trans-α,β-Diformamido-β-(5′-phosphoribosylamino)acrylamide: A Possible New Intermediate in de Novo Purine Biosynthesis†

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ABSTRACT: The nucleotide *trans*-α,β-diformamido-β-(5′-phosphoribosylamino)acrylamide (DAR) has been chemically synthesized and is converted to inosine 5′-phosphate (IMP) by enzyme activities found in chicken, rat, and human liver. The increase in optical density at 250 nm when DAR is converted to IMP is used as the basis of the assay. The K_m values for DAR at pH 7.4 were 2.8 and 4.2 μM with the chicken and rat liver enzymes, respectively. The integrated Michaelis–Menten equation was used to determine the kinetic parameters of the chicken liver enzyme from pH 5.6 to 10.1. The pH–activity profiles show ionizations with pK_a values of 6.1, 7.1, and 8.8. The possibilities that DAR is a substrate analogue or a new intermediate in the pathway of purine biosynthesis de novo are discussed.

During an investigation and purification of the enzyme 10-formyltetrahydrofolate:5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase (EC 2.1.2.3) (AICAR transformylase) from chicken liver, we elected to assay our preparation for inosine-5′-phosphate (IMP) cyclohydrolase (EC 3.5.4.10) in order to determine whether any separation of the two activities had occurred. The assay of IMP cyclohydrolase necessitates the chemical synthesis of its substrate 5′-phosphoribosyl-5-formamido-4-imidazolecarboxamide (formyl-AICAR). Flaks et al. (1957a) and Lukens & Flaks (1963) have described a synthesis of formyl-AICAR based on a procedure developed by Shaw (1950) for the synthesis of 4-formamido-5-imidazolecarboxamide from 4-amino-5-imidazolecarboxamide. Using a similar synthesis, we have obtained a chemical which has properties similar to but not identical with those reported for formyl-AICAR (Flaks et al., 1957a; Lukens & Flaks, 1963). This chemical is converted to IMP by enzyme activities found in chicken, rat, and human liver. The physical, chemical, and biochemical properties of this substrate are presented and a structure is proposed.

Experimental Procedures

Materials

Crystalline AICAR was a gift from Dr. John A. Montgomery (Southern Research Institute) and was purchased from Boehringer Mannheim. *trans*-α,β-Diformamido-β-(5′-phosphoribosylamino)acrylamide (DAR) (Figure 1) was synthesized by the following method. AICAR (free acid) (200 mg) was dissolved in 2 mL of 98% formic acid. To this solution were added 1 mL of acetic anhydride and 200 mg of sodium formate. A slightly exothermic reaction took place. The solution was stirred for 1 h at room temperature and lyophilized and the residue was dissolved in 5 mL of distilled water. The pH was immediately adjusted to 7.0 with 3 N NH_4OH, and 700 mg of barium acetate was added. After the barium salt had dissolved, 3 volumes of ethanol was added (with stirring), and the barium salt of the nucleotide was allowed to precipitate overnight at −20 °C. The white precipitate was collected by centrifugation, washed with 5 mL of acetone, followed by 5 mL of ether, and air-dried.

The resulting white powder (50 mg) was dissolved in 5 mL of water, and the pH was adjusted to 8.0 with 3 N NH_4OH. This solution was loaded on a 1.5 × 30 cm DEAE (Cl form) column, and the column was developed with a linear gradient of 200 mL each of 1 mM Tris-HCl, pH 8.0, and 1 mM Tris-HCl, pH 8.0, and 0.2 M NaCl (Figure 2). Fractions (4.5 mL) 36–45 were pooled and lyophilized and the residue was dissolved in 5 mL of water. To this solution were added 100 mg of barium acetate and 3 volumes of ethanol. The precipitate was collected, washed, and dried as described above. After drying under vacuum, the powder was stored desiccated at −20 °C. Product (30–40 mg) was routinely obtained from the column.

4-Amino-5-imidazolecarboxamide (AICA) was purchased from Sigma Chemical Co. 4-Formamido-5-imidazolecarboxamide (FAICA) was synthesized from AICA by the method described by Shaw (1950). Anal. Calcd for $C_5H_6N_4O_2$: C, 38.96; H, 3.92; N, 36.35. Found: C, 39.22; H, 4.02; N, 36.45.

All other chemicals were of reagent grade or higher purity.

