THE IDENTIFICATION OF PARA-AMINOBENZOYL POLYGLUTAMATES IN EXTRACTS OF *E. COLI*

by

ROBERT ALLAN HENRY FURNESS

The University of Manitoba

A Thesis

submitted to

The Faculty of Graduate Studies and Research

The University of Manitoba

in partial fulfilment

of the requirements for the degree of

MASTER OF SCIENCE

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ABSTRACT

A glutamate-containing component of coenzyme A-disulfide prepared from *E. coli* has been isolated and shown to be a co-purifying contaminant. Physical and chemical techniques were used to characterize the contaminant as a mixture of p-aminobenzoyl poly(γ-L-glutamates) containing 5,6,7 and 8 glutamyl residues with the hexa- and heptaglutamyl forms predominating. Similar distributions of these molecules were present in extracts of both *E. coli* strains B23 and K12 grown either in minimal or enriched medium. A preliminary examination of the cellular pteroyl polyglutamate pool, from which the p-aminobenzoyl polyglutamates are derived, has shown a similar distribution. These results have cast some doubt on the reported size distributions of pteroyl polyglutamates in *E. coli*.

TO BARBARA

and

TO MY PARENTS

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor, Dr. P.C. Loewen, of the Department of Microbiology, University of Manitoba for his support and guidance during this study and, above all, for his friendship. I am also appreciative of his personal concern and sense of fairness demonstrated in our relationship.

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ABBREVIATIONS

A	absorbance
$A_{275/260}$	ratio of absorbance at 275 nm to that at 260 nm
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
^{o}C	degree Celsius
5-CH_3H_4pteglu	N^5-methyl-5,6,7,8-tetrahydropteroylglutamate
5,10-CH-H_4pteglu	N^5,N^{10}-methenyl-5,6,7,8-tetrahydropteroylglutamate
5,10-CH_2H_4pteglu	N^5, N^{10}-methylene-5,6,7,8-tetrahydropteroylglutamate
5-CHO-H_4pteglu	N^5-formyl-5,6,7,8-tetrahydropteroylglutamate
10-CHO-H_4pteglu	N^{10}-formyl-5,6,7,8-tetrahydropteroylglutamate
cm	centimeter
CoA	coenzyme A
$(CoA)_2$	oxidized coenzyme A-disulfide
CoASSG	coenzyme A-glutathione mixed disulfide
DEAE	diethylamino-ethyl
dATP	deoxyadenosine-5'-triphosphate
FPGS	folylpolyglutamate synthetase
g	gram
glu	glutamic acid
glu(γ-glu)$_{n-1}$	poly(γ-glutamic acid) of n residues
gsh	glutathione
h	hours

$H_2pteglu_n$	7,8-dihydropteroylpoly(γ-glutamate)
$H_4pteglu_n$	5,6,7,8-tetrahydropteroylpoly(γ-glutamate)
HCl	hydrochloric acid
Km	Michaelis constant
$LiBH_4$	lithium borohydride
M	molar
min	minute
ml	milliliter
mol	mole
NAD^+	oxidized nicotinamide adenine dinucleotide
nm	nanometer
pABA	para-aminobenzoic acid
pABAglu	N-(p-aminobenzoyl)-glutamic acid
$pABAglu_n$	p-aminobenzoylpoly(γ-glutamate) of "n" glutamyl residues
$pteglu_n$	pteroylpoly(γ-glutamate) of "n" glutamyl residues
PO_4^{3-}	inorganic phosphate ion
Rf	migration relative to the solvent front
RNA	ribonucleic acid
SDS	sodiumdodecylsulfate
TEAB	triethylamine-bicarbonate buffer
Tris	tris-(hydroxymethyl)aminomethane
UTP	uridine-5'-triphosphate
UV	ultraviolet
Vmax	maximum enzymic reaction velocity at saturating substrate concentration
xg	times gravity
Zn	zinc metal

HISTORY

HISTORY

Introduction

During the study of two CoA-containing disulfide compounds found in *E. coli* it became apparent that a co-purifying contaminant existed which contained several glutamyl residues. Several such polyglutamate compounds have been found in nature. Ubiquitous are the pteroyl poly-γ-glutamates, folic acid and its conjugates, and also one of its catabolites, pABA polyglutamate. The polyglutamate portion of $pteglu_n$, which is of variable length (n = 1-7 usually) appears to be of importance in the binding of the folate cofactors to certain enzymes. One exception to this rule is the H_2-$pteglu_6$ found stoichiometrically in bacteriophage T4 tail plate. This molecule is apparently necessary as a structural component for the proper assembly of phage tail plates. The presence of pABA glu_n (in yeast) was first reported in 1946, and it was later established as a breakdown product of $pteglu_n$.

Encapsulated strains of certain *Bacillus spp.* (notably *B. subtilis*, *B. licheniformis* and *B. anthracis*) have as part of their capsule a very large homopolymer of glutamic acid. Like the $pteglu_n$ and pABA glu_n the molecule contains γ-amide links but unlike them, it contains D-glu instead of the naturally predominant form L-glu. It is also much larger, being polymerized to the degree of up to several thousand residues as compared to the short oligopeptides of $pteglu_n$.

Pteroylpolyglutamates

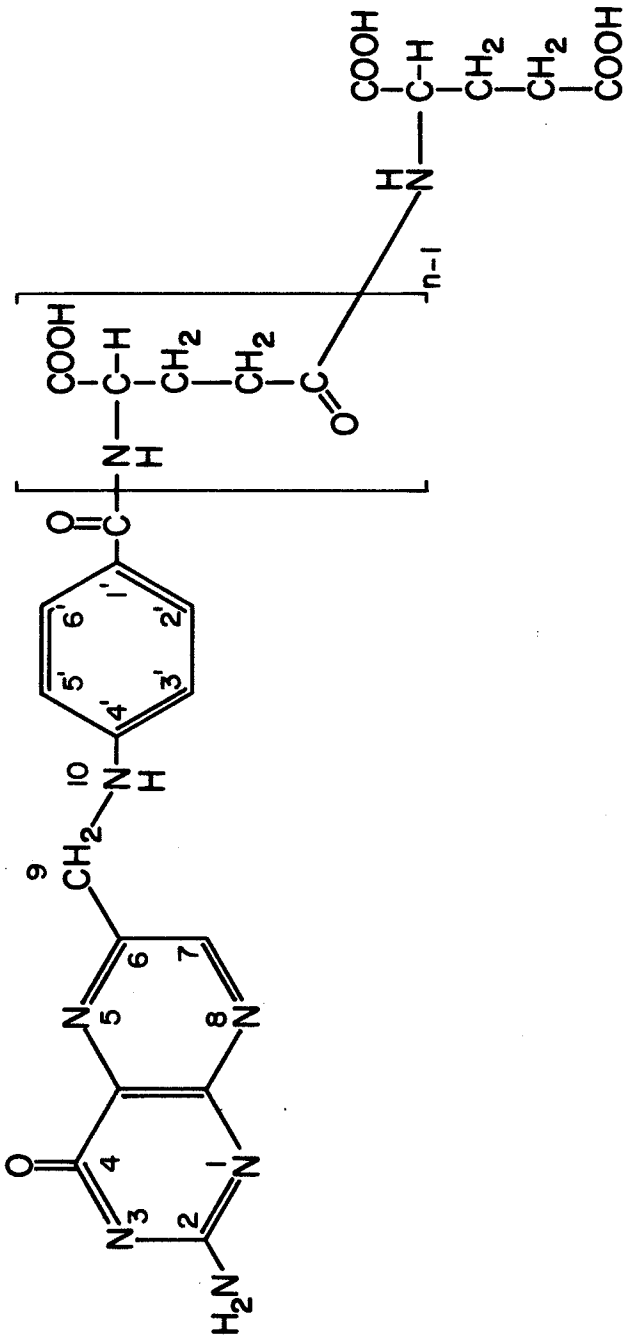
1) Occurrence

The structure of the pteroyl polyglutamates, shown in Figure 1, was elucidated by Angier *et al* (1946). There has been some confusion over nomenclature as folic acid is composed of the pteridine-pABA portion of the molecule and one glutamyl residue and is hence pteroylglutamate. This is the reason for the use of the terminology pteroylpolyglutamates rather than "folyl polyglutamates" or "folic acid conjugates" as was used earlier. The term conjugates referred to the fact that the growth factor folic acid was conjugated to a chain of several glutamyl residues.

The first isolation of $pteglu_n$ was by Pfiffner *et al* (1946), who isolated the pteroyl heptaglutamate from yeast. Chemical analysis along with microbiological assay of the folates gave the ratio of seven γ-glutamyl residues per pteridine. The presence of $pteglu_n$ was established in several organisms, but studies were complicated by several factors. The presence of folate compounds was determined by microbiological assay as with *L. casei*, *S. faecalis* and *P. cerevisiae*, organisms incapable of *de novo* synthesis of folic acid but able to utilize different forms of the pteroyl monoglutamate (i.e. pyrazine oxidation state and N^5-, N^{10}- substituents), $pteglu_2$ or $pteglu_3$ for growth (Schertel *et al* 1965; Johns and Bertino 1965). It is not possible with bioassay to distinguish all forms of these folates without modification; for example 5-CH_3H_4 pteglu and $pteglu_3$ yield the same results. Pteroyl polyglutamates were assayed by cleavage to $pteglu_2$ by

Figure 1. The structure of $pteglu_n$.

The molecule consists of a pteridine ring (1-8) joined to a p-aminobenzoyl (1'-6',10) residue. This is linked by an amide bond to an oligopeptide of (γ-L-glutamic acid) of length n. Co-enzyme activity is determined by oxidation state at C^5 - C^8, the one-carbon constituent carried at N^5 or N^{10} and also the number of glutamyl residues present.



a γ-glutamyl carboxypeptidase ("conjugase") contained in chicken pancreas (Bird *et al* 1945, 1946). Chromatographic properties of natural folate derivatives do not allow easy separation of $pteglu_n$ by size or charge, probably due to interaction of the pteridine moiety with chromatography matrices (Shin *et al* 1972). Also, chemical determinations of glutamate chain length require isolation of the peptide in pure form. For these reasons determinations of the distributions of $pteglu_n$ chain lengths in different organisms was not practical. *Corynebacterium* excreted a $pteglu_3$ into its growth medium (Hutchings *et al* 1948) while *Streptococcus pneumoniae* contained small amounts of pteglu and $pteglu_2$, as well as large amounts of $pteglu_3$ and higher conjugates (Sirotnak *et al* 1963). The presence of $pteglu_n$ was also established in tissues from rat (Bird *et al* 1965), chicken liver (Noronha and Silverman 1962) and human whole blood (Herbert *et al* 1962). Schertel *et al* (1965) identified $pteglu_2$ and $pteglu_3$ in yeast, as well as a polyglutamate form they assumed to be $pteglu_7$ on the basis of the work of Pfiffner *et al* (1946).

Characterization of glutamyl chain length was simplified by cleavage of the C^9-N^{10} bond, either oxidatively using alkaline permanganate (Houlihan and Scott 1972) or reductively with Zn/HCl (Baugh *et al* 1974). Cleavage removes the chromatographically troublesome pteridine portion, leaving pABA glu_n, which can be separated more easily on the basis of glutamyl chain length. The results are not entirely quantitative, as discussed at length by Maruyama *et al* (1978), Baugh *et al* (1979) and Lewis and Rowe (1979), but are much clearer

than those previously obtained. As can be seen from Table 1, the distribution of $pteglu_n$ chain length varies from organism to organism, but the polyglutamate group is apparent in all organisms studied and one chain length predominates in each.

There is still much confusion in the literature over the cellular distribution and even the predominant form of $pteglu_n$ in some organisms. Much of this is a result of inconsistency and inadequacy of techniques, as discussed above. This is true especially in mammalian systems, where most of the folate pool exists as 5-CH_3H_4 $pteglu_n$, due to the failure of both oxidative and reductive cleavage to hydrolyse this form (Lewis and Rowe 1979).

In bacterial systems, however, some of the variation appears to be due to culture conditions. There is, for example *L. casei*, for which Baugh *et al* (1974) reported $pteglu_4$ as the predominant folate, but other authors found mostly $pteglu_8$ (Buehring *et al* 1974; Brody *et al* 1979; Brown *et al* 1974). Bassett *et al* (1976b) attributed this shift in chain length to exogenous folate availability as the only significant difference between the work of Baugh *et al* (1974) and the other groups was the former's use of almost twice the medium folate concentration. This they confirmed.

Another instance of a shift in $pteglu_n$ size profile is in *E. coli* B upon infection with bacteriophage T4D, which contains $H_2pteglu_6$ as a component of its tail plate (Kozloff and Lute 1965). The $H_2pteglu_6$ is a stoichiometric component of the wedge-shaped structures comprising

TABLE I

Size distributions of pteroyl polyglutamates in various organisms.

Organism	Glutamyl Residues in $pteglu_n$ (% of Total) 1	2	3	4	5	6	7	8	9	>9	Reference
Strep. faecalis ATCC 8043	16.5	8.5	20	54.5							Baugh *et al* (1974)
ATCC 8043 methotrexate resist	1	5.7	6.9	81.3	5.1						Baugh *et al* (1974)
8043		1	10	86	2.6						Brody *et al* (1979)
8043			4	61	23						Buehring *et al* (1974)
Lactobacillus casei ATCC 7469	3.2	0	9.3	59.4	23	5.1					Baugh *et al* (1974)
								100			Brody *et al* (1979)
						8	14	42	19		Buehring *et al* (1974)
Corynebacterium spp.				>95							Shane (1980a)
Clostridium acidi-urici			>95								Curthoys *et al* (1972)
Escherichia coli B			>90								Wood *et al* (1968) Kozloff & Lute (1973)
K12			~20	~20	~50						Powers and Snell (1976)
K12	13	14.5	36	12	16	8	1.5				Bassett *et al* (1976b)
NCIB 8109	13	18	30	13	20	5	1				Bassett *et al* (1976b)
Saccharomyces cerevisiae						16	70	12			Bassett *et al* (1976a)
Neurospora crassa					14.5	80	0	5.6			Chan & Cossins (1980)
Rat liver					>95						Shin *et al* (1972) Brody *et al* (1979)
Bacteriophage T_4						100					Kozloff & Lute (1965)

the outer portion of the hexagonal baseplate and is found in close association with the apically located phage gene 11 product (Kozloff *et al* 1975, 1979). Kozloff's group has further shown that the $H_2pteglu_6$ is required as a structural component involved in attachment of the long tail fibers at or near the apices (Kozloff *et al* 1979). This was accomplished using the inhibition of *in vitro* tail fiber attachment with oligo (γ-glutamyl) chains, γ-glutamyl carboxypeptidase and antisera directed against a polyglutamate hapten. They proposed that the presence of the seven anionic charges allows strong tail fiber attachment yet allows the necessary rotation freedom. This does not explain, however, the absolute requirement for $H_2pteglu_6$. Kozloff and Lute (1973) noted a shift from predominantly $pteglu_3$ to $pteglu_6$. This shift was chloramphenicol-sensitive and apparently due to a late phage gene product. T4D gene 28^--infected cells accumulate larger conjugates ($pteglu_9$-$pteglu_{12}$) but little $pteglu_6$. Viable phage are not formed. It appears that $pteglu_6$ is formed from a larger conjugate by cleavage due to a phage gene product. The successful complementation of T4D gene 28^--infected bacterial extracts with the addition of $pteglu_6$ confirmed this (Kozloff *et al* 1973). The mechanism of the shift to larger conjugates is still unclear (Nakamura and Kozloff 1978).

2) Synthesis

The first report of a folylpolyglutamate synthetase (FPGS) was by Griffin and Brown (1964), who described such an activity in *E. coli*.

Subsequent work (Brown *et al* 1974) described folate synthesis in the succession H_2pte, H_2pteglu, H_4pteglu and H_4pteglu$_2$ and reported the extraction of an ATP-dependent FPGS catalyzing the formation of 10 CHO-H_4pteglu$_2$ only (Masurekar and Brown 1975). This led to the postulation of two enzymes for polyglutamate synthesis.

Sakami *et al* (1973) described two FPGS activities in *Neurospora crassa*, one specific for the conversion of H_4pteglu to H_4pteglu$_2$ and one specific for elongation of H_4pteglu$_2$ to H_4pteglu$_3$. These activities could be chemically fractionated and were genetically characterized. Two methionine auxotrophs, *met*-6 and *mac* each were found to be lacking one of these activities (Chan and Cossins 1980).

The most extensive work has been that of Shane (1980a, 1980b, 1980c) on FPGS from *Corynebacterium spp*. He characterized the cellular folate content as to glutamyl chain length, one-carbon substituent and oxidation-reduction state (Shane 1980a) and isolated an FPGS enzyme to greater than 95% purity. This enzyme (Shane 1980b) has a molecular weight of 53 000 by SDS gel electrophoresis a pH optimum of 9.5 or greater, an absolute requirement for L-glutamate and for a monovalent cation (K^+, 200 mM). The activity was Mg ATP-dependent, but dATP or even UTP would suffice to an extent. Folate requirement was variable but monoglutamates were much more effective substrates than higher forms, with H_4pteglu the most effective (Km = 2 μM). Despite the much lower affinity of his enzyme for di- and triglutamates, Shane showed that there was no appreciable difference in activities between the purified enzyme and crude extract, implying that only one and not

two enzymes were present. He speculated that formation of longer glutamyl chains had different optimum conditions. In support of the one-enzyme hypothesis he referred to "preliminary studies" indicating the utilization of $5,10CH_2H_4pteglu_{2-4}$, but this reference was unsupported by data.

