are higher than those of most vertebrate muscles. Thus the maximum activities in the radular muscles of *Buccinum undatum* and *Murex trunculus* are 9.8 and 18.4μmol/min per g fresh wt. of tissue respectively, which compare with 58.0 and 5.0μmol/min per g for locust (*Schistocerca gregaria*) flight muscle and rat heart respectively.

Stability of NAD+-isocitrate dehydrogenase in crude muscle extracts

The effects of ADP or isocitrate on the stability of the enzymes in crude extracts of the radular muscle of the whelk and of the flight muscle of the locust were investigated. An extract of the muscle was prepared as described in the Materials and Methods section and the zero-time activity was measured as soon as possible after sonication (about 2min). The extract was incubated at 0°C and samples were removed at the times indicated and the enzyme activity was measured. The activity from locust muscle decreased within 10min of extraction to about 50% of that at zero time (see Table 1). The presence of ADP or isocitrate had no effect. However, the activity from the radular muscle remained constant for up to 60min in the presence or the absence of added isocitrate or ADP (Table 1).

The presence of high activities, together with the unusual stability of the NAD+-isocitrate dehydrogenase from the radular muscle of the whelk, suggested that the enzyme from this muscle was suitable for a systematic investigation of its properties.

Effect of pH on NAD+-isocitrate dehydrogenase from the radular muscles of the whelk

The pH optimum for activity of this enzyme was 7.1, which was not modified by the presence of ADP (Fig. 1). This is a similar property to that of the enzyme from locust flight muscle (Klingenberg *et al.*, 1965).

Effects of isocitrate concentrations on NAD+-isocitrate dehydrogenase at different concentrations of ADP and Ca^{2+} at different pH values

NAD+-isocitrate dehydrogenase from radular muscle of the whelk. The response of the activity of the enzyme to isocitrate concentration is sigmoid

Table 1. *Effect of incubation of crude sonicated extracts of muscle on the activities of NAD$^+$-isocitrate dehydrogenase from flight muscle of the locust and radular muscle of the whelk*

Crude extracts were obtained as described in the Materials and Methods section. The incubation was started at the end of sonication and it took about 2min from sonication to initiate the first assay. Activities at this time are given as 100%; the actual activities were as follows: for the muscle from the whelk, 3.7, 2.1 and 2.2μmol/min per g fresh wt., and for the locust muscle, 10.6, 13.2 and 11.0μmol/min per g fresh wt., for extract alone, muscle extracted with 2mM-ADP and muscle extracted with 1.5mM-isocitrate respectively.

Time of incubation of crude extracts (min)	NAD$^+$-isocitrate dehydrogenase activity (%)					
	Locust muscle			Whelk muscle		
	No addition	2mM-ADP	1.5mM-isocitrate	No addition	2mM-ADP	1.5mM-isocitrate
2	100	100	100	100	100	100
5	81	61	70	101	100	95
10	60	50	50	101	107	90
15	52	42	46	100	107	95
20	43	35	38	104	109	100
30	24	21	27	101	109	102
60	18	4	14	104	104	102

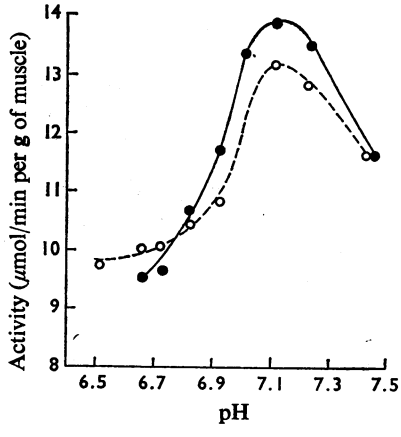

Fig. 1. *Effect of pH on the activity of NAD$^+$-isocitrate dehydrogenase from the radular muscle of the whelk in the presence and absence of ADP*

Assays were carried out as described in the Materials and Methods section at optimum D-isocitrate concentration (1mM). ○, No added ADP; ●, 1.5mM-ADP. No Ca^{2+} was added and the concentration was not controlled by the addition of EGTA.

