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Inhibition of Adenylosuccinate Synthetase and Adenylosuccinate Lyase from Ehrlich Ascites-Tumour Cells by 6-Thioinosine 5'-Phosphate

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6-Mercaptopurine inhibits the conversion of hypoxanthine and of non-cyclic precursors into the guanine and adenine of nucleic acids in a number of micro-organisms and animal tumours (for recent reviews see Davidson, 1960; Carey & Mandel, 1961; Balis, Pecora & Salser, 1963). In an attempt to identify the site or sites of action of 6-mercaptopurine in Ehrlich ascites-tumour cells its nucleotide thio-IMP† was tested as an inhibitor of IMP-NAD oxidoreductase (IMP dehydrogenase; EC 1.2.1.14) from these cells, and was found to be a powerful competitive inhibitor (K_i 3.6 μ M) of oxidation of IMP to xanthosine 5'-phosphate (Atkinson, Morton & Murray, 1963). Thio-IMP has been reported to inhibit formation of adenylosuccinate from IMP with IMP-L-aspartate ligase (GDP) (adenylosuccinate synthetase; EC 6.3.4.4) from *Streptococcus faecalis* (Salser, Hutchison & Balis, 1960). Hampton (1960) reported inhibition of adenylosuccinate synthesis with the enzyme from *Escherichia coli* and found that conversion of adenylosuccinate into adenylyate by adenylosuccinate AMP-lyase (EC 4.3.2.2) from *E. coli* was also inhibited by thio-IMP. The values of K_i and the nature of the inhibitions were not reported by these workers; Hampton (1962) has discussed unpublished results indicating non-competitive inhibition of adenylosuccinate lyase by thio-IMP.

The present paper describes the preparation of adenylosuccinate synthetase and adenylosuccinate lyase of adequate purity for kinetic studies from Ehrlich ascites-tumour cells. The results show that thio-IMP is a non-competitive inhibitor of the conversion of IMP into adenylosuccinate and a

competitive inhibitor of the conversion of adenylosuccinate into adenylyate. In neither case was K_i as low as that found with IMP dehydrogenase from the same cells.

EXPERIMENTAL

Preparation of nucleotides

6-Thioinosine 5'-phosphate. A 25 mM solution of the triethylammonium salt in 1 mM-mercaptoethanol, pH 4, was prepared as described by Atkinson *et al.* (1963); no hydrolysis or oxidation of the nucleotide could be detected after 3 months at -15° . At its final concentration in assay solutions the mercaptoethanol had no inhibitory activity.

Guanosine 5'-triphosphate. Most of the GDP in a commercial sample of GTP (Pabst Laboratories, Milwaukee, Wis., U.S.A.) was removed by chromatography on DEAE-cellulose with a linear gradient (0.50–0.4M) of NH_4HCO_3 . GTP was eluted between 0.21M- and 0.28M- NH_4HCO_3 , and freed of bicarbonate by evaporation. On electrophoresis in 0.04M-sodium citrate buffer, pH 4.2, the product was found to contain 0.02 mol.prop. of GDP. The resultant concentration of GDP in the assay solution for adenylosuccinate synthetase was 3.5 μ M. The addition of GDP to increase the concentration of this compound in the assay solution to 7 μ M caused no inhibition of the initial rate of formation of adenylosuccinate.

Adenylosuccinate. The nucleotide was prepared from adenylyate and fumarate with adenylosuccinate lyase from yeast and was purified by anion-exchange chromatography as described by Carter & Cohen (1956). Only one component that absorbed light at 254 m μ could be detected on chromatography in ammonium sulphate–0.1M-sodium phosphate buffer (pH 6.8)–propan-1-ol (30:50:1, w/v/v; R_F 0.52) and in butan-1-ol–acetic acid–water (2:1:1, by vol.; R_F 0.40). The spectrum at pH 7 was the same as that described by Carter & Cohen (1956); the nucleotide:phosphate ratio, based on the molar extinction coefficient reported by these authors (ϵ 1.96×10^4 at 268 m μ), was 0.92:1.00.

* Deceased 27 September 1963.

† Abbreviation: thio-IMP, 6-thioinosine 5'-phosphate.

