Biochem. J. (1976) 160, 253–262
Printed in Great Britain

Nicotinamide–Adenine Dinucleotide-Linked 'Malic' Enzyme in Flight Muscle of the Tse-tse Fly (*Glossina*) and other Insects

By JAN B. HOEK, DAVID J. PEARSON and NORAH K. OLEMBO
Department of Biochemistry, University of Nairobi, P.O. Box 30197, *Nairobi, Kenya*

(*Received* 12 *May* 1976)

1. A high activity of NAD-linked 'malic' enzyme was found in homogenates of flight muscle of different species of tse-tse fly (*Glossina*). The activity was the same as, or higher than, that of malate dehydrogenase and more than 20-fold that of NADP-linked 'malic' enzyme. A similar enzyme was found in the flight muscle of all other insects investigated, but at much lower activities. 2. A Ca^{2+}-stimulated oxaloacetate decarboxylase activity was present in all insect flight-muscle preparations investigated, in constant proportion to the NAD-linked 'malic' enzyme. 3. A partial purification of the NAD-linked 'malic' enzyme from *Glossina* was effected by DEAE-cellulose chromatography, which separated the enzyme from malate dehydrogenase and NADP-linked 'malic' enzyme, but not from oxaloacetate decarboxylase. 4. The intracellular localization of the NAD-linked 'malic' enzyme was predominantly mitochondrial; latency studies suggested a localization in the mitochondrial matrix space. 5. Studies on the partially purified enzyme demonstrated that it had a pH optimum between 7.6 and 7.9. It required Mg^{2+} or Mn^{2+} for activity; Ca^{2+} was not effective. The maximum rate was the same with either cation, but the concentration of Mn^{2+} required was 100 times less than that of Mg^{2+}. Activity with NADP was only 1–3% of that with NAD, unless very high (>10mM) concentrations of Mn^{2+} were present. 6. It is suggested that the NAD-linked 'malic' enzyme functions in the proline-oxidation pathway predominant in tse-tse fly flight muscle.

Proline constitutes a major substrate for energy supply to the flight muscle of the tse-tse fly (*Glossina*) and some species of beetle (Bursell, 1963; Brosemer & Veerabhadrappa, 1965; De Kort *et al.*, 1973; Hansford & Johnson, 1975). Bursell (1963) proposed a pathway for the conversion of proline into alanine involving a transamination of glutamate, formed from proline, with pyruvate. The regeneration of pyruvate was assumed to occur by a decarboxylation of oxaloacetate, formed by oxidation of α-oxoglutarate via the tricarboxylic acid cycle. Subsequent studies (Bursell, 1966, 1967; Norden & Patersen, 1969; Crabtree & Newsholme, 1970) provided evidence for the occurrence of this pathway in the flight muscle of the tse-tse fly, and a high activity of oxaloacetate decarboxylase (EC 4.1.1.3) was found by Bursell (1965) in water extracts of acetone-treated powders of tse-tse fly flight muscle.

In recent years NAD-linked 'malic' enzymes (EC 1.1.1.39) have been reported in low activity in a variety of higher organisms (Sauer, 1973*a*,*b*; Lin & Davis, 1974). These enzymes have been found to differ from NADP-linked 'malic' enzymes in several respects. Thus they are unable to catalyse the reductive carboxylation of pyruvate (Frenkel, 1975), they do not catalyse the decarboxylation of oxaloacetate (Lin & Davis, 1974; Frenkel, 1975; see also Saz & Hubbard, 1957; Fodge *et al.*, 1972), they are highly specific for NAD when activated with Mg^{2+}, but with Mn^{2+} often show a considerable reactivity with NADP (Sauer, 1973*a*,*b*; Lin & Davis, 1974), they are affected by various activators and inhibitors, such as ATP, pyruvate, succinate and fumarate (Sauer, 1973*a*,*b*; Lin & Davis, 1974; Hansford & Johnson, 1975) and they are located in the mitochondria (Sauer, 1973*a*,*b*; Lin & Davis, 1974). Since an NAD-linked 'malic' enzyme could well be involved in the regeneration of pyruvate from α-oxoglutarate during proline oxidation, we investigated this and related activities in the flight muscle of various selected insects. While this paper was in preparation, Hansford & Johnson (1975) reported a high activity of NAD-linked 'malic' enzyme in flight-muscle mitochondria of the Japanese beetle (*Popillia japonica*), but did not separate the activity from malate dehydrogenase (EC 1.1.1.37) and oxaloacetate decarboxylase so as to identify it as a distinct enzyme.

Materials and Methods

Insects

Glossina morsitans, *G. austeni* and armyworm moths (*Spodoptera exempta*), bred and reared under laboratory conditions, were kindly supplied by the

International Centre of Insect Physiology and Ecology, Nairobi, Kenya. *G. pallidipes* and *G. longipennis*, collected in the field (Nguruman forest, Kenya) by kindness of Dr. J. van Etten and his coworkers, were stored in liquid N_2 until used. Fleshflies (*Sarcophaga tibialis*) were bred and reared in our laboratory. Locusts (*Schistocerca gregaria*) and cockroaches (*Periplaneta americana*) were obtained from the Department of Zoology, University of Nairobi, and bees (*Apis mellifera*) from the National Agricultural Laboratories, Kabete, Kenya. Termites (*Odontotermes* sp.) were of a migratory winged form caught locally immediately after emergence during the rainy season. Dung beetles (*Heliocarpus* sp.), also caught locally, were winged but not in the migratory stage.

