Effect of Environmental Temperature on the Kinetic Properties of Goldfish Brain Choline Acetyltransferase

By CATHERINE HEBB, T. C. STEPHENS* and M. W. SMITH
*Agricultural Research Council Institute of Animal Physiology,
Babraham, Cambridge CB2 4AT, U.K.*

(Received 22 March 1972)

1. Michaelis constants of goldfish brain choline acetyltransferase were found to depend on the concentration of the second substrate present and on the temperature to which the fish had been adapted. 2. Primary plots constructed from results obtained with enzyme prepared from cold-adapted or warm-adapted fish indicated that synthesis of acetylcholine took place by a sequential mechanism. 3. The affinity of choline acetyltransferase for acetyl-CoA was about 100 times that for choline irrespective of whether the enzyme had been prepared from warm-adapted or cold-adapted fish. 4. The maximum rate at which choline acetyltransferase synthesized acetylcholine and the energy of activation for this synthesis remained independent of the previous environmental temperature of the fish. 5. The affinity of choline acetyltransferase for choline and acetyl-CoA showed a complex dependence on temperature. The affinity of the enzyme from cold-adapted fish for substrates increased as the incubation temperature was lowered, whereas that of the enzyme from warm-adapted fish first increased and then decreased. 6. The maximum affinity of choline acetyltransferase for both substrates, from both cold-adapted and warm-adapted fish, occurred at temperatures that corresponded approximately to the respective environmental temperatures of the fish. 7. These changes in enzyme affinity for substrates are not thought to be due to the presence of isoenzymes. Their adaptive significance is unknown, but it could be connected with the maintenance of the enzyme in a stable form.

Direct evidence that compensatory changes occur within the central nervous system of fish in response to changes of environmental temperature has been provided through the work of Prosser & Farhi (1965), who found that the minimum temperature at which a conditioned response was lost depended on the adaptation temperature of the fish. Peripheral nerves and simple reflexes must also function adequately as part of the conditioned response, but it was shown convincingly that adaptation at this level was not responsible for the observed effects (Roots & Prosser, 1962; Prosser & Farhi, 1965). Further work, by Konishi & Hickman (1964), demonstrated that an evoked response in fish midbrain was also subject to temperature compensation, showing again the importance of adaptation within the central nervous system. There seems little doubt that induced changes in the central nervous system alter the capacity of fish brain to integrate nervous impulses and that this explains many of the compensatory changes seen when fish activity is measured at different temperatures (Fry, 1964).

Acetylcholine plays a dominant part in modifying transmission in the central nervous system, and it is

* Present address: Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2 3PX, U.K.

therefore natural to study those enzymes responsible for its synthesis and destruction when searching for an explanation of the gross changes seen to take place in brain function after adaptation. Baldwin & Hochachka (1970) found that an overall increase in brain acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) activity occurred in warm-adapted trout and that two isoenzymes were present having different affinities for acetylcholine. Acetylcholinesterase from fish taken from different thermal environments also shows differences in affinity for choline (Baldwin, 1971).

Less is known about possible changes in choline acetyltransferase, the enzyme responsible for the synthesis of acetylcholine. Earlier work with this enzyme suggested it might also change its affinity for choline, although this conclusion could only be tentative since with the coupled assay then used the concentration of the second substrate, acetyl-CoA, could not be varied (Hebb *et al.*, 1969). More recently a radiometric assay based on the original method of McCaman & Hunt (1965) has allowed a more detailed kinetic examination of this enzyme (Morris *et al.*, 1971). The object of the present work was to use a similar radiometric assay to examine in detail changes in substrate-enzyme affinity that might arise as a

result of temperature adaptation in the brain of goldfish.

Materials and Methods

Animals

Goldfish (*Carassius auratus*), weighing about 100g and 15–18cm long, were purchased from Perry's Hardy Plant Farm, Enfield, Middx., U.K. After 20min immersion in 2% (w/v) NaCl solution to kill any fungus with which they might be infected, the fish were kept for 1 week in an aquarium at room temperature (15–18°C), and were then transferred to aerated acclimatization tanks at 6°C or at 30°C. They were kept in these tanks for 4–5 weeks before being used for experiment. All fish were fed once or twice daily with Duffield's Angler's ground-bait.

