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A KINETIC MODEL FOR THE ACTION OF THE ENZYME ACTIVATED IRREVERSIBLE INHIBITOR 4-AMINO-5-HEXYNOIC ACID *IN VIVO*

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Abstract—The extent of inactivation of three aminotransferases by the enzyme activated inhibitor 4-amino-hex-5-ynoate (acetylenic-GABA) increased with increasing dose in an exponential fashion. Theoretical treatment of the data allowed an estimate of the effective concentration of the drug at its site of action to be made and it was apparent that any rises in substrate concentration produced by the inactivation did not protect the enzyme significantly. Altered diet produced distinct changes in the extent of inactivation of aspartate aminotransferase, but not with ornithine aminotransferase. Cysteine sulphinate, a substrate only of aspartate aminotransferase, also affected the inactivation of ornithine aminotransferase, suggesting that secondary metabolic effects were responsible.

Where inhibition of a particular target enzyme is considered to be therapeutically valuable the synthesis of an appropriate enzyme activated irreversible inhibitor ("suicide" inhibitor) can provide an elegant and promising approach to chemotherapy (for review see [1, 2]). This type of inhibitory mechanism is attractive both because it is potentially highly selective and because enzyme activity should return only as new enzyme is synthesized. The duration of the effect produced by the administration of such a drug might be expected to depend primarily on the rate of replacement of the inactive target enzyme by newly synthesized enzyme protein.

When compared with the covalent steps of normal enzyme catalysis, the irreversible step in this type of inactivation mechanism is often very slow. For example, saturating concentrations of the compound used in the present study, acetylenic-GABA (4-aminohex-5-ynoate), inactivate the intended target enzyme, GABA-transaminase (4-aminobutyrate: 2-oxoglutarate-aminotransferase, EC 2.6.1.19), with a half time of 3 min [3] whereas the enzyme's half reaction with GABA itself has a half time of 0.1 sec. If the rate of inactivation is very much greater than the rate at which the free compound is removed from the site of reaction then inactivation will be almost complete. Conversely the extent of inactivation will be insignificant if the compound is removed much faster than it reacts. Where the two rates are comparable the extent of inactivation achieved would be expected to be a function of both rates, and because the rate of inactivation varies with inhibitor concentration, the extent to which the target enzyme is permanently inactivated *in vivo*, should also be a function of the dose administered.

The rate of inactivation by such an inhibitor is proportional to the fraction of enzyme that is present in a susceptible form. Thus, as with a competitive inhibitor, the effectiveness of the inhibitor *in vivo* will be diminished by increased substrate concentration. This is particularly important when a target enzyme provides the major route for catabolism of its substrate because a significant rise in the steady state concentration of the substrate will automatically follow inhibition of the enzyme. However, the extent of such protection depends on competing kinetic effects. If the increase in substrate concentration occurs relatively slowly it may not protect the enzyme from the first administration of the drug.

This paper describes experiments intended to investigate these effects.

MATERIALS AND METHODS

Acetylenic-GABA was the gift of Dr. Michel Jung, of the Merrel International Research Centre, Strasbourg, France. Cysteine sulphinate and *N*-(2-hydroxyethyl) piperazine-*N*′-3-propane sulphonic acid (EPPS) were purchased from Sigma (Poole, Dorset, U.K.). Other reagents were from BDH (Poole, Dorset, U.K.). Rats were maintained either on a normal diet (Small Animal Diet, Pilsbury Ltd., Birmingham, U.K.) and were necessary were transferred to a high protein diet of textured vegetable protein (Temptein Meat Like Chunks, Brooke Bond, Croydon, U.K.) five days before the start of an experiment.

Enzyme assays. Ornithine aminotransferase (L-ornithine: 2-oxoacid aminotransferase, EC 2.6.1.13) activity in tissue homogenates was assayed by the method of Peraino *et al.* [4] in which the product pyrolline-5-carboxylate is quantified colourimetrically from its reaction with 2-aminobenzaldehyde.

Aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) activity was determined with the method of Karmen [5] using the conditions described by Martinez-Carrion *et al.* [6].

