Studies of the Action of Adenylosuccinase with 6-Thio Analogues of Adenylosuccinic Acid*

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(Received for publication, July 17, 1961)

Recent work has shown that certain growth inhibitory purine analogues undergo anabolism to ribonucleoside 5'-phosphates (1, 2). Ribonucleoside 5'-phosphate derivatives of purine analogues that effect temporary remission of some human leukemias have been synthesized (3-6), and effects of these nucleotides on enzymatic interconversions of naturally occurring purine nucleotides have been studied (7-9). A preliminary communication (7) reported that 6-mercapto-8-b-D-ribofuranosylpurine 5'-phosphate (6-thiosuccinocyanic acid) inhibited conversion of adenylosuccinase (III) to adenosine phosphate by adenylosuccinase. This conversion, described by Carter and Cohen (10), involves reversible cleavage of adenylosuccinic acid to adenosine phosphate and fumaric acid. A possible mechanism of inhibition could be addition of the 6-mercapto group of the nucleotide to the double bond of fumaric acid to form an inhibitory analogue (Fig. 3, IV) of adenylosuccinic acid in which the nitrogen at position 6 is replaced by sulfur. Synthesis of this analogue was desired to investigate this possibility and, in addition, to examine the potentialities of the analogue as a relatively selective inhibitor of adenylosuccinase. The present communication details the synthesis and purification of the 6-thio analogue of adenylosuccinic acid, adenylosuccinacin, adenylosuccinacin is itself, and the xanthine enantiomorphs of each of these nucleotides. An unexpected finding was that the 6-thio analogue is cleaved by the enzyme preparation in a manner analogous to the cleavage of adenylosuccinic acid. From studies of the analogues of adenylosuccinic acid as substrates and inhibitors of adenylosuccinase, and from studies of chemical properties of purine derivatives serving as models, hypotheses are suggested concerning the mechanism of enzymatic cleavage of adenylosuccinate and some of the characteristics of the active site of adenylosuccinase.

EXPERIMENTAL AND RESULTS

Paper chromatography and electrophoresis were carried out as described (3). Pentose in ultraviolet-absorbing spots was determined by the method of Ceriotti (11) with sodium guanosine 5'-phosphate (Pabst Laboratories) as a standard, and total phosphorus by the method of Fiske and SubbaRow (12) for both determinations, equal areas of paper not containing ultraviolet-absorbing material were used to prepare blanks. Optical rotations were measured with a model D attachment (Standard Polimeter Company, New York) to the Beckman model DU spectrophotometer.

6-Chloro- and 6-mercapto-9-b-D-ribofuranosylpurine 5'-phosphates were synthesized (3). Pure d-2-bromosuccinic acid (mp 171°, +76.3°; 6% solution in ethyl acetate) was prepared from d(-)-aspartic acid ([a]D -24.4° in 6 N HCl (California Corporation for Biochemical Research)) by the method of Holmberg (13) and converted to crude L(-)-2-mercaptosuccinic acid (14); crystallization of the latter from methanol isobutyl ketone-benzene, then from ethyl acetate-benzene gave white prisms of the pure L isomer ([a]D 82.2° (5% solution in ethyl acetate)). d(-)-2-Mercaptosuccinic acid ([a]D +82.5°) was similarly prepared from L(-)-aspartic acid. Racemic diethyl 2-mercaptosuccinic acid was prepared according to Barry and McPhee (15). 6-Mercapto-8-b-D-ribofuranosylpurine was purified (10), and 6-chloropurine was obtained in chromatographically homogeneous form after crystallization of the ether-extractable portion from water.

Adenylosuccinase (Fraction IV, fumarase-free (10)) from yeast and adenylosuccinic acid prepared enzymatic reaction of AMP and fumaric acid were kindly provided by Dr. C. H. Carter of Yale University.