Assay of choline acetyltransferase activity

Fish were decapitated, and their brains were dissected out and homogenized by hand at a concentration of 100mg of tissue/ml of 0.3M-sucrose in a Dounce homogenizer. The homogenizer was immersed in crushed ice while the homogenate was made. Three to five brains were pooled to make each preparation; the combined material was then dialysed in Visking tubing against 1 litre of 0.3M-sucrose at 0–4°C; the dialysis solution was renewed twice, first after 30min and again after 60min; the total period of dialysis was 2½h. The dialysed homogenate was stored in separate samples, 0.4ml each, in small Pyrex tubes, at –16°C.

Choline acetyltransferase activity was measured by a modification (Morris *et al.*, 1971) of the McCaman & Hunt (1965) radiometric assay method that involves a single reaction (as opposed to a coupled system) based on the use of [1-¹⁴C]acetyl-CoA as substrate. The incubation medium contained (final concentrations): NaCl, 300mM; sodium phosphate buffer, pH7.5, 33.3mM; eserine sulphate, 0.13mM; choline, 0.01–1.0mM; [1-¹⁴C]acetyl-CoA, 0.57–50µM; 10µl of homogenate; water to give a final volume of 0.12ml. Incubation was carried out in 0.5ml-capacity plastic tubes. The timed incubation was started by the addition of acetyl-CoA after the warming of each tube, containing all the other ingredients, for 15s in the incubation bath to bring it to the same temperature. Incubation was ended by rapidly mixing 0.8ml of 0.15M-HClO₄ with the incubation mixture and cooling the tubes in an ice bath. For the addition of acid and for the initial addition of acetyl-CoA a Vortex mixer was used. The temperature and duration of incubation were varied as described below.

The acidified incubation mixture was quantitatively transferred to the top of a drained column

(10cm×0.65cm) of the anion-exchange resin De-Acidite FF-IP SRA 63 (100–200 mesh; Cl[–] form) (The Permutit Co., London W4 5QE, U.K.) with three washings (0.4ml) of water. The effluent, totalling 1.4ml, was discarded; a further 1.6ml of water was then added and an equivalent volume was collected into 15ml of scintillation fluid. In this way 99–100% of the acetylcholine formed was recovered. As a control, column blanks were prepared for each concentration of [1-¹⁴C]acetyl-CoA used. Incubation mixture, but without active enzyme, was prepared in 0.12ml amounts, HClO₄ was added and the acidified mixture put through a resin column in the usual way. Counting of the radioactivity of these gave a measure of the amount of acetyl-CoA that leaked through into the effluent containing the acetylcholine. The radioactivity of these controls was always low, giving counts of less than twice background, and corresponding to less than 20pmol. The activity of the enzyme is expressed in milliunits, 1 milliunit being equivalent to the formation of 1 nmol of product/min. The maximum activity was found to be about 10 milliunits/g of tissue at 5°C and 150 milliunits/g of tissue at 39°C. Radioactivity was measured as described by Morris *et al.* (1971).

In some species a large part of the choline acetyltransferase present in brain homogenates is in the nerve-ending fraction and must be activated by treatment with ether (Hebb & Smallman, 1956) or detergents (Fonnum, 1966) if maximum rates of synthesis of acetylcholine are to be achieved. To determine whether the enzyme in goldfish brain is similarly bound, two kinds of tests were done. First, samples of the homogenate were treated with ether as described by Bull *et al.* (1970) and their choline acetyltransferase activities compared with those of untreated controls. Secondly, other samples (0.5ml diluted with 0.4ml of 1% NaCl) were centrifuged for 6×10⁶g-min in a Spinco model L preparative ultracentrifuge; the supernatant and pellet were then separately analysed for their choline acetyltransferase activity. The results of both tests indicated that very little, if any, of the enzyme was bound. Ether had no activating effect, thus showing that there is no occluded enzyme comparable with that observed in tests on mammalian brain. Nor was there evidence from centrifuged homogenates of a significant degree of binding; in a homogenate prepared from 30°C-acclimatized fish 78% of the enzyme was recovered in the supernatant; in another, prepared from 6°C-acclimatized fish, 71% was recovered in the supernatant. The difference was not regarded as significant.

