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Regulation of a Metabolic System in Vitro: Synthesis of Threonine from Aspartic Acid†

Mark Szczesiul‡ and D. Eugene Wampler*,§

ABSTRACT: Six enzymes involved in the conversion of aspartate to threonine have been extracted from *Escherichia coli* and separated from each other. Two of these enzymes, aspartokinase and homoserine dehydrogenase, have also been partially purified from *Rhodopseudomonas spheroides*. In an attempt to determine whether small changes in the kinetic properties of individual enzymes are important to the regulation of metabolic flux through a coupled reaction system, the partially purified enzymes were recombined in a variety of ways under reaction conditions designed to resemble the in vivo situation. These conditions include: use of an entire metabolic system rather than a single reaction; high enzyme concentrations at the same relative concentrations as found in the cell; and low, steady-state concentrations of substrates and products. Metabolic flux was followed spectrophotometrically and the concentrations of aspartic semialdehyde, homoserine, *O*-phosphohomoserine, and threonine were measured. The results indicate that the threonine concentration is of major importance in regulating metabolic flux by inhibiting aspartokinase, the first reaction in the pathway. When threonine-insensitive aspartokinases were used, threonine concentrations reached higher levels and the rate of NADPH oxidation remained higher. The fact that neither aspartic semialdehyde nor homoserine accumulated as the threonine concentration increased and the lack of correlation between changes in metabolic flux and ADP/ATP or NADPH/NADP ratios indicate that more subtle forms of metabolic regulation, such as "reverse cascade", secondary feedback sites, or "energy charge", are of little regulatory importance in this isolated, metabolic system. The results also emphasize the need for caution in projecting in vivo control mechanisms from in vitro experiments.

For a number of years biochemists have inferred mechanisms of metabolic control from studies of the catalytic properties of purified enzymes. In gross terms the control mechanisms which have been devised are compatible with growth patterns and nutritional requirements of normal organisms and specific mutants. Synthesis of the aspartic acid family of amino acids (lysine, methionine, threonine, isoleucine, and *ms*-diaminopimelate) has been extensively studied because the highly branched nature of this pathway creates a complex regulatory problem and because different organisms appear to have evolved different mechanisms for regulating flux through this pathway (Datta, 1969). In coliform bacteria, metabolic control employs multiple aspartokinases (EC 2.7.2.4) under separate control (Cohen et al., 1969), while in *Rhodopseudomonas capulatus* (Datta and Gest, 1964) and *Bacillus polymyxa* (Paulus and Gray, 1964) a single aspartokinase is subject to concerted feedback inhibition. Two aspartokinases have been isolated from *Bacillus subtilis*, one subject to concerted feedback inhibition and the other inhibited by diaminopimelate (Rosner and Paulus, 1971). In *Bacillus licheniformis* and *Rhodopseudomonas spheroides* (Datta and Prakash, 1966; Gibson et al., 1962), control appears to be by a "reverse cascade" mechanism in which threonine inhibits homoserine dehydrogenase (EC 1.1.1.3), causing aspartic semialdehyde to accumulate and this aspartic semialdehyde (rather than threonine) inhibits aspartokinase.

In addition to the variety of control patterns observed within the aspartic acid family there have been reports of regulatory interactions between this metabolic family and other metabolic pathways. For example, Klungsoyr et al. (1968) have shown a relationship between lysine-sensitive aspartokinase activity and energy charge and have proposed an interaction between pathways of energy metabolism and amino acid synthesis. Baich and Hagan (1970) reported that hexose monophosphates inhibit a partially purified preparation of aspartokinase and interpreted this observation as indicating a communication between carbohydrate (energy) metabolism and protein synthesis. Kotre et al. (1973) have shown that homoserine inhibits *E. coli* glutamate dehydrogenase (EC 1.4.1.2) and suggested that this is another example of "reverse cascade" in which homoserine impedes aspartate formation by reducing the supply of the primary amino donor, glutamate.

Activation and inhibition patterns observed in experiments with single, purified enzymes may suggest opportunities for control but they can not demonstrate that any specific control

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‡ Present address: Department of Biological Engineering, University of Connecticut, Storrs, Connecticut 06268.
§ Present address: Bio-Gant Corporation, Woodbridge, Connecticut 06525.

mechanism functions within a complete metabolic system. In order to determine which catalytic features are important for metabolic regulation in a complete metabolic system and which are merely incidental properties of purified enzymes, we have undertaken to reconstruct one portion of the biosynthetic network and study its kinetic properties.

Materials and Methods

Cells. Wild type *E. coli* K-12 (CU-1) and a thiaisoleucine-resistant derivative (Tir-8) (Szentirmai et al., 1968) were the gift of Dr. H. E. Umbarger. Cells were grown in a minimal salts medium at 37 °C with 0.5% glucose as the carbon source. *R. spheroides* was the gift of Dr. P. Datta and was grown for us by Dr. R. C. Fuller as described by Datta (1966).

Buffers. Breaking buffer contained: 40 mM Tes[1] (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 70 μM pyridoxal phosphate, and 1 mM each of L-lysine, L-threonine, and L-isoleucine. DEAE buffer had the same composition except that the Tes concentration was 20 mM and the dithiothreitol concentration was 0.1 mM. Specific buffers for the different enzymes were made from a general buffer which contained: 10 mM Tes, 100 mM KCl, 1 mM EDTA, and 0.1 mM dithiothreitol, supplemented with amino acids or pyridoxal phosphate as indicated.

Source of Metabolic Intermediates. DL-Aspartic β-semialdehyde was prepared by the ozonolysis of allylglycine as described by Black and Wright (1955b) and purified as described by Westerik and Wolfenden (1974). L-[^{14}C]Aspartic β-semialdehyde was synthesized enzymatically from L-[^{14}C]aspartate using purified *E. coli* aspartokinase III and aspartic semialdehyde dehydrogenase (EC 1.2.1.11) (25 mM Tris-Cl, pH 7.5, 20 mM aspartate (10^6 cpm/μmol), 50 mM Mg-ATP, 5 mM NADPH, 8 units of aspartokinase III, and 40 units of aspartic semialdehyde dehydrogenase). The reaction was stopped by mixing with 0.2 volume of cold, 50% Cl_3CCOOH. Precipitated proteins were removed by centrifugation and the aspartic semialdehyde was purified as before.

