Amino Acid Sequence of *Escherichia coli* Alkaline Phosphatase. Amino- and Carboxyl-Terminal Sequences and Variations between Two Isozymes†

Philip M. Kelley, Peter A. Neumann, Karen Shriefer, Fiorella Cancedda, Milton J. Schlesinger, and Ralph A. Bradshaw*‡

ABSTRACT: The amino- and carboxyl-terminal fragments of *E. coli* alkaline phosphatase, produced by cyanogen bromide cleavage, have been isolated. The carboxyl-terminal fragment, CB-VI, contains seven amino acid residues in the sequence Lys-Ala-Ala-Leu-Gly-Leu-Lys. The absence of homoserine and the coincidence of carboxyl-terminal lysine in native alkaline phosphatase suggest the carboxyl-terminal assignment. The amino-terminal fragment, CB-VII, contains five and four residues when isolated from isozymes 1 and 3, respectively. CB-VII from isozyme 1 possesses the sequence Arg-Thr-Pro-Glu-HSe. The corresponding peptide from isozyme 3 is devoid of the argininyl residue. Amino-terminal analyses of native alkaline phosphatase suggest the amino-terminal alignment of CB-VII. Analyses for carbohydrate indicate both isozymes are devoid of those sugars commonly found in glycoproteins.

The initial identification of alkaline phosphatase in *E. coli* in 1959 (Horiuchi *et al.*, 1959; Torriani, 1960; Garen and Levinthal, 1960) was followed by a variety of studies that have produced considerable information about genetic control of the protein (Echols *et al.*, 1961), its formation and localization (Malamy and Horecker, 1961; Neu and Heppel, 1964; Schlesinger *et al.*, 1969), its chemical and physical properties (Rothman and Byrne, 1963; Plocke *et al.*, 1962; Schlesinger and Barrett, 1965; Reynolds and Schlesinger, 1967; Applebury and Coleman, 1969; Lazdunski *et al.*, 1969; Csopak, 1969), and its enzymatic mechanism (Schwartz, 1963; Trentham and Gutfreund, 1968; Halford *et al.*, 1969; Halford, 1971; Reid and Wilson, 1971; Lazdunski *et al.*, 1971). However, only meager amounts of data exist on the primary and three-dimensional structure of this protein. Consequently, the determination of the complete amino acid sequence of *E. coli* alkaline phosphatase, whose subunit contains about 410 amino acids, was initiated. At about the same time, a group at Yale University obtained protein crystals suitable for X-ray analysis (Hanson *et al.*, 1970), and an investigation of the crystallographic structure of the enzyme has recently provided a structural map to 7.7-Å resolution (Knox and Wyckoff, 1973).

The strategy for determining the primary structure of this large polypeptide was to first cleave the subunit with cyanogen bromide (CNBr) at the seven methionyl residues and separate the resulting polypeptide fragments. Subsequent papers in this series will describe the purification, composition, and amino acid sequences of the larger CNBr fragments. In this initial communication we report the sequence of two small CNBr peptides which comprise the amino- and carboxyl-terminal segments of the alkaline phosphatase subunit.

In the course of this work, the terminal fragments from two different alkaline phosphatase isozymes were also examined. The presence of electrophoretically distinct isozymes in preparations of alkaline phosphatase, which were otherwise homogeneous by many physical criteria, was reported in some of the earliest work with this protein (Levinthal *et al.*, 1962; Signer, 1963). Subsequently, differences in a tryptic peptide between two isozymes were found (Schlesinger and Andersen, 1968), and more recently, data have been presented that showed different amino-terminal residues associated with different isozymes (Piggott *et al.*, 1972). Independently, our sequence studies indicated that one isozyme has an additional arginine residue at the amino-terminal position of the CNBr fragment assigned to the amino end of the enzyme, whereas the carboxyl-terminal fragments are identical. This difference in amino acid sequence between the isozymes is the only one detected thus far, and no significant amounts of carbohydrate residues were found associated with either isozyme. The possible origin of these isozymes is discussed. Portions of this work have been reported in a preliminary communication (Neumann *et al.*, 1971).