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Cytoplasmic aspartate aminotransferase activity was measured in tissue homogenates after a 10-fold dilution in 0.15 M succinate buffer, pH 6.0 containing 5 mM EDTA and 1 mM 2-oxoglutarate, and incubation at 70° for 2 min.

GABA-transaminase activity was measured in 0.1 M borate buffer, ph 8.4 containing 12.5 mM 2-oxoglutarate and 25 mM GABA at 37°. After 20 min incubation the reaction was stopped by the addition of 20% (w/v) trichloroacetic acid, and the concentration of succinic semialdehyde produced was determined using 3,4-diaminobenzoic acid [8].

Enzyme units are expressed as μmoles product per min except for GABA-transaminase where the units are arbitrarily defined using a standard fluorescein solution (3 μM). One unit is defined as that amount of enzyme that produces in 1 min an amount of product that after complete reaction with diaminobenzoate gives a fluorescence measurement equivalent to 1 nmol of fluorescein.

Analysis of data. Data were analysed by least squares linear regression analysis of the logarithms of enzyme activity. When, with aspartate aminotransferase at the highest doses, more than 10% of the normal enzyme activity remained, so that the limiting value at infinite dose was not clear, a best fit was obtained using a computer program that repeatedly altered an estimate of the extent of inactivation at infinite dose until a maximum value of the correlation coefficient was achieved.

Values of constants from *in vitro* kinetic experiments are given together with associated standard deviations. Values of constants from experiments involving measurements in animals are given together with standard deviations and standard errors. Standard errors of constants derived from combination of several other constants were assessed assuming no covariance between the individual constants.

Preparation of crude liver cytoplasmic aspartate aminotransferase. Rat livers were homogenized in 4 vol. (w/v) 0.15 M EPPS, pH 8.0 containing 0.15 M KCl. One volume of the homogenate was added to 9 vol. of 0.15 M succinate buffer, pH 6.0, containing 5 mM EDTA and 1 mM 2-oxoglutarate. The diluted material was incubated at 70° for 2 min, rapidly cooled and then centrifuged. The supernatant was passed through a small Sephadex G-25 column equilibrated in 0.1 M EPPS, pH 8.0. The material eluting at the void volume of the column was used in subsequent experiments.

THEORY

The inhibition mechanism involves initial reversible binding characterized by a dissociation constant K_i and an irreversible covalent step characterized by a rate constant k_i:

$$E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_i} E_i$$

The rate of enzyme inactivation in such a case is directly proportional to $[EI]$ and if the inhibitor concentration $[I]$ greatly exceeds the initial enzyme concentration $[E_o]$ then the rate of inactivation varies hyperbolically with inhibitor concentration as in equation 1 [7]. The term $[E_a]$ is the concentration of enzyme not yet irreversibly inactivated. This treatment assumes that insignificant amounts of enzyme are protected by combination with substrate although the presence of significant but constant concentrations of substrate will not affect the form of the equation but simply raise the apparent value of K_i.

$$-\frac{d[E_a]}{dt} = \frac{k_i[E_a][I]}{K_i + [I]} \qquad (1)$$

In the simplifying case where $[I] \ll K_i$ equation 1 reduces to equation 2.

$$-\frac{d[E_a]}{dt} = \frac{k_i}{K_i}[E_a][I] \qquad (2)$$

Amounts of inhibitor present in the liver fall by what appears to be a simple exponential process [9]. Assuming that the rise time after injection is negligibly short and that the concentration of inhibitor that the enzyme experiences $[I]$ falls at the same rate as does the amount present in whole liver, then $[I]$ is given by equation (3):

$$[I] = [I_o]\exp(-kt) \qquad (3)$$

$[I_o]$ is the initial concentration of the inhibitor in liver and k is the rate constant governing the exponential fall.

