Adenylosuccinate Synthetase and Adenylosuccinate Lyase from Plant Tissues

By M. D. HATCH

*David North Plant Research Centre, The Colonial Sugar Refining Co. Ltd.,
Indooroopilly, Queensland, Australia*

(Received 30 June 1965)

1. Enzymes that convert IMP into adenylosuccinate (adenylosuccinate synthetase) and adenylosuccinate into AMP (adenylosuccinate lyase) were isolated from wheat germ and pea seeds and their properties are described. 2. These enzymes were purified approx. 200-fold from wheat-germ extracts. 3. A heat treatment provided adenylosuccinate lyase free of adenylosuccinate synthetase but the behaviour of the two enzymes was almost identical in a number of fractionation procedures. The two activities were finally separated by filtration on Sephadex G-100. 4. The identification of these enzymes in plant tissues is discussed in relation to the pathway of purine synthesis.

The pathway for synthesis of purine nucleotides in animal tissues and micro-organisms is well documented and many of the enzymes involved have been isolated and described (Buchanan, 1960; Schulman, 1961). Until recently, little information existed on purine synthesis in plant tissues. Studies on the incorporation of ^{14}C -labelled glycine and 5-aminoimidazole-4-carboxamide into purines of wheat leaves and embryos have now provided some evidence that a similar pathway is operative in plants (Robern, Wang & Waygood, 1965). There are also reports on the synthesis of glycinamide ribonucleotide in wheat-embryo extracts (Kapoor & Waygood, 1962) and the isolation of IMP dehydrogenase from pea seeds (Turner & King, 1961). The present paper describes the partial purification and properties of adenylosuccinate synthetase (IMP-L-aspartate ligase; EC 6.3.4.4) and adenylosuccinate lyase (adenylosuccinate AMP-lyase; EC 4.3.2.2) isolated from wheat germ. These enzymes catalyse the terminal steps for the new synthesis of AMP in liver and micro-organisms (Buchanan, 1960).

METHODS

Preparation of adenylosuccinate synthetase and adenylosuccinate lyase. A 30 g. portion of an acetone-dried powder of wheat germ (Hatch & Stumpf, 1961) was shaken with 120 ml. of 50 mM-potassium phosphate buffer, pH 7.5, for 30 min. at room temperature. The suspension was pressed through fine muslin and the residual material re-extracted twice with 40 ml. of the same buffer. These extracts were combined, cooled to 0° and centrifuged at $25000g$ for 20 min. During the subsequent treatments the temperature was maintained between 0° and 5° . To 170 ml. of the supernatant 15 ml. of 5% (w/v) protamine sulphate was

added slowly and the resulting precipitate was discarded. Small samples of the supernatant were dialysed for 16 hr. against 10 mM-tris-HCl buffer before being assayed for enzyme activity. The remaining supernatant was treated with saturated $(\text{NH}_4)_2\text{SO}_4$, pH 7.0, and the fraction precipitating between 45% and 55% saturation was dissolved in 20 ml. of 10 mM-tris-HCl buffer, pH 7.5, and dialysed against 3 l. of the same buffer. This extract was adjusted to pH 6.1 by the slow addition of 30 mM-HCl and the precipitated protein was discarded. By further addition of HCl the pH was adjusted to 5.5 and the solution allowed to stand for 10 min. at 5° . The precipitated protein was suspended in 10 ml. of 10 mM-potassium phosphate buffer, pH 7.5. Undissolved protein was discarded and the supernatant was applied to a 12 ml. column of calcium phosphate gel with a void volume of 8.5 ml. After washing the column with 20 ml. of 15 mM-potassium phosphate buffer, pH 7.5, the eluate from 16 ml. of 40 mM-potassium phosphate buffer, pH 7.5, was collected. To reduce the volume of this fraction the protein was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to give 65% saturation and then dissolved in 3 ml. of 10 mM-tris-HCl buffer, pH 7.5, containing KCl (50 mM). This fraction was applied to a Sephadex G-100 column 31 cm. long $\times$ 5 cm.² cross-section and eluted with 10 mM-tris-HCl buffer, pH 7.5, containing KCl (25 mM). Fractions of volume 5 ml. were collected at a rate of 0.5 ml./min. The protein content of each fraction was measured, and then 0.25 ml. of serum albumin solution containing 40 mg./ml. was added. Most enzyme studies were conducted with fractions obtained by Sephadex-gel filtration.

