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INHIBITION OF THIAMINE TRANSPORT ACROSS THE BLOOD–BRAIN BARRIER IN THE RAT BY A CHEMICAL ANALOGUE OF THE VITAMIN

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SUMMARY

1. The flux of thiamine from the blood into the brain has been measured using a specially devised technique by which a steady raised level of the vitamin, with or without radioactive labelling, can be achieved rapidly and maintained in the bloodstream. This is done by a continuous injection, given at a rate which is adjusted by a pre-determined programme so as to replace the tracer at the rate at which it has been found to leave the circulation in previous experiments.

2. A further programme was worked out to maintain, in a similar manner by a separate injection, a steady raised level in the bloodstream of a chemical analogue of thiamine, 1-[(4-amino-2-propyl-5-pyrimidinyl)methyl]-2-picolinium chloride HCl (amprolium).

3. In the presence of a high concentration of amprolium the flux of thiamine across the blood–brain barrier was greatly reduced and no longer saturable by raising the blood thiamine concentration up to at least 10 μM. It was concluded that this analogue of thiamine inhibited the saturable component of thiamine transport across the barrier but not the non-saturable component.

4. In a further series of experiments, progressively higher levels of thiamine were maintained in the bloodstream and the influx of the vitamin across the blood–brain barrier was measured. From kinetic analysis of the results, it was clear that the affinity of amprolium for the transport carrier was of a similar magnitude to that of thiamine itself. That the inhibition was competitive was shown by the way in which it could be overcome if the level of thiamine in the blood plasma was raised sufficiently above the normal.

INTRODUCTION

It has been shown by kinetic analysis that the transport of thiamine across the blood–brain barrier is a two-component process, one of which is carrier-mediated transport which is saturable by an excess of thiamine while the other component is a process which does not appear to be saturable by high concentrations of the vitamin in the circulation (Greenwood, Love & Pratt, 1980, 1982). It was not clear whether this second process was passive diffusion or transport by another carrier for which thiamine had only a low affinity. In an attempt to resolve this problem the effect upon thiamine transport across the blood–brain barrier has been investigated of a

close chemical analogue of the vitamin, 1-[(4-amino-2-propyl-5-pyrimidinyl)methyl]-2-picolinium chloride HCl (amprolium). This substance has been found to be a competitive inhibitor of the carrier-mediated transport of thiamine across the mucosa of the small intestine in the chick (Polin, Wynosky & Porter, 1963) as well as that of many other animal species (Rindi & Ventura, 1972), into rat brain slices (Sharma & Quastel, 1965), into isolated rat hepatocytes (Lumeng, Edmondson, Schenker & Li, 1979) and across the cell surface membranes of coccidial schizonts (James, 1980).

A preliminary account of this work has been given (Greenwood & Pratt, 1981).

METHODS

Animals

Young adult Wistar albino rats of either sex were used weighing between 200 and 400 g. Water and food were given without restriction. The diet was a commerical breeding one containing 21·5% of protein and 45% of carbohydrate, with a thiamine content of 18·5 mg kg^{-1}. Under sodium pentobarbitone anaesthesia (B.P.C., 35 mg kg^{-1} of body wt. I.P.), Portex polythene catheters were inserted, one ($1{\cdot}02 \times 10^{-3}$ m o.d.) into a femoral artery for withdrawing blood and one or two ($0{\cdot}63 \times 10^{-3}$ m o.d.) into femoral veins for giving the programmed I.V. injections. [^{14}C]thiamine was given in isotonic NaCl solution, with a specific activity ranging up to 0·899 kBq μmol^{-1}. In some experiments the specific activity was deliberately reduced by adding unlabelled thiamine and the rate of injection was scaled up, so as to maintain the blood level of the vitamin at the desired raised level. Injections of the amprolium were given according to two different procedures. In earlier experiments a single I.V. injection of amprolium was given about 20 s before the start of the continuous I.V. injection of [^{14}C]thiamine, so as to raise the blood amprolium concentration above 0·4 mM for the duration of the influx measurement. In later experiments a programme was worked out so that a continuous I.V. injection of amprolium solution could be given, so as to maintain a steady raised level of this substance in the circulation.

