Dudai, Y., Silman, I., Kalderon, N., and Blumberg, S. (1972a), *Biochim. Biophys. Acta 268*, 138.
Dudai, Y., Silman, I., Shinitzky, M., and Blumberg, S. (1972b), *Proc. Nat. Acad. Sci. U. S. 69*, 2400.
Dunker, A. K., and Rueckert, R. R. (1969), *J. Biol. Chem. 244*, 5074.
Froede, H. C., and Wilson, I. B. (1970), *Israel J. Med. Sci. 6*, 179.
Grafius, M. A., Bond, H. E., and Millar, D. B. (1971), *Eur. J. Biochem. 22*, 382.
Grafius, M. A., Friess, S. L., and Millar, D. B. (1968), *Arch. Biochem. Biophys. 126*, 707.
Grafius, M. A., and Millar, D. B. (1965), *Biochim., Biophys. Acta 110*, 540.
Grafius, M. A., and Millar, D. B. (1967), *Biochemistry 6*, 1034.
Iverson, F., and Mann, A. R. (1969), *Biochemistry 8*, 1889.
Klotz, I. M., and Darnell, D. W. (1969), *Science 166*, 126.
Koelle, G. B. (1963), *Handb. Exp. Pharmacol. 15*, 187.
Kremzner, I. I., and Wilson, I. B. (1968), *Biochemistry 3*, 1902.
Lawler, H. C. (1959), *J. Biol. Chem. 234*, 799.
Leuzinger, W. (1971), *Biochem. J. 123*, 139.
Leuzinger, W., and Baker, A. L. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 446.
Leuzinger, W., Goldberg, M., and Cauvin, E. (1969), *J. Mol. Biol. 40*, 217.
Massoulié, J., Rieger, F., and Tsuji, S. (1970), *Eur. J. Biochem. 14*, 430.
McMeekin, T. L., and Marshall, K. (1952), *Science 116*, 142.
Michel, H. O., and Krop, S. (1951), *J. Biol. Chem. 190*, 119.
Millar, D. B., and Grafius, M. A. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 12*, 61.
Mooser, G., Schulman, H., and Sigman, D. S. (1972), *Biochemistry 11*, 1595.
Nachmansohn, D., and Rothenberg, M. A. (1945), *J. Biol. Chem. 158*, 653.
Pringle, J. R. (1970), *Biochem. Biophys. Res. Commun. 39*, 46.
Rosenberry, T. L., and Bernhard, S. A. (1971), *Biochemistry 10*, 4114.
Rosenberry, T. L., Chang, H. W., and Chen, Y. T. (1972), *J. Biol. Chem. 247*, 1555.
Tanford, C., Kawahara, K., and Lapanje, S. (1967), *J. Amer. Chem. Soc. 89*, 729.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Zaitlin, M., and Hariharasubramanian, V. (1970), *Anal. Biochem. 35*, 296.

The Variable Subunit Structure of Lysine-Sensitive Aspartylkinase from *Escherichia coli* TIR-8†

E. G. Niles*,‡ and E. W. Westhead

ABSTRACT: Lysine-sensitive aspartylkinase (AK III) has been purified to homogeneity by the criteria of disc gel electrophoresis at pH 8.3, electrophoresis in sodium dodecyl sulfate, and by sedimentation velocity. It has been shown to be composed of similar subunits of 48,000 ± 4000 molecular weight by sedimentation equilibrium of reduced, carboxymethylated enzyme in 6 M guanidine hydrochloride and by gel electrophoresis in 0.1% sodium dodecyl sulfate. Equilibrium sedimentation, analytical G-200 Sephadex chromatography, and $s_{20,w}$ plus $D_{20,w}$ determinations have shown that AK III may be obtained in two forms differing in quaternary structure. One form is a fairly stable dimer of about 100,000 molecular weight, $s_{20,w}$ = 6.6 S. The second form appears to be in a dimer–tetramer equilibrium and at higher protein concentration behaves as a tetramer of about 200,000 molecular weight, $s_{20,w}$ = 10.1 S. The partial specific volume of the protein determined by equilibrium sedimentation in D_2O is 0.746 cm³/g. The $E^{1\%}_{280}$ was determined by dry weight to be 3.60. The specific activity of the enzyme increases during log-phase growth and reaches a sharp maximum at the onset of stationary phase. The enzyme purified from late stationary phase exhibits a high uv absorbancy below 280 nm. Lysine addition to native enzyme causes a difference spectrum with a peak at 294 nm. Titration of the native enzyme at 294 nm exhibits an apparent cooperative transition with the half-maximal change occurring at 0.2 mM lysine.

Lysine-sensitive aspartylkinase is one of three aspartylkinases found in *Escherichia coli* K_{12}. Most of our knowledge of these enzymes comes from the work of G. N. Cohen and his collaborators, and this work has recently been definitively reviewed (Cohen, 1969). Two of these enzymes are complex proteins which also carry homoserine dehydrogenase activity. Aspartylkinase I (AK I–HSDH I),[1] which is inhibited by threonine, was isolated by Truffa-Bachi *et al.* (1968) and reported to consist of six similar subunits of 60,000 molecular weight. Recently, Starns *et al.* (1972), studying *E. coli* K_{12} λ, have reported a subunit molecular weight of about 85,000, indicating that AK I–HSDH I is composed of four similar subunits. Aspartylkinase II (AK II–HSDH II), the methionine repressed

† From the Department of Biochemistry, University of Massachusetts, Amherst, Massachusetts 01002. *Received June 5, 1972.* Supported by the National Institutes of Health Grant AM11157. A preliminary account of this work has been given: *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 912 Abstr. (1970). This work is part of the Ph.D. thesis (of E. G. N.) which is available from University Microfilms, Ann Arbor, Mich. 48106.

‡ Present address: Department of Radiobiology, Yale Medical School, New Haven, Conn.

[1] Abbreviations used are: AK III, lysine-sensitive aspartylkinase; Gdn·HCl, guanidine hydrochloride; HSDH, homoserine dehydrogenase.

enzyme, has also been purified in Cohen's laboratory (Falcoz-Kelly *et al.*, 1969) and found to have a molecular weight of 169,000 with four similar subunits of 43,000 each.

The control of the AK III activity has been studied extensively. The enzyme has been shown to be inhibited cooperatively by lysine (Wampler and Westhead, 1968; Truffa-Bachi and Cohen, 1966), leucine, isoleucine, and phenylalanine (Patte and Cohen, 1965), and repressed by lysine (Stadtman *et al.*, 1961). The enzyme has also been shown to be activated by either NH_4^+ or K^+ (Wampler and Westhead, 1968).

