BBA 66052

HYDROLYSIS, RACEMIZATION AND ABSOLUTE CONFIGURATION OF UREIDOGLYCOLATE, A SUBSTRATE OF ALLANTOICASE

E. J. 's-GRAVENMADE, G. D. VOGELS AND C. VAN DER DRIFT

Department of Physical Chemistry and Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)

(Received September 12th, 1969)

SUMMARY

1. A method is given for the accumulation and purification of (−)- and (+)-ureidoglycolate with the aid of allantoicase bound to Ecteola-cellulose. The optical rotation of these compounds was determined as a function of pH, concentration and wavelength. From the sign of the Cotton effects at 208 and 228 mμ it was derived that (−)- and (+)-ureidoglycolate are S- and R-ureidoglycolate.

2. The rates of hydrolysis and racemization were studied as a function of pH; both reactions are susceptible to acid catalysis and the former reaction also to base catalysis. The results were compared with allantoate hydrolysis and glyoxylate hydration and a general reaction mechanism is suggested in which water and urea compete for the double bonds in glyoxylate and allanturate.

3. The hydrolysis of ureidoglycolate is catalyzed by bivalent cations and phosphate ions. The complex formation was studied by the use of conductance measurements, electron paramagnetic resonance and infrared spectra. The mechanism of catalysis is discussed in view of the action mechanism of allantoicase.

INTRODUCTION

Allantoicase (allantoate amidinohydrolase, EC 3.5.3.4) catalyzes the conversion of allantoate (diureidoacetate) into (−)-ureidoglycolate and urea and the conversion of (+)-ureidoglycolate into glyoxylate and urea[1]. Previous papers dealt with the purification[2], the specificity of binding subsites[3], and the role of metal ions in the catalytic action and stability[4] of the enzyme from *Pseudomonas aeruginosa*. To reveal information essential to the interpretation of the enzymic reaction, the chemical reaction catalyzed is studied now and special attention will be given to the bivalent cation-promoted hydrolysis of ureidoglycolate. The results will be compared with the racemization of ureidoglycolate, the hydrolysis of allantoate[5] and the dehydration of glyoxylate hydrate[6], and a reaction mechanism common to these processes will be proposed. A study of the absolute configuration of the optical antipodes of ureidoglycolate reveals information on the steric arrangement of the binding subsites of the enzyme.

EXPERIMENTAL

Materials

Sodium ureidoglycolate was prepared according to VALENTINE AND WOLFE[7], *N*-carbamoyl derivatives of amino acids according to STARK AND SMYTH[8].

Mn^{2+}–ureidoglycolate–glyoxylate complex was prepared by mixing 0.1 mole of sodium ureidoglycolate in 100 ml water with 0.2 mole of $MnCl_2$. The temperature was held at 0–1° and the pH 6–7 was maintained by addition of 0.01 M NaOH. The precipitate formed after several minutes was filtered, washed with alcohol and dried *in vacuo* above P_2O_5.

The optical antipodes of ureidoglycolate were prepared with the aid of allantoicase bound to Ecteola-cellulose. Previously it was reported[2] that the enzyme is readily bound to CM-, DEAE- and Ecteola-cellulose and in the latter instance more strongly when the pH of the eluting fluid was about 8. The enzyme bound could not be removed by any of a large number of methods tested[2] and was very stable during storage at −20° and catalytic action at 30°. (−)-Ureidoglycolate was prepared by suction of a solution of $9 \cdot 10^{-2}$ M sodium allantoate in $5 \cdot 10^{-3}$ M triethanolamine–HCl buffer (pH 7.5) through a column (6 cm × 1.2 cm) containing the enzyme bound to Ecteola-cellulose. The rotation of the eluted solution was −0.22° at 365 mμ. The solution was concentrated about 10 times by lyophilization and 9 vol. of 96% ethanol were added to the chilled residue. On preservation at −20° (−)-ureidoglycolate separated as a clear syrup which contained about 50% optical pure material, calculated on the basis of the specific rotation given previously[9]. The weight of the syrup was reduced to about 50% on heating at 100°, but this process was accompanied by slight browning of the material and a strong increase in the optical rotation, perhaps due to polymerization. (+)-Ureidoglycolate was prepared by suction of a freshly prepared solution of 1 M urea and 0.25 M sodium glyoxylate through the column as given above. The rotation of the eluted solution was +0.56° at 365 mμ. (+)-Ureidoglycolate was isolated as given for (−)-ureidoglycolate.

Allantoate amidohydrolase was the purified enzyme obtained from *Streptococcus allantoicus* and was activated as described previously[10].

Methods

Ureidoglycolate and glyoxylate were determined according to TRIJBELS AND VOGELS[9]. Ammonia was determined according to RICHARD[11] and isocyanate and urea according to VOGELS AND VAN DER DRIFT[5]. Manganese was determined with a Techtron, model AA-100, atomic absorption spectrophotometer.

Optical rotations were measured in a 10-cm cell in a Perkin–Elmer polarimeter, model 141.

Optical rotatory dispersion and circular dichroism spectra were measured as described previously[12].

Conductance measurements were performed with a Wheatstone bridge arrangement in aqueous solutions at 1.7°. A cathode-ray oscilloscope was used as detector. Metal chloride solutions (1.5 M) were added to 10 ml of the ligand solution ($1 \cdot 10^{-2}$ M) by means of a micro hand burette, Metrohm 457, with digital indication.