Purified chicken liver AICAR transformylase was obtained as described before (Baggott & Krumdieck, 1979). Extracts of chicken liver, rat liver, and human liver were obtained by homogenizing the tissues in 2 volumes of 0.25 M sucrose and 10 mM Tris-HCl, pH 7.4, in a Potter-Elvehjem homogenizer and centrifuging for $(6 \times 10^6)g$ min (all operations at 2 °C). The supernatant was dialyzed overnight against 100 volumes of 10 mM Tris-HCl, pH 7.4. The extracts were stored frozen and centrifuged for $(1 \times 10^6)g$ min immediately before use.

Methods

The assay of the enzymatic conversion of DAR to IMP is based on the increase in optical density at 250 nm that accompanies the reaction. A $\Delta\epsilon_{250}$ of 5.5×10^3 at pH 7.4 was obtained by measuring the increase in optical density resulting from the conversion of DAR to IMP and by the use of an ϵ_{250} for IMP of 1.2×10^4. This $\Delta\epsilon_{250}$ was judged to be accurate between pH 5.6 and pH 8.3. At higher pH values, the $\Delta\epsilon_{250}$ was determined by the following equation

$$\Delta\epsilon_{250} = 5.5 \times 10^3 - \frac{(X)}{(X) + (XH)} 2.3 \times 10^3$$

where (X) and (XH) are the concentrations of DAR in the high and low pH forms, respectively (pK_a = 9.80; see Results). The ϵ_{250} of IMP from pH 5.6 to 10.1 does not change. The sample cuvette contained DAR, enzyme, and buffer; DAR was omitted from the reference cuvette.

Initial velocities (v) in terms of optical density units per minute were calculated from linear regression of the data sets

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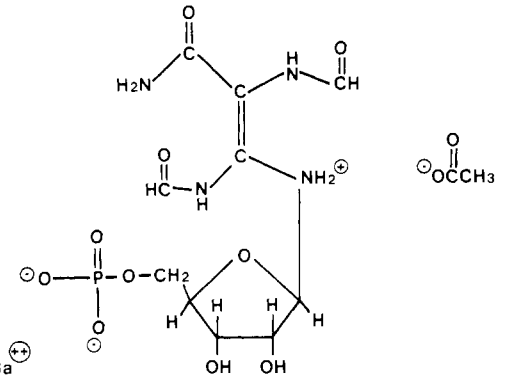

FIGURE 1: DAR (barium acetate salt).

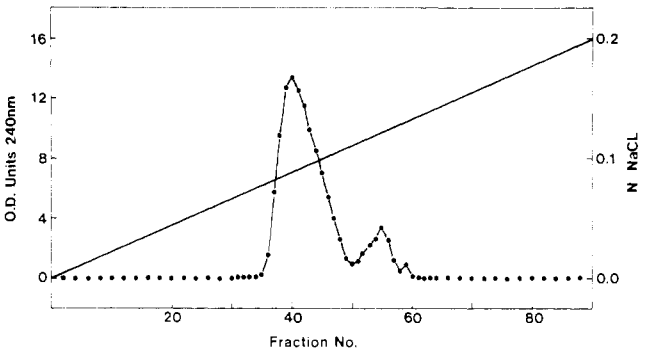

FIGURE 2: DEAE column purification of DAR. Elution profile of DAR from a 30 × 1.5 cm DEAE column eluted with a linear gradient of NaCl. Fractions (4.5 mL) 36–45 were pooled.

during the first 1–3 min of the reaction. Initial velocities were converted to specific activities by use of the $\Delta\epsilon$ for the reaction (correcting for the cuvette path length: 0.2, 1, or 5 cm) and the protein concentration in milligrams per milliliter. Specific activities are reported as nanomoles per minute per milligram. Kinetic constants and their standard errors were calculated from a linear regression fit of the Eadie–Hofstee equation [v vs. $v/(S)$] (Wong, 1975).

Kinetic constants were also calculated from the integrated Michaelis–Menten equation (Walker & Schmidt, 1944). This method involved linear regression fit of the equation

$$\frac{(P)}{t} = \frac{-K_m}{t} \ln\left[\frac{(S_0)}{(S_0) - (P)}\right] + V_m$$

where (S_0) is the initial concentration of substrate and (P) is the concentration of the product at time t. The slope ($-K_m$) and the intercept (V_m) and their standard errors (Wong, 1975) were calculated by using data sets obtained from that portion of the enzymatic rate where mixed zero- and first-order conditions (with respect to S) existed.

Spectrophotometric titrations were performed at room temperature in 0.1 M NaCl with dilute buffers to stabilize the pH. The pH was adjusted between 2 and 12 by the addition of small amounts of concentrated NaOH or HCl. Concentrations of HCl from 0.01 to 0.1 N were adjusted to an ionic strength of 0.1 by using 0.1 M NaCl. DAR was titrated from pH 7 to 12 in 1 mM borate buffer by the addition of small amounts of concentrated NaOH while monitoring the optical density at 262 nm. FAICA was titrated from pH 1 to 11 while the optical density at 269 nm was monitored.

pK_a values of DAR and FAICA were determined by the method of Hofstee (1960). pK_a values of the kinetic parameters were estimated by using the rules of Dixon & Webb (1964).