Materials and Methods

Purification of Isozymes. E. coli alkaline phosphatase isozyme 3 (see Figure 1 for nomenclature) was purified from cells (strain ATCC 27257) grown in a 100-l. Fermacel (New Brunswick) with the following media: 0.12 M Tris-Cl (pH 7.4), 0.2% glucose, 0.4 mM $MgSO_4$, 10 mM NaCl, 2 mM KCl, 10 mM NH_4Cl, 0.13 mM P_i, supplied as pepticase (Sheffield Scientific Company), 0.02 mM $ZnSO_4$, 0.15 mM thiamine hydrochloride, 0.13 mM L-methionine. Isozyme 1 was purified from a culture (ATCC 27257) grown in flasks containing the same media except that, in one case, no $ZnSO_4$ was added, and in another preparation, L-arginine (1.0 mM) was present.

The procedure for obtaining pure protein has been published in detail (Schlesinger and Olsen, 1970). The above culture conditions provide essentially pure isozymes 1 and 3, but we enriched our preparations further by eliminating those fractions from the DEAE column chromatography step which were not electrophoretically homogeneous. A final purification step has been added to the published procedures, namely,

† From the Departments of Microbiology (P. M. K., K. S., F. C., and M. J. S.) and Biological Chemistry (P. A. N. and R. A. B.), Washington University School of Medicine, St. Louis, Missouri 63110. *Received May 4, 1973.* Supported by research grants from the National Institutes of Health (AI-08593 and AM 13362) and the Life Insurance Medical Research Fund.

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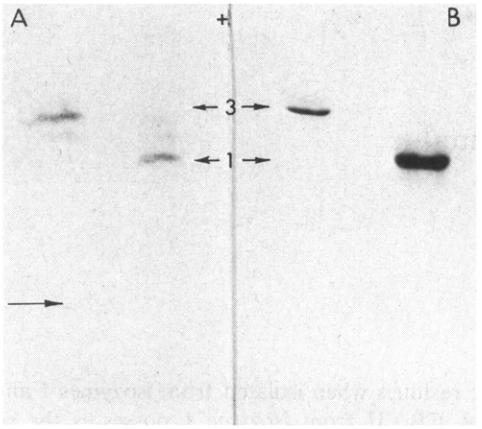

FIGURE 1: Isozyme patterns of purified preparations of *E. coli* alkaline phosphatase separated by starch gel electrophoresis. A Turner 310 starch-gel apparatus was used, and details of the procedure for electrophoresis and staining of samples have been published (Schlesinger and Andersen, 1968). Panel A: samples (2 μl) containing 50 μg/ml of enzyme were applied and subsequently stained for enzymatic activity; panel B: samples (2 μl) containing 2 mg/ml of enzyme were applied and subsequently stained with 0.5% Napthol Blue Black (Allied Chemical) in 7.5% acetic acid. Numbers refer to isozymes analyzed in this paper. Lower arrow was site of application of samples.

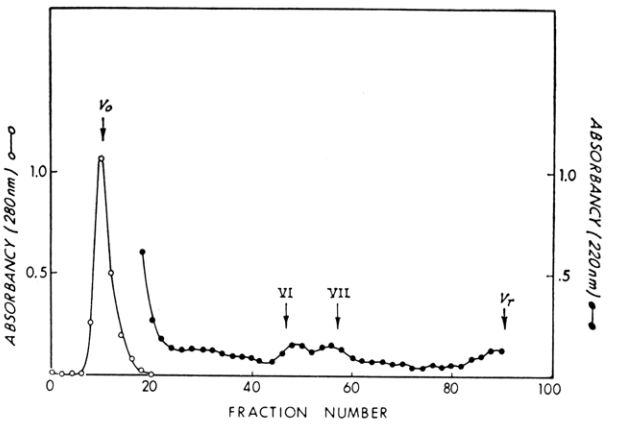

FIGURE 2: Separation of small CNBr fragments on Sephadex G-25. Refer to text for experimental details.

passage of the enzyme in 0.1 M Tris-Cl, pH 7.4, 1 mM $MgCl_2$, and 0.1 mM $ZnSO_4$ over a G-100 Sephadex column (d = 5.0 cm; h = 86 cm). Enzyme was stored as a lyophilized powder after dialysis against distilled water.