Electron paramagnetic resonance spectra were recorded with an AEG X-band spectrometer. Aqueous solutions with a constant concentration of Mn^{2+} ($5 \cdot 10^{-3}$ M)

and variable ligand concentrations were measured. Solutions under investigation were introduced from a small vessel, attached to the wave guide above the resonance cavity. The sample cell was not removed when the solutions were changed and, therefore, the measurements were perfectly reproducible. A ruby crystal adjusted in the cavity was used as reference signal. All measurements were made at room temperature. The variation of the signal intensity was determined both by double integration of the electron paramagnetic resonance signal and by multiplication of the square of the linewidth of one hyperfine component with the amplitude of this component; both methods yielded the same result. The experimental linewidth was measured as the difference (in gauss) between points of maximal slope, corresponding to the peak-to-peak distance in the first derivative spectra.

Infrared spectra were measured with a Perkin–Elmer Infracord spectrophotometer 157, slit N, air as reference and a Hitachi grating infrared spectrophotometer EPI-G2. KBr pellets and ^2H_2O solutions were used as samples; the former contained per 100 mg about 1 mg of the substance investigated.

pH was measured at 30° with a Radiometer, type PHM 4c, pH meter.

Standard assay conditions

The hydrolytic rate of ureidoglycolate was determined at 30° in mixtures containing per ml, 100 μmoles sodium ureidoglycolate and 100 μmoles of the same buffering substances as used before[13]. The amounts of glyoxylate formed at various time intervals were determined. The buffers used did not affect the hydrolytic rate measurably, except the phosphate buffer.

The cation-catalyzed hydrolysis of ureidoglycolate was assayed at 30° in mixtures containing per ml, 20 μmoles sodium ureidoglycolate, 200 μmoles buffering substances and 1 or 5 μmoles Mg^{2+}, Ca^{2+}, Cd^{2+}, Mn^{2+} or Ni^{2+}, or 0.1 or 0.25 μmole Co^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+}, Fe^{3+} or Cr^{3+}.

The anion-catalyzed hydrolysis of ureidoglycolate was assayed in a similar manner in the presence of 0.1 M sodium phosphate, NaCl, $NaNO_3$, Na_2SO_4, $NaClO_3$ or sodium acetate.

The racemization rate of ureidoglycolate was determined at 22° in mixtures containing per ml, 33 μmoles of buffer and 130 μmoles of one of the optical antipodes of ureidoglycolate. The following buffers were used: citrate (pH 1.8–4.5), acetate (pH 4.5–5.5), succinate (pH 6.3–7.2), triethanolamine (pH 7.0–8.0), diethanolamine (pH 8.4) and carbonate (pH 9.4–9.6). Higher pH values were adjusted with KOH.

The logarithms of the observed first-order rate constants (k_{obs}) are given in the figures. $k_{obs} = v_i[\text{ureidoglycolate}]^{-1}$, in which v_i is the initial velocity, given in moles/l per min. The various rate constants are represented as follows: k_a and k_b are the second-order rate constants of the acid- and base-catalyzed reactions, k and k' are the rate constants of the spontaneous reaction of the acid and salt form of the compounds, respectively; k_X is the rate constant of the increment of the reaction rate resulting from the presence of compound X.

RESULTS

Ureidoglycolate hydrolysis as a function of pH

The plot of log k_{obs} as a function of pH (Fig. 1) is composed of parts with slopes

of −1, +1 and 0 due to reactions catalyzed by H_3O^+, OH^- or noncatalyzed reactions. This is expressed in the following equation, in which UGA represents ureidoglycolic acid and UGA^- ureidoglycolate:

$$v_i = (k_a[H_3O^+] + k + k_b[OH^-])\,[UGA] + (k'_a[H_3O^+] + k' + k'_b[OH^-])\,[UGA^-] \quad (1)$$

The acid-catalyzed hydrolysis of UGA^- is assumed to proceed *via* conversion of UGA^- into UGA and the base-catalyzed hydrolysis of UGA *via* conversion of UGA into UGA^-. The dissociation constant of ureidoglycolic acid, K, was calculated from titration data and amounted to $5.9 \cdot 10^{-4}$ M at 16.5° in 0.1 M KCl. Since at pH values below 1, $K \ll [H_3O^+]$, and at pH values above 8, $K \gg [H_3O^+]$, the above equation may be written as:

$$k_{obs} = k_a\,[H_3O^+] + \frac{k\,[H_3O^+] + k'K}{K + [H_3O^+]} + k'_b\,[OH^-] \quad (2)$$

k_a and k'_b were determined from the course of the plot at pH values below 1 and above 9, respectively. From the levels at pH about 6 and 2 and the course of the plot between these pH values, approximate values of k and k' were obtained which were substituted into the three-term equation. The exact values were determined by iterative calculations performed with an IBM 360/50 system computer. The rate constants amounted to $k_a = 0.108\ M^{-1} \cdot min^{-1}$, $k = 4.47 \cdot 10^{-3}\ min^{-1}$, $k' = 3.80 \cdot 10^{-4}\ min^{-1}$ and $k'_b = 158\ M^{-1} \cdot min^{-1}$.

Hydrolysis of ureidoglycolate is a reversible process:

ureidoglycolate $\rightleftharpoons$ glyoxylate + urea

The equilibrium constant

$$K = \frac{[\text{ureidoglycolate}]}{[\text{glyoxylate}]\,[\text{urea}]}$$

was determined at pH 7.5 and amounted to 1.90 at 100°, 3.62 at 70°, 5.60 at 50° and 11.85 M^{-1} at 30°. ΔH of the reaction was calculated from the equation

$$\frac{d \ln K(T)}{dT} = \frac{\Delta H}{RT^2}$$

and amounted to +5.0 kcal/mole.

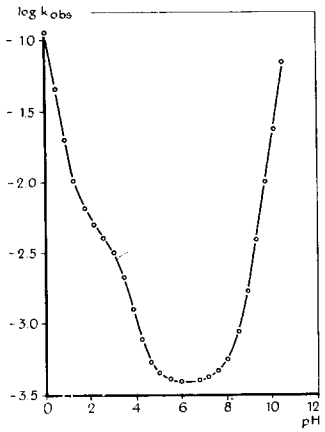
At pH values between −0.2 and 14.3 equal amounts of urea and glyoxylate were formed always. No detectible amounts of isocyanate or ammonia were found. This result contrasted the observations obtained with allantoate, which yielded ammonia and isocyanate instead of urea at pH values above 7 (ref. 5).