(Fig. 2). An increase in Ca^{2+} concentration from 0.001 to 10μM markedly increases this sigmoidicity, so that the apparent K_m value for isocitrate is increased. ADP decreases the sigmoidicity, and the effect of ADP is more pronounced at 10μM-Ca^{2+} (Fig. 2). These properties are similar to those reported for rat heart (Vaughan & Newsholme, 1969). The effects of Ca^{2+} and ADP are presented at the following pH values: 6.5, 6.85, 7.1 and 7.35 (Figs. 2*a*, 2*b*, 2*c*, 2*d*). The effects are similar at the three latter pH values, but the inhibitory effect of Ca^{2+} is most pronounced at pH7.1. However, at pH6.5 there is little effect of Ca^{2+}. Increasing the Ca^{2+} concentration from 0.001 to 10μM increases the apparent K_m for isocitrate only at pH7.1 (in the absence of ADP; see Table 2). Indeed, at pH7.1 for the enzyme from both the whelk and water bug, the apparent K_m value for isocitrate is very similar in all conditions, except 10μM-Ca^{2+} in the absence of ADP (Table 2). Thus at very low Ca^{2+} concentrations the K_m value for isocitrate is very low.

The activation of NAD$^+$-isocitrate dehydrogenase by ADP is shown in Table 2 at the two different concentrations of Ca^{2+}. The percentage activation by ADP at different pH values is very variable and depends on the isocitrate concentration. However, the ratio of the percentage activation by ADP at the two different Ca^{2+} concentrations is usually highest at pH7.1 (see Table 3).

No effect of ADP or Ca^{2+} was detectable on the activity of NADP$^+$-isocitrate dehydrogenase from the whelk muscle.

NAD$^+$-isocitrate dehydrogenase from the flight muscle of the locust and water bug. The responses of the activities of the enzymes from locust and the water bug to isocitrate concentration are also sigmoid, and the effects of Ca^{2+} and ADP are very similar to those observed for the enzyme from the radular muscle of the whelk. Since the responses of the enzyme from the water bug are very similar to those for the enzyme from locust, only the data from the water bug are presented (see Fig. 3).

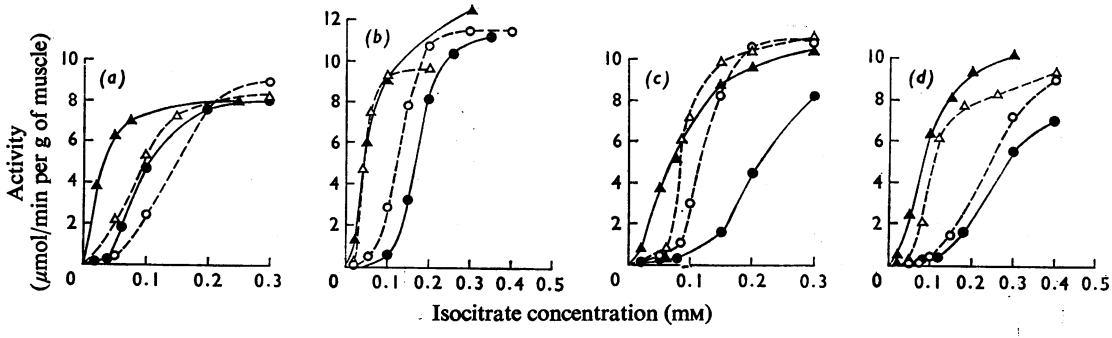

Fig. 2. *Effect of isocitrate concentration on the activity of NAD+–isocitrate dehydrogenase from the radular muscle of the whelk at different concentrations of Ca^{2+} and ADP*

(*a*) pH6.5; (*b*) pH6.85; (*c*) pH7.1; (*d*) pH7.35. The enzyme activity was measured as described in the Materials and Methods section: ○, 0.001 μM-Ca^{2+}; ●, 10 μM-Ca^{2+}; △, 0.001 μM-Ca^{2+} plus 1.5 mM-ADP; ▲, 10 μM-Ca^{2+} plus 1.5 mM-ADP. The concentrations of Ca^{2+} will be higher than 0.001 μM at pH values below 7.1.