Table I: ^{1}H NMR of DAR and AICAR[a]

pattern	position (δ)[b]	integration	assignment[c]
	DAR		
singlet	8.36	0.8	H of CONH-
singlet	8.21	0.9	H of CONH-
doublet	5.64	1.0	1′ ribose
multiplet	3.98	2.2	5′ ribose
singlets	2.22	3.2	acetic acid
	1.91		acetate ion
	AICAR		
singlet	7.51	1.0	2 imidazole
doublet	5.65	1.1	1′ ribose
multiplet	4.01	1.9	5′ ribose

[a] In D_2O; DSS was the internal standard; DAR, 75 mg/mL; AICAR, 60 mg/mL. [b] In parts per million. [c] The structural position of the nonexchangeable hydrogens.

The optical densities at 268 and 254 nm were used to monitor the conversion of DAR to AICAR and IMP in 1 N HCl and 0.5 N NaOH, respectively. Pseudo-first-order rate constants were determined from these optical density measurements by the method of Guggenheim (1926).

Thin-layer chromatography (TLC) was performed using Eastman cellulose plates (6065) and Eastman chromatographic developing apparatus (6071). Spots were visualized under UV light.

Proton magnetic resonance spectra (^{1}H NMR) (Courtesy of Dr. John A Montgomery, Southern Research Institute) were measured at 100 MHz in D_2O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard.

The Bratton–Marshall (Flaks & Lukens, 1963), ninhydrin (Spies, 1957), and fluorescamine (Roche Diagnostics) assays (Udenfriend et al., 1972) were performed as described.

All optical density measurements were performed on a GCA/McPherson Series 700 spectrophotometer equipped with a thermostated cell holder.

Molecular models were constructed from commercially available space-filling atomic units.

Results and Discussion

Physical and Chemical Properties of DAR. DAR was synthesized by a slight modification of a method developed by Shaw (1950) to convert AICA to FAICA. The column chromatographed barium acetate precipitated material was judged to be approximately 85% pure based on its base-catalyzed and enzyme-catalyzed conversion to IMP, its acid-catalyzed conversion to AICAR, and a molecular weight of 579.6 ($C_{10}H_{16}N_4O_{10}P{\cdot}BaC_2H_3O_2$).

The proposed DAR structure is based on the following evidence. The ^{1}H NMR data of DAR and AICAR in D_2O are shown in Table I. The two resonances, δ 8.2–8.4, seen in DAR are not comparable to the imidazole proton of AICAR, δ 7.51. These resonances are approximately correct for a formamido proton (δ 7.9–8.1). Other resonances are comparable with those seen in AICAR and suggest a phosphoribosyl group. The proton integration including an equivalent amount of acetate (acetic acid) is consistent with the proposed structure of DAR.

The UV spectra of DAR in acid or neutral pH is much different from that of AICAR (Flaks et al., 1957b) or FAICA (Figure 3). A strong shoulder is centered about 230 nm. This type of spectrum is not consistent with α,β-unsaturated carbonyls having auxochromes at the α or β position (King & McMillan, 1950; Glickman & Cope, 1945). These compounds have λ_{max} values from 240 to 300 nm with $\epsilon_{max} \geq 5 \times 10^3$. However, a molecular model of DAR indicates that

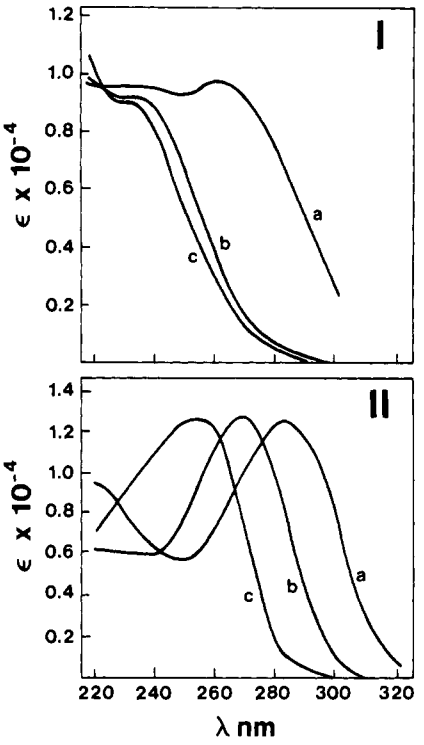

FIGURE 3: UV spectra of DAR and FAICA. (I) The spectra of DAR in 0.01 N NaOH (a), 0.02 M phosphate buffer, pH 7.0 (b), and 0.01 N HCl (c); ϵ = liter per (mole centimeter). (II) The spectra of FAICA in 0.01 N NaOH (a), 0.05 M phosphate buffer, pH 7.0 (b), and 0.1 N HCl (c); ϵ = liter per (mole centimeter).

