Purification of a Diketo Acid Hydrolase from Rat Liver and its Use for the Enzymic Determination of 3,5-Dioxohexanoate (Triacetate)

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1. A diketo acid hydrolase was purified about 100-fold from rat liver and some of its properties were studied. 2. Comparison of its activity with 3,5-dioxohexanoate (triacetate) and fumarylacetoacetate at various stages during the purification suggests that it is identical with 4-fumarylacetoacetate fumarylhydrolase. 3. The acetoacetate formed on cleavage of 3,5-dioxohexanoate by the hydrolase can be determined either with 3-hydroxybutyrate dehydrogenase or with 3-oxo acid CoA-transferase and 3-hydroxyacyl-CoA dehydrogenase. These reactions form the basis of enzymic assays for 3,5-dioxohexanoate.

3,5-Dioxohexanoate (referred to throughout by its trivial name, triacetate) has been identified as the product formed by a purified pigeon liver fatty acid synthetase from acetyl-CoA and malonyl-CoA in the absence of NADPH (Brodie, Wasson & Porter, 1964). Brock & Bloch (1966) showed that with a fatty acid synthetase from *Escherichia coli* the major product was the lactone of 3,5-dioxohexanoic acid (triacetic acid lactone), though the possibility that triacetic acid was lactonized during the isolation procedure could not be ruled out. The occurrence of these compounds in biological systems (Harris, Harris & Light, 1966; Light, Harris & Harris, 1966) and the difficulties encountered in their positive identification suggested the need for a rapid and simple method for their determination.

This paper describes an enzymic method for the determination of triacetate in which it is cleaved to acetoacetate and acetate by a diketo acid hydrolase obtained from rat liver (for a description of the enzyme see Meister, 1949). The acetoacetate formed is measured directly with either 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) (reaction 1):

or with 3-oxo acid CoA-transferase (EC 2.8.3.5) and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) (reactions 2 and 3):

The purification and some properties of the hydrolase, including evidence for its probable identity with 4-fumarylacetoacetate fumarylhydrolase (EC 3.7.1.2), are also described. Difficulties encountered in the development of an enzymic method for triacetic acid lactone are briefly reported.

MATERIALS AND METHODS

Chemicals. Triacetic acid lactone was prepared from dehydroacetic acid by the method of Collie (1891) and recrystallized twice from water. Triacetic acid was prepared from triacetic acid lactone via the ethyl ester by the procedure described by Witter & Stotz (1948). It was recrystallized from diethyl ether–light petroleum and stored at -20° . The concentrations of triacetic acid solutions were checked by the colorimetric method of Witter, Snyder & Stotz (1948), with acetylacetone as a standard. Fumarylacetoacetic acid was prepared enzymically from homogentisic acid by the method of Ravdin & Crandall (1951) and was stored as solid powder at -20° . Concentrations of fumarylacetoacetic acid solutions were determined spectrophotometrically at $330\text{m}\mu$ by using a molar extinction coefficient of 13500 at both pH 1 and 13 (Knox & Edwards, 1955). The lithium salts of acetoacetic acid, 3-oxopentanoic acid and 3-oxohexanoic acid were prepared by the hydrolysis of the corresponding ethyl esters with LiOH by the procedure described for acetoacetic acid by Hall (1962). The 3-oxo acid content of the lithium salts was determined manometrically by decarboxylation with aniline citrate (Edson, 1935). Succinyl-CoA was prepared by the method of Simon & Shemin (1953). Other chemicals were obtained from British Drug Houses Ltd., Poole,

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Dorset, including DL-mevalonolactone, which was converted into the free acid by the method of Hellig & Savard (1965).

Enzymes. 3-Hydroxybutyrate dehydrogenase was prepared from *Rhodopseudomonas spheroides* by the method of Williamson, Mellanby & Krebs (1962) and further purified on DEAE-Sephadex A-50 by the procedure of Bergmeyer, Gawehn, Klotzsch, Krebs & Williamson (1967). The preparations used had a specific activity of 4–6 i.u./mg. of protein. 3-Oxo acid CoA-transferase was prepared from rat heart (D. H. Williamson & M. W. Bates, unpublished work). It had a specific activity of 1 i.u./mg. of protein and was free of triacetate hydrolase activity. A preparation of 3-oxo acid CoA-transferase from pig heart that is also suitable for use in the determination of triacetate has been described by Hersh & Jencks (1967). 3-Hydroxyacyl-CoA dehydrogenase was purchased from Boehringer Corp. (London) Ltd., London, W. 5.