Preparation of CNBr Fragments. Equal amounts, on a weight basis, of protein and CNBr were reacted in 15 ml of 70% formic acid at room temperature in the dark for 24 hr. The reaction was stopped by the addition of a tenfold volume excess of distilled water and the solution lyophilized. It was redissolved in 20 ml of water, lyophilized again, and dissolved in 2 ml of 0.1 M NH_4HCO_3. Separation of the CNBr fragments was achieved by gel filtration on columns of Sephadex eluted with 0.1 M NH_4HCO_3. The amino- and carboxyl-terminal CNBr peptides were readily isolated from the other fragments on a G-25 Sephadex column (d = 2.5 cm, h = 80 cm) with a flow rate of 20 ml/hr (Figure 2).[1]

Sequence Analyses. Edman degradations were used for sequential degradation of the isolated fragment according to methods previously described (Bradshaw *et al.*, 1969). The amino acids removed were identified in the form of the corresponding dansyl derivative (Angeletti *et al.*, 1973), or by differences from aliquots hydrolyzed in 6 N HCl and analyzed.

Carboxypeptidase Hydrolyses. Degradation of the fragments from the carboxyl terminus was performed with carboxypeptidases A and B (Worthington Biochemical Co.). Samples were incubated for the specified times with 5-μl additions of enzyme stock solutions or suspensions at 25°. Samples (usually 0.1 ml) were removed and the reaction was terminated by the addition of sodium citrate buffer (pH 2.2, amino acid analyzer diluter buffer). Samples were analyzed directly without further treatment. Blank reaction mixtures that contained enzyme but no peptide were incubated in parallel and analyzed in the same manner.

Cyanate End-Group Analysis. Amino-terminal analyses of the isozymes were performed by the cyanate method of Stark and Smyth (1963). Blank samples, containing no protein, and protein samples not exposed to urea or cyanate were analyzed in parallel.

Amino Acid Analyses. Samples were hydrolyzed with 6 N HCl under reduced pressure at 110° for 24 hr. They were dried and analyzed on a Spinco 120C amino acid analyzer according to established procedures (Moore and Stein, 1963). Homoserine was usually not converted to the free acid and was estimated only qualitatively.

Results

Identification of Amino- and Carboxyl-Terminal CNBr Fragments of Isozyme 3. All of the sequence studies to be reported, unless otherwise specified, have been carried out on alkaline phosphatase isozyme 3. Treatment of this protein with CNBr results in the formation of five large fragments and three small ones. The latter are easily separated from the longer polypeptides by gel chromatography on Sephadex G-25 (Figure 2).[1] The smallest fragment CB-VIII[2] is not indicated in the figure but it eluted at the total column volume fraction and is a dipeptide, Val-Hse. This fragment has been isolated in variable yields and is located in an interior position of the molecule.[3]

CB-VI was a homogeneous peptide containing seven amino acids and was devoid of homoserine. Its carboxyl-terminal residue, determined from the sequence analysis of the peptide, was lysine. The carboxyl-terminal residue of whole enzyme, determined by carboxypeptidase B hydrolysis, was also lysine (Table I). The yield of CB-VI (0.31 μmol) from one preparation of CNBr fragments was almost equal to that of CB-VII (0.34 μmol) and both represented 60% of the expected yield from a digestion of 0.5 μmol (20 mg) of protein. These results indicate that fragment CB-VI is the carboxyl-terminal segment of the enzyme. The remainder of the sequence of fragment CB-VI was determined by Edman degradation (Table I).

[1] The preferred isolation procedure for obtaining optimal resolution of all the CNBr fragments utilizes columns of Sephadex G-75. In this case the amino- and carboxyl-terminal fragments emerge as a single peak which can be resolved on a column of Dowex 50-X8 (Schlesinger, M. J., Neumann, P. A., and Bradshaw, R. A., manuscript in preparation).

[2] The CNBr fragments of *E. coli* alkaline phosphatase have been designated I–VIII on the basis of their elution from Sephadex G-75. Fragments I–V, designated in order to decreasing molecular weights, range in size from 220 to 42 amino acid residues. Only fragments, VI, VII, and VIII will be discussed in this report.

[3] Neumann, P. A., Bradshaw, R. A., Schlesinger, M. J., and Ericsson, L. H., manuscript in preparation.

