Stereochemistry of the decarboxylation of glyoxylic acid by yeast pyruvate decarboxylase

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Tritiated glyoxylic acid was incubated with pyruvate decarboxylase. The hydroxymethylthiamine diphosphate formed was ozonolysed to give tritiated glycolic acid, the absolute configuration of which was investigated by analysis using glycolate oxidase. The tritiated glycolic acid proved to be racemic. The implications of this result are discussed in relation to models for the mechanism of pyruvate decarboxylase.

Yeast pyruvate decarboxylase (PDC) is the archetypal thiamine diphosphate (TDP)-dependent enzyme. Because the source of the enzyme (baker's or brewer's yeast) is readily and abundantly available and because convenient methods are available for its purification,[1] PDC has been more thoroughly studied than other TDP-dependent enzyme. The generally agreed mechanism by which PDC catalyses decarboxylation of pyruvate is shown in Scheme 1. Deprotonation of TDP **1** generates the ylide **2**[2] which attacks pyruvate **3** to give the 2-lactyl derivative **4**. This undergoes decarboxylation to form the enamine intermediate **5** which is protonated to give hydroxyethylthiamine diphosphate (HETDP) **6**. Base-catalysed elimination regenerates the TDP carbanion **2** with release of acetaldehyde **7**.[3]

In spite of the intense activity in PDC studies, some aspects, and in particular stereochemical aspects, remain obscure. In particular, the absolute configurations of intermediates **4** and **6** are unknown. The configurations shown in Scheme 1 are inferred from molecular modelling studies[4] based on the X-ray crystal structure of PDC determined by Furey and co-workers.[5]

On the basis of experiments with [14C]pyruvate, Holzer and Beaucamp[6] claimed to have isolated 2-lactylthiamine diphosphate **4** from PDC. However, Kluger and co-workers[3a] showed that 2-lactyl-TDP **4** would most probably not have survived the isolation procedure. It seems unlikely, therefore, that 2-lactyl-TDP **4** has ever been isolated from PDC–pyruvate incubations. HETDP (as **5**, Scheme 1) has been isolated from the incubation of pyruvate with the PDC subunit of the E1 component of pyruvate dehydrogenase. The compound was laevorotatory.[7] Hydroxyethylthiamine (HET) has been

resolved.[8] Kluger and co-workers determined the absolute configuration of (+)-HET (**8**, Scheme 2) by conversion into (−)-2-(1-hydroxyethyl)-3,4-dimethyl-5-(2-hydroxyethyl)-1,3-thiazol-3-ium iodide [(−)-**9**, Scheme 2] by reaction with sodium bisulfite followed by iodomethane. This compound was shown by X-ray analysis to have the (*R*)-configuration. Accordingly (+)-HET also has the (*R*)-configuration. Since (+)-HET has been converted into (+)-hydroxyethylthiamine diphosphate [(+)-HETDP] (**10**, Scheme 2),[8a] this compound also has the (*R*)-configuration. The laevorotatory compound isolated from the PDC subunit of pyruvate dehydrogenase accordingly can be assigned the (*S*)-configuration. The significant result was obtained that both enantiomers of HETDP (as **10**, Scheme 2) reactivated wheat germ PDC and were thus converted into PDC-TDP and acetaldehyde.[9] The K_m value for the (*R*)-enantiomer was 1.5 times lower than that of the (*S*)-enantiomer.[10] These values reflect the rate of association of the coenzyme with the apoenzyme.[11] Shiobara *et al.* showed that both enantiomers of HET promote growth in rats and that both are converted into HETDP in rat liver.[12]

As Kluger has pointed out,[9] low stereoselectivity with respect to transformations of the enantiomers of HETDP by PDC does not imply that the enzyme produces racemic HETDP or that the rates of conversion of the enzyme-bound species are similar. The latter inference is based on the assumption that enzyme-bound HETDP is converted into acetaldehyde and PDC-TDP faster than it dissociates. However, given the precise architecture of the active site, these results raise questions as to how PDC manages to process both enantiomers of PDC with nearly equal facility. In their incubation experiments with
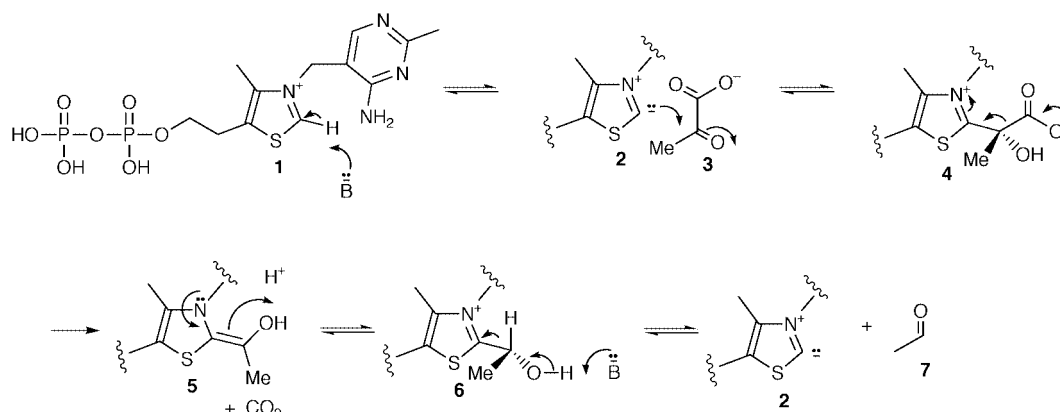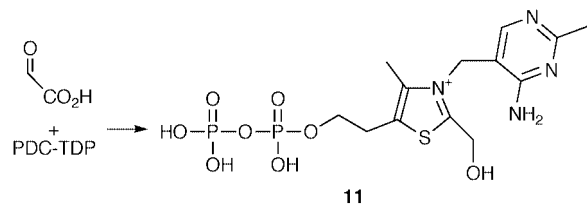

Scheme 1
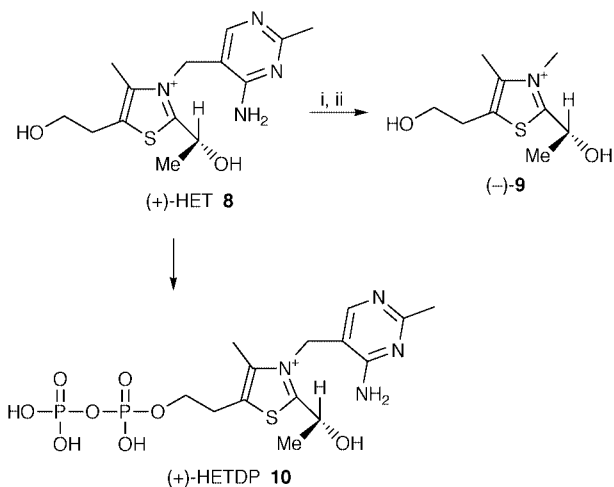
Scheme 2   *Reagents*: i. NaHSO$_3$; ii. MeI.