Preparation of enzymes

Adenylosuccinate synthetase from Ehrlich ascites-tumour cells. The apparent specific activity of this enzyme in extracts of Ehrlich ascites-tumour cells depends on the time for which the cells have been stored at -15° (Fig. 1); low apparent activities in extracts of fresh cells are due to interference in the assay system by the lyase (see the Results and Discussion section). The specific activities reported in the present purification procedure were those obtained with cells that had been kept at -15° for 84 days. Cells were collected and broken as described by Atkinson *et al.* (1963). The supernatant obtained by centrifuging at

Fig. 1. Changes in the apparent activities of adenylosuccinate lyase (A), adenylosuccinate synthetase (B) and IMP dehydrogenase (C) in extracts of Ehrlich ascites-tumour cells after various periods of storage at -15° . For details of assays see the Experimental section. Individual samples were frozen and thawed once for each assay.

20 000g for 15 min. ('first extract', Table 1) was treated with 1 ml. of 5% (w/v) streptomycin sulphate/40 mg. of protein (final vol. about 4 ml.). After 5 min. at 1° the suspension was centrifuged for 10 min. at 1° . The supernatant ('streptomycin supernatant', Table 1) (pH 7.4) was adjusted to pH 5.4 with 0.1 N-acetic acid, placed in a thermostat and stirred while the bath temperature was raised from 23° to 31° during 10 min. The suspension was cooled to 1° and centrifuged at 10 000g for 10 min. The supernatant was brought to pH 7 with N-KOH ('heated enzyme', Table 1) and fractionated with $(\text{NH}_4)_2\text{SO}_4$. Material that was precipitated between 45 and 75% saturation was dissolved in 0.1 M-glycine-NaOH buffer, pH 8 [$(\text{NH}_4)_2\text{SO}_4$ fraction', Table 1]. After dialysis for 16 hr. at 2° against 5 mM-glycine-NaOH buffer, pH 8, insoluble material was removed by centrifuging at 20 000g for 15 min. The supernatant ('dialysed enzyme', Table 1) was used for kinetic studies; its activity was unchanged after storage for 2 days at -15° .

Adenylosuccinate lyase from Ehrlich ascites-tumour cells. The streptomycin supernatant, prepared as described for the synthetase preparation, but from cells that had been kept at -15° for 28 days, was heated at 55° for 10 min., cooled and centrifuged at 10 000g for 10 min. at 1° . The supernatant ('heated enzyme', Table 2) was fractionated with $(\text{NH}_4)_2\text{SO}_4$ at 2° ; material that was precipitated between 20 and 60% saturation of $(\text{NH}_4)_2\text{SO}_4$ was dissolved in 10 mM-sodium phosphate buffer, pH 7.4 [$(\text{NH}_4)_2\text{SO}_4$ fraction', Table 2] and dialysed for 16 hr. at 2° against 5 mM-sodium phosphate buffer, pH 7.4. After removal of insoluble material at 20 000g for 15 min. the supernatant ('dialysed enzyme', Table 2) was used for kinetic studies and did not lose activity during storage for 7 days at -15° .

Adenylosuccinate lyase from yeast. This enzyme was purified as described by Carter & Cohen (1956). For kinetic studies and for preparation of adenylosuccinate a solution of the final $(\text{NH}_4)_2\text{SO}_4$ fraction in water was used. The

Table 1. *Properties of fractions obtained in the purification of adenylosuccinate synthetase from Ehrlich ascites-tumour cells*

Details are given in the Experimental section. Protein content was calculated from $E_{280 \text{ m}\mu}$ and $E_{280 \text{ m}\mu}$ (Warburg & Christian, 1942).

Fraction	Vol. (ml.)	$\frac{E_{280 \text{ m}\mu}}{E_{260 \text{ m}\mu}}$	Protein (mg.)	Adenylosuccinate formed ($\mu\text{m-moles/min.}$)
First extract	44	0.63	343	50
Streptomycin supernatant	54	0.93	226	110
Heated enzyme	64	0.94	172	170
$(\text{NH}_4)_2\text{SO}_4$ fraction	7.4	1.20	47	54.5
Dialysed enzyme	8.5	1.21	30	47.5

Table 2. *Properties of fractions obtained in the purification of adenylosuccinate lyase from Ehrlich ascites-tumour cells*

Details are given in the Experimental section. Protein content was calculated from $E_{280 \text{ m}\mu}$ and $E_{280 \text{ m}\mu}$ (Warburg & Christian, 1942).