The enzyme has been reported to be purified first in microgram quantities by Truffa-Bachi and Cohen (1966) and later in milligram quantities by Niles and Westhead (1970) and by Lafuma *et al.* (1970). Truffa-Bachi and Cohen (1966) reported a molecular weight of the native enzyme of about 100,000. Niles and Westhead (1970) and Lafuma *et al.* (1970) reported a native molecular weight of 177,000 and 133,000, respectively. von Dippe *et al.* (1972) have purified AK III from *E. coli* B and report a subunit molecular weight of 39,000. Also, they report that the enzyme undergoes a dimer–tetramer transition which is affected by lysine.

In this publication, a rapid method is presented for the purification of AK III and partial purification of AK I. The subunit structure of AK III is described and the discrepancy between the molecular weights of the native enzyme previously reported is resolved. Evidence is presented which links the variable physical properties of the enzyme with the rapid decline in AK III activity in early-stationary-phase cells.

Materials

Amino acids, ATP, 2-mercaptoethanol, phosphoenolpyruvate, pyruvate kinase, and lactic dehydrogenase were purchased from Sigma Chemical Co.; dithiothreitol and streptomycin sulfate from Calbiochem; inorganic salts from J. T. Baker Co.; and gel electrophoresis materials from Canalco. Guanidine hydrochloride and ammonium sulfate were Ultra Pure grades from Mann Chemical Co. The following proteins were used as standards for electrophoresis in sodium dodecyl sulfate solution and G-200 Sephadex chromatography: aldolase from Worthington Biochemical Corp.; alcohol dehydrogenase, pyruvate kinase, lactate dehydrogenase, chymotrypsinogen, and cytochrome *c* from Sigma; yeast enolase, prepared by Thomas Shen in this laboratory.

Buffers used were the following, with modifications noted in the text: *GL buffer*, 10 mM potassium phosphate (pH 6.8)–1 mM EDTA–2 mM $MgCl_2 \cdot 6H_2O$–1 mM lysine–0.1 mM dithiothreitol; sonicating buffer, GL buffer–1 mM threonine–100 mM KCl; DEAE buffer, GL buffer–1 mM threonine–0.15 M KCl (low salt) to 0.75 M KCl (high salt); HT buffer, 10 mM (low salt) or 300 mM (high salt) potassium phosphate (pH 6.8)–1.5 mM lysine-HCl–100 mM KCl–0.1 mM dithiothreitol.

E. coli Tir-8 was a generous gift from Dr. H. E. Umbarger. This mutant of K_{12} was isolated by Szentirmai *et al.* (1968), and was reported to be an overproducer for AK I (Janin and Cohen, 1969). Because both AK I and AK III can be copurified, and *E. coli* Tir-8 is also a 2-fold overproducer for AK III (Niles, 1972), Tir-8 was chosen for the isolation of AK III.

Methods

E. coli Tir-8 was grown on the minimal medium of Davis and Mingioli (1950). The methionine derepression studies of Biswas *et al.* (1968) with *E. coli* B were reproduced with *E. coli* Tir-8 (Niles, 1972). Because there was a 2- to 3-fold increase in AK III activity in bacteria grown on 2.5 mM L-methionine, the medium of Davis and Mingioli was supplemented with 5 mM DL-methionine.

To determine the time course of AK III activity, an 11-l. culture was grown at 37° in a New Brunswick fermentor at 175 rpm and under forced aeration. At various times, 200 ml of the culture was removed. The A_{660} was read and the cells were collected by centrifugation at 10,000*g* for 20 min. The cell extract was prepared by sonication of the bacteria, suspended in three volumes of sonicating buffer, by a Bronson Model S-125 sonicator. The cell debris was centrifuged and the AK III activity and protein were measured in the crude extract.

The coupled assay of Wampler and Westhead (1968) containing pyruvate kinase and lactic dehydrogenase was used to follow the aspartokinase activity at 30°. The assay mixture contained (in a final volume of 0.7 ml): 11.4 mM aspartate, 3.3 mM ATP, 5.7 mM $MgCl_2$, 0.31 mM $NADH_2$, 0.57 M KCl, 0.12 M Tris-Cl (pH 7.5), 12 units (as defined by the supplier) of pyruvate kinase, and 25 units of lactic dehydrogenase. Threonine (5 mM) was added to the assay mixture until the DEAE-Sephadex step to inhibit any aspartylkinase I activity. Aspartylkinase II did not interfere because it is repressed when cells are grown in a medium containing 5 mM methionine (Patte *et al.*, 1967).

Until the DEAE-Sephadex step in purification, there is a high initial background oxidation of $NADH_2$ due both to the formation of ADP and the oxidation of $NADH_2$ by other enzymes. This activity subsides after the endogenous substrates for the contaminating enzymes are depleted. To correct for this background activity, we add enzyme to the reaction mixture lacking aspartate and allow the spurious oxidation of $NADH_2$ to continue until the rate decreases to less than 0.03 ΔOD/min. Aspartate is then added and aspartylkinase III activity is measured. The background can be substracted from the apparent rate, or the aspartylkinase III activity can be calculated from the decrease in the rate of $NADH_2$ oxidation after the addition of L-lysine. Throughout the purification and after long periods of storage, the enzyme remains completely inhibited by lysine.

The specific activity is defined as micromoles of $NADH_2$ oxidized per minute per milligram of protein.

Protein Determination. Protein concentrations were determined according to the Lowry method (Lowry *et al.*, 1951) or spectrophotometrically by the method of Warburg and Christian (1942).

Dry Weight Determination. A 0.8 ml sample of pure enzyme was exchanged into 1.0 mM potassium phosphate (pH 6.8)–0.5 mM lysine by passage over a Sephadex G-25 column (1 × 20 cm). The elution profile was read at 280 nm. From the tube with the highest absorbance at 280 nm (1.39), 100-μl aliquots were placed in three preweighed tin drying vessels. Three 100-μl aliquots of buffer were also placed in drying vessels. The solutions were dried over P_2O_5 for 48 hr, then in vacuum for 24 hr. The process was completed by drying for 24 hr at 90°. The vessels were crimped closed in an atmosphere of dry nitrogen and weighed on a microbalance. The weight of the protein was corrected for the weight of the buffer.

Another 100-μl aliquot of the same enzyme was diluted to 1.0 ml and scanned on a Cary 14 and a Gilford 2000 spectrophotometer to determine the specific absorption constant.