[^{14}C]pyruvate,[6] Holzer and Beaucamp isolated in 1% radiochemical yield a material that co-chromatographed with HETDP. However, there have been no reports of the successful quantitative isolation of HETDP from incubations of PDC with pyruvate.
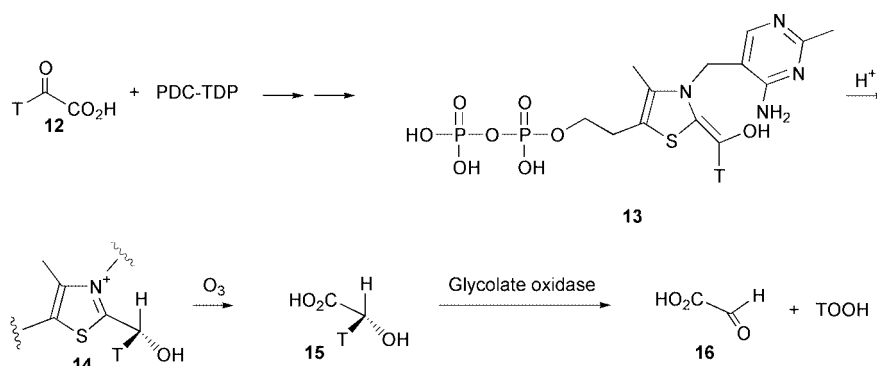
In the face of the apparently insuperable difficulty in isolating HETDP from PDC and the equivocal results obtained from the incubations with (+)- and (−)-HETDP,[9] we attempted to approach the problem of elucidating the stereochemistry of the enamine protonation step in a different way. Uhlemann and Schellenberger[13] had reported that on incubation with glyoxylic acid, yeast PDC was irreversibly inactivated and that the lower homologue of HETDP, hydroxymethyl-TDP (HMTDP, **11**, Scheme 3) could be isolated from the incubation mixture. This

Scheme 3

suggested the approach illustrated in Scheme 4. Incubation of [2-^{3}H]glyoxylic acid **12** with PDC-TDP would generate the enamine **13** after decarboxylation. A detailed analysis using molecular modelling[4] based on the X-ray structure of PDC suggested, by analogy with the hydroxyethyl analogue, that this intermediate should have the *E*-configuration shown. The expected stereospecific protonation on the *si*-face of the enamine would lead to enzyme-bound (*R*)-HMTDP **14**.

Elucidation of the stereochemistry of this process would depend on the development of a method for the chiral analysis of HMTDP **14**. The method that suggested itself was ozonol-

ysis of the adduct **14** to [^{3}H]glycolic acid **15**, the absolute configuration of which could be assigned using the glycolate oxidase analysis developed by Arigoni and co-workers.[14] The procedure described by Weber[14a] gives poor yields of glyoxylic acid. Since it was desirable to maximise the yield for the envisaged study, it was necessary for it to be improved. An increase in yield to 20% was obtained by increasing the concentration of the tris buffer used from 0.1 M to 0.33 M. However, the poor yields in this reaction have been attributed to undesirable side reactions.[15] The enzymatically produced glyoxylic acid is subject to further oxidation by the hydrogen peroxide produced to give formate and carbon dioxide.[16] A slow enzymatic conversion of glyoxylic acid to oxalic acid catalysed by glycolate oxidase also occurs.[17] Seip *et al.*[15] showed that an almost quantitative conversion can be obtained by including catalase in the incubation mixture (to destroy hydrogen peroxide) and ethylenediamine. The amine reacts with glyoxylate to generate the imine, which is then protected from further oxidation. By using this method, an 80% yield of glyoxylic acid was obtained (determined as the 2,4-dinitrophenylhydrazone).

During oxidation of glycolate by glycolate oxidase the pro-*S* hydrogen atom is retained and the pro-*R* hydrogen atom is lost (Scheme 4). The expectation, therefore, based on the molecular modelling studies[4] is that the label introduced into HMTDP **14** would be lost on subsequent conversion into glyoxylate *via* glycolic acid **15** (Scheme 4).

To initiate this investigation, HMTDP **17** was synthesised from TDP and formaldehyde in citrate buffer at pH 5.4 (Scheme 5). A product of approximately 90% purity was
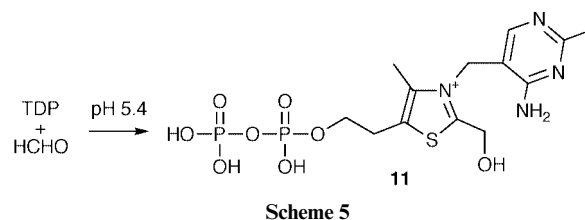
Scheme 5

obtained, the spectroscopic data for which were consistent with the proposed structure. The adduct **11** was unstable in aqueous solution at room temperature, decomposing to the extent of 77% over two days. However, it was stable on storage at −18 °C. Ozonolysis trials were carried out on adduct **11**. A GLC assay for glycolic acid was developed. However, the complexity of the product mixture from the ozonolysis experiments made positive identification and quantitation impossible. To simplify the analysis, an isotope dilution method was developed using [^{3}H]TDP **17** prepared from TDP and glyoxal (Scheme 6). The crude product mixture from ozonolysis of adduct **17** was diluted with unlabelled glycolic acid. The diluted compound was converted into the *p*-bromophenacyl ester **18** which was purified by chromatography and recrystallised to constant activity. Comparison of the specific activity of the product with that of the starting adduct **17** indicated a conversion of 0.15%,
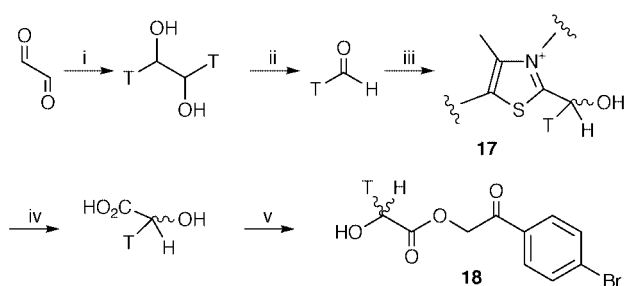
Scheme 4

Scheme 6 *Reagents*: i. Na[³H]BH₄; ii. NaIO₄; iii. TDP; iv. O₂; v. *p*-bromophenacyl bromide.

which was reproducible. To minimise over-oxidation by hydrogen peroxide produced in the ozonolysis, experiments were carried out in the presence of either manganese dioxide or catalase, to decompose hydrogen peroxide. Subsequent investigations with [³H]HMTDP **17** produced from [³H]glyoxylic acid using TDP-PDC indicated that the true conversion was much higher. The low conversions obtained in these experiments are almost certainly attributable to the partial decomposition of the labelled material. However, because of the apparently low yields in the ozonolysis experiments it was considered that glyoxylic acid of as high specific activity as possible would be needed. A simple and direct method for obtaining the required glyoxylic acid was by reduction of oxalic acid with amalgamated magnesium.[18] By minor modification of the published procedure, a 39% yield of glyoxylic acid was obtained in trial experiments. Validation of the method was carried out by reduction in [²H₂]H₂O. NMR analysis of the glyoxylic acid produced indicated a deuterium incorporation of 61%. Finally, repetition of the experiment with [³H₂]H₂O of high specific activity gave sodium [³H]glyoxylate of specific activity 177 MBq mmol⁻¹.