Fraction	Vol. (ml.)	$\frac{E_{280 \text{ m}\mu}}{E_{260 \text{ m}\mu}}$	Protein (mg.)	Adenylosuccinate cleaved ($\mu\text{moles/min.}$)
First extract	86	0.62	1135	2.15
Streptomycin supernatant	108	1.00	518	2.8
Heated enzyme	108	1.12	340	3.4
$(\text{NH}_4)_2\text{SO}_4$ fraction	6.6	1.20	170	2.8
Dialysed enzyme	7.5	1.20	168	2.75

specific activity of this preparation was 3.1 μ moles of adenylosuccinate formed/min./mg. of protein (for details of assay, see below).

Analysis of the products of action of adenylosuccinate synthetase

Synthetase (1.8 mg. of protein; 'dialysed enzyme', Table 1) was kept at 30° for 2 hr. in a solution containing IMP (0.5 μ mole), GTP (0.75 μ mole), L-aspartate (4 μ moles), $MgCl_2$ (16 μ moles) and glycine (125 μ moles) in 5.65 ml. of solution adjusted to pH 8 with NaOH. This solution and a control without enzyme were mixed with 5 ml. of 0.5N-trichloroacetic acid and centrifuged at 2°. The precipitate was washed with 5 ml. of 0.2N-trichloroacetic acid, and the combined supernatants were extracted three times with 50 ml. portions of ether and evaporated to dryness at 30°. The residues from test and control experiments were applied as bands (10 cm. long) to sheets of Whatman 3MM paper and chromatographed in butan-1-ol-acetic acid-water (2:1:1, by vol.) for 21 hr. (descending). Solvent was allowed to drip from the serrated lower edge of the paper. Adenylosuccinate (17.5 cm. movement) and IMP (12 cm. movement) were extracted from the paper with 10 ml. portions of water and the spectra of the extracts were measured with the use of suitable blanks from corresponding regions of the paper. The control contained 0.48 μ mole of IMP, and the test contained 0.24 μ mole of IMP and 0.22 μ mole of adenylosuccinate. Chromatography in the $(NH_4)_2SO_4$ -phosphate-propanol system (see above) showed no AMP (R_F 0.26) as a contaminant in the IMP (R_F 0.55) which had been eluted from the first chromatogram. These nucleotides have the same mobility in the butanol-acetic acid-water system.

Analysis of the products of hydrolysis of adenylosuccinate by adenylosuccinate lyase from ascites-tumour cells

The lyase (1.8 mg. of a dialysed preparation with specific activity 5.8) was kept for 1 hr. at 30° with 0.369 μ mole of adenylosuccinate and 100 μ moles of sodium phosphate buffer, pH 7, in a final volume of 0.6 ml. After chromatography in the butanol-acetic acid-water system and spectrophotometric analysis the product was found to contain 0.24 μ mole of AMP (R_F 0.34), 0.096 μ mole of adenylosuccinate (R_F 0.41); fumarate (R_F 0.82) was detected but the concentration was not measured.

Assay of enzymes

Adenylosuccinate synthetase. For routine assays used in following the purification of the enzyme the extinction of a solution containing synthetase, 0.5 μ mole of IMP, 0.5 μ mole of GTP, 2 μ moles of L-aspartate, 8 μ moles of $MgCl_2$ and 125 μ moles of glycine (final vol. 2.8 ml.; adjusted to pH 7.5 with NaOH) was recorded at 280 m μ . The test cell and a reference cell without aspartate were kept at 25° in the thermostatically controlled cell holder of a Beckman DK-2A spectrophotometer. Rates were constant for about 10 min. after the reaction had been started by addition of aspartate. Specific activities were expressed as μ m-moles of adenylosuccinate formed/min./mg. of protein; the increase of molecular extinction coefficient for the conversion of IMP into adenylosuccinate is 1.17×10^4 at 280 m μ in the pH range 7-8 (cf. Carter & Cohen, 1956; Pabst Laboratories,

