Inducibility and Some Properties of the Threonine Dehydratase of Sheep Liver

By SHAWN DOONAN, DIONA H. KOERNER,* WOLFGANG SCHMUTZLER†
and CHARLES A. VERNON

*The Christopher Ingold Laboratories, Department of Chemistry, University College London,
20 Gordon Street, London WC1H 0AJ, U.K.*

(Received 25 July 1974)

The threonine dehydratase extracted from sheep liver is in an essentially inactive form, referred to here as the precursor, but can be activated by incubation at high temperature in alkaline solution. A method for purification of the activated enzyme to a state approaching homogeneity has been devised. The activated enzyme catalyses the deamination of serine but rapidly loses activity during the process. The enzyme inactivated by incubation with serine can be reactivated under the same conditions as those described for the precursor, the kinetic parameters being the same in both cases. Individual sheep livers differ widely in their content of threonine dehydratase, and the possible role of dietary factors in this variability has been examined. The average amount of the enzyme in the livers of sheep fed on high-plant-protein diets did not differ significantly from that in livers obtained from slaughterhouses, but was higher than that in livers from animals fed only on grass or normal concentrates. Large increases in enzyme concentration were, however, obtained when sheep were injected intraperitoneally with a protein hydrolysate. The enzyme from the livers of these sheep could be purified by the same technique and to the same specific activity as the enzyme from the livers of control sheep.

Threonine dehydratase (EC 4.2.1.16) catalyses the conversion of L-threonine into α -oxobutyric acid and NH_3 . Two forms of the enzyme are found in micro-organisms (see the review by Wood, 1969). One of these, the biodegradative enzyme, is synthesized only in the absence of glucose and O_2 , and is activated by AMP or ADP. The other, so-called biosynthetic enzyme, catalyses the first step in the biosynthesis of isoleucine and is subject to inhibition by the end product; the enzyme is produced constitutively and is not influenced by adenine nucleotides (Umbarger & Brown, 1957). Both enzymes have been extensively studied and shown to depend for activity on pyridoxal 5'-phosphate; the mechanism of action has been discussed by Phillips & Wood (1965). The enzymes act on L-serine, with the production of pyruvate, but become inactivated towards both serine and threonine during the process (Phillips, 1968).

Threonine dehydratase also occurs in mammalian liver; the enzymes from rat liver (Ghazarian &

Borchers, 1973) and sheep liver (Nishimura & Greenberg, 1961; Russell *et al.*, 1960) have been partially characterized, but are less-well understood than the enzymes of microbial origin. The sheep liver enzyme has been shown to depend for activity on pyridoxal 5'-phosphate (Nishimura & Greenberg, 1961) and undergoes inactivation during deamination of serine (Moss *et al.*, 1965; McLemore & Metzler, 1968). Surprisingly, the enzyme as extracted from sheep liver is in an inactive form (referred to here as the precursor) and can be activated by incubation at elevated temperature in alkaline solution (Russell *et al.*, 1960; Moss *et al.*, 1965). Experiments are reported here which indicate that the activation of the precursor and the reversal of inhibition by serine are similar or identical processes. It should be noted that the use of the term precursor is not necessarily meant to imply that the inactive form is of biological significance.

There have been many reports on the inducibility of threonine dehydratase in rat liver. Sayre *et al.* (1956) reported that the activity of the enzyme was increased between four- and five-fold by intraperitoneal injection of threonine or by perfusion of isolated rat livers with threonine. Considerably greater increases (up to 300-fold) have been claimed in response to high-protein diets and to administration of cortisone (Pitot & Peraino, 1964); the effects were abolished

* Present address: Endocrine Research Laboratory, Division of Endocrinology, Department of Medicine, Montefiore Hospital and Medical Center, Bronx, N.Y. 10467, U.S.A.

† Present address: Abteilung Pharmakologie der Medizinischen Fakultät der Technischen Hochschule, Medizinisch-Theoretische Institute, 51 Aachen, West Germany.

by inhibitors of protein synthesis. These observations led us to consider the possibility that the reported wide variations in amounts of threonine dehydratase in sheep livers obtained from slaughterhouses (Nishimura & Greenberg, 1961; Moss *et al.*, 1965) are due to dietary factors. We report here studies on the amounts of threonine dehydratase in the livers of sheep kept under controlled conditions on a variety of diets, and show that the enzyme concentration is markedly increased under certain conditions. A preliminary account of this work has been published (Schmutzler, 1971).

Experimental

Materials

Sheep livers for routine isolation of threonine dehydratase were obtained from City Meat Wholesalers Ltd., Sewardstone Road, London E.4, U.K.; no information could be obtained about age, sex, breed or diet of the animals. Sheep used for feeding experiments were either females or castrated males of the breeds Dorset Horn, Dorset Down, Suffolk and Clun and weighed between 22 and 36 kg. The animals were kept at the Wellcome Veterinary Research Station, Frant, Tunbridge Wells, Kent, U.K., and were fed either on pasture or on normal concentrates before being subjected to special diets. Glutene, zein and casein were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and Ovigest (7%, w/v, solution of horse muscle hydrolysate) from Wellcome Reagents Ltd., Beckenham, Kent, U.K. All buffer materials and general reagents were from BDH Chemicals Ltd., and L-threonine and L-serine were from Sigma (London) Chemical Co., London S.W.6, U.K.

