Biochem. J. (1975) **150**, 105–111
Printed in Great Britain

Activities of Citrate Synthase, NAD⁺-Linked and NADP⁺-Linked Isocitrate Dehydrogenases, Glutamate Dehydrogenase, Aspartate Aminotransferase and Alanine Aminotransferase in Nervous Tissues from Vertebrates and Invertebrates

By P. H. SUGDEN* and E. A. NEWSHOLME†
A.R.C. Unit of Muscle Mechanisms and Insect Physiology, Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K.

(*Received* 15 January 1975)

1. The activities of citrate synthase and NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenases were measured in nervous tissue from different animals in an attempt to provide more information about the citric acid cycle in this tissue. In higher animals the activities of citrate synthase are greater than the sum of activities of the isocitrate dehydrogenases, whereas they are similar in nervous tissues from the lower animals. This suggests that in higher animals the isocitrate dehydrogenase reaction is far-removed from equilibrium. If it is assumed that isocitrate dehydrogenase activities provide an indication of the maximum flux through the citric acid cycle, the maximum glycolytic capacity in nervous tissue is considerably greater than that of the cycle. This suggests that glycolysis can provide energy in excess of the aerobic capacity of the tissue. 2. The activities of glutamate dehydrogenase are high in most nervous tissues and the activities of aspartate aminotransferase are high in all nervous tissue investigated. However, the activities of alanine aminotransferase are low in all tissues except the ganglia of the waterbug and cockroach. In these insect tissues, anaerobic glycolysis may result in the formation of alanine rather than lactate.

The importance of the citric acid cycle in nervous tissue is emphasized by the fact that decrease in O_2 availability causes marked metabolic and functional changes in the brain of mammals (for review see McIlwain & Bachelard, 1971). In addition, measurements of arteriovenous differences across the brains of fed mature humans and rats indicate that these tissues produce very little pyruvate or lactate, so that almost all the glucose taken up is oxidized under these conditions (Rowe *et al.*, 1959; Hawkins *et al.*, 1971). This permits the rate of ATP production to be calculated from measurements of O_2 or glucose uptake. From the data of Hawkins *et al.* (1971), ATP production by the rat brain is calculated to be approximately 17 μmol/min per g fresh wt., which demonstrates the quantitative importance of the cycle for energy production in this tissue. Further, recent work indicates that significant rates of oxidation of ketone bodies occur in nervous tissues and these findings have served to re-emphasize the importance of the cycle (Owen *et al.*, 1967; Hawkins *et al.*, 1971; Sugden & Newsholme, 1973). However, there are very few reported values of activities of enzymes of the citric acid cycle in nervous tissues (see McIlwain & Bachelard, 1971). In the present study the maximum activities of citrate synthase (EC 4.1.3.7), NAD^+-linked isocitrate dehydrogenase (EC 1.1.1.41) and $NADP^+$-linked isocitrate dehydrogenase (EC 1.1.1.42) were measured in nervous tissue from a variety of invertebrate and vertebrate animals. It was considered that these activities could provide some quantitative information on the maximum rates of the tricarboxylic acid cycle in various nervous tissues and that they would provide a basis for comparison with the reported activities of the enzymes of glycolysis in these tissues (Sugden & Newsholme, 1973).

Although an important role of the citric acid cycle in brain is the oxidation of glucose for energy production, certain intermediates of the cycle are important in the aminotransferase reactions. Since amino acid metabolism is known to be important in the function of nervous tissue (for reviews see McIlwain & Bachelard, 1971; Watkins, 1972), the maximum activities of glutamate dehydrogenase (EC 1.4.1.3), aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) were measured and the results are reported in the present paper.

* Present address: Department of Physiology, School of Medicine, Vanderbilt University, Nashville, Tenn. 37212, U.S.A.