O-Phosphohomoserine was prepared enzymatically by a method similar to that described by Skarstedt and Greer (1973). The reaction mixture contained, in a volume of 7 ml: 140 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 70 mM ATP, 140 mM L-homoserine, 300 mM KCl, and 150 unit of purified *E. coli* homoserine kinase (EC 2.7.1.39). The mixture was incubated at 37 °C and the reaction was followed by removing 1.5-μl samples and measuring the ADP which had been formed in a kinase assay mixture (see below) which contained no substrate. When 85–90% of the ATP had been converted to ADP (about 2 h), the reaction was stopped by boiling, precipitated protein was removed by centrifugation, and *O*-phosphohomoserine was purified by ion-exchange chromatography as described by Skarstedt and Greer (1973). Ninhydrin positive fractions from the Dowex-1 column were combined and concentrated to about 10 ml by rotary evaporation at 40 °C. The pH was adjusted to 7.5 with KOH and the potassium was removed by passing the sample over a 2.2 × 22 cm column of Dowex-50W and eluting with water. Ninhydrin positive fractions were again combined and rotary evaporated to a volume of 2–3 ml. *O*-Phosphohomoserine was precipitated from this sample as a barium salt (Flavin and Slaughter, 1960) using 1 M barium acetate. The precipitate was recovered by filtration, washed with several volumes of ethanol, and stored desiccated at 4 °C until use. Stock solutions of *O*-phosphohomoserine were prepared by suspending the powder in a small volume of 50 mM Tes buffer (pH 8.0). For routine assays, excess Ba^{2+} was removed by adding a small quantity of Dowex-50 and decanting the supernatant. For more complete Ba^{2+} removal, the solution was passed over a 1 × 15 cm column of Dowex-50 and eluted with water. In either case the *O*-phosphohomoserine samples were adjusted to pH 8.0 with KOH and assayed for organic phosphate by the method of Chen et al. (1956).

[^{14}C]-α-Ketobutyric acid was synthesized from [^{14}C]-threonine with purified *E. coli* threonine deaminase (EC 4.2.1.16) (107 mM potassium phosphate, pH 8.0, 43 mM NH_4Cl, 9 mM L-threonine (5×10^5 cpm/μmol), and 4 units of threonine deaminase). The product was identified with 2,4-dinitrophenylhydrazine (Greenberg, 1962) and by autoradiography after high-voltage electrophoresis (see below).

Other reagents were from commercial sources.

Assays for Metabolic Intermediates. Amino acids could be separated by thin-layer chromatography on commercial cellulose plates (Baker-flex or Kodak, without fluorescent indicator) using solvent systems similar to those developed by Haworth and Heathcote (1969). The acidic solvent contained 3 parts methyl ethyl ketone, 12 parts ethanol, and 5 parts potassium acetate (pH 4.0). The basic solvent contained 40 parts *tert*-butyl alcohol, 20 parts methyl ethyl ketone, 20 parts acetone, 1 part methanol, 114 parts water, and 5 parts ammonium hydroxide. Amino acids were visualized by spraying either with a ninhydrin spray containing 1 g of ninhydrin, 85 ml of ethanol, 100 mg of $Cd(OAc)_2 \cdot 2H_2O$, and 15 ml of glacial acetic acid or with a ferric chloride spray containing 10 g of $FeCl_3 \cdot 6H_2O$, 10 ml of 2 M HCl, and 95 ml of absolute ethanol. Radioactive amino acids were measured by scintillation counting in a toluene–Triton based scintillation fluid or by strip scanning with a Packard Model 7201 radiochromatogram scanner.

Nonradioactive intermediates were measured quantitatively by a variety of methods. Aspartate or homoserine could conveniently by measured enzymatically using excess aspartokinase or homoserine kinase in the kinase assay (see below) and measuring the extent of the reaction rather than the rate. Aspartic semialdehyde was measured enzymatically with an excess of homoserine dehydrogenase in the homoserine dehydrogenase assay and α-ketobutyrate was measured using an excess of beef heart lactate dehydrogenase (EC 1.1.1.27) in the threonine deaminase assay. β-Aspartyl phosphate was measured as the hydroxamate (Black and Wright, 1955a) and *O*-phosphohomoserine was measured in an organic phosphate assay (Chen et al., 1956).

Enzyme Assays. Aspartokinase activity was measured by a coupled assay as described by Wampler and Westhead (1968). The homoserine kinase assay mixture differed only in the use of 15 mM L-homoserine instead of aspartate. In crude extracts the kinase assays are complicated by ATPases and/or NADPH oxidases so that aspartokinase I is defined as the aspartate-dependent, lysine-independent activity and aspartokinase III as the aspartate-dependent, threonine-independent activity. Homoserine kinase is defined as the homoserine-dependent activity.

Aspartic β-semialdehyde dehydrogenase activity was measured as described by Hegeman et al. (1968), by running the reaction in the reverse direction and following the reduction of NADP at 340 nm.

[1] Abbreviations used are: Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate; EDTA, ethylenediaminetetraacetate; aspartokinase I, threonine-sensitive aspartokinase; homoserine dehydrogenase I, threonine-sensitve homoserine dehydrogenase; aspartokinase III, lysine-sensitive aspartokinase.

Homoserine dehydrogenase activity was measured by following the aspartic semialdehyde dependent oxidation of NADPH at 340 nm as previously described (Truffa-Bachi et al., 1966, as modified by Wampler, 1972).

Threonine synthase (EC 4.2.99.2) activity was measured by the release of inorganic phosphate from *O*-phosphohomoserine. The reaction mixture was a modification of that described by Flavin and Slaughter (1960) and contained, in a volume of 0.3 ml: 50 mM Tes buffer (pH 8.0), 70 μM pyridoxal phosphate, and 1 mM L-*O*-phosphohomoserine.