Cation-promoted hydrolysis of ureidoglycolate

Because metal ions play an essential role in the enzymic catalysis by allantoicase, the influence of metal ions on the hydrolysis of ureidoglycolate was studied. All bivalent cations tested promoted the reaction. The rate of hydrolysis at any pH value was directly dependent of the concentration of the cations (Fig. 2) and may be written as:

$$k_{obs} = k^\circ_{obs} + k_x[X], \quad (3)$$

in which k°_{obs} is measured in the absence of metal ions, and X represents metal ions.



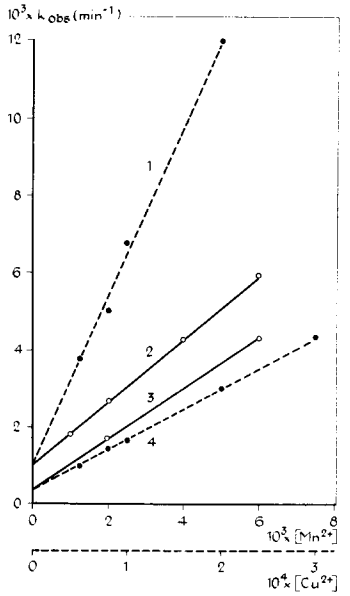


Fig. 1. Velocity of ureidoglycolate hydrolysis as a function of pH. Measurements were performed at 30° as given in the EXPERIMENTAL section.

Fig. 2. Rate of ureidoglycolate hydrolysis at 30° as a function of the concentration of the cations. Curves 1 and 4 refer to Cu^{2+} at pH's 4.0 and 6.5, respectively; Curves 2 and 3 refer to Mn^{2+} at pH's 4.0 and 6.5, respectively.

The values of k_{me} are given in Table I. The catalytic effect was strongly pH-dependent and increased on lowering the pH from 7.5 to about 5. The pH-dependence in this region is about the same as observed for the noncatalyzed reaction in the pH region from 5.5 to about 3.0. Therefore, the catalysis by cations is perhaps a result of a concerted action of H^+ and metal ions on the substrate.

TABLE I

RATE CONSTANTS OF CATION-CATALYZED HYDROLYSIS OF UREIDOGLYCOLATE AS A FUNCTION OF pH
k_{me} values, defined in the text, were determined at 30°.

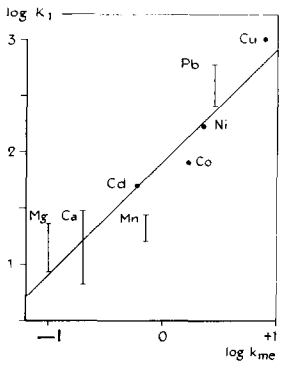
Cation	*k_{me} ($M^{-1} \cdot min^{-1}$) at the indicated pH values*							
	0.5	*1.5*	*2.5*	*3.5*	*4.5*	*5.5*	*6.5*	*7.5*
Fe^{2+}								23.3
Cu^{2+}	0.0	7.5	20	57.5	52.5	41.0	13.1	8.0
Zn^{2+}	0.0	6.5	15.5	21	15.3	10.0	7.0	5.4
Pb^{2+}								2.8
Hg^{2+}								2.6
Ni^{2+}	0.0	1.1	2.8	4.4	3.7	3.0	2.7	2.3
Co^{2+}	0.0	1.0	2.5	4.2	3.6	2.4	2.3	1.7
Cd^{2+}	0.0	0.3	1.0	2.1	1.7	1.3	0.9	0.6
Mn^{2+}	0.0	0.5	0.6	0.9	0.8	0.7	0.7	0.7
Ca^{2+}								0.2
Mg^{2+}								0.1
Fe^{3+}	0.0	30.0	65.0	89.0	111.0	111.0	71.0	23.3
Cr^{3+}	0.0	0.2	0.6	3.8	2.5	1.2	0.6	0.3

Fe^{3+} and Cr^{3+} were catalytically active too, whereas monovalent metal ions, *e.g.* 0.1 M Na^+ and K^+, were without effect.

Metal-ureidoglycolate complexes

The sequence of bivalent cations promoting the hydrolysis of ureidoglycolate (Table I) exposed a strong relationship with the first stability constants of these cations with hydroxy acids (*e.g.* lactic acid[14,15], Fig. 3). This suggested that the catalytic action of these ions involves complex formation with ureidoglycolate and, most probably, with the COO^- and OH groups. Therefore, the complexes of Mn^{2+} and Cu^{2+} with allantoate, ureidoglycolate and glyoxylate were studied.

The stoichiometric composition of the metal–ligand complexes was determined by conductance measurements. Allantoate, ureidoglycolate and glyoxylate formed



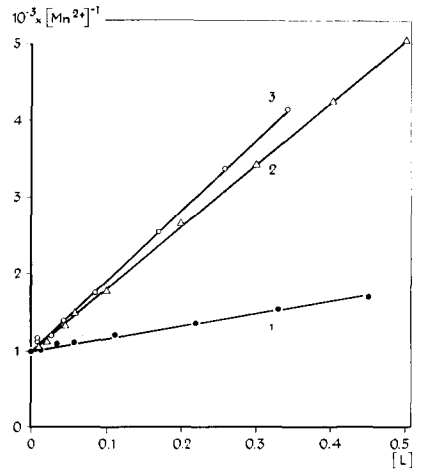


Fig. 3. Comparison of the first stability constants (K_1) of metal–lactic acid complexes with the catalytic effect (k_{me}, defined in the text) of these ions on ureidoglycolate hydrolysis at pH 7.5.