Solutions for paper chromatography were: Solvent A, n-butanol-acetic acid-water (2:1:1); Solvent B, saturated aqueous (NH4)2SO4; Solvent C, 0.1 M aqueous ammonium acetate-isopropanol (79:19:2) (17); Solvent D, 5% Na2HPO4-isoamyl alcohol (18); Solvent E, 5 M aqueous formic acid-n-amy alcohol (1:1) (19); and Solvent F, water. Buffers for paper electrophoresis were 0.05 M ammonium formate-formic acid (pH 3.50) and 0.04 M sodium phosphate (pH 7.15).

Synthesis of 6-(DL-1,2-Dicarboxyethylmercapto)purine (dl-6-Succinomercaptopurine)—A mixture of 6-mercaptopurine (250 mg, 1.66 mmoles), dl-2-bromosuccinic acid (1.65 mmoles), KHCO3 (4.95 mmoles), and dimethylformamide (4 ml) was stirred at room temperature for 4 hours. Volatiles were removed under reduced pressure, and 5 ml of water and 1 ml of 1 N HCl were added to the solid residue; 6-mercaptopurine (30 mg) was removed by filtration, and 4 ml of 1 N HCl were added to the filtrate. The mixture was stored at 2° overnight to complete precipitation, and the crystalline solid (290 mg, 66% yield) was collected and washed with water. Crystallization of the product from 105 ml of boiling water gave 220 mg of cream-colored needles, m.p. 195-198° (decomposition). On paper chromatograms the product gave a single spot, Rf 0.79 in Solvent C, Rf 0.77 in
In 95% ethanol it showed absorption maxima at 215 and 280 m\( \mu \) (Fig. 1), \( \varepsilon_{215} \) 12.5 \( \times \) 10\( ^{4} \) and 17.4 \( \times \) 10\( ^{4} \), respectively.

**Effect of Acid and Base on 6-Succinomercaptopurine and Its Diethyl Ester**—An ethanolic solution containing 0.978 pmole per ml of the diethyl ester (I) was diluted 25-fold with 0.05 N HCl in 95% ethanol. The absorption spectrum of the solution resembled that of the diethyl ester in neutral 95% ethanol (Fig. 1, Curve A) and was unchanged after 4 hours at 25\( ^{\circ} \). With LiOH instead of HCl, the absorbancy progressively decreased at 280 m\( \mu \) and increased at 312 m\( \mu \) for 45 minutes at 25\( ^{\circ} \). The absorption spectrum (Fig. 1, Curve B; \( \varepsilon_{312} \) 19.9 \( \times \) 10\( ^{3} \)) then remained constant for several hours and closely resembled that of 6-mercaptopurine (II) in the same solvent (Fig. 1, Curve C; \( \varepsilon_{312} \) reported (20), 19.6 \( \times \) 10\( ^{3} \)).

\[
\text{H$_2$C$_2$OOC-CH-CH$_2$COOC$_2$H$_5$} \quad \text{Diethyl malate} \\
\text{Diethyl fumarate}
\]

A solution of 34 mg of the diethyl ester in 100 ml of 0.05 N LiOH in 95% ethanol was kept at 25\( ^{\circ} \) until spectrophotometric analysis showed that formation of 6-mercaptopurine was complete (1 hour), refluxed for 5 hours to saponify carboxylic esters, concentrated in a vacuum to approximately 5 ml, and treated with wet Dowex 50-H\( ^{+} \) ion exchange resin until the pH decreased to approximately 1. Chromatograms in Solvent E, together with maleic, maleic, and fumaric acids (RF values 0.35, 0.52, and 0.80, respectively), were sprayed with aqueous potassium permanganate, or with brom thymol blue (21). An area at the RF of fumaric acid had the same ultraviolet absorption, acidic reaction, and behavior toward permanganate, and no other acidic or permanganate-reducing areas were present.

A solution (0.04 M) of the diethyl ester in 0.1 M phosphate buffer, pH 7.4, was incubated at 37\( ^{\circ} \). Paper chromatography (Solvents C and D) showed that after 6 days almost all the diethyl ester (RF values 0.93 and 0 to 0.29, respectively) had decomposed, giving as the major ultraviolet-absorbing component an unidentified substance (RF values 0.87, 0.69) together with a lesser amount of 6-mercaptopurine (RF 0.53, 0.41). The unidentified component gave a weakly acidic reaction toward the brom thymol blue spray on a chromatogram run in Solvent C and from comparison of its RF values with those of 6-succinomercaptopurine and its diethyl ester was tentatively concluded to be a monoethyl ester of 6-succinomercaptopurine.