Table 2. *Effect of Ca^{2+} and ADP on the apparent K_m for D-isocitrate of NAD+–isocitrate dehydrogenase from whelk radular and insect flight muscles*

The enzyme activities were assayed as described in the Materials and Methods section. The ADP concentration was 1.5 mM. The concentration of Ca^{2+} will be higher than 0.001 μM at pH values below 7.1.

		Apparent K_m (mM)			
Muscle	pH	0.001 μM-Ca^{2+}	0.001 μM-Ca^{2+} plus ADP	10 μM-Ca^{2+}	10 μM-Ca^{2+} plus ADP
Whelk radular	6.5	0.13	0.09	0.11	0.02
	6.85	0.13	0.06	0.16	0.04
	7.1	0.12	0.09	0.25	0.08
	7.35	0.27	0.11	0.27	0.09
Water-bug flight	7.1	0.10	0.09	0.34	0.09

Table 3. *Activation of NAD+–isocitrate dehydrogenase from radular muscle of the whelk at different concentrations of Ca^{2+} and isocitrate and at different pH values*

The percentage activation by ADP is calculated from the data in Fig. 2 by comparing the activities in the absence of added ADP with the activities in the presence of ADP. The concentrations of Ca^{2+} will be higher than 0.001 μM at pH values below 7.1.

Concn. of D-isocitrate (mM)	pH	Activation by ADP (%) 0.001 μM-Ca^{2+}	Activation by ADP (%) 10 μM-Ca^{2+}	Activation at 10 μM-Ca^{2+} / Activation at 0.001 μM-Ca^{2+}
0.05	6.5	550	787	1.4
0.05	6.85	2166	6000	2.8
0.05	7.1	175	2800	16.0
0.05	7.35	300	2300	7.6
0.10	6.5	218	134	0.6
0.10	6.85	317	1500	4.7
0.10	7.1	250	1133	4.5
0.10	7.35	1171	2066	1.8
0.15	6.5	146	116	0.8
0.15	6.85	120	247	1.4
0.15	7.1	120	543	4.5
0.15	7.35	473	1012	2.1

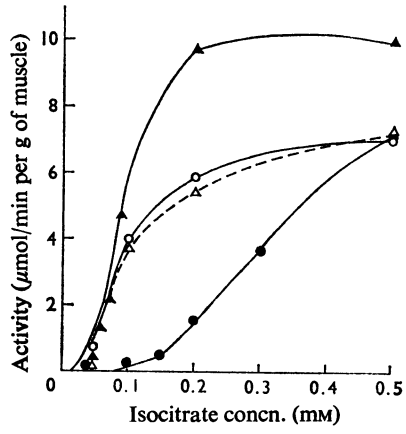

Fig. 3. *Effect of isocitrate concentration on the activity of NAD$^+$-isocitrate dehydrogenase from the flight muscle of the water bug at different concentrations of Ca^{2+} and ADP*

The pH was 7.1. The key is as described in Fig. 2.

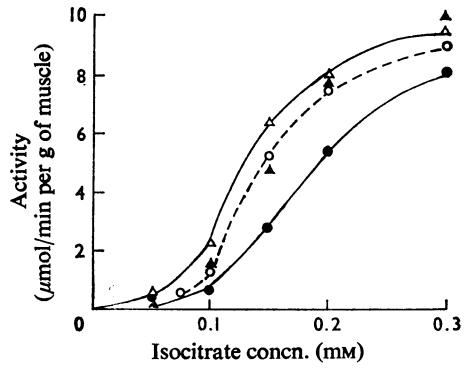

Fig. 4. *Effect of isocitrate concentration on the activity of NAD$^+$-isocitrate dehydrogenase from the radular muscle of the whelk at different concentrations of Ca^{2+} and AMP*

The pH was 7.1 and the activity was measured as described in the Materials and Methods section. ○, 0.001 μM-Ca^{2+}; ●, 10 μM-Ca^{2+}; △, 0.001 μM-Ca^{2+} plus 1.5 mM-AMP; ▲, 10 μM-Ca^{2+} plus 1.5 mM-AMP.