1961). In studies of Michaelis constants and of inhibition by thio-IMP this assay was modified by variation of any one of the reactants and by the inclusion of thio-IMP in both reference and test cells. Test solutions containing thio-IMP but no IMP showed no change of extinction at 280 m μ . For comparison of kinetic parameters at pH 7.01 and 7.76 the glycine was replaced by phosphate (75 μ moles) and the solutions were adjusted to the required pH with NaOH. Michaelis constants (K_m), apparent Michaelis constants in the presence of inhibitor (K_p), inhibitor constants (K_i) and extrapolated maximum velocities in the absence (V) and presence (V_p) of inhibitor, together with their standard errors, were calculated from rates at a number of substrate concentrations by Wilkinson's (1961) statistical method.

Cleavage of adenylosuccinate by adenylosuccinate lyase. For routine assays the extinction of a solution containing the lyase (from ascites-tumour cells or from yeast), 125 μ moles of phosphate and 0.25 μ mole of adenylosuccinate (final vol. 2.5 ml.; adjusted to pH 7.0 with NaOH) was recorded at 280 m μ and 25° against a reference solution containing no adenylosuccinate. Initial rates of hydrolysis were maintained for at least 10 min.

Specific activity was expressed as μ m-moles of adenylosuccinate converted into AMP/min./mg. of protein. The decrease of molecular extinction coefficient on cleavage of adenylosuccinate to AMP and fumarate is 1.07×10^4 at pH 8 (Carter & Cohen, 1956). In experiments where K_m , K_p , V , V_p and the K_i of thio-IMP were measured, four or five concentrations of adenylosuccinate were used in the presence or absence of thio-IMP; the parameters were computed as for the synthetase. For experiments at other pH values the phosphate concentration was kept at 50 mM with suitable mixtures of NaH_2PO_4 and Na_2HPO_4 and the actual pH of each reaction mixture was measured with a glass electrode after the spectrophotometric assay. A second reaction mixture was prepared in each case and the pH was measured as soon as the adenylosuccinate had been added. In no case was the change of pH during the assay period more than 0.02.

In the pH range 5.6-8.4 there is no significant change in the molecular extinction decrement at 280 m μ accompanying the conversion of adenylosuccinate into AMP and fumarate. If all the fumarate formed in the reaction had been converted into malate by fumarase the molecular extinction decrement would have been 1.10×10^4 ; however, no fumarase activity was detected in the samples of lyase used for kinetic studies.

Formation of adenylosuccinate from adenylate with adenylosuccinate lyase. For assay of adenylosuccinate lyase from yeast after the preliminary purification to obtain enzyme for the synthesis of adenylosuccinate, formation of this nucleotide from 0.1 mM-AMP and 4.3 mM-fumarate was measured spectrophotometrically at 25° in 50 mM-sodium phosphate buffer, pH 7.0 (Carter & Cohen, 1956).

Inosine 5'-phosphate dehydrogenase. This was measured as described by Atkinson *et al.* (1963).

RESULTS AND DISCUSSION

As shown in Fig. 1, there are marked changes in the apparent activities of adenylosuccinate lyase, adenylosuccinate synthetase and IMP dehydrogen-

Table 3. *Inhibition of adenylosuccinate synthetase by 6-thioinosine 5'-phosphate*

Expts. A–E were carried out with different batches of synthetase; values of V and V_p for different experiments are not comparable. Expts. A–C were carried out with the glycine buffer system, and Expts. D and E with the tris-phosphate buffer system (for details see the Experimental section). Values in parentheses are the coefficients of variation of the means (for details of calculations see the Experimental section).

Expt.	pH	Concn. of thio-IMP (μM)	K_m of IMP (μM)	K_p (μM)	V (μM -moles/min./mg. of protein)	V_p (μM -moles/min./mg. of protein)	K_i (μM)
A	7.5	0	37 (0.09)	—	2.5 (0.04)	—	—
B	7.5	0	26 (0.17)	—	1.5 (0.08)	—	—
	7.5	228	—	32 (0.17)	—	0.95 (0.08)	394 (0.30)
C	7.5	0	33 (0.11)	—	1.98 (0.04)	—	—
	7.5	92	—	29 (0.13)	—	1.29 (0.05)	172 (0.18)
	7.5	240	—	26 (0.05)	—	0.89 (0.02)	196 (0.08)
D	7.01	0	22 (0.06)	—	0.208 (0.02)	—	—
	7.01	195	—	34 (0.11)	—	0.131 (0.04)	332 (0.12)
E	7.76	0	54 (0.17)	—	0.291 (0.01)	—	—
	7.76	195	—	74 (0.04)	—	0.177 (0.02)	303 (0.05)