Absorption spectra (\( \lambda_{\text{max}} \) 288, 293 m\( \mu \)), respectively, of solutions of 6-succinomercaptopurine in 95% ethanol 0.05 N with

1 Areas of paper containing fumaric or maleic acid reduced permanganate immediately at room temperature, whereas maleic acid required 2 to 5 minutes; all the acids gave rise to yellow or white areas on a red background.
respect to HCl or to LiOH remained unchanged after 4 hours at 25°.

Synthesis of Adenylosuccinic Acid (6-(r-1,2-Dicarboxyethylamino)-9-β-D-ribofuranosyluridine 5'-phosphate)—Model reactions between 6-chloro-9-β-D-ribofuranosyluridine (22) and aspartic acid were analyzed by paper chromatography in Solvents D and F, with the previously prepared ribonucleoside of adenylosuccinic acid (21) as a reference compound. Conditions that gave almost quantitative yields of this nucleoside were equally satisfactory when applied to the synthesis of adenylosuccinic acid itself.

Paper chromatographic and electrophoretic data relating to the four syntheses described below are given in Table I. A solution of barium 6-chloro-9-β-D-ribofuranosyluridine 5'-phosphate dihydrate (25 mg, 46.5 pmoles) in 2 ml of water was percolated through a column of Dowex 50 ion exchange resin (1 ml of the potassium form). The resin was washed with 5 ml of water, and the eluate was evaporated under reduced pressure. To a solution of the residual potassium salt of the nucleotide in 0.32 ml of dimethylformamide-water (2:1) were added 279 μmoles of potassium bicarbonate and 93 μmoles of L(+)-aspartic acid. The solution was kept at room temperature and analyzed at intervals by chromatography in Solvent C; after 3 days 6-chloro-9-β-D-ribofuranosyluridine 5'-phosphate was no longer detectable, and the predominant spot (approximately 90% of the total ultraviolet light-absorbing material) corresponded to adenylosuccinic acid (III); IMP and unidentified material, RF 0.55, were minor components. The bulk of the reaction mixture was chromatographed in Solvent C and the papers air-dried and suspended for several minutes at room temperature in a damp ammonia atmosphere. The portions of paper containing the major component were extracted with water; the solution possessed ultraviolet absorption characteristics in the pH range 1 to 8 indistinguishable from those reported for enzymatically prepared adenylosuccinic acid (10) and contained 6-succinomercapto, phosphorus, and pentose in the ratio 1:1.01:0.91, necessary to reduce by 1.0 per minute the absorbancy at 280 μm of enzyme.

6-(r-1,2-Dicarboxyethylmercaptol)-9-β-D-ribofuranosyluridine 5'-phosphate (V) was similarly obtained by condensation of potassium 6-chloro-9-β-β-D-ribofuranosyluridine 5'-phosphate with α(-)-aspartic acid. The purified nucleotide was unaffected by adenylosuccinase but was identical with natural adenylosuccinic acid in other properties.