Preparations

Flight-muscle homogenates were prepared in 0.25 M-sucrose/1 mM-EGTA [ethanedioxybis(ethylamine)tetra-acetic acid], pH 7.4, except for preparations to be fractionated on DEAE-cellulose, where 10 mM-Tris/HCl/1 mM-EGTA, pH 7.6, was used. The methods applied were essentially those of Van den Bergh (1967). For tse-tse fly, fleshfly, termite, armyworm moth and bee, whole thoraces were pounded in a mortar with ice-cold sucrose/EGTA medium, filtered through four layers of muslin, then carefully homogenized; for locust, cockroach and dung beetle, flight muscles were carefully excised and homogenized in sufficient sucrose/EGTA medium to give a protein concentration of approx. 5 mg/ml.

Assays

NAD-linked 'malic' enzyme was assayed spectrophotometrically at 340 nm in Tris/HCl buffer, pH 7.4 or 7.8 (0.1 M), containing 1 mM-NAD^+, 5 mM-$MgCl_2$, 20 mM-malate and malate dehydrogenase (2.5 units/ml). The malate dehydrogenase reaction was left to equilibrate before the 'malic' enzyme reaction was started by the addition of $MgCl_2$. With partially purified preparations, where no endogenous malate dehydrogenase was present, this enzyme was omitted from the assay system. Where excess of malate dehydrogenase was present in assays of crude preparations, an underestimation of the initial rate of the NAD-linked 'malic' enzyme occurred owing to part of the NADH formed in the reduction of oxaloacetate being trapped. The maximum rate was approached only after the E_{340} had increased by 0.2–0.3 unit. With low activities of NAD-linked 'malic' enzyme, a correction had to be applied for this interference, amounting in the limiting case to 100% of the measured activity (see Pearson, 1965).

Oxaloacetate decarboxylase was assayed at pH 5.9 (see Bursell, 1965) by incubation of a system containing 0.1 M-imidazole buffer, 4 mM-sodium arsenite, 5 mM-Tris/oxaloacetate and 5 mM-$CaCl_2$. Reactions were stopped after different reaction times with $HClO_4$ (3%, v/v, final concentration). After deproteinization, neutralization and removal of $KClO_4$, the extracts were assayed for oxaloacetate and pryuvate by enzymic methods (see Bergmeyer, 1970). For every incubation, a corresponding control incubation was carried out without enzyme preparation, but with an appropriate amount of sucrose/EGTA medium, to correct for non-enzymic decarboxylation of oxaloacetate.

Malate dehydrogenase and α-glycerophosphate dehydrogenase (EC 1.1.1.8) were assayed in 0.1 M-glycine/NaOH buffer, pH 10, containing 1 mM-NAD^+, and either 50 mM-L-malate or 20 mM-DL-α-glycerophosphate. NADP-linked 'malic' enzyme (EC 1.1.1.40) was assayed in 0.1 M-Tris/HCl buffer, pH 7.4, containing 50 μM-$NADP^+$, 5 mM-$MgCl_2$ and 2 mM-L-malate. Citrate synthase (EC 4.1.3.7) was measured by the coupled malate dehydrogenase assay described by Ochoa *et al.* (1951). All enzyme assays were carried out at 30°C. For measurements on crude homogenates possible latency of enzymes was prevented by the addition of Triton X-100 (0.03%) to the assay system. Sonication was avoided because it partially inactivated the NAD-linked 'malic' enzyme. Rotenone (5 μg/ml) was also included in nicotinamide nucleotide-linked assays of crude enzymes. A unit of enzyme activity is taken as the amount required to catalyse the conversion of 1 μmol of substrate into product per min.

Protein was measured by the procedure of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Results

NAD-linked 'malic' enzyme and related enzymes in flight muscle of various insects

The activities of NAD-linked and NADP-linked 'malic' enzymes, malate dehydrogenase and oxaloacetate decarboxylase in the flight muscle of selected insects are shown in Table 1. A remarkably high activity of NAD-linked malic enzyme was observed in the tse-tse fly *Glossina morsitans*. This enzyme had a lower but still considerable activity in all other species except the cockroach, in which only a trace was detectable.

In contrast with the wide variation in activity of NAD-linked 'malic' enzyme, the range of activities found for NADP-linked 'malic' enzyme and malate dehydrogenase was relatively small. The activity of NAD-linked 'malic' enzyme in *G. morsitans* was of the same order of magnitude as that of malate dehydrogenase. In the other species shown in Table 1 the NAD-linked and NADP-linked 'malic' enzyme activities were comparable, one or two orders of magnitude lower than that of malate dehydrogenase.

With the preparation methods used, some contami-

Table 1. *Activities of NAD-linked 'malic' enzyme, NADP-linked 'malic' enzyme, malate dehydrogenase and oxaloacetate decarboxylase in flight-muscle homogenates of various insect species*

The preparation of homogenates and the assay systems are described in the Materials and Methods section. NAD-linked 'malic' enzyme was assayed at pH 7.4. Values quoted are the average of the numbers of preparations indicated in parentheses. Individual activities measured were all within 25% of the mean values shown.

Insect species	Activity (nmol/min per mg of protein)			
	NAD-linked 'malic' enzyme	NADP-linked 'malic' enzyme	Malate dehydrogenase	Oxaloacetate decarboxylase
Diptera				
Tse-tse fly (*Glossina morsitans*) (7)	796	44	882	460
Fleshfly (*Sarcophaga tibialis*) (6)	66	18	2250	78
Orthoptera				
Locust (*Schistocerca gregaria*) (2)	79	20	2290	93
Dictyoptera				
Cockroach (*Periplaneta americana*) (2)	6	13	605	—
Isoptera				
Termite (*Odontotermes* sp.) (2)	107	104	3470	156
Lepidoptera				
Armyworm moth (*Spodoptera exempta*) (2)	30	41	2080	31
Hymenoptera				
Bee (*Apis mellifera*) (2)	40	25	2710	38
Coleoptera				
Dung beetle (*Heliocarpus* sp.) (1)	78	18	608	81

Table 2. *Enzyme activities in flight-muscle homogenates of different species of tse-tse fly (Glossina)*

For details, see Table 1.

