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Activities of Hexokinase, Phosphofructokinase, 3-Oxo Acid Coenzyme A-Transferase and Acetoacetyl-Coenzyme A Thiolase in Nervous Tissue from Vertebrates and Invertebrates

By P. H. SUGDEN and E. A. NEWSHOLME
Agricultural Research Council Unit of Muscle Mechanisms and Insect Physiology, Department of Zoology, University of Oxford, Oxford OX1 3PS, U.K.

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1. The maximum activities of hexokinase and phosphofructokinase in nervous tissue from 18 different animals from different phyla range from 5.1 to 17.6 and from 4.6 to 24.0 μmol/min per g fresh wt. respectively. In any one tissue the activities of these two enzymes are, in general, very similar. The rate of glucose utilization by the brain *in vivo* is much lower than the activities of hexokinase or phosphofructokinase. It is suggested that the high activities of these enzymes indicate a capacity for glycolysis which may be used by the brain during hypoxia or during conditions of extreme neuronal activity. 2. The activities of 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase in the nervous tissues range from 1.1 to 15.3 and from 0.7 to 4.5 μmol/min per g fresh wt. respectively. Unfortunately the activities of these enzymes cannot be used to estimate maximal flux through the ketone-body-utilization pathway, since they may catalyse reactions that are close to equilibrium. Nonetheless, the presence of these enzymes in nervous tissue from a large variety of animals suggests that the importance of ketone bodies as a fuel for nervous tissue may be widespread in the animal kingdom.

The importance of glucose as an energy source for brain and nervous tissue has been recognized for many years (see McIlwain & Bachelard, 1971, for review). Recent measurements of arteriovenous differences across both human and rat brains have firmly established that ketone bodies are utilized by the brain *in vivo* (Owen *et al.*, 1967; Hawkins *et al.*, 1971). In obese patients undergoing prolonged therapeutic starvation, ketone-body utilization accounts for 75% of the oxygen uptake of the brain, and glucose utilization is decreased accordingly (Owen *et al.*, 1967). The utilization of ketone bodies by cerebral-cortex slices of the guinea pig (Rolleston & Newsholme, 1967*a*) and the demonstration of significant activities of ketone-body-utilizing enzymes in homogenates of rat brains (Williamson *et al.*, 1971) provide further evidence that this tissue can utilize ketone bodies. It seemed important to establish that nervous tissue from animals other than mammals possesses the capacity to utilize ketone bodies, and to obtain some quantitative comparative information about the maximum rates of glucose and ketone-body utilization by this tissue. Such information has been obtained by measurement of the maximum activities of hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11), 3-oxo acid CoA-transferase (EC 2.8.3.5) and acetoacetyl-CoA thiolase (EC 2.3.1.9), in homogenates of nervous tissue from a variety of animals, and the results are presented in the present paper. The experimental approach, in which enzyme-activity measurements are used to indicate maximum rates of metabolic pathways, has been described by Crabtree & Newsholme (1972).

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: iodoacetamide and diketen 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) was obtained from Calbiochem Ltd., London W1H 1AS, U.K.; glucose and all inorganic chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K. Pehanon pH-indicator-paper strips were obtained from Camlab Ltd., Cambridge CB4 1TH, U.K.

Sources of animals

Mature animals were obtained from the sources given by Newsholme & Taylor (1969) with the exception or addition of the following: rats were obtained from Charles River (U.K.) Ltd., Ash, Canterbury, U.K., and kept in the animal house of the Department

of Biochemistry; mice were bred in this department; lobsters were obtained from Fisher Bros., London E.C.3, U.K.; earthworms were obtained locally; frogs, newts, snails, lizards, grass snakes and salamanders were obtained from Gerrard and Haig Ltd., East Preston, Sussex, U.K. For at least 24h before the animals were killed they had access to food. Apart from rats and mice, for which only male animals were used, nervous tissue was obtained from male and female animals indiscriminately.

Preparation of homogenates

Animals were usually killed by cervical section 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. For the assay of hexokinase, the extraction medium consisted of 50mM-triethanolamine, 1mM-EDTA, 2mM-$MgCl_2$ and 30mM-2-mercaptoethanol at pH7.5. For the assay of phosphofructokinase, the extraction medium consisted of 50mM-Tris–HCl, 1mM-EDTA and 5mM-$MgCl_2$ at pH8.2. For the assay of acetoacetyl-CoA thiolase and 3-oxo acid CoA-transferase, the extraction medium consisted of 25mM-Tris–HCl and 1mM-EDTA at pH7.4. Before assay of the ketone-body-utilizing enzymes, the homogenates were sonicated for 30s in an MSE 100W ultrasonic disintegrator. Preliminary experiments showed that sonication did not modify the activities of hexokinase or phosphofructokinase. Enzyme assays were carried out as soon as possible after homogenization of the tissue (within 15min).