Methods

Activation of the enzyme precursor. The sample of precursor was incubated at 60°C for 15 min in either borate buffer (0.1 M-sodium borate, pH 9.0) or 0.1 M-2-amino-2-methylpropane-1,3-diol-HCl buffer, pH 9.2. The activity reached was the same under both sets of conditions.

Standard enzyme assays. The substrate solution (4.75 ml) containing 2-amino-2-methylpropane-1,3-diol-HCl buffer (pH 9.2, 0.1 M), or borate buffer (pH 9.0, 0.1 M), and L-threonine (12.5 mM) was incubated at 45°C. The reaction was started by addition of enzyme solution (0.25 ml). A portion (2 ml) was immediately withdrawn and quenched with trichloroacetic acid (4 ml; 15%, w/v). A second portion was withdrawn after 10 min and similarly quenched. With crude enzyme samples, a heavy precipitate formed on quenching with trichloroacetic acid; this was removed by centrifugation in an MSE bench centrifuge. The amount of α -oxobutyric acid formed was determined essentially by the method of

Friedemann & Haugen (1943). Portions (3 ml) of the blank and test solutions were added to 2,4-dinitrophenylhydrazine (1 ml; 0.05%, w/v, in 2 M-HCl) and left for 20 min to allow complete colour development. NaOH solution (5 ml; 2.5 M) was added to both samples and the E_{520} of the test solution was measured in a 1 cm-path-length cell, with the other sample as a blank. Under these conditions, an absorbance of 0.810 corresponded to the production of 1.0 μ mol of α -oxobutyric acid/ml in the assay solution; conversion of observed absorbance into α -oxobutyric acid concentration was made by using a standard calibration curve.

Unit of enzyme activity. This was defined as the amount of enzyme which, under the conditions described above, produced 1 μ mol of α -oxobutyric acid/min.

Protein concentrations. Protein concentration was determined from measurement of the E_{280} of protein solutions in a 1 cm-light-path cell (Layne, 1957) on the assumption that a solution of unit absorbance contains 1 mg of protein/ml. This relationship is obeyed accurately for purified threonine dehydratase samples (Nishimura & Greenberg, 1961).

Specific activity. This is defined as the number of μ mol of α -oxobutyric acid produced/min per mg of protein, where the protein concentration is defined in terms of absorbance as described above.

Assay of sheep livers for threonine dehydratase content. Samples (1 g) of liver were homogenized in 2-amino-2-methylpropane-1,3-diol-HCl buffer (4 ml; 0.1 M, pH 9.2) containing KCl (0.4 M) and the homogenates heated for 15 min at 60°C; the presence of KCl improved coagulation of denatured protein. The samples were cooled to room temperature and clarified by centrifugation in an MSE bench centrifuge. Samples (0.25 ml) of each activated homogenate were assayed as described above. Livers used for purification generally contained 4 units or more of activity/g.

Partial purification of the unactivated precursor.

1. Acetone-dried powder. The liver was minced and homogenized with ice-cold acetone (400 ml/100 g) for 90 s in an MSE Atomix blender. The precipitated material was recovered by filtration and re-homogenized with acetone under the same conditions. The protein cake recovered after filtration was allowed to dry in air at room temperature.

2. Precipitation with $(\text{NH}_4)_2\text{SO}_4$. The acetone-dried powder was dissolved in water (15 ml/g) and $(\text{NH}_4)_2\text{SO}_4$ added to 60% saturation. The suspension was left overnight to coagulate after which the precipitate was recovered by filtration through Whatman no. 1 paper. The protein was suspended in distilled water and $(\text{NH}_4)_2\text{SO}_4$ removed by dialysis.

3. Fractionation with $(\text{NH}_4)_2\text{SO}_4$. The protein in the dialysed solution from step 2 was fractionally precipitated with $(\text{NH}_4)_2\text{SO}_4$. At each step the

precipitate was collected by centrifugation for $\frac{1}{2}$ h at 4500g, suspended in water and dialysed. The bulk of the precursor was precipitated between 40 and 50% saturation with $(\text{NH}_4)_2\text{SO}_4$.

4. Fractionation with ethanol. The active fraction from step 3 was made 0.2M in sodium phosphate buffer (pH 7.0) and further fractionated with ethanol. The ethanol was cooled to -20°C before use and the temperature in the protein solution was maintained at 2°C or less during fractionation. Precursor protein was mainly precipitated between 55 and 70% (v/v) ethanol. The protein was collected by centrifugation, suspended in water and dialysed.

5. Re-fractionation with $(\text{NH}_4)_2\text{SO}_4$. A final re-fractionation with $(\text{NH}_4)_2\text{SO}_4$ was carried out as described for step 3.

At each stage in the procedure, assays were carried out on portions of the fractions which had been reactivated by the method described above.