Enzyme Purification and Characterization. The purification of AK III developed and presented here was based on methods

reported by Truffa-Bachi and Cohen (1966) and Fang (1969). Subsequent to the development of this purification scheme, Lafuma *et al.* (1970) reported the purification of AK III from *E. coli* K_{12}.

Through the streptomycin sulfate step, the purification was carried out at 4°. Each subsequent step was carried out at room temperature. After each step, the pH was adjusted to 6.8 with 0.5 N KOH. All centrifugations were carried out at 4°, at 10,000 rpm in a Sorvall RC-2B centrifuge.

Sonication. Frozen cell paste (500 g) is suspended in three volumes of cold sonicating buffer by homogenization in a Waring blender for 1 min; 280-ml aliquots of the suspension are sonicated in an ice–water bath for two 9-min periods, separated by a 2-min cooling period, using a Bronson Model S-125 sonifier. The temperature was not allowed to go above 20°. The cell suspension is centrifuged and the supernatants are pooled.

Streptomycin Sulfate Precipitation. To the crude extract is added enough streptomycin sulfate, from a 75% solution, to form a final concentration of 3% (w/v). The pH is monitored and maintained at 6.8 by the addition of 1.0 N KOH. The precipitate is allowed to form for 30 min and then collected by centrifugation. The supernatants are pooled and the precipitate was discarded.

Ammonium Sulfate Precipitation. To the streptomycin sulfate supernatant is added enough saturated ammonium sulfate solution (pH 6.8) to bring the final concentration to 25%. The precipitate is allowed to form for 30 min, collected by centrifugation, and discarded.

To the 25% ammonium sulfate supernatant is added enough saturated ammonium sulfate to bring the solution to 45% saturation in ammonium sulfate. The precipitate is again collected by centrifugation after 30-min. The precipitate is dissolved in a volume of sonicating buffer equal to about one-tenth of the crude extract volume. The supernatant is discarded.

The dissolved 45% precipitate is again treated with saturated ammonium sulfate to bring the solution to 20% saturation, ignoring the residual salt concentration of the solution. The precipitate is collected as before, after 30 min.

The supernatant is then brought to 45% saturation and the precipitate was collected by centrifugation. The precipitate is dissolved in a minimal volume of DEAE-Sephadex low salt buffer. The supernatant is discarded.

DEAE-Sephadex. The dissolved 45% ammonium sulfate precipitate is dialyzed into DEAE (lower salt) buffer either by an overnight dialysis against 10 l. of DEAE buffer or by passage through a Sephadex G-25 column (4.5 × 20 cm) preequilibrated with DEAE buffer. The protein solution is put on a DEAE-Sephadex column (4.5 × 20 cm) and eluted with a linear gradient of KCl from 0.15 to 0.75 M. The gradient is constructed from 4 l. of DEAE (low salt) buffer and 4 l. of DEAE (high salt) buffer. A 2-ft pressure head is employed. Fractions of 20 ml are collected at a flow rate of 4 ml/min. Tubes with a specific activity (units/A_{280}) greater than three are pooled and brought to 50% saturation with solid ammonium sulfate. The resulting precipitate is dissolved in a minimal volume of HT (low salt) buffer.

Bio-Gel HT (Bio-Rad). A column of hydroxylapatite (4.5 × 20 cm) is prepared from a slurry of calcium phosphate previously washed with 1 M phosphate buffer (pH 6.8) and equilibrate with HT (low salt) buffer. The protein is passed over G-25 Sephadex as before, adsorbed to the calcium phosphate, and eluted with a linear gradient made from 1 l. of HT (low salt) buffer and 1 l. of HT (high salt) buffer. With a pressure head of 2 ft, 20-ml fractions are collected at a flow rate of 1 ml/min. Tubes with a specific activity greater than 25 (units/A_{280}) are pooled and concentrated to 10 ml in an Amicon concentrator with a UM-10 membrane.

Sephadex G-200. The concentrated protein from the HT column is run on a Sephadex G-200 column (2.5 × 100 cm) in the ascending direction. The protein is eluted with degassed GL buffer at a pressure head of 6 in. Fractions of 5 ml are collected at a flow rate of 0.5 ml/min. Fractions from the constant specific activity region (specific activity about 31) of the enzyme elution profile are pooled and concentrated to at least 5 mg/ml in the Amicon membrane concentrator.

The enzyme, at this point, is greater than 90% pure. This degree of purity was considered adequate for all subsequent experiments except the dry weight determination, the sedimentation equilibrium molecular weight determination and the uv difference spectrum. It is found that if the concentrated enzyme is sterilized by passage thorugh a Millipore filter (0.22 μ) and maintained at a concentration of 5 mg/ml or greater, it remains fully active for a month or more. The enzyme can be recycled on G-200 to remove the 10% impurity if a greater degree of purity is desired.

Disc electrophoresis was run as described in Ornstein and Davis (1964). The gels were pretreated with thioglycolic acid (Brewer, 1967).

Sedimentation Studies. Sedimentation velocity experiments were carried out in a Spinco Model E ultracentrifuge. The enzyme was exchanged into the appropriate buffer by passing the protein through a Sephadex G-25 column (1.1 × 20 cm) preequilibrated with that buffer. Aliquots of 1.0 ml were collected and the protein concentration was determined by measuring the absorbance at 280 nm. The first tube of the elution was used as the blank. The tube which contained the highest concentration of protein was used for the sedimentation.

A 30-mm double-sector centerpiece cell was filled with 0.9 ml of the protein. In concentration-dependence studies, an aliquot of protein was removed after each run, and replaced with the same volume of buffer. The experiments were performed in a temperature range of 18–22° and the temperature was regulated during each run. The sedimentation coefficient was corrected for viscosity and density values. The centrifugation was run at either 48,000 or 52,000 rpm.

Schlieren optics were employed and the distance of migration at each time was determined from developed photographic plates. The bar angle was varied from 45 to 70° depending on the concentration of the protein.

For sedimentation equilibrium experiments, the protein was passed into the appropriate buffer by passage through Sephadex G-25, as before.

In most experiments, the Yphantis meniscus depletion method was used (Yphantis, 1964). The protein was placed in a three-channel Yphantis centerpiece and the rotor was spun at about 14,000 rpm until there was no further change in the base line. The protein concentration was measured by the absorbance at 280 nm as recorded by the scanner with a slow photomultiplier speed and a slit width of 2.0 mm.

The partial specific volume ($\bar{v}$) was determined by sedimentation equilibrium in 91% D_2O according to Edelstein and Schachman (1967). Dialyzed protein in GL buffer and 0.1 M KCl was diluted 1:10 with GL buffer plus 0.1 M KCl in D_2O or H_2O. The samples were sedimented in a three-channel double-sector cell at 14,000 rpm at 16.1° until equilibrium was obtained. The base line was determined by overspeeding at 30,000 rpm for 16 hr.