TABLE I: Carboxyl-Terminal Sequence of *E. coli* Alkaline Phosphatase.[a]

Alkaline phosphatase (moles/44,000 g):
Carboxypeptidase B: 30 min; Lys, 0.33
60 min; Lys, 0.43.
Carboxypeptidase A (no carboxypeptidase B):
only traces of any amino acids

Fragment CB-VI:
Sequence: Lys-Ala-Ala-Leu-Gly-Leu-Lys

Carboxypeptidase B: 5 min; Lys, 1.00
Carboxypeptidase A + B: 15 min; Lys, 1.00; Leu, 1.12; Gly, 0.16; Ala, 0.22
3 hr; Lys, 1.30; Leu, 2.00; Gly, 0.80; Ala, 1.50

[a] Symbols used: →, Edman degradation; ←, carboxypeptidase hydrolysis.

TABLE II: Amino-Terminal Sequence of *E. coli* Alkaline Phosphatase (Isozyme 3).[a]

Alkaline phosphatase:
Amino-terminal analyses:
Cyanate method (moles/44,000 g):[b]
Asp <0.03
Thr 0.85
Glu 0.32
Gly 0.27
Ala 0.12
Protein sequencer:[c]
Thr-Pro-Glu-Met-Pro-Val-Leu-Glu-

Fragment CB-VII:
Sequence: Thr-Pro-Glu-Hse

High voltage electrophoresis, *pH 6.5:* acidic

Tryptic peptide Tp-1:
Sequence: Thr-Pro-Glx-Met-Pro-Val-Leu-Glx-Asx-Arg

[a] Symbols as in Table I. [b] Method of Stark and Smyth (1963). [c] See footnote 4.

TABLE III: Amino Acid Composition of CNBr Fragments CB-VI and CB-VII from *E. coli* Alkaline Phosphatase Isozymes 1 and 3.

Amino Acid	Isozyme 1 CB-VI	Isozyme 1 CB-VII	Isozyme 3 CB-VI	Isozyme 3 CB-VIII
Lysine	1.96		1.63	
Arginine		0.95		
Threonine		1.00		1.00
Glutamic acid		1.37[a]		1.35[a]
Proline		1.09		0.71
Glycine	1.15		1.02	
Alanine	2.11		2.00	
Leucine	2.00		2.10	
Homoserine (+ lactone)		0.89		(+)

[a] Contains some homoserine.

Fragment CB-VII (Figure 2) consisted of four amino acids one of which was homoserine. Three separate lines of evidence suggest that this fragment is the amino-terminal portion of the alkaline phosphatase isozyme 3. First, amino-terminal analysis of whole enzyme by the cyanate procedure (Stark and Smyth, 1963) showed threonine to be the principal end group which is also the amino-terminal residue of fragment CB-VII. Second, analysis of the whole enzyme in the protein sequencer established that the first four residues are identical in sequence with that established for fragment CB-VII. Third, one of the methionine-containing peptides (Tp-1) isolated, following tryptic digestion of whole denatured enzyme, has an amino-terminal sequence identical with fragment CB-VII. Table II summarizes these data and establishes that fragment CB-VII is the amino-terminal CNBr fragment of isozyme 3. Only one of the other large CNBr fragments (CB-II) has an amino-terminal threonine, but its sequence is distinct from that of fragment CB-VII.[3]

Comparison of Isozymes 1 and 3. Constituents of the growth medium of the bacteria are the most important factors in determining the isozyme pattern of alkaline phosphatase. This was first shown by Signer (1963), confirmed by Schlesinger and Andersen (1968), and more recently reported by Piggot *et al.* (1972). By altering the culture conditions, one can heavily enrich for an isozyme and obtain it as a pure electrophoretic species. We have done this (see Methods and Figure 1) and compared CNBr fragments from two isozymes. Analyses of CNBr fragments CB-VI and CB-VII from isozymes 1 and 3 are presented in Table III. They show an additional arginine residue in fragment CB-VII of isozyme 1 while the compositions of CB-VI are the same from both isozymes. Edman degradation of CB-VII from isozyme 1 showed that the extra arginine occupied the amino-terminal position.

As noted above, CB-VII is the amino-terminal portion of the entire protein; thus, isozyme 1 differs chemically from isozyme 3 by virtue of the extra arginine at its amino-terminal position. This difference between the two isozymes accounts for the charge differences observed in gel electrophoresis (Figure 1). The amino-terminal arginine also agrees with the recent report from Bridgen and Secher (1973) who analyzed a preparation of alkaline phosphatase with the protein sequencer. Their data on the first 20 amino-terminal residues are in complete agreement with our analyses[3] for isozyme 3, except for the amino-terminal position which begins with threonine for this isozyme.