Fig. 4. Variation of free Mn^{2+} concentration with changing ligand concentration. Free $[Mn^{2+}]$ was determined from the electron paramagnetic resonance spectra as indicated in the EXPERIMENTAL section. Curve 1, sodium allantoate; Curve 2, sodium ureidoglycolate; Curve 3, sodium glyoxylate.

1:1 and 2:1 complexes with Cu^{2+} and 1:1 complexes with Mn^{2+}. SAKAGUCHI AND TAGUCHI[16] demonstrated the occurrence of 1:1 complexes of glyoxylate with Cu^{2+} and Mn^{2+}.

Electron paramagnetic resonance was used in measurements of the complex constants of Mn^{2+} and the ligands ureidoglycolate, allantoate and glyoxylate. The linear relationship found between $[\text{free } Mn^{2+}]^{-1}$ and the ligand concentration (Fig. 4) was indicative of the formation of 1:1 complexes under the conditions tested, and the complex constants of Mn^{2+} with ureidoglycolate, allantoate and glyoxylate amounted to 8.1, 1.7 and 8.7 M^{-1}, respectively. From the linewidth increment on enhancement of the ligand concentration we could not deduce that outer-sphere complexes were formed[17]. The complex constant of the Mn^{2+}–glyoxylate complex was

of the same order of magnitude as those reported[18] for the Ni^{2+} and Zn^{2+} complexes which amount to 8.7 and 4.4 M^{-1}, respectively.

Attempts to prepare the Mn^{2+}–ureidoglycolate complex yielded a product containing 18.9% C, 9.3% N, 3.8% H and 17.6% Mn. Moreover, on acid hydrolysis of the compound 1.9 moles glyoxylate were formed per mole Mn present. These data are most consistent with the composition Mn^{2+}: ureidoglycolate: glyoxylate: H_2O = 1:1:1:2. The theoretical composition of such a complex would be: 19.0% C, 8.9% N, 3.8% H and 17.5% Mn. In accordance with this view the infrared spectrum of the complex indicated the presence of bound water, since broad bands were observed near 3300 and 1610 cm^{-1}. Besides the C–O stretching band at 1035 cm^{-1} present in ureidoglycolate, a new band was observed in the complex at 1065 cm^{-1} which was also present in the Mn^{2+}–glyoxylate complex[16].

Effect of phosphate and Mn^{2+}–phosphate complex

The catalytic effect exerted by phosphate obeyed Eqn. 3, in which $k_{phosphate}$ is $0.81 \cdot 10^{-2}$ and $1.13 \cdot 10^{-2} M^{-1} \cdot min^{-1}$ at pH's 7.0 and 6.0, respectively. The effect of phosphate was not due to the higher ionic strength of the solution, since NaCl solutions of the same ionic strength were without effect. 0.1 M $NaNO_3$, Na_2SO_4, $NaClO_3$ and sodium acetate did not exert a catalytic effect.

The effects of metal and phosphate ions were not simply cumulative, if both were present simultaneously (Table II). The results suggested that a third term, $k_c[C]$, must be added to Eqn. 3. C represents the Mn^{2+}–HPO_4^{2-} complex, and the dependence of k_{obs} on the phosphate concentration and pH indicated that Mn^{2+}–$H_2PO_4^-$ complexes were much less or not at all catalytically active. Since k_c was 4.1 and k_{Mn} was 0.76, the Mn^{2+}–HPO_4^{2-} complex promoted the reaction 5.4 times more strongly than free Mn^{2+}.

TABLE II

CATALYTIC EFFECT OF Mn^{2+}–HPO_4^{2-} COMPLEX ON THE HYDROLYSIS OF UREIDOGLYCOLATE

Ureidoglycolate hydrolysis was measured at 30° in mixtures containing per ml, 24 μmoles sodium ureidoglycolate, 40 μmoles Tris–HCl buffer (pH 7.0) and the indicated amounts of Mn^{2+} ($[Mn^{2+}]_t$) and of phosphate buffer (pH 7.0, $[phosphate]_t$). From the equation, $k_{obs} = k + k_{Mn}[Mn^{2+}]_f + k_c[C]$, k_c was calculated. k, k_{Mn} and k_c refer to the noncatalyzed, Mn^{2+}-catalyzed and complex-catalyzed reactions, respectively. The concentration of free Mn^{2+}, of free HPO_4^{2-} and of the complex were calculated with the aid of the known values of the second ionization constant of phosphate ($6.46 \cdot 10^{-8}$ M) and $K_c = [Mn^{2+} - HPO_4^{2-}]/[Mn^{2+}][HPO_4^{2-}] = 3.8 \cdot 10^2 M^{-1}$ (ref. 14).

$[Mn^{2+}]_t$ ($10^3 \times M$)	$[Phosphate]_t$ ($10^3 \times M$)	k_{obs} ($10^4 \times min^{-1}$)	$[Mn^{2+}]_f$ ($10^3 \times M$)	$[HPO_4^{2-}]_f$ ($10^3 \times M$)	$[C]$ ($10^3 \times M$)	k_c ($M^{-1} \cdot min^{-1}$)
—	—	3.8	—	—	—	—
—	25	5.8	—	10	—	—
3.0	—	25.7	3.0	—	—	—
3.0	1	32.0	2.79	0.20	0.21	3.81
3.0	1.7	35.8	2.66	0.34	0.34	3.71
3.0	2.5	44.9	2.51	0.51	0.49	4.66
3.0	4.2	51.1	2.22	0.90	0.78	4.00
3.0	8.3	75.4	1.70	2.02	1.30	4.58
3.0	25	98.3	0.75	7.75	2.25	3.98
					Average:	4.1