† Present address: Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

Table 1. *Activities of citrate synthase, NAD⁺-linked isocitrate dehydrogenase and NADP⁺-linked isocitrate dehydrogenase and the maximum glycolytic flux in nervous tissue*

Enzyme activities are presented as means, and ranges of activities and numbers of animals are given in parentheses. Maximum glycolytic flux is obtained from the activities of phosphofructokinase (Sugden & Newsholme, 1973; see Crabtree & Newsholme, 1972), which are multiplied by a factor of 2 so that they are expressed as μmol of pyruvate produced/min per g fresh wt. of nervous tissue at 25°C.

Animal	Nervous tissue	Maximum glycolytic flux	Enzyme activities (μmol/min per g fresh wt of tissue)		
				Isocitrate dehydrogenase	
			Citrate synthase	NAD^+-linked	$NADP^+$-linked
Annelida					
Earthworm (*Lumbricoides terrestris*)	Nerve cord	36.8	3.0 (2.2–3.3) (4)	<0.1 (3)	3.7 (2.3–5.8) (3)
Crustacea					
Lobster (*Homarus vulgaris*)	Cerebral ganglion	19.8	2.6 (1.8–3.5) (4)	<0.1 (3)	1.3 (0.8–1.6) (3)
	Nerve cord and thoracic ganglia	4.6	1.2 (0.7–1.9) (4)	<0.1 (3)	0.6 (0.4–0.9 (4)
Insecta					
Locust (*Schistocerca gregaria*)	Cerebral ganglion	9.2	21.3 (20.0–22.0) (3)	5.8 (5.2–6.3) (3)	7.4 (5.5–10.3) (3)
Cockroach (*Blaberus discoidalis*)	Cerebral ganglion	12.0	23.6 (23.4–24.0) (3)	—	3.4 (2.9–3.8) (2)
Waterbug (*Lethocerus cordofanus*)	Pterothoracic ganglion	48.0	10.8 (10.5–11.0) (3)	4.0 (3.5–4.4) (3)	6.4 (6.0–6.6) (3)
	Cerebral ganglion	30.0	5.6 (4.4–6.9) (4)	3.9 (3.6–4.2) (2)	4.6 (4.4–4.8) (2)
Bumble-bee (queen) (*Bombus terrestris*)	Cerebral ganglion	—	24.1 (16.5–31.6) (2)	—	—
Pisces					
Dogfish (*Scylliorhinus canicula*)	Whole brain	16.4	11.2 (9.8–13.6) (3)	1.0 (0.9–1.0) (2)	4.7 (4.2–5.1) (2)
Rainbow trout (*Salmo gairdneri*)	Whole brain	14.8	9.2 (7.5–12.2) (5)	2.2 (1.2–3.1) (3)	4.5 (2.2–6.5) (4)
Goldfish (*Carassius auratus*)	Whole brain	31.4	14.3 (10.4–16.5) (3)	0.5 (0.4–0.6) (3)	8.3 (7.4–8.9) (3)
Amphibia					
Frog (*Rana temporaria*)	Whole brain	16.4	14.9 (12.3–17.5) (5)	2.6 (2.0–2.9) (3)	4.7 (3.7–5.6) (4)
Salamander (*Salamandra salamandra*)	Whole brain	11.2	5.6 (4.7–6.1) (3)	1.5 (1.3–1.6) (3)	3.7 (3.2–4.4) (3)
European newt (*Triturus vulgaris*)	Whole brain	16.2	7.5 (6.0–8.6) (3)	1.6 (1.0–2.0) (3)	6.9 (5.8–8.2) (3)

Reptilia					
Green lizard (*Lacerta viridis*)	Whole brain	29.8	15.0 (10.5–19.5) (4)	2.8 (2.4–3.6) (3)	8.0 (5.6–12.1) (4)
Grass snake (*Tropidenotus natrix*)	Whole brain	28.6	28.5 (26.9–32.9) (4)	3.9 (3.4–4.3) (3)	6.4 (5.4–7.6) (3)
Aves					
Domestic pigeon (*Columba livis*)	Whole brain	24.4	19.6 (16.4–21.7) (3)	1.1 (0.8–1.5) (3)	2.2 (1.8–3.0) (5)
Domestic fowl (*Gallus gallus*)	Whole brain	27.4	5.7 (4.7–6.4) (3)	0.3 (<0.1–0.4) (3)	2.4 (2.1–2.9) (3)
Pheasant (*Phasianus colchicas*)	Whole brain	19.4	10.8 (7.1–15.7) (5)	2.8 (2.3–3.4) (3)	2.9 (2.5–3.3) (3)
Mammalia					
Laboratory mouse	Whole brain	29.2	24.3 (21.8–29.5) (4)	3.2 (2.5–4.0) (3)	1.8 (1.7–1.8) (3)
Laboratory rat	Whole brain	36.2	13.6 (11.1–15.9) (3)	0.8 (0.5–1.0) (3)	1.1 (0.9–1.3) (3)

Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., except for the following: acetyl-CoA (grade II), DL-isocitric acid (trisodium salt), pyridoxal phosphate and 5,5′-dithiobis-(2-nitrobenzoic acid) 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.; antimycin-A (B grade) and oxaloacetic acid (A grade) were obtained from Calbiochem Ltd., London W1H 1AS, U.K.; aspartic acid, alanine, EDTA and all inorganic chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.

Sources of animals

Animals were obtained from sources given by Sugden & Newsholme (1973). All animals used in this survey were mature and were allowed free access to food and water before death.

Preparation of homogenates

Animals were killed and the brain or nerve cord was removed by careful dissection as rapidly as possible. (Mice and rats were anaesthetized with diethyl ether before cervical section.) Nervous tissue was homogenized in ground-glass homogenizers with 10–20vol. of extraction medium at 0°C. The extraction medium for the assay of citrate synthase consisted of 25mM-Tris–HCl and 1mM-EDTA at pH7.4, and that for the assay of $NADP^+$-linked isocitrate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase consisted of 50mM-triethanolamine, 1mM-EDTA, 5mM-$MgCl_2$ and 30mM-2-mercaptoethanol adjusted to pH7.5 with KOH. For the assay of NAD^+-linked isocitrate dehydrogenase the extraction medium was the same as that for the $NADP^+$-linked enzyme, except for the addition of 2.5mM-ADP. Homogenates were sonicated for 30s at 0°C in an MSE 100W ultrasonic disintegrator before enzyme assays, which were carried out as soon as possible after sonication. Higher enzyme activities were obtained by sonication than by homogenization in ground-glass homogenizers, but other methods of mitochondrial rupture were not tested. After extraction, enzymes were stable for at least 1h.

Assay of enzyme activities

Citrate synthase was assayed by following the rate of change of extinction at 412nm (Srere *et al.*, 1963) and other enzymes by following the change in extinction at 340nm in a Gilford recording spectrophotometer (model 240) at 25°C. The assay medium for

Table 2. *Activities of glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase in nervous tissue*

Enzyme activities are presented as means, and ranges and number of animals are given in parentheses.

