Biochem. J. (1983) **209**, 243–249
Printed in Great Britain

An investigation of the properties of ornithine aminotransferase after inactivation by the 'suicide' inhibitor aminohexynoate and use of the compound as a probe of intracellular protein turnover

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(*Received 17 August 1982/Accepted 11 October 1982*)

Ornithine aminotransferase is shown to bind 1 mol of amino[^{14}C]hexynoate per mol of coenzyme in the 'suicide' inactivation process. At the same time the coenzyme pyridoxal phosphate becomes irreversibly bound to the enzyme protein. Apart from the inactivation, the labelled enzyme is indistinguishable from native ornithine aminotransferase by several separation techniques. Because the rate of degradation of the labelled enzyme is the same as that of the normal enzyme it is concluded that loss of coenzyme does not initiate turnover. Free aminohexynoate is rapidly eliminated from the liver, and 70% of the compound is excreted unchanged in 7.5 h. Inactivated ornithine aminotransferase accounts for 11% of the total labelled liver protein and significant amounts of label are found in aspartate aminotransferase which is also extensively inactivated. The rate of return of enzyme activity is determined and found to be more rapid than expected for a process in which the enzyme is synthesized at a constant rate and degraded in a single, first-order process.

Aminohexynoate, an acetylenic derivative of 4-aminobutyrate, was first synthesized as a 'suicide' inhibitor of 4-aminobutyrate aminotransferase (EC 2.6.1.19; Jung & Metcalf, 1975). Although it does inactivate that enzyme it is slightly more potent towards ornithine aminotransferase (EC 2.6.1.13; Lippert *et al.*, 1977; Jung & Seiler, 1978; John *et al.*, 1979). When the compound is administered to animals, ornithine metabolism is very greatly reduced indicating that the enzyme is extensively inhibited *in vivo* (Jung & Seiler, 1978). The mechanism suggested for the enzyme's inactivation (Jung & Metcalf, 1975; Metcalf *et al.*, 1978) results in covalent binding of the inhibitor both to a nucleophile of the enzyme protein and to the coenzyme pyridoxal phosphate (Fig. 1). Treatment with amino[^{14}C]hexynoate should therefore result in irreversible radioactive labelling of the enzyme and at the same time prevent the coenzyme from being released. Thus the radiolabelled compound might be useful for investigating the process of intracellular degradation of the enzyme by providing a relatively specific means of labelling the ornithine aminotransferase holoenzyme *in vivo*.

Although there are indications that aminohexynoate is much less reactive towards several pyridoxal phosphate-dependent enzymes than towards ornithine aminotransferase (Lippert *et al.*, 1977; Metcalf *et al.*, 1978; John *et al.*, 1979) information on the inhibitor is necessarily restricted to those enzymes investigated. Thus an initial objective was to determine how many liver proteins are labelled by the compound, particularly because, if bound radioactivity was almost exclusively associated with ornithine aminotransferase, aminohexynoate should

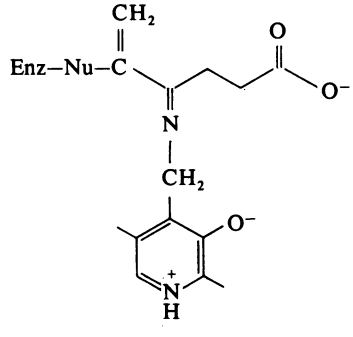

Fig. 1. *Structure proposed for the compound formed between aminohexynoate and ornithine aminotransferase*
Substituents in positions 2 and 5 of the coenzyme ring have been omitted for clarity.

0306-3275/83/010243-07$02.00

provide a useful means for investigating steps in the intracellular degradation of this enzyme.

One hypothesis put forward to explain the widely differing rates of turnover of intracellular proteins is that loss of coenzyme provides the initial step leading to degradation. In support of this hypothesis there is evidence that those enzymes that bind coenzyme most weakly are the most rapidly degraded (Litwack & Rosenfeld, 1973). If inactivation by aminohexynoate does prevent release of coenzyme the hypothesis may be tested directly.

The present paper describes investigations into the stoichiometry of the reaction between ornithine aminotransferase and aminohexynoate and into the extent to which both the compound itself and the coenzyme may be considered permanently bound to the inactive enzyme. The specificity of the compound for ornithine aminotransferase *in vivo* is examined directly and the kinetics of degradation of the labelled inactive enzyme and the return of enzyme activity after inactivation are studied.