Effects of AMP and other nucleotides on the activity of NAD$^+$-isocitrate dehydrogenase from the radular muscle of the whelk

AMP had no effect at 0.001 μM-Ca^{2+}, but it decreased the sigmoidicity of the response to isocitrate at 10 μM-Ca^{2+} (i.e. it lowered the apparent K_m value for isocitrate; see Fig. 4). Thus there is little effect of AMP on the enzyme, so that it is distinct from the enzyme from some other sources (e.g. yeast), on which AMP has a very marked activating effect (see Plaut, 1970). The plot of percentage activation of the enzyme from the whelk radular muscle against AMP concentrations is sigmoid (Fig. 5), but no sigmoidicity is observed with ADP (see below).

The enzyme from the radular muscle was unaffected by GDP, IDP or UDP (2 mM) at a low concentration of isocitrate (0.15 mM-D-isocitrate) at either 0.001 or 10 μM-Ca^{2+}.

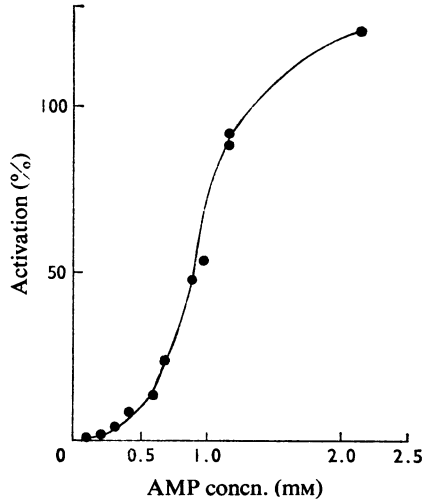

Fig. 5. *Effect of AMP concentrations on the percentage activation of NAD$^+$-isocitrate dehydrogenase from the radular muscle of the whelk*

The concentrations of Ca^{2+} and D-isocitrate were 10 μM and 0.15 mM respectively. The pH was 7.1.

Effect of ADP on the activities of NAD$^+$-isocitrate dehydrogenase from the muscles of the whelk and locust

The percentage activations of NAD$^+$-isocitrate dehydrogenase by ADP for the muscles from both the whelk and the locust are higher in the presence of 10 μM-Ca^{2+} (Table 4). The concentration of ADP that gives 50% of the maximum activation at low isocitrate concentration is decreased from 0.29 to 0.1 mM for the enzyme from the whelk muscle by the higher concentration of Ca^{2+} (10 μM), whereas it is increased from 0.05 to 0.16 mM-ADP for the locust muscle. (The activation effect of ADP is not due to stabilization, since the addition of ADP to a linear assay immediately caused an increase in activity.) The latter increase is at least partly due to the fact that the activation of the locust muscle enzyme by ADP at 10 μM-Ca^{2+} is sigmoidal, which indicates positive co-operativity (i.e. in Fig. 6*b* the double-

Table 4. *Activation by ADP of NAD+-isocitrate dehydrogenase from the radular muscle of the whelk and flight muscle of the locust*

The concentrations of D-isocitrate were 0.1 and 0.5mM for the enzyme from whelk and locust muscle respectively. The pH was 7.1.