Tritiated sodium glyoxylate (**19**, Scheme 7) was incubated with PDC in the presence of pyruvamide in citrate buffer at pH 6.0 for thirty minutes. Protein was precipitated with trichloroacetic acid and filtered off. The filtrate, containing tritiated glycolic acid **20**, was subjected to ozonolysis and the *p*-bromophenacyl ester **21** of glycolic acid was prepared after dilution with unlabelled glycolic acid as before. The derivative was purified to constant specific activity by chromatography followed by recrystallisation. In both the trial experiments and the experiment described here, the specific activity of the material obtained after column chromatography was unchanged after three further recrystallisations.

To prepare for the glycolate oxidase assay the tritiated *p*-bromophenacyl ester was saponified and extracted to remove *p*-bromophenacyl alcohol. The residue was diluted with unlabelled glycolic acid and the radiochemically pure glycolic acid **22** was oxidised with glycolate oxidase. The glyoxylic acid **23** produced was converted into the 2,4-dinitrophenyl-

hydrazone **24** and recrystallised to constant radioactivity. Comparison of the specific activity of the starting sodium glycolate (4.2 kBq mmol⁻¹) with that of the 2,4-dinitrophenyl-hydrazone of glyoxylic acid (2.1 kBq mmol⁻¹) indicated that exactly half of the tritium label had been lost from the glycolic acid. It was concluded that the HMTDP produced in the reaction of PDC with [³H]glyoxylic acid was racemic. This result is susceptible to only a limited number of explanations. For example, it would result from protonation on both *re*- and *si*-faces of the enamine intermediate **5** (Scheme 1). This would require two acidic groups at the active site capable of protonating the enamine at equal rates. This would be unprecedented and in any event quite inconsistent with the molecular modelling studies.[4] The second possibility is that the enamine **5** (Scheme 1) undergoes rapid *Z,E*-isomerisation before protonation. The feasibility of this process cannot be determined experimentally but calculations indicate that the barrier to rotation is high. Friedemann and Breitkopf have calculated a potential energy barrier of >150 kJ mol⁻¹ for the *E→Z* isomerisation in HMTDP[19] which would make it extremely slow at room temperature. (The Gibbs free energy for the rotation is likely to differ from the enthalpy of activation by only a few kJ mol⁻¹ by comparison with the internal rotation in amides.[20]) A third possibility is that reversible elimination–addition of formaldehyde occurs at the active site. In order to examine the possible consequences of this, it is necessary to consider the mechanism of PDC action in some detail. The protonated form of the aminopyrimidine component of TDP has been strongly implicated in the PDC mechanism. The X-ray crystal structure shows that Glu51 is ideally placed to protonate N′1 greatly increasing the acidity of the protons of the 4′-amino group (**25→26**, Scheme 8). We have suggested that deprotonation of the thiazolium system is initiated by deprotonation of a water molecule by an initially ionised Glu477 to give a hydroxide ion, or an incipient hydroxide ion, the formation of which is stabilised by interaction with Asp28 and the 4′-amino group of the protonated pyrimidine component (**26→27→28**, Scheme 8). Glu51 is therefore seen as playing a crucial role in assisting deprotonation of the thiazolium system.[21] Experimental support for this has come from mutagenesis studies in which an E51Q mutant of PDC was shown to bind TDP as strongly as the wild type enzyme, but with only 0.04% the activity of the wild type enzyme and in which the rate of deprotonation of the thiazolium system was markedly decreased.[22]