Experimental

Animals

MRC hooded rats aged 8–16 weeks were fed on a high protein diet, namely the textured soya bean protein 'Temptein Meat-Like Chunks' (Brooke Bond Oxo, Croydon, Surrey, U.K.), for 5 days before beginning any experiment and were maintained on this diet thereafter.

Materials

DL-Amino[2,3-^{14}C]hex-5-ynoic acid (5.2 Ci/mol) and [G-^{3}H]pyridoxine (2.5 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled aminohexynoate was generously given by Dr. Michel Jung of Merrell International Research Centre, Strasbourg, France. Pyridoxal 5'-phosphate and 2-oxoglutaric acid were from Koch-Light, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) was from Hopkin and Williams and 4-(2-hydroxyethyl)-1-piperazine-propanesulphonic acid (Hepps) was from Sigma. Ion-exchange celluloses were from Whatman, Fisofluor 1 was from Fisons and other reagents were from BDH.

[G-^{3}H]Pyridoxal phosphate (77 mCi/mol) was synthesized from [G-^{3}H]pyridoxine according to a method described for pyridoxal phosphate derivatives (see Florentiev *et al.*, 1970, method 4, p. 573 and method 8A, p. 578). Several methods are described and of these we chose the one in which [^{3}H]pyridoxine (2.5 Ci/mmol) was oxidized to the aldehyde with MnO_2. Pyridoxal was isolated as a Schiff base with *p*-phenetidine. This derivative was phosphorylated with polyphosphoric acid for 6 h at 50°C and the [^{3}H]pyridoxal phosphate was isolated after chromatography on Dowex 50 (X4). This preparation gave a single spot on t.l.c. using two different solvent systems.

Ornithine aminotransferase was prepared by the method of Peraino *et al.* (1969) but incorporating the modifications of Williams *et al.* (1982).

Enzyme assays

Ornithine aminotransferase active site concentration was determined by measuring the absorbance of bound coenzyme at 415 nm (Williams *et al.*, 1982). Ornithine aminotransferase activity in crude samples was assayed by the method described by Peraino *et al.* (1969) in which the product pyrroline-5-carboxylate is quantified colorimetrically from its colour reaction with 2-aminobenzaldehyde ($\varepsilon_{440} = 2.71 \cdot mmol^{-1} \cdot cm^{-1}$; Strecker, 1965). One unit is that amount producing 1 μmol of product per min. Amounts of enzyme in crude samples were converted into nmol from the observation that an absorbance change of $0.73\,min^{-1} \cdot nmol^{-1}$ occurred when the pure enzyme was used in the standard assay. This corresponds to a turnover number under assay conditions of $9\,s^{-1}$ and to a specific activity of 0.54 units/nmol.

Aspartate aminotransferase activity was determined by the method of Karmen (1955) using conditions described by Martinez-Carrion (1967). In crude samples the cytoplasmic enzyme was assayed after inactivation of the mitochondrial isoenzyme by heating at 65°C for 4 min in 0.15 M-succinate (pH 6) containing 1 mM-EDTA and 1 mM-2-oxoglutarate. Protein concentrations in the eluates from columns were determined by measuring A_{280}. Apoenzyme and holoenzyme protein concentrations were compared with the method of Lowry *et al.* (1951).

For both enzymes units of activity are $\mu mol \cdot min^{-1}$.

Radioactivity measurements

Radioactivity of samples was measured with an Intertechnique SL4000 liquid-scintillation counter. Scintillation vials contained 15 ml of Fisofluor.

Preparation of apoenzyme and reconstitution with radioactive coenzyme

This method is based on that used by Sanada *et al.* (1976). The enzyme (4.5 ml, 61 μM) in Hepps (pH 8.0) was treated with 1 ml of 0.1 M-ornithine and dialysed against 100 ml of 0.1 M-sodium phosphate (pH 6.6) for 5 h. The enzyme was then dialysed against four changes of 100 ml of sodium phosphate (pH 6.6) containing 5 mM-ornithine. Each dialysis step lasted 10–15 h and at the end the apoenzyme retained 3% of its original activity. The apoenzyme was dialysed into 10 mM-Hepps (pH 8.0) and reconstituted by adding 1.2 mg of [^{3}H]pyridoxal phosphate. The resulting holoenzyme was separated from excess pyridoxal phosphate by repeated cycles

of $(NH_4)_2SO_4$ precipitation and extraction as used in the purification. The second and third cycles gave constant specific radioactivity equal to that of the pyridoxal phosphate used. The specific enzyme activity of the sample was 105% of that of the starting enzyme.