Enzyme assays. Adenylosuccinate synthetase was assayed essentially as described by Lieberman (1965). Reaction mixtures contained enzyme, tris-HCl buffer, pH 8.3 (25 μ moles), potassium phosphate, pH 8.3 (10 μ moles), MgCl_2 (5 μ moles), tricyclohexylamine phosphoenolpyruvate (0.7 μ mole), pyruvate kinase (1.4 units), L-aspartate (1.5 μ moles), ATP (0.08 μ mole), GTP (0.08 μ mole) and IMP (0.5 μ mole) in a total volume of 1 ml. Controls lacking IMP were run for every reaction. Reactions were stopped with

0.7 ml. of 9% (v/v) HClO_4 , and IMP was added to the control systems. After centrifuging to remove precipitated protein the increase in extinction at $275\text{ m}\mu$, compared with controls, was measured. The amount of IMP converted into adenylosuccinate was calculated from the published spectra of IMP (Beaven, Holiday & Johnson, 1955) and adenylosuccinate (Carter & Cohen, 1956). By assaying adenylosuccinate synthetase at pH 8.3 the proportion of the product that underwent further breakdown to AMP remained small with most enzyme fractions. The amount of AMP formed could be calculated from the ratio of the observed extinction changes at $280\text{ m}\mu$ and $270\text{ m}\mu$. For some studies L-[U- ^{14}C]aspartate was used. In this case reactions were stopped by heating at 100° for 2 min., and then samples were chromatographed on Whatman no. 1 paper with propyl acetate–90% (w/v) formic acid–water (11:5:3, by vol.) (Bielecki & Young, 1963) as the developing solvent. Activity was determined by measuring the areas of the peaks traced with a recording strip counter. When necessary these results were confirmed by counting the areas containing aspartate and adenylosuccinate with a Geiger–Müller tube.

Adenylosuccinate lyase was generally assayed by measuring the conversion of AMP into adenylosuccinate in the presence of an excess of fumarate (Carter & Cohen, 1956). Reaction mixtures contained enzyme, potassium phosphate buffer, pH 6.6 ($50\text{ }\mu\text{moles}$), fumarate ($6\text{ }\mu\text{moles}$) and AMP ($0.6\text{ }\mu\text{mole}$) in a total volume of 1 ml. The subsequent procedure including the use of controls was the same as that described for adenylosuccinate synthetase except that activity was measured by the change in extinction at $280\text{ m}\mu$. For some studies the conversion of adenylo[^{14}C]succinate into radioactive fumarate was measured in reaction mixtures containing 40 mM-potassium phosphate buffer, pH 6.6, and enzyme in a total volume of 0.2 ml. Activity was measured by the procedure described for the radiotracer studies with adenylosuccinate synthetase.

Fumarate-hydratase activity was measured in reaction mixtures of the same composition as that described for adenylosuccinate lyase except that AMP was omitted. The decrease in extinction at $275\text{ m}\mu$ was used as a measure of

enzyme activity. The product, malate, was identified by its chromatographic mobility with the three solvents and the detection procedure described by Hatch & Stumpf (1961).

A unit of adenylosuccinate-synthetase or adenylosuccinate-lyase activity is defined as the amount of enzyme required to utilize $1\text{ }\mu\text{mole}$ of substrate/min. under the conditions of assay. All assays were conducted at 30° .

Determination of protein. The protein content of enzyme preparations was determined by the method of Warburg & Christian (1941).

MATERIALS

The following compounds were obtained from commercial sources: ATP, GTP, ADP, IDP, AMP, IMP, CMP, adenosine, inosine, adenine, tricyclohexylamine phosphoenolpyruvate, pyruvate kinase and protamine sulphate from Sigma Chemical Co. (St Louis, Mo., U.S.A.); L-[U- ^{14}C]aspartic acid (6.1 mc/m-mole) from The Radiochemical Centre (Amersham, Bucks.); calcium phosphate gel (hypatite C) from Clarkson Chemical Co. (Williamsport, Pa., U.S.A.); Sephadex G-100 from Pharmacia (Uppsala, Sweden).