Other tests were done on a sample of goldfish brain homogenate to find out whether the tissue contains any hydrolase capable of destroying acetyl-CoA such as that found in rat brain homogenates (Kása *et al.*, 1970). For this purpose 200µl samples of homogenate were incubated at 15°, 25° and 39°C in a

total volume of 1.2 ml containing: NaCl, 300 mM; sodium phosphate buffer, pH 7.5, 33.3 mM; eserine sulphate, 0.13 mM; [$1\text{-}^{14}\text{C}$]acetyl-CoA, 1.75 mM. At hourly intervals 100 μl samples were withdrawn and their acetyl-CoA contents were measured by Chase's (1967) method. Acetyl-CoA was assayed by measuring the release of CoA in the presence of an excess of oxaloacetate, citrate synthase and 5,5'-dithiobis-(2-nitrobenzoic acid). Free CoA was measured by allowing it to react with 5,5'-dithiobis-(2-nitrobenzoic acid) (Morris *et al.*, 1971). No acetyl-CoA was destroyed in the absence of homogenate. With the homogenate present the amount of acetyl-CoA destroyed or hydrolysed/h at 15°C was less than 1%, at 25°C it was 2% and at 39°C it was 2.9%. A parallel test in which the production of acetylcholine was measured at 39°C showed that all but 1.1% of the acetyl-CoA that disappeared at this temperature could be accounted for as acetylcholine formed. When choline was added in saturating concentrations the conversion of acetyl-CoA into acetylcholine at 39°C utilized 23% of the acetyl-CoA present. These results showed that uncontrollable loss of acetyl-CoA would not be sufficient to interfere with the measurements by means of which the kinetic properties of the enzyme were to be studied.

Chemicals

CoA (85–90% pure), *cis*-oxaloacetic acid and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Citrate synthase (EC 4.1.3.7) was supplied by Boehringer Corp., London W.5, U.K.

[$1\text{-}^{14}\text{C}$]Acetyl-CoA was prepared from CoA by treatment with acetic anhydride (Simon & Shemin, 1953). For this purpose [$1\text{-}^{14}\text{C}$]acetic anhydride (specific radioactivity 121 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The procedure for the preparation and purification of labelled acetyl-CoA has been described by Morris *et al.* (1971). An important part of the procedure is application of the acetyl-CoA, after its synthesis and the removal of free acetate, to a DEAE-cellulose column to separate the radioactive acetyl-CoA from a radioactive impurity, which if retained has been found to give high column blanks (C. Hebb, S. P. Mann & J. Mead, unpublished work). Each preparation of acetyl-CoA was adjusted to pH 4–5 and stored at -16°C as described above.

The apparent specific radioactivity (uncorrected for efficiency or quenching) of the acetyl-CoA gave a count of approx. 7000 c.p.m./nmol. The value for one preparation was slightly above this, another slightly below.

Choline chloride and eserine sulphate were supplied by British Drug Houses Ltd., Poole, Dorset, U.K. All other reagents were of analytical grade.

Choline chloride was assayed by titrating the chloride against AgNO_3 , the end point being determined electrometrically (Keynes, 1963).