Fractionation of liver proteins

Livers were homogenized to give 30% (w/v) homogenates in 0.1 M-Hepes (pH 8.0) containing 0.15 M-KCl and were sonicated for 12 min at 10 W/g of tissue. The supernatants after centrifugation (1500 *g*-min) were separated on Sephadex G-25 in 10 mM-sodium phosphate (pH 7.4) and the high-molecular-weight fraction was chromatographed on a column of DEAE-cellulose (1.2 cm × 9.8 cm) equilibrated with 10 mM-sodium phosphate (pH 7.4). The column was eluted with a linear gradient from 0 to 0.4 M-KCl dissolved in 10 mM-sodium phosphate (pH 7.4). The columns were run at room temperature and recoveries of enzyme activity were 95%.

Results and discussion

Properties of the inactive enzyme

Ornithine aminotransferase (50 μM) was treated with amino[^{14}C]hexynoate (0.8 mM, 5.2 Ci/mol) in 10 mM-Hepes buffer (pH 8.0) containing 2-oxoglutarate (5 mM) to reverse the small amount of transamination that occurs (John *et al.*, 1979) and ensure complete inactivation. After 16 h at 30°C no enzyme activity was left and the solution was separated on a column of Sephadex G-25 (57 cm × 2.9 cm) in 10 mM-sodium phosphate buffer (pH 8.0). Recovery of enzyme protein was essentially complete and measurement of radioactivity showed a molar ratio of bound label to enzyme of 1.08. The radioactivity labelled protein was rechromatographed on the same column without any loss of radioactivity or restoration of enzyme activity. In a second experiment the ratio was determined to be 1.18. The ^{14}C label remained bound to the protein after repeated cycles of $(NH_4)_2SO_4$ precipitation, DEAE-cellulose column chromatography and heating to 60°C. These results support the proposal, implicit in the mechanism of Jung & Metcalf (1975), that aminohexynoate binds stoichiometrically to a single amino acid residue of the enzyme protein.

Pyridoxal phosphate is tightly bound to ornithine aminotransferase but when the coenzyme is converted to the pyridoxamine form it is readily removed by dialysis (Sanada *et al.*, 1976). The mechanism proposed by Jung & Metcalf (1975) suggests that after inactivation the coenzyme will be covalently bound to the protein through the inactivating compound (Fig. 1). An absorbance spectrum of the inactive enzyme after extensive dialysis showed a distinct peak at 330 nm (Fig. 2),

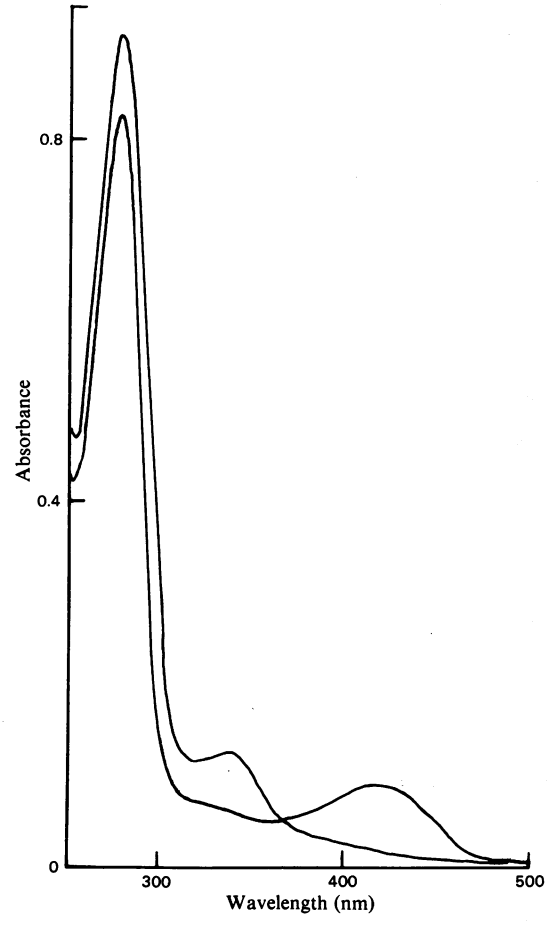

Fig. 2. *Comparison of absorbance spectra of native and aminohexynoate-inactivated ornithine aminotransferase*
Spectra were determined in 10 mM-Hepes, pH 8.0.