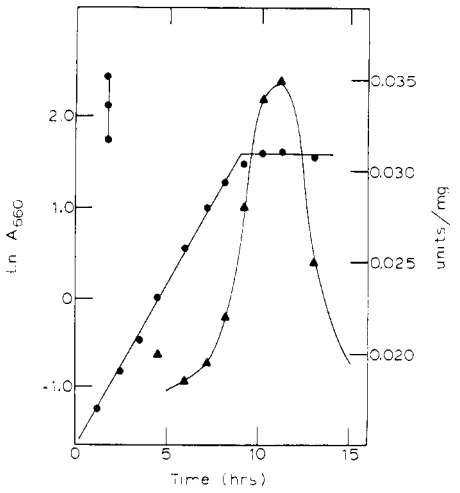

FIGURE 1: Variation on AK III activity during growth. *E. coli* Tir-8 was grown as described in Methods. At time intervals, 200-ml aliquots were removed and the bacteria were isolated. AK III activity was measured in the crude supernatant and the protein was estimated by A_{280}/A_{260}. The growth curve is shown in ln A_{660} *vs.* time (●). The AK III activity is given as units per milligram of protein (▲).

The subunit molecular weight was determined by dissociation of the enzyme in 6 M Ultra Pure guanidine hydrochloride (Mann Chemical Co.) and 0.1 M mercaptoethanol according to Kawahara and Tanford (1966). To 0.5 ml of a protein solution of 5 mg/ml was added enough guanidine hydrochloride and mercaptoethanol to bring the solutions to a final concentration of 6 and 0.1 M, respectively. The enzyme was allowed to dissociate for 72 hr at room temperature. Mercaptoethanol was removed and replaced with 1 mM bromoacetic acid by passage through a Sephadex G-25 column (1.1 × 20 cm) preequilibrated with 6 M guanidine hydrochloride and 1 mM bromoacetic acid. This treatment carboxymethylates exposed sulfhydryl groups and prevents their air oxidation. The protein concentration was determined by the absorbancy at 280 nm using buffer eluted before the protein as a blank.

The sample was centrifuged in a 12-mm double-sector cell at 15.7°. Ultraviolet optics were employed and the sample was centrifuged at 30,000 rpm according to the meniscus depletion technique (Yphantis, 1964). The sample was sedimented for 72 hr, at which time equilibrium was established. The base line was checked by sedimenting at 40,000 rpm for 16 hr.

Electrophoresis. The subunit molecular weight of aspartylkinase III was also determined by electrophoresis in sodium dodecyl sulfate according to the modification by Weber and Osborn (1969) of the method of Shapiro *et al.* (1967).

Analytical Gel Chromatography. A Sephadex G-200 column (1.1 × 40 cm) was prepared according to standard procedures. The following standard proteins were chosen because of their molecular weights and their ease of assay: pyruvate kinase (Sigma), alcohol dehydrogenase (Sigma), cytochrome *c* (Sigma), enolase (Thomas Shen).

The sample, in a volume of 0.50 ml, contained: 200 μg of pyruvate kinase, 50 μg of enolase, 50 μg of alcohol dehydrogenase, 1.0 mg of cytochrome *c*, 50 μg of AK III, and 50 μg of blue dextran. The column was eluted at 10 ml/hr and 1.0-ml fractions were collected. Pyruvate kinase activity was measured by coupling the reaction to lactic dehydrogenase. Alcohol dehydrogenase was assayed by the method of Vallee and Hoch (1955). Enolase activity was measured according to Westhead and McLain (1964). Cytochrome *c* was assayed by its visible absorbance at 415 nm.

Ultraviolet Spectrum. The ultraviolet spectrum of the enzyme was measured on either a Cary 14 or Perkin-Elmer 356 recording spectrophotometer.

The enzyme was prepared for analysis by passing 0.8 ml of enzyme solution through a Sephadex G-25 column (1.1 × 20 cm) equilibrated with the appropriate buffer. Fractions of 1 ml were collected and the enzyme concentration was determined by the protein absorbancy at 280 nm. The first tube of the elution was used as a buffer blank.

Ultraviolet Difference Spectrum. Lysine was removed from the enzyme by passage of the enzyme through a Sephadex G-25 column (1.1 × 20 cm) preequilibrated with GL buffer lacking lysine. The protein (2 mg/ml) was scanned in a Perkin-Elmer 356 spectrophotometer. A protein solution was placed in both the sample and the reference cell and lysine was added to the sample cell to a final concentration of 10 mM. A difference peak was noted at 294 nm. The conformation of AK III was determined by titrating the enzyme with lysine and observing the difference spectrum at 294 nm in a Zeiss PMQ II spectrophotometer.

Results

Bacterial Growth. In the preliminary studies, AK III was isolated from *E. coli* $K_{12}m_2$. After the isolation of Tir-8, a mutant of K_{12} and an overproducer for AK I (Janin and Cohen, 1969; Szentirmai *et al.*, 1968), we routinely used the new strain. The two strains of *E. coli* grow to the same A_{660} when grown under the same conditions, and pure AK III from both strains behaves in a similar manner kinetically. AK III from both strains gives a peak in activity upon reaching the end of log-phase growth in minimal salts–glucose medium.

Growth vs. Specific Activity. Figure 1 depicts the growth of *E. coli* Tir-8 on 1% glucose minimal media supplemented with 5 mM DL-methionine. AK III activity is seen to increase faster than the increase in total protein during log phase which may be due to a gradual derepression of AK III synthesis caused by the depletion of the lysine pools. After the onset of stationary phase the AK III activity declines sharply.

This decline occurs much faster than the average rate of protein degradation, estimated for starved *E. coli*, of 2–5%/hr by Willetts (1967). Since the rate of loss of AK III activity is faster than the rate of random protein degradation, the decrease in AK III activity is not due simply to the repression of AK III synthesis and the random degradation of *E. coli* protein during stationary phase.

The phenomenon shown in Figure 1 is highly reproducible. The specific activity of AK III in stationary-phase cells is from 2- to 6-fold lower than in late-log-phase cells.

Enzyme Purification. Table I is a summary of the purification of the particular sample used for much of the work in this paper, and for the extensive repurification of a radioactive derivative in the following paper. The absolute specific activity at each stage is subject to error because the enzyme is activated by NH_4^+, even above the K^+ concentration in the cuvet, and because oxidation of DPNH interferes in the pre-DEAE stages. The DEAE column yields consistently higher total activity than the ammonium sulfate fraction, possibly because the background activity is eliminated at this step.