Adenylo[^{14}C]succinate was prepared by incubating adenylosuccinate synthetase (0.01 unit) in a 3 ml. reaction mixture of the composition described above except that non-radioactive aspartate was replaced by $1.5\text{ }\mu\text{moles}$ of [U- ^{14}C]aspartate (2.1×10^7 disintegrations/min.). The reaction mixture was incubated at 30° for 2 hr., heated at 100° for 2 min. and then chromatographed as a band on a large sheet of Whatman no. 3MM paper with propyl acetate–90% (v/v) formic acid–water (6:3:1, by vol.) as the developing solvent. The adenylosuccinate band was located by radioautography and eluted from the paper with 5 mM-potassium phosphate buffer, pH 7.0. About 40% of the radioactivity supplied was recovered as adenylosuccinate. Incubation of the product with adenylosuccinate lyase provided evidence for its identity and purity with respect to radioactivity. When low concentrations (0.037 mM) were supplied all the radioactivity was recovered in fumaric acid.

Table 1. *Purification of adenylosuccinate synthetase and adenylosuccinate lyase*

Reaction mixtures contained approx. 1×10^{-3} unit of adenylosuccinate synthetase and approx. 1.5×10^{-3} units of adenylosuccinate lyase. The remaining components of the reaction mixtures and the assay methods are described in the Methods section. The incubation time was 60 min.

Enzyme fraction	Concn. of protein (mg./ml.)	Adenylosuccinate synthetase		Adenylosuccinate lyase	
		Total units	Specific activity (unit/mg. of protein)	Total units	Specific activity (unit/mg. of protein)
25000g supernatant	22	—	—	3.9	0.010
Protamine sulphate supernatant	12	0.68	0.003	4.0	0.017
45–55% satd. $(\text{NH}_4)_2\text{SO}_4$	19	0.54	0.012	3.1	0.07
Ppt. between pH 6.2 and pH 5.5	10.6	0.31	0.03	2.0	0.18
Calcium phosphate-gel fraction	1.4	0.27	0.12	1.8	0.80
Sephadex fraction 11	0.58	0	0	0.52	1.8
Sephadex fraction 12	0.32	0.038	0.24	0.45	2.9
Sephadex fraction 14	0.30	0.082	0.55	0	0

RESULTS

Purification of adenylosuccinate synthetase and adenylosuccinate lyase. Data on the purification are given in Table 1. The specific activities of both enzymes in selected fractions from Sephadex G-100 were more than 200 times those in extracts obtained after treatment with protamine sulphate. The protamine sulphate treatment was included primarily to remove nucleic acids. With the assay procedure employed no adenylosuccinate-synthetase activity was detected in the 25000g supernatant although it was possible to detect activity after treatment with protamine sulphate. An extract of the protamine sulphate precipitate, obtained as described by Mendicino (1960), showed neither adenylosuccinate-synthetase nor adenylosuccinate-lyase activity.

Separation of the two enzymes proved difficult, their behaviour during fractionation by ammonium sulphate and isoelectric precipitation and chromatography on calcium phosphate being almost identical (Table 1). Chromatography at pH 7.4 on DEAE-cellulose was also unsuccessful. Procedures for the quantitative assay of adenylosuccinate synthetase in extracts containing adenylosuccinate lyase are described in the Methods section. The assay of adenylosuccinate lyase in the presence of fumarate hydratase also provided a potential problem. However, under the conditions employed

for the assay only a small fraction of the fumarate supplied was converted into malate. Filtration on Sephadex G-100 provided adenylosuccinate synthetase free of adenylosuccinate lyase and adenylosuccinate lyase free of fumarate hydratase (Fig. 1). Fumarate hydratase moved with the protein fraction that was completely excluded from the gel.