showing that at least part of the coenzyme was still bound. However, as the absorbance coefficient of the new chromophore is unknown, we could not estimate the extent to which the coenzyme might have been released. This point was investigated using ornithine aminotransferase prepared by reconstituting the apoenzyme with [^{3}H]pyridoxal phosphate. Ornithine aminotransferase (3 ml, 65 μM in 10 mM-Hepps, pH 8.0) was treated with 10 μl of 0.5 M-2-oxoglutarate. A sample (1.4 ml) was treated with 1 mg of aminohexynoate and left at 30°C for 45 min, by which time the inactivating reaction was complete. This sample, together with an identical sample of untreated enzyme also radioactively labelled in its coenzyme, was subjected to the dialysis procedure used to separate the coenzyme. After repeated changes of dialysis buffer over 3 days the aminohexynoate-treated sample retained 88% of its

radioactivity whereas the untreated enzyme retained only 5% of its radioactivity. We conclude from these observations that the coenzyme becomes much more tightly bound after covalent modification by aminohexynoate. The fact that the inactive labelled enzyme has a single absorbance maximum at 330nm suggests that electron delocalization is limited to the coenzyme ring (Johnson & Metzler, 1970) and is consistent with the structure proposed (Fig. 1).

The mechanism of inactivation involves the covalent incorporation of a small substrate-like molecule at the normal substrate binding site, a modification that seems unlikely to introduce major changes in the structure of the enzyme. This view is to some extent confirmed by the observations that native and amino[^{14}C]hexynoate-treated enzyme chromatograph identically on DEAE-cellulose. Furthermore, when the labelled inactive enzyme was added to sonicated rat liver mitochondria, radioactivity and enzyme activity paralleled each other throughout the standard purification procedure. In addition an experiment was performed in which labelled inactive enzyme and fully active enzyme were allowed to crystallize slowly from 5% (w/v) $(NH_4)_2SO_4$. Samples of both the supernatant and the sedimented crystals were measured for both enzyme activity and radioactivity. These were found to parallel each other throughout the crystallization.

Elimination of amino[^{14}C]hexynoate from rat liver after intraperitoneal injection

To establish the extent to which aminohexynoate may be considered as a 'pulse' label and to examine the possibility that labelling of the enzyme occurs during the preparation we have measured the rate at which the concentration of the compound in the liver falls over 8h (Fig. 3). We estimate the total liver concentration of aminohexynoate to be 35μM 1h after injection, and in separate experiments in which aminohexynoate was added to liver homogenates to give this concentration, we found only 5% loss of enzyme activity in 30min. We conclude that any inactivation and labelling of the enzyme occurring after death of the animal and during preparation is negligible. Examination of the urine from an animal that had been injected with radiolabelled aminohexynoate showed that 70% of the compound was excreted in 7.5h. Paper electrophoresis (4500V for 45min in 1.25M-pyridine/67mM-acetate, pH6.5) showed the excreted compound to have the same mobility as aminohexynoate.

Specificity of binding of amino[^{14}C]hexynoate to rat liver proteins in vivo

The specific radioactivity of the available amino-[^{14}C]hexynoate was too low to allow analysis of labelled proteins by small-scale electrophoretic techniques. Instead, column chromatography of the whole sonicated liver homogenate on DEAE-cellulose was used to fractionate the proteins. It was clear that not all of the ornithine aminotransferase was labelled at a dose of 12.5mg/kg. The amount of enzyme present in liver varies very little from one animal to another and we estimate that this dose inactivated about half of the liver enzyme. When the dose was increased to 25mg/kg about 70% of the ornithine aminotransferase was inactivated and the amount of radioactivity eluting in the relevant position approximately doubled (Fig. 4*a*). Purification of the inactivated ornithine aminotransferase in this peak to homogeneity as assessed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate showed that no significant amounts of any other radiolabelled proteins eluted in this position.