ase in extracts of Ehrlich ascites-tumour cells that have been stored at -15° . No adenylosuccinate synthetase could be shown by the spectrophotometric assay in the first extracts from cells that had been stored for less than 28 days; this failure to detect the synthetase was due to cleavage of the adenylosuccinate by adenylosuccinate lyase. After cells had been stored for 35 days at -15° traces of adenylosuccinate-synthetase activity could be detected in the extract (curve *B* in Fig. 1), and the real activity of the synthetase was therefore greater than that of the adenylosuccinate lyase ($3\mu\text{M}$ -moles of adenylosuccinate cleaved/min./mg. of protein) at this stage of storage. Even after fractionation as described in the Experimental section the synthetase from fresh cells could not be sufficiently freed of lyase for use in kinetic studies; the small residual activity of lyase in cells that had been kept at -15° for 84 days was easily removed.

The assay conditions and the purity of the synthetase used in these studies seem adequate for the measurement of K_m and the K_i of thio-IMP since the reaction was zero order with respect to substrates during the assay period and since 96 % of the IMP used in a large-scale analysis (see the Experimental section) was recovered unchanged or as adenylosuccinate. In the assay system with glycine buffer (final pH 7.5) K_m for GTP was $96 \pm 16\mu\text{M}$ and K_m for L-aspartate was $53 \pm 8\mu\text{M}$. As shown in Table 3, V_p was always less than V ($P < 0.05$). With the glycine buffer and with the tris-phosphate system at pH 7.76 there was no significant difference between K_m and K_p ($P > 0.2$). With tris-phosphate buffer at pH 7.01 the difference between K_m and K_p was only significant at the 10 % level. Values of K_i , calculated from the expression for non-competitive inhibition:

$$V_p = V/(1 + [\text{thio-IMP}]/K_i)$$

were in the range 0.17–0.39 mM-thio-IMP.

Values of K_m , K_p , V and V_p for cleavage of adenylosuccinate by the lyase at 12 pH values from 5.6 to 8.4 in the presence and absence of thio-IMP are listed in Table 4. There was no evidence of a significant difference between V and V_p at any pH value. The weighted mean value of $V - V_p$, with weights inversely proportional to variance, did not differ significantly from zero within the 95 % fiducial limits. Values of K_i , calculated from the expression:

$$K_p = K_m(1 + [\text{thio-IMP}]/K_i)$$

are also listed in Table 4. The variation of pK_m (i.e. $-\log K_m$), pK_i (i.e. $-\log K_i$) and $\log V$ with pH is shown in Fig. 2. For $\log V$ the solid line describes the relationship of $\log V$ and pH where:

$$V = \tilde{V}/(1 + [\text{H}^+]/K_1)$$

where $\tilde{V}$ is $11.5\mu\text{M}$ -moles of adenylosuccinate cleaved/min./mg. of protein and pK_1 (i.e. $-\log K_1$) is 6.3 (cf. Wilkinson, 1961). Dixon & Webb (1958) have discussed the variation of $\log V$ with pH and have shown that a relationship of this type results from increasing conversion of an active form of the enzyme-substrate complex into an inactive form by protonation at lower pH values.

The unit change of slope at about pH 6.3 (curve *A* in Fig. 2) may be due to protonation of an essential imidazole nitrogen in the enzyme-adenylosuccinate complex, giving an inactive species. If the action of the lyase involved nucleophilic displacement of AMP from bound adenylosuccinate by the imidazole nitrogen of a histidine residue in the active centre a relationship of this kind would be expected, as the imidazolium ion predominating at lower pH values would be a much weaker nucleophile than the uncharged species predominating above the pK of the histidyl residue. However, there is no direct evidence for such a displacement, and other groups such as the phos-