Following deprotonation of the thiazolium system, the enzyme is poised to attack pyruvate (**29**, Scheme 8). Stabilisation of the resulting dianion is available through interaction of the pyruvate carboxylate group with the now protonated Glu477 and of the incipient alkoxide group by the 4′-amino group.[23,24] The essential role of the pyrimidine system and the functionality required for it to participate normally in the PDC system have been elucidated in detail by Schellenberger and co-workers.[24] Proton transfer can then occur to give the α-lactyl
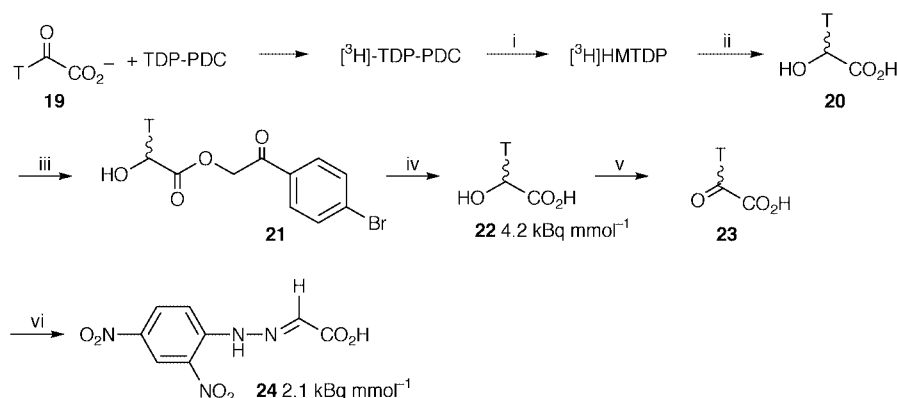
Scheme 7 *Reagents*: i. CCl₃CO₂H; ii. O₃; iii. *p*-bromophenacyl bromide; iv. OH⁻/H₂O; v. glycolate oxidase; vi. 2,4-dinitrophenylhydrazine.
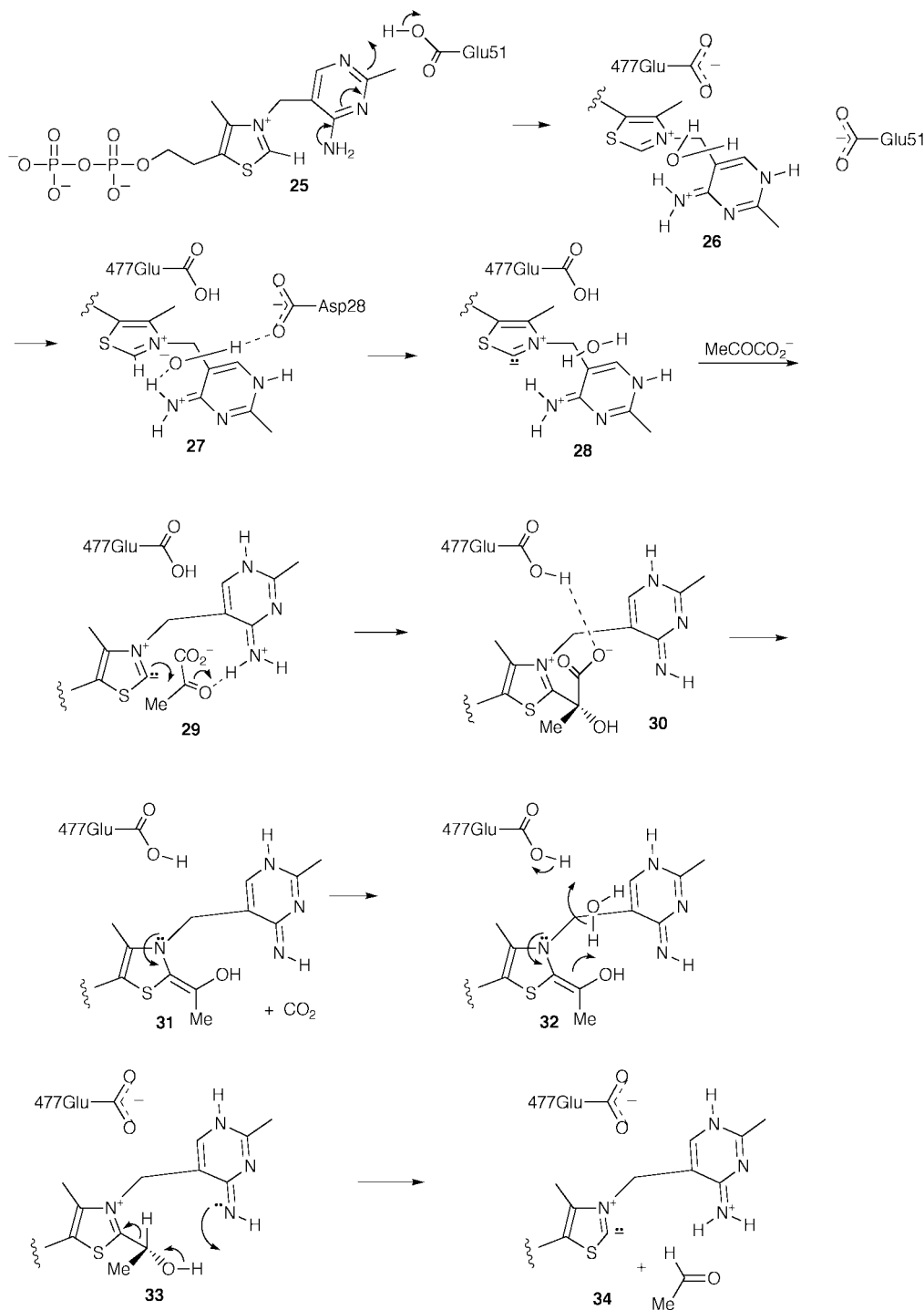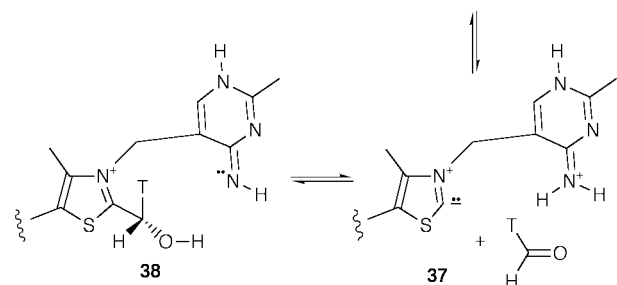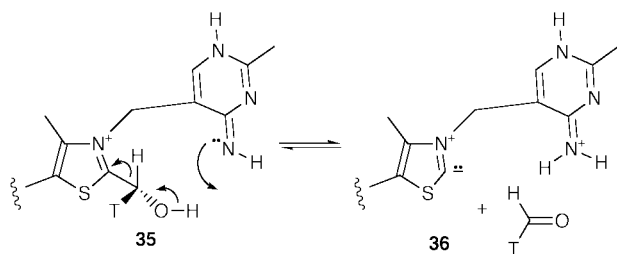
Scheme 8

intermediate **30**. Molecular modelling studies show that the carbon–carboxylate bond is ideally oriented, orthogonal to the thiazolium system, for decarboxylation (**30**→**31**, Scheme 8), to generate the *E*-enamine **32**. Glu477 is now placed to participate, *via* a water molecule, in protonation of the enamine **32** to generate the HETDP **33**, on which the *R*-configuration is imposed by the three-dimensional arrangement of the catalytic groups. The deprotonated 4′-amino group can now deprotonate the hydroxy group of HETDP, catalysing release of acetaldehyde (**33**→**34**). It should be noted that at the end of this sequence, the key catalytic groups (Glu477, the thiazolium and pyrimidine systems) are all in the correct ionisation state to initiate a new catalytic cycle.

The precise choreography of these proton transfer steps would be disrupted if water molecules were able to move freely in and out of the active site. However, experiments

by Washabaugh and co-workers[23] and our own studies[21] have shown that during single turnover in pyruvate decarboxylation and during interaction of HMPDC with acetaldehyde to give lactaldehyde, respectively, the active site is insulated from bulk water.

The most probable explanation for the formation of "racemic" HMTDP lies in the reversibility of the interconversion of intermediates **35** and **36** (Scheme 9), analogous to intermediates **33** and **34** (Scheme 8) in the pyruvate decarboxylation mechanism. Rotation of the [^{3}H]HCHO about the C_2 axis in the active site followed by *re*-face attack would lead to the intermediate **38**, diastereoisomeric with initially produced [^{3}H]HMTDP **35**. That there is ample room for such rotational movement at the active site is known from stereochemical studies of the formation of scalemic products with consistent and reproducible ratios of enantiomers in the formation of

Scheme 9

Scheme 10

acetoin and lactaldehyde in PDC-catalysed reactions.[25] Precise microscopic reversibility of reaction **35**→**36** would require that the adduct **35** would be reformed with the initial configuration. It is probable therefore that the arrangement shown in **36** might represent two different states, one in which the released formaldehyde is associated tightly with the ylide and one in which it can assume a different position and in which it is free to rotate. The second position might be required for the decarboxylation of pyruvate, and comprise the last step in the decarboxylation manifold (using Schowen's terminology[26]) prior to release of acetaldehyde to the medium. It should be noted that the efficiency of an enzymatic reaction requires there to be an effective mechanism for product release. For any enzyme, the required mechanism will be tuned to the natural substrate–product system and will, in general, be less favourable for analogues. In addition, the thermodynamic equilibrium between species **33** and **34** (Scheme 8) may favour the bound form of formaldehyde relative to the corresponding acetaldehyde adduct HETDP.