Purification of the activated enzyme. 1. Homogenization and activation. Sheep liver (800g) was homogenized in 2-amino-2-methylpropane-1,3-diol-HCl buffer (4 litres; 0.1M, pH 9.2) containing KCl (0.4M). The homogenate was heated to 65°C by immersion in a water bath at 70°C , and maintained at this temperature for 15 min. After cooling, precipitated protein was removed by centrifugation at 2000g for $\frac{1}{2}$ h.

2. Fractionation with acetone. Fractionation was carried out with acetone which had been cooled to -20°C . The protein solution was maintained at $0-5^\circ\text{C}$ during fractionation. Two fractions were taken, the first by adding 0.9 vol. of acetone and the second by adding a further 0.9 vol. of acetone. The protein precipitated by the second addition of acetone was collected by centrifugation (2000g for 10 min), suspended in the minimum volume of water, and dialysed against distilled water.

3. Ion-exchange chromatography on DEAE-cellulose. The active fraction from step 2 was concentrated to 50 ml by forced dialysis and then dialysed against Tris-HCl buffer (0.025M, pH 7.45) for 5 h. The sample was applied to a column (1.5 cm $\times$ 30 cm) of DE-11 cellulose (Whatman Biochemicals Ltd., Springfield Mill, Maidstone, Kent, U.K.) equilibrated with the same buffer at 4°C . A flow rate of 60 ml/h was used. A fraction passed down the column without absorption, and the activity was found to be associated with this material.

4. Ion-exchange chromatography on CM-cellulose. The active fraction from step 3 was concentrated to 35 ml by forced dialysis and then dialysed against sodium phosphate buffer (0.02M, pH 6.1) for 5 h. The sample was applied to a column (1.5 cm $\times$ 30 cm) of CM-52 cellulose (Whatman) equilibrated with the same buffer, and the column was eluted at a flow rate of 30 ml/h. After the unabsorbed protein had been eluted, a gradient of increasing ionic strength was

applied. This was formed by withdrawing liquid from a closed mixing vessel (250 ml) containing phosphate buffer (0.02M, pH 6.1) to which was connected a reservoir of the same buffer containing NaCl (0.5M). The active material was associated with the first protein peak eluted after application of the gradient.

5. Repeated ion-exchange chromatography on CM-cellulose. The active fraction from step 4 was re-chromatographed on CM-52 cellulose but with the starting buffer at 0.01M instead of 0.02M. Again the active material was adsorbed on to the column and was eluted by application of a salt gradient (as in step 4 but with 0.01M buffer). The most active fractions were combined and concentrated to 4 ml.

Starch-gel electrophoresis. This was carried out at pH 8.0 by the method of Barrett *et al.* (1962). One-half of the gel was stained for the presence of protein by using nigrosine (0.5%, w/v, in methanol-water-acetic acid, 5:5:1, by vol.). The other half was stained for enzyme activity by immersion in the normal substrate solution for 30 min followed by incubation with 2,4-dinitrophenylhydrazine solution for 10 min. Activity bands appeared as yellow regions on a white background after washing the gel with methanol-water-acetic acid (5:5:1, by vol.).

Kinetics of activation of the precursor. Borate buffer (0.2M, 18 ml) at the desired pH value was incubated in a temperature-controlled water bath and a solution of the purified precursor (2 ml, containing approx. 5 units of activity after complete reactivation) at the same pH value was added at zero time. Portions (about 2.5 ml) were withdrawn at suitable time-intervals and quenched by running them into tubes at -80°C . At the end of the kinetic run, the samples were brought to room temperature and assayed. Assays were carried out either at pH 9.0 and 25°C or at pH 7.0 and 45°C , under which conditions reactivation is slow (see the Results section). The assay solution (9 ml) contained 0.125 mmol of L-threonine. The reaction was started by the addition of the test solution (1 ml), and a sample (1 ml) of the mixture was immediately withdrawn and quenched in trichloroacetic acid. Subsequent samples were removed at 2, 4, 6, 8 and 10 min intervals and similarly quenched. The content of α -oxobutyric acid in the samples was determined as described above. Initial rates of enzymic reaction were determined from plots of α -oxobutyric acid concentration against time.

Sheep feeding experiments. The animals were kept on grass or normal concentrates until they reached body weights of 22–36 kg. They were then subjected to one of the following diets for the last 7 days before slaughter (except for group A). (A) Complete withdrawal of food for the last 4 days; (B) fresh grass *ad libitum*; (C) normal concentrates (450 g/head per day) consisting of 1 part rolled oats, 1 part bran, 1.25 parts ground peanut, 1 part sugar-beet pulp

(molassed); (D) normal concentrates (850g) plus casein (150g) or Ovigest (500ml four times daily, total amino acid content 140g); (E) normal concentrates (850g) plus glutene (75g) and zein (75g); (F) as (E) plus 7g of Ovigest intraperitoneally (3.5%, w/v, solution, 100ml injected twice daily); (G) normal concentrates (800g) plus glutene (75g), zein (75g) and casein (50g); (H) normal concentrates (810g) plus glutene (75g), zein (75g) and $(\text{NH}_4)_2\text{SO}_4$ (40g).