Species	Activities (nmol/min per mg of protein)		
	NAD-linked 'malic' enzyme	NADP-linked 'malic' enzyme	Malate dehydrogenase
G. morsitans (7)	796	44	882
G. austeni (2)	906	55	778
G. pallidipes (4)	1210	79	326
G. longipennis (2)	1040	47	416

nation of the homogenates by tissues other than flight muscle cannot be completely excluded. This may cause some distortion of the values reported, in particular for NADP-linked 'malic' enzyme, which is found in relatively high activity in the fat body in some of these insects.

A close correlation was observed between the activities of NAD-linked 'malic' enzyme and oxaloacetate decarboxylase, the ratio of these activities being practically constant over the range of species investigated. Oxaloacetate decarboxylase activity was measured in the presence of Ca^{2+}. Without added $CaCl_2$ the activity measurable in homogenates of *G. morsitans* flight muscle prepared in the presence of EGTA was less than 10% of that with Ca^{2+} present. Neither Mg^{2+} nor Mn^{2+} could substitute for Ca^{2+}, nor did these cations affect the reaction in the presence of Ca^{2+}.

A positive correlation was also found between the activities of NAD-linked 'malic' enzyme and alanine aminotransferase (results not shown).

Enzyme activities in different species of tse-tse fly are shown in Table 2. The activity of NAD-linked 'malic' enzyme was high in all four species. Malate dehydrogenase activity was found to be rather low in *G. pallidipes* and *G. longipennis*. It should be noted that these two species were caught in the field, and kept in liquid N_2 for 2 weeks before preparation of thorax homogenates, whereas *G. morsitans* and *G. austeni* were bred under laboratory conditions. No significant differences in enzyme activity were found between males and females of the same species.

Separation of 'malic' enzyme and malate dehydrogenase activities

In the commonly used spectrophotometric assay

system, it is not possible to distinguish between a true NAD-linked 'malic' enzyme and a reduction of NAD^+ by malate dehydrogenase coupled to an oxaloacetate decarboxylase activity (see Lin & Davis, 1974; Hansford & Johnson, 1975). In some species the estimated NAD-linked 'malic' enzyme activity of the flight muscle considerably exceeded that of malate dehydrogenase, but an excess of malate dehydrogenase was necessary in the assay of 'malic' enzyme in crude preparations to prevent interference by endogenous malate dehydrogenase.

Convincing evidence for a distinct identity of NAD-linked 'malic' enzyme was obtained by separating it from malate dehydrogenase on DEAE-cellulose (Fig. 1). *G. pallidipes* thoraces (30/7ml) were homogenized in 10mM-Tris/1mM-EGTA, pH7.6. The

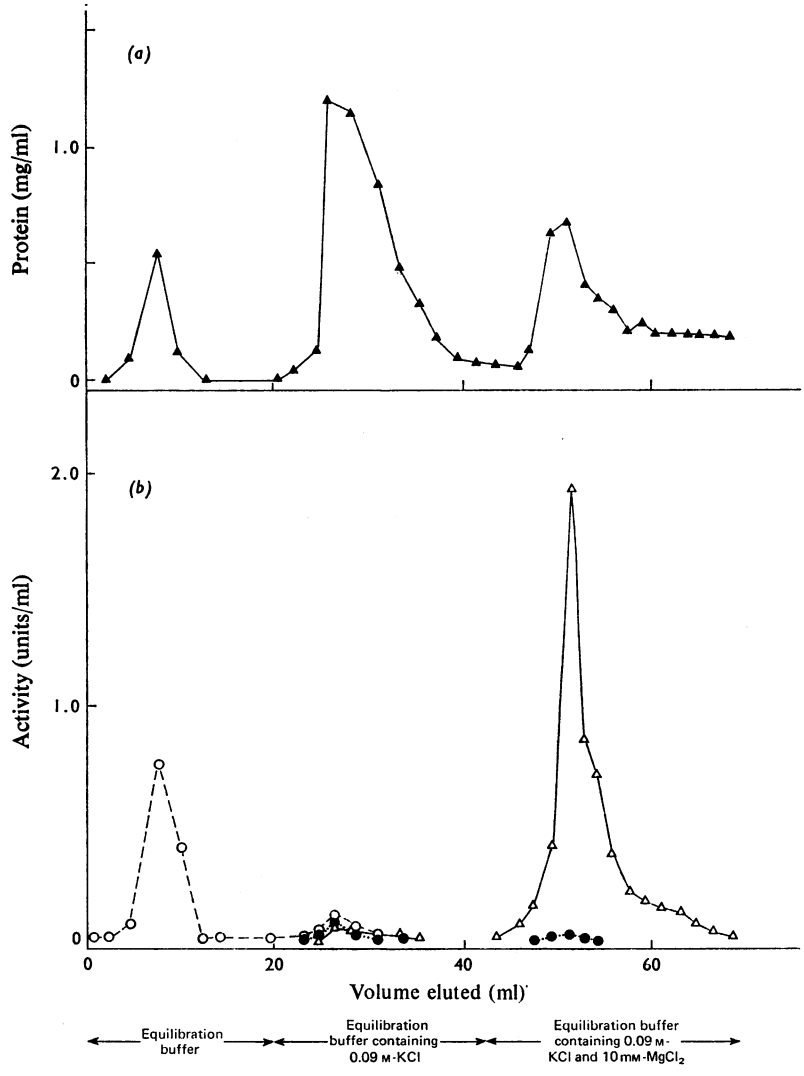


Fig. 1. *Separation of enzyme activities from Glossina flight muscle by DEAE-cellulose chromatography*