Table 4. *Kinetic parameters of the conversion of adenylosuccinate into adenylyate by adenylosuccinate lyase from Ehrlich ascites-tumour cells*

Details are given in the Experimental section. Values in parentheses are the coefficients of variation of the means.

pH	K_m (μM)	K_p (μM)	V (μM -moles/min./ mg. of protein)	V_p (μM -moles/min./ mg. of protein)	K_t (μM)
5.64	5.8 (0.17)	7.5 (0.14)*	1.99 (0.05)	1.87 (0.05)*	750 (0.97)
5.85	4.5 (0.08)	5.7 (0.20)*	2.86 (0.02)	2.62 (0.06)*	825 (1.05)
6.08	4.8 (0.09)	6.6 (0.09)*	4.45 (0.03)	3.90 (0.03)*	587 (0.47)
6.30	5.9 (0.09)	9.8 (0.05)*	5.2 (0.03)	5.4 (0.02)*	333 (0.25)
6.54	8.4 (0.14)	14 (0.02)*	6.9 (0.04)	7.2 (0.01)*	330 (0.38)
6.77	17.5 (0.15)	29 (0.25)*	8.9 (0.05)	9.3 (0.10)*	335 (0.72)
6.94	28 (0.15)	53 (0.12)*	10.5 (0.06)	10.6 (0.06)*	246 (0.39)
7.16	33 (0.04)	55 (0.04)†	10.3 (0.02)	10.2 (0.21)†	270 (0.14)
7.45	57 (0.06)	106 (0.08)†	11.0 (0.03)	10.9 (0.05)†	209 (0.22)
7.75	79 (0.02)	184 (0.13)†	11.8 (0.01)	14.0 (0.08)†	135 (0.23)
7.99	76 (0.19)	159 (0.55)†	10.1 (0.11)	8.6 (0.40)†	165 (1.26)
8.38	71 (0.22)	205 (0.80)†	7.1 (0.11)	7.0 (0.57)†	95 (1.26)

* Measured in the presence of 0.22 mM-thio-IMP.

† Measured in the presence of 0.18 mM-thio-IMP.

Fig. 2. Plots of $\log V$ (A), pK_m (B) and pK_t (C) against pH for the conversion of adenylosuccinate into AMP by adenylosuccinate lyase from Ehrlich ascites-tumour cells in the presence and absence of thio-IMP. For details see Table 4. The continuous curve A was derived from the relationship:

$$V = \bar{V}/(1 + [H^+]/K_t)$$

where $pK_t = 6.3$ (see the text). The 95% fiducial limits of individual points are shown in curves A and B. The line at Y in curve C indicates the mean 95% fiducial limit of the individual values of K_t .

phoryl group of the bound nucleotide might be involved in the change of V with pH.

The variation of pK_m and pK_t with pH is not

described by any of the simple relationships discussed by Dixon & Webb (1958). The increase of K_m from $4.8 \mu\text{M}$ at pH 6.08 to $79 \mu\text{M}$ at pH 7.75 may be the result of a number of dissociations from essential groups in adenylosuccinate, lyase or enzyme-substrate complex, and there is no change of slope corresponding to that in the $\log V$ -pH curve. Dixon & Webb (1958) have pointed out that dissociations, at essential groups with similar pK values, in both enzyme-substrate complex and in enzyme or substrate will have counteracting effects, resulting in no net change in the slope in the pK_m -pH curve.

Similar results were obtained with the lyase from yeast at five pH values from 6.2 to 8.0 (Table 5). It has been claimed that the apparent inhibition of this enzyme by thio-IMP requires the presence of copper and that thio-IMP that had been passed through a chelating resin was not inhibitory (Bridger & Cohen, 1963). The thio-IMP used in the present experiments contained 0.001 atom of copper/molecule of thio-IMP and the final concentrations of copper in assay solutions for lyase from tumour or from yeast were 0.55 and $0.4 \mu\text{M}$ respectively. More than one-half of this copper was introduced with the enzyme in each case. The addition of 20 mM-EDTA to the assay solutions or passage of the thio-IMP through Chelex-100 resin (cf. Bridger & Cohen, 1963) caused less than 9% increase in the apparent value of K_t with the lyase from yeast.