The rotation about the C_2 axis proposed above would not put a limitation on the overall rate of decarboxylation. The rotational frequencies for [³H]HCHO are given by eqn. (1),

$$v = \frac{Kh}{4\pi^2 I_a} \qquad (1)$$

where $K = 0,1,2\ldots$, h is Planck's constant and I_a is the moment of inertia about the C_2 axis. The lowest frequency, corresponding to $K = 1$, given by this equation is 325500 MHz, corresponding to a period of rotation of 3.07 picoseconds. This is far in excess of the frequency needed to ensure complete equilibration between the two isotopomeric forms of the HMTDP. (We are grateful to Professor A. C. Legon for the above analysis.)

It should be noted that the proposed mechanism for equilibration of HMTDP isotopomers is consistent with the observation, noted above, that both enantiomers of HETDP are processed by PDSC.[9] Neither set of experiments proves the hypothesis of free rotation of formaldehyde at the active site coupled to reversible formation–dissociation of HMTDP or HETDP. However, these two different types of observation are internally consistent and together provide strong support for the hypothesis.

The mechanism for deprotonation of the thiazolium system proposed here (**26**→**28**, Scheme 8) differs from that proposed by Schellenberger[3d] who suggests that Glu51 in the ionised form deprotonates the amidine tautomer of the pyrimidine (Scheme 10). However, such amidine tautomers are only observed under
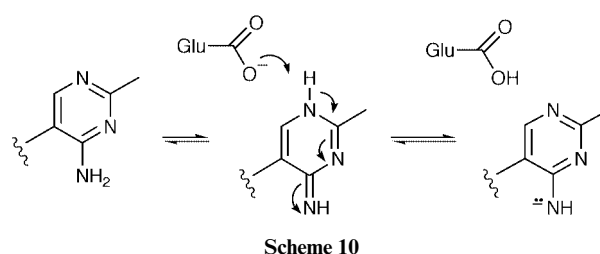
special circumstances and certainly represent high energy species. Deprotonation of such a weak acid also appears unlikely. Washabaugh[27] has suggested that there might be significant proton transfer in the transition state leading to attack on pyruvate (Scheme 11). This suggestion was based on studies
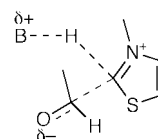
Scheme 11

of retro-acyloin reactions of 2-(1-hydroxyethyl)-3-*R*-4-methyl-thiazolium ions and is summarised in Scheme 11. The mechanism shown in Scheme 8 (**26**→**27**→**28**) would not be inconsistent with this proposal.

PDC is unusual in that it is subject to substrate activation, a phenomenon that gives rise to sigmoidal kinetics. Pyruvamide may replace pyruvate in the activation of the enzyme.[28] (All of the PDC reactions described here were carried out with concentrations of pyruvamide appropriate for maintaining PDC in its catalytically active state.) Kern *et al.*[29] have measured the rates of H–D exchange in TDP by NMR. The rate of exchange in TDP-PDC was accelerated three-fold relative to the deprotonation of free TDP. The rate found was one order of magnitude too small to account for the observed catalytic constant (10 s⁻¹ at 4 °C). However, in the presence of pyruvamide, the rate of deprotonation increased by three orders of magnitude, more than enough to accommodate the observed catalytic constant for wild type PDC. Kern *et al.*[29] accordingly see no need to invoke a concerted mechanism (Scheme 11) as proposed by Washabaugh.[27] However, a *caveat* must be entered because the deprotonation studies[29] were carried out in the absence of substrate. As noted above, during turnover, the active site of PDC is isolated from bulk water. Measurements of the rate of deprotonation in the absence of substrate, while informative, may therefore not correspond to the rate of deprotonation in the presence of substrate.

Experimental

Thiamine diphosphate chloride was purchased from Sigma. Sodium [³H]borohydride (230 µl, 5 mCi, 22.50 mCi cm⁻³) and [³H]water (200 µl, 1.0 Ci, specific activity 5 Ci cm⁻³) were both purchased from Amersham Life Sciences. Pyruvate decarboxylase (PDC) (60 mg) from *Saccharomyces cerevisiae* was provided by Professor G. Hübner (Martin-Luther University, Halle, Germany) as a suspension in 3 M ammonium sulfate. The suspension contained 38 mg cm⁻³ protein with a specific activity of 20 U mg⁻¹ protein. Glycolate oxidase (from sugar beet, suspension in 2.4 M (NH₂)₂SO₄–10 mM Tris pH 8.3 containing 5 mM flavin mononucleotide) was purchased from Sigma. The suspension contained 0.43 mg cm⁻³ protein with a specific activity of 19 U mg⁻¹ protein. Catalase (from bovine liver, suspension in water containing 0.1% thymol) was purchased from Sigma. The suspension contained 84 mg cm⁻³ protein with a specific activity of 44000 U mg⁻¹ protein.

For flash chromatography, 400–230 mesh silica gel 60 (E.Merck No. 9385) was used. TLC analysis was carried out on silica gel plates (Merck 1.05554 Kieselgel 60 F254) and cellulose F coated plastic sheets (Merck No. 5565). Compounds were visualised by UV light (254 nm) and iodine staining. Ozonolysis was carried out using the Boc Mk II ozoniser. Gas chromatography analysis was carried out on a Shimadzu GC-14A gas chromatograph. Scintillation counting on tritium-containing compounds was determined on a Tri-Carb 2000CA liquid scintillation analyser. NMR spectra were obtained at 250 MHz using a Bruker AFC250 spectrometer. Chemical shifts are given in parts per million (δ) and referenced to internal SiMe$_4$. J Values are given in Hz. Mass spectra were determined using a Kratos MS80 spectrometer. All melting points were measured on a Hoover capillary melting point apparatus and are uncorrected.