At the end of the feeding regimen, the animals were slaughtered, their livers removed immediately and transported in a cold insulated box to the laboratory. Samples of each liver were assayed, after activation, by the procedure described above.

Results

Assay of the enzyme before and after activation and identification of products of the reaction

A sample of the partially purified precursor was assayed before and after activation by the standard method. The assay conditions (pH 7.0, 45°C) were chosen to minimize activation during assay. The results are shown in Fig. 1. The precursor showed approx. 5% of the activity of the activated enzyme. It is apparent from the gradually increasing rate of production of α -oxobutyric acid in the former case that a slow activation was occurring during the assay.

Prolonged incubation of the two samples with substrate was carried out. The 2,4-dinitrophenylhydrazones of the products formed were isolated and examined by t.l.c. on silica-gel G plates (E. Merck A.G., Darmstadt, Germany) with the solvent system benzene-methanol-acetic acid (45:8:4, by vol.). In both cases two spots were observed corresponding to the *trans* (R_F 0.74) and *cis* (R_F 0.51) forms of α -oxobutyric acid 2,4-dinitrophenylhydrazone. No other products were observed. The samples were also incubated at pH 7.0, 45°C, with α -oxobutyric acid and the oxo acid was determined at various time-intervals. No decrease in α -oxobutyric acid was observed in either case. These results show that the assay used in this work gives a true measure of threonine dehydratase activity and moreover that the activation phenomenon is not due to the loss by heat inactivation of an enzyme that subsequently removes α -oxobutyric acid produced in the threonine dehydratase reaction.

Assay of sheep livers for threonine dehydratase content

The content of the enzyme in livers obtained from the slaughterhouse varied widely. Each liver was assayed after activation by the methods described, and only those livers showing activities of 4 units or more/g were used for purification of the enzyme.

The enzyme contents of a typical batch of 24 livers, expressed in units of activity/g of liver were: 0.35, 0.50, 0.58, 0.65, 0.82, 0.87, 1.23, 1.35, 1.36, 1.53, 1.70, 1.76, 2.40, 2.41, 2.87, 2.92, 3.08, 3.93, 4.42, 5.02, 5.07, 6.28, 7.28 and 9.40. This batch contained more than the usual number of livers with moderately high activity. In all cases, the observed activities were 10–20-fold lower before activation.

Partial purification of the precursor

A summary of a typical purification procedure is given in Table 1; experimental details are described in the Experimental section. The quoted activity values refer to measurements on activated samples of each fraction. The object of this part of the work was to produce only a partially purified sample of the precursor for studies of the activation process, and no attempt was made to purify the precursor to homogeneity. The product of the procedure was not appreciably activated. On storage at -25°C it lost activity at the rate of about 1%/day.

Purification of the activated enzyme

A summary of the procedure is given in Table 2; experimental details are described in the Experimental section. The first step in the procedure was to heat the homogenate (pH 9.2) at 65°C for 15 min. This step effected complete activation of the precursor as well as removing considerable quantities

Fig. 1. Time-courses of α -oxobutyric acid production by a sample of the precursor before and after activation

●, Before activation; ▲, after activation. Assays were carried out at pH 7.0, 45°C , conditions under which the activation process is slow.

Table 1. *Summary of the procedure for partial purification of the unactivated enzyme precursor*

Protein content was determined from the absorbance at 280nm by using the approximation that a solution with absorbance of unity contained 1mg of protein/ml. For further details see the Experimental section.

Fraction	Vol. (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg)	Total activity (units)	Overall yield (%)	Purification factor (fold)
1. Acetone-dried powder	8640	1.1	21.4	0.05	9460	(100)	(1)
2. 0–60% (NH ₄) ₂ SO ₄ precipitate	2200	4.2	38.2	0.11	9250	98	2.2
3. 40–50% (NH ₄) ₂ SO ₄ fraction	340	15.3	63.6	0.24	5200	55	4.8
4. 55–70% EtOH fraction	339	7.9	8.4	0.94	2660	28	18.8
5. 45–50% (NH ₄) ₂ SO ₄ fraction	31	42.4	20.8	2.04	1300	14	40.8

Table 2. *Summary of the procedure for purification of the activated enzyme*

Protein content was determined as described in Table 1. For further details see the Experimental section.

Fraction	Vol. (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg)	Total activity (units)	Overall yield (%)	Purification factor (fold)
1. Heated homogenate	3300	0.9	18.2	0.05	3000	(100)	(1)
2. 47–65% (v/v) acetone precipitate	380	6.0	18.7	0.32	2300	77	6.5
3. Active fraction from DEAE-cellulose	150	10.0	3.0	3.33	1500	50	68
4. Active fraction from CM-cellulose	40	27.5	1.1	25.0	1100	37	500
5. Active fraction from repeated CM-cellulose fractionation	4	138.0	3.0	46.0	550	18	920