Characterization of reactions. The observed difference spectra for the reactions catalysed by adenylosuccinate synthetase and adenylosuccinate lyase were in close agreement with those calculated from the published spectra of IMP, AMP and adenylosuccinate. For accurate determination of extinction differences below 270m μ further dilution of the perchloric acid extracts of reaction mixtures was necessary owing to the high blank extinction in this region.

The product formed from IMP and L-[U-¹⁴C]-aspartate in the presence of adenylosuccinate synthetase was converted exclusively into radioactive fumarate when incubated with a purified preparation of adenylosuccinate lyase. The latter product co-chromatographed on paper with unlabelled fumarate in three different solvents (Hatch & Stumpf, 1961).

Properties of adenylosuccinate synthetase. The reaction rate was constant for at least 90 min. under the conditions of assay (Table 2). Separate studies with 50mm-potassium phosphate buffer showed the optimum pH for activity was between

Table 2. *Properties of adenylosuccinate synthetase*

The composition of the complete assay system, buffered to pH 8.3 with 25mm-tris-HCl, is described in the Methods section. Buffers described in the Table replaced the above buffer. Each reaction mixture contained an equal sample of fraction 14 from Sephadex G-100 (see Fig. 1), containing 1×10^{-3} unit.

Modification of complete assay system	Reaction time (min.)	Adenylosuccinate-synthetase activity (m μ moles/reaction mixture)
None	30	27
None	60	55
None	90	80
None	120	103
50mm-Glycine-KOH buffer, pH 8.3	60	54
75mm-Tris-HCl buffer, pH 8.3	60	39
25mm-Tris-HCl buffer, pH 7.4	60	63
25mm-Tris-HCl buffer, pH 9.4	60	20
50mm-Potassium phosphate buffer, pH 7.6	60	70
With 3mm-aspartate	60	57
With 1.5mm-asparagine; aspartate omitted	60	0
With 1.5mm-glutamate; aspartate omitted	60	3
With 0.5mm-inosine; IMP omitted	60	0
With 0.5mm-IDP; IMP omitted	60	4
With 0.2mm-GTP; phosphoenolpyruvate omitted	60	37
With 0.2mm-GTP; phosphoenolpyruvate and MgCl ₂ omitted	60	0
GTP omitted	60	0

7.5 and 7.8. However, 25mM-tris-hydrochloric acid buffer, pH 8.3, was used as a routine to minimize the conversion of adenylosuccinate into AMP when adenylosuccinate lyase was present. At this concentration tris-hydrochloric acid buffer did not inhibit the enzyme compared with 50mM-glycine-potassium hydroxide buffer at the same pH. With

75mM-tris-hydrochloric acid buffer a 30% inhibition was observed.

Studies of substrate requirements for adenylosuccinate synthetase are recorded in Table 2. Aspartate was essential for activity. The enzyme was apparently saturated with 1.5mM-aspartate, 3mM-aspartate giving no significant increase in activity. D-Aspartate (in a separate experiment) and asparagine could not replace L-aspartate, and the activity observed with glutamate was barely significant. No activity was observed when IMP was replaced by inosine, and the low activity observed with IDP may have been due to contaminating traces of IMP. The pyruvate-kinase system for generating GTP was apparently essential for maintaining maximum activity since even with 0.2mM-GTP activity was decreased when phosphoenolpyruvate was omitted. Lieberman (1956) reported similar observations. ATP could not replace GTP since no activity was observed when the latter was omitted.

Properties of adenylosuccinate lyase. Under the conditions of assay the rate of the reaction catalysed by adenylosuccinate lyase remained constant for at least 60min. (Table 3). At 60min. 16% of the AMP was utilized and according to the data of Carter & Cohen (1956) approx. 50% would be converted into adenylosuccinate at equilibrium with the concentrations of substrates used. Optimum activity was observed between pH 6.5 and 7.2 and about one-third of maximum activity at pH 5.6 and pH 8.3. Fumarate was essential for activity and increasing its concentration from 6mM to 12mM increased the rate by only 5%. There was no

Fig. 1. Separation of adenylosuccinate synthetase (Δ), adenylosuccinate lyase ($\circ$) and fumarate hydratase ($\blacktriangle$) on Sephadex G-100; the distribution of protein ($\bullet$) is also shown. The column volume was 155ml., the void volume was 45ml. and the volume of each fraction was 5ml. Other details are described in the Methods section. Enzyme activity is expressed in arbitrary units to bring the activity/unit fraction volume to a similar value for each enzyme.