Synthesis of 6-(r-1,2-Dicarboxyethylmercapto)-9-β-D-ribofuranosyluridine 5'-phosphate (IV)—To a solution of 46.5 μmoles of potassium 6-chloro-9-β-D-ribofuranosyluridine 5'-phosphate in 0.15 ml of water were added 279 μmoles of KHCO3 and 93 μmoles of L(-)-2-mercaptosuccinic acid. The pale yellow solution was stored at 25° and analyzed at intervals by paper chromatography (Solvents A and B) and electrophoresis (pH 3.50, 2 hours at 20 volts per cm). After 48 hours, 6-chloro-9-β-D-ribofuranosyluridine 5'-phosphate was no longer detectable, and the only ultraviolet-absorbing components visible were 2-mercaptosuccinic acid, the desired nucleotide, and traces of IMP. A portion of the reaction mixture was diluted with an equal volume of water and subjected to paper electrophoresis at pH 3.50 for 3 hours at 20 volts per cm. The paper was partially dried in warm air and finally for 3 hours at 100° and 10 mm of pressure to remove ammonium formate. The sections of paper containing the desired nucleotide were extracted with water. The spectroscopic properties of the solution at various pH values are shown in Fig. 2. The spectrum was constant from pH 7.4 to pH 9; at pH 7.4 the absorption maximum (291 μm) was the same as that of the aglycone. The solution contained 6-succinonemercapto, total phosphorus, and pentose in the ratio 1:1.01:0.91,
The enzyme concentrations used in these experiments were slightly less than those which ceased to be rate-limiting for the same concentrations of normal substrate. After prolonged enzymatic action (12 to 15 hours), the absorption spectrum showed maxima at 225 and 318 nm and a minimum at 225 nm and resembled that of 6-mercapto-9-β-d-ribofuranosylpurine 5′-phosphate (6-thioinosine 5′-phosphate) in the same buffer.

The molar absorbancy indexes at 290 and 318 μm were determined for 6-thioinosine 5′-phosphate at pH 7.1, and calculations made of the increases in absorbancy at 318 μm which would accompany the observed decreases at 290 μm if the reaction involved conversion of IV to 6-thioinosine 5′-phosphate. The calculated values at 318 μm agreed within 5% with those observed (Table II).

Adenylosuccinase (0.54 unit, 0.50 ml) was incubated at 37° for 30 hours with 0.6 ml of a 2.4 mM solution of IV in 0.02 M phosphate buffer, pH 7.1. The absorption spectrum of a diluted aliquot resembled Curve C of Fig. 4 and showed that at least 85% of IV had undergone conversion. The mixture was heated in boiling water for 5 minutes and clarified by filtration through Celite filter-aid. The solution was concentrated under reduced pressure to approximately 0.1 ml and chromatographed in Solvents E and F together with solutions of malic, fumaric, maleic, and 6-thioinosinic acids in 0.2 M phosphate buffer. The one major ultraviolet-absorbing component (Rf 0 in Solvent E, 0.20 in Solvent F) corresponded to 6-thioinosine 5′-phosphate.

Treatment of the papers with the KMnO₄ spray showed a component with the Rf values and delayed oxidizing characteristic of malic acid; fumaric or maleic acids were not detected. The remainder of the enzymatic digests was concentrated to half volume and subjected to paper electrophoresis at pH 7.1 (20 volts per cm for 65 minutes); the predominant ultraviolet-absorbing component migrated 4 cm towards the anode and when eluted with water and run in Solvent D had the same Rf value (0.79) as that synthesized from L(-)-2-mercaptosuccinic acid and its n-succino enantiomorph (V), and the 6-thio analogue of adenylosuccinic acid (IV) from 6-chloro-9-β-d-ribofuranosylpurine 5′-phosphate. Bonds shown in heavy lines project toward the viewer and depict the absolute stereochemical configuration of the asymmetrical carbon of each succinic acid moiety (see text).

Stereochemical Configurations of Synthetic Nucleotides—The reactions of Fig. 3 involve no asymmetrical carbon atoms, and the resulting nucleotides hence retain the stereochemical configuration of the enantiomorphic aspartic or mercaptosuccinic acid used for their preparation. It has been shown (23) that (−)-2-mercapto succinic acid has the same configuration as (−)-malic acid and that (−)-malic acid (24) and natural aspartic acid (25) have the absolute configuration established (26) for L-glyceraldehyde. Data on the racemization of aspartic acid (27) and of 2-mercapto succinic acid (14) indicate that no significant racemization of these compounds is likely to occur under the conditions used.