2-Hydroxymethylthiamine diphosphate (HMTDP) 11

Thiamine diphosphate chloride (1 g, 2.2 mmol) was dissolved in citrate buffer (0.05 M, pH 5.4, 5 cm^3) and titrated with NaOH (1 M) to pH 5.6. Formaldehyde solution (37% w/v, 1.80 cm^3, 22.2 mmol) was added dropwise and the mixture was stirred at room temperature for 24 h. The HMTDP was isolated from the salts by exclusion chromatography on a Sephadex G-25-80 column (2.5 × 30 cm) using distilled water as the eluant. The product fractions were freeze dried to leave HMTDP **11** as a colourless flaky solid (0.925 g) which was shown by ^{1}H NMR to be 90% HMTDP and 10% TDP, R_f (0.31; EtOH–MeCO$_2$H, 2:1 v/v, cellulose F coated plastic sheet); δ_H (250 MHz; D$_2$O) 2.51 (3 H, s, CH$_3$), 2.55 (3 H, s, CH$_3$), 3.25 (2 H, dd, J 5.0, J 5.0, CH_2CH$_2$O), 4.12 (2 H, dt, J 5.0, J 5.0, CH$_2$OP), 4.97 (1.8 H, s, CH_2OH), 5.41 (2 H, s, CH$_2$N$^+$), 7.99 (0.9 H, s, Ar-H); δ_C (75 MHz; D$_2$O) 11.9 (CH$_3$), 23.8 (CH$_3$), 28.3 (CH$_2$CH$_2$O), 51.2 (CH$_2$OP), 64.8 (CH$_2$OH), 65.4 (CH$_2$N$^+$), 103.2, 107.0, 136.2, 144.0, 152.1, 161.6, 167.3 (Ar) (note: C-1, C-2 and C-5 resonances appear twice due to 10% presence of TDP); m/z (FAB) 477 [(M + Na)$^+$, 19%], 455 [(M + H)$^+$, 21], 437 [(M − OH)$^+$, 100].

Ozonolysis of HMTDP 11

HMTDP (300 mg, 0.65 mmol) was dissolved in deionised water (10 cm^3) with or without manganese oxide (5 mg). The solution was set up for ozonolysis at 0 °C (ice–water bath). The oxygen was supplied to the ozoniser at a controlled flow rate of 0.5 dm^3 min^{-1}. The ozonolysis was followed by TLC by observing the disappearance of the HMTDP (EtOH–acetic acid (2:1 v/v), cellulose F coated plastic sheet or MeOH–EtOAc–water (1:9:1), silica gel coated aluminium sheet).

p-Bromophenacyl ester of glycolic acid (as 18)

Glycolic acid (0.5 g, 6.58 mmol) was dissolved in water (5 cm^3). The solution was brought to pH 7 (0.1 M NaOH). A few drops of very dilute HCl solution were added to the final solution until it was faintly acidic. *p*-Bromophenacyl bromide (1.83 g, 6.58 mmol) dissolved in 95% ethanol in water (20 cm^3) was added and the mixture was boiled under reflux for 1 h. The solution was allowed to cool. The precipitated material was filtered off and washed with a little ethanol and water. Recrystallisation (aqueous ethanol) gave the derivative as colourless flaky crystals (1.44 g, 80%) mp 136–138 °C, R_f (0.25; toluene–EtOAc, 2:1 v/v) (Found: C, 43.5; H, 3.41. C$_{10}$H$_9$O$_4$Br requires C, 43.9; H, 3.30%); δ_H (250 MHz; DMSO-d$_6$) 4.18 (2 H, d, J 6.0, CH_2OH), 5.52 (3 H, s, COOCH$_2$, OH [exchanges with D$_2$O]), 7.79 (2 H, d, J 8.5, Ar-H), 7.90 (2 H, J 8.5, Ar-H); δ_C (75 MHz; DMSO-d$_6$) 59.6 (CH$_2$OH), 66.6 (COOCH$_2$), 128.5, 130.1, 132.4, 133.0 (ArH), 172.5 (COO), 192.5 (COAr); m/z (CI, NH$_3$) 292 [(M + NH$_4$)$^+$, 12%], 290 [(M + NH$_4$)$^+$, 12%], 185 [(CO − C$_6$H$_4$ − ^{81}Br)$^+$, 100], 183 [(CO − C$_6$H$_4$ − ^{79}Br)$^+$, 100].

[³H]Formaldehyde

To a stirred, cooled (ice bath) mixture of glyoxal (40% w/v aqueous solution) (1.28 g, 8.79 mmol) and ethanol (5 cm^3) was added dropwise a solution of sodium borohydride (0.167 g, 4.39 mmol) and sodium [³H]borohydride (in 0.1 M NaOH) (45 μl, 1.0 mCi or 37 MBq, 22.50 mCi cm^{-3}, specific activity 26.0 Ci mmol^{-1}) in water (2 cm^3). On completion of the addition, the mixture was stirred for 1 h at room temperature. Concentrated hydrochloric acid was added dropwise to the stirred solution to bring the pH from 12.2 to about 3–4. Ethanol and water were removed under reduced pressure at 35 °C to leave a white slurry containing ethylene glycol and salts. A solution of sodium periodate (1.68 g, 7.79 mmol) in water (15 cm^3) was added dropwise to the slurry. The mixture was stirred for an additional 45 min. The flask was set up for simple distillation at atmospheric pressure, and several fractions were collected at 98–100 °C. The presence of formaldehyde in each fraction was shown by the spot test with chromotropic acid and the % (w/v) formaldehyde in solution estimated by a quantitative titration method (see below). The fractions containing formaldehyde were combined (10.9 cm^3) and after analysis were shown to contain 2% w/v formaldehyde (0.22 g, 7.27 mmol, 46.4% yield, total activity 27.2 MBq, specific activity 3.74 MBq mmol^{-1}); δ_C (75 MHz; D$_2$O) 84.7 (CH$_2$(OH)$_2$).

Spot test for formaldehyde

A drop of the test solution was mixed with sulfuric acid (6 M, 2 cm^3). A little solid chromotropic acid (1,8-dihydroxynaphthalene-3,6-sulfonic acid) was added and the mixture was heated for 10 min in a water bath at 70 °C. A bright violet colour appeared in the presence of formaldehyde.

Quantitative estimation of formaldehyde [30]

To a 100 cm^3 conical flask, accurately weighed formalin solution (2.5 cm^3) was added. Water (50 cm^3) was added and the mixture was transferred carefully through a small funnel into a 500 cm^3 graduated flask. The conical flask and the funnel were thoroughly rinsed out with water, the solution in the graduated flask made up to the mark and then mixed well. An aliquot (25 cm^3) of this solution was transferred by means of a pipette into a conical flask and iodine solution (0.1 M, 50 cm^3) was added. NaOH solution (10% w/v) was added until the liquid became pale yellow in colour. The mixture was allowed to stand with occasional stirring at room temperature for 10 min. The solution was acidified (dilute HCl) to liberate the remaining iodine which was titrated with sodium thiosulfate solution (0.1 M) using starch as an indicator.

[³H]HMTDP 17

[³H]HMTDP **17** was prepared following the procedure described for the synthesis of unlabelled hydroxymethyl-TDP **11**. The total volume of 2% w/v [³H] formaldehyde (**17**) used in the synthesis was 6.5 cm^3 (0.13 g, 4.33 mmol, 16.2 MBq), together with 0.5 g TDP (1.09 mmol). [³H]HMTDP **17** was isolated as a white solid (682 mg) which was shown by TLC to contain some TDP. The total activity of the product mixture was 5.23 MBq (3.49 MBq mmol^{-1}).