Flight-muscle extract, prepared from *G. pallidipes* as described in the text, was added to a 10ml DEAE-cellulose column (diam. 1.2cm) equilibrated with 10mM-Tris/1mM-EDTA, pH7.6. The enzyme preparation contained 2.55mg of protein, 14.7 units of NAD-linked 'malic' enzyme, 0.57 unit of NADP-linked 'malic' enzyme and 4.05 units of malate dehydrogenase in a volume of 1.5ml. The column was eluted with different buffer mixtures as indicated. Fractions were collected and assayed for NAD-linked 'malic' enzyme (△), NADP-linked 'malic' enzyme (●), malate dehydrogenase (○) and protein (▲) as shown. Ca^{2+} -stimulated oxaloacetate decarboxylase was assayed in pooled fractions obtained with each buffer mixture.

homogenate was frozen overnight and centrifuged (40000*g* for 20min), and the supernatant brought to 80% saturation with $(NH_4)_2SO_4$. The precipitate formed was pelleted by centrifugation (40000*g* for 20min) dissolved in, and dialysed thoroughly against, 10mM-Tris/1mM-EGTA, pH7.6, and added to a 10ml DEAE-cellulose column (1.2cm diam.) equilibrated with the same buffer. Elution with the equilibration buffer resulted in the recovery of about 75% of the original malate dehydrogenase activity in the first few fractions, and no 'malic' enzyme appeared. Subsequent treatment with a buffer mixture of higher ionic strength (0.09M-KCl/10mM-Tris/1mM-EGTA, pH7.6) eluted a considerable amount of the protein, another 10% of the malate dehydrogenase, 50% of the original NADP-linked 'malic'

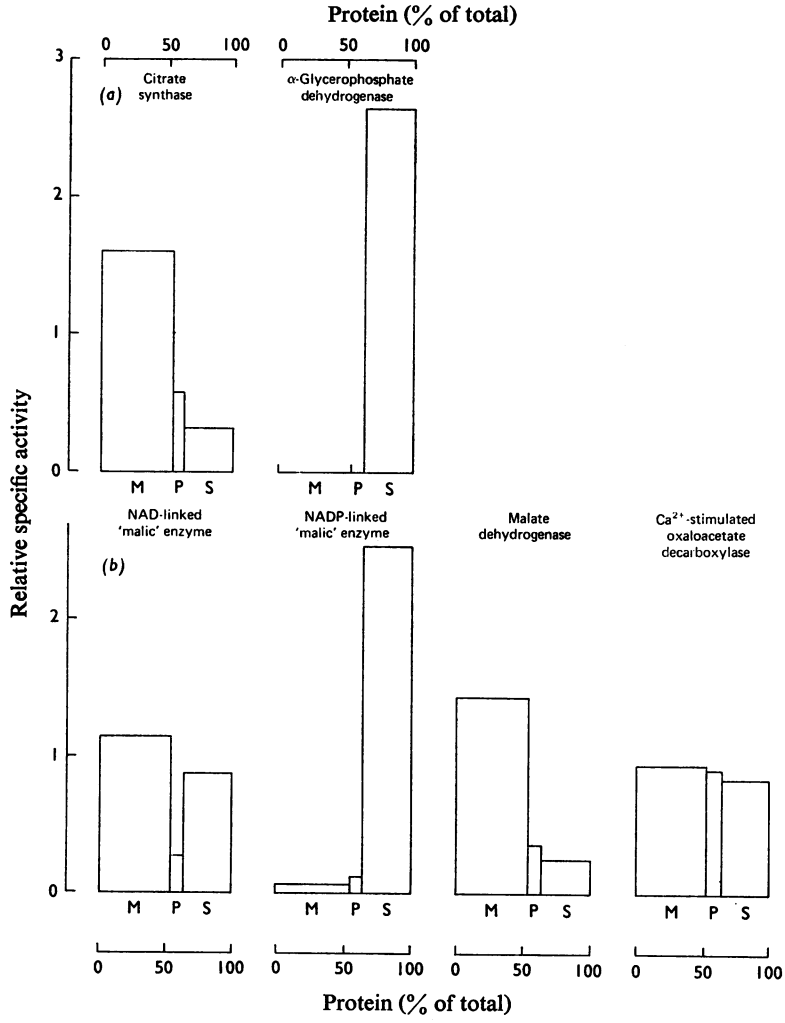


Fig. 2. *Subcellular distribution of enzyme activities of Glossina flight muscle*

A flight-muscle homogenate from 14 *G. morsitans* specimens, prepared in 4ml of 0.25M-sucrose/1mM-EGTA, pH7.4, as described in the Materials and Methods section, was subjected to differential centrifugation. Separation of the nuclei and mitochondria was not attempted. Low-speed centrifugation (10min, 300*g*) sedimented only minor amounts of protein. The sediment obtained after centrifugation for 10min at 8000*g* was washed with 2ml of the medium. This fraction (M) contained 55% of the total protein of the original homogenate. The small-particle fraction (P), collected by centrifugation for 90min at 50000*g*, contained 8% of the total protein, and a further 36% was recovered in the clear supernatant fraction (S). Results are expressed as relative specific activity (percentage of total activity/percentage of total protein; de Duve *et al.*, 1955). Recoveries of enzyme activities ranged from 91–102%.