Concn. of ADP (mM)	Activation (%)			
	Flight muscle		Radular muscle	
	0.001 μM-Ca^{2+}	10 μM-Ca^{2+}	0.001 μM-Ca^{2+}	10 μM-Ca^{2+}
0.02	28	62	12	80
0.05	55	387	32	155
0.10	88	937	56	240
0.25	98	2725	106	340
0.50	108	3125	160	400
1.00	108	3525	205	440
1.40	—	—	214	457

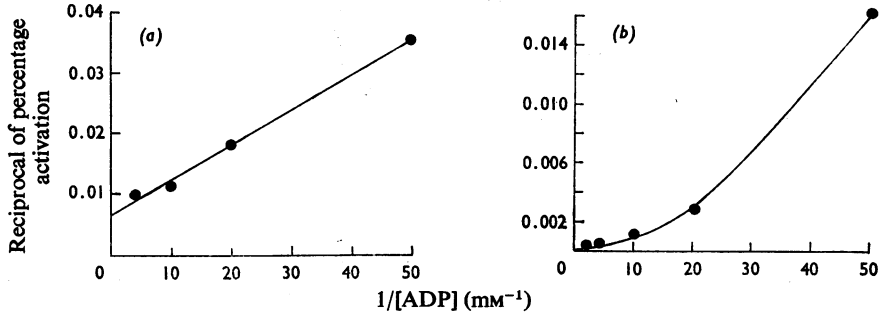

Fig. 6. *Double-reciprocal plots of percentage activation against ADP concentration for NAD+-isocitrate dehydrogenase from locust flight muscle*

(*a*) Ca^{2+} concn. 0.001 μM; (*b*) Ca^{2+} concn. 10 μM. The activity was measured as described in the Materials and Methods section: the isocitrate concentration was 0.5mM-D-isocitrate and the pH was 7.1.

reciprocal plot is concave upwards); there is no indication of co-operativity at 0.001 μM-Ca^{2+} (i.e. in Fig. 6*a* the double-reciprocal plot is linear): similar results have been obtained with the enzyme from the waterbug. However, there is no indication of co-operativity at either Ca^{2+} concentration with the enzyme from whelk muscle (results not shown). It is possible that the positive co-operativity observed with the enzyme from locust flight muscle is due to variations in the chemical nature of ADP (e.g. ADP, ADP-Mg, ADP-Mn) as the concentration of ADP is varied.

Effect of Ca^{2+} concentration of the activities of NAD+-isocitrate dehydrogenase from the radular muscle of the whelk and the flight muscle of the locust and the water bug

An increase in Ca^{2+} concentration from 0.001 to 10 μM causes marked inhibition of the activity at low isocitrate concentrations of the enzymes from all three muscles (Table 5). From plots of percentage inhibition against Ca^{2+} concentration (plots not shown), 50% of maximum inhibition was observed at 0.01, 0.1 and 0.2 μM for the enzymes from the whelk, locust and waterbug muscles respectively.

Effects of NAD+ concentration on the activities of NAD+-isocitrate dehydrogenase from the radular muscle of the whelk

The plot of reciprocal activity against reciprocal of the NAD^+ concentration at 0.001 μM-Ca^{2+} is slightly concave upwards, which suggests positive co-operativity under these conditions (Fig. 7). At 10 μM-Ca^{2+}, the plot is linear but the slope is greater, indicating that Ca^{2+} has increased the apparent K_m value for NAD^+. Thus Ca^{2+} inhibits the enzyme at low concentrations of NAD^+. However, in the presence of ADP, an increase in Ca^{2+} concentration from 0.001 to 10 μM has no effect (Fig. 7).

Effect of ADP and Ca^{2+} on the activity of NAD^+–isocitrate dehydrogenase from brain and kidney cortex of the rat

The response of the activity of NAD^+–isocitrate dehydrogenase from either brain or kidney cortex of the rat to the isocitrate concentration is sigmoid. ADP decreases the sigmoidicity, so that it markedly activates the enzyme at low isocitrate concentrations. However, increasing the Ca^{2+} concentration from 0.001 to 10μM has no effect (Fig. 8). This increase in Ca^{2+} concentration markedly inhibits the enzyme from rat heart (Vaughan & Newsholme, 1969).