Table 3. *Properties of adenylosuccinate lyase*

The composition of the complete assay system, buffered to pH 6.6 with 50mM-potassium phosphate, is described in the Methods section. Buffers described in the Table replaced the above buffer. An equal sample of fraction 12 from Sephadex G-100 (see Fig. 1), containing 1.5×10^{-3} units of activity, was supplied to each reaction mixture.

Modifications of complete assay system	Reaction time (min.)	Adenylosuccinate-lyase activity (μ moles/reaction mixture)
None	20	34
None	40	69
None	60	101
None	80	129
50mM-Potassium phosphate buffer, pH 5.6	60	36
50mM-Potassium phosphate buffer, pH 7.2	60	102
50mM-Potassium glycinate buffer, pH 8.3	60	33
25mM-Tris-HCl buffer, pH 8.3	60	31
Fumarate omitted	60	0
With 12mM-fumarate	60	106
With 1mM-AMP	60	99
With 0.6mM-CMP; AMP omitted	60	0
With 0.6mM-adenosine; AMP omitted	60	0
With 0.6mM-adenine; AMP omitted	60	0

increase in activity when the concentration of AMP was increased to 1mM and no change in extinction was observed when AMP was replaced by CMP, adenosine or adenine.

Michaelis constants. Michaelis constants were calculated from the reciprocals of activity and substrate concentration (Lineweaver & Burk, 1934). The necessity for measuring initial rates with subsaturating concentrations of substrate presented some limitations to accuracy. Calculations were made from extinction values between 0.2 and 0.04. By using the spectrophotometric method the K_m values for IMP and aspartate for the reaction catalysed by adenylosuccinate synthetase were 0.09mM and 0.43mM respectively. With the radiotracer method K_m for aspartate was 0.38mM.

For the reaction catalysed by adenylosuccinate lyase the K_m values for adenylosuccinate, AMP and fumarate were 0.03mM, 0.11mM and 0.65mM respectively. The radiotracer method was used to determine K_m for adenylosuccinate.

Enzyme stability. Heating the enzyme fraction obtained by calcium phosphate-gel chromatography at 60° for 5min. completely inactivated adenylosuccinate synthetase but only 25% of the adenylosuccinate-lyase activity was lost. Both enzymes retained activity when held at pH 5.2 and 5° for 10min. The enzyme activity in all fractions except those obtained by Sephadex-gel filtration was stable for at least 3 weeks when stored at -15°. After 3 weeks at -15° the adenylosuccinate synthetase of fraction 14 and the adenylosuccinate lyase of fraction 12 from Sephadex G-100 lost 15 and 25% respectively of their original activities.

Occurrence in other plant tissues. Adenylosuccinate synthetase and adenylosuccinate lyase were present in a 45-55% saturated ammonium sulphate fraction from pea seeds prepared as described by Hatch & Turner (1960). Failure to detect these enzymes in a 40-60% saturated ammonium sulphate fraction obtained from extracts of immature leaf and stem from sugar-cane, shoots from sugar-cane sets and potato leaves may have been due to the presence of interfering enzymes. All these extracts contained high nucleoside monophosphate-ribosylhydrolase activity but this enzyme was not detectable in wheat and pea preparations.

DISCUSSION

In most essential respects the properties of the adenylosuccinate synthetase and adenylosuccinate lyase from wheat germ were similar to those for the same enzymes isolated from animal tissue and micro-organisms (Carter & Cohen, 1956; Lieberman, 1956; Miller, Lukens & Buchanan, 1959). A marked difference was observed between the wheat-germ adenylosuccinate synthetase and the

bacterial enzyme (Lieberman, 1956) and tumour enzyme (Atkinson, Morton & Murray, 1964) with respect to solubility at low pH. The latter enzymes remained soluble at pH 5.4 and this step was used to remove adenylosuccinate lyase. In the wheat-germ extracts both adenylosuccinate synthetase and adenylosuccinate lyase were completely precipitated as the pH was lowered from 6.2 to 5.5. It is of practical interest that the yeast adenylosuccinate lyase was freed of fumarate hydratase by heating preparations at 60° for approx. 10min. (Carter & Cohen, 1956). Under these conditions wheat-germ fumarate hydratase lost no activity but almost 50% of the adenylosuccinate-lyase activity was destroyed.