Because labelled and native enzyme parallel each other exactly throughout the purification we were able to allow for losses. From these experiments we calculate that inactivated ornithine aminotransferase accounts for 11% of the total liver protein that is radiolabelled by the compound. The majority of the radioactivity is in the early peak composed of proteins that have no affinity for DEAE-cellulose. In

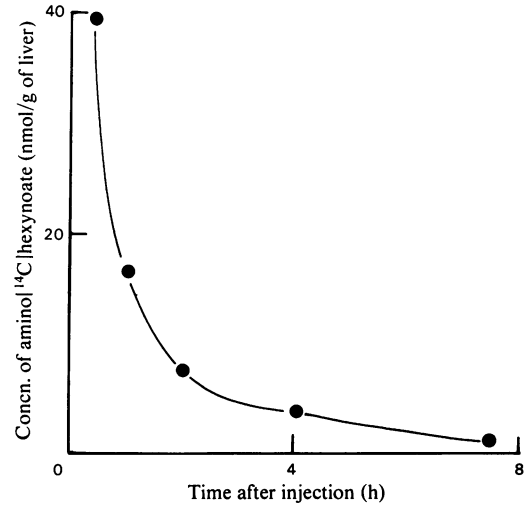

Fig. 3. *Course of elimination of amino[^{14}C]hexynoate from liver*

Animals were injected with 2.5mg of amino[^{14}C]-hexynoate (0.52Ci/mol). Livers were removed at the times indicated and homogenized in 0.1M-Hepes containing 0.15M-KCl, pH8.0. The homogenate was sonicated and the whole preparation was fractionated on Sephadex G-25 in 10mM-Hepes, pH8.0. Free aminohexynoate was determined as radioactivity eluting in the low-molecular-weight fraction.

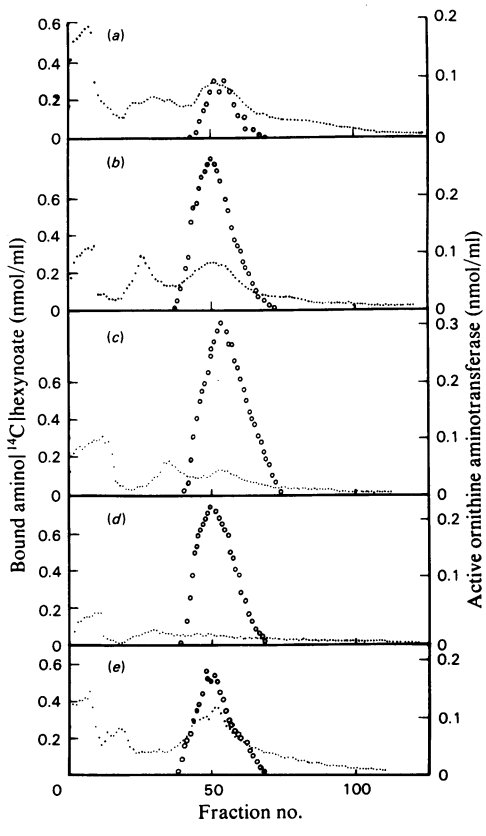

Fig. 4. *Fractionation of proteins labelled by aminohexynoate*

Proteins were fractionated as described in the text. Ornithine aminotransferase activity (O) and radioactivity (····) were measured in each fraction. Fraction volumes were 11 ml for the first nine fractions and 2 ml thereafter. Elutions patterns were obtained from livers removed (*a*) 1 h, (*b*) 1 day, (*c*) 2 days and (*d*) 4 days after injecting 5 mg of amino[^{14}C]hexynoate. The elution pattern (*e*) was obtained from a liver removed 1 h after an injection of amino[^{14}C]hexynoate (5 mg) to an animal treated 1 day earlier with the same quantity of unlabelled inhibitor.