Amino-terminal analyses of native isozyme 1 by the cyanate procedure gave qualitatively similar results. However, two independent analyses gave low recoveries of arginine. It is noteworthy that no threonine was observed in these analyses.

Carbohydrate Analyses of Isozyme 1 and 3. Studies by Signer (1963) with alkaline phosphatase isozymes suggested the possible presence of carbohydrates bound to the protein and we have occasionally found glucosamine in certain preparations of the enzyme. A quantitative analysis of four common sugars in glycoproteins[4] was carried out with pure preparations of isozymes 1 and 3. The data are presented in Table IV and show that neither isozyme has significant amounts of these common sugars.

[4] The carbohydrate analyses were performed by Dr. R. Kornfeld, Department of Medicine, Washington University Medical School.

TABLE IV: Carbohydrate Analyses of *E. coli* Alkaline Phosphatase Isozymes 1 and 3.[a]

Sugar analyzed	Sugar residues/ subunit mol wt = 44,000
Sialic acid	0
N-Acetylglucosamine	0.1
Galactose	0.1
Mannose	0
Fucose[b]	0.3

[a] Preparations of isozymes (0.03 μmol) were dialyzed against distilled water prior to analyses. Procedures for these tests have been published (Kornfeld *et al.*, 1971; Kornfeld and Kornfeld, 1970). The values for both preparations were indistinguishable and are presented as one column. [b] The color test for this sugar is nonspecific and based on absorbancy 427–396 nm. The values of 427-nm absorbancy were abnormally high for the preparations.

Discussion

Genetic studies have established that only a single gene (cistron) exists in the *E. coli* chromosome for determining the structure of the alkaline phosphatase (Garen and Garen, 1963). Thus, changes in primary structure must occur during or after the translational stages of protein synthesis. This is in marked contrast to the situation in higher organisms where subtle modifications in the DNA sequences of redundant genes can lead to microheterogeneity of protein primary structure and can account for isozymic forms.

Four distinct kinds of posttranscriptional events can be proposed to explain the formation of alkaline phosphatase isozymes. One of these is translational ambiguity, which results from infidelity of the protein synthetic machinery involving tRNA, ribosomes, and mRNA. Support for this mechanism comes from recent data of Piggott *et al.* (1972) who showed a correlation between isozyme formation in *E. coli* alkaline phosphatase and the degree of ambiguity caused by mutations in ribosomal proteins.

Another mechanism that could lead to heterogeneity is the nonuniform attachment of carbohydrate to some polypeptides as reported for ribonuclease (Plummer and Hirs, 1964). The data presented show that two alkaline phosphatase isozymes have insignificant amounts of those sugars commonly associated with glycoproteins. Deamidation of asparagine or glutamine residues has also been suggested as the basis for isozymic variations (Glick *et al.*, 1972), and this mechanism may account for some of the alkaline phosphatase isozymes, particularly those occurring during storage at low temperatures (Schlesinger and Andersen, 1966).

The fourth mechanism proposed for producing isozymes consists of limited proteolysis of the polypeptide chain. In *E. coli*, initiation of protein synthesis begins with *N*-formylmethionine yet only one-third to one-half the proteins in an *E. coli* extract have an amino-terminal methionine (Waller, 1963). Cleavage of this methionine certainly occurs for most proteins, leading to other amino-terminal residues. As shown here, proteolysis can account for alkaline phosphatase isozymes. Piggott *et al.* (1972) reported amino-terminal aspartic acid (or asparagine), valine, and threonine for three different alkaline phosphatase isozymes. Two of their isozymes are thus distinct from the ones analyzed here, and this may be the result of using different strains of bacteria grown under different conditions. Bridgen and Secher (1973) failed to find heterogeneity in the amino-terminal position of their preparation of alkaline phosphatase but they did not report the extent of isozymic variations in their enzyme.

Natori and Garen (1970) were among the first to suggest heterogeneity in the amino-terminal residues on the basis of analyses of fragments isolated from amber mutants. The data on tryptic peptides from the fragment (estimated mol wt = 4000: Suzuki and Garen, 1969) do not correspond with the known sequence of 46 amino acids comprising the amino-terminal segment of alkaline phosphatase.[3] It is unlikely that all of their peptide comes from a portion of the enzyme that is cleaved before conversion to the final protein because some of the fragment peptides correspond with lysine-containing peptides obtained from isolated, completed, purified alkaline phosphatase. A possible explanation of Natori and Garen's (1970) data is that their fragment represents reinitiation of translation of the mRNA in a manner similar to that reported for *E. coli lac* repressor protein (Platt *et al.*, 1972).