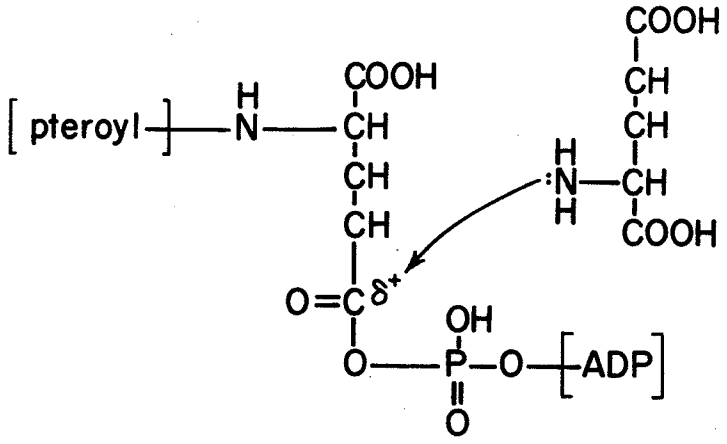
Kinetic studies (Shane 1980c) indicate the reaction proceeds by an Ordered Ter Ter mechanism with substrates joining the enzyme complex in the order MgATP, $H_4pteglu$, glutamate, followed by release of products in the order ADP, $H_4pteglu_2$, PO_4^{3-}. Two speculative mechanisms consistent with this sequence are presented, both involving nucleophilic attack of the free glutamyl α-amine, which would not be ionized at the pH for optimum activity (pH 9.5 or greater). One mechanism proposes nucleophilic attack on the mixed anhydride of a pteroyl (γ-glutamyl-phosphate) intermediate (Figure 2a), while the other proposes a concerted nucleophilic attack of the glutamyl α-amine on a γ-carboxyl carbonium ion of the pteglu coupled with a similar attack of one of the γ-carboxyl oxygens on the γ-phosphorus of ATP (Figure 2b). Both mechanisms are plausible but the evidence at present (Shane 1980c) is at best indirect.

There have been several studies of mammalian FPGS. Gawthorne and Smith (1973) showed $H_4pteglu$, 5-CHO-$H_4pteglu$ and 5-$CH_3H_4pteglu$ to be substrates for enzyme extracts of sheep liver. Taylor and Hanna (1977) showed much lower activity for 5-$CH_3H_4pteglu$ using enzyme from Chinese hamster ovary (CHO) cells. Spronk (1973) and McGuire *et al* (1980) found rat liver FPGS to be inactive with 5-$CH_3H_4pteglu$, as were 10-CHO-$H_4pteglu$ and $H_2pteglu$. Studies with non-mammalian enzymes (Sakami *et al* 1973,

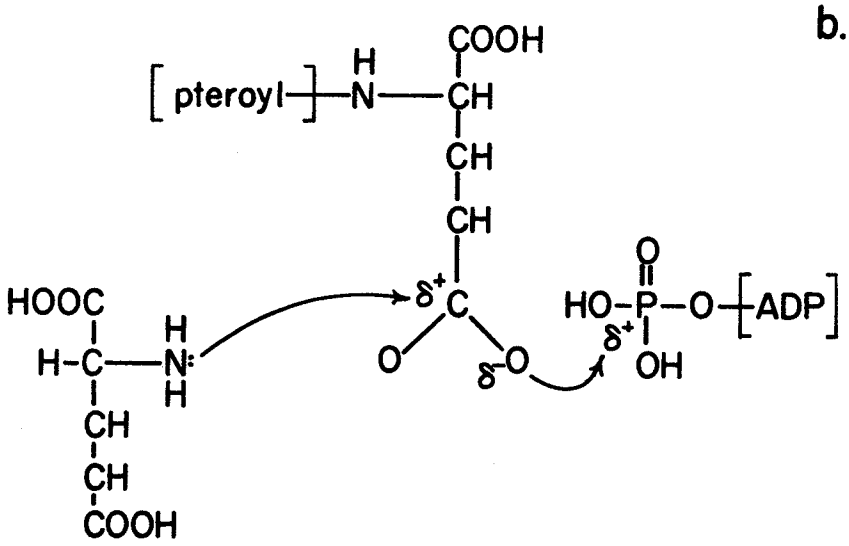
Figure 2. Possible mechanisms of chain elongation by folylpoly-glutamate synthetase.

Two mechanisms of glutamate addition to the free γ-carboxyl of $pteglu_n$ have been proposed by Shane (1980c). One invokes a nucleophilic attack on an active intermediate (a) while the other involves a concerted nucleophilic attack without prior formation of an active form of pteglu (b).

a.


[pteroyl]
H
N
COOH
CH
CH
CH
O=C
δ+
O
OH
P
O
[ADP]
COOH
CH
CH
H
N
CH
COOH

b.


[pteroyl]
H
N
COOH
CH
CH
CH
HOOC
H-C
H
N
δ+
C
O
δ-O
O
HO-P-O-[ADP]
δ+
OH
CH
CH
COOH

Shane 1980b) also reported relative inactivity with 5-CH_3H_4pteglu. These data are not necessarily in conflict with the fact that 5-CH_3H_4pteglu$_n$ are present as a large proportion of mammalian folate pools (Lewis and Rowe 1979) as they may only indicate that conversion to 5-CH_3H_4pteglu$_n$ takes place after polyglutamation.

McBurney and Whitmore (1974) isolated several auxotrophic mutants of CHO cells showing defects in intracellular folate metabolism. Among these were AUX B1 (a glycine, adenosine and thymidine auxotroph) and AUX B3 (a glycine and adenosine auxotroph). Not only did these lines have reduced folate concentrations but AUX B1 contained only monoglutamates while AUX B3 also contained some di- and triglutamates as opposed to the pentaglutamate of the parent line. The authors decided on genetic bases that these mutants had defects in one single locus. Using extracts of these lines Taylor and Hanna (1977) demonstrated that AUX B1 and AUX B3 lacked the parental folylpolyglutamate synthetase activity. Interestingly, they also showed that the number of glutamyl residues added to the substrate is dependent upon the H_4pteglu concentration. At low levels (1 μM) mostly H_4pteglu$_3$ and some H_4pteglu$_4$ are formed while at higher levels (5 μM) mostly H_4pteglu$_2$ is made. At very high levels (100 μM) only H_4pteglu$_2$ results. Taylor and Hanna (1979) further studied FPGS in extracts of revertant lines of AUX B1 and found increased heat sensitivity over wild-type in FPGS activity. This increased lability was apparent when either H_4pteglu$_2$ or H_4pteglu$_4$ synthesis was monitored. This led the authors to conclude that both diglutamate and tetraglutamate activities were on one enzyme.

McGuire *et al* (1980) have partially purified the rat liver FPGS

which shows similarities to the *Corynebacterium* enzyme (Shane 1980b). It requires a monovalent cation, of which K^+ (20 mM) is optimal, has a high pH optimum (8.4), exhibits an absolute requirement for L-glutamate and a less stringent requirement for MgATP that can be alleviated by other nucleotide triphosphates. In contrast, the substrate specifity for folates is broader in that H_2pteglu and 5,10CH-H_4pteglu are utilized much more easily (compared to the primary substrate H_4pteglu) than is the case with the *Corynebacterium* enzyme. Also, the rat liver enzyme catalyses *in vitro* the formation of $pteglu_4$ in large amounts and $pteglu_5$ (the predominant form in rat liver Table I) in an almost equal quantity. As with Taylor and Hanna's (1977) CHO extract the longer products are formed at low H_4pteglu concentrations while at higher concentrations mostly $pteglu_2$ is formed. It was found that $pteglu_5$ was not effective as a substrate and also was a strong inhibitor of the enzyme. This led McGuire *et al* to propose that, on binding of $pteglu_5$ to the enzyme, the length of the glutamyl chain places the terminal γ-carboxyl out of the active site. This suggests that, at least in rat liver, the predominant length of $pteglu_n$ is controlled solely by the size of the active site of FPGS. The binding of the end product $pteglu_5$ to the active site also indicates that feedback inhibition of polyglutamation may be one form of regulation of intracellular folate levels.

3) Significance

The polyglutamate conjugates of folic acid (with the exception of bacteriophage T4 as previously mentioned) appear to be the active coenzyme forms of the vitamin. Kisliuk and Gaumont (1974) showed that $H_4pteglu_3$ and $H_4pteglu_6$ were much better substrates for *L. casei* thymidylate synthetase than was $H_4pteglu$. Polyglutamate forms of folate analogs were strong inhibitors of the enzyme, while $pABAglu_6$ and $glu(\gamma\text{-}glu)_5$ were not bound by the enzyme, indicating that the poly(γ-glu) portion of the molecule enhanced binding but did not determine it. Coward *et al* (1974) demonstrated that dihydrofolate reductase from various mammalian sources has a higher affinity for $H_2pteglu_3$ $H_2pteglu_5$ or $H_2pteglu_7$ than for $H_2pteglu$. Powers and Snell (1976) found that *E. coli* ketopantoate hydroxymethyl transferase (involved in the synthesis of pantothenic acid) had much lower Km values for 5,10-$CH_2H_4pteglu_4$ and 5,10-$CH_2H_4pteglu_5$ than for other glutamyl chain lengths of the coenzyme. Baggott and Krumdieck (1979) obtained similar results with chicken liver 10-formyltetrahydrofolate:5'-phosphoribosyl-5-amino-4-imidazole-carboxamide formyl transferase, an enzyme of purine synthesis. The enzyme had a specificity (as defined by the ratio $^{Vmax}/_{Km}$) for the tetraglutamyl form 250-fold of that for the monoglutamyl form of the coenzyme. In their estimation this would make the tetraglutamate the only active form at the intracellular folate concentration found (1-2 μM).

The work of McBurney and Whitmore (1974) and that of Taylor and

Hanna (1977, 1979) provides both genetic and biochemical evidence that the polyglutamyl forms of folate are necessary for the biosynthesis of glycine, purines and pyrimidines in mammalian cells. Moreover, the ability of AUX B3 FPGS to make $H_4pteglu_3$ but not $H_4pteglu_4$ (Taylor and Hanna 1979) indicates the requirement of the larger oligo(γ-glutamyl) coenzymes.

The work of McBurney and Whitmore (1974) still leaves open the possibility that polyglutamation of folic acid is a means of ensuring cellular retention of the vitamin as the AUX B1 and AUX B3 FPGS mutants contain 10% and 40% of the assayable folic acid of the parental wild-type. This is supported by the observation (Herbert *et al* 1962) that serum contains only pteroyl monoglutamate, indicating it may be only a transport form of the vitamin as suggested by Rabinowitz (1960). However, in light of the enzyme kinetic studies cited above, this must be regarded as an auxiliary mechanism in cellular folate metabolism. As noted in both the bacterial (Bassett *et al* 1976; Powers and Snell 1976) and mammalian (McGuire *et al* 1979; Taylor and Hanna 1977) systems, the degree of polyglutamation is in inverse proportion to available folate levels. This relationship may be a manifestation of metabolic efficiency. When folate is limiting, both retention and reactivity are enhanced at the expense of ATP and glutamic acid. With excess folate, enhancement of activity and retention are not paramount, and ATP and glutamate are conserved. Such controls are quite feasible in the control of so important a metabolite.

Para-aminobenzoylpolyglutamates

At about the same time as the isolation of $pteglu_n$ Ratner *et al* (1946) isolated a $pABAglu_n$ from autolysed yeast. This was identified by the spectral characteristics of the pABA portion, especially the disappearance of the absorption at 273 nm on exposure to acid (pH 1) and by the presence of glutamic acid. The absorption drop is due to ionization of the arylamine, which indicates the peptide link to glutamate is through the pABA carboxyl. This was confirmed by diazotization of the free aryl amine, as opposed to the unreactiveness of blocked amines (e.g. N-acetyl pABA). Chemical analysis yielded a ratio of 10 or 11 glutamates per pABA, all being the L-isomer (bioassay). On the basis of a difference between estimates of glutamic acid nitrogen and α-amino nitrogen the presence of one dicarboxylic amino acid other than glutamic acid was postulated but not determined. The structural analogy between the pABA peptide and folic acid was noted by the authors, but no confirmation was attempted.

Stokstad *et al* (1947) noted that pABA glu was a product of UV photolysis of pteglu. Enzymic degradation of pteglu to pABAglu was demonstrated by Keresztesy and Silverman (1953). Futterman and Silverman (1957) showed the reaction to be dependent upon NAD^+ and ATP and thought it to be non-enzymic oxidation of an unstable reduction product, probably H_4pteglu.

Capsular polyglutamate of *Bacillus spp.*

The presence of a large polymer of glutamic acid in the capsule of *Bacillus spp.* was reported by Ivanovics and Bruckner (1937). It was characterized as possessing a γ-amide linkage between D-glutamyl residues by Bovarnick (1942).

Troy (1973a) demonstrated the synthesis of the polymer from L-glutamic acid by a semi-purified cell-envelope fraction of *Bacillus licheniformis*. This synthesis is not dependent on a RNA template as shown by its retractoriness to inhibition by RNase, chloramphenicol, actinomycin D, puromycin and rifampicin. In a subsequent paper (Troy 1973b) the characterization of the enzymatically synthesized polymer as poly(γ-D-glutamate) was described. Optical configuration was established by chromatography of the diastereoisomeric peptide resulting from derivatization with L-leucine N-carboxyanhydride using standard derivatives of D- and L-glutamic acid. This was confirmed by gas-liquid chromatographic separation of N-triflouro-acetyl-L-prolyl glutamate methyl esters. The amide link was established as γ rather than α by its comparative ease of hydrolysis under mild acid conditions as well and by $LiBH_4$ reduction of the polymer methyl ester followed by acid hydrolysis. The resulting γ-amino-δ-hydroxy-valerate could be separated electrophoretically from the α-amino-δ-hydroxyvalerate yielded by authentic poly(α-D-glutamate). Ultracentrifugal studies showed that the polyglutamates formed are large and polydisperse, ranging in molecular weight from 1.7×10^5 to 3.6×10^5, corresponding roughly to 1000 - 2000 residues. This compares with a

size range of 8.4×10^4 to 1.15×10^6 (500 - 6700 residues) for the native polymer.

Gardner and Troy (1979) have studied the mechanisms of the membrane-bound system and have dissected it into at least two enzyme activities: a glutamate activation and a chain elongation-activity. The membrane-bound L-glu activating system is dependent upon ATP, Mg^{2+}, L-glu (but not D-glu) and K^+(200 mM). This activity was examined by trapping of the activated intermediate as the glutamyl hydroxamate. It was possible also to uncouple the activation activity from elongation of the glutamyl polymer with N-ethylmaleimide, thus indicating the participation of a sulphydryl group subsequent to activation of L-glu. Chemical analyses of the glutamyl hydroxamate showed it to be derived from L-glu activated at the γ-carboxyl, indicating racemization to D-glu occurs after activation. That the activated form was an adenylate was shown by inhibition of the activation reaction by inorganic pyrophosphate and the release of AMP from ATP in the assay system. Polymer synthesis could be inhibited by 5'-AMP, from which the authors inferred the existence of a second activated intermediate, possibly involving the N-ethylmaleimide-sensitive sulphydryl, whose synthesis from γ-L-glu-AMP is reversible. Racemization may occur at this stage or simultaneously with addition to the growing chain.

Pulse-chase labelling with [^{14}C]-glu, followed by N-terminal dansylation, isolation of dansylamino acids and comparison of radio-activity in dansyl glutamate and free glutamate indicated that chain

growth was in the carboxyl to amino direction, unlike ribosomal protein synthesis. Synthesis is believed to involve transfer of the activated glutamyl group to the amino terminus of a membrane-bound acceptor molecule. The acceptor is thought to be endogenous, bound poly(γ-D-glu) as newly incorporated label can only be isolated as very large molecular weight polymers and there is no evidence of small peptide formation. These large molecules can be released under conditions whereby thioesters are liberated, implicating the participation of such a linkage in acceptor binding.

The capsular poly(γ-D-glu) synthesizing system thus involves at least one, possibly two active intermediates, plus a large acceptor molecule all bound to parts of the *Bacillus* cell membrane.

DS Compounds and Polyglutamic Acid

In 1976, Loewen demonstrated the presence of two nucleotides, tentatively labelled DSI and DSII, in *E. coli* cultures undergoing aerobic downshift or reaching stationary phase. Physical and chemical analysis of these compounds (Loewen 1977) revealed that DSI was a coenzyme A-glutathione mixed disulfide while DSII was apparently a modified coenzyme A disulfide. Extensive purification of DSII, involving four chromatographic steps, yielded an anomalous CoA dimer containing glutamic acid. Variation in analysis of glutamic acid after acid hydrolysis (from 1 to 2 residues per adenine) plus the lack of reports in the literature on modified CoA led to the belief that the glutamic acid was a co-purifying contaminant of DSII. As it was thought that

monoglutamic acid would be unlikely to purify with $(CoA)_2$ through the procedures used, it was felt that the glutamic acid may be in the form of a larger polymer.

The object of this study was to isolate and characterize this suspected polyglutamate contaminant.

MATERIALS AND METHODS

MATERIALS AND METHODS

Medium and buffers

E. coli was grown in minimal medium and enriched medium with the following compositions:

Minimal medium:	Tris-HCl	0.1 M pH 7.6
	sodium sulfate	0.16 M
	magnesium sulfate	1 mM
	ammonium sulfate	7.5 mM
	glucose	5.5 mM
	calcium chloride	0.2 mM
	ferric chloride	3 μM
	potassium phosphate	0.64 mM (added after autoclaving)

Enriched medium (LB broth):

yeast extract	5 g
Bactotryptone	10 g
sodium chloride	5 g
distilled water	1000 ml

Triethylammonium bicarbonate (TEAB) pH 7.5 to 8.5

Triethylamine was refluxed 8 h with excess potassium hydroxide and distilled before use. Distilled triethylamine was stored at 4°C. A mixture of 574 ml triethyl amine and 700 ml deionized water was bubbled with carbon dioxide for 18 h. This was diluted to 2 M with deionized water and stored at 4°C.

Chromatography solvents

solvent A: isopropanol: water: formic acid (80:20:4)

solvent B: n-propanol: water (7:3)

solvent C: ammonium sulfate: 0.1 M sodium phosphate pH 7.0: n-propanol (12g: 20 ml: 0.4 ml)

Chromatography

Thin layer chromatography of amino acids was carried out on Brinkmann Polygram CEL 300 UV 254 cellulose plates using solvent A in the first dimension and solvent B in the (optional) second dimension. Glutamic acid had Rf values of 0.46 and 0.39 in the first and second dimensions respectively (Loewen 1977). Amino acids were visualized with a spray of 0.3 g ninhydrin/100 ml 2-methoxy ethanol.

Descending paper chromatography was carried out on Schleicher and Schuell orange ribbon C paper using solvent C. $(CoA)_2$, visualized by UV absorbance, had an Rf value of 0.02 in this system (Loewen 1977).