Measurement of diketo acid hydrolase activity. With triacetate as substrate, the hydrolase activity was measured in a coupled system in which the acetoacetate formed was reduced to 3-hydroxybutyrate with NADH in the presence of 3-hydroxybutyrate dehydrogenase, the rate of oxidation of NADH being a direct measure of the hydrolase activity. The cuvettes contained (in a final volume of 3.1 ml.): potassium phosphate buffer, pH 7.0 (33 mm), triacetic acid (2 mm), NADH (0.25 mm) and 3-hydroxybutyrate dehydrogenase (0.15 i.u.). After equilibration for 2 min. the reaction was started by addition of hydrolase. Suitable controls were included to correct for the slight reduction of triacetate by 3-hydroxybutyrate dehydrogenase alone and, with crude tissue extracts, to correct for non-specific NADH oxidation. With fumarylacetoacetate as substrate the hydrolase activity was measured by following the decrease in E_{366} due to cleavage of the unsaturated diketone. The cuvettes contained (in a final volume of 2.0 ml.): potassium phosphate buffer, pH 7.0 (50 mm), and fumarylacetoacetic acid (0.1 mm). The reaction was started by the addition of the hydrolase preparation.

All enzyme assays were performed at 25° in an Eppendorf photometer (Netheler und Hinz, Hamburg, Germany), with the 366 m μ filter. The molar extinction coefficient at this wavelength for NADH was taken to be 3300 (Hohorst, 1956), and that for fumarylacetoacetic acid was found to be 1980. A unit of hydrolase activity was defined as the amount of enzyme catalysing the hydrolysis of 1 μ mole of triacetate/min. at 25°.

EXPERIMENTAL

Purification of diketo acid hydrolase

Organ distribution of diketo acid hydrolase activity in the rat. Hydrolase activity was detected only in the liver and kidney of the rat, with the highest activity in liver (Table 1). Even with the more sensitive assay with fumarylacetoacetate as substrate no activity was found in heart, brain, spleen or adrenals. These results agree with those of Meister (1949), who in addition found no activity in rat pancreas or skeletal muscle.

Extraction of enzyme from liver. Ten male rats of the Wistar strain were killed by a blow at the back of the neck, and the livers excised immediately and placed on ice. When thoroughly chilled, the livers were passed through an electric meat mincer, and portions of resulting mince (96 g.) were homogenized with 0.25 M-sucrose in 10 mm-tris-hydrochloric acid buffer, pH 7.4 (380 ml.), in a plastic centrifuge tube (capacity 50 ml.) fitted with a loose-fitting glass bulb driven by a low-speed motor. The homogenate was centrifuged at 38 000 g for 1 hr. and the resulting supernatant was collected.

Ammonium sulphate fractionation. Sufficient solid ammonium sulphate (28 g./100 ml.) was added to the above supernatant fluid to bring it to 40% saturation. After stirring for 30 min. the solution was centrifuged and the precipitate discarded. The concentration of ammonium sulphate in the supernatant fluid was brought to 75% saturation by addition of a further 24.6 g./100 ml. The resulting precipitate was collected by centrifugation and dissolved in 125 ml. of 20 mm-potassium phosphate buffer, pH 7.4.

Ethanol fractionation. Ethanol (125 ml.), pre-cooled to –20°, was slowly added to the solution from the previous stage, the temperature of the mixture being kept below –5°. After stirring for 1 hr. the precipitate was centrifuged off at –10° and discarded. The concentration of ethanol in the

Table 1. *Organ distribution of diketo acid hydrolase activity in the rat*

Activities are expressed as μ moles of substrate reacting/min./g. wet wt., and were determined as described in the Materials and Methods section.

Organ	Hydrolase activity		Fumarylacetoacetate/triacetate hydrolysis rate ratio
	Fumarylacetoacetate as substrate	Triacetate as substrate	
Liver	6.3	0.38	16.5
Kidney	2.7	0.17	15.9
Heart	<0.01	<0.01	
Brain	<0.01	—	
Spleen	<0.01	—	
Adrenals	<0.01	—	

supernatant was brought to 75% (v/v) by slow addition over a period of 10 min. and the resulting precipitate was collected by centrifugation at -10° . The precipitate was suspended in 90 ml. of 20 mM-potassium phosphate buffer, pH 7.4, and partially dissolved by exposing it to ultrasonic vibration for two 1 min. periods. Insoluble material was removed by centrifugation and the supernatant fluid dialysed for 6 hr. at 4° against 1.5 l. of 20 mM-potassium phosphate buffer, pH 7.4.