Effect of Ca^{2+} on the activity of mitochondrial glycerol 3-phosphate dehydrogenase from heart, brain and liver of the rat

An increase in the concentration of Ca^{2+} from 0.001 to 10μM increases the activity of mitochondrial glycerol 3-phosphate dehydrogenase from rat heart at low glycerol phosphate concentrations (Table 6). A similar increase in Ca^{2+} concentration has no such effect on the activities of the enzyme from rat brain, liver or kidney cortex. Indeed this increase in Ca^{2+} concentration appears to decrease the $V_{max.}$ value (Table 6).

Table 5. *Inhibition by Ca^{2+} of NAD^+–isocitrate dehydrogenase from radular muscle of the whelk and flight muscles of the locust and water bug*

The concentrations of D-isocitrate were 0.5 and 0.15mM for flight muscles and radular muscle respectively. The pH was 7.1.

Concn. of Ca^{2+} (mM)	Inhibition (%)		
	Radular muscle	Locust flight muscle	Water-bug flight muscle
0.001	0	0	0
0.01	38	0	0
0.1	70	52	27
0.5	—	87	54
1.0	81	100	67
10.0	83	100	67

Discussion

The tricarboxylic acid cycle is particularly important in the provision of energy in muscles that are mechanically active for sustained periods

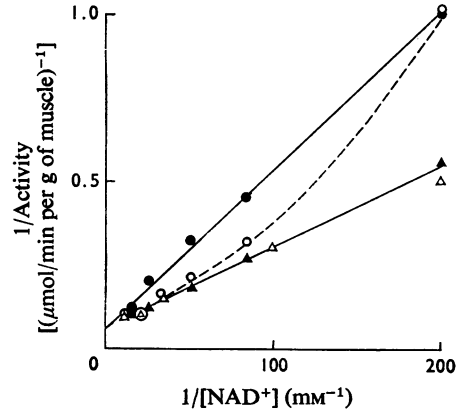

Fig. 7. *Double-reciprocal plot of the activity of NAD^+–isocitrate dehydrogenase from the radular muscle of the whelk against the NAD^+ concentration*

The concentration of D-isocitrate was 1mM and the pH was 7.1. The key is as described in Fig. 2.

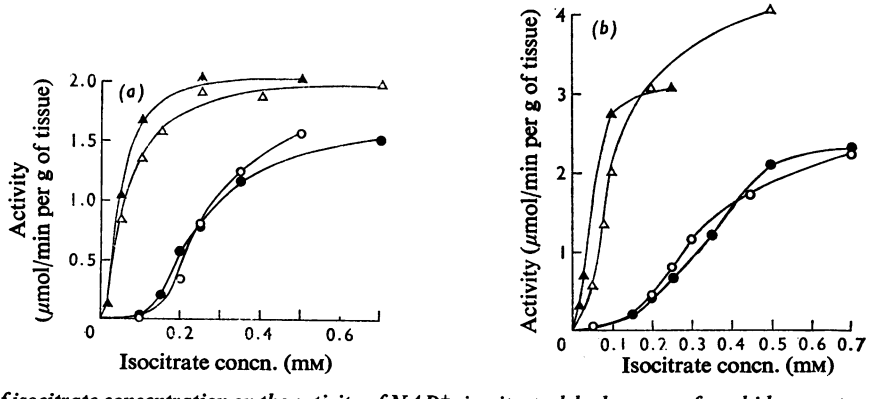

Fig. 8. *Effect of isocitrate concentration on the activity of NAD^+–isocitrate dehydrogenase from kidney cortex (a) and brain (b) of the rat at different concentrations of ADP and Ca^{2+}*

The key is as described in Fig. 2.

Table 6. *Effect of Ca^{2+} on the activity of mitochondrial glycerol 3-phosphate dehydrogenase from rat tissues at different concentrations of glycerol 3-phosphate*

The activities were measured as described in the Materials and Methods section. The activities are expressed in arbitrary units, an activity of unity being assigned to that obtained at 8mM-DL-glycerol 3-phosphate and in the presence of 10μM-Ca^{2+}.