Measurement of influx

The influx, J, that is, the unidirectional movement inwards of thiamine across the walls of the blood vessels into the extracellular space and cells of the brain, was measured by the initial rate at which the tracer entered the tissue from the blood plasma. At the end of the short experimental period (approximately 1 min) during which a steady level of the radioactive tracer was maintained in the blood plasma, the blood was rapidly washed out of the vessels by a forced intra-arterial injection of isotonic saline (Daniel, Love, Moorhouse, Pratt & Wilson, 1974), the head was cut off and the brain removed rapidly and frozen. The influx was calculated from the following equation:

$$J = \frac{R_t C_p}{R_c t}, \tag{1}$$

where R_t is the level of radioactivity in the cerebral tissue, R_c is the mean level of radioactivity in the capillary blood plasma, calculated from that in the arterial blood plasma as described previously (Greenwood *et al.* 1982), C_p is the mean concentration of thiamine in the blood plasma and, t, is the duration of the period during which the radioactive labelling is maintained in the circulation.

Measurement of concentrations of amprolium in blood plasma

A fluorimetric method based upon that of Patrini & Rindi (1980) was used to estimate the sum of the free amprolium and thiamine concentrations. The conditions used were those most appropriate for detection of amprolium, i.e. excitation was done at 370 nm and the emission read at 470 nm. The amprolium concentration was calculated from this result by subtraction from it of the fluorescence at 470 nm, estimated to be due to the relatively small concentration of thiamine present.

Materials

Radioactively labelled (2-[^{14}C]thiazole) thiamine was obtained from the Radiochemical Centre Amersham and thiamine hydrochloride from the Sigma Chemical Company, London. Amprolium was generously provided by Merck, Sharpe & Dohme. All other reagents were of analytical grade whenever available.

Radioative counting

The blood samples were taken into heparinized syringes, the cells spun down, the plasma removed and stored at −20 °C until the radioactivity was assayed. The tissues were dissolved in a solution of an organic base (Soluene-350, Packard Instruments). Glacial acetic acid was then added to neutralize the solution and 15 ml was added of a scintillation mixture containing 5 g of 2,5-diphenyloxazole and 0·3 g of 1,4-bis(2-(4-methyl-5-phenyloxazolyl)) benzene per litre of toluene. Samples of 10 μl of plasma were prepared in a similar way. The radioactivity in the samples was measured in an automatic scintillation spectrometer (Tricarb, Model 2409, Packard Instruments). When necessary, quench corrections were done by the channels–ratio method.

RESULTS

The rate of disappearance of amprolium from the bloodstream was measured by monitoring the change in its blood concentration at intervals after the rapid intra-arterial injection of 3 μmol of amprolium in a small volume of saline. From the rate of disappearance an injection programme was calculated to specify the variable rate at which a continuous I.V. injection had to be given to achieve rapidly and maintain steadily, a predictable blood level of amprolium (Daniel, Donaldson & Pratt, 1975). Despite the close chemical similarity (Fig. 1) the injection protocol for maintaining a steady level of amprolium in the blood plasma differed significantly from that for an injection of thiamine.

The effect of amprolium on thiamine transport across the blood–brain barrier

The influx of thiamine into the brain was measured in a series of experiments in which the concentration of amprolium in the blood plasma was kept greater than 0·4 mM, starting before and continuing throughout the period (less than 1 min) needed to measure the thiamine flux. The measurements were then repeated with progressively higher concentrations of thiamine being maintained in the blood in successive experiments. Under these conditions saturation of the transport system by high thiamine concentrations was no longer evident and the plot of influx against thiamine concentration could be fitted fairly well by linear regression, the line passing close to the origin (Fig. 2).