Preparation of acetoacetyl-CoA

Acetoacetyl-CoA was prepared by the action of diketen on CoA (Wieland & Rueff, 1953; Williamson *et al.*, 1971). A solution of CoA (free acid; approx. 10mg/ml) was neutralized to pH7 with 3M-$KHCO_3$ by the use of Pehanon indicator-paper strips and was cooled in ice. A portion of diketen was added to the solution of CoA (2μl of diketen/mg of CoA) and the mixture was shaken vigorously for 3–5min. The pH was maintained at 7–7.5 by addition of 3M-$KHCO_3$ solution. Any acetoacetate formed was removed by continuous diethyl ether extraction for 2h after the pH had been adjusted to 1–2 by addition of HCl. During ether extraction the acetoacetyl-CoA solution was cooled in ice. After extraction, the pH was adjusted to 6.0 by addition of 3M-$KHCO_3$. Excess of ether was removed by blowing air through the solution at 0°C. The solution of acetoacetyl-CoA was stored at −10°C.

Assay of enzyme activities

Hexokinase was assayed as described by Crabtree & Newsholme (1972). Phosphofructokinase was assayed by a method similar to that described by Newsholme *et al.* (1970). The assay medium consisted of 50mM-Tris–HCl, 6.7mM-$MgCl_2$, 1mM-2-mercaptoethanol, 0.1mM-NADH, 5μg of antimycin A, 2mM-AMP and 1mM-ATP, at pH8.0. Glycerol 1-phosphate dehydrogenase (10μg), triose phosphate isomerase (10μg), aldolase (100μg) and 5–10μl of homogenate were added to each cuvette. The reaction was initiated by addition of a small volume (100μl) of fructose 6-phosphate so that its final concentration in the cuvette was 2mM and the final volume was 2ml. For both assays the change in E_{340} was measured in a Gilford recording spectrophotometer (model 240) at 25°C. The 3-oxo acid CoA-transferase was assayed in the non-physiological direction of acetoacetate formation as described by Williamson *et al.* (1971). Non-specific hydrolysis of acetoacetyl-CoA was measured initially for 3–5min before the addition of succinate. Acetoacetyl-CoA thiolase was assayed in the direction of acetyl-CoA formation by the method of Williamson *et al.* (1971), except that the CoA concentration was 0.35mM. For both assays the rate of decrease in E_{303} was measured in a Gilford recording spectrophotometer (model 240) at 25°C.

The solutions of acetoacetyl-CoA were standardized frequently. This was necessary to determine the precise extinction coefficient, which was found to be very sensitive to small changes in pH. In the present investigation the molar extinction coefficients varied between 23×10^6 and 25.5×10^6 litre·mol^{-1}·cm^{-1}. The concentration of acetoacetyl-CoA was determined as follows. The incubation buffer consisted of 50mM-Tris–HCl and 0.2mM-NADH at pH7.5; a volume of acetoacetyl-CoA solution (10μl) was added to 2ml of the incubation buffer in the cuvette and the decrease in E_{340} was followed after the further addition of 5μg of 3-hydroxyacyl-CoA dehydrogenase in a Gilford recording spectrophotometer.

The pH optima for both 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase from mouse brain are 8.5. The K_m values of 3-oxo acid CoA-transferase from mouse and goldfish brains for acetoacetyl-CoA [obtained by Hofstee (1959) plots at 50mM-succinate and pH8.5] were 55 and 40μM respectively. The K_m values of acetoacetyl-CoA thiolase from both mouse and goldfish brains for acetoacetyl-CoA were approximately 10μM (obtained by Hofstee plots at 0.35mM-CoA and pH8.5). If the K_m values of the other species are similar to those of the mouse and goldfish, the activities of acetoacetyl-CoA thiolase were measured at saturating acetoacetyl-CoA concentrations (i.e. at a concentration 10-fold greater than the K_m value), whereas the 3-oxo acid CoA-transferase activities were measured at concentra-

tions of acetoacetyl-CoA that were less than saturating.

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 different animals. The number of determinations together with the range of activities is given in parentheses in Table 1. Since a systematic study of factors such as season, diet, size, age and sex of the animals was not attempted in this work, precise quantitative considerations based on the results must be made with caution.