From the results in Fig. 1 it is likely that fresh Ehrlich ascites-tumour cells contain less IMP dehydrogenase than adenylosuccinate synthetase and less synthetase than adenylosuccinate lyase. When the assay of the synthetase is corrected for interference by the lyase (see above) the activities

Table 5. *Kinetic parameters of the conversion of adenylosuccinate into adenylate by adenylosuccinate lyase from yeast*

Details are given in the Experimental section. Values in parentheses are the coefficients of variation of the means.

pH	K_m (μM)	K_p (μM)	V (μM -moles/min./ mg. of protein)	V_p (μM -moles/min./ mg. of protein)	K_i (μM)
6.20	8.8 (0.07)	14.2 (0.08)*	61 (0.02)	58 (0.03)*	636 (0.26)
6.46	6.9 (0.04)	12.3 (0.06)*	134 (0.01)	122 (0.02)*	498 (0.09)
7.05	15.7 (0.05)	22.7 (0.12)†	216 (0.02)	215 (0.05)†	404 (0.42)
7.40	28 (0.24)	60 (0.29)*	265 (0.09)	203 (0.15)*	340 (0.69)
7.94	39 (0.16)	88 (0.07)†	248 (0.07)	203 (0.04)†	143 (0.30)

* Measured in the presence of 0.39 mM-thio-IMP.

† Measured in the presence of 0.18 mM-thio-IMP.

of dehydrogenase, synthetase and lyase in extracts of fresh cells are in the approximate proportions 1: > 6:50. The K_m/K_i ratios are 3.9 for the dehydrogenase at pH 8.0 (Atkinson *et al.* 1963), 0.18 for the synthetase at pH 7.76 (Table 3) and 0.47 for the lyase from ascites-tumour cells at pH 7.99 (Table 4). These results suggest that in Ehrlich ascites-tumour cells the conversion of IMP into xanthosine 5'-phosphate might be more readily inhibited by 6-mercaptopurine (through its metabolite thio-IMP) than the conversion of IMP into adenylosuccinate and of adenylosuccinate into AMP. However, the competitive nature of the inhibition of the dehydrogenase would permit reversal of this inhibition if IMP accumulated in the cells. Further experiments with radioactive precursors and with normal and resistant strains of cells will be needed to define the site or sites of inhibition of cell division by 6-mercaptopurine.

SUMMARY

1. The apparent activities of adenylosuccinate synthetase, adenylosuccinate lyase and inosine 5'-phosphate dehydrogenase were measured in extracts of Ehrlich ascites-tumour cells that had been kept at -15° for periods up to 98 days. In extracts of fresh cells the activity of the synthetase was greater than that of the dehydrogenase and less than that of the lyase.

2. Adenylosuccinate synthetase from the ascites-tumour cells was freed of interfering activities and used in kinetic studies of adenylosuccinate synthesis. At pH 7.5 the Michaelis constant (K_m) for guanosine 5'-triphosphate was 0.1 mM, the K_m for L-aspartate was 0.05 mM and the K_m for inosine 5'-phosphate was 0.03 mM. 6-Thioinosine 5'-phosphate was a non-competitive inhibitor of conversion of inosine 5'-phosphate into adenylosuccinate; the inhibitor constant (K_i) was 0.2–0.4 mM.

3. Adenylosuccinate lyase of adequate purity for kinetic studies was prepared from the ascites-tumour cells. Maximum velocities and apparent Michaelis constants for the conversion of adenylosuccinate into adenosine 5'-phosphate in the presence and absence of 6-thioinosine 5'-phosphate were measured at 12 pH values from pH 5.64 to 8.38. 6-Thioinosine 5'-phosphate was a competitive inhibitor; K_i decreased from 0.8 mM at pH 5.64 to 0.1 mM at pH 8.38. The K_m for adenylosuccinate increased from 6 μM to 0.07 mM in the same range. Below pH 8 the increase of maximum velocity (V) with decreasing H^+ ion concentration was described by the relationship:

$$V = \bar{V}/(1 + [\text{H}^+]/K_1)$$

where $\bar{V}$ was 11.5 μM -moles of adenylosuccinate cleaved/min./mg. of protein and $-\log K_1$ was 6.3. Similar results were obtained with adenylosuccinate lyase from yeast.