Results

Stability of choline acetyltransferase

Preparations of choline acetyltransferase from the brains of goldfish adapted to swimming temperatures of 6° or 30°C were incubated with 1 mM-choline and 25 μM -acetyl-CoA over the temperature range 15–45°C for times up to 30 min. Incubation was stopped at known times throughout the incubation and the amount of acetylcholine present was measured. The production of acetylcholine was always linear with time for an initial period of incubation; but when the incubation was prolonged production fell off from linearity at times that depended on the incubation temperature used. Initial rates of reaction could be observed for 4, 8, 12 and 20 min at incubation temperatures of 45°, 35°, 25° and 15°C respectively. Times of incubation were chosen to fall within these limits, and a 15 min incubation period was chosen for the assay of enzyme preparations at incubation temperatures at or below 15°C. The concentrations of choline and acetyl-CoA used in these experiments were high. Deviation from linearity could not be explained by either substrate becoming limiting to the reaction. Essentially similar results were obtained with 0.1 mM-choline with 4.6 μM -acetyl-CoA, both of which were well below saturation concentrations. There was no difference in the stability of enzyme homogenates prepared from 6°C-adapted and 30°C-adapted goldfish.

Choice of substrate concentrations

Goldfish brain homogenates were incubated at 35°C for 8 min together with 1 mM-choline and concentrations of acetyl-CoA ranging from 0.57 to 50 μM . The production of acetylcholine was directly dependent on the amount of acetyl-CoA present over the whole concentration range tested. Acetyl-CoA was used in concentrations that varied between 4.6 and 25 μM in all subsequent experiments.

Similar experiments were carried out with different concentrations of choline (0.01–1 mM) together with acetyl-CoA at a concentration of 25 μM . The amount of acetylcholine synthesized was directly proportional to the amount of choline present over the choline concentration range 0.1–1 mM. This dependence was not found with lower concentrations of choline (0.01–0.1 mM), probably because of some choline release during incubation. Deviation from linearity did not depend on the previous environmental temperature of the fish. Choline was used in concentrations varying from 0.1 to 1 mM in all subsequent experiments.

Effect of environmental temperature on choline acetyltransferase

Preparations of goldfish brain choline acetyltransferase, previously dialysed to remove endogenous free choline, were incubated at 15°C with four fixed concentrations of acetyl-CoA together with various concentrations of choline. The double-reciprocal plots for acetylcholine synthesis measured under these conditions are shown in Fig. 1. The plots were linear and convergent with points of intersection below the abscissa to the left of the ordinate. The apparent K_m for choline depended on the concentration of acetyl-CoA used. This was similar to kinetics found for choline acetyltransferase prepared from mammalian sources (Potter *et al.*, 1968; White & Cavallito, 1970; Morris *et al.*, 1971), and was compatible with the view that the synthesis of acetylcholine occurred via a sequential mechanism. The precise point at which the lines converged, determined by eye or by computer analysis, appeared to depend on the previous environmental temperature of the fish, being further to the left of the ordinate for the cold-adapted animal.

Double-reciprocal plots were also constructed from results obtained with four fixed concentrations of choline and various concentrations of acetyl-CoA. The results obtained are shown in Fig. 2. Again the plots were linear with points of intersection to the left

Fig. 1. *Effect of varying the acetyl-CoA concentration on reciprocal plots for choline with brain choline acetyltransferase prepared from cold-adapted and warm-adapted goldfish*

Goldfish were adapted to 6°C (a) or to 30°C (b). Concentrations of acetyl-CoA used were 4.6 μM ($\circ$), 5.7 μM (Δ), 10.8 μM ($\bullet$) and 25 μM ($\square$). Conditions of incubation were as described in the text.

of the ordinate, the precise point depending on the source of the enzyme.

Secondary plots for the two substrates, derived by obtaining the appropriate intercepts on the ordinates of Figs. 1 and 2 and plotting these against the reciprocal concentrations of second substrate, were constructed for choline acetyltransferase prepared from goldfish adapted to 6°C or to 30°C. The results obtained are shown in Fig. 3. The apparent K_m for choline from the enzyme-acetyl-CoA complex, determined for enzyme obtained from 6°C-adapted goldfish, was 0.17 mM. This increased to 0.33 mM for enzyme prepared from the 30°C-adapted goldfish. The change in the apparent K_m for acetyl-CoA from the enzyme-choline complex was less marked, being 6.5 μM for enzyme prepared from 6°C-adapted fish against 8.3 μM for enzyme prepared from 30°C-adapted goldfish. The maximal rate of acetylcholine synthesis, 24.5 milliunits, was the same for both these preparations measured at an incubation temperature of 15°C.