Animal	Nervous tissue	Enzyme activities (μmol/min per g fresh wt. of tissue)		
		Glutamate dehydrogenase	Aspartate aminotransferase	Alanine aminotransferase
Annelida				
Earthworm (*Lumbricoides terrestris*)	Nerve cord	9.5 (7.8–12.1) (3)	22.7 (16.0–27.8) (3)	1.2 (0.2–2.1) (2)
Crustacea				
Lobster (*Homarus vulgaris*)	Cerebral ganglion	4.3 (3.9–4.5) (3)	21.3 (20.1–22.9) (3)	10.4 (9.2–11.6) (3)
	Nerve cord and thoracic ganglia	2.6 (2.1–3.7) (3)	11.9 (10.1–14.8) (3)	5.6 (3.3–7.7) (3)
Insecta				
Locust (*Schistocerca gregaria*)	Cerebral ganglion	23.3 (16.8–30.6) (3)	50.8 (42.3–66.2) (3)	7.5 (7.3–7.6) (2)
Cockroach (*Blaberus discoidalis*)	Cerebral ganglion	16.9 (12.8–20.5) (3)	39.8 (31.1–53.8) (3)	26.7 (25.8–27.6) (2)
Waterbug (*Lethocerus cordofanus*)	Pterothoracic ganglion	10.1 (8.8–11.3) (2)	23.3 (22.6–23.9) (3)	54.1 (42.9–65.3) (3)
Pisces				
Dogfish (*Scylliorhinus canicula*)	Whole brain	3.9 (3.6–4.2) (2)	33.2 (29.7–36.6) (2)	0.6 (2)
Rainbow trout (*Salmo gairdneri*)	Whole brain	4.4 (3.7–5.5) (3)	56.3 (42.0–77.2) (4)	1.5 (1.1–1.8) (4)
Goldfish (*Carassius auratus*)	Whole brain	23.2 (18.9–30.5) (3)	35.3 (32.8–36.8) (3)	6.1 (5.4–7.5) (3)
Amphibia				
Frog (*Rana temporaria*)	Whole brain	33.6 (31.5–36.0) (3)	38.6 (30.3–41.2) (4)	1.6 (1.3–1.9) (3)
Salamander (*Salamandra salamandra*)	Whole brain	18.8 (15.9–20.4) (3)	42.1 (35.2–48.8) (4)	0.5 (0.4–0.7) (3)
European newt (*Triturus vulgaris*)	Whole brain	33.4 (32.4–34.4) (2)	38.0 (32.9–48.1) (3)	1.6 (1.4–1.9) (3)
Reptilia				
Green lizard (*Lacerta viridis*)	Whole brain	11.5 (7.7–14.6) (4)	46.6 (43.7–51.4) (3)	3.8 (3.5–4.1) (3)
Grass snake (*Tropidenotus viridis*)	Whole brain	34.2 (30.2–39.6) (3)	59.5 (56.6–61.5) (3)	3.8 (3.8–4.3) (3)

Aves				
Domestic pigeon (*Columba livia*)	Whole brain	23.9 (21.0–28.5) (4)	33.1 (24.8–44.2) (4)	1.9 (1.3–2.9) (4)
Domestic fowl (*Gallus gallus*)	Whole brain	14.0 (12.3–16.2) (3)	26.0 (23.3–28.0) (3)	0.3 (0.2–0.4) (3)
Pheasant (*Phasianus colchicas*)	Whole brain	23.8 (15.1–34.8) (4)	29.3 (26.0–32.4) (3)	0.2 (0.2–0.3) (3)
Mammalia				
Laboratory mouse	Whole brain	19.0 (12.6–28.1) (4)	74.1 (66.9–85.2) (3)	0.8 (0.5–1.0) (3)
Laboratory rat (Wistar strain)	Whole brain	10.7 (8.9–12.1) (3)	48.3 (42.0–51.4) (3)	1.5 (1.2–1.9) (3)

citrate synthase consisted of 50mM-Tris–HCl, 0.2mM-5,5′-dithiobis-(2-nitrobenzoic acid), 0.1mM-acetyl-CoA, 0.5mM-oxaloacetate and a volume of suitably diluted homogenate (5–10μl). The final pH was 7.5 and the final volume in the cuvette was 2ml. The assay was initiated by the addition of oxaloacetate, and an equivalent volume of water was added to the controls. The reaction was linear during the period of assay.