If the Mn^{2+}-catalyzed hydrolysis at a certain pH value results from an enhanced hydrolytic rate constant (k^*) of ureidoglycolate bound in the Mn^{2+}–ureidoglycolate complex, then

$$k_{Mn}\,[Mn^{2+}]_t\,[\text{ureidoglycolate}] = k^*\,[Mn^{2+}\text{–ureidoglycolate complex}],$$

in which $[Mn]_t$ refers to the total amount of Mn^{2+} present. Since k_{Mn} is 0.76 $M^{-1} \cdot min^{-1}$ at pH 7.0 and the complex constant of Mn^{2+}–ureidoglycolate complex is 8.1 M^{-1}, k^* is 0.10 min^{-1}. The hydrolytic rate of ureidoglycolate bound in the complex appears to be 260 times higher than that of free ureidoglycolate ($k' = 3.80 \cdot 10^{-4} min^{-1}$). At the moment information is too scant to decide definitely whether the different catalytic effects of the cations result from different complex constants only or from changes in the k^* value for different metal–ureidoglycolate complexes or from a concerted effect. Since the various k_{me} values are rather well proportional to the complex constants of α-hydroxy acids (*cf.* Fig. 3), k^* seems to be rather independent of the nature of the metal ion in the ureidoglycolate complex.

(+)- and (−)-ureidoglycolate

The optical antipodes of ureidoglycolate were prepared with the aid of allantoicase bound to Ecteola-cellulose. The specific rotation of (−)-ureidoglycolate at 365 mμ is given as a function of pH in Fig. 5. On protonation the compound becomes dextrorotatory. The same is true if the wavelength is lowered below 300 mμ (Fig. 6). At low ureidoglycolate concentrations (2–20 mg/ml) the specific rotation at wavelengths ranging from 365 to 589 mμ appeared to obey the equation $[\alpha]_\lambda = [\alpha_0]_\lambda (1 - 10\,c)$, in which c is the concentration of ureidoglycolate (g/ml) and $[\alpha_0]_\lambda$ is the specific rotation at infinite dilution. $[\alpha_0]_\lambda$ is −12.2°, −13.0°, −18.7° and −24.3° for (−)-ureidoglycolate at 578, 546, 436 and 365 mμ, respectively. High concentrations of (−)-ureidoglycolate are dextrorotatory. The rotation of 1.6 g sodium (−)-ureidoglycolate in 10 ml water was +3.33°, 1.67°, 0.755° and 0.615° at 365, 436, 546 and 578 mμ, respectively, and the rotations became −0.314°, −0.243°, −0.168° and −0.157° on

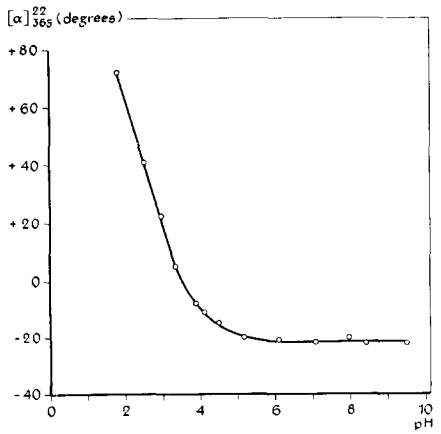


Fig. 5. Specific rotation of (−)-ureidoglycolate as a function of pH. Solutions containing 21 mg (−)-ureidoglycolate per ml were measured.

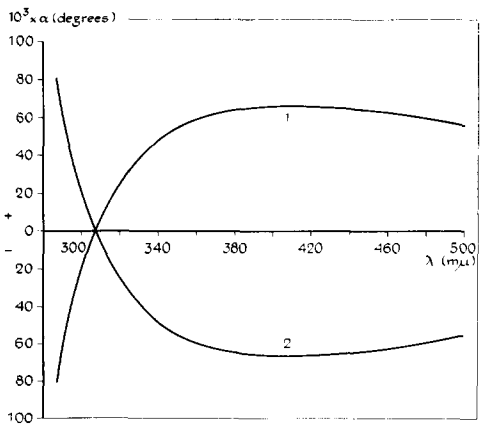


Fig. 6. Rotation of (+)- and (−)-ureidoglycolate as a function of wavelength. Curve 1 refers to (+)-ureidoglycolate; Curve 2 to (−)-ureidoglycolate. pH of the solutions was 7.7. Rotations were measured in a 1-cm cell of solutions containing 50 mg/ml.

10-fold dilution. This effect may be attributed to a strong dipole–dipole interaction between the molecules, as observed for urea[19].

The multiple Cotton effects are shown in Fig. 7. In contrast to the Cotton effect observed at 208 mμ, the Cotton effect at 228 mμ depended strongly on the concentration and the rotation strength was relatively low at high concentrations.

Racemization of ureidoglycolate

The pH–rate profile given in Fig. 8 shows a part with slope −1 at pH 4–6. Above

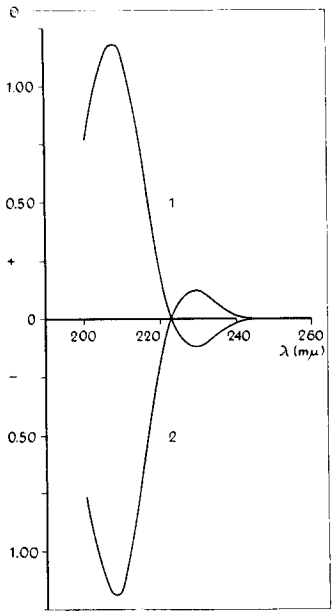


Fig. 7. Circular dichroism of ureidoglycolate at pH 7.7 measured in 1-mm and 0.1-mm cells. The solutions contained 10 mg ureidoglycolate per ml. Curves 1 and 2 refer to (−)- and (+)-ureidoglycolate, respectively.

pH 7 the hydrolysis of ureidoglycolate becomes predominant. The observed rate constant of racemization at pH below 7 obeys the equation:

$$k_{obs} = k_a[H_3O^+] + k\frac{[H_3O^+]}{K + [H_3O^+]} \quad (4)$$

in which K is the dissociation constant of ureidoglycolic acid. k_a for racemization is 160 $M^{-1} \cdot min^{-1}$ and k is 0.72 min^{-1} at 22°.