Chromatography on DEAE-Sephadex. The dialysate was absorbed on a DEAE-Sephadex A-50 column (2 cm. $\times$ 17 cm.), previously equilibrated with 20 mM-potassium phosphate buffer, pH 7.4, and the column was eluted with the same buffer. Fractions (25 ml.) were collected until no further activity was eluted off the column. The active fractions (about 75 ml.) were combined and solid ammonium sulphate (53 g./100 ml.) was added to give 75% saturation. The resulting precipitate was collected by centrifugation and dissolved in 8 ml. of 20 mM-potassium phosphate buffer, pH 7.4. The solution was dialysed for 6 hr. at 4° against 1 l. of the same buffer.

Chromatography on CM-Sephadex. The dialysate was absorbed on a CM-Sephadex column (1.5 cm. $\times$ 10 cm.), previously equilibrated with 20 mM-potassium phosphate buffer, pH 7.4. The column was washed with 50 ml. of the same buffer and the enzyme was subsequently eluted with 50 ml. of 20 mM-potassium phosphate buffer, pH 7.4, containing sodium chloride (50 mM). Active fractions were combined, and the enzyme was precipitated by the addition of solid ammonium sulphate (53 g./100 ml.) to 75% saturation. The protein precipitate was collected by centrifugation, dissolved in 2.5 ml. of 20 mM-potassium phosphate buffer, pH 7.4, and stored at -20° . The purified hydrolase was stable for a period of months at this temperature.

The result of a typical isolation is shown in Table 2.

Properties of the diketo acid hydrolase preparation

Michaelis constant. The apparent Michaelis constant, K_m , for triacetate, as determined by Lineweaver & Burk (1934) plot, was 2.0×10^{-5} M. With fumarylacetoacetate as substrate a saturating concentration was achieved at 0.01 mM; the sensitivity of the assay did not permit an accurate determination of the K_m .

Optimum pH. Potassium phosphate, trihydrochloric acid, tris-maleate and triethanolamine-hydrochloric acid-sodium hydroxide buffers were used for the determination of the pH optimum. No activation or inhibition was found with any of these buffers, since assays carried out with different buffers at the same pH gave the same values for enzyme activity. A plateau of activity was found between pH 6 and 7 with a fairly sharp decline on either side (Fig. 1).

Specificity. The following compounds were tested in the hydrolase assay system at concentrations of 50–100 mM and were found not to give rise

Fig. 1. Effect of pH on diketo acid hydrolase activity. The assays were performed as described in the Materials and Methods section, but the composition of the buffer was varied: ○, tris-maleate (25 μ moles); ●, triethanolamine-HCl-NaOH (50 μ moles); □, potassium phosphate (50 μ moles).

Table 2. *Purification of the diketo acid hydrolase*

Summary of a typical purification of the hydrolase from rat liver. Activities are expressed as μ moles of triacetate reacting/min. at 25° .

Step		Total volume (ml.)	Total activity (units)	Total protein (mg.)	Specific activity (unit/mg.)	Yield (%)
(1)	Extract	340	36.5	10200	0.0036	100
(2)	(NH ₄) ₂ SO ₄ fractionation	125	22.3	2530	0.0088	61*
(3)	Ethanol fractionation	92	20.2	960	0.021	55
(4)	DEAE-Sephadex chromatography	8	11.9	152	0.079	33
(5)	CM-Sephadex chromatography	2.5	6	13	0.46	16

* (NH₄)₂SO₄ interfered with the assay, giving low values.

to acetoacetate: dehydroacetate, triacetic acid lactone, triacetic acid ethyl ester, acetylacetone, mevalonate, mevalonolactone, hydroxymethylglutarate, hydroxymethylglutaryl-CoA, 3-oxopentanoate, 4-oxopentanoate, 3-oxohexanoate, acetoacetic acid ethyl ester, acetoacetyl-CoA and citrate.

Inhibitor studies. Structural analogues of triacetate and tricarboxylic acid-cycle intermediates were tested as possible inhibitors of the hydrolase. Only 3-oxopentanoate, 3-oxohexanoate, malonate, fumarate and 2-oxoglutarate showed inhibition when triacetate was the substrate (Table 3).

Comparison of rates of hydrolysis with fumaryl-

acetoacetate and triacetate. The ratio of the fumarylacetoacetate/triacetate hydrolysis rates was the same with crude extracts obtained from liver and kidney (Table 1). After the first step in the purification of the hydrolase, the ratio increased further in favour of fumarylacetoacetate, and then was virtually constant throughout the rest of the procedure (Table 4).