Threonine deaminase was assayed by a coupled reaction in which the product, α-ketobutyrate, was reduced by beef heart lactic dehydrogenase. The reaction mixture contained, in 0.7 ml: 107 mM potassium phosphate (pH 8.0), 43 mM NH_4Cl, 23 mM L-threonine, 0.26 mM DPNH, and 6 units of beef heart lactic dehydrogenase. The reaction was followed by measuring the decrease in optical density at 340 nm.

For all of the enzymes assayed, a unit of activity is the amount of enzyme required to remove 1 μmol of substrate per minute at 30 °C.

Preparation of Crude Extracts. Tir-8 has a tendency to revert to a faster growing phenotype with reduced enzyme levels so that it is important to test for phenotypic reversion. Because revertants grow faster than Tir-8, each 10-ml starter culture was inoculated with cells from a single small colony picked from cells which had been streaked on a petri dish. When the starter culture had grown up, it was used to inoculate several 25-ml cultures, and one or more of these sister cultures were tested for growth rate and homoserine dehydrogenase activity. Log phase Tir-8 has a doubling time (measured as optical density at 550 nm) of more than 100 min. Homoserine dehyogenase levels were measured by centrifuging a 10-ml sample of known optical density, resuspending the cells in 2 ml of the general buffer (pH 7) supplemented with 0.5 mM L-threonine. Cells were broken by sonic oscillation and a 5-μl sample of the sonic extract was assayed for homoserine dehydrogenase activity. Cells were considered derepressed if the OD_{550} of the original culture was 20–30 times the rate (ΔOD_{340}/min) of the homoserine dehydrogenase reaction.

For measuring intracellular enzyme concentrations, small quantities of cells were grown in 120-ml cultures and harvested by centrifugation. Cells were washed by resuspending them in breaking buffer (50 ml/g of cells) and recentrifuging. Cells were again mixed with breaking buffer (4–5 ml/g of cells) and transferred to a nitrocellulose tube, and acid-washed glass beads (300–500 μm diameter, 1 g of beads/g of cells) were added. The suspension was subjected to sonic oscillation for two 30-s intervals with an intermittant 60-s rest and then centrifuged. The supernatant fluid was saved, the pellet resuspended in the same volume of breaking buffer as before, and the sonic oscillation and centrifugation procedure repeated. This final pellet was washed with a third portion of buffer and recentrifuged and the three supernatant liquids were combined. After carefully measuring the volume, the sample was transferred onto a 3.8-ml polyallomer centrifuge tube and centrifuged for 2 h at 36 000 rpm (106 000*g*) in a SW-45 Ti rotor. This high-speed centrifugation step removed a considerable portion of the background APTase and/or NADH oxidase activity which interfered with kinase assays in crude extracts.

For enzyme purification, cultures of Tir-8 were harvested in late log phase (OD_{550} = 2.5–3.5). Cells were resuspended in breaking buffer (2 ml/g of cells) and broken by sonic oscillation (two 2-min intervals with an intermediate 4-min rest) in 200-ml portions in either a glass rosette or a stainless steel beaker. The extract was centrifuged and the residue resuspended in one-half the original volume of breaking buffer and the sonic oscillation procedure repeated. The extract was again centrifuged and the supernatant liquids were combined.

A crude extract of *R. spheroides* was prepared by two passes through a French Pressure Cell (Amicon Instrument Co.) at 1400 psi. The mixture was centrifuged at 15 000*g* and the supernatant liquid was saved.

Aspartate to Threonine Coupled Reaction. In order to remove ammonium sulfate and place the enzymes in a reaction mixture, the purified enzymes were combined to give a sample (85 μl) containing 0.6 unit of aspartokinase I, 9 units of aspartic semialdehyde dehydrogenase, 4 units of homoserine dehydrogenase, 0.72 unit of homoserine kinase, and 0.18 unit of threonine synthase. This sample was applied to a 2.5-ml column of Sephadex G-25 (course) which had been equilibrated with buffer containing 23 mM Tes (pH 7.5), 114 mM KCl, 6 mM $MgCl_2$, 11.4 mM ATP, and 0.12 μM pyridoxal phosphate. Five drop fractions (~0.14 ml) were collected and assayed for homoserine dehydrogenase. Fractions containing homoserine dehydrogenase were combined and transferred to a 1-ml cuvette thermostated at 30 °C.[2] Fifty microliters of a 100 mM solution of NADPH was added to give a final NADPH concentration of 10 mM. The reaction was started by adding 35 μl of [^{14}C]aspartic acid to give a final volume of 0.5 ml and a final aspartate concentration of 5 or 20 mM. The course of the reaction was followed by measuring NADPH oxidation at 390 nm. At various times after adding aspartate, 75 μl of the reaction mixture was removed and mixed with 25 μl of cold, 40% Cl_3CCOOH containing 20 mM each of L-threonine, DL-aspartic semialdehyde, L-homoserine, and L-*O*-phosphohomoserine. The sample was centrifuged and the clear supernatant liquid decanted. For separation of the metabolic intermediates, 4-μl aliquots were applied to cellulose thin-layer chromatography sheets and the amino acids were separated by flat-bed, high-voltage electrophoresis (Savant Instruments Inc.), followed by thin-layer chromatography in the basic solvent.

Results

Intracellular Enzyme Concentrations. Because we were interested in patterns of repression as well as relative concentrations of the several enzymes, we chose to express enzyme concentration as units per milliliter in the cell and, therefore, had to know the cell density of a given bacterial culture. During logarithmic growth, the parent strain (CU-1) had an OD_{550} doubling time of 60 min, while the thiaisoleucine-resistant derivative (Tir-8) had a doubling time of 140–190 min. At different stages during growth samples were withdrawn, diluted with growth medium, and quickly spread on petri dishes so that each dish contained 100–200 cells. When the cells had developed into colonies, the colonies were counted and a calibration curve was constructed for calculating cell density from optical density at 550 nm. This conversion factor was not a constant, but increased steadily up to an OD_{550} of 4 or 5. Although the factor changed little during CU-1 growth, the factor for Tir-8 was considerably larger (more cells/OD_{550}) and increased significantly as the cells approach stationary phase.