Analogue of Adenylosuccinic Acid as Substrates of Adenylosuccinase—Solutions in phosphate buffer, pH 7.1, of the 6-thio analogue of adenylosuccinic acid (Fig. 3, IV) exhibited an ultraviolet absorption spectrum which remained constant for at least 24 hours at 25°. Addition of adenylosuccinase to such solutions initiated a progressive decrease in absorbancy at 290 μm and a progressive increase and appearance of a new maximum at 318 μm (Fig. 4). The enzyme concentrations used in these experiments were slightly less than those which ceased to be rate-limiting for the same concentrations of normal substrate. After prolonged enzymatic action (12 to 15 hours), the absorption spectrum showed maxima at 225 and 318 μm and a minimum at 225 μm and resembled that of 6-mercapto-9-β-d-ribofuranosylpurine 5′-phosphate (6-thioinosine 5′-phosphate) in the same buffer.

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as 6-thioinosine 5'-phosphate. The remainder of the paper was sprayed with KMnO₄, and only the region between 15 and 23 cm in the direction of the anode reduced permanganate in the manner² of malic acid. Authentic malic acid, run simultaneously, migrated 16 to 23 cm as a streak, whereas fumaric acid migrated 24 cm as a compact spot. There was no ultraviolet-absorbing spot with the mobility of IV.

The rate of attack by adenylosuccinase on the analogue IV was about 7/8 that of the normal substrate at the enzyme concentrations employed. For example, in the presence of 0.043 unit³ of enzyme the rate of disappearance of IV in 2.5 ml of a 46 μM solution was essentially constant at 0.54 mMole per minute for the first 30 minutes, whereas with the same amount of enzyme and the same volume of 51 μM adenylosuccinic acid, the rate was 19.3 mMole per minute over the first 5 minutes.

Attempts to establish equilibrium conditions or to demonstrate reversibility of the enzymatic conversion of IV to 6-thioinosinic acid were unsuccessful. In the presence of 5 mM fumarate, the initial rate of formation of 6-thioinosine 5'-phosphate in 50 μM IV was reduced by 15%, but cleavage proceeded to completion in approximately the same total period of time as in the absence of added fumarate. The analogue differs markedly in this respect from adenylosuccinic acid, which, under the above conditions, yields an equilibrium mixture of 42% unchanged substrate and 58% AMP (calculated from the reported (10) equilibrium constant). Solutions of 6-thioinosinic acid (2.5 ml of 30 μM) were incubated at 37° for several hours with relatively large amounts (0.11 unit) of enzyme in the presence of either 5 mM fumaric acid or 1 mM maleic or maleic acid. The absorbancies at 318 nm were unchanged, and as little as 0.5% decrease in 6-thioinosinate concentration would have been detectable.

The n-succino enantiomorph of IV (Compound VI of Fig. 3) did not serve as a substrate for adenylosuccinase: addition of 0.054 unit of the enzyme to 2 ml of a 45 μM solution of VI in the phosphate buffer produced no change in absorbancy at 290 or 318 nm at 290 and 318 nm, 6,180 and 20,600, respectively.

TABLE II

<table>
<thead>
<tr>
<th>Reaction time</th>
<th>Changes in absorbancy at 290 nm</th>
<th>Increase at 318 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Decrease at 290 nm*</td>
<td>Calculated</td>
</tr>
<tr>
<td>hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.098</td>
<td>0.117</td>
</tr>
<tr>
<td>1.0</td>
<td>0.171</td>
<td>0.205</td>
</tr>
<tr>
<td>2.0</td>
<td>0.242</td>
<td>0.290</td>
</tr>
<tr>
<td>3.0</td>
<td>0.306</td>
<td>0.367</td>
</tr>
</tbody>
</table>

* Corrected for absorption of 6-thioinosine 5'-phosphate at 290 nm.

Fig. 5. Inhibition of adenylosuccinase-mediated cleavage of adenylosuccinic acid by the 6-thio analogue (IV) of adenylosuccinic acid. Adenylosuccinate concentration is proportional to absorbancy at 280 μM (10). Solutions (2.1 ml) of 25 μM adenylosuccinate in 0.05 M phosphate, pH 7.1, contained 0.011 unit of enzyme activity and concentrations of IV as given below; the blank solutions lacked adenylosuccinate. Curve A, rate of decrease in absorbancy at 280 μM in the absence of IV; Curve B, rate in the presence of 50 μM IV; Curve C, rate in the presence of 100 μM IV.