Column chromatography gels and ion exchange resins were pre-equilibrated with the starting buffers used. In order to prevent excessive shrinkage of the analytical 0.5 x 50 cm DEAE A-25 Sephadex column during elution, it was necessary to equilibrate the gel with 1 M sodium chloride, then 0.1 M sodium chloride before pouring the column. This limited shrinkage to 5-10% of column length.

Desalting of concentrated fractions was carried out on a 1.2 x 100 cm column of G-10 Sephadex eluted with 0.05 M TEAB. Nucleotides and polyglutamates eluted with the void volume at 40-45 ml.

Isolation of $(CoA)_2$

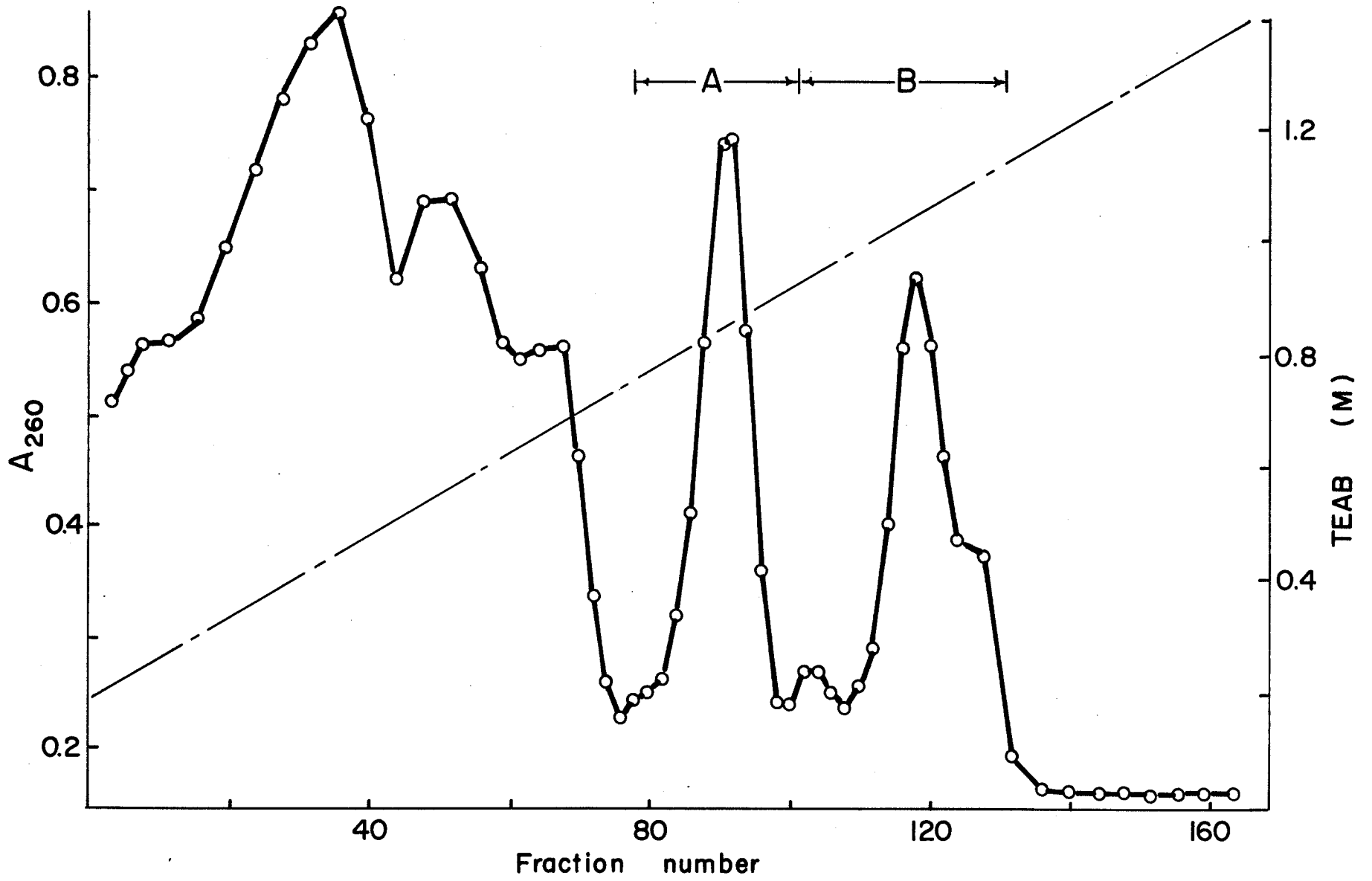
The isolation of $(CoA)_2$ (Loewen 1976) is briefly summarized here for comparison to the isolation of polyglutamate. Stationary phase *E. coli* were concentrated and extracted with 1 M formic acid, followed by neutralization and dilution of the extract. The extract was chromatographed successively on a 2.5 x 30 cm DEAE A-25 Sephadex column eluted with a linear gradient of TEAB, a 1.2 x 100 cm G-50 Sephadex column eluted with 0.05 M TEAB, a 1.0 x 15 cm DEAE A-25 Sephadex column eluted with a linear gradient of sodium chloride in 0.05 M Tris-HCl pH 7.6 and a final 1.2 x 100 cm G-10 Sephadex desalting column.

Isolation of *E. coli* polyglutamate

Isolation procedures closely followed those used for $(CoA)_2$ (Loewen 1976) except modifications were necessary to enhance separation of the polyglutamates from $(CoA)_2$.

Escherichia coli strain B23 was grown at 28°C for 16-18 h in 2000 ml baffled Ehrlenmeyer flasks containing 1000 ml LB broth or minimal medium (1% inoculum). Aeration was by vigorous shaking. Cells were harvested by centrifugation at 20°C (10,000 x g, 10 min). Ten litres of LB culture yielded approximately 75 g cells (wet weight). The cell pellets were resuspended twice in 60 ml 1 M formic acid and allowed to stand 30 min at 0°C each time. After centrifugation at 4°C (10,000 x g, 10 min) the supernatant was adjusted to pH 7.0 with concentrated ammonium hydroxide and diluted with 5 volumes deionized water. The diluted extract was loaded on a 2.5 x 30 cm DEAE A-25 Sephadex column (4°C), washed with 1000 ml 0.2 M TEAB and eluted (Figure 3) with a 500 x 500 ml

Figure 3. DEAE A-25 Sephadex chromatography of formic acid extracts. Cells were extracted with 1 M formic acid and the extracts were neutralized with ammonium hydroxide. The neutralized extracts were diluted five-fold with water and loaded on a 2.5 x 30 cm column of DEAE A-25 Sephadex. The column was washed with 0.2 M TEAB and eluted with a linear gradient of TEAB. Fractions of 6.0 ml were collected. Fraction A contains CoASSG while Fraction B contains $(CoA)_2$ and the contaminating polyglutamate.


A
B
0.8
0.6
0.4
0.2
A_{260}
1.2
0.8
0.4
TEAB (M)
40
80
120
160
Fraction number

linear gradient of 0.2 to 1.4 M TEAB. Fraction B from this column was pooled and evaporated to dryness twice under vacuum. This was then resuspended in 1 ml 0.05 M TEAB and chromatographed at 4°C on a 1.2 x 100 cm column of G-50 Sephadex (Figure 4). Peak A from this column was concentrated by vacuum evaporation to 10 ml and loaded on a 0.5 x 50 cm column of DEAE A-25 Sephadex. This was eluted with a 250 x 250 ml linear gradient of 0.1 to 0.6 M sodium chloride in 0.01 M potassium phosphate pH 7.6 at room temperature (Figure 5). Peaks I-IV were concentrated to dryness, suspended in 1 ml water and desalted using a 1.2 x 100 cm G-10 Sephadex column eluted with 0.05 M TEAB. Each was again concentrated and rechromatographed on the 1.2 x 100 cm G-50 Sephadex column in an effort to reduce cross-contamination of adjacent peaks. Samples were stored in water at -20°C.

Isolation of yeast pABAglu$_n$

Dry baker's yeast (Universal Foods Corp. Milwaukee) (100 g) was resuspended in water per the manufacturer's directions and extracted with formic acid as above. The extract was chromatographed on a 2.5 x 30 cm column of DEAE A-25 Sephadex as was the *E. coli* extract. Fractions from this column corresponding to those of Fraction B (Figure 3) were pooled and processed as for the *E. coli* preparation.

Examination of the *E. coli* pteglu$_n$ pool

For the examination of cellular pteglu$_n$ content the method of Shane (1980a) with modifications, was followed. *E. coli* B23 grown for

Figure 4. G-50 Sephadex chromatography of $(CoA)_2$.

Fraction B of Figure 3 was concentrated and chromatographed on a 1.2 x 100 cm column of G-50 Sephadex eluted with 0.05 M TEAB. Aliquots of the 2.5 ml fractions were assayed with fluorescamine as in Materials and Methods. Both $(CoA)_2$ and the polyglutamate are present in peak A.

○ relative fluorescence at 494 nm

● A_{275}

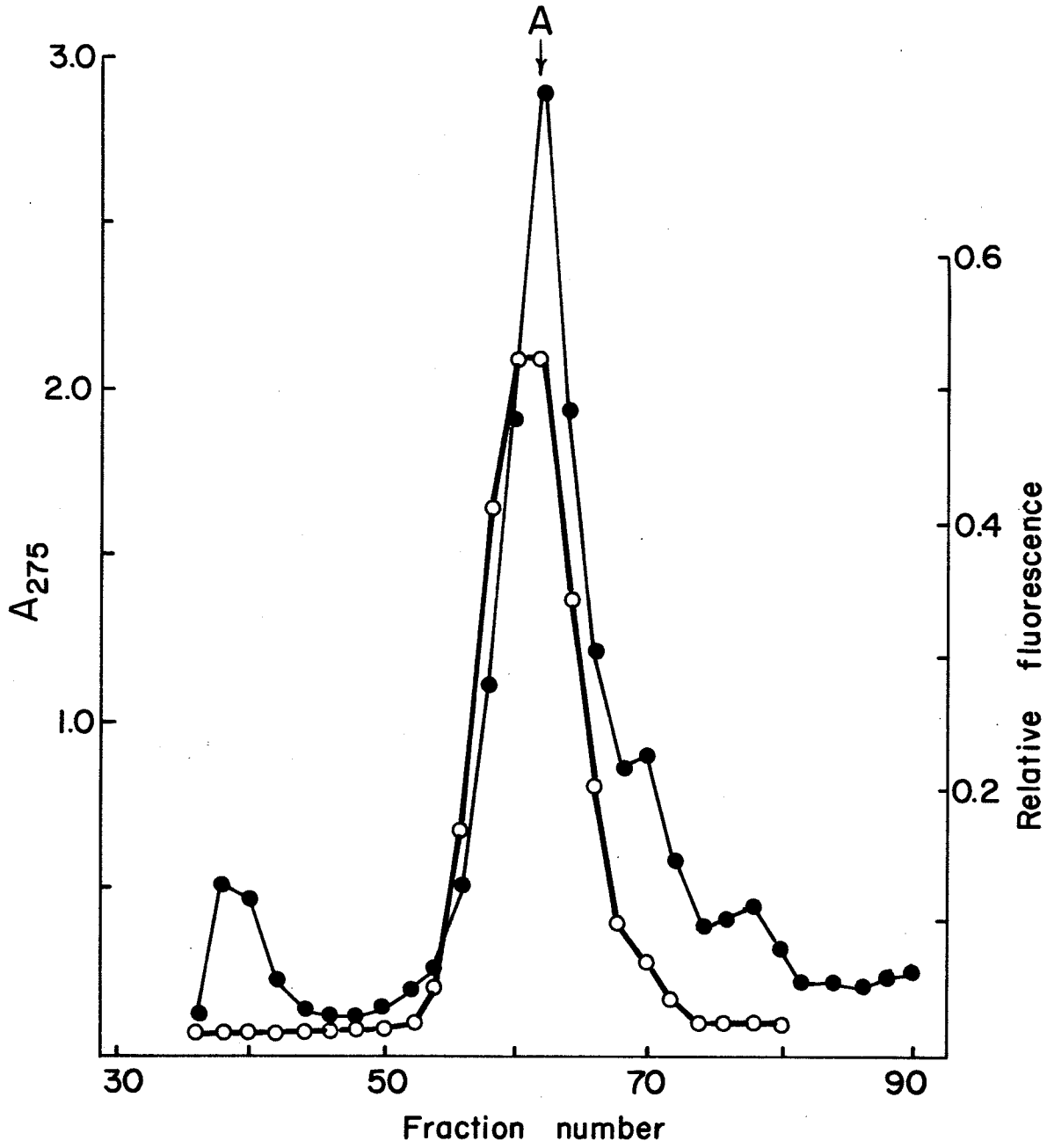
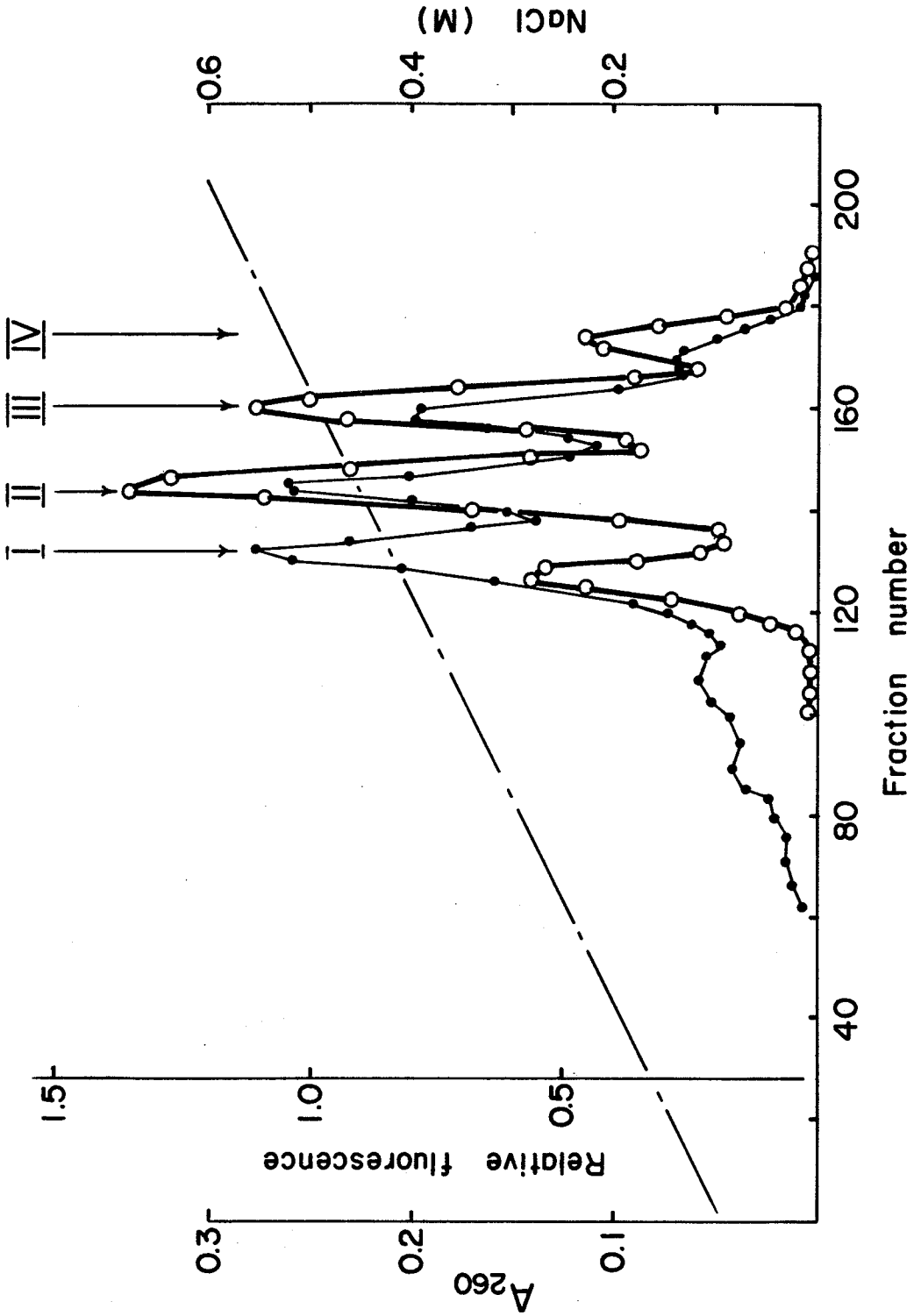

A
3.0
2.0
1.0
A_{275}
0.6
0.4
0.2
Relative fluorescence
30
50
70
90
Fraction number

Figure 5. DEAE A-25 Sephadex separation of $(CoA)_2$ and polyglutamate. Peak A from Figure 3 was chromatographed on a 0.5 x 50 cm column of DEAE A-25 Sephadex eluted with a linear gradient of sodium chloride in 0.01 M potassium phosphate pH 7.6. Fractions of 2.5 ml were collected and assayed with fluorescamine. Most of the $(CoA)_2$ eluted in peak I while the polyglutamate eluted as four peaks (I-IV).

○ relative fluorescence

• A_{260}


NaCl (M)
0.6
0.4
0.2
I
II
III
IV
A260
0.3
0.2
0.1
Relative fluorescence
1.5
1.0
0.5
40
80
120
160
200
Fraction number

16 h at 28°C in LB broth was centrifuged at 10,000 x g for 10 min, and the cell pellet (30 g wet weight) was resuspended in 50 ml 0.1 M potassium phosphate pH 7.6 containing 0.2 M β-mercaptoethanol. The cell suspension was extracted by autoclaving for 5 min, followed by centrifugation of cell debris (15,000 x g, 10 min). The extract was acidified to pH 1 with HCl and allowed to stand at 4°C for 2 h. The extract was then treated with 0.5 ml saturated mercuric chloride to precipitate the mercaptoethanol. Half of the extract was stored at 4°C while the other half was subjected to reductive cleavage of $pteglu_n$ to $pABAglu_n$. The extract was made 0.5 M in HCl and 2.0 g powdered zinc was added with slow stirring at 20°C. After 5 min the mixture was cooled to 4°C and centrifuged (15,000 x g, 5 min). Both the cleaved and uncleaved extracts were diazotized and coupled to naphthylethylene diamine. Dowex AG50 (200-400 mesh) H^+ form (5 ml of a suspension in 0.2 M HCl) was added with stirring to the coupled extract. Five min was allowed for absorption of the azo dyes to the polystyrene resin. The resin was loaded into 1.3 x 3.0 cm columns and washed with 30 ml 0.2 M HCl, followed by 7.5 ml 0.1 M potassium phophsate pH 7.6. The azo dyes were cleaved and the resulting $pABAglu_n$ eluted with 5 mg/ml sodium hydrosulfite (Brody 1976). Fractions containing fluorescamine-reactive material were pooled and loaded on the 0.5 x 50 cm analytical DEAE A-25 Sephadex column and eluted with a linear gradient of NaCl in 0.01 M potassium phosphate pH 7.6.