The separation of the threonine and lysine sensitive aspartylkinases (AK I and III, respectively) takes place on the DEAE-

TABLE I: Purification of Aspartylkinase III from *E. coli* TIR-8.

Step	Units	Protein (mg)	Sp Act. (Units/mg)	Purifcn Factor
Crude extract	6993	36,288	0.19	1
Streptomycin sulfate	6155	9,906	0.62	3.3
25–45% ammonium sulfate	6324	6,680	0.95	5.0
DEAE-Sephadex	6650	518	12.8	68
Hydroxylapatite	3766	293	12.8	68
Sephadex G-200[a]	2540	81	31.5	166

[a] The rest of the enzyme can be recovered by concentrating and recycling the side fractions from the activity peak.

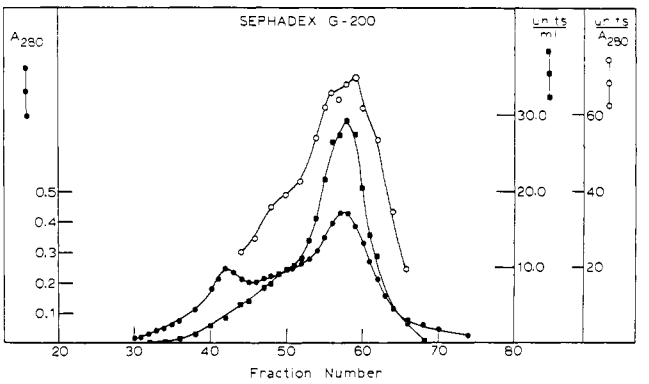

FIGURE 2: AK III was eluted from a G-200 Sephadex column (100 × 2.5 cm) by upward flow. Fractions of 5 ml were collected at 0.5 ml/min. (○) Units/A_{280}, (●) A_{280}, and (■) AK III activity (units/ml).

Sephadex column. AK I elutes at about 0.24 M KCl and AK III at 0.35 M KCl.

In this particular preparation no purification was achieved on hydroxylapatite. More typical preparations show a specific activity (units per milligram) of about 5 after DEAE and 25 or higher after hydroxylapatite. Overall recovery after Sephadex G-200 varies between 30 and 50%.

AK III was shown to be pure after Sephadex G-200 chromatography by disc electrophoresis at pH 8.3, sodium dodecyl sulfate electrophoresis, and sedimentation velocity.

Subunit Molecular Weight. Because there is such a variety of inhibitors and activators for this enzyme, a careful study of the subunit composition of the enzyme was undertaken. The possibility that AK III was composed of unequal subunits was investigated by measuring the molecular weight of the subunits of the carboxymethylated enzyme dissociated in 6 M Gdn · HCl or electrophoresed in 0.1% sodium dodecyl sulfate.

Sedimentation in Guanidine Hydrochloride Solution. The molecular weight of the enzyme purified from log-phase cells was determined by equilibrium sedimentation of AK III dissociated in 6 M Gdn · HCl and reacted with 1 mM bromoacetic acid to carboxymethylate the free sulfhydryl groups. The molecular weight, calculated from a plot of ln C *vs.* r^2, is 47,000 ± 3000. The slope is 1.73 and the data are linear over 4 ln units from 48.7 to 51.2 cm^2 indicating that the protein is a single homogeneous polypeptide.

Sodium Dodecyl Sulfate Electrophoresis. The subunit molecular weight of AK III was also determined by electrophoresis of the enzyme dissociated in 1% sodium dodecyl sulfate plus 1 M mercaptoethanol, according to Weber and Osborn (1969). The marker proteins used and their mobilities are: rabbit muscle pyruvate kinase (0.195), aldolase (0.355), lactic dehydrogenase (0.385), and bovine chymotrypsinogen (0.52). The enzyme prepared from both late-log-phase and stationary-phase cells were electrophoresed on several occasions. The results which varied from 47,000 to 52,000 molecular weight are in excellent agreement with that obtained from the sedimentation equilibrium in Gdn · HCl.

From a combination of the Gdn · HCl sedimentation and sodium dodecyl sulfate electrophoresis, a molecular weight for the AK III was estimated to be 48,000 ± 4000.

Native Molecular Weight. The molecular weight of the native enzyme, purified from several batches of cells grown to either late-log phase or to stationary phase, has been determined by equilibrium sedimentation, by measurement of the sedimentation coefficient, $s_{20,w}$, and the diffusion constant, $D_{20,w}$, and by analytical gel chromatography. By the three analytical methods, there appears to be two forms of the enzyme: one is a stable dimer of about 100,000 molecular weight; the second is a tetramer of about 200,000 molecular weight which dissociates to the dimer species at low protein concentration.

Sephadex G-200 Chromatography. The elution profile of AK III preparations on a Sephadex G-200 column (2.5 × 100 cm) often showed major shifts in elution volume depending on the growth phase of the cells from which the enzyme was purified. In general, the enzyme eluted as a symmetrical peak with an elution volume between 245 and 270 ml for the late-log-phase enzyme and between 280 and 295 ml for the stationary-phase enzyme. The void volumes for the columns were constant at about 175 ml. On occasion a peculiar pattern of elution developed. Figure 2 shows one such peculiar pattern of AK III activity purified from late-log-phase cells. It can be noted that there are 2 components with AK III activity sensitive to lysine. There is a difference of 40 ml in the elution volume between the two forms with a void volume of 175 ml. This corresponds to about a doubling of the apparent molecular weight of AK III.

Partial Specific Volume Determination. The partial specific volume of AK III was measured by the simultaneous equilibrium sedimentation of AK III in D_2O and H_2O. From a plot of ln C *vs.* r^2 both the $\bar{v}$ and the molecular weight can be calculated (Edelstein and Schachman, 1967). The data were as follows: H_2O, the slope is 1.02 and all data are linear from 42 to 44.25 cm^2, ln − 1.5 to ln 1.0; D_2O the slope is 0.83 and the data are linear from 35.5 to 38 cm^2, ln −1.2 to ln 1.0. The $\bar{v}$ is calculated to be 0.746 cm^3/g and this value was used in all calculations. This can be compared to that calculated from the amino acid composition, 0.735 cm^3/g, by Lafuma *et al.* (1970).

The enzyme sedimented as a single species of 96,000 molecular weight, indicating that the enzyme used in this experiment was in a dimeric form.