enzyme and a minor fraction of the NAD-linked 'malic' enzyme. By including 10mM-$MgCl_2$ in the elution medium, the major peak of the NAD-linked 'malic' enzyme was eluted together with some NADP-linked 'malic' enzyme activity (30% of the original activity), but no further malate dehydrogenase was detectable. The recovery of all activities and protein was satisfactory (65–85%). Similar separations of malate dehydrogenase and NAD-linked 'malic' enzyme were achieved with preparations from thoraces of *G. morsitans* and *Sarcophaga*. The Ca^{2+}-stimulated oxaloacetate decarboxylase activity, recovered in the combined fractions containing NAD-linked 'malic' enzyme, was about 45% of that applied to the column, and no activity was recovered in other fractions.

The two peaks of NADP-linked 'malic' enzyme were investigated kinetically to assess their relationship to each other and to the NAD-linked activity. The activity eluted with KCl/Tris/EGTA buffer had an apparent K_m for $NADP^+$ in the presence of 2mM-L-malate of less than 10μM (accurate determination was not possible with the amounts of enzyme eluted), whereas the activity eluted with $MgCl_2$-containing buffer had an apparent K_m for $NADP^+$ of more than 0.2mM. Moreover, with 0.75mM-$NADP^+$, the latter activity was stimulated about 30% by increasing the malate concentration from 2mM to 8mM, whereas the former activity was not. The two activities were clearly catalysed by different proteins. The first peak eluted was presumed to be a true NADP-linked 'malic' enzyme, probably of cytosolic origin (see below), whereas in view of its high K_m for both $NADP^+$ and malate, the peak eluted with the $MgCl_2$-containing buffer was presumed to be due to the NAD-linked enzyme, with which it was coincident.

Intracellular distribution

In Fig. 2 the distribution of NAD- and NADP-linked 'malic' enzymes, Ca^{2+}-stimulated oxaloacetate decarboxylase and malate dehydrogenase between different fractions obtained by differential centrifugation of a *G. morsitans* flight-muscle homogenate is compared with that of citrate synthase and α-glycerophosphate dehydrogenase, marker enzymes for mitochondria and cytosol respectively. NAD- and NADP-linked 'malic' enzyme activities showed a very different distribution pattern. The NADP-linked activity followed the distribution of α-glycerophosphate dehydrogenase, and hardly any activity was detectable in the mitochondrial fraction. Of the NAD-linked activity about 65% was recovered in the mitochondrial fraction and 30% in the cytosolic fraction. The distribution of Ca^{2+}-stimulated oxaloacetate decarboxylase closely followed that of NAD-linked 'malic' enzyme. Some 87% of the mitochondrial marker enzyme citrate synthase was recovered in the mitochondrial fraction, together with 80% of the malate dehydrogenase.

The relatively high proportion of NAD-linked 'malic' enzyme recovered in the supernatant might be held to suggest that the enzyme has a dual distribution in the cell. There are, however, some indications that the enzyme is relatively easily released from the mitochondria. After repeated freezing and thawing of a mitochondrial fraction, followed by centrifugation (40000*g* for 30min), 32% of the NAD-linked 'malic' enzyme was recovered in the supernatant, as compared with only 5% of the malate dehydrogenase. In similar studies with fleshfly flight-muscle preparations, the distribution of NAD-linked 'malic' enzyme followed more closely that of citrate synthase, and the enzyme was less easily released from the mitochondrial fraction by freezing and thawing.

In the absence of Triton X-100 in the assay system, about 70% of the total NAD-linked 'malic' enzyme activity in a freshly prepared *G. morsitans* homogenate was found to be latent, and was detectable only after repeated freezing and thawing, sonication or treatment with detergent (Table 3). This suggests that the enzyme is located predominantly in the mitochondrial matrix space.

Properties of the partially purified NAD-linked 'malic' enzyme

The fractions from the DEAE-cellulose column containing the highest activities (see Fig. 1) were pooled and used for preliminary studies on the properties of the NAD-linked 'malic' enzyme. For different preparations, the specific activity of the pooled

Table 3. *Effect of different treatments on the latency of enzyme activities in flight-muscle mitochondria from Glossina morsitans*

A mitochondrial fraction was prepared by the procedure described in the legend to Fig. 2, and assayed in the absence of Triton X-100 except where otherwise indicated. NAD-linked 'malic' enzyme was assayed at pH7.4. Activities are expressed as a percentage of the activity with 0.03% Triton X-100 in the assay medium. Sonication was for three 20s periods, using a Branson sonifier (model W185). Freezing and thawing was repeated three times.

	Activity (%)		
Treatment	NAD-linked 'malic' enzyme	Malate dehydrogenase	Citrate synthase
Untreated	38	36	40
Sonication	67	114	97
Freezing–thawing	89	79	80
Triton X-100	100	100	100

fractions varied between 15 and 24 μmol/min per mg of protein at 30°C.

Kinetic parameters. The apparent K_m values for NAD^+ and L-malate, measured at concentrations of the second substrate normally used in our assay system, were 0.21 and 2.5 mM respectively. We have failed to detect the reverse reaction, the reductive carboxylation of pyruvate, even with high concentrations of pyruvate and bicarbonate present (20 and 50 mM, respectively).

pH optimum. The enzyme had a pH optimum between 7.6 and 7.9 (Fig. 3). Replacement of the Tris buffer by phosphate buffer decreased activity by about 40% over the whole pH range, but had no effect on the position of the pH optimum. It should be noted that in the comparative study (Tables 1 and 2) assays were performed at pH 7.4, so that the tabulated activities are underestimated by 20–30%.