Substitution and integration gives equation (4):

$$\log_e \frac{[E_a]}{[E_o]} = \frac{k_i[I_o]}{kK_i}\left\{\exp(-kt) - 1\right\} \qquad (4)$$

This equation predicts that as time increases the extent of enzyme inactivation will approach a limiting value which may be predicted by setting t in equation (4) at infinity to give equation (5):

$$\log_e [E_a] = \log_e [E_o] - \frac{k_i}{kK_i}[I_o] \qquad (5)$$

If a linear relationship exists between $[I_o]$ and the injected dose D, such that $[I_o] = \Phi D$ then the amount of enzyme remaining should fall exponentially as the dose increases (equation 6):

$$[E_a] = [E_o]\exp\left(-\frac{k_i}{kK_i}\Phi D\right) \qquad (6)$$

If the constants k, k_i and K_i are known from separate experiments then the constant relating dose (mg/kg) to concentration (μM) achieved in the environment of the enzyme may be determined by best fitting data to equation 6.

RESULTS

Ornithine aminotransferase

The amount of this enzyme is increased in liver approximately fourfold by feeding the animals on a high protein diet [9]. Figure 1 shows the effects of increasing dose of acetylenic-GABA on ornithine aminotransferase in rats on either high protein or normal diets. The simple exponential expression of equation 6 provides a reasonable fit to the data for each group of animals suggesting that there is a linear relationship between the administered dose of acetylenic-GABA and the concentration of inhibitor

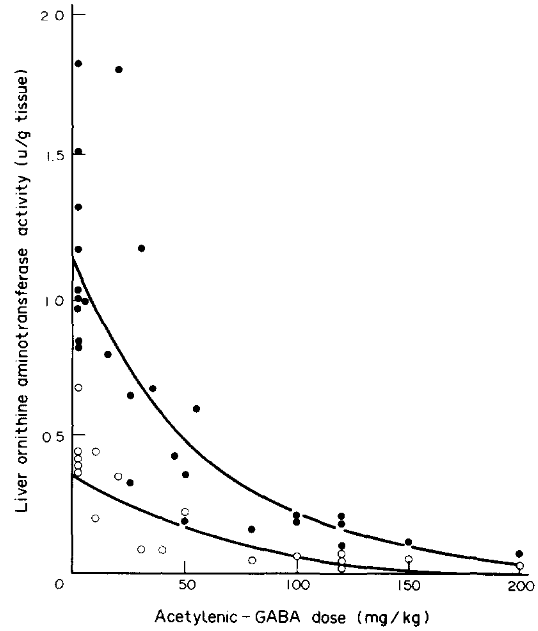

Fig. 1. The inactivation of liver ornithine aminotransferase by acetylenic-GABA *in vivo*. Acetylenic-GABA dissolved in water was administered to rats maintained on either a high protein diet (solid symbols) or the normal diet (open symbols) by intraperitoneal injection. One hour later the animals were killed and their livers were removed. The livers were homogenized in 4 vol. (w/v) of 0.1 M EPPS, pH 8.0, containing 0.15 M KCl. The homogenates were assayed for ornithine aminotransferase activity as described in the text. The solid lines are predicted by equation 6 using values given in the text.

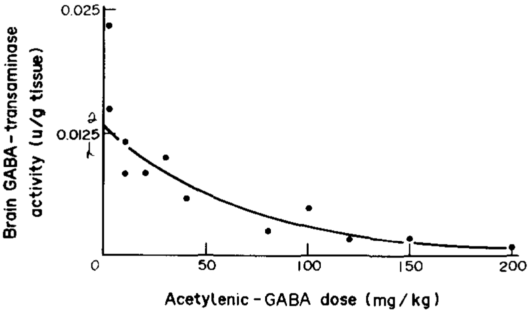

Fig. 2. The inactivation of brain GABA transaminase by acetylenic-GABA *in vivo*. Both the treatment of animals maintained on the high protein diet and the processing of their brains were as described in Fig. 1. The solid line is that predicted by equation 6 using values given in the text.

achieved at the site of action. This simple behaviour also implies that the concentration of inhibitor is not high enough to saturate the enzyme significantly in the preliminary reversible binding step characterized by K_i and that the concentrations of the enzyme's substrates do not rise sufficiently to protect the enzyme. Linear regression analysis of the logarithms of the data of Fig. 1 gave a slope of 0.018 mg^{-1} kg (S.D. = ±0.01 mg^{-1} kg; S.E. = ±0.002 mg^{-1} kg) for animals receiving high protein diet or 0.019 mg^{-1} (S.D. = ±0.016 mg^{-1} kg; S.E. = 0.003 mg^{-1} kg) for animals maintained on the normal diet. These almost identical values show that the difference in diet had no effect on the susceptibility of this enzyme to the drug.