of protein impurities. It should be noted that the recorded overall purification factor (920-fold) is based on the specific activity after heating the homogenate and is hence probably underestimated by a factor of 4 to 5 if the protein contaminants removed during heating are taken into account. The peak of active protein eluted after the first passage through CM-cellulose was incompletely resolved from a peak of contaminating protein that was eluted immediately afterwards. It was found that this contaminant could be largely removed by repeated chromatography on CM-cellulose at a lower initial buffer concentration. An alternative procedure was to fractionate the active material from the first passage through CM-cellulose by gel filtration on Sephadex G-200 equilibrated with NaCl (0.1 M); the threonine dehydratase emerged from the column before the contaminating protein. Starch-gel electrophoresis at pH 8.0 (Barrett *et al.*, 1962) of the final product from the purification described (Table 2) showed one major component, migrating slowly towards the anode, and one minor more rapidly migrating component. Activity stains (see the Experimental section) showed unambiguously that threonine dehydratase was the major component. By assuming equal intensity of staining for the two proteins, the threonine dehydratase was estimated to account for greater than 90% of the sample.

Kinetic studies of the activation process

Studies of the activation process were carried out by using a sample of precursor purified as described above. Assuming that the active enzyme E is formed from the precursor in a first-order process, then the integrated rate equation will have the form

$$k_1 = \frac{2.303}{t} \log \left(\frac{E_\infty - E_0}{E_\infty - E_t} \right)$$

where E_0 , E_t and E_∞ are the amounts of active enzyme at the start of the activation process, at time t , and at complete activation respectively. Since the amount of active enzyme is directly proportional to the initial rate of α -oxobutyric acid formation v , the equation may also be written:

$$k_1 = \frac{2.303}{t} \log \left(\frac{v_\infty - v_0}{v_\infty - v_t} \right)$$

In Fig. 2, $-\log(v_\infty - v_t)$ is plotted against time for a typical activation process; the conditions were pH 9.0, 45°C. The linear relationship up to 75% reaction is indicative of a true first-order process. In this particular case, v_0 and v_∞ were 0.002 and 0.027 $\text{mm} \cdot \text{min}^{-1}$ respectively, i.e. the precursor had 7.4% of the final activity after activation.

First-order rate constants (k_1) for the activation process under a variety of conditions are given in

Table 3. The value given for pH 9.0 and 45°C is the average of four independent determinations; in two cases assay of the activated samples was carried out at pH 9 and 25°C (k_1 values of 0.0322 min⁻¹ and 0.0303 min⁻¹) and in the other two, assays were at pH 7 and 45°C (k_1 values of 0.0329 min⁻¹ and 0.0326 min⁻¹). Since both sets of assay conditions led to essentially the same result, in subsequent experiments only assays at pH 9 and 25°C were used.

The data in Table 3 include three values of k_1 obtained at a fixed pH (9.00) but at various temperatures. An Arrhenius plot (log k_1 against 1/T°K) of the data gave a good straight line, from the slope of which the activation energy of the reaction was found to be 115.8 kJ·mol⁻¹. Similarly, values of k_1 were obtained at fixed temperature (45.0°C) at various pH values. A plot of -log k_1 against pH gave a straight line of slope -0.6, i.e. k_1 is proportional to [H⁺]^{-0.6}. The addition of pyridoxal 5'-phosphate (75 μM) increased the first-order rate constant at pH 9.00 and 45°C by approx. 50%. At lower pH and higher pyridoxal 5'-phosphate concentrations, much larger increases in rate of activation were observed, but interference of pyridoxal 5'-phosphate in the assay of enzyme activity precluded quantitative measurements.

Comparison of the kinetics of activation of the precursor and of the enzyme inhibited with serine

A sample of the precursor, purified by the techniques described above, was activated by the usual method. Precipitated protein was removed by centrifugation and the sample transferred to phosphate buffer (0.05M, pH 7.0) by gel filtration

Fig. 2. First-order plot of data for the activation process at pH 9.0, 45°C

v_∞ and v_t are the initial rates of α -oxobutyric acid production by the completely activated precursor and after activation for time t respectively. The first-order rate constant obtained from these data was 0.0322 min⁻¹.

through a column of Sephadex G-25 equilibrated in this buffer. L-Serine was added to a final concentration of 0.05M and the sample stored at 2°C for 72h. Excess of serine was removed by gel filtration through a column of Sephadex G-25 equilibrated in phosphate buffer. Assays at pH 7.0, 45°C, of the enzyme inhibited with serine before and after reactivation showed that the inhibited enzyme retained only 5% of the original activity but that 95% of the original activity was regained by the normal reactivation process.

Measurements were made of the rate of activation of the enzyme inhibited with serine and of the precursor protein, with the same amount of protein in both cases, at pH 9.00 by using borate buffer (0.05M) containing KCl (0.2M). The reactions were strictly first-order and the rate constants obtained are given in Table 4. It can be seen that the values of k_1 for the precursor and the enzyme inhibited with serine are the same both at 37.0°C and at 45.0°C.