Nicotinamide nucleotide specificity and the effect of bivalent metal ions. The NAD-linked 'malic' enzyme activity was completely dependent on the presence of Mg^{2+} or Mn^{2+}. Ca^{2+} was not effective and caused partial inhibition with Mg^{2+} present. Replacement of

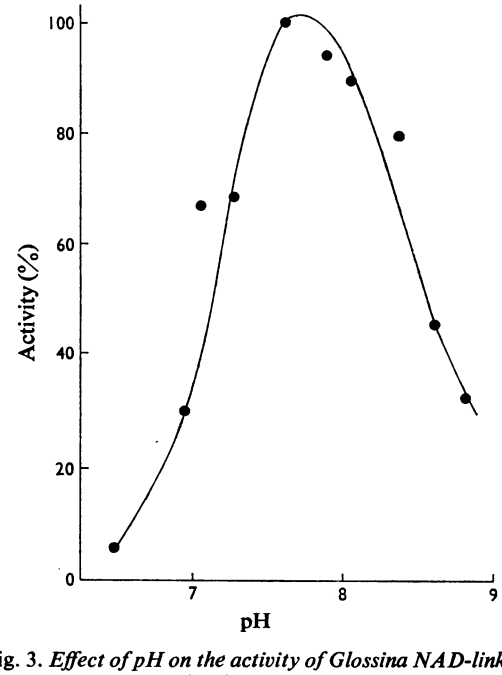


Fig. 3. *Effect of pH on the activity of Glossina NAD-linked 'malic' enzyme*

A partially purified enzyme preparation from *G. pallidipes* was used. Assays were performed as described in the Materials and Methods section, in Tris/HCl buffer of different pH values. Activities are expressed as a percentage of the activity at pH 7.8.

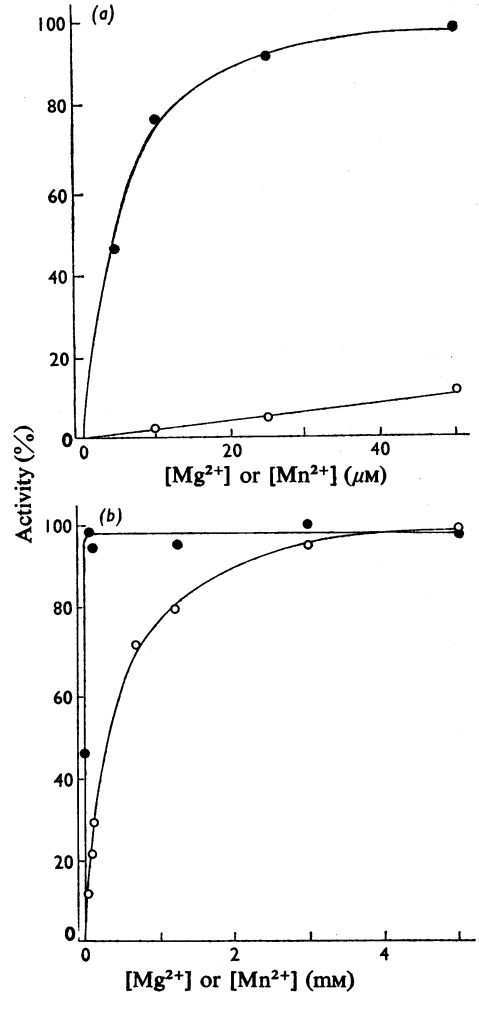


Fig. 4. *Activity of Glossina NAD-linked 'malic' enzyme at (a) low and (b) high concentrations of Mg^{2+} and Mn^{2+}*

A partially purified enzyme from *G. pallidipes* was used. Assays were performed at pH 7.8. Activities are expressed as a percentage of the activity in media containing 5 mM-Mg^{2+}. ○, Mg^{2+} present; ●, Mn^{2+} present.

$MgCl_2$ by $MgSO_4$ had no effect on activity. Although the maximum activity of the enzyme was the same in the presence of either Mg^{2+} or Mn^{2+} (Fig. 4), the concentration of the two cations required for full activation was remarkably different, 5 mM in the case of Mg^{2+} but only 50 μM in the case of Mn^{2+}. At very high concentrations (30 mM) some slight further activation was observed with Mn^{2+} but not with Mg^{2+} (results not shown).

With 0.5 mM-$NADP^+$, and Mg^{2+} as the activator, activity reached about 1.5% of that found with

NAD^+, but, again, maximum activation required a metal ion concentration of about 5mM. By contrast, the same activation of the NADP-dependent reaction is given by very low concentrations of Mn^{2+} (about 50μM), and higher concentrations of Mn^{2+} cause a marked further increase in the NADP-linked activity; no saturation was obtained with concentrations of Mn^{2+} up to 50mM, where the rate of the NADP-dependent reaction had reached almost 20% of that with NAD.