In a further series of experiments, the effect of increasing blood concentrations of amprolium upon the influx of thiamine into the brain was measured. A steady level of amprolium was maintained in the blood plasma, starting 2 min before and continuing throughout the period (less than 1 min) needed to measure the thiamine flux. The amprolium level was progressively increased in successive experiments from zero to approximately 0·5 mM. The influx of thiamine into the brain fell sharply at first as the amprolium concentration was raised up to about 50 μM and then somewhat less rapidly to little more than 10 % of the rate in the absence of amprolium. The effect upon influx into the cerebellum is shown (Fig. 3) but substantially similar degrees of inhibition were seen for the influx of thiamine across the blood–cerebral

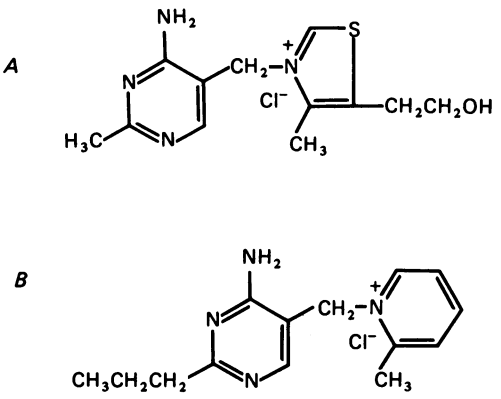

Fig. 1. The molecular structures *A*, of thiamine and *B*, of its close chemical analogue amprolium.

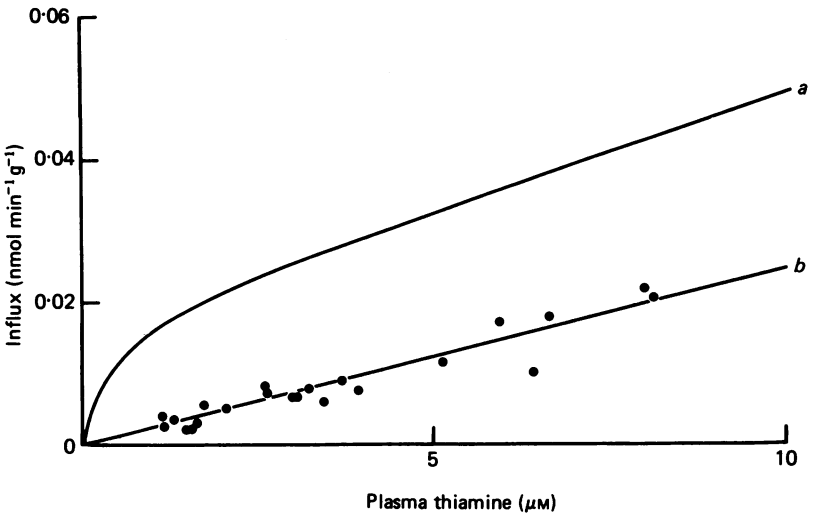

Fig. 2. To show the relation between the influx of thiamine across the blood–brain barrier and its concentration in the blood plasma *a*, with no inhibitor present; curve obtained by fitting eqn. (2) to the data of Greenwood *et al.* (1982) and *b*, in the presence of a concentration of at least 0·4 mM of amprolium in the blood plasma. The best straight line has been fitted to the data points by linear regression of influx upon thiamine concentration.

barrier in other brain regions. The inhibitory effect of amprolium upon thiamine flux across the barrier could be overcome if the concentration of thiamine in the blood plasma was also raised sufficiently (by injecting [^{14}C]thiamine of suitably low specific activity). Thus in the presence of 6 μM of amprolium the influx of thiamine into the brain was reduced to some 30 % of the normal (Fig. 3). In two further experiments, in which this inhibitory level of amprolium was present, when the concentration of free thiamine in the blood plasma was also raised about four times from approximately 1 to 3·1 or 4·5 μM, the influx was doubled, reaching approximately 60 % of the normal value.