Ozonolysis of [³H]HMTDP 17 and isolation of the *p*-bromophenacyl ester of glycolic acid 18

General procedure. Ozonolysis of an aqueous solution of [³H] HMTDP **17** (100 mg, 0.77 MBq) with unlabelled HMTDP **1** (100 mg), total activity (1.75 MBq mmol^{-1}), was carried out three times either in the presence (5 mg) or absence of MnO$_2$ or with catalase (from bovine liver, 185000 U).

On completion of the reaction (TLC), solid MnO$_2$ was removed by filtration (Celite), glycolic acid (100 mg, 1.32 mmol)

was added and the solution was neutralised with NaOH (0.1 M). The mixture was freeze dried and the solid obtained (460 mg) was dissolved in distilled water (5 cm³) and a few drops of very dilute HCl solution were added to make the solution faintly acidic. *p*-Bromophenacyl bromide (0.57 g, 2.05 mmol) dissolved in 95% ethanol in water (7 cm³) was added and the mixture was boiled under reflux for 1 h. The resulting solution was concentrated under reduced pressure to give a dark orange–brown solid which was added to distilled water (20 cm³). The mixture was extracted with ethyl acetate (2 × 20 cm³). The combined organic extracts were dried (MgSO₄) and evaporated to leave a creamy yellow solid. The *p*-bromophenacyl ester of glycolic acid was isolated by flash chromatography (R_f 0.25; toluene–EtOAc, 2 : 1 v/v). Recrystallisation (aqueous ethanol) gave a colourless flaky solid of uniform specific activity; in the presence of MnO₂ (135 mg, 1150 Bq, 2.33 kBq mmol⁻¹); in the absence of MnO₂ (145 mg, 1127 Bq, 2.12 kBq mmol⁻¹); with catalase (161 mg, 1258 Bq, 2.13 kBq mmol⁻¹).

Hydrolysis of the *p*-bromophenacyl ester of glycolic acid (as 18)

The ester (100 mg, 0.37 mmol) was dissolved in ethanol (5 cm³). To the solution was added NaOH (1 M, 1 cm³, 1 mmol). The mixture was stirred for 30 min. The solvents were removed under reduced pressure and the solid obtained was dissolved in water (20 cm³). The solution was extracted with dichloromethane (2 × 20 cm³). HCl (1 M) was added to neutralise the aqueous solution. The cream coloured solid which precipitated on addition of acid, was removed by filtration under suction. The filtrate was freeze dried to give a white coloured solid (136 mg) containing sodium glycolate and NaCl.

Enzymatic conversion of glycolic acid into glyoxylic acid

Glycolic acid (20 mg, 0.26 mmol) was dissolved in a solution of ethylenediamine (0.33 M, 1 cm³). To this solution was added glycolate oxidase (from sugar beet, suspension in (NH₄)₂SO₄ (2.4 M) containing Tris buffer (10 mM, pH 8.3) and containing flavin mononucleotide (5 mM) [240 µl, 2 U]) and catalase (from bovine liver) (5 µl, 18000 U). The mixture was stirred at 30 °C under oxygen (O₂ balloon) for 24 hours. The yield of glyoxylate formed from the reaction (80%) was determined by the preparation of its dinitrophenylhydrazone derivative (see below). The mass of the dinitrophenylhydrazone derivative obtained was 53 mg (80% yield).

2,4-Dinitrophenylhydrazone of glyoxylic acid (as 24)

To 2,4-dinitrophenylhydrazine (100 mg, 0.51 mmol) was added conc. H₂SO₄ (1 cm³) and water (1 cm³). The mixture was swirled to dissolve the reagent, cooled and added to the aqueous solution of glyoxylic acid. The solid formed was filtered with suction, washed with aqueous methanol (1 : 1, 5 cm³) and dried.

Glyoxylic acid from the reduction of oxalic acid [18]

Oxalic acid dihydrate (1.26 g, 10 mmol) was dissolved in HCl (1 M, 10 cm³) and the solution was placed in an ice–salt bath. Saturated aqueous mercuric chloride solution (5 drops) was added followed by Mg turnings (500 mg, 20 mmol), added in small portions, with constant stirring, over a period of 45 min. The pH was maintained at 3.0 by dropwise addition of conc. HCl. The grey paste of magnesium salts was filtered off (Celite). To the clear filtrate was added an equal volume of a freshly prepared 20% solution of sodium bisulfite. The solution was stirred and 10 cm³ of 95% ethanol was carefully layered over the solution without mixing. When crystals of the bisulfite addition compound began to form at the interface, the mixture was vigorously stirred, left at 4 °C overnight and filtered. The addition product was decomposed by dissolving it in HCl (1 M, 5 cm³), evaporating the solution to 2.5 cm³, adding 2.5 cm³ of water and repeating the evaporation to ensure complete

removal of SO₂. NaOH (1 M) was added to bring the solution to pH 8 and the solution was evaporated to dryness under reduced pressure to give a colourless solid mixture of sodium glyoxylate and sodium chloride (1.32 g). The glyoxylate was derivatised with 2,4-dinitrophenylhydrazine (0.7 g, 3.54 mmol) as described above. The mass of the dinitrophenylhydrazone obtained was 524 mg (2.06 mmol) corresponding to 198 mg of glyoxylic acid obtained (21%); mp 186–188 °C (Found: C, 37.2; H, 2.47; N, 21.7. C₈H₆O₆N₄ requires C, 37.8; H, 2.38; N, 22.0%); δ_H (250 MHz; DMSO-d₆) 7.97 (1 H, s, CH=N), 7.98 (1 H, d, *J* 9.5, Ar-H), 8.43 (1 H, dd, *J* 9.5, *J* 2.4, Ar-H), 8.81 (1 H, d, *J* 2.4, Ar-H), 11.83 (1 H, s, NH); δ_C (75 MHz; DMSO-d₆) 117.6 (C-1), 122.6, 130.0, 131.4, 138.9, 144.0, 164.4 (Ar); (EI) *m/z* 254 [(M)⁺, 21%].

Glyoxylic acid from the reduction of oxalic acid: modified procedure

Oxalic acid dihydrate (0.126 g, 1 mmol) was dissolved in distilled water (1 cm³) and HCl (10 M, 0.1 cm³) and solution was placed in an ice–salt bath. One drop of a saturated aqueous mercuric chloride solution was added followed by Mg turnings (50 mg, 2 mmol), added in small portions, with constant stirring, over a period of 45 min. The solution was stirred for a further 30 min. The grey paste of magnesium salts was filtered off (Celite) and the clear filtrate was passed through Dowex 50W-X8 ion exchange resin (7 g, 12 cm high). The fractions collected were combined, one drop of phenolphthalein solution was added and the solution was titrated with NaOH (1 M) until the solution acquired a permanent faint pink colour. The water was removed under reduced pressure at 35 °C. A white solid remained (165 mg) containing sodium glyoxylate and NaCl.