Enzymic determination of triacetate with purified hydrolase

Procedure A. Triacetate can be determined enzymically in a similar system to that described in the Materials and Methods section for the assay of hydrolase activity. The essential difference is that in this case the substrate is the limiting factor. Cuvettes (1cm.) contained potassium phosphate buffer, pH 7.0 (100 μ moles), NADH (0.3 μ mole), triacetic acid sample (0.01–0.10 μ mole) and water to 2.0ml. The initial E_{340} was read and the reaction started by addition of 5 μ l. of 3-hydroxybutyrate dehydrogenase (0.15 i.u.; 30 μ g. of protein) and 10 μ l. of purified hydrolase (0.02 i.u.; 50 μ g. of protein). The decrease in extinction was followed until the reaction was complete (about 20 min.). Fumarylacetoacetate also reacted quantitatively and at a considerably faster rate (about 5 min.) in this system. The recoveries of various amounts of triacetate and fumarylacetoacetate by this procedure are shown in Table 5. With fumarylacetoacetate the measurements were made at E_{366} so as to minimize its contribution to the extinction changes and the calculations were made on the basis of molar extinction coefficient of 5280 for NADH plus fumarylacetoacetic acid (see the Materials and Methods section). As mentioned above, 3-hydroxybutyrate dehydrogenase catalyses the reduction of triacetate (but not fumarylacetoacetate) by NADH at a low rate. Although this is of no importance with pure solutions in the assay system described above, it could be a source of error if acetoacetate and triacetate were present in the same sample, and 3-hydroxybutyrate dehydrogenase was added

Table 3. *Effect of various compounds on diketo acid hydrolase activity*

The compounds listed were tested with either triacetate or fumarylacetoacetate as substrate in the assay systems described in the Materials and Methods section. The results are expressed as percentages of the activity with substrate alone.

Addition	Activity (%)	
	Triacetate (1.5 mm)	Fumarylacetoacetate (0.1 mm)
Acetylacetone (5 mm)	100	—
Dehydroacetate (5 mm)	100	—
Triacetic acid lactone (5 mm)	100	—
Triacetic acid ethyl ester (5 mm)	100	—
3-Oxopentanoate (2.5 mm)	70	100
3-Oxohexanoate (2.5 mm)	70	100
4-Oxohexanoate (5 mm)	100	—
Mevalonate (1.5 mm)	100	—
Hydroxymethylglutarate (1.5 mm)	100	—
Malonate (2.5 mm)	60	100
Oxalate (5 mm)	100	—
Maleate (1.5 mm)	100	100
Fumarate (1.5 mm)	80	80
Fumarate (0.1 mm)	100	100
Citrate (5 mm)	100	—
Succinate (2.5 mm)	100	100
Pyruvate (2.5 mm)	95	100
2-Oxoglutarate (5 mm)	50	100

Table 4. *Comparison of triacetate and fumarylacetoacetate hydrolysis rates with different diketo acid hydrolase fractions*

Activity measurements are expressed as μ moles/min. and were carried out as described in the Materials and Methods section. Hydrolase fractions are those given in Table 2.

Fraction	Activity with		Fumarylacetoacetate/triacetate hydrolysis rate ratio
	Triacetate	Fumarylacetoacetate	
(1) Extract	14.0	230	16.5
(2) $(\text{NH}_4)_2\text{SO}_4$ precipitate	3.6	296	82
(3) DEAE-Sephadex chromatography	6.1	420	69
(4) CM-Sephadex chromatography	6.4	500	78

Table 5. *Recovery of triacetate and fumarylacetoacetate by procedure A*

Various amounts of triacetate and fumarylacetoacetate were analysed by procedure A.

Expt. no.	Compound	Calculated (μ moles)	Found (μ moles)
1	Triacetate	99	96
2	Triacetate	49	53
3	Triacetate	29	27
4	Triacetate	10	11
5	Triacetate + triacetic acid lactone (80 μ moles)	25	22
6	Triacetate + triacetic acid lactone (200 μ moles)	19	20
7	Fumarylacetoacetate	50	48
8	Fumarylacetoacetate	10	12

before the hydrolase to determine the acetoacetate initially present. Consequently, an alternative method for the analysis of mixtures of triacetate and acetoacetate was developed in an attempt to overcome this difficulty.