an attempt to separate and identify the labelled components of this fraction it was applied to a column (4.2 cm × 4.8 cm) of CM-cellulose and eluted with 10 mM-sodium acetate adjusted to pH 5.4 with acetic acid and containing 0.5 M-KCl. About 50% of the radiolabel applied to this column eluted with this solution and this eluate was fractionated on a column (2.2 cm × 40 cm) of CM-cellulose using a linear gradient from 0 to 0.5 M-KCl with the pH maintained at 5.4 by 10 mM-sodium acetate that had been adjusted with acetic acid. Several well-separated proteins were eluted, only one of which was radioactive and this coincided with aspartate aminotransferase activity. When a rat liver homogenate, from which the mitochondria had been removed by centrifugation, was fractionated in this way aspartate aminotransferase again was eluted in this position suggesting that this part of the label is bound to the cytoplasmic isoenzyme of aspartate aminotransferase. Furthermore, the enzyme eluting in this position was stable after heating to 70°C for 5 min, confirming that it was the cytoplasmic and not the mitochondrial form of the enzyme (Huynh *et al.*, 1980). We estimate that 5 nmol of liver cytoplasmic aspartate aminotransferase becomes labelled after an injection of aminohexynoate (12.5 mg/kg). In separate experiments at a higher dose level (50 mg/kg) cytoplasmic aspartate aminotransferase was found to be decreased from 14.1 ± 1.6 to 6.2 ± 0.7 units/g of liver. This inactivation of aspartate aminotransferase was unexpected because in experiments *in vitro* (John *et al.*, 1979) the compound has been shown to be only 5% as effective towards this enzyme as towards ornithine aminotransferase. The discrepancy is perhaps due to a higher concentration of the compound in the cytoplasm than in the mitochondria, although other factors such as protection by substrate may play a part. We have not identified the other proteins labelled by aminohexynoate.

It became clear from experiments to be described below that radioactivity in ornithine aminotransferase declined more rapidly than the total protein-bound radioactivity. Such behaviour presumably reflects the relatively rapid turnover of the enzyme and suggested that it may be of use in raising the proportion of label that is incorporated into ornithine aminotransferase. The property was exploited by treating a rat with unlabelled aminohexynoate (25 mg/kg) and then, 24 h later, with the same dose of the radioactive compound. Any extensively inactivated but slowly regenerating enzymes would be much less susceptible to the second treatment but significant amounts of newly synthesized ornithine aminotransferase should be labelled. This strategy produced an increase of 50% in the proportion of total bound label that was present in ornithine aminotransferase (Fig. 4*e*).

The elution patterns from rats killed at intervals after administration of amino[^{14}C]hexynoate (Fig. 4) show enzyme activity increasing as radiolabelled enzyme decreased. It was noticeable that enzyme activity returned faster than expected for a process in which enzyme synthesis remained constant and in which degradation is characterized by a rate constant of 0.4 day^{-1}.

Rate of degradation of the labelled enzyme

The rate of intracellular degradation of the inactivated enzyme was investigated by purifying it

to homogeneity from separate livers removed from rats at intervals after intraperitoneal injection (25 mg/kg) of the amino[^{14}C]hexynoate. To increase the bulk of the inactive enzyme, thereby improving recoveries, 20 nmol of inactivated but unlabelled pure enzyme was added to each preparation. The inevitable losses of about 30% were quantified by measuring losses of the accompanying enzymically active protein which, it had been established, exactly parallels the labelled enzyme throughout the purification procedure. Fig. 5 shows these data, which fit reasonably well to the line predicted by the well established observed degradative rate constant of 0.4 day^{-1} (Chee & Swick, 1976). We conclude therefore that the irreversible covalent binding of the coenzyme to ornithine aminotransferase does not significantly alter the rate of degradation of the enzyme and that loss of coenzyme does not initiate degradation of ornithine aminotransferase.

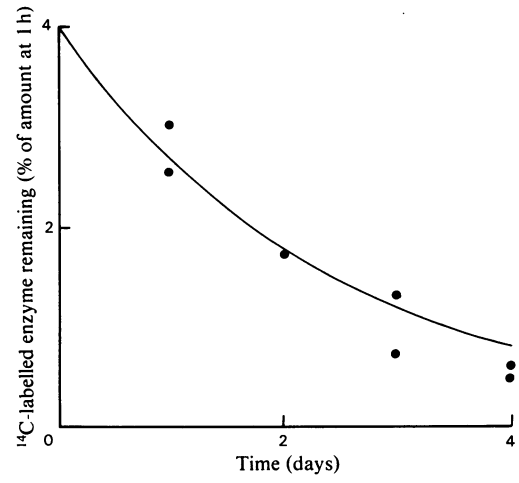

Fig. 5. *Degradation of ^{14}C-labelled ornithine aminotransferase*
The amount of labelled inactive enzyme remaining in the liver was determined as described in the text. The line is that predicted for a first order degradation having a rate constant of 0.4 day^{-1}.