the two formamido groups cannot lie in the same plane as the acrylamide backbone which would reduce π-orbital overlap and result in a shift of the λ_{max} to a shorter wavelength. The strong absorbance at $\lambda \leq 240$ nm in the acid and neutral spectra of DAR results from the simple summation of the contributions of the α,β-unsaturated amide and the two formamido chromophores which should have λ_{max} values with large molar extinction coefficients ($\epsilon \geq 5 \times 10^3$) at or below 220 nm. The marked change in the spectrum in alkaline pH with a pK_a of 9.80 is consistent with the ionization of a secondary amino group. The uncharged amino group is an auxochrome while the charged group is not.

The UV spectra of FAICA are much different from those of DAR (Figure 3). FAICA has spectrophotometric pK_a values of 2.53 and 9.65 (Figure 4); the acidic pK_a is most likely the ionization of the imidazole ring while the basic pK_a may be the ionization of the formamido group (Magrath & Brown, 1957). FAICA behaves as a buffer in solution in the pH range of 9–10; therefore, the spectrophotometric pK_a does represent the ionization of a proton.

DAR gives only a weakly positive Bratton–Marshall test, indicating the absence of a primary aromatic amino group (Table II). Both DAR and FAICA give weak ninhydrin and fluorescamine tests, indicating the absence of a primary aliphatic amino group. The slightly positive ninhydrin test may result from decomposition of DAR under the conditions of the assay (15 min at 100 °C; pH 4.5). The fluorescamine test is performed at room temperature.

DAR is converted to AICAR by heating in dilute acid (Figure 5). AICAR was identified by its UV spectrum (Flaks et al., 1957b), TLC (Table III), and a strongly positive Bratton–Marshall test which is in agreement with that expected for the conversion of DAR to AICAR (Table II). DAR is converted to AICAR at 37 °C in 1 N HCl with a pseudo-first-order rate constant of 0.052 min^{-1} determined by the

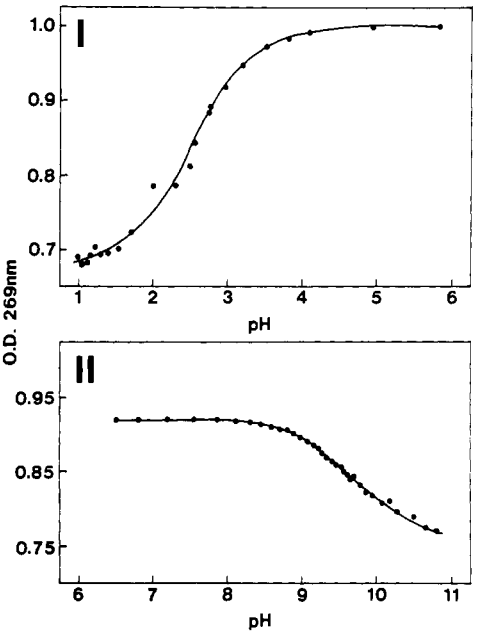

FIGURE 4: Spectrophotometric titrations of FAICA. (I) FAICA in 0.1 M NaCl and 1 mM acetate was titrated at room temperature with concentrated HCl from pH 6 to 2. Titration from pH 2 to 1 was performed by diluting a stock solution of FAICA in the appropriate concentration of HCl–NaCl, ionic strength 0.1. (II) FAICA in 0.1 M NaCl, 0.2 mM phosphate, and 0.2 mM borate was titrated at room temperature with concentrated NaOH.

Table II: Chemical Tests

compd	Bratton–Marshall (OD_{540nm}/mol)/L ± SD
DAR	1 570 ± 200
DAR (acid-treated)[a]	19 000 ± 800
AICAR (acid-treated)[a]	19 600 ± 300

compd	ninhydrin (OD_{570nm}/mol)/L ± SD
DAR	5 360
AICAR	1 760
FAICA	600
glycine	20 200 ± 500

compd	fluorescamine[b] (fluorescent units/μmol)/L ± SD
DAR	14
AICAR	2
FAICA	0
glycine	152 ± 7

[a] Heated in 0.1 N HCl at 80 °C for 30 min. [b] In 0.2 M borate buffer, pH 9.5.