Procedure B. This method is based on the finding that triacetate is not a substrate for 3-oxo acid CoA-transferase. The acetoacetate initially present is converted into acetoacetyl-CoA with excess of succinyl-CoA and 3-oxo acid CoA-transferase (eqn. 2). The acetoacetyl-CoA is reduced to L-3-hydroxybutyryl-CoA with NADH in the presence of 3-hydroxyacyl-CoA dehydrogenase (eqn. 3); the amount of NADH oxidized is equivalent to the acetoacetate originally present. The triacetate is then determined by the amount of acetoacetate formed on addition of the hydrolase. The cuvettes (1 cm.) contained potassium phosphate buffer, pH 7.0 (100 μ moles), NADH (0.3 μ mole), sample containing acetoacetate and triacetic acid (0.01–0.10 μ mole), succinyl-CoA (1.8 μ moles), 3-hydroxyacyl-CoA dehydrogenase (0.1 i.u.) and water to 2.0 ml. The initial E_{340} was read and 10 μ l. of 3-oxo acid CoA-transferase (0.1 i.u.) was added. The decrease in extinction (reaction complete in 10–20 min.) was a measure of the acetoacetate present. The further decrease in E_{340} on addition of purified hydrolase (0.020 i.u.; 50 μ g. of protein) was equivalent to the acetoacetate formed from triacetate. A comparison of the analysis of various triacetate and acetoacetate mixtures by procedures A and B is shown in Table 6. It is clear that at low concentrations of triacetate (0.1 μ mole/cuvette and below) the two methods give similar recoveries. This is because 3-hydroxybutyrate dehydrogenase has a relatively poor affinity for triacetate (K_m 6.7×10^{-3} M) in the reduction reaction.

Table 6. *Comparison of procedures A and B for the analysis of triacetate (or fumarylacetoacetate) and acetoacetate mixtures*

The analyses were carried out as described under procedures A and B, except that 3-hydroxybutyrate dehydrogenase was added first in procedure A.

Mixture	Calculated (μ moles)	Found (μ moles)
Procedure A		
1. Triacetate + acetoacetate	250	185
2. Triacetate + acetoacetate	25	20
3. Fumarylacetoacetate + acetoacetate	104	102
	100	97
Procedure B		
1. Triacetate + acetoacetate	9	9.5
2. Triacetate + acetoacetate	0	0
3. Triacetate + acetoacetate	10	8
4. Triacetate + acetoacetate	9	7.5
5. Triacetate + acetoacetate	10	8
6. Triacetate + acetoacetate	17.5	15
	10	8
	9	9
	20	17.5
	90	83
	100	97

Development of a method for the enzymic determination of triacetic acid lactone

Witter & Stotz (1948) reported that rat liver homogenates contained a lactonase that converted triacetic acid lactone into triacetate. Coupling of this enzyme with either of the assay systems for triacetate could provide a combined determination of triacetic acid lactone and triacetate. The lactonase activity was found to be mainly associated with the particulate fraction of rat liver homogenates, and could not be fully solubilized by treatment with either ultrasonic vibration or deoxycholate. In addition, it could not be freed from appreciable hydrolase activity. These facts, together with the finding that the apparent K_m of the lactonase for triacetic acid lactone was 1.0×10^{-2} M, argued against its application for the enzymic determination of triacetic acid lactone.

DISCUSSION

An enzyme capable of hydrolysing triacetate to acetoacetate and acetate has been described in cat liver (Breusch & Ulusoy, 1947) and in rat liver and kidney (Witter & Stotz, 1948; Meister, 1949). It has been purified about 100-fold from ox liver (Connors & Stotz, 1949). The method of purification

reported in the present paper for the enzyme from rat liver is similar to that recorded for ox liver in the early steps, but differs in the employment of column chromatography at later stages. The properties of the enzymes from ox and rat liver are similar in most respects, but an important point of difference is the higher affinity of the rat liver enzyme for triacetate (K_m 2.0×10^{-5} M versus 1.38×10^{-3} M for ox liver).

The purified (about 120-fold) enzyme from rat liver was found to be active with triacetate and fumarylacetoacetate. It has previously been suggested that 4-fumarylacetoacetate fumarylhydrolase, an enzyme involved in tyrosine degradation, was identical with the triacetate hydrolase (Ravdin & Crandall, 1951). This suggestion was based on the apparent rarity of enzymes capable of cleaving C-C bonds in ketones, and the fumarylhydrolase had never been isolated. The present work supports the presumed identity of the two enzymes; the ratio of the fumarylacetoacetate/triacetate hydrolysis rates remains constant during purification of the hydrolase. Application of purified hydrolase to the measurement of low concentrations (0.01 – $0.10 \mu\text{mole}$) of triacetate proved effective, but its usefulness is somewhat restricted by the inability to distinguish between fumarylacetoacetate and triacetate. However, the fact that triacetate can be determined in the presence of acetoacetate and triacetic acid lactone suggests that the hydrolase may prove a valuable tool in metabolic studies involving these compounds.

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