Sedimentation Equilibrium. AK III was sedimented to equilibrium under a variety of speeds, temperatures and buffer conditions. The Yphantis meniscus depletion technique (Yphantis, 1964) was generally employed but on occasion, low-speed equilibrium sedimentation was used. In all cases, the enzyme was sedimented in GL buffer plus 0.1 M KCl.

A summary of the data generated states that the stationary-phase enzyme sedimented as a stable dimer of mol wt 88,000–

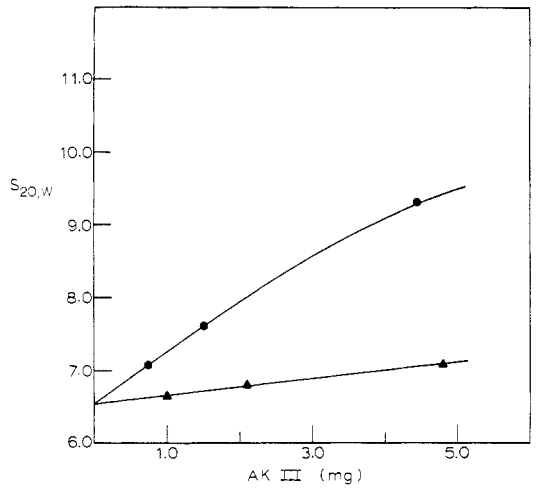

FIGURE 3: The protein concentration dependence of the $s_{20,w}$ of AK III is shown. (●) Mid-log-phase enzyme (Table II); (▲) stationary-phase enzyme (Table II). The samples were sedimented at 20°, 52,000 rpm in GL buffer plus 0.1 M KCl. All plots of the logarithm of the distance sedimented *vs.* time were linear over the complete range of sedimentation.

106,000. The late-log-phase enzyme results varied from 88,000 to 210,000 molecular weight. The protein concentration in all cases was between 0.8 and 1.8 mg per ml and the enzyme data were linear over at least 3 ln units and 2 r^2 units.

Analytical Gel Chromatography. The apparent molecular weight of AK III was determined by passage of the enzyme over a G-200 Sephadex column with standard protein markers. As with the other molecular weight determinations, there was a 2-fold variance in the results.

The apparent molecular weight varied from 115,000 to 210,000 with enzyme isolated from different preparations of *E. coli*.

The effect of the monovalent cation activators, NH_4^+ and K^+ (Wampler and Westhead, 1968; Niles, 1972), on the quaternary structure of AK III was tested by passage of AK III, isolated from late-log cells, through Sephadex G-200 pre-equilibrated with GL buffer plus either 0.2 M KCl or 20 mM NH_4Cl. The standard markers used were pyruvate kinase, alcohol dehydrogenase, enolase, and cytochrome *c*. In the absence of additional monovalent salt, AK III displayed a molecular weight of 150,000. In the presence of 20 mM NH_4Cl or 0.2 M KCl AK III eluted as a protein of 188,000 or 210,000, respectively.

In the parentheses following each enzyme the v_e/v_0 calculated for each protein eluted in GL buffer, GL buffer plus 20 mM NH_4Cl, or GL buffer plus 0.2 M KCl. The data are as follows: pyruvate kinase (1.43, 1.43, 1.43), alcohol dehydrogenase (1.57, 1.57, 1.57), enolase (1.86, 1.79, 1.79), cytochrome *c* (2.50, 2.43, 2.43), and AK III (1.57, 1.50, 1.46).

In the presence of either 20 mM NH_4Cl or 0.2 M KCl the enzyme elutes as higher molecular weight species, indicating that the monovalent cation activators stabilize the association of dimers. It is not yet known, however, if this stabilization is responsible for the kinetic activation of AK III by K^+ and NH_4^+ (Wampler and Westhead, 1968).

All of our sedimentation experiments were carried out in 0.1 M KCl, and both 100,000 and 200,000 molecular weight species were seen in different preparations. Therefore, it is apparent that this monovalent cation ion effect is not the only factor which stabilizes the high molecular weight form of the enzyme.

TABLE II: Comparison of the Properties of Mid-Log-Phase AK III and Late-Stationary-Phase AK III.

Parameter	Mid-Log Phase	Stationary Phase
A_{280}/A_{260}	1.6	1.2
$D_{20,w}$	7.02×10^7	8.3×10^7
$s_{20,w}$ (S)	9.3–6.6	7.0–6.6
Molecular weight[a]	144,000	94,000
G-200 (v_e/v_0)	1.47	1.65
Specific activity	27.0	21.0

[a] Calculated from the sedimentation and diffusion constants at a concentration of 4.8 mg/ml.

Diffusion Constant. The $D_{20,w}$ was measured on two samples of AK III with different $s_{20,w}$ values. The $D_{20,w}$ measured with a laser light-scattering photometer, as described by Ford *et al.* (1972), are presented in Table II. From the $D_{20,w}$ and $s_{20,w}$ values for each enzyme sample, molecular weights of 144,000 and 94,000 can be calculated for the mid-log-phase enzyme and late-stationary-phase enzyme, respectively.

Sedimentation Velocity. Figure 3 shows the dependence on protein concentration of $s_{20,w}$ for two AK III preparations, the mid-log-phase and late-stationary-phase samples of Table II. Both samples show an increase in $s_{20,w}$ with increasing protein concentration but the concentration dependence for mid-log-phase enzyme is much greater than for the other sample. This change in $s_{20,w}$ with protein concentration indicates an association–dissociation equilibrium between the dimeric and tetrameric forms of the enzyme.

Ultraviolet Spectrum. The uv spectrum of AK III was determined for a number of pure preparations of AK III and appreciable variation was found in the absorbtion below 280 nm. The spectra of pure AK III isolated from mid-log-phase cells and late-stationary-phase cells, where the AK III activities are high and low, respectively, are presented in Figure 4. The spectrum of the late-stationary-phase enzyme was normalized to the spectrum of the mid-log-phase enzyme by the ratio of the absorbance of the stationary-phase enzyme to the mid-log-phase enzyme above 289 nm. There appears to be a modification of stationary-phase AK III which increases the absorbance of the enzyme below 280 nm. The shape of the spectrum above 280 nm, including the shoulder at 289 nm, remains the same.

Comparison of Enzyme Prepared from Mid-Log-Phase and Late-Stationary-Phase Cultures. The data in Table II compare properties of two preparations of the enzyme from different phases of growth. Both preparations were homogeneous upon gel electrophoresis and sedimentation velocity centrifugation. It appears that the difference in ultraviolet absorption spectrum noted above may be related to the changes in other physical properties shown in Table II.