If the model described in the theoretical section of the present paper is correct then this is the value of the expression $\Phi\ k_i/kK_i$ in equation 6. Using values $k_i/K_i = 3.8\ M^{-1}sec^{-1}$ (S.D. = 1.1 $M^{-1}sec^{-1}$) determined independently on the pure enzyme [3] and k = 0.00014 sec^{-1} (S.D. = 0.00004 sec^{-1}; S.E. = 0.00002 sec^{-1}) determined for liver by using radiolabelled acetylenic-GABA [9] the value of Φ was estimated to be 6.7 μM/mg kg (S.D. = 8.2 μM/mg kg; S.E. = 2.6 μM/mg kg). This value indicates that an increase in dose of 1 mg/kg produces an increase in concentration at the site of action of 6.7 μM. At the highest dose given (200 mg/kg) we estimate therefore that the concentration achieved at the enzyme's location in liver mitochondria was 1.2 mM (S.E. = ±0.4 mM).

GABA-aminotransferase

Figure 2 shows how the extent of inactivation of brain GABA-aminotransferase depends on the dose of intraperitoneally administered acetylenic-GABA. The data fit reasonably to the simple exponential expression of equation 6 suggesting that in this case there is also a linear relationship between the administered dose of acetylenic-GABA and the concentration of inhibitor achieved at the site of action. Linear regression of the logarithms of the data of Fig. 2 gave a slope of 0.017 mg/kg (S.E. = ±0.0018 mg/kg; S.D. = ±0.009 mg/kg) but we could not estimate the value of the constant Φ because a good estimate of the rate of removal of acetylenic-GABA from brain was not available.

GABA-aminotransferase is also present in liver and is inactivated by acetylenic-GABA *in vivo*. The same dose range as in Fig. 2 was used but enzyme inactivation was so nearly complete at all doses that a fit to equation 6 was not possible.

Aspartate aminotransferase

This enzyme also increases when rats are transferred from a normal to a high protein diet, an observation which we have not seen reported previously. The effects of acetylenic-GABA on groups of animals fed different diets are shown in Fig. 3. Rats fed a high protein diet have approximately three times as much cytoplasmic aspartate aminotransferase as rats on normal diets and the enzyme is clearly susceptible to inactivation by the inhibitor. Analysis of the data gave a slope of 0.0075 mg^{-1} kg (S.E. = ±0.0017 mg^{-1} kg; S.D. = 0.006 mg^{-1} kg). Although, because of the scatter of the data, this value is less certain than those for the other enzymes, it is clear that as aspartate aminotransferase is extensively inactivated despite the fact that *in vitro* the enzyme is much less sensitive to acetylenic-GABA than are the other two enzymes.

In separate experiments *in vitro* (Fig. 4) we have found that the inactivation of rat liver aspartate aminotransferase is characterized by k_i/K_i = 0.14 $M^{-1}s^{-1}$ (S.E. = 0.006 $M^{-1}s^{-1}$; S.D. = 0.017 $M^{-1}s^{-1}$)