Variation in threonine dehydratase activity in response to dietary conditions

Data on the variation in activity of threonine dehydratase as a function of diet are given in Table 5. The mean value of the activities of 48 livers obtained from the slaughterhouse is also given for comparison; the maximum variation in activity

Table 3. First-order rate constants (k_1) for activation of the precursor under various conditions

For details see the text.			
pH	Temperature (°C)	Added reagents	$10^2 \times k_1$ (min ⁻¹)
9.00	45.0	—	3.20
9.00	35.8	—	1.00
9.00	60.2	—	27.0
8.48	45.0	—	1.59
9.48	45.0	—	7.22
9.00	45.0	Pyridoxal 5'-phosphate 4.87 (75 μM)	

Table 4. First-order rate constants (k_1) at pH 9.0 for activation of the precursor and of the enzyme inhibited with serine

For details see the text.		
Sample	Temperature (°C)	$10^2 \times k_1$ (min ⁻¹)
Precursor	45.0	2.60
	37.0	0.76
Enzyme inhibited with serine	45.0	2.70
	37.0	0.76

Table 5. *L-Threonine dehydratase activities in livers of sheep kept under various dietary conditions*

Unless stated otherwise, animals were maintained on the diet for 7 days before slaughter. For further details see the Experimental section.

Group	Experimental conditions	Number of livers tested	Units of activity ($\mu\text{mol}/\text{min}$ per g of liver)	
			Means $\pm$ S.E.M.	Range
—	Livers from slaughterhouse	48	1.88 ± 0.24	0.33–9.40
(A)	Starved for 4 days	6	1.70 ± 0.34	0.75–2.70
(B)	Fresh grass	20	0.99 ± 0.15	0.35–3.41
(C)	Normal concentrates	12	1.06 ± 0.29	0.28–4.00
(D)	Normal concentrates plus casein or Ovigest	17	1.07 ± 0.17	0.22–2.70
(E)	Normal concentrates plus zein and glutene	27	1.71 ± 0.66	0.19–15.00
(F)	As for group (E) plus injections of Ovigest	8	9.08 ± 2.72	2.00–26.00
(G)	Normal concentrates plus zein, glutene and casein	10	0.76 ± 0.10	0.22–1.29
(H)	Normal concentrates plus zein, glutene and $(\text{NH}_4)_2\text{SO}_4$	4	0.68 ± 0.07	0.50–0.84

was approximately 30-fold, with 10% of the livers showing activities considerably greater than the mean.

In animals kept under controlled conditions on diets consisting either of fresh grass (group B) or normal concentrates (group C) the liver threonine dehydratase activity was consistently low and significantly lower than that in livers obtained from the slaughterhouse ($P < 0.01$ and $P < 0.05$ respectively). Complete withdrawal of food during the last 4 days before slaughter (group A) caused a loss of body weight ranging from 8 to 18%, but threonine dehydratase activity was not significantly altered compared with that of animals maintained on normal diets. Similarly, supplement of the normal concentrate diet with casein or with the protein hydrolysate Ovigest (group D) had no effect on enzymic activity.

On the other hand, supplement of the normal concentrates with plant proteins, zein and glutene (group E), caused a distinct increase in liver threonine dehydratase activity in about 15% of the animals. The activity remained low in the majority of animals in this group and hence the mean value is not significantly different from that of the controls. It is, however, noteworthy that the mean value, variability and maximum activity in this group corresponds remarkably well with those from livers obtained from the slaughterhouse. A dramatic increase (approximately tenfold, $P < 0.001$) was observed in the mean enzymic activity if the animals received intraperitoneal injections of Ovigest (group F) in addition to the normal concentrate diet supplemented with zein and glutene. One of the livers in this batch contained the highest activity so far recorded. Conversely, no increase in enzyme activity was observed when the diet of concentrates, zein and glutene was further supplemented with casein (group G) or $(\text{NH}_4)_2\text{SO}_4$ (group H).

In all these experiments, the activity of threonine dehydratase was determined after activation and by

using standard assay conditions as described in the Experimental section. It had already been established (see above) that under these conditions only threonine dehydratase activity was measured and there was no interference from other enzymes, for example threonine aldolase. The threonine dehydratase from several of the livers that showed elevated enzyme activities was purified by the technique described for the activated enzyme. The enzymes from livers obtained from slaughterhouses and from livers of sheep maintained on controlled diets behaved identically during purification and the same specific activities were attained. This demonstrates clearly that the increased ability to degrade threonine was due to an increased content of threonine dehydratase.

Discussion

Fig. 1 demonstrates clearly that the threonine dehydratase extracted from sheep liver is in a largely inactive form but can be activated 10–20-fold by heating at alkaline pH. The degree of activation obtainable is independent of the absolute activity in any particular liver. The existence of the enzyme in an inactive form, referred to here as the precursor, has not been recognized by previous authors, but examination of their results reveals phenomena attributable to the activation process. For example, Nishimura & Greenberg (1961) used heat denaturation at pH 7.2 during their purification procedure. Recoveries of activity of 116 and 124% were recorded in the first two heat treatments, and these high recoveries must represent increases from activation superimposed on the usual losses due to heat inactivation. The overall purification factor of 1500-fold claimed by Nishimura & Greenberg (1961) is therefore considerably overestimated, since it included the increase in specific activity due to the activation process. Conversely, the purification

factor obtained during purification of the activated enzyme in the present work (920-fold, Table 2) was calculated on the basis of the specific activity after initial heat activation, and is hence probably too small by a factor of three or four, since a large amount of protein was precipitated by the heat treatment.