Using these conversion factors, we could calculate the number of cells which were broken to give the high-speed supernatant and from this estimate the intracellular enzyme

[2] Approximately 20% of the activity was lost in the leading and trailing edges of the elution profile so that the final enzyme concentrations (in 0.5 ml) were about 1/10 that found in log phase CU-1.

TABLE I: Enzyme Levels for the Aspartate to Threonine Pathway in Wild Type *Esceerichia coli* K12 (CU-1) and a Derepressed Mutant (Tir-8).[a]

Growth Stage (OD_{550})	CU-1		Tir-8	
	1.4	5.0	1.4	6.1
Aspartokinase III	19	35	21	18
Aspartic semialdehyde dehydrogenase	250	690	240	300
Aspartokinase I	19	16	61	55
Homoserine dehydrogenase	130	115	410	390
Homoserine kinase	19	15	104	84
Threonine synthase	9	9	13	25

[a] Activity is expressed as units/ml in the cell, assuming that enzyme extraction was complete, that the cell volume was 8×10^{-13} cm^3, 80% water, and that the enzyme was uniformly dissolved in the water. Breaking efficiency was 98%. This number was obtained by spreading a sample of the pellet from the final low-speed centrifugation step and counting the colonies which developed.

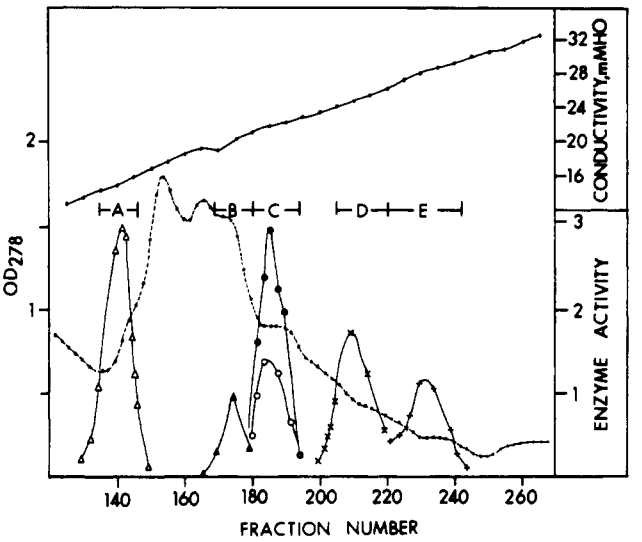

FIGURE 1: Separation of pathway enzymes on DEAE-Sephadex. Fractions were combined to give five samples as follows: (A) homoserine kinase; (B) threonine synthase; (C) homoserine dehydrogenase (●) and aspartic semialdehydehydrogenase (○); (D) aspartokinase III; and (E) threonine deaminase. The symbols used are: (— • —) conductivity in $m\Omega^{-1}$; (- - • - -) optical density at 278 nm; (—) enzyme activity. Enzyme activity is presented in arbitrary units which can be converted to units/ml by multiplying the points under C by 5, dividing the points under A and D by 2, E by 5, and B by 10.

concentrations. The results are presented in Table I. Enzymes involved in the synthesis of threonine from aspartate fall into two groups: those which increase in activity as wild type cells go into stationary phase (aspartokinase III and aspartic semialdehyde dehydrogenase) and those which are derepressed in Tir-8 (the aspartokinase–homoserine dehydrogenase I complex, homoserine kinase, and threonine synthase). Szentirmai et al. (1968) had previously shown that isoleucine pathway enzymes were derepressed in this mutant and, since the synthesis of the threonine-sensitive aspartokinase–homoserine dehydrogenase complex is coordinately controlled by threonine and isoleucine (Freundlich, 1963), this polyfunctional enzyme was also found to be derepressed in Tir-8 (Szentirmai, 1968). The results in Table I extend this derepression pattern to homoserine kinase and threonine synthase, an observation which is compatible with the genetic evidence that these three proteins are coded on a single operon (Thèze and Saint-Girons, 1974; Thèze et al., 1974b). The observation that lysine-sensitive aspartokinase and aspartic semialdehyde dehydrogenase activities fluctuate together, but not with the other enzymes of threonine synthesis, again agrees with the genetic evidence (Boy and Patte, 1972; Thèze et al., 1974b). Smith and Smith (1967) and Niles and Westhead (1973) had shown that lysine-sensitive aspartokinase activity increased as cells went into stationary phase under certain culture conditions. The results in Table I extend this observation to aspartic semialdehyde dehydrogenase. In the case of the lysine-sensitive aspartokinase, Niles and Westhead (1973) presented evidence that the increase in activity was related to the adenylylation of the enzyme. Whether a similar post-translational modification occurs with aspartic semialdehyde dehydrogenase is not known.

Enzyme Separation. In order to study the metabolic pathway from aspartate to threonine in vitro, the five enzymes (six activities) were separated from each other. Except for threonine synthase, all of these enzymes had previously been identified in *E. coli* and purified to a greater or lesser extent. The object of this purification was to give a rapid, clean separation of all threonine biosynthetic enzymes rather than to achieve maximum specific activity. Tris was occasionally substituted for Tes in the preparation of buffers and in one case all operations involving threonine synthase were made at 4 °C. Although Tris inhibits threonine synthase activity, neither of these changes resulted in a significant difference in fractionation behavior or yield. Protein concentrations were estimated from OD_{278} and samples were concentrated by adding solid ammonium sulfate (388 g/l.) if the OD_{278} was greater than 0.5. When the OD_{278} was less than 0.5, the enzymes were concentrated by applying to a small column of DEAE-Sephadex and eluting with buffer containing 0.5 M KCl. All column chromatography was done at room temperature.

The crude cellular extract was made 3% in streptomycin sulfate by predissolving the streptomycin in a small volume of breaking buffer and slowly adding this solution to the crude extract with stirring at 4 °C for 1 h. The precipitate was removed by centrifugation and discarded. Solid ammonium sulfate (388 g/l.) was slowly added to the supernatant fluid and stirred for at least 2 h. The precipitate was removed by centrifugation and the supernatant fluid was discarded.