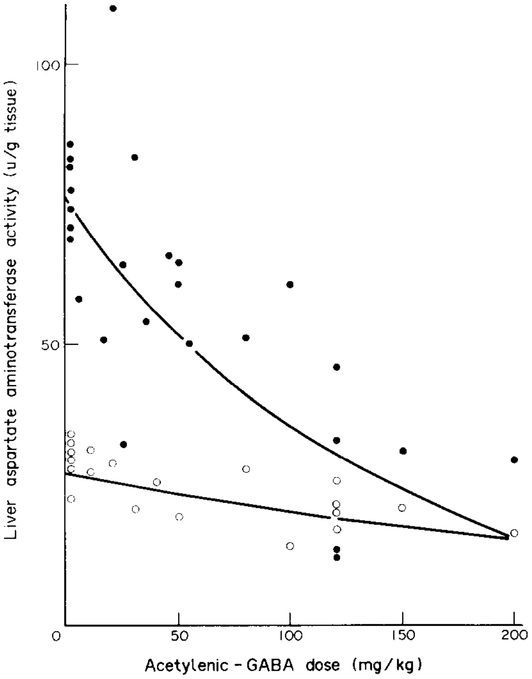

Fig. 3. The inactivation of liver cytoplasmic aspartate aminotransferase by acetylenic-GABA *in vivo*. Animals maintained of either a high protein diet (solid symbols) or the normal diet (open symbols) were given acetylenic-GABA and their livers processed as described in the legend to Fig. 1. Cytoplasmic aspartate aminotransferase activity was measured in the liver homogenates as described in the text. The solid lines are those predicted by equation 6 using the values given in the text.

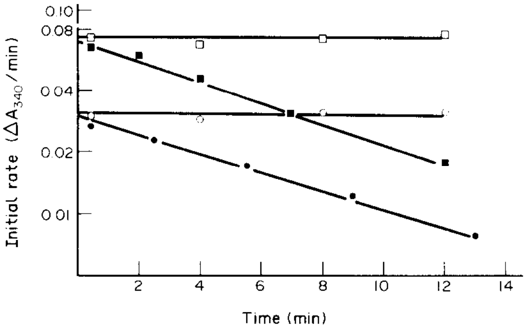

Fig. 5. The time course of the inactivation of liver cytoplasmic aspartate aminotransferase. Rats were maintained on either a high protein diet (square symbols) or on the normal diet (circles). Partially purified enzyme from the two groups of animals was inactivated with acetylenic-GABA (25 mM) as described in the legend to Fig. 4. Incubations were also carried out in the absence of inhibitor (open symbols).

and using the method described earlier we determine that in order to achieve the extent of inactivation observed, cytoplasmic aspartate aminotransferase must be exposed to a much higher concentration of acetylenic-GABA than the mitochondrial ornithine aminotransferase. Using the same rate constant for removal of the compound from liver, assuming that flux between the two compartments of the liver cell is rapid compared with the loss of the compound from the liver as a whole, we calculate a value of Φ of $7.5\ \mu M\ mg^{-1}\ kg$ (S.E. = $\pm 3.2\ \mu M\ mg^{-1}\ kg$; S.D. = $\pm 10.1\ \mu M\ mg^{-1}\ kg$). This value indicates that at the maximum dose used the effective concentration achieved in liver cytoplasm was 1.5 mM (S.E. = ±0.7 mM).

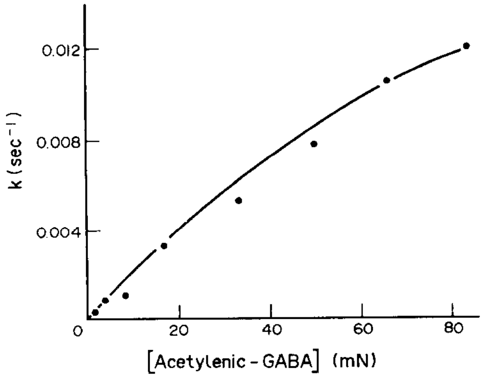

Fig. 4. The inactivation of liver cytoplasmic aspartate aminotransferase by acetylenic-GABA *in vitro*. Crude enzyme (100 μl) was incubated at 37° in 0.1 M EPPS pH 8.0.

The change in diet altered the susceptibility of this enzyme to inactivation by acetylenic-GABA *in vivo*, since the slope of the line in Fig. 3 for animals fed the normal diet was found to be $0.0030\ mg^{-1}\ kg$ (S.D. = $0.00069\ mg^{-1}\ kg$; S.E. = $0.002\ mg^{-1}\ kg$). This difference in susceptibility was not due to a difference in the reactivity of the aspartate aminotransferase in the two groups of animals, because enzyme from both groups was inactivated *in vitro* at similar rates (Fig. 5).