TABLE II

* Calculated from the assumptions that the enzyme concentration in these experiments (0.0052 unit per ml) was rate-limiting, and that the presence of adenylosuccinic acid caused no reduction in the rate.

Figure 5

* Downloaded from http://www.jbc.org/ on October 29, 2017
Carter and Cohen (10) showed spectrophotometrically that the pKₐ values of the two carboxyl groups and of the substituted amino group of adenylosuccinic acid are within the range 2.3 to 5.1 and potentiometric titration of the corresponding ribonucleoside is in agreement (21). The pKₐ value for the secondary phosphoryl dissociation is 8.8 (10). Therefore, in solutions at pH 7.1 adenylosuccinic acid carries a negative charge at each carboxyl group, no charge at the substituted amino group, and 1.7 negative charges at the phosphorol group. The electrophotographic mobility (Table I) at this pH of the 6-thio analogue of adenylosuccinic acid (Fig. 3, IV) was indistinguishable from that of adenylosuccinic acid under conditions in which differences of 4% were detectable. Electrophotographic mobility is known to be a function of molecular charge, weight, and configuration. The 6-thio analogue is virtually identical to adenylosuccinic acid in molecular weight and configuration, hence at pH 7.1 it must also be identical with adenylosuccinic acid in its number and distribution of negative charges. The major configurational difference between the two nucleotides could reside in the bond angles of the nitrogen and sulfur atoms in the 6-position, the angle in the normal substrate being approximately 108° (simple amines) and that in the analogue 92° (thioethers).

The products of cleavage of the analogue IV by partially purified adenylosuccinase were 6-thioinosinic 5'-phosphate and malic acid. In obtaining this result, a prolonged incubation period was necessary for complete cleavage, and it is possible that the primary product was fumaric acid which was subsequently converted to malic acid by fumarase, even though the adenylosuccinase used was fumarase-free under normal assay conditions (10). The formation of fumaric acid from IV could be analogous to the cysteine and homocysteine desulfhydrases for which initial addition of cofactor glutathione to the ethylenic bond of the substrate complex in which the negative charges associated with the site occupied by the normal substrate. These findings virtually discount the possibility that prior formation of IV could be involved in the inhibition of adenylosuccinase by 6-thioinosinate (7).

Cleavage of IV could involve substitution by a nucleophile (X⁻) on the a-succino carbon (Reaction 1a) to initiate formation of 6-thioinosinate (RS⁻) followed by elimination of XIH to give fumaric acid (Reaction 1b), or displacement of X by hydroxyl ion to give maleic acid (Reaction 1c).

\[
\begin{align*}
HOOC-CH(SR)-CH_2-COOH & \xrightarrow{X^-} HOOC-CH(X)-CH_2-COOH + RS^- \\
X^- + HOOC-CH(OH)-CH_2-COOH & \xrightarrow{(a)} HOOC-CH(OR)-CH_2-COOH
\end{align*}
\]

Alternatively, the nucleophile could induce removal of a proton from the β carbon (Reaction 2a) to initiate formation of 6-thioinosinate and fumaric acid by a β elimination mechanism (Reaction 2b). For either reaction, activation of IV by neutralization of the negative charges present at each of its carboxyl groups would seem to be necessary. The diethyl ester (I) of the aglycone of IV was synthesized in order to serve as a model for the situation in which ionization of both carboxyls of IV is completely suppressed. Comparison of this diester with its nonesterified counterpart showed that both were stable under acidic or neutral conditions, but that the diester alone was unstable under alkaline conditions; rapid and quantitative cleavage to 6-mercaptopurine occurred (Fig. 1). After a saponification treatment, fumaric acid (Reaction 1b) or displacement of X by hydroxyl ion to give maleic acid (Reaction 1c).