Effect of temperature on choline acetyltransferase kinetics

Experiments similar to those reported in the preceding section, with the same preparations of choline acetyltransferase, were repeated at various incubation temperatures within the range 5–45°C. The maximal rates of acetylcholine synthesis at these different temperatures, calculated from the intercepts of secondary plots similar to those shown in Fig. 3,

Fig. 2. *Effect of varying the choline concentration on reciprocal plots for acetyl-CoA with brain choline acetyltransferase prepared from cold-adapted and warm-adapted goldfish*

Goldfish were adapted to 6°C (a) or to 30°C (b). Concentrations of choline used were 0.1 mM ($\circ$), 0.15 mM (Δ), 0.3 mM ($\bullet$) and 1.0 mM ($\square$). Conditions of incubation were as described in the text.

Fig. 3. Secondary intercept plots derived from Figs. 1 and 2

The intercepts on the ordinates of Figs. 1 and 2 are plotted against the reciprocal of the concentration of choline (○) or acetyl-CoA (●) used. Results were obtained with brain choline acetyltransferase obtained from goldfish adapted to 6°C (a) or to 30°C (b).

are shown in Fig. 4. The logarithm of acetylcholine synthesis was related linearly to the reciprocal of the absolute temperature over the temperature range 5–40°C. The energy of activation for the reaction was 61.1 kJ/mol (14.6 kcal/mol) and the Q_{10} 2.1. It was not possible to distinguish between the enzyme prepared from 6°C-adapted fish and that from 30°C-adapted fish by using this parameter.

The apparent K_m values for both substrates were also determined at different incubation temperatures. These results are shown in Fig. 5. All the Arrhenius plots were curvilinear, showing upward deflexions at high incubation temperatures, the points of transition depending on whether the enzyme had been prepared from warm-adapted or cold-adapted fish. Thus the apparent K_m for choline of choline acetyltransferase from brains taken from 30°C-adapted fish showed little dependence on the temperature of incubation when this was varied from 5° to 30°C, but a high temperature-dependence was apparent over the temperature range 30–45°C. The enzyme from cold-adapted fish showed similar changes in apparent K_m for choline, but the temperature range over which K_m was markedly temperature-dependent now extended from 15° to 45°C, the temperature-independent region being confined to the temperature range 5–15°C.

The apparent K_m values of choline acetyltransferase for acetyl-CoA also showed temperature-sensitivities that depended both on the range of incubation temperature used and on the previous environmental temperature of the fish. The changes seen were very similar to those found for choline, suggesting that they might have a common origin.

The effect of temperature on dissociation constants (K_s values) for acetylcholine and acetyl-CoA have been calculated from Figs. 1 and 2. The point at which the lines of a double-reciprocal plot intersect has a co-ordinate on the abscissa equal to the negative reciprocal of the dissociation constant for the complex between free enzyme and whichever substrate is used as the variable substrate in the construction of the plot (Florini & Vestling, 1957). The validity of K_s values in describing the true kinetic behaviour of the enzyme depends on its mode of operation. For the most general random two-substrate mechanism (Alberty, 1953) these values give true dissociation constants, but for an ordered mechanism the constant is strictly meaningful for only the leading substrate. In fact K_s values changed with temperature in a way similar to that found for K_m values (Fig. 6). Dissociation constants for choline and for acetyl-CoA increased rapidly as the temperature of incubation rose from 30° to 45°C, irrespective

of whether the enzyme had been prepared from fish swimming at 6° or 30°C. At lower incubation temperatures, however, the dissociation constants continued to decrease with enzyme prepared from cold-adapted fish whereas those for the enzyme from warm-adapted fish increased at incubation temperatures below 20°C. There was therefore an approximate correlation between the incubation temperature giving minimal dissociation constants and the previous environmental temperature of the fish, 5°C for the 6°C-adapted goldfish and 20–25°C for the 30°C-adapted goldfish.