The ammonium sulfate precipitate was dissolved in a minimum volume of DEAE buffer and was dialyzed at 4 °C against at least two changes of this buffer, usually overnight. Debris was removed from the dialyzed sample by centrifugation and the sample was diluted with DEAE buffer which contained no KCl until the conductivity (measured at room temperature) was below 8 $m\Omega^{-1}$. The sample was then applied to a DEAE-Sephadex column (15 ml of bed/g of cells), preequilibrated with DEAE buffer. Enzymes were eluted with a 0.1–0.6 M KCl gradient (7–10 ml/ml of bed) in DEAE buffer at a flow rate of 3 ml/min and 8–12-ml fractions were collected. The elution pattern from DEAE-Sephadex is given in Figure 1 to show the amount of overlap that one can normally expect. In this particular experiment the two dehydrogenases were not separated while in other experiments aspartic semialdehyde dehydrogenase sometimes preceded and sometimes followed homoserine dehydrogenase. This difference in elution pattern changed the way fractions were combined for subsequent columns but did not change the final purity or yield. Fractions were combined as shown in Figure 1 to give five

TABLE II: Elution Pattern of Pathway Enzymes from the Three Chromatography Columns Used.[a]

Enzyme	$m\Omega^{-1}$	P_i (mM)	Mol Wt
Aspartokinase III	23–24	54	150 000
Aspartic semialdehyde dehydrogenase	20–21	22	70 000
Aspartokinase–homoserine dehydrogenase I	21–22	20	340 000
Homoserine kinase	12–13		60 000
Threonine synthase	18–20	5	36 000
Threonine deaminase	24–26		204 000

[a] Elution position is indicated by conductivity ($m\Omega^{-1}$) for DEAE-Sephadex, phosphate (P_i) concentration for hydroxylapatite, and approximate molecular weight for gel filtration.

samples containing: homoserine kinase, threonine synthase, the two dehydrogenases (with aspartokinase I), aspartokinase III, and threonine deaminase. Because homoserine kinase was so well separated from the other enzymes, it was not subjected to hydroxylapatite chromatography. Similarly, threonine deaminase contained only a small aspartokinase III contamination which was readily removed by gel filtration so the hydroxylapatite step was omitted for this enzyme too.

The other three samples from the DEAE-Sephadex column were applied separately to columns of hydroxylapatite (Bio-Gel HT, 2–5 ml of bed/mg of protein) which had been equilibrated with the general buffer supplemented with 70 μM pyridoxal phosphate for threonine synthase, 0.5 mM L-threonine for aspartokinase–homoserine dehydrogenase I and with 1 mM L-lysine for aspartokinase III. Enzymes were eluted with a 0–0.1 M potassium phosphate gradient (3–5 ml/ml of bed volume) at a flow rate of 2–5 ml/min.

Combined fractions from hydroxylapatite were concentrated by ammonium sulfate precipitation, dialyzed for an hour or more to bring the whole sample into solution and reduce the density of the sample, and centrifuged to remove debris. This concentrated sample (2–5 ml) was applied to a 2.5 × 90 cm column of Sephadex G-100 or G-200. Elution was by downward flow at a rate of 15 ml/h and 5-ml fractions were collected. The two dehydrogenases are finally separated by Sephadex G-200 chromatography and the other enzymes are given a final purification on Sephadex G-100 or G-200. After gel filtration, active fractions were combined and concentrated by ammonium sulfate precipitation. The enzymes were stored as slurries in 60% saturated ammonium sulfate at 4 °C. The chromatographic behavior of these enzymes is summarized in Table II and the concentration of each enzyme in each of the final samples is given in Table III.

Aspartokinase and homoserine dehydrogenase were purified from *R. spheroides* by essentially the same methods used for the *E. coli* enzymes. The crude extract was treated with streptomycin sulfate, proteins were precipitated with ammonium sulfate, and the dialyzed, redissolved ammonium sulfate precipitate was passed twice through a column of Sephadex G-200. Aspartokinase and homoserine dehydrogenase eluted with considerable overlap (homoserine dehydrogenase leading). Both activities were reasonably well separated from aspartic semialdehyde dehydrogenase by the first gel filtration column and were very well separated from the contaminating ATPases and/or DPNH oxidases. A second pass over G-200 removed the small aspartic semialdehyde dehydrogenase contamination.[3]

Kinetic Properties of Threonine Synthase. Since this is the first report of threonine synthase purification from *E. coli*, a brief examination of the kinetic properties of this enzyme was conducted. In the general buffer (pH 8) supplemented with 70 μM pyridoxal phosphate, purified threonine synthase was quite stable, exhibiting a half-life of 2 weeks at 20 °C and 13 h at 50 °C. At 60 °C there was a rapid (5 min) initial loss of 75% of the activity followed by a slower loss of the remaining activity. Substrate saturation was cooperative with a Hill coefficient of 1.4 and with 0.23 mM *O*-phosphohomoserine giving one-half maximum activity. In contrast to the other enzymes of this operon, threonine synthase does not show a monovalent cation requirement. Indeed, at higher substrate concentrations (>10 mM), Na^+, K^+, and $(CH_3)_4N^+$ become inhibitors. Tris is also an inhibitor so that reaction mixtures using Tris as the buffer exhibit only 30% of the activity as compared with Tes. Using glycylglycine, Tes, and Bistris as buffers, threonine synthase exhibits a pH optimum between 8.0 and 8.5 with ½ maximum activity at pH 6.8 and 9.4. Gel filtration over Sephadex G200, using cytochrome *c* (12 500 daltons), chymotrypsinogen (25 000 daltons), and lactate dehydrogenase (150 000 daltons) as markers, indicated a molecular weight of 36 000 for threonine synthase.

Chemical Stability of Intermediates. The main difficulty in trying to measure the concentrations of all metabolic intermediates was the instability of β-aspartyl phosphate and aspartic semialdehyde. The problem was further complicated by the fact that aspartic semialdehyde is most stable at low pH, where β-aspartyl phosphate is very unstable (Black and Wright, 1955a). Because we wanted to test the possibility that aspartic semialdehyde is involved in regulating threonine synthesis, we decided to stop the reaction under acid conditions where aspartic semialdehyde is stable and allows β-aspartyl phosphate to be hydrolyzed back to aspartic acid.