Table III: R_f Values

compd	R_f system 1[a]	R_f system 2[b]
DAR	0.73	0.79
AICAR	0.49	0.53
IMP	0.53	0.57
DAR (acid-treated)[c]		0.52
DAR (base-treated)[d]	0.52	
DAR (enzyme-treated)[e]	0.53	0.56

[a] Saturated ammonium sulfate. [b] Saturated ammonium sulfate–1 M ammonium acetate (90:10 v/v). [c] Heated in 0.1 N HCl at 80 °C for 30 min. [d] Refluxed in 0.5 M $KHCO_3$ for 30 min. [e] For conditions see Figure 5.

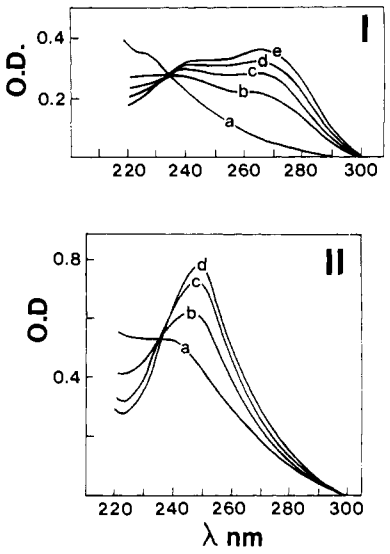

FIGURE 5: (I) Acid-catalyzed conversion of DAR to AICAR. DAR (0.13 mg/mL) in 0.1 N HCl was heated at 80 °C for 0 (a), 5 (b), 10 (c), 15 (d), and 30 min (e). Spectra were scanned of cooled solutions (25 °C) in a 0.2-cm path length cell; isosbestic point is 235 nm. (II) Enzyme-catalyzed conversion of DAR to IMP. To a solution of DAR (0.23 mg/mL) in 50 mM Tris-HCl (pH 7.4) was added purified chicken liver AICAR transformylase to a final concentration of 0.01 mg of protein/mL. Scans a, b, c, and d are immediately before addition of the enzyme and 50, 90, and 130 min after the addition of the enzyme, respectively. Scan time was approximately 3 min in a 0.2-cm cell, 25 °C; isosbestic point is 236 nm.

method of Guggenheim (1926).

DAR is converted to IMP by refluxing for 30 min in 0.5 M potassium bicarbonate. IMP was identified by its UV spectra at neutral and basic pH values and by TLC (Table III). DAR is converted to IMP at 37 °C in 0.5 N NaOH with a pseudo-first-order rate constant of 0.025 min^{-1} determined by the method of Guggenheim (1926).

DAR has certain properties which are similar to but not identical with those reported for formyl-AICAR (Flaks et al., 1957a; Lukens & Flaks, 1963). Formyl-AICAR is reported to have a UV spectrum at pH 7 which has a single absorption maximum at 270 nm with a molar extinction coefficient of 1.13×10^4, and in this respect it does not resemble DAR. On the other hand, formyl-AICAR is reported to be quantitatively converted to IMP by heating at 38 °C in 0.1 N NaOH for 30 min and quantitatively converted to AICAR by heating at 100 °C in 0.2 N H_2SO_4 for 3 min; these base-catalyzed and acid-catalyzed conversions of formyl-AICAR are similar to those observed with DAR.

Structure of DAR, AICAR, and Formyl-AICAR. The data presented is consistent with the structure of DAR shown in Figure 1 but does not provide unequivocal proof. An imine-type linkage to the ribose is possible. The acid-catalyzed conversion of DAR to AICAR involves hydrolysis of a formamido group and ring closure (dehydration). The linear Guggenheim plot which was obtained and the isobestic point at 235 nm (Figure 5) suggest either that these two processes are concerted or that one proceeds much more rapidly than the other. A rapid equilibrium that is reasonable is that of ring closure. Base-catalyzed conversion of DAR to IMP most likely involves one ring closure in a slow step preceded or followed by the rapid closure of the other ring.