Discussion

A possible criticism of the work described here is that all of the measurements were made on enzyme in crude extracts of brain. The kinetics of mammalian choline acetyltransferase have usually been studied on tissue extracts that have been put through two or more stages of purification, including $(\text{NH}_4)_2\text{SO}_4$ fractionation, so as to increase the amount of the enzyme relative to the total protein concentration. Two points require comment here. The first is that the specific activity of the goldfish brain enzyme in the crude extracts used in this research was found to be approx. 4 milliunits/mg of protein when incubated at 15°C, which compares favourably with values reported in the literature for mammalian enzyme after two stages of purification. These include 3.0 and 8.3 milliunits/mg of protein respectively for cat and for rat

Fig. 4. *Temperature-dependence of acetylcholine production by brain choline acetyltransferase prepared from cold-adapted and warm-adapted goldfish*

The maximal rate of acetylcholine production at a single incubation temperature was obtained as the reciprocal of the intercept on the ordinate of secondary plots similar to those shown in Fig. 3. Measurements were made at incubation temperatures varying from 5° to 45°C with choline acetyltransferase prepared from goldfish adapted to 6°C (○) or to 30°C (●).

Fig. 5. *Temperature-dependence of the apparent K_m values for choline and for acetyl-CoA of brain choline acetyltransferase prepared from cold-adapted and warm-adapted goldfish*

The apparent K_m values for choline (a) and for acetyl-CoA (b) were obtained for choline acetyltransferase prepared from goldfish adapted to 6°C (○) or to 30°C (●).

Fig. 6. Temperature-dependence of dissociation constants (K_s values) for choline acetyltransferase-substrate complexes

The K_s values for choline and acetyl-CoA were determined at different incubation temperatures by estimating the point of intersection of double-reciprocal plots similar to those shown in Figs. 1 and 2. The range of incubation temperatures used was 5–45°C. Values given for acetyl-CoA (a) and choline (b) were determined for brain choline acetyltransferase prepared from goldfish adapted to 6°C (o) or to 30°C (●).

brain incubated at 37°C (Fonnum, 1970), 2–4 milliunits/mg of protein for ox caudate nucleus incubated at 37°C (White & Cavallito, 1970) and approx. 3–4 milliunits/mg of protein for human placenta incubated at 37° and 39°C respectively (Schuberth, 1966; Morris *et al.*, 1971). The second point is that there is no evidence to show that further purification of the enzyme improves the accuracy of the determination of the kinetic constants; in fact the K_m values for choline, 777 μM , and for acetyl-CoA, 17.5 μM , for crude rat brain (Kaita & Goldberg, 1969) are very similar to the corresponding values of 800 and 16 μM for ox striatal enzyme purified to a specific activity of 2–4 milliunits/mg of protein (White & Cavallito, 1970) and of 750 and 10 μM for the same tissue purified to a specific activity of 116 milliunits/mg of protein (Glover & Potter, 1971). The differences are no more than could be expected from differences in methodology.

There are, on the other hand, positive reasons for studying choline acetyltransferase from goldfish and other cold-blooded animals without purification. First, the enzyme is unstable, as the initial experiments reported here show, and this instability could be expected to become greater with each successive stage of purification. The enzyme from cold-blooded animals is generally very unstable (see Hebb & Ratković, 1964; Prince & Hide, 1971), and in this it differs from the mammalian enzyme. Secondly, use

of homogenates without further purification is preferred because it avoids the danger of a selective loss of the enzyme. Loss of activity might be acceptable if the enzyme were truly homogeneous and one could be sure that the losses that did occur due to denaturation or to other causes had a uniform effect, but if the enzyme exists in more than one form (Malthe-Sørensen & Fonnum, 1972) this can no longer be assumed to be true. With such uncertainty existing about the nature of the system we were dealing with, we felt obliged to take the least number of steps to change that system, so preferring to study it in relatively crude form.