Conjugase preparation

Chicken pancreas conjugase was prepared following the method of Bird *et al* (1965). Two chicken pancreata (obtained from the Department of Animal Science, University of Manitoba) were homogenized with two volumes of 0.1 M potassium phosphate pH 8.0 and allowed to autolyse for 18 h at 32°C. The autolysate was centrifuged (15,000 x g, 15 min) and the supernatant was differentially precipitated with ammonium sulfate. The 40-80% precipitate was redissolved in 2 ml cold 0.05 M potassium phosphate pH 8.0 and dialyzed against 1 L of this buffer at 4°C for 16 h. To the 2 ml conjugase was added 5 µmol calcium chloride, followed by incubation at 37°C for 4 h and then dialysis at 4°C for 4 h against 0.025 M potassium phosphate pH 8.0 containing 50% glycerol. The preparation was frozen at -76°C in small aliquots. Digestion of polyglutamate samples was carried out by mixing 250 µl sample, 0.5 µmol calcium chloride, 25 µmol Tris-HCl pH 7.6 and 5 µl of the frozen pancreas conjugase in a total volume of 300 µl, with incubation at 37°C. The reaction was stopped by boiling for 1 min.

Glutamate dehydrogenase

Glutamate dehydrogenase activity was measured as described by Strecker (1955). Assays (1 ml total volume) contained 100 µmol Tris-acetate pH 9.0, 1.0 µmol NAD^+, 0.28 units enzyme and approximately 100 nmol glutamic acid. Actual concentrations of glutamic acid were measured with an amino acid analyzer. The enzymic reaction was followed at room temperature by monitoring the change in absorbance at 340 nm due to the reduction of NAD^+ and initial rates were measured as slopes

of the ΔA_{340} vs time plots.

Analytical separation of $pABAglu_n$

Samples of $pABAglu_n$ for size analysis were partially hydrolyzed at 100°C for 45 min in 1 M HCl, then evaporated to dryness under vacuum twice to remove the acid before chromatography.

The hydrolysates were separated on an analytical column (0.5 x 50 cm) of DEAE A-25 Sephadex eluted with a 250 x 250 ml linear gradient of 0.1 to 0.6 M sodium chloride in 0.01 M potassium phosphate buffer pH 7.6

The resultant peaks of intermediate sizes were concentrated, desalted on the 1.2 x 100 cm G-10 Sephadex column and chromatographed on the 1.2 x 100 cm G-50 Sephadex column in preparation for quantitation of pABA and glutamic acid content. Standard samples of glutamic acid, pABA and pABAglu were chromatographed separately.

Quantitation of pABA

pABA was quantitated by measuring the difference in absorption at 273 nm between samples in water and 0.1 M HCl (Ratner *et al* 1946). Using authentic pABAglu a molar extinction coefficient for this change was calculated to be 1.32×10^4 M^{-1} cm^{-1}.

Quantitation of glutamic acid

Samples were hydrolyzed at 100°C for 6 h in 1 M HCl and evaporated to dryness twice under vacuum. Hydrolyzed samples were resuspended in

0.1 M HCl and compared to standards using a Technicon NC-2P Autoanalyzer amino acid analyzer operated by the Department of Chemistry, University of Manitoba.

Ultraviolet absorption measurements

Column eluate was assayed for UV absorbance using a Gilford model 2400 spectrophotometer. This instrument was used for glutamate dehydrogenase assays and for all concentration measurements based on UV absorption. Absorption spectra were measured on a Beckman Acta III dual beam scanning spectrophotometer using water or 0.1 M HCl for blanks as required.

Fluorescamine Assay

Fluorescamine (4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione) is a reagent that specifically reacts with primary amines. A modification of the technique of Udenfriend *et al* (1972) was used to assay columns for the presence of polyglutamates. Fluorescamine (3 mg) was dissolved in 1 ml anhydrous acetone (dried over calcium carbonate) and was prepared fresh before use. Aliquots of column eluates were assayed by direct addition of fluorescamine solution with vigorous mixing and then allowed to stand for 15 min to allow hydrolysis of unreacted fluorscamine. It was noted that authentic glutamic acid and poly(glutamic acid) reacted poorly with fluorescamine unless the pH of the sample was more alkaline. For this reason 100 µmol sodium borate (pH 9.0) was added to aliquots of column eluate when standards of poly(glutamic acid) were run. The omission of the borate in analytical chromatography of pABAglu$_n$

hydrolysates effectively reduced background and false peaks due to poly(glutamic acid) byproducts of hydrolysis.

For 50 µl and 100 µl sample aliquots 5 µl of reagent was used while 15 µl reagent was used for 1000 µl samples. Small reaction mixtures were diluted to 1 ml before measurements were made. Fluorescence of the product was measured at 494 nm with excitation at 398 nm using an Aminco Bowman spectrophotofluorometer.

Azo dye formation

Diazotization and coupling of polyglutamates was accomplished using the method of Bratton and Marshall (1939) as modified by Brody *et al* (1979). Samples (1-2 ml) were made 0.1 M in HCl followed by addition of 0.5 ml sodium nitrite (0.5%). After two min, 0.5 ml ammonium sulfamate (2.5%) was added and the mixture allowed to stand a further two min. The azo dyes were formed by adding 0.25 ml 0.1% naphthylethylene diamine and allowing 20 min for colour development. Absorbance was measured at 556 nm. Naphthylethylene diamine was prepared by dissolving 0.1 g dye in 1-2 ml 95% ethanol and diluting to 100 ml with deionized water. This solution was stored in a dark foil-wrapped bottle for a maximum of two weeks.

Chemicals

The following chemicals were purchased from the Sigma Chemical Co.: N-(para-aminobenzoyl)glutamic acid; Tris, poly(γ-L-glutamic acid), DEAE A-25, G-10 and G-50 Sephadex, fluorescamine, D- and L-glutamic acid, glutamate dehydrogenase.

The following were purchased from various companies: naphthylethylene diamine and 2-methoxyethanol (Eastman Organic Chemicals), yeast extract and Bacto-Tryptone (Difco Laboratories), poly(α-L-glutamic acid) (Miles-Yeda Laboratories), Dowex AG-50 (200-400 mesh) (Bio-Rad Laboratories).

All other chemicals were reagent grade or better.

RESULTS

RESULTS

Isolation of glutamate component of $(CoA)_2$

$(CoA)_2$ isolated from *E. coli* was found to contain glutamic acid on acid hydrolysis, even after extensive chromatographic purification (as summarized in Materials and Methods). In an attempt to free the $(CoA)_2$ from non-covalently bound glutamate it was subjected to DEAE Sephadex A25 chromatography in the presence of 7 M urea (Figure 6). The resultant $(CoA)_2$, when hydrolysed in acid and examined with two-dimensional cellulose thin layer chromatography in solvents A and B, showed no glutamic acid, indicating that the glutamate content of $(CoA)_2$ was indeed a co-purifying contaminant.

In an effort to isolate this glutamate-containing contaminant, $(CoA)_2$ previously eluted from the 2,5 x 30 A-25 and 1,2 x 100 cm G-50 Sephadex columns (as described in Materials and Methods) was rechromatographed on a long (0.5 x 50 cm) DEAE A-25 Sephadex column. This was eluted with a linear gradient of NaCl and assayed for primary amines with fluorescamine. Figures 7 and 8 show a pattern of four fluorescent peaks in the region of $(CoA)_2$, one of which overlaps with $(CoA)_2$. There is some UV absorbing material present in the fluorescamine-reactive peaks and it can be seen that the $A_{275/260}$ ratio is considerably higher under these peaks than under the purely UV absorbing peak. The principal UV absorbing peak is $(CoA)_2$ as shown by paper chromatography in solvent C. Acid hydrolysis showed $(CoA)_2$ isolated in this manner contained some glutamate, presumably from the overlapping

Figure 6. DEAE A-25 Sephadex-urea purificiation of $(CoA)_2$. *E. coli* $(CoA)_2$ isolated by the method of Loewen (1976) (outlined in Materials and Methods) was subjected to chromatography on a column of DEAE A-25 Sephadex eluted with a linear gradient of sodium chloride in 0.05 M Tris-HCl containing 7 M urea. Fractions of 2.5 ml were collected. The resulting $(CoA)_2$ proved to be free of any glutamic acid.

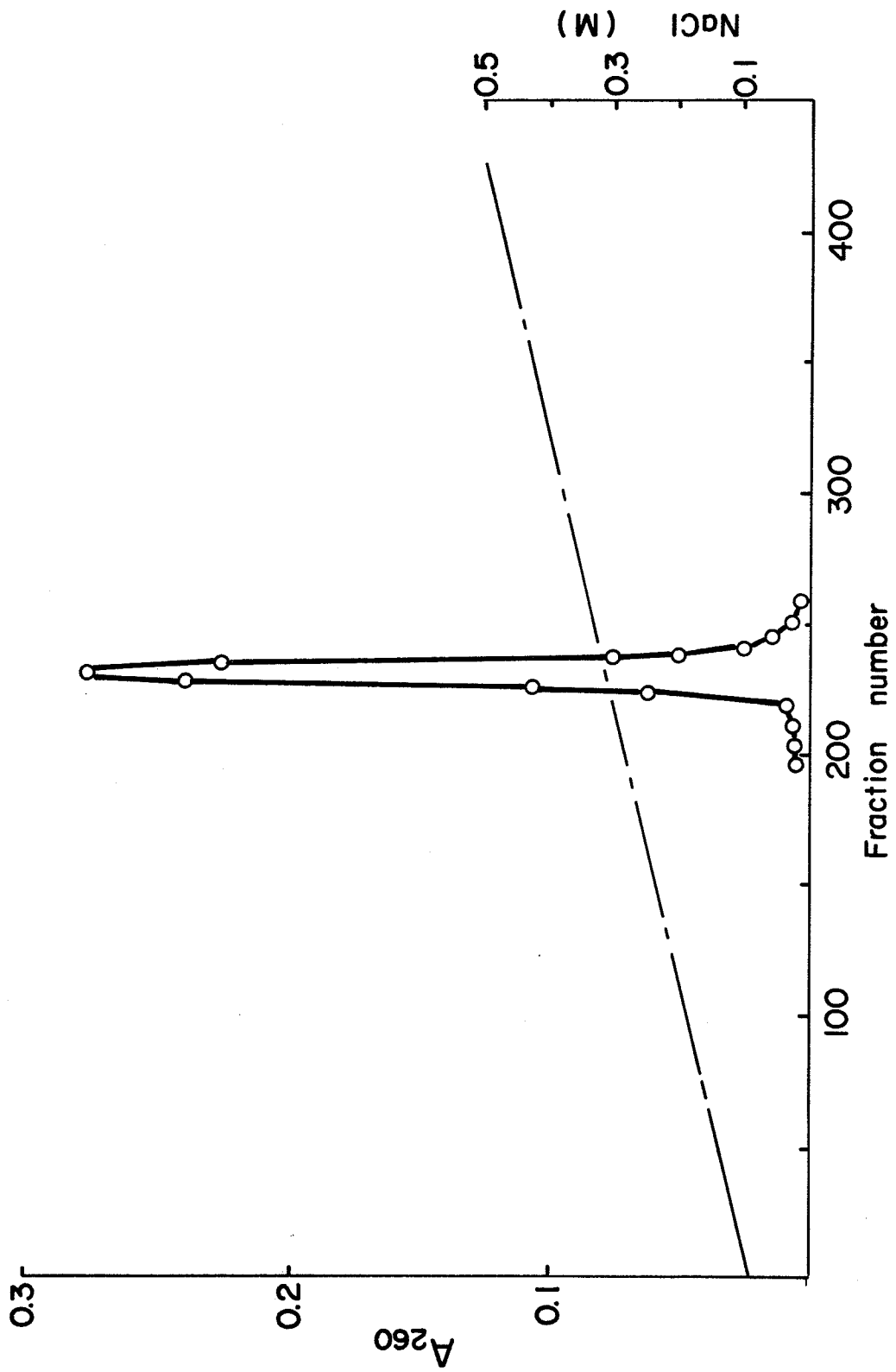

NaCl (M)
0.5
0.3
0.1
0.3
0.2
0.1
A260
100
200
300
400
Fraction number

Figure 7. DEAE A-25 Sephadex separation of $(CoA)_2$ and glutamate-containing contaminant.

$(CoA)_2$ extracted from *E. coli* B grown in LB broth was chromatographed on a 0.5 x 50 cm column of DEAE A-25 Sephadex eluted with a linear gradient of sodium chloride in 0.01 M phosphate buffer pH 7.6. Fractions (2.5 ml) were collected and 200 µl aliquots assayed with fluorescamine as in Materials and Methods.

- ○ relative fluorescence at 494 nm
- △ A_{260}
- ● A_{275}
- □ $A_{275/260}$

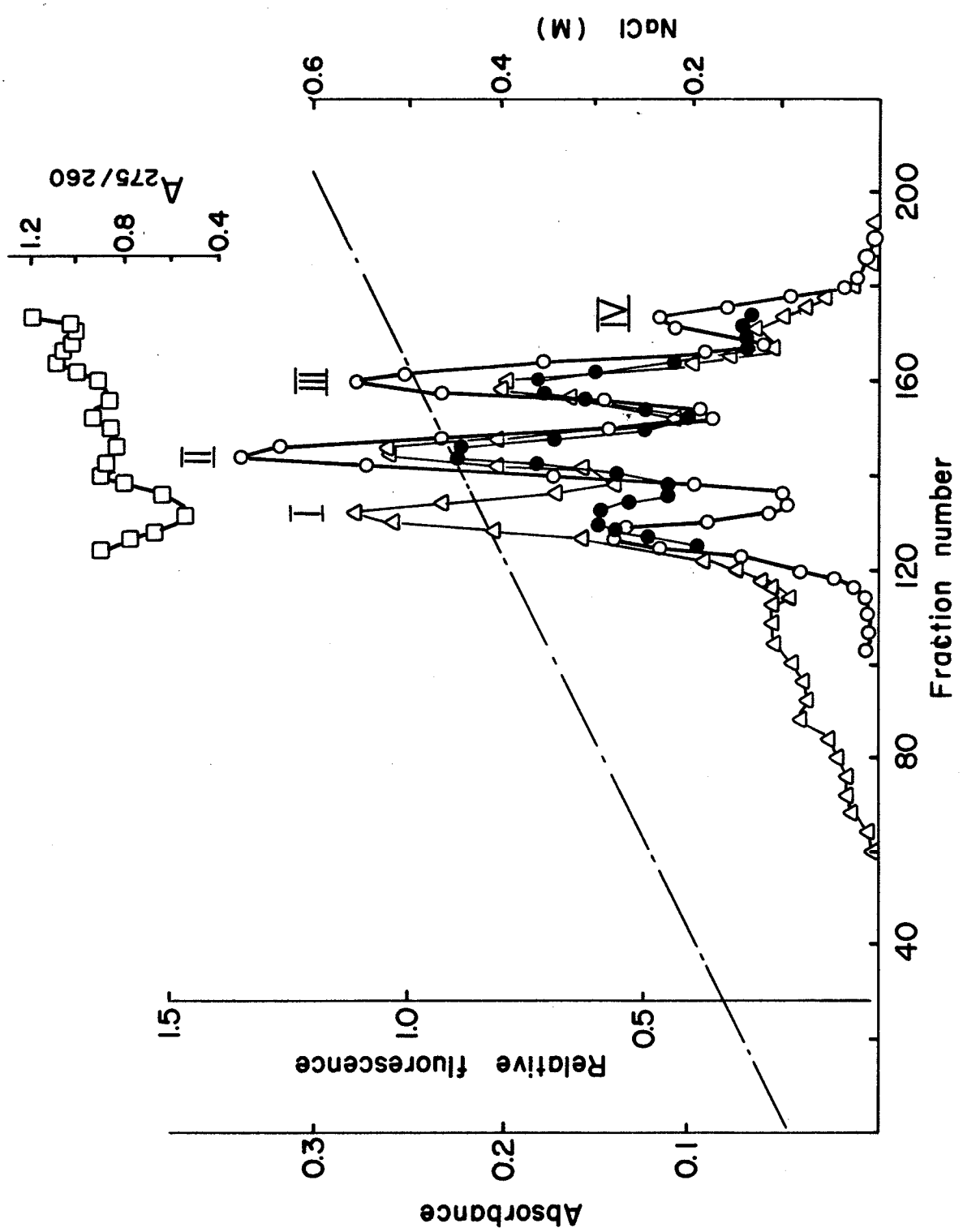
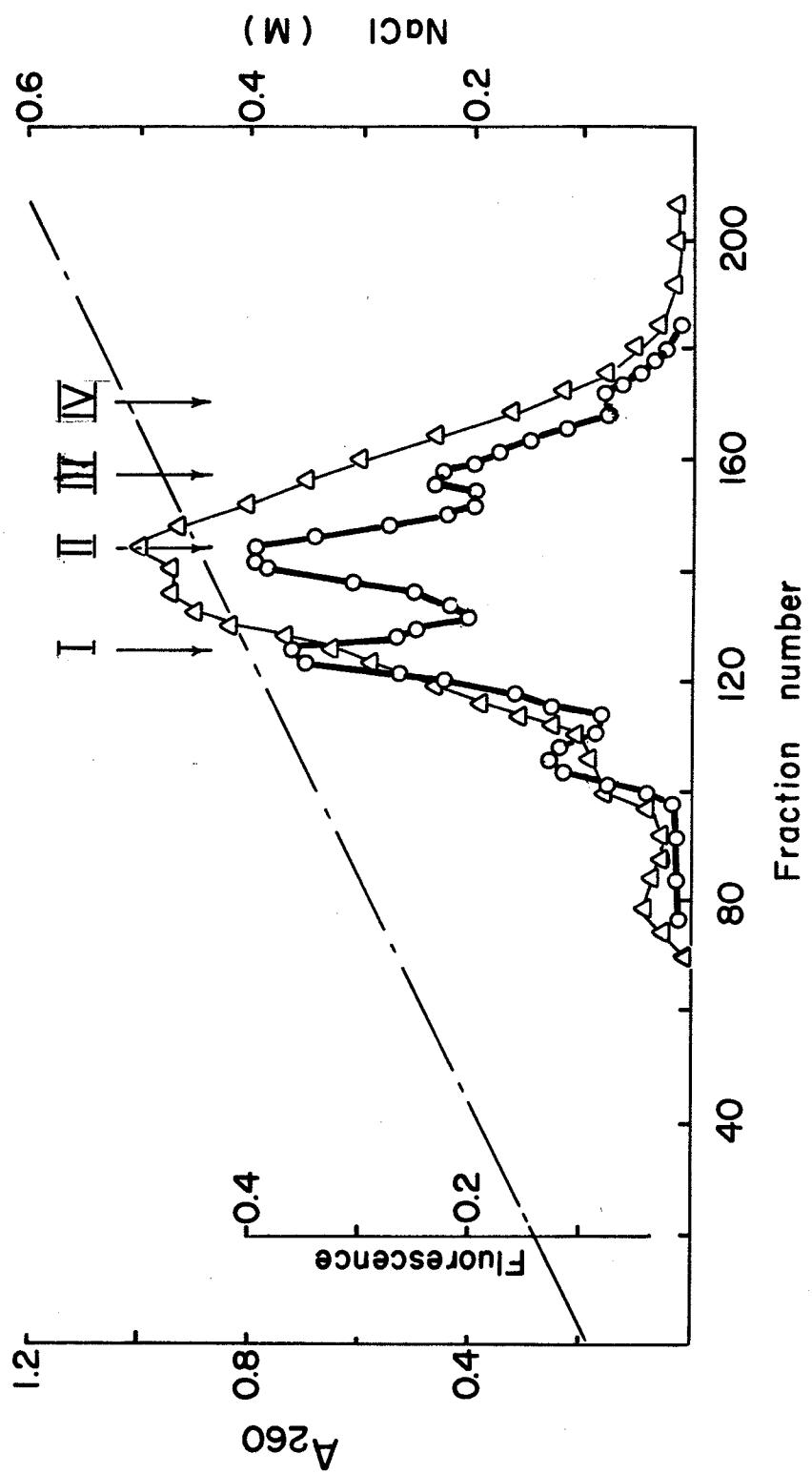