The acid-catalyzed conversion of DAR to AICAR and the base-catalyzed conversion of DAR to IMP indicate that DAR is a high-energy unstable compound. The molecular model of formyl-AICAR also indicates that this compound is (should be) a high-energy unstable species. Indeed, the formamido and carboxamide moieties of formyl-AICAR cannot both be coplanar (because of steric hindrance) with the imidazole ring, thus reducing the possibility of π-orbital overlap. Unlike the molecular model of formyl-AICAR, the molecular models of AICA, AICAR, and FAICA indicate that the carboxamide and the amino or formamido moieties can both lie coplanar with the imidazole ring, which results in the formation of an intramolecular hydrogen bond between the nitrogen-bonded proton (amino or formamido group) and the carbonyl oxygen of the carboxamide moiety. Thus, formyl-AICAR is not structurally similar to AICA, AICAR, or FAICA. The molecular model of DAR indicates that although the two formamido groups are not coplanar with the acrylamide backbone there are three possible intramolecular hydrogen bonds involving the three carbonyl oxygens. The formation of these hydrogen bonds may result in the DAR structure being more stable than the formyl-AICAR structure which, if present in solution, would exist in very small amounts.

The data above demonstrate that the chemical formylation of the 5-amino group of AICAR destabilizes the imidazole ring, resulting in hydrolytic cleavage. The acid-catalyzed hydrolysis of the formamido group reverses this process. Base-catalyzed formation of IMP most likely proceeds with ring closure to the pyrimidine, followed by rapid ring closure to form the now stabilized imidazole moiety. A hypothesis, which will require further testing, is that enzyme-catalyzed formylation of the 5-amino group of AICAR also results in hydrolytic cleavage of the imidazole ring.

Biochemical Properties of DAR. (1) Assay and Kinetics. The enzymatic conversion of DAR to IMP is monitored by observing the increase in optical density at 250 nm. IMP was identified by its UV spectrum (Figure 5) and TLC (Table III). The validity of the assay was established by the fact that the rate of formation of IMP from DAR catalyzed by extracts of chicken, rat, and human liver was proportional to the protein concentration. Using initial concentration of DAR of 50 μM in 50 mM Tris-HCl buffer and 150 mM KCl, pH 7.4 at 37 °C, we found specific activities (± SD) of 121 ± 14, 4.84 ± 0.25, and 0.24 ± 0.02 nmol of IMP formed per min per mg of protein in extracts of chicken, rat, and human liver, respectively. In the absence of enzyme there is no change in optical density at 250 nm and therefore no production of IMP at 37 °C over the pH range of 5.6–9 during the time period required to assay the enzyme or measure the kinetic parameters, which was a maximum of 30 min (see Methods and Figure 6). Above pH 9 a small increase in the optical density at 250 nm was observed in a 50 μM solution of DAR containing no enzyme. The enzyme concentration was adjusted such that this nonenzymatic rate was less than 5% of the enzyme-catalyzed rate and this relatively small nonenzymatic conversion of DAR to IMP did not confound the measurement of the kinetic parameter at alkaline pH.

This spectrophotometric assay is sensitive and specific for DAR. The conversion of as little as 0.04 nmol of DAR per mL per min at pH 7.4 could be accurately measured by using a 5-cm path length cuvette. FAICA is not a substrate for the enzyme and does not inhibit the enzymatic conversion of DAR to IMP when tested at concentrations from 50 to 700 μM (pH 7.4; data not shown). Assays were run in 150 mM KCl in order to approximate the intracellular potassium ion concentration and to maintain constant ionic strength.

The K_m values for DAR of the chicken liver and rat liver enzymes were remarkably low with both the initial velocity measurements and the integrated Michaelis–Menten equation yielding equivalent kinetic parameters for the chicken liver

Table IV: Kinetic Parameters[a]

source (buffer, salts)	K_m ± SE (μM) integrated M-M[b]	K_m ± SE (μM) initial velocity[c]	V_m ± SE (nmol/min)/mg integrated M-M[b]	V_m ± SE (nmol/min)/mg initial velocity[c]
chick liver (50 mM Tris-HCl, 150 mM KCl)	2.79 ± 0.15	2.75 ± 0.50	158 ± 1	177 ± 17
chick liver (20 mM sodium phosphate, 150 mM KCl)	2.96 ± 0.09		168 ± 1	
chick liver (50 mM Tris-HCl, 100 mM NaCl, 50 mM KCl)	2.76 ± 0.08		165 ± 1	
chick liver (50 mM Tris-HCl, 2 mM EDTA)	2.34 ± 0.17		168 ± 1	
rat liver (50 mM Tris-HCl, 150 mM KCl)		4.25 ± 0.85		6.60 ± 0.72

[a] pH 7.4, 37 °C. [b] Determined by use of the integrated Michaelis-Menten equation (see Methods); initial concentration of DAR was 50 μM. [c] Determined from initial velocity measurements and the Eadie-Hofstee equation.