The convergent primary double-reciprocal plots found with goldfish brain choline acetyltransferase are similar to others obtained with enzyme prepared from rat and calf brain (Potter *et al.*, 1968; White & Cavallito, 1970). They indicate that the synthesis of acetylcholine proceeds through a sequential mechanism whereby both substrates combine with the enzyme before any products are released. It further appears, from inhibition studies with human placental choline acetyltransferase, that the type of sequential mechanism involved is Theorell-Chance (Morris *et al.*, 1971) and that, in brain, acetyl-CoA might act as the leading substrate (White & Cavallito, 1970). There is as yet no evidence to suggest species or tissue variation in choline acetyltransferase, and one can probably assume, for the purpose of discussion,

that a similar sequential mechanism operates for the goldfish brain enzyme. In this case the simple determination of K_m as a measure of affinity of enzyme for substrate will be valid only for the final reaction between the enzyme-acetyl-CoA complex and choline, and the true dissociation constant for the binding of acetyl-CoA to the enzyme is given by the K_s value. It is these values that have therefore been used to describe adaptational changes in enzyme-substrate affinity. Fortunately both constants show essentially the same response to temperature, so that even without knowing for certain whether the enzyme is governed by a random or ordered mechanism or, in the latter case, which is the leading substrate, it is still possible to discuss the effect of temperature adaptation on true enzyme-substrate affinity.

Choline acetyltransferase from warm-adapted fish has an affinity for acetyl-CoA (K_s value) that reaches a minimum at about 20°C. This U-shaped dependence of affinity on temperature changes on adaptation to cold, so that the minimum K_s value now corresponds to the lowest temperature of incubation (5°C). The affinity of the enzyme-acetyl-CoA complex for choline (K_m value) is about 100-fold less than that of the free enzyme for acetyl-CoA, but the temperature-dependence of the affinity for choline and the effect of changing adaptation temperature on this dependency are strikingly similar to those for acetyl-CoA. This characteristic U-shaped dependence of affinity on temperature has also been found for fish brain acetylcholinesterase (Baldwin & Hochachka, 1970; Baldwin, 1971), but it is by no means restricted to enzymes operating within the central nervous system. In many cases complex U-shaped K_m -temperature curves can be shown to be due to the presence of more than one isoenzyme, each having a different affinity for its substrate. This has been shown to be true for lactate dehydrogenases (Hochachka & Somero, 1968), pyruvate kinases (Somero & Hochachka, 1968), fructose diphosphatases (Behrlich, 1969) and isocitrate dehydrogenases (Moon & Hochachka, 1971). Changes in the affinity of choline acetyltransferase for its substrates could be explained by the presence of more than one isoenzyme, but this seems unlikely. Malthe-Sørensen & Fonnum (1972) have found that the mammalian enzyme can exist in different forms, but the kinetic properties of each form are the same. It has been suggested that enzymes which change their properties on adaptation do so to stabilize their catalytic rates over a wide range of temperature (Hochachka & Somero, 1968). At high environmental temperatures increases in V_{max} would then be balanced by a fall in enzyme affinity for its substrate so that the rate at which enzyme product was formed would stay constant, provided that the concentration of substrate remained unchanged. It is, however, more difficult to imagine how a decrease in enzyme affinity for its substrate at low environmental

temperatures could possibly stabilize reaction rates. Indeed, if this were the only concern of adaptation there would seem to be no need to change from the cold- to the warm-variant form of the enzyme in the first place. Baldwin (1971) has suggested that something as non-specific as changes in membrane lipids might be responsible for the characteristic shapes of the temperature-affinity plots, but this seems equally improbable. Though it may be relevant to some of the enzymes studied previously, it can hardly be a factor for others, like choline acetyltransferase, which are not normally found in a membrane-bound form. The affinity of choline acetyltransferase for its substrates decreases as the incubation temperature rises. It would be unwise to draw too close a parallel between the behaviour of choline acetyltransferase in the whole animal and that in a crude tissue homogenate, but it is possible that a decreased affinity represents the first stage of a process leading to the eventual inactivation of the enzyme. In such a case some fish might be able to synthesize a new type of enzyme, or modify that already present, so that it will resist the damaging effects of a change in temperature. As Dixon & Webb (1964) have pointed out, the presence of substrates may protect an enzyme from denaturation by binding with the active centre and so maintaining its tertiary structure. It follows that the most stable state of an enzyme will be that having a high affinity for its substrates. The appearance of an enzyme on adaptation having a maximal affinity for substrate at a temperature near to the new environmental temperature could then be seen, not as a means of maintaining catalytic rates constant, but rather as a way of stabilizing enzyme structure in a changed environment.