There is an apparent decrease in the size of AK III in the enzyme isolated from stationary-phase cells with a slight change in specific activity. This decrease in size is consistent with an increase in the $D_{20,w}$, decrease in the $s_{20,w}$ and molecular weight and an increase in the elution volume from G-200. Concomitant with this decrease in size is an increase in the absorption at 260 nm.

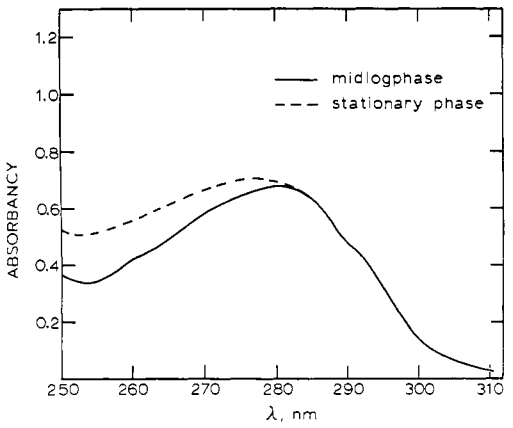

FIGURE 4: Comparison of two uv spectra of AK III purified from either mid-log-phase cells or late-stationary-phase cells. The protein was dialyzed in GL buffer and the spectra were taken at 24°. (——) Mid-log-phase cells; (– – – –) late-stationary-phase cells.

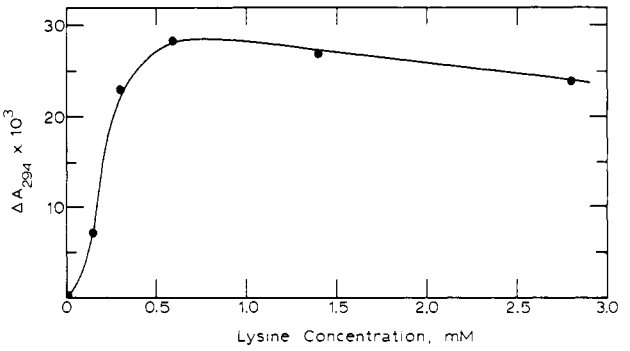

FIGURE 5: Absorption difference due to lysine. The difference in absorption at 294 nm is obtained by the addition of lysine to a protein sample (2 mg/ml) and compared to a lysine-free protein sample (2 mg/ml). The average of ten readings was taken at each concentration of lysine. The enzyme was in GL buffer lacking lysine and the absorbancies were observed at room temperature, 23°.

A kinetic study was undertaken to compare the mid-log-phase and late-stationary-phase species of AK III. Only slight differences were noted in monovalent and divalent cation activation and a 2-fold decrease in the lysine K_I for the stationary-phase AK III. The apparent K_M values for aspartate remained constant. These studies were by no means complete, and the fact that no large kinetic differences was found does not eliminate the possibility that a kinetic difference exists, under conditions not yet explored.

Lysine Difference Spectrum. A difference spectrum, obtained by the addition of 10 mM lysine to a protein sample (2 mg/ml), was measured on a Perkin-Elmer 356 spectrophotometer. To the reference protein solution was added the same volume of buffer. A slight peak was apparent at 294 nm.

This indication of a conformational change in the enzyme was examined in more detail by titrating the enzyme with lysine. With equal volumes of enzyme in sample and reference cuvets, small volumes of lysine solution were added to the sample cuvet and an equal volume of buffer to the reference cuvet. Figure 5 shows that an apparent conformational change is induced cooperatively by lysine, with a half-maximal change at 0.2 mM lysine. This is close to the value for the K_I of lysine, 0.3 mM.

Discussion

Growth Dependence of AK III. In Figure 1, it can be seen that the specific activity of AK III in crude extracts increases during log-phase growth. This may be due to a gradual derepression of the enzyme's synthesis or to an activation of the enzyme subsequent to its synthesis. However, at the onset of stationary phase due to glucose limitation, AK III activity declines rapidly. Since the decline of AK III activity is much more rapid than the turnover in total protein (Willetts, 1967), AK III activity appears to be selectively eliminated. A decrease in activity is not seen for AK I from *E. coli* K_{12} (Fang, 1969) nor for AK III from *E. coli* Tir-8 grown on limiting nitrogen or on glycerol as the carbon source (E. M. Tecson, manuscript in preparation). It appears that this elimination of AK III is linked specifically to glucose limitation and not to either carbon or nitrogen limitation.

It has been noted by Pastan and Perlman (1970) that there is an increase in the levels of certain catabolic enzymes related to carbohydrate metabolism, *i.e.*, α-galactosidase, galactokinase, glycerolkinase, and amino acid metabolizing enzymes, *i.e.*, D-serine deaminase and tryptophase, at the onset of glucose starvation. The induction of these enzymes has been related to the sudden increase in cellular cyclic AMP concentrations at that point in growth. The possible relationship between the decrease of AK III and elevation of cyclic AMP levels is being studied at the present time.

The activity of aspartylkinase from other bacterial strains also shows a peak in activity at the onset of stationary phase. Aspartylkinase from *Bacillus licheniformis* (Gray and Bernlohr, 1969) and *Pseudomonas putida* (Robert-Gero *et al.*, 1970) is inactivated at the end of log-phase growth. *Bacillus subtilis* has two AK activities; one, sensitive to *meso*-diaminopimelic acid, is synthesized throughout the growth cycle; the other enzyme, sensitive to lysine and threonine, is inactivated at the end of log-phase growth (Rosner and Paulus, 1971). *B. subtilis* and *B. licheniformis* need aspartylkinase activity in order to produce dipicolinic acid, a necessary constituent of their spore coat.

Physical Properties. Aspartylkinase III, as isolated, has been shown to be composed of similar subunits of mol wt 48,000 ± 4000 as determined by sedimentation equilibrium in guanidine hydrochloride and by electrophoresis in sodium dodecyl sulfate. The native enzyme can be isolated in two forms. One specie, isolated from stationary-phase cells, shows only a slight tendency to form tetramers. The other form, purified from log-phase cells, readily forms tetramers at higher protein concentrations, as shown by sedimentation velocity (Figure 4). The formation of tetramers is influenced by the ionic environment. Enzyme activators NH_4^+ and K^+ increase the average molecular weight to about 200,000.

von Dippe *et al.* (1972) has recently shown that the AK III from *E. coli* B also undergoes a dimer tetramer transition and that the presence of lysine favors the tetrameric form. This finding is of interest in light of the fact that lysine causes a uv spectral shift in AK III (Figure 5). This shift might be due to a dimerization of AK III rather than a cooperative transition. This fact is still in doubt since all of the sedimentation experiments and the Sephadex G-200 chromatography were carried out with 1.0 mM lysine which is five times the *s* of 0.5 for the uv shift shown in figure 5. If the uv shift seen in Figure 5 were due simply to formation of tetramers, then the tetramer should have been the predominant specie in the sedimentation and chromatography experiments.