Absorbance
Relative fluorescence
NaCl (M)
Fraction number
A275/260
I
II
III
IV

Figure 8. DEAE A-25 Sephadex separation of $(CoA)_2$ and glutamate-containing contaminant.

$(CoA)_2$ extracted from *E. coli* B23 grown in minimal medium was rechromatographed on a 0.5 x 50 cm column of A-25 Sephadex eluted with a linear gradient of sodium chloride in 0.01 M phosphate buffer pH 7.6. Fractions of 2.5 ml were collected and aliquots assayed with fluorescamine.

○ relative fluorescence at 494 nm

△ A_{260}


NaCl (M)
0.6
0.4
0.2
I
II
III
IV
A260
1.2
0.8
0.4
Fluorescence
0.4
0.2
40
80
120
160
200
Fraction number

fluorescent peak.

It should be noted that the pattern of four fluorescent peaks is present in cells grown both in medium containing yeast extract (Figure 7) and in minimal medium (Figure 8).

Characterization of fluorescamine-reactive components

1) Presence of pABA

Peaks I-IV were desalted on G10 Sephadex eluted with 50 mM TEAB and concentrated before UV absorption spectra were determined (Figure 9). Peaks I and II were largely adenosine-like in the absence or presence of 0.1 M HCl, while peaks III and IV showed a strong acid-labile absorption maximum at 273 nm. The disappearance of the maximum at 273 nm in 0.1 M HCl is characteristic of pABA. In fact, the spectra of peaks III and IV were very similar to that of a pABA glu_n isolated from yeast (Ratner *et al* 1946) and identical to that determined for authentic pABA glu (Figure 11). A molar extinction coefficient of 1.45 X 10^4 M^{-1} cm^{-1} at 275 was reported for pABA glu by Ratner *et al* (1946) but, as the acid-lability of the maximum at 273 nm for authentic pABA-glu was not complete (Figure 10) the difference in molar absorption coefficient upon acidification was used in concentration measurements. Using authentic pABA glu this was determined to be (1.32 ± 0.01) X 10^4 M^{-1} cm^{-1} at 273 nm (average ± s.d. of 5 determinations).

2) Optical configuration of glutamic acid

The glutamate yielded by acid hydrolysis was shown to be in the

Figure 9. Ultraviolet absorption spectra of isolated *E. coli* polyglutamates.

The UV absorption spectra of peaks I-IV from Figure 7 were determined in water and in 0.1 M HCl and are represented in panels I-IV.

——— pH 7

– – – pH 1

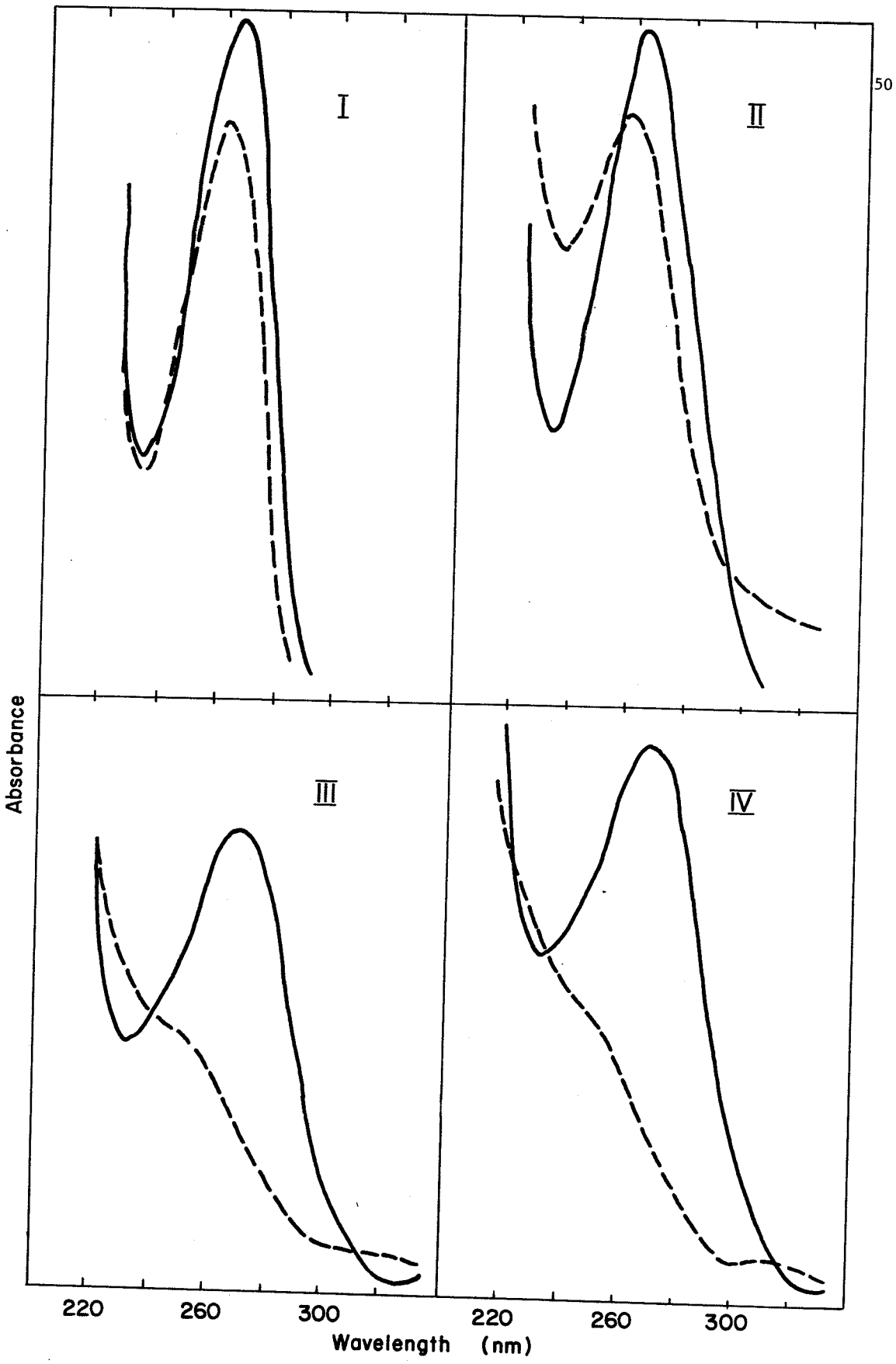
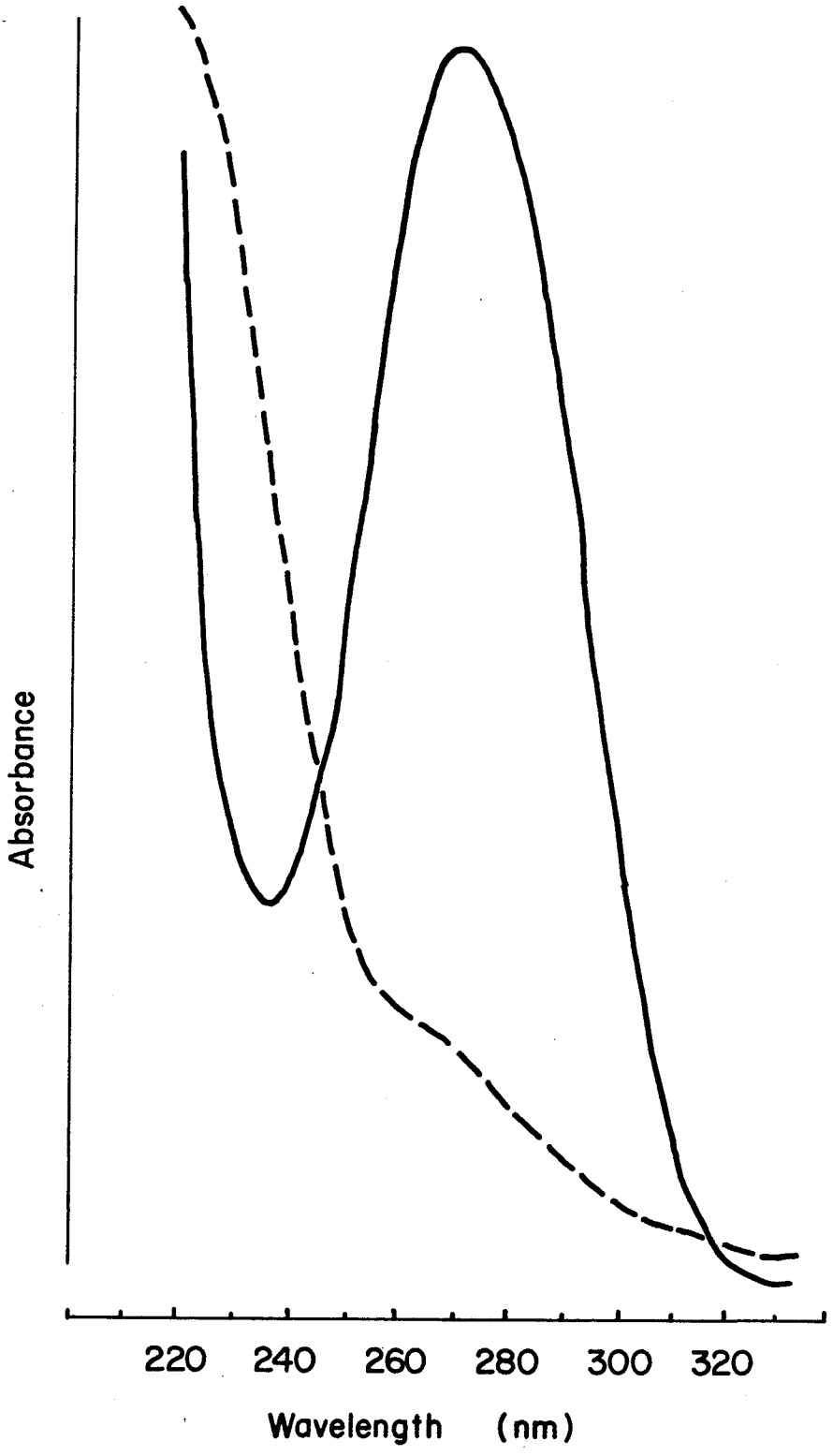

I
II
III
IV
Absorbance
220
260
300
220
260
300
Wavelength (nm)

Figure 10. Ultraviolet absorption spectrum of pABAglu.

The UV absorption spectrum of authentic pABAglu was determined in the presence and absence of 0.1 M HCl.

——— pH 7

— — — pH 1


Absorbance
220
240
260
280
300
320
Wavelength (nm)

L-configuration (Table 2). D-Glutamate is not a substrate for and is a competitive inhibitor of L-glutamate dehydrogenase (Strecker 1955) but the polymer-derived glutamate was equally as active a substrate as authentic L-glutamate. Also, it is noted that neither D- nor L-glutamate are substrates for the respective amino acid oxidases (Burton 1955; Ratner 1955).

3) Mode of peptide linkage

The mode of peptide linkage was determined to be γ rather than α by several criteria. The isolated polymer was sensitive to a chicken pancreas conjugase prepared as by Bird *et al* 1965 which contains in addition a γ-glutamyl carboxypeptidase (Rosenberg and Neumann 1974). An incubation period of five hours succeeded in releasing most of the glutamate, as determined by two dimensional thin layer chromatography in solvents A and B. The facility of hydrolysis under acid conditions also suggested that the glutamyl residues were linked through a γ-peptide bond (Waley 1955; Troy 1973b). Hydrolysis in 1 M HCl for two hours was sufficient to completely hydrolyse the polymer and an authentic poly(γ-glutamate), while authentic poly(α-glutamate) required more than six hours under these conditions to effect complete hydrolysis. Analytical elution profiles of partial hydrolysates of these standards are shown in Figure 11.

4) Linkage of pABA

pABA was shown to be linked to the amino terminus of the peptide through its acid carboxyl. This was inferred from the fact that when

TABLE 2

Susceptibility of glutamate samples to oxidation by glutamate dehydrogenase.[a]

Substrate	Concentration[b] (μM)	Initial Velocity (ΔA_{340} min^{-1})	Relative Velocity $\Delta A_{340} min^{-1} \mu mol\ glu^{-1}$
D-Glu	200	0.00	0.00
L-Glu	87.5	0.046	0.52
acid hydrolyzed polymer	97.9	0.049	0.50

[a]Reaction conditions as in Materials and Methods.

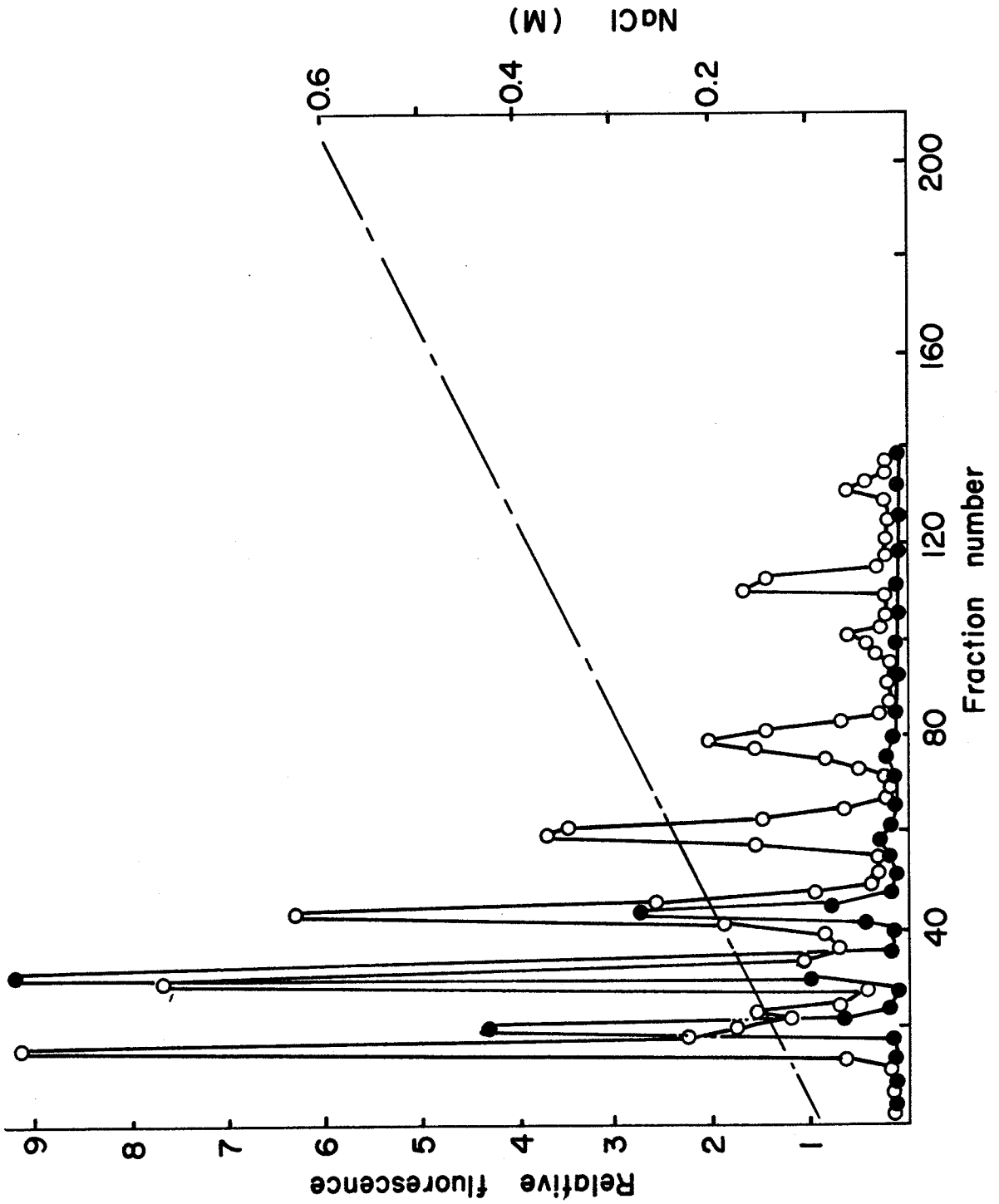
[b]Concentrations determined using amino acid analyzer.