Discussion

Although a high activity of NAD-linked 'malic' enzyme has been reported before in insect flight muscle (Hansford & Johnson, 1975), the present study emphasizes the general importance of the enzyme in a variety of insects, and has established its distinct identity in at least three species by a complete separation from malate dehydrogenase. It is noteworthy that Lewis & Price (1956), who demonstrated an NADP-linked 'malic' enzyme in the flight muscle of the blowfly (*Calliphora erythrocephala*), failed to detect an NAD-linked activity in aqueous extracts of an acetone-dried powder of thoraces from this insect. Qualitatively, the properties of the partially purified *Glossina* enzyme resemble in many respects those of NAD-linked 'malic' enzyme from rabbit heart (Lin & Davis, 1974), beetle flight muscle (Hansford & Johnson, 1975), helminth muscle (Fodge *et al.*, 1972) and cauliflower (Macrae, 1971). Thus the reaction is dependent on the presence of Mg^{2+} or Mn^{2+}, the enzyme is rather specific for NAD^+, especially in the presence of Mg^{2+}, the apparent K_m for NAD^+ is relatively high (cf. Lin & Davis, 1974) and the enzyme cannot catalyse the reverse reaction (Frenkel, 1975). The specific activity of the partially purified *Glossina* preparation (15–24 units/mg of protein) is comparable with that of the highly purified helminth enzyme (Fodge *et al.*, 1972) and tenfold higher than reported for the partially purified rabbit heart enzyme (Lin & Davis, 1974).

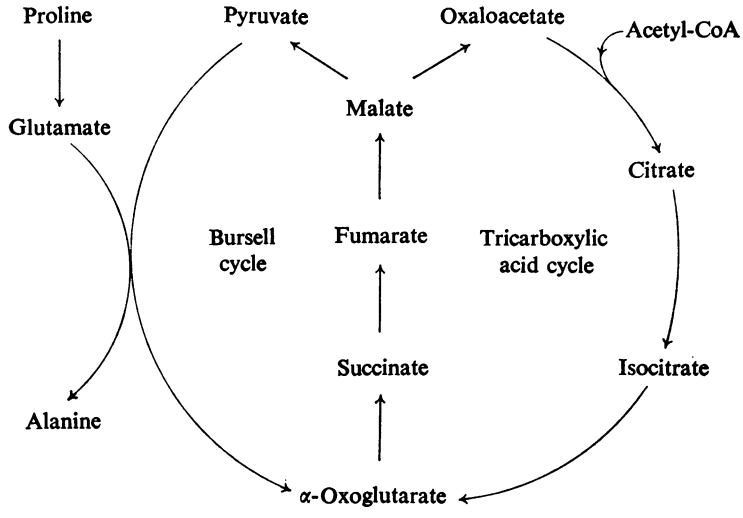
Certain features of the *Glossina* enzyme call for comment. Unlike the helminth enzyme, it is not inactivated by $(NH_4)_2SO_4$. Indeed, the partially purified enzyme stored at 4°C as an $(NH_4)_2SO_4$ precipitate lost only 50% of its activity in 1 month. Its affinity for Mg^{2+} seems to be similar to that reported for other NAD-linked 'malic' enzymes, but its high sensitivity to Mn^{2+} is remarkable, full activation being achieved with a concentration less than one-tenth the apparent K_m value reported for the rabbit heart enzyme.

Another noteworthy property of the insect NAD-linked 'malic' enzymes, is the apparent ability to decarboxylate oxaloacetate, a feature that would classify them as EC 1.1.1.38 rather than EC 1.1.1.39. However, with the partially purified preparation studied here, the possibility that the Ca^{2+}-stimulated oxaloacetate decarboxylase activity is due to a distinct protein cannot be excluded, particularly since the cation requirement for the two activities is different. On the other hand, the fact that the two enzyme activities are largely purified together on DEAE-cellulose fractionation, their comparable cellular distribution and their relatively constant ratio in different insects strongly suggest that they reside in one protein. This interpretation is supported by the observation that, with a partially purified preparation, which was stored for about 4 months at −8°C, a parallel loss of activity of NAD-linked 'malic' enzyme and Ca^{2+}-stimulated oxaloacetate decarboxylase occurred. An NAD-linked 'malic' enzyme with oxaloacetate decarboxylase activity has been reported in *Lactobacillus arabinosus* (Korkes *et al.*, 1950). Other NAD-linked 'malic' enzymes have been reported not to catalyse the decarboxylation of oxaloacetate (see Fodge *et al.*, 1972; Lin & Davis, 1974; Frenkel, 1975), but it should be noted that tests have usually been carried out in the absence of Ca^{2+} and at pH4.5 (Ochoa, 1955; Fodge *et al.*, 1972; Lin & Davis, 1974).

Bursell (1965) investigated the effect of different cations on tse-tse fly oxaloacetate decarboxylase and observed considerable activity even in the absence of added Ca^{2+}, which was stimulated only twofold by $CaCl_2$. However, no EGTA was present in his experiments, which may explain why his findings differed from those reported here. Certainly, the relationship between oxaloacetate decarboxylase and NAD-linked 'malic' enzyme requires a more detailed investigation.

The comparative survey of NAD-linked 'malic' enzyme activities in different insects clearly illustrates the outstanding position of the tse-tse fly, which has a 8–20-fold higher activity than any of the other species investigated. In the other insects, there is no obvious correlation between the NAD-linked 'malic' enzyme activity and either the type of fuel used by the flight-muscle mitochondria or the type of flight muscle, synchronous or asynchronous. Thus comparable activities were found in the synchronous flight muscle of the termite and the asynchronous flight muscle of the fleshfly, both of which rely mainly on carbohydrate oxidation (J. B. Hoek & N. K. Olembo, unpublished observations; N. Abo-Khatwa, unpublished observations), as well as in the synchronous flight muscle of the locust, which can oxidize fatty acids. The beetle species in our survey did not show the high 'malic' enzyme activity found in the Japanese beetle by Hansford & Johnson (1975). It is possible, however, that the development of beetle flight muscle for migration involves large modifications in metabolic pattern (cf. De Kort & Bartelink, 1972), with changes in the activity of this enzyme.