Results

For 18 species of animals from many phyla the activities of phosphofructokinase and hexokinase are, in general, very similar in the same nervous tissue from the same animal (see Table 1). The exceptions include the pterothoracic ganglion of the waterbug and the nerve cord of the earthworm, in which hexokinase activities are much lower than phosphofructokinase activities, and the nerve cord plus thoracic ganglia of the lobster, in which the hexokinase activities are much higher than those of phosphofructokinase. Further, if these particular tissues are excluded, the activities of the individual enzymes in nervous tissues from different species are similar: the ranges of mean hexokinase and phosphofructokinase activities are 5.1–17.6 and 4.6–24.0 μmol/min per g fresh wt. of nervous tissue respectively (Table 1).

The range of mean activities for the ketone-body-utilizing enzymes appears to be somewhat greater than that for the carbohydrate-utilizing enzymes: the range of mean 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase activities are 1.1–15.3 and 0.7–4.5 μmol/min per g fresh wt. respectively (Table 1). In general, the activities of the two enzymes are similar in the same tissue from the same animal, although in two species the differences are fairly large (i.e. lizard and snake; see Table 1).

Discussion

The demonstration that the enzymes 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase are present in nervous tissue from many different species indicates that the capacity of this tissue to use ketone bodies is widespread in the animal kingdom. Indeed the presence of enzymes utilizing ketone bodies in all of the nervous tissues studied suggests that ketone bodies may be a significant source of energy under some conditions.

In muscle, Crabtree & Newsholme (1972) showed that the maximum activities of hexokinase and phosphofructokinase *in vitro* can provide a reasonable quantitative indication of the maximum rates of glucose utilization. Since hexokinase and phosphofructokinase catalyse reactions far displaced from equilibrium in some nervous tissues (Lowry *et al.*, 1964; Rolleston & Newsholme, 1967*b*), it has been assumed that the maximum activities of these enzymes *in vitro* provide quantitative information about the maximum capacity for glucose utilization in nervous tissue. The rate of glucose utilization can be calculated from a knowledge of the arteriovenous differences and blood flow across the brain. Unfortunately, this information is available only for rat and human brains (Owen *et al.*, 1967; Hawkins *et al.*, 1971). The rates of glucose utilization are approximately 0.5 and 0.12 μmol/min per g fresh wt. for rat and human brain respectively (at 37°C). For the rat, this is only about 2% of the maximum capacity of the glycolytic pathway, as indicated by the maximum activities of hexokinase and phosphofructokinase at 37°C. This confirms that the rate of glycolysis is severely inhibited under the conditions of the arteriovenous measurements and that, in some situations, the rate of glucose utilization by brain can be increased very markedly (see McIlwain & Bachelard, 1971, for review). Glucose utilization by guinea-pig cerebral-cortex slices is approximately 0.3 μmol/min per g, and this is increased to almost 2.0 by the addition of CN^- (Rolleston & Newsholme, 1967*b*). The rate of glucose utilization by the ischaemic mouse brain is 6.5 μmol/min per g (Lowry *et al.*, 1964), or about 25% of the maximum capacity of the glycolytic pathway. Thus the large capacity of the glycolytic pathway in nervous tissue may provide a short-term mechanism for the production of energy by anaerobic glycolysis if the brain should become hypoxic. Alternatively, the large glycolytic capacity may be required to provide energy at a sufficient rate to supply the demands of maximal neuronal activity. However, under normal conditions only small areas of the brain may be maximally active at any one time, so that the overall utilization of glucose by the whole brain is relatively small.

For the ketone-body-utilization pathway (in muscle and kidney), it has been shown that the enzymes catalyse reactions that are close to equilibrium (Krebs, 1969; Williamson & Hems, 1970). This suggests that the maximum activities of these enzymes *in vitro* (measured in either direction) will be very much higher than the maximum flux through the reaction *in situ* (Newsholme & Crabtree, 1973). The rates of ketone-body utilization by rat and human brains *in situ* are approximately 0.2 μmol/min per g at 37°C in starved animals, as calculated from arteriovenous differences (Owen *et al.*, 1967; Hawkins *et al.*, 1971). These values are considerably lower than the activities of the enzymes reported in Table 1. Unfortunately, therefore, the activities of these

Table 1. *Activities of hexokinase, phosphofructokinase, 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase in nervous tissue*

Enzyme activities are presented as means, with the range of activities and the number of animals given in parentheses. For further details see the Materials and Methods section; all activities were measured at 25°C.