Figure 11. Analytical ion exchange chromatography of partial hydrolysates of authentic poly(glutamic acid).

Samples (5 mg) of authentic poly(γ-L-glu) and poly(α-L-glu) were hydrolyzed in 1 M HCl at 100°C for 45 min and 4 h respectively. After removal of acid samples were chromatographed separately on a 0.5 x 50 cm DEAE A-25 Sephadex column eluted with a linear gradient of NaCl in 0.01 M potassium phosphate pH 7.6. Fractions of 2.5 ml were collected and 1 ml aliquots were assayed with fluorescamine.

○ poly(γ-L-glu)

● poly(α-L-glu)


NaCl (M)
0.6
0.4
0.2
Relative fluorescence
1
2
3
4
5
6
7
8
9
40
80
120
160
200
Fraction number

partially hydrolysed authentic poly(γ-glutamate) was chromatographed on the same 0.5 x 50 cm A25 Sephadex column much larger quantities (1-5 mg, before hydrolysis) were required for an observable fluorescamine reaction (pH 9.0) than were present in entire preparations of the isolated peptide (75 - 100 μg). Indeed, glutamic acid yielded a comparatively poor fluorescent product with fluorescamine, exhibiting 3000-fold less fluorescence than pABA or pABA-glu fluorescamine products at pH 7.6 (Table 3). This suggested that pABA was linked to the amino terminus of the polyglutamate.

Other evidence indicated that the pABA amine was free. Table 3 shows that the isolated polymer can be diazotized and coupled to a dye by the method of Bratton and Marshall (1939), which is specific for aryl amines. The reactivity of pABA, pABA-glu and glu are also shown for comparison. A good indicator of the availability of the pABA amine is the disappearance of the UV absorbance maximum at 273 nm (Figure 10). This is due to the ionization of the arylamine in acid (Ratner *et al* 1946) and shows that it is unbound. In addition the pattern of partial acid hydrolysis products from polymers I-IV did not correlate with the partial hydrolysis pattern of authentic poly(γ-glutamate) shown in Figure 11. This would indicate that the polymers I-IV are not strictly polyglutamates but do have pABA attached at the amino terminus.

5) Size of the glutamate chain

All four fractions I-IV shown in Figures 7 and 8 were subjected

TABLE 3

Reactivity of primary amines to specific reagents.

Amine-specific reagent[a]	Amine-containing Compound	Relative Reactivity[b]
fluorescamine	L-glu (ph 7.6)	0.0003
	L-glu (pH 9.0)	0.06
	pABAglu (pH 7.6)	1.16
	E. coli polymer[c]	1.08
diazotization/ dye coupling	L-glu	0
	pABAglu	1.00
	E. coli polymer[c]	0.91

[a] reaction conditions and protocols as in Materials and Methods, units measured are A_{556} for azo dye formation, "relative fluorescence" at 494 nm for fluorescamine reaction.

[b] reactivity relative to pABA, calculated per μmol free amine.

[c] isolated *E. coli* polymer concentration based on content of pABA measured by acid-labile A_{270}.

to partial acid hydrolysis and chromatographed on the 0.5 x 50 cm DEAE Sephadex A-25 column as shown in Figures 12-15. The locations of authentic pABA and pABA glu are shown and correspond well with two of the hydrolysis products. The elution position of free glutamate is also shown. By simply counting the number of hydrolysis products relative to pABA monoglutamate, it can be seen that peaks I-IV correspond to $pABAglu_5$ - $pABAglu_8$ respectively. In addition, the positions of peaks I-IV in Figure 7 align with the four larger products of hydrolysis of peak IV, again indicating a range of glutamate chain length of from 5 to 8.

As chicken pancreas conjugase contains primarily an endopeptidase activity yielding $pteglu_2$ and $glu_{(n-2)}$ from $pteglu_n$ (Leichter *et al* 1977), a limited digest was not possible. However, conjugase digestion for one hour, as shown in Figure 16, yielded a product corresponding well to the expected elution position of $pABAglu_2$.

In addition, all of the fractions were hydrolysed completely in acid and their glutamate content was determined with an amino acid analyzer. As shown in Table 4, the ratios of glutamate to pABA thus determined correlate with those assigned from the partial hydrolysis elution profiles.

Comparison With Yeast $pABAglu_n$

An attempt was made to isolate the $pABAglu_{10-11}$ reported by Ratner *et al* (1946) for comparison to the *E. coli* pABA glu_{5-8}. Isolation was as for the *E. coli* $pABAglu_n$, and the final DEAE Sephadex A-25

Figure 12. Analytical ion exchange chromatography of partial hydrolysis products of peak I.

Peak I (45 nmol) was hydrolyzed in 1 M HCl at 100°C for 45 min and freed of acid. The partial hydrolysate was chromatographed on a 0.5 x 50 cm DEAE A-25 Sephadex column eluted with a linear gradient of sodium chloride in 0.01 M phosphate buffer pH 7.6. 2.5 ml fractions were collected and 1 ml aliquots assayed with fluorescamine. Elution positions of standard L-glu, pABA and pABAglu are shown. Beginning with pABAglu, the peaks eluting at progressively higher salt concentrations are $pABAglu_2$, $pABAglu_3$, $pABAglu_4$ and $pABAglu_5$ A small amount of $pABAglu_6$, present as a cross-contaminant from peak II, is also apparent.

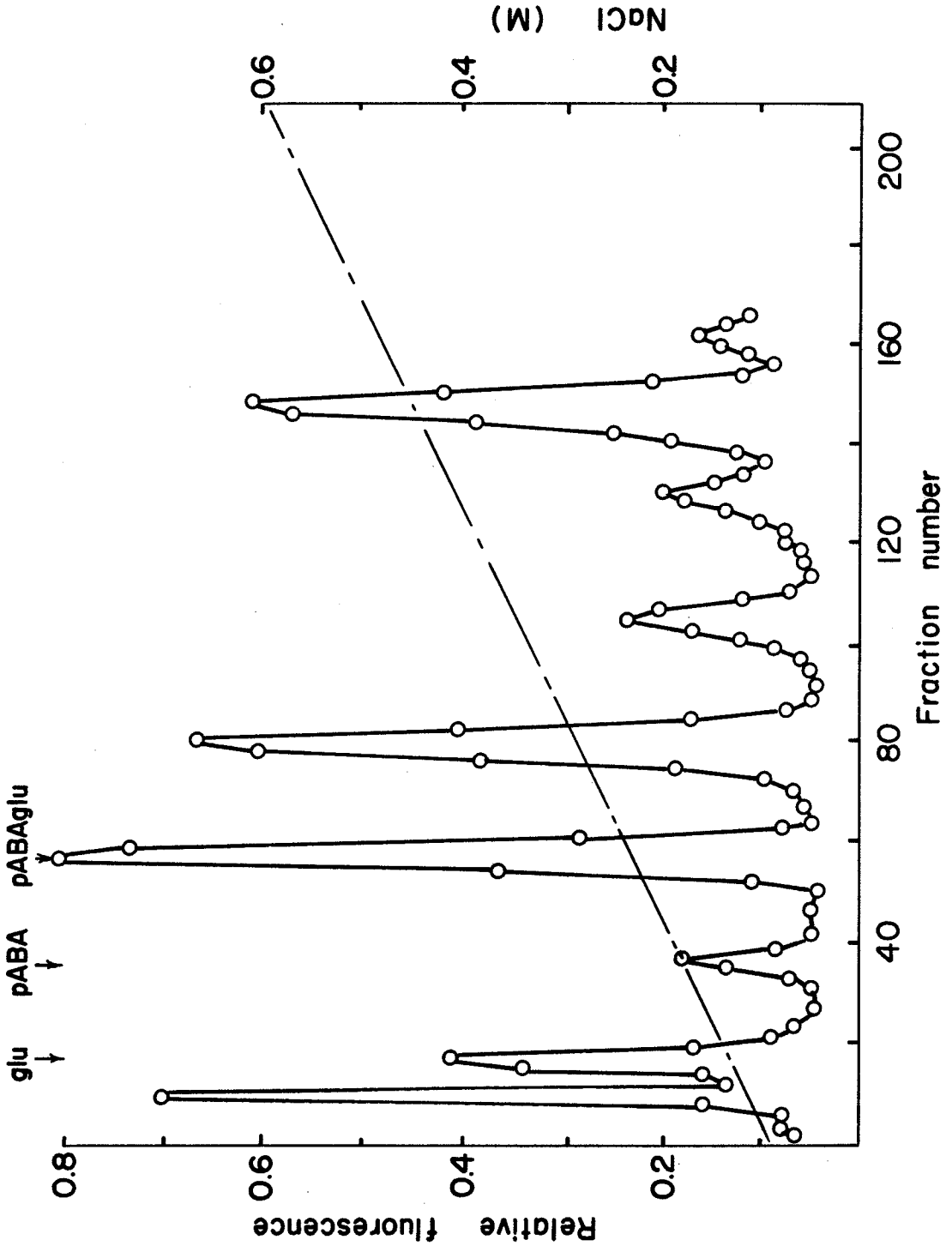

Relative fluorescence
0.8
0.6
0.4
0.2
glu
pABA
pABAglu
40
80
120
160
200
Fraction number
NaCl (M)
0.6
0.4
0.2

Figure 13. Analytical ion exchange chromatography of partial hydrolysis products of peak II.

Peak II (60 nmol) was partially hydrolyzed and chromatographed on the analytical DEAE A-25 Sephadex column as described in the legend to Figure 12. The major fluorescamine-reactive peaks represent pABA glu-pABAglu$_6$.

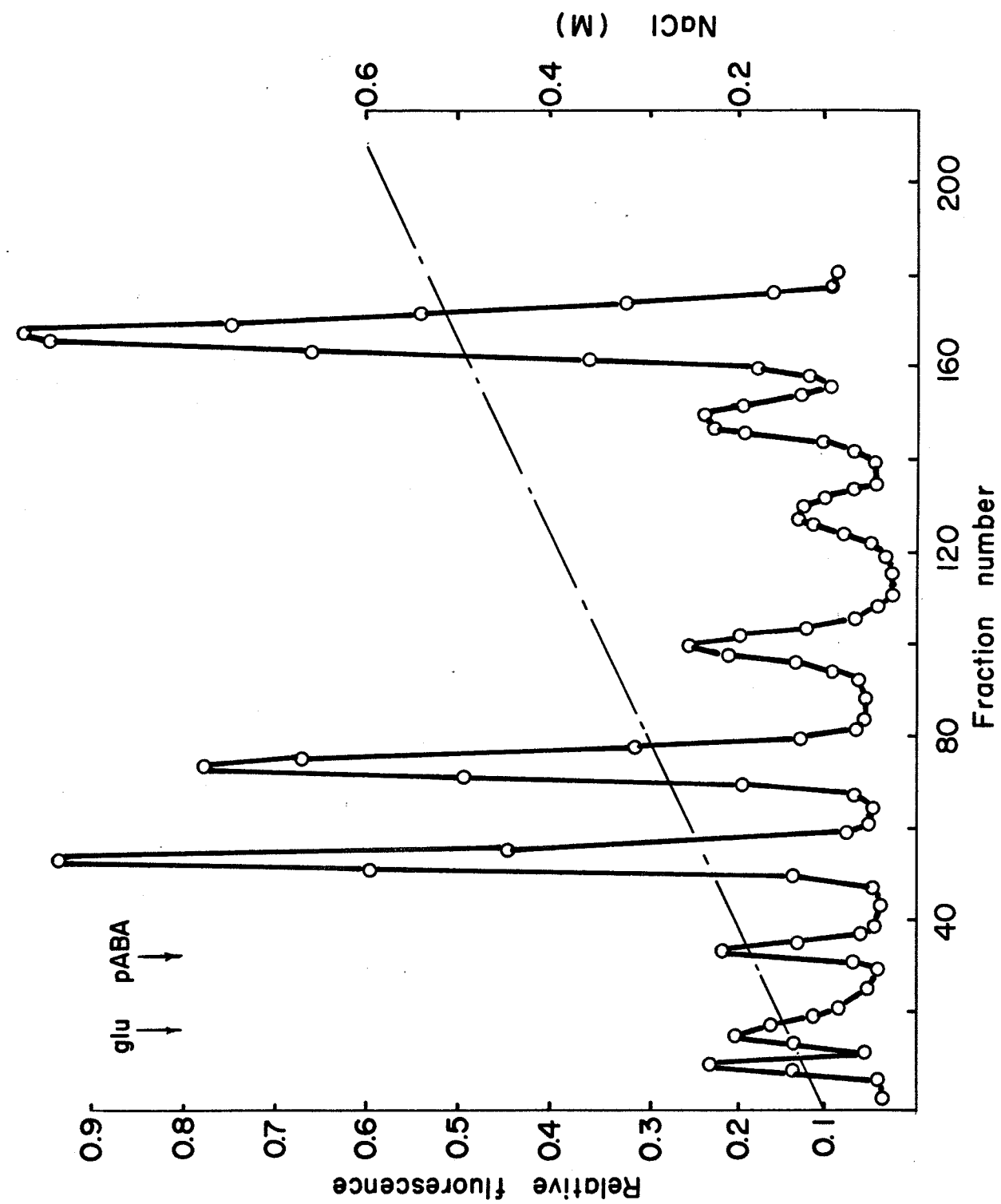

NaCl (M)
0.6
0.4
0.2
Relative fluorescence
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
glu
pABA
40
80
120
160
200
Fraction number

Figure 14. Analytical ion exchange chromatography of partial hydrolysis products of peak III.

Peak III (70 nmol) was partially hydrolyzed and the hydrolysate chromatographed on the analytical DEAE A-25 Sephadex column as described in the legend to Figure 12. The major fluorescamine-reactive peaks represent pABAglu - $pABAglu_7$.

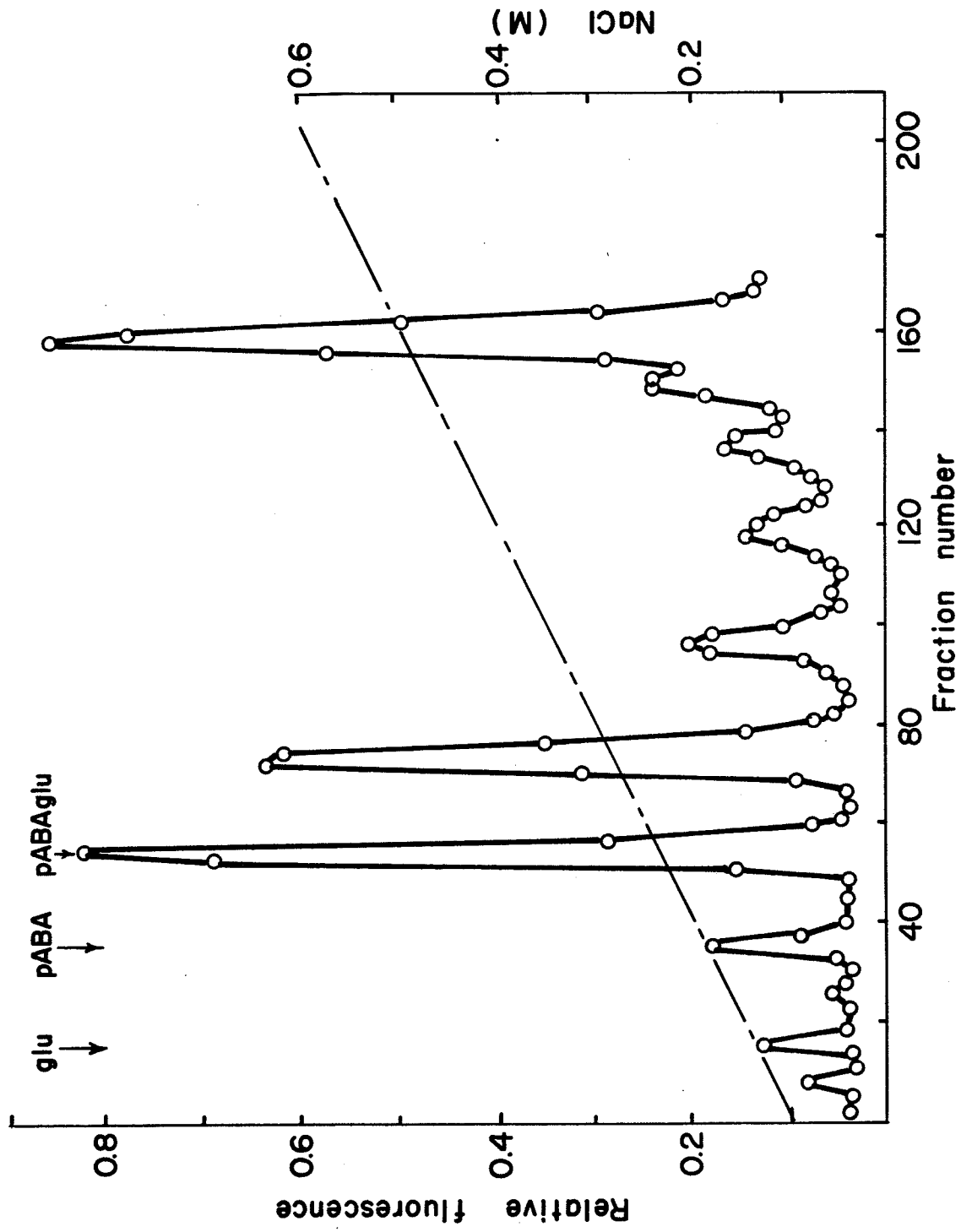

Relative fluorescence
0.8
0.6
0.4
0.2
glu
pABA
pABAglu
40
80
120
160
200
Fraction number
NaCl (M)
0.6
0.4
0.2

Figure 15. Analytical ion-exchange chromatography of partial hydrolysis products of peak IV.