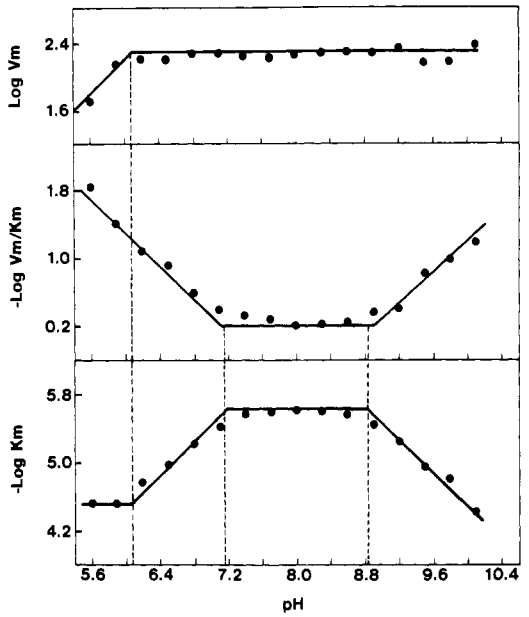

FIGURE 6: Kinetic parameters–pH profiles. Log V_m, -log V_m/K_m, and -log K_m at 37 °C are plotted vs. the pH. The following buffers were used: pH 5.6, 0.04 sodium acetate; pH 5.9–7.4, 0.02 M sodium phosphate; pH 7.4–8.9, 0.05 M Tris-HCl; pH 9.2–10.1, 0.02 M sodium borate. All buffers contained 150 mM KCl; ionic strength was 0.16–0.21. Initial concentration of DAR was 50 μM. Kinetic parameters were determined from the integrated Michaelis–Menten equation (see Methods). Dashed lines show the position of the pK_a values.

enzyme (Table IV). Unlike AICAR transformylase, a potassium ion activation of the enzyme at pH 7.4 could not be demonstrated (Baggott & Krumdieck, 1979) (Table IV). Plots of the integrated Michaelis–Menten equation (see Methods) for the rat liver enzyme were not linear, suggesting that product inhibition and/or destruction of the substrate was occurring. Linear plots of the integrated Michaelis–Menten equation for the chicken liver enzyme were obtained from pH 5.9 to 10.1. At pH 5.6 a deviation from linearity was apparent after 50% of the initial DAR had been converted to IMP; therefore, the first portion of the reaction was used to determine K_m and V_m.

(2) Mechanism of Enzyme Catalysis. The pH–activity profiles (Figure 6) were plotted as suggested by Dixon & Webb (1964) and show three pK_a values from pH 5.6 to 10.1. Following Dixon's rules (Dixon & Webb, 1964), we found that the pK_a of 6.1 appears to be associated with the enzyme–substrate complex while those of 7.1 and 8.8 are associated with the free enzyme or substrate. The pK_a values of 6.1 and 7.1 are consistent with the deprotonation of the imidazole ring of histidine. The second ionization of the phosphate moiety of DAR should also have a pK_a of 6–7, and it is possible that this ionization is responsible for the pK_a of V_m and K_m of 6.1. However, if a histidine is responsible for both of these pK_a values, then the binding of the substrate would decrease its pK_a by 1 unit (7.1 to 6.1), and this alteration in pK_a would be reasonable if the histidine were located at the active site. This interpretation is also consistent with the observed slope of +1 in the -log K_m vs. pH plot from pH 6.2 to 7.1 since deprotonation of a histidine in concert with substrate binding would produce this slope (Dixon & Webb, 1964).

The pK_a at 8.8 is most likely associated with the free enzyme since no group on the enzyme–substrate complex (log V_m vs. pH plot) or the substrate has a pK_a with that value. This ionization is consistent with a sulfhydryl group (Dixon & Webb, 1964) which can deprotonate in the free enzyme but not in the enzyme–substrate complex. A reasonable explanation for the data would be the formation of a covalently bound substrate linked to that sulfhydryl. It is also reasonable that the ionization of a sulfhydryl could perturb the binding of DAR to the enzyme since between pH 7 and ~9.5 DAR would exist with a net charge of -1, while above pH 9.8 it would have a net charge of -2. Therefore, if a sulfhydryl group is present at the active site, it would have a formal negative charge at pH ~8.8 or above which could have an unfavorable interaction with a negatively charged DAR. This unfavorable interaction could result in the increase in K_m observed above pH 8.8. This type of interpretation is not without precedent; Gerwin et al. (1966) found a pK_a of 8.4 in the -log K_m vs. pH plot which was absent in the log V_m vs. pH plot for purified streptococcal proteinase, and Lucas & Williams (1969) found a pK_a of 8.3–8.6 for papain which was associated with the V_m/K_m parameter but not with V_m. Both of these enzymes contain a sulfhydryl at the active site and form an acyl enzyme linked to that sulfhydryl during catalysis. Obviously, the above interpretation represents one simple explanation which is consistent with the data but does not exclude others.