To be sure that we could accurately measure aspartic semialdehyde in a reaction mixture, a brief study of the chemical stability and recoverability of this intermediate was made. At pH 6.2 or below, aspartic semialdehyde has a half-life ($t_{1/2}$) of a week or more. In 1 M HCl it can be stored at −20 °C for a year or more without significant loss of substrate activity. At pH 7.5 there is a rapid initial loss in activity ($t_{1/2} < 20$ min) and then a slow decay with a half-life of about 25 days. After 1 day at pH 7.5, a drop of 6 M HCl was added to 200 μl of the sample (bringing the pH below 2) and its aspartic semialdehyde concentration was measured. The rapid drop in pH did not immediately change the concentration of aspartic semialdehyde but 4 days later the substrate activity had returned to that of the original sample. The fact that substrate activity was recovered slowly when the pH was lowered is consistent with the suggestion of Westerik and Wolfenden (1974) that this amino acid is in a pH-dependent equilibrium among other molecular forms.

[3] Attempts to identify homoserine kinase either in crude extracts of *R. spheroides* or in fractions from the G-200 column were unsuccessful. An attempt was also made to identify homoserine kinase in crude extracts and partially purified preparations of *Crithidia fasiculata*, without success, although weak threonine synthetase activity was observed following gel filtration of a *C. fasiculata* extract. Kidder and Dewey (Kidder, G. W., and Dewey, V. C. (1972), *J. Protozool. 19*, 93) have shown that this organism can synthesize threonine but that homoserine comes from α-keto-γ-hydroxybutyrate rather than from aspartate. The synthesis of threonine from homoserine was presumed to be by homoserine kinase and threonine synthetase. The inability to detect homoserine kinase from these two organisms may be because ATP is not the phosphoryl donor. Other possible sources of the phosphoryl group were not tested.

TABLE III: Enzymatic Composition of the Partially Purified Enzymes from *Escherichia coli* Used in the Coupled Reactions.

Enzyme Fraction	Enzyme Act. (units/ml)[a]						
	AK-I	AK-III	ASADH	HSDH	HSK	TS	TD
Aspartokinase–homoserine dehydrogenase I (AK-I, HSDH)	115	0	2	745	0	0	0
Aspartokinase III (AK-III)	0.6	85	2	5	0	0	0
Aspartic semialdehyde dehydrogenase (ASADH)	0	0.4	317	0	0.4	0	0
Homoserine kinase (HSK)	0	0	3	0.5	350	0	0
Threonine synthase (TS)	0	0.1	0	0.1	0.2	30	0.1

[a] "0" activity indicates less than 0.1% of the principal activity in that fraction.

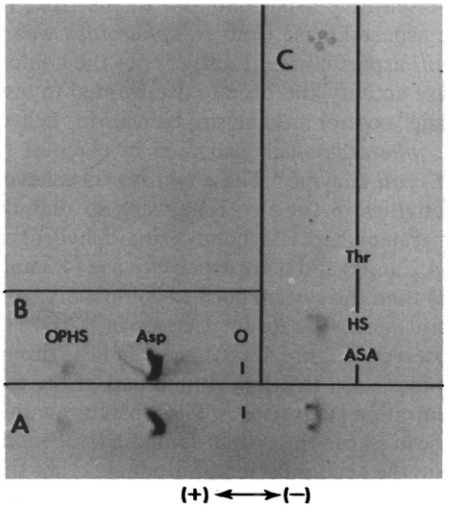

FIGURE 2: Autoradiogram of [^{14}C]aspartate and its metabolites after 5.5 min in the hybrid reaction mixture (see Figure 6A). The identifiable spots are: origin (O), aspartate (Asp), aspartic semialdehyde (ASA), homoserine (HS), *O*-phosphohomoserine (OPHS), and threonine (Thr). High-voltage electrophoresis was in the horizontal direction using wicks made of Wattman No. 1 filter paper. Electrophoresis was for 20 min at 3.5 K_v in 58 mM acetic acid adjusted to pH 3.3 for Kodak TLC plates and to pH 3.6 for Bakerflex plates. Under these conditions, α-ketobutyrate, *O*-phosphohomoserine, and aspartate moved to the anode while threonine, aspartic semialdehyde, and homoserine moved together toward the cathode. The bottom strip (A) was cut off and sprayed with ninhydrin to locate the three principal spots. The remaining sheet was cut vertically and the area right of the origin was subjected to thin-layer chromatography in the basic solvent (see Materials and Methods). Two 2-in. wide strips (B and C) were then cut off, and radioactivity was measured on a strip scanner. The four small circles on strip C are samples of known radioactivity used for calibration.

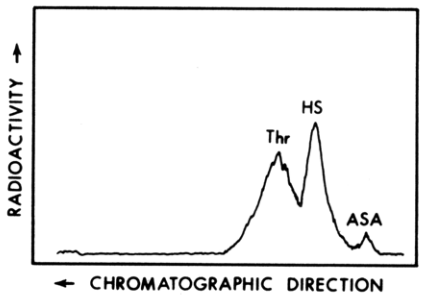

FIGURE 3: Strip scanner recording of radioactive threonine (Thr), homoserine (HS), and aspartic semialdehyde (ASA) by thin-layer chromatography after separation from aspartate by electrophoresis. Conditions are given in Figure 2.

Separation of Intermediates. Initial work on separating intermediates concentrated on thin-layer chromatography. A two-dimensional system similar to that developed by Haworth and Heathcote (1968) worked well with simple mixtures of amino acids and was particularly useful for separating threonine from homoserine. However, this system had two problems. The first was that aspartate, *O*-phosphohomoserine, and α-ketobutyrate were poorly separated. The second problem was that, when samples were taken from reaction mixtures (which contained some protein and 0.15 M salt), the amino acids smeared badly. To overcome these problems we developed a separation system which used high-voltage electrophoresis in the first direction and thin-layer chromatography in the second. After electrophoresis, the thin-layer sheet was cut in two and the lower sample was sprayed with ninhydrin (Section A, Figure 2). Using the spots on this strip as a guide, the upper portion of the sheet was cut vertically between the origin and those amino acids which had a net positive charge. These positively charged amino acids were then separated by chromatography in the basic solvent. The two large sheets were then cut down to 2-in. wide strips (bands B and C, Figure 2) and radioactivity was measured on the strip scanner. After strip scanning, the sheet could be recomposed and an autoradiogram made (Figure 2) to confirm the identity of the radioactive peaks.