An equation was derived in previous work for the relation between the transport of thiamine across the blood–brain barrier and the concentration of free thiamine in the blood plasma:

$$J = \frac{J_{max} C_p}{C_p + K_t} + D'C_p, \tag{2}$$

where J_{max} and K_t represent the kinetic parameters of transport, maximum influx and Michaelis constant, respectively and D' is an apparent transfer constant for

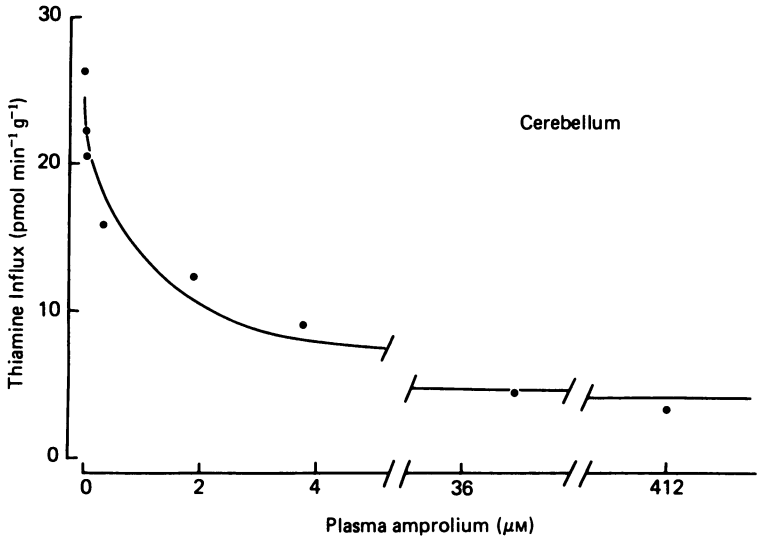

Fig. 3. The reduction in influx of thiamine across the blood–brain barrier of the cerebellum produced by amprolium. In successive experiments in which the concentration of thiamine in the blood plasms was approximately 1 μM, the concentration of amprolium in the blood plasma was raised progressively from zero to over 0·4 mM. A curve has been fitted to the points using eqn. (3) and the method of maximum likelihood (Bard, 1974).

diffusion. The effect of amprolium was to increase the value of the denominator in eqn. (2) as follows:

$$J = \frac{J_{max} C_p}{C_p + K_t(1 + i/K_i)} + D'C_p, \tag{3}$$

where, i is the concentration of the inhibitor, amprolium, in the blood plasma and, K_i is its affinity for the carrier. The data were fitted reasonably well by eqn. (3) by the method of maximum likelihood (Bard, 1974) giving an estimate of the value of K_i for amprolium of $0{\cdot}28 \pm 0{\cdot}01$ μM.

DISCUSSION

Our results provide support for the existence of two separate components in the transport of thiamine across the blood–brain barrier: a carrier-mediated saturable process and a non-saturable process, probably passive diffusion (Greenwood *et al.*

1982). It seems clear that only the carrier-mediated process is susceptible to inhibition by the chemical analogue of thiamine, amprolium, and that, in the presence of a sufficiently high concentration of the inhibitor, thiamine can only be transported across the blood–brain barrier to any appreciable extent by the non-saturable process not apparently affected by the inhibitor.

That this is so is confirmed by consideration of the kinetics. The straight line relating the influx, in the presence of a high concentration of the inhibitor, to the thiamine concentration in the blood plasma (Fig. 2) corresponds fairly closely with the line representing the non-saturable component of thiamine flux in the absence of inhibitor (Greenwood *et al.* 1982). The slopes of these lines, which are $3{\cdot}3 \pm 0{\cdot}2$ and $2{\cdot}4 \pm 0{\cdot}1$ μl min^{-1} g^{-1} respectively, measure the apparent transfer constant for diffusion, D' in eqn. (2), which has thus been estimated in two different ways. In previous work (Greenwood *et al.* 1982) an estimate was obtained by kinetic analysis by fitting eqn. (2) to data relating thiamine influx across the blood–brain barrier with the blood concentration of the vitamin. A further estimate has now been obtained by measuring the influx in the presence of a high concentration of the inhibitor amprolium (Fig. 2) so that the large value of i makes the first term in eqn. (3) negligibly small. It seems likely that these are two estimates of the same transport process which is subject neither to saturation nor to competitive inhibition and therefore, very probably, is passive diffusion with a mean apparent transfer constant of $2{\cdot}85$ μl min^{-1} g^{-1}.