Partial purification of the precursor has been carried out; the techniques used (Table 1) were chosen to minimize activation during purification and the final product showed the same ratio of activity before and after activation as did the original extract. The kinetics of the activation process were studied and the results are given in Table 3. It had previously been reported that threonine dehydratase is inactivated during its action on L-serine (Nishimura & Greenberg, 1961; McLemore & Metzler, 1968; Phillips & Wood, 1965; Phillips, 1968). We have compared the kinetics of activation of the precursor and the enzyme inhibited with serine and have shown that the first-order rate constants for activation of the two species are the same at two different temperatures (Table 4). These observations provide strong, although perhaps not conclusive, evidence that the precursor and the enzyme inhibited with serine are the same species. The mechanism of the inhibition by serine has not been finally established, but the work of Phillips (1968) suggests that an aminoacrylate residue produced by dehydration of serine alkylates the side chain of a cysteine residue in the protein. A similar interpretation of inactivation of aspartate aminotransferase during β -elimination of serine *O*-sulphate has been given (John & Fasella, 1969; John *et al.*, 1973). Final confirmation of the hypothesis that the precursor arises from inhibition by serine would require isolation of the pure precursor in large quantity and identification of the C_3 compound derived from aminoacrylate which would be liberated during activation.

Threonine dehydratase is found in the inactive form in homogenates of freshly removed liver immediately after homogenization at 4°C. This, combined with the fact that homogenates are unlikely to contain sufficient free serine to cause complete conversion of active into inactive enzyme, indicates that the precursor is the major form of the enzyme *in vivo*. There is then a problem concerning the activation process. Reactivation at pH 7 and 37°C is slow (half-life of about 10h from the data in Tables 3 and 4), although the rate could be increased by high local concentrations of pyridoxal 5'-phosphate. In addition, the concept of pH is of doubtful validity when applied to the interior of a cell, and it may be that regions of high effective basicity exist where reactivation would be rapid. The possibility remains, however, that there is a presently unrecognized physiological reactivation mechanism;

this obviously merits further study. It is noteworthy that the enzyme from rat liver is rapidly inactivated by heating at pH 9 and 60°C (S. Doonan, unpublished work) in marked contrast with the behaviour of the enzyme from sheep liver.

The threonine dehydratase of rat liver has been shown to be inducible in response to a variety of stimuli, e.g. by feeding with amino acids or proteins (Sayre *et al.*, 1956; Goldstein *et al.*, 1962; Pitot & Peraino, 1964; Yamashita *et al.*, 1968), by injection of glucocorticoids (Peraino, 1967; Peraino *et al.*, 1966; Ku *et al.*, 1969), by starvation (Pitot *et al.*, 1958) or by experimental diabetes (Ishikawa *et al.*, 1965). Conversely, the enzyme activity is decreased by feeding on high carbohydrate and fat diets (Nakano & Ashida, 1970), by administration of insulin (Yamashita *et al.*, 1969) and by treatment with thyroxine (Ku *et al.*, 1969). In general it seems that the enzyme activity is increased by gluconeogenic stimuli and that the main function of the enzyme in the rat is to supply α -oxobutyric acid for carbohydrate synthesis *de novo*.

The inducibility of the enzyme in the rat suggested to us that the frequently observed variation in activity of threonine dehydratase in sheep liver (Sayre & Greenberg, 1956; Nishimura & Greenberg, 1961; Moss *et al.*, 1965) is due to dietary factors. We have, therefore, carried out a series of experiments in which sheep were maintained on controlled diets, after which the activity of threonine dehydratase was measured. The results are recorded in Table 5. Starvation of the animals (group A) did not result in increased threonine dehydratase activities as it did in the rat (Pitot *et al.*, 1958); the different response is perhaps not surprising, since the rat is unusually susceptible to starvation (Baldwin *et al.*, 1966). Similarly, diets containing large amounts of casein or a protein hydrolysate (group D) did not cause an increase in enzyme activity compared with diets of fresh grass or normal concentrates (groups B and C). On the other hand, some sheep fed with plant proteins zein and glutene (group E) responded with an increased enzyme activity. In interpreting these results, it must be borne in mind that proteins such as casein are extensively degraded by the ruminal bacteria to volatile fatty acids and ammonia (El-Shazly, 1952*a,b*), whereas bacterial degradation is less pronounced with plant proteins, which undergo at least in part a normal digestive process in the lower intestinal tract (Annison, 1956). Since the animals showed a great variation in response to feeding with plant proteins, a group of sheep (group F) received intraperitoneal injections of a protein hydrolysate in order to circumvent the intestinal tract. The mean activity of threonine dehydratase in the livers of animals so treated was very high; one liver contained 26 units of enzyme/g, the highest value so far recorded. Hence it appears

that threonine dehydratase is inducible by amino acids and protein in the sheep provided that these substances escape rumenal digestion. It is interesting that the group of sheep (group G) fed with casein in addition to zein and glutene showed consistently low enzyme activities. It may be that the low-molecular-weight fatty acids produced by metabolism of casein stimulated the secretion of insulin, which in turn repressed production of threonine dehydratase; insulin has this effect in the rat, as mentioned above (Yamashita *et al.*, 1969).