The studies described in the present paper provide further support for the conclusion of Robern *et al.* (1965) that the pathway for the new synthesis of purine nucleotides in plants is similar to that operative in animal tissues and micro-organisms, the terminal steps for AMP synthesis being catalysed by adenylosuccinate synthetase and adenylosuccinate lyase. There is now strong evidence that the conversion of adenylosuccinate into AMP and the cleavage of 5-aminimidazole-4-*N*-succinocarboxamide ribotide, an earlier step in purine synthesis, are catalysed by the same protein entity in liver, moulds and micro-organisms (Flaks & Lukens, 1963). A similar double function for the plant enzyme appears likely.

The demonstration of adenylosuccinate synthetase in plants is of interest with respect to the possible mode of action of hadacidin (*N*-formyl-hydroxyaminoacetic acid) as an inhibitor of growth of sugar-cane (P. E. Robinson, personal communication) and many other plants (Gray, Gauger, Dulaney, Kaczka & Woodruff, 1964). Growth-inhibitory effects of hadacidin on tumour cells (Kaczka, Gitterman, Dulaney & Folkers, 1962) and bacteria (Shigeura & Gordon, 1962a) has been attributed entirely to inhibition of adenylosuccinate synthetase (Shigeura & Gordon, 1962b,c).

REFERENCES

- Atkinson, M. R., Morton, R. K. & Murray, A. N. (1964). *Biochem. J.* **92**, 398.
- Beaven, G. H., Holiday, E. R. & Johnson, E. A. (1955). In *The Nucleic Acids*, vol. 1, p. 493. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Bielecki, R. L. & Young, R. E. (1963). *Analyt. Biochem.* **6**, 54.
- Buchanan, J. M. (1960). In *The Nucleic Acids*, vol. 3, p. 304. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Carter, C. E. & Cohen, L. H. (1956). *J. biol. Chem.* **222**, 17.
- Flaks, J. G. & Lukens, L. N. (1963). In *Methods in Enzymology*, vol. 6, p. 88. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

- Gray, R. A., Gauger, G. W., Dulaney, E. L., Kaczka, E. A. & Woodruff, H. B. (1964). *Plant Physiol.* **39**, 204.
- Hatch, M. D. & Stumpf, P. K. (1961). *J. biol. Chem.* **236**, 2879.
- Hatch, M. D. & Turner, J. F. (1960). *Biochem. J.* **76**, 556.
- Kaczka, E. A., Gitterman, C. O., Dulaney, E. L. & Folkers, K. (1962). *Biochemistry*, **1**, 340.
- Kapoor, M. & Waygood, E. R. (1962). *Biochem. biophys. Res. Commun.* **9**, 7.
- Lieberman, I. (1956). *J. biol. Chem.* **223**, 327.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Mendicino, J. (1960). *J. biol. Chem.* **235**, 3347.
- Miller, R. W., Lukens, L. N. & Buchanan, J. M. (1959). *J. biol. Chem.* **234**, 1806.
- Robern, H., Wang, D. & Waygood, E. R. (1965). *Canad. J. Biochem.* **43**, 225.
- Schulman, M. D. (1961). In *Metabolic Pathways*, vol. 3, p. 389. Ed. by Greenberg, D. M. New York: Academic Press Inc.
- Shigeura, H. T. & Gordon, C. N. (1962a). *Cancer Res.* **22**, 1356.
- Shigeura, H. T. & Gordon, C. N. (1962b). *J. biol. Chem.* **237**, 1932.
- Shigeura, H. T. & Gordon, C. N. (1962c). *J. biol. Chem.* **237**, 1937.
- Turner, J. F. & King, J. E. (1961). *Biochem. J.* **79**, 147.
- Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.