Concn. of DL-glycerol 3-phosphate (mM)	Glycerol 3-phosphate dehydrogenase activities							
	Heart		Brain		Liver		Kidney cortex	
	0.001μM-Ca^{2+}	10μM-Ca^{2+}	0.001μM-Ca^{2+}	10μM-Ca^{2+}	0.001μM-Ca^{2+}	10μM-Ca^{2+}	0.001μM-Ca^{2+}	10μM-Ca^{2+}
0.26	0.05	0.28	—	—	0.63	0.59	0.15	0.15
0.66	0.23	0.54	0.86	0.75	1.16	0.94	0.43	0.43
1.33	0.29	0.73	1.09	0.91	1.19	0.94	0.83	0.85
2.66	0.70	0.87	1.18	0.95	1.38	1.00	—	—
4.00	0.80	0.98	1.34	0.98	—	—	1.29	0.87
8.00	0.96	1.00	1.54	1.00	1.56	1.00	1.45	1.00

(e.g. heart, aerobic vertebrate skeletal muscle, radular retractor muscle of the prosobranchs) and in muscles that utilize energy at a very high rate (e.g. insect flight muscles). There is some evidence that NAD+–isocitrate dehydrogenase plays a role in the regulation of the cycle in these muscles (see Goebell & Klingenberg, 1964; Johnson & Hansford, 1975). Consequently, the properties of this enzyme are important in providing the basis for a theory of the cycle in muscle.

The inhibition of NAD+–isocitrate dehydrogenase by low concentrations of Ca^{2+} was first reported by Vaughan & Newsholme (1969). However, the instability of the enzyme in crude (or mitochondrial) extracts of muscle (see Table 1) deterred any further work on the detailed properties of the enzyme, particularly the interaction between the effects of Ca^{2+} and ADP. Thus within 10min of extraction at least 50% of the activity is lost, and the remaining activity is completely lost within 2h. Consequently, a kinetic study that lasts longer than a few hours is very difficult to perform with an extract from a given muscle. The discovery that the radular muscles of prosobranchs possess high activities of NAD+–isocitrate dehydrogenase which are more stable permitted a detailed analysis of the effects of Ca^{2+} and ADP on the activity of this enzyme. The results of this study demonstrate that the properties of the enzyme from the whelk are very similar to those of the enzyme from insect flight muscles. The response of the activity to the isocitrate concentration is sigmoid, which is exaggerated by increasing the Ca^{2+} concentration to 10μM. Conversely, ADP decreases the sigmoidicity. Thus Ca^{2+} increases the apparent K_m value of the enzyme for isocitrate, though this value is decreased by ADP. The results demonstrate that Ca^{2+} modifies the response of the enzyme to ADP, so that it may be more sensitive to the changes in the concentration of ADP at 10μM-Ca^{2+} (see below).

These effects of Ca^{2+} may be important in the regulation of enzyme activity during the contraction cycle of the muscle (see below). The effects of Ca^{2+} on the activities of NAD+–isocitrate dehydrogenase from two non-muscle aerobic tissues were also studied. The brain and kidney cortex of the rat were chosen, since Vaughan & Newsholme (1969) had demonstrated an inhibitory effect of Ca^{2+} on the enzyme from the heart of the rat. The enzyme from these tissues in the rat was not affected by 10μM-Ca^{2+}. This finding emphasizes the possible physiological significance of this effect of Ca^{2+} in the control of energy formation in contracting muscle.