Peak IV (55 nmol) was partially hydrolyzed and chomatographed on the analytical DEAE A-25 Sephadex column described in the legend to Figure 12. The major fluorescamine-reactive products are pABAglu to $pABAglu_8$.

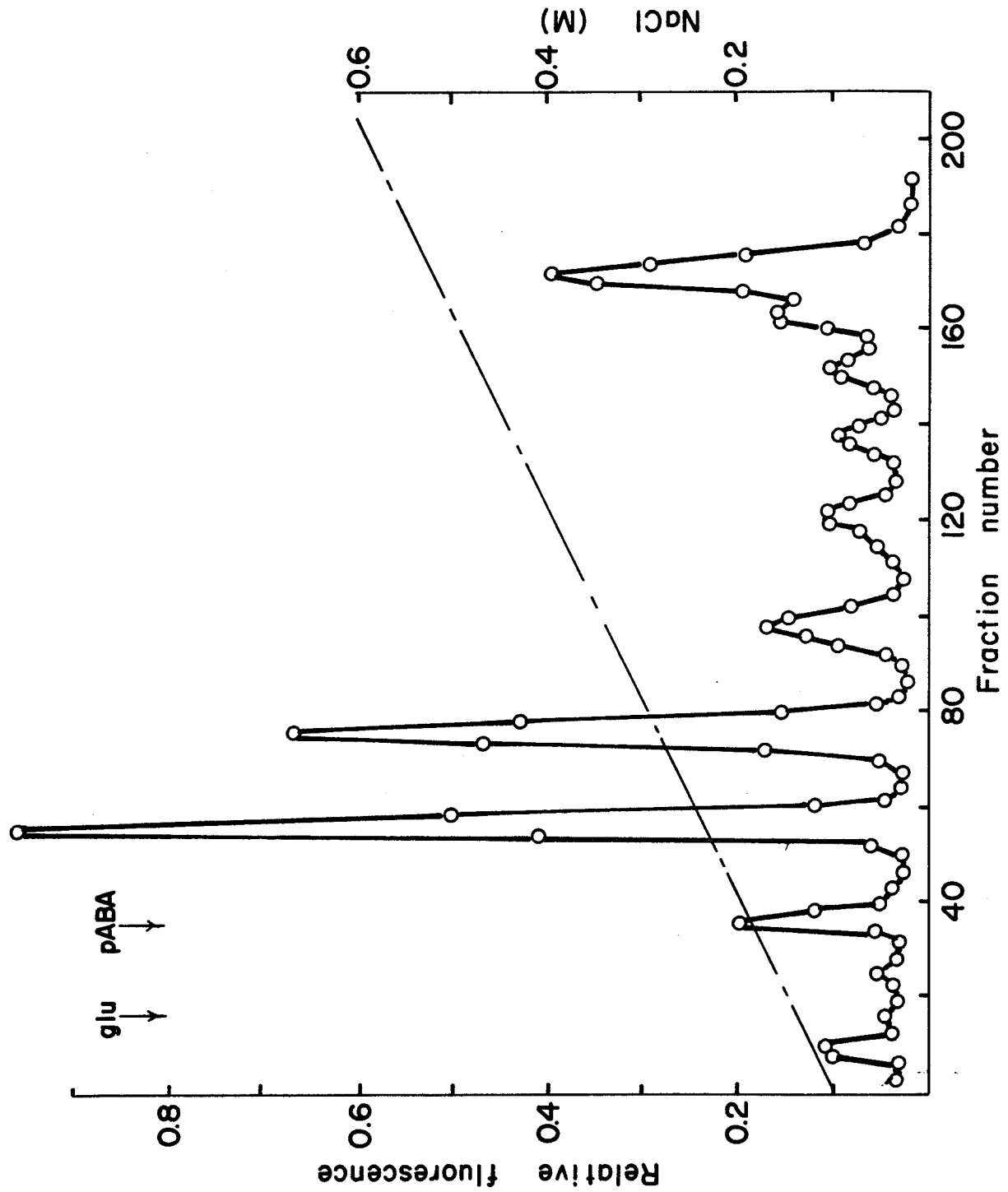

Relative fluorescence
0.2
0.4
0.6
0.8
glu
pABA
40
80
120
160
200
Fraction number
0.2
0.4
0.6
NaCl (M)

Figure 16. Analytical ion exchange chromatography of products of conjugase digestion of peak I.

Peak I (70 nmol) was subjected to digestion by chicken pancreas conjugase at 37°C for 5 h. The mixture was boiled and chromatographed on a 0.5 x 50 cm DEAE A-25 Sephadex column eluted with a linear gradient of sodium chloride in 0.01 M potassium phosphate pH 7.6. Aliquots (1 ml) of the 2.5 ml fractions were assayed with fluorescamine. The elution positions of standard glu, pABA and pABAglu are marked. The other peaks correspond to $pABAglu_2$ and $pABAglu_5$

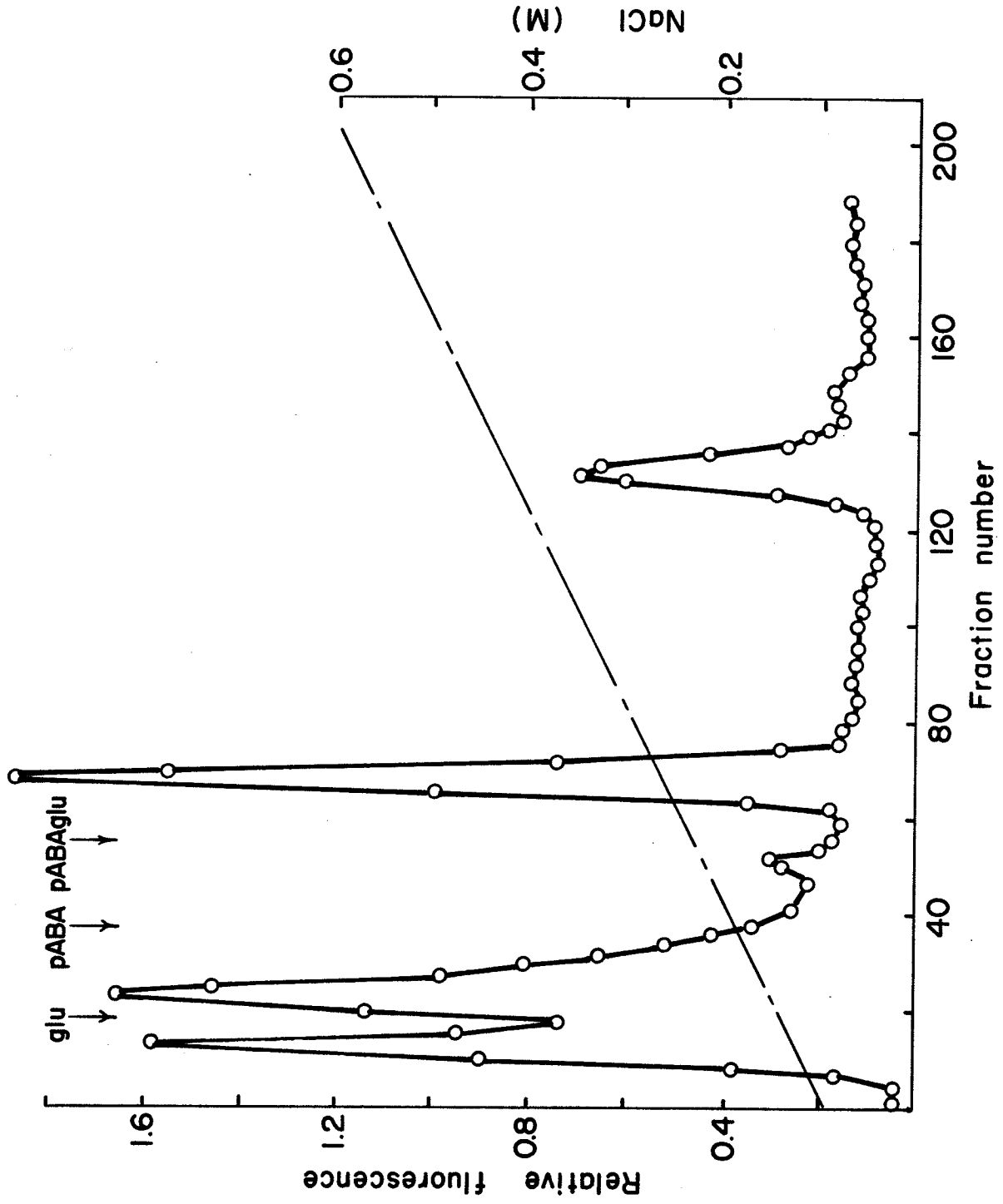

Relative fluorescence
0.4
0.8
1.2
1.6
glu
pABA
pABAglu
40
80
120
160
200
Fraction number
NaCl (M)
0.2
0.4
0.6

TABLE 4

Quantitation of glutamic acid/pABA ratios in pABA glu_n[a].

Sample		molar ratio glutamic acid/pABA
yeast (Figure 17)		6.97
E. coli (Figure 7)	I	_[b]
	II	_[b]
	III	6.81
	IV	8.01
limit product of conjugase digest (Figure 16)		2.08
acid hydrolysis products Figure 12-15)	1	1.07
	2	2.37
	3	3.26
	4	7.29
	5	8.23
	6	6.02

[a]Results are averages of several determinations carried out as described in Materials and Methods.

[b]The pABA content of peaks I and II could not be readily determined due to the presence of large amounts of $(CoA)_2$.

column pattern is shown in Figure 17. There is one major peak of fluorescamine-reactive material. This has a shoulder indicating that material of a smaller size is also present. The presence of pABA is indicated by its UV absorption spectrum (Figure 18). The peak was subjected to partial acid hydrolysis (Figure 19) and amino acid analysis for comparison of glutamate to pABA content (Table 4). Both procedures indicate that the material is predominantly $pABAglu_7$, in conflict with the value reported by Ratner *et al* (1946) but consistent with the fact that yeast folates are predominantly heptaglutamates (Pfiffner *et al* 1946; Bassett *et al* 1976a).

E. coli $pteglu_n$ content

A soluble extract of stationary phase *E. coli* was made using a procedure designed to protect cellular folates from degradation (Shane 1980a). The extracted $pteglu_n$ were reductively cleaved to their corresponding $pABAglu_n$ and the size distribution of these was examined by analytical ion exchange chromatography (Figure 20). While there are several lengths of $pABAglu_n$ apparent, it should be noted that the pattern of $pABAglu_5$-$pABAglu_8$ (peaks D-G) is very similar to that found for $pABAglu_5$-$pABAglu_8$ co-purifying with $(CoA)_2$ (peaks I-IV, Figures 7 and 8). Also the amount of fluorescamine-reactive material per gram bacteria in peaks D-G (Figure 20) is almost equal to that in peaks I-IV (Figure 7). These data are not inconsistent with the conjecture that the $pABAglu_n$ co-purifying with $(CoA)_2$ are derived from their corresponding $pteglu_n$. Shorter chain lengths, with the exception of the diglutamate,

Figure 17. Isolation of yeast $pABAglu_n$ on DEAE A-25 Sephadex. Dried baker's yeast was extracted with formic acid and the extract chromatographed on a 2.5 x 30 cm DEAE A-25 Sephadex column eluted with TEAB as described in Materials and Methods. The peak from the 2.5 x 30 cm column corresponding to *E. coli* $(CoA)_2$ was rechromatographed on a 0.5 x 50 cm column of DEAE A-25 Sephadex eluted with a linear gradient of sodium chloride in 0.01 M potassium phosphate pH 7.6. Fractions of 2.5 ml were collected and 50 µl aliquots were assayed with fluorescamine.

○ relative fluorescence

● A_{275}

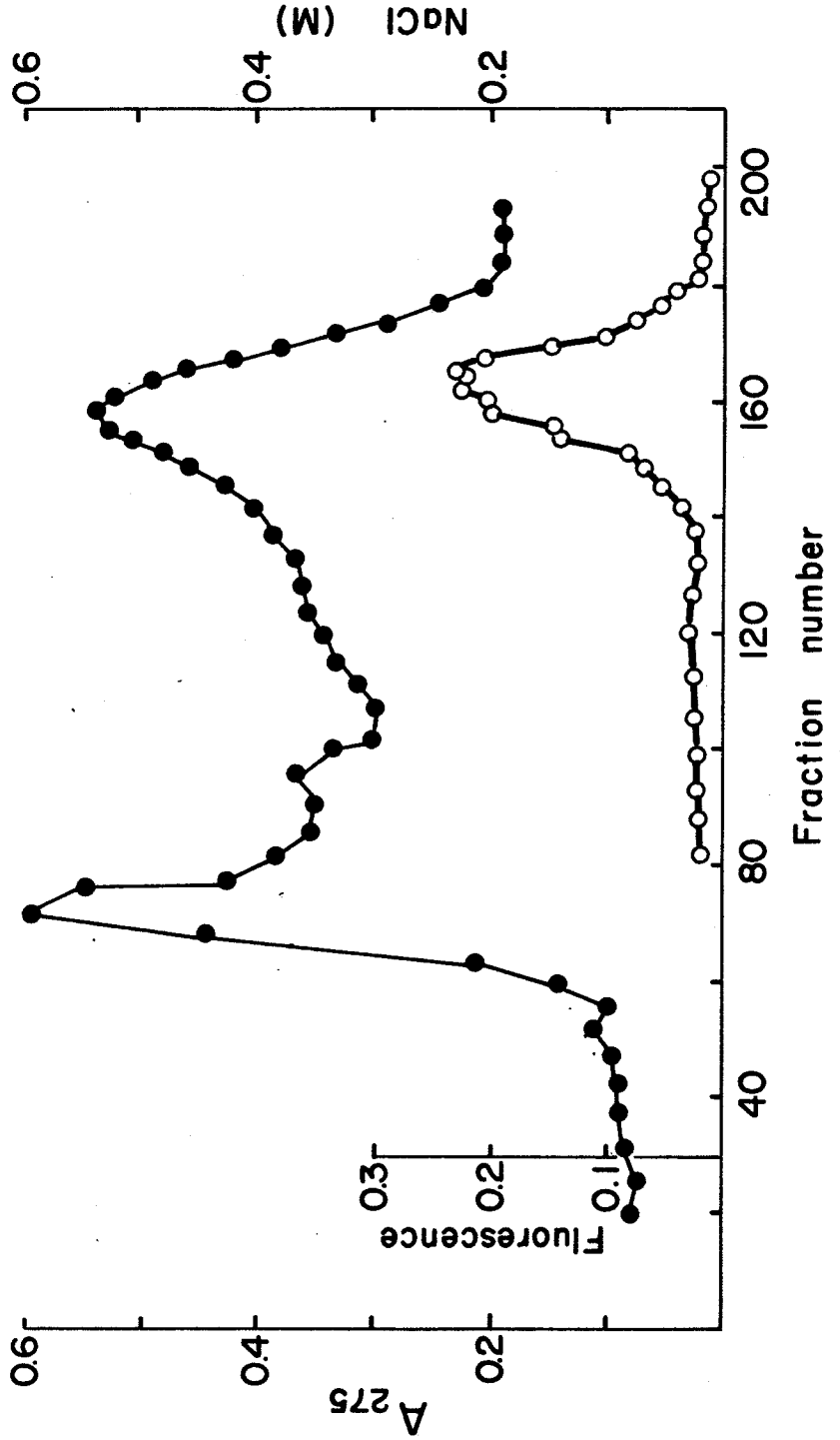

NaCl (M)
0.6
0.4
0.2
A 275
0.6
0.4
0.2
Fluorescence
0.3
0.2
0.1
40
80
120
160
200
Fraction number

Figure 18. Ultraviolet absorption spectrum of yeast $pABAglu_n$

The UV absorption spectrum of the fluorescamine-reactive peak of Figure 17 was determined in water and in 0.1 M HCl.

——— pH 7

– – – pH 1

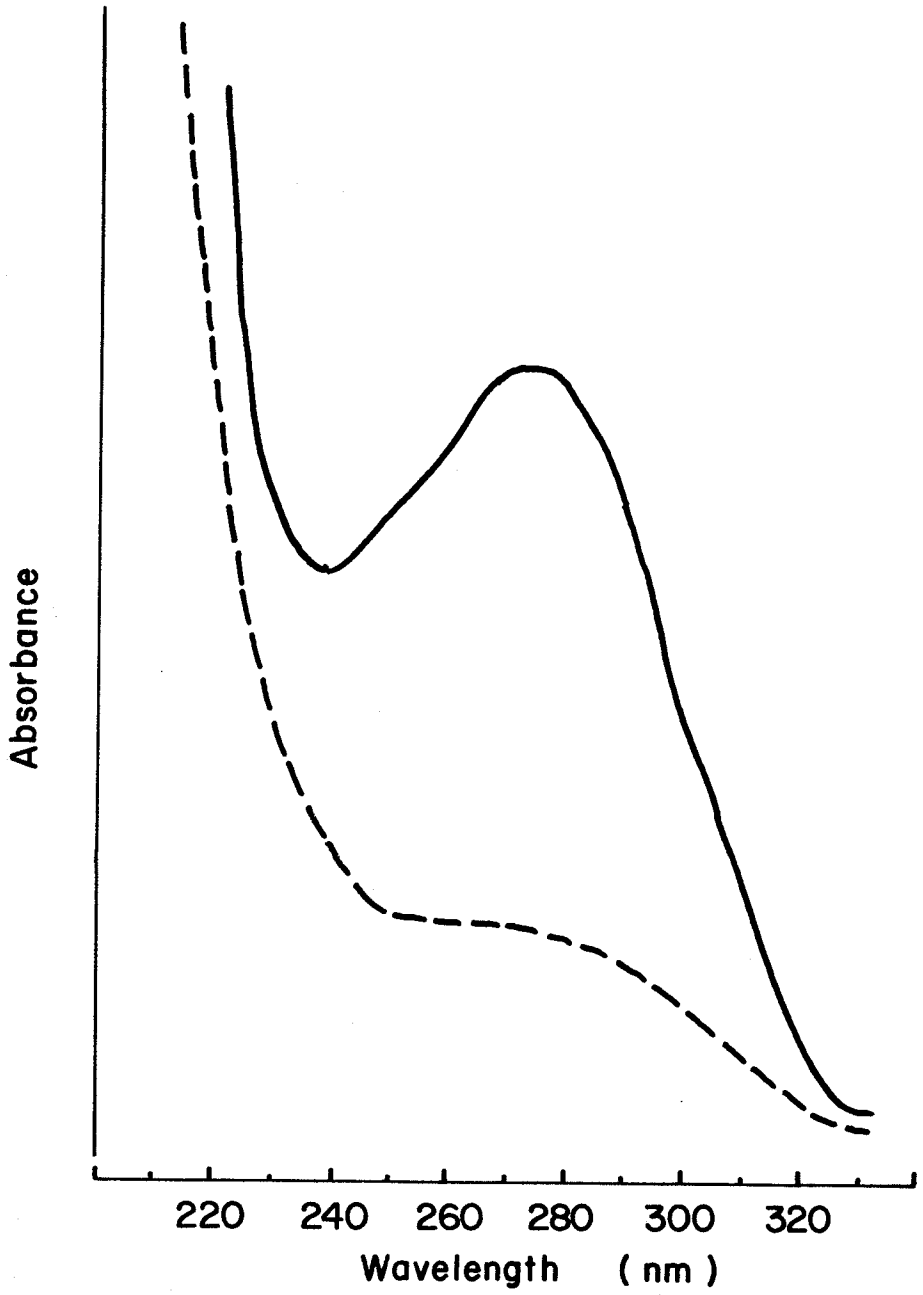

Absorbance
220
240
260
280
300
320
Wavelength (nm)

Figure 19. Analytical ion-exchange chromatography of partial hydrolysis products of isolated yeast $pABAglu_n$.