That the inhibition by amprolium, of the saturable, carrier-mediated transport of thiamine across the blood–brain barrier, is competitive in character is suggested by the way in which the inhibitory effect of a particular concentration of amprolium in the blood plasma can be overcome if the level of the substrate, thiamine, is also raised sufficiently above normal. The effect of increasing the concentration of amprolium in the blood plasma (Fig. 3) seems to be to inhibit competitively, by raising the apparent value of K_t which is increased by the factor $(1 + i/K_i)$, eqn. (3). This effect corresponds closely with competitive inhibition between chemically related amino acids sharing a common carrier for their transport across the blood–brain barrier (Pratt, 1979). That amprolium competes with thiamine for a site on a transport carrier seems reasonable in view of the close chemical similarity between the two substances (Fig. 1). Competitive inhibition between the two substances for a transport carrier has been reported across the intestinal border and the cell membranes of micro-organisms (Rindi & Ventura, 1972; James, 1980).

Amprolium is used commercially in chicken rearing as a routine addition to the diet to inhibit coccal infections (Ryley & Betts, 1973). Although there do not appear to have been any reports of adverse effects arising from accidental human ingestion of amprolium, the possibility exists that amprolium or a similar chemical analogue of thiamine, if ingested or formed by some means in the body and present in high enough concentration in the blood over a long enough period, might adversely affect the transport of the vitamin across the blood–brain barrier. There is evidence that such a condition occurs in cattle by thiaminase in the rumen splitting dietary thiamine at the methylene bridge, allowing the pyrimidine moiety to link up with an organic amine to form an amprolium-like analogue of the vitamin, which, it has been suggested (Edwin & Jackman, 1973), may cause or potentiate cerebrocortical

necrosis, a naturally occurring thiamine-deficiency disease of cattle. Evidence in support of this hypothesis was obtained experimentally by showing that amprolium given to calves produced, in either pre-ruminant (Markson, Lewis, Terlecki, Edwin & Ford, 1972) or ruminant (Markson, Edwin, Lewis & Richardson, 1974) animals, a condition closely resembling cerebrocortical necrosis, even though in the weaned ruminant animals amounts of the vitamin normally adequate for nutrition are synthesised in the rumen. The low brain thiamine levels in the calves in which the disease was induced by giving amprolium (Markson *et al.* 1974) suggest that the analogue was not only interferring with absorption of the vitamin from the food but also inhibiting its transport across the blood–brain barrier. Consistent with our finding of the competitive nature of the inhibition of thiamine transport across the blood–brain barrier, is the stress laid by Markson *et al.* (1972, 1974) on the importance of the amprolium to thiamine ratio as a factor in the development of cerebrocortical necrosis.

If amprolium, present in the blood, caused transport inhibition at the blood–brain barrier and thus produced a thiamine deficiency selectively affecting the central nervous system, a moderately large dose of the vitamin, which raised the blood level considerably above normal, would almost certainly suffice to overcome this potentially harmful transport inhibition. Thus our work explains why, in the calf experiments of Markson *et al.* (1972, 1974), the development of the disease due to thiamine deficiency in the central nervous system could be prevented by giving extra thiamine together with the amprolium. Conversely, harmful effects are more likely to occur when the blood thiamine level is below normal, for example, in the malnourished, chronic, alcoholic patient, in whom there is commonly interference with alimentary absorption of the vitamin (Thomson, Baker & Leevy, 1970). An increase in the blood concentration of thiamine raises its flux into the brain by two separate effects: by overcoming the competitive inhibition and by increasing the rate of entry by the non-saturable component of the transport. This conclusion may have a bearing upon the nutritional treatment of various conditions, including chronic alcoholism (Thomson, 1982) and Leigh's disease (Pincus, Solitaire & Cooper, 1976).

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