Biochemical Significance. It is clear that DAR must either be a naturally occurring substrate or a substrate analogue of an enzyme-catalyzed reaction in de novo purine biosynthesis. A low K_m, an equilibrium greatly in favor of its conversion to IMP, a large difference in the specific activity of the enzyme when uricotelic and ureotelic animals are compared, and an optimum pH for the enzyme-catalyzed reaction of 8.0 are consistent with the hypothesis that DAR is a biological substrate. The K_m of 2–3 μM (pH 7.4; chick liver enzyme) is approximately 10 times lower than the K_m of AICAR transformylase for AICAR under similar conditions (Baggott & Krumdieck, 1979). The large differences in specific activities of the enzyme of birds and mammals are explained by the very large differences in the rate of uric acid synthesis per kilogram of body weight existing between uricotelic and ureotelic animals. We have calculated that uric acid synthesis in birds may be 10^4 times higher than in mammals.

The substrate activity of DAR is similar to but not identical with the substrate activity of formyl-AICAR. Formyl-AICAR (Flaks et al., 1957a) is converted to IMP by an enzyme activity

present in chicken liver. The pH optimum of this enzyme activity is 7.4 and, unlike AICAR transformylase, shows no requirement for potassium ions. The kinetic parameters of this enzyme activity have not been reported.

The putative nucleotide product of the AICAR transformylase catalyzed reaction is formyl-AICAR. There is a report of a mutant of *Salmonella typhimurium* which lacks the enzyme IMP cyclohydrolase but has measurable amounts of AICAR transformylase (Gots et al., 1969). Formyl-AICAR, however, was not isolated from these cultures but its presence was inferred indirectly by an increase in aryl amines, measured by the Bratton–Marshall test, following acid hydrolysis. Since our data demonstrates the acid-catalyzed formation of AICAR from DAR (Table II and III), the presence of a diazotizable aryl amine detected by the Bratton–Marshall test is insufficient to unambiguously demonstrate the accumulation of formyl-AICAR in this mutant.

It is possible that DAR may exist in rapid equilibrium with a very small amount of an aromatic nonpurine nucleotide (i.e., a pyrimidine or imidazole compound) which binds to the enzyme and represents the true substrate for IMP cyclohydrolase. Indeed, any number of rapid chemical equilibria preceding the binding of the true substrate by the enzyme may be postulated. With due regard for the above considerations, we hypothesize that DAR is most likely either a naturally occurring substance formed as the nucleotide product of the AICAR transformylase catalyzed reaction or merely a substrate analogue of IMP cyclohydrolase. Experiments are now underway to test these possibilities.

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Inhibition of Lactate Transport and Glycolysis in Ehrlich Ascites Tumor Cells by Bioflavonoids†

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ABSTRACT: Bioflavonoids are potent inhibitors of lactate transport in Ehrlich ascites tumor cells. The most effective bioflavonoids have four to five hydroxyl groups. Sugar substitution at carbon three, or reduction of the double bond between carbons two and three, decreases their inhibitory activity. Quercetin, the most extensively studied of these compounds, inhibits lactate efflux by 50% at 0.1 μg/mg of protein. On additon of quercetin to glycolyzing Ehrlich ascites tumor cells, lactate accumulates inside the cell and the intracellular pH drops. Total lactate production is also inhibited. Nigericin prevents the internal acidification that occurs in the presence of quercetin and also reduces the inhibition of glycolysis. Thus, it appears that inhibition of lactate efflux can affect glycolysis through a lowering of the intracellular pH. The inhibitory effect of quercetin on glycolysis can be explained by its effect on lactate efflux and its previously reported effect on the Na^+–K^+ ATPase [Suolinna, E.-M., et al. (1974) *J. Natl. Cancer Inst. 53*, 1515].

Cells that produce more lactic acid than they can metabolize contain a transport system for monocarboxylic acids (cf. Harold & Levin, 1974). Spencer & Lehninger (1976) have shown that lactate is rapidly excreted by Ehrlich ascites tumor cells via a lactate–proton symport mechanism. Human erythrocytes excrete lactate by the same mechanism (Dubinsky & Racker, 1978). Since the ascites tumor cells, like all other malignant tumors, produce large amounts of lactate, even under aerobic conditions, a study of effects of intracellular accumulation of lactate and protons seemed of interest. To this purpose, Thomas et al. (1979) have developed a procedure that permits continuous measurement of intracellular pH in ascites tumor cells and they have correlated pH changes with

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