Many facts remain to be established concerning control of threonine dehydratase in sheep. For example, the time-course of induction was not investigated in the present work. It is also not known whether the increase in activity results from increased synthesis or decreased degradation of the enzyme or both (Schimke & Doyle, 1970). It appears, however, that the greater threonine-metabolizing ability of livers of sheep maintained on special diets is due to the same enzyme as that normally present, since the product of the reaction is solely α -oxobutyric acid and the enzyme can be purified by the same techniques and to the same specific activity as the enzyme from control sheep. It also seems likely that the variation in activity of livers obtained from the slaughterhouse is due to dietary factors, since the livers of animals fed on concentrates plus plant proteins and those from slaughterhouses showed a close similarity in distribution of threonine dehydratase activities.

The authors are indebted to the Director of the Wellcome Veterinary Research Station for the gift of sheep and to Mr. G. L. Shearer and Mr. K. Flodgell who carried out the feeding experiments. Financial support from the Rockefeller Foundation (D. H. K.) and the Deutsche Forschungsgemeinschaft and the Paul-Martini-Stiftung (W. S.) is gratefully acknowledged.

References

- Annisson, E. F. (1956) *Biochem. J.* **64**, 705–714
- Baldwin, R. L., Ronning, M., Radanovics, C. & Plange, G. (1966) *J. Nutr.* **90**, 47–55
- Barrett, R. J., Friesen, H. & Astwood, E. B. (1962) *J. Biol. Chem.* **237**, 432–439
- El-Shazly, K. (1952a) *Biochem. J.* **51**, 640–647
- El-Shazly, K. (1952b) *Biochem. J.* **51**, 647–653
- Friedemann, T. E. & Haugen, G. E. (1943) *J. Biol. Chem.* **147**, 415–442
- Ghazarian, H. G. & Borchers, R. (1973) *Int. J. Biochem.* **4**, 537–541
- Goldstein, L., Knox, W. E. & Behrman, E. J. (1962) *J. Biol. Chem.* **237**, 2855–2860
- Ishikawa, E., Ninagawa, T. & Suda, M. (1965) *J. Biochem. (Tokyo)* **57**, 506–513
- John, R. A. & Fasella, P. (1969) *Biochemistry* **8**, 4477–4482
- John, R. A., Bossa, F., Barra, D. & Fasella, P. (1973) *Biochem. Soc. Trans.* **1**, 862–864
- Ku, Y., Rogers, Q. R. & Harper, A. E. (1969) *Proc. Soc. Exp. Biol. Med.* **130**, 556–563
- Layne, E. (1957) *Methods Enzymol.* **3**, 447–454
- McLemore, W. O. & Metzler, D. E. (1968) *J. Biol. Chem.* **243**, 441–445
- Moss, G., Russell, D. H., Thain, E. M. & Vernon, C. A. (1965) *Biochem. J.* **95**, 3p
- Nakano, K. & Ashida, K. (1970) *J. Nutr.* **100**, 208–216
- Nishimura, J. S. & Greenberg, D. M. (1961) *J. Biol. Chem.* **236**, 2684–2691
- Peraino, C. (1967) *J. Biol. Chem.* **242**, 3860–3867
- Peraino, C., Lamar, C. & Pitot, H. C. (1966) *J. Biol. Chem.* **241**, 2944–2948
- Phillips, A. T. (1968) *Biochim. Biophys. Acta* **151**, 523–526
- Phillips, A. T. & Wood, W. A. (1965) *J. Biol. Chem.* **240**, 4703–4709
- Pitot, H. C. & Peraino, C. (1964) *J. Biol. Chem.* **239**, 1783–1788
- Pitot, H. C., Dent, J. H. & Feary, T. (1958) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **17**, 290
- Russell, D. H., Thain, E. M. & Vernon, C. A. (1960) *Proc. Chem. Soc. London* 255–256
- Sayre, F. W. & Greenberg, D. M. (1956) *J. Biol. Chem.* **220**, 787–799
- Sayre, F. W., Jensen, D. & Greenberg, D. M. (1956) *J. Biol. Chem.* **219**, 111–117
- Schimke, R. T. & Doyle, D. (1970) *Annu. Rev. Biochem.* **39**, 929–976
- Schmutzler, W. (1971) *Arzneim. Forsch.* **21**, 1509–1510
- Umbarger, H. E. & Brown, B. (1957) *J. Bacteriol.* **73**, 105–112
- Wood, W. A. (1969) *Curr. Top. Cell. Regul.* **1**, 161–182
- Yamashita, M., Eto, T., Fujimaki, M. & Sakurai, Y. (1968) *Agr. Biol. Chem.* **32**, 374–378
- Yamashita, M., Arai, S., Eto, T., Fujimaki, M. & Sakurai, Y. (1969) *Agr. Biol. Chem.* **33**, 1007–1012