We thank Mrs. J. Mead for her skilled technical assistance throughout this work.

References

- Alberty, R. A. (1953) *J. Amer. Chem. Soc.* **75**, 1928
- Baldwin, J. (1971) *Comp. Biochem. Physiol.* **40B**, 181
- Baldwin, J. & Hochachka, P. W. (1970) *Biochem. J.* **116**, 883
- Behrlich, H. W. (1969) *Biochem. J.* **115**, 687
- Bull, G., Hebb, C. & Ratković, D. (1970) *J. Neurochem.* **17**, 1505
- Chase, J. F. A. (1967) *Biochem. J.* **104**, 503
- Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., p. 148. Longmans, Green and Co. Ltd., London
- Florini, J. R. & Vestling, C. S. (1957) *Biochim. Biophys. Acta* **25**, 575
- Fonnum, F. (1966) *Biochem. J.* **100**, 479
- Fonnum, F. (1970) *J. Neurochem.* **17**, 1095
- Fry, F. E. J. (1964) *Adapt. Environ. (Handb. Physiol. Sect. 4)*, 715
- Glover, V. A. S. & Potter, L. T. (1971) *J. Neurochem.* **18**, 571
- Hebb, C. O. & Ratković, D. (1964) *Proc. Int. Neurochem. Symp.* **5th**, p. 347

- Hebb, C. & Smallman, B. N. (1956) *J. Physiol. (London)* **134**, 385
- Hebb, C., Morris, D. & Smith, M. W. (1969) *Comp. Biochem. Physiol.* **28**, 29
- Hochachka, P. W. & Somero, G. N. (1968) *Comp. Biochem. Physiol.* **27**, 659
- Kaita, A. A. & Goldberg, A. M. (1969) *J. Neurochem.* **16**, 1185
- Kása, P., Mann, S. P. & Hebb, C. (1970) *Nature (London)* **226**, 812
- Keynes, R. D. (1963) *J. Physiol. (London)* **169**, 690
- Konishi, J. & Hickman, C. P., Jr. (1964) *Comp. Biochem. Physiol.* **13**, 433
- Malthe-Sørensen, D. & Fonnum, F. (1972) *Biochem. J.* **127**, 229
- McCaman, R. E. & Hunt, J. M. (1965) *J. Neurochem.* **12**, 253
- Moon, T. W. & Hochachka, P. W. (1971) *Biochem. J.* **123**, 695
- Morris, D., Maneckjee, A. & Hebb, C. (1971) *Biochem. J.* **125**, 857
- Potter, L. T., Glover, V. A. S. & Saelens, J. K. (1968) *J. Biol. Chem.* **243**, 3864
- Prince, A. K. & Hide, E. J. J. (1971) *Nature (London)* **234**, 222
- Prosser, C. L. & Farhi, E. (1965) *Z. Vergl. Physiol.* **50**, 91
- Roots, B. I. & Prosser, C. L. (1962) *J. Exp. Biol.* **39**, 617
- Schuberth, J. (1966) *Biochim. Biophys. Acta* **122**, 470
- Simon, E. J. & Shemin, D. (1953) *J. Amer. Chem. Soc.* **75**, 2520
- Somero, G. N. & Hochachka, P. W. (1968) *Biochem. J.* **110**, 395
- White, H. L. & Cavallito, C. J. (1970) *Biochim. Biophys. Acta* **206**, 343