The effect of $3.5 \cdot 10^{-3}$ M $CuSO_4$ and $3 \cdot 10^{-2}$ M $MnSO_4$ on the rate of racemization was examined in $3.3 \cdot 10^{-2}$ M acetate buffer (pH 5). The small enhancement of the disappearance of (−)-ureidoglycolate could be fully accounted for by the increase of the hydrolytic rate caused by these ions. The same is true for the effect of 0.13 M phosphate, shown in Fig. 8. The disappearance of (−)-ureidoglycolate in the presence of phosphate obeys the equation

$$k_{obs} = k^\circ_{obs} + k_{phosphate}\,[phosphate] \quad (5)$$

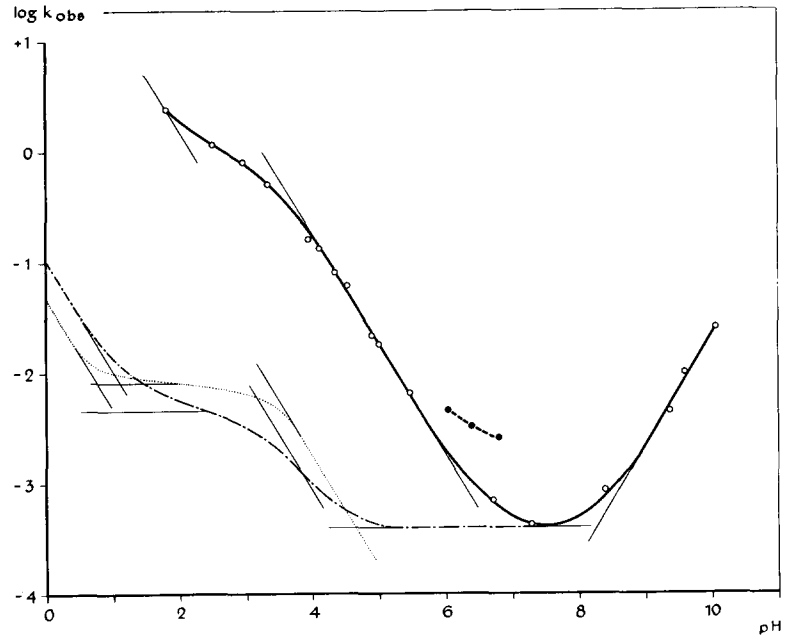


Fig. 8. pH–rate profile of ureidoglycolate racemization (———). The logarithm of the observed reaction rate was determined as given in the EXPERIMENTAL section. Moreover, k_{obs} of racemization measured in the presence of 0.13 M phosphate (●– – –●) and k_{obs} of ureidoglycolate hydrolysis (— . — . —) and of allantoate hydrolysis (. . . . . .) are given.

in which $k°_{obs}$ is the observed rate constant in solutions buffered by $3.3 \cdot 10^{-2}$ M succinate. $k_{phosphate}$ is equal to the reaction constants of the catalysis of the hydrolysis by phosphate given previously. Therefore, bivalent cations and phosphate exposed no measurable effect on the racemization of ureidoglycolate.

Comparison of rate and equilibrium constants

Table III represents the rate constants of ureidoglycolate hydrolysis and racemization, which are compared with earlier reported values for glyoxylate hy-

TABLE III

COMPARISON OF RATE CONSTANTS

k and k' refer to the neutral and anionic forms of the compounds, respectively.

	Temp.	k	k'	*Dimension*
Glyoxylate. hydrate dehydration	22°	2.3*	0.43	min^{-1}
Glyoxylate hydration	22°	70	0.48	$M^{-1} \cdot min^{-1}$
Ureidoglycolate formation	30°	89	0.28	$M^{-1} \cdot min^{-1}$
Ureidoglycolate hydrolysis	30°	$4.47 \cdot 10^{-3}$	$3.80 \cdot 10^{-4}$	min^{-1}
Ureidoglycolate racemization	22°	0.72	$< 2 \cdot 10^{-4}$	min^{-1}
Allantoate hydrolysis	30°	$8.0 \cdot 10^{-3}$	$< 1.6 \cdot 10^{-6}$	min^{-1}

* This value is extrapolated from the rates in the pH region 4–6.

dration[6] and allantoate hydrolysis[5]. The values were calculated with the aid of the equilibrium constants of glyoxylate hydration[20], allantoate hydrolysis[5] and ureidoglycolate hydrolysis.

It appeared that the rate constants of glyoxylate hydration and ureidoglycolate formation are about equal, which indicates that urea and water are bound with almost the same velocity. Urea production from allantoate proceeded about twice as rapidly as from ureidoglycolate. This result is perhaps due to the presence of two ureido groups in allantoate.

DISCUSSION

Absolute configuration of (+)- and (−)-ureidoglycolate

In order to decide which of the ureido groups of allantoate is split off by allantoicase, which produces (−)-ureidoglycolate, the absolute configuration of the latter compound must be determined. Thus, the steric arrangement of the subsites of the active center of allantoicase can be established.

Allantoate amidohydrolase from *Streptococcus allantoicus* transforms allantoate into (−)-ureidoglycolate, CO_2 and ammonia. This enzyme transforms *N*-carbamoyl-L-asparagine into asparagine, CO_2 and ammonia, but the D-isomer is not attacked[21]. These results suggested that the intermediary ureidoglycine and (−)-ureidoglycolate are related to the L-families of amino and hydroxy acids and must be denominated[22,23] S-ureidoglycine and R-ureidoglycolate, respectively. However, the occurrence of an optical inversion in the enzymic interconversion[24] of the latter compounds could not be excluded.