The assay medium for NAD^+-linked isocitrate dehydrogenase consisted of 70mM-Tris–HCl, 2mM-NAD^+, 2mM-ADP, 1mM-$MnCl_2$, 8mM-$MgCl_2$, 22.5mM-citrate (trisodium salt), 3mM-DL-isocitrate (trisodium salt) and 5μg of antimycin-A (added as 5μl of the ethanolic solution) and a suitable volume of homogenate (10–20μl) (see Plaut, 1969). Antimycin-A was added to inhibit NADH oxidase activity. The final pH was 7.5 and the final volume was 2ml. The assay was initiated by the addition of citrate–DL-isocitrate, and an equivalent volume of water was added to the controls. The assay medium for $NADP^+$-linked isocitrate dehydrogenase was identical with that for the NAD^+-linked enzyme except that ADP and antimycin-A were omitted and NAD^+ was replaced by 0.5mM-$NADP^+$. (Preliminary studies with extracts of mouse brain indicated that NADPH oxidase activity was negligible.) Isocitrate–citrate was omitted from one control and the homogenate was omitted from a second control. (The second control is necessary since reduction of $NADP^+$ occurs occasionally in the absence of homogenate.) The assay medium for glutamate dehydrogenase consisted of 70mM-Tris–HCl, 0.2mM-NADH, 105mM-ammonium acetate, 2mM-ADP, 5μg of antimycin-A (added as 5μl of ethanolic solution), 8mM-2-oxoglutarate (free acid, adjusted to pH7.5 with $KHCO_3$) and a small volume (5–10μl) of suitably diluted homogenate (Williamson *et al.*, 1967). The final pH was 7.5 and the final volume was 2ml. 2-Oxoglutarate was omitted from controls. The reaction was linear during the period of the assay. The assay medium for aspartate aminotransferase consisted of 75mM-potassium phosphate buffer (an equimolar mixture of K_2HPO_4 and KH_2PO_4), 10mM-2-oxoglutarate (free acid adjusted to pH7.5 with $KHCO_3$), 50μg of pyridoxal phosphate, 0.2mM-NADH, 5μg of antimycin-A, and various concentrations of L-aspartate (17.5, 35 or 70mM of free acid, adjusted to pH7.5 with $KHCO_3$). Three different concentrations of L-aspartate were used because of the relatively high K_m value of aspartate aminotransferase for aspartate (Bergmeyer & Bernt, 1965*a*). Maximum activities of the enzyme were obtained by extrapolation to infinite substrate concentration by means of a reciprocal plot. Malate dehydrogenase (10μg in glycerol) and a volume (5–10μl) of suitably diluted homogenate were added to each cuvette. The final pH was 7.5 and the final volume was 2ml. The

reaction was initiated by the addition of 2-oxoglutarate, and an equivalent volume of water was added to the controls. The reaction was linear with time during the period of the assay. The assay medium for alanine aminotransferase was the same as that for aspartate aminotransferase (see above) except that L-aspartate was replaced by DL-alanine (20, 40 or 80mM) and malate dehydrogenase was replaced by 10 μg of lactate dehydrogenase (in glycerol) (see Bergmeyer & Bernt, 1965*b*). The reaction was linear with time during the period of the assay.

Expression of results

All enzyme activities are expressed as μmol of substrate utilized/min per g fresh wt. of nervous tissue at 25°C. The values reported represent the means of a number of determinations (on tissue from different animals) which are given together with the range in parentheses in Tables 1 and 2. Since a systematic study of factors such as season, diet, sex or age of the animals was not attempted, precise quantitative interpretations based on these reported activities must be made with caution. Optimum conditions for each enzyme assay were found by preliminary studies with the enzymes from mouse brain. It is assumed that these conditions are optimum for enzymes from other tissues.

Results

For 19 species of animals from several phyla, the activities of citrate synthase and NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenase show considerable variation (Table 1). Activities of citrate synthase range from 1.2 to 28.5 μmol/min per g fresh wt. (nerve cord plus thoracic ganglia of the lobster and the brain of the grass snake respectively). Activities of NAD^+-linked isocitrate dehydrogenase are not detectable in nervous tissue from the earthworm and lobster, whereas the highest activity is 5.8 μmol/min per g fresh wt. in the cerebral ganglion of the locust. Activities of $NADP^+$-linked isocitrate dehydrogenase range from 0.6 to 8.3 μmol/min per g fresh wt. (nerve cord plus thoracic ganglia of the lobster and the brain of the goldfish respectively).