The importance of the sarcoplasmic reticulum in the control of the intracellular distribution of Ca^{2+} in muscle is widely accepted. However, recent work suggests that mitochondria may play a supplementary

or a complementary role to the reticulum in controlling the Ca^{2+} concentration during the contraction–relaxation cycle in some muscles. In other muscles, in which the sarcoplasmic reticulum is poorly developed (e.g. heart, smooth muscle, insect fibrillar muscles), the mitochondria may play a more prominent role in the control of the sarcoplasmic concentration of Ca^{2+} (see Carafoli, 1973; Lehninger, 1974, for reviews). In order to play such a role, mitochondria must release Ca^{2+} during the contractile phase of the cycle and take up Ca^{2+} during the relaxation phase. Consequently, the direction of change of the intramitochondrial Ca^{2+} concentration would be expected to be opposite to that of the sarcoplasmic Ca^{2+}. Since glycerol phosphate dehydrogenase is localized on the outer face of the inner mitochondrial membrane, it would be expected that this enzyme (from muscle) would be activated by Ca^{2+}. The properties of the NAD^+–isocitrate dehydrogenase are consistent with stimulation of the enzyme activity during the contractile phase of the cycle. Thus at low concentrations of Ca^{2+} the apparent K_m value of the enzyme for isocitrate is low (in either the presence or the absence of ADP; see Table 2), so that the enzyme will be catalytically active. Thus it is possible to suggest that intramitochondrial concentration changes in Ca^{2+} play a much more important role in regulation of the activity of this enzyme during contraction than do changes in that of ADP.

It is known that muscles will require energy for functions other than contraction (e.g. biosynthesis, ion transport) and this energy will be required during periods of rest. Consequently, it is notable that (at pH7.1) only at the high concentration of Ca^{2+} (i.e. that assumed to be present in the mitochondria when the muscle is at rest) does ADP lower the apparent K_m value for isocitrate.

Further, the enzymes from both the locust flight muscle and the whelk radular muscle are more sensitive to changes in the concentration of ADP at $10\mu M$-Ca^{2+}. [The latter enzyme is half-maximally activated at a lower ADP concentration, whereas the enzyme from the locust exhibits positive co-operativity to ADP at $10\mu M$-Ca^{2+}. Positive co-operativity increases the sensitivity of an enzyme to a change in concentration of a regulator (see Newsholme & Start, 1973).] Thus it is suggested that the activation of NAD^+–isocitrate dehydrogenase by ADP may be important in stimulating energy formation in muscle during non-contractile conditions. This suggestion is consistent with the findings that NAD^+–isocitrate dehydrogenase from non-contractile tissues is activated by ADP but Ca^{2+} has no effect (Fig. 8).

The activating effect of Ca^{2+} on mitochondrial glycerol phosphate dehydrogenase was observed for the enzyme from muscle but not from non-contractile tissues (e.g. brain, liver, or kidney cortex). This emphasizes the physiological importance of the effect of Ca^{2+} on the enzyme from muscle. Since this enzyme resides on the outer surface of the inner mitochondrial membrane, the opposite effects of Ca^{2+} on this enzyme and isocitrate dehydrogenase are consistent with the proposed changes in intracellular distribution of this ion, so that the activities of both enzymes are increased to generate energy for the contractile activity. However, the activating effects of Ca^{2+} in the concentration range $0.1–10\mu M$ on the activity of another mitochondrial enzyme, pyruvate dehydrogenase phosphatase, are not consistent with increased energy formation during the contraction phase in muscle. A decrease in the intramitochondrial concentration of Ca^{2+} would result in a fall in the activity of the phosphatase and hence in that of pyruvate dehydrogenase, so that the oxidation of pyruvate and hence glucose would be decreased. It is suggested that the effects of Ca^{2+} on the pyruvate dehydrogenase complex may be of regulatory importance only in tissues in which the activity of the phosphatase is modified by hormones (e.g. adipose tissue, liver, kidney cortex). Indeed, it is known that whereas pyruvate oxidation by isolated mitochondria is inhibited instantaneously by the addition of Ca^{2+} (Hansford, 1972), maximum activation of pyruvate dehydrogenase by Ca^{2+} occurs only after 20min (Shuster & Olson, 1974). In the present study, changes in the activity of NAD^+–isocitrate dehydrogenase by Ca^{2+} were observed within 5s. The rapidity of this effect on NAD^+–isocitrate dehydrogenase is consistent with the role of Ca^{2+} in the control of energy generation for the contractile process in muscle.

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