Definite evidence for the configuration was derived from the optical rotatory dispersion and circular dichroism spectra. Comparison of the Cotton effects with those of compounds containing the identical chromophore in the same conformational environment, *e.g.* R- and S-carbamoylamino acids, revealed that the Cotton effect at 208 mμ originates from the carboxyl group and the Cotton effect at 228 mμ must be ascribed to the ureido group[12,25]. It was concluded from the sign of the Cotton effects that (−)-ureidoglycolate possesses the S-configuration and (+)-ureidoglycolate the R-configuration (Fig. 9).

This result implicates that an optical inversion occurred in the transformation of ureidoglycine to ureidoglycolate by allantoate amidohydrolase.

The steric arrangement of the subsites of allantoicase is depicted in a preceding

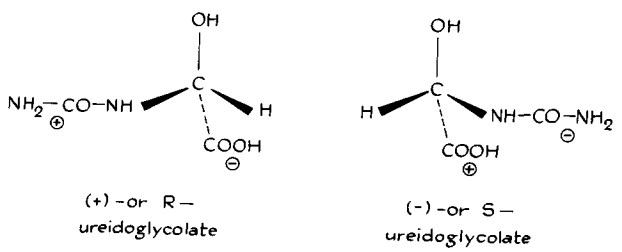
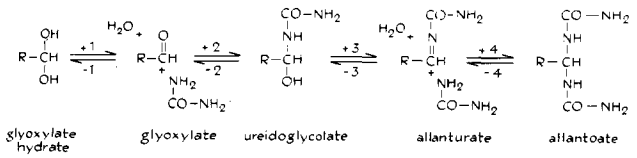


Fig. 9. Absolute configuration of (+)- and (−)-ureidoglycolate. The signs of the Cotton effects originating from the ureido and carboxylate groups are indicated.

report[3] and it was assumed that optical inversion did not take place in the action of this enzyme.

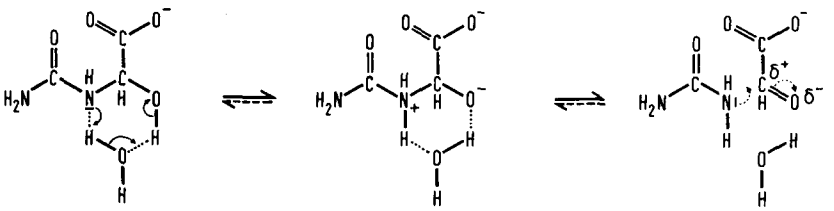
Hydrolysis and racemization of ureidoglycolate

The reaction sequence involved in the hydrolysis and racemization of ureidoglycolate, both described in this paper, and in the hydrolysis of allantoate[5] and the hydration of glyoxylate[6] is given in the reaction sequence below, in which R represents COOH or COO^-.



The name allanturate is chosen in analogy to PONOMAREW[26] and BEILSTEIN[27].

The four reactions proceed most probably along the same reaction mechanism given here for the hydrolysis of ureidoglycolate:



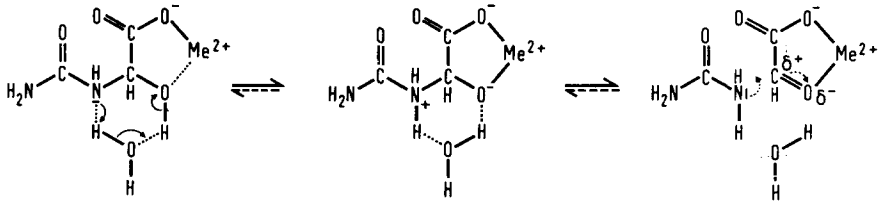
The reaction is an example of known processes in which nucleophilic agents, urea and water in the above reactions, act on the carbon atom of a C=O group or a C=N bond. The second and third reaction represent a Schiff base formation, which proceeds *via* a carbinolamine intermediate[28,29]. These reactions result in the establishment of an equilibrium, and the formation constants of the carbinolamine products of glyoxylate with glycine, β-alanine, and α-aminoisobutyric acid are 57, 19 and 10, respectively, at 25° according to LEUSSING AND HANNA[18]. The formation constant of ureidoglycolate is about 13 under these conditions.

The mechanism of racemization in this view results from the reversible formation of allanturate, accompanied by a *cis–trans* isomerization of this compound. From the equilibrium constant [allantoate][H_2O]/[ureidoglycolate][urea] = 440 (ref. 6), and the rate constants of ureidoglycolate racemization and allantoate hydrolysis, given in Table III, it appeared that urea is bound about 5 times more rapidly to allanturate than water.

Cation-promoted hydrolysis of ureidoglycolate

Metal and phosphate ions affected neither the hydrolysis of allantoate[5] nor the racemization of ureidoglycolate but did promote the hydrolysis of ureidoglycolate and the hydration of glyoxylate. The results suggested the involvement of the OH

group in the catalytic action. It is assumed that metal ions are bound by the carboxylate group and by sharing the electron pair of the α-O-atom, thus increasing the reaction rate.



This view is corroborated by the fact that complexes are formed between metal ions with both glyoxylate and ureidoglycolate, and by the relationship between the complex constants of α-hydroxy acids and the catalytic enhancement exerted by the cations. A similar reaction mechanism was proposed for the metal-ion-catalyzed decarboxylation of acetonedicarboxylate[30].