The high activity of NAD-linked 'malic' enzyme in



Scheme 1. *Alternative pathways of energy metabolism in Glossina flight muscle*

tse-tse flies is correlated with high activities of proline dehydrogenase, alanine aminotransferase and glutamate dehydrogenase (Crabtree & Newsholme, 1970; Norden & Patersen, 1969; Bursell, 1975), enzymes which are apparently involved in the proline-oxidation pathway predominant in these insects (Bursell, 1963, 1975; see also Sacktor & Childress, 1967; De Kort *et al.*, 1973; Hansford & Johnson, 1975). This strongly suggests a functional role of NAD-linked 'malic' enzyme in proline oxidation, presumably in supplying pyruvate for transamination with glutamate, the primary product of proline oxidation. This assumption raises three questions. (1) Is there a distinct role for both NAD-linked 'malic' enzyme and oxaloacetate decarboxylase? The latter activity could be thought to function by trapping oxaloacetate derived from malate, and diverting it into pyruvate if required. However, its low pH optimum (Bursell, 1965) and its requirement for relatively high concentrations of oxaloacetate make it doubtful whether this activity could ever be significant under physiological conditions. (2) What factors regulate the fate of intramitochondrial malate, which can choose between two equally active malate dehydrogenases, one directing the carbon skeleton into the tricarboxylic acid cycle, the other into the 'Bursell cycle' (see Scheme 1)? The answer to this question requires a study of the regulatory properties of these enzymes. (3) What is the role of NAD-linked 'malic' enzymes in other insects? Proline oxidation has been proposed to function in carbohydrate-feeding Diptera in the supply of tricarboxylic acid-cycle intermediates for pyruvate oxidation at the onset of high respiratory activity [see Sacktor (1970), for a review]. An NAD-linked 'malic' enzyme, if active in this situation, would clearly be incompatible with this concept, as it would bring about a depletion of cycle intermediates. The enzyme could, of course, be active only during resting. Alternatively, it might become important during periods of relative carbohydrate shortage, when a proline-oxidation pathway similar to that occurring in tse-tse flies may be called on to utilize fatty acids from the fat-body as a source of energy (cf. McCabe & Bursell, 1975).

We thank Mr. V. Dixit and Miss V. Shitakha for their enthusiastic help in part of the experimental work, and the staff of the International Centre of Insect Physiology and Ecology (Director: Professor T. Odhiambo), in particular, Dr. J. van Etten, for their co-operation in the supply of tse-tse flies.

References

Bergmeyer, H. U. (ed.) (1970) *Methoden der Enzymatischen Analyse*, Verlag Chemie, Weinheim
Brosemer, R. W. & Veerabhadrappa, P. S. (1965) *Biochim. Biophys. Acta* **110**, 102–112
Bursell, E. (1963) *J. Insect Physiol.* **9**, 439–452
Bursell, E. (1965) *Comp. Biochem. Physiol.* **16**, 259–266
Bursell, E. (1966) *Comp. Biochem. Physiol.* **19**, 809–818
Bursell, E. (1967) *Comp. Biochem. Physiol.* **23**, 825–829
Bursell, E. (1975) *Insect Biochem.* **5**, 289–297
Crabtree, B. & Newsholme, E. A. (1970) *Biochem. J.* **117**, 1019–1021
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, E. (1955) *Biochem. J.* **60**, 604–617

De Kort, C. A. D. & Bartelink, A. K. M. (1972) *Comp. Biochem. Physiol. B***43**, 1009–1017
De Kort, C. A. D., Bartelink, A. K. M. & Schuurmans, R. R. (1973) *Insect Biochem.* **3**, 11–17
Fodge, D. W., Gracy, R. W. & Harris, B. G. (1972) *Biochim. Biophys. Acta* **268**, 271–284
Frenkel, R. (1975) *Curr. Top. Cell. Regul.* **9**, 157–181
Hansford, R. G. & Johnson, R. N. (1975) *Biochem. J.* **148**, 389–401
Korkes, S., Del Campillo, A. & Ochoa, S. (1950) *J. Biol. Chem.* **187**, 891–905
Lewis, S. E. & Price, G. M. (1956) *Nature (London)* **177**, 842–843
Lin, R. C. & Davis, E. J. (1974) *J. Biol. Chem.* **249**, 3867–3875
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Macrae, A. R. (1971) *Biochem. J.* **122**, 495–501
McCabe, C. T. & Bursell, E. (1975) *Insect Biochem.* **5**, 781–789
Norden, D. A. & Patersen, D. J. (1969) *Comp. Biochem. Physiol.* **31**, 819–827
Ochoa, S. (1955) *Methods Enzymol.* **1**, 739–753
Ochoa, S., Stern, J. R. & Schneider, M. C. (1951) *J. Biol. Chem.* **193**, 691–700
Pearson, D. J. (1965) *Biochem. J.* **95**, 23c–24c
Sacktor, B. (1970) *Adv. Insect Physiol.* **7**, 268–347
Sacktor, B. & Childress, C. C. (1967) *Arch. Biochem. Biophys.* **120**, 583–588
Sauer, L. A. (1973*a*) *Biochem. Biophys. Res. Commun.* **50**, 524–531
Sauer, L. A. (1973*b*) *FEBS Lett.* **33**, 251–255
Saz, H. J. & Hubbard, J. A. (1957) *J. Biol. Chem.* **225**, 921–933
Van den Bergh, S. G. (1967) *Methods Enzymol.* **10**, 177–182