Animal	Nervous tissue	Enzyme activities (μmol/min per g fresh wt. of nervous tissue)			
		Hexokinase	Phosphofructokinase	3-Oxo acid CoA-transferase	Acetoacetyl-CoA thiolase
Annelida:					
Earthworm (*Lumbricoides terrestris*)	Nerve cord	3.2 (2.6–3.6) (3)	18.4 (15.6–25.4) (3)	1.3 (0.8–1.7) (3)	1.5 (0.3–2.7) (3)
Crustacea:					
Lobster (*Homarus vulgaris*)	Cerebral ganglion	16.0 (15.1–16.8) (3)	9.9 (7.8–12.0) (3)	1.6 (1.5–1.7) (3)	1.4 (0.8–1.7) (3)
	Nerve cord and thoracic ganglia	12.2 (8.8–16.4) (5)	2.3 (2.1–2.4) (3)	1.1 (0.7–1.3) (4)	0.7 (0.2–1.0) (4)
Insecta:					
Locust (*Schistocerca gregaria*)	Cerebral ganglion	5.6 (5.3–6.0) (4)	4.6 (2.8–6.4) (5)	2.7 (0.8–4.9) (5)	1.2 (0.7–2.9) (5)
Cockroach (*Blaberus discoidalis*)	Cerebral ganglion	5.1 (4.6–5.8) (3)	6.0 (5.4–6.4) (3)	4.0 (3.4–5.2) (3)	3.8 (3.4–4.4) (3)
Waterbug (*Lethocerus cordofanus*)	Pterothoracic ganglion	0.7 (0.3–1.1) (5)	24.0 (17.5–31.4) (3)	—	—
Pisces:					
Dogfish (*Scylliorhinus canicula*)	Whole brain	10.7 (11.8–9.6) (2)	8.2 (7.4–8.9) (2)	2.7 (2.3–2.9) (3)	3.4 (3.3–3.5) (3)
Rainbow trout (*Salmo gairdneri*)	Whole brain	9.9 (9.7–10.3) (3)	7.4 (6.7–8.1) (3)	2.8 (2.4–3.2) (3)	4.0 (3.7–4.2) (3)
Goldfish (*Carassius auratus*)	Whole brain	16.2 (14.5–17.3) (3)	15.7 (15.4–16.6) (3)	8.7 (7.7–10.7) (3)	3.8 (3.3–4.3) (3)
Amphibia:					
Frog (*Rana temporaria*)	Whole brain	11.9 (9.7–14.5) (3)	8.2 (7.5–8.7) (3)	1.5 (1.1–1.8) (4)	4.0 (1.1–7.7) (5)
Salamander (*Salamandra salamandra*)	Whole brain	9.6 (7.0–14.3) (3)	5.6 (4.7–6.1) (3)	3.9 (3.7–4.3) (3)	1.0 (0.7–1.1) (3)
European newt (*Triturus vulgaris*)	Whole brain	7.6 (6.6–8.2) (3)	8.1 (7.7–8.7) (3)	1.7 (1.3–2.3) (3)	0.8 (0.7–1.0) (3)
Reptilia:					
Green lizard (*Lacerta viridis*)	Whole brain	16.4 (14.5–18.5) (3)	14.9 (1.3–17.2) (3)	13.9 (10.8–16.5) (3)	2.8 (2.4–3.5) (4)
Grass snake (*Tropidonotus natrix*)	Whole brain	17.6 (14.5–22.5) (3)	14.3 (11.3–18.0) (3)	15.3 (14.5–16.5) (3)	4.5 (4.3–4.7) (2)
Aves:					
Domestic pigeon (*Columba livia*)	Whole brain	13.4 (8.7–18.5) (4)	12.2 (8.2–15.1) (4)	3.2 (2.7–3.7) (3)	2.3 (1.6–3.0) (3)
Domestic fowl (*Gallus gallus*)	Whole brain	14.9 (14.2–15.6) (3)	13.7 (12.7–15.7) (3)	2.9 (1.9–3.7) (3)	2.1 (1.0–3.0) (3)
Pheasant (*Phasianus colchicas*)	Whole brain	10.1 (9.7–10.7) (3)	9.7 (9.3–10.0) (3)	2.9 (2.6–3.1) (3)	2.3 (2.2–2.4) (3)
Mammalia:					
Laboratory mouse	Whole brain	14.8 (11.3–19.8) (4)	14.6 (13.9–15.6) (3)	9.8 (8.6–12.4) (4)	2.7 (2.0–3.3) (4)
Laboratory rat (Wistar strain)	Whole brain	13.9 (13.4–14.2) (3)	18.1 (17.6–18.4) (3)	4.8 (3.1–7.0) (4)	1.8 (1.4–2.1) (4)

enzymes *in vitro* provide little quantitative information about the maximal rate of ketone-body utilization *in situ*. Nonetheless, the activities do indicate the widespread existence of the ketone-body-utilization pathway in nervous tissue and suggest that ketone bodies can provide an important energy-source for oxidation.

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