\[
\begin{align*}
HOOC-CH(OR)-CH_2-COOH & \xrightarrow{(a)} HOOC-CH(OR)-CH_2-COOH + XH \\
HOOC-CH(SR)-CH_2-COOH & \xrightarrow{X^-} HOOC-CH(SR)-CH_2-COOH
\end{align*}
\]

These findings tend to support the view that enzymatic cleavage of the 6-thio analogue involves formation of an enzyme-substrate complex in which the negative charges associated with the carboxyl groups of the substrate are neutralized, conceivably by salt formation with basic centers in the enzyme. The activated nucleophile can then be subject to nucleophilic attack by a group located on the enzyme or by an anion present in solution. Several lines of evidence argue that cleavage of IV is mediated by adenylosuccinase rather than by some other enzyme present. That IV has affinity for adenylosuccinase is revealed by its ability to inhibit enzymatic cleavage of adenylosuccinate (Fig. 5);
the a-succino enantiomorph of IV, which by this criterion has less affinity than IV for adenylosuccinate, is not cleaved by the enzyme preparation. This parallelism between affinity and cleavage applies also to a-succino adenylosuccinate (V), which was neither a marked inhibitor of adenylosuccinase nor substrate for the enzyme preparation. The aglycone of IV was likewise not a substrate, thus further implicating adenylosuccinase as the catalytic agent for IV, since adenylosuccinase has a specific requirement for nucleoside 5’-phosphates (10). Miller, Lukens, and Buchanan (38) reported that the 6-thio analogue used in the present studies also inhibits enzymatic cleavage of fumaric acid from N-succinomercaptopurine is stable to both hydrogen and hydroxyl ions. But with hydroxyl ions readily yields 6-mercapto-


molecules for nucleophilic attack at the OL or /3 succinocarbon with subsequent formation of neutralized, thereby activating the molecules for nucleophilic attack at the carboxyl groups of both normal substrate and analogue are 31. DE LA HABA, G., AND CANTONI, G. L., Federation Proc., 16, (1938).


Since the 6-thio analogue evidently undergoes cleavage at the active site of adenylosuccinase, the mechanism previously discussed could therefore operate during cleavage of the normal substrate. The salt-forming groups on the enzyme are presumably not metal cations, since the activity of adenylosuccinase is unchanged by 0.01 M ethylenediaminetetraacetate (10). It may be noted that Reactions 1 and 2 are directly applicable to the formation of AMP if RH2— is substituted for RS—, that is, if the substituted amino group of adenylosuccinate is in the protonated form in the enzyme-substrate complex.

SUMMARY

Syntheses are described of adenylosuccinic acid, of its a-succino enantiomorph, and of the d- and L-enantiomorphs of the 6-thio analogue of adenylosuccinic acid, the aglycone of the latter, and its diethyl ester.

The L-succino form of the 6-thio analogue of adenylosuccinic acid inhibits cleavage of adenylosuccinic acid by partially purified adenylosuccinase, and is itself slowly and irreversibly cleaved to 6-thioinosinic acid and either fumaric or malic acid. The remaining analogues of adenylosuccinic acid were neither substrates nor marked inhibitors.

The diethyl ester of 6-succinomercaptopurine is stable toward hydrogen ions, but with hydroxyl ions readily yields 6-mercaptopurine and either fumaric or malic acid, whereas nonesterified 6-succinomercaptopurine is stable to both hydrogen and hydroxyl ions.

Cleavage of the 6-thio analogue of adenylosuccinic acid probably occurs at the active site of adenylosuccinase. It is suggested that in the enzyme-substrate complexes the negative charges on the carboxyl groups of both normal substrate and analogue are neutralized, thereby activating the molecules for nucleophilic attack at the α or β succinocarbon with subsequent formation of fumaric (or with the analogue malic) acid.

Acknowledgments—The author thanks Dr. Charles E. Carter for valuable discussions as well as generous gifts of adenylosuccinic acid and adenylosuccinate. He is also indebted to Dr. George Bosworth Brown for helpful suggestions and continued encouragement, and to Miss Joan M. Griffiths for skilled assistance.

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