4. From a comparison of the activities and kinetic parameters of the dehydrogenase, synthetase and lyase in Ehrlich ascites-tumour cells it seems likely that the first of these activities is the most sensitive to inhibition by 6-thioinosine 5'-phosphate, a metabolite of the inhibitory drug 6-mercaptopurine.

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The Relationship between Iron and the Activity of Aconitase Purified from the Leaves of Mustard (*Sinapis alba*)

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The activity of aconitase from iron-deficient tissues of both animals (Beutler, 1959) and plants (Bacon, DeKock & Palmer, 1961*b*) has been found to be lower than that from normal tissues. Such studies had been prompted by reports that the enzyme purified from animal tissues is dependent on Fe^{2+} ions and cysteine for maximum activity (Dickman & Cloutier, 1951; Morrison, 1954*b*; Herr, Sumner & Yesair, 1956). There is no report of the purification of aconitase from plant sources.

In the present work aconitase has been purified from mustard leaves in an attempt to find out the part played by iron in the mechanism of the plant enzyme. A preliminary account of this work has been published (Palmer, 1963).

MATERIALS AND METHODS

Plants. Mustard plants were grown in the gardens or greenhouses of this Institute.

Substrates. Potassium dihydrogen (+)-isocitrate was prepared by the method of Vickery & Wilson (1960) from leaves of *Sedum prealtum*, and *cis*-aconitic anhydride by the method of Malachowski & Maslowski (1928). Sodium citrate was a commercial preparation (analytical-reagent grade). Solutions were made up by dissolving the salts in water and adjusting the pH to 8.0 with *N*-NaOH.

Tris buffers. All buffers used in the preparation of the enzyme were prepared by dissolving tris and sodium citrate in glass-distilled water and adjusting to the required pH with 0.2*N*-HCl. Traces of metals were removed by extraction with 1% (w/v) 8-hydroxyquinoline in chloroform. A stock solution of 1*M*-tris buffer, pH 8.0, was extracted with 8-hydroxyquinoline, and portions were diluted to 0.1*M* with water for use in the assay of the enzyme. The untreated buffer (1*M*) was shown to contain less than 0.5 $\mu\text{g. atom}$ of iron/ml.

Diethylaminoethylcellulose. DEAE-cellulose (Whatman

DE50) was suspended at room temperature in 5 mm-tris buffer containing sodium citrate (1 mm), pH 7.0, and the fine particles were removed by repeated decantation. The slurry was then packed into a column by means of a stainless-steel plunger and the column washed with the same buffer in a cold room (5°) until the effluent from the column was pH 7.0.

Gel-filtration. This was carried out on columns of Sephadex G-25 (medium grade; Pharmacia, Uppsala, Sweden). The gel was suspended at room temperature in 5 mm-tris buffer containing sodium citrate (1 mm), pH 7.0, and the fine particles were removed by decantation. The columns were placed in the cold room overnight before use.

Columns of Sephadex G-100 were prepared from gel equilibrated with 50 mm-tris buffer containing sodium citrate (5 mm), pH 7.0, for at least 24 hr. at room temperature. Buffer was run through the columns overnight in the cold room before use.

Estimation of iron. The method of Peterson (1953) or the method of Massey (1957) was used. All glassware used for the estimation of iron was rinsed with conc. HCl followed by water distilled from an all-glass apparatus. Iron was also measured spectrochemically together with zinc, copper, manganese, magnesium and aluminium. The sample was evaporated to dryness, oxidized with HNO_3 and then ashed at 450° overnight. The residue was taken up in a small volume of 0.64*N*- HNO_3 containing lithium and chromium as internal standards, and the metals were estimated by a porous-cup-spark method (Scott, 1960).

Enzyme assays. Aconitase was measured essentially as described by Bacon, Palmer & DeKock (1961*a*), except that sucrose was omitted from the incubation medium. Readings were made every minute for 10 min. except for the initial extract (every 5 min. for 30 min.), with final concentrations of potassium dihydrogen (+)-isocitrate and citrate of 10 and 30 mm respectively. A unit of enzyme activity is defined as the amount of enzyme that formed 1 μmole of *cis*-aconitate in 10 min. under these conditions. Specific activity is defined as the number of units/mg. of protein.

Protein. This was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

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