Yeast $pABAglu_n$ (60 nmol) was partially hydrolyzed in 1 M HCl at 100°C for 45 min and freed from acid. The hydrolysate was chromatographed on a 0.5 x 50 cm column of DEAE A-25 Sephadex eluted with a linear gradient of sodium chloride in 0.01 M potassium phosphate pH 7.6. 2.5 ml fractions were collected and 1 ml aliquots assayed with fluorescamine. The positions of standard glu and pABA are shown. The major fluorescamine-reactive products correspond to pABAglu to $pABAglu_7$.

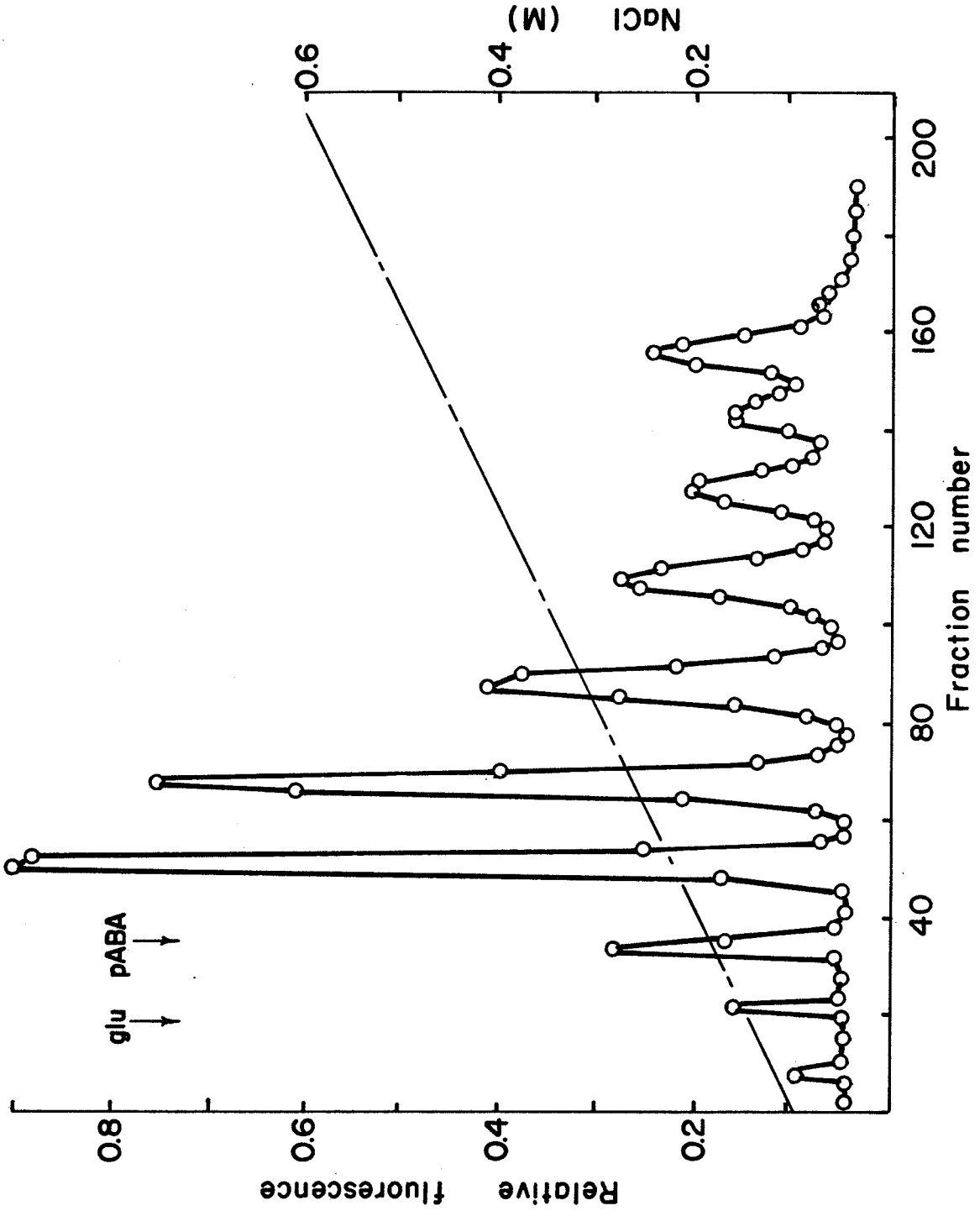
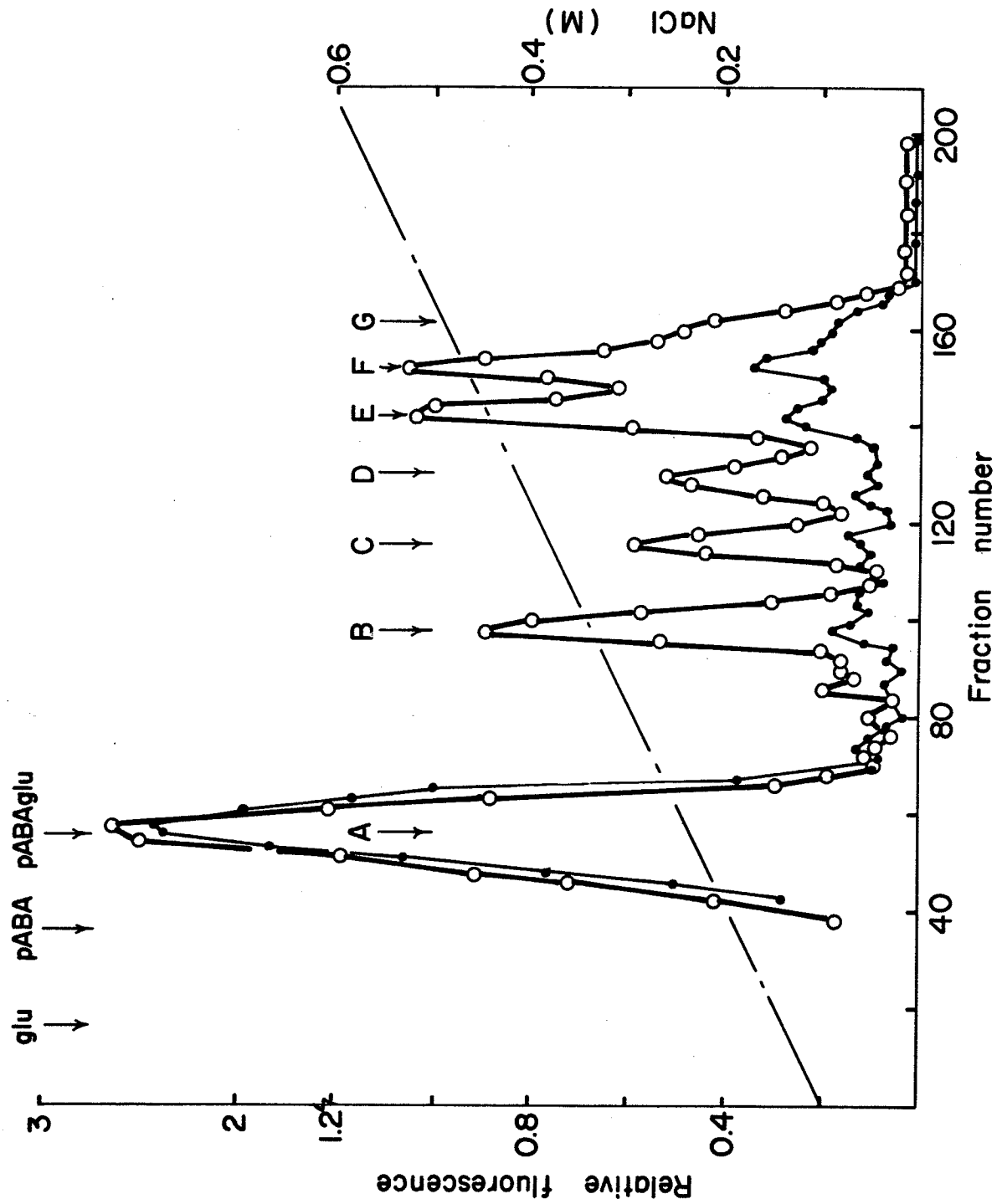

Relative fluorescence
0.2
0.4
0.6
0.8
glu
pABA
40
80
120
160
200
Fraction number
NaCl (M)
0.2
0.4
0.6

Figure 20. Analytical ion-exchange chromatography of $pABAglu_n$ derived from reductive cleavage of *E. coli* $pteglu_n$.

As described in Materials and Methods, *E. coli* B (30 g) grown in LB broth was extracted in the presence of 0.2 M β-mercaptoethanol to preserve cellular folates. Half of the extract was treated with zinc in HCl to cleave $pteglu_n$ to their corresponding pABAglu. These $pABAglu_n$ were isolated, as were endogenous $pABAglu_n$ in the untreated half of the extract, and chromatographed on the analytical DEAE A-25 Sephadex column as described in the legend to Figure 19. Aliquots of 1 ml were assayed with fluorescamine.

○ cleaved extract

● uncleaved extract


glu
pABA
pABAglu
A
B
C
D
E
F
G
Relative fluorescence
3
2
1.2
0.8
0.4
NaCl (M)
0.6
0.4
0.2
40
80
120
160
200
Fraction number

are also present in the folate pool but do not co-chromatograph with $(CoA)_2$.

The distribution of $pteglu_n$ chain lengths shown in Figure 20 is in disagreement with other published data (Table 1), which indicate very small amounts of $pteglu_6$ and $pteglu_7$ and no detectable $pteglu_8$.

It is also apparent from Figure 20 that while $pABAglu_n$ are present in uncleaved cell extracts their quantity is insufficient to account for all of the $pABAglu_n$ in the $(CoA)_2$ preparation. This pABAglu must, therefore, be in large part a product of $pteglu_n$ degraded during the isolation procedure and not a component of the extracted cells.

DISCUSSION

DISCUSSION

Loewen (1976, 1977) reported the presence of a glutamate-containing CoA-disulfide compound in *E. coli*. Because of a large degree of variation in glutamate content it was felt that this component may have been a co-purifying contaminant. This study has confirmed this suspicion, and characterized the contaminant. It consists of a mixture of p-aminobenzoyl poly(γ-L-glutamates) containing 5,6,7 and 8 glutamate residues. These compounds were present in both *E. coli* B and a strain of *E. coli* K12 (821, a *gsh* mutant of AB1157). The pABA glu_{5-8} were present in *E. coli* grown in both a rich yeast-extract-containing medium and a minimal glucose-salts medium. Qualitatively the results are similar although there are quantitative differences, especially in the content of pABA-glu_7 (Figures 7 and 8). This may possibly be due to the presence of pABAglu_7 in the yeast extract, but this was not investigated as the difference was only quantitative.

The characterization of the compounds was based primarily on their ultraviolet light absorption and amino acid analysis. The UV spectrum indicated the presence of pABA at the amino terminus of the peptide. The ionization of the arylamine in acid (inferred from the spectral characteristics) and the chemical reactivity of the arylamine confirmed this. Glutamic acid was shown to be present by thin layer chromatography and through the use of an automated amino acid analyzer. It was shown to be L-glutamic acid by utilizing the stereochemical specificity of

L-glutamic acid dehydrogenase. The peptide linkage was shown to involve the α-amine and γ-carboxyl groups of neighbouring glutamyl residues by virtue of its facility of hydrolysis in acid.

The determination of polyglutamate chain lengths was based on the fact that each increment of chain length results in a change in charge of -1 at a pH greater than about 3(pK'αCOOH = 2.19, (Lehninger 1970)).

Each compound was subjected to a limited acid hydrolysis which yielded $pABAglu_n$ of intermediate sizes ranging down to $pABAglu_1$. These were separated on an analytical anion exchange column and glutamate chain length was determined by counting the number of intermediate hydrolysis products (Figures 12-15). Under the conditions used, the amine-specific fluorometric assay was much more sensitive to fragments with an amino terminal pABA than to polyglutamates alone, so the latter products gave little interference with this method. This procedure proved to be precise (as only integer results were possible) as well as reproducible. Yeast $pABAglu_7$ was isolated and the presence of seven glutamyl residues was confirmed with this technique, in agreement with the work of Pfiffner *et al* (1946) and Bassett *et al* (1976a).

Confirmation of these results using chemical and physical quantitation of glutamic acid and pABA was also attempted (Table 4). The inability to free $pABAglu_5$ and $pABAglu_6$ from substantial amounts of $(CoA)_2$ is shown by the strong adenosine character of their UV absorption spectra (Figure 9). This interfered with the quantitation of pABA in these two cases and hence an inability to confidently determine the ratio of pABA to glutamate. In determinations of chain length of partial acid hydrolysis

products, the results for intermediates $pABAglu_3$ to $pABAglu_5$ were higher than expected (Table 4). It is possible that the other products of partial hydrolysis, particularly glu-$(\gamma$-glu$)_3$ to glu-$(\gamma$-glu$)_5$ are present in these fractions (Figure 11). Although these contaminants would not interfere significantly in the hydrolysate elution profile they would likely co-chromatograph with the $pABAglu_n$ through the G-10 and G-50 Sephadex gel filtration columns used to prepare the samples for amino acid analysis. Such extra polyglutamate would indeed result in high glutamate: pABA ratios. In addition there was a steady decline in absorbance at 273 nm of samples that had been stored at -20^oC. This would indicate a decomposition, at least of the pABA portion of the molecule, but not necessarily of the poly(γ-glutamate) portion. Such a differential loss of assayable material would lead to fractional overestimation of the glutamate: pABA ratio by these menas. However, because the loss is not great it is hard to imagine the loss of an entire molecular population. Any remaining $pABAglu_n$ would yield a correct glutamate: pABA ratio if subjected to partial hydrolysis and analytical ion exchange chromatography.

In general, $pABAglu_n$ are catabolites of their corresponding $pteglu_n$ resulting from cleavage of the C^9-N^{10} bond (Stokstad *et al* 1947). For this reason the cellular $pteglu_n$ pool was examined. Glutamate chain length of the extracted folates was determined by reductive cleavage to $pABAglu_n$, and analytical ion exchange chromatography. The reductive cleavage does not result in cleavage of γ-glutamyl peptide bonds (Lewis and Rowe, 1979). The resulting $pABAglu_n$ profile (Figure 20) is of interest from several viewpoints. First, the folate-derived $pABAglu_{5-8}$

are present in the same proportions and amounts as are the $pABAglu_{5-8}$ contaminating preparations of $(CoA)_2$. This would indicate that the $(CoA)_2$ contaminants are indeed derived from $pteglu_n$ that have been broken down during the isolation procedure.

Other folates, particularly $pteglu_3$ and $pteglu_4$, are represented in Figure 20 but do not appear as contaminants of $(CoA)_2$ preparations. These are assumed to be excluded during the preparation because of their size and charge differences.

The $pteglu_n$ distribution derived for *E. coli* is in marked disagreement with that reported by other authors. Powers and Snell (1976) stated that half of the cellular folates are present as $pteglu_5$ while the only other major forms are $pteglu_3$ and $pteglu_4$. Bassett *et al* (1976b) found the predominant form to be $pteglu_3$, while all other forms containing up to seven glutamyl residues were present in smaller quantities. This study has revealed that the major pteroyl polyglutamates are $pteglu_6$ and $pteglu_7$. Also more $pteglu_3$ than $pteglu_4$ or $pteglu_5$ is present. It is possible that the lower values reported in the literature are a result of the breakage of γ-glutamyl peptide bonds during the isolation or treatment of folate extracts. It has also been suggested that the glutamyl chain length of cellular $pteglu_n$ shows an inverse relationship to the environmental availability of folic acid or its precursor pABA. In the studies mentioned above $pteglu_n$ were examined using radioactively labelled folic acid or pABA in the culture medium in low but significant concentrations. In this study, *E. coli* grown in glucose-salts medium (with no folic acid present) showed the existence of the larger $pteglu_n$

by the presence of their catabolites $pABAglu_{5-8}$. However the cells grown in an enriched medium (for which the folate concentration is not known) also contain the larger $pteglu_{5-8}$. It should be noted that in this study the $pteglu_n$ distribution has only been examined in cultures that have reached the end of the logarithmic growth phase. It is not known whether the distribution remains constant throughout the growth cycle. The reasons for the discrepancies among reported $pteglu_n$ distributions are therefore not clear. Further examination of this problem may prove to be enlightening with regard to the control of folate metabolism.

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