For 18 species of animals from several phyla, the activities of glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase also show considerable variation (Table 2). The activities of aspartate aminotransferase range from 11.9 to 74.1 μmol/min per g fresh wt. (nerve cord plus thoracic ganglia of the lobster and the brain of the mouse respectively). The activities of alanine aminotransferase range from 0.2 to 54.1 μmol/min per g fresh wt. (brain of the pheasant and the pterothoracic ganglion of the waterbug respectively). Maximum activities of glutamate dehydrogenase range from 2.6 to 34.2 μmol/min per g fresh wt. (nerve cord plus thoracic ganglia of the lobster and the brain of the grass snake respectively). The effect of ADP on the activities of glutamate dehydrogenase from nervous tissues from a selected number of animals was investigated by omitting ADP from the assay medium for the enzyme. The results demonstrated that in all tissues studied, ADP (1 mM) caused marked activation (results not given).

Discussion

Comparison of maximum activities of enzymes in a pathway can be used to identify reactions that are near-to or far-displaced from equilibrium (see Newsholme & Start, 1973, for review). In the present work the activities of citrate synthase are greater than those of the total isocitrate dehydrogenase (i.e. NAD^+-linked plus $NADP^+$-linked) in higher animals, whereas they are similar in animals from the lower phyla. This suggests that, at least in higher animals, isocitrate dehydrogenase catalyses a reaction that is far-displaced from equilibrium. A comparison of the maximum flux *in vivo* through a pathway with the maximum activities of the enzyme provides an alternative means of indicating reactions far-displaced from equilibrium: a non-equilibrium reaction is indicated if the maximum activity of the enzyme is similar to the maximum flux (see Crabtree & Newsholme, 1975). The arteriovenous difference of glucose across the rat brain (Hawkins *et al.*, 1971) indicates a glycolytic flux of approximately 0.5 μmol of glucose/min per g fresh wt. of brain (at 37°C). The activities of citrate synthase, NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenase *in vitro* at 37°C (calculated from Table 1, by assuming that the activity doubles for a 12°C rise in temperature) are 30, 1.5 and 2.0 μmol/min per g fresh wt. respectively. These data support the suggestion that the isocitrate dehydrogenase reaction is far-displaced from equilibrium in brain tissue of this animal.

If the total isocitrate dehydrogenase activities represent the maximum flux through the tricarboxylate cycle, the latter can be compared with the maximum glycolytic fluxes in the nervous tissues studied (see Table 1). In the nervous tissues of most of the animals studied the maximum glycolytic flux is considerably greater than that of the cycle. This suggests that in these tissues energy formation can increase above the limitation imposed by O_2 supply, so that, under some conditions, anaerobic glycolysis may be important for energy provision in nervous tissue (see also Sugden & Newsholme, 1973). At the present time the importance of glycolysis and the tricarboxylate cycle in the neuronal and the glial cells is not known. When satisfactory techniques for isolation of these two classes of cells are available, the activities of the glycolytic and tricarboxylate-cycle enzymes may provide a better understanding of energy provision in the nervous system.

The activities of glutamate dehydrogenase are high in most nervous tissues, although they are rather low in the lobster, dogfish and trout. The reason for these differences is not known. Aspartate aminotransferase activities are high in all tissues investigated, which indicates the importance of this enzyme in metabolism in nervous tissue. However, alanine aminotransferase activities are low in most tissues except the ganglia of the waterbug and cockroach. This suggests that anaerobic glycolysis in these insect nervous tissues may result in the formation of alanine rather than lactate. The activities of aspartate aminotransferase in the nervous tissue of all the species studied are much greater than the maximum fluxes through the citric acid cycle (as indicated by the activities of NAD^+-linked plus $NADP^+$-linked isocitrate dehydrogenase). However, since the aminotransferase is likely to catalyse a reaction close to equilibrium in nervous tissue (Sugden, 1973), the activities of this enzyme cannot be used to provide quantitative information about the flux through this reaction (see Crabtree & Newsholme, 1975). Thus it is not possible to comment on the quantitative relationship between flux through the cycle and the fluxes through some of the pathways of amino acid metabolism. Nonetheless the possible metabolic roles of these enzymes might become clearer if their activities could be measured separately in the neuronal and glial cells of the individual nervous tissues.

We thank Professor J. W. S. Pringle, F.R.S., for his interest and encouragement. P. H. S. was a recipient of a Medical Research Council Training Scholarship.

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