Reduction of the effectiveness of acetylenic-GABA by cysteine sulphinate

Cysteine sulphinate is a substrate for aspartate aminotransferase, producing pyruvate, sulphite and the pyridoxamine form of the enzyme after reaction. Administration of this compound along with acetylenic-GABA might therefore be expected to protect aspartate aminotransferase against inactivation by the inhibitor, since the pyridoxamine form of the enzyme would be unable to react. We hoped that the protection would be selective because cysteine sulphinate is not a substrate for ornithine aminotransferase.

Accordingly, rats were given either acetylenic-GABA alone or acetylenic-GABA and cysteine sulphinate together (Table 1). In animals fed a normal diet there was little difference in the cytoplasmic aspartate aminotransferase activity measured in the two groups. No protection of ornithine aminotransferase was seen in these animals. By contrast, cysteine sulphinate protected both aspartate aminotransferase and ornithine aminotransferase in rats fed a high protein diet. The protection of ornithine

Table 1. Effects of cysteine sulphinate

	Ornithine aminotransferase (U/g tissue)		Aspartate aminotransferase (U/g tissue)	
Treatment	Normal	High protein	Normal	High protein
Saline control	0.39 (100%)	1.14 (100%)	31.1 (100%)	77.1 (100%)
Acetylenic-GABA	0.036 (9.1%)	0.18 (15.0%)	19.5 (61.0%)	26.3 (34.1%)
Acetylenic-GABA and cysteine sulphinate	0.027 (6.8%)	0.59 (52%)	19.6 (61.1%)	44.8 (59.0%)

Animals were given either acetylenic-GABA (120 mg/kg body weight) or acetylenic-GABA and cysteine sulphinate (both 120 mg/kg body weight) and processed as described in the legend to Fig. 1. The liver homogenates were assayed for the two enzymes using methods described in the text.

aminotransferase was unexpected, because cysteine sulphinate is not a substrate for this enzyme. The reason for this protection is unclear but the indirect protection of ornithine transaminase must be due to secondary metabolic effects, possibly mediated through changes in the glutamate/ketoglutarate ratio.

DISCUSSION

The simple exponential approach to zero enzyme activity observed with all three enzymes with increasing dose of acetylenic-GABA indicates that any rise in substrate concentration brought about by the inactivation is either too slow or too slight to afford protection to any of the enzymes. Ornithine and GABA concentrations rise considerably when the respective enzymes are inactivated with acetylenic-GABA *in vivo* [11, 12]. The rise must lag sufficiently behind inactivation such that it does not interfere with the inactivation. In this situation a single high dose would be expected to be more effective than multiple smaller doses repeated at intervals.

The constant Φ, that relates drug dose to the extent of enzyme inactivation may be used predictively, but the level of confidence that can be placed on the prediction deserves consideration. The statistical analysis shows that, despite the large variability in the enzyme activity initially present in individual animals, the constant itself is subject to much less variation. Thus, a prediction of how much enzyme activity will remain in a particular animal after treatment with the drug will be very uncertain. However, as the main source of this uncertainty is the variability in the amounts of enzyme present initially in the animal population, rather than any variability in the constant, a much more accurate prediction of the fractional inactivation of the enzyme present originally in an individual animal can be made.

Metabolic factors cannot be ignored as modifiers of these effects. Feeding rats a high protein diet changed both the susceptibility of aspartate aminotransferase to acetylenic-GABA inactivation, and the extent of the protection seen with cysteine sulphinate. One possible explanation of these effects is that the high protein diet elevates the tissue concentrations of keto acids, altering the pyridoxamine–pyridoxaldimine equilibria in favour of the aldimine forms of the various transaminases. These changes would favour the increased reaction of aspartate aminotransferase with acetylenic-GABA.

The protection of ornithine aminotransferase by cysteine sulphinate was unexpected, as this compound is not a substrate for this enzyme. The protection may also be explained by elevated tissue levels of keto acids. The cysteine sulphinate would be rapidly transaminated by apartate aminotransferase, and 2-oxoglutarate quickly converted to glutamate. This transient increase in glutamate concentration is sufficient to protect both enzymes, via their pyridoxamine forms, against reaction with acetylenic-GABA.

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