Ureidoglycolate hydrolysis by Mn^{2+}–HPO_4^{2-} complex and by allantoicase

The ratio between the catalytic effects exerted by phosphate, Mn^{2+} and Mn^{2+}–HPO_4^{2-} is about 1:100:500. This indicates that ureidoglycolate bound to Mn^{2+}–HPO_4^{2-} is more labile than ureidoglycolate in the Mn^{2+}–ureidoglycolate complex. This might result from an enhanced stability constant of the ternary complex. Relatively high stability constants of ternary complexes were reported by SIGEL *et al.*[31] for 2,2′-bipyridyl–cation complexes and by LEUSSING AND HANNA[18] for Ni^{2+}– and Zn^{2+}–glyoxylate complexes.

The specific activity of highly purified allantoicase against (+)-ureidoglycolate is about 650 units per mg protein[2,3]. Preliminary results indicate that about 1 mmole Mn^{2+} is present per 15 000 mg of protein. If the catalytic action of allantoicase involves formation of the ternary ureidoglycolate–Mn^{2+}–allantoicase complex, then k^* of this complex will be $1 \cdot 10^4$, the turnover number of the enzyme. This number is $2.5 \cdot 10^7$ times higher than the k' value of the noncatalyzed reaction and 10^5 times higher than the k^* value of the Mn^{2+}–ureidoglycolate complex.

The stability constant of the enzyme–substrate complex amounted to 40 (ref. 3), which is 5 times higher than the stability constant of the Mn^{2+}–ureidoglycolate complex. Therefore, the gap between the enzymic and nonenzymic catalysis is about a factor 20 000, if one only reckons with ureidoglycolate stability in the complex. This factor might be lower if one takes into account the concerted action of metal and hydrogen ions on the hydrolytic rate of ureidoglycolate.

ACKNOWLEDGMENTS

We wish to thank Dr. J. Van Broekhoven for performance of the EPR measurements, Mr. L. Van der Ven (Technical University, Eindhoven) for the performance of the infrared measurements and Miss C. Van Pelt for skillful technical assistance.

REFERENCES

1 F. TRIJBELS AND G. D. VOGELS, *Biochim. Biophys. Acta*, 132 (1967) 115.
2 E. J. 'S-GRAVENMADE AND G. D. VOGELS, *Antonie van Leeuwenhoek*, 35 (1969) 463.
3 G. D. VOGELS, *Biochim. Biophys. Acta*, 185 (1969) 186.
4 C. VAN DER DRIFT AND G. D. VOGELS, *Biochim. Biophys. Acta*, 198 (1970) 339.
5 G. D. VOGELS AND C. VAN DER DRIFT, *Rec. Trav. Chim.*, 88 (1969) 951.
6 G. D. VOGELS AND C. VAN DER DRIFT, *Anal. Biochem.*, in the press.
7 R. C. VALENTINE AND R. S. WOLFE, *Biochem. Biophys. Res. Commun.*, 5 (1961) 305.
8 G. R. STARK AND D. G. SMYTH, *J. Biol. Chem.*, 238 (1963) 214.
9 F. TRIJBELS AND G. D. VOGELS, *Biochim. Biophys. Acta*, 113 (1966) 292.
10 C. VAN DER DRIFT AND G. D. VOGELS, *Biochim. Biophys. Acta*, 139 (1967) 162.
11 C. RICHARD, *Ann. Inst. Pasteur*, 109 (1965) 516.
12 E. J. 'S-GRAVENMADE, G. D. VOGELS AND C. VAN PELT, *Rec. Trav. Chim.*, 88 (1969) 929.
13 G. D. VOGELS, F. E. DE WINDT AND W. BASSIE, *Rec. Trav. Chim.*, 88 (1969) 940.
14 L. G. SILLÉN AND A. E. MARTELL, *Stability Constants of Metal-ion Complexes*, The Chemical Society, London, 1964, pp. 185, 390.
15 N. C. LI, W. M. WESTFALL, A. LINDENBAUM, J. M. WHITE AND J. SCHUBERT, *J. Am. Chem. Soc.*, 79 (1957) 5864.
16 T. SAKAGUCHI AND K. TAGUCHI, *Yakugaku Zasshi*, 86 (1966) 26.
17 L. BURLAMACCHI AND E. TIEZZI, *J. Mol. Struct.*, 2 (1968) 261.
18 D. L. LEUSSING AND E. M. HANNA, *J. Am. Chem. Soc.*, 88 (1966) 696.
19 G. C. KRESHECK, *J. Phys. Chem.*, 73 (1969) 2441.
20 J. KŮTA AND P. VALENTA, *Collection Czech. Chem. Commun.*, 28 (1963) 1593.
21 C. VAN DER DRIFT, *Thesis*, Nijmegen, 1968.
22 R. S. CAHN AND C. K. INGOLD, *J. Chem. Soc.*, (1951) 612.
23 R. S. CAHN, C. K. INGOLD AND V. PRELOG, *Experientia*, 12 (1956) 81.
24 C. VAN DER DRIFT, F. E. DE WINDT AND G. D. VOGELS, *Arch. Biochem. Biophys.*, in the press.
25 E. J. 'S-GRAVENMADE AND G. D. VOGELS, *Anal. Biochem.*, 32 (1969) 286.
26 I. PONOMAREW, *J. Russ. Phys. Chem. Soc.*, 11 (1879) 12.
27 F. K. BEILSTEIN, *Handbuch der Organischen Chemie*, Vol. III, Springer, Berlin, 1942, p. 388.
28 R. L. REEVES, *J. Am. Chem. Soc.*, 84 (1962) 3332.
29 E. H. CORDES AND W. P. JENCKS, *J. Am. Chem. Soc.*, 84 (1962) 832.
30 J. E. PRUE, *J. Chem. Soc.*, (1952) 2331.
31 H. SIGEL, K. BECKER AND D. B. MCCORMICK, *Biochim. Biophys. Acta*, 148 (1967) 655.