To demonstrate that aspartic semialdehyde could be identified in a mixture of radioactive intermediates, L-[^{14}C]aspartic semialdehyde was made enzymatically from L-[^{14}C]aspartate and radioactive threonine and homoserine were added to the reaction mixture. A sample of this mixture was processed as described in the previous paragraph and a tracing of the strip scan of the three amino acids which exhibit a small net positive charge is given in Figure 3. Even though there was ten times as much radioactivity in threonine and homoserine, aspartic semialdehyde formed a clearly separate peak.

Coupled Reaction. The five enzymes were combined in several ways to form a variety of coupled reactions. The first reaction contained 20 mM aspartate and enzyme contractions were approximately $\frac{1}{10}$ the intracellular concentration of CU-1 growing in exponential phase. Under these conditions the initial rate of TPNH consumption was quite rapid and decreased rapidly for the first 3–4 min (Figure 4, open circles). After about 3.5 min there was an abrupt change in the rate of optical density decrease so that the rate was relatively constant for the next 10–20 min. Analysis of the amino acid composition (Figure 5A) showed that the rate stabilized at the same time as the L-threonine concentration reached about 1 mM. The concentrations of the three intermediates (aspartic semialdehyde, homoserine, and *O*-phosphohomoserine) increased

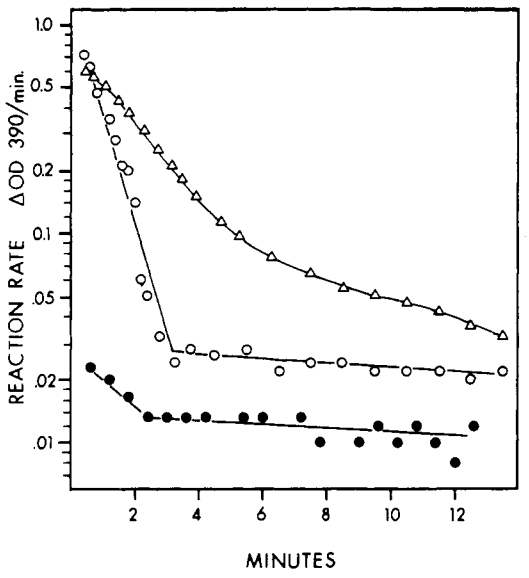

FIGURE 4: Rate of NADPH oxidation (ΔOD_{390}/min) during the coupled reaction. Rates were calculated from tangents drawn to the recording of OD_{390} as a function of time. The enzymes used were isolated either from *E. coli* (circles) or from both *E. coli* and *R. spheroides* (△). The reaction mixture contained the standard reactants described in the text (open symbols) or was supplemented with 1 mM L-threonine (●).

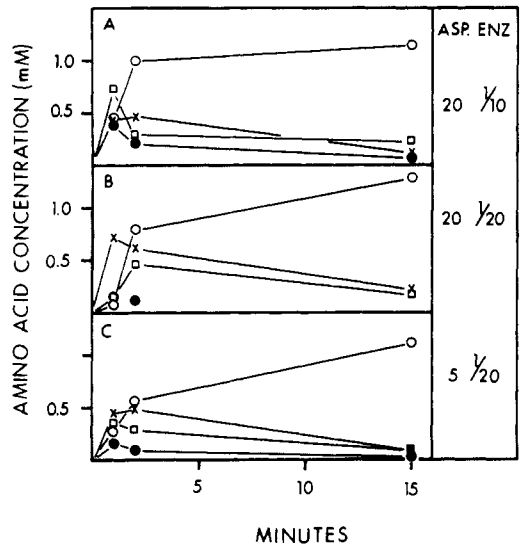

FIGURE 5: Concentrations of four amino acids in the coupled reaction mixture using only *E. coli* enzymes. The initial mM aspartate concentrations (Asp) and the enzyme concentrations expressed as a fraction of the estimated intracellular concentration (Enz) are given in the right panel. Symbols used are: (●) aspartic semialdehyde; (X) homoserine; (□) *O*-phosphohomoserine; (○) threonine.

during the first 1 or 2 min and then declined to very low levels. Aspartic semialdehyde maintained the lowest concentration with radioactivity not much greater than the noise level of the strip scanner (in these experiments corresponding to about 0.1 mM). Other reaction mixtures were composed with different concentrations of substrate and/or enzymes, although the relative enzyme concentrations were not changed. Under these conditions the rate of NADPH consumption showed the same pattern that it did at the higher concentration, that is, a rapid decrease in reaction rate followed by a stable, slower reaction. Changes in amino acid concentration were also similar (Figure 5B,C). In the last case, when both aspartate and enzyme concentrations were low, the threonine concentration rose more slowly, although the break in the reaction rate curve still occurred at about 4 min. This behavior is probably due to the fact that threonine inhibition of aspartokinase I is competitive with aspartate (Stadtman et al., 1961).

To test the influence of threonine concentration more directly, a reaction mixture was composed containing 1 mM L-threonine in the initial reaction mixture. When this was done, the rate of NADPH oxidation was slow from the beginning (Figure 4, filled circles), supporting the idea that feedback inhibition by L-threonine at about 1 mM caused the decrease in reaction rate.