Sodium [2-²H]glyoxylate

The procedure was identical to the one described above but using [²H₂O] (1 cm³) instead of distilled water to give a colourless solid mixture of [2-²H] sodium glyoxylate and sodium chloride (180 mg). The glyoxylate was derivatised as above to give the 2,4-dinitrophenylhydrazone (96 mg) (corresponding to 36 mg glyoxylic acid [37%]); δ_H (250 MHz; DMSO-d₆) 7.98 (1.32 H, d, *J* 9.5, H-3, superimposed s, H-3), 8.43 (1.05 H, dd, *J* 9.5, *J* 2.4, H-4), 8.81 (0.80 H, d, *J* 2.4, H-5), 11.83 (1 H, s, H-2); (EI) *m/z* 255 [(M)⁺, 21%]. On average, integration yielded $(1.0 + 0.80 + 1.05)/3 = 0.95$ arbitrary units per proton, showing that $(1.32 − 0.95) = 0.37$ of the integral for the superimposed signal at 7.98 ppm can be assigned to the partly deuteriated position. Therefore the hydrogen content in this position is $(0.37/0.95) \times 100 = 38.9\%$, deuterium content $(100 − 38.9) = 61.1\%$.

Sodium [2-³H]glyoxylate

The procedure was identical to the one described above for the synthesis of unlabelled sodium glyoxylate (modified procedure) using tritiated water (0.2 cm³, 1.0 Ci or 37 GBq, specific activity 5 Ci cm⁻³), distilled water (0.7 cm³) and HCl (10 M, 0.1 cm³). A white solid was obtained (175 mg, 1.8 mCi or 66.3 MBq), containing sodium chloride and sodium glyoxylate ([2-³H]), 177 MBq.

PDC-catalysed formation of [³H]HMTDP from sodium [2-³H]-glyoxylate

The incubation mixture contained in 3.60 cm³ final volume, sodium [2-³H]glyoxylate (10 mM) (16.82 mg mixture of salts, 3.46 mg glyoxylate, 36 µmol, activity 6.37 MBq, dissolved in citrate buffer (0.1 M, pH 6.0, 1 cm³), pyruvamide (80 mM [25.1 mg, 288 µmol, dissolved in citrate buffer (0.1 M, pH 6.0, 1 cm³)]) and PDC from baker's yeast in ammonium sulfate (3 M, 1.6 cm³, 38 mg/1.0 cm³, total protein 60.8 mg, 1 µmol). The mixture was incubated at room temperature for 30 min. Trichloroacetic

acid (5% in distilled water, 0.1 cm³, 0.185 g) was added. After 20 min the precipitated protein was removed by centrifugation. The solution was filtered and neutralised with NaOH (0.1 M).

Ozonolysis of [³H]HMTDP 20

Ozonolysis of the [³H]HMTDP solution (3.7 cm³) to which had been added unlabelled HMTDP **11** (100 mg, 0.22 mmol) was carried out in the presence of catalase (from bovine liver, 200 µl, 185000 U) overnight. On completion of reaction (TLC), unlabelled glycolic acid (50 mg, 0.66 mmol) was added and the solution was neutralised (0.1 M NaOH). The mixture was freeze dried and the solid obtained (1.12 g) was dissolved in distilled water (5 cm³). A few drops of very dilute HCl solution were added to make the solution faintly acidic. *p*-Bromo-phenacyl bromide (0.45 g, 1.62 mmol) dissolved in ethanol in water (95%, 10 cm³) was added and the mixture was boiled under reflux for 1 h. The resulting solution was concentrated under reduced pressure to give a dark orange–brown solid which was added to distilled water (20 cm³). The solution was extracted with ethyl acetate (2 × 20 cm³). The combined organic extracts were dried (MgSO₄) and evaporated to leave a creamy yellow solid. Flash silica gel chromatography (R_f 0.25; toluene–EtOAc, 2:1 v/v) gave the *p*-bromophenacyl ester of [2-³H]-glycolic acid (**21**) (69 mg, 12.09 kBq, 47.8 kBq mmol⁻¹). Two recrystallisations (aqueous ethanol) gave a derivative of constant specific activity (42 mg, 7.63 kBq, 49.60 kBq mmol⁻¹).

Hydrolysis of the *p*-bromophenacyl ester of [2-³H]glycolic acid

The *p*-bromophenacyl ester **21** of [2-³H]glycolic acid (14 mg, 0.05 mmol, 2.54 kBq) was dissolved in ethanol (3 cm³) and to the solution was added NaOH (1 M, 0.1 cm³, 0.1 mmol). The mixture was stirred for 0.5 h. The solvent was evaporated under reduced pressure. The solid obtained was dissolved in water (10 cm³) and washed with dichloromethane (2 × 10 cm³). The solution was brought to pH 7 (1 M HCl). The cream coloured solid which precipitated was filtered off. To the filtrate was added unlabelled glycolic acid (40 mg, 0.53 mmol) and the pH was adjusted to 8.0 (1 M NaOH). The solution was freeze dried to give a colourless solid (120 mg) containing sodium glycolate (56 mg, 0.57 mmol, 4.20 kBq mmol⁻¹) and NaCl (total activity 2.39 kBq).

Enzymatic conversion of [2-³H]glycolate 22 into [2-³H]glyoxylate 23

To a solution of ethylenediamine (0.33 M, 1 cm³) was added 58 mg of the mixture containing sodium [2-³H]glycolate (27 mg, 0.276 mmol, 4.20 kBq mmol⁻¹). To this solution were added glycolate oxidase (from sugar beet, suspension in 2.4 M (NH₂)₂SO₄, in 10 mM Tris buffer, pH 8.3, and containing 5 mM flavin mononucleotide) (240 µl, 2 U) and catalase (from bovine liver) (5 µl, 18000 U). The mixture was stirred at 30 °C under oxygen (O₂ balloon) for 24 h. The solution was brought to pH 2 (1 M HCl). The product was derivatised with 2,4-dinitro-phenylhydrazine (110 mg, 0.55 mmol) according to the general procedure described above to give the 2,4-dinitrophenyl-hydrazone **24** of [2-³H]glyoxylic acid 56 mg (0.22 mmol, 80%, activity 427 Bq). The derivative was recrystallised three times (ethanol) to give each time a solid of constant specific activity (2.10 kBq mmol⁻¹).

Acknowledgements

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