Recovery of enzyme after inactivation

If the enzyme is synthesized at a constant, zero-order rate and degraded in a single first-order step then the course of the rise in activity after inactivation or after induction is governed by the degradation rate constant (Segal & Kim, 1963; Berlin & Schimke, 1965; Swick *et al.*, 1968). Enzyme activity E, is predicted by eqn. (1) in which k represents the degradative rate constant, E_0 is the residual enzyme immediately after inactivation and E_∞ is the steady state level of enzyme activity ($E_\infty = v/k$ were v is the rate of synthesis):

$$E = E_0 + (E_\infty - E_0)(1 - e^{-kt}) \qquad (1)$$

Fig. 6 shows the recovery of enzyme activity during 4 days after the administration of aminohexynoate (50 mg/kg). The broken line is that expected for a process in which the enzyme is synthesized at a constant rate and degraded in a first order process ($k = 0.4$ day^{-1}).

It is clear that the data do not fit this model. The model can only be made to fit if k is taken to be increased to 0.9 day^{-1} and, in order to account for recovery of enzyme activity to normal levels, the rate of synthesis must be simultaneously and proportionally increased. Such coupled increases in synthesis and degradation seem improbable. Furthermore the evidence of Fig. 5 shows that the labelled inactivated enzyme continues to be degraded with a rate constant close to 0.4 day^{-1} in the same period that new enzyme is being synthesized rapidly. Chee & Swick (1976) reported a similar discrepancy in that the rate constant they measured by assuming the simple model and measuring changes in enzyme levels induced by dietary change was significantly higher than that measured by pulse labelling with $NaH^{14}CO_3$. Chee & Swick (1976) also demonstrated a transient fall in the rate of degradation when they carried out pulse-labelling experiments during the period of diet-induced transition. They concluded that the new enzyme level was estab-

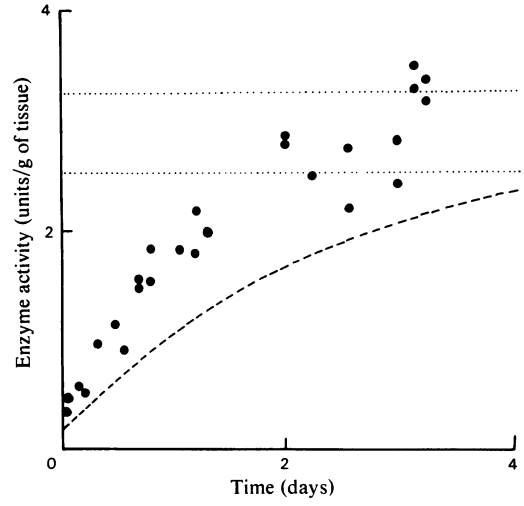

Fig. 6. *Return of enzyme activity after inactivation*
Animals were treated with aminohexynoate (50 mg/kg) and the ornithine aminotransferase activity in their livers was measured at the intervals indicated. The broken line shows the course of recovery of enzyme activity predicted by eqn. (1) when $k = 0.4$ day^{-1} and $E_0 = 0.26$ units/g. The horizontal dotted lines indicate the standard error of the enzyme activity of 19 control rats injected with KCl (50 mg/kg).

lished as a result of an initial, transient decrease in degradation rate constant and maintained by a slowly developing increase in synthesis. If the observation that aminohexynoate-inactivated enzyme continues to be degraded at its normal rate during regeneration may be taken as evidence that the degradative process is unaltered, then the discrepancy reported in the present paper cannot be explained in this way. One possible explaination is that there is a very rapid but transient rise in the rate of synthesis caused by the changed metabolic circumstances arising from the treatment itself.

We thank the Medical Research Council and the Wellcome Trust for financial support, Merrel International Research Centre for their gift of aminohexynoate and Mr. Geoffrey Ward for expert assistance with the animals. E. D. J. thanks the Science Research Council for a Studentship.

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