To test the possibility that feedback inhibition was specific for aspartokinase I, two reaction mixtures were composed using threonine-insensitive aspartokinase. In the first, threonine-insensitive aspartokinase from *R. spheroides* was used. Because *E. coli* aspartokinase I also carries the homoserine dehydrogenase activity and because we wanted to test the "reverse cascade" control mechanism, homoserine dehydrogenase I from *R. spheroides* was also used in place of the corresponding *E. coli* enzyme.[4] These two enzymes have different relative activities in the two organisms so that the hybrid metabolic system had less homoserine dehydrogenase (2.5 instead of 4.2 units) and more aspartokinase (4.3 units instead of 0.6 unit) than the system derived completely from *E. coli*. In this hybrid system the abrupt decrease in NADPH oxidation was not observed (Figure 4, triangles) and the threonine concentration increased to 2.5 mM, one-half of the total amino acid concentration (Figure 6A). These results again show that the decrease in rate in the system composed of *E. coli* enzymes is not due to the accumulation of products other than L-threonine or depletion of substrates as the reaction approaches chemical equilibrium. The other significant observation is that aspartic semialdehyde does not accumulate sufficiently to inhibit the *R. spheroides* aspartokinase.

The second test of threonine control was to add aspartokinase III to the *E. coli* system so that there would be a by-pass of the threonine-sensitive aspartokinase reaction. In this system, threonine accumulation did not stop at 1 mM, but continued to rise to 1.8 mM after 15 min (Figure 6B). Although this accumulation is not as great as in the *R. spheroides* hybrid system (Figure 6A), it is nearly twice as much as in the *E. coli* system with only aspartokinase I (Figure 5C).

Discussion

Post-translational regulation of metabolic flux through the aspartic acid "family tree" is complicated by the large number of branches to a variety of metabolic products. The clearest control is feedback inhibition of aspartokinase, the first energy consuming reaction in the pathway. More subtle, secondary control sites (e.g., at the first committed step for each product) have been proposed, based on kinetic studies of individual enzymes. Results presented in this report bear on three of these control mechanisms.

The observation that aspartic semialdehyde does not accumulate as the threonine concentration increases, in any of the systems tested, argues against the metabolic importance of a "reverse cascade" mechanism. Indeed, when the *R. spheroides* enzymes were used, the aspartic semialdehyde concentration did not exceed 0.2 mM (Figure 6A) which is 1/10 the concen-

[4] The threonine sensitivity of these two enzymes is equivalent in our hands. Both are 80% inhibited by 5.6 mM L-threonine in the standard homoserine dehydrogenase assay.

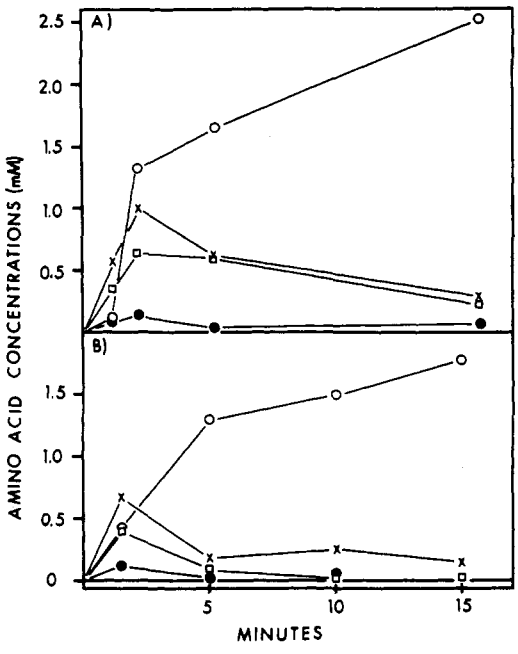

FIGURE 6: Concentrations of four amino acids in the coupled reaction mixture using (A) aspartokinase and homoserine dehydrogenase from *R. spheroides* and the other enzymes from *E. coli*, (B) all enzymes from *E. coli* with both aspartokinase I and aspartokinase III. Symbols have the same meaning as in Figure 5.

tration required for 30% inhibition of purified *R. spheroides* aspartokinase, assayed at 5 mM aspartate (Datta and Prakash, 1966). The failure to detect significant aspartokinase inhibition at low metabolite concentrations suggests that the regulation of threonine synthesis may be under less rigid control in *R. spheroides* than in *E. coli*.

The suggestion by Thèze et al. (1974a) that threonine inhibition of homoserine kinase may be important for channeling metabolism from threonine to methionine is likewise not supported by experiments with the coupled reaction since homoserine does not accumulate as the concentration of threonine increases. In fact the opposite situation is observed. Even when aspartokinase inhibition is bypassed with threonine-insensitive enzymes, the concentrations of all three intermediates "run out" as the mixture settles down to a steady-state reaction or approaches chemical equilibrium.

Finally, these results give information on the relationship between energy metabolism and threonine synthesis. Because the coupled reaction described in this paper is a closed system, the ATP/ADP ("energy charge") and the NADPH/NADP ratios are also changing. Although these changes may play a part in decreasing flux through the coupled reactions, they cannot be the whole explanation for the decrease in flux seen in Figure 4 since: (a) addition of threonine to the original reaction mixture resulted in decreased flux from the beginning and (b) use of threonine-insensitive aspartokinases resulted in a sustained, elevated rate of NADPH oxidation and higher threonine accumulations.

Biochemists have always been aware that experiments with purified enzymes under optimal reaction conditions, while convenient to handle, do not represent intracellular conditions. In recent years investigators have begun to study enzymatic activity in situ (Kornberg and Malcovati, 1973; Reeves and Sols, 1973; Weitzman, 1973; Wyman et al., 1975) and the kinetics of coupled reactions (Rolleston, 1972; Savageau, 1972; Srere et al., 1973; Bunow, 1974). Despite these advances in our ability to simulate and analyze in vivo conditions, many proposals for mechanisms of metabolic regulation have been based on subtle changes in initial velocities of single enzymes under nonphysiological concentrations of enzyme and substrates. The reaction conditions described in this report are still far from physiological in terms of enzyme concentrations and metabolic complexity, but they are an important improvement since (a) a metabolic system instead of a single reaction was studied, (b) the enzyme concentrations were closer to their physiological concentrations (5–15 times the concentrations used in studies with single enzymes), (c) the relative enzyme concentrations are the same as those found in the cell, and (d) the reactant and product concentrations were their low, steady-state concentrations rather than the high concentrations used to give optimum activity. The results reported here emphasize the need for conducting experiments under conditions which are as nearly physiological as possible and the need for caution in using in vitro results for building models of metabolic regulation in vivo.

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