The amino-terminal residues of both alkaline phosphatase isozymes are buried within the tertiary structure and only react with coupling reagents after denaturation of the protein.[5] Therefore, isozymes of this protein are probably generated during or very soon after formation of the polypeptide chain, possibly before secretion of this protein from the cytoplasm to the outer envelope of the cell where the final stages of subunit assembly occur (Schlesinger *et al.*, 1969).

Finally, we must also consider the possibility that the extra arginine residue of isozyme 1 has been added to the protein by an arginine-transfer enzyme such as described by Soffer (1968). Detailed analyses of the nascent peptides of alkaline phosphatase which contain the removed residues will be required to resolve this last possibility.

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Cooperativity in Human Erythrocyte Phosphofructokinase†

Lilian M. Y. Lee, R. M. Krupka,‡ and R. A. Cook*,§

ABSTRACT: A kinetic study of purified phosphofructokinase has revealed intermediary plateau regions in substrate saturation curves for fructose 6-phosphate and ATP at the optimal pH, 8.4. At pH 6.8 similar behavior was seen with one substrate, fructose 6-phosphate, but not ATP. Cyclic 3′,5′-AMP and AMP activated the enzyme at pH 6.8 but not 8.4. At the lower pH cyclic 3′,5′-AMP eliminated the intermediary plateau region, producing simple substrate saturation curves. Photooxidation treatment of the enzyme with Methylene Blue abolished cyclic 3′,5′-AMP activation, and also abolished the plateau regions at either pH. The plateau regions and cyclic 3′,5′-AMP activation are therefore likely to depend on closely linked mechanisms. On the other hand, inhibition by high concentrations of ATP is apparently an independent phenomenon, since it persists after photooxidation treatment. In both cases where the plateau regions were eliminated—with cyclic 3′,5′-AMP and after photooxidation—the new K_m was intermediate between the two apparent K_m values of the unmodified enzyme. This circumstance suggests a mechanism involving several sites which exhibit positive cooperativity and possibly negative cooperativity as well, rather than two or more independent sites.

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) catalyzes the nearly irreversible phosphorylation of fructose 6-phosphate by ATP to form fructose 1,6-bisphosphate ($FruP_2$)[1] and ADP. It is recognized as a key enzyme in the regulation of glycolysis (Newsholme and Randle, 1961; Lowry *et al.*, 1964; Vinuela *et al.*, 1963). Phosphofructokinase isolated from heart (Mansour, 1965), brain (Passoneau and Lowry, 1963), muscle (Ling *et al.*, 1965), yeast (Ramaiah *et al.*, 1964), *Escherichia coli* (Atkinson and Walton, 1965), and the liver fluke (Mansour and Mansour, 1962) have similar characteristics. All are inhibited by high concentrations of ATP and the structurally unrelated compound, citrate. The inhibitions of the enzyme by ATP and citrate are reduced in the presence of NH_4^+, orthophosphate, FruP, and 5′-AMP.

Mansour and Mansour (1962) first reported an activation of the enzyme by cyclic 3′,5′-AMP (using the enzyme from liver fluke). A catalytically active form of the enzyme from guinea pig heart and a smaller, catalytically inactive form, possibly a monomer, were later demonstrated in sedimentation experiments (Mansour, 1965). The "inactive" form regained activity at slightly alkaline pH and in the presence of ADP, FruP, or $FruP_2$. With crystalline sheep heart phosphofructokinase, Mansour *et al.* (1966) and Mansour and Ahlfors (1968) suggested that regulation of enzyme activity depended on a

† From the Department of Pathological Chemistry, University of Western Ontario, London, Ontario, Canada. *Received January 15, 1973.* This work was supported by operational grants from the Defense Research Board of Canada and the Medical Research Council of Canada to Dr. Y. S. Brownstone, Department of Pathological Chemistry, University of Western Ontario, London, Canada.

‡ Agricultural Research Institute, London, Ontario, Canada.
§ Department of Biochemistry, University of Western Ontario.

[1] Abbreviations used are: FruP, fructose 6-phosphate; $FruP_2$, fructose 1,6-bisphosphate; *n*, interaction coefficient in the Hill equation.