Truffa-Bachi and Cohen (1966) previously reported a molecular weight for this enzyme of about 100,000 as determined by Sephadex G-200 chromatography. Lafuma *et al.* (1970) reported a molecular weight, determined by high-speed sedimentation equilibrium, of 133,000 and an $s_{20,w}$ of 6.9 S. The report of Truffa-Bachi and Cohen (1966) is in good agreement with the dimer species reported here. Lafuma *et al.* (1970) apparently calculated the molecular weight only over a distance of $0.6r^2$ units. It is apparent from the results reported here that their enzyme sample was not a single homogeneous protein species and the value of 133,000 is the result of equilibrium between the tetramer and dimer forms.

The enzyme also exhibits a variable uv spectrum (Figure 4), which in the accompanying publication (Niles and Westhead, 1973) is shown to be due to the presence of a covalently bound 260-nm-absorbing moiety. It is certain that this 260-nm-absorbing species is covalently bound, since the enzyme, denatured in guanidine hydrochloride and carboxymethylated with bromoacetic acid, maintains the same high 260-nm absorption as the native enzyme.

The data in this paper show that the activity of this enzyme varies in a striking manner during the growth of cells on glucose. They further show that enzyme purified from different stages of exponential growth differs in both average molecular weight and in its content of a 260-nm-absorbing moiety. No substantial kinetic differences between preparations of pure enzyme have been found. Kinetic data are obtained at such low enzyme concentrations, however, that all enzyme samples would be expected to be in the lower molecular weight form. Kinetic differences at *in vivo* enzyme concentrations, where the dimer–tetramer equilibrium might be more important, would not necessarily be observed at normal assay concentrations.

Correlations among stage of growth, uv spectra and molecular weight are only suggestive at this point. In a number of preparations of the protein we have found variation in comparing the 280 nm:260 nm absorption ratio to the apparent molecular weight. We have not found growth curves to be reproducible enough to make any fine correlation between stage of growth and molecular properties. Only gross differences, such as shown in this paper, have been regularly reproducible. We are currently determining growth conditions that will lead to reproducible forms of the enzyme in continuous culture.

Acknowledgment

We thank Mrs. Nancy MacLeod for valuable help with this work.

References

Biswas, D. K., Mazumder, R., and Biswas, C. (1968), *J. Biol. Chem. 243*, 3655.
Brewer, J. M. (1967), *Science 156*, 256.
Clark, R. B., and Ogilivie, S. (1972), *Biochemistry 11*, 1278.
Cohen, G. N. (1969), *Curr. Top. Cell. Regul. 1*, 184.
Davis, B. D., and Mingioli, E. (1950), *J. Bacteriol. 60*, 17.
Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242*, 306.
Falcoz-Kelly, F., van Rapenbusch, R., and Cohen, G. N. (1969), *Eur. J. Biochem. 8*, 146.
Fang, T., S.-J. (1969), Masters Thesis, University of Massachusetts, Amherst.
Ford, N. C., Gabler, F. R., and Karasz, F. E. (1972), *Advan. Chem.* (in press).
Gray, B. H., and Bernlohr, R. W. (1969), *Biochim. Biophys. Acta 178*, 248.
Janin, J., and Cohen, G. N. (1969), *Eur. J. Biochem. 11*, 320.
Kawahara, K., and Tanford, C. (1966), *J. Biol. Chem. 241*, 3228.
Lafuma, C., Gros, C., and Patte, J.-C. (1970), *Eur. J. Biochem. 15*, 111.
Lowry, H. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Niles, E. G. (1972), Ph.D. Thesis, University of Massachusetts, available from University Microfilms, Ann Arbor, Mich.
Niles, E. G., and Westhead, E. W. (1970), *Fed. Proc. Fed. Amer. Soc. Exp. Biol. 28*, 912 Abstr.
Niles, E. G., and Westhead, E. W. (1973), *Biochemistry 12*, 1723.
Ornstein, L., and Davis, B. J. (1964), Academic Printer, Distillation Products Industries (Division of Eastman Kodak Company).
Pastan, I., and Perlman, R. (1970), *Science 169*, 339.
Patte, J.-C., and Cohen, G. N. (1965), *C. R. Acad. Sci., Ser. A, 259*, 1255.
Patte, J.-C., LeBras, G., and Cohen, G. N. (1967), *Biochim. Biophys. Acta. 136*, 245.
Robert-Gero, M., Poiret, M., and Cohen, G. N. (1970), *Biochim. Biophys. Acta 206*, 17.
Rosner, A., and Paulus, H. (1971), *J. Biol. Chem. 246*, 2965.
Shapiro, A. L., Viñuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Stadtman, E. R., Cohen, G. N., LeBras, G., and de Robichon-Szulmajster, J. (1961), *J. Biol. Chem. 236*, 2033.
Starnes, W. L., Munk, P., Maul, S. B., Cunningham, G. N., Cox, D. J., and Shive, W. (1972), *Biochemistry 11*, 677.
Szentirmai, A., Szentirmai, M., Umbarger, H. E. (1968), *J. Bacteriol. 95*, 1672.
Truffa-Bachi, P., and Cohen, G. N. (1966), *Biochim. Biophys. Acta 113*, 531.
Truffa-Bachi, P., van Rapenbusch, R., and Cohen, G. N. (1968), *Eur. J. Biochem. 5*, 83.
Truffa-Bachi, P., van Rapenbusch, R., Janin, J., Gros, C., and Cohen, G. N. (1969), *Eur. J. Biochem. 7*, 401.
Vallee, B. L., and Hoch, F. L. (1955), *Proc. Nat. Acad. Sci. U. S. 41*, 327.
von Dippe, P. J., Abraham, A., Nelson, C. A., Smith, W. G. (1972), *J. Biol. Chem. 247*, 2433.
Wampler, D. E., and Westhead, E. W. (1968), *Biochemistry 7*, 1661.
Warburg, O., and Christian, W. (1942), *Biokhimiya 310*, 384.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Westhead, E. W., and McLain, G. (1964), *J. Biol. Chem. 239*, 2464.
Willetts, N. S. (1967), *J. Biochem. 103*, 453.
Yphantis, D. (1964), *Biochemistry 3*, 297.